Estudi dels mecanismes moleculars implicats en el dany vascular induït per l'activitat catalítica de la SSAO en el context de l'Angiopatia Cerebral Amiloide associada a la demència tipus Alzheimer

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Isaac Newton

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ÍNDEX

ABREVIATURES

1-Br	1-Bromoheptà
5-HT	*5-hydroxytriptamine* (Serotonina)
4-MP	4-Metilpirazol
βPEA	β-feniletilamina
A	Adrenalina
aa	amino àcids
A7r5	Línia cel·lular immortalitzada de múscul llis d'aorta de rata
$A\beta_{1-40}$ D	Beta Amiloide 1-40 amb la mutació Dutch (E22Q)
ACE	*Angiotensin-converting enzyme*
AD	*Alzheimer's Disease* (Malaltia d'Alzheimer)
ADAM	*a disintegrin and metalloprotease*
AGEs	*Advanced glycation end products*
AIF	*Apoptosis-inducing factor*
AMC	*7-amino-4-metilcumarina*
AO	Amino oxidasa
Apaf-1	*Apoptosis protease-activating factor-1*
APH-1	*Anterior pharynx-defective-1*
ApoE	Apolipoproteina E
APS	*Ammonium persulfate*
APP	*Amyloid precursor protein*
ATP	*Adenosine triphosphate*
BACE-1	*β-site APP cleaving enzyme 1*
BH3-only	*Bcl-2-homology domain 3 only*
BHE	Barrera hematoencefàlica
BSA	*Bovine serum albumine*
BSAO	*Bovine serum amine oxidase*
Bz	Benzilamina
Bzdh	Benzaldehid
CAA	*Cerebral Amyloid Angiopathy*
CAD	*Caspase-activated DNase*
CADASIL	*Cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy*
CARD	*Caspase-recruitment domain*
Cat	Catalasa
Chl Hyd	Cloral hidrat
Clor	Clorgilina
CO_2	Diòxid de carboni
Co-IP	Immunoprecipitació de complexes proteics
Cpms	Comptes per minut
Cyan	Cianamida
DA	Dopamina
DAO	Diamino oxidasa
DD	*Death domain*
DED	*Death effector domain*

Dep	Deprenil
DH5α	Soca del bacteri *Eschericha Coli* utilitzada per a l'amplificació de plasmidis
DISC	*Death-inducing signaling complex*
DM	*Diabetes mellitus*
DMEM	*Dulbecco's modified eagle's medium*
DMSO	Dimetilsulfòxid
DNA	*Deoxyribonucleid acid*
dpm	Desintegracions per minut
DTT	*Dithiothreitol*
EAE	Encefalomielitis al·lèrgica experimental
ECACC	*European collection of cell cultures*
EDTA	Àcid etilendiaminotetraacètic
ELISA	*Enzyme-linked immunosorbent assay*
FA	Formaldehid
FAD	Dinucleòtid de flavina i adenina
FADD	*Fas associated protein with death domain*
FBS	*Fetal bovine serum*
G418	Geneticina, antibiòtic de selecció utilitzat en el manteniment de les línies cel·lulars transfectades de forma estable
GAPDH	*Glyceraldehyde 3 phosphate dehydrogenase*
G-CSF	*Granulocyte colony-stimulating factor*
GDS	*Global deterioration scale*
GFP	*Green fluorescent protein*
GLUT1 (o 4)	*Glucose transporter 1 (or glucose transporter 4)*
GRP78	*Glucose regulated protein 78*
H_2O_2	Peròxid d'hidrogen
H_2Od	Aigua destil·lada
HCHWA-D	*Hereditary cerebral hemorrhage with amyloidosis dutch type*
HPAO	*Human placenta amine oxidase*
HRP	*Horse radish peroxidase*
HtrA2/Omi	*High temperature requirement protein A2*
HUVEC	*Human umbilical vein endothelial cells* (línia cel·lular immortalitzada d'endoteli de vena de cordó umbilical humà)
IAPs	*Inhibitors of apoptosis proteins*
ICAM-1	*Intracellular adhesion molecule-1*
IDE	*Insulin-degrading enzyme*
IGFr Iβ	*Insulin growth factor receptor, subunit 1β*
IF	Immunofluorescència
IL	Interleucina
iNOS	Òxid nítric sintasa induïble
IP	Immunoprecipitació
IRS	*Insulin-receptor substrate proteins*
IUBMB	*International union of biochemistry and molecular biology*
JCBN	*Joint comission on biochemical nomenclature*
KO	*Knock out*
LB	*L-Broth* (Medi utilitzat per al cultiu de bacteris)

LO	Lisil oxidasa
LPS	Lipopolisacàrid bacterià
LRP1	*Lipoprotein receptor related protein 1*
LTQ	Lisil-tirosil quinona
LXR	Receptor X del fetge
MA	Metilamina
MAO A	Monoamino oxidasa A
MAO B	Monoamino oxidasa B
MAPK	*Mitogen-activated protein kinase*
MD	*Mixed dementia*
MDL72974A	[(E)-2-(4-fluorophenethyl)-3-fluoroallylamine hydrochloride]
MCI	*Mild cognitive impairment*
MIP-1α	*Macrophage inflammatory protein 1 alpha*
MMSE	*Mini mental state examination*
MPTP	*Mitochondrial permeability transition pore*
mRNA	Àcid ribonucleic missatger
MTT	*3,(4,5-dimethylthiazol-2-yl)2,5-diphenyl-tetrazolium bromide*
NA	Noradrenalina
NCT	Nicastrina
NF-κB	*Nuclear factor kappa-light-chain-enhancer of activated B cells*
NMDA	*N-methyl D-aspartate*
PAO	Poliamino oxidases
PARP	*Poly (ADP-ribose) polymerase*
PBS	*Phosphate buffered saline*
PEI	*Polycation polyethyleneimine*
PEN-2	*Presenilin enhancer 2*
PET	Tomografia per emissió de positrons
PI3K	*Phosphoinositide 3-kinase*
Pif-α	Pifitrina alfa
PPO	*2,5-difeniloxazol*
PS-1, PS-2	Presenilina 1, presenilina 2
PVDF	*Polyvinylidene difluoride*
RAGE	*Receptor for advanced glycation end products*
RAO	*Retina-specific amine oxidase*
Rpm	Revolucions per minut
Sc	Semicarbazida
SDS	*Sodium dodecyl sulfate*
SEM	*Standard error of mean*
Smac/DIABLO	*Second mitochondria-derived activator of caspase/direct inhibitor of apoptosis-binding protein with low pI*
SRF	*Serum response factor*
SSAO	*Semicarbazide sensitive amine oxidase*
TBS	*Tris buffered saline*
TETA	Trietilentetraamina
TNF-α	*Tumor necrosis factor α*
TTBS	*Tween-tris buffered saline*
Tyr	Tiramina
TPK	Tampó fosfat de potassi

TPQ	Topaquinona o *2,4,5-trihydroxyphenylalanine* en la seva forma quinona
UPR	*Unfolded protein response*
VaD	*Vascular dementia*
VAP-1	*Vascular adhesion protein-1*
VCAM-1	*Vascular cell adhesion molecule-1*
VCI/VCD	*Vascular cognitive impairment/Vascular cognitive disorder*
VEGF	*Vascular endothelial growth factor*
WB	*Western blot*
WT	*Wild Type*
XBP	*X-box binding protein*

RESUM

L'amino oxidasa sensible a semicarbazida (SSAO) és un enzim localitzat en el teixit adipós, en el múscul llis i endoteli del sistema vascular, i en el plasma sanguini en forma soluble. A banda de la seva activitat enzimàtica, en la que metabolitza amines primàries, desenvolupa també una funció insulinomimètica en teixit adipós i múscul llis, i una funció d'adhesió limfocitària a l'endoteli en situacions inflamatòries, on és coneguda com a VAP-1 (proteïna d'adhesió vascular 1). El metabolisme dels seus substrats genera productes tòxics com el peròxid d'hidrogen, l'amoni o diferents aldehids en funció de l'amina metabolitzada. Aquests productes han estat associats a l'aparició de dany vascular en diverses malalties en les que s'han detectat nivells incrementats d'activitat soluble i/o d'expressió tissular de la SSAO, com són la diabetis, les aturades cardíaques o la malaltia d'Alzheimer (AD), però els mecanismes responsables d'aquest increment i les seves conseqüències no són del tot coneguts.

Per tal de poder estudiar aquesta proteïna *in vitro*, s'han generat i caracteritzat bioquímicament dues línies cel·lulars vasculars d'endoteli i múscul llis, transfectades de forma estable amb el gen humà de la SSAO/VAP-1, ja que l'expressió d'aquesta proteïna es perd en cèl·lules en cultiu. Aquestes línies cel·lulars han estat utilitzades per estudiar els efectes dels productes generats durant el metabolisme de la metilamina, un dels principals substrats fisiològics de la SSAO, així com per estudiar a nivell molecular la seva possible implicació en el dany vascular associat a l'AD.

Els resultats han mostrat que els productes generats per l'activitat de la SSAO transmembranal transfectada són capaços d'induir la mort apoptòtica de les cèl·lules de múscul llis, que mostren una activitat SSAO més elevada que les endotelials. Aquesta mort és dependent de l'activitat transcripcional de p53, la qual indueix efectes sobre la mitocòndria produint una desregulació del balanç Bax/Bcl-2, una inducció de l'expressió de Puma-α i una activació de les caspases 9 i 3. D'altra banda, s'ha utilitzat el tractament amb el pèptid beta amiloide de 40 amino àcids amb la mutació Dutch ($A\beta_{1\text{-}40}$ D) com a model d'Angiopatia Cerebral Amiloide associada a l'AD (CAA-AD), patologia que cursa amb dipòsits d'Aβ vasculars i degeneració vascular. En aquest model s'ha observat que l'Aβ provoca un increment de la quantitat d'SSAO en les cèl·lules endotelials, que es tradueix en l'aparició de citotoxicitat associada al metabolisme de la metilamina. S'ha confirmat també que l'activitat SSAO incrementa el dipòsit d'Aβ, i s'ha afegit una nova implicació de la SSAO en aquesta patologia, que consisteix en promoure el dipòsit d'Aβ mitjançant mecanismes independents de la seva activitat catalítica.

Vista a nivell molecular la possible contribució de la SSAO en la degeneració vascular en condicions de CAA-AD, i havent estat descrit prèviament un increment d'expressió de la SSAO i de la seva activitat plasmàtica en pacients afectats per AD, es van determinar els nivells d'activitat SSAO plasmàtica en una mostra més gran de pacients d'AD. Aquests resultats van confirmar que l'augment plasmàtic d'SSAO nomes es produeix en condicions de demència severa, i que és independent de l'edat i del sexe dels individus. A més, la determinació de l'activitat SSAO plasmàtica en altres demències amb afectació vascular, com la demència vascular o la demència mixta no van mostrar canvis significatius en l'activitat SSAO amb la gravetat de la demència; tot i així, aquests resultats no descarten una possible implicació de la SSAO en aquestes patologies ja que no va ser possible disposar d'un grup de pacients amb demència severa en aquestes condicions patològiques. De la mateixa manera, el baix número de mostres analitzades de pacients amb demència frontotemporal no permet extreure conclusions sobre la implicació de la SSAO en aquesta patologia.

Existeix, doncs, una implicació directa de la SSAO tant en la degeneració vascular associada a l'AD com en la deposició de l'Aβ, la qual és capaç de promoure l'augment d'activitat SSAO alimentant així el cercle viciós d'inducció de dany vascular. Aquests efectes poden ser rellevants durant tot el procés de la malaltia, tant en l'inici promovent la deposició d'Aβ, com en les etapes més avançades, en les que s'ha detectat un increment de la seva activitat en el plasma que contribuirien al dany vascular. Tot i així, seran necessaris més estudis amb mostres de pacients, per poder determinar la seva possible implicació relacionada amb el dany vascular en altres tipus de demències.

ABSTRACT

Semicarbazide sensitive amine oxidase (SSAO) is an enzyme localized in adipocytes, in the smooth muscle and endothelial cells of vascular system, and in blood plasma as a soluble form. Besides its enzymatic function of primary amines metabolism, SSAO activity has also insulinomimetic effects in adipose and in smooth muscle tissues, and it behaves as vascular adhesion protein 1 (VAP-1) in endothelial cells under inflammatory conditions. The SSAO substrates oxidation generates the toxic products hydrogen peroxide, ammonia and different aldehydes depending on the metabolized amine. These products have been related to the vascular damage observed in diseases where increased levels of SSAO plasma activity and/or tissue expression have been detected, as diabetes, congestive heart failure or Alzheimer's disease (AD). The mechanisms that induce this increase and its consequences, however, are not fully understood.

In order to perform *in vitro* studies with this protein, an endothelial and a smooth muscle cell line were transfected in a stable form with the human SSAO/VAP-1 gene, due to the loss of SSAO expression in cell cultures. These cell lines were characterized at biochemical level and used to analyze the effect of methylamine oxidation products, as methylamine is one of the main physiological SSAO substrates. Results showed that the products generated by the transfected SSAO activity were able to promote the apoptotic cell death of the smooth muscle cells, which displayed higher SSAO activity levels than endothelial cells. This apoptotic cell death was p53 transcriptional activity-dependent, and it involved the mitochondrial apoptotic pathway through the Bax/Bcl-2 ratio deregulation, the Puma-α expression induction and the caspases 9 and 3 activation.

On the other hand, the possible involvement of SSAO in the vascular damage related to AD was also analyzed with the developed cell lines. Treatment with the 40 amino acids length amyloid beta peptide containing the Dutch mutation ($A\beta_{1\text{-}40}$ D) was used as a model of Cerebral Amyloid Angiopathy related to AD (CAA-AD), which is characterized by vascular Aβ deposits and vascular degeneration as well. Results showed that Aβ treatment induced an increase of SSAO/VAP-1 protein amounts in endothelial cells, which induced the appearance of methylamine oxidation-dependent cytotoxicity. Moreover, besides the confirmation that SSAO activity enhances the Aβ deposition, which was recently described, results revealed a new involvement of SSAO in the Aβ deposition process through enzymatic activity-independent mechanisms.

Finally, given the possible contribution of SSAO in CAA-AD vascular degeneration at molecular level, and the described increase of SSAO expression and plasma activity in AD patients, a higher population of AD patients was analyzed. The obtained results confirmed the increase of plasma SSAO activity only at severest dementia conditions, and that it was age and sex-independent. Moreover, SSAO activity levels did not show differences in increasing conditions of vascular dementia or mixed dementia, as other types of dementia with vascular affectation. However, these results do not allow ruling out a possible involvement of SSAO in these pathologies, since severe groups of demented patients were not available. In the same way, the low number of samples of frontotemporal dementia patients analyzed does not allow to conclude an involvement of SSAO in this pathology.

In summary, the obtained results in this work show that SSAO is directly involved in CAA-AD-related vascular degeneration as well as in Aβ deposition by activity-dependent and independent mechanisms. Moreover, the Aβ deposition increases the SSAO availability, thus reinforcing its toxic effect as a vicious circle. These effects can become important during all the disease progression, enhancing the Aβ deposition at early stages, but also contributing to the vascular damage at advanced ones when SSAO activity is increased. Even so, it would be necessary to perform more studies on plasma human samples in order to determine its possible involvement in the vascular damage observed in other dementia conditions.

I. INTRODUCCIÓ

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1. Amino Oxidases: Classificació Enzimàtica

Els enzims es poden classificar funcionalment, mitjançant un sistema que té en compte les reaccions que aquests catalitzen, i de forma independent a l'estructura de la molècula catalítica. La "*IUBMB Enzyme List*" (http://www.chem.qmul.ac.uk/iubmb/enzyme/) conté aquesta classificació, la qual és elaborada i actualitzada pel Comitè de Nomenclatura de la IUBMB (*International Union of Biochemistry and Molecular Biology*) i la JCBN (*Joint Comission on Biochemical Nomenclature*). Segons aquesta classificació, cada enzim o grup enzimàtic que catalitza una mateixa reacció està identificat per un codi de quatre components; el primer indica la classe a la qual pertany l'enzim, el segon la subclasse, el tercer la sub-subclasse i el quart és un nombre de sèrie dins de la sub-subclasse.

El grup enzimàtic de les amino oxidases està inclòs en la classe 1, que correspon a les oxidoreductases, la subclasse 4, que correspon a les oxidoreductases que actuen sobre els grups CH-NH_2 com a donadors, i la sub-subclasse 3, la qual té l'oxigen com a acceptor. Les amino oxidases (AOs) són un grup enzimàtic heterogeni que catalitza desaminacions oxidatives de diferents tipus d'amines, tant endògenes, com obtingudes de la dieta o xenobiòtics (revisat per (Strolin *et al.* 2007)), generant l'aldehid corresponent a l'amina metabolitzada, peròxid d'hidrogen (H_2O_2) i amoni (NH_3), segons la següent reacció:

$$RCH_2NH_2 + O_2 + H_2O \text{ --> } RCHO + NH_3 + H_2O_2$$

Monoamines, diamines i poliamines esdevenen substrats específics de cada amino oxidasa, tot i que hi ha substrats comuns a diversos d'aquests enzims.

El grup de les amino oxidases, s'ha dividit tradicionalment en dos subgrups, atenent al cofactor que contenen associat (figura 1). D'una banda es troben els enzims que contenen FAD (dinucleòtid de flavina i adenina), com ara les poliamino oxidases (PAO) [EC 1.5.3.11], o les monoamino oxidases (MAO) [EC 1.4.3.4] tots ells enzims intracel·lulars; d'aquestes últimes se'n distingeixen dues isoformes, la MAO A i la MAO B, en base a la seva selectivitat de substrat i a la seva sensibilitat a diferents inhibidors (Knoll and Magyar 1972). D'altra banda, hi ha els que contenen coure i topaquinona (TPQ), com ara la diamino oxidasa (DAO) [EC 1.4.3.22] o l'amino oxidasa sensible a

semicarbazida (SSAO) [EC 1.4.3.21] (Janes and Klinman 1995), estudiada al llarg d'aquest treball. Dins aquest segon grup s'inclou també la lisil oxidasa (LO) [EC 1.4.3.13], la qual conté coure com les anteriors, però difereix en l'altre cofactor, tenint la lisil-tirosil quinona (LTQ) enlloc de la TPQ (Wang *et al.* 1997).

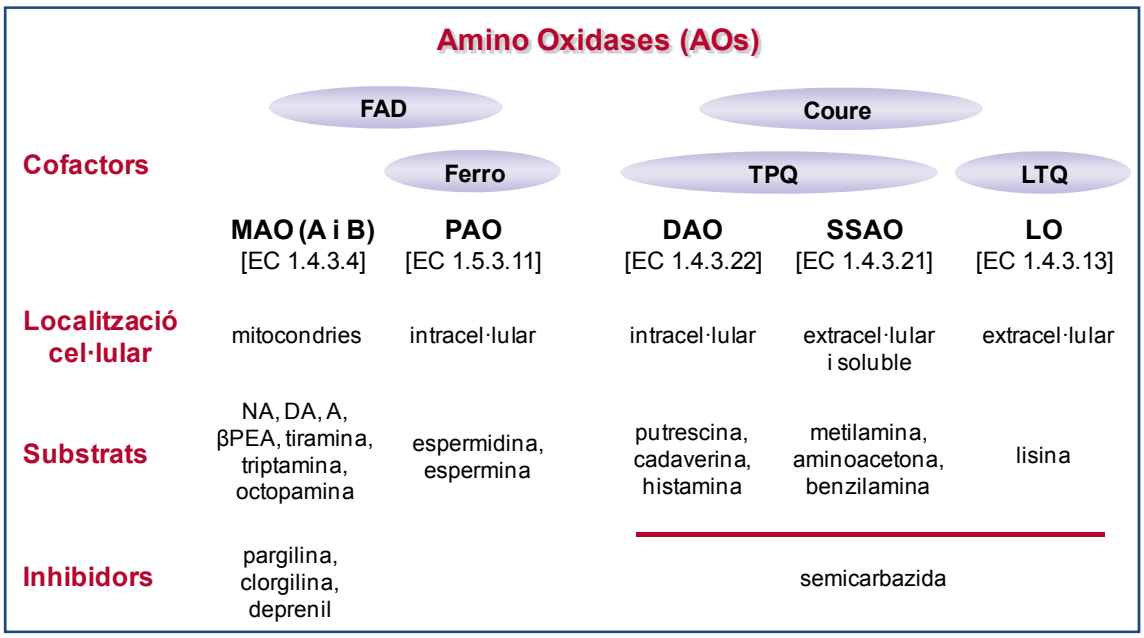


Figura 1. Classificació de les principals AOs. FAD, dinucleòtid de flavina i adenina; TPQ, topaquinona; LTQ, lisil-tirosil quinona; NA, noradrenalina; DA, dopamina; A, adrenalina; βPEA, β-feniletilamina. Adaptació de (Jalkanen and Salmi 2001).

Tot i aquesta divisió, les amino oxidases que contenen FAD i les que contenen coure difereixen no només en el cofactor sinó també en l'especificitat de substrat, la sensibilitat a inhibidors, la funció o la localització tant tissular com subcel·lular.

De les amino oxidases que contenen FAD,

- Les **MAOs A i B** es troben en tots els teixits humans excepte en els eritròcits, però ambdues isoformes es distribueixen de diferent forma en alguns d'ells: per exemple, la MAO A predomina en la placenta, mentre que la MAO B es troba preferentment en plaquetes i limfòcits. Aquests dos enzims es localitzen subcel·lularment en la membrana mitocondrial externa, on tenen com a substrats amines primàries, secundaries i terciàries, i són inhibits per compostos acetilènics, com ara la clorgilina, l'l-deprenil o la pargilina. Una de les seves funcions més ben establertes és el seu paper en el metabolisme dels neurotransmissors, com la

noradrenalina, i d'altres amines biògenes com l'adrenalina o la tiramina (Shih *et al.* 1999).

- Les **PAOs** es troben també pràcticament en tots els teixits humans (Suzuki *et al.* 1984), i subcel·lularment en el citosol i peroxisomes. Tenen com a substrat amines secundàries, com ara l'espermina i l'espermidina. La principal funció que se'ls ha associat és la regulació del creixement cel·lular (Seiler 1990).

En referència a les amino oxidases que contenen coure, totes elles es caracteritzen per ser inhibides per reactius que contenen grups carbonil, com ara la semicarbazida; per això aquest grup ha estat anomenat tradicionalment família d'amino oxidases sensibles a semicarbazida, encara que en formessin part identitats enzimàtiques diferents com són la LO, la DAO o la SSAO pròpiament dita.

- La **LO** difereix bastant de la resta de membres del grup: a part de no tenir a la TPQ com a cofactor juntament amb el coure, es tracta d'una proteïna sintetitzada en forma de pre-proLO, que a través d'una sèrie de modificacions post traduccionals arriba a ser un proenzim de 50 KDa, el qual és secretat al medi extracel·lular en forma monomèrica de 32 KDa (Kagan and Trackman 1991). Catalitzant la desaminació oxidativa de residus de lisina i hidroxilisina, la LO participa en l'entrecreuament de proteïnes de la matriu extracel·lular com ara el col·lagen i l'elastina, assegurant l'estabilitat de la matriu. Per aquest motiu, és essencial en el manteniment de les característiques de tensió i elasticitat dels teixits connectius dels sistemes esquelètic, pulmonar i cardiovascular entre altres (Kagan and Li 2003; Lucero and Kagan 2006). Recentment ha estat cristal·litzada una LO que conté TPQ en el llevat *Pichia Pastoris* (Duff *et al.* 2003), la qual tot i no estar relacionada a nivell de seqüència amb la LO de mamífers, presenta la mateixa especificitat de substrat.

- La DAO i la SSAO han estat durant molt temps classificades dins la mateixa identitat enzimàtica ([EC 1.4.3.6]), ja que els dos enzims contenen el mateix cofactor, i es solapen en diversos aspectes com ara la localització tissular o els substrats que metabolitzen. Aquest fet comporta que en algunes publicacions antigues no quedi del tot clar quin dels dos enzims s'estava estudiant; en altres, la única distinció utilitzada era que la SSAO tenia una baixa activitat en front a diamines com la histamina, la putrescina o la cadaverina, que en canvi són bons substrats de la DAO.

- La **DAO** es troba preferentment en la placenta, ronyons i intestí, i és un enzim majoritàriament intracel·lular, que es localitza en vesícules associades a la membrana plasmàtica (Schwelberger *et al.* 1998); també es troba en una forma

secretada, que és capaç d'unir-se a les cèl·lules endotelials de manera dependent d'heparina (Biebl *et al.* 2002). Els seus substrats més importants són les diamines putrescina i la histamina (Buffoni 1966), i referent a aquest últim, participa en la regulació de processos d'inflamació i reaccions al·lèrgiques. Se l'ha relacionat amb el control de la proliferació cel·lular (Quash *et al.* 1979), i s'ha descrit també com una proteïna d'unió a amiloride en el ronyó humà (Barbry *et al.* 1990).

- La **SSAO** presenta una activitat més elevada en múscul lliss del sistema cardiovascular, en teixit adipós i en pulmons. A nivell cel·lular, es presenta en forma transmembranal amb una petita porció intracel·lular, i també en forma soluble. Només és capaç de metabolitzar amines primàries, com la metilamina, l'aminoacetona o la benzilamina.

Recentment, i davant el coneixement que la SSAO i la DAO tenen diferents activitats enzimàtiques, el grup enzimàtic EC 1.4.3.6 ha estat substituït per dos enzims diferents, que conformen ara els grups EC 1.4.3.22 (DAO) i EC 1.4.3.21 (SSAO). A més, ja que el nom SSAO era utilitzat moltes vegades per nombrar tota la família, ja que inclou les "amino oxidases inhibides per semicarbazida", actualment s'anomena l'enzim EC 1.4.3.21 (tradicionalment la SSAO) com a "*primary-amine oxidase*". En aquest treball però, s'utilitzarà el terme SSAO per facilitar la concordança amb la bibliografia existent.

2. Amino Oxidasa Sensible a Semicarbazida (SSAO)

2.1. La SSAO en diferents espècies

Les SSAOs es troben àmpliament distribuïdes en la natura, i han estat purificades de microorganismes, plantes i mamífers. En alguns casos, han estat emmarcades sota el nom genèric d'"amino oxidases que contenen coure", englobant probablement també l'activitat DAO.

Fins a l'actualitat, aquestes proteïnes s'han purificat en diverses espècies de **FONGS**:

- *Aspergillus niger AKU 3302*: (Frebort *et al.* 1996)
- *Gibberella fujikuroi*: (Frebort *et al.* 1997)
- *Aspergillus oryzae*: (Matsumura *et al.* 2004)

de **BACTERIS**:

- *Klebsiella aerogenes*: (Sugino *et al.* 1992)
- *Arthrobacter strain P1*: (Zhang *et al.* 1993)
- *Arthrobacter globiformis*: (Tanizawa *et al.* 1994)
- *Escherichia coli*: (Parsons *et al.* 1995; Roh *et al.* 1994)
- *Klebsiella oxytoca*: (Hacisalihoglu *et al.* 1997)
- *Mycobacterium sp. Strain JC1 DSM 3803*: (Lee *et al.* 2008; Ro *et al.* 2006)

de **LLEVATS**:

- *Hansenula polymorpha*: (Bruinenberg *et al.* 1989)
- *Kluyveromyces marxianus*: (Corpillo *et al.* 2003)

de **PLANTES**:

- *lentil seedling* (llenties): (Rossi *et al.* 1992)
- *Arabidopsis thaliana*: (Moller and McPherson 1995)
- *pea seedling* (pèsols): (Kumar *et al.* 1996; Tipping and McPherson 1995)

i de **MAMÍFERS**:

La primera SSAO de mamífers que es va clonar va ser la de sèrum boví (BSAO), encara que es va clonar a partir del fetge (Mu *et al.* 1994). Més tard es va demostrar, també en teixit boví, l'existència d'almenys 3 gens que codifiquen per diferents amino oxidases que contenen coure. Actualment es coneix també la seqüència de la SSAO en els rosegadors rata (Morris *et al.* 1997; Ochiai *et al.* 2005) i ratolí (Bono *et al.* 1998a; Moldes *et al.* 1999).

En humans, la SSAO va ser clonada per dos grups independents sota diferents noms: "*human placenta amine oxidase (HPAO)*" (Zhang and McIntire 1996) i "*human vascular adhesion protein 1(VAP-1)*" (Smith *et al.* 1998). En el primer treball, s'estudiava l'amino oxidasa que conté coure de la placenta humana, identificant-la com un homòleg de la BSAO, i diferenciant-la clarament de la DAO. En el segon treball s'estudiava la proteïna d'adhesió vascular-1 (VAP-1), la qual participa en l'adhesió limfocitària als vasos sanguinis en situacions d'inflamació i a més té activitat monoamino oxidasa; en conèixer la seqüència completa de la VAP-1 es va concloure que era la mateixa proteïna que la SSAO clonada de la placenta humana. Ambdós grups van localitzar la seqüència en el cromosoma 17q21. Més tard es va identificar una forma d'SSAO que es va denominar com a específica de retina (RAO) (Imamura *et al.* 1997), amb la

seqüència localitzada també en el cromosoma 17q21, i que presentava una variant d'*splicing* (Imamura *et al.* 1998); avui es sap, però, que la RAO s'expressa també en altres teixits (Heniquez *et al.* 2003; Su *et al.* 2002). Finalment es va descriure una altra seqüència en el genoma humà relacionada amb la SSAO, essent aquest cop un pseudogen amb dues variants d'splicing, localitzada com les anteriors en el cromosoma 17q21 (Cronin *et al.* 1998).

Tot i trobar-se majoritàriament codificada pels mateixos gens en diverses espècies de mamífers, existeixen diferències que determinen el nivell d'activitat SSAO que es troba en el plasma d'aquestes (Schwelberger 2007). Els gens que codifiquen per les amino oxidases que contenen coure en mamífers s'han identificat com a AOC1-4: l'AOC-1 codifica per la DAO; els AOC2, 3 i 4 es localitzen en tàndem en el mateix cromosoma (17q21) i codifiquen per la SSAO específica de retina (AOC2), la proteïna d'adhesió vascular VAP-1 (AOC3) i en el cas del gen AOC4, el producte que codifica difereix segons l'espècie: en rosegadors i humans, es tracta d'un pseudogen que codifica per una proteïna truncada precoçment i que no és funcional; en canvi, en altres mamífers com ara el porc, vaca o gos, el gen AOC4 codifica per una proteïna de 762 aminoàcids amb una homologia del 93% amb la SSAO/VAP-1, la qual presenta una seqüència peptídica de secreció enlloc d'un domini transmembrana a l'extrem N-terminal. Aquesta proteïna és sintetitzada al fetge, però és secretada directament al plasma, i és responsable de la majoria d'activitat SSAO plasmàtica en espècies que tenen el gen AOC4 funcional, mentre que en les altres, la SSAO plasmàtica deriva del processament de la proteïna transmembranal (Abella *et al.* 2004; Stolen *et al.* 2004b). Aquest fet explica la diferència en els nivells d'activitat SSAO que es troba en el plasma de diferents espècies de mamífers, essent dels nivells de milers de μU/ml en la majoria de mamífers, de centenars en humans, i només de 20-30 μU/ml en rosegadors (Boomsma *et al.* 2003).

2.2. Seqüència i estructura de la SSAO (AOC3)

Cal destacar l'elevat grau d'homologia de seqüència que existeix entre les SSAOs de diferents espècies de mamífer, essent d'un 80% entre la proteïna humana i la bovina (BSAO), d'un 83% entre la humana i la de ratolí i d'un 80% entre la humana i la de rata. En canvi, existeix només un 64% d'homologia entre la forma humana de la SSAO i la forma humana específica de retina (RAO). Tot i que l'homologia va disminuint en espècies més allunyades filogenèticament dels mamífers, és important notar que

existeixen uns motius altament conservats en les seves seqüències, corresponents al centre actiu de la proteïna, i que l'estructura tridimensional de la proteïna també és extremadament similar.

A partir del clonatge del gen i promotor de la SSAO de ratolí (Bono *et al.* 1998b) es va descriure que el gen que codifica per la SSAO és un gen de còpia única que es composa de 4 exons separats per 3 introns. Aquest treball va descriure també l'existència de múltiples llocs d'inici de la transcripció, els quals poden donar lloc a transcrits d'mRNA de diferent mida, explicant així l'observació de dos transcrits de diferent mida en adipòcits humans (Heniquez *et al.* 2003). A més, es suggereix que el fet que hi hagi diferents llocs d'inici de la transcripció pot ser el responsable de diferències en la regulació de la transcripció de la proteïna entre teixits, com ara l'observada entre endoteli i múscul llis. En aquest treball també es van identificar llocs d'unió de diversos factors de transcripció en el promotor del gen murí, com ara el d'NF-κB entre altres.

En la figura 2 es mostren els principals motius conservats en la seqüència de les SSAOs de la majoria d'espècies. Les posicions d'aquests varien segons la llargada de la proteïna, que difereix lleugerament entre espècies. En mamífers, s'ha determinat una longitud de 762 aa per la forma soluble bovina, amb un pes molecular de 84.75 KDa (Mu *et al.* 1994); 763 aa i 84.6 KDa per a la SSAO de placenta humana (Zhang and McIntire 1996) i VAP-1 (Smith *et al.* 1998); 765 aa i 84.5 KDa per la SSAO de ratolí (Bono *et al.* 1998b) i 763 aa i 85 KDa per la SSAO de rata (Ochiai *et al.* 2005).

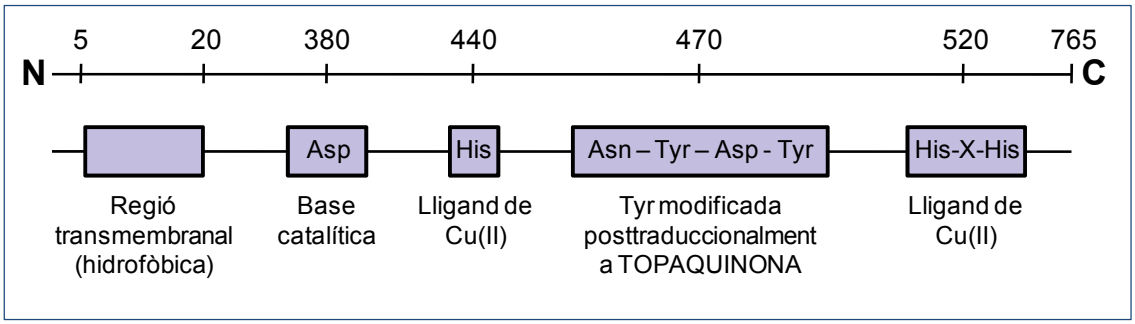


Figura 2. Motius conservats entre SSAOs de diferents espècies. Senyal transmembranal a l'extrem N-terminal. Posició de la base catalítica, histidines que coordinen els àtoms de coure i la seqüència de quatre aminoàcids que conté la tirosina que es modifica a TPQ. Adaptació de (Jalkanen and Salmi 2001).

El centre actiu de les SSAOs s'ha conservat al llarg de l'evolució mitjançant unes seqüències determinades importants per la seva activitat catalítica. Un exemple són les tres histidines que funcionen com a lligands de l'àtom de coure que es troba en cada proteïna. També es conserva la seqüència de quatre aminoàcids que conté la tirosina que es modifica per donar lloc al cofactor topaquinona (Klinman and Mu 1994; Salminen *et al.* 1998); aquesta modificació està duta a terme per la proteïna d'unió a coure, i requereix l'àtom de coure i consum d'oxigen (Janes *et al.* 1990; Mu *et al.* 1992). L'àcid aspàrtic conservat participa també en la reacció catalítica. Existeixen nombrosos punts de glicosilació conservats en la seqüència, els quals són importants per a la funcionalitat de la proteïna (Salmi and Jalkanen 1996a).

En mamífers, l'estructura de la SSAO es descriu com una sialo-glicoproteïna transmembranal de tipus II amb l'extrem N-terminal curt intracel·lular, de només 4 aminoàcids, seguit d'una regió hidrofòbica transmembranal d'uns 23 aminoàcids, i un extrem C-terminal gran exposat extracel·lularment, el qual conté el centre actiu i nombrosos llocs d'O i N-glicosilacions (Smith *et al.* 1998). La proteïna transmembranal es presenta en forma de dímer d'uns 170-180 KDa dependent del grau de glicosilacions (Salmi and Jalkanen 1996b); es troba també una forma soluble en plasma, la qual deriva en rosegadors i humans d'un tall de la transmembranal i per tant perd els aminoàcids transmembranals i intracel·lulars (Abella *et al.* 2004; Stolen *et al.* 2004b). Cada monòmer conté un àtom de coure, i l'estructura dimèrica es sustenta mitjançant interaccions covalents i no covalents.

L'estructura de la SSAO ha estat estudiada mitjançant la cristal·lització de la proteïna de diferents espècies. La primera SSAO cristal·litzada va ser la de la bactèria *E. Coli* (Parsons *et al.* 1995). Després es van cristal·litzar també les SSAOs de *Pisum sativum* (Kumar *et al.* 1996), *Hansenula polymorpha* (Li *et al.* 1998b) i *Arthobacter globiformis* (Wilce *et al.* 1997). La primera SSAO de mamífer cristal·litzada va ser la VAP-1 humana completa (Nymalm *et al.* 2003), i més tard es van cristal·litzar una forma truncada de la proteïna humana, sense l'extrem N-terminal transmembranal (Jakobsson *et al.* 2005a), i la forma bovina soluble (BSAO) (Lunelli et al., 2005). L'estructura teòrica del centre catalític de la SSAO/VAP-1 humana, però, havia estat prèviament descrita mitjançant modelatge estructural (Salminen *et al.* 1998).

Tot i que l'homologia de la seqüència de les SSAOs de diferents espècies varia, essent des d'un 25% entre espècies més llunyanes fins a un 90% entre espècies més semblants, l'estructura de totes elles es manté molt homogènia. L'estructura de totes les SSAOs cristal·litzades fins al moment mostra un plegament similar, en forma de

bolet, contenint tres dominis anomenats D2, D3 i D4 (figura 3). En la proteïna d'*E. Coli* s'observa a més un domini D1 el qual incrementa la interfase del dímer mitjançant interaccions entre els dominis D1 dels monòmers (Parsons *et al.* 1995). De tots ells, el domini central en "*β-sandwitch*" D4 és el més conservat ja que conté els residus implicats en la generació de la topaquinona i/o en la reacció catalítica. Cada domini D4 s'estabilitza mitjançant un pont disulfur, i forma la interfase principal del dímer en forma de braços que abracen a l'altre monòmer (Jakobsson *et al.* 2005b).

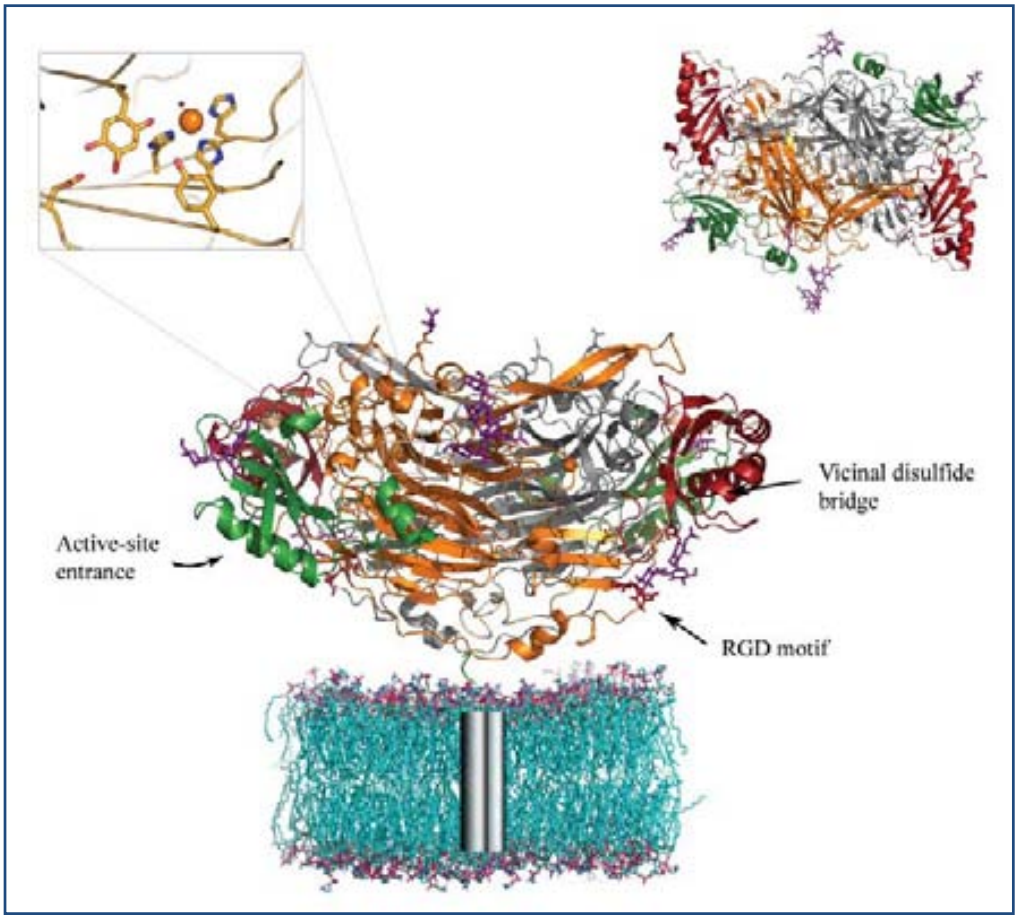


Figura 3. Visió general de l'estructura de la SSAO humana, figura extreta de (Jakobsson *et al.* 2005b). Es mostra l'estructura en forma de bolet de l'homodímer en la forma transmembranal. Els dos cilindres grisos representen les hèlix que insereixen la molècula a la bicapa lipídica. S'observen els dominis D2 en verd, els D3 en vermell i els D4 en gris (monòmer A) o en taronja (monòmer B). Els carbohidrats es veuen en lila. La petita il·lustració inserida a la part superior esquerra mostra el centre actiu de la SSAO, i la de la part superior dreta mostra una visió de l'homodímer des de dalt.

El centre actiu es situa a l'interior de l'estructura, accessible a través d'un canal de delimitat per residus conservats del domini D3, residus variables del D4 i un dels braços de l'altra subunitat. En la SSAO humana, la TPQ del centre actiu es pot trobar en dues conformacions diferents: "*on-copper*" o "*off-copper*". En la primera, la TPQ està en contacte amb l'ió de coure, fet que provoca que la leucina 469 bloquegi l'entrada de lligands al centre actiu; en la conformació "*off-copper*", la leucina 469 deixa lliure l'accés al centre actiu, actuant així com un "guardià" regulant l'accés dels substrats al centre actiu (Airenne *et al.* 2005). Aquest residu de leucina és un residu variable del motiu conservat que envolta la TPQ, i desenvolupa un paper important a l'hora de definir l'especificitat de substrat entre els membres de la família de les amino oxidases, i també entre SSAOs de diferents espècies. Per exemple, altres SSAOs tenen en aquesta posició una glicina (*E. Coli*, *Arthrobacter globiformis*), la qual deixa una entrada més gran; tant la SSAO humana com la bovina tenen una leucina, però en conformació diferent, la qual resulta en una entrada més estreta en la humana (Jakobsson *et al.* 2005b). L'especificitat de substrat entre espècies es determina mitjançant aquesta i altres petites diferències estructurals (Marti *et al.* 2004; Yraola *et al.* 2009). A més de les cavitats d'entrada i sortida del substrat, la proteïna té altres cavitats on es poden unir molècules per modular-ne l'activitat (Holt *et al.* 2004; Holt *et al.* 2008).

Altres trets diferencials en l'estructura de la proteïna humana són el motiu RGD, que es troba en el domini D4 (arginina-glicina-àcid aspàrtic, residus 726-728) (Salminen *et al.* 1998; Smith *et al.* 1998), o la presència de tres ponts disulfur que estabilitzen la part C-terminal de la proteïna. El motiu RGD és important per a la funcionalitat de la proteïna, ja que la seva deleció disminueix l'adhesió de limfòcits (Salmi *et al.* 2000). A més, s'ha postulat que aquest motiu, degut a que és una seqüència d'unió d'integrines, podria actuar reclutant metaloproteases tipus ADAMs (disintegrines i metaloproteases), les quals també tenen motius RGD, i aquestes podrien ser les responsables del tall de la SSAO transmembranal per ser alliberada al torrent sanguini (Jakobsson *et al.* 2005b). D'altra banda, la majoria d'SSAOs eucariotes es troben glicosilades: en aquest sentit, la SSAO humana presenta llocs d'O i N-glicosilació amb àcids siàlics als extrems, importants per l'adhesió limfocitària que desenvolupa la SSAO (Salmi and Jalkanen 1996a). Alguns d'aquests carbohidrats, juntament amb els 4 últims residus de l'extrem C-terminal afecten també a la mida de l'entrada al centre actiu en la proteïna humana.

2.3. Activitat catalítica de la SSAO

Les SSAOs catalitzen la desaminació oxidativa d'amines primàries alifàtiques i aromàtiques (Klinman and Mu 1994; Wilmot *et al.* 1999), segons la reacció:

$$R\text{-}CH_2\text{-}NH_2 + O_2 + H_2O \dashrightarrow R\text{-}CHO + H_2O_2 + NH_3.$$

Aquesta reacció de desaminació segueix un mecanisme ping-pong (Mure *et al.* 2002). En la primera mitja-reacció (reductora), el cofactor de l'enzim és reduït per l'amina primària (substrat) i aquesta és oxidada a l'aldehid corresponent, que s'allibera. En la segona mitja-reacció (oxidativa), el cofactor és re oxidat per l'oxigen molecular, el qual és reduït a peròxid d'hidrogen que s'allibera juntament amb l'amoni. Durant el procés reductor es produeixen de forma seqüencial una sèrie d'estats de transició entre els quals es forma una base de Schiff covalent però transitòria entre l'enzim i el substrat (Dooley *et al.* 1991; Hartmann *et al.* 1993).

2.3.1 Substrats de la SSAO

Amines primàries alifàtiques i aromàtiques són substrats de la SSAO, però no poliamines ni diamines. Està àmpliament acceptat, que la benzilamina és el substrat preferit de la majoria d'SSAOs en mamífers (Boomsma 2000) (Figura 4.a), encara que aquest substrat no és fisiològic i també és substrat de la MAO B (Strolin Benedetti M.S. and Dostert P 1985). En aquest sentit, cal destacar que existeixen diferències d'especificitat de substrat entre diferents espècies, i també entre diferents teixits de la mateixa espècie, com s'ha comentat en referència a petites variacions estructurals.

A part de la benzilamina, altres **amines primàries aromàtiques** identificades com a substrats de la SSAO inclouen la tiramina (Rucker and Goettlich-Riemann 1972; Young *et al.* 1982) (exògena), o la β-feniletilamina (Young *et al.* 1982), la histamina (Banchelli *et al.* 1994), la dopamina (Lizcano *et al.* 1991a) i la triptamina (Rucker and Goettlich-Riemann 1972; Young *et al.* 1982) (endògenes). D'aquestes, la histamina és un substrat dèbil en la majoria d'espècies, però és relativament actiu en porc, i també en cor humà, on es creu que podria substituir la inexistent activitat DAO (Pino *et al.* 1998); de forma similar, la tiramina, β-feniletilamina i triptamina són substrats més importants per a l'enzim de conill, i la dopamina per al de conill i de plasma de rumiants (Lyles 1996; Sharman *et al.* 1983). A part de la histamina i la dopamina, altres neurotransmissors com la noradrenalina i la serotonina no són bons substrats per la

majoria d'SSAOs, però s'ha observat que la serotonina és un bon substrat de l'enzim de polpa dental humana i porcina (Norqvist *et al.* 1981); a més, s'ha observat que pacients amb la malaltia de Norrie, els quals tenen una deleció del gen de la MAO, excreten en l'orina concentracions normals dels metabòlits desaminats de serotonina i dopamina (Murphy *et al.* 1991), fet que ha suggerit que la SSAO pugui ser la responsable de la desaminació d'aquests substrats en aquest cas, encara que no se'n tenen proves definitives.

Entre les principals **amines primàries alifàtiques** que són substrats de la SSAO es troben la metilamina (MCEWEN, Jr. 1965; Precious *et al.* 1988) i l'aminoacetona (Lyles and Chalmers 1992), ambdues endògenes, però també són substrats de la SSAO l'etilamina, n-propilamina, n-butilamina (Yu 1990), n-pentilamina (Guffroy *et al.* 1983), hexilamina, heptilamina, octilamina, nonilamina i decilamina (Smeraldi *et al.* 2001).

D'altra banda, s'ha observat que la desaminació dels metabòlits 3-Iodotironamina (T(1)AM) i 3,3',5-triiodotironamina (T(3)AM) (**metabòlits de la hormona tiroxina** (T4)) és duta a terme per una activitat amino oxidasa inhibida per *iproniazid*, un inhibidor comú a SSAO i a MAO, però no s'ha acabat de confirmar si es tracta d'una amino oxidasa o l'altra (Wood *et al.* 2009).

La SSAO també és capaç de catalitzar la desaminació oxidativa d'alguns **xenobiòtics** (substàncies normalment exògenes a l'organisme) (Strolin *et al.* 2007; Tipton KF and Strolin Benedetti M 2001). Alguns exemples en són l'al·lilamina, que és una amina alifàtica insaturada que prové de l'activitat industrial, i és convertida en l'aldehid altament tòxic acroleïna per acció de la SSAO (Boor *et al.* 1990), o la metilamina, que a part de ser una amina endògena pot provenir també d'origen exogen a través de la inhalació del fum del tabac (Yu and Deng 1998) o de productes de la ingesta (Zeisel *et al.* 1983), i és metabolitzada a formaldehid, també altament tòxic. A més, el metabolisme de fàrmacs com la mescalina, l'antimalàric "primaquina", o el tresperimus (Cellimis) (un agent immunosupressor), depèn de l'activitat SSAO (Claud *et al.* 2001).

Molts dels substrats de la SSAO són també substrats d'altres amino oxidases. En aquest sentit, la metilamina i l'aminoacetona (figura 4.b i c) són els substrats més exclusius de la SSAO. La metilamina pot provenir de diverses reaccions catabòliques, com ara del metabolisme de la sarcosina, de la lecitina, de la colina i la creatina o també del metabolisme de l'adrenalina, el qual és dut a terme per la MAO A (Yu *et al.* 1997; Yu and Deng 2000; Zeisel *et al.* 1983). La relació SSAO-MAO A és rellevant en situacions d'estrès, durant les quals s'alliberen elevades quantitats d'adrenalina, que

és metabolitzada per la MAO A generant metilamina, i aquesta és alhora metabolitzada per la SSAO generant formaldehid (Yu *et al.* 1997). La creatina també pot estar present en quantitats elevades en casos en els que aquesta es prengui com a complement nutricional, generant metilamina en el seu procés catabòlic (Yu and Deng 2000). L'aminoacetona, per la seva banda, pot provenir del metabolisme de la treonina o de la glicina, i és oxidada per la SSAO per generar metilglioxal (Mathys *et al.* 2002), un agent citotòxic i mutagènic.

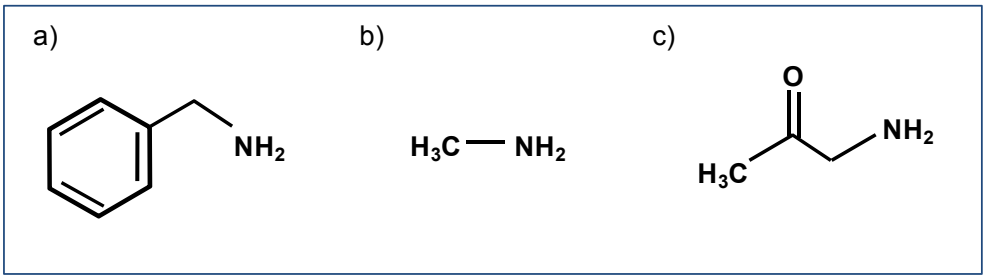


Figura 4. Estructura dels principals substrats de la SSAO: la Benzilamina (exogen) (a), la Metilamina (b) i l'Aminoacetona (c) (endògens).

Recentment s'estan buscant noves molècules que puguin ser substrats de la SSAO amb finalitat terapèutica per als pacients amb diabetis, donat l'efecte insulinomimètic que mostra l'activitat SSAO. Mitjançant modelatge per homologia del domini catalític de la SSAO de ratolí i la cerca per bases de dades de substàncies químiques, s'han identificat nous substrats de la SSAO (Marti *et al.* 2004), amb diferent afinitat per diferents espècies d'SSAOs (Yraola *et al.* 2006). Amb el mateix objectiu, s'han sintetitzat sals de vanadi que combinen els efectes insulinomimètics de l'administració de substrats de la SSAO i vanadat (Yraola *et al.* 2007b). Dades provinents d'aquests estudis poden permetre el disseny de substrats més específics per les SSAOs que per altres amino oxidases, i també per les SSAOs humanes en front a les d'altres espècies (Yraola *et al.* 2009).

2.3.2 Inhibidors de la SSAO

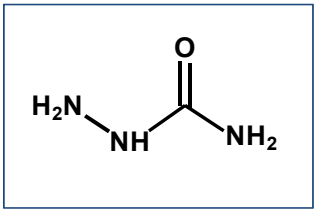
Degut al solapament de substrats existent entre la SSAO i les isoformes de la MAO, és important disposar d'inhibidors selectius dels diferents enzims per poder estudiar els efectes de l'activitat de cadascun d'ells. Durant molt temps, però, el disseny i síntesi d'inhibidors específics de la SSAO no ha estat un tema d'interès, de manera que els

existents clàssicament eren compostos desenvolupats amb altres finalitats, la majoria afectant també l'activitat MAO. D'altra banda, i com ocorre amb els substrats, molts inhibidors de la SSAO tenen diferent capacitat inhibitòria dependent de l'espècie (Lyles 1996). Per aquest motiu és important determinar les constants d'inhibició en diferents teixits i espècies.

Els inhibidors clàssics de la SSAO es poden classificar en els grups principals següents: hidrazina i els seus derivats, arilalquilamines, haloalilamines i haloalquilamines (revisat per(Lyles 1996) i (Matyus *et al.* 2004)).

Menció a part mereix la **semicarbazida**, el compost de tipus hidrazina que ha definit clàssicament les SSAOs (figura 5) (TABOR *et al.* 1954), la qual a concentracions de 0.1 – 1 mM inhibeix la SSAO sense tenir gaires efectes sobre la MAO (Lewinsohn *et al.* 1978). Així mateix, les SSAOs s'han definit també per ser insensibles a la inhibició per les amines aromàtiques acetilèniques pargilina, clorgilina i deprenil (selegilina) a concentracions 0.1 – 1 mM, a les quals produeixen una inhibició completa i irreversible d'ambdues formes de la MAO (Lyles 1984). Tot i així, s'ha observat que la clorgilina i el deprenil 1 mM són capaços d'inhibir la SSAO plasmàtica humana (Eriksson and Fowler 1984; Lewinsohn *et al.* 1978), i que la clorgilina 0.1 – 0.8 mM pot actuar com a inhibidor reversible competitiu dèbil de la SSAO plasmàtica bovina purificada (Houslay and Tipton 1975).

Figura 5. Estructura de la Semicarbazida.



Un altre compost no derivat d'hidrazina més potent i selectiu que la semicarbazida és la hidroxilamina (NH_2-OH), la qual inhibeix la SSAO d'aorta de rata a 1 μM, sense afectar les activitats MAOs fins a concentracions de 10 mM (Lyles and Flagg 1984).

Altres inhibidors de la SSAO **derivats d'hidrazina** són: l'aminoguanidina (inhibidor de la òxid nítric sintasa; utilitzada en el tractament del la diabetis mellitus) (Tang *et al.* 1989); la benserazida i la carbidopa (inhibidors de la DOPA descarboxilasa utilitzats en el tractament del Parkinson) (Andree and Clarke 1982; Lewinsohn *et al.* 1978; Lyles and Callingham 1982); els compostos nialamida, iproniazida, fenelzina i fenilhidrazina (inhibidors irreversibles de les MAOs utilitzats en el tractament de la depressió)

(Barrand and Callingham 1982; Lizcano *et al.* 1996); la hidralazina (agent antihipertensiu) (Lyles *et al.* 1983); la procarbazina (agent carcinostàtic) (Holt and Callingham 1994); o la isoniazida (fàrmac antituberculós) (BLASCHKO 1962).

Els derivats d'hidrazina mencionats, tot estar entre els mes potents inhibidors de la SSAO, generen controvèrsia a l'hora de ser utilitzats per a aplicacions terapèutiques degut a la seva toxicitat a nivell hepàtic, sobretot en administracions a llarg termini.

Dins els **derivats d'arilalquilamines** es troba el B24 (3,5-etoxi-4-aminometilpiridina), un anàleg piridínic de la benzilamina que va resultar un inhibidor potent i selectiu de la SSAO a concentracions micromolars (Banchelli *et al.* 1990); més tard es va observar que en realitat és un substrat dèbil de l'enzim (Buffoni *et al.* 1998). Els anàlegs del fàrmac antiarítmic metilexina van resultar també potents inhibidors de la SSAO, encara que no prou específics i essent la metilexina, alhora, un inhibidor reversible competitiu per les MAOs (Lyles 1984). Alguns d'aquests anàlegs són el FLA 336(-), potent inhibidor de la SSAO, els FLA 788(+) i FLA 668(+), menys potents i selectius, o l'FLA 1402, potent inhibidor en cor i pulmó de rata però inactiu per a la SSAO plasmàtica humana (Eriksson and Fowler 1984; Fowler *et al.* 1984).

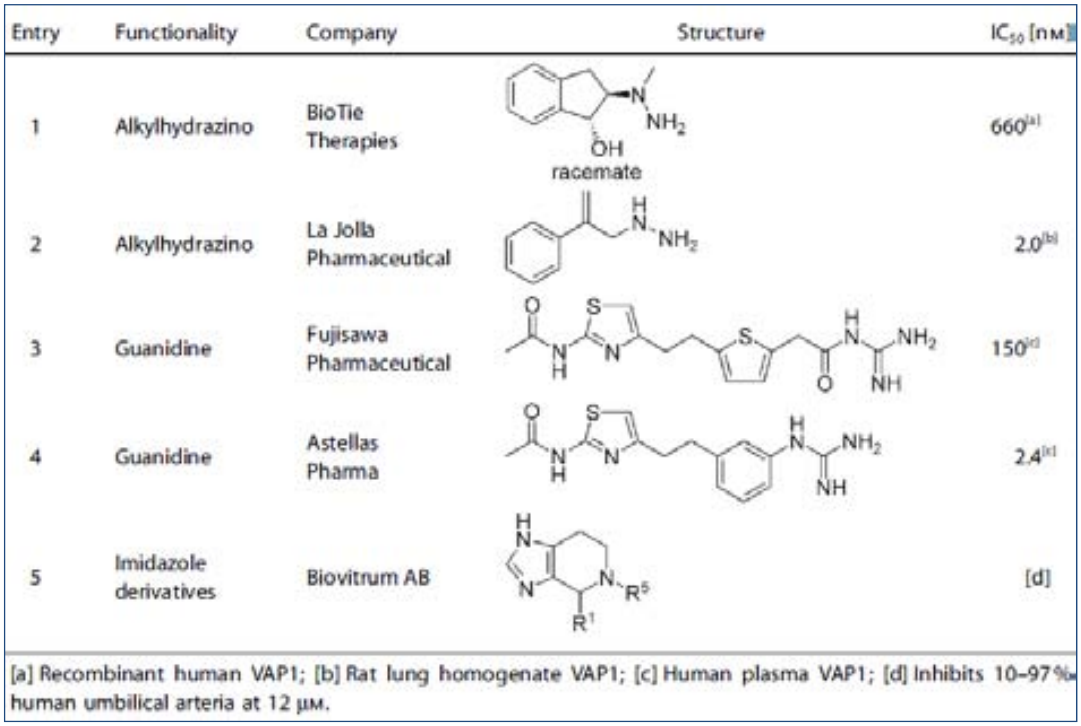
Un altre grup de compostos amb capacitat d'inhibir la SSAO són els **derivats d'haloalilamina** (propenilamines i propargilamines), els quals van ser sintetitzats originàriament buscant inhibidors irreversibles de les MAOs. Alguns exemples són la propargilamina, que inhibeix la SSAO de plasma humà (Rando and De 1974) o l'MDL 72145, que és un potent inhibidor de la SSAO d'aorta de rata, però menys potent de la de plasma i arteria umbilical humans (Yu *et al.* 1994b) i que també inhibeix la MAO B (Zreika *et al.* 1984). En aquest grup destaca l'MDL 792974A [(E)-2-(4-fluorofenetil)-3-fluoroalilamina], inhibidor irreversible de la SSAO tant en teixits vasculars com en plasma, i 1000 vegades més efectiu que la semicarbazida, amb una IC_{50} de 10^{-7} M per a la SSAO plasmàtica i 10^{-8} M per a la tissular (Meszaros *et al.* 2000; Palfreyman *et al.* 1994). Presenta també, però, una inhibició irreversible de la MAO B (Lyles *et al.* 1987; Yu and Zuo 1992).

Per una altra banda es troben els **derivats d'haloalquilamines**: els compostos 2-Bromoetilamina i 3-Bromopropilamina, son potents i inhibidors de la SSAO (Kinemuchi *et al.* 2000; Kinemuchi *et al.* 2001). El primer d'aquests es considera el primer inhibidor selectiu de la SSAO, sense actuar sobre la MAO; a més, ha mostrat també efectivitat *in vivo* (Yu *et al.* 2001). Es un inhibidor irreversible de tipus suïcida, i es considera una eina útil en l'estudi de la SSAO.

A part dels grups detallats anteriorment, cal mencionar també la capacitat inhibitòria que tenen alguns compostos antidepressius sobre la SSAO. La imipramina és el més potent d'aquests, seguit d'altres com la maprotilina, zimeldina i nomifensina (Obata and Yamanaka 2000b). En aquest cas està en dubte si la capacitat d'inhibició de la SSAO per aquests compostos té o no relació amb la seva acció farmacològica.

Degut a la implicació de la SSAO en processos patològics, sobretot en diabetis i en patologies inflamatòries, la cerca d'inhibidors potents i selectius de la SSAO s'ha incrementat. Aquesta activitat aportat nous pèptids sintètics derivats d'hidrazina dissenyats en base a models moleculars del centre actiu de la SSAO (Lazar *et al.* 2004; Yegutkin *et al.* 2004), o derivats d'1,3,4-oxadiazina (Fülöp *et al.* 2004). Entre aquests compostos es troben el BTT-2027, altament selectiu (Koskinen *et al.* 2004), els BTT-2042 i BTT-2052, els quals ja han estat utilitzats *in vivo* (Marttila-Ichihara *et al.* 2006; Stolen *et al.* 2004a), la sèrie de molècules com l'LJP 1207 (O'Rourke *et al.* 2007; Salter-Cid *et al.* 2005; Wang *et al.* 2006) i l'LJP 1586 (O'Rourke *et al.* 2008) o la molècula U-V002, que reacciona amb la topaquinona de la SSAO (Noda *et al.* 2008a). També s'han aconseguit nous inhibidors mitjançant la transformació de substrats coneguts, els anomenats "*substratelike*" (Bertini *et al.* 2005). D'altra banda, una de les últimes estratègies emprades per al desenvolupament de nous inhibidors de la SSAO ha estat la generació d'anticossos que s'uneixen al domini extracel·lular de la proteïna, bloquejant la seva funció inflamatòria però no l'enzimàtica (Kirton *et al.* 2005), el tractament amb els quals ja ha estat avaluats assajos clínics en fase I (Vainio *et al.* 2005).

L'interès creixent en el desenvolupament de nous inhibidors de la SSAO (revisat per (Yraola *et al.* 2007a)) queda pal·lès en el fet que nombroses companyies farmacèutiques s'hi troben implicades (figura 6), i en el nombre de patents publicades referents a aquests compostos (Caldirola *et al.* 2002; Kivi *et al.* 2008; Smith D.J. *et al.* 2003; Smith *et al.* 2002b; Smith *et al.* 2002a; Takayuki I *et al.* 2004; Takayuki I *et al.* 2006).



Entry	Functionality	Company	Structure	IC_{50} [nM]
1	Alkylhydrazino	BioTie Therapies	racemate	660[a]
2	Alkylhydrazino	La Jolla Pharmaceutical		2.0[b]
3	Guanidine	Fujisawa Pharmaceutical		150[c]
4	Guanidine	Astellas Pharma		2.4[c]
5	Imidazole derivatives	Biovitrum AB		[d]

[a] Recombinant human VAP1; [b] Rat lung homogenate VAP1; [c] Human plasma VAP1; [d] Inhibits 10–97% human umbilical arteria at 12 μM.

Figura 6. Molècules inhibidores de la SSAO/VAP-1 desenvolupades en els darrers anys per diverses companyies farmacèutiques. Es mostra el tipus d'inhibidor, companyia farmacèutica, estructura i IC_{50} de cada molècula. Figura extreta de l'article (Yraola *et al.* 2007a).

2.4. Distribució de la SSAO

La SSAO és un enzim àmpliament distribuït en la natura com s'ha descrit anteriorment, essent present en plantes, microorganismes i mamífers, incloent l'espècie humana. En mamífers, la SSAO es troba en un gran nombre de teixits de diversos òrgans i també en forma soluble en el plasma sanguini (Lyles 1996). La presència i activitat de l'enzim en els diferents teixits i en el plasma però, difereix entre teixits i entre espècies (Andres *et al.* 2001): de forma general, per exemple, s'ha observat que els teixits humans són més rics en SSAO que els teixits de rata o porc (Boomsma *et al.* 2000b).

2.4.1 SSAO tissular

La distribució tissular de la SSAO en mamífers ha estat estudiada en diverses ocasions (veure (Andres *et al.* 2001; Boomsma *et al.* 2000b; Lewinsohn *et al.* 1978; Salmi *et al.* 1993) per revisions extenses), tant per mètodes immunohistològics com per determinació de l'activitat enzimàtica. En treballs en els que s'han utilitzat ambdues

tècniques s'ha observat bona correlació entre la detecció immunohistològica i la mesura de l'activitat (Andres *et al.* 2001).

De forma general, la SSAO es troba en la majoria de teixits perifèrics humans, associada fonamentalment a la vasculatura, (múscul llis i endoteli, sobretot l'associat als òrgans limfoides) i al teixit adipós blanc i marró (Barrand and Fox 1984; Lewinsohn *et al.* 1978; Salmi *et al.* 1993). Altres teixits no vasculars amb notable expressió i/o activitat SSAO són el pulmó, intestí, placenta, bufeta biliar, glàndula adrenal i ronyó; amb baixos o indetectables nivells d'SSAO es troben el cor, fetge, melsa, pàncrees i tiroides. Aquestes dades es corresponen amb les obtingudes en la determinació de l'RNA de la proteïna en diferents teixits humans (Smith *et al.* 1998; Su *et al.* 2002) (figura 7).

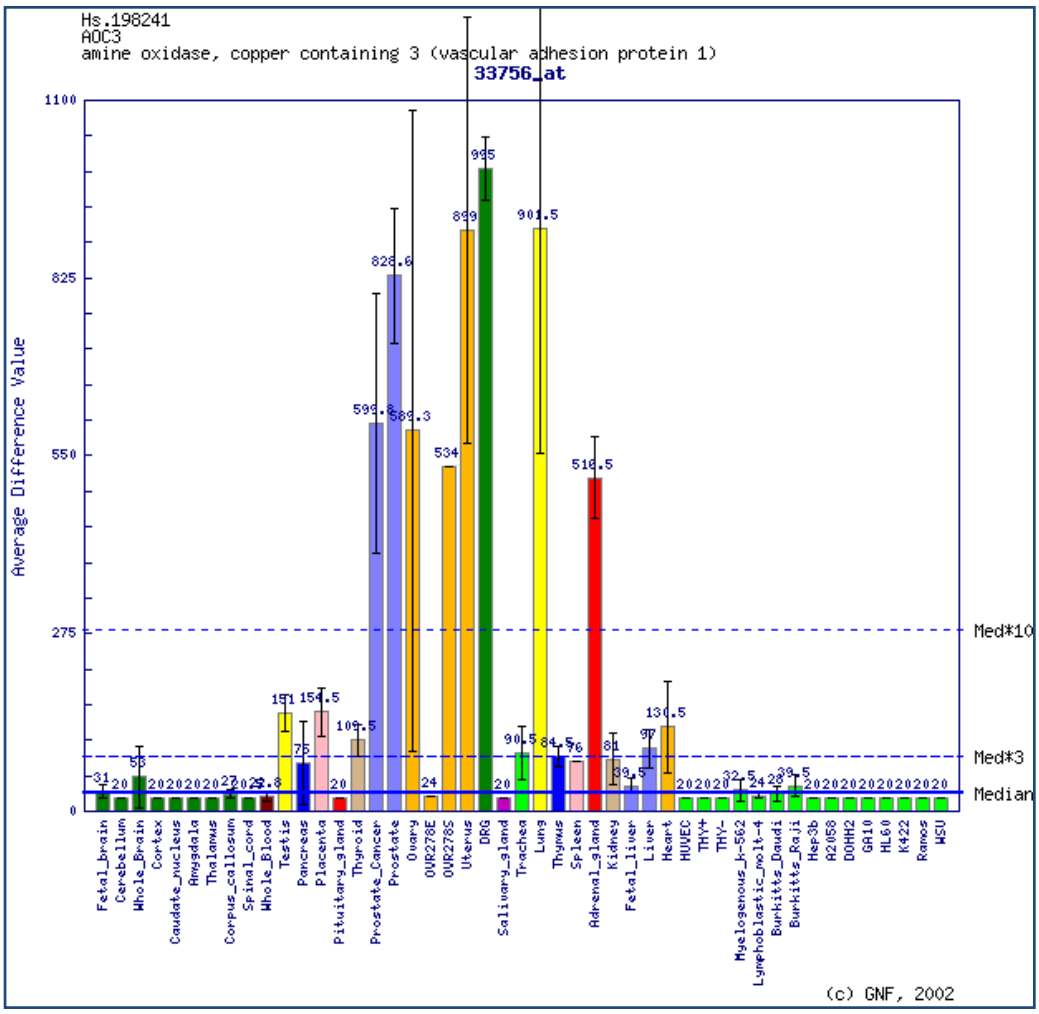


Figura 7.Nivells d'expressió de l'RNA del gen AOC3de la SSAO humana en diferents teixits. Extret de la pàgina web http://expression.gnf.org, creada pels autors de l'article (Su *et al.* 2002).

De forma més específica i dependent de l'espècie s'ha identificat també la presència d'SSAO en condròcits del cartílag articular en rata (Lyles and Bertie KH 1987), en odontoblasts de polpa dental porcina (Norqvist *et al.* 1981) i humana (O'Sullivan *et al.* 2002), en diferents parts de l'ull boví (Fernandez de *et al.* 1991), en les cèl·lules dendrítiques dels centres germinals en humans (Salmi *et al.* 1993), i en l'úter, urèter i conducte deferent humans (Lewinsohn 1981), i recentment en macròfags de rata (Vega *et al.* 2004).

En referència a la vasculatura perifèrica, la SSAO es troba majoritàriament associada a la capa de múscul llis de la túnica media i a la capa íntima endotelial (on es va descriure com a VAP-1). En els vasos sanguinis propers als nodes limfàtics i del cordó umbilical humà, l'expressió de la SSAO/VAP-1 és notable en la superfície de les cèl·lules endotelials (Salmi *et al.* 1993), mentre que en la resta de teixit vascular endotelial s'expressa preferentment en vesícules intracel·lulars, i s'indueix la seva translocació en condicions d'inflamació (Bono *et al.* 1998a; Salmi *et al.* 1993).

En el sistema vascular cerebral humà i boví, tant les cèl·lules del múscul llis de les meninges (túnica media i adventícia) com les cèl·lules endotelials de la capa íntima de les meninges i els microvasos mostren expressió i activitat SSAO (Airas *et al.* 2008; Castillo *et al.* 1998). L'expressió cerebrovascular de la SSAO ha estat observada també en rata (Obata and Yamanaka 2000a) i en primats (Obata and Yamanaka 2000b). Diferències entre espècies mostren que el teixit cerebrovascular humà presenta una activitat més elevada que el boví, i diferències entre teixits, mostren que les meninges tenen més activitat que els microvasos en ambdues espècies. A més, cal destacar que la constant cinètica K_m de la SSAO humana obtinguda en teixits cerebrovasculars és notablement més baixa que la d'altres teixits perifèrics humans (Castillo *et al.* 1998; Unzeta *et al.* 2007). A part de la vasculatura, l'expressió d'SSAO/VAP-1 sembla absent en la resta de teixit cerebral (Castillo *et al.* 1998), encara que s'ha trobat expressió de l'enzim en un tipus de teixit nerviós, concretament en les fibres nervioses de la polpa dental (O'Sullivan *et al.* 2002).

D'altra banda, estudis recents sobre l'expressió de la SSAO/VAP-1 a nivell de desenvolupament humà (Salmi and Jalkanen 2006) i de ratolí (Valente *et al.* 2008) destaquen una expressió regulada espaial i temporalment, amb rellevància en els processos de desenvolupament i maduració del sistema vascular. La proteïna expressada a nivell fetal és també activa enzimàticament. En aquest sentit, tot i l'aparent rellevant paper que jugaria la SSAO en desenvolupament, animals *knock out* per la SSAO/VAP-1 no presenten anormalitats de desenvolupament evidents, encara

que no s'han fet estudis estructurals o funcionals durant aquest procés en aquests animals (Stolen *et al.* 2005).

Cal mencionar també que la majoria de línies cel·lulars immortalitzades no presenten expressió ni activitat de la SSAO (BLASCHKO 1962; Owens 1995; Yu and Zuo 1993), i només alguns cultius primaris expressen baixos nivells d'SSAO intracel·lular (Salmi and Jalkanen 1995).

2.4.2 Localització subcel·lular de la SSAO tissular

La SSAO es localitza majoritàriament associada a la membrana plasmàtica de les cèl·lules (Lyles 1996), i distribuïda bàsicament en les estructures anomenades caveoles (figura 8) (Aboulaich *et al.* 2004; Jaakkola *et al.* 1999; Souto *et al.* 2003). Les caveoles conformen un subtipus de *lipid rafts* (rais lipídics), subdominis membranals de composició lipoproteica específica (Brown and London 2000). Les caveoles són invaginacions de la membrana plasmàtica, d'uns 50 - 100 nm, de composició lipídica específica rica en esfingolípids i colesterol, amb funcions específiques en el transport lipídic entre altres; les proteïnes caveolines són les que els confereixen aquesta estructura.

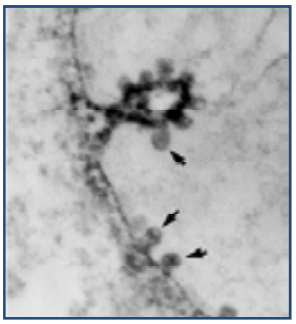


Figura 8. Immunolocalització de la SSAO en les caveoles de les cèl·lules de múscul llis mitjançant microscòpia electrònica. Figura extreta de l'article (Jaakkola *et al.* 1999).

En alguns casos, la SSAO s'observa a nivell intracel·lular en cèl·lules endotelials (Jaakkola *et al.* 1999; Salmi and Jalkanen 1995) (figura 9), i en adipòcits, en aquestes últimes associada a vesícules del compartiment endosomal juntament amb el transportador de glucosa GLUT4 (Enrique-Tarancon *et al.* 1998; Morris *et al.* 1997). D'altra banda, s'ha observat també tinció immunohistològica en el citosol de diversos tipus cel·lulars (Andres *et al.* 2001). En moltes ocasions, però, la seva localització subcel·lular s'ha definit a partir de preparacions microsomals, amb les quals no es pot distingir clarament la membrana plasmàtica de les vesícules o orgànuls intracel·lulars

(Barrand and Callingham 1982; Lizcano *et al.* 1990a; Norqvist and Oreland 1989; Wibo *et al.* 1980).

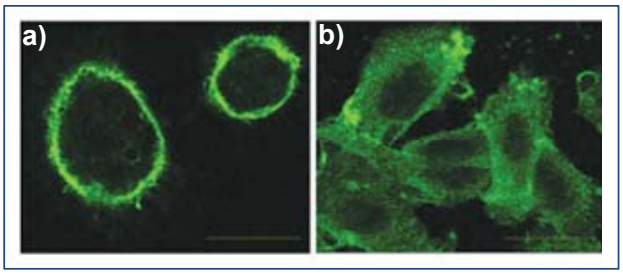


Figura 9. Immunolocalització de la SSAO/VAP-1 a la membrana (**a**) (cèl·lules permeabilitzades amb saponina) i al citosol (**b**) de cèl·lules CHO transfectades amb la VAP-1. Figura extreta de l'article (Yegutkin *et al.* 2004)

2.4.3 Diferències entre SSAOs vasculars (endoteli i múscul llis)

Tot i ser codificada pel mateix gen, la SSAO expressada en diferents teixits es comporta diferent, essent-ne un clar exemple la SSAO expressada en múscul llis i endoteli (VAP-1) del teixit vascular (Jaakkola *et al.* 1999).

A nivell de regulació de l'expressió, les dues formes de la proteïna necessiten un microambient intacte per ser expressades, ja que no s'observen nivells d'expressió notables de la SSAO ni en línies cel·lulars ni en cultius primaris de cèl·lules provinents de l'endoteli o del múscul llis (Jaakkola *et al.* 1999; Salmi and Jalkanen 1995). La SSAO/VAP-1 endotelial, però, és induïble en condicions inflamatòries (Salmi *et al.* 1993), a diferència de la SSAO del múscul llis vascular que s'expressa de forma constitutiva, i no modifica la seva expressió en zones pro inflamatòries, com ara les pròximes a una placa d'ateroma (Jaakkola *et al.* 1999). Aquestes dades indiquen que la regulació de l'expressió de la SSAO en les cèl·lules endotelials i del múscul llis és diferent.

A nivell estructural, l'homodímer endotelial mostra una mobilitat electroforètica més baixa que el corresponent a cèl·lules de múscul llis. A més, en les cèl·lules musculars s'observa una forma trimèrica de la proteïna, que no es detecta en les endotelials. D'altra banda, ambdues proteïnes contenen modificacions post traduccionals en forma d'àcids siàlics, O-glicans, i N-glicans (Maula *et al.* 2005), però en diferent proporció (Jaakkola *et al.* 1999).

A nivell funcional, ambdues proteïnes presenten activitat SSAO. En canvi, només la SSAO/VAP-1 endotelial participa en el procés inflamatori (Smith *et al.* 1998) ja que la proteïna muscular no és capaç de facilitar la unió de limfòcits a la seva superfície per permetre'n l'extravasació als teixits (Jaakkola *et al.* 1999).

2.4.4 SSAO plasmàtica

La forma soluble de la SSAO/VAP-1 es troba en el plasma sanguini. Durant molt temps hi ha hagut controvèrsia sobre l'origen d'aquesta forma soluble, considerant-se que podia venir codificada per un gen diferent a la forma transmembranal, o que podia provenir d'un tall de la forma transmembranal que l'alliberés com a proteïna soluble. Avui es coneix que ambdues hipòtesis són certes, encara que poden o no coexistir.

D'una banda, algunes espècies de mamífers com cabra, ovella, porc, cavall, vaca o gos presenten dues entitats diferents d'SSAO en el plasma (Boomsma *et al.* 2000b), una de les quals sembla que està codificada per un gen diferent al de la SSAO/VAP-1 tissular, que no és funcional en espècies que només presenten una SSAO plasmàtica (pseudogen AOC4), com ara rosegadors i humans (Schwelberger 2007). La presència d'ambdues formes enzimàtiques es tradueix en uns nivells més elevats d'activitat SSAO present en el plasma d'aquestes espècies; així mateix, els rosegadors rata i ratolí tenen els nivells més baixos d'activitat SSAO plasmàtica, per sota dels humans (Boomsma *et al.* 2000b).

D'altra banda, la forma d'SSAO plasmàtica que prové del gen AOC3 (la única observada en rosegadors i en humans) prové d'un tall proteolític de la forma transmembranal, probablement dut a terme per una activitat metal·loproteasa (Abella *et al.* 2004; Gokturk *et al.* 2003; Stolen *et al.* 2004b). Confirma aquesta hipòtesi el fet que la seqüència N-terminal de la SSAO soluble purificada del plasma humà és idèntica a la seqüència distal a la membrana de la seva forma transmembranal (Kurkijarvi *et al.* 2000). En condicions fisiològiques s'ha observat que la font tissular de la SSAO podria ser l'endoteli, però els adipòcits podrien alliberar-ne també en condicions d'estrès biològic (Stolen *et al.* 2004b). Les cèl·lules del múscul llis també són capaces d'alliberar la forma soluble de la SSAO (Gokturk *et al.* 2003).

Els estudis destinats a quantificar els nivells d'SSAO plasmàtica s'han dut a terme majoritàriament determinant l'activitat enzimàtica de la SSAO, però en alguns s'han utilitzat també tècniques d'ELISA, que han mostrat una bona correlació entre ambdues mesures (Salmi and Jalkanen 2006). Aquests estudis han permès associar que aproximadament 1 ng de VAP-1/SSAO pot convertir 1.6 pmols de benzaldehid per minut (Boomsma *et al.* 2003).

Respecte als nivells basals d'activitat SSAO plasmàtica en humans, no s'han trobat diferències significatives entre gèneres (Boomsma *et al.* 2003; del Mar Hernandez *et*

al. 2005). Tanmateix, ratolins mascles amb sobreexpressió endotelial de la SSAO/VAP-1 presenten nivells més elevats d'activitat SSAO plasmàtica que les femelles (Stolen *et al.* 2004b). Aquests autors suggereixen que situacions de sobreexpressió de la SSAO/VAP-1 podria accentuar diferències petites reals entre gèneres, i associen aquest fet a la incidència més elevada d'aterosclerosi en homes que en dones. Tot i així, dades d'activitat SSAO de pacients d'Alzheimer, els quals tenen un increment de l'activitat plasmàtica d'SSAO, no mostren tampoc diferències entre gèneres (del Mar Hernandez *et al.* 2005).

L'edat sí que s'ha associat a canvis en els nivells plasmàtics (Lewinsohn *et al.* 1980). En primer lloc, el sèrum fetal conté una activitat SSAO lleugerament més elevada que el sèrum adult (Salmi and Jalkanen 2006). A més, els nivells d'SSAO plasmàtica són més elevats en la infantesa i adolescència, i a partir dels 16 anys d'edat cauen aproximadament un 50% per mantenir-se en nivells baixos fins als 50-60 anys d'edat, moment en que incrementen a poc a poc una altra vegada (Boomsma *et al.* 1999; Boomsma *et al.* 2003; Tryding *et al.* 1969). En adults sans, l'activitat SSAO plasmàtica intraindividual varia poc. Interindividualment, els valors d'activitat varien entre 350 – 400 mU/L ± 100 mU/L, corresponent 1 mU/L als picomols de benzaldehid generats per mil·lilitre per minut (Boomsma *et al.* 2003). Tot i així, existeixen diverses condicions patològiques en les quals l'activitat SSAO plasmàtica es troba modificada (veure Introducció, apartat 2.7).

2.5. Funcions de la SSAO

Durant molt temps, el paper biològic de la SSAO en mamífers ha estat poc clar i restringit a la destoxificació d'amines (Lyles 1996), però més tard se li han anat atribuint altres funcions. La SSAO ha estat definida com una proteïna "multifuncional" dependent del teixit on s'expressa. La majoria d'aquestes múltiples funcions venen determinades pels productes de la seva activitat catalítica, així que es podria definir com un enzim amb activitat amino oxidasa, els productes de la qual tenen diferents efectes segons el teixit on es trobin.

Com a amino oxidasa, la SSAO participa en el metabolisme d'amines endògenes així com de diversos xenobiòtics com la mescalina o l'al·lilamina (Tipton KF and Strolin Benedetti M 2001). En aquest sentit, l'elevada activitat SSAO present en el pulmó contribueix al metabolisme de la metilamina inhalada (en forma de contaminant

ambiental) i d'altres amines volàtils (Lizcano *et al.* 1998). D'altra banda, els productes formats durant la reacció enzimàtica també tenen funcions fisiològiques. El peròxid d'hidrogen, per exemple, pot actuar com a "missatger intra o intercel·lular" en concentracions baixes (Burdon 1995; Kunsch and Medford 1999), però nivells crònicament elevats són una font d'espècies reactives d'oxigen que pot causar citotoxicitat (Kanno *et al.* 1999), així com l'amoni o els aldehids (Conklin *et al.* 1999; Deng *et al.* 1998; Mathys *et al.* 2002; Yu 1998; Yu and Deng 2000).

Altres funcions fisiològiques que han estat atribuïdes a la forma transmembranal de la SSAO/VAP-1 són el seu paper en l'adhesió dels limfòcits i granulòcits a l'endoteli i extravasació d'aquests (Smith *et al.* 1998), el seu efecte insulinomimètic (El Hadri *et al.* 2002; Enrique-Tarancon *et al.* 1998), la participació en la regulació del creixement i maduració d'alguns tipus cel·lulars (Moldes *et al.* 1999), el seu paper en el manteniment i deposició de la matriu extracel·lular en el múscul llis (Langford *et al.* 2002), el tràfic lipídic en els adipòcits (Souto *et al.* 2003) o la unió a fàrmacs (Novotny *et al.* 1994); aquestes funcions es detallen a continuació.

2.5.1 Adhesió i tràfic limfocitari i granulocitari a través de l'endoteli.

La SSAO endotelial es va identificar com a proteïna d'adhesió vascular (VAP-1), i en clonar-la es va veure que es tractava de la mateixa SSAO (Smith *et al.* 1998). La VAP-1 es va identificar com una glicoproteïna que promou l'adhesió dels limfòcits i granulòcits a les cèl·lules endotelials en situacions d'inflamació, i la recirculació normal d'aquests de manera independent al lligand leucocitari L-selectina (Salmi *et al.* 1993; Salmi *et al.* 1997; Tohka *et al.* 2001). En les cèl·lules endotelials, sembla que la SSAO/VAP-1 es troba emmagatzemada en gränuls intracel·lulars, i en condicions inflamatòries transloca a la superfície luminal de l'endoteli (Jaakkola *et al.* 2000b), però els mecanismes moleculars implicats en aquesta procés són encara desconeguts.

El procés d'extravasació de les cèl·lules immunitàries del torrent sanguini cap als teixits en condicions d'inflamació és un procés amb múltiples passos en els que hi participen tant l'endoteli com els leucòcits (Springer 1994). Primer, les selectines i els seus lligands promouen els processos anomenats *tethering* i *rolling*, que fan que es redueixi la velocitat dels leucòcits pel flux sanguini. Després succeeix el *triggering,*en el que les quimiocines i els seus receptors activen les integrines dels leucòcits, permetent l'adhesió ferma de la cèl·lula a l'endoteli. Finalment, les integrines i altres molècules

promouen la *transmigració* del leucòcit a través de les unions intercel·lulars de l'endoteli (figura 10). La SSAO/VAP-1 participa com a "ecto-enzim" en els processos de *rolling*, *adhesió ferma* i *transmigració* contribuint al reclutament leucocitari (Jalkanen and Salmi 2008; Stolen *et al.* 2005; Yegutkin *et al.* 2004).

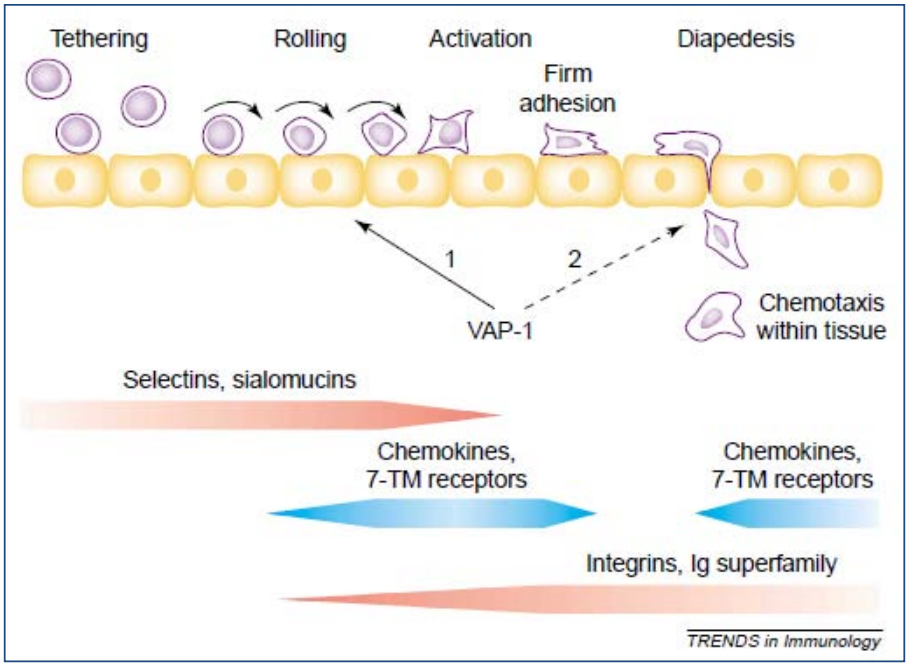


Figura 10. Esquema dels múltiples passos que constitueixen el procés d'extravasació leucocitària. S'indiquen les diferents fases de migració i les famílies proteiques més rellevants implicades en cadascuna. 7-TM receptor (receptor serpentina de 7 segments transmembrana acoblada a proteïna G). Figura extreta de l'article de revisió (Salmi and Jalkanen 2001).

El mecanisme pel qual la SSAO/VAP-1 participa en aquest procés sembla ser dual. D'una banda, de forma enzimàtica-independent la VAP-1 funcionaria com una molècula d'adhesió per ella mateixa a través d'epítops específics (Koskinen *et al.* 2004; Salmi and Jalkanen 1996b). S'ha postulat que alguns d'aquests epítops podrien ser carbohidrats presents en la superfície dels leucòcits (Salmi *et al.* 1997) o L-lisines (Olivieri *et al.* 2007). D'altra banda, l'activitat enzimàtica també contribuiria a la unió del leucòcit a l'endoteli (Koskinen *et al.* 2004), i vindria determinada per la unió covalent transitòria que es produeix entre l'enzim i el seu substrat durant la reacció enzimàtica (Dooley *et al.* 1991; Hartmann *et al.* 1993). Els seus substrats serien grups amino lliures de sucres o proteïnes de la superfície del leucòcit (Yegutkin *et al.* 2004). A més, s'ha postulat que la SSAO podria oxidar també lligands de les E-selectines en els leucòcits i lligands de les L-selectines en les cèl·lules endotelials, estabilitzant la unió amb els seus lligands (Ley and Deem 2005) (figura 11).

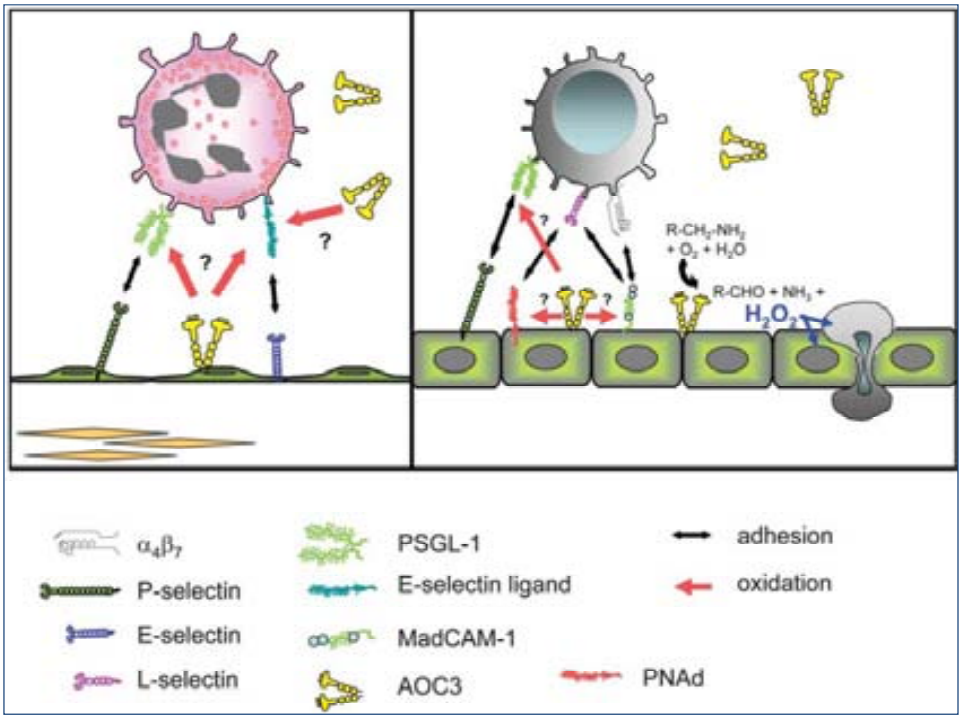


Figura 11. Esquema de la possible modulació que exerciria la SSAO/VAP-1 sobre la unió leucòcit-endoteli a través de la oxidació de lligands d'E i L selectines. L'H_2O_2 generat durant la reacció enzimàtica podria contribuir també a les senyals necessàries per a la transmigració leucocitària. Figura extreta de l'article (Ley and Deem 2005).

A més d'aquests efectes "directes", els productes biològicament actius derivats de la reacció enzimàtica, principalment el peròxid d'hidrogen, tenen efectes en el microambient local (Okayama *et al.* 1998; True *et al.* 2000). En aquest sentit, s'ha observat que l'activitat SSAO activa vies de senyalització que indueixen la transcripció i traducció de les selectines E i P endotelials o la ICAM-1 en models *in vitro* i *in vivo*, les quals promouen l'adhesió limfocitària (Jalkanen *et al.* 2007). En l'endoteli hepàtic les vies moleculars que, induïdes per l'activitat SSAO generen aquesta activació endotelial, passen per l'activació de la via de la PI3K o de la MAPK – p38, que indueixen la translocació al nucli del factor de transcripció NF-κB, el qual promou l'activació de gens pro inflamatoris. Els gens que s'han vist activats en aquest sistema han estat la E-selectina, la ICAM-1 i també la secreció de la IL-8 (Lalor *et al.* 2007). En endoteli pulmonar s'ha observat també que l'activitat pro inflamatòria generada pels productes de la SSAO incrementa els nivells de citocines pro inflamatòries com MIP-1α, G-CSF, TNF-α i IL-6 (Yu *et al.* 2006).

D'altra banda, la utilització d'anticossos anti-VAP-1 en models animals de malalties inflamatòries impedeix parcialment l'extravasació leucocitària als teixits inflamats (Martelius *et al.* 2004; O'Rourke *et al.* 2007; Tohka *et al.* 2001), i alleuja la inflamació en models de colitis (Salter-Cid *et al.* 2005), inflamació de la pell (Koskinen *et al.* 2004), sèpsies (Kinemuchi *et al.* 2004), artritis (Marttila-Ichihara *et al.* 2006), encefalomielitis al·lèrgica experimental (EAE) (O'Rourke *et al.* 2007), inflamació pulmonar (Kinemuchi *et al.* 2004) o isquèmia cerebral (Xu *et al.* 2006). Aquests efectes s'observen també en ratolins KO per la VAP-1, els quals presenten defectes en la recirculació limfocitària fisiològica i en la resposta inflamatòria (Bour *et al.* 2009; Marttila-Ichihara *et al.* 2006; Stolen *et al.* 2005).

2.5.2 Participació en l'homeòstasi de la glucosa: efecte insulinomimètic.

La oxidació de substrats de la SSAO té un efecte insulinomimètic, exercit per l'H_2O_2 generada. Aquest es produeix mitjançant l'increment de la recaptació de glucosa per part dels adipòcits madurs i de les cèl·lules de múscul llis a través del reclutament de receptors GLUT4 i GLUT1 respectivament a la superfície cel·lular (El Hadri *et al.* 2002; Enrique-Tarancon *et al.* 1998; Enrique-Tarancon *et al.* 2000; Marti *et al.* 1998). Aquest efecte s'ha vist acompanyat en alguns models d'una reducció moderada de la hiperglucèmia in vivo (Marti *et al.* 2001; Morin *et al.* 2002) i d'un efecte antilipolític (Iglesias-Osma *et al.* 2004; Morin *et al.* 2001; Visentin *et al.* 2003). L'efecte antilipolític podria donar-se també pel control que exerciria la SSAO sobre els nivells d'histamina (la qual indueix lipòlisi) (Raimondi *et al.* 1993).

L'addició de vanadat és necessària en alguns sistemes perquè es produeixi l'efecte insulinomimètic, però no és el cas dels cultius d'adipòcits humans (Morin *et al.* 2001) ni de les cèl·lules de múscul llis d'aorta de rata (El Hadri *et al.* 2002). El mecanisme pel qual el vanadat és necessari en alguns sistemes i no en altres es desconeix. Es creu que l'H_2O_2 formada durant la reacció enzimàtica de la SSAO reaccionaria amb el vanadat, formant-se probablement peroxovanadat, un potent agent insulinomimètic que seria el responsable final de l'efecte (Marti *et al.* 2001). El peroxovanadat format estimularia proteïnes tirosina cinasa o inhibiria tirosina fosfatases, causant l'activació del receptor d'insulina, d'altres substrats del receptor d'insulina (IRS) i de la proteïna PI3 cinasa, fet que provocaria el reclutament dels receptors GLUT4 a la superfície cel·lular, els components finals que induirien la recaptació de glucosa (figura 12.A). La producció d'H_2O_2 és imprescindible, ja que l'addició de catalasa aboleix completament

l'efecte (Enrique-Tarancon *et al.* 1998; Marti *et al.* 1998). La producció de peroxovanadat, en canvi, no està clara ja que s'han observat diferències entre els efectes moleculars del peroxovanadat i els del vanadat en combinació amb els substrats de la SSAO (Castan *et al.* 1999; Heffetz *et al.* 1992; Zorzano *et al.* 2003). Això, juntament amb el fet que el vanadat no sigui necessari en certs models suggereix que altres productes es deuen formar en combinació entre vanadat i H_2O_2, o entre H_2O_2 i altres molècules. D'altra banda, un model teòric sense vanadat ha estat proposat, el qual integra les activitats MAO i SSAO en el metabolisme successiu d'adrenalina i metilamina per la generació d'H_2O_2 (figura 12.B) (McDonald *et al.* 2007).

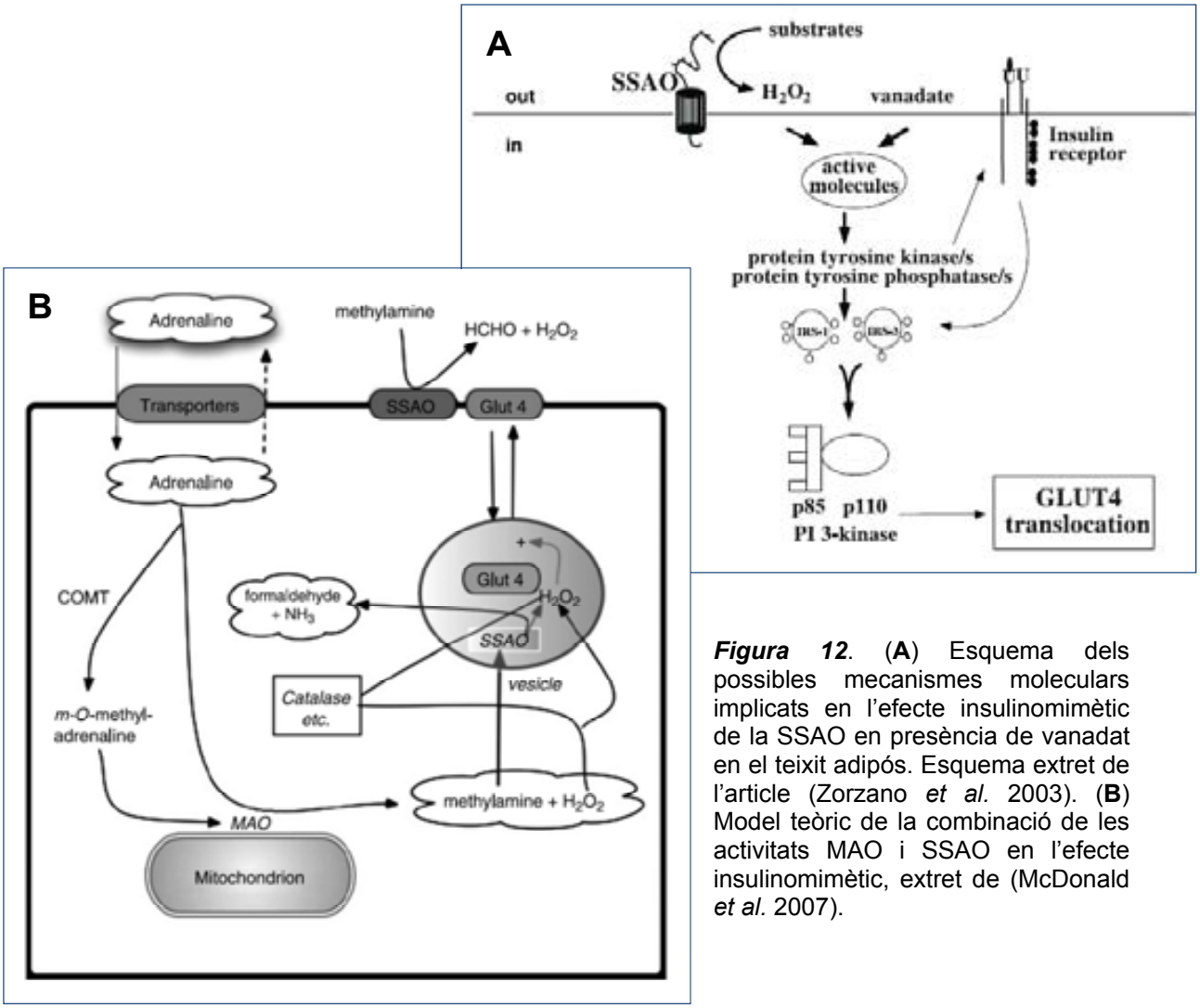


Figura 12. (**A**) Esquema dels possibles mecanismes moleculars implicats en l'efecte insulinomimètic de la SSAO en presència de vanadat en el teixit adipós. Esquema extret de l'article (Zorzano *et al.* 2003). (**B**) Model teòric de la combinació de les activitats MAO i SSAO en l'efecte insulinomimètic, extret de (McDonald *et al.* 2007).

De totes maneres, el fet que la combinació de substrats de la SSAO i vanadat indueixi la recaptació de glucosa en els teixits ha fet que alguns autors suggereixin que la seva administració pot constituir un agent potencial per al tractament o prevenció de la diabetis mellitus (Visentin *et al.* 2005; Yraola *et al.* 2009). En aquest sentit, la inducció

de la patologia diabètica en animals que sobreexpressen la SSAO en múscul llis provoca un increment de la SSAO de teixit adipós i plasma, mimetitzant l'efecte observat en humans i constituint un bon model d'estudi del paper i regulació de la SSAO en aquestes condicions (Gokturk *et al.* 2004). A més, l'administració crònica de benzilamina i baixes dosis de vanadat a rates diabètiques normalitza els seus nivells de glicèmia, recluta receptors GLUT4 a la superfície cel·lular i incrementa l'expressió basal d'aquest receptor (Marti *et al.* 2001).

2.5.3 Regulació del creixement i maduració cel·lulars.

S'ha descrit que l'activitat SSAO participa de forma important en la maduració d'alguns tipus cel·lulars com els adipòcits o les cèl·lules del múscul llis.

En els preadipòcits, pràcticament no s'observa expressió de la SSAO, la qual va incrementant a mesura que maduren a adipòcits (Bour *et al.* 2007; Moldes *et al.* 1999). A més, l'addició de diversos substrats de la SSAO és capaç d'induir la maduració dels adipòcits de forma dosi dependent, i els antioxidants impedeixen aquest efecte, fet que suggereix que aquesta funció és també duta a terme mitjançant el peròxid d'hidrogen generat durant l'acció catalítica (El Hadri *et al.* 2002; Fontana *et al.* 2001; Mercier *et al.* 2001; Subra *et al.* 2003). Aquesta funció s'ha descrit de forma semblant en el cas de la maduració de les cèl·lules de múscul llis (El Hadri *et al.* 2002; Owens 1995).

De la mateixa manera, en estudis de desenvolupament amb embrions de ratolí s'ha observat una elevada expressió d'SSAO en cèl·lules en procés de diferenciació durant la vasculogènesi de diferents òrgans, i també en diferenciació dels teixits adipós i cartilaginós (Valente *et al.* 2008). Aquesta elevada expressió disminueix en començar el procés d'ossificació en el cas del teixit cartilaginós, fet que concorda amb dades publicades anteriorment (Lyles and Bertie KH 1987).

2.5.4 Deposició i manteniment de la matriu extracel·lular en el múscul llis vascular.

S'ha descrit que la SSAO participa en la deposició i manteniment de la matriu extracel·lular de les cèl·lules del múscul llis (Langford *et al.* 2002). Estudis desenvolupats inhibint la SSAO en cultius de cèl·lules del múscul llis del cor de rata

han mostrat aberracions en la deposició de col·lagen i elastina en la matriu extracel·lular d'aquestes cèl·lules (Langford *et al.* 2002). Els mateixos autors han mostrat també que existeix una desorganització de l'arquitectura de l'elastina en la capa mitja muscular de l'aorta en rates amb l'activitat SSAO inhibida, causant una dilatació de l'aorta (Langford *et al.* 1999). Així mateix, animals que sobreexpressen la SSAO en cèl·lules de múscul lliss mostren una arquitectura anormal de l'elastina en les grans artèries: es suggereix que una activitat SSAO excessiva podria incrementar el *cross-linking* de l'elastina degut als productes resultants de la reacció enzimàtica, que podrien canviar l'estructura terciària de l'elastina (Gokturk *et al.* 2003). Aquestes observacions donen suport al paper de la SSAO en l'estructura d'aquesta matriu extracel·lular del múscul lliss. Tot i així, altres autors no troben diferències en el cross-link d'elastina ni de col·lagen en ratolins KO per la SSAO (Mercier *et al.* 2006), o consideren que la lisil oxidasa podria contribuir en gran part a aquesta funció (Mercier *et al.* 2009), fet que s'hauria d'estudiar més profundament amb inhibidors específics de cadascuna d'aquestes amino oxidases.

2.5.5 Tràfic lipídic.

Diversos estudis mostren la presència de la SSAO en les caveoles de la membrana plasmàtica (Aboulaich *et al.* 2004; Jaakkola *et al.* 1999; Souto *et al.* 2003), estructures membranals implicades en el flux lipídic cap a dins i fora dels adipòcits (Parton 1996). El fet que s'hagi observat que les caveoles adipocitàries de rata contenen una composició proteica limitada, contenint només el receptor *scavenger* de lipoproteïnes CD36 i la SSAO (Souto *et al.* 2003) ha suggerit que la SSAO també participi en aquest procés. Aquesta hipòtesi està recolzada per l'observació que els Receptors X del fetge (LXRs), els quals responen a concentracions intracel·lulars de colesterol i estan implicats en l'activació de gens que participen en el tràfic lipídic, mostren un efecte inhibitori sobre l'expressió de la SSAO (Dai *et al.* 2008).

2.5.6 Unió a substàncies de tipus imidazòlic.

La SSAO es comporta, com les isoformes de la MAO, com una proteïna amb capacitat d'unió a amiloride i altres substàncies tipus imidazòlic i guanidínic (Novotny *et al.* 1994), però la localització extracel·lular de la SSAO en front a la intracel·lular de les MAOs li dóna més rellevància en aquest procés. Aquestes substàncies són inhibidors

dels sistemes transportadors de sodi, i són utilitzats com a agents diürètics i hipertensors. La unió de la SSAO a aquestes molècules és reversible i és capaç de modular l'activitat amino oxidasa (Holt *et al.* 2004). La capacitat d'unió a aquests compostos, però, difereix entre enzims de diferents procedències (Lizcano *et al.* 1998), i es desconeix si aquest tipus d'unions té algun significat biològic (Tipton *et al.* 2003).

2.5.7 Altres funcions de la SSAO transmembranal.

La SSAO es troba àmpliament expressada en el sistema vascular. Això i el fet que generi productes vasoactius com l'H_2O_2 (Swei *et al.* 1997) ha fet suposar que la SSAO es trobi implicada en el **control del to muscular** (Callingham *et al.* 1990). En aquest sentit, alguns autors suggereixen que el mecanisme d'acció de la hidralazina i la hidroxilamina com a agents vasodilatadors i hipotensors es deu en part a que són inhibidors de la SSAO (Vidrio *et al.* 2003; Vidrio and Medina 2007). Segons aquesta hipòtesi, l'H_2O_2 generada en condicions fisiològiques per l'activitat SSAO actuaria com a agent vasoconstrictor i hipertensor (Conklin *et al.* 2004; Vidrio 2003). Estudis realitzats amb animals transgènics que sobreexpressen la SSAO, en els que s'ha observat una desregulació de la pressió arterial, donen suport a aquesta teoria, explicant-se en part per l'augment en la generació d'H_2O_2 i en part pel paper de la SSAO en la deposició de la matriu extracel·lular arterial (Gokturk *et al.* 2003; Stolen *et al.* 2004a). D'altra banda, animals *knock out* per la SSAO no mostren alteracions en la funcionalitat contràctil vascular (Mercier *et al.* 2006), resultats que contradiuen la hipòtesi anterior.

Tot i així s'ha de tenir en compte que l'H_2O_2 pot exercir efectes que incrementen el to vascular (Okatani *et al.* 1997; Rodriguez-Martinez *et al.* 1998), però també pot induir la relaxació del múscul llis arterial (Beny and von der Weid 1991; Burke and Wolin 1987). De la mateixa manera, amines o altres metabòlits poden també tenir un efecte sobre la pressió arterial: la metilamina per ella mateixa, per exemple, tendeix a baixar la pressió arterial (Vidrio *et al.* 2003). D'altra banda, en la regulació del to vascular hi estan implicats molts altres factors com ara les endotelines o l'òxid nítric, els quals també poden respondre a estímuls com l'H_2O_2. En aquest sentit, s'ha observat que l'H_2O_2 inhibeix la síntesi de la iNOS en macròfags i cèl·lules de múscul llis (Guikema *et al.* 2005; Vega *et al.* 2004), disminuint doncs la producció del vasodilatador òxid nítric, mentre que amines com la metilamina i la histamina incrementen la producció d'NO en teixit el adipós (Fracassini *et al.* 2008). Pot ser doncs, el balanç entre tots aquests

elements reguladors el que determini el comportament final del vas sanguini en cada moment.

Una altra funció en la que s'ha implicat a la SSAO ha estat el **control de la ingesta**. Considerant que malalties com la diabetis o l'Alzheimer van acompanyades d'increments en l'activitat SSAO plasmàtica (Boomsma *et al.* 1999; del Mar Hernandez *et al.* 2005), increments de metilamina (Yu *et al.* 2003b) o trastorns alimentaris (Muñoz *et al.* 2006), s'ha postulat que la SSAO, mitjançant la regulació dels nivells de metilamina, participaria en el control de la ingesta de manera independent al circuit de la leptina (Raimondi *et al.* 2007). Aquesta hipòtesi es basa en resultats que mostren que la metilamina té propietats anorexigèniques en ratolins, tant en normals com en diabètics (Cioni *et al.* 2006; Pirisino *et al.* 2004), i dependent de la dosi en rates té efectes hiper o hipofàgics (Raimondi *et al.* 2007). Es creu que aquest efecte es produiria mitjançant la inducció per part de la metilamina, de l'increment d'NO o dopamina a l'hipotàlem, probablement a través de la interacció amb els canals de potassi Kv1.6, induint efectes hiper o hipofàgics respectivament (Pirisino *et al.* 2004). La metilamina es comportaria doncs com un modulador endogen de la producció central d'NO, ja que en situacions com la diabetis o l'Alzheimer, en les quals es dóna una inflamació de la barrera hematoencefàlica, aquesta amina pot travessar la barrera i entrar al sistema nerviós central (Mitchell and Zhang 2001). La SSAO participaria en aquest procés controlant els nivells de metilamina.

2.5.8. Funcions específiques de la SSAO soluble.

La forma soluble de la SSAO posseeix també activitat enzimàtica, per tant, es creu que una de les seves funcions recau en la destoxificació d'amines circulants en el torrent sanguini. A més, també s'ha associat a la funció inflamatòria mitjançant la seva possible participació en la modulació de l'adhesió i transmigració limfocitàries (Kurkijarvi *et al.* 1998), a través de la generació de senyals positives per a l'activació del limfòcit (Ley and Deem 2005).

En altres espècies de mamífers s'han descrit altres funcions per la SSAO soluble; per exemple, la SSAO del plasma boví és capaç de catalitzar l'oxidació de grups amino lliures de proteïnes com la lisozima, la ribonucleasa o la polilisina (Wang *et al.* 1996). El mateix enzim és també capaç d'activar els canals de potassi voltatge-dependents

en una línia cel·lular de neuroblastoma (Wu *et al.* 1996). El significat biològic d'aquests processos, però, no es coneix encara (Nocera *et al.* 2003).

D'altra banda, la SSAO soluble es troba incrementada en algunes malalties, (veure Introducció, apartat 2.8.2), però en la majoria d'elles no es coneix la funció que desenvoluparia aquest increment. Probablement està relacionat amb la inducció de la resposta inflamatòria, el control del nivell de certes amines o la generació dels productes metabòlics.

2.6. Regulació de l'expressió i activitat de la SSAO

La SSAO es pot regular fisiològicament tant a nivell d'expressió, mitjançant mecanismes reguladors de la transcripció gènica; com a nivell d'activitat, mitjançant la presència d'activadors/inhibidors endògens; com també a nivell de localització cel·lular, afavorint la seva localització i manteniment a la membrana plasmàtica; o a nivell de modificacions post traduccionals, controlant-ne els nivells de glicosilació. Encara són pocs, però, els mecanismes moleculars coneguts que s'encarreguen d'aquestes regulacions i les senyals sota les quals aquests actuen.

En referència a l'expressió de la SSAO/VAP-1, la caracterització del seu promotor en ratolí ha permès conèixer alguns dels seus elements reguladors (Bono *et al.* 1998b). En primer lloc, la identificació de diversos llocs d'inici de la transcripció suggereix l'existència de transcrits d'mRNA de diverses mides en diferents teixits. D'altra banda, en la regió promotora de la SSAO/VAP-1 s'han identificat potencials llocs d'unió dels **factors de transcripció** NF-κB, Sp1, AP-2 o AP-3, entre altres. No es coneix, però, quins d'aquests són utilitzats i quan, o si n'existeixen més. Entre aquests,el factor de transcripció NF-κB participa també en la inducció d'altres proteïnes pro inflamatòries com VCAM-1, E-selectina i ICAM-1 mitjançant l'acció de la IL-1β o TNF-α. En aquest sentit, s'ha descrit que la SSAO/VAP-1 s'indueix en endoteli mitjançant molècules associades a la resposta inflamatòria com ara interleucines 1 i 4, interferó-γ, LPS o TNF-α (Abella *et al.* 2004). La regulació per TNF-α, però, no es produeix sempre a l'alça ja que la mateixa molècula produeix l'efecte contrari en teixit adipós (Mercier *et al.* 2003). Això confirma la diferent regulació de l'expressió de la SSAO/VAP-1 en diferents teixits i diferents tipus cel·lulars (Arvilommi *et al.* 1997), com és el cas d'altres molècules inflamatòries com la VCAM-1 (Iademarco *et al.* 1993).

Diferents **hormones** també són capaces de modular l'expressió de la SSAO. Els nivells d'insulina, per exemple, correlacionen negativament amb els nivells d'SSAO plasmàtica (Abella *et al.* 2004; Dullaart *et al.* 2006). Es creu, però, que algun altre factor a part de la insulina està implicat en aquesta regulació, ja que aquest increment d'SSAO plasmàtica es produeix també en diabetis tipus II (no dependent d'insulina), i que no s'observen canvis d'expressió de la SSAO en teixit adipós en resposta a la insulina (Enrique-Tarancon *et al.* 1998). D'altra banda, s'han detectat increments d'SSAO plasmàtica en condicions d'hiperinsulinèmia (Stolen *et al.* 2004b), tot i que l'increment de glucosa en sang no sembla ser un estímul suficient per induir l'increment de la SSAO (Salmi *et al.* 2002). La hormona tiroïdal també podria regular els nivells d'SSAO en plasma, ja que els seus nivells són baixos en pacients hipotiroïdeus, i alts en hipertiroïdeus, retornant als nivells normals després del tractament adequat (Boomsma *et al.* 2005). Una altra situació hormonal en la que els nivells d'SSAO es veuen modificats és en dones que utilitzen contraceptius de tipus esteroide (Tryding *et al.* 1969), o en situacions d'embaràs (Sikkema *et al.* 2002), en les que s'han detectat nivells elevats d'SSAO plasmàtica. En el mateix sentit, s'ha observat que agonistes sintètics dels Receptors X del fetge (LXRs) inhibeixen l'expressió i activitat de la SSAO en nombrosos teixits en ratolins; aquests receptors són membres de la família de receptors nuclears d'hormones i responen a la concentració intracel·lular de colesterol (Lewis and Rader 2005), promovent l'expressió de gens implicats en el tràfic de colesterol i inhibint la inflamació *in vivo* (Castrillo *et al.* 2003). Alguns autors han suggerit que els efectes ateroprotecors d'aquests agonistes podrien estar relacionats amb aquesta inhibició de la SSAO (Dai *et al.* 2008).

D'altra banda, la correlació observada entre la SSAO plasmàtica i l'activitat de l'enzim convertidor d'angiotensina (ACE) ha suggerit també una regulació comú d'aquests dos enzims, probablement a nivell del **tall de la proteïna tissular**, ja que l'ACE s'allibera al plasma també d'aquesta forma (Boomsma *et al.* 2005).

La **presència de substrat** podria regular també els nivells de l'enzim. Segons això, es creu que la SSAO incrementaria en patologies on substrats de la SSAO es troben incrementats: seria el cas de la metilamina i aminoacetona en diabetis mellitus, de la metilamina en insuficiència renal crònica, de la creatinina en urèmia, o de l'adrenalina en situacions d'estrès (Matyus *et al.* 2004). Com a efecte contrari, s'ha descrit una inhibició de l'activitat SSAO per altes concentracions del substrat benzilamina, efecte que no es dóna en el cas d'altres substrats com la metilamina, β-PEA o histamina (Lizcano *et al.* 2000). Les conseqüències fisiològiques d'aquest fenomen serien en

principi limitades ja que la benzilamina no és un substrat fisiològic, però hi poden haver altres substrats encara no identificats que es comportin de forma semblant. En aquest sentit s'ha descrit també una activació de l'enzim per temperatura, possiblement mitjançant un canvi conformacional, que també afecta només al substrat benzilamina, indicant l'existència de diferències en els mecanismes cinètics dels diferents substrats (Lizcano *et al.* 2000).

Els **nivells elevats d'activitat SSAO** poden actuar com a autoreguladors de l'enzim. En aquest sentit, s'ha observat que la inducció de diabetis en animals que sobreexpressen la SSAO específicament en múscul llis, i que responen a la diabetis amb un increment de l'activitat SSAO en plasma i teixit adipós, mostren també una disminució de l'mRNA en teixit adipós. Els resultats observats en aquest treball mostren una combinació de tres tipus diferents de regulació: (a) l'increment observat en teixit adipós, tot i la disminució de l'mRNA, s'atribueix a modificacions post traduccionals, ja que nivells elevats de glicosilació alenteixen la degradació de les proteïnes incrementant la seva vida mitja (Gokturk *et al.* 2004). (b) La disminució de l'mRNA pot ser conseqüència d'un feedback inhibitori produït per nivells elevats d'activitat SSAO; d'altra banda, l'efecte contrari no es produeix: la inhibició de la proteïna no incrementa els seus nivells d'expressió (Gokturk *et al.* 2004; Nordquist *et al.* 2002). (c) L'increment observat al plasma pot provenir del tall de la forma tissular: aquest efecte s'ha observat també en isquèmia cerebral, on s'ha detectat un augment de la SSAO plasmàtica juntament a la disminució a nivell tissular en el costat ipsilateral de la isquèmia (Airas *et al.* 2008). A més, tant en isquèmia com en diabetis augmenta el TNF-α (Sotgiu *et al.* 2006), el qual incrementa el tall i alliberament al plasma de la SSAO tissular (Abella *et al.* 2004), fet que corrobora la hipòtesi.

S'ha descrit també una possible modulació de l'expressió de la SSAO en relació amb l'**expressió d'altres amino oxidases**. És el cas per exemple de l'increment d'expressió de la RAO per compensar la deleció del gen de la SSAO en ratolins (Marttila-Ichihara *et al.* 2006), o l'increment d'activitats MAO A i MAO B observat després de la inhibició de la SSAO (Kinemuchi *et al.* 2004). No s'han observat fins al moment fenòmens de compensació entre la LO i la SSAO (Mercier *et al.* 2006). D'altra banda, el tractament amb inhibidors no selectius de la MAO també és capaç d'induir un increment en l'activitat SSAO (Fitzgerald and Tipton 2002; Kinemuchi *et al.* 2004), compensació que es podria donar en la malaltia de Norrie, on tot i la deleció del gen de la MAO s'observen metabòlits d'aquesta activitat en el líquid cefaloraquidi, suposadament deguts a l'activitat SSAO (Fitzgerald *et al.* 1998).

Finalment, la **modulació de l'activitat enzimàtica** constitueix un altre possible mecanisme de regulació de la proteïna. En aquest sentit, s'ha descrit l'existència d'un inhibidor endogen de la SSAO present en plasma humà (Banchelli *et al.* 1988; Buffoni *et al.* 1983), no caracteritzat fins al moment; d'un inhibidor present en cervell de rata, induïble mitjançant estrès per immobilització (Obata 2006; Obata and Yamanaka 2000a); i d'un altre present en rates amb tumors de mama malignes (Lizcano *et al.* 1990b; Lizcano *et al.* 1991b). D'altra banda, s'han descrit també com a activadors endògens de la SSAO un component plasmàtic de baix pes molecular, probablement el fosfolípid lisofosfatidilcolina (Dalfo *et al.* 2003), i el bicarbonat, també present en plasma (Hernandez-Guillamon *et al.* 2007). A més, s'ha descrit que la SSAO presenta nombrosos llocs d'unió a compostos que poden modificar l'activitat de l'enzim, com ara les substàncies de tipus imidazòlic o l'amoni mateix generat durant l'acció catalítica (Holt *et al.* 2004; Holt *et al.* 2008; Kelly *et al.* 1981). Els nivells d'aquests activadors i inhibidors podrien estar modificats en situacions patològiques, contribuint a les alteracions observades en l'activitat SSAO en certes malalties.

2.7. La SSAO en condicions patològiques

L'expressió i/o activitat de la SSAO s'ha trobat alterada en nombroses situacions patològiques en humans, tant en la seva forma soluble com en la lligada als teixits (per revisions, veure (Boomsma *et al.* 2003; Lyles 1996; O'Sullivan *et al.* 2004)). En la majoria dels casos no es coneix la causa de l'alteració, si és una conseqüència del procés patològic o si contribueix a la pròpia patologia. La seva modificació com a conseqüència de la patologia inicial, sembla vàlida en el cas de processos inflamatoris, on la SSAO es veu incrementada, ja que té una funció definida en inflamació. D'altra banda, el fet que els productes resultants de la seva activitat siguin potencialment tòxics suggereix que també pot contribuir a la pròpia patologia en casos d'increment de la seva activitat.

2.7.1. Alteracions en la SSAO transmembranal.

Les alteracions en la SSAO transmembranal que s'han observat en situacions patològiques es mostren resumides a la taula 1.

Patologia	Alteració	Referència
Malaltia d'Alzheimer	Increment	(Ferrer *et al.* 2002)
Diabetis (rata)	Increment	(Lyles 1996) (Gokturk *et al.* 2004)
Psoriasi (i altres malalties inflamatòries humanes i experimentals)	Increment	(Arvilommi *et al.* 1996) (Madej *et al.* 2007) (Jaakkola *et al.* 2000b)
Malalties inflamatòries renals	Increment	(Holmen *et al.* 2005)
Aterosclerosi	Increment	(Anger *et al.* 2007)
Miopaties	Increment	(Olive *et al.* 2004)
Isquèmia	Increment/ Disminució	(Airas *et al.* 2008; Jaakkola *et al.* 2000a)
Tumors de mama (rata)	Disminució	(Lizcano *et al.* 1991b)

Taula 1. Situacions patològiques en les que s'observen canvis en l'activitat o en els nivells de proteïna de la SSAO en diferents localitzacions anatòmiques. Si no s'especifica, es refereix a l'enzim humà.

En patologies cerebrovasculars, la sobreexpressió de la SSAO podria indicar una contribució a la degeneració vascular degut a l'estrès oxidatiu generat. En la **malaltia d'Alzheimer**, la sobreexpressió de la SSAO s'ha observat entre la capa íntima i muscular dels vasos sanguinis, colocalitzant amb dipòsits de beta amiloide i amb zones de sobreexpressió de l'enzim superòxid dismutasa (Ferrer *et al.* 2002). D'altra banda, la sobreexpressió d'SSAO s'ha observat també en vasos sanguinis de pacients amb CADASIL (*cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy*), suposadament associada a fenòmens d'estrès oxidatiu (Ferrer *et al.* 2002).

En situacions de **diabetis** s'han detectat increments d'activitat SSAO en ronyó i teixit adipós en rates i ratolins, però no en pulmó, pàncrees o aorta (Gokturk *et al.* 2004; Lyles 1996).

Moltes malalties en les que s'observa un increment en la immunoreactivitat tissular contra la SSAO tenen un component **inflamatori** important, com la psoriasi (Madej *et*

al. 2007), la dermatitis al·lèrgica (Arvilommi *et al.* 1996) o l'excema atòpic (Madej *et al.* 2006). En diversos models experimentals de malalties inflamatòries s'ha observat també un increment en l'expressió d'SSAO, com ara en artritis i dermatitis experimentals (Jaakkola *et al.* 2000b). És el cas també de malalties que inclouen vasculitis del sistema renal, com ara la granulomatosi de Wegener (Holmen *et al.* 2005). En aquests casos, l'increment de proteïna sembla estar associat a la seva funció d'adhesió.

L'**aterosclerosi** mostra també un gran component inflamatori; en aquest sentit, s'ha observat sobreexpressió de la SSAO i d'altres proteïnes inflamatòries en plaques arterioscleròtiques i en processos de calcificació i estenosi de les vàlvules aòrtiques (Anger *et al.* 2007). Així mateix, soques de ratolí més susceptibles a patir aterosclerosi mostren nivells basals més elevats d'activitat SSAO (Yu and Deng 1998). La SSAO ha estat també relacionada en processos de vasoespasme, el qual està associat a aterosclerosi i hipertensió, mitjançant el metabolisme de l'al·lilamina a la paret dels vasos sanguinis (Conklin *et al.* 2001; Conklin *et al.* 2006).

En casos de **miopaties** humanes s'ha observat també un increment en la immunoreactivitat contra la SSAO, en aquest cas en múscul esquelètic, on no s'expressa la SSAO en condicions normals. Es creu que aquesta expressió és deguda a la situació d'inflamació del múscul esquelètic. Les miopaties estudiades inclouen: miositis amb cossos d'inclusió, miopatia relacionada amb la desmina, dermatomiositis, miopatia granulomatosa, denervació i renervació muscular, i rabdomiolisi (Olive *et al.* 2004).

Finalment, en estudis en mostres *post-mortem* humanes de pacients amb **isquèmia** cerebral s'ha descrit una disminució de la SSAO tissular en el costat ipsilateral de l'infart, que podria anar a compte de l'increment observat en el plasma. No s'observen canvis però en el costat contralateral (Airas *et al.* 2008). D'altra banda, en infarts coronaris s'ha observat un increment de l'expressió d'SSAO en els vasos sanguinis de les àrees infartades (Jaakkola *et al.* 2000a), així com un increment en el teixit pulmonar després d'un procés d'isquèmia i reperfusió d'aquest (Ucar *et al.* 2005). Aquests diferents resultats poden ser deguts a una diferent regulació segons el teixit, o a una temporalitat diferent en l'anàlisi de les mostres després del procés isquèmic.

2.7.2. Alteracions en la SSAO soluble.

El plasma és una font de fàcil obtenció per a la determinació de paràmetres bioquímics relacionats amb situacions patològiques en humans. Per això la majoria d'alteracions en la SSAO s'han descrit mitjançant l'anàlisi del plasma de pacients. Aquestes alteracions es mostren en la taula 2.

Substrats de la SSAO com la metilamina o l'aminoacetona es troben incrementats en **diabetis**; es creu que l'increment dels substrats podria donar lloc a l'increment de l'activitat SSAO, i aquesta podria contribuir al desenvolupament de complicacions diabètiques degut als productes tòxics generats (Deng and Yu 1999). A més, aquests increments d'activitat SSAO plasmàtica es correlacionen amb la severitat de la malaltia i amb la presència de complicacions característiques de la diabetis com la nefropatia, la retinopatia i la neuropatia (Boomsma *et al.* 1999; Garpenstrand *et al.* 1999a; Gronvall-Nordquist *et al.* 2001; Meszaros *et al.* 1999; Nunes *et al.* 2008). D'altra banda, s'ha observat que la SSAO no està elevada en estats pre-diabètics, confirmant la necessitat d'un agent desencadenant inductor de l'increment de la SSAO (Ruotsalainen *et al.* 2008), però també que l'increment d'activitat SSAO precedeix l'aparició de complicacions vasculars, indicant la seva possible implicació en l'inici d'aquestes (Boomsma *et al.* 1997). A més, s'han observat complicacions característiques de la diabetis, com aterosclerosi o glomerulosclerosi, en animals que sobreexpressen la SSAO, sense patir diabetis, fet que dóna suport a la implicació de la SSAO en la mateixa patologia (Stolen *et al.* 2004a). Tot i així, la situació d'increment d'SSAO no és irreversible ja que s'han descrit casos de diabetis insipidus en dones en estat d'embaràs, en les que l'activitat SSAO plasmàtica incrementada retorna als nivells normals després del part (Boomsma *et al.* 2003).

Encara que sovint, la **obesitat** va lligada a la diabetis, increments d'SSAO plasmàtica s'han detectat també en casos d'obesitat no lligada a diabetis. Es creu que en aquests pacients, l'increment d'SSAO podria ser un marcador de risc cardiovascular (Weiss *et al.* 2003). En aquest sentit, s'ha descrit una lleu disminució de la deposició de greix en rates tractades amb inhibidors de la SSAO, indicant un possible paper d'aquesta proteïna en l'obesitat (Prevot *et al.* 2007). Contràriament a aquests resultats, però, s'ha descrit que ratolins KO per la SSAO mostren un lleuger increment de la massa de teixit adipós (Bour *et al.* 2009), mentre que altres estudis no han observat canvis en l'activitat soluble de la SSAO en pacients obesos (Visentin *et al.* 2004), indicant que l'increment observat anteriorment pot ser un reflex de patologia associada i no pròpiament de l'estat d'obesitat.

Patologia	Alteració	Referència
Diabetis mellitus (tipus I i II)	Increment	(Boomsma *et al.* 1995; Boomsma *et al.* 1999; Meszaros *et al.* 1999)
Obesitat no lligada a diabetis	Increment/ No canvi	(Weiss *et al.* 2003)
Malalties cardíaques	Increment	(Boomsma *et al.* 1997)
Malalties hepàtiques cròniques	Increment	(Kurkijarvi *et al.* 1998)
Disfunció renal crònica /Urèmia	Increment	(Lin *et al.* 2008; Nemcsik *et al.* 2007)
Aterosclerosi	Increment	(Meszaros *et al.* 1999) (Karadi *et al.* 2002; Li *et al.* 2009)
Psoriasi i altres malalties inflamatòries	Increment	(Ling *et al.* 2009; Madej *et al.* 2006; Madej *et al.* 2007)
Esclerosi Múltiple	Increment	(Airas *et al.* 2006)
Malaltia d'Alzheimer	Increment	(del Mar Hernandez *et al.* 2005)
Infart/vessament cerebral	Increment/ Disminució	(Ishizaki 1990) (Airas *et al.* 2008)
Càncer	Increment/ Disminució	(Ekblom *et al.* 1999) (Lewinsohn 1984)
Depressió severa	Disminució	(Roessner *et al.* 2006)
Hèrnia diafragmàtica congènita	Disminució	(Boomsma *et al.* 2003)
Cremats severs	Disminució	(Lewinsohn 1977)

Taula 2. Situacions patològiques en les que s'observen canvis en l'activitat de la SSAO plasmàtica humana.

En casos de **malalties cardíaques cròniques** o **aturada cardíaca** també s'han observat correlacions entre l'increment d'activitat SSAO plasmàtica i la gravetat de la malaltia (Boomsma *et al.* 1997; Boomsma *et al.* 2000a). A més, l'increment és additiu en casos que presenten diabetis i aturada cardíaca alhora (Boomsma *et al.* 2003).

En les **malalties hepàtiques cròniques** com la cirrosi hepàtica o situacions inflamatòries hepàtiques (Kurkijarvi *et al.* 1998; Kurkijarvi *et al.* 2000), l'increment observat es correlaciona també amb la severitat (Boomsma *et al.* 2000a). En aquest tipus de patologies, els valors d'activitat SSAO plasmàtica poden recuperar els nivells normals després d'un transplantament de fetge (Boomsma *et al.* 2003). En referència als transplantaments, s'ha observat un increment d'activitat SSAO en casos de rebuig al transplantament de ronyó (Kurkijarvi *et al.* 2001), on s'ha postulat que la SSAO facilitaria l'entrada de leucòcits a l'òrgan rebutjat.

La **urèmia** es caracteritza per un estrès oxidatiu elevat i acumulació de compostos potencialment tòxics degut a una **insuficiència renal**. La metilamina es troba elevada en el plasma d'aquests pacients (Baba *et al.* 1984), i es creu que és la causa de l'increment d'SSAO plasmàtica en aquestes situacions (Nemcsik *et al.* 2007). En situacions de malalties renals cròniques, l'activitat plasmàtica de la SSAO també es correlaciona amb la gravetat de l'estat patològic (Lin *et al.* 2008).

L**'aterosclerosi** és un tret comú a moltes situacions patològiques on l'activitat SSAO està incrementada (diabetis, patologia coronària, demència, etc.). A més, s'ha observat correlació entre l'increment d'SSAO plasmàtica i la gravetat de plaques carotídies i els nivells de colesterol totals en situacions no diabètiques (Karadi *et al.* 2002). El fet que hi hagi correlació entre diversos factors de risc de patir aterosclerosi i l'activitat SSAO plasmàtica suggereix que la SSAO pot tenir un paper en el desenvolupament d'aquesta situació (Meszaros *et al.* 1999). A més, recentment s'ha descrit que l'increment d'SSAO plasmàtica produït després de l'administració oral de glucosa correlaciona amb un increment d'estrès oxidatiu i d'AGEs en plasma, que podria contribuir al desenvolupament de l'arteriosclerosi (Li *et al.* 2009).

Altres malalties amb un component inflamatori en les que la SSAO plasmàtica s'ha trobat incrementada són la **psoriasi** (Madej *et al.* 2007), l'**excema atòpic** (Madej *et al.* 2006), la **osteoartritis** (Ling *et al.* 2009) o l'**esclerosi múltiple** (Airas *et al.* 2006), en aquesta última essent l'increment major en malalts amb component inflamatori. Tot i l'increment d'SSAO observat en moltes patologies inflamatòries, la inflamació no és una condició necessària perquè la SSAO incrementi, ni pot utilitzar-se aquesta alteració com a marcador general d'inflamació ja que existeixen malalties inflamatòries en les que la SSAO mostra nivells comparables als normals, com ara en artritis reumàtica o malalties inflamatòries intestinals (Kurkijarvi *et al.* 1998).

En el cas de la **malaltia d'Alzheimer**, l'increment d'SSAO plasmàtica s'observa només en estats avançats de la malaltia (del Mar Hernandez *et al.* 2005), podent contribuir al dany vascular mitjançant estrés oxidatiu.

Els resultats dels nivells d'SSAO plasmàtica analitzats en situacions de vessaments o infarts cerebrals es contradiu en la bibliografia. En ocasions, aquesta s'ha trobat disminuïda després d'episodis de **vessament cerebral** i subaracnoidal, però en canvi s'ha trobat incrementada després d'**infarts cerebrals** (Airas *et al.* 2008; Ishizaki 1990), o en altres estudis no s'observen canvis després d'infarts cerebrals (Garpenstrand *et al.* 1999b). Aquests resultats contradictoris s'han associat a l'heterogeneïtat en l'etiologia de la condició dels malalts utilitzats. En aquests casos es postula que l'increment en la SSAO plasmàtica podria respondre a un increment en el tall de la proteïna en la zona cerebral infartada, ja que es detecta una disminució en la proteïna tissular ipsilateral (Airas *et al.* 2008). D'altra banda, el tractament amb inhibidors de la SSAO en models animals ha mostrat prevenir el dany microvascular posterior a la isquèmia-reperfusió tant cerebral com pulmonar (Cockroft *et al.* 1996; Kiss *et al.* 2008; Ucar *et al.* 2005; Xu *et al.* 2006).

En diversos casos de **càncer**, la SSAO plasmàtica es troba incrementada, i d'altres es troba disminuïda. Es troba incrementada en el plasma de càncers de pròstata amb metàstasis òssies (Ekblom *et al.* 1999), en els quals l'increment es relaciona amb processos de creixement (mimetitzant una situació de creixement en l'adolescència) (Boomsma *et al.* 2003). Es troba disminuïda, en canvi, en el plasma de tumors sòlids (Lewinsohn 1984), i en microvasos intratumorals de melanoma humà, correlacionant la disminució amb la severitat del càncer (Forster-Horvath *et al.* 2004). En càncers colorectals s'ha observat també un lleuger increment de la SSAO plasmàtica, de manera que els nivells més baixos d'SSAO correlacionen amb un pitjor pronòstic incloent l'aparició de metàstasis limfàtiques i hepàtiques (Toiyama *et al.* 2009). D'altra banda, en càncers de pulmó de cèl·lules no petites, s'observa correlació amb el factor angiogènic VEGF, però no amb la severitat del càncer (Garpenstrand *et al.* 2004).

De totes les alteracions de la SSAO plasmàtica en situacions patològiques, les menys freqüents són les disminucions d'activitat, observades per exemple en situacions de **depressió severa**, però la poca quantitat de pacients amb els que està fet l'estudi i l'heterogeneïtat de tractaments d'aquests fa que no es pugui concloure un paper definit de la SSAO en aquesta patologia (Roessner *et al.* 2006). En casos **d'hèrnia diafragmàtica congènita**, l'activitat SSAO plasmàtica es troba també baixa, però recupera els nivells normals després de la correcció quirúrgica. Així mateix, el

manteniment d'aquests nivells baixos correlaciona amb un pitjor pronòstic o amb l'aparició de complicacions, mentre que la recuperació dels nivells normals s'associa a un bon pronòstic (Boomsma *et al.* 2003).

2.8. Efectes tòxics resultants de l'activitat catalítica de la SSAO

Tot i el teòric paper de la SSAO com a destoxificadora d'amines, els productes resultants d'aquesta activitat catalítica són sovint més tòxics que les amines inicials. Les amines més estudiades en aquest aspecte han estat l'al·lilamina, l'aminoacetona i la metilamina, el metabolisme de les quals genera acroleïna, metilglioxal i formaldehid respectivament, a mes del peròxid d'hidrogen i l'amoni.

Tant el peròxid d'hidrogen (H_2O_2) com l'amoni (NH_3), són productes potencialment tòxics. D'una banda, l'H_2O_2 és una espècie reactiva d'oxigen (ROS), i a partir de determinades concentracions és capaç d'induir dany cel·lular mitjançant estrés oxidatiu i generació de radicals lliures com els ions hidroxil (Gutteridge 1994). A més, en combinació amb el formaldehid també és capaç d'induir la formació de radicals lliures (Lichszteld K. 1979). Tot i així, a baixes concentracions l'H_2O_2 és una molècula transductora de senyals, i regula la funció de factors de transcripció com NF-κB (Bogdan *et al.* 2000; Bradley *et al.* 1993; Finkel 1998; Kunsch and Medford 1999; Suzuki *et al.* 1997), a part de ser responsable de moltes de les funcions associades a la SSAO. D'altra banda, l'NH_3 és també un compost tòxic que pot alterar la glicosilació proteica (Seiler 2002; Yang and Butler 2002).

Dels tres productes generats durant l'activitat catalítica de la SSAO, però, els aldehids acostumen a ser els més tòxics dels tres, ja que són molècules altament reactives. Entre ells, tant el metilglioxal com el formaldehid són capaços d'induir l'entrecreuament de proteïnes (Nagaraj *et al.* 1996), i l'increment de formació de productes de glicosilació avançada (AGEs) (Langford *et al.* 1999; Mathys *et al.* 2002; Stolen *et al.* 2004a; Yu and Zuo 1993; Yu and Zuo 1997). A més, es coneix que l'entrecreuament de proteïnes induït pel formaldehid o "formilació" afecta principalment a residus de lisina i arginina de les proteïnes (Yu *et al.* 2006).

El lloc de formació d'aquests productes té especial importància, ja que incrementa el potencial efecte tòxic d'aquests. Les amines substrats de la SSAO poden viatjar pel torrent sanguini, on es troba també la SSAO ja que és una proteïna majoritàriament extracel·lular, i els productes generats s'alliberen al torrent sanguini. Baixes quantitats de formaldehid produït intracel·lularment podrien ser destoxificades mitjançant

deshidrogenases ubicades dins de les cèl·lules (Teng *et al.* 2001). El fet de ser produït en el plasma, però, on no es troba la formaldehid deshidrogenasa (Helander and Tottmar 1987), pot fer que la destoxificació del formaldehid s'alenteixi o s'impedeixi, ja que es requereix el seu transport a l'interior de les cèl·lules per degradar-lo. En elevar-se'n els nivells i no tenir prou capacitat de destoxificació, el formaldehid podria actuar a nivell dels enzims mitocondrials, inhibint la respiració mitocondrial i augmentant els nivells d'espècies reactives d'oxigen intracel·lulars (Teng *et al.* 2001), i també generant adductes irreversibles entre proteïnes o el DNA (Yu 1998). D'altra banda, l'elevada reactivitat del formaldehid augmentaria la possibilitat d'induir dany a les cèl·lules vasculars veïnes, o d'afectar la funcionalitat de proteïnes plasmàtiques per la formació d'adductes irreversibles entre elles (Gronvall *et al.* 2000).

La toxicitat d'aquests aldehids ha estat descrita de forma activitat SSAO-dependent per l'acroleïna en cèl·lules vasculars en cultiu (Conklin *et al.* 1998; Hysmith and Boor 1988; Ramos *et al.* 1988), pel metilglioxal en diversos tipus cel·lulars (Amore *et al.* 2003; Du *et al.* 2000; Okado *et al.* 1996), i pel formaldehid en cultius de cèl·lules vasculars endotelials (Tyihak *et al.* 2001; Yu and Zuo 1993) i del múscul llis (Hernandez *et al.* 2006). A més, aquest efecte tòxic sobre cèl·lules vasculars en cultiu causa danys semblants als canvis patològics observats en processos d'aterosclerosi (Lyles 1996; Lyles and Pino 1998), els quals són abolits mitjançant la inhibició de l'activitat enzimàtica de la SSAO (Conklin *et al.* 1998; Conklin *et al.* 1999; Yu 1998). En el cas de cèl·lules vasculars de múscul llis, s'ha descrit en un treball que aquestes són resistents a la toxicitat induïda pel metabolisme de la metilamina (Langford *et al.* 2001); sembla però, que l'obtenció d'aquests resultats podria estar condicionada a la poca expressió d'SSAO en les cèl·lules utilitzades, ja que en altres ocasions sí que s'ha descrit l'efecte citotòxic del metabolisme de la metilamina en cèl·lules vasculars de múscul llis (Hernandez *et al.* 2006).

Aquests efectes nocius s'han observat també en animals, on l'administració de substrats de la SSAO indueix l'acumulació dels productes resultants en el teixit vascular *in vivo*, principalment en els ronyons de rates diabètiques (Gubisne-Haberle *et al.* 2004; Yu and Zuo 1996), així com un increment dels nivells d'aldehids i de productes terminals de la peroxidació lipídica com el malondialdehid en l'orina d'aquests animals (Deng *et al.* 1998). En aquest sentit, l'administració d'inhibidors de la SSAO en rates diabètiques provoca una disminució de la nefropatia associada (Yu and Zuo 1997), en ratolins s'incrementa la metilamina excretada per l'orina, disminuint-se la quantitat de formaldehid i malondialdehid circulant, així com les lesions arterioscleròtiques (Yu *et al.* 2002), i en conills alimentats amb una dieta rica en

colesterol es redueix la formació de plaques d'ateroma (Panagiotopoulos *et al.* 1998). D'altra banda, en estudis amb ratolins transgènics que sobreexpressen la SSAO/VAP-1 en endoteli s'ha observat com la inhalació de metilamina és capaç de generar una important resposta inflamatòria pulmonar amb la corresponent patologia associada, fet que no s'observa en animals WT, suggerint que l'increment d'activitat SSAO juga un paper clau en l'aparició de vasculopatia, més que l'increment del nivell de substrats (Yu *et al.* 2006). En el mateix sentit, aquests animals transgènics mostren una expressió basal diferencial de proteïnes hepàtiques sensores d'estats oxidatius respecte els animals WT, indicant una resposta a l'elevada propensió d'aquests animals a la generació d'H_2O_2 com a agent oxidatiu (Stolen *et al.* 2004b).

Respecte als teixits humans, s'ha detectat també una acumulació de productes AGE i d'adductes formats per aldehids en situacions patològiques com l'arteriosclerosi, les disfuncions renals, les complicacions vasculars associades a la diabetis, la insuficiència renal o la malaltia d'Alzheimer (Brownlee 2000; Lovell *et al.* 2001; Mathys *et al.* 2002; Sakata *et al.* 2003; Yu and Zuo 1997). En aquest aspecte, les observacions anteriors juntament amb els resultats obtinguts *in vitro* i els estudis amb animals han fet que s'associï l'excessiva activitat de la SSAO a l'inici o acceleració del dany vascular observat en aquestes mateixes condicions patològiques (Garpenstrand *et al.* 1999a; Yu *et al.* 1994a; Yu *et al.* 1997; Yu 1998; Yu 2001). D'altra banda, el mateix procés de resposta inflamatòria pot comportar l'acumulació de metabòlits tipus amina alliberats de les cèl·lules danyades, accelerant així el dany tissular ja iniciat (Smith *et al.* 1998). En aquest sentit, s'ha postulat que la inhibició de l'activitat SSAO podria reduir la producció d'agents angiotòxics i de complicacions vasculars (Ekblom 1998; Kinemuchi *et al.* 2004; Yu 1998).

2.9. Estratègies terapèutiques que inclouen la SSAO

Les estratègies terapèutiques més importants que inclouen modificacions de l'activitat SSAO estan relacionades amb la seva funció pro inflamatòria i insulinomimètica. D'altra banda, el fet que els nivells d'SSAO plasmàtica es trobin modificats en diverses patologies, i que correlacionin amb la seva severitat ha suggerit un possible paper de la SSAO en el diagnòstic i pronòstic d'aquestes.

2.9.1. Inhibició de la SSAO en situacions d'inflamació.

Com s'ha descrit anteriorment, moltes malalties inflamatòries cursen amb un increment de l'expressió/activitat de la SSAO/VAP-1. En aquest sentit, s'ha demostrat àmpliament el paper que té la SSAO en promoure l'adhesió i entrada dels leucòcits als teixits, tant mitjançant mecanismes dependents com no-dependents de l'activitat enzimàtica (Jalkanen and Salmi 2008; Koskinen *et al.* 2004). Un dels mecanismes pels quals la inflamació mateixa pot generar complicacions és l'excessiva acumulació de leucòcits en els teixits on es produeix la inflamació. A més, els productes resultants de l'activitat SSAO, fonamentalment l'H_2O_2, poden contribuir també a la regulació a l'alça d'altres proteïnes pro inflamatòries, o fins i tot incrementar el dany subsegüent a l'estat inflamatori (Jalkanen *et al.* 2007; Lalor *et al.* 2007; Yu *et al.* 2006). Per tots aquests factors, s'ha suggerit que la SSAO/VAP-1 podria ser una diana terapèutica interessant en teràpia antiinflamatòria.

Així, l'objectiu principal d'aquesta teràpia consistiria en bloquejar les funcions d'adhesió de la proteïna per prevenir la transmigració leucocitària als teixits inflamats, i alleujar-ne la condició inflamatòria. Experimentalment, aquest efecte s'ha intentat dur a terme tant mitjançant anticossos específics contra el domini d'adhesió de la SSAO/VAP-1, com mitjançant inhibidors específics de l'activitat enzimàtica, donada la doble implicació de l'enzim en aquesta funció d'adhesió. La inhibició de l'activitat enzimàtica, a més d'atenuar l'adhesió, prevé també l'alliberament dels productes actius del seu metabolisme, contribuint a la disminució de la resposta inflamatòria i de la toxicitat generada, la qual pot ser una font de complicació de moltes malalties on els substrats de l'enzim es troben també incrementats. L'aplicació d'aquesta possible teràpia en models d'inflamació ha resultat en l'atenuació de la resposta inflamatòria *in vivo*, tant pel tractament amb anticossos (Bonder *et al.* 2005; Merinen *et al.* 2005; Tohka *et al.* 2001), com amb inhibidors (Kinemuchi *et al.* 2004; Kiss *et al.* 2008; Koskinen *et al.* 2004; Marttila-Ichihara *et al.* 2006; Noda *et al.* 2008a; Noda *et al.* 2008b; O'Rourke *et al.* 2007; Salter-Cid *et al.* 2005; Xu *et al.* 2006). Tot i l'eficàcia d'ambdós estratègies, de cara a la comercialització sembla més factible l'administració oral de molècules inhibidores que l'administració intravenosa d'agents biològics com els anticossos.

A més de les patologies en les que ja s'ha descrit un efecte beneficiós del bloqueig/inhibició de la SSAO, altres situacions patològiques en les que aquesta estratègia terapèutica ha estat proposada són l'obesitat (Bour *et al.* 2007; Raimondi *et al.* 2007), o la psoriasi (Madej *et al.* 2007). De forma semblant, i degut al fet que s'ha

observat un increment de la SSAO en situacions de rebuig d'òrgans trasplantats (Boomsma *et al.* 2003; Kurkijarvi *et al.* 2001; Martelius *et al.* 2004; Martelius *et al.* 2008), s'ha proposat la inhibició de la SSAO com a mecanisme immunosupressor en aquestes situacions.

Respecte a la seguretat del tractament, és important mencionar que ratolins KO per la SSAO/VAP-1 presenten nivells reduïts d'interacció leucòcit-endoteli i resistència a la inducció de patologies inflamatòries (Stolen *et al.* 2005), però no mostren més susceptibilitat que els WT a patir infeccions espontànies (Koskinen *et al.* 2007). D'altra banda, s'ha comprovat que la inhibició de forma aguda de la SSAO en malalties inflamatòries no compromet la capacitat de combatre infeccions (Koskinen *et al.* 2007). Aquests resultats i els obtinguts amb anticossos desenvolupats contra la SSAO/VAP-1 per a ús terapèutic en humans (Kirton *et al.* 2005), els quals estan essent avaluats en assajos clínics en Fase I i no han produït efectes adversos fins al moment (Vainio *et al.* 2005), confirmen la seguretat en l'aplicació del tractament. Tot i així, s'han d'extremar les precaucions en aquest tipus de tractaments ja que s'han observat casos en els que s'ha associat la inhibició de la SSAO amb el desenvolupament de vasculotoxicitat i dilatació de l'aorta toràcica en rates (Langford *et al.* 1999). Així mateix, assajos clínics desenvolupats amb anticossos contra altres receptors d'adhesió s'han hagut de suspendre temporalment degut a l'aparició d'infeccions virals del sistema nerviós central en alguns dels pacients (Yousry *et al.* 2006).

2.9.2. Modulació de l'activitat SSAO en diabetis.

La SSAO participa en el metabolisme de la glucosa mitjançant la seva funció insulinomimètica, però l'increment dels seus substrats i de la seva activitat en situacions diabètiques és en part responsable de les complicacions microvasculars com la retinopatia, la nefropatia o la neuropatia associades a aquesta patologia (Garpenstrand *et al.* 1999a; Gronvall-Nordquist *et al.* 2001; Mathys *et al.* 2002).

Inicialment, la intervenció sobre la SSAO en el tractament de la diabetis es va plantejar com una inhibició de la seva activitat enzimàtica per tal de minimitzar els efectes tòxics dels productes generats (Ekblom 1998). Més tard es va observar que l'administració crònica d'inhibidors de la SSAO no tenia cap efecte positiu sobre la hiperglucèmia, en comparació amb la millora després de l'administració de substrats de la SSAO (Visentin *et al.* 2005). A més, l'administració de substrats no induïa l'aparició d'efectes

tòxics, ja que aquests desplaçaven l'oxidació de la metilamina i per tant disminuïen la generació de formaldehid. Així doncs, l'estratègia terapèutica va canviar, i es centra actualment en la possible administració de substrats de la SSAO com a agents antidiabètics (Yraola *et al.* 2006; Yraola *et al.* 2009). Com en el cas dels inhibidors en el possible tractament antiinflamatori, actualment s'estan desenvolupant noves molècules que actuïn com a substrats específics de la SSAO, amb capacitat insulinomimètica per utilitzar-los com a agents antidiabètics (Dunkel *et al.* 2008; Garcia-Vicente *et al.* 2007; Yraola *et al.* 2007b; Yraola *et al.* 2009; Zorzano *et al.* 2009).

D'altra banda, una altra estratègia descrita per a la prevenció de l'aparició de complicacions associades a la diabetis consisteix en l'administració d'un quelant de coure (trietilentetraamina (TETA)). Els primers resultats han mostrat que l'administració d'aquest compost disminueix els nivells de proteïna SSAO al ronyó de rates diabètiques, i prevé l'aparició de nefropatia diabètica (Gong *et al.* 2008).

2.9.3. Possible utilització de la SSAO en diagnòstic i pronòstic.

Ja que els nivells d'SSAO plasmàtica es troben modificats en certes patologies, i en moltes d'aquestes correlacionen amb la seva severitat o amb la possible aparició de complicacions, s'ha suggerit que la determinació dels nivells d'SSAO seria d'utilitat en el diagnòstic i pronòstic d'aquestes.

Per exemple, s'ha postulat que la mesura de l'activitat plasmàtica de la SSAO pot ser un biomarcador d'etapes inicials d'aterosclerosi i un predictor de l'aparició de complicacions macrovasculars diabètiques (Karadi *et al.* 2002). En aquest sentit, recentment s'ha descrit que després de l'administració oral de glucosa es produeix un increment agut d'SSAO plasmàtica que correlaciona amb factors de risc d'arteriosclerosi com l'increment d'estrès oxidatiu i d'AGEs en plasma. Aquests resultats han suggerit que els nivells d'increment d'SSAO assolits després de l'administració de glucosa es podrien utilitzar com un marcador d'arteriosclerosi induïda per hiperglucèmia (Li *et al.* 2009).

És el cas també de l'osteoartritis. En aquesta patologia s'utilitza el concepte de "signatura proteica" en referència a l'ús de la determinació dels nivells de certes proteïnes en diferents estadis de la patologia: pre-diagnòstic per raigs X, diagnòstic i establiment/prevalença de la patologia. En aquest sentit, la SSAO forma part del grup

de proteïnes que es troben incrementades en els tres estadis, de manera que es podria utilitzar juntament amb altres proteïnes, com a indicador de pronòstic i predictor de la resposta inflamatòria (Ling *et al.* 2009).

Això també seria aplicable a situacions de càncer colorectal, on s'observen nivells plasmàtics d'SSAO lleugerament superiors en aquests pacients que en els controls, i els nivells d'SSAO van disminuint amb la progressió de la malaltia. En aquest cas, els nivells més baixos d'SSAO en situacions preoperatòries correlacionarien amb un pitjor pronòstic, incloent l'aparició de metàstasis hepàtiques i limfàtiques, encara que aquestes no s'hagin detectat en el moment de la intervenció quirúrgica. La determinació dels nivells d'SSAO en aquests pacients podria proveir d'informacions molt valuoses en termes de predicció de l'evolució dels pacients (Toiyama *et al.* 2009).

D'altra banda, s'ha plantejat també la possible utilització de la SSAO com a molècula diana per localitzar i estudiar processos d'inflamació *in vivo* mitjançant tècniques d'imatge (Jaakkola *et al.* 2000b). En aquest sentit, recentment s'ha descrit un pèptid traçador radioactiu (^{68}Ga-DOTAVAP-P1 (*gallium-68-labeled 1,4,7,10-tetraazacyclododecane-N',N'',N''',N''''-tetraacetic acid-peptide targeted to VAP-1*)) capaç d'unir-se a la SSAO/VAP-1 i ser observat, mitjançant PET (tomografia per emissió de positrons). Aquest sistema permetria determinar llocs d'inflamació o infecció *in vivo*, com s'ha observat en models animals d'osteomielitis difusa (Lankinen *et al.* 2008).

3. Mort Cel·lular

La mort cel·lular, tant a nivell fisiològic com patològic, és un procés programat a nivell cel·lular, i sovint és un procés actiu ja que requereix activació genètica i síntesi de proteïnes. A nivell fisiològic, la mort cel·lular és necessària per al correcte desenvolupament de l'organisme i requereix un control tan regulat com els processos de creixement i proliferació. Aquesta vessant de la mort cel·lular es pot anomenar "mort cel·lular fisiològica", i va ser descrita durant el desenvolupament d'organismes vertebrats per Carl Vogt, el 1842. D'altra banda, l'excés de mort cel·lular pot ser el reflex d'alteracions fisiològiques associades a la patogènesi d'una malaltia, com per exemple les malalties degeneratives, o l'absència de mort també pot representar una patologia, com ara el càncer.

L'inici, progressió o inhibició de la mort cel·lular està regulada per diferents estímuls i intensitats d'aquests, i pot transcórrer a través de diverses vies de senyalització molecular. Aquestes vies de senyalització, a més, es comuniquen entre elles per mecanismes moleculars, generant connexions que poden modificar la progressió del procés de mort.

3.1. Principals mecanismes de mort cel·lular: apoptosi, necrosi, autofàgia i altres

Actualment hi ha un elevat consens en considerar l'existència de tres grans tipus principals de mort cel·lular: apoptosi, necrosi i autofàgia. Tot i així, existeixen altres formes de mort cel·lular, algunes no considerades com tipus de mort diferenciats, i altres poc caracteritzades bioquímicament o específiques de tipus cel·lulars concrets. L'NCCD (*Nomenclature Commitee on Cell Death*) formula periòdicament recomanacions per la correcta nomenclatura dels diferents tipus de mort, considerant aspectes morfològics, enzimològics, funcionals o immunològics (Kroemer *et al.* 2009), on a part dels tres mencionats anteriorment, s'inclouen la "catàstrofe mitòtica", la "paraptosi", la "oncosi", la "piroptosi", la "pironecrosi", l'"excitotoxicitat", l'"anoikis", la "degeneració Walleriana", la "cornificació" o l'"entosi". La figura 13 mostra les principals característiques d'algunes d'aquestes formes de mort.

La **catàstrofe mitòtica** es produeix en resposta a un procés mitòtic anormal, i sovint s'acompanya de micronucleació. Hi ha autors que la consideren un procés dins de la mort apoptòtica o necròtica (Vakifahmetoglu *et al.* 2008), i altres que la consideren un tipus de mort diferent (Castedo *et al.* 2004). Durant el seu procés intervenen cinases específiques del cicle cel·lular, p53, caspases i membres de la família de Bcl-2 (Castedo *et al.* 2004).

La **paraptosi** és una mort cel·lular caspasa-independent controlada per proteïnes cinasa activades per mitògens (MAPKs), que transcorre amb vacuolització citoplasmàtica, inflament mitocondrial i absència de canvis nuclears com la condensació de la cromatina o la fragmentació del DNA (Sperandio *et al.* 2000). És morfològica i bioquímicament diferent de l'apoptosi i s'ha identificat en processos del desenvolupament i també en models transgènics de malaltia de Huntington i esclerosi lateral amiotròfica (Dal Canto and Gurney 1994; Turmaine *et al.* 2000).

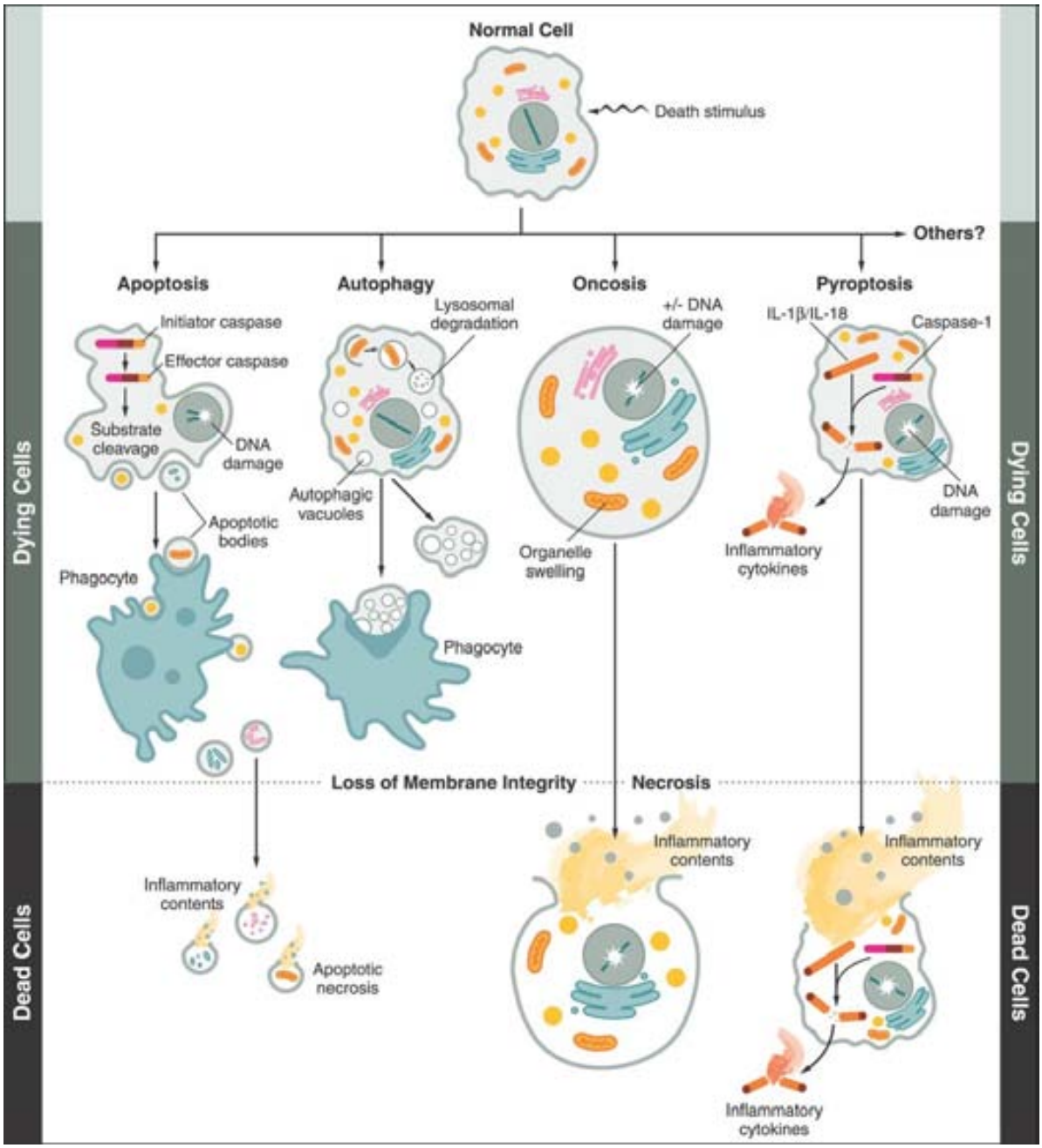


Figura 13. Diferents tipus de vies que porten a la mort cel·lular, amb les seves principals característiques. Figura extreta de la revisió (Fink and Cookson 2005).

La **oncosi** és una via de "premort" que condueix a la necrosi, caracteritzada per l'inflament dels orgànuls intracel·lulars i l'increment de la permeabilitat membranal (Majno and Joris 1995). El procés de la oncosi porta a l'esgotament de les reserves d'energia cel·lulars i a la fallada de les bombes iòniques de la membrana plasmàtica, generant un estat pro inflamatori degut al trencament de la permeabilitat membranal.

Aquest tipus de mort s'ha descrit en processos d'isquèmia així com d'infecció per diversos patògens (Dacheux *et al.* 2000; Majno and Joris 1995; Perez *et al.* 1998).

La **piroptosi** és una mort cel·lular dependent de la caspasa 1, una caspasa no involucrada normalment en el procés apoptòtic (Hilbi *et al.* 1997). Aquesta via s'ha descrit en macròfags infectats per patògens, els quals activats per la infecció activen la caspasa 1, que activa les citocines inflamatòries IL-1β i IL-18 i aquestes provoquen la mort de la cèl·lula hoste (Monack *et al.* 2000). El procés transcorre amb l'activació d'inflamació, fet que la diferencia de l'apoptosi. A part del sistema immunitari (Shi *et al.* 1996), la piroptosi s'ha observat també en tipus cel·lulars del sistema nerviós central (Zhang *et al.* 2003) o del sistema cardiovascular (Frantz *et al.* 2003).

La **pironecrosi** sembla una variant de la piroptosi, però se'n diferencia per no requerir l'activació de la caspasa 1, a diferència de la piroptosi (Willingham *et al.* 2007).

L'**excitotoxicitat** s'activa en neurones quan aminoàcids excitadors com el glutamat indueixen l'obertura dels canals NMDA, fet que provoca l'entrada de calci al citosol, i l'activació de vies de senyalització de mort (Orrenius *et al.* 2003). La seva regulació es solapa amb els mecanismes apoptòtics i necròtics, de manera que no es considera una forma diferent de mort (Kroemer *et al.* 2009).

L'**anoikis** és l'apoptosi induïda per la pèrdua de l'adhesió d'una cèl·lula al substrat o a les cèl·lules veïnes (Grossmann 2002). Els mecanismes moleculars que la regulen semblen els clàssics de l'apoptosi.

La **degeneració Walleriana** ocorre en el sistema nerviós, i es produeix quan una part d'una neurona o axó degenera sense afectar el cos cel·lular principal (Raff *et al.* 2002). No es considera estrictament un tipus de mort ja que les cèl·lules que pateixen aquest procés sovint segueixen vives (Kroemer *et al.* 2009).

La **cornificació** o **queratinització** és una forma específica de mort cel·lular programada que té lloc a l'epidermis (Candi *et al.* 2005). Duu a la formació dels "corneòcits", que són queratinòcits morts contenint una amalgama de proteïnes específiques com la queratina i de lípids com ceramides, necessaris per mantenir la seva elasticitat, impermeabilitat i estabilitat estructural.

L'**entosi** és una nova modalitat de mort cel·lular descrita, en la que una cèl·lula engull una altra cèl·lula veïna que està viva, la qual mor dins el fagosoma de la primera (Overholtzer *et al.* 2007). S'ha observat en limfoblasts de pacients amb la malaltia de

Huntington, i també en cèl·lules MCF-7 (línia cel·lular de càncer de mama). Es creu que és una via emmascarada per defecte, ja que només s'observa quan altres mecanismes estan suprimits (per exemple en el cas de les MCF-7, que són apoptosi i autofagia-incompetents).

La **necrosi** s'ha descrit clàssicament com un procés de mort cel·lular no programat i passiu, o representant el pas final d'altres processos de mort, com ara de l'apoptosi o la oncosi. Es caracteritza per un inflament cel·lular i lisi d'aquesta, amb la pèrdua de la integritat de membrana i la corresponent alliberació del contingut intracel·lular a l'exterior, generant inflamació. El fet que sigui un procés passiu, però, està essent qüestionat davant l'observació de processos de mort amb morfologia necròtica però subjectes a un programa de mort actiu i regulat, caspasa dependent o independent, als que s'ha anomenat "mort cel·lular programada *necrotic-like*" (Edelstein *et al.* 1999; Kitanaka and Kuchino 1999; Zong and Thompson 2006).

L'**autofàgia** consisteix en la degradació de components cel·lulars sense el trencament de la membrana citoplasmàtica, i per tant sense generar inflamació al seu voltant (Cohen 1991; Noda *et al.* 2002). Dependent del tipus cel·lular pot transcórrer amb o sense condensació nuclear (Jones 2000). En l'autofàgia, el citoplasma i els seus orgànuls són segrestats en vesícules multimembranals que es fusionen amb els lisosomes per ser degradats en un procés dependent de la síntesi de proteïnes i de la presència d'ATP. Es creu que aquest procés s'activa en principi com un mecanisme de supervivència per obtenir energia de la degradació dels propis orgànuls, però davant l'absència d'una font d'energia alternativa portaria finalment a la mort cel·lular. Aquest tipus de mort s'ha observat durant el desenvolupament, en malalties humanes i en respostes cel·lulars a la deprivació de nutrients.

A part dels tipus de mort cel·lular explicats anteriorment, i de l'apoptosi, que s'explica més en detall a continuació, existeixen altres tipus de mort que comparteixen trets distintius de diverses formes de mort alhora. L'"aponecrosi", per exemple, és un terme introduït per definir un tipus de mort amb característiques dinàmiques, moleculars i morfològiques d'apoptosi i de necrosi alhora (Formigli *et al.* 2000). Així mateix existeix relació entre l'apoptosi i l'autofàgia, ja que ambdues comparteixen mecanismes bioquímics com la regulació d'Akt (proteïna cinasa B), o el control per Bcl-2 (Pattingre *et al.* 2005). Aquesta interconnexió existent entre els diferents tipus de mort apoptòtica, necròtica i autofàgica suggereix que tots ells representen diferents expressions morfològiques d'una xarxa bioquímica compartida, en la que la ruta de mort cel·lular

depèn de diversos factors com ara el medi que les envolta, l'estat de desenvolupament, el tipus de teixit i l'origen de la senyal de mort (Zeiss 2003).

3.2. Apoptosi

L'apoptosi és un dels tipus més estudiats de mort cel·lular programada. Va ser observada ja a finals del segle XIX per Flemming durant la maduració dels fol·licles ovàrics, i la va anomenar *cromatolisi*. Després se li han anat atribuint altres noms, fins que al 1972, Kerr i col·laboradors van dur a terme la caracterització de la seqüència fenotípica del procés (Kerr *et al.* 1972).

Els mecanismes moleculars implicats en l'apoptosi estan força conservats a nivell evolutiu (Metzstein *et al.* 1998), i es van començar a estudiar mitjançant el model de desenvolupament del nematode *Caenorhabditis elegans* (Horvitz 1999), durant el qual es generen 1090 cèl·lules somàtiques, 131 de les quals moren en punts concrets del procés per apoptosi. De la mateixa manera que en el desenvolupament d'aquest nematode, l'apoptosi també ocorre durant el desenvolupament i creixement dels mamífers com a mecanisme homeostàtic fisiològic per mantenir les poblacions cel·lulars dels teixits. A més, també s'activa com un mecanisme de defensa en les reaccions immunològiques o quan les cèl·lules estan danyades com a conseqüència d'una patologia o d'un agent tòxic (Norbury and Hickson 2001).

3.2.1. Característiques de l'apoptosi.

Les cèl·lules que moren per un procés apoptòtic mostren unes característiques definides, algunes de les quals poden ser comuns a altres vies de mort. Les característiques morfològiques, descrites inicialment per Kerr i col·laboradors es representen a la figura 14 i inclouen:

- Arrodoniment de la cèl·lula, amb retracció dels pseudopodis i reducció del volum cel·lular
- Condensació de la cromatina (o *pyknosis*)
- Fragmentació nuclear (o *karyorhexis*)
- Pocs o absents modificacions ultraestructurals dels orgànuls citosòlics

- Embutllofament de la membrana plasmàtica amb manteniment de la seva integritat fins a les etapes finals del procés
- Formació dels cossos apoptòtics i engulliment pels fagocits residents *in vivo* mitjançant els fagolisosomes

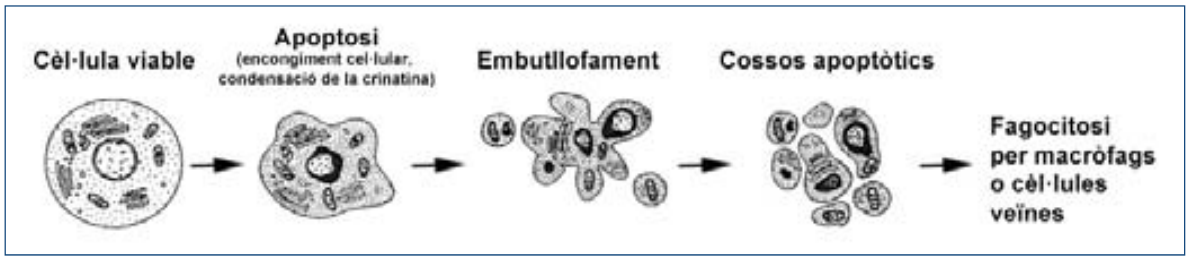


Figura 14. Il·lustració del procés apoptòtic segons Majno i Joris (1995); modificació de la figura extreta de l'article (Van and Van Den 2002).

A nivell bioquímic, els mecanismes apoptòtics són complexos i sofisticats, i conformen una cascada molecular dependent d'energia. Les cèl·lules apoptòtiques mostren unes característiques bioquímiques definides:

- Talls proteolítics mitjançant activitats caspasa o altres (Cohen 1997)
- *Cross-linkings* entre proteïnes (Nemes, Jr. *et al.* 1996)
- Trencament del DNA per les endonucleases en fragments d'uns 180-200 parells de bases (Bortner *et al.* 1995)
- Exhibició de marcadors de superfície per al reconeixement fagocític (Bratton *et al.* 1997)

Les cèl·lules apoptòtiques mostren diverses de les característiques citades, encara que poden no manifestar-les totes. Per exemple, es pot desenvolupar una mort apoptòtica sense fragmentació del DNA.

3.2.2. Principals vies apoptòtiques.

L'apoptosi es pot activar i dur a terme per diferents vies moleculars (intrínseca, extrínseca, dependent del reticle, ...) i amb la participació de diferents grups proteics (p. ex. caspasa dependent o independent), existint també camins de comunicació i transactivació entre aquestes vies moleculars (Igney and Krammer 2002).

A partir de l'estudi amb cèl·lules en cultiu s'han identificat senyals intracel·lulars que condueixen a l'apoptosi mitjançant dues principals vies apoptòtiques: la extrínseca i la intrínseca, detallades més endavant. La via extrínseca s'inicia mitjançant l'estimulació dels receptors de mort, com per exemple el receptor de Fas o el de Tnf-α, situats a la membrana plasmàtica (Yuan 1997). La via intrínseca es promou mitjançant l'alliberament de molècules proapoptòtiques de la mitocòndria al citosol, on activen l'execució del procés apoptòtic (Li *et al.* 1997).

A part de les vies extrínseca i intrínseca existeixen altres vies apoptòtiques descrites més recentment, com són la via de l'estrès reticular i la via de la perforina/granzima. La via de l'estrès reticular, s'activa en el reticle endoplasmàtic en resposta a un mal plegament de proteïnes o a alteracions en l'homeòstasi del calci (Rao *et al.* 2002) (figura 15).

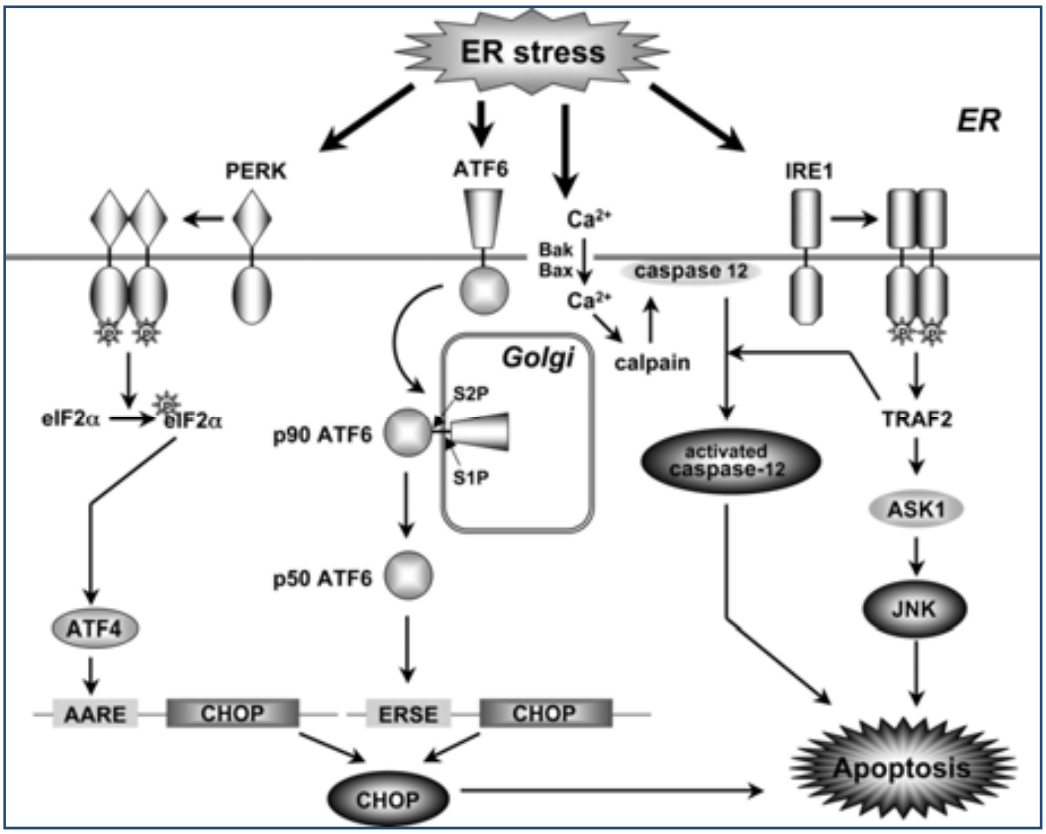


Figura 15. Esquema de la inducció de la UPR proapoptòtica per la via de l'estrès reticular. L'activació de la via de PERK-eIF2 indueix el factor de transcripció ATF4. ATF4 indueix l'expressió de CHOP (pro apoptòtic) mitjançant l'activació de l'element de resposta a amino àcid (AARE). La via d'ATF6 i IRE1 indueix també l'expressió de CHOP mitjançant l'activació d'ERSE. L'estrès reticular activa també la caspasa 12 (o caspasa 4 en humans), localitzada a la membrana del reticle, mitjançant la interacció amb IRE1 i TRAF2, fet que promou l'apoptosi. La interacció entre IRE1 i TRAF2 també permet el reclutament i activació d'ASK1, i JNK, la qual està implicada en una gran varietat de senyals proapoptòtiques. Esquema extret de (Kitamura 2008).

Aquest estrés en el reticle endoplasmàtic indueix l'activació de la UPR (*Unfolded Protein Response*), la qual en primer lloc intenta superar la situació d'estrès, però en mantenir-se l'estímul pot conduir a la mort de la cèl·lula. En la UPR hi participen diferents vies moleculars que porten a la inducció de l'expressió de xaperones per ajudar al correcte plegament de les proteïnes, a l'atenuació general de la traducció o a l'activació d'una via de degradació de proteïnes associada al reticle endoplasmàtic. Hi intervenen proteïnes com la caspasa 12 (Nakagawa *et al.* 2000) o el seu ortòleg humà caspasa 4 (Hitomi *et al.* 2004), GRP78 o proteïnes d'unió a "X-box" (XBP) (Reimold *et al.* 2000).

La via de la perforina/granzima és utilitzada pels limfòcits T citotòxics per induir la mort de cèl·lules tumorals o infectades (figura 16).

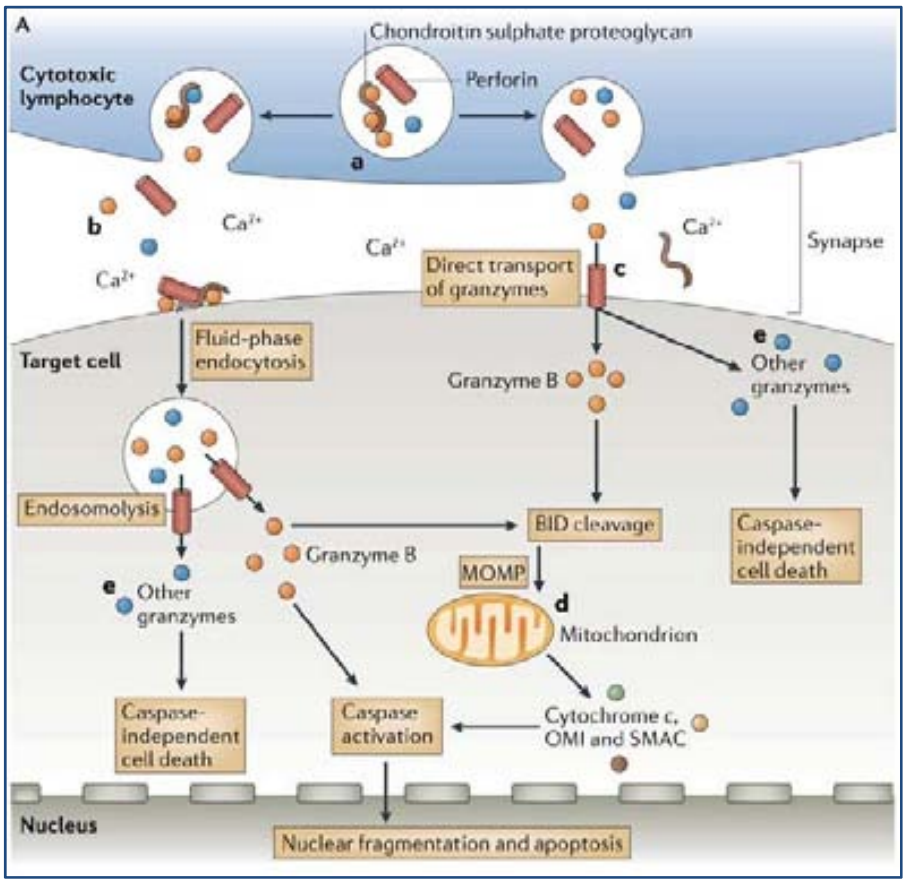


Figura 16. Esquema de la via apoptòtica activada per les perforines/granzims. Els granzims (proteïnes bàsiques) s'emmagatzemen associats a proteoglicans, i se'n dissocien quan s'alliberen a l'escletxa de contacte, on el pH és neutre, s'uneixen a la membrana i són endocitats. D'altra banda, les perforines s'uneixen a la membrana de la cèl·lula diana, i quan arriben a una concentració prou elevada, polimeritzen formant porus transmembranals de 10-20 nm de diàmetre a través dels quals els granzims poden difondre a la cèl·lula diana. Quan els granzims arriben al citosol de la cèl·lula diana, indueixen apoptosi preferencialment activant la via intrínseca o mitocondrial (granzima B) o també activant altres processos pro apoptòtics caspasa-independents (granzima A). Esquema extret de l'article (Voskoboinik *et al.* 2006).

Aquesta via consisteix en la secreció de la perforina per part dels limfòcits, la qual forma porus transmembrana entre ambdues cèl·lules a través dels quals el limfòcit introdueix a l'altra cèl·lula gränuls citoplasmàtics, compostos bàsicament de granzima A i B (Pardo *et al.* 2004; Trapani and Smyth 2002). La granzima B pot activar la procaspasa 10, la via mitocondrial o activar directament la caspasa 3 a la cèl·lula diana (Russell and Ley 2002). La granzima A, d'altra banda, activa processos independents de caspases com la degradació del DNA (Lieberman and Fan 2003).

3.2.2.1. Via intrínseca o mitocondrial

La via apoptòtica intrínseca o mitocondrial s'inicia mitjançant una sèrie d'estímuls no dependents de receptors, els quals generen senyals intracel·lulars que inicien processos a nivell de la mitocòndria. Aquests estímuls poden actuar de manera positiva o negativa, segons si activen directament la via apoptòtica, o si indueixen una fallada dels mecanismes antiapoptòtics.

Els processos activats indueixen canvis en la membrana mitocondrial interna que resulten en una obertura dels porus de permeabilitat transitoris (MPTP – *Mitochondrial Permeability Transition Pore*), amb la conseqüent pèrdua del potencial de membrana mitocondrial i l'alliberament de dos grups de proteïnes pro-apoptòtiques al citosol, les quals normalment romanen a l'espai intermembranal mitocondrial (figura 17) (Garrido *et al.* 2006; Saelens *et al.* 2004).

- El primer grup de proteïnes alliberat activa la via mitocondrial dependent de caspases i inclou citocrom *c*, Smac/DIABLO i la serin-proteasa HtrA2/Omi. El citocrom *c* s'uneix i activa a Apaf-1 i a la procaspasa 9, formant l'"apoptosoma" (Chinnaiyan 1999). Smac/DIABLO i HtrA2/Omi promouen l'apoptosi inhibint l'activitat de les IAPs (proteïnes inhibidores de l'apoptosi) (van Loo *et al.* 2002).

- El segon grup de proteïnes alliberat inclou AIF, l'endonucleasa G i CAD (DNasa activada per caspases), i es produeix de forma més tardana en el procés apoptòtic. AIF transloca al nucli i causa la fragmentació del DNA en fragments de 50 a 300 Kb (Joza *et al.* 2001). L'endonucleasa G també transloca al nucli, on talla la cromatina per formar fragments oligonucleosomals de DNA (Li *et al.* 2001). Les dues anteriors funcionen de manera caspasa independent. CAD també transloca al nucli on, després de

ser tallada per la caspasa 3 s'activa i indueix la fragmentació oligonucleosomal del DNA (Enari *et al.* 1998).

La regulació d'aquests processos a nivell de la mitocòndria es produeix per part de membres de la família de Bcl-2 (Cory and Adams 2002) i de la proteïna supressora de tumors p53, entre altres (Schuler and Green 2001).

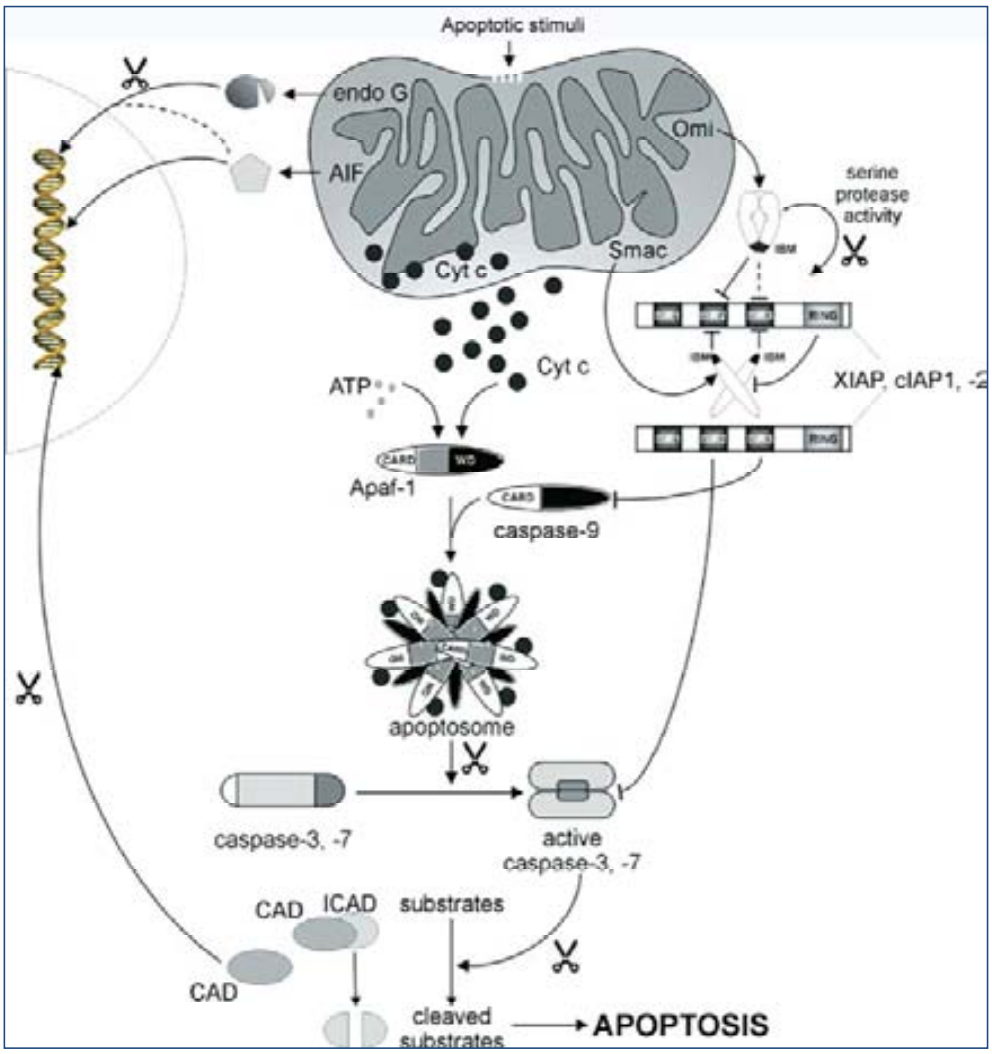


Figura 17. Alliberament de proteïnes mitocondrials durant l'activació de la via intrínseca de l'apoptosi. Modificació de l'esquema extret de l'article (Saelens *et al.* 2004).

3.2.2.2. Via extrínseca o de receptors de mort

La via extrínseca de l'apoptosi s'activa per interaccions de lligands amb receptors transmembrana (figura 18). Aquests receptors s'anomenen "receptors de mort", i

alguns són membres de la superfamília gènica del receptor del factor de necrosi tumoral (TNF) (Locksley *et al.* 2001). Es caracteritzen per compartir dominis extracel·lulars similars, rics en cisteïnes, i per tenir un domini citoplasmàtic d'uns 80 aminoàcids anomenat "domini de mort" (*death domain*) (Ashkenazi and Dixit 1998). Els lligands/receptors de mort més coneguts inclouen els FasL/FasR, TNF-α/TNFR1, Apo3L/DR3, Apo2L/DR4 i Apo2L/DR5 (Peter and Krammer 1998; Suliman *et al.* 2001). D'aquests, FasL/FasR i TNF-α/TNFR1 són dels més estudiats.

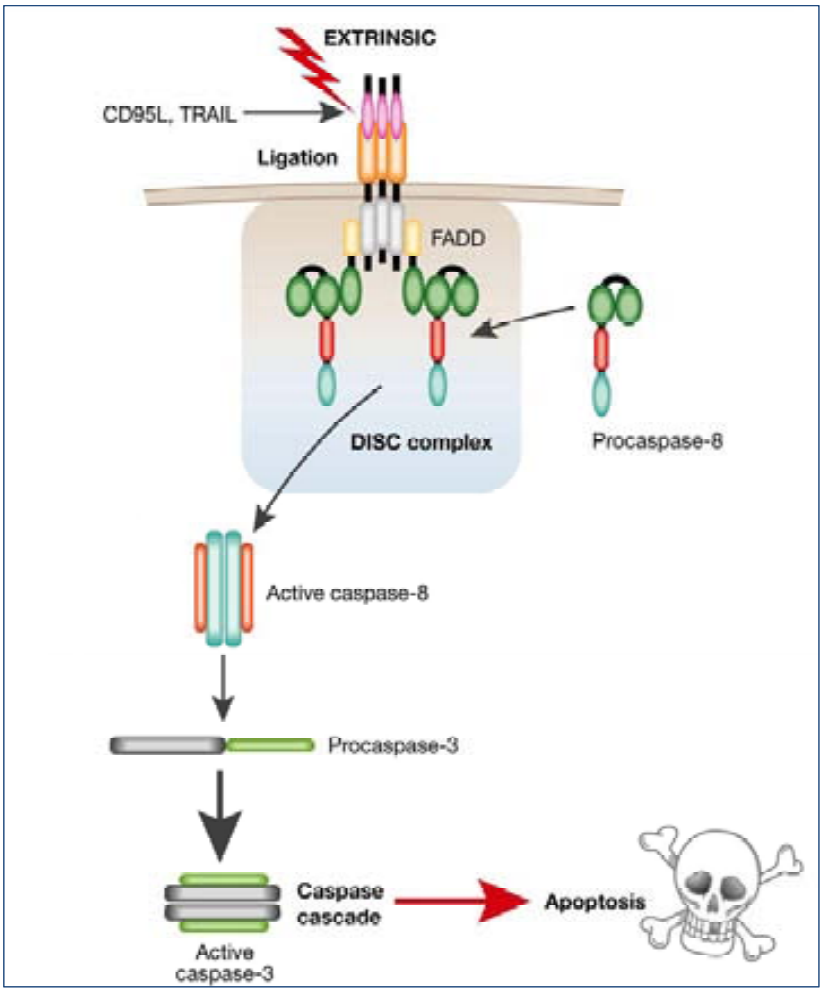


Figura 18. Esquema d'activació de la via apoptòtica extrínseca. Després de la unió del lligand al receptor de mort s'activa la procaspasa 8 mitjançant el seu reclutament cap al complex trimeritzat lligand-receptor (DISC) a través de la molècula adaptadora FADD. Esquema modificat de l'article (MacFarlane and Williams 2004).

En aquests models es produeix una associació de receptors després de la unió del lligand trimèric, i seguidament té lloc un reclutament de proteïnes adaptadores citosòliques que tenen dominis de mort (FADD i TRADD) (Hsu *et al.* 1995; Wajant

2002). FADD s'associa després amb la procaspasa 8 mitjançant la dimerització del domini efector de mort, formant-se el complex de senyalització inductor de mort (DISC – *death-inducing signaling complex*) que activa la procaspasa 8 per auto catàlisi (Kischkel *et al.* 1995), iniciant-se la fase executora de l'apoptosi. L'apoptosi dependent dels receptors de mort es pot inhibir per diferents mecanismes, com ara mitjançant la proteïna c-FLIP, que s'uneix a FADD i a la caspasa 8 deixant-les inefectives (Kataoka *et al.* 1998), o mitjançant la proteïna Toso, que inhibeix el processament de la procaspasa 8 (Hitoshi *et al.* 1998).

Existeixen mecanismes d'entrecreuament entre la via mitocondrial i la de receptors de mort, que permeten que una moduli l'activació de l'altra. Un exemple n'és el trencament de Bid per part de la caspasa 8: la caspasa 8 s'activa per la via de receptors de mort, i és capaç de trencar Bid, una proteïna pro apoptòtica mitocondrial de la família de Bcl-2 (Li *et al.* 1998a).

3.2.2.3. Fase d'execució de l'apoptosi: les caspases

Les vies de senyalització que condueixen a l'apoptosi convergeixen en la fase d'execució, considerada el camí final cap a l'apoptosi. La fase d'execució es duu a terme majoritàriament mitjançant l'activació de la família de proteases anomenades *"caspases"*, tot i que també pot ser caspasa-independent en el cas de ser executada per proteïnes com els granzims o les calpaïnes.

Les caspases són un tipus de cisteïna proteases (*cysteine-aspartate-proteases*) altament conservades en el procés evolutiu, que es caracteritzen per tallar proteïnes per residus d'àcid aspàrtic (Chowdhury *et al.* 2008). Es troben expressades de forma constitutiva en la majoria de cèl·lules en forma de zimògens o proenzims, i necessiten un processament proteolític per ser activades. Estan formades per tres dominis: un domini central de 17-21 KDa, anomenat domini p20, el qual conté el centre actiu; un domini petit C-terminal de 10-13 KDa, anomenat p10; i un domini N-terminal de 3-24 KDa anomenat domini de mort (DD – *death domain*). Els dominis DD de les caspases poden ser llargs o curts, i els llargs poden contenir dos subdominis: el DED (*death effector domain*) o el CARD (*caspase-recruitment domain*).

La família de les caspases en mamífers es pot subdividir en 3 subfamílies, segons l'estructura del domini N-terminal, les condicions en les que s'activi i el seu paper en la cascada apoptòtica (Lincz 1998) (figura 19). Dins el grup I trobem les caspases

implicades en el processament de citocines i la resposta inflamatòria, que normalment no participen en l'apoptosi; aquestes tenen el prodomini llarg i són les caspases 1, 4, 5, 11, 12, 13 i 14. El grup II conté les caspases iniciadores de l'apoptosi, que també tenen el prodomini llarg, i són les caspases 2, 8, 9 i 10. El grup III són les caspases executores de l'apoptosi, tenen prodominis curts i són les caspases 3, 6 i 7.

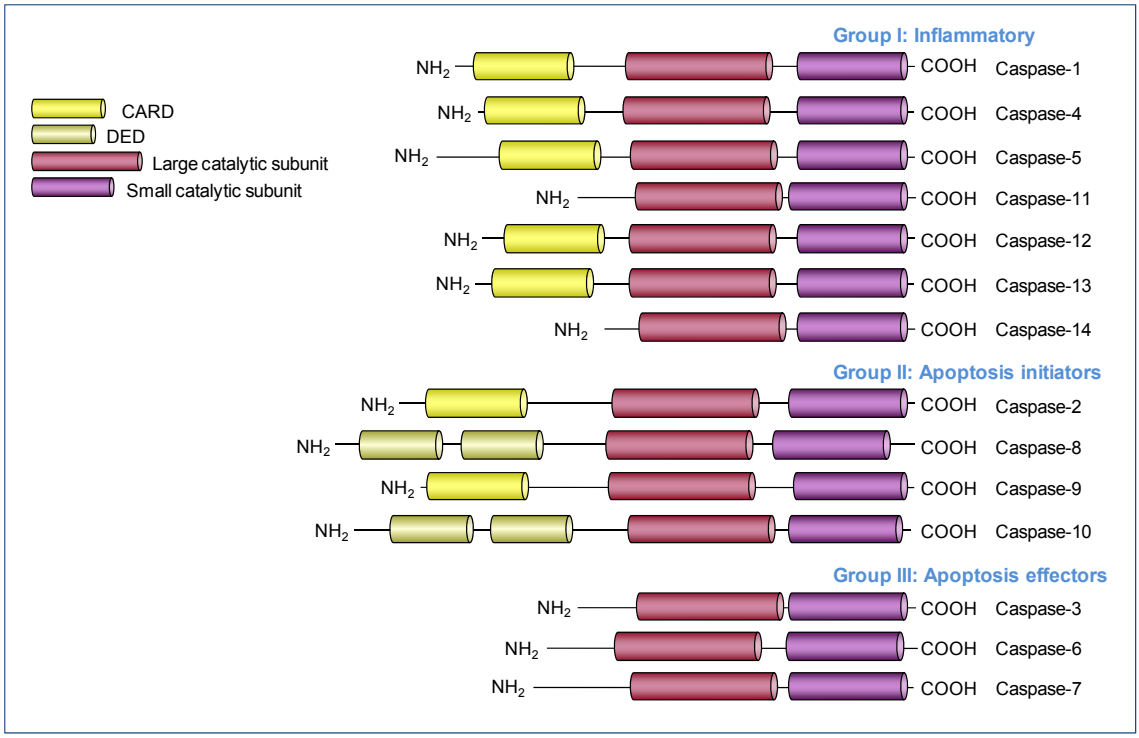


Figura 19. Classificació de la família de les caspases, on es presenten els tres grups en els que es troben agrupades. Grup I o de caspases inflamatòries; grup II o de caspases iniciadores de l'apoptosi; i grup III o de caspases efectores de l'apoptosi. S'indiquen els dominis CARD, DED, p20 (subunitat catalítica llarga) i p10 (subunitat catalítica petita) de cadascuna. Esquema adaptat de l'article (Rupinder *et al.* 2007).

En l'apoptosi, la forma d'activació de les caspases iniciadores i les efectores és diferent (Shi 2004). Les efectores són homodímers activats per les caspases iniciadores a través de processament proteolític, el qual els produeix un canvi de conformació que permet la unió del substrat al centre actiu. Les iniciadores, en canvi, estan com a zimogen en forma de monòmers, i s'activen amb l'ajuda de complexos adaptadors que estimulen la seva homodimerització, i aquesta els causa un canvi conformacional que fa que s'activin (Boatright *et al.* 2003). El factor més important, però, per a l'activació de les caspases iniciadores és el seu reclutament en plataformes

d'activació de caspases com ara l'apoptosoma, en la via intrínseca o el DISC, en la via extrínseca.

En el procés apoptòtic, l'activació de les caspases iniciadores promou l'activació de les caspases efectores. Les caspases efectores, després, degraden proteïnes estructurals i reguladores, i activen endonucleases que degraden el DNA, processos que causen finalment els canvis morfològics i bioquímics característics de les cèl·lules apoptòtiques. L'endonucleasa CAD és una de les que s'activen durant aquest procés: la caspasa 3 l'activa específicament, trencant el seu inhibidor (ICAD) al qual està acomplexat i alliberant-la així. Entre els substrats de les caspases efectores es troben citoqueratines, la proteïna PARP (*poly (ADP-ribose) polymerase*) implicada en la reparació del DNA (Kaufmann *et al.* 1993), les proteïnes del citoesquelet α-fodrina i gelsolina, o la proteïna nuclear NuMA, entre altres (Kothakota *et al.* 1997; Slee *et al.* 2001).

Un altre fenomen que té lloc en aquest procés és l'externalització de fosfatidilserina a la superfície de les cèl·lules apoptòtiques, fet que *in vivo* indueix el reconeixement fagocític de la cèl·lula apoptòtica de forma no inflamatòria. Es creu que aquest procés es dóna per la pèrdua de l'activitat aminofosfolípid translocasa, que indueix un "flip-flop" inespecífic de fosfolípids de diverses classes. Sembla que intervenen en aquest procés Fas, la caspasa 8 i la caspasa 3 (Bratton *et al.* 1997)

3.2.3. Proteïnes reguladores de l'apoptosi.

Les vies de mort desemboquen en processos catastròfics per la cèl·lula, i per això la seva activació està finament regulada. Existeixen diversos tipus de proteïnes que regulen aquestes vies a diferents nivells.

3.2.3.1. Proteïnes de la família de Bcl-2

Les proteïnes de la família de Bcl-2 controlen la integritat mitocondrial i l'activació de la cascada de mort intrínseca (Danial and Korsmeyer 2004). Aquestes proteïnes comparteixen dominis conservats, els dominis BH (*Bcl-2 Homology Domains*), i es subdivideixen funcionalment en pro i anti apoptòtiques. Les anti apoptòtiques inclouen

Bcl-2, Bcl-x_L, Bcl-w, BAG, Mcl-1 i A1, i es caracteritzen per contenir 4 dominis BH (BH1-4). Les pro apoptòtiques inclouen Bcl-10, Bax, Bak, Bid, Bad, Bim, Bik i Blk.

Les proteïnes anti apoptòtiques actuen a la membrana mitocondrial externa neutralitzant les pro apoptòtiques com Bax i Bak. Un cop activada la senyalització de mort, proteïnes BH3-only (membres de la família de Bcl-2 que només tenen una de les regions d'homologia BH, la BH3) com Bim, Bid, Puma, Bad, Noxa, Bmf, Hrk o Bik, s'uneixen a les proteïnes anti apoptòtiques per alliberar les pro apoptòtiques o, en el cas de Bim, Bid i Puma, poden activar directament Bax i Bak. Un cop alliberades o activades, Bax i Bak indueixen la permeabilització de la membrana mitocondrial externa i l'alliberament de les proteïnes apoptòtiques que romanen a l'espai intermembranal mitocondrial. Diferents tipus d'estrès poden activar diferents proteïnes BH3-only, les quals en condicions normals es troben no expressades o inactivades. Per exemple, el dany al DNA indueix l'expressió via p53 de Noxa i Puma (Villunger *et al.* 2003).

3.2.3.2. IAPs

Després de l'activació de les caspases, no sempre es produeix la mort de la cèl·lula. De fet, les caspases tenen altres funcions a part de la inducció de la mort cel·lular, com ara participar en la regulació del dinamisme de l'actina, en la immunitat innata, en la proliferació cel·lular, diferenciació i supervivència (Lamkanfi *et al.* 2007), i per això la seva activitat està regulada per diferents factors.

Les proteïnes que regulen algunes de les caspases un cop activades són membres de la família de proteïnes inhibidores de l'apoptosi (IAPs), encara que no totes les IAPs participen en la regulació de l'apoptosi. Les IAPs s'encarreguen de neutralitzar les caspases, efecte que aconsegueixen associant-s'hi i a través de la seva activitat ubiquitin lligasa. Aquesta neutralització desapareix quan una cèl·lula rep un estímul apoptòtic, gràcies a l'activitat dels antagonistes de les IAPs. En mamífers, aquests antagonistes es troben segrestats a la mitocòndria (Smac/DIABLO i Omi/HtrA2) o al reticle endoplasmàtic (GSPT1/eRF3), i s'alliberen al citosol en rebre l'estímul apoptòtic.

A part d'inhibir les caspases, les IAPs promouen la supervivència cel·lular mitjançant altres vies, com per exemple modulant l'activació d'NF-κB o aturant la l'activació de JNK per una estimulació perllongada de TNFα i TGFβ (Meier and Vousden 2007).

3.2.3.3. p53

La proteïna supressora de tumors p53 és una altra proteïna important en la regulació de l'apoptosi, ja que en resposta a determinats estímuls inicia accions per tal d'aturar el cicle cel·lular o induir una mort per apoptosi. Diversos tipus d'estrès cel·lular activen p53, com la deprivació de factors de creixement, l'expressió d'oncogens o el dany al DNA (Vogelstein *et al.* 2000). En

L'activitat funcional de p53 es troba regulada per diversos mecanismes com ara per la unió al seu repressor Mdm2 o per modificacions post traduccionals de tipus fosforilació o acetilació. La fosforilació de la serina 15 de p53 és una de les modificacions que s'ha associat a l'activació del procés apoptòtic dependent de p53 (Unger *et al.* 1999). Un cop activada, p53 és un factor de transcripció que indueix la transcripció de gens que determinaran el destí de la cèl·lula.

La mort apoptòtica dependent de p53 deriva en l'activació de caspases (Woo *et al.* 1998) a través, majoritàriament, de l'alliberament de factors pro apoptòtics de la mitocòndria per modulació de l'activitat de membres de la família de Bcl-2. Per tal d'activar la via mitocondrial, p53 pot actuar per mecanismes dependents de la transcripció gènica, induint la transcripció de gens com Bax, Noxa i Puma (Nakano and Vousden 2001), o de forma independent a l'activació de la transcripció, actuant directament sobre la mitocòndria (figura 20) (Caelles *et al.* 1994; Chen *et al.* 1996; Haupt *et al.* 1995) (revisat per (Haupt *et al.* 2003) i (Chipuk and Green 2006)). D'altra banda, també s'ha observat que p53 pot modular l'expressió de receptors de mort com CD95/Fas/APO-1 i TRAIL receptor 2/KILLER/DR5, formant així una connexió entre les vies apoptòtiques extrínseca i intrínseca (Bennett *et al.* 1998; Muller *et al.* 1997; Owen-Schaub *et al.* 1995).

Puma, un dels gens transcripcionalment activats per p53 (Jiang *et al.* 2006; Nakano and Vousden 2001), pertany a la família de proteïnes BH3-only. Aquestes proteïnes poden interaccionar amb altres proteïnes que continguin dominis BH3, com Bcl-2 i Bcl-XL, per promoure l'alliberament de citocrom *c* i altres factors pro apoptòtics de la mitocòndria. Puma també pot activar Bax induint la seva translocació i multimerització a la membrana externa mitocondrial per induir l'alliberament de factors pro apoptòtics mitocondrials (Yu *et al.* 2003a).

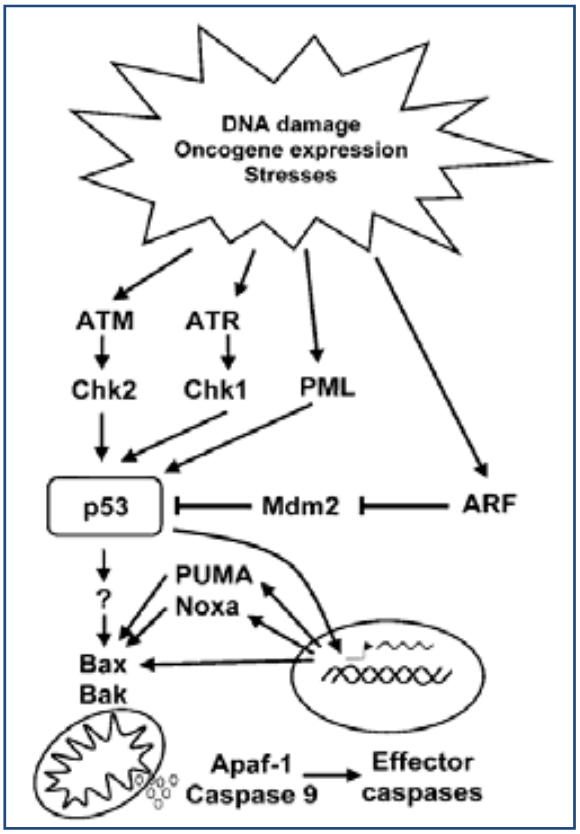


Figura 20. Esquema de les vies d'activació de l'apoptosi de p53. Després de la dissociació del seu repressor Mdm2 es pot modificar post transcripcionalment per estabilitzar-se i activar-se. Després pot actuar com a activador transcripcional, afavorint l'expressió de proteïnes pro apoptòtiques de la família de Bcl-2. També pot actuar de forma independent a la transcripció, actuant directament sobre proteïnes mitocondrials per permetre l'alliberament de factors apoptogènics, que facilitarien l'activació de caspases efectores a través de la formació de l'apoptosoma. Esquema extret de l'article (Schuler and Green 2001).

4. La malaltia d'Alzheimer i altres demències vasculars

L'augment de l'esperança de vida als països desenvolupats ha portat també a un increment de la incidència de diverses patologies, com per exemple la malaltia de Parkinson o la malaltia d'Alzheimer. Aquest allargament en la l'esperança de vida no es correlaciona doncs amb la preservació de la qualitat de vida. És per això que actualment s'inverteixen recursos per a la investigació d'aquestes malalties, tant en termes de prevenció, pal·liació o curació com en la descripció dels factors que les provoquen i dels mecanismes que actuen en el seu desenvolupament.

4.1. La malaltia d'Alzheimer (AD)

La malaltia d'Alzheimer (AD – *Alzheimer's Disease*) és actualment una de les demències més prevalent en la nostra societat, essent d'aproximadament un 1.5% als 65 anys d'edat, i incrementant amb l'edat fins arribar a valors del 30% als 85 anys d'edat (Barranco-Quintana *et al.* 2005; Ferri *et al.* 2005). Va ser descrita al 1906 per Alois Alzheimer, com un desordre heterogeni amb afectació neuronal i vascular. En

l'actualitat, la seva etiologia multifactorial encara no totalment coneguda juntament amb l'absència d'una cura efectiva la converteixen en una epidèmia difícil d'estudiar i d'eradicar.

4.1.1. Principals característiques de la malaltia d'Alzheimer.

La malaltia d'Alzheimer és un desordre neurodegeneratiu progressiu, que cursa en símptomes clínics com la pèrdua de memòria, dificultats en la parla, confusió, canvis en la personalitat, desorientació i dificultat a l'hora de desenvolupar les activitats de la vida diària (Ferri *et al.* 2005).

Anatomopatològicament, els cervells de pacients d'Alzheimer mostren una pèrdua neuronal i sinàptica, majoritàriament de tipus glutamatèrgic, evidenciada per l'atròfia principalment de l'hipocamp i dels còrtex frontal i tempoparietal. Aquesta atròfia cerebral s'acompanya de dos trets característics: la presència de plaques de pèptid beta amiloide (Aβ), i els cabdells neurofibrilars (figura 21). Sovint, a més d'aquesta patologia neuronal apareixen també lesions vasculars característiques com l'Angiopatia Cerebral Amiloide (CAA), la degeneració microvascular o les lesions periventriculars en la substància blanca (De la Torre J.C. and Hachinski V. 1996; Kalaria 1999).

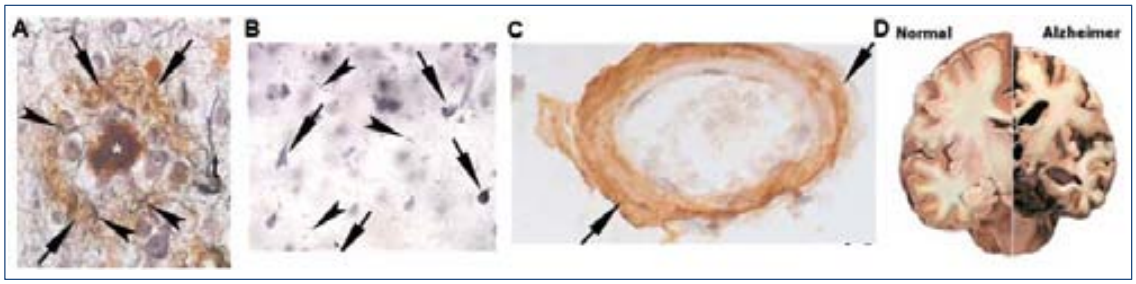


Figura 21. **A:** visió d'una placa neurítica; l'Aβ es veu en marró i els cabdells neurofibrilars en negre (t). El core d'amiloide es situa al centre, envoltat per dipòsits d'Aβ més difusos (fletxes). **B:** cabdells neurofibrilars. **C:** Vas sanguini d'un cervell amb CAA; s'observen dipòsits d'Aβ a la capa media del vas sanguini, els quals han destruït la paret vascular substituint les cèl·lules del múscul llis (fletxes). **D:** Evident pèrdua de volum cerebral en els cervells amb Alzheimer (part dreta) respecte un cervell normal (part esquerra). Figura adaptada de l'article (Thal *et al.* 2008b).

Les plaques d'amiloide són acumulacions extracel·lulars de dipòsits de pèptid beta amiloide (Aβ), i es poden trobar tant en el parènquima cerebral com en els vasos

sanguinis cerebrals. Els cabdells neurofibrilars (NFT – *Neurofibrillary Tangles*) són agregats anormals intraneuronals formats per l'acumulació de la proteïna associada als microtúbuls "Tau" hiperfosforilada (Grundke-Iqbal *et al.* 1986; Lee *et al.* 2001). La forma característica de trobar les plaques d'amiloide parenquimals és com a "plaques neurítiques", les quals estan formades per un core amiloidogènic envoltat per la presència de neurites distròfiques contenint o no NFT, micròglia activada i astròcits reactius (Selkoe 2004).

L'Aβ és un pèptid derivat del processament de la proteïna precursora de l'amiloide (APP). L'APP és una glicoproteïna transmembranal de tipus I, altament conservada entre espècies, expressada de forma constitutiva en la majoria de tipus cel·lulars en mamífers, incloent les cèl·lules vasculars. Fisiològicament, l'APP participa en l'adhesió entre cèl·lules i a la matriu (Mattson 1997), en la migració de precursors neuronals durant el desenvolupament cerebral (Young-Pearse *et al.* 2007), en la regulació de la senyalització intracel·lular per calci actuant com a receptor d'ApoE o del mateix Aβ (Leissring *et al.* 2002; Reinhard *et al.* 2005), i en el creixement dendrític (Dawson *et al.* 1999; Sabo *et al.* 2003; Small *et al.* 1994). L'APP es pot processar per dues vies diferents (figura 22):

- La via no amiloidogènica és la predominant en condicions normals. En aquesta via, l'APP és tallat en primer lloc per l'activitat enzimàtica de l'α-secretasa, un enzim pertanyent a la família de les ADAMs (*a disintegrin and metalloprotease*) (Buxbaum *et al.* 1998; Lammich *et al.* 1999). L'α-secretasa talla l'APP dins el domini de l'Aβ, impedint que aquest s'alliberi. La part C-terminal restant és posteriorment tallada per l'activitat enzimàtica γ-secretasa, duta a terme per un complex multiprotèic que conté la presenilina 1 (PS-1) o la presenilina 2 (PS-2) com a unitat catalítica, i la nicastrina (NCT), APH-1 (*anterior pharynx-defective-1*) i PEN-2 (*presenilin enhancer 2*) com a proteïnes adaptadores (Edbauer *et al.* 2003).

- La via amiloidogènica és la que genera el pèptid Aβ, mitjançant un primer tall de l'APP per la β-secretasa (BACE-1 – *β-site APP cleaving enzyme 1*) (Vassar *et al.* 1999) i un segon per la γ-secretasa (Selkoe 1998; Wolfe *et al.* 1999). L'Aβ generat pot tenir diverses longituds, dependent del tall en el seu extrem C-terminal, essent les formes majoritàries les de 40 i 42 aminoàcids, de les quals l'$A\beta_{1-42}$ té més capacitat d'agregació (Muñoz *et al.* 2002). En condicions normals existeix un equilibri entre la formació i la degradació

d'Aβ, duta a terme per l'enzim degradador d'insulina (IDE) i la neprilisina (Leissring 2006) majoritàriament. En aquest sentit, i segons la hipòtesi de la cascada amiloide, es creu que una desregulació en el balanç de producció/degradació d'Aβ seria el responsable de l'inici de la cascada d'agregació de l'Aβ. Els pèptids generats en excés s'agregarien adquirint una estructura de plegament en forma de làmina beta, i polimeritzarien formant primer oligòmers neurotòxics i després agregats organitzats cada vegada més grans, fins a formar les fibres insolubles que constitueixen les plaques d'amiloide. Això portaria finalment a la pèrdua sinàptica i a la degeneració neuronal característiques de la patologia d'AD (Hardy and Selkoe 2002). Encara no es coneixen, però, els factors que induïren aquesta desregulació ni el motiu pel que individus que presenten dipòsits d'Aβ no mostren els símptomes de la malaltia d'AD.

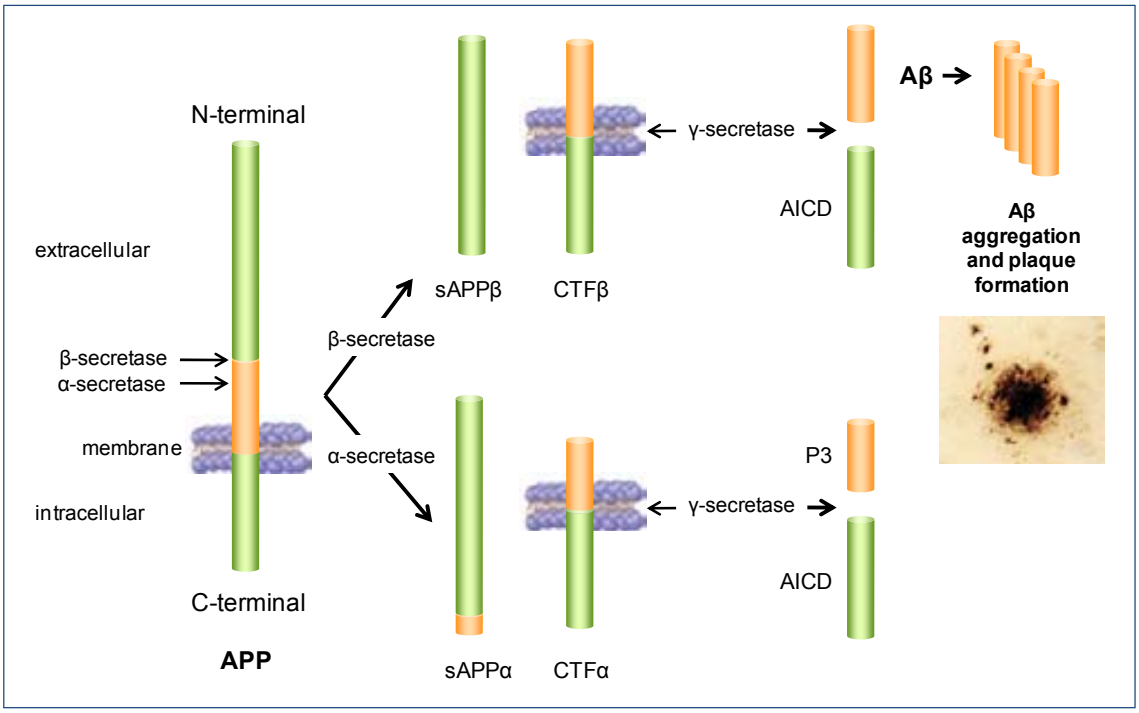


Figura 22. Diagrama esquemàtic del processament proteolític de l'APP. L'APP és una proteïna transmembranal de tipus I, la qual és processada per la via no amiloidogènica (α-secretasa), o per l'amiloidogènica (β-secretasa i γ-secretasa).

A nivell cel·lular, sembla que el processament de l'APP per una via o per l'altra difereixen en el lloc on es produeixen. En aquest sentit s'han observat dues localitzacions de l'APP en la membrana plasmàtica, una part associada als lipid rafts i l'altra fora d'ells. Així mateix, l'α-secretasa s'associa a la membrana no-*lipid raft*,

mentre que la β-secretasa, responsable de la generació de l'Aβ, s'associa als lipid rafts (Cordy *et al.* 2006; Fassbender *et al.* 2001; Simons and Ehehalt 2002). Aquest processament s'ha observat també en els endosomes, on la BACE actuaria millor degut al pH àcid d'aquests (Small and Gandy 2006).

Mutacions en l'APP, així com en les presenilines PS1 i PS2 són responsables del desenvolupament de la malaltia d'Alzheimer familiar, d'aparició precoç i d'herència autosòmica dominant. Les causes genètiques de desenvolupament de la malaltia d'Alzheimer representen només un 10% del total dels casos. Aquestes mutacions poden alterar la proporció d'$A\beta_{1-40}$/$A\beta_{1-42}$, accelerant la producció de la forma amb més capacitat d'agregació, l'$A\beta_{1-42}$ (Selkoe 1999).

4.1.2. Factors de risc i etiologia de la malaltia d'Alzheimer esporàdica.

Fins al moment s'han identificat nombrosos factors de risc per a desenvolupar la malaltia d'Alzheimer que inclouen l'edat, l'arteriosclerosi, la hipertensió, la diabetis, les lesions cerebrals, la hiperhomocisteïnèmia, la hipercolesterolèmia, factors trombogènics, malalties cardíaques i la presència i dosi gènica de l'al·lel ε4 del gen que codifica per l'apoproteïna E (ApoE) (Corder *et al.* 1993; Gorelick 2004; Saunders *et al.* 1993), una lipoproteïna plasmàtica que participa en la recaptació del colesterol i altres lípids; en canvi la presència de l'al·lel ε2 representa un factor de protecció contra el risc de patir la malaltia (Corder *et al.* 1994).

Molts d'aquests factors de risc ho són també per a patologies vasculars. Al principi es creia que la malaltia d'Alzheimer es tractava d'una patologia exclusivament neuronal, però cada vegada hi ha més indicis que el sistema vascular juga un paper important tant en l'etiologia com en la progressió de la malaltia, així com en el deteriorament cognitiu (Bell and Zlokovic 2009; de la Torre 2004; Zlokovic 2005). Per exemple, l'arteriosclerosi a la base del cervell (cercle de Willis) i altres processos que cursen amb una reducció del flux sanguini cerebral estan associades a l'aparició i estadis primaris de la malaltia d'Alzheimer (Hirao *et al.* 2005; Johnson *et al.* 2005; Roher *et al.* 2003); a més, s'ha observat que individus amb MCI o probable AD abans de manifestar-se presenten una reducció en la recaptació de glucosa cerebral, indicant un problema vascular previ a la neurodegeneració (Drzezga *et al.* 2003; Hunt *et al.* 2007). D'altra banda, el fet d'haver tingut infarts cerebrals previs requereix menys presència de patologia tipus Alzheimer per mostrar símptomes de demència (Snowdon 2003).

Tot i l'associació d'aquests factors amb l'aparició de la malaltia, l'etiologia a nivell bioquímic de la malaltia d'Alzheimer esporàdica no és ben coneguda encara. Es creu que la desregulació entre el nivell de producció i degradació del pèptid Aβ podria ser una de les primeres alteracions, però no es coneixen els mecanismes que poden portar a aquesta descompensació, i que probablement passarien per un increment en la producció del pèptid o per una disminució en la degradació d'aquest.

D'altra banda, l'estrès oxidatiu és un tret comú a molts dels factors de risc mencionats i precedeix les manifestacions neuropatològiques de la malaltia d'Alzheimer, de manera que s'ha suggerit que pot estar implicat en l'inici de la patologia. En aquest sentit, existeix una desregulació en el balanç entre anti i pro oxidants en el procés normal de l'envelliment que porta a l'acumulació d'espècies reactives d'oxigen (ROS) (Ames *et al.* 1993). Això i evidències experimentals que mostren marcadors oxidatius en lípids, proteïnes i àcids nucleics en pacients d'AD han donat lloc a la hipòtesi de que l'estrès oxidatiu juga un paper important en l'aparició de la malaltia d'Alzheimer (Behl 2005; Miranda *et al.* 2000). També s'ha observat que el dany oxidatiu en pacients amb síndrome de Down precedeix la deposició d'Aβ (Nunomura *et al.* 2000), i que pacients amb MCI (*mild cognitive* impairment) o en estadis lleus d'Alzheimer presenten nombrosos marcadors d'estrès oxidatiu així com disminució dels mecanismes antioxidants (Pratico *et al.* 2002; Rinaldi *et al.* 2003). A més, s'ha de tenir en compte que el cervell és especialment vulnerable al dany per radicals lliures ja que conté una gran quantitat d'àcids grassos peroxidables, una taxa de consum d'oxigen elevada i una menor proporció de mecanismes antioxidants que els teixits perifèrics. De la mateixa manera, factors que disminueixin els radicals lliures, com les vitamines C i E, l'estrogen, els antiinflamatoris no esteroides, estatines o altres s'associen a una menor incidència d'Alzheimer (Nunomura *et al.* 2006). En aquest sentit, agents oxidants com l'H_2O_2 són capaços d'incrementar la producció d'Aβ en neurones (Paola *et al.* 2000) i en cèl·lules no neuronals (Coma *et al.* 2008; Frederikse *et al.* 1996; Tong *et al.* 2005), actuant com un mecanisme de retroalimentació positiva en la producció d'Aβ. En aquest sentit, s'ha proposat que el procés de formació de plaques podria representar un mecanisme de protecció contra l'estrès oxidatiu, ja que les formes monomèriques d'$Aβ_{1-40}$ i $_{1-42}$ mostren una certa capacitat antioxidant i quelant d'ions metàl·lics (Bishop and Robinson 2003; Cuajungco *et al.* 2000; Zou *et al.* 2002). Tot i així, altes concentracions d'Aβ així com l'estat oligomèric d'agregació generen estrés oxidatiu en diversos sistemes biològics (Caughey and Lansbury 2003; Walsh and Selkoe 2004).

4.1.3. Aspectes vasculars de la malaltia d'Alzheimer: Angiopatia Cerebral Amiloide.

L'angiopatia cerebral amiloide (CAA – *Cerebral Amyloid Angiopathy*) és la deposició anòmala de proteïna amiloidogènica en les parets dels vasos sanguinis cerebrals, induint la degeneració de les cèl·lules que els formen (Vinters 1987). En el cas de la malaltia d'Alzheimer, la proteïna amiloidogènica implicada és l'Aβ, però existeixen altres tipus de CAA amb altres proteïnes amiloidogèniques com per exemple ABri (*Amyloid-British protein*), ADan (*Amyloid-Danish protein*), PrP (*Prion Protein*), cistatina C, gelsolina o transtiretina (Revesz *et al.* 2003; Yamada 2000).

La CAA relacionada amb la malaltia d'Alzheimer es pot manifestar de forma esporàdica o genètica, com la malaltia d'Alzheimer. En el cas de la forma esporàdica, la seva incidència i severitat s'associen a l'edat, el síndrome de Down i la malaltia d'Alzheimer. De fet, la CAA es manifesta en la majoria de casos de la malaltia d'Alzheimer (figura 23). Un dels principals factors de risc per a manifestar la CAA de forma esporàdica és la presència dels al·lels ε4 i ε2 de l'ApoE, factors de risc també per a la malaltia d'Alzheimer i per a l'arteriosclerosi respectivament (Chalmers *et al.* 2003).

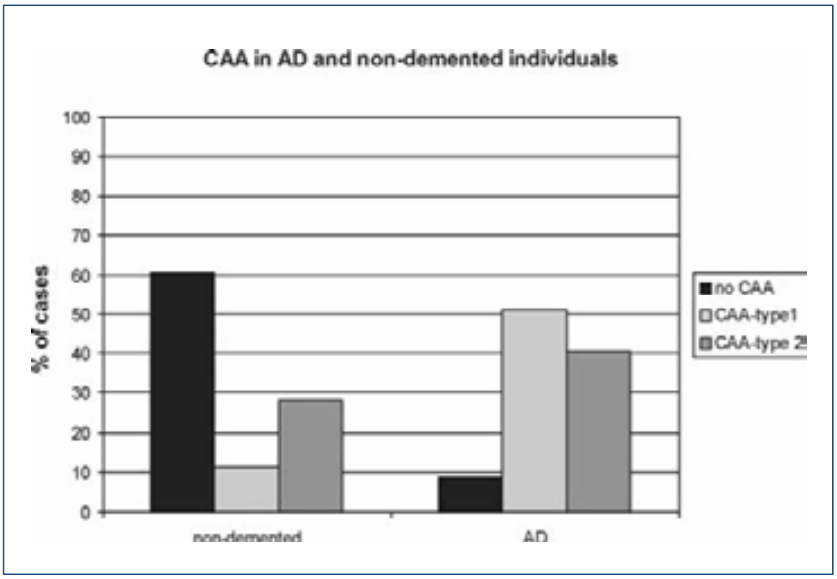


Figura 23. Prevalença de la CAA en malalts d'Alzheimer i controls sense demència. En els controls sense demència, la prevalença de CAA és baixa (39,6%), i els individus que en presenten, la forma sense afectació dels capil·lars (tipus II) és més freqüent (28,2%) que la que té afectació dels capil·lars (tipus I) (11,4%). En canvi, la majoria dels pacients d'AD presenten CAA (91%), essent un 51% corresponent al tipus I (amb afectació capil·lar) i un 40% corresponent al tipus II. Gràfica extreta de l'article (Thal *et al.* 2008b).

Des del punt de vista genètic, s'han identificat diverses mutacions en la seqüència de l'Aβ associades a la CAA, com ara la mutació Flemish (A21G) (Hendriks *et al.* 1992), la mutació Dutch (E22Q) (Levy *et al.* 1990) la mutació Italian (E22K) (Tagliavini *et al.* 1999), la mutació Arctic (E22G) (Nilsberth *et al.* 2001) o la mutació Iowa (D23N) (Grabowski *et al.* 2001) (figura 24). Aquestes mutacions poden implicar canvis en la càrrega de l'aminoàcid, fet que els confereix un comportament diferent.

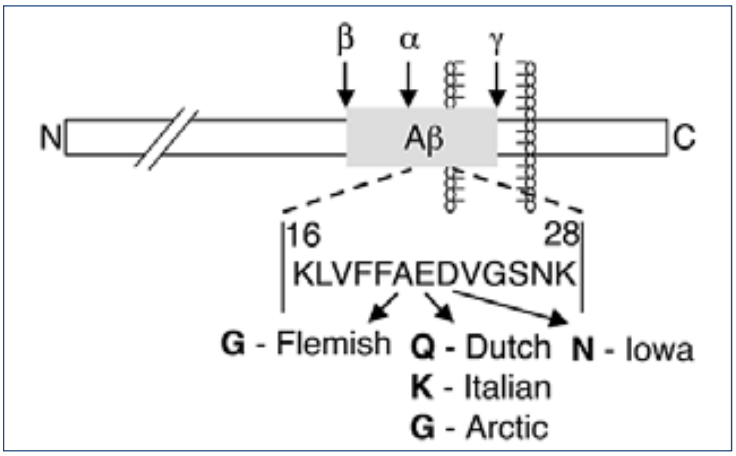
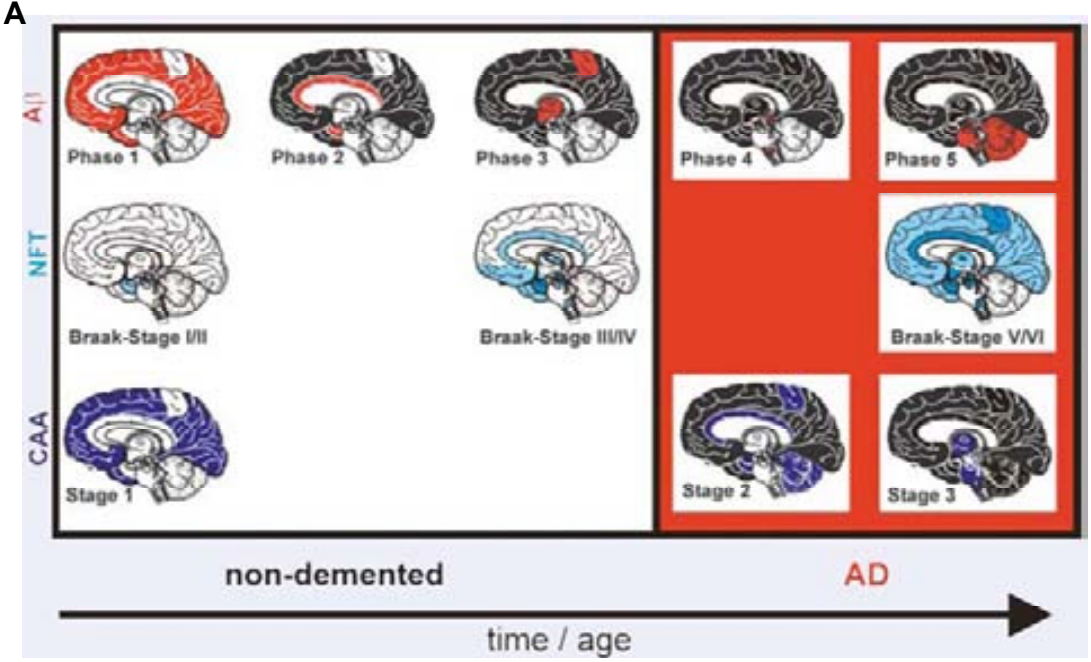


Figura 24. Esquema de la proteïna APP en relació a la membrana, on es mostren sobre l'esquema els punts de tall de les secretases α, β i γ. La seqüència del pèptid Aβ està amplificada en els aminoàcids 16-28, on es mostren les principals mutacions observades en aquesta regió. Esquema extret de l'article (Nilsberth *et al.* 2001).

En els casos que presenten alguna d'aquestes mutacions es desenvolupa una CAA d'origen precoç que cursa amb hemorràgies cerebrals. La mutació Dutch, la qual genera un Aβ més fibrilogènic i tòxic (Davis and Van Nostrand 1996; Melchor *et al.* 2000; Wisniewski *et al.* 1991), provoca la HCHWA-D (*hereditary cerebral haemorrhage with amyloidosis of the Dutch type*), que cursa amb CAA severa amb plaques d'amiloide difuses en el parènquima cerebral, però sense NFT o plaques neurítiques; clínicament, es caracteritza per hemorràgies i infarts cerebrals recurrents, sovint amb símptomes de demència, i es produeix una mort a edats primerenques (Bornebroek *et al.* 1997).

A nivell temporal, es poden distingir tres estats de CAA, que correlacionen amb les fases de la deposició de plaques d'Aβ i el grau de demència. En el desenvolupament d'aquests tres estats, la deposició d'Aβ vascular segueix una expansió cap a diferents regions cerebrals (Figura 25 A i B) (Thal *et al.* 2003; Thal *et al.* 2008b). A més, alguns autors suggereixen que l'afectació dels capil·lars, associada a la presència de l'al·lel ε4 de l'ApoE, representa una forma de CAA més severa (Olichney *et al.* 2000; Thal *et al.* 2002), ja que cursa amb una oclusió capil·lar que contribueix a la disfunció neuronal en l'AD (Thal *et al.* 2008a).

A



B

Brain region	CAA	capillary involvement
Frontal Cortex	Stage 1	+
Parietal Cortex	Stage 1	+
Temporal Cortex	Stage 1	+
Occipital Cortex	Stage 1	+
Hippocampal formation	Stage 2	CA1/Subiculum + CA4
Insular Cortex	Stage 2	-
Cingulate Cortex	Stage 2	+
Entorhinal Cortex	Stage 2	+
Amygdala	Stage 2	+
Hypothalamus	Stage 2	+
Midbrain	Stage 2	+
Cerebellum	Stage 2	+
Thalamus	Stage 3	+
Basal ganglia	Stage 3	-
Basal forebrain Nuclei	Stage 3	+
Pons	Stage 3	+
Medulla oblongata	Stage 3	-

Figura 25. **A:** L'expansió dels dipòsits d'Aβ en la patologia d'AD (5 fases, on les zones vermelles són les que mostren els nous dipòsits i les negres els anteriors). L'expansió de les plaques d'Aβ va acompanyada per la de les NFTs, indicada per l'estat Braak (Braak and Braak 1991) (les zones blau clar són les noves). En paral·lel té lloc també l'expansió de la CAA (les zones blaves són les noves), que comença en regions neocorticals, seguida de regions al·locorticals incloent regions mesencefàliques i cerebel i finalment afectant a la protuberància, ganglis basals i tàlem. Figura extreta de l'article (Thal *et al.* 2008b). **(B):** Taula que mostra les principals zones afectades en cada estadi de CAA, i si mostren o no afectació capil·lar. Figura extreta de l'article (Thal *et al.* 2008c).

A nivell histològic, l'Aβ es diposita extracel·lularment en la CAA, inicialment en la capa mitja i adventícia de les petites artèries, arterioles i capil·lars corticals, subcorticals i leptomeníngis, i més rarament en les venes (Frackowiak *et al.* 1994; Mandybur 1986). En incrementar la severitat, l'Aβ s'infiltra en totes les capes de la paret del vas, i es produeix una pèrdua de les cèl·lules musculars; finalment, l'arquitectura del vas es trenca formant-se un microaneurisme de doble barrera (figura 26). En els capil·lars, la CAA es presenta en forma de fines capes de dipòsits d'Aβ en la membrana basal perivascular, de dipòsits globulars en la paret del capil·lar i sovint de dipòsits en el neuròpil adjacent i en la glia limitant en el cas de vasos intracorticals (Attems 2005).

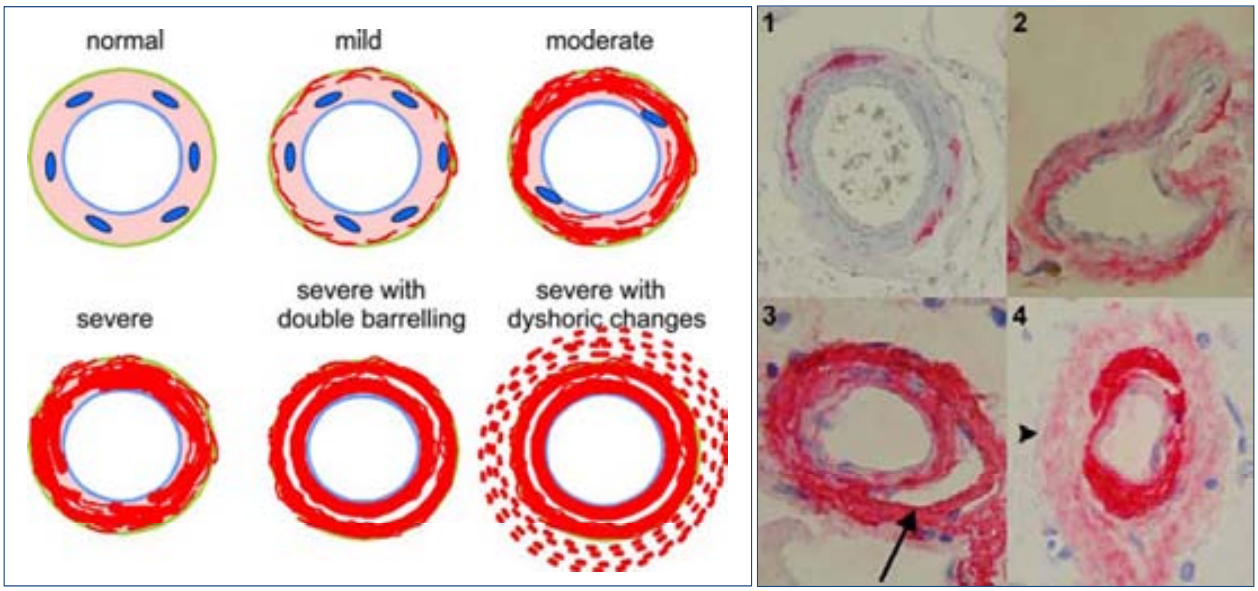


***Figura 26*:** Progressió de la CAA a nivell dels vasos. (**esquema esquerre**), en la fase més lleu l'Aβ es diposita en la capa adventícia del vas, i en la seva progressió cap a un estat més moderat colonitza totes les capes, amb pèrdua de cèl·lules musculars. En un estat sever, l'Aβ substitueix la paret del vas i es poden presentar situacions de "doble barril" o de dipòsits en el neuròpil adjacent (canvis discòrics). En les micrografies de la **dreta** s'observen tincions dels dipòsits d'Aβ (color rosat) de vasos en diferents fases de la CAA: (1) lleu, (2) moderat, (3) sever amb "doble barril" i (4) sever amb afectació del neuròpil. Figura extreta de l'article (Attems 2005).

La deposició d'Aβ al teixit vascular comporta una destrucció de les cèl·lules del múscul llis, que incrementa la fragilitat del vas sanguini, i augmenta la probabilitat de que aquest es trenqui i provoqui hemorràgies intracerebrals o infarts (Mandybur 1986; Vonsattel *et al.* 1991). En els petits capil·lars, els dipòsits d'Aβ poden arribar a ocluir-los, impedint l'aport d'oxigen i nutrients a través de la barrera hematoencefàlica, i contribuint així a la neurodegeneració (Thal *et al.* 2008b; Zarow *et al.* 1999); de fet,

existeix una bona correlació entre l'acumulació d'Aβ microvascular i la demència (Attems and Jellinger 2004; Thal *et al.* 2003). A més, la degeneració dels vasos sanguinis cerebrals comporta alteracions en la permeabilitat de la barrera hematoencefàlica (Vinters *et al.* 1988), i tot aquest procés genera una resposta vascular neuroinflamatòria, en la que hi estan implicats l'endoteli, la micròglia perivascular, els pericits i els astròcits (Zlokovic 2005), la qual contribueix també a la degeneració vascular mitjançant l'alliberació de citocines i altres factors inflamatoris.

4.1.4. Origen de l'Aβ vascular.

Hi ha certa controvèrsia sobre l'origen de l'Aβ dipositat als vasos sanguinis. D'una banda, es creu que es tracta d'Aβ neuronal, que arribaria a la circulació sanguínia perifèrica mitjançant mecanismes de drenatge perivascular cap al líquid cefaloraquidi o directament travessant la barrera hematoencefàlica mitjançant el receptor LRP1 (*low density lipoprotein receptor related protein 1*). La saturació dels mecanismes de drenatge per un excés de producció d'Aβ neuronal juntament amb la degeneració vascular normal dels individus adults podria promoure'n l'acumulació als vasos sanguinis (Mackic *et al.* 1998; Preston *et al.* 2003; Weller *et al.* 1998). Aquesta teoria pren força per la presència de dipòsits d'Aβ vasculars en ratolins que sobreexpressen l'APP en neurones (Van *et al.* 2000). D'altra banda, es creu que l'Aβ vascular pot provenir de les mateixes cèl·lules vasculars, ja que s'ha demostrat que tant les cèl·lules del múscul llis com els pericits vasculars són capaces de generar Aβ, predominantment $A\beta_{1-40}$, que és el majoritari en els dipòsits vasculars (Castano *et al.* 1996; Frackowiak *et al.* 1995; Frackowiak *et al.* 2005). En aquest sentit, les cèl·lules musculars en situacions de proliferació o degeneració també produeixen Aβ (Wisniewski *et al.* 1994). A més, s'ha observat que la matriu extracel·lular de les cèl·lules del múscul llis té una gran capacitat fibrilogènica (Van Nostrand *et al.* 2000), per tant, les cèl·lules vasculars mateixes poden promoure l'agregació de l'Aβ en aquest teixit tant si prové d'elles mateixes com del flux cervell-sang, encara que la producció d'Aβ només del sistema vascular no sembla suficient per causar la patologia de la CAA (Thal *et al.* 2008c).

Les cèl·lules vasculars, juntament amb la glia, participen en el procés de drenatge i eliminació de l'Aβ neuronal, així com en el flux d'Aβ a través de la barrera hematoencefàlica (BHE), per tant, el seu mal funcionament pot contribuir a la progressió de la CAA i de l'AD. A més, desregulacions en el transport de l'Aβ a través

de la BHE poden tenir conseqüències importants en l'acumulació de l'Aβ al parènquima cerebral (Thal *et al.* 2008c). Això atorga un paper al sistema cerebrovascular no només d'observador en la patologia neuronal de l'AD sinó també de modulador d'aquesta. L'LRP1 i el receptor RAGE (*receptor for advanced glycation end products*) són els principals controladors d'aquest flux, del cervell a la sang i de la sang al cervell respectivament (Deane *et al.* 2004b; Zlokovic 2004). Després de la unió de l'Aβ a l'LRP (preferentment l'$A\beta_{1-40}$ (Deane *et al.* 2004a)) amb l'ajuda de xaperones com l'ApoE, l'ApoJ o l'α2-Macroglobulina, aquest és endocitat per les cèl·lules endotelials i alliberat al torrent sanguini.

4.1.5. Mecanismes patològics de l'Aβ en el sistema vascular.

L'efecte tòxic de l'Aβ ha estat estudiat majoritàriament en neurones. En aquest tipus cel·lular, sembla que la toxicitat de l'Aβ depèn del seu estat d'agregació (Pike *et al.* 1993), essent els oligòmers les espècies més tòxiques (Bhatia *et al.* 2000; Watson *et al.* 2005); a més, s'ha observat que en certes condicions *in vitro*, la toxicitat induïda per l'Aβ té lloc en conjunció amb altres factors com els ions metàl·lics, l'estrès oxidatiu o l'excés d'aminoàcids excitadors (Morgan *et al.* 2004). En el cas del sistema vascular, s'ha descrit que l'Aβ indueix una degeneració de les cèl·lules vasculars (endotelials, del múscul llis i pericits) en els pacients d'AD (Kawai *et al.* 1993; Wisniewski *et al.* 1992). En estudis *in vitro* s'ha observat un efecte tòxic en cèl·lules vasculars endotelials (Miravalle *et al.* 2000) i del múscul llis (Van Nostrand *et al.* 1998) tant de l'$A\beta_{1-42}$ com de l'$A\beta_{1-40}$Dutch, tot i l'absència de toxicitat de l'$A\beta_{1-40}$WT a les mateixes concentracions.

El mecanisme pel qual l'Aβ indueix citotoxicitat és diferent segons el tipus cel·lular. En aquest sentit, s'ha observat que l'Aβ és capaç d'induir citotoxicitat mitjançant la producció d'estrés oxidatiu en cèl·lules neuronals (Behl *et al.* 1994) i endotelials (Coma *et al.* 2005), però no en cèl·lules vasculars del múscul llis (Jung and Van Nostrand 2002). L'Aβ és capaç de generar radicals lliures (Barnham *et al.* 2004; Butterfield and Boyd-Kimball 2005), i a la vegada, els oligòmers d'aquest poden interaccionar amb el receptor RAGE induint l'increment de la producció intracel·lular de ROS. Com a conseqüència de l'increment de ROS s'activa NF-κB, o altres vies dependents de MAP cinases, així com també s'indueix dany a les proteïnes i lípids de membrana i als àcids nucleics (Mark *et al.* 1997; Miranda *et al.* 2000; Yan *et al.* 2000). A més, s'ha observat que el fragment C-terminal citosòlic de 31 aminoàcids és neurotòxic a quantitats

elevades (Lu *et al.* 2000), i que l'Aβ pot induir l'apoptosi de les cèl·lules neuronals mitjançant l'activació de caspases i calpaïnes (Lee *et al.* 2000; Nakagawa *et al.* 2000; Troy *et al.* 2000).

Així, els efectes finals de la deposició de l'Aβ a nivell vascular serien, d'una banda, la degeneració teixit vascular mitjançant la toxicitat de l'Aβ, amb la corresponent alteració de la funcionalitat del sistema vascular i l'aparició de patologia associada com ara vasculitis, hemorràgies o ictus. D'altra banda però com a conseqüència de la primera es produiria una alteració del flux sanguini cerebral que contribuiria a la degeneració neuronal mitjançant la reducció de l'aport d'oxigen i nutrients al teixit cerebral.

4.2. Altres demències amb afectació vascular

A part de la CAA, altres disfuncions del sistema vascular poden donar lloc o estar relacionades amb processos de demència. Aquestes disfuncions estan englobades actualment dins el terme "*Vascular Cognitive Impairment (VCI)*" (Bowler 2005) o "*Vascular Cognitive Disorder (VCD)*" (Roman *et al.* 2004), però anteriorment havien estat parcialment descrites com a "*arteriosclerotic dementia*", "*multi-infarct dementia (MID)*","*poststroke dementia*" o "*vascular dementia*" (Jellinger 2008).

L'VCI/VCD comprèn un gran grup de desordres cognitius heterogenis que presumptament comparteixen una causa vascular. Molts dels pacients amb VCI mostren un perfil cognitiu semblant, en el que la memòria està preservada però existeix una disfunció en la funció executora (O'Brien *et al.* 2003). Existeixen nombroses etiologies capaces de donar lloc a un desordre cognitiu d'origen vascular (Roman *et al.* 2004), que poden fer referència a situacions d'hipoperfusió vascular, hemorràgies, embòlies, desordres hematològics, causes tòxiques, processos inflamatoris no infecciosos o autoimmunes, infarts cerebrals secundaris a infeccions, anomalies genètiques o altres, i aquestes situacions, a la vegada, poden ser causades per diversos processos patològics. Aquest gran nombre de situacions patològiques i la seva heterogeneïtat tant en la causa com en el patró de dèficits cognitius fa molt difícil la unificació dels criteris de diagnòstic (Moorhouse and Rockwood 2008).

L'VCI inclou tres categories: VCI sense demència, demència vascular i demència mixta (demència vascular + demència neurodegenerativa (sovint AD)), segons aspectes clínics complementats per estudis de neuroimatge. El terme "VCI sense demència" seria l'equivalent a MCI (*mild cognitive impairment*) d'origen vascular (Bowler 2005;

Roman *et al.* 2004), es a dir, comprèn els individus els símptomes dels quals no estan associats a una disfunció funcional substancial, que majoritàriament han patit una isquèmia subcortical. La demència vascular (VaD) inclou desordres com la demència post-ictus, la demència multi-infart o la demència subcortical entre altres. La demència mixta (MD), d'altra banda, es refereix als pacients que presenten característiques clíniques i neuropatològiques típiques de la malaltia d'Alzheimer i de la demència vascular conjuntament.

A nivell de fisiopatologia, es pot dividir l'VCI segons si la seva patologia s'associa a vasos sanguinis grans o petits. El tipus associat als vasos grans normalment deriva d'un ictus, de múltiples infarts més petits o menys freqüentment de vasculitis cerebral afectant a les regions irrigades per les grans artèries; les causes d'aquests poden ser arteriosclerosi, èmbols provinents del cor, angiopaties inflamatòries o genètiques, o estenosi i oclusió d'artèries, entre altres. El tipus associat als vasos petits deriva de microinfarts predominantment, amb afectació de la substància blanca i estructures subcorticals com el tàlem o els ganglis basals; es pot dividir en subtipus segons la localització, i pot estar causat per les causes clàssiques d'infarts, processos de perfusió reduïda o episodis d'hipotensió, canvis microvasculars deguts a processos d'arteriosclerosi amb o sense oclusió, o disfuncions de la barrera hematoencefàlica, entre altres. A part d'aquests dos tipus, es classifiquen de forma independent encefalopaties post-isquèmiques, demència hemorràgica, i desordres esporàdics o genètics associats a hemorràgies, com la CAA.

5. La SSAO i les demències vasculars

La majoria de demències vasculars inclouen processos de degeneració vascular. Un dels casos de degeneració vascular associats a demència més estudiats és l'Angiopatia Cerebral Amiloide relacionada amb la malaltia d'Alzheimer (CAA-AD). La SSAO/VAP-1, com s'ha vist anteriorment, es localitza en el cervell, associada al teixit vascular (Castillo *et al.* 1998), i com a conseqüència de la seva activitat catalítica genera productes tòxics com el formaldehid, el metilglioxal o l'H_2O_2, que han mostrat toxicitat i capacitat d'induir processos d'entrecreuament covalent o *cross*-linkings entre proteïnes i DNA (Heck *et al.* 1990; Nagaraj *et al.* 1996), així com la capacitat d'incrementar l'estrès oxidatiu. Aquests processos estan estretament lligats a l'envelliment i als desordres vasculars crònics com ara les complicacions diabètiques (p. ex. retinopatia, nefropatia).

A partir de les observacions anteriors es va suggerir una hipòtesi que relacionava l'activitat SSAO amb els desordres vasculars de la CAA-AD (Yu 2001). Ja es coneixien els efectes en la degeneració vascular induïts per l'activitat SSAO en la diabetis, els quals s'assemblen als que tenen lloc en la CAA, i a més, en la formació de les plaques d'Aβ es donen processos d'entrecreuament intra i intermoleculars, així com de glicació avançada (Kapurniotu *et al.* 1998), que podrien ésser promoguts per l'activitat de la SSAO. La localització dels dipòsits vasculars d'Aβ també coincideix amb la localització de la SSAO ja que les deposicions projecten des de la membrana basal cerebrovascular (Miyakawa 1997; Perlmutter *et al.* 1994; Zarow *et al.* 1997). D'altra banda, la degeneració vascular i els productes de l'activitat SSAO generen estrés oxidatiu i inflamació, la qual indueix l'expressió de la SSAO (Salmi *et al.* 1993), que genera més productes tòxics, creant així un cercle viciós. Aquesta hipòtesi es resumeix en la figura 27.

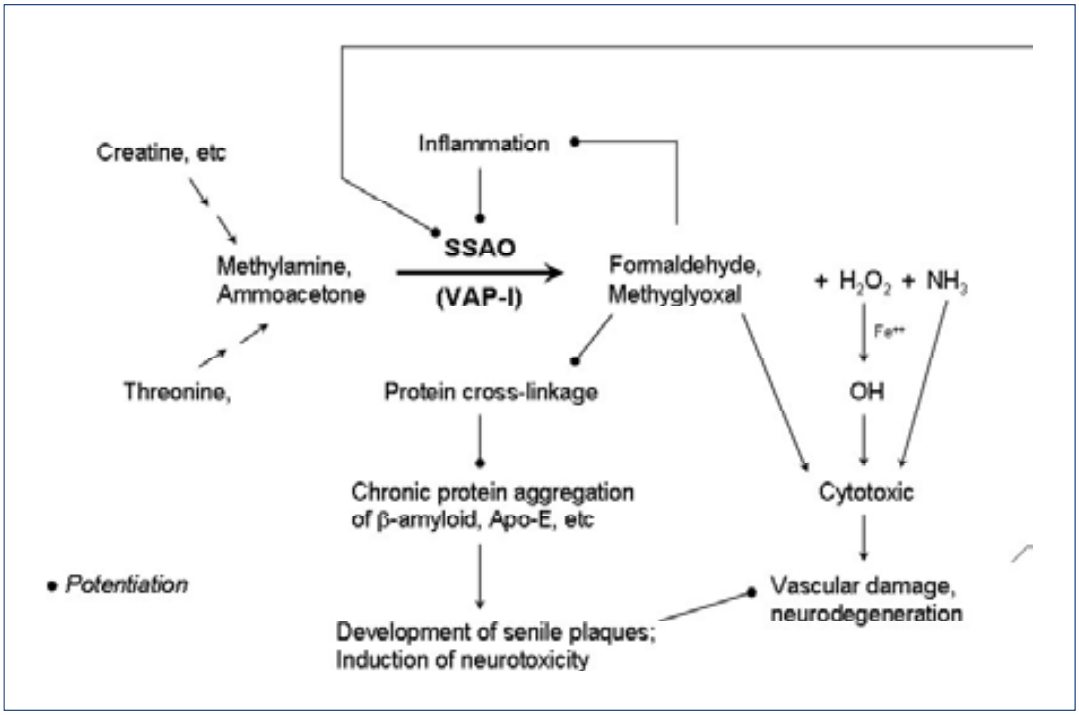


***Figura 27*:** Esquema que resumeix la hipòtesi suggerida (Yu 2001), que relaciona l'activitat catalítica de la SSAO amb la patogènesi de la malaltia d'Alzheimer.

Més tard es va analitzar l'expressió de la SSAO en models de malalties humanes amb CAA i CADASIL:

- D'una banda, es van fer estudis immunohistològics d'expressió de la SSAO en mostres cerebrals de pacients d'AD i CADASIL (*cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy*); el CADASIL es caracteritza per l'acumulació de material basofílic i osmiofílic entre les cèl·lules del múscul llis vascular, amb engruiximent i fibrosi de les parets de les arteries cerebrovasculars petites i mitjanes (Kalimo *et al.* 1999). En aquest estudi es va observar un increment selectiu en la immunoreactivitat contra la SSAO en els vasos sanguinis que contenien dipòsits d'Aβ en AD, colocalitzant amb ells, així com un increment en la SSAO en els vasos en CADASIL, de forma pròxima als dipòsits granulars i en associació amb marcadors d'estrés oxidatiu (Ferrer *et al.* 2002). A més, purificacions de meninges i microvasos de mostres post-mortem de cervells humans afectats d'AD van confirmar aquest increment d'SSAO observat immunohistològicament (Unzeta *et al.* 2007).

- D'altra banda, es va estudiar si la forma plasmàtica de la SSAO també es trobava alterada en la malaltia d'AD, tal com s'havia observat en la forma tissular (Ferrer *et al.* 2002) i en el plasma d'altres malalties que cursen amb degeneració vascular (Boomsma *et al.* 1999; Yu *et al.* 2003b). En aquest estudi es va observar com l'activitat SSAO plasmàtica incrementa en estats avançats de la malaltia d'Alzheimer (moderat-greu i greu), però no en estats lleus o moderats, ni es correlaciona amb la concentració plasmàtica d'Aβ1-40 o 1-42. Els resultats van suggerir que l'increment d'SSAO plasmàtica observat devia provenir del tall de la sobreexpressió observada en el teixit, i que igualment podria contribuir al dany vascular en AD mitjançant la seva acció catalítica (del Mar Hernandez *et al.* 2005).

Els resultats obtinguts a partir de mostres de pacients amb AD estaven a favor de la hipòtesi prèviament postulada. A més, l'extensió de l'estudi a mostres provinents de pacients amb CADASIL, ampliava la implicació de la SSAO en altres tipus de demències. Fins al moment, però, no s'ha estudiat la SSAO en altres demències vasculars.

En referència a la implicació de la SSAO en la patologia de la CAA-AD, més tard es van fer estudis sobre la implicació dels diferents aldehids generats a partir de l'activitat SSAO en l'agregació de l'Aβ. En aquests estudis es mostra com aquests aldehids són capaços de potenciar la formació d'estructures de fulles beta de l'Aβ, la seva

oligomerització i fibrilogènesi i la formació d'agregats estables grans entre l'Aβ i altres proteïnes (Chen *et al.* 2006; Chen *et al.* 2007). També es mostra com l'activitat SSAO és capaç d'incrementar la deposició d'Aβ en seccions de cordó umbilical humà (Jiang *et al.* 2008). Aquests resultats, doncs, suggereixen un paper actiu de la SSAO en aquesta patologia, a banda d'una mera resposta inflamatòria induïda pel dany vascular.

En resum, la SSAO i els seus substrats endògens es troben incrementats en diverses malalties que cursen amb dany i degeneració vascular com la diabetis o l'arteriosclerosi. Aquestes, a la vegada, són factors de risc per a desenvolupar la malaltia d'Alzheimer, en la qual també es troba un increment d'SSAO tant a nivell del teixit cerebrovascular com del plasma. D'altra banda, l'activitat SSAO genera productes tòxics capaços d'induir estrés oxidatiu, mort de les cèl·lules vasculars i entrecreuament covalent entre proteïnes i DNA, tots ells observats en la malaltia d'Alzheimer. La relació entre la SSAO i l'Alzheimer, vist tot l'anterior, sembla evident en aspectes com la inflamació, la promoció de l'agregació de l'Aβ i la inducció del dany vascular. Encara no està clar, però, si l'activitat SSAO és una de les causes de l'inici de la malaltia d'AD o si és només una de les conseqüències del dany observat, actuant com un mecanisme amplificador d'aquest. Tot i així, estudis recents suggereixen que la disfunció cerebrovascular precedeix els dèficits cognitius i l'inici dels canvis neurodegeneratius en la malaltia d'Alzheimer, per tant, mecanismes capaços d'induir dany vascular s'haurien de tenir en compte en aquest aspecte.

El treball dut a terme en aquesta tesi intenta conèixer una mica més la relació entre la SSAO i la malaltia d'Alzheimer, els mecanismes que alteren el patró d'expressió de la SSAO en aquesta patologia i com afecten aquests al desenvolupament de la mateixa. Així mateix s'intenta aclarir també la seva possible implicació en l'inici i progressió del procés patològic.

II. OBJECTIUS

II. OBJECTIUS

L'objectiu general d'aquest treball de tesi ha estat aprofundir en l'estudi del possible paper de l'amino oxidasa sensible a semicarbazida (SSAO) en el desordre vascular associat a la malaltia d'Alzheimer.

Per tal d'abordar aquest objectiu general ens hem fixat els següents objectius concrets:

1. Caracteritzar la línia cel·lular de múscul lliss transfectada de forma estable amb el gen de la SSAO/VAP-1 humana (A7r5 hSSAO/VAP-1) generada prèviament en el nostre laboratori, per avaluar la seva utilitat en l'estudi de la proteïna SSAO/VAP-1.

2. Generar una línia cel·lular endotelial transfectada de forma estable amb el gen de la SSAO/VAP-1 humana i caracteritzar-la bioquímicament amb el mateix objectiu anterior.

3. Avaluar el possible efecte tòxic generat per la pròpia activitat catalítica de la SSAO en aquestes dues línies cel·lulars i determinar les vies moleculars activades en la mort cel·lular induïda.

4. Estudiar l'efecte de la SSAO/VAP-1 en un model *in vitro* d'angiopatia cerebral amiloide, mitjançant el tractament de cèl·lules vasculars amb el pèptid beta amiloide 1-40 contenint la mutació Dutch.

5. Determinar els nivells plasmàtics d'activitat SSAO en pacients afectats per demències amb component vascular i comparar-los amb els obtinguts en pacients d'Alzheimer per tal d'avaluar el possible us de l'activitat SSAO com a marcador de dany o disfunció vascular.

II. OBJECTIVES

The broad objective of this work has been to study in depth the possible role of semicarbazide-sensitive amine oxidase (SSAO) in the Alzheimer's disease associated vascular disorder.

To tackle this objective we have set the following specific objectives:

1. To characterize the smooth muscle cell line transfected in a stable form with the human gene of SSAO/VAP-1 (A7r5 hSSAO/VAP-1), which was previously generated in our laboratory, in order to assess its usefulness in the study of the SSAO/VAP-1 protein.

2. To develop a new endothelial cell line transfected in a stable form with the human gene of SSAO/VAP-1, and to characterize it for the same purpose that the previously mentioned.

3. To evaluate the possible toxic effect induced by the catalytic activity of SSAO on the cell lines previously characterized, and to determine the molecular pathways involved in the induced cell death.

4. To study the effect of SSAO/VAP-1 in an *in vitro* model of cerebral amyloid angiopathy based on treatments of vascular cells with beta amyloid 1-40 peptide containing the Dutch mutation.

5. To determine the plasma SSAO activity levels in patients afflicted by different types of dementia with vascular affectation, and to compare them with the obtained from AD patients in order to assess the possible usefulness of SSAO activity measurement as a marker of damage or vascular dysfunction.

III. MATERIALS I MÈTODES

III. MATERIALS I MÈTODES

1. MATERIALS BIOLÒGICS UTILITZATS

1.1. Línies cel·lulars

En aquest treball s'han utilitzat les següents línies cel·lulars immortalitzades no tumorals d'origen vascular:

- ***A7r5 WT***: Línia cel·lular de múscul llis d'aorta de rata, adherent i amb morfologia fibroblàstica (figura 28.A). L'anomenem WT (*Wild Type*) perquè no li hem induït cap canvi a nivell genètic. És comercial, i va ser adquirida a l'ECACC (*European Collection of Cell Cultures*).

- ***A7r5 hSSAO/VAP-1***: Línia cel·lular obtinguda al nostre laboratori a partir de la transfecció estable de l'anterior amb el vector pcDNA3.1(+) contenint l'inserit de la proteïna humana SSAO/VAP-1. El procediment d'obtenció d'aquesta línia cel·lular està descrit en una tesi doctoral realitzada anteriorment al nostre grup (Hernandez 2005).

- ***HUVEC WT***: Línia cel·lular d'endoteli de vena de cordó umbilical humana, adherent i amb morfologia empedrada (figura 28.B). En aquest cas també l'anomenem WT ja que no li hem modificat l'expressió de gens. Aquesta línia cel·lular va ser cedida al nostre laboratori pel Dr. Francisco J. Muñoz, investigador del Departament de Ciències Experimentals i de la Salut de la Universitat Pompeu Fabra (Barcelona).

- ***HUVEC hSSAO/VAP-1***: Línia cel·lular obtinguda al nostre laboratori a partir de la transfecció estable de l'anterior amb el vector pcDNA3.1(+) contenint l'inserit de la proteïna humana SSAO/VAP-1. El procediment d'obtenció d'aquesta línia cel·lular s'explica més endavant en aquest treball (veure materials i mètodes, apartat 2).

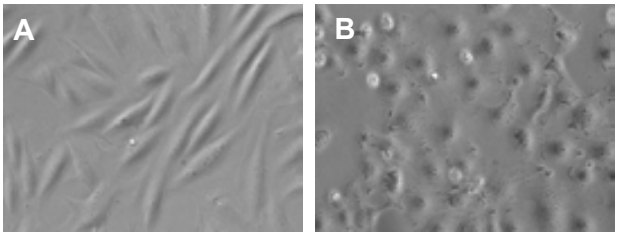


Figura 28: Micrografies de contrast de fase de les cèl·lules musculars (A7r5) (**A**) i endotelials (HUVEC) (**B**). Tant les WT com les transfectades mostren la mateixa morfologia.

1.2. Plasma humà

En aquest treball s'han utilitzat mostres de plasma humà, de pacients afectats per diversos tipus de demències i graus d'afectació, com són l'MCI (*Mild Cognitive Impairment*), la malaltia d'Alzheimer (AD - *Alzheimer's Disease*), la demència Vascular (VaD), la demència Mixta (MD - demència tipus Alzheimer acompanyada de demència tipus vascular) o la demència Frontotemporal (FTD). A més, s'han utilitzat també mostres de plasma de controls sans d'edats similars a les dels pacients amb demència.

Aquestes mostres de plasma es van obtenir del centre d'assistència ACE de Barcelona (Fundació ACE, Institut Català de Neurociències Aplicades). Les mostres de sang es van recollir mitjançant venopunció en tubs contenint una solució de citrat sòdic de 0.129 mol/L; aquestes es van centrifugar a 2500 x *g* durant 15 minuts i se'n van obtenir així les mostres de plasma, que es van aliquotar i emmagatzemar a -80 ºC fins al moment del seu ús.

2. PROCEDIMENT D'OBTENCIÓ DE LA LÍNIA CEL·LULAR HUVEC hSSAO/VAP-1

2.1. Constructe utilitzat: amplificació i purificació

El constructe utilitzat per a l'obtenció de la línia cel·lular HUVEC hSSAO/VAP-1 va ser el mateix que per a l'obtenció de les cèl·lules A7r5 hSSAO/VAP-1 (Sole *et al.* 2007). El vector pcDNA3.1(+), conté l'inserit de la proteïna humana SSAO/VAP-1, contenint també gens que confereixen resistència als antibiòtics ampicil·lina (per selecció en bacteris) i neomicina (per selecció en cèl·lules eucariotes).

L'amplificació del plasmidi es realitza a partir d'un clon de cèl·lules bacterianes DH5α, del qual ja es coneix que conté el plasmidi d'interès. Primer, es preinoculen 10 µl

d'aquesta colònia, obtinguda prèviament i que resta guardada a 4ºC (Hernandez 2005), en 5 ml de medi LB líquid contenint l'antibiòtic de selecció ampicil·lina (150 µg/ml) (Sigma). El medi LB conté 5 g/L d'extracte de llevat (Pronadisa), 10 g/L NaCl (Sigma), 10 g/L de triptona (Pronadisa), i s'ajusta a pH 7.2, abans d'autoclavar-lo per tal d'esterilitzar-lo. Aquest cultiu es manté a 37 ºC en agitació constant (aproximadament a 200 RPMs) durant tota una nit, en un incubador per a cultiu bacterià (*Incubator Shaker Series INNOVA®44*, New Brunswick Scientific). L'endemà, s'inoculen 150 µl del preinòcul obtingut en 100 ml de medi LB líquid amb l'antibiòtic de selecció, i es deixa créixer en un erlenmeyer de 500 ml, en agitació constant a 37ºC durant tota la nit.

L'extracció i purificació del material genètic s'efectua mitjançant el kit *QUIAGEN®Plasmid maxi kit* (Quiagen), seguint les instruccions del proveïdor. Un cop obtingut el material genètic, aquest es quantifica mitjançant un aparell Qubit (Invitrogen), i el kit de quantificació de DNA *Quant-iT™ DNA Assay Kit, Broad Range* (Invitrogen), seguint les especificacions del proveïdor. En el nostre cas s'obtingué una concentració de 3.125 µg/µl de DNA, amb una ratio 260/280 d'1.82. Un cop obtinguda la concentració del plasmidi, es porta fins a una concentració d'1 µg/ml amb H_2O destil·lada estèril, i es guarda a 4ºC fins al seu ús per a la transfecció de cèl·lules eucariotes. Una part del material obtingut es guarda congelat a -80 ºC i una altra part es diposita en un paper de filtre per a la seva conservació durant llargs períodes de temps.

2.2. Transfecció de la línia cel·lular HUVEC WT

Per a la transfecció de la línia cel·lular HUVEC WT amb el plasmidi que conté el DNA de la proteïna SSAO/VAP-1 humana es va utilitzar el mètode de transfecció amb PEI linial de 25 KDa (*Polyethylenimine*) (Polysciences, Inc).

Per a preparar la solució stock de PEI, se'n dissol la quantitat necessària en H_2O destil·lada a una concentració d'1 mg/ml. S'ajusta el pH de la solució a 7.0 i s'esterilitza mitjançant filtració a través d'un filtre de 0.11 µm. La solució obtinguda s'aliquota i es guarda congelada a -80 ºC.

Per poder ajustar les condicions de transfecció de les cèl·lules HUVEC amb aquest mètode es va utilitzar un vector amb la proteïna verda fluorescent (GFP) inserida (*Vitality® phrGFP-C Mammalian Expression Vector*, Stratagene). La transfecció

transitòria de les cèl·lules amb aquest plasmidi permet conèixer de forma fàcil i ràpida el percentatge de cèl·lules transfectades en diferents condicions de transfecció, i permet escollir la millor mescla de transfecció. Les cèl·lules que incorporen el plasmidi emeten fluorescència verda en ser excitades adequadament com a resultat de l'expressió de la proteïna GFP (figura 29).

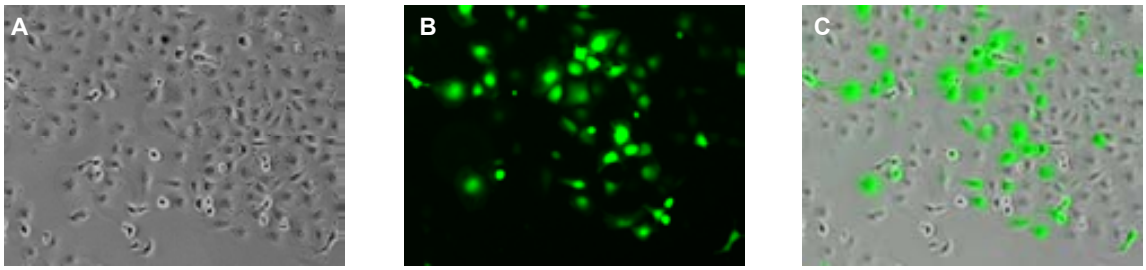


Figura 29: Micrografies de les cèl·lules endotelials (HUVEC), transfectades de forma transitòria mitjançant PEI, amb el vector phrGFP-C. (**A**), contrast de fase; (**B**), cèl·lules transfectades amb GFP; (**C**), superposició d'A i B.

Per a la transfecció cel·lular s'utilitzen plaques de petri de 100 mm de diàmetre, les quals han estat sembrades un o dos dies abans, per estar a una confluència entre el 50 i el 80 % el dia de la transfecció. Unes 3 - 4 hores abans d'iniciar el procés es canvia el medi de les cèl·lules per medi fresc complet (10% FBS). Per a la transfecció, es preparen tantes mescles de transfecció com plaques a transfectar. En primer lloc, es dissol el DNA en medi sense sèrum, en un volum de medi equivalent a una desena part del medi que conté la placa a transfectar; després s'afegeix el PEI a aquesta solució. La ratio DNA:PEI (µg:µl) que va donar un millor resultat entre les assajades va ser la 5:52,5. Cada solució DNA:PEI preparada es vorteja 3 vegades durant 3 segons, i després es deixen incubar durant 10 minuts a temperatura ambient. Finalment, s'afegeix la mescla a la placa de petri: amb una pipeta d'1 ml es deixa caure la mescla sobre la placa gota a gota des de la màxima altura possible, perquè cada gota impacti sobre el medi de la placa. Es retorna la placa a l'incubador, i s'incuba durant 2 - 3 dies, temps després del qual es procedeix a seleccionar les cèl·lules que han incorporat el plasmidi.

2.3. Selecció i amplificació de les cèl·lules transfectades

Per a realitzar la selecció de les cèl·lules que han incorporat el plasmidi, s'utilitza el medi complet de les cèl·lules endotelials amb l'addició de l'antibiòtic de selecció G418 (Gibco-Invitrogen), a una concentració final de 400 µg/ml. Aquesta concentració va ser

escollida a partir d'una corba de toxicitat realitzada prèviament amb concentracions creixents de l'antibiòtic durant 7 dies de tractament. L'antibiòtic G418 és un compost anàleg al sulfat de neomicina, per tant, ja que el plasmidi transfectat conté un gen de resistència a la neomicina, totes les cèl·lules que incorporin el plasmidi sobreviuran al tractament amb G418.

El tractament de les cèl·lules transfectades amb altes concentracions de l'antibiòtic (400 µg/ml) es manté durant dues setmanes, canviant el medi cada 2 - 3 dies, temps en el qual les cèl·lules no transfectades van morint. Després d'aquest temps, es tripsinitzen les cèl·lules que han sobreviscut i es sembren en altres plaques de 100 mm a una densitat molt baixa, mantenint encara el tractament amb altes concentracions de G418. En aquest pas, es deixa que les cèl·lules proliferin, generant en la placa petites colònies cel·lulars, provinents cadascuna d'una única cèl·lula.

Havent passat dues setmanes aproximadament, es seleccionen 24 colònies d'una mida entre 2 i 3 mm de diàmetre cadascuna, per repicar-les i sembrar-les separadament en pous de 24. Per repicar les colònies, primerament es localitzen mitjançant visualització amb un microscopi invertit, i es delimita el seu diàmetre per la part de sota de la placa. La placa es renta amb PBS 1X, i utilitzant una pipeta de 10 µl, s'agafen 5 µl de medi, i es dipositen sobre la colònia desitjada. Amb la punta de la pipeta es rasca una mica la placa per desenganxar-ne les cèl·lules i es recull el volum de medi dipositat, amb les cèl·lules desenganxades, per sembrar-les en un pou d'una placa de 24 prèviament preparada amb medi complet.

Al cap de 24 hores, temps durant el qual les cèl·lules s'adhereixen a la placa, s'afegeix l'antibiòtic G418 al medi, aquest cop a una concentració final de 100 µg/ml. Aquesta concentració és a la que es va utilitzar a partir d'aquest moment per al manteniment de les línies cel·lulars transfectades. Els clons independents es deixen créixer amb canvis de medi complementat amb G418 100 µg/ml cada 3 - 4 dies durant un aproximadament mes. Durant aquest temps, quan arriben al 80-90% de confluència es subcultiven en plaques de 6 pous, i més tard es divideix cada clon en 3 plaques de 60 mm de diàmetre.

De les 3 plaques sembrades per cada clon, una es segueix subcultivant per amplificar-lo, i les altres dues es llisen, una per analitzar-ne l'activitat SSAO (veure materials i mètodes, apartat 3.2) i l'altra per analitzar l'expressió de la SSAO/VAP-1 mitjançant western blot (WB) (veure materials i mètodes, apartat 7). Un cop analitzada l'activitat i expressió de la proteïna transfectada en cadascun dels clons, es congelen tres vials de cada clon seleccionat per mantenir-los en estoc en nitrogen líquid (veure materials i

mètodes, apartat 3.3.1). Els clons seleccionats són aquells que expressen la SSAO/VAP-1, i en els que aquesta és enzimàticament activa, ja que no tots els clons resistents a l'antibiòtic de selecció expressen finalment la proteïna transfectada.

3. MANTENIMENT, SUBCULTIU I EMMAGATZEMATGE DE LES LÍNIES CEL·LULARS

3.1. Manteniment i subcultiu de les cèl·lules musculars (A7r5 WT i hSSAO/VAP-1)

Els dos tipus cel·lulars musculars es mantenen en creixement en medi complet, contenint: medi DMEM (*Dulbecco's Modified Eagle's Medium – high glucose*, Sigma), 2 mM d'L-glutamina (Invitrogen), 10% d'FBS (Invitrogen), 100 u/ml de penicil·lina i 100 µg/ml d'estreptomicina (PAN Biotech). En les cèl·lules A7r5 hSSAO/VAP-1, s'afegeix al medi anterior una concentració final de 100 µg/ml de l'antibiòtic de selecció G418, amb l'objectiu de conservar el plàsmid introduït en aquestes cèl·lules, que conté un gen de resistència a aquest antibiòtic. Les cèl·lules es deixen créixer amb aquest medi en un incubador amb atmosfera humida amb un 95 % d'aire i un 5 % de CO_2 a 37 ºC.

Les cèl·lules es subcultiven quan la superfície del flascó es troba més o menys a un 90% de confluència (normalment es fa una dilució 1/4 cada setmana), amb canvis de medi cada 2 - 3 dies, i es treballa amb elles fins al passatge 30 com a màxim. Entenem per subcultiu l'aixecament de les cèl·lules de la superfície on es troben adherides per tal de comptar-les i ressembrar-les a una densitat (cèl·lules/ml) coneguda.

El protocol de subcultiu és el següent: es fan dos rentats de la placa amb PBS 1X (Sigma), complementat amb 200 u/ml penicil·lina + 200 µg/ml d'estreptomicina + 30 mM D(+)Glucosa, per eliminar completament les restes de sèrum (FBS) del medi. S'afegeixen després uns 20 µl de tripsina-EDTA/cm^2 (Sigma), i s'incuba la placa durant 1 minut a 37 ºC (procés de tripsinització). Seguidament es para l'acció de la tripsina afegint a la placa un volum de medi complet tres vegades el de tripsina, es recull la suspensió i es centrifuga durant 5 minuts a 800 x *g* per tal de recuperar-ne les cèl·lules. Es ressuspèn el *pellet* cel·lular en un volum de medi complet conegut, i es realitza un comptatge de les cèl·lules mitjançant una cambra de Neubauer. Per a realitzar aquest comptatge, es barregen 20 µl de la suspensió cel·lular amb 20 µl de blau de tripà, afegint 10 µl de la mescla a cada costat de la cambra de Neubauer. Es compten les cèl·lules viables (les que exclouen el colorant) dels quatre quadrants

externs de la cambra i s'obté la densitat cel·lular (cèls/ml) de la suspensió multiplicant la mitja de les cèl·lules comptades en cada quadrant per la dilució que s'ha fet amb el blau de tripà (2) i per 10^4 (ja que el volum d'un quadrant és de 0.1 µl). Les cèl·lules es dilueixen en medi complet a la densitat desitjada i es sembren en les plaques adequades (les plaques utilitzades per al cultiu cel·lular han estat obtingudes de Corning, Falcon, Sarstedt o TPP).

3.2. Manteniment i subcultiu de les cèl·lules endotelials (HUVEC WT i hSSAO/VAP-1)

El medi de cultiu complet utilitzat per al manteniment d'ambdós línies cel·lulars endotelials conté: medi M199 (Gibco), 1.2 mM d'L-glutamina, 2.2 g/l de bicarbonat (Fluka), 5 % d'FBS, 100 u/ml de penicil·lina i 100 µg/ml d'estreptomicina. Com en el cas de les cèl·lules musculars transfectades, en les cèl·lules HUVEC hSSAO/VAP-1 també s'afegeix al medi una concentració final de 100 µg/ml de l'antibiòtic de selecció G418. Les cèl·lules es deixen créixer en les mateixes condicions que les anteriors, en un incubador amb atmosfera humida amb un 95 % d'aire i un 5 % de CO_2 a 37 ºC.

Aquestes cèl·lules també es subcultiven quan es troben a un 90 % de confluència, amb canvis de medi cada 2 - 3 dies, i fins al passatge 40 com a màxim. En aquest cas, es fa una dilució 1/10 cada setmana aproximadament, ja que aquestes cèl·lules tenen una taxa de duplicació més alta que les musculars. El protocol de subcultiu és exactament el mateix que per a les cèl·lules musculars (veure materials i mètodes, apartat 3.1), només canviant el tipus de medi utilitzat.

3.3. Emmagatzematge de les línies cel·lulars musculars i endotelials: congelació i descongelació

Les línies cel·lulars es poden emmagatzemar congelades a -80 ºC (durant períodes no superiors a tres mesos), o en nitrogen líquid (per llargs períodes de temps). L'emmagatzematge de les cèl·lules es fa en forma de banc: s'amplifica el vial original en diversos vials, un dels quals es descongela per comprovar l'absència de contaminants bacterians, llevats o paràsits. Després s'obtenen entre 10 i 20 vials més de cadascun dels primers, que conformen el banc principal i que es manté congelat en nitrogen líquid. Cada vial es descongela quan és necessari per a realitzar els

experiments desitjats, utilitzant les cèl·lules sempre dins del mateix rang de passatges, per mantenir les característiques de la línia cel·lular.

3.3.1. Protocol de congelació:

Quan les cèl·lules que es volen congelar arriben a un 90 % de confluència aproximadament, es tripsinitzen de la mateixa manera que per a realitzar un subcultiu rutinari. Després de la centrifugació, es ressuspèn el *pellet* en medi de congelació, a una densitat aproximada d'un milió de cèl·lules/ml. El medi de congelació conté el medi basal propi de cada línia cel·lular amb un 20 % d'FBS i un 10 % de DMSO (dimetilsulfòxid) (Sigma), aquest últim actuant com a agent crioprotector. El DMSO genera calor en dissoldre's en solucions aquoses, així doncs, és convenient preparar el medi de congelació uns 5 - 10 minuts abans del seu ús. Cal dir que al medi de congelació no se li afegeix l'antibiòtic de selecció utilitzat per al manteniment de les cèl·lules transfectades, per maximitzar així el nombre de cèl·lules viables durant el procés de congelació.

Un cop les cèl·lules han estat ressuspeses amb aquest medi, es deixa un període d'equilibrat perquè el crioprotector pugui penetrar a les cèl·lules, d'entre 10 i 20 minuts (en cap cas més de 30 minuts), i es fan alíquotes d'1 ml que es dipositen en criotubs de 2 ml de capacitat. Els criotubs es col·loquen en una caixa amb sistema de congelació isopropílic (Cryo Freezing container, Nalgene) en un congelador de -80 ºC, aconseguint així que les cèl·lules es congelin a una taxa de descens de la temperatura d'1 ºC/min. Després de 24 hores a -80 ºC es passen els tubs al tanc de nitrogen líquid per al seu emmagatzematge, essent estables allí durant llargs períodes de temps.

3.3.2. Protocol de descongelació:

A diferència del procés de congelació, que requereix un cert temps d'equilibrat, en el procés de descongelació de les cèl·lules s'ha de procedir de forma ràpida. Abans de retirar les cèl·lules del tanc de nitrogen es prepara un flascó per a cultiu cel·lular de 75 cm^2 amb uns 12 ml de medi complet (el corresponent segons el tipus cel·lular) temperat a 37 ºC. En el menor temps possible, es descongela el criotub submergint-lo parcialment en un bany a 37 ºC i es transfereix tot el contingut del criotub al flascó. Al cap d'unes hores, tan bon punt les cèl·lules estan adherides, es canvia el medi del flascó per medi complet fresc, per tal d'eliminar les cèl·lules no viables i el DMSO que

contenia el medi de congelació, el qual pot ser tòxic per a les cèl·lules si es manté durant moltes hores. És en aquest primer canvi de medi, quan es torna a afegir l'antibiòtic de selecció a les cèl·lules transfectades.

4. TRACTAMENT DELS CULTIUS CEL·LULARS SEGONS EL DISSENY EXPERIMENTAL

Per a tots els dissenys experimentals que es descriuen a continuació, les cèl·lules musculars (A7r5 WT i hSSAO/VAP-1) s'han sembrat a una densitat de 40000 cel/ml i les cèl·lules endotelials (HUVEC WT i hSSAO/VAP-1) a una densitat de 50000 cel/ml ja que tenen una mida inferior a les musculars. Ambdós tipus cel·lulars s'han sembrat en medi complet, entre 24 i 48 hores abans de realitzar els tractaments corresponents. S'ha utilitzat un volum de sembra de 500 µl per pou en cas de les plaques de 24 pous, mantenint la relació volum/superfície per a la resta de tipus de plaques.

- **Experiments de caracterització de les línies cel·lulars transfectades:** per aquests experiments no s'efectua cap tractament; les cèl·lules es deixen créixer durant 3 - 4 dies fins arribar al 80-90 % de confluència en medi complet, i es recullen per analitzar-ne diferents característiques en condicions normals de proliferació.

- **Experiments d'inducció de l'expressió de la SSAO/VAP-1 i MAO A:** en aquest cas, les cèl·lules WT es sembren en medi complet contenint clorgilina (Clor, 10^{-7} M), i semicarbazida (Sc, 10^{-3} M) les cèl·lules hSSAO/VAP-1. El tractament es manté durant 10 dies per posteriorment analitzar-ne les activitats SSAO i MAO A.

- **Experiments per analitzar la toxicitat cel·lular i les vies de senyalització activades:** per aquests experiments es depriven les cèl·lules amb medi amb un baix contingut en sèrum (0.2 % d'FBS) una hora abans del tractament. En cas d'utilitzar inhibidors, aquests s'afegeixen 30 minuts abans del tractament inductor de la mort.

- **Experiments de citotoxicitat per analitzar l'efecte del bicarbonat en el medi de cultiu:** les cèl·lules es tracten amb medi de deprivació (0.2 % d'FBS) contenint diferents concentracions de bicarbonat (0.1, 1, 2, 3 i 3.7 g/l), i metilamina (MA) 3

mM. Prèviament al tractament s'ajusta el pH dels medis amb les diferents concentracions de bicarbonat per evitar interferències de les diferències de pH en l'experiment.

- **Experiments amb beta amiloide (Aβ):** el beta amiloide utilitzat en aquest treball és la forma de 40 aminoàcids, amb la mutació tipus Dutch, en la que l'àcid glutàmic en posició 22 és canviat per una glutamina (E22Q): $A\beta_{1-40}D$ (Anaspec). S'ha utilitzat aquesta forma del beta amiloide ja que la forma de 40 aminoàcids és la forma majoritària observada en el sistema vascular (Mayeux *et al.* 2003), i també perquè aquesta mutació indueix HCHWA-D (*Hereditary Cerebral Hemorrhage With Amyloidosis Dutch type*), una patologia relacionada amb l'Angiopatia Cerebral Amiloide (CAA), una de les condicions patològiques que s'estudien en aquest treball. L'$A\beta_{1-40}D$ (0.5 mg) liofilitzat es dissol en 1150 µl de PBS per obtenir una solució estoc de 100 µM, que es guarda a -80 ºC. En aquest treball, l'Aβ s'ha utilitzat sempre en forma soluble per als tractaments.

Tots els compostos utilitzats en els tractaments es dissolen en H_2O destil·lada, si no es diu el contrari, i s'esterilitzen, si és necessari, per filtració amb filtres de 0.2 µm per fer les solucions estoc. Les solucions estoc es preparen 100 vegades més concentrades que les concentracions de tractament.

Els compostos utilitzats han estat els següents, juntament amb les seves concentracions finals a l'assaig: metilamina, rang 1-3 mM (Sigma); semicarbazida, 0.1 - 1 mM (Sigma); clorgilina, 1 µM (Sigma); peròxid d'hidrogen (H_2O_2), formaldehid (FA), i amoni (NH_3) (Sigma), rang 0.1 - 2 mM; catalasa (Cat), 100 u/ml (Sigma); MDL72974A ((E)-2-(4-fluorophenethyl)-3-fluoroallylamine hydrochloride), 1 µM (obsequi del Dr. P. H. Yu, University of Saskatchewan, Saskatchewan, Canadà); 4-metilpirazol (4-MP), cianamida (Cyan), cloral Hidrat (Chl Hyd) i 1-bromoheptà (1-Br), rang 10-500 µM (Sigma); pifitrina-α (Pif-α), rang 1-10 µM (Sigma) (dissolta en DMSO); beta amiloide 1-40 Dutch ($A\beta_{1-40}$ D), rang 0.125-10 µM (Anaspec) (dissolt en PBS).

5. OBTENCIÓ I PREPARACIÓ DE MOSTRES

5.1. Obtenció de mostres per a la determinació de les activitats enzimàtiques SSAO, MAO A i MAO B

5.1.1. Mostres de medis de cultiu:

L'objectiu de determinar activitats enzimàtiques dels medis de cultiu recau en el fet que la SSAO és una proteïna transmembranal que pot ser alliberada al medi en la seva forma soluble (Abella *et al.* 2004). Per això es recuperen els medis condicionats, es a dir, que han estat en contacte amb les cèl·lules en cultiu (amb o sense tractament) i es comparen amb altres medis condicionats o no condicionats, es a dir, extrets directament de l'ampolla.

Per a obtenir aquestes mostres de medi i poder analitzar-les, es recull el medi de la placa, es centrifuga a 800 x *g* durant 5 minuts per descartar les cèl·lules mortes que hi puguin haver flotant, i es guarda a -80 ºC fins al moment del seu ús.

5.1.2. Mostres de cèl·lules en cultiu:

Per a mesurar les activitats SSAO, MAO A i MAO B de cèl·lules en cultiu es llisen les cèl·lules en el mateix tampó en el que es realitzi posteriorment l'assaig de determinació de les activitats enzimàtiques citades. A aquest tampó se li afegeix també un 1 % d'un còctel comercial d'inhibidors de proteases per a cèl·lules de mamífer i extractes cel·lulars (Sigma), per evitar la degradació de les proteïnes.

- En el cas de l'assaig de determinació de l'activitat SSAO, s'utilitza un tampó Tris 100 mM pH 9.0: 12.114 g/L de Tris-HCl (USB) en H_2Od, ajustant el pH amb HCl (Panreac).

- En el cas dels assajos de determinació de les activitats MAO A i MAO B, s'utilitza un tampó de fosfat potàssic (TPK) 50 mM pH 7.4: solució 50 mM de $K_2HPO_4 \cdot 3H_2O$ (Panreac) (11.411 g/L en H_2Od, solució bàsica), que es porta a pH 7.4 afegint una solució 50 mM KH_2PO_4 (Panreac) (6.845 g/L en H_2Od, solució àcida).

Per a obtenir les mostres, un cop finalitzat el tractament de les cèl·lules, en cas que s'hagi realitzat, es renten les plaques amb PBS 1X dues vegades per eliminar les restes de medi. S'aspira bé el PBS restant, i s'afegeix, amb les plaques sobre gel, un volum del tampó amb 1 % d'inhibidors de proteases de 150 µl per placa de 10 cm de diàmetre. Per plaques d'altres mides, es manté la relació de volum de tampó utilitzat segons la superfície de la placa. Les plaques es deixen en gel durant 10 minuts, i es recullen les cèl·lules amb l'ajuda d'un raspador (*scrapper*) (Sarsted). Les suspensions es recullen en tubs d'1.5 ml, es soniquen uns 8 - 12 segons cadascuna i es guarden a -80 ºC per, posteriorment, determinar-ne la concentració de proteïna (veure materials i mètodes 5.7) i analitzar-ne les activitats enzimàtiques citades (veure materials i mètodes 6).

5.2. Obtenció de mostres per a l'anàlisi per Western Blot (WB)

5.2.1. Mostres de medis de cultiu

Per als WB de medis de cultiu cel·lulars, aquests es recullen de la mateixa manera que per determinar-ne l'activitat enzimàtica, centrifugant-los per eliminar-ne les possibles cèl·lules flotants. En aquest cas, però, a més s'han de concentrar ja que la quantitat de proteïnes excretades que conté el medi queda molt diluïda. Per concentrar els medis, aquests es centrifuguen a 4000 x *g* i a 4 ºC en tubs Centricon® (Millipore) de 10 KDa de porus, fins quedar reduït el volum de medi a una quarta part de l'inicial, amb el que s'aconsegueix concentrar les proteïnes del medi 4 vegades. Alternativament, volums més grans de medi s'han concentrat mitjançant liofilització amb un liofilitzador *Freeze Dryer Modulyo* (IMA Edwards, Dongen, Holanda) i una posterior dissolució del liofilitzat obtingut en un volum conegut més petit que l'inicial.

Un cop obtingudes les mostres, aquestes s'han de preparar per poder ser analitzades per WB. En el cas dels medis de cultiu, aquests es comparen entre ells per volum, és a dir que no se'n determina la concentració de proteïna. Simplement s'afegeix a un mateix volum de cada mostra de medi, el volum necessari de tampó de càrrega concentrat 4 vegades (4X) perquè quedi 1X. El tampó de càrrega utilitzat conté 250 mM de Tris pH 6.8, 4% d'SDS (Sigma) i 40% de Glicerol (USB). Just en el moment d'utilitzar-lo se li afegeix també un 10% de β-Mercaptoetanol (Sigma). Després d'afegir el tampó de càrrega a les mostres, aquestes es bullen en un bany sec a 99 ºC durant 3 minuts i es centrifuguen un pols per fer baixar el vapor generat de les parets. Les

mostres ja estan preparades per realitzar el WB, o es poden conservar a -80 ºC fins realitzar l'assaig de WB, tornant-les a bullir just abans del seu ús.

5.2.2. Mostres de llisats cel·lulars complets

Per obtenir mostres de cultius cel·lulars per ser analitzades per WB, un cop finalitzat el tractament es renten les plaques dues vegades amb PBS 1X. Seguidament s'afegeix a la placa un volum de tampó de llisi de 100 µl per placa de 10 cm de diàmetre, mantenint la relació volum/superfície per plaques de diferents mides. En cas d'haver-hi cèl·lules mortes flotant, es centrifuga el medi de la placa 5 minuts a 800 x *g* per recuperar-les i ajuntar-les amb la suspensió cel·lular obtinguda de la placa. Les plaques es deixen 10 minuts en gel, i es recullen les suspensions cel·lulars amb l'ajuda d'un *scrapper*, per ser després sonicades durant 8 - 12 segons i conservades a -80 ºC fins al moment del seu ús. En aquest treball s'han utilitzat dos tipus de tampons de llisi diferents, segons si l'anàlisi de les mostres es durà a terme en condicions desnaturalitzants o no:

- El tampó de llisi que s'ha utilitzat per condicions desnaturalitzants conté un 1 % de Tritó X-100 (Probus), 0.01 M d'EDTA (Sigma) i 0.05 M de Tris-HCl pH 7.5.

- El tampó de llisi que s'ha utilitzat per a condicions no desnaturalitzants no conté Tritó X-100, i les mostres no es soniquen un cop obtingudes.
  Ambdós es poden conservar a 4 ºC i abans del seu ús se'ls addiciona un 1 % del còctel d'inhibidors de proteases.

Per tal de preparar aquestes mostres per poder ser analitzades per WB, és necessari determinar-ne la concentració de proteïna (veure materials i mètodes, apartat 5.7). Un cop fet això, es calculen els volums requerits de cada mostra per tenir la quantitat de proteïna desitjada: normalment es carreguen 20 µg de proteïna per gel, de cada mostra. A aquest volum de mostra se li afegeix una quarta part del volum total de tampó de càrrega 4X, i s'iguala el volum de totes les mostres amb tampó de llisi. El tampó de càrrega utilitzat normalment en condicions desnaturalitzants és el mateix que l'utilitzat per als medis de cultiu (250 mM de Tris pH 6.8, 4% d'SDS i 40% de Glicerol,

més un 10% de β-Mercaptoetanol afegit al moment d'utilitzar-lo); en condicions no desnaturalitzants, el tampó no conté SDS ni se li afegeix β-mercaptoetanol.

Un cop preparades les mostres, aquestes es bullen 3 min a 99 ºC (aquest pas no es fa en condicions no desnaturalitzants), i es centrifuguen un pols per fer baixar el vapor d'aigua de les parets. En aquest punt ja es poden carregar al gel d'electroforesi, o reservar-les a -80 ºC fins al seu ús, tornant-les a bullir just abans d'utilitzar.

5.3. Fraccionament subcel·lular per a l'obtenció de mostres enriquides en membranes

Per a obtenir preparacions cel·lulars enriquides en membranes, es parteix d'una placa de 15 cm de diàmetre per a cadascun dels subfraccionaments a realitzar, al voltant del 80 % de confluència. Acabat el tractament, si n'hi ha, es retira el medi de les plaques i es renten les cèl·lules dues vegades amb PBS 1X, per afegir-hi després 250 µl per placa de tampó de llisi. Aquest tampó de llisi conté 10 mM d'Hepes (Sigma), 1.5 mM $MgCl_2$ (Fluka) i 10 mM KCl (Panreac), i s'ajusta el pH a 7.9; prèviament al seu ús, se li afegeix un 1 % del còctel d'inhibidors de proteases. Després de mantenir uns 5 minuts les plaques en gel, se'n recullen les cèl·lules amb l'ajuda d'un *scrapper*, i es llisa cada mostra mitjançant l'homogeneïtzació d'aquestes en un *Weathon douncer* de 7 ml, unes 40 vegades cadascuna, mantenint la mostra en gel.

En acabar, els llisats es deixen en gel durant 10 minuts més i seguidament se'n centrifuga una part de cadascun durant 15 minuts a 2000 x *g* i 4 ºC, guardant la resta del llisat com a fracció total. D'aquesta centrifugació s'obté un *pellet*, que conté les cèl·lules no trencades i els nuclis. El sobrenedant obtingut s'ultracentrifuga a 4 ºC, a 100000 x *g* durant 30 minuts, en el cas de les cèl·lules endotelials, o 1 hora en el cas de les cèl·lules musculars. D'aquí s'obté, en el sobrenedant, la fracció corresponent als citosols de les cèl·lules, i en el *pellet*, la fracció enriquida en membranes. La fracció citosòlica es pot guardar a -80 ºC fins al moment del seu ús. El *pellet* membranal es renta dues vegades amb tampó de llisi per eliminar les restes de citosol, i després se li afegeixen 100 µl del tampó de llisi per a poder ressuspendre'l mitjançant sonicació. Després d'això ja es pot guardar a -80 ºC fins que sigui utilitzat.

Aquestes mostres es poden analitzar per diferents tècniques, però prèviament, és convenient de determinar-ne la concentració de proteïna per poder comparar els resultats entre diferents mostres (veure materials i mètodes, 5.7).

5.4. Fraccionament subcel·lular per a l'obtenció de Rais Lipídics (*lipid rafts*) de membrana

Els *lipid rafts* de membrana són regions específiques de les membranes cel·lulars que es caracteritzen per la seva composició lipídica i proteica diferent de la d'altres regions de la membrana, la qual els dóna la capacitat de ser insolubles en detergents del tipus Tritó X-100 (Brown 1992).

Per a obtenir fraccions cel·lulars riques en aquestes regions membranals es parteix de dues plaques de cèl·lules de 15 cm de diàmetre al 80 % de confluència aproximadament. Les plaques es renten dues vegades amb PBS 1X, i es recullen en 5 ml de PBS amb l'ajuda d'un *scrapper*. Les cèl·lules es recuperen mitjançant una centrifugació de 5 minuts a 800 x *g*, i es ressuspenen en 1.5 ml de tampó de llisi Tris/HCl. Aquest tampó de llisi està compost per 50 mM Tris/HCl, 150 mM NaCl, 1 mM EDTA (Sigma), 1 % del detergent Brij 98 (Sigma) i 1 % del còctel d'inhibidors de proteases (afegit en el moment d'utilitzar-lo), ajustat a un pH de 7.2.

La suspensió cel·lular s'incuba durant 15 minuts a 37 ºC en agitació, per permetre que el detergent actuï, i es centrifuga 10 minuts a 2000 x *g* per descartar els nuclis i restes cel·lulars massa grans. El sobrenedant es recupera i se li afegeixen 1.5 ml del tampó de llisi Tris/HCl amb un 90 % de sucrosa (Scharlau), quedant la mescla en 3 ml finals al 45 % de sucrosa. Aquests 3 ml es situen al fons d'un tub per a ultracentrifugació (*Ultra-Clear TM* (Beckman), de 12 ml de capacitat (14x89 mm), sobre els quals es disposa la resta del gradient de sucrosa que permetrà la separació dels *lipid rafts* de la resta de membrana: 6 ml de Tris/HCl al 35 % de sucrosa a la part central, i 3 ml de Tris/HCl al 5 % de sucrosa a la part superior del tub.

Aquestes mostres es centrifuguen a 38000 rpm en un rotor SW41 (en una ultracentrífuga Sorvall Combi), durant unes 18 - 20 hores. Durant aquesta centrifugació, els *lipid rafts*, no solubilitzats pel detergent, es desplacen en el gradient de sucrosa fins situar-se en la seva densitat, que és aproximadament d'un 16 % de sucrosa. La resta de membrana, que sí que s'ha solubilitzat per l'acció del detergent, resta a densitats elevades, mantenint-se al fons del tub. Passades les 18 - 20 hores de centrifugació es recullen 12 fraccions d'1 ml cadascuna, i es preparen per analitzar-les mitjançant WB, amb anticossos contra proteïnes específiques de *lipid rafts* i de fracció soluble de membrana.

Per analitzar aquestes mostres per WB, no és necessari determinar-ne la concentració de proteïna, ja que cada fracció obtinguda té una composició proteica diferent.

L'avaluació d'aquesta diferència mitjançant WB permet comprovar si el fraccionament ha estat realitzat correctament. Així doncs, per preparar aquestes mostres per WB simplement es mesclen 75 µl de cada fracció amb 25 µl del tampó de càrrega 4X, es bullen 5 minuts a 99 ºC en un bany sec, i es centrifuguen un pols per fer baixar el vapor condensat de les parets. D'aquesta manera ja es poden carregar les mostres en un gel d'electroforesi, o guardar a -80 ºC per al seu posterior ús.

5.5. Obtenció de mostres per a immunoprecipitació (IP)

Un cop finalitzats els tractaments de les cèl·lules en cultiu que seran utilitzades per a assajos d'immunoprecipitació, es renten les plaques amb PBS 1X dues vegades i es deixen 15 minuts en gel amb 100 µl de tampó d'IP amb 1 % d'inhibidor de proteases per placa de 10 cm de diàmetre. El tampó d'immunoprecipitacions conté un 1 % de Tritó X-100 en 100 mM de Tris-HCl pH 7.5. Després es recullen amb l'ajuda d'un *scrapper*, es disgreguen bé pipetejant les mostres varies vegades, i es mantenen 10 minuts més en gel. Un cop acabat això, ja es poden guardar a -80 ºC fins al moment del seu ús. Prèviament a fer l'assaig d'IP (veure materials i mètodes, apartat 11), se'n determina la concentració de proteïna pel mètode de Bradford (veure materials i mètodes 5.7).

5.6. Obtenció de mostres per a la determinació de l'activitat caspasa-3

Després del tractament corresponent en plaques de 10 cm de diàmetre, les cèl·lules i el medi de la placa es recullen de forma conjunta amb l'ajuda d'un *scrapper*. Les suspensions obtingudes es centrifuguen 5 minuts a 800 x *g*, després es renten els *pellets* obtinguts amb 1 ml de PBS 1X per eliminar les restes de medi, i es tornen a centrifugar 3 minuts a 8000 x *g* per recuperar les cèl·lules. Es retira completament el PBS del tub, deixant el *pellet* cel·lular al fons del tub, el qual es congela directament a -80 ºC fins al moment d'efectuar l'assaig de mesura de l'activitat caspasa 3 (veure materials i mètodes, apartat 9).

5.7. Determinació de la concentració de proteïna pel mètode de Bradford

Aquest assaig de determinació de la concentració de proteïna es basa en el mètode de Bradford (Bradford 1976). El procediment consisteix primerament en preparar una

recta patró amb concentracions conegudes de proteïna, per després poder comparar les mostres problema amb aquesta recta i així determinar-ne la concentració de proteïna. La recta patró es prepara a partir d'una solució de BSA (*Bovine Serum Albumine)* a una concentració d'1 mg/ml (Bio-Rad), la qual es dilueix amb H_2Od per obtenir les concentracions de 0.042, 0.0625, 0.083, 0.125, 0.167, 0.25, 0.333 i 0.5 mg/ml.

En una microplaca de 96 pous es disposen per duplicat 10 µl de cada punt de la recta patró, incloent un punt a concentració 0 mg/ml (H_2Od). Seguidament, es disposen per triplicat, 10 µl de cadascuna de les mostres problema en la dilució adequada: normalment es fan dilucions 1/10 dels llisats cel·lulars, i 1/200 dels plasmes humans, per tal que entrin en el rang de la recta patró. S'afegeixen a cada pou que conté mostra 200 µl del reactiu de Bradford (Bio-Rad) diluït 1/5 en H_2Od. Després es procedeix a la lectura de l'absorbància de les mostres a 595 nm mitjançant un lector de microplaques d'absorbància, fluorescència i luminiscència tipus Synergy HT (Bio-Tek®), i s'analitzen els resultats amb el software d'anàlisi de dades incorporat KC4 ™ (Bio-Tek®). Amb la lectura i anàlisi de les dades s'obté l'equació de la recta patró, que és utilitzada per determinar la concentració de les mostres problema a partir de la seva absorbància mesurada.

6. MÈTODE RADIOMÈTRIC PER A LA DETERMINACIÓ DE LES ACTIVITATS ENZIMÀTIQUES SSAO, MAO-A I MAO-B

L'assaig utilitzat en aquest treball per a determinar activitats enzimàtiques d'amino oxidases es basa en el mètode radiomètric desenvolupat per Otsuka i Kobayashi (OTSUKA and KOBAYASHI 1964). Aquest mètode és altament sensible, depenent aquesta sensibilitat de la radioactivitat específica de cada substrat utilitzat. Les activitats enzimàtiques es determinen mitjançant la utilització de substrats específics dels diferents enzims, marcats radioactivament amb l'isòtop carboni 14 (^{14}C). Durant la incubació dels enzims amb els substrats corresponents es generen els aldehids, productes de les reaccions enzimàtiques, que resten marcats radioactivament. Aquests es poden separar dels substrats i quantificar mitjançant el comptatge de la radioactivitat del producte amb un comptador de centelleig líquid.

Depenent de l'amino oxidasa de la qual s'avaluï l'activitat, s'han de tenir en compte diverses peculiaritats i s'han d'aplicar petites variacions en el procediment:

- El substrat marcat que s'utilitza pot ser diferent segons l'enzim. En el cas de la determinació de les activitats SSAO i MAO B, s'utilitza 7-^{14}C-Benzilamina (^{14}C-Bz) (^{14}C-*Benzylamine hydrochloride*, 55 mCi/mmol (Amersham)) a una concentració 100 µM i una activitat específica de 2 µCi/µmol. Per a determinar l'activitat de l'enzim MAO A s'utilitza com a substrat ^{14}C-Serotonina (^{14}C-5-HT) (*5-(2-^{14}C)-hydroxytriptamine binoxalat*, 45.9 mCi/mmol (Perkin Elmer)) a una concentració 100 µM i una activitat específica de 0.5 µCi/µmol. Per preparar els substrats, els estocs comercials marcats es dilueixen en H_2Od amb la corresponent quantitat de substrat fred (no radioactiu) per obtenir la concentració i activitat específica desitjada i es conserven a -20 ºC.

- Com que hi ha amino oxidases que comparteixen substrats, com per exemple la SSAO i la MAO B, abans de determinar qualsevol de les dues activitats és necessari fer una preincubació de la mescla de reacció amb l'inhibidor de l'altre enzim. D'aquesta manera s'assegura que en afegir el substrat, aquest només serà metabolitzat per l'enzim d'interès. Per determinar l'activitat SSAO, doncs, es preincuba la mescla amb L-Deprenil (Sigma), inhibidor de la MAO B, a una concentració final 1 mM. Per a determinar l'activitat MAO B es preincuba la mescla amb Semicarbazida (inhibidor de la SSAO) a una concentració final 1 mM.

- Aquesta preinhibició també és útil per comprovar que tota l'activitat detectada és deguda a l'enzim que s'està avaluant. En aquest cas s'utilitza la Semicarbazida (10^{-3} M) o l'MDL72974A (10^{-6} M) per inhibir la SSAO, l'L-Deprenil (10^{-3} M) per la MAO B i la Clorgilina (10^{-6}M) per la MAO A.

- Normalment s'utilitzen entre 200 i 300 µg de proteïna/mostra en cada assaig (o 50 µl, en el cas del plasma), i cada mostra s'analitza com a mínim per duplicat. El temps d'incubació amb el substrat depèn del tipus de llisat, de si té molta o poca activitat enzimàtica. Ambdós, ja sigui la quantitat de proteïna o el temps d'incubació, han d'estar dins el rang en el que el metabolisme del substrat és lineal amb la proteïna i amb el temps.

- El tampó utilitzat en la determinació de l'activitat SSAO és diferent del de les altres amino oxidases. Per a l'activitat SSAO es realitza la reacció en un tampó Tris-HCl 100 mM pH 9.0, ja que aquest pH bàsic és òptim per a

determinar l'activitat SSAO (Castillo *et al.* 1998). En canvi, per a les activitats MAO A i MAO B s'utilitza un tampó TPK 50 mM pH 7.4.

6.1. Procediment de l'assaig

Els assajos radiomètrics de determinació d'activitats amino oxidasa es realitzen en minivials de plàstic de fons pla, de 5 ml de capacitat. En aquests minivials es disposa la mescla de reacció, que conté en un primer moment la mostra (200-300 µg de llisat o 50 µl de plasma), 20 µl de cadascun dels inhibidors necessaris 10X (o H_2Od si no hi ha preinhibició) i el tampó adequat per arribar a un volum final de 200 µl per tub. Es fan duplicats o triplicats de cada mostra i duplicats del blanc de reacció, el qual conté també 100 µl d'una solució d'àcid cítric 2M, utilitzada per aturar la reacció. El blanc ens dóna el valor de la radioactivitat inespecífica.

Els tubs es mantenen en un bany a 37 ºC en agitació constant durant 30 minuts com a preincubació per permetre que els inhibidors actuïn. Passat aquest temps, s'inicia la reacció afegint 25 µl del substrat corresponent, i es mantenen els tubs a 37 ºC durant el temps necessari, que normalment és de 90 minuts per a les activitats SSAO i MAO B, i de 30 minuts per a l'activitat MAO A. Un cop finalitzat el temps d'incubació s'atura la reacció afegint a cada mostra 100 µl de la solució d'àcid cítric 2M.

Seguidament s'inicia el procés d'extracció del producte de la reacció. Per això s'afegeixen a cada minivial 4 ml d'una solució de toluè:etilacetat 1:1 (v/v), que conté també un 0.6% (p/v) de PPO (2,5-difeniloxazol) (Sigma). Es tapen els tubs i s'agiten vigorosament durant 1 minut per tal que el producte format a la fase d'incubació (aquosa) passi a la fase orgànica (toluè:etilacetat). Els aldehids formats no tenen càrrega, i durant aquest procés d'agitació passen a la fase orgànica que té un pH àcid mentre que les amines que resten com a substrat no oxidat es queden a la fase aquosa (Fowler and Tipton 1981).

Després de l'extracció, els vials es deixen 20 minuts a -80 ºC, per permetre que la fase aquosa es congeli al fons del tub; d'aquesta manera, es pot separar la fase orgànica que conté el producte radioactiu de la reacció decantant-la en un tub nou. La radioactivitat que contenen aquests nous tubs es mesura en un comptador de centelleig líquid Tri-Carb 2810TR (Perkin Elmer), amb el software Quanta Smart versió 3.0 (Perkin Elmer) un programa específic per a mesurar ^{14}C, i un temps de comptatge d'1 minut per vial.

6.2. Càlcul de l'activitat enzimàtica

L'activitat enzimàtica específica de cada enzim es calcula a partir de les dpm (desintegracions per minut), dades obtingudes en el comptador de centelleig. S'utilitza la següent fórmula,

$$dpm \cdot (100/X) \cdot Y \cdot (1/min) \cdot (1/\mu l \text{ proteïna}) \cdot (1000/P) = pmol/min \cdot mg \text{ proteïna}$$

on,

- X és el quocient d'extracció de l'aldehid en la fase orgànica (Fowler and Oreland 1980): 86.8 % en el cas del benzaldehid i 74.4 % en el cas de l'aldehid de la serotonina (5-hidroxiindol acetaldehid),
- Y és el factor de conversió de dpm a pmol,
- i P és la concentració de proteïna utilitzada en l'assaig, expressada en mg/ml i determinada mitjançant el mètode de bradford (veure materials i mètodes, apartat 5.7).

Y depèn de l'activitat del substrat (μCi/μmol), i es calcula segons:

$$Y = (\mu mol/\mu Ci) \cdot (1\mu Ci / 2.22 \cdot 10^6 \text{ dpm}) \cdot (10^6 \text{ pmol}/ 1\mu mol) = pmol/dpm$$

En el cas de la benzilamina, que s'ha utilitzat a una concentració 100 μM i amb una activitat específica de 2 μCi/μmol, s'obté un valor per a Y = 0.22; en el cas de la serotonina, que s'ha utilitzat a una concentració de 100 μM i amb una activitat específica de 0.5 μCi/μmol, s'obté un valor per a Y = 0.9.

7. MÈTODOLOGIA DEL WESTERN BLOT

El Western Blot és una tècnica d'anàlisi de proteïnes àmpliament utilitzada. Es basa en la detecció específica de proteïnes mitjançant interaccions antígen-anticòs, i permet identificar proteïnes concretes entre una mescla de gran quantitat de proteïnes diferents. El procediment té diversos passos ben diferenciats, el primer dels quals és la separació de les proteïnes d'una mostra segons el seu pes molecular i la seva càrrega en un gel d'acrilamida (electroforesi). Després d'això té lloc el *blotting*, en el que es transfereixen les proteïnes separades en el gel a una membrana. Finalment, la incubació d'aquesta membrana amb els anticossos corresponents permet la detecció de la proteïna d'interès d'una forma quantitativa i depenent de l'especificitat de l'anticòs.

En aquest treball s'han seguit dos tipus de procediments diferents per a l'anàlisi de proteïnes per WB. Un d'ells ha estat utilitzat per detectar el pèptid beta-amiloide (Aβ), el qual presenta variacions respecte al procediment estàndard degut a la dificultat de detectar proteïnes de baix pes molecular com l'Aβ. L'altre procediment ("estàndard") ha estat utilitzat per detectar la resta de proteïnes. Els dos es detallen de forma separada ja que difereixen tant la metodologia com la composició dels tampons.

7.1. Electroforesi

La majoria d'electroforesis de tipus "estàndard" que s'han realitzat en aquest treball han estat en condicions desnaturalitzants; puntualment, però, algunes s'han fet en condicions no desnaturalitzants. Les diferències existents en el procediment d'ambdues condicions han estat la preparació de les mostres, explicada anteriorment (veure materials i mètodes, apartat 5.2), i la composició del gel d'acrilamida, el qual no conté SDS en condicions no desnaturalitzants. La resta del procediment és igual en ambdós casos, i es detalla a continuació.

7.1.1. Electroforesi estàndard

L'electroforesi es duu a terme en un sistema de minigel vertical del tipus *Mini-Protean III Cell and Systems* (Bio-Rad), on les plaques que contenen el gel estan submergides en tampó d'electroforesi. Aquest tampó d'electroforesi està compost per 25 mM de Tris-HCl, 192 mM de Glicina (USB) i 20% d'SDS, ajustant el pH a 8.3.

El gel que separa les proteïnes està format per dos fases, la fase concentradora a dalt, i la separadora a baix. El gel separador es prepara a diferents percentatges d'acrilamida (7.5, 10, 12 o 15) segons la proteïna a analitzar (*30% Acrylamide/Bis Solution 37.5:1 (2.6 % C)*, Bio-Rad), tampó tris (1.5 M, pH8.8; 375 mM final), i SDS 20% (0.25 % final) (sense SDS en condicions no desnaturalitzants); el concentrador es prepara al 5 % d'acrilamida-bisacrilamida, en H_2Od, tampó tris (1 M, pH6.8; 65 mM final) i SDS 20 % (0.1 % final) (sense SDS en condicions no desnaturalitzants). Per a la polimerització dels gels s'afegeixen a la solució APS 10 % (persulfat d'amoni, Sigma) i Temed (*N,N,N',N'-Tetramethylethylenediamine*, Sigma).

Un cop polimeritzats els gels, s'introdueixen en la cubeta d'electroforesi amb la cambra interior (entre els dos gels) plena de tampó i l'exterior plena fins més o menys la

meitat. Es carreguen entre 20 i 40 µg de proteïna de cada mostra en cadascun dels pous, prèviament bullides durant 3 minuts a 99 ºC. En un dels pous es carreguen també 7 µl del marcador de pes molecular (*Precision Plus Protein™ Standard All* Blue (Bio-Rad)), que permetrà després saber el pes molecular de les proteïnes detectades. L'electroforesi es realitza a 40 mA en amperatge constant, fins que el front arriba a la part final del gel.

7.1.2. Electroforesi per a detectar el pèptid Aβ

El pèptid Aβ té un pes molecular d'aproximadament 4 KDa; aquesta mida tant petita fa que la seva detecció tingui certes dificultats. Per això s'utilitza un protocol de WB modificat, que permet la detecció de proteïnes de baix pes molecular.

El gel separador es prepara al 12 % d'acrilamida (*Acrylamide/Bis Solution 19:1 (5 % C)*, Bio-Rad), en H_2Od, tampó del gel 4X (1.6 M Tris, 0.4 M H_2SO_4 (Merck), pH8.1) i SDS 20 % (0.1 % final); el gel concentrador es prepara al 6 % d'acrilamida, en H_2Od, tampó del gel 2X (0.8 M Bistris (Fluka), 0.2 M H_2SO_4, pH6.7), SDS 20 % (0.1 % final) i 50 µl del colorant *pyronin Y* 0.1 % (Sigma). Aquest colorant, a diferència del colorant blau de bromofenol que conté el tampó de càrrega, marca la posició real del front, fet important en analitzar proteïnes de baix pes molecular per evitar que aquestes s'escapin del gel. S'utilitza el mateix sistema de minigel vertical que l'esmentat anteriorment. En aquest cas, s'utilitzen dos tampons d'electroforesi, un es diposita a la cambra interior (limitada pels gels), i l'altre en la cambra exterior. El tampó de la cambra interior (càtode) conté 0.2 M Bicina (Sigma), 0.1 M NaOH, 0.25 % w/v SDS, ajustant el pH a 8.2; el tampó de la cambra exterior (ànode) conté 0.2 M Tris, 0.05 M H_2SO_4 i s'ajusta el pH a 8.1.

La preparació de les mostres és la mateixa que en el cas de realitzar un WB estàndard, bullint també les mostres 3 minuts a 99 ºC abans de ser carregades en el gel d'electroforesi. Juntament amb aquestes mostres es carrega un pou amb 7 µl del marcador per a proteïnes de baix pes molecular (*Kaleidoscope Polypeptide Standard*, Bio-Rad). L'electroforesi es duu a terme a 100 V en voltatge constant, fins que el front arriba a la part final del gel.

7.2. Transferència de proteïnes a una membrana

En aquest procediment, les proteïnes que s'han separat en l'electroforesi es transfereixen a una membrana de nitrocel·lulosa (Schlecher & Schüll) o de PVDF (*Polyvinylidene difluoride, Hybond-P PVDF* Membrane, Amersham), per tal de poder-hi detectar les proteïnes d'interès mitjançant els anticossos adients. Per tal de transferir les proteïnes a la membrana s'utilitza el sistema *Mini Trans-Blot® Electrophoretic Transfer Cell* (Bio-Rad). En aquest sistema es posen en contacte el gel i la membrana dins un sandvitx situat entre dos elèctrodes, i mitjançant l'aplicació de voltatge s'aconsegueix que les proteïnes migrin del gel a la membrana, on es queden adherides.

De la mateixa manera que amb l'electroforesi, en la transferència també s'utilitzen dos procediments diferents, un per a les proteïnes en general, el qual es fa sobre una membrana de nitrocel·lulosa, i l'altre específicament per detectar el pèptid Aβ, el qual es fa sobre una membrana de PVDF.

7.2.1. Transferència de proteïnes a una membrana de nitrocel·lulosa (procediment estàndard)

Un cop acabada l'electroforesi es munta el sistema de transferència, que posa en contacte el gel i la membrana de nitrocel·lulosa, prèviament submergida en tampó de transferència. El tampó de transferència utilitzat conté 25 mM de Tris, 192 mM de Glicina i 20 % de Metanol (J. T. Baker) en H_2Od. S'omple tota la cubeta amb tampó de transferència, juntament amb una peça congelada que ajuda a mantenir la temperatura del tampó, i en agitació constant per homogeneïtzar la temperatura d'aquest. Per tal que les proteïnes migrin del gel a la membrana, s'aplica un voltatge constant de 80 V durant 2 hores o de 100 V durant 1.5 hores.

Un cop acabada la transferència, es desmunta el sandvitx i la membrana es submergeix en una solució de Pounceau S (Sigma) durant uns segons. Després, es va destenyint mitjançant rentats amb H_2Od. Aquesta solució tenyeix de vermell totes les proteïnes que s'han transferit a la membrana, i permet saber si la transferència s'ha realitzat correctament. Després d'aquesta comprovació s'acaba de destenyir la membrana amb un parell de rentats amb TTBS (TBS, tampó tris salí (20 mM Tris, 137 mM NaCl) amb 0.1 % de Tween 20 (Sigma)).

7.2.2. Transferència de proteïnes a una membrana de PVDF (procediment per a detectar el pèptid Aβ)

En aquest procediment s'utilitzen membranes de PVDF en comptes de nitrocel·lulosa, ja que són més adients per a proteïnes de baix pes molecular. Prèviament al muntatge del sistema de transferència és necessari activar les membranes de PVDF, a diferència de les de nitrocel·lulosa, que no necessiten aquest pas. Per activar les membranes de PVDF, es submergeixen durant uns 30 segons en metanol, després en H_2Od per eliminar les restes de metanol, i seguidament es deixen submergides en el tampó de transferència utilitzat fins al muntatge del sistema. És necessari repetir aquest pas d'activació amb metanol cada cop que la membrana s'assequi. Un cop acabada l'electroforesi, el gel també es manté uns 5 minuts submergit en el tampó de transferència per tal d'equilibrar-lo abans de muntar el sistema de transferència.

El sistema de transferència utilitzat és el mateix que en el cas anterior. En aquest cas, però, el tampó de transferència és tampó CAPS 1X (el tampó CAPS 10X és 100 mM de CAPS (Sigma), ajustant el pH a 11) amb un 10 % de metanol. Per tal que les proteïnes migrin a la membrana, en aquest cas s'aplica un voltatge constant de 90 V durant 1,5 hores. En acabat aquest procés, i per tal d'exposar els antígens de la membrana, aquesta es renta amb H_2Od i després es submergeix durant 5 minuts en PBS 1X (sense Tween 20) acabat de bullir.

7.3. Bloqueig i detecció de les proteïnes de la membrana

7.3.1. Bloqueig

El bloqueig de la membrana és un procés que s'utilitza per tal de cobrir tots els epítops de la membrana i així minimitzar les unions inespecífiques dels anticossos amb les proteïnes que aquesta conté. El bloqueig es realitza tant en les membranes de nitrocel·lulosa com en les de PVDF, però amb una solució diferent segons el procediment de WB estàndard o el d'Aβ:

- La solució de bloqueig utilitzada en el procediment estàndard, en el que s'han utilitzat les membranes de nitrocel·lulosa, és una solució al 5 % (w/v) de llet en pols desnatada en TTBS.

- En el cas del procediment per detectar l'Aβ, amb les membranes de PVDF, s'utilitza una solució al 10 % de llet en pols desnatada (w/v) i al 0.1 % (w/v) de BSA en PBS-0.05 % Tween 20, i s'ajusta el pH entre 7.4 i 7.6.

En ambdós procediments, les membranes es mantenen submergides en les solucions de bloqueig corresponents, en agitació suau, durant 1 hora.

7.3.2. Incubació amb anticossos

Després del bloqueig, les membranes s'incuben amb els anticossos primaris corresponents, l'antigen dels quals és la proteïna que es vol detectar. Les membranes s'incuben amb les dilucions adequades dels anticossos en solució de bloqueig durant tota la nit a 4 ºC, en agitació constant; en el cas dels WB d'Aβ, els anticossos primaris es dissolen en PBS-0.05 % Tween i 0.1 % BSA, en comptes de la solució de bloqueig.

Després de la incubació amb l'anticòs primari es fan 3 rentats de 10 minuts amb TTBS en el cas del procediment estàndard, o amb PBS-0.05 % Tween en el cas de la detecció d'Aβ. Seguidament es realitza la incubació de les membranes amb l'anticòs secundari conjugat a peroxidasa, a una dilució 1:2000 (1:5000 en el cas de proteïnes molt abundants com ara els controls de càrrega) en la solució de bloqueig corresponent, durant 1 hora a temperatura ambient i en agitació constant. En acabat, es fan 3 rentats més d'uns 10 minuts cadascun amb TTBS o PBS-0.05 % Tween; en el cas de la detecció d'Aβ, a més d'aquests 3 rentats se'n fan 4 més de 10 minuts.

7.3.3. Detecció de les proteïnes d'interès sobre la membrana

La detecció de la proteïna d'interès es fa gràcies a la peroxidasa que porta conjugada l'anticòs secundari, el qual està unit a l'anticòs primari que resta unit a la proteïna d'interès. La senyal de la peroxidasa es revela mitjançant el mètode quimioluminiscent d'ECL® (Amersham), i la màquina de revelat *Medical Film Processor FPM 100-A* (Fuji).

Les membranes es cobreixen amb la solució de revelat quimioluminiscent durant 1 minut. Seguidament es retira l'excés de solució de revelat, i les membranes es dipositen entre dos fulls transparents en un casset de revelat (*Amersham Hypercassette™ Autoradiography* Cassette, Amersham). En una cambra fosca,

s'introdueix en el casset i sobre la membrana un film per a revelat (*Amersham Hyperfilm* ECL, Amersham), el qual queda velat en la posició on rep la senyal luminiscent de la peroxidasa i es fa evident en passar el film per la màquina de revelat.

7.4. "Stripping" de membranes

Aquest procediment s'utilitza en cas de voler reincubar una membrana ja utilitzada amb un anticòs primari diferent, sobretot si les dues proteïnes a detectar son de pesos moleculars semblants, o si tenen el mateix anticòs secundari.

Després del revelat de la membrana amb el primer anticòs, es fan 3 rentats de la membrana amb TTBS i s'incuba aquesta en una caixa tancada amb tampó de *Stripping*, en un bany a 50 ºC durant 20 minuts, i en agitació constant. El tampó de *Stripping* conté 100 mM de β-Mercaptoetanol, 2 % (p/v) SDS i 62.5 mM Tris-HCl pH 6.7. Després d'això es fan rentats amb TTBS, durant 2 - 3 hores fins que ja no es senti olor de β-Mercaptoetanol, i a partir d'aquí s'incuba amb el següent anticòs primari i es segueix el protocol explicat anteriorment.

7.5. Anticossos utilitzats per a WB

Els anticossos primaris utilitzats per a la tècnica de WB han estat:

Rabbit anti-rec d'IGF I (subunitat β) (Santa Cruz Biotech); mouse anti-GAPDH (Ambion); rabbit anti fosfo-p53 Ser 15, rabbit anti-Bax, rabbit anti-Caspasa 3 fragmentada en Asp 175, rabbit anti-Caspasa 9 fragmentada i rabbit anti-PUMA (Cell Signaling); mouse anti-p53 (Abcam); mouse anti-Bcl-2, mouse anti-Grp 78, mouse anti-flotilina (BD Biosciences); mouse anti-receptor de transferrina (ZYMED); mouse anti-β-actina (Sigma-Aldrich) i mouse anti-APP 20.1 (generat a partir dels hibridomes 20.1 cedits pel Dr. W. E. Van Nostrand (New York) (veure annex II).

Per a la detecció de la proteïna SSAO/VAP-1 s'han utilitzat els anticossos primaris: goat anti-VAP-1 C-terminal E-19 i rabbit anti-VAP-1 H-43 (Santa Cruz Biotech); rabbit anti-SSAO de pulmó boví (purificat al nostre laboratori (Lizcano *et al.* 1998)).

Els anticossos secundaris utilitzats han estat:

HRP anti-rabbit IgG (BD Biosciences); HRP anti-mouse IgG (DakoCytomation) i HRP anti-goat IgG (Pierce).

Per a més informació sobre els anticossos utilitzats i les condicions d'utilització, veure l'annex I.

7.6. Anàlisi semi quantitatiu de les dades obtingudes mitjançant la tècnica de WB

La semi quantificació de les dades obtingudes per WB s'ha dut a terme mitjançant la densitometria dels films corresponents amb el programa Image J 1.34 s (*National Institutes of Health, USA*). Els nivells de proteïna de cada WB es corregeixen pels corresponents controls de càrrega, o pels nivells de proteïna total en cas d'analitzar una forma fosforilada. Després es comparen els nivells de proteïna de les mostres tractades amb els corresponents controls no tractats per a poder realitzar un anàlisi estadístic.

8. MÈTODES DE DETERMINACIÓ DE LA VIABILITAT CEL·LULAR

8.1. Mètode de reducció d'MTT

El mètode de reducció d'MTT (Mosmann 1983) és un mètode àmpliament utilitzat per a la quantificació de la viabilitat cel·lular. Es basa en la reducció de l'MTT (3,(4,5-dimethylthiazol-2-yl)2,5-diphenyl-tetrazolium bromide), una sal de tetrazoli hidrosoluble, en formazà, que és un compost de color lila insoluble en solucions aquoses. Aquesta reducció del compost la duen a terme les deshidrogenases mitocondrials de les cèl·lules viables, per tant, la mesura de la producció de formazà esdevé un valor que correlaciona amb la proliferació/viabilitat cel·lular. Cal tenir en compte, però, que l'assaig no és una mesura directa de la mort cel·lular, sinó que és un reflex de l'activitat mitocondrial, la qual normalment indica l'estat en el que es troben les cèl·lules, però no s'ha de confondre.

Per dur a terme aquest assaig, les cèl·lules es sembren en plaques de 24 pous i es tracten. Un temps abans de la finalització del tractament (1.5 hores per a les cèl·lules musculars i 4 hores per a les endotelials) s'afegeix a cada pou una quantitat de solució estoc d'MTT (dissolt en PBS 1X calent, conservat a -20 ºC i protegit de la llum), tal que la concentració final assolida al pou sigui de 0.5 mg/ml d'MTT. Les plaques es deixen a l'incubador fins a la finalització del tractament, temps durant el qual es redueix l'MTT a

formazà en les mitocòndries funcionals de les cèl·lules. Passat aquest temps, s'aspira el medi i es substitueix per 250 µl/pou de DMSO, el qual dissoldrà el precipitat de formazà format. Seguidament es llegeix l'absorbància del precipitat a 560 i a 620 nm en un lector de microplaques Synergy HT (Bio-Tek®).

L'absorbància del precipitat és la de 560 nm, però es fa també la lectura a 620, a la qual no absorbeix el precipitat, per tal de descartar possibles interferències degudes a restes cel·lulars solubilitzades i al plàstic de la placa. El valor que resulta de la diferència entre les absorbàncies a 560 i a 620 per a cada pou és el que s'utilitza per a expressar el percentatge de viabilitat respecte el control no tractat.

8.2. LIVE/DEAD® viability/cytotoxicity kit (Molecular Probes)

Aquest kit comercial permet determinar la viabilitat directament de les cèl·lules en cultiu, sense necessitat de llisar-les o alterar-les d'alguna manera. La mesura s'obté a partir de la fluorescència generada, i es pot detectar per microscòpia de fluorescència o bé per mesura d'aquesta fluorescència mitjançant un fluorímetre lector de plaques. En aquest treball, la utilització d'aquest kit ha estat paral·lela a l'avaluació de la viabilitat cel·lular mitjançant la reducció de l'MTT, la qual ja s'ha comentat que no és una mesura directa de mort cel·lular.

El kit conté dues sondes: la calceïna AM i l'homodimer d'etidi-1. La calceïna AM és substrat de l'activitat esterasa intracel·lular, i en ser hidrolitzada dóna lloc a un producte fluorescent verd, la calceïna, la qual roman a l'interior de les cèl·lules indicant la seva viabilitat. L'homodimer d'etidi-1 només és capaç de penetrar en les cèl·lules que han perdut la integritat de la membrana, unint-se als àcids nuclèics d'aquestes i conferint-los fluorescència vermella, per tant, esdevé un marcador de cèl·lules no viables. La relació entre els dos tipus de fluorescència és la mesura de la viabilitat o mort cel·lular.

El procediment que es segueix és el recomanat pel proveïdor. Breument, les cèl·lules es sembren en plaques de 24 pous i es tracten normalment. Un cop finalitzat el tractament, es prepara una solució de medi de cultiu sense sèrum amb 1 µl de la solució de calceïna AM i 2 µl de la solució d'homodimer d'etidi-1. S'afegeix la mateixa quantitat d'aquesta solució a cadascun dels pous, evitant així treure el medi que tenen i que pot contenir cèl·lules mortes flotant. La placa s'incuba durant 15 minuts a 37 ºC i després es poden avaluar les diferències, ja sigui quantificant-les amb el lector de

microplaques de fluorescència Synergy HT, o bé mitjançant el microscopi invertit de fluorescència Nikon Eclipse TE 2000-E, amb emissió a 515 nm en verd i a 635 nm en vermell, amb una càmara Hamamatsu C-4742-80-12AG i el software Metamorph® Imaging System.

9. MÈTODE PER A LA MESURA FLUORIMÈTRICA DE L'ACTIVITAT CASPASA 3

La determinació de l'activitat caspasa 3 s'utilitza de forma complementària al WB de la proteïna fragmentada, en l'estudi dels processos de mort cel·lular apoptòtica. Per tal de mesurar l'activitat caspasa 3 de cèl·lules tractades, les mostres es recullen com s'ha detallat anteriorment (veure materials i mètodes, apartat 5.6.), i es segueixen les instruccions del proveïdor del kit utilitzat (Molecular Probes).

Breument, els *pellets* obtinguts es llisen en 65 µl de tampó de llisi cadascun, mitjançant un procés de congelació-descongelació de les mostres submergint-les en etanol amb gel sec durant 5 minuts. Un cop descongelades, aquestes es centrifuguen durant 5 minuts a 3000 x *g* i a 4 ºC per descartar-ne el material cel·lular insoluble. Després es transfereixen 50 µl del sobrenedant de cada mostra a una microplaca opaca negra, afegint un blanc amb només tampó de llisi, i reservant la resta de cada llisat per a determinar-ne la concentració de proteïna (veure materials i mètodes, apartat 5.7.). En la microplaca, s'afegeixen a cada mostra 50 µl de la "solució de treball", la qual conté el pèptid Z-DEVD-AMC com a substrat de la caspasa 3, i es deixa incubar la placa, protegida de la llum, durant 30 minuts. Com a recta patró, s'utilitza una dilució seriada de l'estàndard de referència AMC (7-amino-4-metilcumarina) a la mateixa placa.

Durant el temps d'incubació, la caspasa 3 activa de cada mostra hidrolitza el substrat afegit, resultant en un producte fluorescent (excitació/emissió ~342/441 nm) que es mesura després mitjançant el lector de microplaca de fluorescència Synergy HT i el software d'anàlisi de dades KC4™. A partir de la recta patró, s'obté per a cada mostra la concentració d'AMC que s'ha hidrolitzat. Les unitats finals d'activitat caspasa 3 s'expressen com a µM d'AMC/µg de proteïna en reacció, i es refereixen les unitats de les mostres tractades a les del control no tractat.

10. IMMUNFLUORESCÈNCIA (IF)

La immunofluorescència és una tècnica basada, com en el cas del WB, en la interacció entre un antigen i un anticòs, i la posterior detecció de l'anticòs mitjançant un fluorocrom. Aquesta tècnica es realitza directament sobre les cèl·lules, prèviament fixades per tal de mantenir-ne l'estructura, i per tant permet localitzar la proteïna detectada en un compartiment cel·lular concret. La tècnica és compatible amb la visualització simultània de diferents proteïnes, mitjançant l'ús de diferents fluorocroms. La utilització del microscopi confocal en comptes d'un microscopi de fluorescència convencional permet precisar més la localització de la proteïna en la cèl·lula.

Per tal de poder realitzar una immunofluorescència en un cultiu cel·lular, aquest ha d'estar a una confluència baixa per poder observar millor la morfologia de les cèl·lules. Les cèl·lules es sembren a una baixa densitat (20000 cèls/pou les A7r5 i 15000 cèls/pou HUVEC) en plaques de 12 pous amb cobreobjectes de vidre, tractades prèviament amb poli-D-lisina (Sigma) 10 µg/ml durant 1 hora a Tº ambient, o amb colàgen I 50 µM (*collagen I rat tail*, BD Biosciences) durant 1 hora a Tº ambient. Les cèl·lules es deixen créixer entre 2 i 3 dies, i es tracten si és el cas.

10.1. Fixació dels cultius cel·lulars

Un cop acabat el tractament, si n'hi ha, es fixen les cèl·lules perquè mantinguin la seva estructura i es puguin incubar amb els anticossos corresponents. Els mètodes de fixació per a les cèl·lules musculars (A7r5) i per a les endotelials (HUVEC) és diferent:

- En el cas de les cèl·lules A7r5, es fan dos rentats amb PBS 1X i la fixació es realitza afegint una solució al 2 % de paraformaldehid (Sigma) en PBS durant 10 minuts a 4 ºC. Després de la fixació es fan dos rentats més amb PBS 1X.

- En les cèl·lules HUVEC, després dels dos rentats amb PBS 1X, la fixació es duu a terme afegint la solució de Carnoy's (Metanol:Àcid acètic glacial (Panreac) 3:1 durant 20 minuts a -20 ºC. Després també es fan dos rentats amb PBS 1X.

Les cèl·lules fixades es poden guardar a 4 ºC durant uns dies abans de continuar amb el procediment. Per continuar, si les proteïnes a detectar són la SSAO o l'Aβ es realitza un pas que ajuda a exposar els antígens; aquest pas consisteix en fer un tractament de les cèl·lules fixades, amb àcid fòrmic (Panreac) al 15 % en H_2Od durant 1 minut. Després es fan 3 rentats de 5 minuts cadascun amb PBS 1X per eliminar les restes d'àcid fòrmic.

10.2. Incubació amb anticossos i tinció amb Hoechst

Seguidament (tant si s'ha fet el pretractament amb àcid fòrmic com si no), es realitzen 3 rentats de 5 minuts amb PBS-Tritó (0.1 %) i es fa el bloqueig incubant les plaques durant 30 minuts a Tº ambient en solució de bloqueig. La solució de bloqueig està formada per PBS 1X, contenint un 0.2 % de gelatina (Panreac), un 0.1 % de Tritó, 20 mM de Glicina i un 5 % d'FBS. Un cop fet el bloqueig, s'incuba amb l'anticòs primari a la dilució adequada en la mateixa solució de bloqueig però amb un 3 % d'FBS en comptes d'un 5 %. La incubació amb l'anticòs primari es realitza normalment durant tota la nit a 4 ºC, però per certs anticossos es pot fer una incubació de 2 hores a Tº ambient, o una incubació de tota la nit a Tº ambient. En cas de realitzar immunofluorescències de més d'una proteïna, la incubació amb el següent anticòs primari (de diferent espècie que el primer) es pot realitzar en aquest moment, o després de la incubació amb el primer secundari. Després es fan 5 rentats de 5 minuts en PBS-Tritó (0.1 %).

A continuació es realitza la incubació amb l'anticòs secundari conjugat a un fluorocrom, en el mateix tampó en el que s'ha realitzat la incubació amb el primari, durant 1 hora a Tº ambient, normalment a una dilució 1/1000. En cas d'haver incubat les mostres amb dos anticossos primaris diferents, es poden afegir els dos secundaris alhora; en cas d'incubar amb un altre primari després del primer secundari, es repeteix el procés explicat.

Durant la incubació amb els anticossos secundaris es pot realitzar també un contrast de nuclis amb Hoechst. Per això s'afegeix a la solució que conté l'anticòs secundari una dilució 1/1000 d'un estoc de Hoechst 33258 a 0.5 mg/ml. El Hoechst s'intercala en el DNA, i li confereix fluorescència blava en ser excitat amb llum ultraviolada. El contrast de nuclis és útil per a localitzar les cèl·lules en la preparació, especialment en casos en que hi hagi una senyal baixa de les proteïnes detectades. D'altra banda, la tinció dels nuclis amb Hoechst és un mètode que s'utilitza també de forma aïllada per a

detectar cèl·lules que estan en procés d'apoptosi, ja que durant aquest procés es produeix la condensació de la cromatina, que en ser tenyida amb Hoechst emet una senyal més brillant que el material genètic en estat normal.

Després d'efectuar les tincions desitjades, les preparacions es munten per poder ser observades en un microscopi de fluorescència, o en un microscopi confocal. Per muntar-les, els cobreobjectes s'inverteixen sobre un portaobjectes amb una gota del medi de muntatge (Mowiol (Fluka) o Fluorescent Mounting Medium (Dako)). Les preparacions es guarden a -20 ºC fins que son visualitzades en un microscopi de fluorescència invertit Nikon Eclipse TE 2000-E, amb una càmera Hamamatsu C-4742-80-12AG i el software *Metamorph® Imaging System*, o en un microscopi confocal Leica TCS SP2 AOBS (Servei de Microscòpia de la UAB).

10.3. Anticossos utilitzats

Els anticossos primaris utilitzats per a la tècnica d'IF han estat:

Goat anti-VAP-1 C-terminal (E-19) i rabbit anti-VAP-1 (H-43) (Santa Cruz Biotech); i mouse anti-Aβ Clon Bam10 (Sigma-Aldrich).

Els anticossos secundaris utilitzats per a la tècnica d'IF han estat:

Alexa Fluor® 594 goat anti-rabbit, Alexa Fluor® donkey anti-goat 488 i Alexa Fluor® 594 goat anti-mouse (Molecular Probes).

Per a més informació sobre els anticossos utilitzats i les condicions d'utilització, veure l'annex I.

11. IMMUNOPRECIPITACIÓ DE COMPLEXOS PROTÈICS (IP)

Aquesta tècnica permet estudiar si dues o més proteïnes estan unides formant un complex, ja sigui de forma permanent o transitòria. Mitjançant la unió específica d'una de les proteïnes del complex a un anticòs, unit a la vegada a una resina, es poden aïllar els complexos proteics de la resta de proteïnes o concentrar una proteïna concreta.

11.1. Preparació de la resina

S'utilitza com a resina la proteïna G-agarosa (Roche). La resina ve diluïda 1:1 en metanol, el qual s'ha de retirar abans d'iniciar el procés d'immunoprecipitació. Per això s'agafa el volum necessari de resina, que correspon a 25 µl de proteïna A-agarosa per assaig (per tant, 50µl de la mescla amb metanol) més un volum més per evitar que en falti: [50 µl de resina x nº mostres] + 1. S'afegeix a la resina 1 ml del tampó de llisi utilitzat per a obtenir els homogenats cel·lulars i es centrifuga a 12000 x *g* durant 30 segons; aquest pas es repeteix 3 vegades per eliminar el metanol completament. Després de retirar el sobrenedant de l'últim rentat, s'afegeix el mateix volum de tampó de llisi que el que hi hagi de resina. El volum total es divideix entre el nombre d'assajos + 1, i és el que s'afegeix a cada assaig.

11.2. Procediment de la immunoprecipitació

Acabat el tractament, si n'hi ha, les mostres s'obtenen com s'ha explicat en l'apartat 5.5. de materials i mètodes. Es calcula la concentració de proteïna de les mostres, i s'utilitza per a cada assaig de 500 µg a 1 mg de proteïna en 1 ml de volum final (ajustat amb tampó de llisi amb un 1 % d'inhibidors de proteases). La resta de llisat es prepara per ser analitzat mitjançant WB com a fracció total.

Després es fa el pas de "preclarat" de les mostres. Aquest pas consisteix en afegir a cada mostra el volum necessari per assaig de resina i es deixen les mostres a la roda vertical, a 4 ºC durant 1 - 3 hores. Amb el preclarat es pretén eliminar del llisat les proteïnes que s'uneixin inespecíficament a les boles d'agarosa. Un cop passat el temps de preclarat, es centrifuguen les mostres a 1000 rpm durant 2 minuts per separar el sobrenedant i la resina.

Els *pellets* de resina es renten 3 vegades amb tampó de llisi, se'ls afegeix 40 µl de tampó de càrrega a cadascun (el mateix que s'utilitza per a WB) amb 10 mM de DTT (dithiothreitol, Sigma) i es bullen 3 minuts a 99 ºC per ser analitzats per WB.

Els sobrenedants obtinguts són els llisats preclarats de les mostres; se'ls afegeix a cadascun 1 µg/assaig de l'anticòs corresponent i s'incuben a la roda vertical, a 4 ºC durant 2 hores. Després se'ls afegeix el volum necessari de resina nova (preparada prèviament juntament amb la utilitzada per al preclarat de les mostres) i s'incuben en una roda giratòria a 4 ºC de 2 a 6 hores més. En acabat, es centrifuguen les mostres a 1000 rpm durant 3 minuts per recuperar la resina, que tindrà unit l'anticòs juntament

amb el seu antigen i altres possibles proteïnes unides. Els sobrenedants es poden conservar a -20 ºC i analitzar posteriorment per WB. Els *pellets* es renten 3 vegades amb tampó de llisi, se'ls afegeix a cadascun 40 µl de tampó de càrrega amb 10 mM de DTT i es bullen 3 minuts a 99 ºC per posteriorment ser analitzats mitjançant WB (veure material i mètodes, apartat 7) i així comprovar si la IP s'ha realitzat correctament, i si existeixen complexos entre les proteïnes d'interès i altres.

11.3. Anticossos utilitzats

Els anticossos primaris utilitzats per a la tècnica d'IP han estat:

Rabbit anti-Aβ (Sigma-Aldrich).

Per a més informació sobre els anticossos utilitzats i les condicions d'utilització, veure l'annex I.

12. ANÀLISI ESTADÍSTIC DE LES DADES

Els resultats presentats en aquest treball s'expressen com a mitjana ± l'error estàndard de la mitjana (SEM). Per a la comparació de les dades obtingudes es realitza la prova *t* de Student, o l'ANOVA d'un factor acompanyat d'un test *post hoc* de *Dunnett*, *Bonferroni* o *Newman-Keuls*. Els valors es consideren significatius si $p < 0.05$. L'anàlisi estadístic de les dades, així com les seves representacions en gràfiques s'han dut a terme mitjançant els programes *Graph-Pad Prism 3.0* (San Diego, CA, USA) o el paquet *Statistical Package for the Social Sciences* (SPSS) 15.0.1 (Chicago, IL, USA).

IV. RESULTATS

Capítol I:

"Characterization of A7r5 cell line transfected in a stable form by hSSAO/VAP-1 gene (A7r5 hSSAO/VAP-1 cell line)"

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Characterization of A7r5 cell line transfected in a stable form by hSSAO/VAP-1 gene (A7r5 hSSAO/VAP-1 cell line)

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Summary A smooth muscle cell line (A7r5) was stably transfected with the human SSAO/VAP-1 (hSSAO/VAP-1) gene. The expressed protein was located solely in the membrane fraction of the cell. However it was also shown to be released into the cell-culture medium. Both the membrane-bound and released, soluble, forms had SSAO enzyme activity. Although MAO-A is present in wild-type A7r5 cells, it was undetectable in the transfected cells.

Keywords: Monoamine oxidase, semicarbazide-sensitive amine oxidase, smooth muscle cells, gene transfection, vascular-adhesion protein 1

Abbreviations

Clor	clorgyline
Dep	deprenyl
MAO	monoamine oxidase
Sc	semicarbazide
SSAO	semicarbazide-sensitive amine oxidase
VAP-1	vascular-adhesion protein 1

Introduction

Semicarbazide sensitive amine oxidase (SSAO, [E.C 1.4.3.6]), is a multifunctional enzyme (O'Sullivan et al., 2004) found in almost all mammalian tissues (Andres et al., 2001), specially in fat and in highly vascularized tissues (adipocytes, endothelial and smooth muscle cells). It is a glycoprotein that has different biological roles, which depend on the tissue where it is expressed. SSAO metabolizes primary amines (Lyles, 1996), generating hydrogen peroxide (H_2O_2), ammonia (NH_3) and the corresponding aldehyde as final products. In adipocytes, SSAO shows an insulinomimetic effect (Enrique-Tarancon et al., 1998) and it behaves as an inducible vascular-adhesion protein (VAP-1) under inflammatory conditions in endothelial cells (Salmi et al., 2001). However, its physiological function is still far from clear. SSAO/VAP-1 exists as a membrane-bound form and also as a soluble enzyme in plasma. It has been postulated (Abella et al., 2004) that the soluble form is derived from the membrane-bound one by a metalloprotease-dependent shedding process. Although high expression of SSAO/VAP-1 is observed *in vivo*, cultured cells lose this phenotype. In order to characterize the enzyme present in smooth muscle cells, we have stably transfected a smooth muscle cell line (A7r5) with the human SSAO/VAP-1 (hSSAO/VAP-1) gene.

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Materials and methods

Construct

Human SSAO/VAP-1 cDNA contained in pCMV-SPORT6 vector (ATCC; Image Clone ID: 6193046, GenBank ID: BC050549) was subcloned into pcDNA3.1(+) vector (Invitrogen; Barcelona, Spain). Recombinant vector was introduced in transformed *E. coli* DH5a cells. Cells were allowed to grow in LB medium plus ampicillin (50 μg/ml), and positive clones were selected and amplified to extract DNA. Plasmid DNA was confirmed by restriction enzyme digestions with *Eco*RI, *Not*I and *Spe*I and agarose gel electrophoresis.

Cell culture and transfection

The immortalized non-tumorigenic rat aortic smooth muscle cell line A7r5 (ECACC) was grown in DMEM supplemented with 2 mM glutamine,

50 U/ml penicillin, 50 μg/ml streptomycin, and 10% FBS (Gibco BRL; Grand Island, NY, USA). Cultures were maintained at 37°C in a humidified incubator, containing 95% room air and 5% CO_2 atmosphere. Transfections were performed using Fugene6 (Roche) in 50% confluent 100 mm dishes. Cells were allowed to grow for 1–2 months in medium containing the selection antibiotic geneticine (G418; 400 μg/ml). Cells were then diluted and allowed to grow in the presence of 100 μg/ml G418 in isolated colonies, which were amplified separately.

Total cell lysates, subcellular fractionation and concentrated culture media

To obtain total cell lysates, cells were washed twice with PBS, scrapped into a buffer containing 1% Triton X-100, 10 mM EDTA, 50 mM Tris-HCl, pH 7.5, supplemented with protease inhibitor cocktail (Sigma Aldrich; St. Louis, Mo, USA) and sonicated for 10 s. For subcellular fractionation, the buffer contained 10 mM Hepes, 1.5 mM $MgCl_2$ and 10 mM KCl, pH 7.9, supplemented with protease inhibitor cocktail. Cells were lysed by Dounce homogenization and centrifuged at 2,000 *g* for 15 min at 4°C to pellet unlysed cells and nuclei. The supernatant was centrifuged at 100,000 *g* (Sorvall Discovery M120 SE) for 1 h at 4°C to separate a soluble cytosolic fraction from a membranous pellet. Culture medium was collected from the cells and concentrated by means of successive centrifugations at 20,000 *g* in 10 KDa porus Centricon® centrifugal filter units.

Western blot analysis

Cell lysate protein determinations were made using the Bradford method. Samples were separated by SDS-PAGE, for culture media, the maximum volume was loaded, and transferred onto nitrocellulose membrane. Membranes were incubated with specific antibodies for VAP-1 (1:500) (C-terminal E-19 from Santa Cruz Biotech; Heidelberg, Germany), IGF-I receptor (1:1000) (Santa Cruz Biotech; Heidelberg, Germany), or GAPDH (1:20000) (Ambion; Cambridgeshire, UK) overnight at 4°C and developed using ECL® detection reagents, from Amersham Pharmacia Biotech (Piscataway, NJ, USA).

SSAO, MAO-A and MAO-B activity determination

A modification of the radiochemical method of Otsuka and Kobayhashi (1964) was used. SSAO and MAO-B activities were determined using 100 μM (^{14}C)-benzylamine (2 mCi/mmol, Amersham, UK), and MAO-A activity using 5-(2-^{14}C)-hydroxytryptamine binoxalate (0.5 mCi/mmol, Perkin Elmer, USA) as substrates. 1 μM *l*-deprenyl was used to inhibit MAO-B, 1 μM semicarbazide to inhibit SSAO and 1 μM clorgyline to inhibit MAO-A. Cell lysate activities are expressed as pmol product/min · mg protein and culture medium activities as pmol product/min · ml medium.

Statistics

Results are given as means ± SEM. Statistical analysis were done by one-way ANOVA and further Newman-Keuls Multiple Comparison Test, using the program Graph-Pad Prism 3.0. Significance was accepted at $P < 0.05$.

Results

SSAO/VAP-1 expressed in A7r5 hSSAO/VAP-1 transfected cells is localized in the plasma membrane and released into culture medium

Although high expression of SSAO/VAP-1 is observed *in vivo*, cells lose this phenotype in culture. In order to study the membrane-bound form of this enzyme present in smooth muscle cells, it was necessary to obtain a smooth muscle cell line transfected in a stable form with the human SSAO/VAP-1 gene. To achieve this, the human SSAO gene was subcloned into pcDNA3.1 vector, which allows selection in eukaryotic cells, and the recombinant vector obtained was checked by means of restriction enzyme digestions (data not shown). The A7r5 smooth muscle cells were then transfected using the FuGene6 method (Roche) and selected with the antibiotic G418.

After isolation and amplification of cell colonies, different clones were checked and selected by their SSAO expression. The specific markers: IGF-1 receptor for membrane fraction and GAPDH for cytosolic fraction were used in order to elucidate the subcellular localization of transfected SSAO in the cytosolic and membrane fractions of A7r5 hSSAO/VAP-1 cells. Figure 1A shows the presence of SSAO only in the membrane fraction. Because it has been postulated that the soluble SSAO is derived from the membrane-bound form, the possibility that the clones released SSAO into culture medium was investigated. Western-blot analysis (Fig. 1B) showed that SSAO

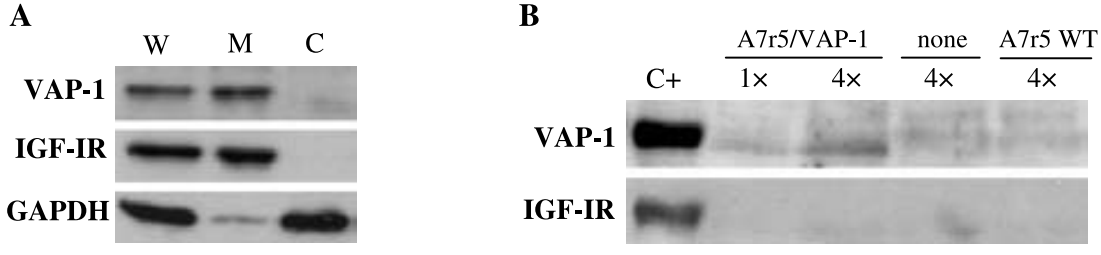

Fig. 1. Distribution of SSAO/VAP-1 in A7r5 hSSAO/VAP-1 cells. **A** SSAO/VAP-1 is present in the cell membrane but not in cytosol. Whole lysate (*W*), membrane (*M*) and cytosolic (*C*) subcellular fractions. IGF-IR and GAPDH were used as membrane and cytosolic subcellular fraction markers. **B** The enzyme is released into the culture medium from transfected cells. hSSAO/VAP-1 presence in: A7r5 hSSAO/VAP-1 cell lysate (C+) and conditioned medium from A7r5 hSSAO/VAP-1 cells [not concentrated (1×) and 4 times concentrated (4×)], control medium, and from A7r5/WT cells. IGF-IR was used as control for any lysed cells in the medium

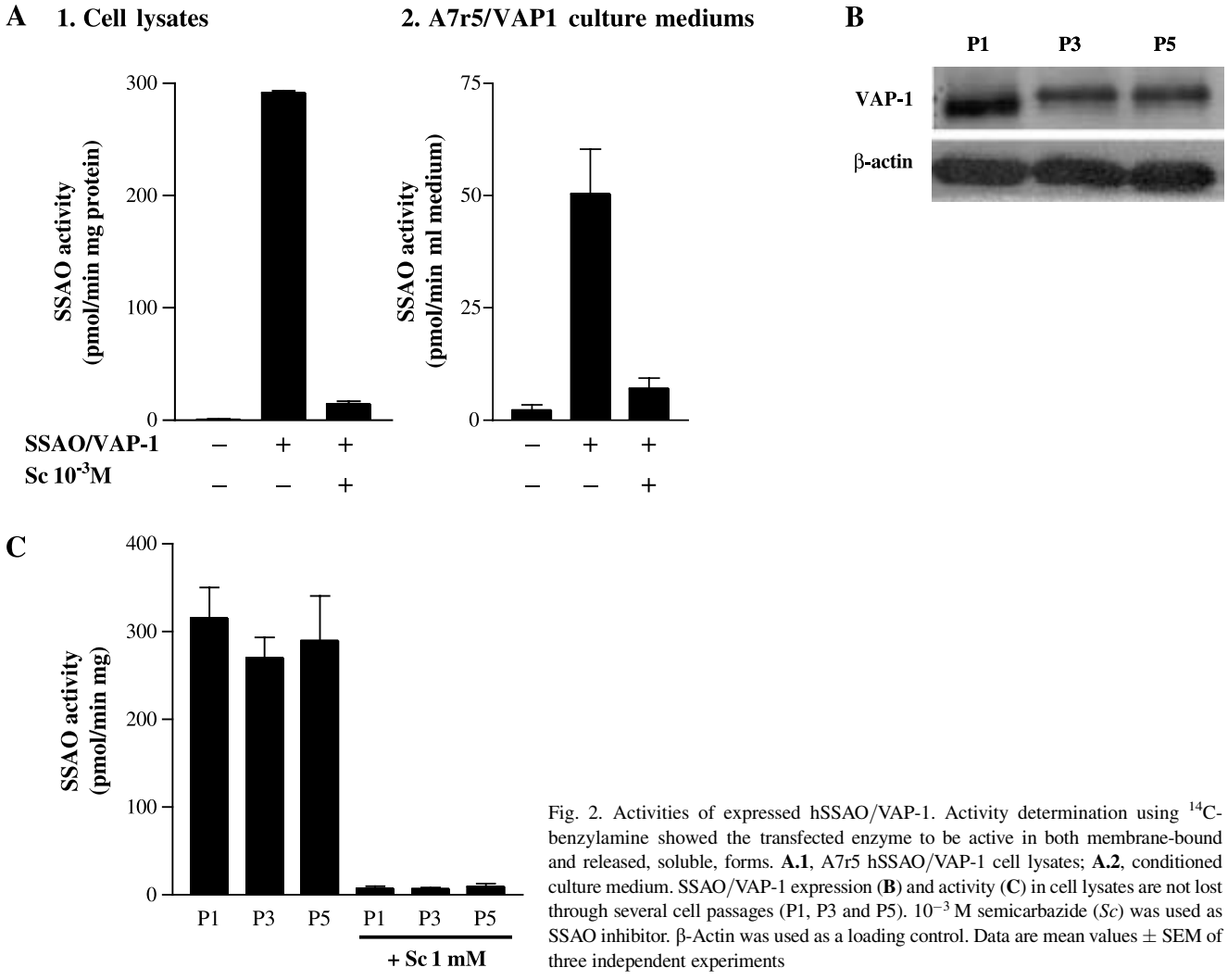

Fig. 2. Activities of expressed hSSAO/VAP-1. Activity determination using ^{14}C-benzylamine showed the transfected enzyme to be active in both membrane-bound and released, soluble, forms. **A.1**, A7r5 hSSAO/VAP-1 cell lysates; **A.2**, conditioned culture medium. SSAO/VAP-1 expression (**B**) and activity (**C**) in cell lysates are not lost through several cell passages (P1, P3 and P5). 10^{-3} M semicarbazide (*Sc*) was used as SSAO inhibitor. β-Actin was used as a loading control. Data are mean values ± SEM of three independent experiments

was present in the medium from transfected cells [not concentrated (1×) and four times concentrated (4×)], but was absent from control medium or medium from wild-type (WT) cells.

Transfected hSSAO/VAP-1 is enzymatically active in both cell lysates and culture medium

After checking the protein expression in different clones, the enzyme activity towards benzylamine as substrate was determined. Both cell lysates and culture medium from transfected cells showed SSAO activity, which was completely inhibited by the specific SSAO inhibitor semicarbazide (Fig. 2A), whereas neither cell lysates, nor culture media of WT cells showed any SSAO activity. To check the stability of SSAO expression in transfected cells, successive cell passages of different clones were analyzed for SSAO expression (Fig. 2B) and activity (Fig. 2C). There was no loss of expression or activity through several passages.

A7r5 WT and A7r5 hSSAO/VAP-1 have different amine oxidase pattern activities

MAO-A and MAO-B activities were also assayed in the transfected and WT cells. There was significant MAO-A activity in WT cells (Fig. 3A), but SSAO and MAO-B activities were absent. Surprisingly, this pattern changed in transfected cells (Fig. 3B), which showed SSAO activity, as a consequence of the transfection, but a lack of MAO-A activity. These results suggest a possible modulation between SSAO and MAO-A activities. However, treatment of WT cells with the MAO-A inhibitor, clorgyline (10^{-7} M) for ten days did not induce SSAO activity,

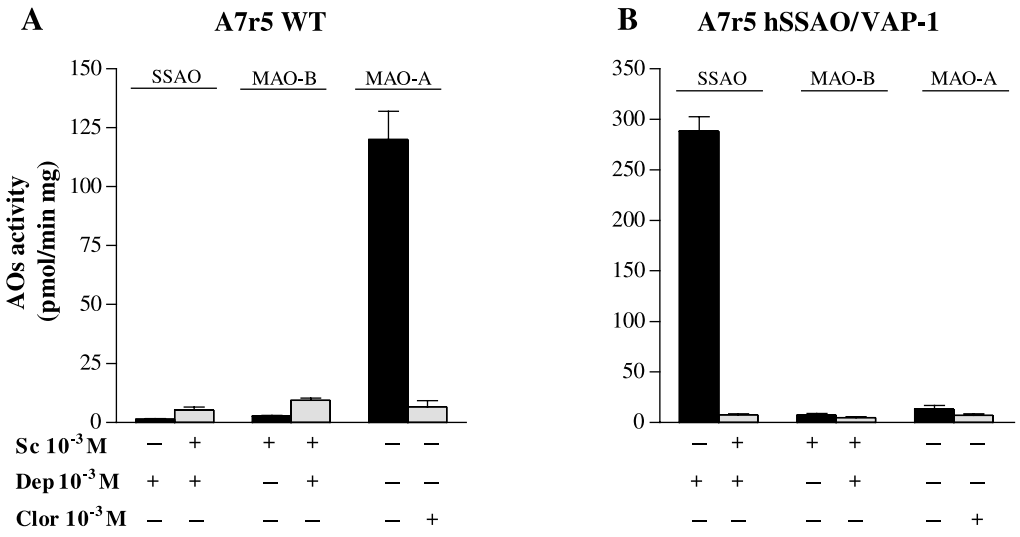

Fig. 3. Enzyme activities of SSAO, MAO-B and MAO-A in A7r5 WT and hSSAO/VAP-1 cell lysates. SSAO and MAO-B activities were measured with 100 μM ^{14}C-benzylamine, and MAO-A with 100 μM ^{14}C-5-HT. Total inhibition of SSAO, MAO-B and MAO-A activities with their specific inhibitors semicarbazide (*Sc*), deprenyl (*Dep*) and clorgyline (*Clor*), respectively, confirmed the specificity of the activities detected. **A**, MAO-A activity was detected in WT cells, but MAO-B and SSAO activities were not present. **B**, SSAO activity was detected in hSSAO/VAP-1 cells, but there was no MAO-A or -B activity. Data are mean values ± SEM of three independent experiments

and treatment of transfected cells with semicarbazide (10^{-3} M) was unable to induce MAO-A activity (data not shown).

Discussion

In order to elucidate the role of SSAO in cerebrovascular tissue in pathological conditions such as in cerebral amyloid angiopathy linked to Alzheimer's disease (CAA-AD), and its contribution to vascular damage, it was necessary to work with vascular cells that express SSAO/VAP-1. Although SSAO is constitutively expressed in vascular smooth muscle cells (Conklin et al., 1998), A7r5 cell line and HVSM (human vascular smooth muscle) cells did not exhibit any detectable SSAO activity or expression. This may result from the difficulty of maintaining a differentiated contractile phenotype in culture. It has been widely reported that vascular SMC show an *in vivo* and *in vitro* plasticity, which allow them to change its phenotype in response to environmental changes (Langford et al., 2001). Moreover, the loss of SSAO/VAP-1 expression in other smooth muscle and endothelial cell types has been previously observed by other authors (Blaschko, 1962; Owens, 1995; Yu and Zuo, 1993).

The results obtained in this study showed SSAO/VAP-1 expression in cell lysates of transfected A7r5 cells and in its culture medium, and this expression correlated with the catalytic activity towards ^{14}C-benzylamine. This activity was of the same order as that previously reported in aorta (Andres et al., 2001). Since WT cells did not show any SSAO activity in cell lysates or in culture medium, our results suggest that overexpression of membrane-bound SSAO in the transfected cells enhances the enzyme release into the culture medium. These data are in agreement with studies reported using transgenic mice overexpressing SSAO/VAP-1 in smooth muscle (Gokturk et al., 2003), endothelial cells and adipocytes (Stolen et al., 2004a, b).

Although SSAO expression and activity were not lost in transfected cells through several cell passages, they showed the same morphology than A7r5 WT cells. After subcellular fractionation, SSAO/VAP-1 expression was only observed in the membrane fraction indicating the same subcellular localization as *in vivo* conditions.

Some authors have suggested that it could exist a compensatory activity between MAO-A/MAO-B and SSAO (Fitzgerald et al., 1998; Fitzgerald and Tipton, 2002). Although the transfected cell line showed high SSAO/VAP-1 activity, MAO-A and MAO-B activities were not detected. In contrast, WT A7r5 cells showed significant levels of MAO-A, but no SSAO and MAO-B activities. When activities were determined in human aorta homogenates, we detected high SSAO/VAP-1 activity but no MAO-A activity. This amine oxidase activity pattern, also observed in our transfected cell line, could suggest a possible modulation between the enzymes. We tried to induce SSAO expression in WT cells and MAO-A expression in transfected cells by means of clorgyline and semicarbazide treatments, respectively, but this was not successful. The

possible modulation between these enzymes may be explained by another, activity independent, mechanism, probably at the gene expression level.

Taken together, these results show that the hSSAO/VAP-1-transfected A7r5 cell line is a valid model for studying the membrane-bound form of SSAO/VAP-1 enzyme *in vitro*.

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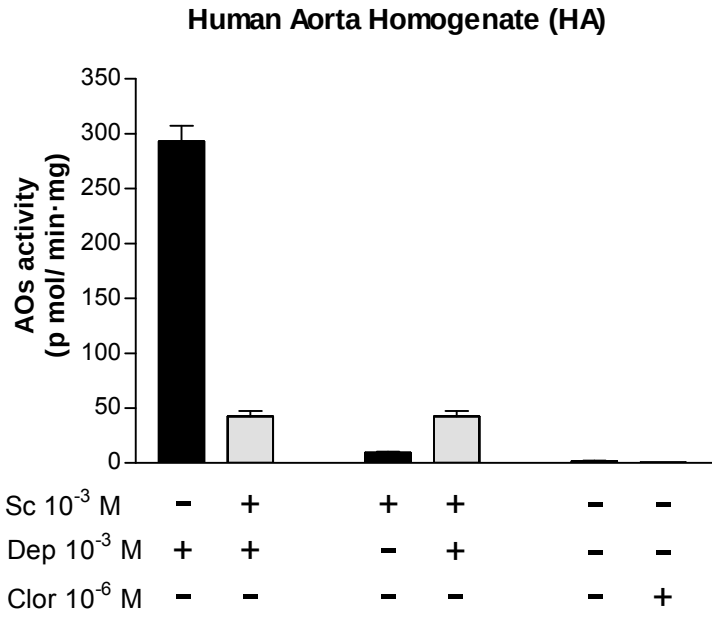
1. Annex Capítol I.

1.1. Supplementary figure A.

Since we obtained different amine oxidase activity patterns in A7r5 WT or hSSAO/VAP-1 cells, we analyzed this situation in aortic human tissue as a reference of the normal condition. MAO A, MAO B and SSAO activities were determined in potassium phosphate buffer pH 7.4 or Tris buffer pH 9 using ^{14}C-serotonine or ^{14}C-benzylamine, respectively (supplementary figure A).



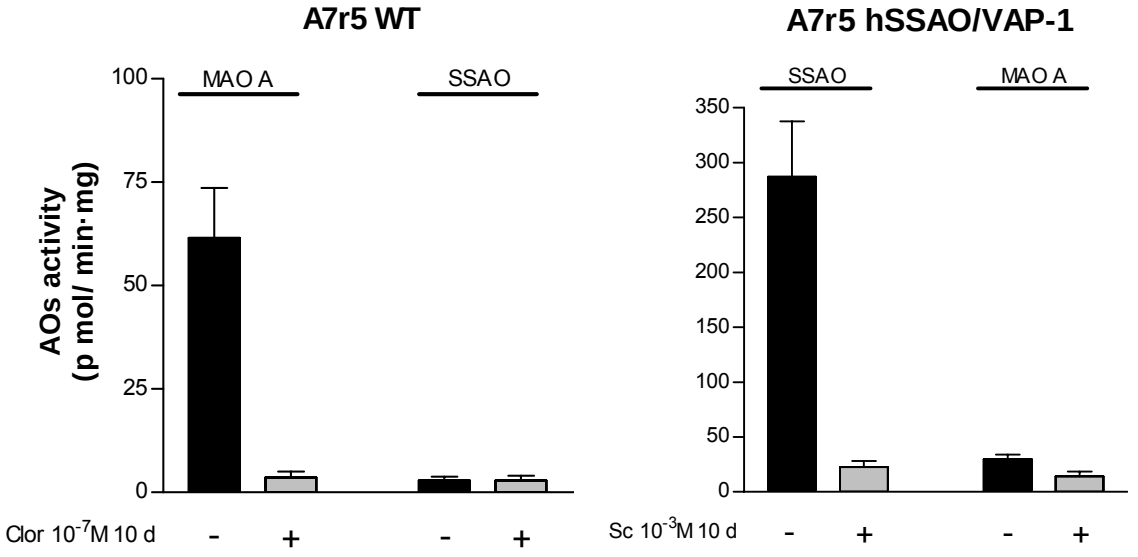
Supplementary figure A. Amine oxidase activity pattern of human aorta homogenate. Enzymatic activities were determined radiometrically with 100 µM ^{14}C-benzylamine or ^{14}C-5-HT. The selective inhibitors semicarbazide (Sc), deprenyl (Dep) and clorgyline (Clor) were used during in the activity determination to confirm the specificity of the measured activities.

The analyses of the three amine oxidase activities in human aortic tissue showed the same pattern to those observed in the A7r5 hSSAO/VAP-1 cells, with high SSAO/VAP-1 expression and almost undetectable MAO A and MAO B activities. These results suggest that after the transfection of the SSAO/VAP-1 protein into A7r5 cells, there were restored the physiological conditions in terms of amine oxidase presence. Moreover, it is possible that the MAO A activity observed in WT cells would be the result of the lost of SSAO expression by the culture conditions, as it has been

described to happen in response to SSAO inhibition (Kinemuchi *et al.* 2004). However the cause of the SSAO/VAP-1 disappearance by the culture conditions remains to be elucidated.

1.2. Supplementary figure B.

A7r5 hSSAO/VAP-1 cells and WT cells have different AOs activity pattern, showing WT cells a significant MAO A activity that is lost after SSAO/VAP-1 expression in transfected cells. Since a possible modulation between these amine oxidase activities has been suggested (Fitzgerald and Tipton 2002; Kinemuchi *et al.* 2004), we tried to induce one of these activities through the inhibition of the other (supplementary figure B). However, neither treatment of WT cells with clorgyline as MAO A inhibitor, nor treatment of SSAO/VAP-1 cells with semicarbazide as SSAO inhibitor during 10 days was able to induce SSAO and MAO A activities respectively.



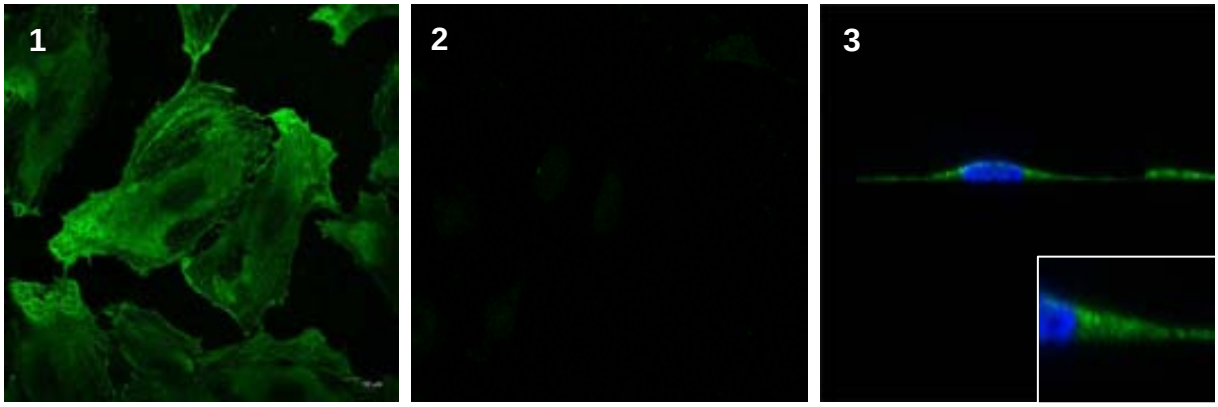
Supplementary figure B. A7r5 WT and hSSAO/VAP-1 cells were maintained in culture during 10 days in presence of clorgyline (Clor) (10^{-7} M) or semicarbazide (Sc) (10^{-3} M) respectively. After this time, cells were collected in Tris buffer pH 9, and SSAO and MAO A enzymatic activities were determined radiometrically with 100 µM ^{14}C-benzylamine or ^{14}C-5-HT. The inhibitors Sc and Clor were used to confirm the specificity of the activities detected.

An increase of plasma SSAO has been observed in Norrie disease patients, which have a deletion of genes encoding MAOs or dysfunctional MAOs (Murphy *et al.* 1991).

Additionally, an increase of SSAO activity after an *in vivo* treatment with the non-selective MAO inhibitor tranylcypromine was observed by Fitzgerald DH and collegues (Fitzgerald *et al.* 1998), but not with the selective MAO A and MAO B inhibitors clorgyline and lazabemide, respectively. These authors suggested that changes in SSAO activity to compensate for a loss of MAO activity may occur only when both MAO A and B activities are mostly inhibited. Therefore, we decided to use only clorgyline in our experiment because MAO B activity was not detected in WT or in hSSAO/VAP-1 cells. On the other hand, an induction of MAO A and B activities after *in vivo* inhibition of SSAO has been observed, but it has not been possible to reproduce these results *in vitro* (Kinemuchi *et al.* 2004). These results indicate that the genetic deletion of MAOs or SSAO/VAP-1 is not indispensable to see a compensatory effect. However, since we did not obtain MAO induction after SSAO inhibition or neither the inverse effect, it is possible that specific signals from other tissues would be necessary to induce these effect, as in both cases it has been observed at *in vivo* conditions, and the results have not been reproduced *in vitro*.

1.3. Supplementary figure C.

SSAO/VAP-1 is localized both in cytoplasmic granules and in the surface of endothelial cells. Its localization in smooth muscle cells of human tissues has been described in the cell surface, associated to caveolae (Jaakkola *et al.* 1999), but also intracellularly (Andres *et al.* 2001). Moreover, SSAO expression is not detected in different smooth muscle cell lines (Hernandez *et al.* 2006; Jaakkola *et al.* 1999). In A7r5 hSSAO/VAP-1 cells, we localized the transfected SSAO/VAP-1 in the membrane fraction, but this type of fractionation does not allow us to exclude its presence in cytoplasmic granules. In order to analyze the possible cytoplasmic presence of SSAO in our smooth muscle transfected cell line A7r5 hSSAO/VAP-1, we analyzed its distribution by immunofluorescence confocal microscopy.



Supplementary figure C. A7r5 hSSAO/VAP-1 and WT cells were seeded on collagen I coated coverslips and fixed with 2% paraformaldehyde for 10 minutes at 4ºC. Primary anti- VAP-1 (E19) (Santa Cruz Biotechnology) was added at 1/100 dilution and incubated over night. Secondary Alexa Fluor® 488-conjugated donkey anti-goat (Molecular Probes) and Hoechst 0.5 mg/ml were added both at 1/1000 dilution and incubated for 1h at room temperature. Preparations were mounted in Fluorescent Mounting Medium (Dako). Preparations were analysed with a Leica TCS SP2 AOBS confocal microscope at the *Servei de Microscòpia de la UAB*. **1**: A7r5 hSSAO/VAP-1 cells; **2**: A7r5 WT cells; **3**: reconstruction of different images throug Z axis of a hSSAO/VAP-1 cell, with magnification of the perinuclear zone.

The results showed that SSAO was expressed in the membrane but it was also present in the cytosol of A7r5 hSSAO/VAP-1 cells (1) while WT cells were devoid of signal (2); a reconstruction of several micrographs of a transfected cell at different Z-levels showed its presence in membrane and cytosol (3 and insert). SSAO/VAP-1 subcellular localization in smooth muscle cells has been poorly studied, and there have been reported distinct results about it in the literature. Moreover, several approaches have been done through fractionations that give total membrane preparations. Our results suggest that smooth muscle SSAO could be associated to intracellular membranes in the A7r5 hSSAO/VAP-1 cell line, as it has been described for smooth muscle cells *in vivo* (Andres *et al.* 2001), but more experiments should be done in order to confirm its specific location in intracellular granules. We have to consider also that we introduced the protein exogenously, and this process could interfere with its physiological localization. This effect could be observed especially in cases of protein overexpression, but this not seems to be our situation, since the SSAO activity levels of these transfected cells are comparable to the observed in human smooth muscle tissue homogenates (Andres *et al.* 2001).

Capítol II:

“Characterization of HUVEC cell line transfected in a stable form by hSSAO/VAP-1 gene (HUVEC hSSAO/VAP-1 cell line) and comparison with A7r5 hSSAO/VAP-1”

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Characterization of HUVEC cell line transfected in a stable form by hSSAO/VAP-1 gene (HUVEC hSSAO/VAP-1 cell line) and comparison with A7r5 hSSAO/VAP-1

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Running title: Endothelial cell line stably transfected with SSAO

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Abstract

Semicarbazide-sensitive amine oxidase/vascular adhesion protein 1 (SSAO/VAP-1) is an amine oxidase mainly expressed in adipocytes and endothelial and smooth muscle vascular cells. Its *in vitro* study displays some difficulties since primary cultures or immortalized cell lines lose the expression of this protein. In order to obtain a tool which would allow performing basic studies on this protein, an endothelial cell line (human umbilical vein endothelial cells - HUVEC) was transfected in a stable form with the SSAO/VAP-1 gene. The transfected protein was enzymatically active, it maintained a regular expression during passages and it was located in the membrane cellular fraction. The activity of the monoamine oxidases A and B (MAO A – MAO B) was also determined in these cells. Moreover, SSAO/VAP-1 expression in HUVEC hSSAO/VAP-1 cells was compared with its expression in a previously developed smooth muscle cell line transfected with hSSAO/VAP-1 gene (A7r5 hSSAO/VAP-1). Its level of expression, kinetic constants, membrane subtype localization, dimerization and behavior after cell subculture were analyzed. In summary, the analysis of the transfected SSAO/VAP-1 behavior in HUVEC cells showed that this new cellular model can be used in the *in vitro* biochemical study of SSAO/VAP-1 protein. Moreover, these models can be useful tools for the basic study of the role that SSAO/VAP-1 plays in vascular pathologies or associated disorders such as atherosclerosis, Alzheimer's disease, or diabetes-associated vascular complications.

Keywords: Semicarbazide-sensitive amine oxidase, endothelium, monoamine oxidase, transfection, lipid rafts

Abbreviations: A7r5, rat aortic smooth muscle cell line; AOs, Amine Oxidases; Clor, clorgyline; Dep, deprenyl; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HUVEC, human umbilical vein endothelial cells; MAO, monoamine oxidase; MDL, MDL72974A: [(E)-2-(4-fluorophenethyl)-3-fluoroallylamine hydrochloride]; Sc, semicarbazide; SSAO, semicarbazide-sensitive amine oxidase; Tf Rec, transferrin receptor; VAP-1, vascular adhesion protein 1; WT, wild type.

Introduction

Semicarbazide sensitive amine oxidase (SSAO, [E.C 1.4.3.21]) is an enzyme found in almost all mammalian tissues (Andres *et al.* 2001). It metabolizes primary amines (Lyles 1996), generating hydrogen peroxide (H_2O_2), ammonia (NH_3) and the corresponding aldehyde, and thus contributing to the monoamine detoxification from the organism. As a consequence of its enzymatic activity, SSAO plays other different roles depending on the tissue where it is expressed (O'Sullivan *et al.* 2004).

In adipocytes and in smooth muscle cells its activity has an insulinomimetic effect, being able to stimulate glucose transport (El Hadri *et al.* 2002; Enrique-Tarancon *et al.* 1998), and it is also involved in cellular differentiation processes (El Hadri *et al.* 2002; Mercier *et al.* 2001). In endothelial cells, SSAO was described as vascular adhesion protein-1 (VAP-1) (Smith *et al.* 1998) where it regulates lymphocyte rolling, firm adhesion and transmigration under inflammatory conditions (Jalkanen and Salmi 2008). SSAO/VAP-1 is also found in plasma, supposedly derived from the transmembrane protein by a metalloprotease-dependent shedding process (Abella *et al.* 2004). In plasma, SSAO/VAP-1 presence has been suggested to modulate the adhesive function of the transmembrane protein by generating positive signals on lymphocyte surface (Kurkijarvi *et al.* 1998; Ley and Deem 2005). Moreover, alterations in plasma SSAO activity levels are associated with pathological conditions such as diabetes (Boomsma *et al.* 1995), congestive heart failure (Boomsma *et al.* 1997), atherosclerosis (Karadi *et al.* 2002; Meszaros *et al.* 1999), inflammatory liver disease (Kurkijarvi *et al.* 1998), Alzheimer's disease (del Mar Hernandez *et al.* 2005) and others (Airas *et al.* 2006; Boomsma *et al.* 2003; Ekblom *et al.* 1999; Lewinsohn 1977; Madej *et al.* 2006; Madej *et al.* 2007; Nemcsik *et al.* 2007; Roessner *et al.* 2006).

Although high expression and activity of SSAO/VAP-1 are observed *in vivo*, primary cultured cells of SSAO/VAP-1 positive tissues progressively lose its expression (Arvilommi *et al.* 1997). Moreover, most immortalized cell lines do not show any SSAO/VAP-1 expression or activity (Jaakkola *et al.* 1999; Salmi and Jalkanen 1995). In certain endothelial models as HUVEC primary cultures or E1Hy-926 cell line, a very low SSAO/VAP-1 expression has been observed in cytoplasmic granules (Salmi and Jalkanen 1995), however, some authors describe that only the surface-expressed protein is enzimatically active (Yegutkin *et al.* 2004). In the same way, although several molecules are known to be involved in the regulation of endothelial SSAO/VAP-1 expression *in vivo*, they have failed in inducing its expression *in vitro*, both in

endothelial cell lines or in primary HUVEC cells and appendix organ cultures (Arvilommi *et al.* 1997; Salmi *et al.* 1993; Salmi and Jalkanen 1995). By contrast, it has been possible to induce SSAO/VAP-1 expression in human tonsil organ cultures by treatment with some pro inflammatory mediators (Arvilommi *et al.* 1997), indicating the existence of different regulation mechanisms of SSAO/VAP-1 expression in different tissues or organs, and the requirement of an intact tissue architecture to induce its expression. Regarding the non-endothelial SSAO/VAP-1 expression *in vitro*, it has been observed in a pre-adipocyte cell line and in smooth muscle primary cultures during its differentiation process (El Hadri *et al.* 2002; Mercier *et al.* 2001).

Since it is not possible to study certain aspects involving this protein *in vivo*, and it is difficult to study SSAO/VAP-1 *in vitro* by its lack of expression, several studies have been done inducing its expression in cell lines by transfection methods (Smith *et al.* 1998; Sole *et al.* 2007). SSAO/VAP-1 stable transfected COS-7 (monkey fibroblasts), CHO (chinese hamster ovary), Ax (rat high endothelial venules) and A7r5 (rat aortic smooth muscle) cells have been used to study SSAO/VAP-1 functionality, structure or activity (Smith *et al.* 1998; Sole *et al.* 2008; Yegutkin *et al.* 2004).

In this work, a HUVEC immortalized cell line has been stably transfected with the human SSAO/VAP-1 cDNA. The new cell line (HUVEC hSSAO/VAP-1) has been characterized in terms of SSAO expression, localization, activity and expression relative to other amine oxidases (MAO A and MAO B). Moreover, this cell line has been compared with the smooth muscle cell line stably transfected with hSSAO/VAP-1 (A7r5 hSSAO/VAP-1) that has been recently developed by our laboratory (Sole *et al.* 2007). This new cell line will provide us a new tool for the study of this particular enzyme.

Materials and methods

Cell culture and treatments.

The immortalized HUVEC cell line was a kind gift from Dr. F.J. Muñoz, from *Universitat Pompeu Fabra* (Barcelona, Spain). HUVEC cells were cultured in M199 medium (Invitrogen, Grand Island, NY, USA), supplemented with 1.2 mM Glutamine, 2.2 g/l sodium bicarbonate, 100 U/ml penicillin, 100 µg/ml streptomycin and 5% (v/v) FBS. WT and hSSAO/VAP-1 A7r5 smooth muscle cells were obtained from ECACC (European Collection of Cell Cultures, Wiltshire, UK) or as described (Sole *et al.* 2007),

respectively. A7r5 cells were grown in high glucose (4.5 mg/l) DMEM (Sigma-Aldrich, St. Louis, Mo., USA), supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin and 10% (v/v) FBS. Both cells were maintained at 37ºC in a humidified atmosphere containing 5% CO_2. Geneticin (G418, 100 µg/ml, Invitrogen) was added to the culture medium to ensure hSSAO/VAP-1 DNA maintenance.

For Amine Oxidases (AOs) induction experiments, cells were cultured during 10 days in medium containing clorgiline (Clor) 10^{-6} M, semicarbazide (Sc) 10^{-4} M or MDL72974A (MDL) 10^{-6} M (a kind gift from Dr. P.H. Yu, University of Saskatchewan, Canada). For experiments studying the stability of the protein during the culture process, cells were collected at different times after trypsinization.

Construct and transfection method

The vector pcDNA3.1(+) containing the human SSAO/VAP-1 cDNA (Sole *et al.* 2007) was transfected in HUVEC cells plated in 100 mm dishes at 50-80% confluency to obtain the HUVEC hSSAO/VAP-1 cell line. Transfection was performed by mixing the vector containing the hSSAO/VAP-1 cDNA and PEI (polyethylenimine (Polysciences Europe GmbH, Eppelheim, Germany)) in serum free medium, which was incubated during 10 min and added to the cell plates. Cells were selected allowing them to grow for 1-2 months in medium containing the selection antibiotic G418 (400 µg/ml). Isolated surviving cell colonies grown in presence of 100 µg/ml G418 were amplified and checked for SSAO/VAP-1 expression.

Total cell lysates and concentred culture medium

Total cell lysates for western blot were obtained homogenising the cells in a buffer containing 1% Triton X-100, 10 mM EDTA, 50 mM Tris-HCl pH7.5 and protease inhibitors (protease inhibitor cocktail, Sigma-Aldrich, St. Louis, Mo., USA). In case of AOs activity determinations, the buffers used were 100 mM Tris-HCl pH9 for SSAO activity or 50 mM potassium phosphate buffer pH 7.4 for MAO A and B activities. Lysates were sonicated for 10 s. Culture mediums were recovered after cell treatments and centrifuged at 800 x *g* to eliminate possible dead cells. Mediums were concentrated by lyophilisation in a freeze dryer (Edwards Freeze Dryer Modulyo, C.R. Mares, Barcelona, Spain) and resuspended in a smaller known volume.

Subcellular fraccionations

To obtain enriched membrane preparations, cells were recovered in a buffer containing 10 mM Hepes, 1.5 mM $MgCl_2$ 10 mM KCl and protease inhibitors, at pH 7.9. After

Dounce homogenization, samples were centrifuged at 2000 x *g* for 15 min at 4ºC and the supernatant was then ultracentrifuged at 100000 x *g* (Sorvall Discovery M120 SE) for 30 min at 4ºC to separate the soluble cytosolic fraction from the membranous pellet.

To obtain membranal lipid rafts, two 15 cm dishes of cells were scrapped in PBS and recovered by centrifugation for 5 min at 800 x *g*. The cellular pellet was resuspended in 1.5 ml of buffer containing 50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1% Brij 98 and protease inhibitors, at pH 7.2. Samples were then incubated for 15 min at 37ºC in a shaker and centrifuged for 10 min at 2000 x *g* to discard nuclei. The supernatant was mixed with 1.5 ml of 90% sucrose in Tris-HCl buffer to obtain a fraction of 45% sucrose, which was deposited in the bottom of the tube. Two additional fractions of 35% and 5% sucrose were added on the former to create a sucrose gradient, and samples were centrifuged for 18-20 h at 100000 x *g*. Finally, 12 fractions of 1 ml each were recovered and analyzed by western blot to identify the lipid raft or the soluble membrane enriched fractions.

Western blot assay

Protein concentration of total cell lysates was determined by the Bradford method. An equal amount of protein (20 µg/lane) or an equal volume of medium was separated by SDS-polyacrylamide gel electrophoresis using the Bio-Rad Mini-PROTEAN 3 system, and transferred onto nitrocellulose membranes. Polyacrylamide gels without SDS were used to separate the proteins in non-denaturing conditions. Membranes were blocked for 1h with TBS-0.1% Tween buffer plus 5% (w/v) non fat dry milk and incubated overnight at 4ºC with the corresponding primary antibodies. After incubation with the corresponding secondary antibodies, blots were developed using ECL® Chemoluminiscent detection reagents and High Performance Chemiluminiscence Films (Amersham-GE Healthcare, UK). The antibodies used were: goat anti-VAP-1 (E19) (1:500) (Santa Cruz Biotechnology, Heidelberg, Germany), rabbit anti-bovine SSAO (1:1000) (Lizcano *et al.* 1998), mouse anti-β-actin (1:20000) (Sigma-Aldrich), mouse anti-GAPDH (1:40000) (Ambion-Invitrogen, Barcelona, Spain), mouse anti-transferrin receptor (1:1000) (ZYMED, California, USA), mouse anti-flotillin (1:1000) (BD Biosciences, Madrid, Spain), HRP anti-rabbit IgG (1:2000) (BD Biosciences, Madrid, Spain), HRP anti-mouse IgG (1:2000) (Dako, Barcelona, Spain) and HRP anti-goat IgG (1:2000) (Pierce, Madrid, Spain).

Immunofluorescence

Cells were grown on collagen I coated glass coverslips during 2 days in complete medium. HUVEC cells were fixed during 20 min at -20ºC in Carnoy's solution (Methanol:Acetic acid, 3:1 (v/v)), and A7r5 cells were fixed for 10 min at 4ºC in 2% paraformaldehyde. After fixation, cells were treated during 1 min with 15% formic acid to improve the antigen exposition. Primary antibody (rabbit anti-VAP-1 (H43), Santa Cruz Biotech) was added at 1/100 dilution in PBS containing 0.2% gelatine, 0.1% triton, 20 mM glycine and 5% FBS overnight at 4ºC. Alexa Fluor® 594 Goat anti-rabbit secondary antibody (Invitrogen) and Hoechst 0.5 mg/ml were incubated then at 1/1000 dilution for 1 h at room temperature, and cells were mounted in Fluorescent Mounting Medium (Dako). Preparations were observed in a Nikon Eclipse TE 2000-E inverted fluorescence microscope, with a Hamamatsu C-4742-80-12AG camera and Metamorph® Imaging System software.

Enzymatic analyses

Amino oxidase (MAO A, MAO B and SSAO) activities were determined radiochemically using a modification of Otsuka and Kobayashi method (OTSUKA and KOBAYASHI 1964). 100 µM (^{14}C)-Benzylamine Hydrochloride (2mCi/mmol, Amersham) was used for MAO B and SSAO activity determination, and 100 µM 5-(2-^{14}C)-hydroxytryptamine binoxalate (0.5 mCi/mmol, Perkin Elmer, USA) was used for MAO A activity determination. For SSAO and MAO B activity determinations, a 30 min inhibitory pre treatment of the samples was performed at 37ºC with 1 mM deprenyl (Dep) or 1 mM Sc respectively. Moreover, 1 mM Sc, 1 mM Dep or 1 µM Clor were used to specifically inhibit SSAO, MAO B or MAO A activities respectively. Standard reactions were performed at 37ºC for 90 min (SSAO and MAO B) or 30 min (MAO A) in a final volume of 200 µl of 100 mM Tris-HCl pH9 buffer (SSAO) or 50 mM potassium phosphate buffer pH 7.4 (MAO A and MAO B), and the subsequent addition of 25 µl of substrate. 200-300 µg of protein or 50 µl of medium were used in each vial, and triplicates were done for each sample. The catalytic reaction was stopped adding 100 µl of 2M citric acid. The aldehyde products of the reaction were extracted into 4 ml of toluene:ethylacetate (1:1) solution containing 0.6% (w/v) of diphenyloxazole per vial. The amount of ^{14}C-aldehyde was quantified using a liquid scintillation counter Tri-Carb 2810TR (Perkin Elmer) and the Quanta Smart 3.0 software (Perkin Elmer). Cell lysate activities are expressed as pmol/min·mg protein and culture medium activities as pmol/min·ml medium.

The SSAO kinetic parameters (K_m and V_{max}) of the transfected cell lines were calculated with the Graph-Pad Prism 3.0 software, based on the Michaelis-Menten equation. The curves were obtained by activity determinations of 30 min incubations in potassium phosphate buffer pH7.4 with different ^{14}C-Benzylamine concentrations: 10, 25, 50, 100, 200 and 300 µM for A7r5 cell line and: 10, 25, 50, 100, 200, 300, 400, and 500 µM for HUVEC cell line.

Results

Endothelial cells express SSAO/VAP-1 *in vivo*, but its expression is lost in primary cultures or in cell lines. In order to obtain an endothelial cell model to study the SSAO/VAP-1 *in vitro*, we have transfected an immortalized HUVEC cell line with human SSAO/VAP-1 protein. The PEI method of transfection was used, and cells were allowed to grow for 2 months in high concentrations of the selection antibiotic G418. After cellular selection, individualized cells were amplified forming colonies, and some of them were analyzed for SSAO expression and activity. Figure 1.A shows the presence of the SSAO/VAP-1 protein in cell lysates of different HUVEC transfected clones. Although all these clones were resistant to the selection antibiotic, they showed different levels of SSAO/VAP-1 expression. However, the activity levels were proportional to those of expression. These different clones were compared to HUVEC wild type cells (WT), which did not show any SSAO/VAP-1 protein expression or activity. Moreover, the HUVEC clones were also compared to A7r5 hSSAO/VAP-1 cells (A7r5), showing the latest higher levels of SSAO activity.

The stability of the protein expression during the cell line culture was assessed determining the SSAO enzymatic activity at different cell passages (figure 1.B). Results showed few variations between passages 1 and 12 of HUVEC transfected cells. Moreover, the specificity of the activity was evaluated through its inhibition by semicarbazide. It can be observed in figure 1.C that all the activity measured through benzylamine oxidation was inhibited by the SSAO inhibitor semicarbazide, and that WT cells were not able to oxidize benzylamine at all.

After this, SSAO subcellular localization in HUVEC transfected cells was studied through subcellular fractionation (figure 1.D). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and Transferrin receptor (Tf Rec) were used as cytosol and

membrane fraction controls, respectively. SSAO/VAP-1 was detected in both whole cell lysate and membrane fractions, but not in cytosolic fraction.

It has been demonstrated that endothelial cells can be a source of plasma SSAO *in vivo* (Stolen *et al.* 2004). To assess whether these HUVEC transfected cells were able to release also the soluble form of SSAO, its enzymatic activity was determined in conditioned culture mediums of HUVEC WT and hSSAO/VAP-1 cells (figure 1.E). Practically undetectalbe levels of SSAO activity were observed in both mediums, which were inhibited by semicarbazide. They could come to the serum of the medium since none differences were observed between both mediums. However, it could be possible that due to its lower SSAO expression compared to A7r5 hSSAO/VAP-1, released HUVEC SSAO levels would be too low to be detected in culture medium. For this reason, we concentred conditioned culture mediums of WT and transfected HUVEC cells and analysed its SSAO presence by western blot (figure 1.F). Conditioned mediums were concentred through lyophilisation, and resuspended in 1/30 part of the initial volume. 30x concentred medium of transfected cells showed higher SSAO presence than WT cells medium. However, enzymatic activity determination of the 30x mediums did not display any SSAO activity (data not shown). The presence of flotillin was analyzed to ensure that cellular remains were not the source of this SSAO signal.

To characterize the amine oxidase activity pattern of HUVEC WT and transfected cells, MAO A and MAO B activities were also determined (figure 2.A). Results showed a distinct pattern between both cells, with a moderate MAO A activity detected in WT cells that disappeared in SSAO expressing cells. MAO B activity, however, was not detected in none of these cells. A possible compensatory activity between SSAO and the amine oxidases MAO A and MAO B has been suggested by several authors (Fitzgerald and Tipton 2002; Kinemuchi *et al.* 2004). To further study this possible modulatory effect between MAO A and SSAO activities in these cells, we performed a chronic inhibition of each activity in WT and SSAO/VAP-1 cells in culture and then assayed their activities (figure 2.B). However, neither the 10 days MAO A inhibition by clorgyline in HUVEC WT cells induced the SSAO activity, nor the SSAO inhibition in HUVEC hSSAO/VAP-1 cells induced the MAO A activity.

SSAO/VAP-1 is expressed in endothelial and smooth muscle cells under the same gene (AOC3). However, the study of SSAO in these different cell types has revealed some functional, structural and regulatory differences (Jaakkola *et al.* 1999). Since we have recently developed the A7r5 smooth muscle cell line transfected with SSAO/VAP-1 (Sole *et al.* 2007), we compared in this work some features between these and the

HUVEC hSSAO/VAP-1 cells. SSAO/VAP-1 has been described to possess amine oxidase activity in both cell types; in this concern, we have also detected amine oxidase activity after SSAO/VAP-1 transfection in both A7r5 and HUVEC cells. However, A7r5 smooth muscle cells show 3 times higher activity than HUVEC cells (figure 3.A). This different expression levels can be observed also by immunofluorescence (figure 3.B). To study whether there are differences in the SSAO enzymatic behaviour between both cells, the kinetic parameters Km and Vmax have been determined for each cells (table 1). The K_m values did not show significant differences between both cells. However, V_{max} and catalytic efficiency (represented as V_{max}/K_m) of A7r5 hSSAO/VAP-1 cells was significantly higher than those of HUVEC hSSAO/VAP-1 cells. These results confirm that the surrounding environment can modify the SSAO/VAP-1 behaviour although the DNA sequence would be the same.

In smooth muscle cells, the membranal SSAO has been observed to be concentred in the caveolae, small bottle-shaped invaginations that constitute microdomains of the plasma membrane (Jaakkola *et al.* 1999). We performed membrane fractionations to separate the lipid rafts and the TritonX-100 soluble membrane fractions of the plasma membrane in both A7r5 and HUVEC cells (figure 4.A). As it has been described, we observed in A7r5 cells the presence of SSAO/VAP-1 mainly in the fractions corresponding to lipid rafts, which were identified by its flotillin content. Few SSAO detection was observed in soluble TritonX-100 soluble fractions, identified by its transferrin receptor presence, which could result from the intracellular membrane associated SSAO or from excessive solubilization time, as they contain also some flotillin. In HUVEC cells, the transfected hSSAO/VAP-1 was also observed in the lipid raft fractions, showing therefore the same behaviour in terms of localization than smooth muscle cells.

SSAO/VAP-1 is expressed as a homodimer of two ~90-KDa subunits. In addition, trimeric forms have been observed in smooth muscle cells but not in endothelials (Jaakkola *et al.* 1999). To study these protein associations in our cells, lysates of A7r5 and HUVEC transfected cells were analyzed by western blot under non denaturing conditions (figure 4.B). In both HUVEC and A7r5 hSSAO/VAP-1 cells, SSAO was detected over the 150 KDa marker, which corresponds to the observed homodimer weight (160-185 KDa) (Jaakkola *et al.* 1999). However, trimeric forms were not observed in smooth muscle cells. Another faint band was observed near the 250 KDa marker in HUVECs, but it was also present WT cells, suggesting that it was unspecific. An additional band near 100 KDa was also observed in A7r5 cells, where three different clones with increasing SSAO/VAP-1 expression (clones A-C) were analyzed,

which could correspond to the monomeric SSAO/VAP-1 that may migrate different due to the non reducing conditions. Under reducing conditions, the monomeric SSAO/VAP-1 was detected near 100 KDa, and it was showed as a double band in HUVEC cells, not evident in A7r5 cells. Moreover, at high expression levels (A7r5 clone C), a specific band was observed over 250 KDa, which could correspond to high molecular weight oligomeric forms that may not be completely denatured.

Differences in electrophoretic mobility between smooth muscle and endothelial SSAO/VAP-1 have been also described (Jaakkola *et al.* 1999), displaying the endothelial protein a slightly slower electrophoretic mobility. This variability is possibly due to differences in posttraductional modifications. We did not observe this phenomenon between the A7r5 and HUVEC transfected cells at monomeric level. However, in non denaturing conditions it was also observed this little difference previously described (figure 4.C).

Finally, we analyzed the behaviour of the SSAO/VAP-1 protein regarding the subculture conditions. For this purpose, we trypsinized both cells as by routine, plated them and lysed at different times after trypsinization up to 72 h. At 0 h, we lysed the cells immediately after its trypsinization. Smooth muscle SSAO/VAP-1 was more affected by the trypsinization process than the endothelial SSAO/VAp-1, since the detected signal was lower at 0 h in A7r5 cells compared to later times. However, this expression was rapidly recovered. In HUVEC cells, fewer variations were observed between the different times of culture after trypsinization.

Discussion

SSAO/VAP-1 is expressed in endothelial cells *in vivo*, but it is difficult to study this protein *in vitro*, since cells in culture do not express SSAO/VAP-1. SSAO/VAP-1 is also expressed in smooth muscle cells *in vivo*, but its expression is also lost in cultured cells. Due to this handicap in studying SSAO/VAP-1, we have previously developed and characterized a smooth muscle cell line which expresses in a stable form the human SSAO/VAP-1 protein (Sole *et al.* 2007). The main objective of the present work was to develop an endothelial cell line with a stable SSAO/VAP-1 expression, which could be used in the future for the study of SSAO in this cellular environment.

Since HUVEC cells have been widely used as endothelial cellular models, we used a HUVEC immortalized cell line to transfect with the human SSAO/VAP-1 gene. To assess the validity of the model, we characterized the SSAO/VAP-1 expression in this new cell line: HUVEC hSSAO/VAP-1. We observed that although we obtained several positive clones, not all of them were expressing the same protein levels. However, the different levels of SSAO specific activity correlated with the protein expression amounts, indicating that all the synthesized protein was enzymatically active. This correlation between the protein levels and the specific activity has been observed in other studies (Salmi and Jalkanen 2006), but not in situations in which an enzyme modulation would be occurring (Obata 2006), thus our results suggest that none endogenous inhibitor was present in our culture conditions. On the other hand, the protein expression was stable through different cell passages, allowing the use of these cells without losing SSAO activity at least until passage 12. Moreover, the transfected enzyme responded to inhibition by semicarbazide, as the *in vivo* protein does. The localization of the protein was observed to be in the membrane by subcellular fractionations, but since total membrane enriched preparations were obtained, it could not be determined by this method whether some of this protein was associated to intracellular membranes, as it has been described before (Salmi and Jalkanen 1995; Yegutkin *et al.* 2004).

The soluble form of SSAO is believed to come from the shedding of the membrane protein (Abella *et al.* 2004), so we evaluated if the HUVEC hSSAO/VAP-1 cells were able to release the protein to the culture medium. Specific activity of HUVEC SSAO/VAP-1 conditioned cultured medium displayed very low activity levels, not different from that observed in WT medium. Since SSAO/VAP-1 is present in the foetal bovine serum used for cell culture, it is feasible to think that this activity could come from this source. However, to ensure that none SSAO was being released from cells, conditioned culture mediums were concentrated. After lyophilisation and western blot analyses of 30 times fold concentred mediums (30x), we observed higher SSAO/VAP-1 detection in HUVEC hSSAO/VAP-1 medium than in HUVEC WT. These results pointed out to the possibility that HUVEC cells could be releasing small amounts of SSAO/VAP-1 protein. However, the same 30x concentred mediums did not display any SSAO activity after lyophilisation, indicating a possible enzymatic inactivation due to this process.

A possible SSAO/VAP-1 expression modulation has been suggested with relation to the expression of other amine oxidases. For example, an increase of MAO A and MAO B activities was described after SSAO inhibition (Kinemuchi *et al.* 2004). On the other

hand, treatment with non-selective MAO inhibitors induce an increase of SSAO activity (Fitzgerald and Tipton 2002; Kinemuchi *et al.* 2004). Moreover, there have been detected normal MAO activity metabolites in Norrie's disease patients, which have a deletion or non functional MAO A and MAO B proteins (Lizcano *et al.* 1991). In this concern, we detected different amine oxidase activity patterns in the HUVEC WT and hSSAO/VAP-1 cells, showing WT cells a moderate MAO A activity which was lost after the SSAO transfection in hSSAO/VAP-1 cells. This effect was also observed in A7r5 cells (Sole *et al.* 2007), which have higher MAO A activity than HUVEC cells. To test whether it would be able to modify these activities towards the initial situation and know the nature of this effect, MAO A and SSAO activities were inhibited during 10 days in HUVEC WT and hSSAO/VAP-1 cells in culture respectively. However, neither MAO A inhibition was able to induce SSAO activity, nor SSAO inhibition was able to recover the MAO A activity. These results suggested that something else than the enzymatic inhibition would be necessary to induce the other activity appearance. Moreover, since it has not been possible to reproduce none of the previous cited similar situations in cultured cells (Kinemuchi *et al.* 2004), it is possible that signals coming from other tissues would be needed to led to this modulatory effect.

Although SSAO/VAP-1 is expressed in both vascular smooth muscle and endothelial cells, its comparative study has revealed structural, functional and regulatory differences (Jaakkola *et al.* 1999). Giving the fact that we have developed smooth muscle and endothelial stable transfected cell lines, its comparison at different levels has been done to study possible differences in SSAO characteristics due to the different environment. The first one was the different level of SSAO expression and specific activity towards benzylamine, showing smooth muscle cells above three times more expression and activity than endothelial cells. These results were in close agreement with previous that showed over 338 ± 139 $pmol \cdot min^{-1} \cdot mg\ prot^{-1}$ for aorta as smooth muscle rich tissue, and 133 ± 43 $pmol \cdot min^{-1} \cdot mg\ prot^{-1}$ for lung as endothelial rich tissue (Andres *et al.* 2001). Also in cerebrovascular tissue, meninges show higher activity than microvessels, as smooth muscle or endothelial enriched tissues respectively (Castillo *et al.* 1998). However, the reasons for these differential levels of expression are not known.

Regarding the kinetic constants of the enzyme in both cell types, K_m was not statistically different between cells, but V_{max} and the catalytic efficiency were significantly higher in smooth muscle cells. These data are in agreement with previous observed concerning the K_m values (Holt *et al.* 2008; Lyles *et al.* 1990), however, the distinction of the kinetic constants between the endothelial and smooth muscle SSAO

has not been deeply studied. Previous studies done in our group have shown similar results that the obtained here, displaying the smooth muscle SSAO higher V_{max} and catalytic efficiency values than the endothelial enzyme (Castillo *et al.* 1998; Unzeta *et al.* 2007), although the kinetic constants for cerebrovascular SSAO show lower values than those described for other tissues (Castillo *et al.* 1998; Lyles 1996). These data reinforces the hypothesis that the molecular environment can modify the behaviour of the same protein.

On the other hand, both SSAOs are localized in the lipid raft fractions of the membrane, as it has been described for adipocytes and smooth muscle cells (Aboulaich *et al.* 2004; Jaakkola *et al.* 1999; Souto *et al.* 2003), but not specified for endothelial cells. Since lipid rafts are specialized regions of the membrane, which develop functions related to lipid trafficking in and out the cells (Parton 1996), and SSAO has been associated with lipid trafficking (Dai *et al.* 2008), it could be possible that SSAO would participate in some of these lipid raft-attributed functions.

At structural level, the SSAO western blot analyses of A7r5 and HUVEC transfected cells in denaturing conditions showed in both cases a band near 100 KDa. Moreover, in HUVEC cells this band was seen as a double band, which was less evident in smooth muscle cells. This effect is seen also in other studies, but its cause has not been studied; it could be due to different glycosilated populations. SSAO is mostly found as a homodimer, however, a trimeric form of SSAO was also observed in smooth muscle cells in contrast to its lack in endothelial cells (Jaakkola *et al.* 1999). By western blot in non denaturing conditions, we observed the band corresponding to the dimeric form near 150 KDa, as described (Jaakkola *et al.* 1999). However, it was not detected the trimeric form in smooth muscle cells; this could be due by too low amounts of protein analyzed, since the trimeric form is minoritary. By contrast, a high molecular weight band was observed in high activity clones of smooth muscle cells in denaturing conditions, which could correspond to non denaturalised multiproteic complexes. Variations in glycosilation levels can be also the responsible of the different electrophoretic mobility described between smooth muscle and endothelial cells (Jaakkola *et al.* 1999). In our cell lines, a little slower mobility was observed in HUVEC hSSAO/VAP-1 cells compared to A7r5 at dimeric level, but not in the monomer. The small difference could be seen only by the addition of two monomers, but it may be imperceptible in monomeric situations. However, more studies should be done in order to confirm that the enzyme is differently processed in these two cell types. These or some other processing differences could be also the responsible of the higher resistance to the trypsinization process observed in HUVEC cells. A7r5 cells, however,

showed lower SSAO levels after the tripsinization, which were therefore rapidly recovered. Its association with the extracellular matrix in smooth muscle cells, which is in part degraded by trypsin, could act in favour of its lost.

In conclusion, the characterization of the HUVEC hSSAO/VAP-1 cells stably transfected with the human SSAO/VAP-1 cDNA developed in this work showed that this new cell line could become a good model for the *in vitro* study of the SSAO/VAP-1 protein under an endothelial environment and also to study the differences observed with the smooth muscle cell environment. However, regulatory expression modulations cannot be studied in this model since the SSAO/VAP-1 expression is constitutively regulated by a different SSAO/VAP-1 promoter. Given the role that SSAO/VAP-1 has in some diseases, the study of this protein in cellular models could be useful for the knowledge of the SSAO/VAP-1-dependent mechanisms involved in atherosclerosis, diabetes-associated vascular complications or vascular damage associated to inflammatory processes or Alzheimer's disease. Moreover, screening of new molecules designed for the treatment of these diseases concerning the SSAO/VAP-1 role can be easily assayed in a cellular model.

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Figure Legends

Table 1. SSAO Kinetic parameters K_m and V_{max} in HUVEC and A7r5 transfected cells. SSAO was determined radiochemically using ^{14}C-benzylamine as substrate (10, 25, 50, 100, 200 and 300 μM for A7r5 and 25, 50, 100, 200, 300, 400 and 500 μM for HUVEC). Values represent the mean ± SEM of three different experiments performed in triplicate. (**) $p < 0.01$, (***) $p < 0.001$ statistically significant differences by Student's *t* test. NS, not statistically significant differences ($p > 0.05$).

Figure 1. SSAO expression, activity and localization in positive transfected clones of HUVEC cells. (**A**) Activity and expression SSAO/VAP-1 levels are shown by several clones, compared with the absence of activity in *wild type* (WT) cells, and with the higher activity of A7r5 hSSAO/VAP-1 cells (A7r5). (**B**) The SSAO activity is not lost through consecutive cell passages in HUVEC hSSAO/VAP-1 cells. (**C**) The transfected SSAO is sensible to semicarbazide. (**D**) Subcellular fractionations of HUVEC hSSAO/VAP-1 cells show the SSAO/VAP-1 localization in the whole (W) and membrane (M) fractions, but not in cytosol (C). GAPDH and transferrin receptor (Tf Rec) are used as specific cytosolic and membanal fraction markers. (**E**) Conditioned cultured mediums of HUVEC WT and transfected cells show low levels of SSAO activity. (**F**) 30 times fold concentred cultured mediums show higher levels of SSAO detection in hSSAO/VAP-1 medium. SSAO activity was measured with 100 μM ^{14}C-Benzylamine as substrate, and inhibited by 10^{-3} M semicarbazide. Data are mean ± SEM values of three independent experiments.

Figure 2. Enzymatic activities of SSAO, MAO B and MAO A in HUVEC WT and hSSAO/VAP-1 cell lysates (**A**) show presence of MAO A activity in WT cells which is lost in transfected cells. SSAO and MAO B activities were measured with 100 μM ^{14}C-Benzylamine, and MAO A with 100 μM ^{14}C-5-HT. Inhibition of SSAO, MAO B and MAO A activities were performed by 10^{-3} M semicarbazide (Sc), 10^{-3} M deprenyl (Dep) or 10^{-6} M clorgyline (Clor). Inhibition of MAO A in WT cells or inhibition of SSAO in transfected cells did not induce SSAO or MAO A activities respectively (**B**). The long amine oxidase inhibition was performed maintaining WT cells in culture in the presence of clorgyline 10^{-6} M for MAO A, or maintaining transfected cells in the presence of

semicarbazide 10^{-4} M or MDL 10^{-6} M during 10 days. Data are mean ± SEM values of three independent experiments.

Figure 3. Comparison of A7r5 hSSAO/VAP-1 and HUVEC hSSAO/VAP-1 levels of SSAO/VAP-1 activity and expression by western blot (**A**) or expression by immunofluorescence (**B**). SSAO activity was measured by 100 μM ^{14}C-Benzylamine. Activity data are mean ± SEM values of three independent experiments.

Figure 4. (**A**) SSAO/VAP-1 localization in lipid raft fractions *vs* Triton X-100 soluble fractions. The protein is mainly localized in lipid raft enriched fractions in both cell types. Flotillin and transferrin receptor were used to identify lipid raft and soluble fractions respectively. (**B**) The presence of dimeric or trimeric forms was analyzed in HUVEC and A7r5 transfected cells under denaturing (D) or non denaturing (ND) conditions, both showing only the dimeric form under ND conditions. (**C**) A slight electrophoretic mobility difference is observed between dimeric forms of A7r5 (A7) and HUVEC (H) hSSAO/VAP-1 cells under ND conditions, but not between monomeric forms under D conditions. (**D**) The endothelial SSAO/VAP-1 is less sensitive to the trypsinization process than the smooth muscle protein. Cells were seeded and tripsinized at different times, to that SSAO/VAP-1 was analyzed by western blot. GAPDH was used as loading control. Blots are representative experiments of a minimum of three with similar results.

Table 1. Kinetic constants of SSAO in HUVEC and A7r5 hSSAO/VAP-1 cells.

	K_m (μM)	**V_{max} (pmol/min·mg)**	**Catalytic efficiency (V_{max}/K_m)**
HUVEC hSSAO/VAP-1	276.6 ± 14.57	183.8 ± 5.604	0.665 ± 0.022
A7r5 hSSAO/VAP-1	228.4 ± 22.48 (NS)	583.3 ± 76.84**	2.53 ± 0.154 ***

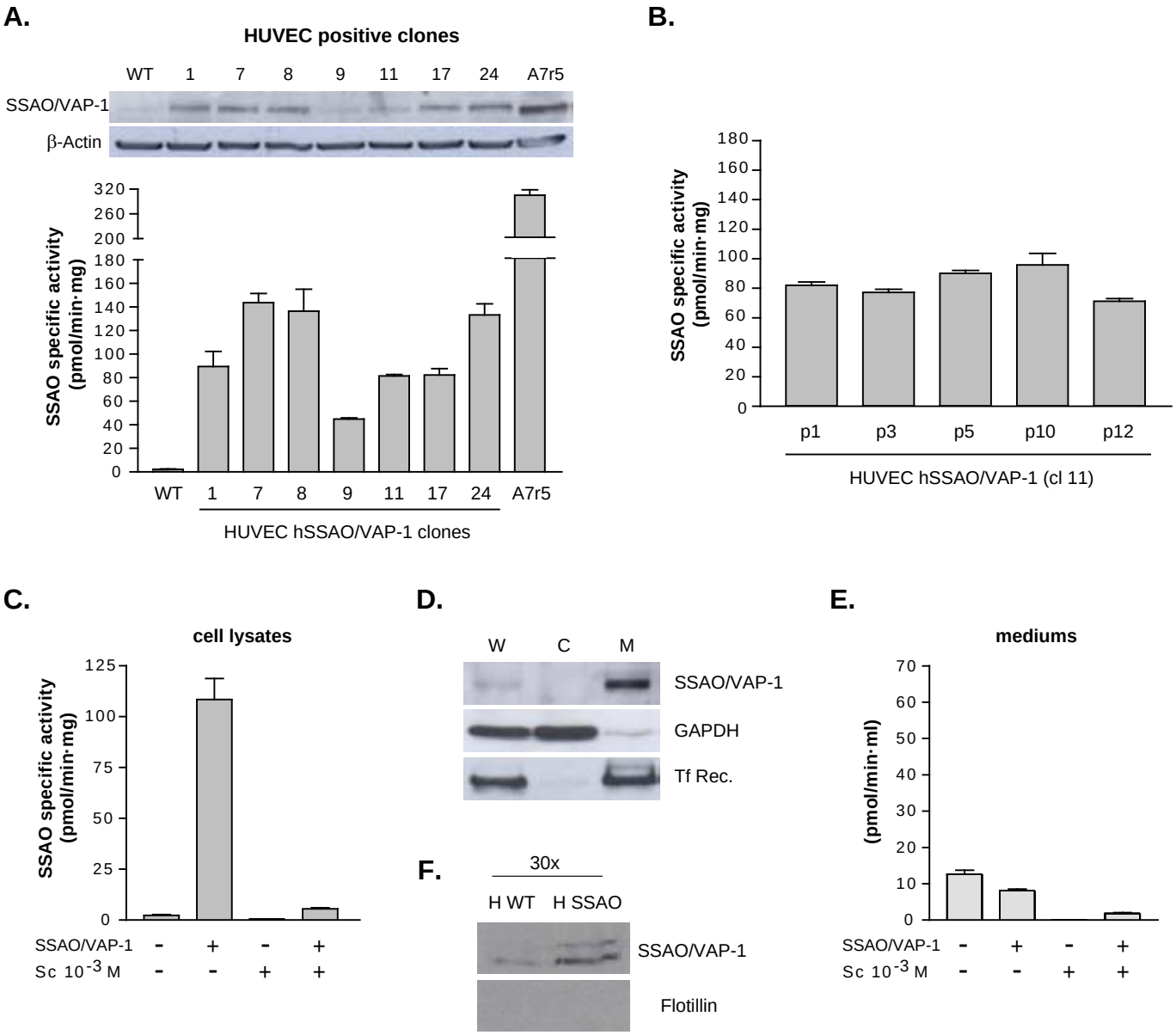

Figure 1 - *Solé et al.*

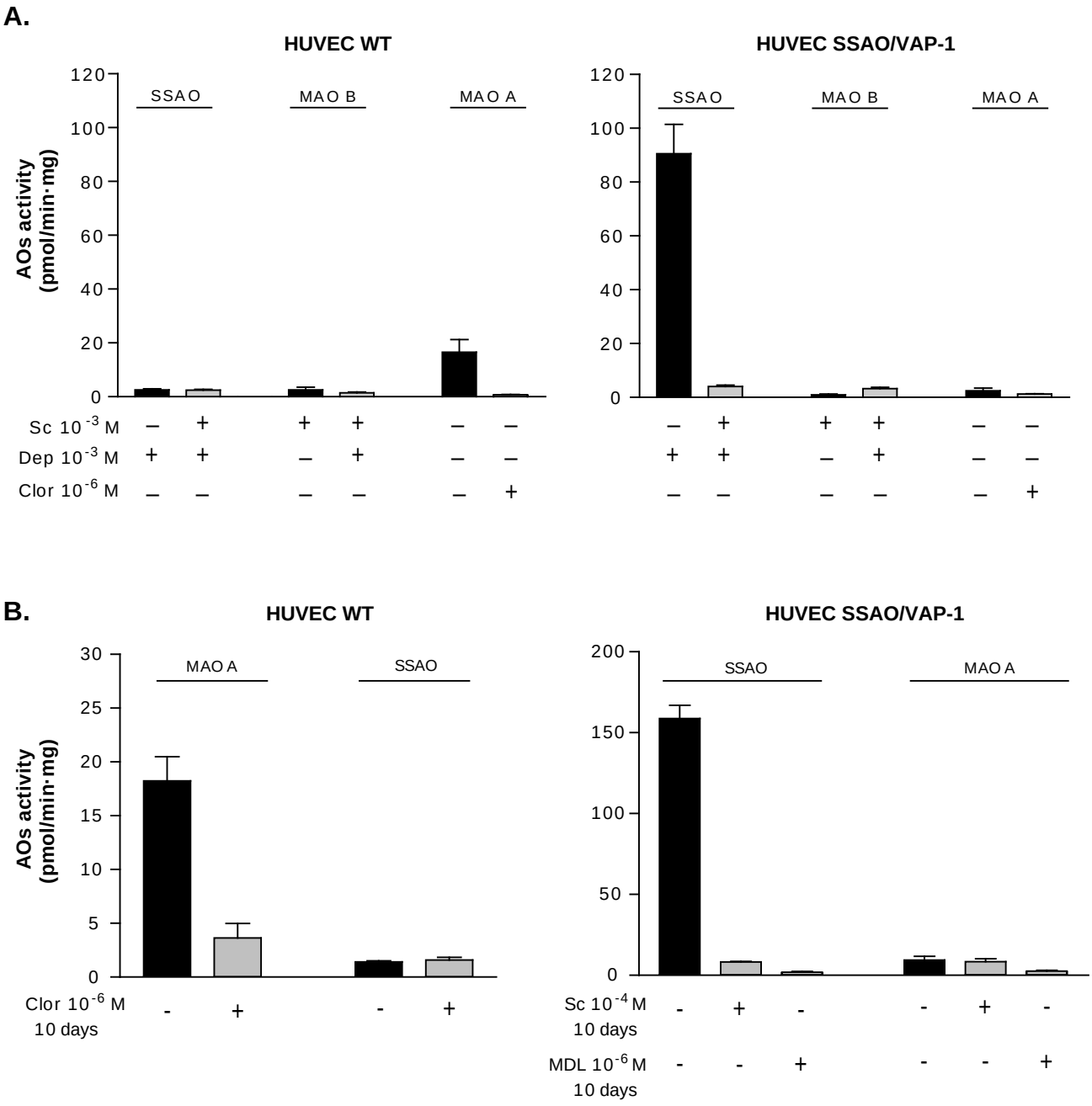

Figure 2 - *Solé et al.*

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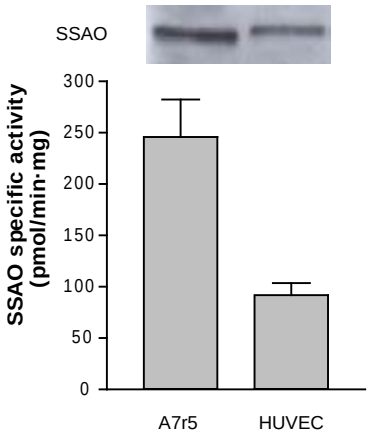

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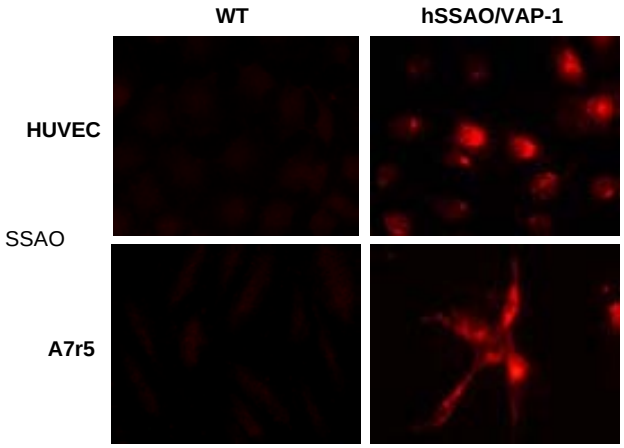

Figure 3 - *Solé et al.*

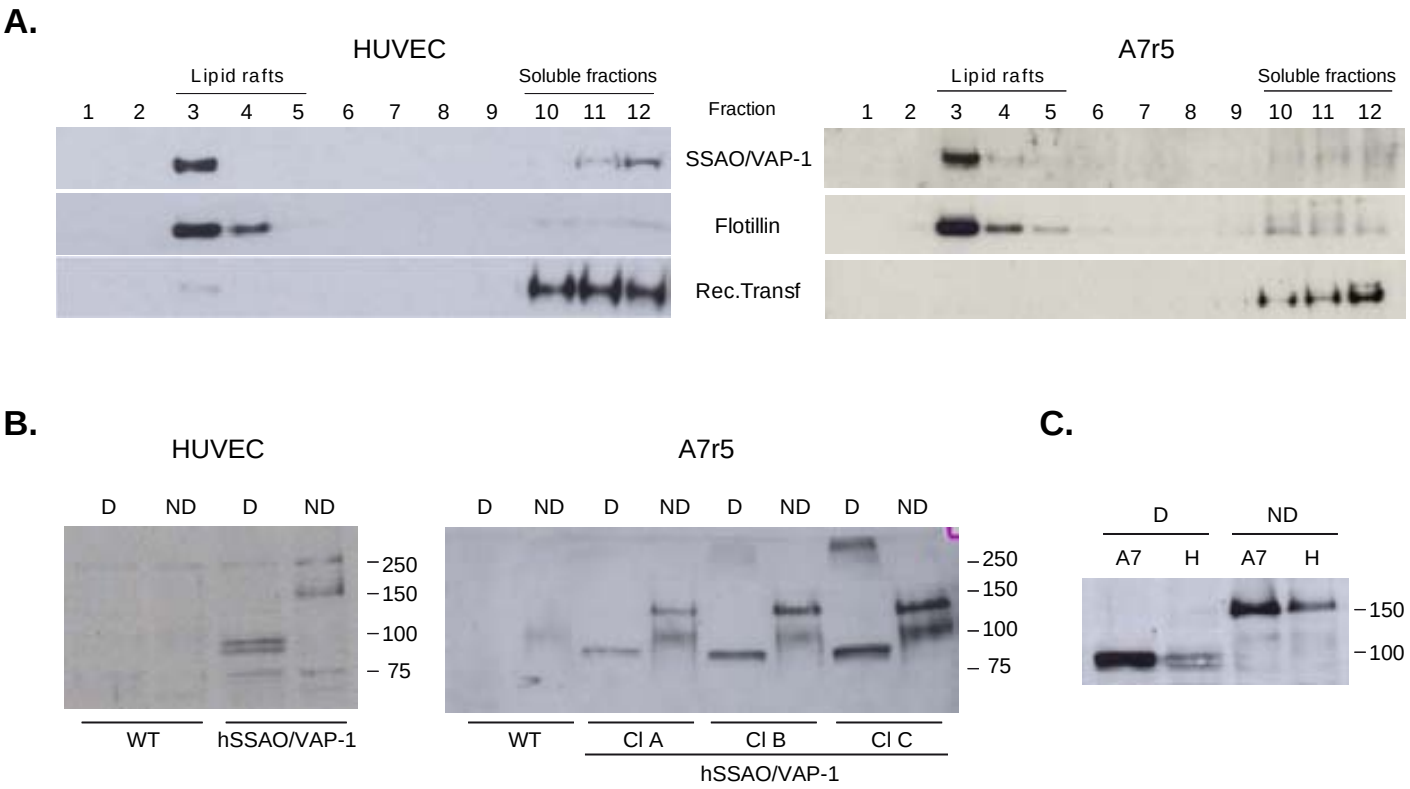

Figure 4 - *Solé et al.*

Capítol III:

“p53 phosphorylation is involved in vascular cell death induced by the catalytic activity of membrane-bound SSAO/VAP-1”

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p53 phosphorylation is involved in vascular cell death induced by the catalytic activity of membrane-bound SSAO/VAP-1

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ABSTRACT

Semicarbazide sensitive amine oxidase (SSAO) is a multifunctional enzyme present mainly in adipocytes, endothelial and smooth muscle cells. It metabolizes primary aliphatic and aromatic amines generating products able to contribute to cellular oxidative stress. SSAO is expressed in a membrane-bound form and is also present as a soluble enzyme in plasma. Both isoforms are increased in several pathologies, and the catalytic products generated by the soluble enzymatic activity can induce cytotoxicity of vascular cells in culture. We have analyzed whether the transmembrane form of the enzyme is able to produce a cytotoxic effect through methylamine oxidation. Since cells in culture lose the expression of this enzyme, we used an SSAO stably transfected smooth muscle cell line. Herein we report that cell treatment with the substrate methylamine induced a dose and time dependent cytotoxic effect. The tumor suppressor protein p53 played an important role in the molecular pathway involved in this cell death. Moreover, we also observed the induction of PUMA-α expression with mitochondrial Bcl-2 family proteins being affected, and final effector caspases being activated.

1. Introduction

Semicarbazide Sensitive Amine Oxidase [E.C.1.4.3.6, oxidoreductase (deaminating) (copper-containing), SSAO] constitutes a large family of enzymes which are inhibited by semicarbazide [1,2]. SSAO is localized in almost all mammalian tissues [3], mainly in fat and in highly vascularized tissues, where it is associated with both plasma membranes [4] and cytoplasmic granules [5,6]. SSAO is also present as a soluble form in blood plasma [2,7], which is thought [8,9] to come from the membrane-bound form through metalloprotease-dependent shedding [10]. Membrane-bound SSAO shows high activity in smooth muscle and endothelial cells from blood vessels [1–3]. However, in those cell types, SSAO seems to be a different molecular entity [4] exhibiting different regulation and probably different behavior. In fact, SSAO is considered as a multifunctional protein whose function varies depending on the tissue where it is expressed [11]. In adipocytes and smooth muscle cells, SSAO activity stimulates glucose transport, mimicking the insulin effect [12,13], while in endothelial cells, where it was also described as vascular adhesion protein-1 (VAP-1) [14], it is involved in leukocyte trafficking and extravasation under inflammatory conditions [14–16].

The catalytic action of SSAO/VAP-1 requires oxygen and generates ammonia, hydrogen peroxide (H_2O_2) and the corresponding aldehyde as a result of the oxidative deamination of primary aromatic and aliphatic amines. Aminoacetone and methylamine (MA) are considered the physiological SSAO substrates [17], and methylglioxal and formaldehyde are their respective metabolites [18]. In this context, the metabolic end products of the catalytic activity of SSAO have been considered a potential risk factor for stress-related angiopathy [19,20] and vascular degeneration. H_2O_2 is widely implicated in several diseases, as it is a major reactive oxygen species and the main generator of oxidative stress. Moreover, formaldehyde is a very reactive aliphatic aldehyde, considered as a powerful inflammatory agent [21]. At different levels, these toxic products potentially induce cell damage [22–25], activating molecular pathways that lead to a programmed cell death. As a result, plasma SSAO activity through MA oxidation has been shown to induce cytotoxicity in endothelial cells [22,26], and apoptosis in cultured smooth muscle cells [27]. However, the molecular mechanism by which the SSAO catalytic products could activate apoptotic processes and induce vascular damage remains unknown.

Our previous studies showed that membrane-bound SSAO was overexpressed in the cerebrovascular tissue of Alzheimer's Disease (AD) patients [28] and that soluble SSAO was also found to be increased in plasma from severe AD patients [29]. In this context, the aim of the present work was to confirm whether the overexpression of membrane-bound SSAO was able to induce vascular cell death through its own catalytic action on MA as substrate, and to elucidate the molecular pathway activated in such vascular damage.

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In this study we report that a methylamine (MA) treatment of SSAO/VAP-1 stable transfected smooth muscle cell line (A7r5) [30] induces an apoptotic cell death mediated by the SSAO substrate oxidation products. The study of different molecular pathways involved in the process revealed the key role of the tumor suppression protein p53 through the mitochondrial pathway in this mediated cell death.

2. Materials and methods

2.1. Cell culture and treatments

A7r5 WT rat aortic smooth muscle cell line was obtained from the ECACC (European Collection of Cell Cultures, Wiltshire, UK). A7r5 hSSAO/VAP-1 cell lines were obtained by means of stable transfection of A7r5 WT cell line with SSAO/VAP-1 human cDNA [30], and different positive clones were amplified as different cell lines to use in this work. Moreover, VAP-1/SSAO stable expression in the A7r5 hSSAO/VAP-1 cell line was periodically confirmed by Western Blot using the antibodies cited in Protein detection by Western Blot analyses (data not shown). All cell lines were grown in high glucose (4.5 mg/l) DMEM (Sigma-Aldrich, St. Louis, Mo., USA) supplemented with 10% (v/v) FBS (Invitrogen, Grand Island, NY, USA), 2 mM L-glutamine (Invitrogen, Grand Island, NY, USA), 1000 U/ml penicillin and 1000 U/ml streptomycin (PAN Biotech GmbH, Aidenbach, Germany) and maintained at 37 °C in a humidified atmosphere containing 5% CO_2. Geneticin (G418, 100 μg/ml, Invitrogen (Grand Island, NY, USA)) was added to the culture medium to ensure SSAO/VAP-1 DNA maintenance. For experiments, cells were seeded at 40000 cells/ml, grown for 24 h and starved with DMEM containing 0.2% (v/v) FBS for 1 h before treatment. For treatments, semicarbazide was coincubated with MA; catalase was added 30 min before MA, H_2O_2 or FA addition; formaldehyde dehydrogenase inhibitors were added 30 min before FA addition and pifithrin-α was added 1 h before MA addition. Semicarbazide, MA, H_2O_2, FA, catalase, pifithrin-α and formaldehyde dehydrogenase inhibitors (4-methylpyrazole, 1-bromoheptane, cyanamide, chloral hydrate) were purchased from Sigma-Aldrich (St. Louis, Mo., USA).

2.2. Cell viability assays

Cell viability was determined by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) reduction assay or by LIVE/DEAD Viability/Cytotoxicity Kit (Molecular Probes-Invitrogen (Grand Island, NY, USA)). For MTT estimations, cells were plated in 24-well plates, and MTT solution (0.5 mg/ml, final concentration) was added to cells 90 min before the end of the treatments; after these 90 min of incubation at 37 °C the medium was replaced with dimethyl sulfoxide to dissolve the formazan blue precipitate formed, which was quantified at 560 and 620 nm in a microplate reader (Labsystems multiskan RC) [31]. For LIVE/DEAD Viability/Cytotoxicity Kit cells were plated in 24-well plates and after treatments, 0.2 μM of Calcein/AM and ethidium homodimer-1 (EthD-1) was added with fresh medium. Cells were incubated for 15 min at 37 °C and then examined using an inversed microscope Nikon Eclipse TE 2000-E and a Hamamatsu C-4742-80-12AG camera. Calcein/AM is adsorbed by living cells and becomes a substrate for cytosolic esterases, which convert it into a green fluorescent product (Excitation 495 nm/Emission 530 nm), whereas EthD-1 is only able to enter cells with compromised cell membrane integrity, and becomes red fluorescent after its attachment to nucleic acids (Excitation 495 nm/Emission 635 nm).

2.3. Protein detection by Western Blot analysis

Cells were plated in 60 mm dishes, and after treatments, cells were washed in cold PBS and lysed in a buffer containing 1% Triton X-100, 10 mM EDTA, 50 mM Tris–HCl pH 7.5 and protease inhibitors (protease inhibitor cocktail, Sigma-Aldrich, Mo., USA). The lysates were incubated 10 min on ice, sonicated and centrifuged at 8000 ×*g* for 15 min at 4 °C. Protein concentration was determined by the Bradford method. An equal amount of protein (20 μg/lane) was separated by SDS-polyacrylamide gel electrophoresis using the Bio-Rad Mini-PROTEAN 3 system, and transferred onto nitrocellulose membranes. Membranes were blocked for 1 h with TBS-Tween buffer (66 mM Tris, 41.44 mM NaCl, 0.1% Tween 20) containing 5% (w/v) non fat dry milk, and incubated overnight at 4 °C with the corresponding primary antibodies diluted 1:1000 in the blocking buffer. The primary antibodies used were anti-cleaved caspase 3, anti-cleaved caspase 9, anti-Bax, anti PUMA and anti-phospho p53 antibodies from Cell Signaling (Beverly, MA, USA), anti-Bcl-2 from BD Biosciences Pharmingen (San Diego, CA, USA), anti-β-actin from Sigma-Aldrich (St. Louis, Mo., USA), anti-p53 from Abcam (Cambridge, UK), anti-SSAO E-19 and anti-SSAO H-43 from Santa Cruz Biotechnology (Heidelberg, Germany). After incubation with the appropriate secondary antibodies (HRP anti-rabbit igG from BD Biosciences Pharmingen or HRP anti-mouse IgG from Dako Cytomation (Glostrup, Denmark)) in the blocking buffer for 1 h, blots were developed using ECL® chemoluminiscent detection reagent and High Performance Chemiluminiescence Films from Amersham Pharmacia Biotech (GE Healthcare, UK). Semi-quantitative

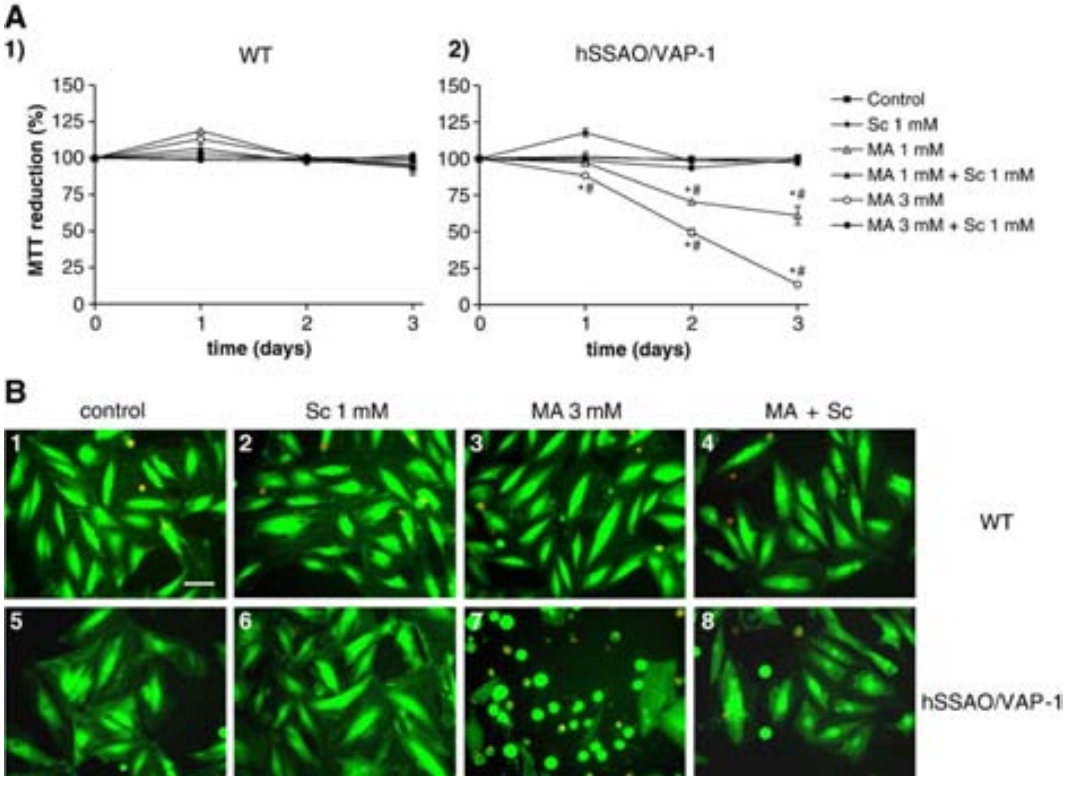

Fig. 1. MA oxidation by the transmembrane form of hSSAO/VAP-1 induces cytotoxicity in hSSAO/VAP-1 cells, shown as two different cell viability assays. (A) WT (1) and hSSAO/VAP-1 (2) cells were treated during 1, 2 or 3 days with methylamine (MA) 1 or 3 mM, semicarbazide (Sc) 1 mM, or both, and cell viability by measuring MTT reduction was determined. Data are the mean ± SEM of three independent experiments performed in triplicate. (*) $p<0.01$ vs control, (#) $p<0.01$ vs Ma+Sc by a one-way ANOVA test and the addition of Newman–Keuls Multiple Comparison test. (B) Cells were treated for 2 days with MA 3 mM (3 and 7), Sc 1 mM (2 and 6), both (4 and 8) or none (1 and 5). Cell viability was assessed with the fluorescence Viability/Cytotoxicity Kit which dyes live cells in green and dead cells in red. Scale bar = 100 μm. Representative images were taken by each treatment performed in triplicate for three separate experiments.

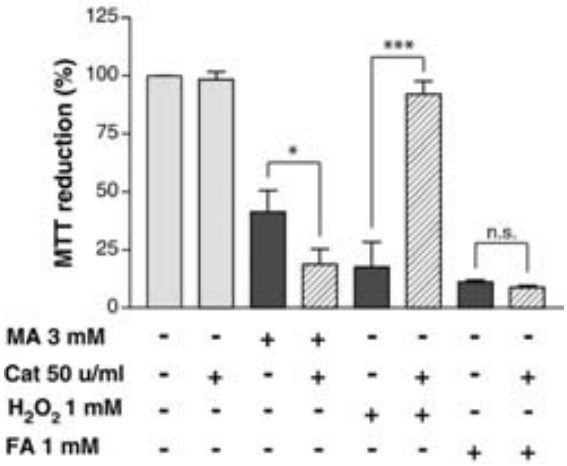

Fig. 2. Formaldehyde is the main contributor of the cytotoxicity induced by methylamine's oxidation. hSSAO/VAP-1 cells were treated for 2 days with MA (3 mM), hydrogen peroxide (H_2O_2) (1 mM) or formaldehyde (FA) (1 mM) alone or with a 30 min pretreatment of catalase (Cat) (50 U/ml) as well. Cell viability was determined by MTT reduction method. Data are the mean ± SEM of three independent experiments performed in triplicate. (***) $p<0.001$, (*) $p<0.05$, (n.s.) $p>0.05$ by a one-way ANOVA test and the addition of Newman–Keuls Multiple Comparison Test.

analysis of Western Blots was performed by densitometry, using the Image J 1.34 s software (National Institutes of Health, USA), and protein levels were corrected for corresponding loading control.

2.4. Caspase 3/7 activity determination

Caspase 3/7 activity was measured by EnzChek® Caspase-3/7 Assay Kit (Molecular Probes-Invitrogen (Grand Island, NY, USA)). Cells were seeded in 100 mm dishes and after treatments, samples were lysed according to the kit manufacturer's instructions. The Caspase 3/7 activity was determined by the amount of AMC released (Excitation 342 mn/Emission 441 nm), which was measured using the fluorescent microplate reader Synergy HT and date analysis software KC4 ™ (Bio-Tek® Instruments Inc. Winooski, USA). AMC concentration generated by each sample was then corrected by the corresponding protein concentration. Data is expressed as fold caspase 3/7 activity relative to untreated samples.

2.5. Statistics

Results are given as means ± SEM of independent experiments. Statistical analysis was done by one-way ANOVA and further Newman–Keuls Multiple Comparison Test. A p value of less than 0.05 was considered to be statistically significant. Statistical analysis and graphic representations were obtained using Graph-Pad Prism 3.0 program.

3. Results

3.1. MA oxidation induces cytotoxicity in A7r5 hSSAO/VAP-1 stably transfected cells

SSAO/VAP-1 expression has been shown to be lost in cultured cells [13,22]. In this work, a transformed smooth muscle cell line (A7r5 hSSAO/VAP-1), which expresses the transmembranal human SSAO/VAP-1 protein in a stable manner [30], was used to study the effect of methylamine (MA) oxidation by this enzymatic form in cell culture. Untransfected cells (A7r5 WT) were used as the negative control for treatments.

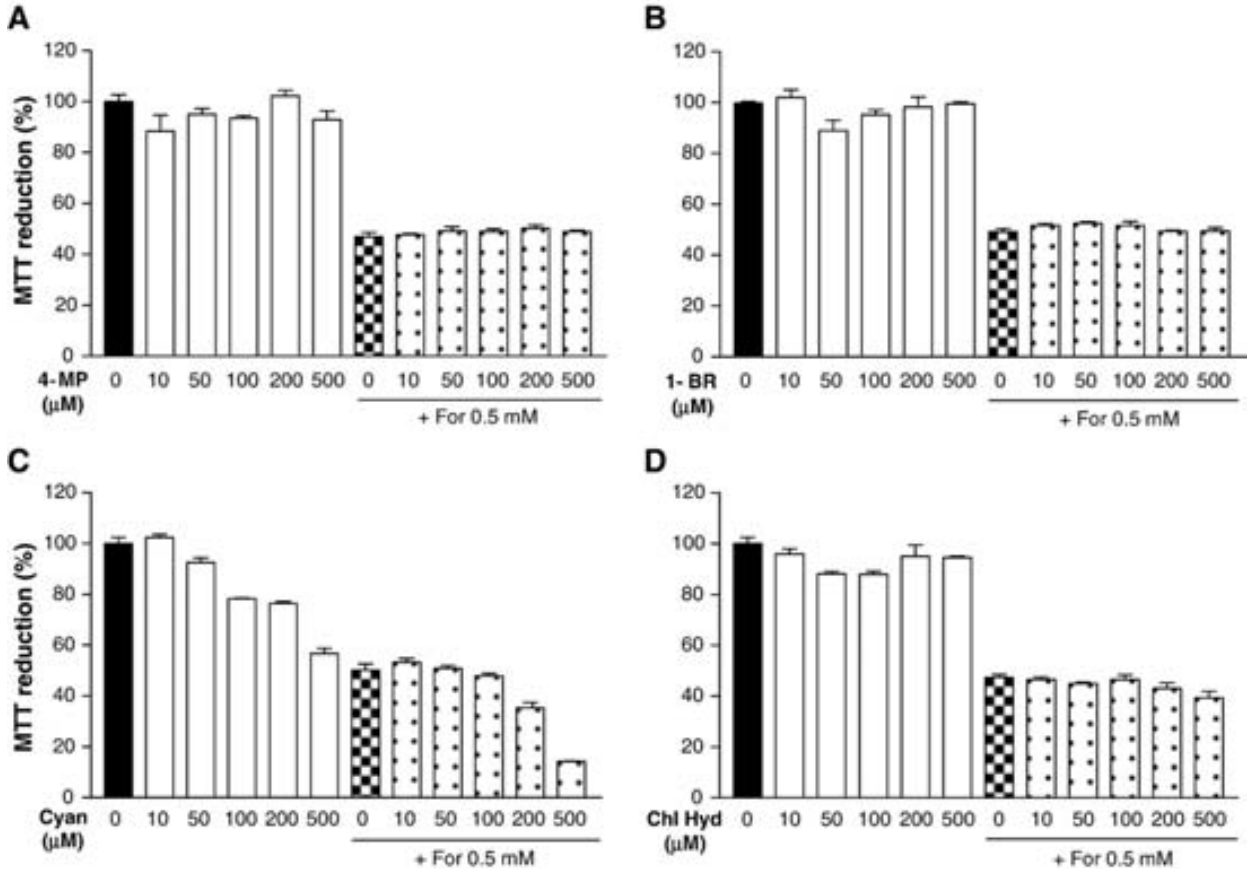

Fig. 3. Intracellular FA dehydrogenases do not modify FA induced toxicity. hSSAO/VAP-1 cells were treated for 18 h with increasing concentrations of different formaldehyde dehydrogenase inhibitors alone or plus FA (0.5 mM). A 30 min pretreatment was done with inhibitors before FA addition. Cell viability is expressed as MTT reduction percentages. Inhibitors used were: (A) 4-methylpyrazole (4-MP), as alcohol dehydrogenase 1 (ADH 1) inhibitor; (B) 1-bromoheptane (1-BR), as GSH-dependent formaldehyde dehydrogenase (ADH 3) inhibitor; (C) cyanamide (Cyan), as aldehyde dehydrogenase 2 (ALDH 2) inhibitor; and (D) chloral hydrate (Chl Hyd), as aldehyde dehydrogenase 2 (ALDH 2) inhibitor. Data are the mean ± SEM of three independent experiments performed in triplicate.

To study the possible toxicity produced by SSAO activity on MA metabolism, both cells were treated with different concentrations of this substrate. WT cells, which do not express SSAO/VAP-1 protein [30], and hSSAO/VAP-1 cells were treated with MA (1 and 3 mM) during 1 to 3 days, and semicarbazide (Sc) was used as SSAO inhibitor. Fig. 1.A shows the cell viability results obtained after the corresponding treatments measured by the MTT reduction assay, as previously described in Materials and methods. In WT cells, neither MA nor Sc had any effect on cell viability (Fig. 1.A.1). However, a dose and time-dependent cytotoxic response was observed in hSSAO/VAP-1 cells after MA treatment (Fig. 1.A.2), with a maximum 40% decrease in MTT reduction after 72 h treatment with 1 mM MA and an 85% decrease in MTT reduction after 72 h treatment with 3 mM MA. The total recovery of the cell viability in hSSAO/VAP-1 cells after MA and Sc cotreatment, confirmed that the metabolic products obtained from MA oxidation by transmembranal SSAO/VAP-1 mediated the cytotoxic process. Fig. 1.B shows the effect of 3 mM MA, 1 mM Sc or their cotreatment in WT and hSSAO/VAP-1 cells using the fluorescence Viability/Cytotoxicity Kit, which allows live and dead cells to be stained simultaneously in green and red fluorescence, respectively. After 48 h treatment with 1 mM Sc (Fig. 1.B.2), 3 mM MA (Fig. 1.B.3), 1 mM Sc plus 3 mM MA (Fig. 1.B.4) or untreated cells (Fig. 1.B.1), no changes were observed in WT cells, which showed a morphological integrity and emitted mainly green fluorescence. Although hSSAO/VAP-1 cells did not show any effect by 1 mM Sc treatment (Fig. 1.B.6), 3 mM MA treatment induced clear cell shrinkage and shedding, allowing EthD-1 to enter. Moreover, cotreatment of 3 mM MA and 1 mM Sc prevented the cell degeneration observed with MA alone, indicating that SSAO activity was the only cause of the cell damage observed.

3.2. FA is the main contributor to the cytotoxicity mediated by MA oxidation in hSSAO/VAP-1 cells

Since MA oxidation induced a clear cytotoxic effect when it was incubated with those cells that expressed transmembrane SSAO, we next studied the contribution of each MA oxidation product to the toxic process observed. Both cell types were treated with increasing concentrations of the final products: NH_4, H_2O_2 or Formaldehyde (FA), from 0.1 to 1 mM during 24 h (data not shown) and cell viability results indicated similar behavior for both cell types, expressing or not SSAO/VAP-1. Whereas NH_4 did not produce any effect in any of the assayed concentrations (0.1–1 mM), H_2O_2 produced a decrease of 60% in MTT reduction only in the maximum concentration assayed (1 mM). FA induced 40% cell viability loss at low concentration (0.1 mM), and reached an 80–90% of cell death at 0.25 mM, indicating that FA was the most toxic metabolic product in the conditions assayed.

To ensure that FA was the main cause of the toxicity observed, hSSAO/VAP-1 cells were treated for 2 days with 3 mM MA, 1 mM H_2O_2 and 1 mM FA alone or with 50 U/ml of catalase, in order to determine the H_2O_2 contribution to the overall cytotoxic effect of MA oxidation (Fig. 2). Although catalase pretreatment succeeded in recovering the

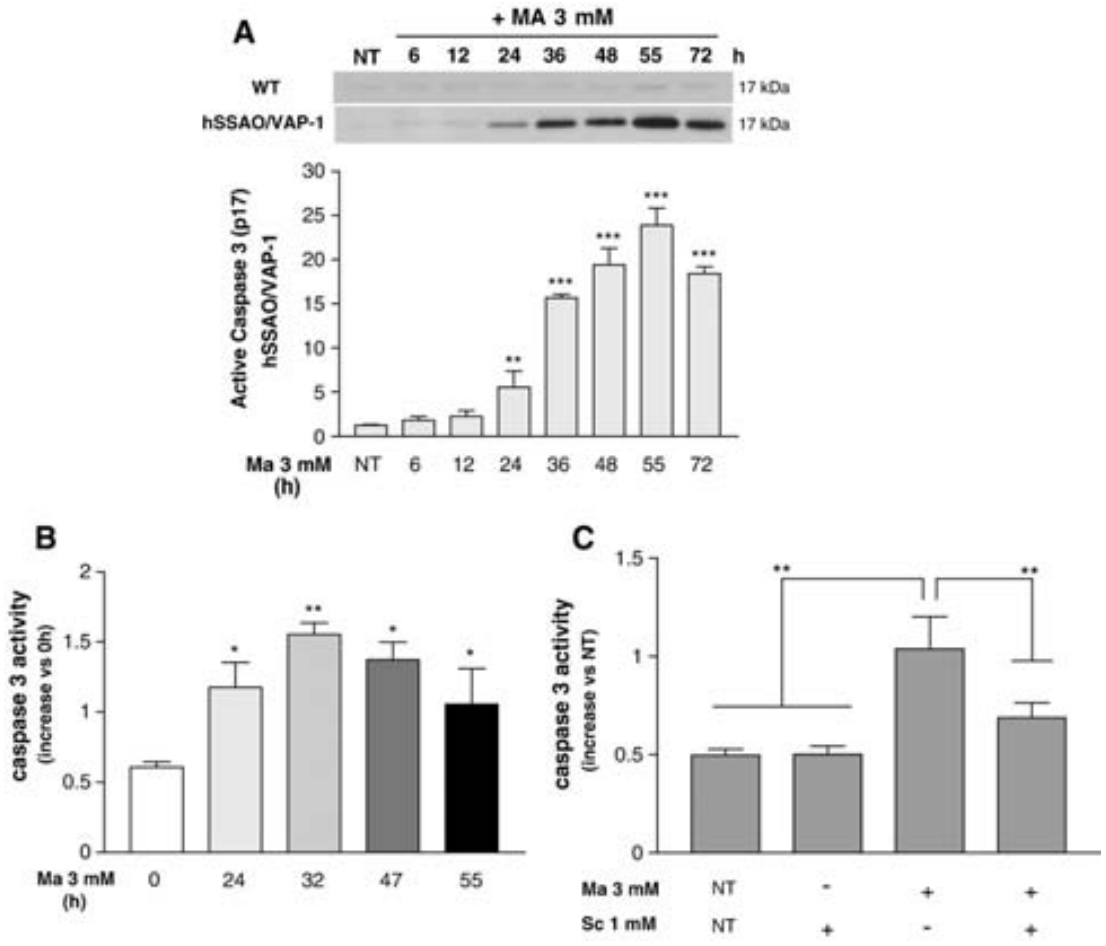

Fig. 4. Methylamine oxidation by transmembrane hSSAO/VAP-1 induces caspase 3 activation. (A) Representative immunoblot of 17 KDa caspase 3 active fragment after different times of MA 3 mM treatment in WT and hSSAO/VAP-1 cells. Values of densitometric analysis of the 17 KDa band corresponding to active caspase 3 in MA treated hSSAO/VAP-1 cells are expressed in arbitrary units versus untreated cells (NT) and represent the mean±SEM of three independent experiments. (B) Caspase 3 activity levels after different times of MA 3 mM treatment in hSSAO/VAP-1 cells. (C) Caspase 3 activity levels after 32 h treatment of hSSAO/VAP-1 cells with MA 3 mM, Sc 1 mM or both. Data in B and C are expressed in fluorescence units versus untreated cells (0 h or NT) and represent the mean±SEM of three independent experiments. (***) $p<0.001$, (**) $p<0.01$, (*) $p<0.05$ by a one-way ANOVA test and the addition of Newman–Keuls Multiple Comparison Test.

cell viability loss induced by H_2O_2, the treatment was not able to protect cells after MA treatment. Moreover, catalase alone did not produce any change in hSSAO/VAP-1 cell viability by itself and was not able to recover the cytotoxicity after FA treatment either. Surprisingly, catalase treatment enhanced the cell death caused by MA oxidation.

In order to elucidate whether FA exerted its cytotoxic effect inside or outside the cells, they were preincubated for 30 min with different aldehyde dehydrogenase inhibitors before 18 h FA treatment (Fig. 3). Increasing concentrations of intracellular dehydrogenase inhibitors were used alone or plus 0.5 mM FA. The inhibitors used were 4-Methylpyrazole (4-MP) (Fig. 3.A), as an alcohol dehydrogenase 1 (ADH 1) inhibitor; 1-Bromoheptane (1-BR) (Fig. 3.B), as a GSH-dependent formaldehyde dehydrogenase (ADH 3) inhibitor and Cyanamide (Cyan) (Fig. 3.C) and chloral hydrate (Chl Hyd) (Fig. 3.D) as aldehyde dehydrogenase 2 (ALDH 2) inhibitors. All of them were assayed in a range from 0 to 500 μM. If FA toxic effect had been triggered inside the cells, the expected results would show an increase of FA cytotoxicity at higher dehydrogenase inhibitor concentrations. However, our results showed no differences in cell viability loss between FA alone or when it was incubated in the presence of different concentrations of the dehydrogenase inhibitors assayed: 4-MP (Fig. 3.A), 1-BR (Fig. 3.B), Cyan (Fig. 3.C) or Chl Hyd (Fig. 3.D). In the case of cyanamide (Fig. 3.C), a loss of cell viability was observed in correlation with increasing cyanamide concentrations. This effect was also observed with cyanamide alone, indicating that the inhibitor was toxic by itself. So, these results suggested that FA toxicity was not produced inside the cells.

3.3. MA oxidation by membrane-bound hSSAO/VAP-1 induces an apoptotic cell death

Caspase 3 is one of the main executer caspases in the apoptotic process, and its cleavage is one of the classic apoptotic features. To evaluate whether MA oxidation products were leading to an apoptotic cell death, caspase 3 cleavage was assayed by Western Blot and its activity by using EnzChek® Caspase-3/7 Assay Kit (Molecular Probes) in hSSAO/VAP-1 cells treated with MA. Fig. 4.A shows a representative immunoblot of caspase 3 cleavage. The 17-KDa active fragment of caspase 3 appeared after 24 h of MA treatment in hSSAO/VAP-1 cells, but not in WT cells, confirming the previous cell viability results (Fig. 1) and indicating that MA by itself does not induce apoptotic cell damage. The 17 KDa active fragment of caspase 3 appeared to increase significantly from 24 to 55 h of treatment.

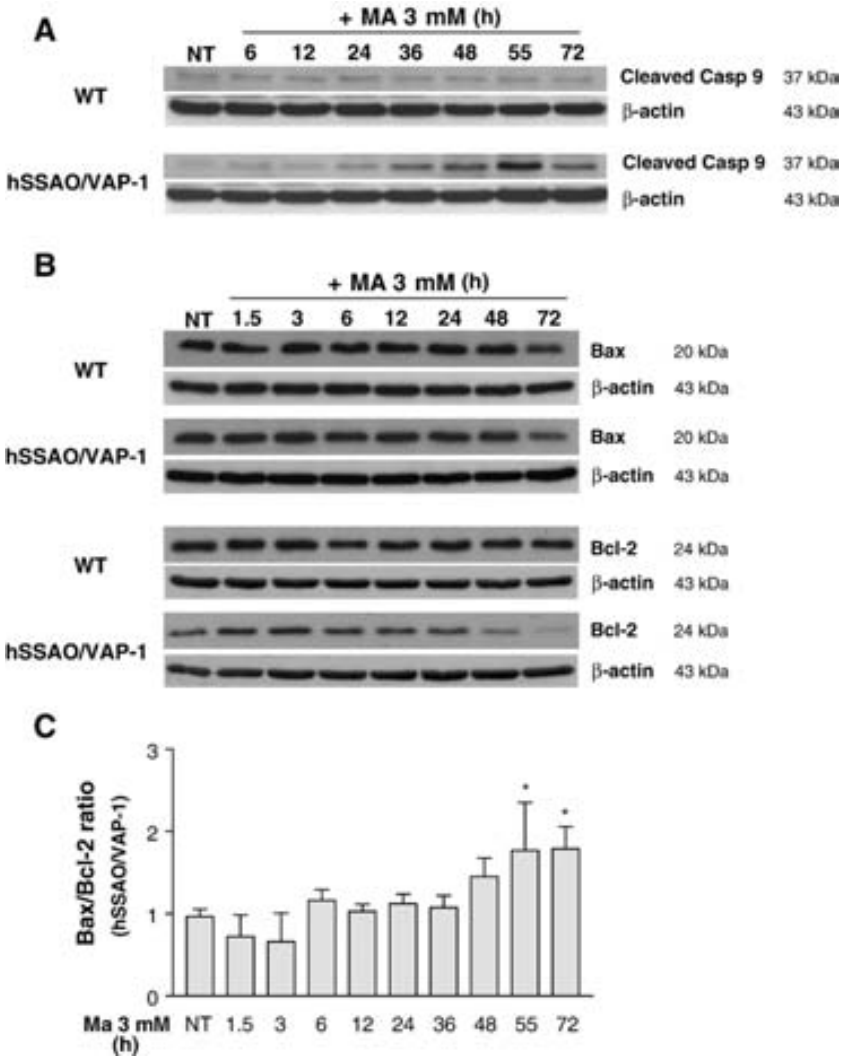

Fig. 5. Methylamine oxidation by transmembrane hSSAO/VAP-1 activates the mitochondrial apoptotic pathway including caspase 9 cleavage and Bax/Bcl-2 ratio increase. (A) Representative immunoblot of 37 KDa caspase 9 active fragment after different times of MA 3 mM treatment in WT and hSSAO/VAP-1 cells. (B) Representative immunoblots of Bax and Bcl-2 protein levels after different times of MA 3 mM treatment in WT and hSSAO/VAP-1 cells. (C) Bax/Bcl-2 ratio after different times of MA 3 mM treatment in hSSAO/VAP-1 cells. Data are expressed in densitometric units versus untreated cells (NT) and represent the mean ± SEM of three independent experiments. β-actin was used as loading control. (*) $p<0.05$ by a one-way ANOVA test and the addition of Newman–Keuls Multiple Comparison Test.

When caspase 3 activity was measured using the EnzChek ® Caspase-3/7 Assay Kit, results showed that 24 h MA treatment induced a two fold increase in caspase 3 activity versus control (Fig. 4B). The maximum rate was reached at 32 h treatment, and after that, it began to decline. A cotreatment of MA with semicarbazide at the maximum activation point blocked this caspase 3 activity and confirmed that MA oxidation was the responsible of such effect (Fig. 4.C).

After the caspase 3 cleavage and activation was confirmed, the possibility that caspase 9 was the responsible of activating caspase 3 was assessed. As shown in Fig. 5.A, caspase 9 cleavage was determined by Western Blot analysis after 3 mM MA treatment at different time points. The presence of the 37 kDa caspase 9 fragment increased in a time-dependent manner after MA treatment in hSSAO/VAP-1 cells, reaching a maximum cleavage at 55 h. On the other hand, no cleaved form was detected under the same conditions in WT cells. Caspase 3 and caspase 9 cleavages showed a similar timing pattern, so is reasonable to suggest that caspase 9 could act as one of the caspase 3 activator molecules.

3.4. Cytotoxicity mediated by MA oxidation activates the mitochondrial apoptotic pathway

Caspase 9 is involved in two important apoptotic signaling pathways: mitochondrial (or intrinsic) pathway [32] and reticular stress (ER) pathway [33,34]. To investigate their involvement in the caspase 9 activation process, some of the main characteristic protein markers of these two pathways were analyzed by Western Blot.

The GRP78 chaperone plays a crucial role in the ER misfolding protein process [35]. Its protein levels were determined by means of Western Blot, but results did not show significant differences after MA treatment in hSSAO/VAP-1 or WT cells (data not shown), so the ER stress apoptotic pathway was rejected as the cause of caspase 9 activation.

The other main apoptotic pathway which requires caspase 9 cleavage is the intrinsic or mitochondrial pathway [36,37]. The mitochondrial apoptotic pathway is regulated by the ratio of anti to pro-apoptotic molecules, which includes the Bcl-2 family [38]. Representative Western Blots of Bax and Bcl-2 protein levels are shown in Fig. 5.B. MA treatment in WT cells did not modify the pro-apoptotic protein Bax. Comparably, Bax protein levels were not affected by MA oxidation products generated in hSSAO/VAP-1 cells after MA treatment. However, in hSSAO/VAP-1 cells only, the anti-apoptotic protein Bcl-2 showed a significant reduction in protein levels after 48 h of MA treatment. Fig. 5.C shows the Bax/Bcl-2 ratio in those cells, confirming its alteration after 48 h of MA treatment, and indicating the probably involvement of the mitochondrial pathway in the cell death studied.

3.5. p53 phosphorylation and PUMA-alpha are increased in hSSAO/VAP-1 cell death under MA oxidation conditions

P53 is related to the mitochondrial apoptotic pathway activation through different cell damaging insults [39]. To assess whether p53 had any role in MA oxidation mediated toxicity, its phosphorylation on serine 15 was determined, since this residue has been described as an apoptosis-related phosphorylation site [40]. Fig. 6.A shows representative immunoblots of MA treated WT and hSSAO/VAP-1 cells for phospho-p53 in serine 15 and total p53 protein. No p53 phosphorylation in ser-15 residue was observed in WT cells. On the contrary, blots corresponding to hSSAO/VAP-1 cells showed an increase of phospho-p53 in ser-15, which began at 12 h and reached a maximum rate at 36–48 h of MA treatment, decreasing after this point. Even so, none of the blots showed changes in total p53 protein levels, indicating the presence of phosphorylated protein without de novo synthesis.

PUMA expression induced by p53 transcriptional activity has been demonstrated to be required for p53-mediated cell death through the mitochondrial pathway under numerous p53-dependent apoptotic stimuli [41–43]. In our study, PUMA α and β protein levels were determined by Western Blot in MA treated cells (Fig. 6.B). In WT cells, neither PUMA α, nor PUMA β protein levels changed during MA treatment: whereas PUMA α was almost undetectable, PUMA β isoform

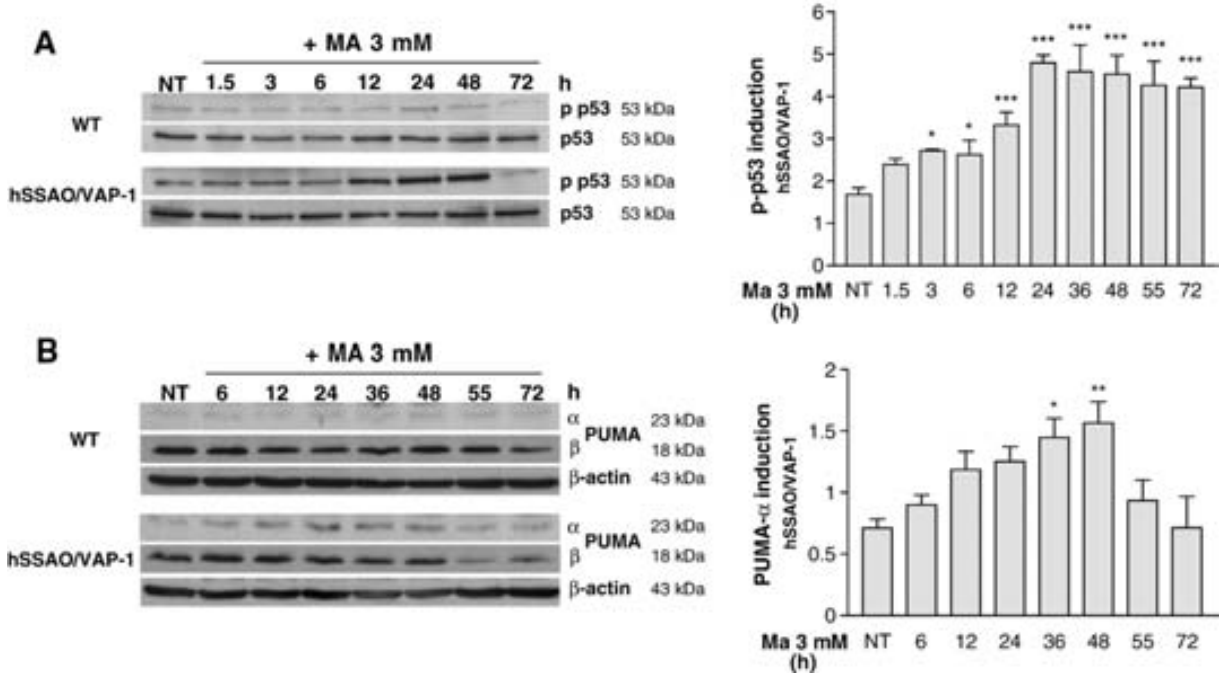

Fig. 6. Methylamine oxidation induces p53 phosphorylation of serine-15 residue and PUMA-α expression in hSSAO/VAP-1 cells. (A) Representative immunoblots of phospho-p53 (serine 15) and total p53 after different time treatments of WT and hSSAO/VAP-1 cells with MA 3 mM. P53 phosphorylation is increased in hSSAO/VAP-1 cells after 12 h of MA treatment without changes of total p53 protein levels. Data of phospho-p53 induction in hSSAO/VAP-1 cells are expressed in densitometric units versus non-treated cells (NT). (B) Representative immunoblots of PUMA (isoforms α and β) after MA treatment. PUMA-α levels increased in hSSAO/VAP-1 cells after 12 h of treatment, and were maintained until 48 h; in contrast, PUMA-β levels decreased in hSSAO/VAP-1 cells at 55 and 72 h, but they did not change in WT cells. Quantification of PUMA-α induction in hSSAO/VAP-1 cells is expressed as densitometric units versus non-treated cells (NT). β-actin was used as loading control. In both cases, densitometric values represent the mean ± SEM of three independent experiments. (***) $p<0.001$, (**) $p<0.01$, (*) $p<0.05$ by a one-way ANOVA test and the addition of Newman–Keuls Multiple Comparison Test.

expression was markedly expressed. However, a significant induction of PUMA α at 36–48 h was observed in MA treated hSSAO/VAP-1 cells at 24 h of treatment, which was maintained until 48 h. On the other hand, PUMA β expression was not increased after MA treatment in transfected cells, showing slower protein levels at longer time treatments (55 and 72 h). These results suggested a possible involvement of p53, through PUMA α expression induction, on the mitochondrial deregulation observed in MA oxidation mediated toxicity.

3.6. p53 activity mediates hSSAO/VAP-1 cell death under MA oxidation conditions

To test whether p53 activation was mediating the apoptotic cell death through a transcription-dependent mechanism, pifithrin-α [44], a p53 transcriptional activity inhibitor, was coincubated with MA. Different concentrations of pifithrin α (1, 5 and 10 μM) were added 1 h before the 48 h MA treatment (Fig. 7). Cell viability determinations showed that pifithrin-α produced a dose-dependent cell viability recovery of MA induced toxicity in hSSAO/VAP-1 cells obtaining a complete cytoprotection with 10 μM pifithrin-α (Fig. 7.B). This dose-dependent recovery was observed also at 72 h treatment in hSSAO/VAP-1 cells (data not shown). WT cells did not show any cytotoxicity with MA treatment, and pifithrin-α treatment produced a slight cytotoxicity on these cells (Fig. 7.A). The total cell death recovery after pifithrin α pretreatment evidenced the role of p53 in the early and advanced SSAO mediated apoptotic process.

Finally, to ensure that mitochondrial alterations, as Bax/Bcl-2 ratio increase, were induced by the activation of p53 pathway, hSSAO/VAP-1 cells were treated with MA at different time points, with or without pifithrin-α, and Bax and Bcl-2 protein expression was analyzed by Western Blot (Fig. 7.C). Treatment of hSSAO/VAP-1 cells with pifithrin-α blocked Bcl-2 decrease, and did not change Bax protein levels. These

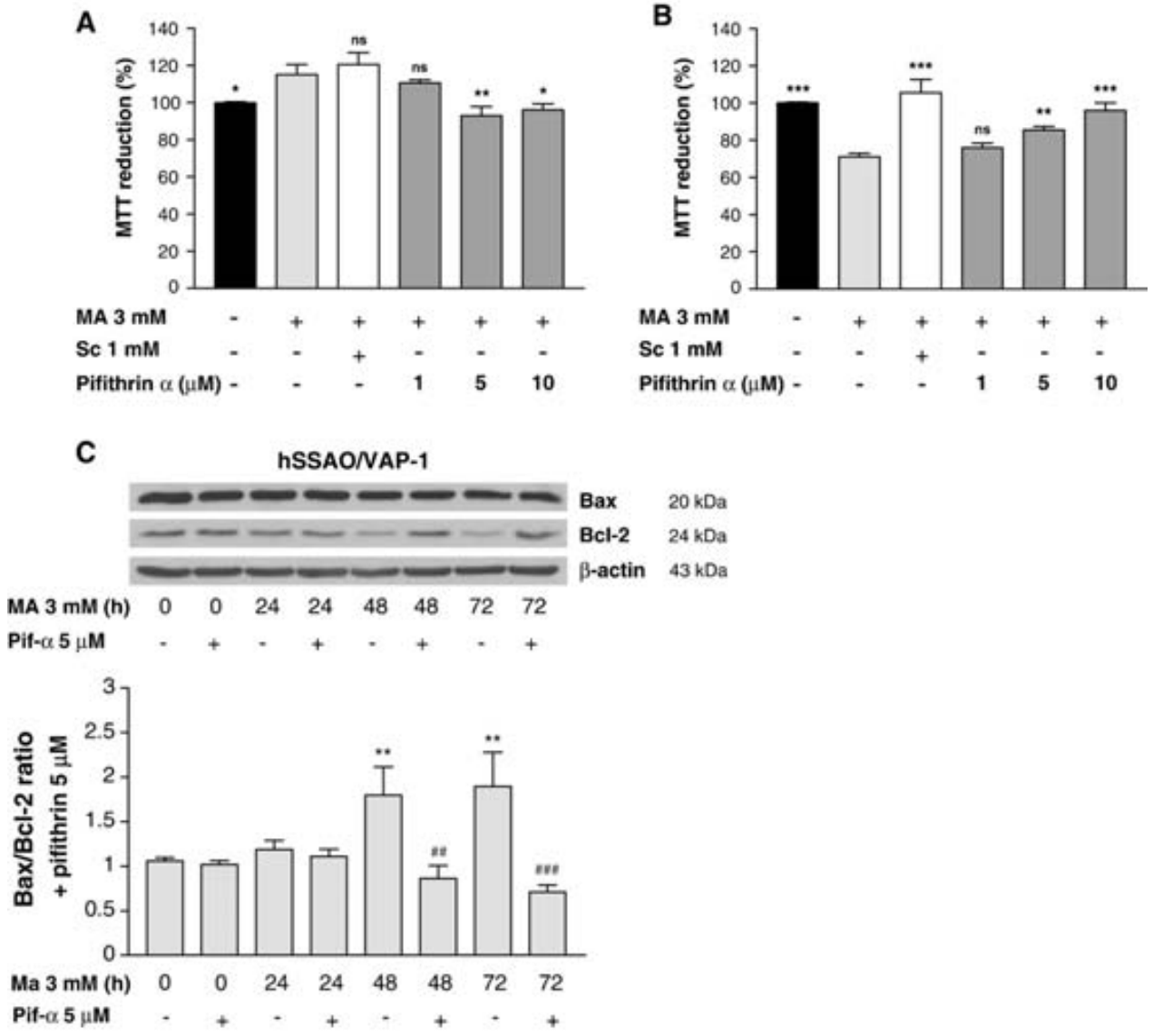

Fig. 7. p53 transcriptional activity inhibition with pifithrin-α reverts hSSAO/VAP-1 cell death and Bax/Bcl-2 ratio increase induced by MA oxidation products. WT cells (A), or hSSAO/VAP-1 cells (B) were treated with MA 3 mM, Sc 1 mM or/and pifithrin α (1, 5 or 10 μM) during 48 h. Pifithrin-α was added 1 h before MA and Sc treatment. Pifithrin-α produced dose-dependent cell viability recovery of MA treated hSSAO/VAP-1 cells. Pifithrin-α was not significantly toxic by itself at assayed concentrations (data not shown). Cell viability was determined by MTT reduction method. Data are the mean ± SEM of three independent experiments performed in triplicate. (***) $p<0.001$, (**) $p<0.01$, (*) $p<0.05$, (n.s.) $p>0.05$ by a one-way ANOVA test and the addition of Newman–Keuls Multiple Comparison Test, where all columns were compared with MA 3 mM values. (C) hSSAO/VAP-1 cells were treated with MA 3 mM during different times with or without pifithrin 5 μM. Representative immunoblots for Bax and Bcl-2 proteins are shown. Bax/Bcl-2 ratio after treatments in hSSAO/VAP-1 cells represents the mean ± SEM of three independent experiments. Data are expressed in densitometric units versus untreated cells (0h). β-actin was used as loading control. (**) $p<0.01$ vs non-treated cells; (###) $p<0.001$ and (##) $p<0.01$ vs MA 3 mM, by a one-way ANOVA test and the addition of Newman–Keuls Multiple Comparison Test.

effects leaded to prevent the increase of the Bax/Bcl-2 ratio, confirming that the activation of the p53-dependent pathway was directly involved in the apoptotic process induced by MA oxidation.

4. Discussion

SSAO activity is altered in several pathological conditions. MA and plasma SSAO are increased in patients suffering from diabetes type I and II [45,46], but increased plasmatic SSAO activity has been found also in patients afflicted by many other pathologies related with vascular system and/or inflammatory conditions [46–49]. Increased SSAO activity has also been related to diseases affecting the nervous system, as Alzheimer's disease (AD) [50] or multiple sclerosis [51]. In this context, we have previously reported that circulating SSAO activity is increased in plasma from severe AD patients [29], and that membrane-bound SSAO is overexpressed in the cerebrovascular tissue of AD and CADASIL (Cerebral Autosomal Dominant Arteriopathy with Subcortical Infarcts and Leukoencephalopathy) patients, with the subsequent perturbation of the brain vasculature [28].

Increased SSAO activity can lead to a rise in its metabolic products, such as methylglioxal, formaldehyde and hydrogen peroxide, which are involved in producing covalent cross-links with proteins and DNA [24,52,53], generating advanced glycation end products (AGEs) [54,55], and inducing oxidative stress by producing free radicals [23,56]. In fact, soluble SSAO mediated amine oxidation in endothelial [22,57] and smooth muscle cells in culture [27,58,59] have both shown a cytotoxic effect. Given that the membrane-bound enzymatic form has also been shown to be overexpressed in pathologic situations, we have studied whether the metabolism of the cellular enzyme is a potential source of vascular cell damage. Although tissues show elevated SSAO levels, cells in culture stop expressing this protein [13,22]. In order to study the metabolism by membrane-bound SSAO, we have used a transformed smooth muscle cell line which stably expresses the human SSAO protein [30].

In this study, we show that MA at both 1 and 3 mM produces a cytotoxic effect only in cells expressing SSAO (hSSAO/VAP-1 cells), but not in untransfected (WT) cells. Since our model is based on acute treatments of cultured cells, the MA levels used in this work are higher than those reported in physiological conditions for human plasma [60], even so, this MA concentration range has been widely used in many other cell culture studies [13,22,58]. The SSAO activity levels obtained from the transfected cell line are similar to those observed in smooth muscle tissue [30], suggesting that acute exposures to SSAO substrates could produce a chronic vascular damage by toxic substances accumulation. In fact, cytotoxic effects appear after 1–2 days cell treatments, whereas other studies have not observed this toxic effect after short term exposures (hours) to MA [13]. Moreover, several studies have reported increased vascular damage in animals treated with the SSAO substrates aminoacetone or methylamine [55,61]. Vascular complications like AGE formation, hypertension, atherosclerosis, glomerulosclerosis or retinopathy also have been observed in transgenic mice overexpressing human VAP-1 [61,62]. Moreover, an increase of reactive aldehydes generated by SSAO and other amine oxidases has been found in neurodegenerative diseases, and may contribute to the development and progression of these disorders [63].

Our results show that MA plus catalase cotreatment does not recover cell death induced by MA oxidation, confirming the principal role of formaldehyde in the toxicity process, in agreement with previous reports [22,27]. On the contrary, this cotreatment increased the cytotoxicity. This effect could be explained by the fact that H_2O_2 removal may shift the MA oxidation reaction in the direction of product generation, which would increase the formaldehyde concentration, and thus raising the cytotoxic effect. Alternatively, we cannot rule out that subtoxic H_2O_2 doses may induce pro-survival molecular pathway activation, such as the PKB/AKT pathway [64], which might induce more resistance to the toxic insults.

The mechanism by which formaldehyde induces a cytotoxic effect in our in vitro system remains unknown. Teng et al. [65] reported that pretreating hepatocytes with different intracellular formaldehyde dehydrogenase inhibitors before treating with formaldehyde enhanced its toxicity, showing that the aldehyde was permeable to the cell membrane and acted inside the cytosol. However, treatments with formaldehyde dehydrogenase inhibitors do not enhance formaldehyde toxicity in our model. These results suggest that formaldehyde may act from outside the cell membrane, since it is a very reactive molecule able to induce extracellular protein crosslinkings, thus activating intracellular molecular pathways. Possible recognition of formaldehyde by extracellular receptors could not be ruled out. Even so, it should be considered that formaldehyde detoxification enzyme systems are different between cell types, and this fact could explain the different results obtained with the formaldehyde dehydrogenase pretreatments.

Cytotoxic damage induced in smooth muscle cells by MA oxidation products leads to the activation of caspase 3, one of the main apoptosis executers, as was shown previously by soluble SSAO activity [27]. The study of the signaling pathways that could lead to this apoptotic fate in our system reveals that the MA treatment of hSSAO/VAP-1 cells does not modify the constitutive expression of pro-apoptotic protein Bax, but it significantly decreases the anti-apoptotic protein Bcl-2 levels, leading to deregulation of the pro/anti-apoptotic protein balance. Alterations of the ratio between these proteins, such as Bax/Bcl-2, are considered determinant events for apoptosis induction by mitochondrial pathway [38]. In addition, a p53 phosphorylation has been observed after same treatment in transfected cells. Supporting these results, it has been previously described that p53 activity can downregulate the expression of Bcl-2 [66]. Moreover, although Bax is a transcriptional target of p53 activity [67], its induction has been shown not to be necessary for p53-dependent apoptosis, since p53 is able to induce Bax oligomerization and this promotes mitochondrial outer membrane permeabilization (MOMP) [68,69]. Another evidence that these mitochondrial alterations are key events in the apoptotic progression is the activation of caspase 9 in MA treated transfected cells. Its activation takes place after the mitochondrial factors release [70]. Although caspase 9 is also activated in late stages of reticular stress apoptotic pathway, this signaling cascade was rejected to take part in our model since protein levels of GRP78, a key chaperone in the Unfolded Protein Response (UPR) [71], are not altered after MA treatment.

p53 is activated under conditions such as oncogene expression, DNA damage or other sources of cellular stress [72]. In response to these cell insults, phosphorylation or other posttranslational modifications can stabilize p53 and let it act through transcription-dependent or independent mechanisms of apoptosis (reviewed by [73] and [39]). Serine 15 phosphorylation seems to be the p53 stabilization mechanism in SSAO mediated cytotoxicity in smooth muscle cells. After this, p53 could have direct effects on the mitochondria decreasing Bcl-2 levels, or activate the transcription of its target genes. As well as *BAX*, the pro-apoptotic mitochondria-related genes *PUMA, NOXA* and *BID* are also induced by p53 transcriptional activity [41,74,75]. The total recovery of cell death with pifithrin-α treatment suggests that, although p53 is able to induce apoptosis without transcription or protein synthesis in certain conditions [76], this activity is essential to the cytotoxicity mediated by SSAO. Moreover, the prevention of the Bax/Bcl-2 increase after pifithrin-α pretreatment in MA treated cells suggested the involvement of this single apoptotic pathway in mediating this cell death. PUMA could be a candidate to mediate such activity, since PUMA-α is increased after MA treatment in hSSAO/VAP-1 cells. Although PUMA has several isoforms with different regulations [41], α form has been shown to be induced in certain types of p53-dependent apoptosis, in contrast to PUMA-β [77]. PUMA is able to bind to anti-apoptotic proteins like Bcl-2 or Bcl-$_{XL}$ through its BH3 domain, antagonizing its

repressor action on Bax or other pro-apoptotic proteins [41,78,79]. Taking all, the increase of the PUMA-α isoform observed in this work suggests that it could be participating in this cell death pathway. However, since there are multiple p53-inducible genes, other proteins might be involved in the process.

In summary, the protein analyses performed in this work demonstrate that the mitochondrial pathway is involved in cell death induced by MA oxidation. In the signaling sequence, p53 activation induced by MA oxidation products might lead to decrease Bcl-2 levels, thus allowing Bax oligomerization and inducing mitochondrial permeabilization, which in turn activates caspase 9 and caspase 3. This pathway could be reinforced by p53 mediated PUMA expression, which may contribute to the over-availability of pro-apoptotic proteins by anti-apoptotic proteins sequestration. Additionally, the toxicity recovery in the presence of pifithrin-α, a p53 transcriptional activity inhibitor extensively used in blocking p53 pathway [44,80,81], suggests a principal role for p53 in mediating this cell death.

Our study supports the idea that in conditions in which membrane-bound SSAO is overexpressed, its chronically elevated activity could lead to vascular cell damage through its catalytic products. Recently, several studies have been developed new SSAO inhibitor molecules to target potential treatments for inflammatory diseases [82–84]. As far as this is concerned, our findings contribute to the idea that SSAO inhibition would be useful in blocking the cell damage source induced by SSAO activity in such pathologies.

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1. Annex Capítol III.

1.1. Supplementary figure A.

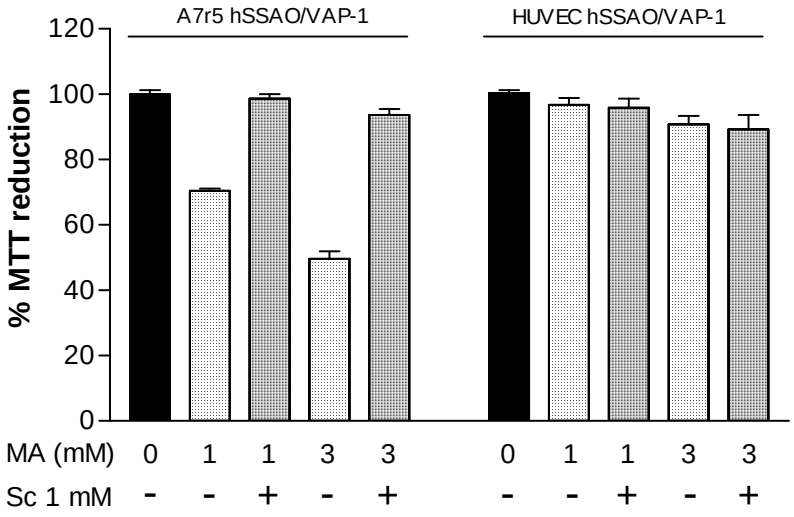
The metabolic products of SSAO activity through methylamine (MA) are hydrogen peroxide (H_2O_2), ammonia (NH_3) and formaldehyde. All of them are potentially toxic molecules that have been considered potential risk factors for vascular damage and degeneration in situations with increased SSAO activity (Yu *et al.* 2003). Moreover, the toxicity induced by soluble SSAO mediated methylamine oxidation has been proved to be toxic to vascular cells (Hernandez *et al.* 2006). We have described the molecular pathway involved in methylamine induced toxicity through its metabolism by the transmembrane SSAO protein transfected into A7r5 smooth muscle cells (Sole *et al.* 2008). Endothelial cells, however, are also exposed to the toxic products generated by SSAO metabolism, so we evaluated the possible toxic effect of methylamine oxidation by the transmembrane SSAO transfected into HUVEC cells (supplementary figure A).

The treatment of A7r5 hSSAO/VAP-1 cells with MA induced a significant reduction in their cell viability, which was completely recovered pretreating cells with the SSAO inhibitor semicarbazide. By contrast, the same treatment in HUVEC hSSAO/VAP-1 cells only induced a slight reduction in cell viability, which was not prevented by semicarbazide pre treatment, indicating that it was possibly due to MA itself toxicity (supplementary figure A.1). Moreover, higher MA concentrations (until 5 mM) or longer time of treatment (until 72h) showed similar results (data not shown). To assess if these cells were also sensitive to MA oxidation toxicity, different concentrations of soluble SSAO (BSAO) plus MA were added (supplementary figure A.2). The 24 h incubation with BSAO and MA induced a high toxic effect in HUVEC cells, as it was previously demonstrated for A7r5 smooth muscle cells (Hernandez *et al.* 2006). Thus, HUVEC cells were more sensible to MA oxidation toxicity than A7r5 cells, however, the transfected transmembrane SSAO was not able to induce its toxicity.

We suppose that the lower levels of activity observed in HUVEC hSSAO/VAP-1 cells compared to those observed in A7r5 hSSAO/VAP-1 cells are the responsible of this lack of toxicity. In agreement, it has been suggested that a threshold of activity may be necessary to be exceeded to induce cytotoxicity (Yu *et al.* 2006). Therefore, the levels of SSAO activity that show the HUVEC transfected cells may not reach this threshold. However, we think that the SSAO activity of endothelial cells may be an important

source of vasculotoxicity *in vivo*, as it has been demonstrated in the endothelial SSAO overexpressing mice (Stolen *et al.* 2004).

1.



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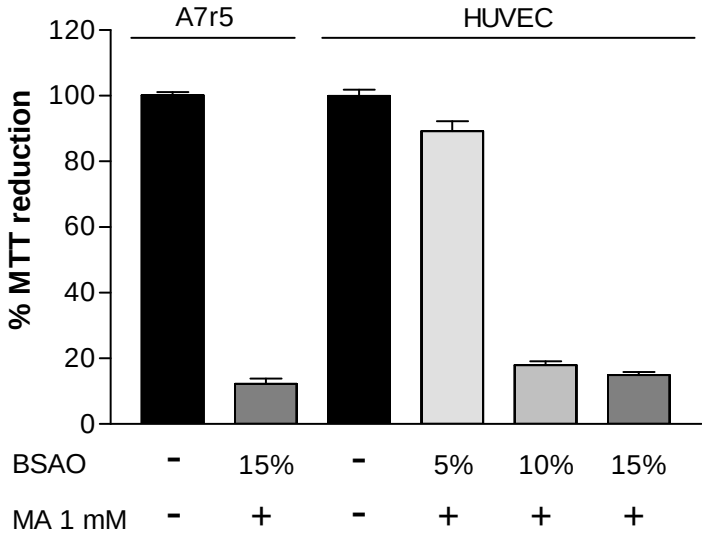
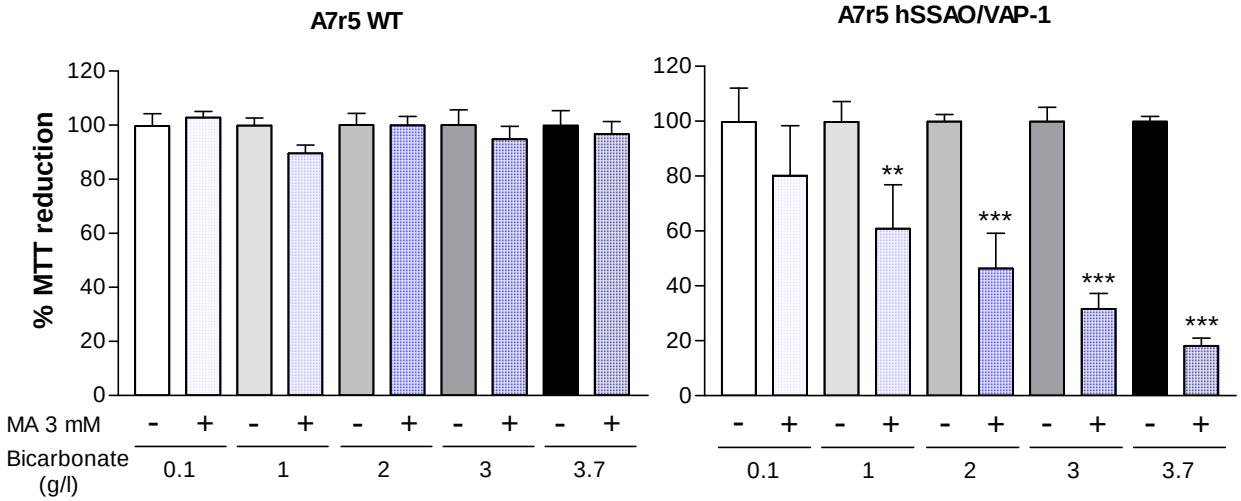


Supplementary figure A. A7r5 and HUVEC hSSAO/VAP-1 cells were treated during 48 h with methylamine (MA) 1 and 3 mM with or without 1 mM semicarbazide (Sc) pre treatment, and cell viability was evaluated by MTT reduction method (**1**). A dose-response cytotoxic effect was observed in A7r5 cells, which was prevented by Sc pre treatment. However, HUVEC cells did not decrease its MTT reduction rate. To evaluate whether or not the HUVEC cells were sensible to MA oxidation products, cells were treated during 24h with soluble SSAO (BSAO) plus 1 mM MA (**2**). HUVEC cells were as sensitive as A7r5 cells to the toxicity induced by SSAO mediated MA oxidation. Data are mean ± SEM of values of three independent experiments.

1.2. Supplementary figure B.

The enzymatic SSAO activity is regulated by several mediators. We have recently described that bicarbonate is able to enhance the SSAO activity by a modulation that is abrogated by a dialysis procedure (Hernandez-Guillamon *et al.* 2007). This effect is dependent on bicarbonate concentration and it is induced on both the soluble and the transmembrane forms of SSAO. Moreover, a similar modulation has been described for histaminase activity of rat adipocytes (Raimondi *et al.* 1997). Bicarbonate is present in physiological conditions in human plasma, where it functions as a buffering molecule. Therefore, variations in blood concentrations of bicarbonate may induce, as consequence, variations in SSAO activity. To evaluate the possible consequences of an increase of bicarbonate concentration in terms of the toxic products generated and its effect on vascular cell culture, it was analyzed the cell viability after exposure to different concentrations of bicarbonate in presence of MA as SSAO substrate (supplementary figure B).



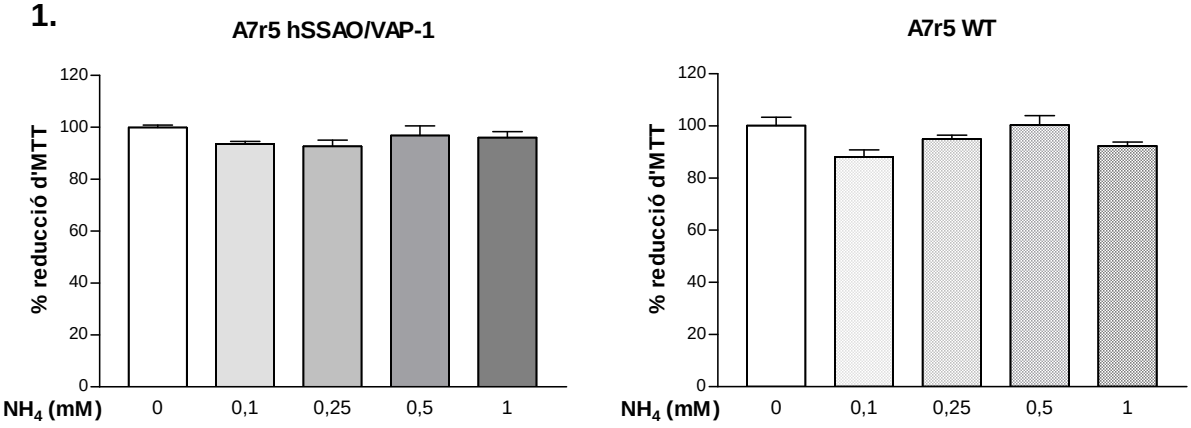
Supplementary figure B. A7r5 WT and hSSAO/VAP-1 cells were treated during 72 h with methylamine (MA) 3 mM with different concentrations of bicarbonate in the culture medium. Culture medium was prepared without bicarbonate, the indicated amounts of sodium bicarbonate were added to the mediums and the pH of the mediums was adjusted at 7.2 to avoid possible variations due to the different pH. Cell viability was quantified by MTT reduction method. (**) $p<0.01$ and (***) $p<0.001$ *vs* the same bicarbonate concentration without MA. Data are mean ± SEM of values of three independent experiments.

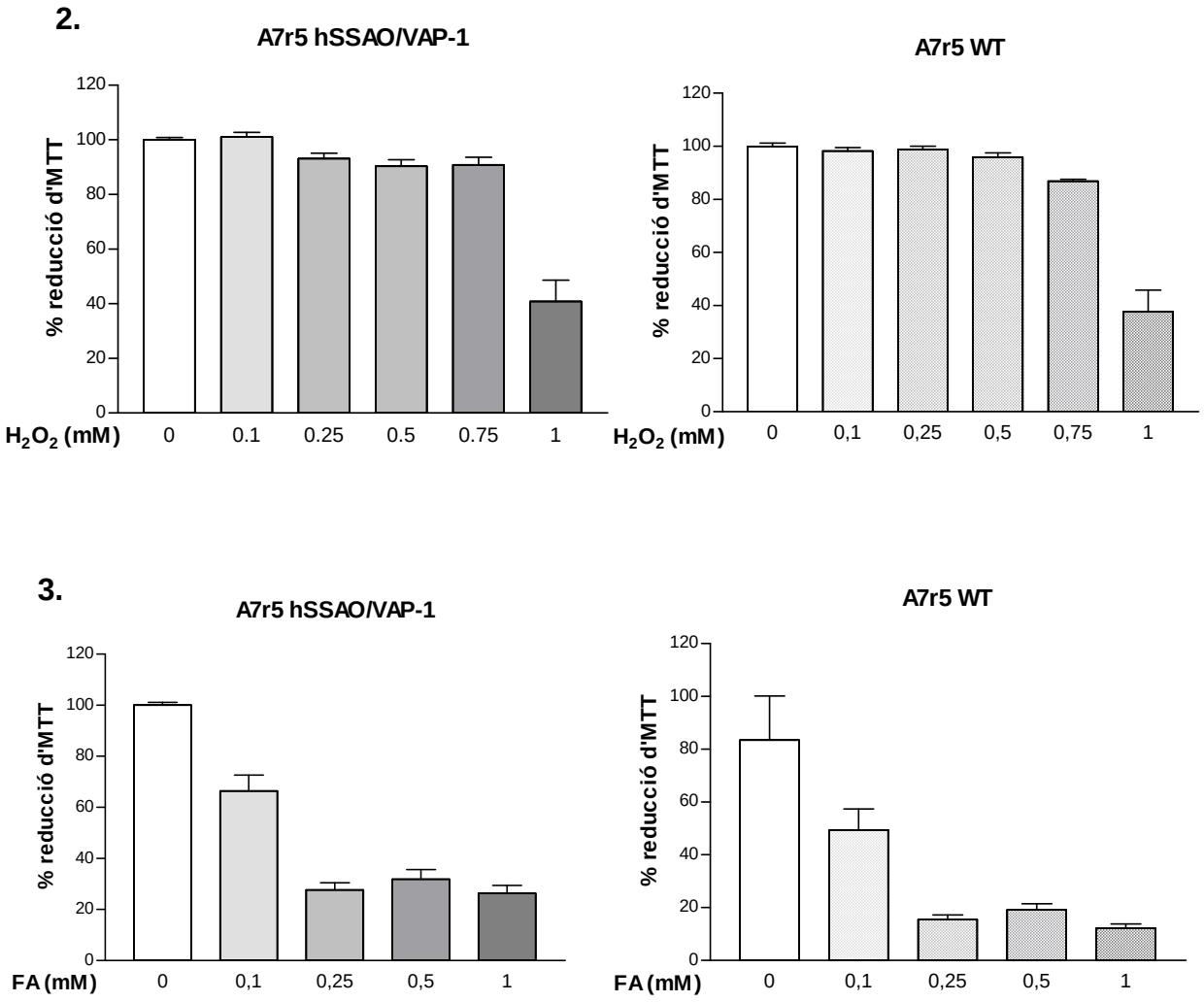
After MA treatments in medium with different sodium bicarbonate concentrations, A7r5 hSSAO/VAP-1 cells showed bicarbonate concentration-dependent decrease in cell viability, while A7r5 WT cells did not modify it. These results indicated that changes in SSAO activity would have a drastic effect on its induced toxicity. Moreover, the concentration of sodium bicarbonate present in blood plasma is about 1.4 g/l, at which SSAO activity induces cytotoxicity. Thereby the results here obtained are in the physiological bicarbonate range, and demonstrate that this process can occur at physiological conditions. In addition, few increases in SSAO activity observed in several pathological situations should be considered as a cause of their vasculotoxic processes. The 3.7 g/l bicarbonate concentration value reached in the study represents its concentration in the culture medium, and the viability values obtained agree with the reported in this work. This experiment was not done with HUVEC hSSAO/VAP-1 cells because its medium contains 2.2 g/l of sodium bicarbonate, concentration that exceeds the observed in blood plasma; since none cytotoxic effect was observed at these concentrations, it would not be observed either at lower bicarbonate concentrations.

1.3. Supplementary figure C.

To study the contribution of each methylamine oxidation product to the toxic process observed, A7r5 WT and hSSAO/VAP-1 cell types were treated with increasing concentrations of NH_4, H_2O_2 or formaldehide (FA) during 24 h (supplementary figure C).

1.





Supplementary figure C. A7r5 WT and hSSAO/VAP-1 cells were treated during 24 h with increasing concentrations of the methylamine oxidation products: NH4 (**1**), H2O2 (**2**) or formaldehyde (For) (**3**). Cell viability was determined by MTT reduction. Data are mean ± SEM of values of three independent experiments.

Treatment of A7r5 WT and hSSAO/VAP-1 cells with the MA oxidation products gave different responses depending on the product, but the same effects in both cells were observed. NH_4 did not induce toxicity even at high concentrations. H_2O_2 induced a moderate toxicity but only at highest concentrations assayed. However, formaldehyde displayed the most powerful effect, inducing high levels of toxicity at low concentrations. Therefore, formaldehyde seems to be the main contributor to the toxic effect seen after SSAO mediated MA oxidation. Both formaldehyde and methylglioxal (the aldehyde formed from SSAO mediated aminoacetone oxidation) are potent cross-link inducers (Nagaraj *et al.* 1996) and contribute to the increase of advanced glycation

end products (AGEs) production (Yu and Zuo 1993). Moreover, direct proves of its activity have been observed in animal models (Langford *et al.* 1999; Mathys *et al.* 2002; Stolen *et al.* 2004; Yu and Zuo 1997), and in human tissues of various pathological conditions (Lovell *et al.* 2001; Sakata *et al.* 2003; Yu 1998) demonstrating that they action has real consequences in the physiopathology.

Capítol IV:

“Role of semicarbazide-sensitive amine oxidase (SSAO) in amyloid beta$_{1-40}$ Dutch effects on vascular cells”

Article pendent d’enviar a publicar

Role of semicarbazide-sensitive amine oxidase (SSAO) in amyloid beta $_{1-40}$ Dutch effects on vascular cells

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Running title: SSAO toxicity enhances beta amyloid deposition

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Abstract

Cerebral Amyloid Angiopathy (CAA) is present in most cases of Alzheimer's disease (AD), and it is characterized by the deposition of beta amyloid (Aβ) in cerebral cortical and meningeal blood vessels, inducing the degeneration of vascular cells. Semicarbazide-sensitive amine oxidase (SSAO) is a multifunctional enzyme present in endothelial and smooth muscle cerebrovascular cells, and as soluble form in blood plasma. SSAO metabolizes primary amines, generating hydrogen peroxide, ammonia and the corresponding aldehyde. These products are able to induce apoptosis, to enhance the advanced glycation end-products generation and to increase the Aβ aggregation as well. An association between SSAO and CAA has been suggested due to the observed overexpression of SSAO colocalizing with Aβ deposits in the cerebrovascular tissue of CAA-AD patients, and to the increased SSAO activity observed in plasma of severe AD patients. In order to study the SSAO involvement in this neurodegenerative disorder and the possible causes of the SSAO overexpression we used vascular cell models expressing SSAO, previously described in our group. Aβ treatment increased the SSAO-induced cytotoxicity, which was accompanied by an increase of SSAO protein only in endothelial cells. Moreover, SSAO was able to enhance the Aβ deposition on the cells by activity-dependent and activity-independent mechanisms. Results suggest that Aβ could be one of the factors that contribute to the SSAO overexpression in AD, which in turns enhance the toxic effect of SSAO activity. In addition, these results suggest a role of SSAO in Aβ deposition that could reinforce the SSAO overexpression and contribute to the vascular dysfunction observed in CAA-AD.

Keywords: Semicarbazide-sensitive amine oxidase, beta amyloid, apoptosis, aldehydes.

Abbreviations: A7r5, rat aortic smooth muscle cells; Aβ, amyloid beta peptide; AD, Alzheimer's disease; APP, amyloid precursor protein; CAA, cerebral amyloid angiopathy; HUVEC, human umbilical vein endothelial cells; MA, methylamine; Sc, semicarbazide; SSAO, semicarbazide-sensitive amine oxidase; VAP-1, vascular adhesion protein 1; WT, wild type.

Introduction

Alzheimer's disease (AD) is a progressive degenerative heterogeneous disorder that includes both neuronal and vascular affectations. Several authors support the view that vascular disorders are involved in the aetiology and progression of AD (Bell and Zlokovic 2009; de la Torre 2004). Vascular aspects of AD include cerebral amyloid angiopathy (CAA), which is found in most cases of AD. CAA is characterized by the deposition of beta-amyloid (Aβ) in cerebral cortical and meningeal blood vessels, inducing the degeneration of vascular cells (Kawai *et al.* 1993). Familial forms of CAA have also been found, which some of them are produced by point mutations inside the Aβ sequence of the amyloid precursor protein (APP). Among them, the Dutch mutation results in a glutamic acid to glutamine substitution at residue 22 of Aβ sequence (E22Q) (Levy *et al.* 1990), which induces the rare disorder named hereditary cerebral haemorrhage with amyloidosis-Dutch type (HCHWA-D). This disorder causes severe CAA with haemorrhagic strokes of mid-life onset and dementia (Bornebroek *et al.* 1997).

Semicarbazide-sensitive amine oxidase (SSAO) [E.C. 1.4.3.6] is an enzyme strongly expressed in adipocytes and in vascular endothelial and smooth muscle cells, and is also present in blood plasma as a soluble form (Lyles 1996). In brain, SSAO is localized in the cerebrovascular system, associated to membranes of endothelial and smooth muscle cells in microvessels and meninges (Castillo *et al.* 1998). S enzyme, SSAO catalyses the oxidative deamination of primary amines as methylamine or aminoacetone generating hydrogen peroxide (H_2O_2), ammonia (NH_3) and the corresponding aldehyde (Lyles 1996; Precious *et al.* 1988). SSAO activity, however, has different effects depending on the tissue where it is expressed, having insulinomimetic effects in adipocytes and smooth muscle cells (El Hadri *et al.* 2002; Enrique-Tarancon *et al.* 1998), or promoting lymphocyte trafficking in endothelial cells (Salmi *et al.* 1993), where is also known as Vascular adhesion protein 1 (VAP-1) (Smith *et al.* 1998) and its expression is induced under inflammatory conditions (Salmi *et al.* 1993). Moreover, the molecules resulting from SSAO activity are able to induce cytotoxicity on vascular cells *in vitro* (Hernandez *et al.* 2006; Ramos *et al.* 1988; Sole *et al.* 2008; Tyihak *et al.* 2001; Yu and Zuo 1993).

SSAO/VAP-1 protein levels and activity have been found to be increased in numerous pathologies (Airas *et al.* 2006; Boomsma *et al.* 1995; Boomsma *et al.* 1997; Karadi *et al.* 2002; Kurkijarvi *et al.* 1998; Olive *et al.* 2004), and as consequence, SSAO-dependent associated vasculotoxicity has been observed in several of them

(Garpenstrand *et al.* 1999; Stolen *et al.* 2004b; Yu *et al.* 2002; Yu and Zuo 1997). Regarding the AD pathology, we have reported an overexpression of cerebrovascular SSAO/VAP-1 colocalizing with Aβ deposits and that this overexpression correlates with an increase of plasma SSAO activity in advanced AD-dementia (del Mar Hernandez *et al.* 2005; Ferrer *et al.* 2002; Unzeta *et al.* 2007). Moreover, the molecules generated during SSAO activity have been shown to contribute to the oxidative stress, advanced glycation end-products generation (Gubisne-Haberle *et al.* 2004), and Aβ aggregation (Chen *et al.* 2006). In this context, a possible contribution of SSAO activity in the vascular pathology observed in AD has been largely suggested. However, the reason of the SSAO/VAP-1 overexpression found in AD is still far from clear, and the role of SSAO/VAP-1 in AD pathology is not fully understood. Moreover, since cell lines or primary cultures of vascular cells do not express SSAO/VAP-1, the possible involvement of this protein in the vascular pathology of AD has not been taken into account in previous *in vitro* studies.

The aims of this work have been to study the possible effects of SSAO/VAP-1 in Aβ-dependent pathology, and also the possible effects of Aβ in SSAO/VAP-1-dependent physiopathology. For this purpose, we used vascular smooth muscle and endothelial cell lines stably transfected with hSSAO/VAP-1 previously developed in our laboratory (Sole *et al.* 2007)(Sole, manuscript in preparation), and the same wild type cell lines that do not express the SSAO/VAP-1 protein. We treated the cells with the $A\beta_{1-40}$ Dutch mutated form of Aβ, since the 40 amino acids form it is the major form deposited in vascular vessels (Castano *et al.* 1996), and because the Dutch mutation is targeted to the vasculature and induces a severe form of CAA (Herzig *et al.* 2004). Our results show an Aβ-dependent increase in SSAO-mediated cytotoxicity produced through an increase of SSAO/VAP-1 protein availability in endothelial cells. Moreover, our results not only confirm the involvement of SSAO activity in Aβ aggregation, but also show a new role of SSAO in Aβ deposition that is mediated through activity-independent mechanisms.

Materials and methods

Cell culture and treatments

The A7r5 and HUVEC WT cells were obtained from ECACC (European Collection of Cell Cultures, Wiltshire, UK) or were a kind gift from Dr. F.J. Muñoz, from *Universitat Pompeu Fabra* (Barcelona, Spain), respectively. The A7r5 and HUVEC hSSAO/VAP-1

cells were obtained as described previously (Sole *et al.* 2007) or (Solé *et al.*, manuscript in preparation), respectively. A7r5 cells were cultured in high glucose (4.5 mg/l) DMEM (Sigma-Aldrich, St Louis, Mo, USA) with 10% (v/v) FBS, and HUVEC cells were grown in M199 medium (Invitrogen, Grand Island, NY, USA) with 5% (v/v) FBS and 2.2 g/l sodium bicarbonate; both mediums were supplemented with Glutamine (2 mM for A7r5 and 1.2 mM for HUVEC), 100 U/ml penicillin and 100 µg/ml streptomycin. All cells were maintained at 37ºC in a humidified atmosphere containing 5% CO_2. Geneticin (G418, 100 µg/ml, Invitrogen) was added to the culture medium of both hSSAO/VAP-1 cells to ensure the hSSAO/VAP-1 DNA maintenance.

Before cell treatments, A7r5 and HUVEC cells were starved for 1 h in its corresponding medium with 0.2% (v/v) or 0% FBS, respectively. All treatments were performed during 48h, if not specified different, and pre-treatments with catalase (Sigma-Aldrich) or with SSAO inhibitors semicarbazide (Sc) (Sigma-Aldrich) and MDL72974A (MDL) (a kind gift from Dr. P.H. Yu, University of Saskatchewan, Canada) were added 30 min before the treatment. In methylamine (MA) and β-amyloid (Aβ) co-treatments, MA (Sigma-Aldrich) was added 30 min before Aβ ($A\beta_{1-40}$ Dutch, Anaspec, Fremont CA, USA). Aβ was dissolved in sterile PBS, aliquoted and stored at -80ºC until its use.

MTT reduction method

For MTT reduction assays, cells were seeded in 24-well plates at 40000 cells/ml, and allowed to grow for 24 h before treatments. MTT (3-(4,5-dimethylthiazol-2-yl)2,5-diphenyl-tetrazolium bromide) (Sigma-Aldrich) solution (0.5 mg/ml, final concentration) was added to cells 90 min (A7r5) or 4 h (HUVEC) before the end of the treatments. At the end of the treatment, the medium was replaced by dimethyl sulfoxide (DMSO, Sigma-Aldrich) to dissolve the formazan blue precipitate formed, which was quantified at 560 and 620 nm in a microplate reader (Synergy HT and data analyses software KC4™, Bio-Tek® Instruments Inc. Winooski, USA).

Total cell lysates, subcellular fractionations and concentred culture medium

Total cell lysates for western blot were obtained by cell homogenisation in a buffer containing 1% Triton X-100, 10 mM EDTA, 50 mM Tris-HCl pH7.5 and protease inhibitors (protease inhibitor cocktail, Sigma-Aldrich). In case of SSAO activity determinations, 100 mM Tris-HCl pH 9 buffer was used, with added protease inhibitors. Lysates were sonicated for 10 s and stored at -80ºC until their use. To obtain enriched membrane preparations, cells were recovered in a buffer containing 10 mM Hepes, 1.5 mM $MgCl_2$ 10 mM KCl and protease inhibitors (Sigma-Aldrich), at pH 7.9. After Dounce

homogenization, samples were centrifuged at 2000 x *g* for 15 min at 4ºC and the supernatant was then ultracentrifuged at 100000 x *g* (Sorvall Discovery M120 SE) for 30 min at 4ºC to separate the soluble cytosolic fraction from the membranous pellet. Culture mediums were recovered after cell treatments, centrifuged at 800 x *g* to eliminate possible dead cells, lyophilisated in a freeze dryer (Freeze Dryer Modulyo, IMA Edwards, Dongen, The Netherlands) and resuspended in a smaller volume.

SSAO activity determination

SSAO activity was determined radiochemically using a modification of Otsuka and Kobayashi method (OTSUKA and KOBAYASHI 1964). Before the addition of the substrate, samples (200 – 300 µg of protein/sample) were preincubated during 30 min at 37ºC in presence of deprenyl (10^{-3} M) to inhibit the possible presence of MAO B. Reactions were performed at 37ºC during 90 min, in a final volume of 200 µl of 100 mM pH 9 tris buffer. 25 µl of 100 µM (^{14}C)-Benzylamine Hydrochloride (2mCi/mmol, Amersham-GE Healthcare, UK) were added to each vial as SSAO substrate. The catalytic reaction was stopped adding 100 µl of 2 M citric acid. The aldehyde products of the reaction were extracted into 4 ml of toluene:ethylacetate (1:1) solution containing 0.6% (w/v) of diphenyloxazole per vial. The amount of ^{14}C-aldehyde was quantified using a liquid scintillation counter Tri-Carb 2810TR (Perkin Elmer) and the Quanta Smart 3.0 software (Perkin Elmer). Cell lysate activities are expressed as pmol/min·mg of protein, being the protein concentration determined by the Bradford method.

Western blot assay

Protein concentration of total cell lysates was determined by the Bradford method. An equal amount of protein was separated by SDS-polyacrylamide gel electrophoresis using the Bio-Rad Mini-PROTEAN 3 system, and transferred onto nitrocellulose membranes (Schleicher & Schüll, Germany). For Aβ detection, 12 % tris/bicine polyacrylamide gels were used, and proteins were transferred onto PVDF membranes (Amersham). Nitrocellulose membranes were blocked for 1 h with TBS-0.1% Tween buffer plus 5% (w/v) non fat dry milk; PVDF membranes were incubated for 10 min in boiled PBS and blocked for 1h with PBS-0.05% Tween buffer plus 10% (w/v) non fat dry milk and 0.1% (w/v) BSA, pH 7.2. Membranes were then incubated overnight at 4ºC with the corresponding primary antibodies diluted in blocking buffer. After incubation with the corresponding secondary antibodies, blots were developed using ECL® Chemoluminiscent detection reagents and High Performance Chemiluminiscence Films (Amersham). The antibodies used were: goat anti-VAP-1 (E19) (1:500) (Santa Cruz Biotechnology, Heidelberg, Germany), rabbit anti-bovine

SSAO (1:1000) (Lizcano *et al.* 1998); mouse anti-β-actin (1:20000) (Sigma-Aldrich); mouse anti-GAPDH (1:40000) (Ambion-Invitrogen, Barcelona, Spain); rabbit anti-phospho p53 (Ser 15) (1:1000), rabbit anti-cleaved caspase 3 (1:1000), rabbit anti-cleaved caspase 9 (1:1000) (Cell Signaling); mouse anti-transferrin receptor (1:1000) (ZYMED, California, USA); mouse anti-flotillin (1:1000), mouse anti Bcl-2 (1:1000) (BD Biosciences, Madrid, Spain); mouse anti-APP 20.1 (1:1000) (generated by the 20.1 hybrydoma cell line, a kind gift from Dr. W.E. Van Nostrand , NY, USA); HRP anti-rabbit IgG (1:2000) (BD Biosciences, Madrid, Spain); HRP anti-mouse IgG (1:2000) (Dako, Barcelona, Spain) and HRP anti-goat IgG (1:2000) (Pierce, Madrid, Spain). Semi-quantitative analysis of western blots were performed by densitometry, using the Image J 1.34 s software (National Institutes of Health, USA), and protein levels were corrected for the corresponding loading control.

Immunofluorescence

Cells were grown on collagen I coated glass coverslips during 2 days in complete medium. HUVEC cells were fixed during 20 min at -20ºC in Carnoy's solution (Methanol:Acetic acid, 3:1 (v/v)), and A7r5 cells were fixed for 10 min at 4ºC in 2% paraformaldehyde. After fixation, cells were treated during 1 min with 15% formic acid to improve the antigen exposition. Primary antibody mouse anti-Aβ Clone Bam10 (Sigma-Aldrich) was added at 1/200 dilution in PBS containing 0.2% gelatine, 0.1% triton, 20 mM glycine and 5% FBS overnight at 4ºC. Alexa Fluor® 594 Goat anti-mouse secondary antibody (Invitrogen) and Hoechst 0.5 mg/ml were incubated then at 1/1000 dilution for 1 h at room temperature, and cells were mounted in Fluorescent Mounting Medium (Dako). Preparations were observed in a Nikon Eclipse TE 2000-E inverted fluorescence microscope, with a Hamamatsu C-4742-80-12AG camera and Metamorph® Imaging System software.

Immunoprecipitation

Cells were lysed in a buffer containing 100 mM Tris-HCl pH 7.5, 1% (v/v) Triton X-100 and protease inhibitors. 500 µg of protein lysates were incubated in a rocking platform for 2 h at 4ºC with 1 µl of primary antibody (anti-Aβ, Sigma-Aldrich), in 900 µl of final volume. Then, 100 µl of protein G-agarose (Roche Appl. Sci., Mannheim, Germany) were added to each sample, and were incubated for other 2 - 6h at 4ºC. The agarose-antibody-antigen complexes were collected by 1000 x *g* for 3 min centrifugation and washed for 3 times with lysis buffer. The final pellets were resuspended in 40 µl of loading buffer. After denaturing its proteins by heating to 100ºC for 3 min, the protein

G-agarose was removed by centrifuging the suspensions at 1000 x *g* for 3 min. The samples were loaded in SDS-polyacrilamide gels and analysed by western blot.

Statistical analyses

Data are expressed as mean ± standard error of mean (SEM) of independent experiments. Statistical analyses of biochemical data were performed by the one-Way ANOVA test followed by Newman-Keuls Multiple Comparison test, elaborating experimental data by means of Graph-Pad 3.0 software (San Diego, CA, USA). Differences with $p < 0.05$ were considered significant.

Results

Effect of $A\beta_{1-40}$ Dutch treatment on smooth muscle and endothelial cells viability.

Vascular aspects of Alzheimer's disease include the Aβ peptide accumulation in cerebrovascular vessel walls, named Cerebral Amyloid Angiopathy (CAA). The 40 amino acids beta amyloid peptide ($A\beta_{1-40}$) is the main form deposited in vascular system, and distinct mutations in Aβ sequence such as the Dutch mutation led to earlier and more severe phenotypes of CAA. Moreover, vascular deposits involve vascular cell degeneration and death. To evaluate the possible toxic effect of the Dutch mutated form of Aβ on our vascular smooth muscle and endothelial cell lines, we treated them with the peptide ($A\beta_{1-40}$ D) during 48 h and determined the cell viability by MTT reduction method (figure 1.A). Results showed a dose-response toxic effect of $A\beta_{1-40}$ D treatment on A7r5 and HUVEC wild type (WT) cells; the same results were obtained with SSAO-expressing cells (data not shown).

It has been previously described that Aβ toxicity is mediated through hydrogen peroxide (H_2O_2) production in both neuronal and endothelial vascular cells (Behl *et al.* 1994; Muñoz *et al.* 2002). However, it seems that this mechanism does not occur in cerebrovascular smooth muscle cells (Jung and Van Nostrand 2002). To evaluate the role of H_2O_2 in Aβ mediated toxicity in our cell lines, cells were treated during 48 h with 0.5 μM $A\beta_{1-40}$ D, with or without a pre-treatment with the enzyme catalase. Catalase acts as an H_2O_2 scavenger, so its pre-treatment would prevent the possible H_2O_2 mediated toxicity generated by Aβ. Aβ treatment induced approximately a 50 % loss of MTT reduction ability, and catalase pre-treatment prevented the $A\beta_{1-40}$ D induced toxicity in HUVEC cells, but it had little effect in A7r5 smooth muscle cells (figure 1.B).

These results indicated a different mechanism of Aβ induced toxicity in both cells, although the sensibility to Aβ toxicity was similar in both cell types. Moreover, these differences cannot be explained by different catalase effectiveness, since its pre-treatment was able to recover the cell death induced by H_2O_2 added to both cells (data not shown).

$Aβ_{1-40}$ D enhances the SSAO-mediated toxicity through MA oxidation in vascular cells

The toxic effect of SSAO enzymatic activity has been demonstrated in numerous cellular and animal models (Conklin *et al.* 1998; Deng *et al.* 1998; Gubisne-Haberle *et al.* 2004; Hernandez *et al.* 2006; Kinemuchi *et al.* 2004; Sole *et al.* 2008; Yu 1998; Yu and Zuo 1993). In order to study the influence of SSAO activity in $Aβ_{1-40}$ D induced toxicity on vascular cells, they were co-treated with Aβ and MA as SSAO substrate in the presence or absence of SSAO, using hSSAO/VAP-1 or WT cells, respectively (figure 2.A). None differences in the induced toxic effect were observed after $Aβ_{1-40}$ D treatment between WT and transfected cells, indicating that the presence of SSAO does not modify the toxic effect of Aβ. However, in A7r5 and HUVEC hSSAO/VAP-1 cells, the addition of MA to Aβ treatment significantly decreased the cell viability comparing with the Aβ treatment, an effect that was not observed in A7r5 or HUVEC WT cells. MA treatment alone induced a cell viability decrease in A7r5 hSSAO/VAP-1 cells, as it had been previously described (Sole *et al.* 2008).

To analyze whether this increase in the toxicity observed with the addition of MA was due to the SSAO activity, hSSAO/VAP-1 cells were pre-treated with the SSAO inhibitors semicarbazide and MDL before adding the Aβ-MA co-treatment (figure 2.B). In both cases, SSAO inhibitor pre-treatments prevented the MA-dependent toxicity increase. As previously observed, catalase pre-treatment was able to prevent the Aβ induced toxicity in HUVEC cells. Moreover, the SSAO inhibitors by themselves did not modify the Aβ induced toxicity (data not shown).

To further confirm that cell viability was really compromised under these conditions, several proteins known to be involved in the SSAO mediated apoptosis in smooth muscle cells (Sole *et al.* 2008) were analyzed (figure 3). An increase in p53 phosphorylation, a decrease in bcl-2 expression and an increase in caspase 3 and 9 cleavage were observed in both MA and Aβ-MA treatments in A7r5 hSSAO/VAP-1 cells. These pro-apoptotic changes were prevented by semicarbazide and MDL pre-treatments. Aβ treatment alone, however, was only able to induce significant changes

in p53 phosphorylation. Intriguingly, Aβ-MA co-treatments apparently displayed lower caspase activation levels than MA treatments.

$A\beta_{1-40}$ D induces an increase of SSAO activity in HUVEC hSSAO/VAP-1 cells

The results showing that SSAO activity was the responsible of the increase in cell death after Aβ-MA co-treatment compared to MA suggested us that Aβ treatment could be an SSAO activity modulator. To test this hypothesis, SSAO activity was determined in A7r5 and HUVEC hSSAO/VAP-1 cells after Aβ treatment (figure 4.A). Surprisingly, different results were obtained for both cell types. While HUVEC cells displayed an increase in SSAO activity after Aβ treatment, A7r5 cells did not show differences in SSAO activity. To know whether this activity increase was due to a modulation of the enzymatic activity or was a reflection of a protein increase, SSAO protein levels were analyzed by western blot after Aβ treatment (figure 4.B). Results showed the same pattern than activity levels, with an increase of SSAO/VAP-1 protein levels in HUVEC hSSAO/VAP-1 cells, but not in A7r5 cells. Moreover, the increase observed in SSAO activity was proportional to the protein level, indicating that the latest was the responsible of the activity increase. Treatments were done also in WT cells, in order to know whether Aβ treatment was able to induce the SSAO/VAP-1 expression, but none SSAO/VAP-1 expression was observed (data not shown).

Because the concentration of Aβ used to see the SSAO/VAP-1 increase effect could not be the optimal, we assayed also lower concentrations of Aβ, since higher ones could become too toxic (figure 4.C). The results showed a dose-dependent increase, with a maximum level reached at 0.25 μM Aβ treatment. However, since 0.25 and 0.5 μM Aβ concentrations were not statistically different, the study was continued with 0.5 μM Aβ treatments. Moreover, longer times of treatment were assayed (4 and 6 days), but none of them resulted in higher SSAO/VAP-1 increases than the obtained at 48h (data not shown).

Aggregates are formed after $A\beta_{1-40}$ D treatment in cultured cells

Looking at the obtained results, in which an SSAO/VAP-1 alteration was observed under the influence of Aβ, we were interested in study whether SSAO/VAP-1 could modify the behaviour of Aβ on these vascular cultured cells. Since Aβ is able to form aggregates *in vivo*, which became the amyloid plaques found in tissues of Alzheimer's

disease patients, we first studied the behaviour of $A\beta_{1-40}$ D in our cultured cells. We observed the status of Aβ after different concentration treatments and different culture times. In one hand, we treated A7r5 and HUVEC WT cells with different concentrations of $A\beta_{1-40}$ D, fixed them and analyzed by Aβ immunofluorescence (figure 5.A). Results showed the presence of large protein aggregates attached on the cell surface stained with an anti-Aβ antibody. Moreover, the analysis of Aβ presence in cell lysates after different culture times (figure 5.B) showed the time-course of Aβ deposition on cell monolayers. In both A7r5 and HUVEC WT cells, Aβ started to aggregate at significant amounts on the cells at 30 min after its addition, reaching the maximum level after 1 – 3 h of incubation and maintaining similar levels during the rest of the culture, until 48h. These results indicated that the Aβ aggregation was a quick process, and that its aggregation on cells may have effects soon after its addition.

Next, we analyzed the localization of Aβ deposits on the cultured cells, since it has been described that $A\beta_{1-40}$ is endocytosed by cerebrovascular endothelial cells (Deane *et al.* 2003; Deane *et al.* 2004; Kandimalla *et al.* 2009). To analyze the subcellular localization of the exogenously added $A\beta_{1-40}$ D, treated A7r5 and HUVEC WT cells were processed to obtain membrane and cytoplasm enriched fractions (figure 5.C and 5.D). Results showed that Aβ was preferentially membrane-associated, since none signal was detected in cytosolic fractions (figure 5.C). However, these results does not allow us to rule out that little amounts of Aβ would be internalised, or that little Aβ-containing cytoplasmic vesicles would be purified as membrane fraction. GAPDH and transferrin receptor were used as controls for purity of the fractions (figure 5.D).

SSAO/VAP-1 enhances the $A\beta 1_{1-40}$ D deposition on cultured vascular cells

It has been recently described that the toxic products generated during SSAO activity can contribute to the Aβ aggregation (Chen *et al.* 2006; Chen *et al.* 2007). To confirm these results in our model, the involvement of SSAO activity regarding the Aβ deposition on these cells was studied. The hSSAO/VAP-1 cells were co-treated with Aβ-MA, with or without a pre-treatment with semicarbazide as SSAO inhibitor, and cell lysates were analyzed by western blot for Aβ presence after treatments (figure 6.A). Results showed an increase in Aβ deposition with MA treatment, which was prevented by semicarbazide pre-treatment. These results were observed in both A7r5 and HUVEC cells, and thus confirmed the previous obtained using umbilical artery sections (Jiang *et al.* 2008). On the other hand, to analyze if SSAO/VAP-1 presence has any role in Aβ deposition, A7r5 and HUVEC cells were treated with Aβ, and its deposition

on hSSAO/VAP-1 cells was compared with the corresponding WT cells (figure 6.B). Results showed an increase in Aβ deposition in SSAO-expressing cells compared to WT. This increase may be due to the presence of SSAO protein, since these cells were not treated with SSAO substrates. The quantification of these results obtained by western blot are shown in figure 6.C, in which are also represented the results obtained for WT cells. A clear significant increase in Aβ deposition on A7r5 and HUVEC hSSAO/VAP-1 cells compared to WT cells is observed. Moreover, this increase is higher with the addition of MA as SSAO substrate, which in turn is prevented by semicarbazide pre-treatment. WT cells show fewer changes in Aβ deposition after MA or semicarbazide treatments that are not significantly different from the Aβ treated cells.

To confirm that the increase of Aβ found in hSSAO/VAP-1 cells compared to WT was due to an increase in Aβ deposition, it was also analyzed the remaining Aβ found in the culture medium of treated cells (figure 6.D). Since low concentrations of Aβ were used in these experiments, it was necessary to concentrate the culture mediums in order to detect it. After western blot analysis of Aβ in 30-fold concentred mediums of HUVEC cells, a higher amount of remaining Aβ was detected in medium of WT cells compared to hSSAO/VAP-1 cells, confirming that less Aβ had been deposited on WT cells. Moreover, flotillin was analyzed in these concentred mediums to ensure that the Aβ detected was not from dead cells or membrane debris origin, and none flotillin signal was detected in the concentred mediums.

The results obtained confirmed the role of SSAO in Aβ deposition by activity-dependent mechanisms, but also indicated a new role in this process by activity-independent mechanisms. The fact that SSAO would be able to induce the Aβ deposition by an activity-independent way suggested us the possibility of SSAO acting as an Aβ receptor-like. To study whether the activity-independent mechanism involved a direct interaction between Aβ and SSAO, HUVEC WT and hSSAO/VAP-1 cells treated with Aβ were subjected to Aβ immunoprecipitation. Then, immunoprecipitates were analyzed for SSAO presence (figure 6.E). Semi-quantitative analyses referred to total immunoprecipitated APP showed not statistically significant differences in SSAO/VAP-1 immunoprecipitated from Aβ treated cells versus non treated HUVEC hSSAO/VAP-1 cells. Some SSAO/VAP-1 positive signal was also observed in the immunoprecipitate of non-treated cells, indicating probably a non-specific binding of SSAO/VAP-1 or maybe another type of interaction involving APP, since the antibody used for Aβ immunoprecipitation is also able to immunoprecipitate APP. These types of associations, however, require more experimental analyses.

Discussion

SSAO/VAP-1 is an amino oxidase highly expressed in the vascular system, which has been found to be overexpressed in cerebrovascular tissue of AD patients colocalizing with vascular Aβ deposits (Ferrer *et al.* 2002), and increased in plasma of patients in advanced AD conditions (del Mar Hernandez *et al.* 2005). However, it is not known whether the SSAO/VAP-1 increase is produced before or after the Aβ deposition. Moreover, its activity increase has been associated to the vascular degeneration observed in diseases such as diabetes or atherosclerosis (Garpenstrand *et al.* 1999; Meszaros *et al.* 1999; Stolen *et al.* 2004a), due to the toxicity of the products generated through its enzymatic activity. Interestingly, these pathologic conditions constitute risk factors for AD, as also cerebral lesions, hypertension or cardiac diseases, pathological situations in which plasma SSAO has been observed also increased (Boomsma *et al.* 1997; Conklin *et al.* 2006; Ishizaki 1990). Therefore, the possible toxic effect of SSAO mediated monoamine oxidation in Alzheimer's disease has been largely postulated (Unzeta *et al.* 2007; Yu 2001).

In order to elucidate the role of SSAO in the vascular degeneration observed in AD, our study was firstly centred in the evaluation of the $A\beta_{1\text{-}40}$ D effect on our smooth muscle and endothelial cell line cultures. 48 h treatments with soluble Aβ were performed in order to simulate a complete physiopathological Aβ aggregation process. It was considered that it was important to have all the aggregation process in culture, because the different size aggregates show different effects on vascular cells (Gonzalez-Velasquez and Moss 2008; Miravalle *et al.* 2000; Muñoz *et al.* 2002; Van Nostrand *et al.* 1998). After treatments, a similar dose response cytotoxic effect was observed in both cell types, measured by MTT reduction method. The results obtained were similar to those reported by other authors (Eisenhauer *et al.* 2000; Miravalle *et al.* 2000). Moreover, the Aβ-dependent toxic effect was mediated by different mechanisms in the smooth muscle or in the endothelial cells. An H_2O_2-dependent toxic effect was observed in endothelial cells, while it was H_2O_2-independent in smooth muscle cells. This different toxicity mechanism has been previously observed, with Aβ acting through H_2O_2 generation-dependent mechanisms in neuronal and endothelial cells (Behl *et al.* 1994; Coma *et al.* 2005), but not in cerebrovascular smooth muscle cells (Jung and Van Nostrand 2002).

When SSAO/VAP-1-expressing vascular cell lines were used in order to study the role of SSAO/VAP-1 in Aβ mediated toxicity, none significant changes were observed regarding the Aβ effect in WT or in transfected cells. However, when the SSAO/VAP-1-

expressing cells were treated with $A\beta_{1-40}$ D in the presence of methylamine as SSAO substrate, an increase in the SSAO-dependent toxicity was observed, confirmed by its prevention by SSAO inhibitors pre-treatment. To evaluate the relevance of this effect in cell death, the main apoptotic pathways described to be involved in SSAO mediated apoptosis in smooth muscle cells (Sole *et al.* 2008) were also analyzed. All p53 phosphorylation, bcl-2 decrease, caspase 9 and caspase 3 cleavages were clearly increased by MA treatment. Among them, p53 phosphorylation and Bcl-2 decreased levels were maintained with Aβ-MA co-treatment. However, caspase 9 and 3 cleavage levels were slightly lower in the Aβ-MA co-treatment compared to MA treatment. This effect could be due to the increase in cell toxicity observed by MTT reduction: since caspases activation displays an activation curve in this model, decreasing its levels after reaching a maximum level (Sole *et al.* 2008), a higher toxic insult as the induced by Aβ-MA co-treatment would probably advance in time the activation curve of caspases. This hypothesis, however, would need to be confirmed by analyzing the caspases activation at shorter times of co-treatment.

Moreover, it can be observed that Aβ treatment of A7r5 cells significantly activated p53 phosphorylation, it did not affect Bcl-2 levels or caspase 9 cleavage, and only a slight tendency to increase was observed in caspase 3 cleavage. In this regard, p53 dependent apoptosis has been detected in cerebrovascular tissues of AD patients (de la Monte *et al.* 2000), or in cerebrovascular cells after Aβ treatment (Hsu *et al.* 2007), but at the moment, the molecular pathways involved in Aβ mediated vascular cell death have not been fully elucidated, so it could be possible that the other proteins analyzed would not be involved in Aβ mediated cell death. Alternatively, it has to be considered that the MTT reduction method is based on the mitochondrial metabolic activity, so it cannot be excluded that the low concentrations of Aβ used in this work would be able to induce some toxic effect, as demonstrated by p53 phosphorylation, but not enough to induce the final death of cells. However, the cytotoxic effect of MA and Aβ-MA correlated with cell death since high levels of apoptosis executer caspases were found after treatments.

The results showing that the increase in cell death induced by Aβ-MA co-treatment was SSAO activity-dependent suggested us a possible modulation of SSAO activity by Aβ. However, after analyzing SSAO activity and protein levels in Aβ treated cells, a different behaviour was observed again in both cell types, showing HUVEC cells an increase of SSAO activity due to an increase in SSAO protein amounts, but neither effect was observed in A7r5 cells. At this point, the increase in cell death observed by

MTT reduction after Aβ-MA co-treatment versus Aβ treatment could be explained by different mechanisms in both cells, although the cell death was induced in both cases.

In A7r5 cells, where SSAO activity levels did not change after Aβ-MA co-treatment but it induced a higher toxic effect, this toxicity increase may be due to a joint effect of the toxic products generated from MA metabolism and the Aβ. This effect would be important *in vivo*, since cerebrovascular smooth muscle cells constitutively express high levels of SSAO (Castillo *et al.* 1998) and high levels of aldehydes as acrolein or methylglioxal, or ammonia, which can be generated through SSAO metabolism, have been detected in cerebrospinal fluid or blood of AD patients (Ahmed *et al.* 2005; Lovell *et al.* 2001; Seiler 2002; Wood *et al.* 2007). Under these conditions, the toxic effect of SSAO metabolism in the presence of Aβ would enhance the degeneration of smooth muscle cells observed in cerebral amyloid angiopathy (Mandybur 1986; Vinters 1987).

In HUVEC cells, however, an increase of SSAO/VAP-1 protein was observed after Aβ treatment. This increase in SSAO activity was the responsible of the enhanced toxicity observed after Aβ-MA co-treatment compared to Aβ alone, since it was prevented by SSAO inhibitors. In this concern, small increases of SSAO/VAP-1 have been reported to induce toxicity in other studies (Yu *et al.* 2006), where it was postulated that a threshold of SSAO activity is required to induce toxicity, so the increase observed in this study would reach this threshold. On the other hand, SSAO/VAP-1 is constitutively stably expressed in our cells, so protein expression increases in response to Aβ treatment would not be expected. An unspecific reaction of the constitutive promoter was discarded since the same promoter in A7r5 cells did not respond in the same way. Thus, we hypothesize that the increase of SSAO/VAP-1 protein observed after Aβ treatment in HUVEC hSSAO/VAP-1 cells may be due to a decrease in the protein degradation. This effect has been also observed in SSAO/VAP-1 overexpressing mice, where higher SSAO protein levels were observed in adipose tissue after diabetes induction although its decreased mRNA (Gokturk *et al.* 2004). These authors suggest that the SSAO/VAP-1 increase observed may be explained by post-transcriptional changes probably associated to diabetes, where an excess flux of glucose would enhance the glycosylation of proteins, a process that protects them from degradation. This process could be occurring also in our cultures, but also the production of formaldehyde could be involved, inducing cross-links between proteins, decreasing the membrane mobility and impairing protein degradation, including SSAO/VAP-1, in an unspecific manner. The latest process, however, was discarded since analysis of flotillin which is localized in membrane lipid rafts with SSAO/VAP-1 did not display this increase after Aβ treatment (data not shown), indicating a specific mechanism involved.

Alternatively, SSAO degradation could be impaired by specific mechanisms, but confirming this hypothesis would require more exhaustive studies. We cannot rule out either the possibility of an inactive SSAO pool would become activated.

The results obtained with HUVEC cells, thereafter, are in agreement with previous observations in which an SSAO/VAP-1 increase was detected in cerebrovascular tissue of AD patients (Ferrer *et al.* 2002; Jiang *et al.* 2008). Moreover, a higher increase was observed in microvessels, mainly composed by endothelial cells, compared to meninges, with higher content in smooth muscle cells (Unzeta *et al.* 2007). On the other hand, results showing that endothelial SSAO/VAP-1 is induced under several inflammatory conditions but not the smooth muscle SSAO, support the different regulation of the protein in both cell types (Arvilommi *et al.* 1997; Jaakkola *et al.* 1999).

Aβ forms aggregates which can be deposited on cells *in vitro* (Eisenhauer *et al.* 2000). We have confirmed the $A\beta_{1\text{-}40}$ D extracellular deposition on our cultured vascular cells, and observed that the aggregation process was produced in the first hours after its addition. Moreover, after the first 1 – 3 hours, the amount of deposited Aβ remained more or less stable until the last time studied (48 h). Fractionations of treated cells showed that this Aβ was mainly located in the membrane enriched fraction, as extracellular Aβ deposits. However, some authors have described that endothelial cells can endocytate Aβ (Kandimalla *et al.* 2009); in this regard, our results do not allow us to discard that some Aβ would be internalized, since intracellular vesicles may be included in the membrane enriched fraction. Transferrin receptor is an example of endocytated protein through the endosomal system, as is Aβ (Kandimalla *et al.* 2009), and it was exclusively detected in membranal fractions of our preparations, so another experimental approximation would be necessary to study the possible endocytosis of Aβ in these cells.

The modulation of the Aβ aggregation is relevant, since Aβ toxicity in vascular cells has been correlated with the aggregate formation process, showing the Aβ Dutch form a higher toxic effect due to its elevated propensity to self-assemble compared to other forms (Folin *et al.* 2005; Murakami *et al.* 2002; Van Nostrand *et al.* 1998). In this concern, SSAO has been shown to have a role in Aβ deposition through its enzymatic activity (Jiang *et al.* 2008). In the present study, a higher increase in Aβ deposition with MA treatment and the prevention with SSAO inhibitor pre-treatment confirmed the previously reported results, mediated through an enzymatic activity-dependent mechanism. In addition, the deposition of Aβ on the vascular cells showed differences

depending on the SSAO/VAP-1 expression, in which the presence of the SSAO/VAP-1 protein increased the Aβ deposition on both A7r5 and HUVEC cells. Moreover, the lower remaining Aβ amount found in the medium of SSAO/VAP-1 expressing cells confirmed this result. Thus, the increase of Aβ deposition in SSAO/VAP-1 cells compared to WT cells without MA treatment indicated an involvement of SSAO in this process by an enzymatic activity-independent mechanism, which was not previously described due to the absence of a non-expressing SSAO tissue.

In this concern, the SSAO activity-dependent mechanism would probably act through the generation of aldehydes, since aldehydes derived from SSAO-mediated deamination are able to induce Aβ misfolding (Chen *et al.* 2006; Chen *et al.* 2007). On the other hand, it has been postulated that SSAO/VAP-1 could use free amino groups of proteins as substrates in order to bind leucocytes to the endothelium during the inflammatory process (Jalkanen and Salmi 2008; Salmi *et al.* 2001; Yegutkin *et al.* 2004). Moreover, the ability of SSAO/VAP-1 to bind peptides displaying free amino groups, for example of lysine residues, has been demonstrated (Yegutkin *et al.* 2004). This process could explain the SSAO activity-independent mechanism of Aβ enhancement deposition, since Aβ contains arginine, lysine and glutamine residues that are suitable for interacting with proteins due to its disposition faced outside the folded structure of Aβ. Moreover, $A\beta_{1\text{-}40}$ D has an additional glutamine residue, also suitable for interacting with SSAO/VAP-1. In this regard, it would be interesting to study if there are differences between $A\beta_{1\text{-}40}$ WT and $A\beta_{1\text{-}40}$ D deposition on hSSAO/VAP-1 and WT vascular cells, in order to study the involvement of this residue in this hypothesized process.

To confirm this possible direct interaction between Aβ and SSAO/VAP-1, Aβ immunoprecipitations were performed with Aβ treated cells, but Aβ immunoprecipitates showed few amounts of bound SSAO/VAP-1. The SSAO/VAP-1-Aβ binding mechanism cannot be excluded after this result, since it is possible that the weakness of the interaction would not support the immunoprecipitation process. However, it cannot be ruled out the possibility that the SSAO activity-independent mechanism of Aβ enhancement deposition would depend on other proteins, whose expression could be modified by SSAO/VAP-1 presence. In this concern, alterations in the expression of several proteins, including redox-sensitive proteins as superoxide dismutase-Mn have been observed after SSAO/VAP-1 overexpression in mice (Stolen *et al.* 2004b). Regarding this, it would be interesting to study if the expression of proteins involved in the APP cleavage or in the Aβ binding systems are altered in the SSAO/VAP-1 expressing cells.

In summary, our results show that $A\beta_{1-40}$ D treatment can modify the toxicity induced by SSAO-mediated metabolism, and that this effect is mediated by an increase in the SSAO/VAP-1 protein availability in endothelial cells. Moreover, our results confirm that SSAO/VAP-1 affects the $A\beta_{1-40}$ D deposition by an activity-dependent mechanism, but also include a new role of SSAO in this process by an activity-independent mechanism. The observed effects could be relevant *in vivo,* in Alzheimer's disease conditions, since Aβ and SSAO would meet in blood vessels. High levels of Aβ could interact with SSAO, inducing the modifications here described and generating a vicious circle in which SSAO could promote Aβ deposition, which could increase the SSAO protein and activity, becoming its activity more toxic to vascular cells and contributing to the vascular degeneration observed in AD. On the other hand, oxidative stress conditions, which are detected at previously or at early stages of AD, could induce the SSAO/VAP-1 expression, and this protein could participate in Aβ cerebrovascular deposition in early stages of AD, thus contributing to the early progression and aggravation of the disease.

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Figure Legends

Figure 1. Effect of $A\beta_{1-40}$ D treatment on A7r5 and HUVEC cell viability. (**A**) A7r5 and HUVEC WT cells were treated at 0.2% serum (A7r5) or in absence of serum (HUVEC) during 48 h with increasing concentrations of $A\beta_{1-40}$ D and cell viability compared to non treated cells (NT) was determined by MTT reduction method. A dose-response cytotoxic effect was observed in both cell types. (**B**) A7r5 and HUVEC WT cells were treated for 48 h with 0.5 μM $A\beta_{1-40}$ D with or without a 30 min pre-treatment of 100 U/ml catalase. Catalase pre-treatment prevented the Aβ-dependent toxicity in HUVEC cells, but it had a fewer effect on Aβ-dependent toxicity in A7r5 cells. (***) $p < 0.001$ *vs* non treated cells; ($^{\#}$) $p < 0.05$ and ($^{\#\#\#}$) $p < 0.001$ *vs* Aβ treated cells. Data represent mean ± SEM of three independent experiments.

Figure 2. Effect of Aβ and MA co-treatment on A7r5 and HUVEC cell viability. (**A**) A7r5 and HUVEC WT and hSSAO/VAP-1 cells were treated at 0.2% serum (A7r5) or in absence of serum (HUVEC) during 48 h with 1 or 3 mM MA, 0.5 μM $A\beta_{1-40}$ D or with Aβ-MA co-treatment. Cell viability was determined by MTT reduction. MA treatment induced a 50% decrease in MTT reduction in A7r5 hSSAO/VAP-1 cells while none effect was induced in HUVEC hSSAO/VAP-1 cells. Aβ-MA co-treatment induced an increase of the toxic effect compared with Aβ treatment in both hSSAO/VAP-1 cells. (**B**) A7r5 and HUVEC hSSAO/VAP-1 cells were treated with 1 or 3 mM MA, 0.5 μM $A\beta_{1-40}$ D or a MA-Aβ co-treatment during 48 h. 1 mM Sc and 1 μM MDL were used as SSAO inhibitors, and 100 U/ml catalase (Cat) was used as H_2O_2 scavenger. SSAO inhibitor pre-treatments prevented the increase of toxicity observed after Aβ-MA co-treatment compared to Aβ treatment. Catalase prevented the Aβ mediated toxicity in HUVEC cells. (*) $p < 0.05$; (**) $p < 0.01$ and (***) $p < 0.001$ *vs* Aβ + MA treated cells. Data represent mean ± SEM of at least three independent experiments.

Figure 3. Western blot analysis of proapoptotic proteins involved in SSAO-mediated cell death in A7r5 hSSAO/VAP-1 cells after Aβ-MA co-treatment. A7r5 hSSAO/VAP-1 cells were treated at 0.2% serum during 48 h with 1 mM MA, 0.5 μM $A\beta_{1-40}$ D, Aβ-MA co-treatment or Aβ-MA co-treatment with SSAO inhibitors pre-treatment. SSAO inhibitors used were 1 mM Sc and 1 μM MDL. (**A**) Representative western blot analyses of p53 phosphorylated on Ser-15, Bcl-2 and cleaved caspases 9 and 3 after the detailed treatments. β-actin was used as loading control. (**B**) Densitometric analysis of western blots of three independent experiments was performed, and data for each protein were normalized for β-actin as loading control. Phospho-p53 levels were increased after MA, Aβ and Aβ-MA treatments, and the increase was prevented by

SSAO activity inhibitors. Bcl-2 protein levels were decreased after MA and Aβ-MA treatments, effect that was prevented by Sc pre-treatment but only partially by MDL pre-treatment. Caspases 9 and 3 cleavages were increased after MA and Aβ-MA treatments, and prevented by SSAO inhibitors pre-treatment; Aβ treatment alone induced a non significative, slight increase of caspases cleavage. (*) $p < 0.05$; (**) $p < 0.01$ and (***) $p < 0.001$. Data represent mean ± SEM of three independent experiments.

Figure 4. SSAO activity and protein levels in A7r5 and HUVEC hSSAO/VAP-1 were determined after $A\beta_{1-40}$ D treatment. A7r5 and HUVEC hSSAO/VAP-1 cells were treated at 0.2% serum or in absence of serum respectively during 48 h with 0.5 μM $A\beta_{1-40}$ D. After treatments, SSAO activity was determined by the radiometric method using ^{14}C-Benzylamine as substrate, and western blot analysis of SSAO protein were performed. (**A**) Aβ treatment induced an increase in SSAO activity in HUVEC hSSAO/VAP-1 without modification in A7r5 hSSAO/VAP-1 cells. (**B**) Semi-quantifications of SSAO/VAP-1 western blots after Aβ treated cells were related to β-actin levels as loading control and represented. Representative western blots are showed. Aβ treatment induced an increase in the SSAO/VAP-1 protein amounts in HUVEC hSSAO/VAP-1 cells. (**C**) HUVEC hSSAO/VAP-1 cells were treated for 48 h with increasing concentrations of $A\beta_{1-40}$ D (0.125, 0.25 and 0.5 μM) and SSAO activity was determined. Results showed a dose-dependent SSAO activity increase after Aβ treatments, with a maximum level at 0.25 μM not statistically different from 0.5 μM. (*) $p < 0.05$; (**) $p < 0.01$ and (***) $p < 0.001$. Data represent mean ± SEM of at least three independent experiments.

Figure 5. $A\beta_{1-40}$ D forms extracellular aggregates on vascular cells in culture. (**A**) A7r5 and HUVEC cells were treated with the indicated increasing concentrations of $A\beta_{1-40}$ D during 48 h at 0.2% serum (A7r5) or without serum (HUVEC). Cells were fixed and an anti-Aβ immunofluorescence was performed as described in materials and methods. Presence of Aβ aggregates were detected on the cultures at the different Aβ concentrations. (**B**) Cells were treated with 0.5 μM $A\beta_{1-40}$ D during different times, and presence of Aβ on lysates, indicating Aβ deposition, was analyzed by western blot using tris/bicine gels. A representative western blot is showed, and the semi-quantification of three independent experiments is represented, with data related to GAPDH values as loading control. Data showed that the majority of Aβ deposition occurred during the first 3 h of treatment, and it was maintained at similar levels until 48 h of treatment. (**C**) Subcellular fractionations were performed with cells treated during 48 h with 0.5 μM $A\beta_{1-40}$ D. Samples of two different fractionations were loaded as whole

lysate (W), cytosolic fractions (C) or membrane fractions (M). Aβ presence was detected in whole lysate and membrane fractions in both cells. (**D**) Representative western blots showing the purity of the cytosolic and membranal fractions. GAPDH and transferrin receptor (Tf Rec) were used as cytosolic and membrane fraction controls.

Figure 6. SSAO/VAP-1 increases the $A\beta_{1-40}$ deposition on vascular cells. (**A**) A7r5 and HUVEC hSSAO/VAP-1 cells were treated during 48 h with 0.5 μM $A\beta_{1-40}$ D, with Aβ plus 1 mM MA or with Aβ-MA co-treatment with 1 mM Sc pre-treatment. Aβ presence in cell lysates was determined by western blot using tris/bicine gels and representative western blots are showed, using GAPDH as loading control. Results showed an increase of Aβ deposition with Aβ-MA co-treatment compared with Aβ. Moreover, Aβ deposition levels were decreased with Sc pre-treatment. (**B**) A7r5 and HUVEC WT and hSSAO/VAP-1 cells were treated in 0.2% serum (A7r5) or without serum (HUVEC), during 48 h with 0.5 μM $A\beta_{1-40}$ D. A7r5 and HUVEC hSSAO/VAP-1 cells showed increased Aβ deposition compared to their respective WT cells. (**C**) Summary of the semi-quantitative densitometry of the Aβ bound to the cell monolayers. Results indicated statistical differences in Aβ deposition in hSSAO/VAP-1 cells compared to WT cells. Moreover, MA treatment in hSSAO/VAP-1 cells showed significant increases of Aβ deposition, prevented with Sc pre-treatment. (*) $p < 0.05$ and (**) $p < 0.01$ *vs* Aβ treated WT cells. ($^{\#}$) $p < 0.05$ and ($^{\#\#}$) $p < 0.01$ *vs* the indicated treatment. Data represent mean ± SEM of three independent experiments. (**D**) Representative western blot of 30 times concentred mediums of HUVEC WT and hSSAO/VAP-1 cells treated during 48 h with $A\beta_{1-40}$ D. Mediums were concentred by lyophilisation, and reconstituted in the appropriate volume of distilled water. A significant decrease of Aβ presence in HUVEC hSSAO/VAP-1 medium compared to HUVEC WT medium was observed. Flotillin detection was used as control of absence of cell rests. (**E**) Aβ treated HUVEC cells were used to immunoprecipitate Aβ aggregates, and a posterior western blot analysis of SSAO presence was performed. Representative experiments are shown of three with similar results. None SSAO signal was observed in WT cells. In Aβ treated HUVEC hSSAO/VAP-1 cells, a moderate SSAO signal was detected, but not different from the observed in non treated cells as indicates the densitometry graph.

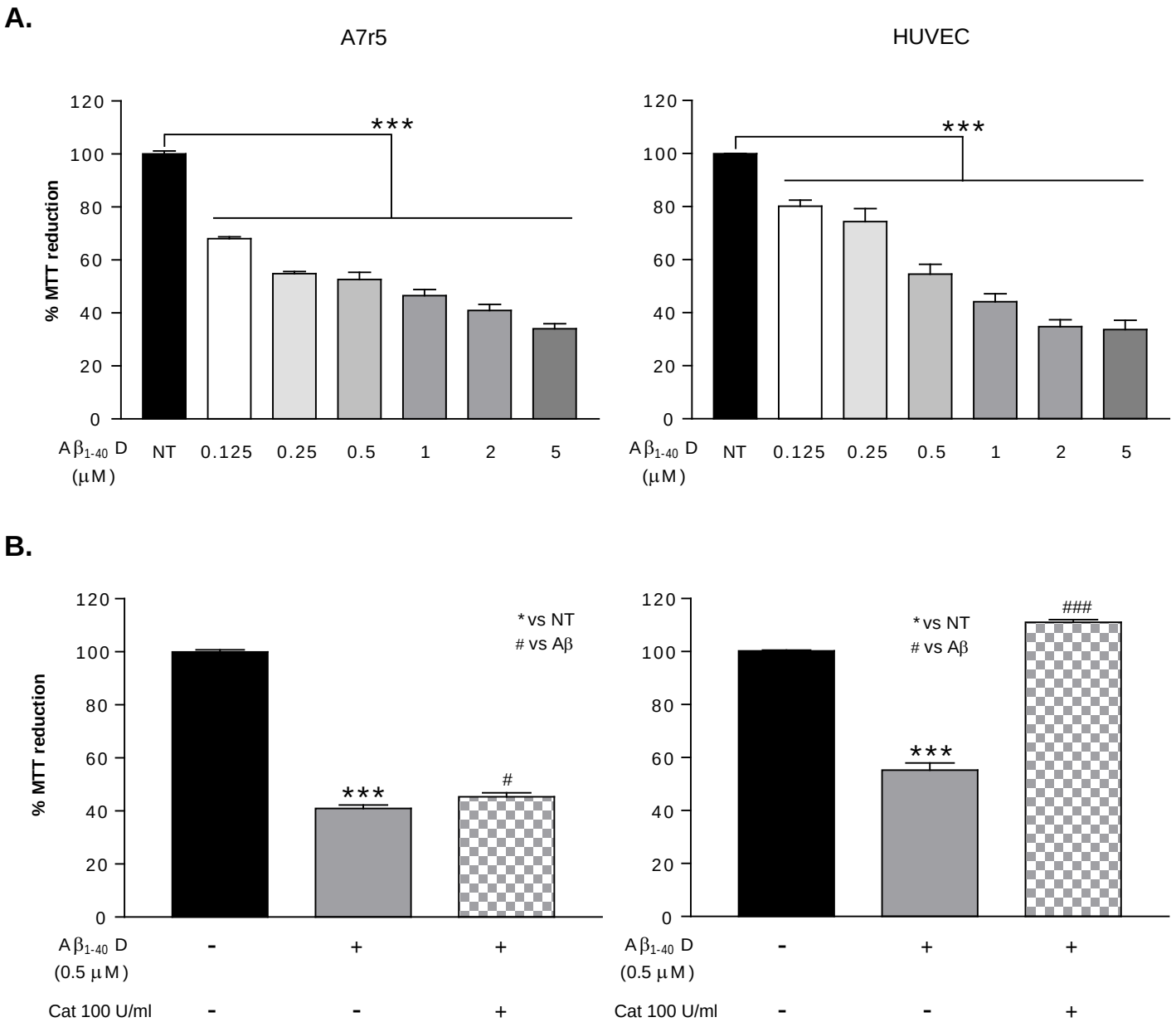
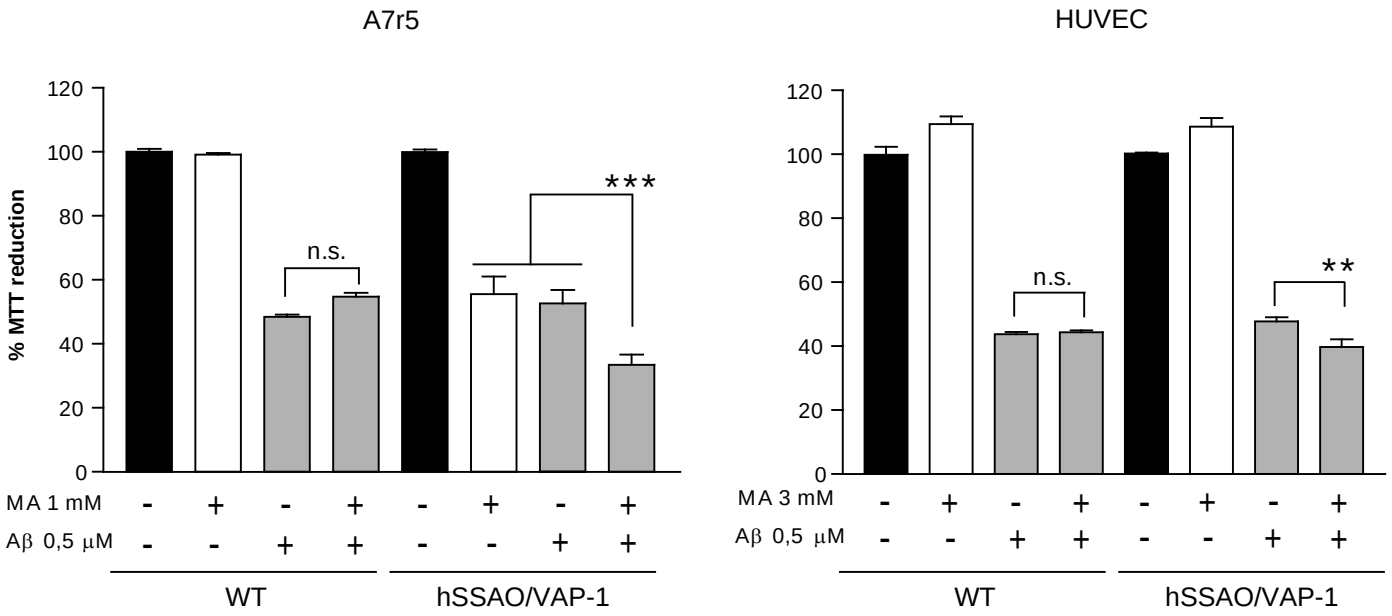


Figure 1 - *Solé et al.*

A.



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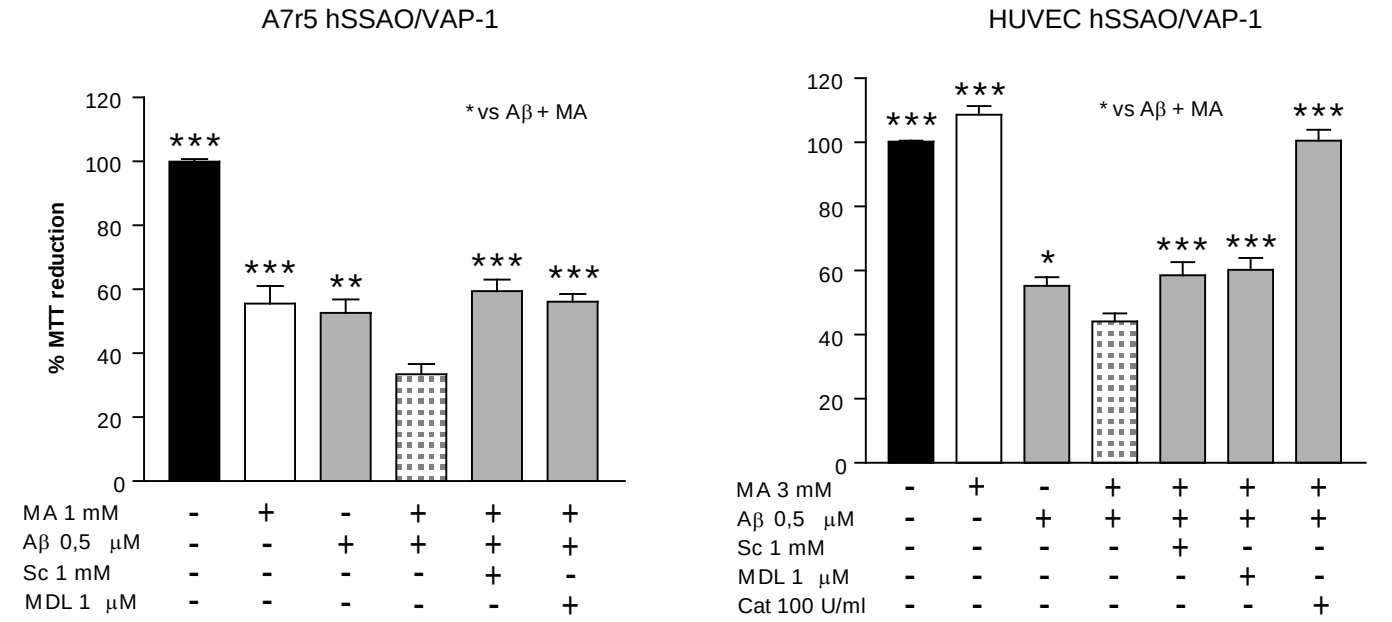
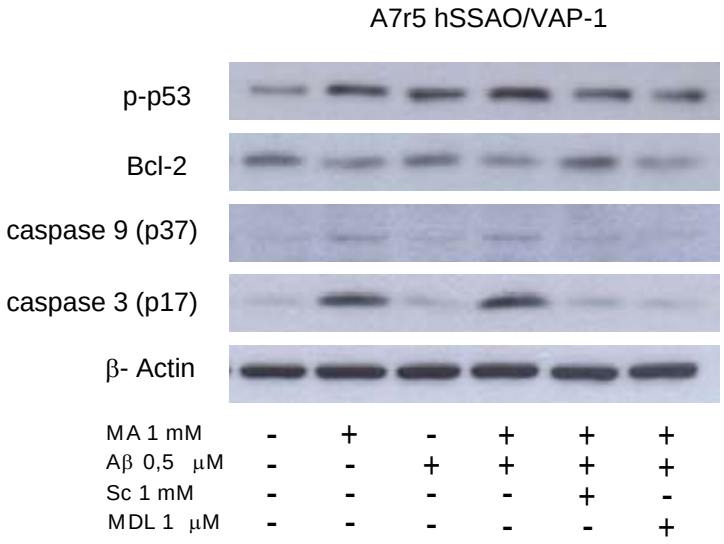
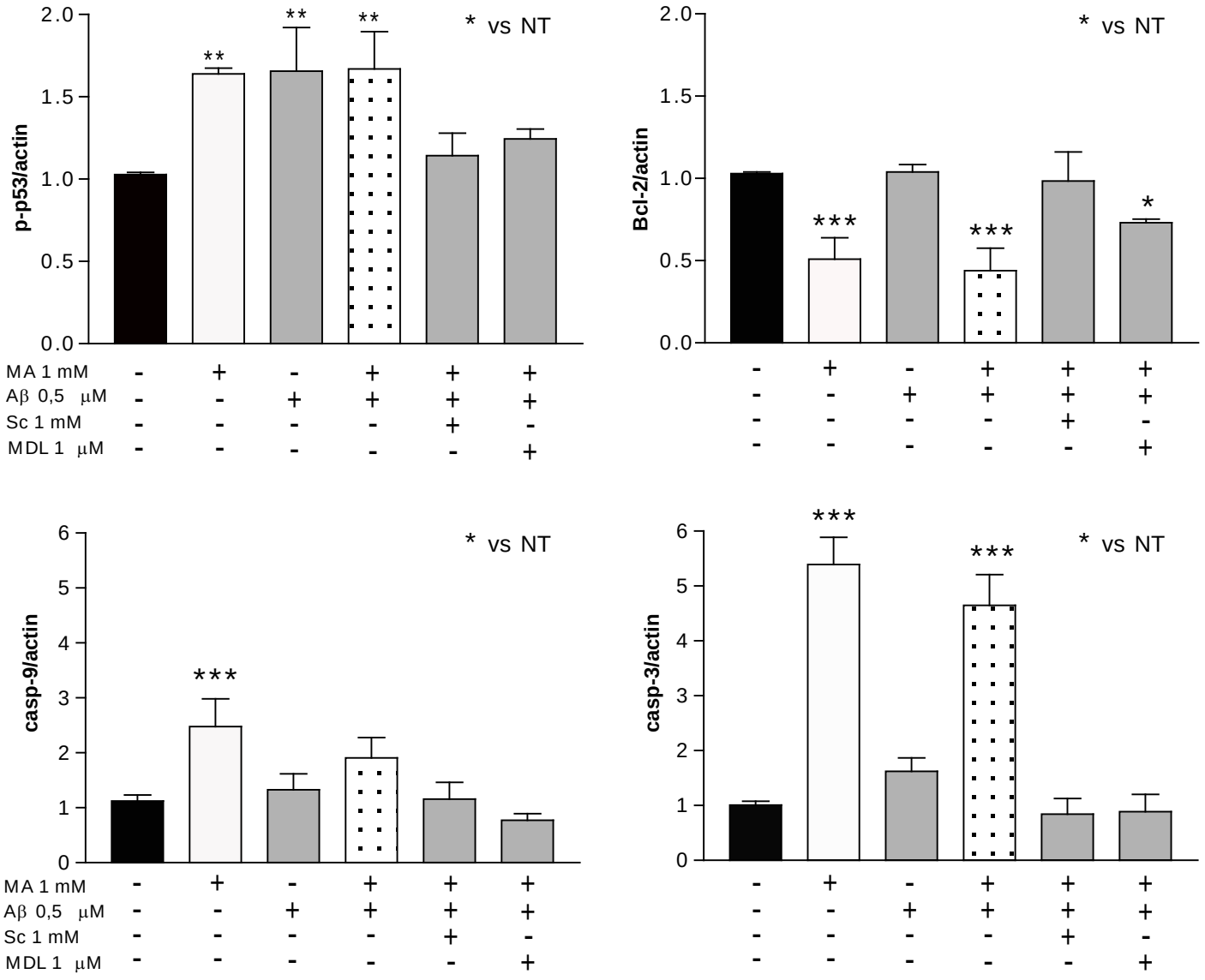


Figure 2 - *Solé et al.*

A.


A7r5 hSSAO/VAP-1
p-p53
Bcl-2
caspase 9 (p37)
caspase 3 (p17)
β- Actin
MA 1 mM - + - + + +
Aβ 0,5 μM - - + + + +
Sc 1 mM - - - - + -
MDL 1 μM - - - - - +

B.


p-p53/actin
Bcl-2/actin
casp-9/actin
casp-3/actin
* vs NT
MA 1 mM - + - + + +
Aβ 0,5 μM - - + + + +
Sc 1 mM - - - - + -
MDL 1 μM - - - - - +

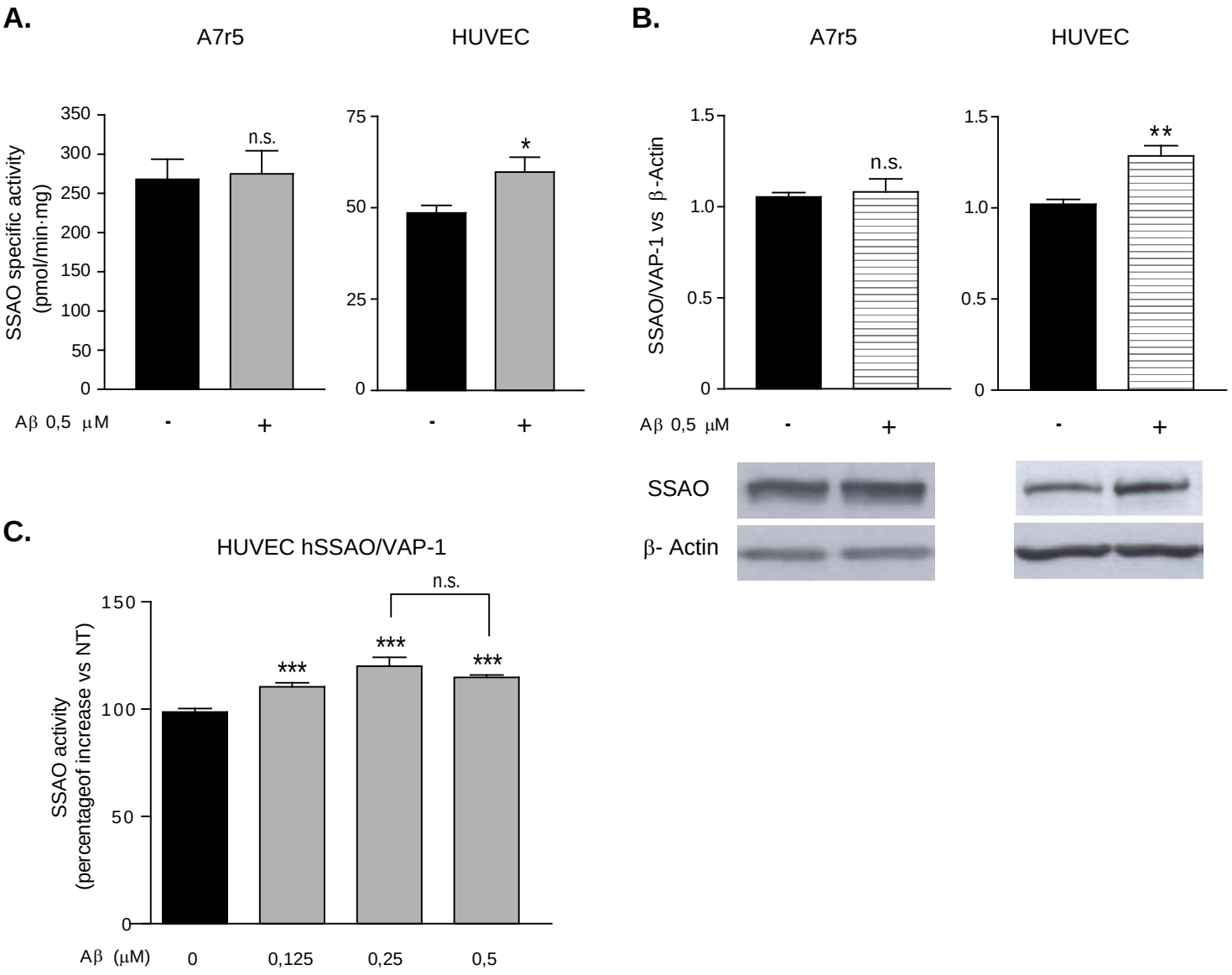


Figure 4 - *Solé et al.*

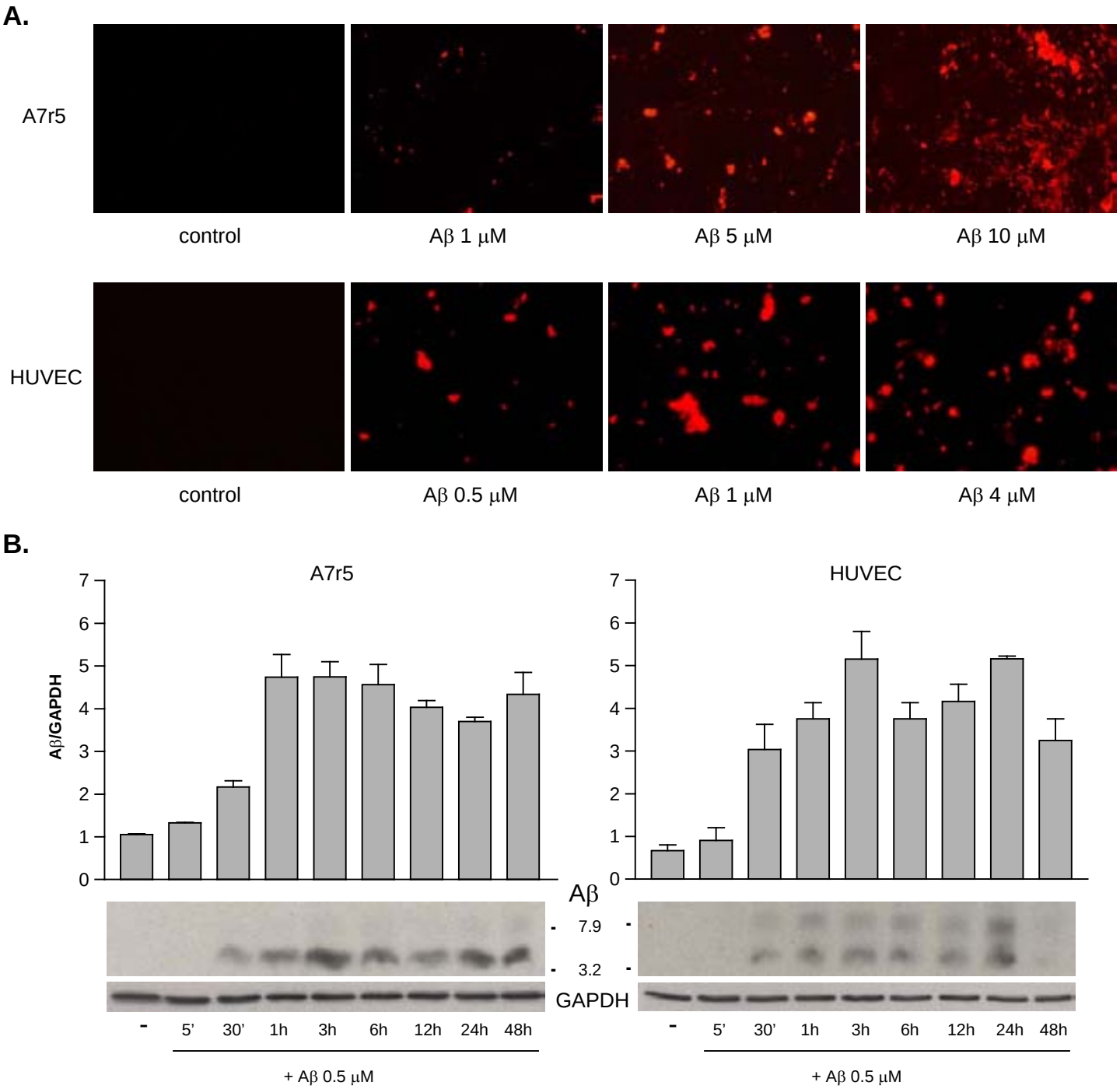


Figure 5 - *Solé et al.*

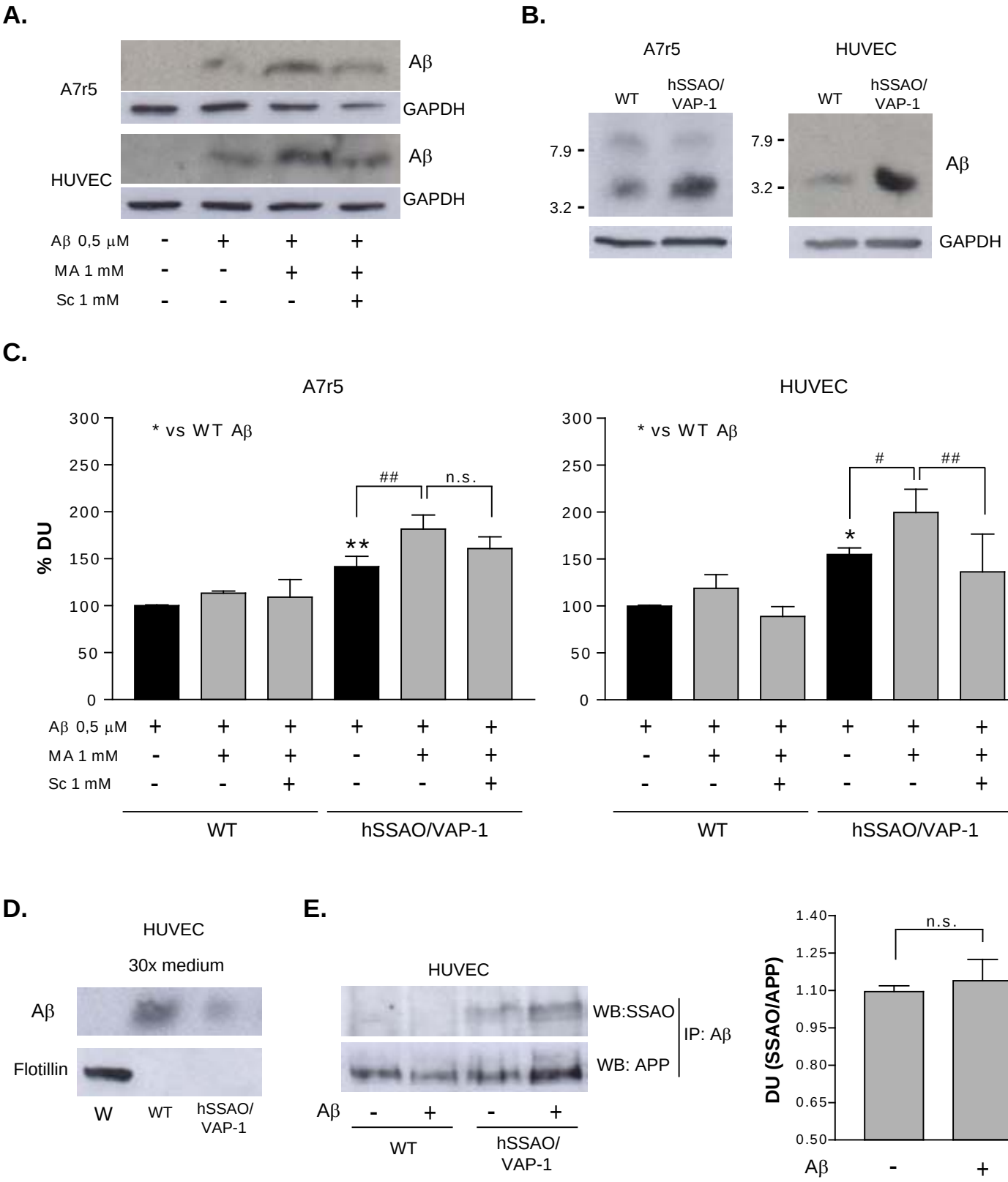


Figure 6 - *Solé et al.*

Capítol V:

“Plasma Semicarbazide Sensitive Amine Oxidase (SSAO) activity in Alzheimer’s disease and other dementias”

Article pendent d’enviar a publicar

Plasma Semicarbazide Sensitive Amine Oxidase (SSAO) activity in Alzheimer's disease and other dementias

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Running title: Plasma SSAO activity in dementias

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Abstract

Semicarbazide-sensitive amine oxidase (SSAO) is expressed in the endothelial and the smooth muscle layers of blood vessels in the cerebrovascular system. It metabolizes primary amines generating the toxic products hydrogen peroxide, ammonia, or aldehydes as formaldehyde or methylglioxal, all able to induce cell toxicity, protein cross-linking and to contribute to the generation of advanced glycation end products. The cleavage of the tissue-bound protein releases an active soluble form of the enzyme into blood plasma. An increase of the plasma SSAO activity has been detected in several pathologies, such as diabetes type I and II, congestive heart failure, renal and hepatic failure, multiple sclerosis or Alzheimer's disease (AD). Moreover, it has been described an increase of its protein expression in the cerebrovascular tissue of CADASIL and AD patients. Therefore, its activity has been associated to the vascular damage observed in these pathologies. As AD or CADASIL, other dementias display vascular damage and involve the cerebrovascular system in its aetiology. Thus, the aim of this work was to determine the plasma SSAO activity levels in patients afflicted by distinct grades of AD, mixed dementia, vascular dementia or frontotemporal dementia to know whether SSAO activity could have a role in the vascular dysfunction associated to these disorders. Results showed that plasma SSAO activity was significantly increased in the severest cases of AD, but not in mixed or vascular dementias. However, the lack of a severe demented group of plasma samples in vascular and mixed dementias does not allow us to conclude that SSAO is not involved in the vascular damage of these pathologies.

Keywords: Semicarbazide-sensitive amine oxidase, plasma, Alzheimer's disease, vascular dementia, mixed dementia.

Abbreviations: AD, Alzheimer's disease; DM, diabetes mellitus; FTD, frontotemporal dementia; GDS, global deterioration scale; HTA, hypertension; MCI, mild cognitive impairment; MD, mixed dementia; MMSE, mini mental state examination; SSAO, semicarbazide-sensitive amine oxidase; VaD, Vascular dementia; VAP-1, vascular adhesion protein 1.

Introduction

Semicarbazide sensitive amine oxidase (SSAO) [E.C. 1.4.3.21], also known as vascular adhesion protein 1 (VAP-1) in endothelium (Smith *et al.* 1998) is a monoamine oxidase enzyme widely distributed in nature. In mammals, including human, SSAO/VAP-1 is found in most organs and tissues (Andres *et al.* 2001; Lyles 1996), but its expression is higher in adipocytes and in smooth muscle and endothelial cells of blood vessels (Barrand and Fox 1984; Castillo *et al.* 1998; Lewinsohn 1984). A soluble form of the protein has also been found circulating in blood plasma (Lyles 1996). In rodents and in humans, some evidences suggest that the soluble SSAO/VAP-1 comes from a proteolytic shedding of the tissue-bound protein, probably through a metalloprotease activity (Abella *et al.* 2004; Gokturk *et al.* 2003; Stolen *et al.* 2004). All adipocytes, endothelial or smooth muscle cells have been shown the ability to release the soluble form of SSAO/VAP-1 in various conditions (Gokturk *et al.* 2003; Stolen *et al.* 2004).

As monoamine oxidase, SSAO catalyzes the oxidative deamination of primary aromatic and aliphatic amines, being methylamine and aminoacetone its main physiological substrates (Lyles and Chalmers 1992; Precious *et al.* 1988). Its activity generates ammonia, hydrogen peroxide (H_2O_2) and the corresponding aldehyde: formaldehyde from methylamine or methylglioxal from aminoacetone (Precious *et al.* 1988). A part from its amine oxidase activity, other functions have been attributed to SSAO/VAP-1 depending on the tissue, but often associated to the amine oxidase activity. In adipocytes and smooth muscle cells, SSAO activity stimulates glucose transport through the generated H_2O_2, mimicking the insulin effect (El Hadri *et al.* 2002; Enrique-Tarancon *et al.* 1998); in endothelial cells, SSAO/VAP-1 is involved in lymphocytes trafficking (Smith *et al.* 1998), and its expression in these cells is induced during inflammatory conditions (Arvilommi *et al.* 1997; Salmi *et al.* 1993).

SSAO/VAP-1 expression and/or activity have been found altered in some pathological conditions, in its tissular or plasmatic form. Plasma SSAO is increased in type I and II diabetic patients (Boomsma *et al.* 1999; Meszaros *et al.* 1999), correlating with the severity of the diabetic complications as retinopathy, nephropathy or neuropathy (Garpenstrand *et al.* 1999; Gronvall-Nordquist *et al.* 2001; Nunes *et al.* 2008). Elevated plasma SSAO activity is found also in non diabetic morbidity obese patients (Weiss *et al.* 2003), in congestive heart failure patients (Boomsma *et al.* 1997), in hepatic chronic diseases (Kurkijarvi *et al.* 1998), in chronic renal failure (Lin *et al.* 2008; Nemcsik *et al.* 2007), and it has been implicated in atherosclerosis (Karadi *et al.* 2002; Li *et al.* 2009).

Moreover, SSAO activity is altered in several other inflammatory conditions, probably due to its pro inflammatory function (Airas *et al.* 2006; Ling *et al.* 2009; Madej *et al.* 2006; Madej *et al.* 2007). As in most pathologies in those SSAO is found increased it correlates with the severity of the condition, and the products generated by its activity are toxic, it has been suggested by numerous authors that the enzymatic SSAO activity contributes to the vascular pathology observed in these diseases (Boomsma *et al.* 1997; Garpenstrand *et al.* 1999; Yu *et al.* 1994; Yu 1998).

We have previously described that plasma SSAO activity is increased in moderately-severe and severe Alzheimer's disease (AD) patients (del Mar Hernandez *et al.* 2005). Moreover, the membrane-bound SSAO/VAP-1 is also overexpressed in this neurodegenerative disease and in cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL) patients (Ferrer *et al.* 2002). Both disorders display vascular degeneration, and it has been suggested that SSAO activity could contribute to this vascular pathology through its enzymatic activity (Jiang *et al.* 2008; Unzeta *et al.* 2007; Yu 2001).

Besides AD, other dementias such as vascular dementia (VaD) or mixed dementia (MD) display vascular dysfunctions, and others as frontotemporal dementia (FTD) display neuronal protein inclusions different from the observed in AD. Moreover, conditions as diabetes, atherosclerosis, cerebral lesions or cardiac pathology, in which SSAO is increased, constitute risk factors for the development of several neurodegenerative diseases. Therefore, the aims of the present study were to confirm the results obtained before for AD patients increasing the number of analyzed patients, and to determine the plasma SSAO activity levels in the other cited types of dementia, in order to elucidate whether SSAO could be also involved in their vascular degeneration.

Materials and methods

Patients and plasma samples

AD patients analyzed in the study (N=281) included 84 men and 197 women, with ages ranging from 66 to 94 years. These patients were diagnosed of sporadic AD, according to the NINCS-ADRDA criteria (Dubois *et al.* 2007; McKhann *et al.* 1984), and classified in four groups, based on the Global Deterioration Scale (GDS) (Reisberg *et al.* 1982) and the Mini Mental State Examination (MMSE) (Folstein *et al.* 1975). The patients were divided in mild (GDS = 3 – 4 and MMSE > 20), moderate (GDS = 4 – 5 and

MMSE < 20), moderately severe (GDS = 6) and severe demented (GDS = 7). Patients suffering from Mild Cognitive Impairment (MCI) were included as a pre-demented group of AD patients, since it has been suggested that MCI constitutes a pre-AD condition (Galasko *et al.* 1994). MCI patients (N=44) were diagnosed considering criteria described in (Petersen 2004), and included 13 men and 31 women, with ages ranging from 67 to 87 years.

VaD patients analyzed (N=119) included 63 men and 56 women, with ages ranging from 66 to 90 years. These patients were diagnosed according to the NINCS-AIREN criteria (Roman *et al.* 1993), and distributed in three groups, based on the GDS scale, that were mild (GDS = 3 – 4), moderate (GDS = 4 – 5) and moderately severe demented (GDS = 6). MCI patients with vascular aetiology criteria (N= 53) were included as pre-demented VaD group, and included 33 men and 20 women, with ages ranging from 67 to 90 years.

MD patients analyzed (N=114) included 44 men and 70 women, with ages ranging from 67 to 94 years. These patients were diagnosed of AD, but they showed also VaD criteria. MD patients were distributed in three groups based on the GDS scale: mild (GDS = 3 – 4), moderate (GDS = 4 – 5) and moderately severe demented (GDS = 6).

FTD patients included in the study (N=9) included 4 men and 5 women, with ages ranging from 70 to 78 years. These patients were diagnosed of FTD according to consensus criteria described in (Cairns *et al.* 2007; Neary *et al.* 1998).

Control patients (N=26) free of neurological disease (GDS = 1 – 2) included 13 men and 13 women, with ages ranging from 66 to 90 years.

Plasma samples of patients and controls were obtained from Fundació ACE (Barcelona, Spain). Blood samples were collected by venipuncture in sterile plunger tubes containing sodium citrate (0.129 M) as anticoagulant. Samples were centrifuged at 2500 x *g* during 20 min to separate the plasma, that was aliquoted and frozen at -80 ºC until its use.

Plasma SSAO activity determinations

SSAO activity of plasma samples was determined radiochemically as previously described (Fowler and Tipton 1981). Briefly, 50 µl of plasma were used for assays, which were performed in 100 mM Tris-HCl buffer, pH 9 in a final volume of 200 µl. A pre incubation of samples with 1 mM L-deprenyl during 30 min was done before adding the substrate in order to inhibit possible MAO B contamination from platelets. The

substrate used was 100 µM (^{14}C)-benzylamine hydrochloride (2 mCi/mmol, Amersham-GE Healthcare, UK). 25 µl of substrate were added to each vial, and reactions were performed at 37ºC during 90 min. Reactions were stopped by adding 100 µl of 2 M citric acid. Radiolabelled products were extracted from the reaction mixture into 4 ml of toluene/ethyl acetate (1:1) (v/v), containing 0.6% (w/v) 2,5-diphenyloxazole (PPO). The organic phase containing the radiolabelled products of the reaction were analyzed in a liquid scintillation counter Tri-Carb 2810TR (Perkin Elmer) and the Quanta Smart 3.0 software (Perkin Elmer), in order to determine the amount of ^{14}C-aldehyde from each sample. The protein concentration of plasma samples was determined by the Bradford method. The SSAO specific activity is expressed as pmol/min·mg of protein.

Statistical analyses

Data are expressed as mean ± standard error of mean (SEM). Statistical analyses of biochemical data were performed by the one-Way or two-Way ANOVA tests followed by Newman-Keuls Multiple Comparison test, elaborating experimental data by means of Graph-Pad 3.0 software (San Diego, CA, USA) or Statistical Package for the Social Sciences (SPSS) 15.0.1 software (Chicago, IL, USA). Differences with $p < 0.05$ were considered significant.

Results and Discussion

AD patients' details are resumed in Table 1. Plasma SSAO specific activity values of AD patients at different severity conditions were compared with those of control subjects and MCI patients. SSAO activity of control samples showed no significant differences compared to MCI patients or mild, moderate or moderately severe demented AD patients. However, a significant increase in SSAO activity was observed in plasma of severe demented AD patients compared to controls and to the other disease conditions. Moreover, plasma SSAO activity of the moderate demented group was also statistically different from MCI group (figure 1). These results coincide with the previous reported (del Mar Hernandez *et al.* 2005) confirming the increase of SSAO plasma activity in the severest cases of sporadic AD dementia, but not in moderately severe demented patients.

Since age is a known risk factor for suffering AD, age of the different states of dementia was compared to non demented controls. Results showed that age of moderate, moderately severe and severe demented patients was statistically higher than those of control, MCI and mild demented subjects (figure 2.A). No correlation was found when

the age of control subjects was compared with SSAO activity (data not shown), as previously described (del Mar Hernandez *et al.* 2005; Salmi *et al.* 2002). Moreover, the SSAO activity did not correlate with age in severe-demented patients (figure 2.B) (Pearson $r = 0.125$).

Other pathologies have been associated with increased plasma SSAO activity, such as diabetes mellitus (DM) type I or II, heart failure or cerebrovascular pathology, and an additive increase in plasma SSAO activity has been observed in patients suffering from two of these pathologic conditions, such as diabetes and heart failure, at the same time (Boomsma *et al.* 2003). Among them, however, only DM was associated with higher plasma SSAO activity in our study population (figure 3.A); cardiopathies or cerebrovascular accidents (CVA), reported to show increased plasma SSAO activity (Airas *et al.* 2008; Boomsma *et al.* 1997), were not associated with this increase in our population (figure 3.B and C). The different aetiology of the cardiopathy in our study could explain the lack of agreement with other studies in this concern, in which only the congestive heart failure has been reported to be associated with high plasma SSAO activity. Moreover, the results obtained for cerebrovascular accidents depend on the type of cerebrovascular disease (Ishizaki 1990). Other vascular risk factors such as hypertension (HTA) or hypercholesterolemia/dyslipidemia, which have not been associated to SSAO increases, were not associated either in our population (figure 3.D and E).

Since the prevalence of these pathologies is high in aged subjects, our study population contained several patients afflicted by these associated pathologies. Thus, in order to confirm that AD pathology was the responsible of the SSAO increase, the different groups of AD patients were separated depending on their associated pathology. Patients suffering from DM, cardiopathy, or CVA were considered in the first group, since it has been reported an SSAO increase in these pathologies (Airas *et al.* 2008; Boomsma *et al.* 1997; Boomsma *et al.* 1999; Ishizaki 1990; Meszaros *et al.* 1999). Patients without cardiovascular risk factors or with cardiovascular risk factors not associated with increased levels of plasma SSAO activity constituted the second group. Patients suffering only from HTA or hypercholesterolemia/dyslipidemia (Chol/dys) were included in the second group, since it has not been reported any increase in plasma SSAO activity levels associated to this conditions alone, although the correlation exists when found in combination with DM or chronic heart failure (Boomsma *et al.* 2003; Karadi *et al.* 2002; Meszaros *et al.* 1999). When plasma SSAO activity was determined in different groups of AD patients without risk factors of increased SSAO, the level of significance increased between severe demented AD

patients and the other groups (figure 4.A), but significant differences were not observed between the other groups. These results confirmed that the increased SSAO levels were associated to AD pathology. However, the presence of DM, cardiopathy or cerebrovascular accidents increased the plasma SSAO activity levels in all AD groups (figure 4.B). A significant SSAO increase was observed in moderately-severe demented patients with SSAO increased associated pathology compared to patients without SSAO increased associated pathology. Unfortunately, this comparison could not be done in severe demented patients, since only one of these patients showed SSAO increased associated pathology.

Gender differences in plasma SSAO activity levels have not been observed in other studies with human subjects (del Mar Hernandez *et al.* 2005; Karadi *et al.* 2002; Kurkijarvi *et al.* 1998). However, gender differences have been described in transgenic mice overexpressing human SSAO/VAP-1, with higher plasma SSAO/VAP-1 levels detected in males (Stolen *et al.* 2004). Since non transgenic mice did not display these gender differences, the authors suggest that SSAO/VAP-1 overexpression conditions may accentuate pre existing real gender differences. Severe AD pathology is a situation in which SSAO is overexpressed in humans, so we analyzed if gender differences were evidenced under this condition (figure 5). Two-way ANOVA (gender and severe AD dementia) revealed a significant effect of dementia, but no effect of gender, neither interaction between these two factors in SSAO activity increase. The one-way ANOVA revealed that only demented women had significantly higher plasma SSAO activity than control women. The lack of significance obtained in men, however, could be due to the low number of severe demented men. Although it was not statistically significant, women tended to have higher plasma SSAO activity levels than men, which was different from results obtained with transgenic mice (Stolen *et al.* 2004). These different results could be due to specie differences, since SSAO has different levels of tissue expression, different plasma SSAO activity levels and even different substrate specificity between species, so it could have also different expression and shedding regulation systems. Moreover aging could also be involved in the differences observed, since levels of different proteins or disease conditions can change with aging depending on the gender, especially those related with vascular risk factors (Azad *et al.* 2007; Khalil 2005; Mercuro *et al.* 2003; Sattler *et al.* 2005).

The results obtained by the analyses of plasma SSAO activity in different severities of AD dementia confirmed the obtained in previous reports showing an increase of plasma SSAO activity in the severest AD dementia conditions (del Mar Hernandez *et al.* 2005). Moreover, the increase of plasma SSAO activity correlated with the increase

of tissue SSAO expression, which was observed at less severe conditions of AD (Ferrer *et al.* 2002). Thus, the increased levels of plasma SSAO activity observed in AD and in other pathologies could be a reflex of the increasing SSAO expression and cleavage in tissues. Although the signals that would induce the expression and shedding of SSAO/VAP-1 under these conditions are not fully known, the inflammatory environment present in advanced cerebral AD tissue could be one of the involved factors, since it has been described that endothelial SSAO/VAP-1 is induced under inflammatory conditions (Salmi *et al.* 1993). In addition, the activity of both forms of the protein is able to induce the apoptosis of vascular cells through the products generated during its enzymatic activity (Hernandez *et al.* 2006; Sole *et al.* 2008), so we suggest that the increase of SSAO observed in AD could contribute to the vascular damage observed in cerebrovascular AD tissue. Thus, the increase of plasma SSAO activity could be a marker of vascular damage or vascular dysfunction. In order to confirm this hypothesis, the plasma SSAO activity was analyzed in other types of dementia that show vascular damage.

Vascular dementia (VaD) is originated by vascular damage that led to brain injure through reduced cerebral blood flow and deprivation of oxygen and nutrients to the brain. Several vascular dysfunctions such as atherosclerosis, embolic stroke, diabetes or hypertension can induce VaD (Strachan *et al.* 2008). Interestingly, many of these pathologic vascular conditions have been also associated with high levels of SSAO activity. Moreover, oxidative stress signals have been detected in AD and VaD, indicating a common link between these pathologies (Bennett *et al.* 2009; Pratico 2008), and also with SSAO, since its activity induces oxidative stress. Although vascular damage is often evident in VaD, when plasma SSAO activity of these patients at different levels of dementia was determined, none increase was observed related to the severity of the dementia (figure 6.A). On the other hand, mixed dementia (MD) is an AD-type dementia that shows also vascular aetiology criteria, so vascular risk factors are also associated with MD. Similarly to VaD, when plasma SSAO activity of the MD groups was compared with control subjects, any significant increase in SSAO activity was detected (figure 6.B).

In addition, plasma samples of frontotemporal dementia (FTD) patients were analyzed. FTD shows neuronal protein inclusions different from the observed in AD, that are enriched in ubiquitin and TDP-43, but do not contain tau or α-synuclein. When plasma SSAO activity was analyzed in different grades of FTD, significant differences between control subjects and demented patients were not observed (figure 6.C). The low

number of samples could be however determinant in this case, since only 9 samples were available.

The results obtained with VaD and MD patients indicated that SSAO/VAP-1 does not seem to be useful as a vascular damage marker, since although these dementias, as AD, show clear signs of vascular dysfunction, they do not display increases in plasma SSAO activity. The increase in plasma SSAO activity did not correlate either with the severity of the dementia in the three types of dementia analyzed besides AD. Therefore, the results showing an increase in plasma SSAO activity of severe AD patients suggest that a specific factor found in AD but not in other dementias may be the responsible of the SSAO induction. If this hypothesis was correct, since MD shows also AD characteristics, a lower increase in SSAO activity should be observed in MD patients compared to AD patients; however, the non-AD component of MD could hide this effect. On the other hand, we cannot rule out that the severity of the condition would be a relevant factor, since we do not had at our disposal plasma samples of severe demented VaD, MD or FTD patients due to the difficulty to obtain them. Therefore, the cause or function of plasma SSAO/VAP-1 release in AD remains to be elucidated, and it would be necessary to extend the study with severe demented patients of VaD, MD and FTD, as well as to increase the number of FTD samples analyzed in order to definitively rule out the increase of plasma SSAO in these pathologies.

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Figure legends

Table 1. Characteristics of the AD patients included in the study. Patients are classified according to the severity of the AD condition, and the number of patients (N), the male/female ratio (m/f), the mean age and SSAO activity (pmol/min·mg) are specified. The percentage of patients with pathologies associated to increased plasma SSAO activity (diabetes mellitus (DM), cardiopathy or cerebrovascular accidents) is detailed for each condition.

Figure 1. SSAO specific activity of plasma samples corresponding to control subjects, non-demented subjects with mild cognitive impairment (MCI) or different conditions of AD dementia (mild, moderate, moderately-severe or severe). An increase of SSAO activity is observed in samples of severe demented patients compared to controls, MCI and the other levels of AD dementia. ($^{\#}$) $p < 0.05$; (**) $p < 0.01$ and (***) $p < 0.001$ *vs* severe demented samples. Data represent mean ± SEM.

Figure 2. (**A**) Scatter plot of age distribution in control samples, MCI samples and the different groups of AD dementia. Age correlates with the severity of the AD dementia. Statistically significant differences are shown as * *vs* control and MCI samples or as $^{\#}$ *vs* mild demented AD samples. (*) $p < 0.05$; (**) $p < 0.01$ and (***) $p < 0.001$. (**B**) No significant correlation was observed between age and SSAO specific activity in severe demented patients (Pearson r = 0.125).

Figure 3. Association of vascular risk factors and SSAO activity in the whole population analyzed in the study. The plasma SSAO activity of different risk factors or vascular pathologies was compared with non afflicted patients by Student's *t* test. Only Diabetes Mellitus (DM) (**A**) is associated with increased levels of SSAO activity in the study population. The other analyzed, cardiopathy (**B**), cerebrovascular accidents (CVA) (**C**), hypertension (HTA) (**D**) or hipercholesterolemia/dyslipidemia (Chol/dis) (**E**) did not show association. The mean values of SSAO activity of the different groups, the number of samples analyzed in each group (N) and the *p* value of the Student's *t* test is shown on the corresponding graph. (**) $p < 0.01$; n.s. non significant.

Figure 4. (**A**) Scatter plot of SSAO specific activity in controls, MCI and groups with increasing severity of AD dementia, considering only patients without associated pathologies that show increased levels of plasma SSAO activity. Severe demented patients display a significant increase in plasma SSAO specific activity compared to the other groups analyzed. (**B**) Differences in plasma SSAO specific activity in each group of dementia, separating patients with or without associated pathology that show increased SSAO activity levels. Higher levels of SSAO activity are observed in patients with associated pathology in each group, although the difference is statistically significative only in moderately-severe demented group. (*) $p < 0.05$ and (***) $p < 0.001$, by a one-way ANOVA test followed by Newman-Keuls multiple comparison test.

Figure 5. Gender is not associated with SSAO activity increase neither in controls, nor in severe demented AD patients, analyzed by two-way ANOVA test. Significant differences were observed only when female severe demented group was compared with its control. (**) $p < 0.01$ vs control with same gender; n.s., non significant, by a one-way ANOVA test.

Figure 6. (**A**) Scatter plot of SSAO activity in control subjects, MCI with vascular aetiology subjects and different conditions of vascular dementia patients. No differences in SSAO activity were observed between plasma of controls and the different levels of vascular dementia (VaD). (**B**) Scatter plot of SSAO activity in control subjects and different levels of mixed dementia (MD) or (**C**) different levels of frontotemporal dementia (FTD). Statistically significant differences in SSAO activity between controls and demented groups were not detected.

Table 1. Characteristics of the AD patients included in the study

	Controls	**MCI**	**Mild**	**Moderate**	**Mod-Sev**	**Severe**
N	26	44	104	104	48	25
Sex (m/f)	13/13	13/31	42/62	25/79	13/35	4/21
Age ± S.E.M.	73.8±1.0	74.5±0.8	76.2±0.5	78.6±0.8	80.8±0.8	80.1±1.5
SSAO Act. ± S.E.M.	3.16±0.19	2.91±0.16	2.99±0.10	3.42±0.11	3.48±0.18	4.35±0.23
DM (%)	7.8	11.3	8.6	19.2	6.2	0
Cardiopathy (%)	0	4.5	16.3	9.6	14.5	4
Cerebrovascular accident (%)	0	0	1.9	2.8	0	0

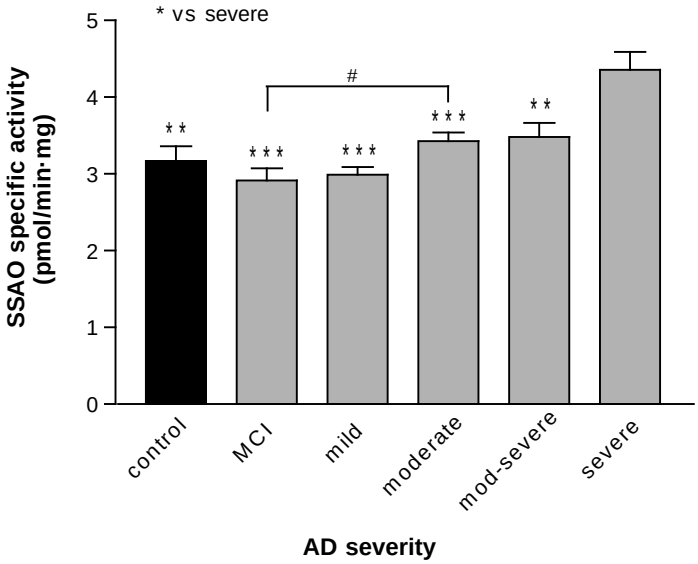
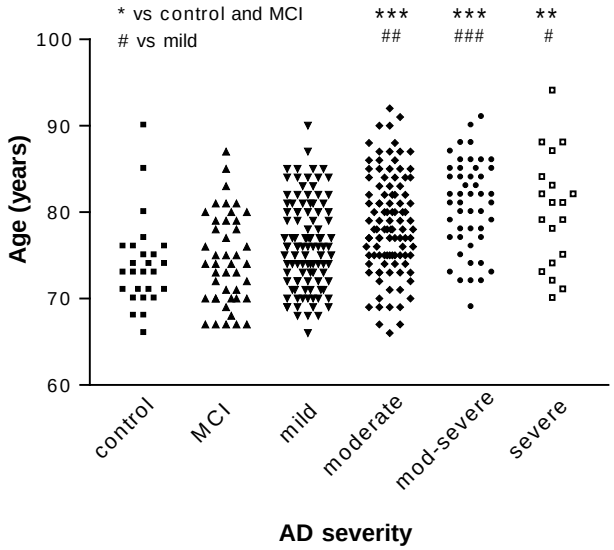
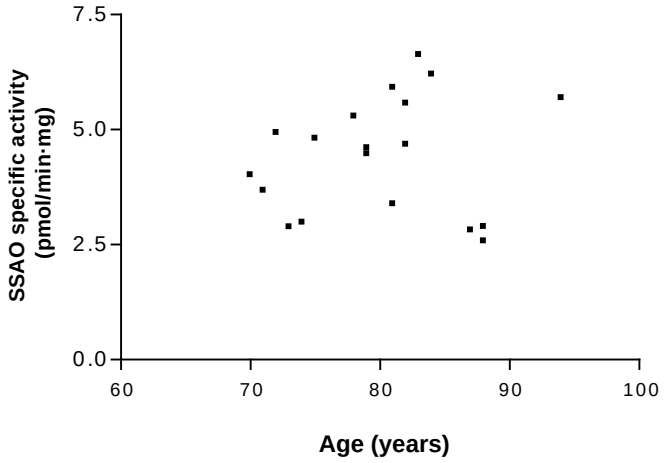


Figure 1 - *Solé et al.*

A.


* vs control and MCI
# vs mild
***
##
***
###
**
#
100
90
80
70
60
Age (years)
control
MCI
mild
moderate
mod-severe
severe
AD severity

B.


7.5
5.0
2.5
0.0
SSAO specific activity
(pmol/min·mg)
60
70
80
90
100
Age (years)

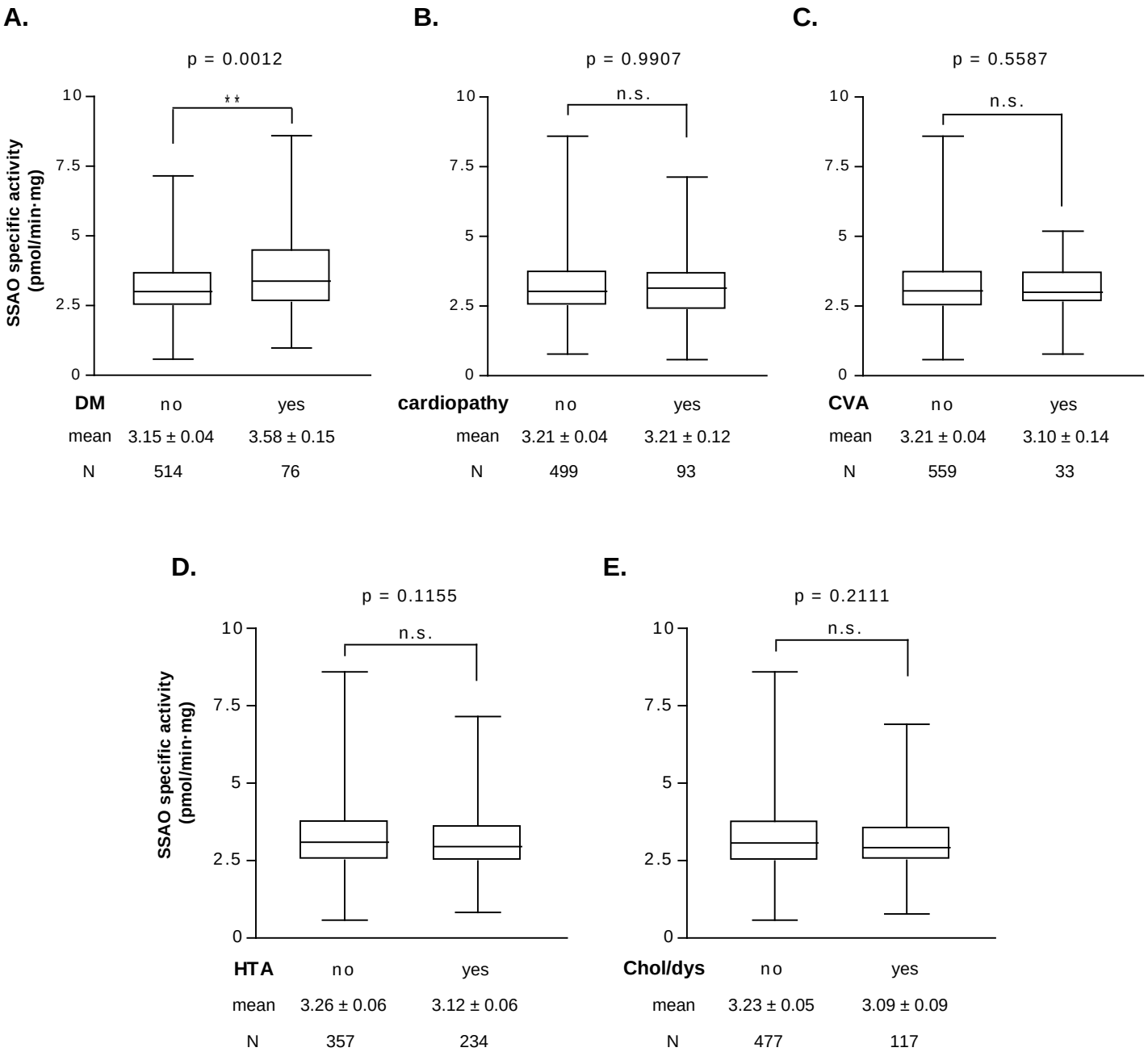


Figure 3 - *Solé et al.*

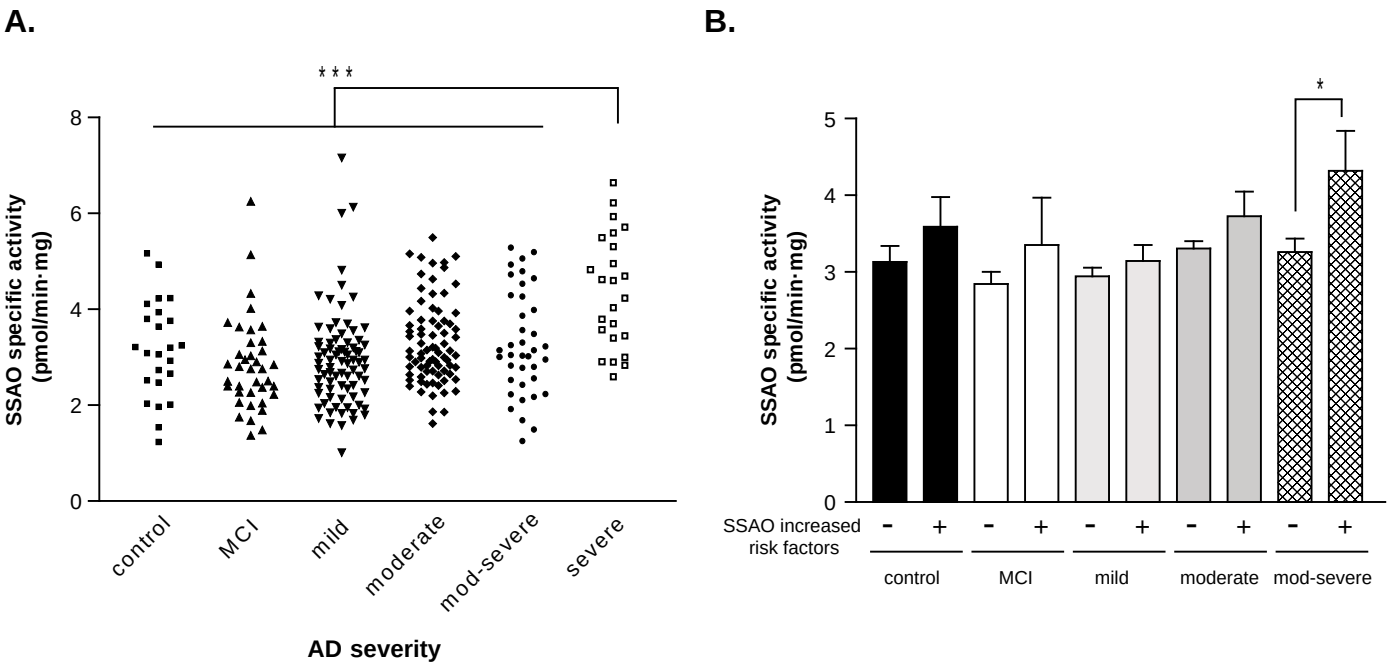


Figure 4 - *Solé et al.*

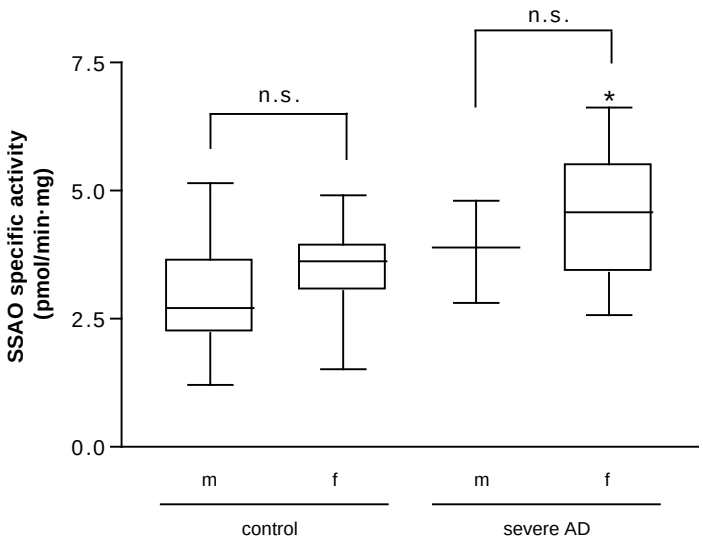
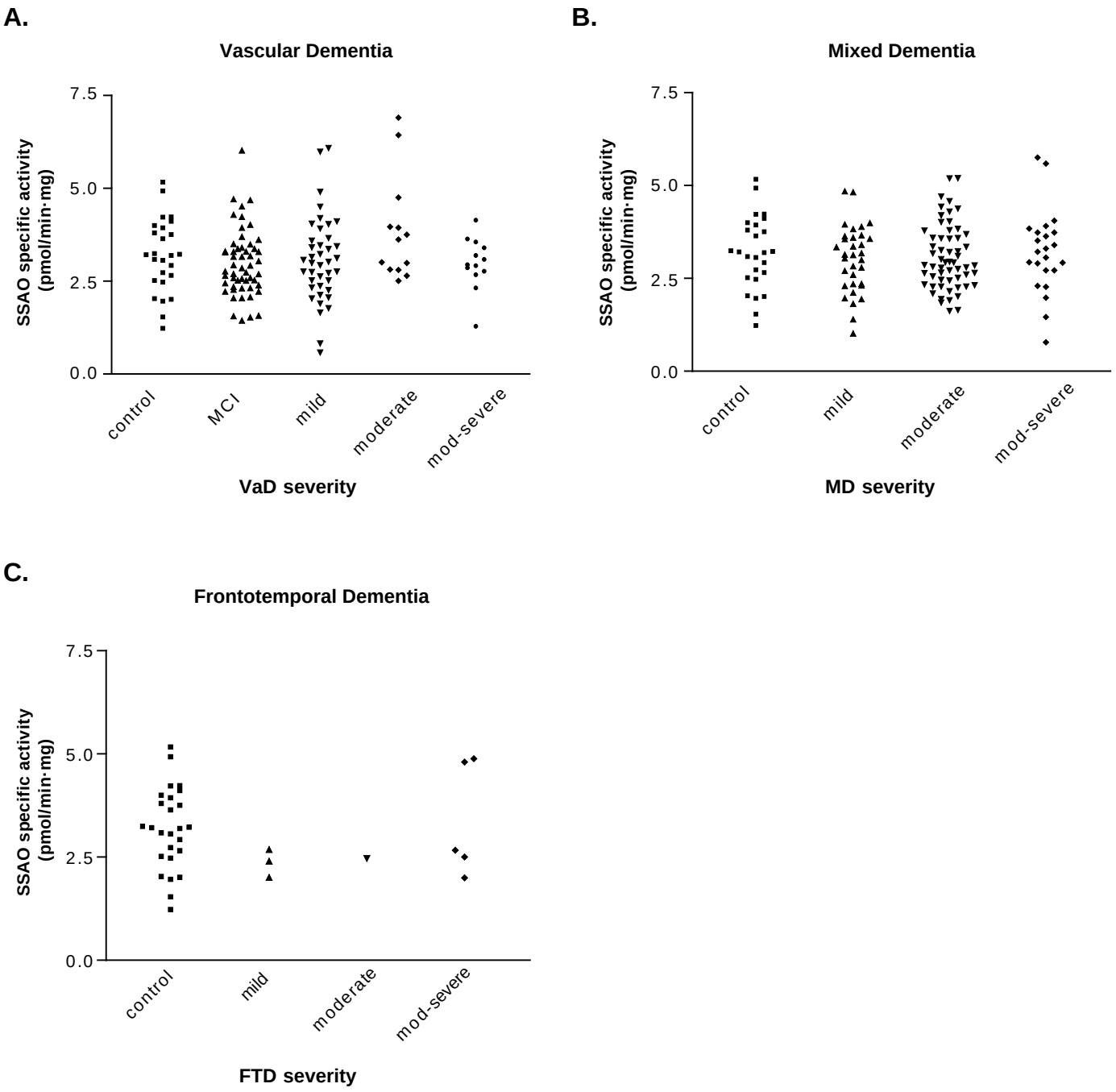


Figure 5 - *Solé et al.*


A.
Vascular Dementia
SSAO specific activity (pmol/min·mg)
7.5
5.0
2.5
0.0
control
MCI
mild
moderate
mod-severe
VaD severity
B.
Mixed Dementia
SSAO specific activity (pmol/min·mg)
7.5
5.0
2.5
0.0
control
mild
moderate
mod-severe
MD severity
C.
Frontotemporal Dementia
SSAO specific activity (pmol/min·mg)
7.5
5.0
2.5
0.0
control
mild
moderate
mod-severe
FTD severity

V. DISCUSSIÓ

V. DISCUSSIÓ

Semicarbazide-Sensitive Amine Oxidase (SSAO) is an amine oxidase widely present in nature. In mammals, it is found in almost all tissues and also in plasma, as a soluble form. In humans and in other mammals as rodents, the plasma SSAO is believed to come from the shedding of the membrane-bound form (Abella *et al.* 2004), although different tissues can originate it depending on the situation (Gokturk *et al.* 2003; Stolen *et al.* 2004). SSAO is highly expressed in adipocytes, smooth muscle cells and endothelial cells, especially from blood vessels and from endothelial venules of peripheral lymph nodes. As amine oxidase, SSAO metabolizes primary amines, being methylamine and aminoacetone the main physiological substrates, and allylamine and benzylamine the non physiological ones. In this concern, SSAO may be involved in part of the amine detoxification in the body, a function that may be significant in the lung, where SSAO is highly expressed, in order to detoxify the volatile amines that could enter to the organism by breathing.

Besides this apparent main function of SSAO, other functions have been attributed to this particular enzyme, often associated to the enzymatic activity, as it happens with several other enzymes such as NADPH, CD73 or CD157 (Jalkanen and Salmi 2008). Moreover, this variety of functions is associated with different tissues: in adipocytes and in smooth muscle cells, SSAO activity promotes the glucose uptake as an insulinomimetic effect, through the recruitment of GLUT4 and GLUT1 receptors to the cell surface (El Hadri *et al.* 2002; Enrique-Tarancon *et al.* 1998). In endothelial cells, SSAO functions as a Vascular Adhesion Protein-1 (VAP-1) (Smith *et al.* 1998), participating in lymphocyte adhesion to the endothelium by enzymatic activity-dependent and independent mechanisms (Koskinen *et al.* 2004; Salmi *et al.* 1993; Salmi *et al.* 1997).

Although the widespread distribution and the high expression of SSAO/VAP-1 *in vivo*, its *in vitro* study displays some difficulties because cell lines do not express SSAO/VAP-1 and primary cultures progressively lose its expression (BLASCHKO 1962; Owens 1995; Salmi and Jalkanen 1995; Yu and Zuo 1993). *In vitro* studies constitute a useful tool for preliminary or screening studies, which serve as bridge to start with more physiological or with animal models. These studies become of great interest when involving proteins related with human diseases, as can be SSAO for diabetes, inflammatory diseases or Alzheimer's disease. For this reason one of the first objectives of this work was to obtain cellular models that would allow studying the

SSAO/VAP-1 *in vitro*. The development and characterization of the smooth muscle cell line A7r5 hSSAO/VAP-1 and the endothelial cell line HUVEC hSSAO/VAP-1 have provided us with a new tool that fulfil the basic criteria for the SSAO study in terms of protein behaviour: the protein is expressed in the appropriate levels, localized as in physiological conditions, and it is enzymatically active. In addition, its localization in the lipid raft domains in these cells, as it does in physiological conditions, suggest that it has a specific role in this functional unit that could be analyzed in the future, as well as the relationship with its molecular partners. Moreover, the use of a smooth muscle and an endothelial cell line has allowed us to compare the SSAO/VAP-1 in these two different environments, in which it has been demonstrated to be structurally and functionally different *in vivo* (Jaakkola *et al.* 1999). However, despite the usefulness of these cellular models, they do not allow to study all the aspects of SSAO behaviour such as the regulation of its expression, for which an animal model or another type of vector construction containing the SSAO/VAP-1 promoter would be necessary.

Even so, the SSAO/VAP-1 transfection has showed to restore the amine oxidase activity pattern to the physiological conditions observed in the vascular tissue, with low MAO A activity. This result indicates that the SSAO/VAP-1 expression can modify the expression of other proteins, and that this regulatory mechanism could be studied in part with this model. In this regard, it would be interesting to study in depth the compensation phenomenon observed between MAOs and SSAO activities, also observed in some conditions *in vivo,* as in Norrie's disease (Fitzgerald and Tipton 2002; Kinemuchi *et al.* 2004; Marttila-Ichihara *et al.* 2006; Murphy *et al.* 1991). In addition, the induction of hepatic expression of redox-sensitive proteins observed in transgenic mice overexpressing SSAO/VAP-1 (Stolen *et al.* 2004) could be tested in our models, as it can involve regulatory mechanisms that would be interesting to study. Since the products generated during SSAO activity contain potentially toxic compounds, such as NH_3, H_2O_2, formaldehyde or methylglioxal, these molecules or their derivates may be the responsible of these effects.

Although at low concentrations, the products generated by SSAO activity can mediate transduction signals (Bradley *et al.* 1993; Finkel 1998; Suzuki *et al.* 1997), higher concentrations of these compounds can be dangerous for cells. Specifically, SSAO enzymatic activity has been shown to induce toxicity in the surrounding cells through the products generated (Conklin *et al.* 1998; Hernandez *et al.* 2006; Hysmith and Boor 1988; Ramos *et al.* 1988; Yu and Zuo 1993). Moreover, the aldehydes generated can contribute to the formation of advanced glycation end products, irreversible adducts between proteins and DNA or to alter the protein glycosylation (Gronvall *et al.* 2000;

Nagaraj *et al.* 1996; Seiler 2002; Yang and Butler 2002; Yu and Zuo 1996). These toxic effects have been shown to be relevant in pathologic processes as arteriosclerosis, diabetic vascular complications, renal dysfunctions or Alzheimer's disease (Chen *et al.* 2007; Mathys *et al.* 2002; Panagiotopoulos *et al.* 1998; Sakata *et al.* 2003; Stolen *et al.* 2004; Yu and Zuo 1997). Given the importance of the toxic effect induced by the catalytic activity of SSAO, and its consequences in pathological situations, another objective of the present work was to study the molecular mechanisms involved in the SSAO-mediated cell death.

For this purpose, the SSAO/VAP-1 transfected cell lines were used, and treatments with methylamine as substrate or with SSAO inhibitors were performed to evaluate the effects of the generated products on the vascular cultured cells. The first unexpectedly obtained result was that cell death was induced in smooth muscle cells but not in endothelial cells. We think that this difference is only due to the distinct levels of SSAO expression in both cells, which correlate with the observed *in vivo* (Andres *et al.* 2001). Moreover, the hypothesis that it is needed to exceed a threshold of activity in order to generate enough products to be toxic (Yu *et al.* 2006) is in agreement with these results. In addition, considering the possible role of SSAO in the vascular damage observed in Cerebral Amyloid Angiopathy (CAA) related to AD, it is also observed that endothelial cells remain undamaged at more advanced conditions of the dementia (Attems 2005). However, many other stimuli may be implicated in the vascular degeneration in AD apart from SSAO activity. Thus, the molecular mechanisms involved in SSAO mediated toxicity were studied only in the smooth muscle cell model.

Results showed a clear role of p53 in mediating SSAO dependent apoptosis through its transcriptional activity, since cell death was prevented by pifithrin-α. Moreover, it was clear that p53 was acting on the mitochondria by inducing a deregulation of Bax/Bcl-2 balance, which in turn may activate the caspases 9 and 3. The level of PUMA-α involvement in the pathway, however, was not clearly elucidated in this work although it has been involved in apoptosis in other models (Chipuk *et al.* 2005; Tampio *et al.* 2009): since it is one of the multiple p53-inducible genes, and it is increased after methylamine treatment, it could participate in the observed effects, but the requirement of its induction was not assessed. Moreover, other p53-inducible genes could be involved in the pathway. At this point, any possible therapeutic mechanism focused to inhibit the toxic effects of SSAO activity products at molecular level displays clear difficulties, since p53 is involved in tumour suppression through cell cycle regulation, and its inhibition has been related with many types of cancer progression. In this concern, inhibiting the SSAO enzymatic activity or enhancing the H_2O_2 and aldehyde

scavenger ability seems to be more feasible to avoid the apoptosis induced by SSAO activity.

In this regard, it would be interesting to determine the responsible of the p53 activation to potentiate the appropriate scavenger activity. Since formaldehyde has been shown to have the highest toxic activity among the three products generated, and the metabolism of other amines such as tyramine or benzylamine is less toxic than methylamine (Hernandez *et al.* 2006), it seems the main candidate, although the generation of secondary products from a reaction between the aldehydes, H_2O_2 and NH_3, or with other molecules cannot be excluded. Moreover, formation of DNA-DNA or DNA-protein adducts can be induced by formaldehyde, and DNA damage is a p53 activator (Vogelstein *et al.* 2000). However, to induce DNA damage, formaldehyde should enter inside the cell, and results obtained with different formaldehyde dehydrogenase inhibitors do not support this hypothesis. Alternatively, formaldehyde is a very reactive compound, which can easily interact with proteins or lipids in the membrane, inducing cellular signals able to activate p53 through a DNA damage-independent pathway, such as oxidative stress (Fuchs *et al.* 1998; Hsu *et al.* 2006; Niizuma *et al.* 2009). In this concern, direct DNA analyses focused on DNA adducts search after methylamine metabolism could be performed in order to elucidate this point.

SSAO activity products have been associated with several pathologic conditions, as it has been previously mentioned. Moreover an increase of the SSAO/VAP-1 expression and/or activity has been also detected in most of these pathologies, indicating not only the possibility of inducing damage at basal conditions, but also the enhancement of these toxic effects under these pathologic situations. The reason of the SSAO expression or activity increase is not known in most of these diseases, but since SSAO/VAP-1 is induced under inflammatory conditions in endothelium, this increase may be explained by this mechanism, for example in inflammatory liver diseases (Kurkijarvi *et al.* 1998), multiple sclerosis (Airas *et al.* 2006) or psoriasis (Madej *et al.* 2007). In this concern, we have fixed another objective of the present work in studying the toxic consequences of SSAO activity in the context of AD and its associated vascular pathology as CAA, since an increase of SSAO expression and activity has been described under these conditions (del Mar Hernandez *et al.* 2005; Ferrer *et al.* 2002). Moreover, we have been also interested in studying a possible relationship between SSAO and beta amyloid (Aβ) deposits found in cerebral blood vessels, since SSAO is specifically expressed in this cerebral tissue.

Alzheimer's disease (AD) has been classically considered a neuronal pathology. However, several authors have remarked the importance of the vascular system dysfunction in AD aetiology and progression, until the extreme of considering AD as a vascular pathology (de la Torre 2004; Zlokovic 2005). This hypothesis is based in observations that describe an AD onset after a previous arteriosclerosis in the circle of Willis (Roher *et al.* 2003) or after cerebral blood flow reduction processes (Hirao *et al.* 2005; Johnson *et al.* 2005). Moreover a reduction in cerebral glucose uptake has been observed in preceding stages of AD, before the onset of neurodegeneration, indicating a previous vascular dysfunction (Drzezga *et al.* 2003; Hunt *et al.* 2007). Thus, the importance of studying possible causes of vascular dysfunction, as is SSAO activity, would help us understanding the origins of this multifactorial pathology, and may provide us with new tools to fight against this disorder.

In CAA, Aβ forms deposits around arterioles and capillaries of the cerebral cortex and leptomeninges, where SSAO is expressed *in vivo* (Castillo *et al.* 1998; Jellinger 2002; Yamada 2000). Therefore, vascular degeneration is induced through cell death and the subsequent disruption of blood vessels. Interestingly, cellular dysfunction with p53 activation, protein oxidation or increased content of aldehyde adducts have been observed in cerebrovascular tissue of AD, as well as increased formation of ammonia in plasma and cerebral tissue of AD patients (de la Monte *et al.* 2000; Hensley *et al.* 1995; Lovell *et al.* 2001; Seiler 2002). All these features coincide with SSAO activity effects. To study the effects and behaviour of SSAO in CAA conditions, an *in vitro* model that consists of treating vascular cells with Aβ has been used. We have used the 40 amino acids length Aβ, since it is the major form deposited on blood vessels rather than the 42 length (Castano *et al.* 1996), and we have used it with the Dutch mutation, since it has been associated to severe CAA pathology (Herzig *et al.* 2004).

Results showed that Aβ toxicity was not modified by SSAO presence or activity. However, Aβ presence increased the cell toxicity observed after methylamine oxidation by SSAO activity on endothelial and smooth muscle cells. This phenomenon may be probably mediated by different mechanisms in the two vascular cell types, since an increase in SSAO protein and activity was observed in endothelial cells but not in smooth muscle cells. In smooth muscle cells, the Aβ-induced toxicity could join to the toxicity produced by the SSAO activity products, thus inducing a higher toxic effect. By contrast, the metabolism of methylamine did not induce toxicity on endothelial cells, suggesting that another mechanism could mediate this effect. The increase of the SSAO protein and activity observed after Aβ treatment in endothelial cells, suggested that this mechanism could be related to the previously cited hypothesis about the

importance of exceeding a threshold of activity to induce toxicity (Yu *et al.* 2006). Thereby, the increase in the SSAO activity produced by the Aβ presence would induce a generation of enough toxic products to exceed this threshold and promote cell toxicity.

Increases in protein expression are not expected to occur in this model, since SSAO expression is under a constitutive expression promoter. However, in a similar way, an increase in SSAO/VAP-1 protein availability was also described under transfected conditions in transgenic mice, where it was explained by an increase of the glycosylation process that would reduce the rate of protein degradation (Gokturk *et al.* 2004). Thus, the cause of the different behaviour observed in both vascular cells could be related with the induction of different levels of glycosylation in response to Aβ treatment, or also to the generation of oxidative stress by this treatment, which appears only in endothelial cells. Thus, we hypothesize that the presence of Aβ itself could induce part of the increase of the SSAO protein observed in the vascular tissue of CAA-AD patients, by reducing its degradation process. This increase would probably occur at advanced pathological situations, but also at earlier conditions during the process of Aβ deposition, enhancing the potential SSAO toxicity and contributing to the initial steps of vascular degeneration. This mechanism alone, however, does not explain the higher increase of SSAO observed in all the cerebrovascular tissue of CAA-AD patients, so other mechanisms of SSAO expression induction may be also involved.

In this regard, the inflammatory stimulus induced by Aβ or by a massive oxidative stress could contribute to the SSAO/VAP-1 expression increase. As example, Aβ has shown to induce the activity of Nf-κB (Behl *et al.* 1994), which has a binding site in the SSAO/VAP-1 promoter (Bono *et al.* 1998) and in other proinflammatory proteins as VCAM-1, ICAM-1 or E-selectin. Moreover, this induction of SSAO/VAP-1 expression has been observed in endothelial cells *in vivo,* in response to the inflammatory signals IL-1, IL-4, IFN-γ, LPS or TNF-α, but smooth muscle SSAO does not respond to these signals (Arvilommi *et al.* 1997). Again, different mechanisms might explain the SSAO induction in endothelial and in smooth muscle cells in CAA-AD conditions. Since SSAO in endothelial and in smooth muscle cells has shown several differences, including the induction of its expression, it is possible that stimulus that do not induce the expression of endothelial SSAO/VAP-1 would induce the expression of the smooth muscle SSAO. Moreover, these stimulus may not be proinflammatory, since smooth muscle SSAO does not have the ability to bind lymphocytes (Jaakkola *et al.* 1999). In this concern, the upregulation of the transcription factors myocardin and serum response factor

(SRF), which has been observed in cerebral smooth muscle cells in AD conditions, could explain the SSAO expression increase in these cells. The upregulation of these molecules induces arterial hypercontractility, potentiating a reduction of the cerebral blood flow (Chow *et al.* 2007), an abnormality that has been observed in mild cognitive impairment (MCI) patients and in early AD pathogenesis through magnetic resonance imaging (Johnson *et al.* 2005). It has been also associated to a decrease in Aβ clearance through lipoprotein receptor related protein 1 (LRP1) transcriptional repression. Myocardin acts a co-activator of SRF that binds to a CArG box localized in the promoter of several genes involved in the vascular smooth muscle cell differentiation program (Chen *et al.* 2002; Li *et al.* 2003). SSAO is involved in smooth muscle differentiation (El Hadri *et al.* 2002; Owens 1995), and it has been shown to contain the CArG box in its gene promoter (Sun *et al.* 2006). Moreover, the upregulation of myocardin and SRF is not due to oxidant stress, inflammation or Aβ-related smooth muscle cell injury, which is in agreement with the different regulation of SSAO expression in endothelial and in smooth muscle cells. By contrast, myocardin and SRF upregulation has been shown to respond to hypoxic conditions (Reynolds *et al.* 2004). In this regard, it would be interesting if SSAO expression is induced in hypoxic conditions.

This work has shown the involvement of SSAO in CAA-AD through the toxicity induced by the products generated during its enzymatic activity, but also through its ability to modify the aggregation of Aβ. Results have shown an increase in Aβ deposition on cell cultures by the presence and activity of SSAO/VAP-1. The activity-dependent enhancement of Aβ deposition was observed before (Jiang *et al.* 2008), and the involved mechanism seems to be related with the production of cross-linking molecules such as formaldehyde. In this concern, formaldehyde is able to enhance Aβ β-sheet formation, oligomerization and fibrillogenesis, and also advanced stable aggregates with other proteins (Chen *et al.* 2006).

The activity-independent mechanism of Aβ-deposition enhancement, however, was not described before. In this concern, Aβ binding to SSAO could be mediated by free NH_3^+ residues as lysine, arginine or glutamine as it was suggested to happen with free amino sugars of proteins in SSAO/VAP-1-leukocyte binding (Jalkanen and Salmi 2008; Salmi *et al.* 2001), although this hypothesis needs to be confirmed. The demonstration of the ability of SSAO/VAP-1 to bind peptides displaying free amino groups of lysine, however, supports this hypothesis (Yegutkin *et al.* 2004). This dual function of SSAO in promoting Aβ aggregation could take importance in CAA development, where Aβ could increase the SSAO expression by different mechanisms, and SSAO would enhance

the Aβ deposition, all in an environment in which Aβ clearance would be decreased and high concentrations of Aβ would be found locally, enhancing its toxic effects and the toxic effects of SSAO activity, as it has been shown by the results obtained. Regarding this results, it would be interesting to study whether there are differences in SSAO dependent Aβ deposition between wild type Aβ and Dutch Aβ, since the second has an added glutamine residue suitable for interact with SSAO.

The results obtained *in vitro* and the previous results obtained by plasma SSAO activity determination in AD patients suggested us that SSAO activity could be a marker of dementia or vascular damage. To probe this hypothesis, another objective of the work was to increase the number of analyzed AD patients to confirm the previous results obtained (del Mar Hernandez *et al.* 2005), and to determine the plasma SSAO activity levels in other types of dementias with associated vascular pathology. Results obtained with a higher population of AD patients confirmed that plasma SSAO activity is increased only in the severest dementia conditions. Moreover, SSAO activity was age and sex-independent, although women displayed a trend to have higher SSAO activity. Opposite results regarding the association of SSAO activity with sex were obtained in transgenic mice overexpressing SSAO/VAP-1, where males displayed higher plasma SSAO activity (Stolen *et al.* 2004). This difference could be explained by species difference; however, the age of the individuals could also be involved, since several proteins related with the cardiovascular system are lower in young women than in young men, but this situation is inverted at advanced ages, after women reaching the menopause (Azad *et al.* 2007; Khalil 2005; Mercuro *et al.* 2003; Sattler *et al.* 2005).

The differentiation of the AD patients analyzed considering their affectation by other associated pathologies in which plasma SSAO has been shown increased (such as diabetes, congestive heart failure or cerebrovascular pathology), confirmed the association of plasma SSAO activity with AD severity. Moreover, these results showed also an additive effect of SSAO activity increase in patients that were afflicted by advanced conditions of AD dementia and vascular pathology associated to SSAO increases. These results suggested that AD severity is higher in patients with specific vascular pathology, which is associated to an SSAO increase, thus involving this enzyme in the pathology. However, SSAO usefulness as AD biochemical marker is discarded since the increase is only seen at very severe conditions.

On the other hand, the analyses of plasma SSAO activity performed in vascular dementia (VaD) and in mixed dementia (MD) patients did not show significant differences depending on the severity of the dementia conditions, even though these

pathologies show clear vascular damage. These results indicated that plasma SSAO activity was not associated to vascular damage, but the lack of a severe demented group of patients does not allow us to definitively rule out this hypothesis, since only the severe demented group of AD patients shows plasma SSAO activity increase. It would be interesting to analyze VaD and MD severe demented patients as well as to analyze the SSAO/VAP-1 protein amounts in the cerebrovascular tissue of patients afflicted by these dementias, since the absence of plasma SSAO increase does not exclude its involvement in vascular damage under these conditions. In the same way, the determination of plasma SSAO activity in frontotemporal dementia (FTD) patients does not allow us to conclude any role of SSAO in this pathology due to the low number of samples available. It would be necessary to increase in the number of analyzed samples in the future in order to obtain more concluding results.

Taken together, the major achievements of this work can be resumed in the followings:

Two models of vascular cells have been developed and characterized, and they can be a useful tool to study SSAO biochemical aspects but also the SSAO involvement in human diseases at basic levels.

The molecular mechanism of SSAO induced cytotoxicity has been partially described and these data can be taken into account in future studies involving the vascular system.

An association between SSAO/VAP-1 and Aβ has been found, which contribute to elucidate part of the role of this enzyme in AD, and opens new perspectives in the study of SSAO/VAP-1 in AD and in other cerebrovascular and systemic diseases. These studies will be necessary in order to know the causes that induce the expression of this protein in specific pathological conditions.

As conclusion, the role of SSAO/VAP-1 in CAA-AD pathology is summarized in the following diagram (figure 30):

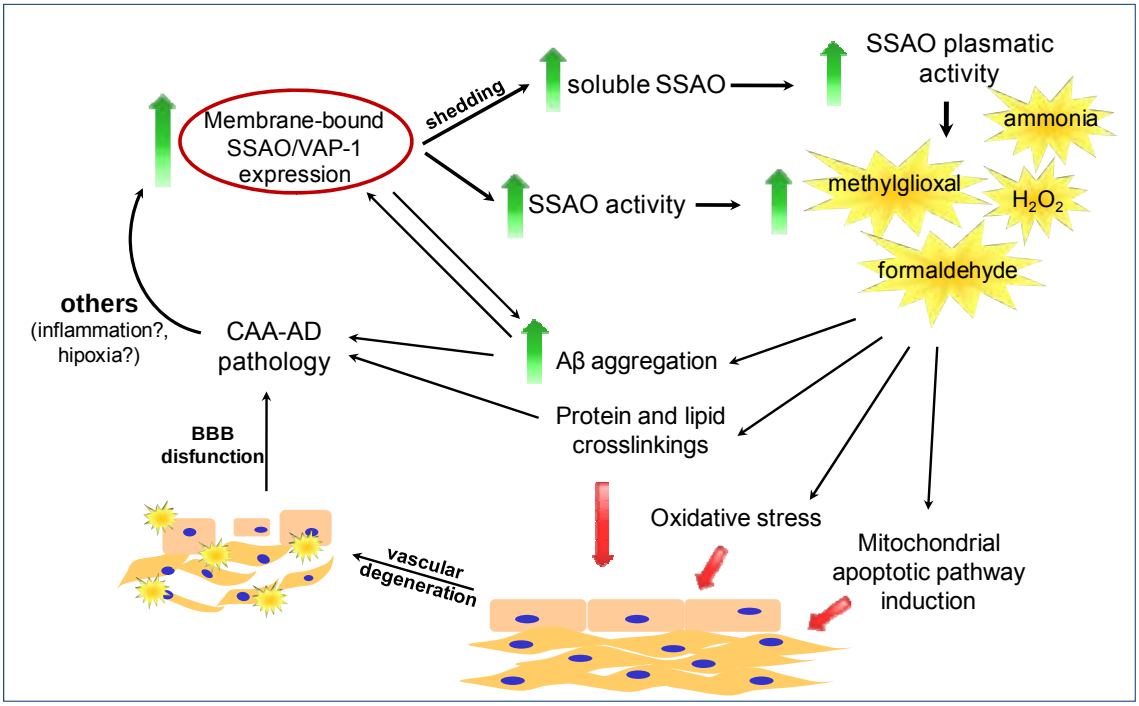


Figure 30. Diagram of the hypothesized role of SSAO/VAP-1 in vascular damage associated to CAA-AD pathology. Membrane-bound SSAO/VAP-1, and its released soluble form would generate toxic metabolites through its enzymatic activity; these metabolites would induce toxic effects on vascular cells, as well as increase the Aβ aggregation, both contributing to the vascular degeneration process observed in CAA-AD pathology; on the other hand, Aβ aggregation would increase the SSAO/VAP-1 protein availability, that would reinforce its toxic effects and also enhance the Aβ deposition on blood vessels by activity-independent mechanisms. In addition, other mechanisms would induce the cerebrovascular SSAO/VAP-1 expression, reinforcing all the explained process; inflammatory signals or hypoxia conditions, could mediate this transcriptional-dependent SSAO/VAP-1 increase although others could be involved. SSAO/VAP-1, semicarbazide sensitive amine oxidase/vascular adhesion protein 1; CAA-AD, cerebral amyloid angiopathy related to Alzheimer's disease; H_2O_2, hydrogen peroxide; Aβ, beta amyloid; BBB, blood brain barrier.

VI. CONCLUSIONS

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Les conclusions generals que es poden extreure del treball presentat són les següents:

1. La SSAO transfectada en les línies cel·lulars A7r5 i HUVEC mostra uns nivells d'expressió, activitat i localització subcel·lular equivalents als observats en condicions fisiològiques, per tant, aquestes línies cel·lulars poden constituir un model d'estudi de la SSAO en múscul llis i endoteli respectivament.

2. La compensació d'activitat enzimàtica observada entre la MAO A i la SSAO en les cèl·lules *wild type* i hSSAO/VAP-1 no s'aboleix mitjançant la inhibició d'aquests enzims.

3. L'activitat de la SSAO transfectada en les cèl·lules A7r5 és capaç d'induir toxicitat en aquestes cèl·lules mitjançant el metabolisme del seu substrat metilamina, essent el formaldehid el component més nociu. Aquest efecte, però, no es produeix en les cèl·lules HUVEC transfectades, en les que nivells més baixos d'activitat no semblen suficients per arribar a induir toxicitat.

4. L'activitat SSAO transfectada en les cèl·lules A7r5 respon a l'activació per bicarbonat, la qual incrementa l'efecte tòxic del seu metabolisme.

5. La toxicitat induïda pel metabolisme de la metilamina en les cèl·lules A7r5 hSSAO/VAP-1 genera una mort apoptòtica d'aquestes cèl·lules que és depenent de l'activació de la proteïna p53. En aquest model, la proteïna p53 actua sobre la mitocòndria produint una desregulació del balanç entre Bax i Bcl-2, una expressió de Puma-α, i una activació de les caspases 9 i 3.

6. El tractament de les cèl·lules A7r5 i HUVEC amb $A\beta_{1-40}$ D indueix citotoxicitat depenent de la generació de peròxid d'hidrogen en les cèl·lules endotelials però independent d'aquest en les de múscul llis.

7. El cotractament amb $A\beta_{1-40}$ D i metilamina produeix un increment de la toxicitat induïda per l'activitat de la SSAO en les cèl·lules A7r5 hSSAO/VAP-1, i indueix l'aparició de toxicitat depenent de l'activitat SSAO en les cèl·lules HUVEC hSSAO/VAP-1.

8. El tractament amb $A\beta_{1-40}$ D indueix un increment de l'activitat SSAO i de la quantitat d'aquesta proteïna en les cèl·lules HUVEC hSSAO/VAP-1, fet que podria explicar l'aparició de toxicitat pel metabolisme de la metilamina, però aquests canvis no es produeixen en les cèl·lules A7r5 hSSAO/VAP-1, en les quals l'increment de toxicitat s'hauria d'explicar per un efecte conjunt dels productes tòxics generats i l'$A\beta_{1-40}$ D.

9. El tractament amb $A\beta_{1-40}$ D genera dipòsits extracel·lulars d'Aβ en els cultius de cèl·lules A7r5 i HUVEC, els quals es formen majoritàriament durant les tres primeres hores de tractament.

10. La SSAO/VAP-1 afavoreix el dipòsit d'$A\beta_{1-40}$ D sobre els cultius per mecanismes dependents i independents de la seva activitat catalítica sobre la metilamina.

11. L'activitat SSAO del plasma de pacients afectats per la malaltia d'Alzheimer amb demència severa presenta uns nivells més elevats comparats amb el plasma de subjectes control o en estadis més lleus de la malaltia. Aquest increment no correlaciona amb l'edat ni amb el sexe dels pacients, però és additiu en situacions en les que la demència tipus Alzheimer s'acompanya de patologia en la que s'ha descrit un increment de la SSAO, com la diabetis o les aturades cardíaques.

12. Els nivells d'activitat plasmàtica de la SSAO no mostren diferències entre subjectes control i diferents graus de demència vascular, demència mixta o demència frontotemporal. Tot i així, per descartar la implicació de la SSAO en aquestes patologies seria necessari ampliar l'estudi aconseguint mostres de pacients amb demència severa afectats per aquestes patologies, així com incrementar el nombre de mostres en el cas de la demència frontotemporal,

13. La SSAO està implicada en la demència tipus Alzheimer a diferents nivells, contribuint a l'agregació de l'Aβ, a la generació d'estrès oxidatiu i a la degeneració vascular a través de la seva activitat catalítica. De totes maneres, serà necessari prosseguir amb aquests estudis per poder determinar la rellevància d'aquesta implicació en els diferents estadis de la malaltia.

VI. CONCLUSIONS

The results obtained in this work allow us to draw the following conclusions:

1. Expression levels, activity levels, and subcellular localization of the transfected SSAO/VAP-1 protein in A7r5 and HUVEC cell lines display similar features to the observed in physiological conditions, indicating its possible utilization as endothelial and smooth muscle cellular models in which to study the SSAO/VAP-1 protein.

2. The enzymatic activity compensation observed between MAO A and SSAO in wild type and transfected cells is not suppressed through enzymatic inhibition.

3. The activity of the transfected SSAO is able to induce cytotoxicity in A7r5 cells through the metabolism of its substrate methylamine, being formaldehyde the most harmful generated product. The lower SSAO activity levels observed in HUVEC transfected cells, however, seem to be insufficient to induce the same effect.

4. The activity of the transfected SSAO in A7r5 cells responds to activation by bicarbonate, which increases the toxic effect induced by its metabolic activity.

5. The SSAO-dependent methylamine metabolism in A7r5 transfected cells induces its apoptotic cell death, which is dependent on p53 activation. In this model, p53 has effects on the mitochondria inducing a Bax/Bcl-2 ratio deregulation, Puma-α expression and a subsequent activation of caspases 9 and 3.

6. A7r5 and HUVEC $A\beta_{1-40}$ D treatment induces a hydrogen peroxide-dependent cytotoxicity in HUVEC cells but it is hydrogen peroxide-independent in A7r5 cells.

7. Methylamine and $A\beta_{1-40}$ D co treatment induces an increase of the SSAO-dependent toxicity in A7r5 transfected cells, and induces the appearance of SSAO-dependent toxicity in HUVEC transfected cells.

8. $A\beta_{1-40}$ D treatment induces an increase of the SSAO activity and protein amount in transfected HUVEC cells. This effect could be the responsible of the appearance of SSAO-dependent toxicity after Aβ-methylamine co treatment. However, these changes do not occur in A7r5 cells, in which the toxicity increase observed after the co treatment may be explained by a joint effect of the toxic products and the $A\beta_{1-40}$ D.

9. The $A\beta_{1-40}$ D treatment forms extracellular Aβ deposits on A7r5 and HUVEC cultures, which are mainly formed during the first three hours of treatment.

10. SSAO/VAP-1 enhances the $A\beta_{1-40}$ D deposition on cellular cultures by both activity-dependent and independent mechanisms.

11. The plasma SSAO activity of severe demented Alzheimer's disease patients show increased activity levels compared to control subjects or less severe demented patients. This increase is not associated to age or gender, but it is additive in conditions where Alzheimer's disease pathology is accompanied by disorders in which an increase in plasma SSAO activity has been described, such as diabetes or congestive heart failure.

12. The plasma SSAO activity levels do not show significant differences between control subjects and increasing conditions of vascular dementia, mixed dementia or frotntotemporal dementia. However, it would be necessary to extend the study by obtaining samples of severe demented patients afflicted by these pathologies, and to increase the number of frontotemporal samples in order to rule out the involvement of SSAO in these pathologies.

13. SSAO is involved in AD at distinct levels, contributing to the Aβ aggregation, to the oxidative stress generation and to the vascular degeneration through its catalytic activity. However, it would be necessary to follow with these studies in order to determine the importance of this involvement in the different phases of the pathology.

VII. BIBLIOGRAFIA

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VIII. ANNEXOS

ANNEX I: Relació dels anticossos utilitzats en aquest treball.

Relació d'anticossos primaris utilitzats en aquest treball

Antígen	Espècie	Utilització	Dilució	Casa comercial	Referència
Aβ	mouse	IF	1:200	Sigma	A5213
Aβ	rabbit	IP	1 µl/assaig	Sigma	A8326
APP 20.1	mouse	WB	1:1000	W.E. Van Nostrand	-
β-Actina	mouse	WB	1:20000	Sigma	A1978
Bax	rabbit	WB	1:1000	Cell Signaling	2772
Bcl-2	mouse	WB	1:1000	BD Biosciences	610538
Casp-3 cleav	rabbit	WB	1:1000	Cell Signaling	9661
Casp-9 cleav	rabbit	WB	1:1000	Cell Signaling	9507
Flotilina	mouse	WB	1:1000	BD Biosciences	610820
GAPDH	mouse	WB	1:40000	Ambion	4300
GRP78	mouse	WB	1:1000	BD Biosciences	610978
IGFrec-Iβ	rabbit	WB	1:1000	Sta. Cruz	sc-713
p-p53 $^{Ser\ 15}$	rabbit	WB	1:1000	Cell Signaling	9284
p53	mouse	WB	1:1000	Abcam	Ab28
PUMA	rabbit	WB	1:1000	Cell Signaling	4976
SSAO bovina	rabbit	WB	1:1000	(Lizcano *et al.* 1998)	-
Rec. Transferrina	mouse	WB	1:1000	ZYMED	136800
VAP-1 C-term	goat	WB-IF	1:500-1:100	Sta. Cruz	sc-13741
VAP-1 H43	rabbit	WB-IF	1:1000-1:100	Sta. Cruz	sc-28642

Relació d'anticossos secundaris utilitzats en aquest treball

Antígen	Conjugat	Utilització	Dilució	Casa comercial	Referència
goat IgG	HRP	WB	1:2000	Pierce	HB987316
mouse IgG	HRP	WB	1:2000 o 1:5000*	Dako Cytomation	P0161
rabbit IgG	HRP	WB	1:2000	BD Biosciences	554021
rabbit IgG	Alexa Fluor® 594	IF	1:1000	Molecular Probes	A11012
goat IgG	Alexa Fluor® 488	IF	1:1000	Molecular Probes	A11055
mouse IgG	Alexa Fluor® 594	IF	1:1000	Molecular Probes	A11005

* En cas de proteïnes molt abundants, com ara les utilitzades per fer controls de càrrega.

ANNEX II: Procediment per a l'obtenció de l'anticòs anti-APP 20.1 a partir dels hibridomes 20.1

(gentilment cedits pel Dr. W.E. Van Nostrand, Stony Brook University, NY, USA)

Manteniment, subcultiu i emmagatzematge dels Hibridomes 20.1

Els hibridomes 20.1 creixen majoritàriament en suspensió, però una part s'adhereix a la superfície de la placa. El medi de cultiu utilitzat per al manteniment i creixement d'aquests hibridomes és *Dulbecco's Modified Eagle's Medium* (DMEM) amb un alt contingut en glucosa (4.5 g/l) (D-5796, Sigma-Aldrich), suplementat amb un 10 % (v/v) de sèrum específic per hibridomes (FCI) (*Fetal Clone I*, HyClone Labs), un 1% d'aminoàcids no essencials (Sigma-Aldrich), 100 U/ml de penicil·lina i 100 µg/ml d'estreptomicina (PAN Biotech). Les cèl·lules es deixen créixer amb aquest medi en un incubador amb atmosfera humida amb un 95% d'aire i un 5% de CO_2 a 37ºC.

Les cèl·lules es subcultiven cada 2-3 dies, fent una dil·lució 1:4 d'aquestes. Com que creixen en suspensió, no cal utilitzar tripsina per al subcultiu, ja que les cèl·lules es separen de la superfície d'adhesió en colpejar la placa de cultiu; per tant, un cop aixecades les cèl·lules es recullen en un tub per centrifugar-les a unes 800 x *g* durant 5 min, es ressuspenen en medi i es sembra la quantitat adequada en una placa nova amb medi fresc.

Per tal de generar un estoc d'hibridomes congelats, s'amplifica el cultiu fins obtenir el numero de plaques desitjat, a una densitat semblant a que es troba en el moment de fer el subcultiu. Es procedeix de la mateixa manera que per fer un subcultiu, però després de recuperar les cèl·lules per centrifugació, aquestes es ressuspenen directament en Sèrum Boví Fetal (FBS, Invitrogen) amb un 10% (v/v) de dimetilsulfòxid (DMSO, Sigma-Aldrich). La mescla FBS-DMSO es prepara minuts abans del contacte amb les cèl·lules per evitar que l'escalfament que es genera durant el contacte del DMSO amb solucions aquoses afecti les cèl·lules. Un cop resuspeses les cèl·lules, amb uns 2 ml per placa, es dipositen en vials de congelació (criotubs) de 2 ml de capacitat, amb només 1 ml de cèl·lules per vial. Havent transcorregut entre 10 i 20 minuts des de l'addició del medi, els criotubs es col·loquen en una caixa amb sistema de congelació isopropílic (Cryo Freezing container, Nalgene), i aquesta s'introdueix en un congelador a -80ºC perquè les cèl·lules es congelin a una taxa de descens de la temperatura d'1 ºC/min. Després de 24 h a -80 ºC es passen els tubs al tanc de nitrogen líquid per al seu emmagatzematge per llargs períodes de temps.

La descongelació es duu a terme submergint parcialment el criotub en un bany a 37 ºC fins que es descongeli, i introduint el contingut del vial en una placa amb uns 10 ml de medi complet. El medi es canvia a les 24 h per retirar les restes de DMSO.

Protocol d'obtenció de medi amb anticossos anti-APP 20.1 a partir del cultiu dels hibridomes 20.1.

Per obtenir el medi ric en anticòs anti-APP 20.1, un cop les cèl·lules estan per subcultivar es recuperen mitjançant centrifugació com si es procedís per al subcultiu, però en comptes de ressuspendre-les en medi complet es ressuspenen en medi al 0 % d'FCI i es tornen a sembrar totes en una placa amb uns 15 ml de medi al 0% d'FCI. Les cèl·lules es deixen a l'incubador durant 4 dies amb aquest medi per tal que alliberin l'anticòs al medi. Un cop passat aquest temps, es centrifuga el medi d'aquestes per descartar-ne les cèl·lules, se li afegeix PMSF (*phenylmethanesulphonylfluoride*) (0.1 mM final en isopropanol) com a inhibidor de proteases, es torna a centrifugar durant 5 min a unes 800 x *g* i es guarda el medi a -80ºC. Petits volums de medi es poden conservar a 4ºC per al seu ús immediat. El medi s'utilitza com si fos anticòs purificat, es a dir, una dilució 1/1000 del medi en el tampó corresponent es fa servir per exemple per incubar membranes de western blot.

ANNEX III: Altres publicacions

ANNEX III.1

“Soluble Semicarbazide Sensitive Amine Oxidase (SSAO) catalysis induces apoptosis in vascular smooth muscle cells”

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Soluble Semicarbazide Sensitive Amine Oxidase (SSAO) catalysis induces apoptosis in vascular smooth muscle cells

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Abstract

Semicarbazide sensitive amine oxidase (SSAO) metabolizes oxidative deamination of primary aromatic and aliphatic amines. It is selectively expressed in vascular cells of blood vessels, but it is also circulating in blood plasma. SSAO activity in plasma is increased in some diseases associated with vascular complications and its catalytic products may cause tissue damage. We examined the effect of the oxidation of the SSAO substrate, methylamine, on cultured smooth muscle cells. Cell incubation with methylamine plus soluble SSAO, contained in bovine serum, resulted toxic to rat aorta A7r5 and human aortic smooth muscle cells, as measured by MTT reduction. This effect was completely reverted by specific SSAO inhibitors, indicating that the toxicity was mediated by the end products generated. Moreover, SSAO-mediated deamination of methylamine induced apoptosis in A7r5 cells, detected by chromatin condensation, Caspase-3 activation, PARP cleavage and cytochrome *c* release to cytosol. Formaldehyde, rather than H_2O_2, resulted to be a strong apoptotic inducer to A7r5 cells. Taken together, the results suggest that increased plasma SSAO activity in pathological conditions, could contribute to apoptosis in smooth muscle cells, leading to vascular tissue damage.

Keywords: Semicarbazide Sensitive Amine Oxidase; Methylamine; Formaldehyde; Hydrogen peroxide; Apoptosis; Aortic smooth muscle cell

1. Introduction

Semicarbazide Sensitive Amine Oxidase [E.C.1.4.3.6, oxidoreductase (deaminating) (copper-containing), SSAO] constitutes a large family of enzymes present in almost all mammalian species studied. All these enzymes are inhibited by semicarbazide [1,2]. SSAO catalyses the oxidative deamination of primary aromatic and aliphatic amines. Its catalytic action requires oxygen and generates ammonia, hydrogen peroxide (H_2O_2) and the corresponding aldehyde. Aminoacetone and methylamine (MA) are considered the physiological substrates of SSAO [3] and their oxidation generates the toxic end products, methylglioxal and formaldehyde, respectively [4]. SSAO is associated with cell membranes and it is also present in blood plasma [2,5]. Membrane-bound SSAO is predominantly expressed in adipocytes, smooth muscle and endothelial cells from blood vessels [1,2,6].

The physiological role of SSAO is still far from clear and it is considered to be a multifunctional enzyme, depending on the tissue where it is expressed [7]. In adipocytes, SSAO activity stimulates glucose transport, mimicking the insulin effect through the H_2O_2 generated during the catalytic process [8]. In addition, SSAO, also known as vascular adhesion protein-1 (VAP-1) [9], is involved in lymphocytes trafficking [10] and its expression in endothelial cells is induced during inflammation.

Some authors have proposed that soluble SSAO is derived from the membrane-bound enzyme [11,12]. Recently, it has been reported that soluble SSAO is shed from the membrane by a metalloprotease activity in adipocytes [11]. Furthermore, transgenic mouse models expressing human VAP-1 in

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endothelial cells showed that VAP-1 from vascular cells could be the major source of circulating SSAO in mice [12].

Plasma SSAO activity is increased in several pathological conditions; diabetes type I and II [13], congestive heart failure [14] and non diabetic morbid obesity [15], and it has also been implicated in atherosclerosis [16,17]. The products generated by SSAO, formaldehyde and H_2O_2, have been considered a potential risk factor for stress-related angiopathy [18,19]. H_2O_2, a major reactive oxygen species, is the principal generator of oxidative stress, which is widely implicated in several diseases. On the other hand, formaldehyde is a highly reactive aliphatic aldehyde, which is considered to be a powerful inflammatory agent [20]. The combined effect of these products could contribute to vascular degeneration associated to several disease states. Alzheimer's disease (AD) patients exhibit significant cerebrovascular pathology, such as microvascular degeneration affecting smooth muscle cells and endothelial cells, hyalinosis and fibrosis. In this context, we have previously reported that membrane-bound SSAO is overexpressed in the cerebrovascular tissue of AD and CADASIL (Cerebral Autosomal Dominant Arteriopathy with Subcortical Infarcts and Leukoencephalopathy) patients, with the subsequent perturbation of the brain vasculature [21]. Moreover, we have recently reported that soluble SSAO activity is increased in patients suffering of severe AD [22]. There is a need to examine if the increase in SSAO activity is correlated to the vascular damage in this pathology.

Since MA and plasma SSAO levels are increased in certain diseases [23,24], we examined whether soluble SSAO, through its catalytic action, induces cell death in cultured smooth muscle (SMC). As A7r5 cells and HASMC (Human Aortic Smooth Muscle Cells) do not show SSAO activity or expression, bovine serum (BS) with high SSAO activity was used as the source of soluble enzyme. Different amines; MA, tyramine (TYR) and benzylamine (BZ), were used as SSAO substrates.

2. Methods

2.1. Materials

Dulbecco's modified Eagle's medium, fetal bovine serum (FBS), L-glutamine, penicillin, streptomycin and trypsin were obtained from Gibco BRL (Grand Island, NY, USA). Bovine Serum (BS) was from Biosystems (Barcelona, Spain). Methylamine, tyramine, benzylamine, semicarbazide, H_2O_2, formaldehyde, Hoechst 33258 and other chemicals were purchased from Sigma Aldrich (St. Louis, Mo., USA). MDL72974A ((E)-2-(4-fluorophenethyl)-3-fluoroallylamine hydrochloride) was a kind gift from Dr. P.H. Yu (University of Saskatchewan, Saskatchewan, Canada). The primary antibodies used were anti-cleaved Caspase-3 antibody from Cell Signaling (Beverly, MA, USA), anti-PARP from Upstate Biotechnology, Inc. (Lake Placid, NY, USA), anti-cytochrome *c* from BD Biosciences Pharmingen (San Diego, CA, USA) and anti-β-actin from Sigma Aldrich (St. Louis, Mo., USA). The secondary antibodies used were goat anti-rabbit IgG Alexa Fluor 594 from Molecular Probes (Eugene, OR, USA), HRP anti rabbit IgG from BD Biosciences Pharmingen and HRP anti-mouse IgG from Dako Cytomation (Glostrup, Denmark).

2.2. SSAO activity determination

SSAO activity was determined radiochemically at 37 °C as previously described [25] using 100 μM [^{14}C]-benzylamine (3 mCi/mmol, Amersham, UK) as substrate. Samples were preincubated for 30 min at 37 °C with 1 μM L-deprenyl to inhibit possible platelet MAO B contamination. The reaction was carried out at 37 °C in a final volume of 225 μl 50 mM Tris–HCl buffer, pH 9, and stopped by the addition of 100 μl 2 M citric acid. Radiolabelled products were extracted into toluene/ethyl acetate (1:1, v/v) containing 0.6% (w/v) 2,5-diphenyloxazole (PPO) before liquid scintillation counting. Different BS batches were tested and selected only when SSAO specific activity was 60 pmol/min mg protein. SSAO activity in cell treatments is expressed as U/ml (1 Unit produces 1 μmol/min of the catalytic product).

SSAO kinetic constants towards benzylamine, methylamine and tyramine (0.1–10 mM) as substrates were determined using a continuous spectrophotometric method coupled to peroxidase [26]. 4-Aminoantipyrine is oxidized by the hydrogen peroxide formed during amine oxidation and then condenses with vanillic acid to give a red quinone imine dye. The absorbance at 498 nm is proportional to the amount of hydrogen peroxide generated. Product concentrations were measured using a Cary spectrophotometer and K_m and V_{max} values were calculated using the Graph-Pad Prism 3.0 program.

2.3. Cell culture

Human Aortic Smooth Muscle Cells (HASMC) from normal adult thoracic aortas were obtained from control donor heart transplants. Samples were provided by Hospital de la Vall d'Hebron, Barcelona, according to the Rules and Procedures of its Ethics Committee. The smooth muscle cells from aorta were isolated by the explant method and cultured as described previously [27]. Cells were used at passages 3–8 and characterized as smooth muscle by morphologic criteria and expression of smooth muscle -actin. Rat aortic smooth muscle cells A7r5 were obtained from ATCC. Cells were grown in high glucose (4,500 mg/l) DMEM supplemented with 10% (v/v) FBS, 2 mM L-glutamine, 1000 U/mL penicillin, 1000 μg/mL streptomycin. Cells were seeded at 50000 cell/ml and grown at 37 °C in a humidified atmosphere containing 5% CO_2. For experiments, cells were grown for 2 days and starved with DMEM containing 0.2% (v/v) FBS for H_2O_2 or formaldehyde treatments. For MA treatments, cells were replaced with DMEM (0% FBS) containing 5, 10 or 15% (v/v) of Bovine Serum (BS), which corresponds to 3×10^{-4}, 6×10^{-4} and 9×10^{-4} U/ml of SSAO activity, respectively. SSAO inhibitors, semicarbazide or MDL72974A, were coincubated with MA in DMEM supplemented with BS.

2.4. Cell viability

Mitochondrial activity was estimated by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) reduction assay. After treatments, MTT (0.5 mg/ml) was added to cells and after 90 min incubation at 37 °C the medium was replaced by dimethyl sulfoxide. The amount of formazan blue formed after MTT reduction was quantified spectrophotometrically at 560 nm [28].

2.5. Active Caspase-3 immunocytochemistry and detection of apoptotic nuclei using Hoechst 33258 staining

Analysis of active Caspase-3 was performed on treated A7r5 cells seeded on coverslips. Cells were fixed with 4% paraformaldehyde for 30 min at room temperature and washed in PBS containing 0.1% Tween 20. Coverslips were then incubated with a blocking solution containing 1% (w/v) Bovine Serum Albumin. Anti-cleaved Caspase-3 diluted 1:100 in blocking buffer was incubated overnight at 4 °C, washed in PBS/0.1% Tween 20, and thereafter incubated with the secondary antibody anti-rabbit IgG Alexa Fluor 594 diluted 1:1000 for 1 h. For nuclear staining, coverslips were washed in PBS and incubated with Hoechst 33258 (1 μg/ml) for 10 min at room temperature in the dark. Three independent experiments were performed and three fields of each treatment in duplicate were counted. At least 1000 cells were counted for each treatment.

2.6. Detection of PARP cleavage and cytochrome c release by Western Blot analysis

For cleaved PARP detection in total cell lysates, cells were washed in cold PBS and lysed in a buffer containing 50 mM Tris–HCl pH 6.8, 10%

glycerol, 2% SDS, 10 mM dithiothreitol and 0.01% bromofenol blue. To detect cytochrome *c* release, cells were harvested in 250 μl of ice-cold buffer containing 50 mM Tris–HCl (pH 7.4), 1 mM EDTA, 1 mM dithiothreitol, complete protease inhibitor, and 250 mM sucrose, after washing once in cold PBS. Cells were incubated for 30 min on ice and then disrupted by douncing 10 times with a tight pestle in a 7 ml Weathon douncer. After centrifugation at 800×*g* for 10 min at 4 °C, supernatants were centrifuged at 20,000×*g* for 40 min at 4 °C. The resulting supernatants were saved as cytosolic extracts. Protein determinations were made using the Bradford method. Samples were size fractionated by SDS-PAGE and transferred to nitrocellulose membranes. Membranes were blocked for 1 h with Tris-buffered saline (TBS), 0.1% Tween 20 and 5% (w/v) defatted powdered milk, and incubated overnight with the corresponding antibody diluted 1:1000 in blocking buffer. Membranes were developed using ECL® detection reagents from Amersham Pharmacia Biotech (Piscataway, NJ, USA).

2.7. Statistics

Results are given as means±S.E.M. Statistical analysis was done by one-way ANOVA and further Newman–Keuls Multiple Comparison Test using the program Graph-Pad Prism 3.0. A *P* value of less than 0.05 was considered to be statistically significant.

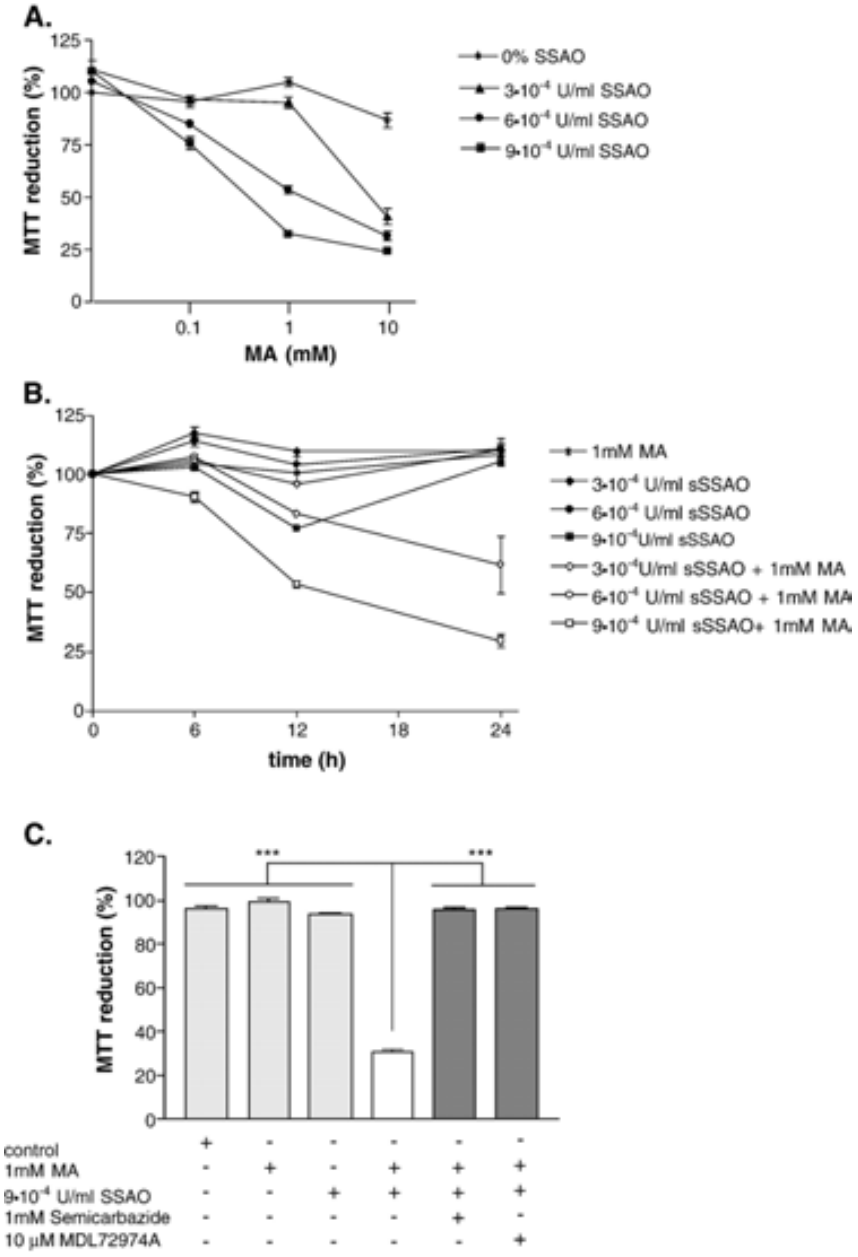


Fig. 1. MA oxidation by soluble SSAO induces cytotoxicity in A7r5 cells expressed as MTT reduction percentages. Cells were incubated with (A) different methylamine (MA) and soluble SSAO concentrations for 24 h (B) 1 mM MA and different soluble SSAO concentrations for 6, 12 and 24 h, and (C) 1 mM MA plus 9×10^{-4} U/ml of soluble SSAO and SSAO inhibitors, 1 mM semicarbazide and 10 μM MDL72974A, for 24 h. Data are mean±S.E.M. values of three separate experiments performed in triplicate. ***$P<0.001$ by a one-way ANOVA test and the addition of Newman–Keuls Multiple Comparison Test.

3. Results

3.1. MA oxidation by soluble SSAO induces cytotoxicity in A7r5 cells

Although the smooth muscle cell line A7r5 showed some MAO-A activity (100 pmol/min mg protein) determined radiochemically by serotonin oxidation, cells did not present MAO-B or SSAO activity assayed towards benzylamine as substrate. These conditions allowed us to study the soluble SSAO catalytic action on vascular cells. For this purpose, a bovine serum (BS) was used as the enzyme source, due to the large homology (81%) between soluble bovine amine oxidase and human SSAO [29]. We first studied methylamine (MA) oxidation by soluble SSAO on A7r5 cell viability, because this aliphatic amine is considered as a common substrate of bovine and human plasma SSAO [30].

A7r5 cells were treated with increasing concentrations of soluble SSAO and MA, and cell viability was measured by the MTT reduction assay. Soluble SSAO (3×10^{-4}, 6×10^{-4} and 9×10^{-4} U/ml) and 1 mM MA did not affect cell viability by themselves. However, 1 mM MA plus 9×10^{-4} U/ml of soluble SSAO induced a 70% decrease of total MTT reduction after 24 h treatment (Fig. 1A). The toxic effect was dose-dependent, obtaining a significant decrease in MTT reduction with 0.1 mM MA plus 6×10^{-4} U/ml or 9×10^{-4} U/ml of soluble SSAO (20% and 25% respectively on MTT reduction percentages). On the other hand, cell viability decrease was time dependent in the range studied (6, 12 and 24 h) (Fig. 1B). In order to confirm that cell viability loss was mediated by the SSAO activity contained in the BS, the experiment was performed using two specific SSAO inhibitors. Enzymatic activity was completely inhibited by 1 mM semicarbazide and 10 μM MDL-72974A, but cell viability was not affected at these concentrations. These SSAO inhibitors completely reverted the loss of cell viability caused by 1 mM MA plus 9×10^{-4} U/ml of soluble SSAO (Fig. 1C).

3.2. Effect of benzylamine and tyramine oxidation by soluble SSAO on A7r5 cells

Other SSAO substrates, benzylamine (BZ) and tyramine (TYR), were tested in the same experimental conditions. Incubation of A7r5 cells with 1 mM TYR and 9×10^{-4} U/ml of soluble SSAO resulted in a 90% decrease of total MTT reduction after 24 h. This effect was almost totally reverted by the presence of SSAO inhibitors (Fig. 2A). MAO inhibition by 1 μM clorgyline did not affect the cell viability, which ruled out the involvement of other amine oxidases activity in the process. In contrast, the non-physiological substrate BZ 1 mM, showed a slight toxic effect by itself, but no significant changes were observed in the presence of soluble SSAO (Fig. 2B). Inhibition of MAO or SSAO activity did not protect the cell death induced by BZ, demonstrating that they were not involved in the cytotoxicity. The enzyme kinetic parameters were determined towards the different SSAO substrates; MA, BZ and TYR. BZ was the best bovine serum SSAO substrate in terms of V_{max}/K_m ratio (Fig 2C), as previously described [30], followed by TYR and MA. In this context, the non cytotoxic effect observed after BZ metabolism suggests that the aldehyde produced by SSAO, rather than H_2O_2 or ammonia, is the main factor responsible for the reduction in cell viability.

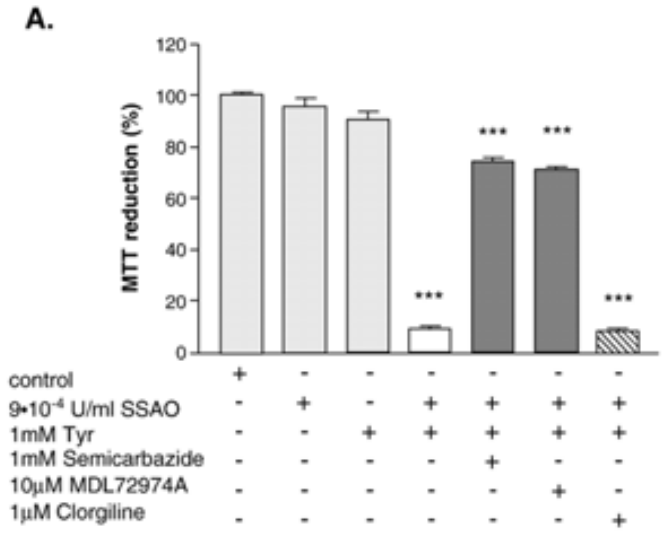
3.3. MA oxidation by SSAO induces apoptosis in A7r5 cells

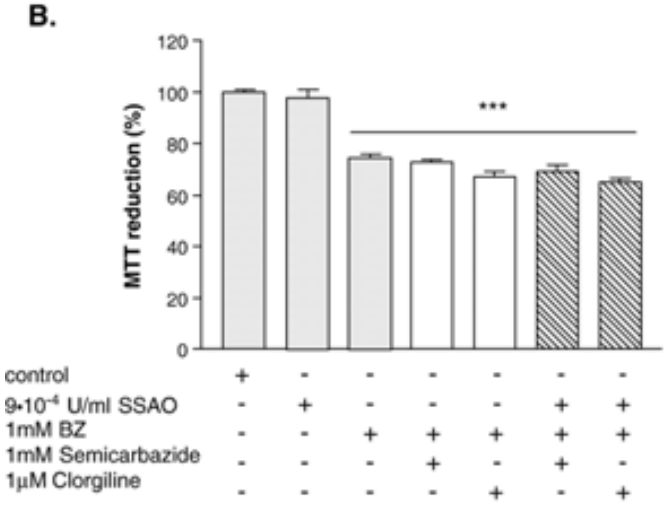
We then evaluated whether apoptosis would be involved in such toxic effect. Fig. 3 shows the double staining with Hoechst 33258 and anti-cleaved Caspase-3 antibody. Cleavage of the executer Caspase-3 into the active form is considered as a classical apoptotic feature. On the other hand, Hoechst 33258 staining display apoptotic cells with condensed, crescentic-aggregated, segmented or fragmented nuclei characteristic of apoptosis. Cell incubations with 1 mM MA or 9×10^{-4} U/ml of soluble SSAO separately for 24 h did not show stained positive cells (Fig. 3D and E). However, co-incubation with 1 mM MA and 9×10^{-4} U/ml of soluble SSAO resulted in numerous cells displaying a strong cytoplasmic red staining for cleaved Caspase-3 (Fig. 3B). The detailed micrograph shows that stained positive cells overlap with condensed nuclei stained with Hoechst 33258 (see Fig. 3B inset). Although 1 mM semicarbazide had no toxic effect by itself (Fig. 3F), it significantly diminished the number of cleaved Caspase-3-positive cells, indicating that the amine oxidase activity mediated the apoptotic process (Fig. 3C). The high percentage of active Caspase-3-positive cells (Fig. 3G) indicated that apoptosis was the main factor responsible for the cell death observed.

We also evaluated other apoptotic features; Poly (ADP-ribose) polymerase (PARP) is one of the essential substrates cleaved by executioner caspases and it is involved in maintaining DNA stability and repair. Western blot analysis revealed PARP cleavage only in cells treated with 1 mM MA plus 9×10^{-4} U/ml of soluble SSAO for 24 h (Fig. 4A), confirming the Caspase-3 activation observed. On the other hand, cytosolic fractions of cells showed a time-dependent increase in cytochrome *c* levels (Fig. 4B), as a consequence of its release from mitochondria to cytosol. Cytochrome *c* appeared after 12 h of soluble SSAO and MA co-treatment, indicating that it could be an early event in the intrinsic apoptosis pathway, before Caspase-3 activation and PARP cleavage determined at 24 h.

3.4. Effect of SSAO catalytic products on A7r5 cells

In order to study the mechanism involved in the toxicity observed, we next analyzed the direct effect of the final products generated by SSAO catalytic activity: H_2O_2, ammonia and different aldehydes (formaldehyde or benzaldehyde, which are the products of MA or BZ oxidation respectively). Considering the oxidative deamination stoichiometry (1:1) of primary amines [30] and our previous assays, which were performed at 1 mM MA, the products concentration range selected for treatments was from 0.1 to 1 mM. When cells were incubated in the presence of ammonia, no toxic effect was observed (Fig. 5A). In contrast, cells incubated with H_2O_2 for 24 h presented a





C.

SSAO substrates	Vmax (nmol/min)	Km (mM)	Vmax/ Km
Methylamine	7.32 ± 0.44	1.04 ± 0.28	7.04
Benzylamine	86.79 ± 5.22	0.85 ± 0.13	102.11
Tyramine	11.85 ± 0.73	0.42 ± 0.11	28.21

Fig. 2. Effect of different SSAO substrates oxidation in A7r5 cells. Cells were incubated for 24 h with (A) 9×10^{-4} U/ml of soluble SSAO plus 1 mM benzylamine (BZ) and SSAO inhibitors, 1 mM semicarbazide and 10 µM MDL72974A, or MAO-A inhibitor, 1 µM clorgyline, (B) 9×10^{-4} U/ml of soluble SSAO plus 1 mM tyramine (TYR) and 1 mM semicarbazide, 10 µM MDL72974A or 1 µM clorgyline. Data are mean±S.E.M. values of three separate experiments performed in triplicate. Statistically differences expressed as ***$P<0.001$ by a one-way ANOVA test and the addition of Newman–Keuls Multiple Comparison Test compared to control samples. (C) Kinetic constants of soluble SSAO towards BZ, MA, TYR as substrates (0.1, 0.5, 1, 5, 7.5, 10 mM) determined spectrophotometrically following the H_2O_2 generated.

slight decrease in cell viability, reaching a 40% decrease of total MTT reduction at 1 mM H_2O_2 (Fig. 5B). When formaldehyde was assayed at the same concentration range, the percentage of MTT reduction decreased drastically at much lower concentrations, compared to H_2O_2 treatment (Fig. 5C). Formaldehyde 0.5 mM induced almost 100% loss of cell viability. On the other hand, benzaldehyde treatment did not show changes in cell viability by itself (Fig 5D), demonstrating that BZ oxidation did not induce cytotoxicity because of the inert effect of the aldehyde generated.

We then examined whether the cytotoxic products induced apoptosis in A7r5 cells. Although it has been widely reported

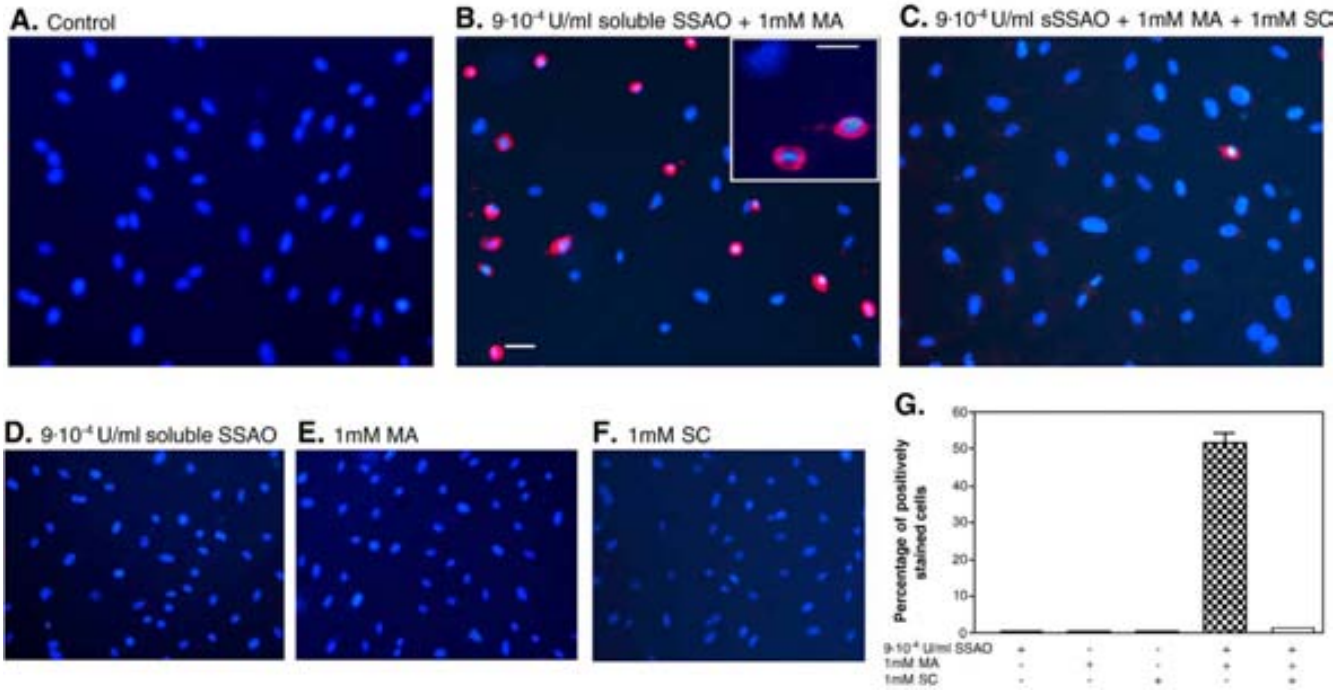


Fig. 3. MA oxidation by soluble SSAO induces Caspase-3 activation and nuclei condensation in A7r5 cells. Representative immunostaining with anti-cleaved Caspase-3 antibody and Hoechst 33258 of (A) control, (B) 9×10^{-4} U/ml of soluble SSAO+1 mM MA, (C) 9×10^{-4} U/ml of soluble SSAO+1 mM MA+1 mM semicarbazide (SC), (D) 9×10^{-4} U/ml of soluble SSAO, (E) 1 mM MA, and (F) 1 mM SC, for 24 h. Scale bar=50 μm in A–E and F and 25 μm in B inset. (G) Percentages of cleaved Caspase-3-positive cells. Data are mean±S.E.M. values of two separate experiments performed in triplicate.

that H_2O_2 induces such a cell death in many cell types, including smooth muscle cells [31], it was not known if formaldehyde was able to induce apoptosis in this cell type. 0.5 mM formaldehyde treatment for 24 h induced a 75% cleaved Caspase-3 stained cells (Fig. 6A). Hoechst 33258 staining of positive cells also revealed the characteristic condensed morphology of apoptotic nuclei. In contrast, when cells were treated with H_2O_2 in the same experimental conditions, only 10% of cells were positive for cleaved Caspase-3 cells (Fig. 6B). On the other hand, formaldehyde treatment for 24 h showed a clear PARP cleavage at all concentrations studied (Fig. 7A). Since this is one of the latest events in the apoptosis pathway, cells treated with H_2O_2 only showed PARP cleavage at longer time (42 h) (Fig. 7B).

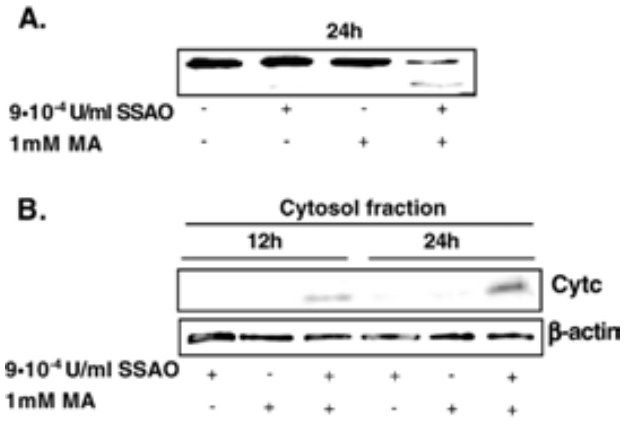


Fig. 4. MA oxidation by soluble SSAO induces apoptosis in A7r5 cells. Representative immunoblot of (A) PARP cleavage after 24 h treatment, and (B) time-dependent (12 and 24 h) cytochrome *c* release to cytosol. β-Actin is used as loading control.

3.5. MA oxidation by soluble SSAO induces cytotoxicity in HASMC

To examine whether the toxic effect induced by MA oxidation was attributable to the cell line, cell viability experiments were performed on primary cultures of smooth muscle cells from human aorta (HASMC). When cells were incubated in the presence of 1 mM MA plus 9×10^{-4} U/ml of soluble SSAO, only a 35% decrease of total MTT reduction was observed at 24 h (Fig. 8), in comparison with the 75% observed on A7r5 cells. These results indicate that cells from the primary culture are more resistant to the toxicity mediated by SSAO metabolic products. SSAO inhibitors, semicarbazide 1 mM and MDL72974A 10 μM, had no toxic effect by themselves, but recovered the loss of cell viability induced by SSAO catalysis. Since these cells do not present membrane-bound SSAO activity, MA oxidized by soluble SSAO was again the only factor responsible for the cell damage.

4. Discussion

Plasma levels of SSAO and its substrate MA are increased in certain diseases [13,22–24]. Although oxidation of the synthetic

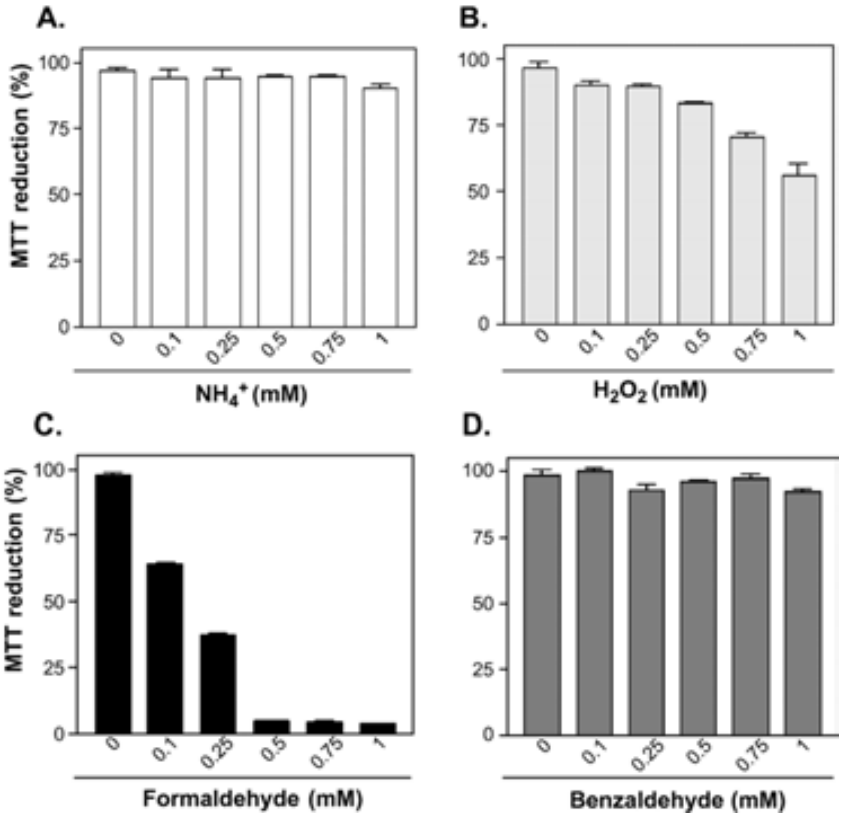


Fig. 5. Catalytic products generated by SSAO activity induce cytotoxicity in A7r5 cells expressed as MTT reduction percentages obtained from the incubation with different concentrations of (A) H_2O_2, (B) NH_4^+, (C) formaldehyde and, (D) benzaldehyde for 24 h. Data are mean±S.E.M. values of three separate experiments performed in triplicate.

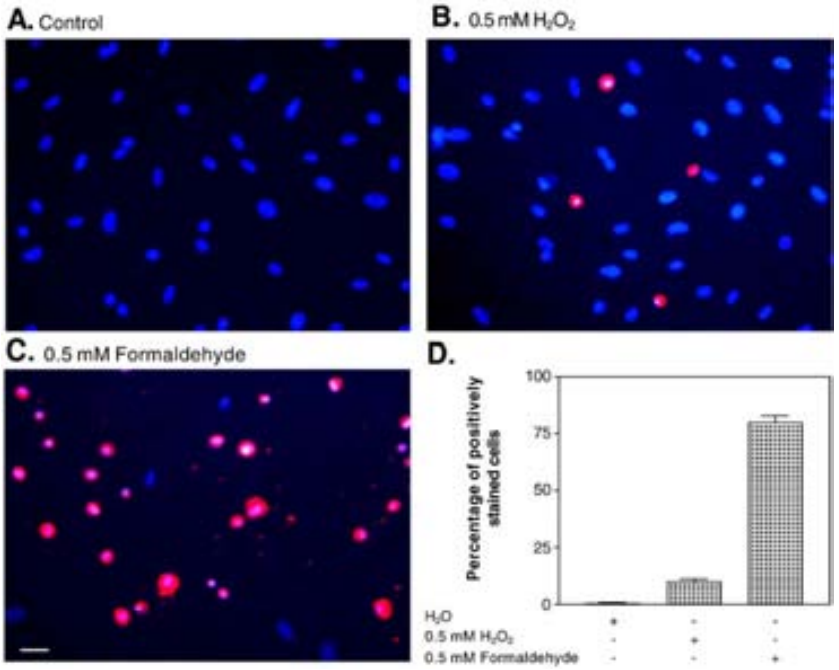


Fig. 6. SSAO cytotoxic catalytic products (H_2O_2 and formaldehyde) induce Caspase-3 activation and nuclei condensation on A7r5 cells. Representative immunostaining with anti-cleaved Caspase-3 antibody and Hoechst 33258 of (A) control, (B) 0.5 mM H_2O_2 and (C) 0.5 mM formaldehyde, after 24 h treatment. Scale bar=50 μm. (D) Percentages of cleaved Caspase-3-positive cells. Data are mean±S.E.M. values of two separate experiments performed in triplicate.

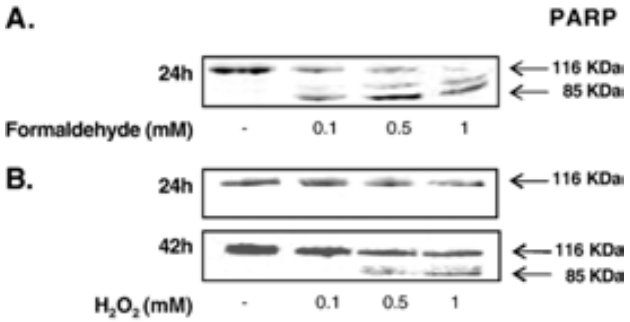


Fig. 7. H_2O_2 and formaldehyde induce apoptosis on A7r5 cells. Representative immunoblot of PARP cleavage after formaldehyde treatment for 24 h and H_2O_2 treatment for 24 and 42 h.

aliphatic amine allylamine is cytotoxic in smooth muscle cells (SMC) [32,33], these cells are resistant to MA toxicity [34], probably because of the low basal SSAO activity in cultured SMC. However, MA was toxic to endothelial cells in the presence of soluble SSAO [35]. Here we examine the effect of MA deamination by circulating SSAO on SMC as a potential risk factor of vascular damage, when the enzyme level is significantly increased in pathological conditions.

Although SSAO is constitutively expressed in vascular smooth muscle [2], A7r5 and HASMC do not show SSAO activity or expression. This could be explained by the difficulty of maintaining a differentiated contractile phenotype in culture. It has been widely reported that vascular SMC show plasticity in vivo and in vitro, which allows them to change phenotype in response to environmental changes [36]. Moreover, the loss of SSAO/VAP-1 expression in vascular cells has also been reported [35,37].

In this study, we show that soluble SSAO plus 1 mM MA was cytotoxic in A7r5 and HASMC cells. Because our experimental model is based on cultured cells, we studied an acute MA treatment for 24 h, using a higher amine concentration than those previously reported in human plasma [23]. Although this MA concentration is outside the physiological range, it has been widely used in cell culture studies [33–35,37]. The cytotoxicity observed was reverted when cells were preincubated with the specific SSAO inhibitors, semicarbazide or MDL 72974A, showing that cell death induction was due to the SSAO catalytic action, discarding other damage sources in the BS used. Furthermore, the catalytic products assayed separately, formaldehyde and H_2O_2, were cytotoxic, while ammonia was not. Formaldehyde, generated by MA oxidation, was much more toxic than H_2O_2 in the same experimental conditions, as reported in endothelial cells [35]. However, free radicals can be generated from formaldehyde and H_2O_2, which may contribute synergically to oxidative stress [38] and vascular damage. Only the oxidation of physiological substrates, MA and TYR, was toxic to A7r5 cells. In contrast, oxidation of the non-physiological substrate, BZ, by SSAO did not affect cultured cells. Because SSAO showed the highest affinity towards BZ, the differences in the cytotoxicity may be attributed to the specific reactivity and the chemical structure of the aldehyde generated. This hypothesis was confirmed by the lack of cytotoxicity obtained with benzaldehyde in comparison with formaldehyde.

Apoptosis of vascular SMC occurs during normal blood vessel development and maturation, but it has also been implicated in vascular disease [39]. Biogenic amines appear to be important for apoptosis triggering through the catalytic action of mitochondrial monoamine oxidase [40]. However, up to now, no direct evidence had shown that circulating SSAO induces apoptosis in SMC. In the present study apoptosis induced by MA oxidation was observed by chromatin condensation, Caspase-3 activation and PARP cleavage. Moreover, the release of mitochondrial cytochrome *c* to cytosol suggests that mitochondrial-mediated apoptosis is involved, probably through the oxidative stress generated by SSAO catalytic action. However, further studies are needed to elucidate whether other mechanisms or apoptotic pathways are also involved.

In pathological conditions, such as neurodegenerative diseases, stroke, traumatic brain injury, atherosclerosis and hypertension, oxidative stress contributes to apoptosis. Among the various factors that can induce oxidative damage, H_2O_2 plays a key role because it is generated in nearly all sources of oxidative stress and can diffuse freely in and out of cells and tissues [41]. In this study, we show that formaldehyde is a stronger inducer of apoptosis than H_2O_2. Formaldehyde can generate covalent interactions with macromolecular constituents in biological samples [42], altering cellular structures and inducing cell death. The ability of formaldehyde to generate cross-linking with proteins, DNA and other macromolecules, could explain the alteration of mitochondrial membrane structures, inducing the opening of the mitochondrial transition pore, promoting cytochrome *c* release to the cytosol, caspase activation and cell death. The apoptotic rate obtained after formaldehyde treatment is consistent with reports of the formation of glucose degradation products, such as methylglyoxal and

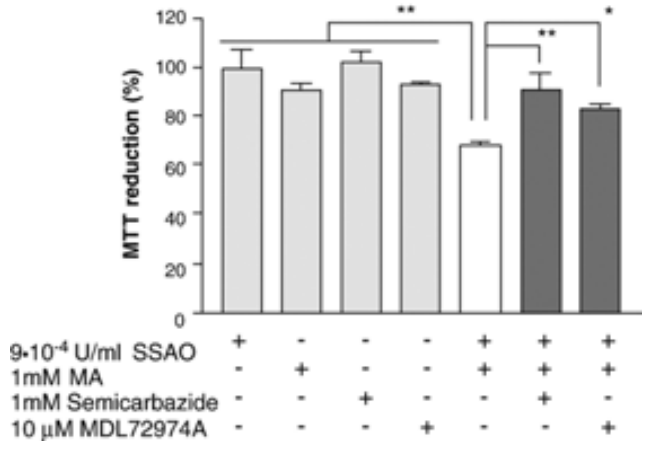


Fig. 8. MA oxidation by soluble SSAO induces cytotoxicity in HASMC expressed as MTT reduction percentages obtained with the incubation of 9×10^{-4} U/ml of soluble SSAO plus 1 mM MA and SSAO inhibitors, semicarbazide and MDL72974A, for 24 h. Data are mean±S.E.M. values of three separate experiments performed in triplicate. $^{**}P<0.01$, $^{*}P<0.05$ by a one-way ANOVA test and the addition of Newman–Keuls Multiple Comparison Test.

formaldehyde, which induced apoptosis in mesothelial cells [43].

Transgenic mice models that express full-length human VAP-1/SSAO in smooth muscle cells [44], endothelial cells or adipose tissue [12], show an increase in hVAP-1/SSAO in serum. This increase is dramatically higher when transgenic animals are treated with experimental diabetes inducers [12]. These results are in agreement with those data previously reported in human dysfunctions [13].The increase of blood MA and the resulting blood formaldehyde is potentially harmful because of the absence of formaldehyde dehydrogenase in blood plasma [45]. In vivo studies corroborate the toxic consequences of MA: chronic MA administration to SSAO/VAP-1 transgenic mice produced vascular complications related to diabetes pathology [46]. Moreover, there is evidence that increased SSAO-mediated deamination of MA contributes to protein deposition, formation of plaques and inflammation [47]. In this concern, transgenic mice overexpressing VAP-1/SSAO in SMC present pathological changes in the elastic fibers of aorta, suggesting the contribution of tissue-bound SSAO in the development of vascular damage [44].

Herein, we report for the first time, at molecular level, that plasma SSAO, through its catalytic action on the physiological substrate MA, induces apoptosis in SMC. The formaldehyde generated by SSAO seems to be the main contributor to cell death, by altering the mitochondria homeostasis and inducing apoptosis. However, a synergic effect of formaldehyde and H_2O_2 cannot be rule out.

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ANNEX III.2

“Semicarbazide Sensitive Amine Oxidase (SSAO) and its possible contribution to vascular damage in Alzheimer’s disease”

Unzeta M, **Solé M**, Boada M and Hernandez M

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Semicarbazide-sensitive amine oxidase (SSAO) and its possible contribution to vascular damage in Alzheimer's disease

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Summary One of the key pathological features of the progressive neurodegenerative disorder Alzheimer's disease (AD) is cerebral amyloid angiopathy (CAA). CAA is present in most cases of AD, and it is characterized by the deposition of β-amyloid (Aβ) in brain vessels, inducing the degeneration of vascular smooth muscle cells and endothelial cells. Herein we report that semicarbazide-sensitive amine oxidase (SSAO) is overexpressed in cerebrovascular tissue of patients with AD-CAA, and that it colocalizes with β-amyloid deposits. This over-expression correlates with high SSAO activity in plasma of severe AD patients. In addition, we have observed that the catalytic activity of SSAO is able to induce apoptosis in smooth muscle cells *in vitro*. Taken together, these results allow us to postulate that SSAO may contribute to the vascular damage associated to AD.

Keywords: β-Amyloid, Alzheimer's disease, cerebral amyloid angiopathy, formaldehyde, hydrogen peroxide, methylamine, semicarbazide-sensitive amine oxidase

Abbreviations

Aβ	β-amyloid
AD	Alzheimer's disease
AGE	advanced-glycation end products
CAA	cerebral amyloid angiopathy
SSAO	semicarbazide-sensitive amine oxidase
VAP-1	vascular-adhesion protein 1

Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disorder of the central nervous system, associated to cognitive impairment and dementia. β-Amyloid (Aβ) accumulation produces the senile plaques in the brain parenchyma characteristic of AD and the vascular deposits of cerebral amyloid angiopathy (CAA). CAA is present in most cases of AD and it is characterized by the deposition of Aβ in the tunica media and adventitia of leptomeningeal vessels and intracortical microvessels, thus producing the degeneration of vascular smooth muscle cells and endothelial cells (Vinters et al., 1988). The fact that AD and cerebrovascular diseases share risk factors supports the common view that there is a link between vascular degeneration and AD. It has been suggested that the accumulation of Aβ in the vessel wall causes the functional deterioration of the blood brain barrier, which is essential for the correct transport and clearance of Aβ from parenchyma (Deane et al., 2004; Zlokovic, 2005).

Semicarbazide-sensitive amine oxidase [E.C.1.4.3.6, amine:oxygen oxidoreductase (deaminating) (copper-containing), SSAO], also known as vascular adhesion protein-1 (VAP-1) (Salmi and Jalkanen, 1992), constitutes a large family of enzymes present in almost all mammalian species studied. SSAO is associated with cell membranes, and it is also present as a soluble form in blood plasma (Precious and Lyles, 1988; Lyles, 1996). The physiological role of SSAO is still far from clear. It is considered as a multifunctional enzyme whose function varies depending on the tissue where it is expressed (O'Sullivan et al., 2004). In adipocytes, SSAO activity stimulates glucose transport, mimicking the effects of insulin (Enrique-Tarancon et al.,

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1998), while in endothelial cells it is involved in lymphocyte trafficking (Smith et al., 1998).

SSAO catalyses the oxidative deamination of primary aromatic and aliphatic amines, producing ammonia, hydrogen peroxide (H_2O_2) and the corresponding aldehyde. Aminoacetone and methylamine are considered the physiological SSAO substrates (Precious et al., 1988), and their oxidation generates and methylglioxal formaldehyde, respectively (Dar et al., 1985). The products generated by SSAO have been considered a potential risk factor for stress-related angiopathy (Yu et al., 1997, 2003; Yu, 2001), due to their capacity to induce lipid peroxidation and the formation of advanced-glycation end products (AGE), as well as to increase the oxidative stress. The combined effect of these products could be important as risk factors in diseases related to vascular degeneration. In this context, it would be relevant to elucidate the role of human cerebrovascular SSAO in both physiological and pathological conditions.

Characterization of human cerebrovascular SSAO/VAP-1 in physiological and pathological conditions related to cerebral amyloid angiopathy (CAA) and Alzheimer's disease (AD)

The presence of SSAO in microvascular tissue and cerebral parenchyma has been a controversial issue (Andree and Clarke, 1981; Dostert et al., 1989; Zuo and Yu, 1994). We have found by biochemical and immunohistochemical approaches that SSAO is present in human microvessels and meningeal vessels, whereas it is absent in neurons and glia (Castillo et al., 1999). The SSAO detected in human microvessels and meningeal vessels, by immunoblotting with polyclonal anti-bovine SSAO antibodies corresponded to a single 100 kDa band, similar to that described for bovine lung membrane-bound SSAO (Lizcano et al., 1998). In addition, we showed immunohistochemically that SSAO is localized in the tunica media and tunica intima of meningeal membranes (Castillo et al., 1998).

The expression of SSAO in human cerebrovascular tissue under physiological conditions suggests that the enzyme could contribute, by its own catalytic action, to the oxidative stress and vascular damage associated to some pathologies. Because oxidative stress has been linked to AD (Behl et al., 1994), we proceeded to characterize the expression of SSAO in microvessels and meningeal vessels from patients afflicted with the disease.

Human brain samples were obtained from the Banc de Teixits Neurològics de l'Hospital Clínic de Barcelona, and meningeal vessels and microvessels were prepared as previously described (Mrsulja et al., 1976). The kinetic behavior of SSAO, performed radiochemically towards

Table 1. *Kinetic constants of SSAO in microvessels and meningeal vessels from AD-CAA patients and controls. SSAO activity was determined radiochemically using benzylamine as a substrate (0, 5, 10, 50 and 100 μM).* $^{**}p < 0.01$*; statistically significant differences, and (NS); not statistically significant differences* ($p > 0.05$) *(one-way ANOVA test followed by the Newman-Keuls multiple comparison test)*

	K_m (μM)	V_{max} (pmol/min · mg)
Microvessels control ($n = 4$)	11.41 ± 0.53	11.20 ± 2.04
Microvessels AD ($n = 4$)	13.86 ± 2.18 (NS)	18.59 ± 2.04**
Meningeal vessels control ($n = 2$)	16.55 ± 1.16	452.65 ± 85.75
Meningeal vessels AD ($n = 4$)	19.25 ± 5.89 (NS)	391.68 ± 87.85 (NS)

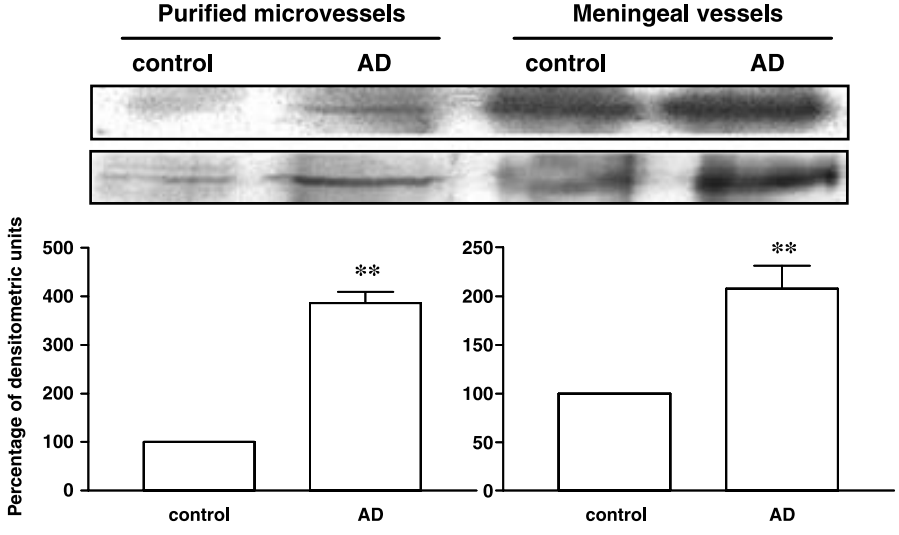

Fig. 1. Western-blot analysis of SSAO in microvessels and meningeal vessels from AD-CAA patients ($n = 2$) and controls ($n = 2$). SSAO contents are expressed as the percentage respect to controls evaluated by a densitometric analysis

benzylamine as substrate, revealed that the V_{max} value for SSAO was about 40 times higher in human meningeal vessels than in pure microvessels preparations, whereas the K_m values were similar (Table 1). The catalytic efficacy, expressed as the V_{max}/K_m ratio, was higher in meningeal vessels than in microvessels, pointing to the possibility of two different forms of SSAO in the human cerebrovascular system. In meningeal vessel preparations, neither SSAO K_m nor V_{max} values were statistically different in AD-CAA patients and controls. In contrast, the V_{max} in microvessel preparations was higher in AD-CAA samples (18.590 ± 2.043 pmol/min · mg protein, $n = 4$) than in controls (11.198 ± 2.043 pmol/min · mg protein, $n = 4$), although the K_m values did not differ (Table 1). SSAO expression in brain vascular preparations was also studied by Western-blotting using polyclonal anti-SSAO antibodies (see Fig. 1). Results, from 2 AD-CAA samples and 2 controls, showed that SSAO was overexpressed in both human meningeal vessels (a 2-fold increase) and microvessels (a 4-fold increase) in AD-CAA patients.

In order to confirm these results, we assessed the expression of SSAO immunohistochemically in 10 *post-mortem* samples from AD patients and 8 control samples (Banc de Teixits Neurològics de l'Hospital Clínic de Barcelona). A moderate to strong selective increase in SSAO immunoreactivity was seen in the AD samples between the intima and the muscular layer of arteries containing amyloid deposits (Ferrer et al., 2002). Double labeling for SSAO and βA4 confirmed the co-localization of the increased SSAO immunoreactivity and the abnormal amyloid deposition, the latter being distributed at the periphery of SSAO deposits. Increased Cu/Zn superoxide dismutase 1 (SOD1) immunoreactivity was also observed, indicating the presence of oxidative stress. Because a strong link between βA4 amyloid, oxidative stress and neurodegeneration has been proposed in AD (Hensley et al., 1995), the results obtained by Western-Blot analysis allow us to suggest that the overexpression of SSAO could contribute to the oxidative stress and the vascular damage related with AD-CAA.

Alteration of human plasma SSAO activity in AD

Soluble SSAO activity is altered in several pathological conditions. Plasma SSAO is increased in patients suffering from diabetes type I and II (Boomsma et al., 1995), congestive heart failure (Boomsma et al., 1997), non-diabetic obesity (Meszaros et al., 1999), and it has been implicated in atherosclerosis (Gronvall-Nordquist et al., 2001; Karadi et al., 2002). Serum SSAO activity is also altered in inflammatory liver diseases (Garpenstrand et al., 1999; Kurkijarvi et al., 2000). Here we studied whether the overexpression of SSAO detected in cerebrovascular tissue of AD-CAA patients (Ferrer et al., 2002) was correlated with altered SSAO in plasma. Previous results reported by our group showed a clear increase of plasma SSAO activity in moderate-severe and severe AD patients (del Mar et al., 2005). The study presented here was performed in a larger number of patients and confirmed the results obtained previously.

Human plasma samples were provided by the Fundació ACE, Institut Català de Neurociències Aplicades (Barcelona, Spain), and SSAO activity was determined radiochemically towards benzylamine as substrate. Controls and AD patients ($n = 164$) did not have any chronic metabolic disease or congestive heart failure, and their ages ranged from 65 to 94 years. All patients were suffering from sporadic Alzheimer dementia, according to NINCDS-ADRA criteria (McKhann et al., 1984). They were distributed in 5 groups based on the Global Deterioration Scale (GDS) (Reisberg et al., 1982): Age-matched control cases ($n = 29$), who were free of neurological disease, mild-D ($n = 52$, GDS = 3–4), moderate ($n = 41$, GDS = 5), moderate-severe ($n = 24$, GDS = 6) and severe dementia ($n = 18$, GDS = 7).

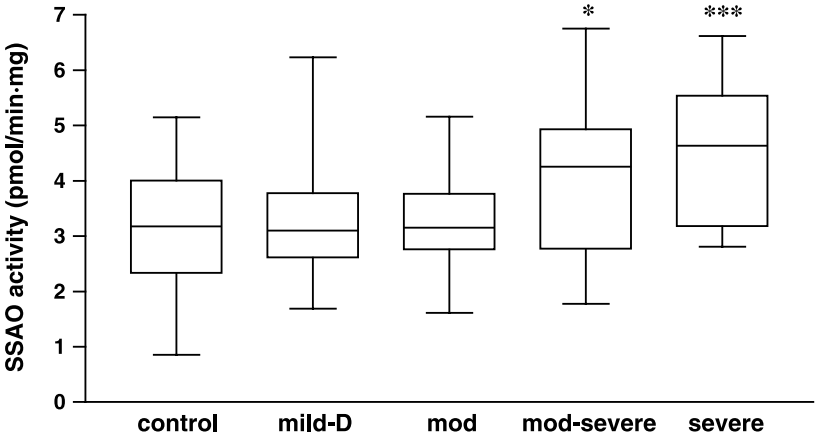

Fig. 2. SSAO activity in controls ($n = 29$) and groups with different level of AD severity classified by GDS criteria; mild-D ($n = 52$), moderate ($n = 41$), moderate-severe ($n = 24$) and severe ($n = 18$). Statistically significant differences are shown as $^{***}p < 0.001$, $^{*}p < 0.05$ (one-way ANOVA test followed by the Newman-Keuls multiple comparison test)

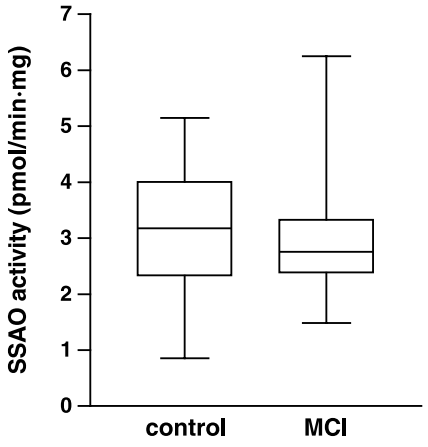

Fig. 3. SSAO activity in controls ($n = 29$) and the mild cognitive impairment group (MCI) ($n = 35$). There are no statistically significant differences between groups

Plasma SSAO specific activity from control samples showed no significant difference compared to mild or moderate patients. However, we observed a clear increase in SSAO activity in plasma from moderate-severe ($p < 0.05$) and severe AD patients ($p < 0.001$) (see Fig. 2), with patient age being an independent correlative factor (data not shown). A number of patients categorized as mild cognitive impairment (MCI) (Jack et al., 1999; Petersen and Morris, 2005) ($n = 35$) were also included in the study (see Fig. 3). MCI includes individuals who are not cognitive normal for age and yet have not evident dementia; MCI is equivalent to a GDS rating of 3. As expected, their plasma SSAO activity was not increased with respect to controls.

Altogether, our results open the possibility that the SSAO overexpression found in AD brain vessels could be the responsible for the increased plasma SSAO activity observed in severe AD patients. This increase may result from increased shedding of SSAO from the cell membrane, since it has been proposed that soluble SSAO is derived from the membrane-bound enzyme (Abella et al., 2004; Stolen et al., 2004b). It has also been reported, from studies with adipocytes, that soluble SSAO is shed from the membrane by a metalloprotease activity (Abella et al., 2004). Furthermore, transgenic mouse models expressing human VAP-1 in endothelial cells showed that VAP-1 from vascular cells can be the major source of circulating SSAO in mice (Stolen et al., 2004b). These data support a possible link between the overexpression of vascular membrane-bound SSAO and alterations of soluble protein levels in pathological conditions, such as AD associated to CAA.

Since SSAO is an adhesion protein whose expression is induced under inflammatory conditions (Arvilommi et al., 1997), an increased SSAO activity in advanced AD could be the result of vascular degeneration and inflammation. Overexpression of membrane bound SSAO by vascular cells, and its release into plasma, could amplify the oxidative stress and contribute to vascular damage in AD. However, further studies are required to elucidate the molecular mechanism that controls the shedding of the membrane-bound enzyme and the possible pathological agents involved.

The catalytic action of SSAO induces apoptosis in smooth muscle cells in culture

To investigate whether the increase of membrane-bound SSAO expression and plasma SSAO activity in AD play a role in cerebrovascular damage, we performed toxicity studies *in vitro*. Under pathological conditions, when both methylamine levels (Yu and Zuo, 1993; Hernandez et al., 2006) and SSAO activity are elevated, its own catalytic action could be an important source of toxicity through the products generated, such as H_2O_2 and formaldehyde. H_2O_2 is a major reactive oxygen species and is the principal generator of oxidative stress, which is widely implicated in several diseases. On the other hand, formaldehyde is a very reactive aliphatic aldehyde, which is considered to be a powerful inflammatory agent (d'A Heck, 1988; Yu and Deng, 1998).

We studied the cytotoxic effect of soluble SSAO, through its catalytic action, on rat aorta A7r5 smooth muscle cells, and in primary cultures of human aorta smooth muscle cells. Bovine serum, which contains high SSAO activity was used as a soluble enzyme source and different amines, methylamine, tyramine and benzylamine, were used as SSAO substrates (Hernandez et al., 2006). Our results confirmed that methylamine and tyramine oxidation by soluble SSAO catalytic action induced cytotoxicity and apoptosis in rat and human smooth muscle cells, which was prevented by SSAO inhibitors, semicarbazide and MDL-72974A. In contrast, benzylamine, a non-physiological SSAO substrate, did not show any deleterious effects. We next evaluated the effect of each of the SSAO reaction products, H_2O_2, formaldehyde and ammonia. Whereas H_2O_2 and formaldehyde were extremely cytotoxic, ammonia was not toxic at any concentration assayed, suggesting that the ammonia produced does not contribute to cell damage. It is very well known that oxidative stress is an underlying factor that contributes to the apoptotic process. Among the diverse factors capable of inducing oxidative stress, H_2O_2 plays a key role because it is generated in nearly all sources of oxidative stress and can diffuse freely in and out of cells and tissues (Barbouti et al., 2002).

However, we found that formaldehyde had a higher apoptotic effect than H_2O_2, suggesting that the formaldehyde produced during methylamine oxidation would be the main contributor to the cell death. Formaldehyde is an extremely reactive aldehyde capable of covalent interactions with macromolecules (Gubisne-Haberle et al., 2004), thus altering cellular structures. It has been previously described as a strong apoptotic inducer in other cell types (Teng et al., 2001; Tyihak et al., 2001). However, under alkaline conditions, free radicals can be generated from formaldehyde and H_2O_2, which may contribute synergistically to oxidative stress (Lichszteld, 1979) and vascular damage.

In order to confirm the cytotoxic effects of SSAO overexpression, we studied whether the methylamine oxidation was able to induce apoptosis in smooth muscle cells transfected stably with hSSAO/VAP-1 gene (Sole et al., manuscript in preparation). Our results confirm that both an increase of soluble SSAO levels and the overexpression of the membrane-bound enzyme were toxic to cells in culture. Furthermore, we observed that the transfection of SSAO produced an increase of soluble SSAO in the culture medium, confirming that in smooth muscle cells the soluble form was a product of the overexpressed protein. Our results are consistent with studies that demonstrated that the administration of methylamine to transgenic mice, overexpressing VAP-1/SSAO to produce vascular complications, increased AGE levels and modificated the progression of atherosclerosis (Stolen et al., 2004a).

In summary, the alteration of SSAO expression and the increase of its physiological substrate levels, in pathological conditions, are important factors that may amplify the vascular damage associated to certain diseases. Taken together, our results prompt us to suggest that an augmented expression and the concomitant increase in catalytic activity of SSAO isoforms observed in severe Alzheimer's disease may play an important role in the vascular damage underlying the AD-CAA disorder. Further studies must be performed in order to elucidate the mechanisms that induce such SSAO overexpression in this neurodegenerative disorder.

Acknowledgements

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ANNEX III.3

“Sodium bicarbonate enhances membrane-bound and soluble human semicarbazide sensitive amine oxidase *in vitro*”

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Sodium Bicarbonate Enhances Membrane-bound and Soluble Human Semicarbazide-sensitive Amine Oxidase Activity *In Vitro*

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Semicarbazide-sensitive amine oxidase (SSAO) is a multifunctional enzyme with different biological roles that depend on the tissue where it is expressed. Because SSAO activity is altered in several pathological conditions, we were interested in studying the possible regulation of the human enzyme activity. It has been previously reported that SSAO activity is increased in the presence of Dulbecco's modified Eagle medium (DMEM) *in vitro*. The aim of the present work was to investigate the effects of the different constituents of DMEM on human SSAO activity. We found that sodium bicarbonate was the only component able to mimic the enhancement of both human aorta and plasma SSAO activity *in vitro*, suggesting a possible physiological role of bicarbonate as an intrinsic modulator of the human enzyme. Failure to take this activating effect into account could also result in inaccuracies in the reported tissue activities of this enzyme.

Key words: activity enhancement, dulbecco's modified eagle medium, semicarbazide-sensitive amine oxidase, sodium bicarbonate, vascular adhesion protein-1.

Abbreviations: DMEM, Dulbecco's modified Eagle medium; MAO, monoamine oxidase; SSAO, semicarbazide-sensitive amine oxidase.

The term 'semicarbazide-sensitive amine oxidase' (SSAO) is generally used to describe those enzymes classified as E.C.1.4.3.6 [amine: oxygen oxidoreductase (deaminating) (copper-containing)]. Semicarbazide inhibition allows SSAOs to be distinguished from monoamine oxidases (MAOs) [amine: oxygen oxidoreductase (deaminating) (flavin-containing); E.C.1.4.3.4 (MAO), which are sensitive to acetylenic inhibitors, such as clorgyline and L-deprenyl, but are less affected by semicarbazide. The substrate specificities of MAO and SSAO overlap to some extent but, whereas MAO catalyzes the oxidative deamination of primary, secondary and some tertiary amines, SSAO activity appears to be restricted to primary amines. Methylamine, which arises from the metabolism of adrenaline, lecithin, sarcosine and creatinine, is metabolized by SSAO from many sources. It has been proposed that methylamine and aminoacetone, which are not MAO substrates, are important physiological SSAO substrates (*1*, *2*).

SSAO is associated with cell membranes in mammalian tissues and is also present in blood plasma (*3*). Membrane-bound SSAO shows high activity in endothelial and smooth muscle cells of blood vessels (*4*, *5*). The soluble SSAO in blood plasma is believed to be derived from the membrane-bound enzyme, and it has recently been reported that soluble SSAO is shed from the adipocyte membrane by a metalloprotease activity (*6*). The physiological roles of SSAO are still far from clear, and it has been described as an enzyme with multifunctional behaviour that depends on the tissue where it is expressed (*7*). SSAO is also known as vascular adhesion protein-1 (VAP-1), which is involved in lymphocytes trafficking, and its expression in endothelial cells is induced during an inflammatory response (*8*).

SSAO activity has been shown to be altered in several pathological conditions. Plasma SSAO is increased in patients suffering from diabetes types I and II (*9*), in patients afflicted by congestive heart failure (*10*), in non-diabetic morbid obesity (*11*), in inflammatory liver diseases (*12*) and in severe Alzheimer's disease (*13*). It has also been implicated in atherosclerosis (*14*) and in the development of diabetic complications (*15*). Furthermore, it has been shown that plasma SSAO can induce apoptosis in smooth muscle cells through its catalytic action on methylamine as substrate, which might contribute to vascular cell damage (*16*) and in the development of diabetic retinopathy (*15*).

Although some factors in human plasma have been reported to modulate platelet MAO activity (*17*, *18*), little is known about the possible modulation of SSAO under physiological conditions.

We have previously described the activation of membrane-bound SSAO from human lung by a low molecular weight component present in human plasma (*19*). In addition, it has also been reported that the standard cell culture medium, Dulbecco's modified Eagle medium

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(DMEM), enhances the SSAO activity present in foetal calf serum *in vitro* (*20*).

The aim of the present work was to investigate the effects of the DMEM and its constituents on human SSAO activity *in vitro*. Here we report, for the first time, that sodium bicarbonate ($NaHCO_3$) is the only component of DMEM able to enhance SSAO activity. The kinetic behaviour of this modulator is reported and its possible physiological role suggested.

MATERIAL AND METHODS

Chemicals—[^{14}C]-Benzylamine was from Amersham (Amersham, UK). MDL72974A ((E)-2-(4-fluorophenethyl)-3-fluoroallylamine hydrochloride) was a kind gift from Dr P. H. Yu (University of Saskatchewan, Saskatoon, Saskatchewan, Canada). DMEM, with or without $NaHCO_3$ (3.7 mg/ml), methylamine, semicarbazide, L-deprenyl and other chemicals were from Sigma-Aldrich (St Louis, MO, USA).

Human Samples—The Ethical Committee of Universitat Autònoma de Barcelona approved the experimental protocol used for human samples in this study.

Dialyzed human plasma

Human plasma samples were obtained from Hospital Universitari de la Vall d'Hebron, Servei d'Hematologia, Barcelona, Spain, and stored at −20°C until use. Plasma was thawed at 37°C and dialyzed against fresh saline solution (1 : 500) overnight at 4°C. Dialyzed samples were stored in aliquots at −20°C.

Human aorta homogenates

Human aorta was obtained from Hospital Universitari de la Vall d'Hebron, Servei de Transplantaments, Barcelona, Spain, and stored in PBS at –80°C until use. For the homogenization process, the tissue was thawed and the tunica media was detached and saved from the rest of the tissue. The endothelial cell layer was removed by rubbing the luminal side of the vessel with a cell scraper. The final homogenate was prepared in phosphate buffer (10 ml:1 g tissue) with a polytron homogenizer. The homogenate was then stored, in aliquots, at −20°C until use.

SSAO activity determination—SSAO activity towards benzylamine as substrate was determined radiometrically at 37°C as previously described (*21*), using 100 μM [^{14}C]-benzylamine (2 mCi/mmol). Samples were pre-incubated for 30 min at 37°C with 1 μM L-deprenyl to inhibit any possible platelet MAO B contamination. The reaction was carried out at 37°C in a final volume of 225 μl in 50 mM phosphate buffer (pH 7.2) and stopped by the addition of 100 μl 2 M citric acid. Radiolabelled products were extracted into toluene/ethyl acetate (1 : 1, v/v) containing 0.6% (w/v) 2,5-diphenyloxazole before liquid scintillation counting.

SSAO activity towards methylamine 500 μM as substrate was determined by following H_2O_2 formation, using a peroxidase-coupled continuous spectrophotometric method (*22*). In this system, 4-aminoantipyrine is oxidized by the hydrogen peroxide (H_2O_2) formed during amine oxidation and then condenses with vanillic acid to give a red quinone imine dye. The absorbance at 498 nm, which was monitored using a Cary spectrophotometer, is proportional to the amount of H_2O_2 generated. SSAO activity is expressed as pmol/min mg protein. All assays were performed in the presence of L-deprenyl 1 μM to ensure the inhibition of any MAO activity. Protein was measured by the method of Bradford, using bovine-serum albumin as standard.

Kinetic studies—The effects of $NaHCO_3$ concentration (0–1 g/l) on SSAO activity towards benzylamine (25–400 μM) were determined without pre-incubation with the enzyme. The pH of $NaHCO_3$ solution was adjusted to 7.0–7.2 with HCl at the beginning of each experiment.

Reversibility studies—The reversibility of the SSAO activation by $NaHCO_3$ was determined by dialysis. Enzyme samples were pre-incubated for 30 min at 37°C with 2 g/l $NaHCO_3$. Samples were then dialyzed using a Centricon Centrifugal Filter (2 ml capacity, 3.0 Molecular Weight-Limit Membrane; Millipore, USA), following the manufacturer's instructions. Briefly, three consecutive washings were performed and samples were centrifuged at 4°C for 30 min between washings. Total protein was measured and SSAO activity was determined as described previously.

Analysis and Statistics—Results were given as means ± SEM. Statistical analysis was done by one-way ANOVA and further Newman–Keuls Multiple Comparison Test using the program Graph-Pad Prism 3.0. A P value of < 0.05 was considered to be statistically significant. K_m and V_{max} values were determined by non-linear regression, using the same program. The double-reciprocal plot is used only for illustrative purposes.

RESULTS

The ability of complete DMEM to enhance SSAO activity towards benzylamine was tested, using two different enzyme sources; circulating SSAO from human plasma and membrane-bound SSAO from human aorta. The basal SSAO activities of the aorta homogenates and plasma were 915.2 ± 95.5 and 0.73 ± 0.02 pmol/min mg protein, respectively. Pre-incubation in the presence of DMEM for 30 min increased the activity of the plasma SSAO 2.48 ± 0.10 times and that of the membrane-bound SSAO 3.43 ± 0.38 times (Fig. 1). In order to elucidate which specific component(s) of DMEM was responsible for the activation effect, each constituent, shown in Table 1, was tested alone. A concentration range of each component, including the corresponding dose present in DMEM, was incubated for 30 min with human plasma or human aorta homogenate before the SSAO activity was assayed towards benzylamine as substrate. $NaHCO_3$ was the only constituent that caused activation, and this was the same as that obtained with complete DMEM. Other inorganic salts, including those containing the sodium cation, did not show any effect on SSAO activity (data not shown). Since the pH of DMEM is 7, all compounds tested were prepared at this pH to avoid possible alterations in the activity determination caused by pH differences.

To confirm that $NaHCO_3$ was the only component responsible for the activation, the same experiments were performed using a mixture equivalent to DMEM

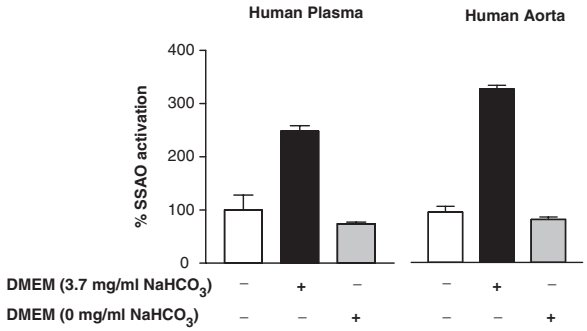


Fig. 1. **$NaHCO_3$ is the only component contained in DMEM responsible of SSAO activity enhancement.** Human aorta and human plasma were pre-incubated for 30 min with 50 µl of DMEM, with or without $NaHCO_3$. SSAO activity was determined towards 100 µM benzylamine as substrate in 50 mM phosphate buffer (pH 7.2). Data are mean ± SEM of three different experiments.

Table 1. **DMEM composition.**

Amino acid (g/l)		Vitamins (g/l)	
L-Arginine·HCl	0.084	Choline chloride	0.4
L-Cysteine·2HCl	0.0626	Folic acid	0.004
Glycine	0.03	Myo-inositol	0.0072
L-Histidine·HCl·H_2O	0.042	Niacinamide	0.004
L-Isoleucine	0.105	D-Pantothenic acid	0.004
L-Leucine	0.105	Pyridoxine·HCl	0.004
L-Lysine·HCl	0.146	Riboflavin	0.0004
L-Methionine	0.03	Thiamine·HCl	0.004
L-Phenylalanine	0.066		
L-Serine	0.042		
L-Threonine	0.095		
L-Tryptophan	0.016		
L-Tyrosine·2Na·2H_2O	0.10379		
L-Valine	0.094		
Inorganic Salts (g/l)		**Others (g/l)**	
$CaCl_2$ ·2H_2O	0.265	D-glucose	1.0
$Fe(NO_3)_3$ ·9H_2O	0.0001	Phenol Red·Na	0.015
$MgSO_4$	0.09767	Pyruvic Acid·Na	0.11
KCl	0.4		
$NaHCO_3$	3.7		
NaCl	6.4		
NaH_2PO_4	0.109		

Note: All components of DMEM were tested separately as possible SSAO modulators and $NAHCO_3$ was the only compoundable to enhance its activity.

with or without $NaHCO_3$. Figure 1 shows that the presence of $NaHCO_3$ in the medium is necessary to enhance both membrane-bound and plasma SSAO activity, suggesting this inorganic compound to be a, previously unrecognized, modulator of the SSAO activity.

Because there are significant concentrations of $NaHCO_3$ in human plasma (about 23 m Eq/l, which corresponds to 1.4 g/l), a prior dialysis process was required to study the net effect of this compound. The SSAO activity in dialyzed human plasma (0.31 ± 0.01 pmol/min mg protein) was lower than that determined without dialysis

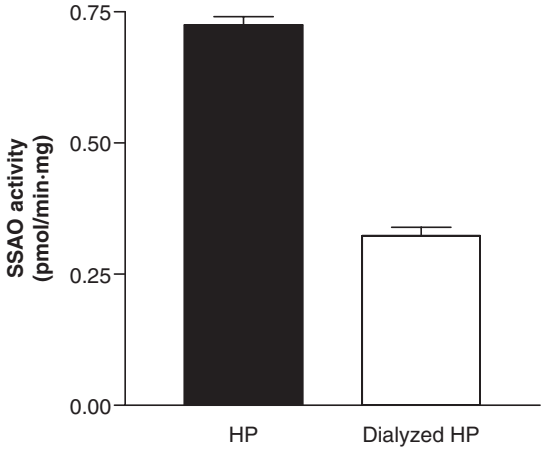


Fig. 2. **Dialysis of human plasma decreases SSAO specific activity.** Human plasma (HP) was dialyzed towards saline solution. SSAO activity present in dialyzed and non-dialyzed human plasma was assayed towards 100 µM benzylamine as substrate in 50 mM phosphate buffer (pH 7.2). Data are mean ± SEM of three different experiments.

(0.73 ± 0.02 pmol/min mg protein) (Fig. 2). These results suggest that the $NaHCO_3$ itself, as one of the components of human plasma, could be able to interact physiologically with the circulating enzyme.

Figure 3 shows the effects of varying concentrations of DMEM and $NaHCO_3$ on SSAO activity from dialyzed human plasma. The $NaHCO_3$ concentrations tested were equivalent to those contained in the different DMEM volumes used. The presence of the SSAO inhibitor, MDL 72974A, in the reaction mixtures completely destroyed the activity, confirming it to be due to SSAO. The activation of SSAO was sigmoidally dependent on the $NaHCO_3$ concentration, reaching a maximum at about 2 g/l NaHCO3 (Fig. 4). Fitting the data to the Hill equation (not shown) gave a Hill constant of 3.2 ± 0.7.

The possible time dependence of the activation was studied using high concentrations of $NaHCO_3$ (Fig. 4). The assay was initiated by the addition of the substrate to the mixture, containing the enzyme and $NaHCO_3$ that had been pre-incubated for 30 or 0 min. The enhancement of SSAO activity from dialyzed human plasma by $NaHCO_3$ was not time dependent, and, as shown in Fig. 5, this activation was completely reversible by dialysis.

The kinetic behaviour of $NaHCO_3$ towards plasma SSAO activity was determined from the initial rates in the presence of different amounts of the modulator (0–1 g/l) and increasing concentrations of the substrate, benzylamine (25–400 µM). $NaHCO_3$ behaved as a competitive activator of SSAO, as shown in the Lineweaver–Burk plot (Fig. 6A). The K_m values decreased as the amount of $NaHCO_3$ increased, whereas the V_{max} values remained constant (Fig. 6B). The decline in K_m was not a simple hyperbolic function of the $NaHCO_3$ concentration, as might be expected from the dependence shown in Figs. 3 and 4, and therefore a Ka value was not determined.

NaHCO3 also enhanced membrane-bound SSAO activity towards the physiological substrate methylamine (Fig. 7) and the presence of the classical SSAO inhibitor, semicarbazide, inhibited the enzyme activity completely.

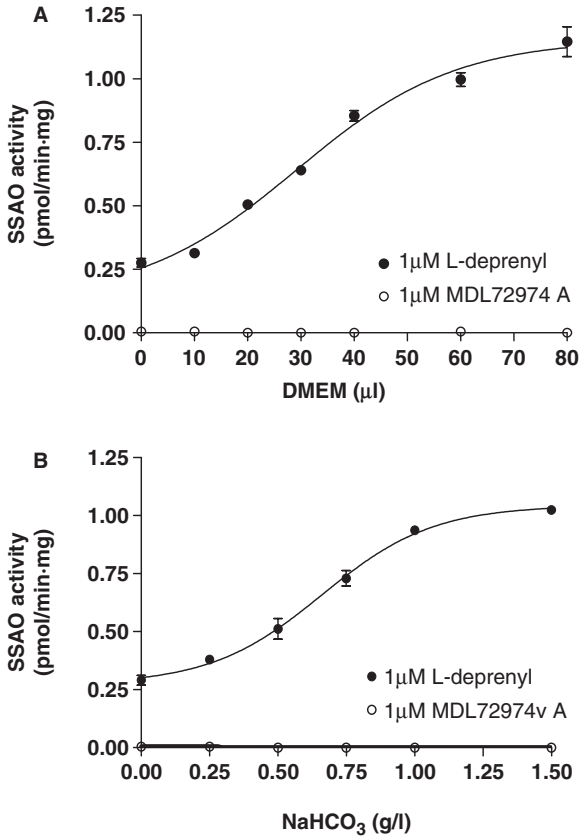


Fig. 3. **DMEM and $NaHCO_3$ enhance SSAO activity.** Dialyzed human plasma was pre-incubated with **(A)** DMEM (pH 7.0) or **(B)** $NaHCO_3$ (pH 7.2) in 50 mM phosphate buffer (pH 7.2) until a final volume of 200 µl for 30 min before adding 100 µM benzylamine as substrate. $NaHCO_3$ final concentration contained in DMEM was 3.7 g/l. Samples were previously inhibited with 1 µM L-deprenyl (black figures) or 1 µM MDL71974A (empty figures). Data are mean ± SEM of three different experiments.

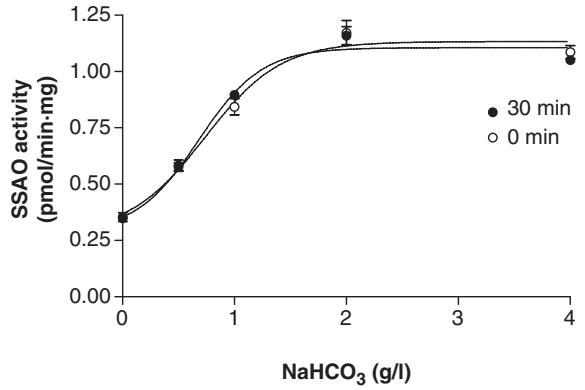


Fig. 4. **$NaHCO_3$ enhances SSAO activity in a non-time-dependent manner**. Dialyzed human plasma was pre-incubated with different $NaHCO_3$ solutions (pH 7.2) for 0 min (empty figures) or 30 min (black figures) before adding 100 µM benzylamine as substrate in 50 mM phosphate buffer (pH 7.2). Data are mean ± SEM of three different experiments.

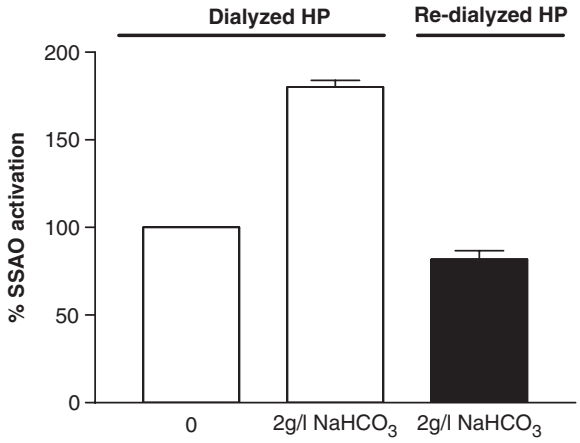


Fig. 5. **$NaHCO_3$ enhances SSAO activity in a reversible manner**. Dialyzed human plasma (Dialyzed HP) was pre-incubated for 30 min at 37°C with 2 g/l $NaHCO_3$ and then dialyzed again (see 'Materials and Methods'). Three consecutive washings were performed and samples were centrifuged at 4°C for 30 min between washings. Activity was measured by adding 100 µM benzylamine as substrate in 50 mM phosphate buffer (pH 7.2). Empty figures: dialyzed human plasma samples as control. Black figures: Samples of dialyzed human plasma that were dialyzed again after the 30 min SSAO activation process by $NaHCO_3$. Data are mean ± SEM of three different experiments.

However, the percentage of activation with methylamine was smaller than the enhancement observed using benzylamine as substrate.

DISCUSSION

SSAO activity has been reported to be altered in several pathological conditions (*7*), but little is known about the factors that may modulate its activity under physiological conditions. We have previously described the activation of human lung SSAO by a low molecular weight molecule present in human plasma, which had no effect on either MAO A or MAO B (*19*). Raimondi *et al*. (*23*) have reported bicarbonate to activate the histaminase activity of rat adipocytes at elevated pH values. Trent *et al*. (*20*) reported that culture medium was able to enhance the SSAO activity present in foetal calf serum, but they did not identify the component(s) responsible for the activation.

The present results show that DMEM enhances the activities of both the plasma and the tissue-bound forms of human SSAO and that $NaHCO_3$ is the sole component of DMEM that is responsible for this activation.

The activating effect on SSAO from dialyzed human plasma by $NaHCO_3$ was reversible and not time dependent. Kinetic studies showed the activation to be apparently competitive. Because amines can react with CO_2 to form carbamates (*24*), it is possible that these derivatives are better substrates than the free amines. Such a system is illustrated in Scheme 1A. This might account for the greater degree of activation seen with benzylamine than with methylamine because the ease of carbamate formation depends on the physico-chemical properties of the amine (*25*). However, it would be expected to give rise to complicated dependence of activity on both amine and bicarbonate concentrations (*26*). Furthermore, SSAO is

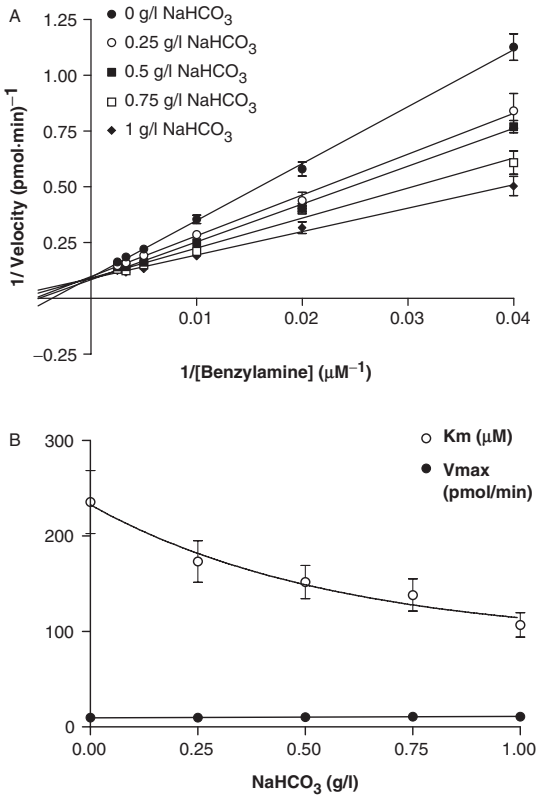


Fig. 6. **Kinetic behaviour of SSAO activation by $NaHCO_3$.** (A) Double reciprocal plots (Lineweaver–Burk transformation) of SSAO activation by $NaHCO_3$ towards benzylamine as substrate and (B) their corresponding kinetic constants towards $NaHCO_3$ concentration. Enzyme samples from dialyzed human plasma were incubated in the absence or presence of $NaHCO_3$ (0–1 g/l, pH 7.2) and, immediately, different benzylamine concentration (25–400 μM) were added to the reaction mixture in 50 mM phosphate buffer (pH 7.2). Data are mean ± SEM of six different experiments.

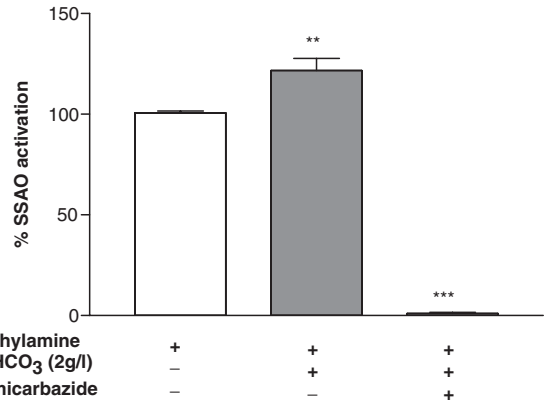
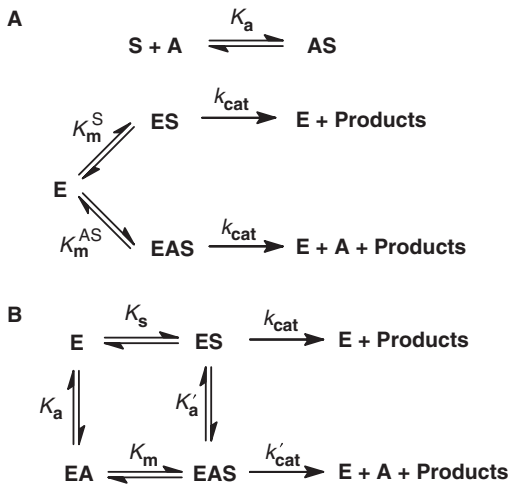


Fig. 7. **$NaHCO_3$ enhances SSAO activity towards methylamine as substrate.** Human aorta homogenate was preincubated with $NaHCO_3$ (2 g/l) in 50 mM phosphate buffer for 30 min and SSAO activity was assayed spectrophotometrically towards methylamine 500 μM as substrate. Semicarbazide (SC) 1 μM was used as SSAO inhibitor. Data are mean ± SEM of three different experiments; $^{***}P < 0.001$, $^{**}P < 0.01$ by a One-way ANOVA test and the addition of Newman–Keuls Multiple Comparison test versus control.

reported not to be active towards N-substituted amines. An alternative explanation, shown in Scheme 1B, would involve the binding of bicarbonate to the free enzyme resulting in a species (EA) with a higher affinity for substrate (S) without affecting the rate of product formation.



Under rapid-equilibrium conditions, this mechanism would result in competitive activation if $K_s > K_m$ and $k_{cat} = k'_{cat}$. Steady-state treatment of the above mechanism would, however, yield a complex equation containing squared reactant concentration terms. This might account for the apparently sigmoid dependence of activation on the concentration (Figs. 3 and 4). Interaction of more than one bicarbonate molecule with the enzyme might also contribute.

It has been reported that aminopeptidase A (PepA) from *Escherichia coli* is activated 10-fold by bicarbonate when L-leucine *p*-nitroanilide is used as substrate (*27*). In this case, the authors proposed that an exogenous bicarbonate anion as a catalytic group in an enzyme mechanism. Although our results would also seem to be consistent with such a process, more detailed protein structure studies would be necessary to investigate this hypothesis. It is also possible that activation might result from carbamoylation of lysine side chains in the enzyme itself, as a carbamoylated lysine has been shown to be essential for the activity of some class-D β-lactamases (*28*).

The report that bicarbonate increases the rate of oxidative deamination of histamine by SSAO from rat adipocyte [*24*] suggests that this activation may be a general phenomenon. It would be interesting to test such effect using different SSAO substrates.

Under physiological conditions, $NaHCO_3$ is an important buffering molecule in human plasma. The extent to which variations in the blood concentrations of bicarbonate might modulate the activity of the enzyme, enhancing the metabolism of circulating amines in respiring peripheral tissues, merits further investigation. This phenomenon could also result in inaccuracies in the SSAO activities previously reported because of the effects of dissolution of varying amounts of atmospheric CO_2 in the assay medium.

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ANNEX III.4

“SSAO/VAP-1 protein expression during mouse embryonic development”

Valente T, **Solé M** and Unzeta M

Dev Dyn, 2008, 237(9):2585-93

SSAO/VAP-1 Protein Expression During Mouse Embryonic Development

Tony Valente*, Montse Solé, and Mercedes Unzeta

SSAO/VAP-1 is a multifunctional enzyme depending on in which tissue it is expressed. SSAO/VAP-1 is present in almost all adult mammalian tissues, especially in highly vascularised ones and in adipocytes. SSAO/VAP-1 is an amine oxidase able to metabolise various endogenous or exogenous primary amines. Its catalytic activity can lead to cellular oxidative stress, which has been implicated in several pathologies (atherosclerosis, diabetes, and Alzheimer's disease). The aim of this work is to achieve a study of SSAO/VAP-1 protein expression during mouse embryogenesis. Our results show that SSAO/VAP-1 appears early in the development of the vascular system, adipose tissue, and smooth muscle cells. Moreover, its expression is strong in several epithelia of the sensory organs, as well as in the development of cartilage sites. Altogether, this suggests that SSAO/VAP-1 enzyme could be involved in the differentiation processes that take place during embryonic development, concretely in tissue vascularisation. ***Developmental Dynamics 237:2585–2593, 2008.*** **© 2008 Wiley-Liss, Inc.**

Key words: SSAO/VAP-1; vasculogenesis; embryo; smooth muscle cells; myogenesis; cartilage development; vascular cells

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INTRODUCTION

Semicarbazide Sensitive Amine Oxidase [E.C.1.4.3.6, oxidoreductase (deaminating) (copper-containing), SSAO], also known as vascular adhesion protein-1 (VAP-1) (Salmi and Jalkanen, 1992), constitutes a family of enzymes present in almost all mammalian species (Lewinshon, 1984). SSAO/VAP-1 is encoded by the amine oxidase cooper-containing gene, AOC3 (Jalkanen and Salmi, 2001). SSAO/VAP-1 is a homodimeric 180-kDa glycoprotein, mainly located in fat and highly vascularised tissues (endothelial and smooth muscle cells). SSAO/VAP-1 metabolizes primary amines, generating hydrogen peroxide (H_2O_2), ammonia (NH_3), and the corresponding aldehyde (Lyles, 1996). Aminoacetone and methylamine are considered physiological SSAO/VAP-1 substrates (Precious et al., 1988). SSAO/VAP-1 is considered a multifunctional enzyme whose function varies depending on the tissue where it is expressed (O'Sullivan et al., 2004). Moreover, plasma SSAO/VAP-1 activity is altered in several pathological conditions: diabetes type I and II (Boomsma et al., 1995), congestive heart failure (Boomsma et al., 1997), non-diabetic obesity (Mészáros et al., 1999), atherosclerosis (Grönvall-Nordquist et al., 2001; Karádi et al., 2002), inflammatory liver diseases (Kurkijärvi et al., 2000; Garpenstrand et al., 1999; Salmi et al., 1993), and Alzheimer's disease (del Mar et al., 2005). Moreover, SSAO/VAP-1 is found up-expressed in cerebrovascular amyloid angiopathy (AD-CAA) (Ferrer et al., 2002). Recently, Salmi and Jalkanen (2006) described the presence of SSAO/VAP-1 protein in some foetal human tissues. However, little is known about the expression of this protein during the development of mammalian embryos. The aim of this study is to characterize the expression pattern of SSAO/VAP-1 protein during the development of the mouse embryo.

Additional Supporting Information may be found in the online version of this article.

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RESULTS AND DISCUSSION

Specificity of the hSSAO/VAP-1 and bSSAO/VAP-1 Antibodies

First, we compared the specificity of two different types of SSAO/VAP1 antibodies. bSSAO/VAP-1 is a rabbit polyclonal antibody raised against bovine SSAO/VAP-1 from lung microsomal membranes produced in our laboratory (Lizcano et al., 1998), whereas hSSAO/VAP-1 is a commercial antibody raised against the C-terminal extracellular domain of human SSAO/VAP-1 (H-43, Santa Cruz Biotechnology). When bSSAO/VAP-1 antibody was used, two molecular forms were detected in the lysates of the rat and mouse embryos: the trimeric glycoprotein (250–265 kd band) and the monomeric protein (90–100 kd band) (Fig. 1A, lane 1, rat embryo lysate, and lane 2, mouse embryo lysate). The same results were obtained when the bSSAO/VAP-1 antibody was used (Fig. 1A, lane 4, rat embryo lysate, and lane 5, mouse embryo lysate). The stable rat A7r5 cell line that overexpressed human SSAO/VAP-1 was used as positive control (Sole et al., 2007). In this case, both antibodies strongly detected the 90–100-kd band (Fig. 1A, lanes 3 and 6). All bands observed were correlated with those previously reported by Jaakkola et al. (1999) using human lysates from muscle samples and the human SSAO/VAP-1 antibody produced by themselves. Under immu-

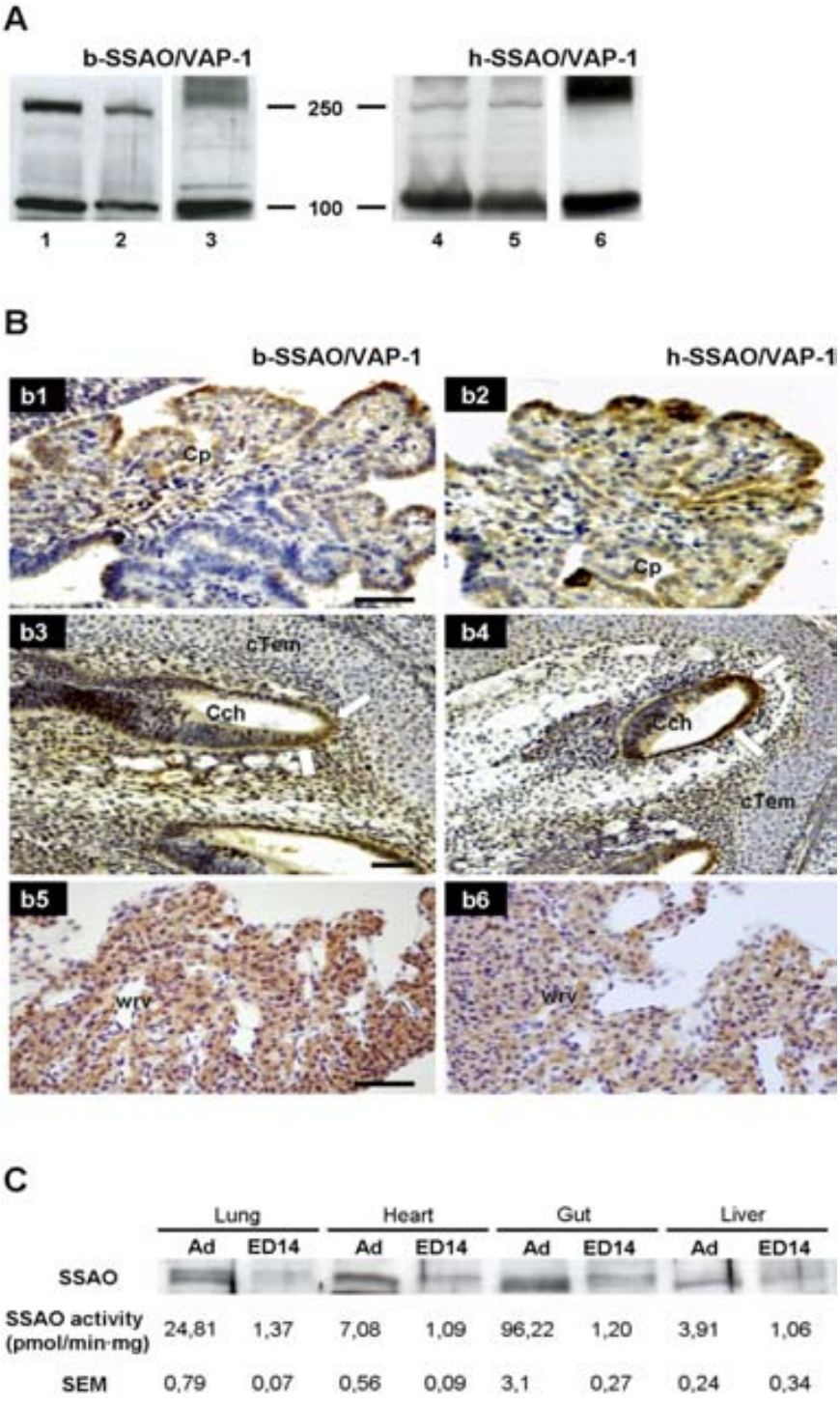


Fig. 1. Immunospecificity of SSAO/VAP-1 antibodies by immunoblot and immunohistochemistry techniques. **A:** Immunoblot of SSAO/VAP-1 antibodies in human, rat, and mouse lysates. bSSAO/VAP-1 antibody (Lizcano et al., 1998) strongly detected two forms of SSAO/VAP-1 protein, the trimeric (250 kd) and monomeric (100 kd) in the lysates from rat and mouse whole embryos (lanes 1 and 2, respectively). In adult mouse spinal cord lysate, only the monomeric form was weakly detected by bSSAO/VAP-1 antibody (lane 3). The monomeric form was the main form shown (lane 4) when using bSSAO antibody in the human A7r5 stable cell line that overexpresses the human SSAO/VAP-1. The hSSAO/VAP-1 antibody (H-43, Santa Cruz Biotechnology), strongly detected the monomeric form of SSAO/VAP-1 protein, whereas in rat and mouse whole embryo lysates, only a weakly trimeric form was detected (lanes 5 and 6). Similar bands were observed in adult mouse spinal cord lysate (lane 7). In the A7r5 SSAO/VAP-1 cell line lysate, the trimeric and monomeric forms were strongly detected by hSSAO/VAP-1 antibody (lane 8). **B:** Immunohistochemistry of SSAO/VAP-1 antibodies in mouse embryo sections. Both bSSAO/VAP-1 and hSSAO/VAP-1 antibodies are strongly expressed in the choroids plexus of the fourth ventricle (B1 and B2, respectively), in the inner ear (B3 and B4, respectively), and in the heart (B5 and B6, respectively) of mouse embryos after 14 days of gestation. **C:** bSSAO/VAP-1 Western blotting in heart, liver, lung, and gut from embryonic and adult mice lysates. Low levels of SSAO/VAP-1 protein are found in embryo tissues whereas high levels are observed in adult tissues. These levels of SSAO/VAP-1 protein are correlated with their enzymatic activity (see bottom panel). A: Lanes: 1 and 5, rat whole embryo lysates; 2 and 6, mouse whole embryo lysates; 3 and 7, adult mouse spinal cord lysates; 4 and 8, A7r5 SSAO/VAP-1 cell line lysates. B: Scale bar = 50 μm. Cch, cochlea; cTem, cartilage of temporal bone; wrv, wall of right ventricle of the heart.

nohistochemical analysis, both antibodies showed the same expression pattern during mouse embryonic development (Fig. 1B). However, the intensity of the immunostaining was higher with the bovine antibody (Fig. 1B, b1, b3, and b5), than the human SSAO/VAP-1 antibody (Fig. 1B, b2, b4, and b6). In addition, in rat embryo sections similar SSAO/VAP-1 expression pattern was observed (data not shown). Moreover, no staining was detected when SSAO/VAP-1 antibodies were preabsorbed with the SSAO/VAP-1 (see Fig. 4B) peptide or when SSAO/VAP-1 was replaced by foetal bovine serum (Fig. 2J). These data allow us to conclude that in mammalians both antibodies b-SSAO/VAP-1 and h-SSAO/VAP-1 detected specifically the same protein: SSAO/VAP-1. Moreover, in embryonic mouse sections, the expression pattern of SSAO/VAP-1 observed is the same with both antibodies. The expression of SSAO protein during mouse embryonic development was described in detail in the Table 1.

Low levels of SSAO/VAP-1 Protein Are Found Early in Embryonic Development

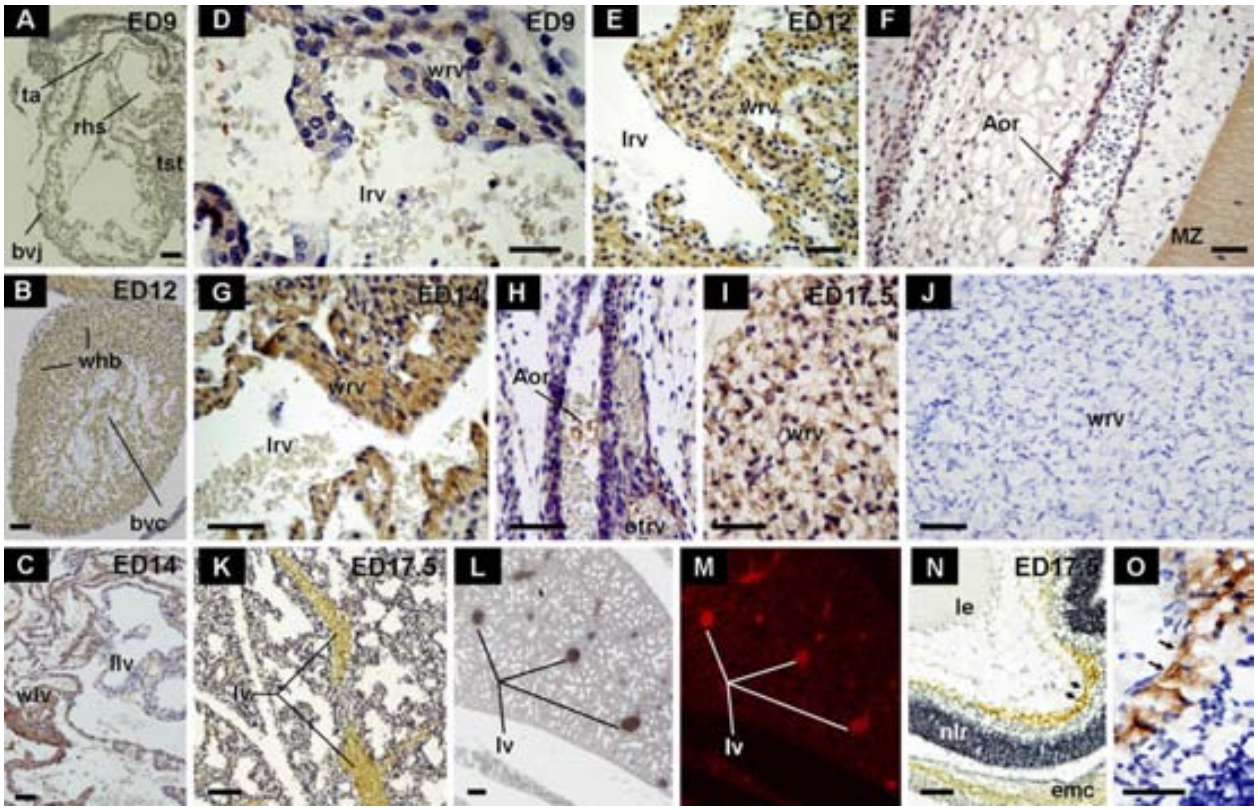
SSAO begins to express weakly in early embryonic development, at ED9 (Fig. 2A and Table 1). Its expression in embryos reaches moderate levels between ED12 and ED14 (Fig. 2B,C and Table 1). However, although its expression during embryonic development is weak to moderate, in adult its expression is very high. This is confirmed by Western blot analysis using heart, gut, and lung lysates from both ED14 and adult samples (Fig. 1C). Moreover, we used the same lysates for measurement of SSAO/VAP-1 enzyme activity and this activity in embryonic development was very low (Fig. 1C). The low levels of SSAO/VAP-1 protein during embryogenesis compared with the high levels detected in adult mice are correlated with the corresponding activities. The SSAO/VAP-1 activities from different embryonic and adult mouse tissues reported here are similar to those described by Salmi and Jalkenen (2006) in human foetal tissues. SSAO/VAP-1 is expressed early during embryogenesis and its expression is moderate in embryos with 14 days of gestation as observed by immunohistochemistry. Therefore, the early expression of the SSAO/VAP-1 in embryos doesn't seem to be only related to their enzymatic activity, suggesting that the SSAO/VAP-1 could have another role in development not yet described. Further studies will be necessary to reveal the role of SSAO/VAP-1 enzyme in early development.

Heart and Vascular Associated System

In general, the expression of SSAO/VAP-1 in embryonic development is very low on embryonic day 9, ED9, and moderate on embryonic day 12 and 14, ED12 and ED14 (Fig. 2A–C). In lower magnifications, SSAO/VAP-1 protein was detected at ED12 (Fig. 2B); however, at higher magnifications its expression was found at ED9 (Fig. 2D). Thus, low levels of SSAO/VAP-1 protein were detected in the myocardial progenitor cells of the ventricular wall of the heart at embryonic day nine, ED9 (Fig. 2D). At this embryonic stage, the heart is in a differentiated process. The outflow tract of the heart is differentiating and at this stage it is possible to distinguish the aortic-pulmonary septum formation. At ED12, the expression of SSAO/VAP-1 protein increases in the heart (Fig. 2E), when the differentiation of the vascular system begins to define the asymmetry of the branchial (pharyngeal and aortic) arch arterial system, and in this case it is possible to see the aorta (Fig. 2F). During subsequent embryonic stages, the vessels of the ascending aorta and the pulmonary trunk are very well defined and the pulmonary circulation is established. Thus, at ED14, the immunolocalization of SSAO/VAP-1 protein was found in the heart (Fig. 2G), aorta, and pulmonary vessels (Fig. 2H), as well as in the vessels and arteries of the lung (Fig. 2K,L). Moreover, this expression of SSAO/VAP-1 in the vessels and arteries colocalize with MECA-32, a common endothelial cell marker, as it was observed in the lung (Fig. 2L,M). At ED17.5, the expression of SSAO/VAP-1 decreases in the heart (Fig. 2I) and it is located in the endothelial layer and in the vascular smooth muscle cells. No staining was observed in the heart when the SSAO/VAP-1 antibody was replaced by foetal bovine serum (Fig. 2J). On the other hand, many vascular smooth muscle cells exhibit a wide range of different phenotypes at different stages of development and these cells are not completely differentiated in adult organisms (Owens et al., 1996; Owens and Wise, 1997). During embryonic development, the SSAO/VAP-1 protein is expressed parallel to the myogenesis processes of the vascular smooth muscle cells, and its expression persists in adult mammalian tissue, where SSAO/VAP-1 plays an important role in the vascular system (Ochiai et al., 2005; Jaakkola et al., 1999; Castillo et al., 1998; Salmi et al., 1993). Under inflammatory conditions in endothelial cells, SSAO/VAP-1 behaves as an inducible vascular adhesion protein involved in lymphocyte trafficking (Smith et al, 1998). Moreover, SSAO/VAP-1 has been described as being most active in the vascular smooth muscle cells and endothelial cells of the mammalian adult aortic wall (Langford et al., 1999). Functional study of arteries from adult SSAO/VAP-1 KO mice suggests that SSAO/VAP-1 might contribute to arterial wall remodelling (Mercier et al., 2006). Therefore, the expression of SSAO/VAP-1 protein in embryos seems to follow vasculogenesis of the heart and the associated vascular system, which suggests that this protein could play a role during the cardiogenesis. Further experimental work is necessary in order to confirm the role of SSAO/VAP-1 in heart and vascular system development.

Smooth Muscle

In the smooth muscle tissue, the expression of SSAO/VAP-1 appeared weakly at ED12 in the skin and moderately at ED14 in the gut and tail. However, the high levels of SSAO/VAP-1 were observed in the smooth cells of the digestive tract and oesophagus at ED16 (Fig. 3E), and in lower levels at ED17.5. Moreover, at this embryonic stage moderate levels of SSAO/VAP-1 protein were detected in the smooth muscle cells of the lips (Fig. 3H,I), skin (Fig. 4A and I), and gut (Fig. 4G,H), as well as in the smooth muscle cells of the tail (Fig. 3A), abdominal cavity, and reproductive organs (data not shown). No staining was found in the smooth muscle cells when SSAO/VAP-1 antibody is preabsorbed previously with SSAO/VAP-1 peptide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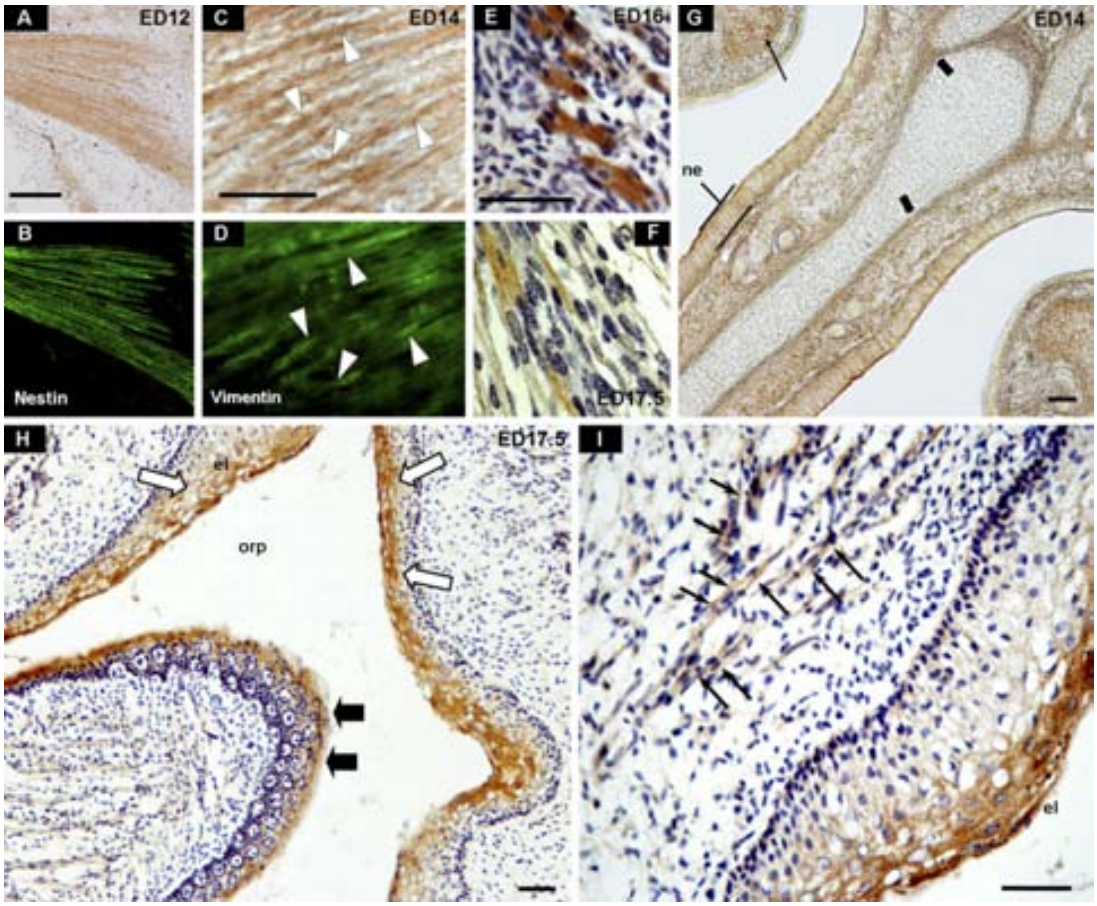
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Fig. 3.

(Fig. 4B). Moreover, smooth muscle cells of the tail co-expressed SSAO/VAP-1 and nestin (Fig. 3A–C) at ED12, while the smooth muscle cells of the lips co-expressed SSAO/VAP-1 and vimentin (Fig. 3C,D) at ED14. Nestin is a marker of multi-lineage progenitor cells (neural progenitors, myogenic cells, epithelial cells, and so on) that include smooth muscle progenitor cells (Wiese et al., 2004), and vimentin is an intermediate filament highly expressed during the differentiation process of smooth muscle cells (Van Muijen et al., 1987; Pixley et al., 1984). Smooth muscle precursor cells mostly derive from the mesoderm (gut), but also from the neuroectoderm (skin and tail) and endoderm (lips). The growth and differentiation of smooth muscle cells start at different times in different organs and many growth factors and neurohumoral agents regulate smooth muscle growth and differentiation. SSAO/VAP-1 expression during the growth and differentiation of smooth muscle from different tissues could be related to the myogenesis of smooth muscle.

Adipose Tissue

The expression of the SSAO/VAP-1 protein is observed weakly in adipose tissue of embryos with 14 days of gestation. At ED17.5, the SSAO/VAP-1 protein is strongly expressed in the embryonic adipose tissue (Fig. 4C), at a developmental stage where high adipocyte differentiation occurs. In adults, high levels of SSAO/VAP-1 protein were observed at the caveolae of differentiating and mature adipocytes (Souto et al., 2003) and could be involved in triggering terminal adipocyte differentiation (Fontana et al., 2001). In adults, SSAO/VAP-1 activity stimulates glucose transport by mimicking the insulin effect (Enrique-Tarancón et al, 1998), Therefore, the SSAO/VAP-1 could play a role in differentiating adipocytes during development just as it does in adults.

Sensory Organs

In the eye, the expression of SSAO/VAP-1 protein was found weakly in the vascular cells of the hyaloid cavity surrounding the internal part of the neural layer of the retina at ED14, and increased at ED17.5 (Fig. 2K,L). During development, the angiogenic mesenchyme forms the hyaloid artery and vein, which later become the central artery and vein of the retina (Lang, 1997), and during later embryonic stages both were immunolabelled with SSAO/VAP-1. We previously described SSAO/VAP-1 activity in the optic nerve, choroid, iris, and retina of adult bovine eyes, and the role of this enzyme in the metabolism of dopamine in retinal tissue (Fernandez de Arriba et al., 1991). Therefore, SSAO/VAP-1 enzyme could be required for the vascularisation that takes place during eye development. In the nasal and oral cavities, the expression of SSAO/VAP-1 protein was detected in several epithelia of the embryo between 14 and 17.5 days of gestation (Fig. 3). SSAO/VAP-1 immunostaining was found in the epithelial cells of the marginal layer of the oral cavity, mainly in the lips (Fig. 3H,I), where during development the external surface of the lip passes through a transitional zone to merge with the oral mucosa on the inner surface of the lip (Kang and Svoboda, 2005). Its expression is also found in the epithelium of the tongue (Fig. 3H), specifically at the specialized lingual papillae, which is strongly vascularised in adulthood. Moreover, SSAO/VAP-1 protein is expressed in the epithelium of the nasal cavity (Fig. 3G) and in the epithelia of the organ of Corti and the cochlea of the inner ear (Fig. 1B3,4). During inner ear development, the vestibulocochlear artery supplies the cochlea at the same time that the epithelia of the cochlear region and the organ of Corti begins to express SSAO/VAP-1 protein (ED14). Therefore, SSAO/VAP-1 expression appeared when the oral cavity began to mature during the later embryonic stages, just before birth, in parallel with both the vascular system and the smooth muscle cells in the nasal and oral cavities. All these findings suggest that SSAO/VAP-1 could play a role in vasculogenesis and/or myogenesis of the sensory organs.

Fig. 2. Expression pattern of SSAO/VAP-1 protein in the vascular system of mouse embryo. SSAO/VAP-1 is expressed in the heart of immunohistological sections of embryonic day 9, ED9 (**A**), 12 (**B**), and 14 (**C**). In the wall of ventricles (**D, E, G, I**), SSAO protein is detected weakly at embryonic day 9, ED9 (D), strongly between ED12 and 14 (E, G), and moderately at ED17.5 (I). At ED12 and ED14, strong expression of SSAO/VAP-1 protein is detected in the wall of aorta (**F** and **H**, respectively). No immunostaining is found when the primary antibody is replaced by foetal bovine serum (**J**). SSAO/VAP-1 is found in liver vessels of embryos of 17.5 days of gestation (**K,L**). Immunostaining for MECA-32 in liver vessels at ED17.5 (**M**). In the eye, many immunostained SSAO/VAP-1 cells were observed in the vascular hyaloid layer of the retina (arrows in **N,O**). Sagital sections. Scale bar = 50 μm in A–O. Aor, aorta; bvc, bulbo-ventricular canal; bvj, bulbo-ventricular junction; emc, eye muscle cells; hv, hyaloid vascular cells; le, lens; llv, lumen of left ventricle; lrv, lumen of right ventricle; lv, lung vessels; mz, marginal zone of spinal cord; nlr, neural layer of retina; otrv, outflow tract of right ventricle (pulmonary trunk leading to ductus arteriosus); rhs, right horn of sinus venosus; smg, submandibular gland; ta, truncus arteriosus; tst, tissue of septum transversal; whb, wall of heart at bulbo-ventricular junction; wlv, wall of left ventricle; wrv, wall of right ventricle.

Fig. 3. Expression of SSAO/VAP-1 protein in the epithelia of nasal and oral cavities in mouse embryos. At ED12, the smooth cells of the tail co-expressed SSAO/VAP-1 and nestin (**A** and **B**, respectively). At ED14, the smooth cells of lips co-expressed SSAO/VAP-1 and vimentin (arrowheads in **C** and **D**). At ED16, the expression of SSAO/VAP-1 is very strong in the smooth cells of the digestive tract, oesophagus (**E**), and its expression decrease at ED17.5 (**F**). At ED17.5, SSAO/VAP-1 is detected in the epithelium of the nasal cavity (**G**) and in the epithelial layer of lips and tongue (white and black solid arrows, respectively, in **H**). Similar expression is observed in the smooth muscle cells of the most internal part of lips (arrows in **I**). Sagital sections. Scale bar = 50 μm. el, epithelium of lip; ne, nasal epithelium; orp, orporopharynx.

Cartilage Development

At ED12 and ED14, SSAO/VAP-1 expression was found in the chondrocytes showing active differentiating processes, mainly in the vertebrae and ribs (Fig. 4D,E), as well as in the vascular cells that surround the cartilage primordium of these skeletal sites (Fig. 4D). However, no immunostaining for SSAO/VAP-1 protein was observed at the cartilage sites of the head, concretely in the nasal area (Fig. 3G) and limbs (data not shown). At ED17.5, the ossification process is predominant and many cartilages are replaced by bones. At the same time, SSAO/VAP-1 protein is expressed very weakly in the resting chondrocytes (Fig. 4F). Therefore, SSAO/VAP-1 expression in the skeletal ele-

TABLE 1. SSAO/VAP-1 Expression Pattern in Abryonic Tissues

Embryonic area	Embryonic day ED9	ED12	ED14	ED17.5
Vascular system				
Heart				
Wall of ventricular chamber of heart	+	++	+++	++
Lumen of ventricular chamber of heart	−	−	+/−	+/−
Umbilical vein		+	++	+++
Umbilical artery		+/−	+	+
Aorta		+	+	++
Lung				
Vessels		−	−	+/−
Artery		−	−	+
Brain				
Hypothalamus			+/−	+
Optic chiasma			+/−	+
Choroid plexus differentiating from roof of fouth ventricle		+	++	+
Pituitary gland				
Infundibulum of pituitry (future pars nervosa)		++	++	
Vascular differentiation in anterior wall of Rathke's pouch		++	++	
Eye[a]				
Hyaloid artery			+/−	+
Cartilage				
Cartilage primordium of head		−	−	−
Cartilage primordium of limbs		−	−	−
Cartilage primordium of ribs and vertebrae		++	+	+/−
Ossification sites of ribs and vertebrae		−	−	+/−
Epithelia of sensory organs				
Cochlear sensory epithelium		+	+++	++
Olfactory epithelium		++	+++	++
Tongue epithelium			+/−	++
Digestive system				
Epithelium of lips			+/−	++
Epithelium of oropharynx			+/−	++
Stomach			−	−
Liver			−	−
Gut				
Lumen			−	+/−
Smooth muscle			+/−	++
Adipose tissue			+	++
Skin[a]				
Cutaneous muscle			+	+
Outer layer			+	++

[a]Sensory organs.

ments of the mouse embryo shows a regional pattern during differentiation process. Previous studies showed the expression of SSAO/VAP-1 protein in adult rat chondrocytes from articular cartilage (Lyles and Bertie, 1987). However, the role of SSAO/VAP-1 in chondrogenic sites is still unknown. Chondrocytes are a dynamic cell type that plays an important role in bone maintenance. In embryo, the vertebrate skeleton is formed from mesodermic cells for endoskeleton primarily in the trunk (ribs, vertebrae, and limbs), and from neural crest cells for exoskeleton and endoskeleton of the head and branchial region. The expression of SSAO/VAP-1 is found essentially in the vertebrae and ribs (endoskeleton from mesodermic cells) and concretely in endochondral bones, since its expression decreases with the osteogenesis process. Therefore, the regional expression of SSAO/VAP-1 and its basal expression after ossification in the resting chondrocytes suggests that this enzyme could be involved in the development and/or maintenance of cartilage sites.

Conclusion

SSAO/VAP-1 expression is spatially and temporally regulated in several mouse embryonic tissues, first in the vascular system and later in the sensory organs, smooth muscle tissue, and skeletal elements. In parallel, its enzymatic activity was low during mouse embryonic development. SSAO/VAP-1 could play a different role during mouse development beyond its role in amine metabolism and vascular adhesion described in adult mammalians. Further studies will be necessary in order to

Developmental Dynamics

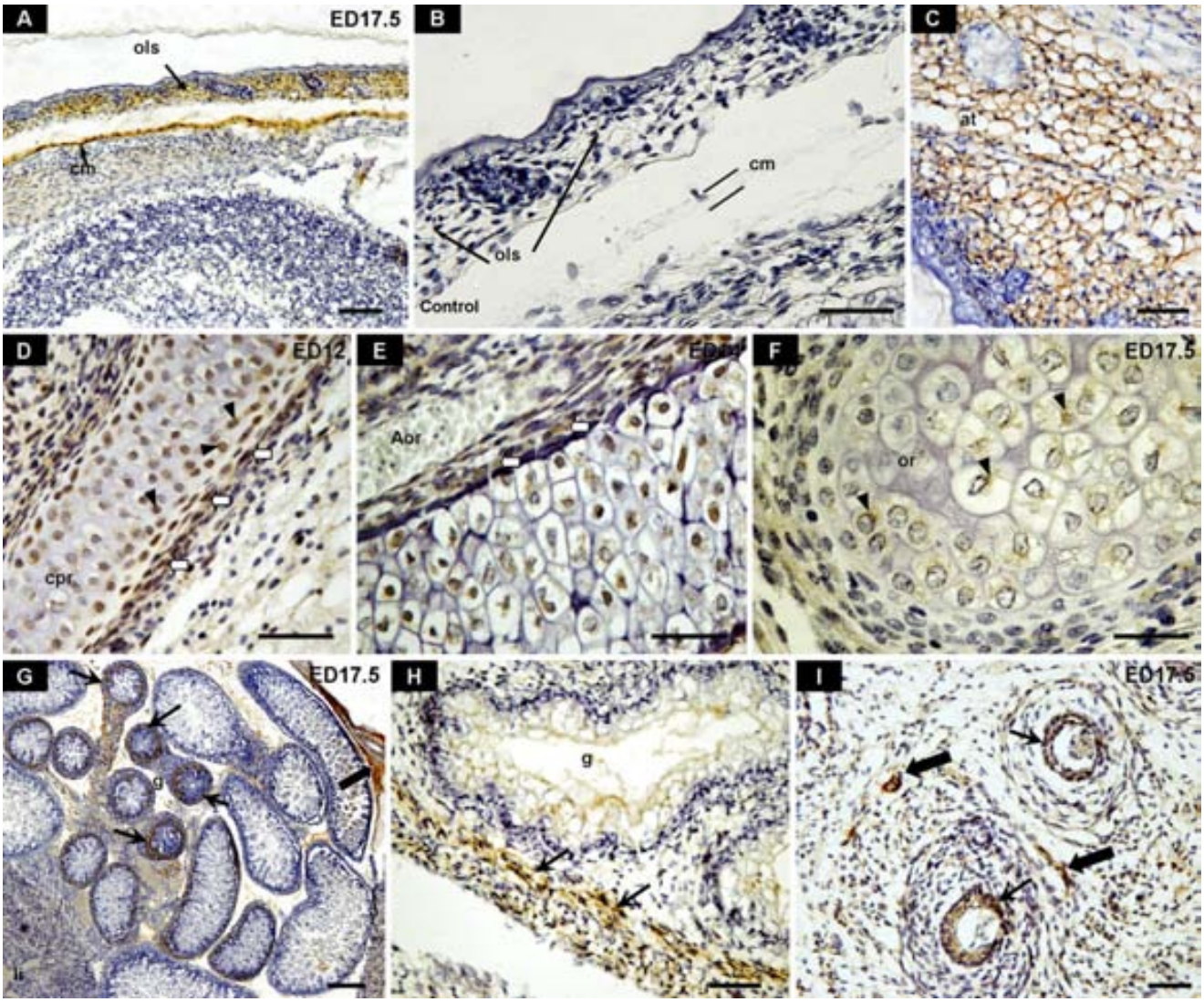

Fig. 4. SSAO/VAP-1 protein expression in the smooth muscle cells, chondrocytes and adipocytes. At embryonic day 17.5, ED17.5, the cutaneous muscle of the skin and many cells of the outer layer of skin expressed SSAO/VAP-1 (**A**). No SSAO/VAP-1 expression is found in the skin when the SSAO/VAP-1 antibody is preadsorbed with SSAO/VAP-1 peptide (**B**). Strong expression of SSAO/VAP-1 protein is detected in the adipose tissue of the dorsal part of the embryo at 17.5 days of gestation (**C**). SSAO/VAP-1-positive chondrocytes are observed at ED12 (arrowheads in **D**) only in the cartilage of vertebrae and ribs, whereas in the subsequent developmental stages, this immunolabelling progressively decreased in the differentiating chondrocytes (D, **E**) and at ED17.5 only some resting chondrocytes expresses SSAO/VAP-1 protein (arrowheads in **F**). Strong SSAO/VAP-1 expression is detected in the perichondrium cells of these cartilage sites at early embryonic stages (white solid arrows in D and E). At ED17.5, many smooth muscle cells of the gut are strongly immunolabelled for SSAO/VAP-1 protein (arrows in **G, H**). Strong expression of SSAO/VAP-1 protein is also observed in the umbilical vein (solid arrow in G), in the vibrissae follicles (arrows in **I**), and in the face muscle cells that surround the vibrissae follicles (solid arrows in I). Sagital sections. Scale bar = 50 μm. Aor, aorta; at, adipose tissue; cm, cutaneous muscle cells; cpr, cartilage primordium of ribs; g, gut; li, liver; ols, outer layer of skin; or, ossification of ribs.

clarify the role that SSAO/VAP-1 protein expression plays during mouse embryogenesis.

EXPERIMENTAL PROCEDURES

Animals

We used embryos of NMRI mice and Sprague-Dawley rats (Iffa Credo, Lyon, France).

The day on which a vaginal plug was detected was considered embryonic day 0 (E0). E1 began 24 hr later. The fetal animals (E8, E9, E10, E11, E12, E14, E15, E16, E17.5, and E18.5) were removed from the mother under anaesthesia by intraperitoneal injection of ketamine (100 mg/Kg) and Xylazine (10 mg/Kg). All animals were perfused with 4% paraformaldehyde in 0.1 M phosphate buffer (PFA) and processed for paraffin-embedded and for frozen PFA-fixed samples. The paraffin-embedded samples were cut into 5-μm sagital sections and frozen samples were cut into 25-μm sagital free-floating sections. The animals were kept under controlled temperature, humidity, and light conditions and were treated according to European Community Council Directive 86/609/EEC.

Immunohistochemistry

Paraffin and free-floating sections were processed for immunohistochemistry in

accordance with the Valente protocol (Valente et al., 2005). The sections were incubated with rabbit polyclonal antibodies against bovine SSAO/VAP-1 protein at 1:200 (Lizcano et al., 1998) or human SSAO/VAP-1 protein at 1:50 (H-43, Santa Cruz Biotechnologies). Thereafter, sections were sequentially incubated with biotinylated goat anti-rabbit antibody (1:200) and with the avidin-biotin-peroxidase complex (ABC, 1:200). Peroxidase was developed with 0.05% diaminobenzidine in 0.1 M PB and 0.01% H_2O_2, and immunoreacted sections were mounted onto gelatinised slides. Alternatively, some sections were counterstained with hematoxylin. SSAO/VAP-1 immunohistochemical controls were made. First, we performed immunohistochemistry replacing the primary antibody by bovine foetal serum; second, we performed immunohistochemistry using the SSAO-VAP-1 antibody previously preadsorbed 1 hr at room temperature with the SSAO/VAP-1 peptide used for antibody production (Lizcano et al., 1998). For double immunohistochemistry and immunofluorescence, the sections immunostained with SSAO/VAP-1 were incubated with a second primary antibody: mouse anti-Nestin (1:200, Hybridoma Bank); mouse anti-Vimentin (1:500, DAKO); and a rabbit anti-MECA-32 (1:100, Hybridoma Bank). After, several washes, the sections were incubated 1 hr at room temperature with the respective secondary antibody for immunofluorescence: goat anti-mouse Alexa 488 (1:1,000, Invitrogene). The sections were mounted in mowiol medium.

Sections were photographed in a NIKON Eclipse 901 microscope/Nikon digital sight camera, using a 10× and 20× objective lens. Embryonic anatomy was defined and termed according to *The Atlas of Mouse Development* by Kaufman (1999).

Preparation of Embryonic Extracts and Western Blotting

Whole embryos with ED16 from rat and mouse were homogenized in Ripa buffer at 4°C to obtain a total cellular fraction. Total extracts (20 μg/lane) were prepared in sample buffer (250 mM Tris-HCl, pH 6.8, 10% SDS, 30% Glycerol, and 2,5% β-mercaptoethanol) and were resolved on 7.5% SDS-PAGE gels (using the Bio-Rad Mini-PROTEAN 3 system) and transferred to nitrocellulose membranes for Western blot. The membranes were incubated with rabbit polyclonal against SSAO/VAP-1 protein (Lizcano et al 1998) or with rabbit polyclonal anti-SSAO/VAP-1 (Santa Cruz Biotechnologies). The bands were visualized using the ECL chemiluminescence system (Amersham Pharmacia Biotech).

Measure of SSAO/VAP-1 Activity

A modification of the Otsuka and Kobayhashi (1964) method was used to determine the SSAO activity. SSAO activity was determined radiochemically using 100 μM (14°C)-benzylamine (2 mCi/mmol, Amersham, UK) as substrate. L-deprenyl (1 μM) was used to pre-inhibit MAO B. Cell lysate activities are expressed as pmol product/min · mg protein.

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