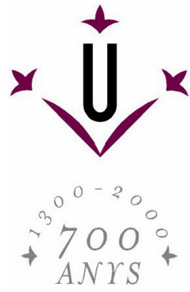

Universitat de Lleida

Departament de Ciències Mèdiques Bàsiques

IMPLICATION OF TNF SUPERFAMILY RECEPTORS AND THEIR FUNCTIONAL ANTAGONISTS IN NEURONAL APOPTOTIC CELL DEATH

Raffaella Gozzelino

Lleida, 19 de Noviembre 2007

Departament de Ciències Mèdiques Bàsiques,

Universitat de Lleida

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RAFFAELLA GOZZELINO

19 de Noviembre 2007

Thesis co-directors:

Joan X. Comella Carnicé and Victor J. Yuste Mateos

Universitat de Lleida

IRB Lleida
Institut de Recerca Biomèdica

hospital universitari
arnau de vilanova
InstitutCatalàdelaSalut

Joan X. Comella Carnicé, doctor in *Medicine and Surgery* and Professor of Cellular Biology in the Faculty of Medicine at the *Universitat Autònoma de Barcelona* and Head of the *Cell Signaling and Apoptosis Group*, and **Victor J. Yuste Mateos**, doctor in *Medicine and Surgery* and researcher under the *Ramón y Cajal* Program at the *Institut de Neurociències* of the *Universitat Autònoma de Barcelona*, both attached to the *Departament de Ciències Mèdiques Bàsiques* of the *Universitat de Lleida* during the course of the thesis experimental work,

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hereby states that,

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Lleida, August 31st, 2007

Dr. Carme Gallego González, *PhD*

Joan X. Comella Carnicé, Doctor en *Medicina i Cirurgia* i Catedràtic de Universitat de Biologia Cel·lular de la Facultat de Medicina de la *Universitat Autònoma de Barcelona* i coordinador del *Grup de Senyalització Cel·lular i Apoptosi*, i **Victor J. Yuste Mateos**, Doctor en *Medicina i Cirurgia* i investigador *Ramón y Cajal* en el *Institut de Neurociències* de la *Universitat Autònoma de Barcelona*, ambdós adscrits al Departament de Ciències Mèdiques Bàsiques de la Universitat de Lleida durant la direcció experimental del treball exposat a l'actual tesi,

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Dra. Carme Gallego González, *PhD*

Ao meu melhor amigo

Hassana

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Abbreviations

ActD	Actinomycin D
AD	Alzheimer Disease
AIF	Apoptosis Inducing Factor
APAF-1	Apoptosis Protease Activation Factor 1
ASK1	Apoptosis Signal-Regulating Kinase 1
BDNF	Brain Derived Neurotrophic Factor
BSA	Bovine Serum Albumin
CARD	Caspase Recruitment Domain
CHX	Cycloheximide
CMV	Cytomegalovirus
CNS	Central Nervous System
CO	Carbon Monoxide
CRD	Cystein Rich Domain
DcR	Decoy Receptor
DD	Death Domain
DED	Death Effector Domain
DISC	Death Inducing Signalling Complex
DIV	Days *in Vitro*
DMEM	Dulbecco’s modified Eagle’s medium
DMSO	Dymethilsulfoxide
DNA	Desoxirribonucleic Acid
DR	Death Receptor
DTT	1,4-Dithiothreitol
EAE	Experimental Autoimmune Encephalomyelitis
EDTA	Ethylene Diamine Tetra Acetic Acid
ERK	Extracellular Regulated Kinase

Abbreviations

FADD	Fas Associated Death Domain
FAIM	Fas Apoptosis Inhibitory Molecule
FBS	Fetal Bovine Serum
FHC	Ferritin Heavy Chain
FKHR	Forkhead Transcription Factor
FLICE	FADD-like Interleukin-1β-converting enzyme
FLIP	Fas-Asscociated Death Domain-like Interleukin-1-beta-converting enzyme-inhibitory protein
HBSS	Hank's Balanced Salt Sodium
HO-1	Heme Oxygenase I
HS	Horse Serum
Htt	Huntingtin Protein
IAP	Inhibitor of Apoptosis Protein
IBM	IAP Binding Motifs
IKK	I-κB-Kinase
ILK	Integrin-linked Kinase
iNOS	Inducible Nitric Oxide Synthase
IPC	Ischemic Preconditioning
JNK	c-jun N-terminal Kinase
kDa	kiloDalton
LDH	Lactate Deidrogenase
LFG	Lifeguard
LTR	Long Terminal Repeat
MAPK	Mitogen Activated Protein Kinase
MCS	Multiple Cloning Site
MEK	MAP/ERK Kinase
MEKK1	MAP/ERK Kinase Kinase

MEM	Minimum Essential Medium
MKP	MAPK Phosphatase
MnSOD	Manganese Superoxide Dismutase
MOMP	Mitochondrial Outer Membrane Permeabilization
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MS	Multiple Sclerosis
MTT	Methyltiazolyldiphenyl-tetrazolium Bromide
NAC	N-acetyl-L-cystein
NEMO	NF-κB Essential Modifier
NF-κB	Nuclear Factor Kappa B
NGF	Nerve Growth Factor
NIK	NF-κB Inducing Kinase
NLS	Nuclear Localization Signal
NO	Nitric Oxide
NOS	Nitric Oxide Synthase
NMDA	N-methyl-D-aspartate
PAK2	p21-Activated Kinase
PARP	poly-ADP Ribose Polimerase
PBS	Phosphate Buffer Saline
PCD	Programmed Cell Death
PCR	Polimerase Chain Reaction
PD	Parkinson Disease
PDK	Phosphoinositide-dependent Kinase
PEI	Polyethylenimine
PFA	Paraphormaldeyde
PIP_3	Phosphatidylinositol 3,4,5-trisphosphate

Abbreviations

PI-3K	Phosphatydil Inositol 3-Phospate Kinase
PKC	Protein kinase C
PVDF	Polyvinilydene difluoride
RIP	Receptor Interacting Protein
RNA	Ribonucleic Acid
ROCK I	Rho-Associated Kinase I
ROS	Reactive Oxigen Species
RT	Room Temperature
SCI	Spinal Cord Injury
shRNA	Short Hairping RNA
siRNA	Small Interference RNA
SMAC	Second Mitchondria-Derived Activator of Caspase
SODD	Silencer of Death Domains
TBI	Traumatic Brain Injury
TEMED	N,N,N',N'-tetramethylethylendiamine
tBID	Truncated Bid
TNF	Tumor Necrosis Factor
TRADD	TNFR-Associated Death Domain
TRAF	TNFR-Associated Factor
TRAIL	TNF-Related Apoptosis Inducing Ligand
TSE	Transmissible Spongiform Encephalopathies (TSE)
UV	Ultraviolet
ZF	Zing Finger

Abstract

Apoptotic cell death is triggered by several different stimuli, among which death receptors. To induce apoptosis, TNFα needs the cooperation of RNA transcription or protein synthesis inhibitor, i.e. ActD and CHX. In this study we demonstrate that ActD renders rat PC12 cells and primary mouse cortical neurons susceptible to the cytotoxic effect of TNFα by the activation of the initiator caspase 8, generation of tBid and activation of pro-caspase-9 and -3. Proteins involved in TNFα receptor signaling complex are not affected by TNFα/ActD stimulation. However, the analysis of anti-apoptotic proteins, e.g. FLIP, IAPs and Bcl-2 family members, demonstrates that Bcl-x_L is the endogenous regulator of neuronal sensitivity to TNFα-induced apoptosis and that it operates in a NF-κB-independent manner. Bcl-x_L overexpression completely protects against TNFα/ActD-induced apoptosis, whereas its endogenous decrease sensitizes to TNFα cytotoxic effect promoting JNK-dependent cell death. To point out the relevance of Bcl-x_L in TNFα signaling pathway, endogenous decrease of the main anti-apoptotic Bcl-2 family members, e.g. Bcl-2, Bcl-x_L, Bcl-w and Mcl-1, was performed in HeLa cell line in which, contrarily to PC12, these proteins are expressed. The results obtained demonstrate that Bcl-x_L is the most important Bcl-2-cytoprotective protein in regulating TNFα cytotoxicity.

Moreover, TNFα-induced cell death is promoted by high levels of free heme accumulation. Heme sensitizes Hepa cell line to TNFα-triggered apoptosis enhancing ROS production and ROS-mediated damage. This results in JNK and pro-caspase-3 activation. Oxidative stress-promoted apoptosis induced by heme/TNFα treatment is inhibited by the overexpression of HO-1 and H-Ferritin cytoprotective proteins.

Abstract

L'apoptosi pot ser induïda a través de nombrosos estímuls, entre els quals hi ha els receptors de mort. Per induir apoptosi TNFα necessita la participació d'inhibidors de la transcripció d'ARN o de la síntesi proteica, com són ActD i CHX. En aquest estudi demostrem com la citotoxicitat de TNFα en cèl·lules PC12 i en neurones corticals sensibilitzades amb ActD es dóna a través de l'activació de la caspasa iniciadora 8, de la generació de tBid i de la conseqüent activació de les pro-caspases-9 i -3. A més, el tractament amb TNFα/ActD no indueix diferències detectables en l'expressió de proteïnes involucrades en el complex de senyalització de TNFα. L'anàlisi de les principals proteïnes antiapoptòtiques, com són FLIP, IAPs i els membres de la família de Bcl-2, demostra que Bcl-x_L endogen és capaç de regular l'apoptosi induïda per TNFα, sense afectar l'activació de la via del factor de transcripció NF-κB. La sobreexpressió de Bcl-x_L dóna resistència a la mort promoguda per TNFα i ActD, i la reducció dels seus nivells indueix mort cel·lular per mitjà de TNFα, a través de l'activació de JNK. Per confirmar la rellevància de Bcl-x_L en el senyal promogut per TNFα, es va avaluar l'efecte de la reducció dels nivells basals de proteïnes antiapoptòtiques com Bcl-2, Bcl-x_L, Bcl-w i Mcl-1, en el model cel·lular HeLa, on aquestes s'hi troben expressades de forma fisiològica, contràriament al que passa en les cèl·lules PC12, demostrant que Bcl-x_L és la proteïna antiapoptòtica més rellevant en la protecció de la mort induïda per TNFα.

Per altra banda, l'apoptosi induïda per TNFα pot ser deguda a l'acumulació d'elevats nivells de hemo lliure. El grup hemo sensibilitza les cèl·lules Hepa a l'acció citotòxica de TNFα a través de la inducció d'estrès oxidatiu, que provoca un dany cel·lular que porta a l'activació de la via de JNK y de pro-caspasa-3. La producció de ROS i el dany induït per estrès oxidatiu, així com la mort induïda per TNFα, conjuntament amb elevats nivells del grup hemo lliure, poden inhibir-se amb l'expressió de proteïnes protectores com HO-1 y H-Ferritina.

La apoptosis puede ser inducida a través de numerosos estímulos, entre los cuales los receptores de muerte. Para promover la apoptosis, TNFα necesita la colaboración de inhibidores de la trascripción del RNA o de la síntesis proteica, como ActD y CHX. En este estudio demostramos como la citotoxicidad de TNFα en células PC12 y en neuronas corticales sensibilizadas con ActD ocurre a través de la activación de la caspasa iniciadora 8, la generación de tBid y la activación de las pro-caspasas-9 y -3. Además no se detectan diferencias de expresión, inducidas por TNFα/ActD, de proteínas involucradas en la formación del complejo de señalización de TNFα. El análisis de las principales proteínas antiapoptóticas, como FLIP, IAPs y miembros de la familia de Bcl-2, demuestra que Bcl-x_L es la molécula endógena capaz de regular la apoptosis promovida por TNFα, sin afectar la activación de la vía del factor de trascripción NF-κB. La sobre-expresión de Bcl-x_L confiere resistencia a la muerte apoptótica mediada por TNFα y ActD, y su disminución forzada es capaz de inducir muerte celular únicamente tratando con TNFα por activación de JNK. Para confirmar la relevancia de Bcl-x_L en la señal promovida por TNFα, la represión de proteínas anti-apoptóticas como Bcl-2, Bcl-x_L, Bcl-w y Mcl-1 ha sido evaluada en el modelo de células HeLa, donde estas se expresan fisiológicamente al contrario que en las células PC12, demostrando que Bcl-x_L es la proteína anti-apoptótica más importante en la protección de la muerte inducida por TNFα.

Por otra parte, la apoptosis mediada por TNFα puede ser promovida por la acumulación de elevados niveles del grupo hemo libre. El grupo hemo sensibiliza las células Hepa a la acción citotóxica de la citoquina TNFα a través de la inducción de estrés oxidativo, cuyo daño resulta en la activación de la vía de JNK y de pro-caspasa-3. La producción de ROS y el daño inducido por estrés oxidativo, así como la muerte inducida por elevados niveles del grupo hemo libre y de TNFα, pueden inhibirse por la sobre-expresión de proteínas protectoras como HO-1 y H-Ferritina.

Introduction

4.1 *APOPTOSIS: historical background and morphological classification*

Apoptosis is a physiological mechanism in which cell death and proliferation are kept in balance and it is essential to control the homeostasis. A malfunctioning in apoptosis leads to a multitude of pathological conditions, including degenerative disorders, cancer or autoimmune diseases (Zimmermann K.C. et al, 2001).

Although ishemia, stroke, Parkinson, Alzheimer, etc., present different development and evolutionary mechanisms which define the pathologies, they share as common feature the upregulation of proteins important for the execution of this named "controlled suicide pathway". Therefore, these degenerative disorders are associated to an excessive apoptosis rate. On the contrary, the upregulation of proteins whose function is to avoid the apoptotic process is detected in cancer and autoimmune disease (Cory S. et al, 2003).

The term Apoptosis was coined for the first time by Kerr, Wyllie and Currie to describe a cell death with typical morphological characteristics found repetively in different cell types and upon exposure to different stimuli (Kerr, 1969; Kerr at al, 1972; Lockshin and Zakeri, 2001; Cory S. et al 2003). This observation suggested the existence of a conserved death program, which is the essential requirement for the correct development of the organisms (Hengartner O., 2000).

Apoptotic cells preserve their membrane integrity while undergoing plasma membrane blebbing, cytoplasmatic and chromatine condensation, nuclear fragmentation and the consequent loss of adhesion (Choen et al, 1994, Zimmermann K. et al., 2001). The final result is the removal of the apoptotic cells by macrophages. This occurs through the recognition of phosphatidylserine, a phospholipid normally localized in the inner leaf of the plasma membrane and externalized during the first phases of the apoptotic process. Ingestion of apoptotic cells by macrophages avoids the release of intracellular content and the consequent inflammatory reaction that are typical features of the necrotic cell death (Marchetti L. et al, 2004).

The first effort to classify the apoptotic program was based on morphological analysis, through which Schweichel and Merker identified three types of cell death e.g. type 1, 2 and 3. The features which characterize type 1 apoptotic cell death are the same observed by Kerr. These include nuclear condensation and piknosis, cell shrinkage, late cell fragmentation, and the formation of the so-called apoptotic bodies which are subsequently engulfed by macrophages. Type 2 is also known as "autophagic degeneration" and is characterized by strong and compact cytoplasm

vacuolization, without chromatine condensation. Type 3 is also named “cytoplasmatic cell death” and is associated with the complete disintegration and deletion of cell organelles (Schweichel J.U. et al, 1973).

Apoptotic cell death is a strictly controlled process in which every single step is highly regulated by proteins, i.e. caspases, whose function is to carry on the whole suicide program (Shi Y. et al, 2002).

4.2 *THE APOPTOTIC SIGNAL TRANSDUCTION MACHINERY*

4.2.1 *Caspases functional classification*

The proteins required for the initiation and the execution of the apoptotic process belong to a family of highly conserved enzymes known as caspases. The function of the caspases was discovered in *Caenorhabditis elegans*, in which the apoptotic cell death is mainly controlled by two pro-apoptotic genes (*ced-3* and *ced-4*, caspase-3 and Apaf-1, respectively in mammals) and one anti-apoptotic gene (*ced-9*, Bcl-2 in mammals) (Yuan J. et al, 1993). Their homologues are reported in different species e.g. *Drosophila melanogaster*, *Spodoptera frugiperda*, and even *Saccharomyces cerevisiae* (Hengartner O., 2000).

Caspases are cystein proteases, synthetized as zymogens which include an N-terminal prodomain, a large subunit (p20) containing the cystein active site and a C-terminal small subunit (p10) present in mature enzyme. Their catalytic activity is regulated by a cystein active site by which caspases cleave their protein substrates at aspartic acid residue (Zimmermann K. et al, 2001).

Caspase classification is based on their mechanisms of activation (Figure 1). Caspases are divided in two groups: initiator caspases, e.g. caspase-2, -8,-9, and -10, whose function is to initiate the apoptotic process. These are characterized by the presence of a caspase recruitment domain (CARD) (caspases 2 and 8) or a death effector domain (DED) (caspases 8 and 10) in their N-terminal which governs their activation. Moreover, initiator caspases have a high substrate specificity allowing target recognition (Fuentes-Prior P. et al, 2004). Once initiator caspases are activated, they induce the activation of downstream executor caspases, caspase-3, 6 and -7, which are the responsible for morphological and biochemical features typical of this cell death (Hengartner O., 2000; Shi Y. et al, 2002).

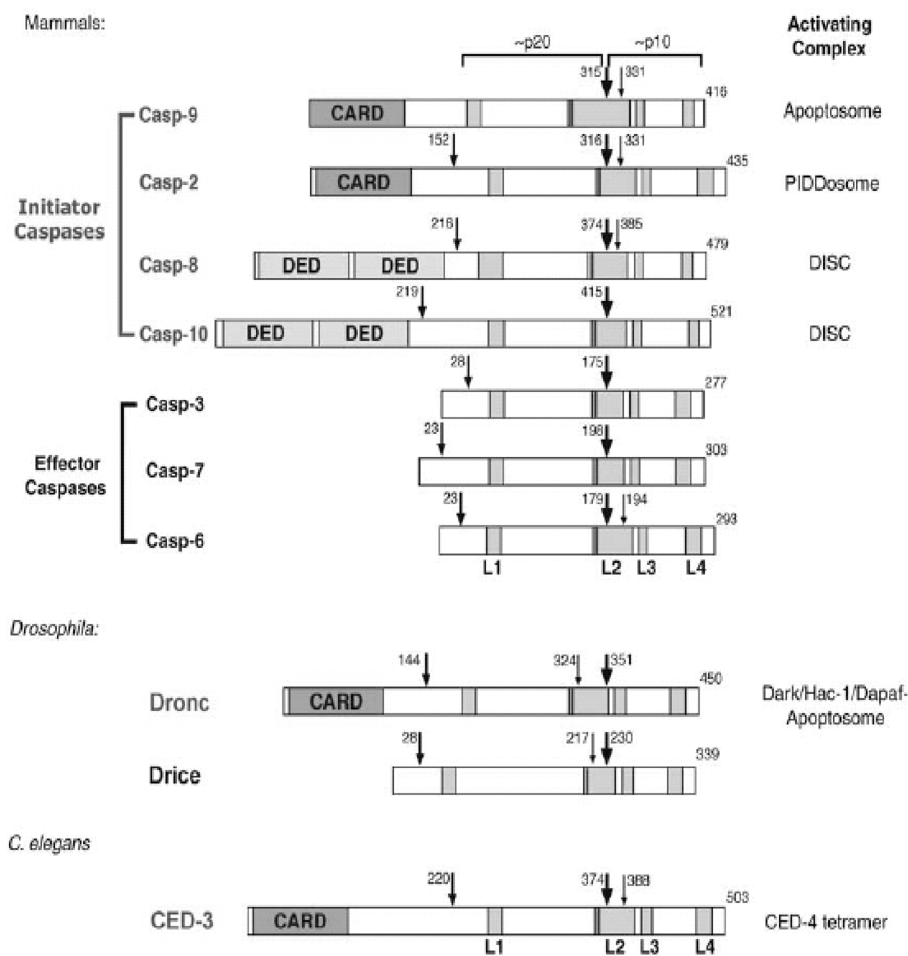


Figure 1. Initiator and effector caspases. The large arrow represents the first intrachain cleavage sites (between the large and the small subunit), whereas medium and small arrows show additional cleavages. The prodomains CARD and DED of initiator caspases are indicated, as well as the four loops, L1 to L4, which form the active caspase site. Moreover, the catalytic cystein residue is shown with a red line (adapted from Bao Q. and Shi Y, 2007).

Another group of caspases that includes caspase 1, -4, -5, -11, -12 and -13, do not trigger apoptosis, being mainly pro-inflammatory enzymes (Yakovlev AG, 2004; Scott A.M. et al, 2007).

4.2.2 *Caspase activation*

Three mechanisms were proposed to explain caspase activation (Figure 3):

1) The activation of effector caspases, i.e. caspase-3, -6 and -7, is induced through a proteolytic cleavage by an active upstream caspase. As the cleavage sites occur at the aspartic acid residues, the possibility of an autocatalytic activation is not completely discarded, although caspase precursors are present in cells in such conformation that should prevent this phenomenon (Thornberry N.A. et al, 1998). Once

activated, effector caspases are responsible for the proteolitic cleavage of cellular targets which lead to cell death associated to the typical apoptotic morphology.

2) The second mechanism proposed explains the activation of initiator caspases, i.e. caspase-8. To be activated it requires the assembly of a multi-component complex formed upon exposure to death stimuli (Riedl JS, et al, 2004). This results in the recruitment of high concentrations of pro-caspase 8 which, although presenting low protease activity, are sufficient to induce its own cleavage and activation by protein-protein interactions. This mechanism based on proximity activation was also proposed for other caspases e.g. caspase-2.

3) The third model is to explain the activation of pro-caspase 9 which takes place through its association to a regulatory subunit. To be activated caspase-9 requires to be bound to two other proteins, e.g. Apaf-1 and cytochrome c, to form a complex known as Apoptosome. As a consequence, caspase-9 acquires its complete functionality promoting the activation of the executor caspase-3 (Hengartner O., 2000). Contrarily to other caspases, the proteolytical activity of pro-caspase 9 is not crucial for its activation.

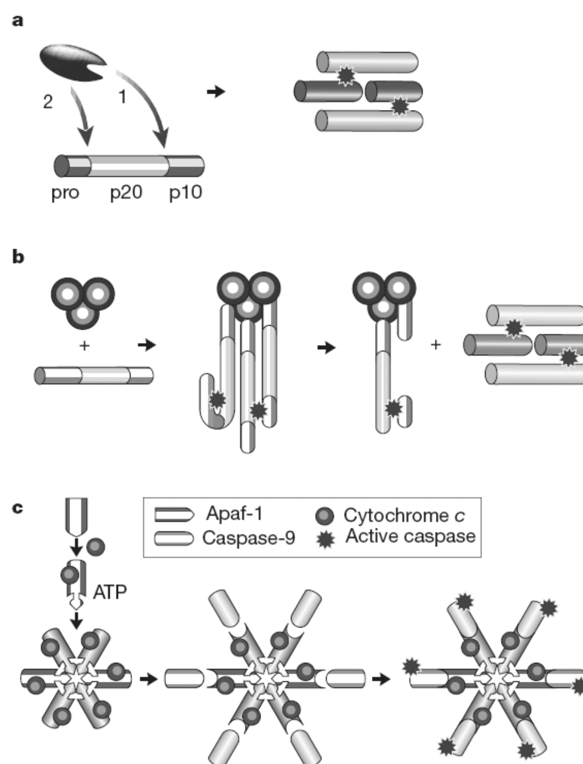


Figure 3. Three mechanisms were proposed to explain caspase activation. **a.** Proteolitic cleavage by an upstream caspase, which explains the activation of downstream effector caspases. **b.** Recruitment and proximity activation, which is the model proposed for caspase-8 activation. **c.** Association with regulatory subunits formed by cytochrome c and Apaf-1 which, in presence of ATP, allows the recruitment of procaspase-9 into the apoptosome complex. Activation of caspase-9 is mediated by conformational change and not proteolysis (adapted from Hengartner M.O., 2000).

4.2.3 *Caspase substrates*

The analysis of single caspase substrates, performed by target proteins cleavage site map screening, use of non-cleavable mutants, generation of gene knockout or non cleavable gene knockin, rendered possible the identification of about 400 proteins targeted by caspases. The role of some of them in the apoptotic cell death remains to be elucidated (Lüthi A.U. et al, 2007).

In the attempt to classify the multitude of reported caspase substrates, these were divided in proteins whose cleavage promotes a gain of function and proteins whose caspase processing results in a loss of function. This subdivision is related to conformational and/or molecular changes in the intrinsic properties of the cleaved molecule, e.g. the removal of a regulatory domain, or the zymogen activation, or the dissociation of active domains (Timmer J.C. et al, 2007).

The most relevant caspase substrates whose cleavage promotes a gain of function are:

- Executioner procaspase-3 and -7, the best known and well characterized caspase substrates. The initiator caspases, e.g. caspase-8, -9 and -10, are highly specific proteases which mediate the cleavage and activation of downstream effector caspases, leading to the propagation of the apoptotic stimuli (Fuentes-Prior P. et al, 2004). Initiator caspase-9 is cleaved at two sites that, although not essential for its activation, play an important role in regulating the apoptotic process. The first cleavage, mediated by caspase-9 itself, produces a N-terminal motif required for XIAP inhibition, whereas the second is promoted by executioner caspases and induces the removal of XIAP inhibitory effect, leading to the proceeding of the apoptotic cell death (Stennicke H.R. et al, 1999; Srinivasula S.M. et al, 2001).
- Cytosolic protein Bid, processed by active caspase-8 and -10 upon death receptor stimulation. Its cleavage is essential for the amplification and propagation of the apoptotic signal which results in the activation of the intrinsic mitochondrial apoptotic pathway (see Figure. 2) (Bossy-Wetzel E. et al, 1999; Timmer J.C. et al, 2007). The caspase-mediated processing of pro-apoptotic Bcl-2 family members, e.g. Bad and Bim, increases their cytotoxic ability, whereas the cleavage of Bcl-x_L originates a C-terminal fragment with pro-apoptotic effect (Lüthi A.U. et al, 2007).
- Retinoblastoma-associated protein (RB). RB is a tumor suppressor that inhibits cell cycle progression into S-phase by suppressing DNA synthesis through the inactivation of the DNA replication complex C large subunit. RB must be inactivated for the apoptotic progression (Chau B.N. et al, 2002).

- IAP binding motif (IBM) generated in caspase-7 and -9 and induced by not yet identified proteins. IBM allows the recruitment of IAPs, providing the inhibition of the apoptotic signal. However, other caspase substrates, e.g. SMAC and HtrA2/Omi, present a IBM in their sequence. Therefore, SMAC and HtrA2/Omi, becoming IAP antagonists, relieve the inhibition these proteins promote on caspases, leading to the proceeding of the apoptotic cascade (Huang Y. et al, 2003; Timmer J.C. et al, 2007).
- p21-activated protein kinase gamma-PAK, known also as PAK2. PAK2 is a kinase activated in response to several stimuli, e.g. DNA damage and serum starvation. During apoptosis PAK2 is cleaved by caspase-3 (Roig J., et al 2001). Removal of the N-terminus regulatory domain of PAK2 promotes its activation. Overexpression of the C-terminal fragment of PAK2 is sufficient *per se* to promote nuclear and cytoplasmatic condensation and phosphatidylserine externalization. Interestingly, caspase inhibitors do not avoid the alteration induced by the constitutive active PAK2, demonstrating that this enzyme functions as a downstream executioner in the process which leads to the apoptotic cell death (Lee et al, 1997; Rudel et al, 1997).
- MEKK1, the apical kinase of JNK/SAPK signaling transduction pathway (Cardone et al, 1997). Ectopic expression of the truncated MEKK1 induces apoptosis. Aspartic mutation in the MEKK1 cleavage site confers resistance to cell death and abolishes the activation of caspase-7 (Cardone et al, 1997).
- PKCδ. It undergoes proteolytic cleavage by caspase-3, leading to the removal of the N-terminus inhibitory domain from the kinase sequence. This results in PKCδ activation (Emoto et al, 1995). Overexpression of the catalytic fragment of PKCδ in HeLa cell line, besides contributing to the induction of the morphological changes typical of the apoptotic process, inhibits cell adhesion promoting the further loss of cell viability (Ghayur et al, 1996; Datta et al, 1997).

The caspase substrates whose cleavage promotes a loss of function are:

- DFF45/ICAD, the inhibitor of DFF40/CAD which is the nuclease responsible for DNA cleavage. In the absence of stimuli, the caspase – activated dnase (CAD) exists in an inactive complex bound to its inhibitor ICAD, whose human homologues are DFF40 and DFF45, respectively. When the apoptotic machinery is activated, CAD is released from ICAD through caspase-3 mediated cleavage. This nuclease induces the cleavage of genomic DNA between nucleosomes, generating approximately 180 and multiples base pairs length (Hengartner O., 2000, Fuentes-Prior P. et al, 2004; Timmer J.C. et al 2007).

- Poly (ADP-ribose) polymerase (PARP). It is a nuclear protein involved in DNA repair (Duriez P.J. et al, 1997). Cleavage of PARP by caspase-3 and -7, generates a protein product which can not synthetize ADP-ribose polymers in response to DNA damage. Mice expressing a mutated PARP caspase cleavage site develop normally, but are resistant to endotoxic shock as well as ischemia-reperfusion injury (Tewari M. et al, 1995; Petrilli V. et al, 2004).
- RIP. It is the protein engaged by TNFR1 and Fas receptor complexes that mediates NF-κB activation (Ting A.T. et al, 1996; Kelliher M.A. et al, 1998; Lee T.H. et al, 2004). Its proteolytic inactivation by caspase-8 results in a loss of function, which promotes the proceeding of the apoptotic stimuli (Lin Y. et al, 1999; Martinon F. et al, 2000).

Caspase-3-mediated proteolytic cleavage of fodrin, gelsolin, tubulin, actin, myosin, etc., responsible for cytoskeleton disassembly and blebbing, is the result of the apoptotic process (Kerr J.F. et al, 1972; Lüthi A.U. et al, 2007). The disruption of nuclear lamins is mediated by caspase-3 and -6, even though contribution of caspase-6 in nuclear shrinkage and progressive fragmentation is still not clear (Kothakota et al, 1997; Slee E.A. et al, 2001; Zimmermann K. et al, 2001). To better elucidate nuclear dismembering mechanism, several studies were focused on the serine/threonine kinase rho-associated kinase I (ROCK I). Caspase-mediated ROCK I cleavage induces to a constitutive active kinase that promotes an increase contractility of actin-myosin filaments (Coleman M.L. et al, 2001; Croft D.R. et al, 2005). Together with nuclear lamins degradation, increased cytoskeleton contractility causes a debilitation of nuclear envelope associated to nucleus disruption (Rao L. et al, 1996). Moreover, ROCK I also plays an important role in membrane blebbing through a reorganization of actin-myosin cytoskeleton, once caspase-dependent cleavage relieves its C-terminal auto-inhibitory region (Figure 4) (Mills J.C. et al, 1998; Sebbagh M. et al, 2001).

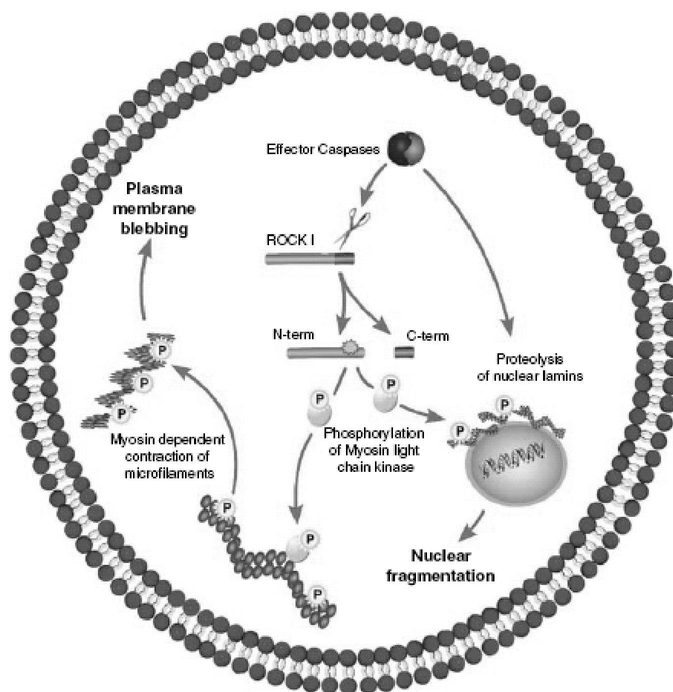


Figure 4. Caspase-dependent proteolysis of ROCK-I contributes to plasma membrane blebbing and nuclear fragmentation. Once the regulatory C-terminal portion of ROCK I is removed, the resulting active kinase promotes myosin light chain kinase phosphorylation, which regulates the rearrangements of myosin-dependent actin microfilaments (adapted from Lüthi A.U. et al, 2007).

4.2.4. *Chemical inhibitors of the apoptotic cell death*

The use of caspase inhibitors is an important tool to study, better understand and characterized the mechanism of the apoptotic program. Some of these compounds are effective in distinct animal disease models, e.g. stroke, myocardial ischemia/reperfusion injury, liver disease and traumatic brain injury (Yakovlev A. et al, 1997; Yaoita H. et al, 1998).

Caspase inhibitors are designed with modification on the N-terminal or C-terminal portion of the protein, or on the Asp acid residue in order to have reversible or irreversible antagonists respectively (Figure 5) (Degterev A. et al, 2003). Among the reversible caspase inhibitors are identified aldheydes, nitriles and ketones, which act as pseudosubstrates for caspase enzymes, without chemically altering them (Ekert P.G. et al, 1999). On the other hand, examples of irreversible caspase inhibitors include diazomethyl ketones, acyloximethyl ketones and alomethyl ketones. The tyomethyl ketone II originated by the interaction of these molecules with the caspase cystein active site results in the total and irreversible inhibition of the enzyme (Garcia-Calvo M. et al, 1998; Ekert P.G. et al 1999).

Caspase inhibitors present a tetrapeptide recognition motif, correspondent to their substrate specificity, which confers the ability to bind and inhibit specific caspases.

The action of inflammatory and effector caspases is suppressed by aldheydes molecules which present as tetrapeptide recognition motif Ac-WEHD-CHO and Ac-DEVD-CHO respectively, whereas initiator caspases are inhibited by Ac-IETD-CHO. This results in the synthesis of reversible membrane permeable peptides e.g. IETD-fmk and DEVD-fmk, commonly used to inhibit caspase-8 and caspase-3 respectively (Garcia-Calvo M. et al, 1998).

Based on the aminoacids substrate specifity of caspases, a broad spectrum enzyme activity blocker, the fluoromethyl ketone z-VAD-fmk, was identified as a competitive and irreversible inhibitor of the caspase-mediated apoptotic pathway (Garcia-Calvo M, et al, 1998). As short peptides, fluoromethyl ketone inhibitor compounds are much more permeable than aldheyde based inhibitors, conferring protection against caspase activation although used at very low doses (Ekert P.G., et al, 1999). The efficacy of z-VAD in disease animal models is due to its efficient capacity to inhibit different caspase enzymes, being important in attenuate ischemic brain damage in rodent (Loddick S.A. et al, 1996; Hara H. et al, 1997).

Toxicity, low half-life and poor cell permeability are some of the limitations reported in the use of these caspase–antagonists. However, the main disvantage is represented by the reported modest selectivity and low specificity, as they can poorly discriminate among different caspase family members (Degterev A. et al, 2003).

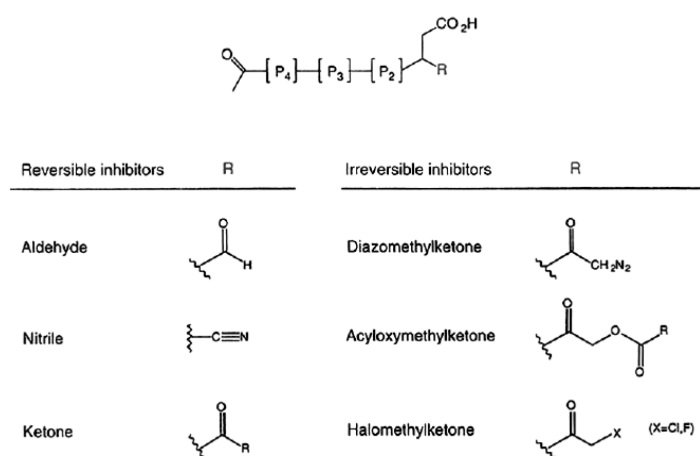


Figure 5. Chemical caspase inhibitors classification based on the enzymes recognition sequences (adapted from Thornberry N.A. et al, 1998).

4.3. *THE APOPTOTIC SIGNALING PATHWAYS*

The apoptotic process can be classified into three distinct phases: initiation induced by death signals, execution mediated by caspase activation and termination in which the apoptotic bodies are engulfed by phagocytes (Krueger A, 2001). Apoptosis

can take place through two different pathways: the intrinsic and the extrinsic (Riedl JS. et al, 2004), which lead to the final activation of executor caspases (Figure 6).

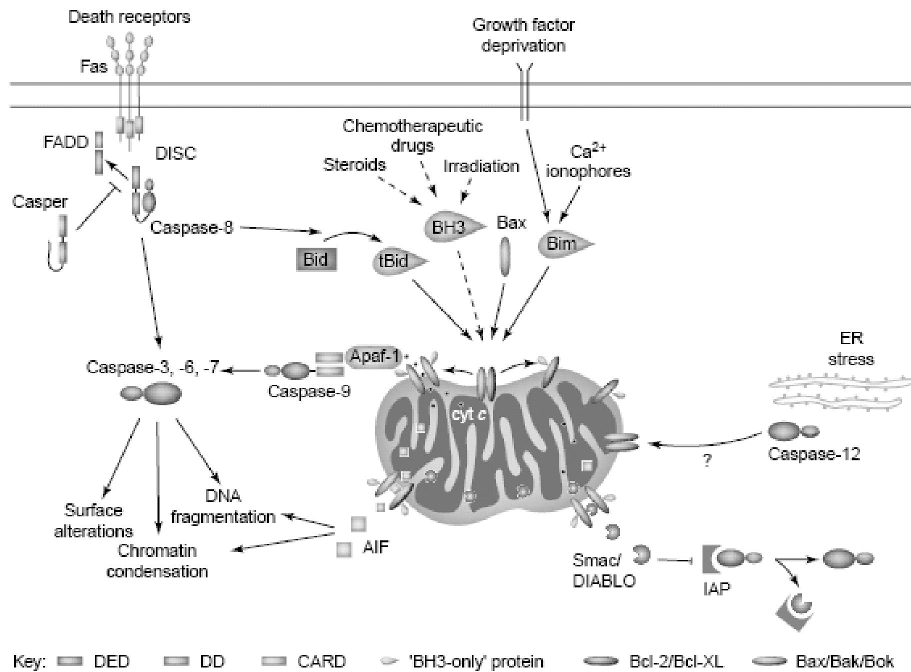


Figure 6. Extrinsic and intrinsic apoptotic signaling pathway in mammalian cells (adapted from Joza N. et al, 2002).

The "intrinsic" apoptotic pathway requires the activation of the mitochondria and the formation of the so-called apoptosome, whereas the "extrinsic" is mediated by cell-surface receptors.

4.3.1. *Intrinsic Pathway*

Some cell types undergo apoptosis through the activation of the intrinsic apoptotic pathway, in which the key role of the entire process is played by the mitochondria. This organelle can be injured by different stress stimuli, e.g. UV-radiation, γ-irradiation, heat, DNA damage, viruses, chemotherapeutic drugs, endoplasmic reticulum-induced stress, etc. (Khosravi-Far R. et al, 2004; Gupta S. et al, 2005). The net result is an alteration of the outer mitochondrial membrane permeability, according to which some proteins normally retained inside this organelle are released and free to diffuse into the cytosol (Figure 7). Among these, cytochrome c plays a central role in promoting caspase activation. Cytochrome c is normally bound to the inner mitochondrial membrane through the association with cardiolipin, a mitochondrial lipid rich in unsaturated fatty acids. Even though the mechanism remains unclear, it was suggested that cytochrome c release proceeds in two different steps. Peroxidation

of cardiolipin induced by the death stimuli promotes the dissociation of cytochrome c from the lipid present in the inner mitochondrial membrane. Cytochrome c is rapidly released consequently to swelling of the mitochondrial matrix and ropture of the outer mitochondrial membrane (Zhao K. et al, 2004; Orrenius S. et al, 2005; Kroemer G. et al, 2007). Once released, cytochrome c binds the apoptosis protease activator factor -1 (Apaf-1) inducing the apoptosome formation. This is the essential step for the recruitment and the activation of pro-caspase-9 within this complex, which subsequently can cleave and activate the executor caspases, e.g. caspase-3 and -7.

Depending on the stimuli, it is reported that through a feed-back amplification loop active caspase-3 can cleave and consequently activate at least four other caspases (-2, -6, -8 and -10), promoting an exacerbation of the apoptotic cascade (Yakovlev AG, 2004).

Besides cytochrome c, there are other proteins released from mitochondria whose function is also to promote apoptotis. Among them are the Second Mitochondria-Derived Activator of Caspases (SMAC/DIABLO) and High-Temperature-Requirement Protein A2 (OMI/HTRA2), which inhibit IAP family members (Garrido C. et al, 2004). Smac/DIABLO is a pro-apoptotic mitochondrial protein released into the cytosol upon activation of the intrinsic apoptotic pathway (Wang X., 2001). The removal of the mitochondrial target sequence, present in its N-terminal portion, converts SMAC in a mature form characterized by the exposure of a hydrophobic tetrapeptide. This motif is the essential requirement for SMAC pro-apoptotic action as inhibitor of IAPs, explaining why only this mature form is functional in cells (Verhagen A.M. et al, 2000; Bratton S.B., 2003). Smac functions as a IAP antagonist, as binding c-IAP1, c-IAP2, XIAP and survivin, removes their inhibition over caspases. The main target of SMAC/DIABLO is XIAP (Bratton SB. Et al, 2003; Shiozaki E.N. et al, 2004). Three splicing isoforms, which lack both the mitochondrial targeting sequence and the IAP binding motif, were identified. They are known as Smac-β, Smac-γ, and Smac-δ and due to their molecular structure, they localize in the cytosol. Little is known about these isoforms, although it is described that Smac-β seems to promote apoptosis as efficiently as the full-length Smac (Roberts D.L. et al, 2001).

Even though Smac can sensitize cells to death stimuli, e.g. UV irradiation, its overexpression is not sufficient to trigger apoptotic cell death (Du C. et al, 2000; Hunter A.M. et al, 2003). Moreover, Smac-null mice seems to be as sensitive as wild-type to death receptor-induced apoptosis (Okada H., 2001). As Smac-deficient cells respond normally to the apoptotic stimuli, this suggests the possible existence of other

molecules and/or pathways which cope with the absence of Smac (Okada H. et al, 2002)

HtrA2/Omi is a serine protease whose function in healthy cells is to maintain mitochondria homeostasis. Upon apoptotic stimuli, the release of HtrA2/Omi from mitochondria promotes cell death both through IAP inhibition and a caspase-independent mechanism based on its serine-protease activity (Garrido C. et al, 2004; Yang Q.H. et al, 2003).

The mitochondrial release of the apoptosis inducing factor (AIF) and of the EndonucleaseG (EndoG) upon death stimuli is associated with a sever damage of the mitochondria itself (Reidl JS et al, 2004). Induction of apoptosis promotes AIF translocation from the mitochondria to the nucleus where it interacts with the DNA causing its caspase-independent degradation. On the other hand, once released, EndoG translocates to the nucleus where it induces oligonucleosomal DNA fragmentation also when caspases are inhibited (Garrido C. et al, 2004).

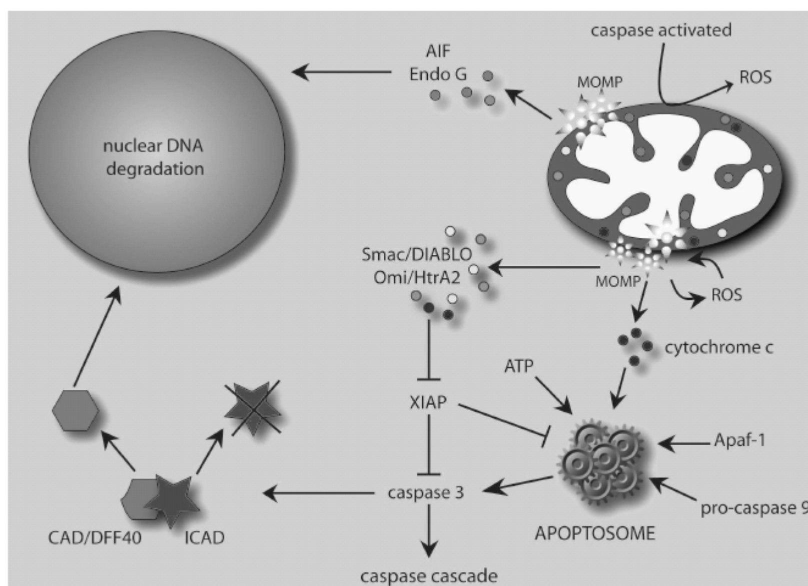


Figure 7. The mitochondria can be activated by several distinct death stimuli, which result in mitochondrial outer membrane permeabilization (MOMP) and subsequent release of pro-apoptotic proteins including cytochrome c, Smac/Diablo, AIF, EndoG, and Omi/HtrA2 (adapted from Bras M. et al, 2005).

The intrinsic or mitochondrial apoptotic pathway is governed by the Bcl-2 family members. These proteins play crucial roles in either promoting the proceeding of the cell death or inhibiting it, as they present both pro- and anti-apoptotic functions. The balance between Bcl-2 pro- and anti-apoptotic family members decides whether cells live or die upon the death stimuli.

4.3.2. *Bcl-2 family members*

Several Bcl-2-related proteins were identified in mammals and based on their structural similarity and functionality they were classified in three groups (Figure 8).

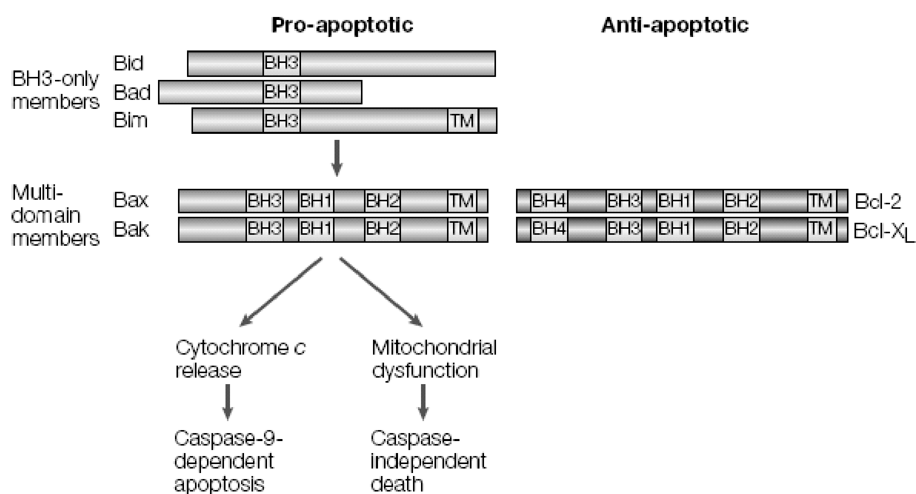


Figure 8. Most characterized Bcl-2 family members. The antiapoptotic proteins share sequence conservation throughout all four Bcl-2 homology domains (BH1-4). The proapoptotic members can be divided in multidomain proteins, which show homology with Bcl-2 antiapoptotic molecules in BH1-3, and BH3-only proteins. Many of these members possess a transmembrane domain, which allows them to interact with the endoplasmic reticulum, nuclear envelope and mitochondrial membrane (adapted from Jesemberg V. et al, 2002).

The first group includes proteins with anti-apoptotic activity, e.g. Bcl-2, Bcl- x_L and Bcl-w, which share structural similarity, and A1 and Mcl-1, which present distinct molecular structure. They protect cells from a broad range of citotoxic stimuli and are characterized by four conserved domains known as Bcl-2 homology (BH) domains (BH1-4) (Adams and Cory, 1998, Gross et al, 1999; Hengartner O., 2000, Cory and Adams 2002; Cory S. et al 2003). These proteins are localized in the outer mitochondrial membrane through their C-terminal portion (Huang et al 1997a; Cory S. et al 2003). The main function of these anti-apoptotic Bcl-2 family members is to inhibit the release of pro-apoptotic factors, i.e. cytochrome c, from the mitochondria, avoiding the proceeding of the apoptotic cascade (Hengartner O., 2000).

The second group is composed by proteins whose function is to promote apoptosis. These include Bax, Bak, Bad, Bok and Bcl-x_S. They present similar structure to the anti-apoptotic proteins but are composed by three of the four conserved domains, BH1, BH2 and BH3 (Muchmore et al, 1996; Suzuki et al, 2000; Petros et al, 2001; Hinds et al, 2003; Cory et al, 2003). They act in a pro-apoptotic manner inducing a destabilization of the mitochondrial membrane potential, which as a consequence promotes the release of pro-apoptogenic molecules e.g. cytochrome c (Cory et al, 2003). Once in the cytosol, citochrome c induces the formation of the apoptosome and

the subsequently activation of effector caspases (Wang X. 2001; Potts P.R. et al, 2003).

Upon stimuli, the pro-apoptotic protein Bax translocates to the outer mitochondrial membrane where it interacts with Bak. This results in the loss of mitochondrial integrity and change in its membrane potential (Wolter et al, 1997; Hsu and Youle, 1998; Griffiths et al, 1999; Cory et al, 2003). Different mechanisms were hypothesized to explain how Bax and Bak induce membrane mitochondrial permeabilization (Figure 9) (Harris and Thompson, 2000; Tsujimoto and Shimizu, 2000; Martinou and Green, 2001; Hardwick and Polster, 2002; Cory et al 2003). According to the first proposal, Bax and Bak can provoke the formation of pores, originating membrane channels whose dimensions are large enought to release cytochrome c and other molecules (Muchmore et al, 1996; Antonsoon et al, 2000; Saito et al, 2000; Pavlov et al, 2001; Roucou et al, 2002; Kuwana et al, 2002; Cory et al, 2003). The second theory is that Bax through the interaction with other proteins disrupts the mitochondrial membrane, mediating in this way the release of cytotoxic molecules retained in the inner compartment (Karbowski et al, 2002; Cory et al, 2003).

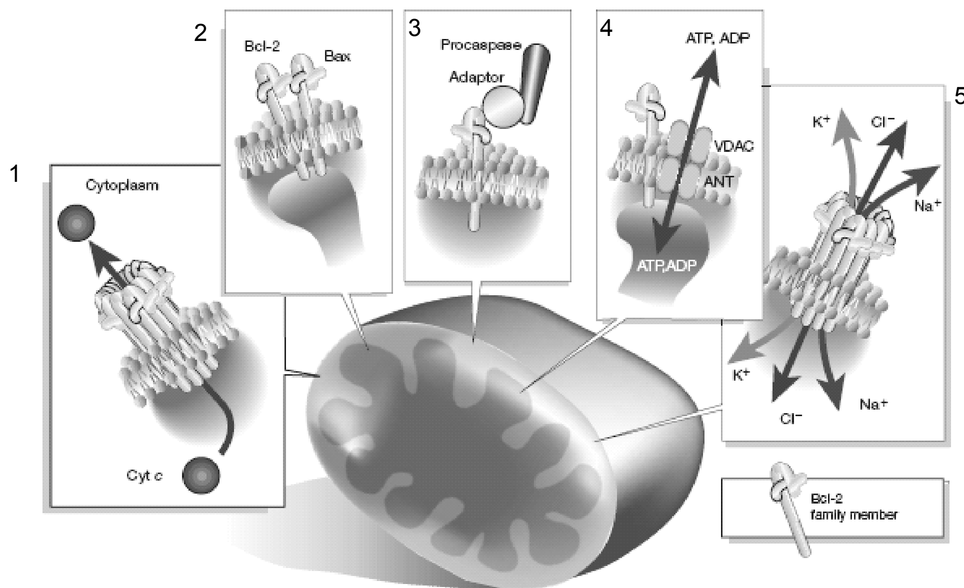


Figure 9. Bcl-2 family members play essential roles in controlling apoptosis, including 1. formation of a pore, through which cytochrome c (Cyt c) and other intermembrane proteins are released; 2. heterodimerization between pro- and anti-apoptotic family members; 3. direct regulation of caspase via adaptor molecules; 4. interaction with other mitochondrial proteins, e.g. VDCA and the adenosine nucleotide transporter (ANT), either to generate a pore for cytochrome c release or to modulate mitochondrial homeostasis; 5. oligomerization to form a weakly selective ion channel (adapted from Hengartner M.O., 2000)

The last group of the Bcl-2 family members is formed by the so-called “BH3-only proteins”, as their molecular structure presents just the conserved BH3 domain.

These proteins include Bid, Bik, Bad, Bim, Hrk, Puma and Noxa. Their function is to promote the correct proceeding of the apoptotic cell death. The BH3 motif is required for binding to other Bcl-2 family members, resulting in the activation of the pro-apoptotic Bak and Bax whose absence confers resistance to the death stimuli (Huang and Strasser, 2000; Puthalakath and Strasser, 2002; Cheng et al, 2001; Zong et al, 2001; Cory et al, 2003; Galonek H.L. et al, 2006).

To explain how BH3-only proteins induce the apoptotic program, two main theories were proposed. According to the first model, known as "direct model", BH3-only proteins bind directly and inactivate the anti-apoptotic Bcl-2 family molecules. Accordingly to this hypothesis, Bak and Bax are retained in an inactive state by their anti-apoptotic homologoues. The activation of BH3-only proteins leads to the disruption of Bax /Bak–anti-apoptotic Bcl-2 family members association. This promots the release of Bax and Bak which result free to mediate the proceeding of the apoptotic cascade (Figure 10) (Sattler et al, 1997; Petros et al, 2000; Liu 2003, Cory et al, 2003; van Delft M.F. et al, 2006; Galonek H.L. et al, 2006).

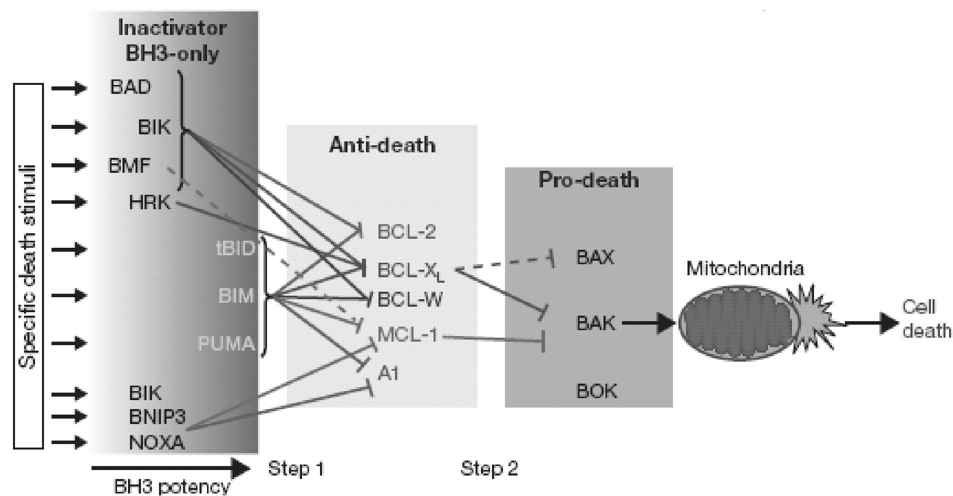


Figure 10. "Direct model". Step 1: BH3-only proteins are activated as a consequence of death stimuli. Their function is to bind and inactivate anti-apoptotic members of the Bcl-2 family, which in healthy cells inhibit Bax and Bak activation. Step 2: The activation of BH3-only proteins disrupts the association between Bax/Bak and Bcl-2 anti-apoptotic molecules, leading to Bax and Bak release, which mediate the proceeding of the apoptotic program (modified from Galonek H.L. et al 2006).

The second model proposed to elucidate Bax and Bak activation is the "hierarchy model". This suggests a classification of BH3-only proteins in two groups: inactivators of anti-apoptotic Bcl-2 family molecules and promoters of Bax and Bak activation. It was hypothesized that specific BH3-only proteins, represented by Bad and NOXA, directly bind anti-apoptotic Bcl-2 family members, leading to the release of tBid, Bim and PUMA. These BH3-only molecules, considered as real "activators" of the apoptotic machinery, promote the activation of Bax and Bak by direct binding or by

action on other cellular proteins, i.e. VDAC2, which act as negative regulators (Figure 11) (Kim H. et al, 2006; Galonek H.L. et al, 2006).

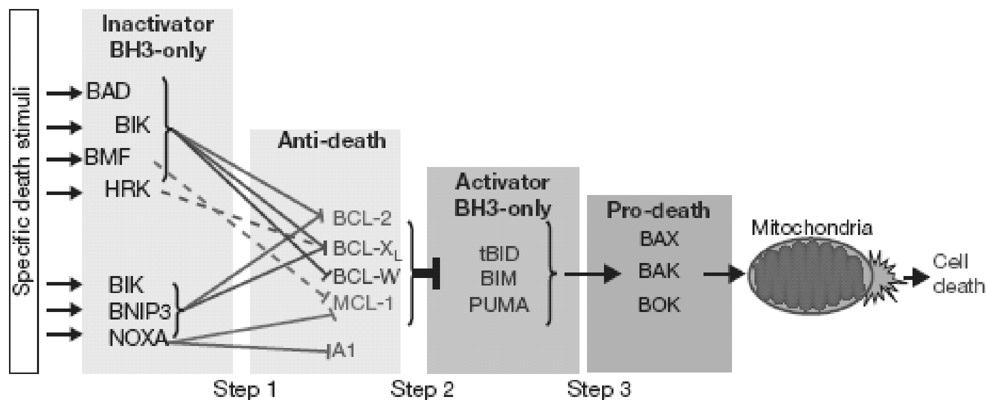


Figure 11. "Hierarchy model". Step 1: specific BH3-only proteins bind and inactivate Bcl-2 anti-apoptotic family members in response to death stimuli. Step 2: inactivators proteins promote the disruption of the association between anti-apoptotic molecules of Bcl-2 family and tBid, Bim and PUMA, leading to their release. Step 3; Identified as "activators", tBid, Bim and PUMA mediate Bax and Bak activation (modified from Galonek H.L. et al, 2006).

Both models imply that activation of Bax and Bak results in the destabilization of the mitochondrial membrane potential. As a consequence, cytochrome c is released and free to mediate the proceeding of the apoptotic cascade. The models proposed differ on the distinct BH3-only proteins interactions with the anti-apoptotic Bcl-2 family members. Moreover, it was demonstrated that Bim-E_L as well as PUMA can trigger cytochrome c release. Similarly to tBid, these proteins can displace Bak from the outer mitochondrial membrane channel VDAC2, leading to its activation (Cheng E.H.Y. et al, 2003; Kim H. et al 2006; Galonek H.L. et al, 2006)

4.3.3. *Extrinsic pathway*

The extrinsic apoptotic pathway is mediated by the activation of the so-called "death receptors", cell surface receptors which transmit the apoptotic signal after binding with their specific ligand. The extrinsic apoptotic cell death is governed by Tumor Necrosis Factor (TNF) family which includes about 20 different homologues ligands and their correspondent receptors. Among these ligands TNFα, Fas and TRAIL are the most characterized (Figure 12) (Darnay B.G. et al, 1999; MacEwan D.J. 2002; Gaur U. et al, 2003).

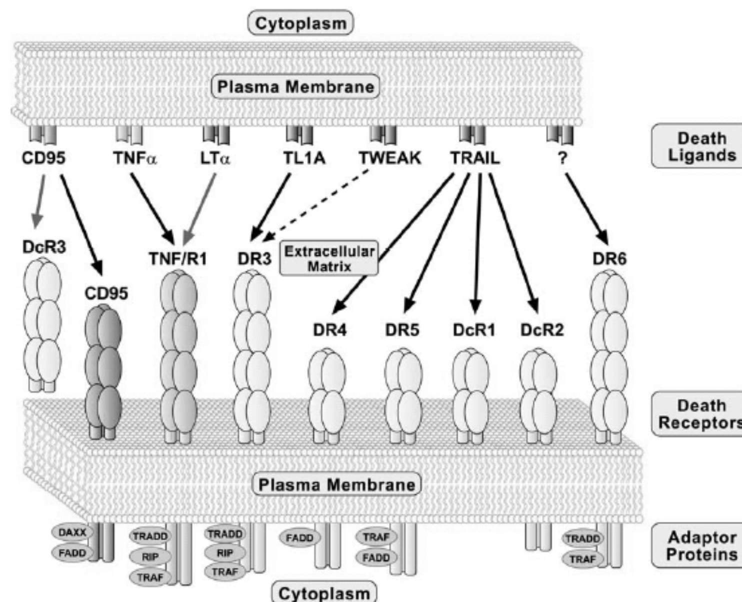


Figure 12. Death receptors and their correspondent ligands. Adaptor proteins recruited for the signal transmission are indicated in the figure (adapted from Curtin J.F., et al, 2003).

TNFα is a pleiotropic proinflammatory cytokine, mainly produced by macrophages, involved in many biologic functions e.g. cellular proliferation, differentiation as well as apoptosis (MacEwan DJ, 2002). Even though TNFR-1 is expressed on a variety of different cell types, TNFR-1-expressing cells are normally resistant to TNFα cytotoxic effect. Therefore, we decided to investigate in the nervous system the mechanism responsible for TNFα resistance to apoptosis.

TNFα is synthesized as a transmembrane protein and released through the action of the metalloprotease TNFα converting enzyme (TACE). The cleaved form, a 17kDa protein, is the responsible for all the processes in which the cytokine is involved (Wajant H. et al, 2003). TNFα achieves its biological functions through the interaction with TNFR-1 (also known as p55TNFR, p60, CD120a, TNFRSF1a) and TNFR2 (also known as p75TNFR, p80, CD120b, TNFRSF1b), which share only the 28% of homology mostly in their extracellular portion. The main structural difference between these two receptors is the presence in the intracellular part of TNFR-1 of a stretch of about 60-80 aminoacids called Death Domain (DD) that is absent in TNFR-2 (Tartaglia L.A. et al, 1992; Yang L. et al, 2002; MacEwan D.J., 2002). This motif is essential for the interaction and the recruitment of proteins required for the transmission of the apoptotic signal. Therefore, TNFR-1 is mainly involved in the apoptotic cell death, whereas TNFR-2 primarily mediates survival signaling triggering prolonged NF-κB activation.

4.3.4. *Adaptor proteins essential in TNFα signaling pathway*

Activation of the extrinsic pathway is triggered via the binding of an extracellular “death ligand” to its cell-surface “death receptors”. The ligands are present in cells as homotrimers. Once interacting with the receptors, they induce their homotrimerization, originating a homotrimeric complex ligand-receptor. This step is required for the recruitment of scaffold proteins named “adaptors” which are essential for the initiation of the signaling pathway (Tang P. et al, 1996; Locksley R.M. et al, 2001; Wajant H. et al, 2003).

Among the adaptor proteins required to mediate TNFα signal, particular relevance has TNFR–Associated Death Domain (TRADD) (Hsu H. et al, 1995). TRADD acts like a platform which allows the recruitment of other DD-adaptor proteins to the receptor. The relevance of this protein was demonstrated with TRADD-deficient animals in which NF-κB signaling transduction pathway and caspase-8 activation, both induced by TNFα, are completely impared and almost abrogaded (Zheng L. et al, 2006).

Other important adaptors involved in the transmission of TNFα signaling include TNF Receptor–Associating Factors (TRAFs) family. TRAF family members play essential roles in several biological functions including proliferation and cell death (Figure 13).

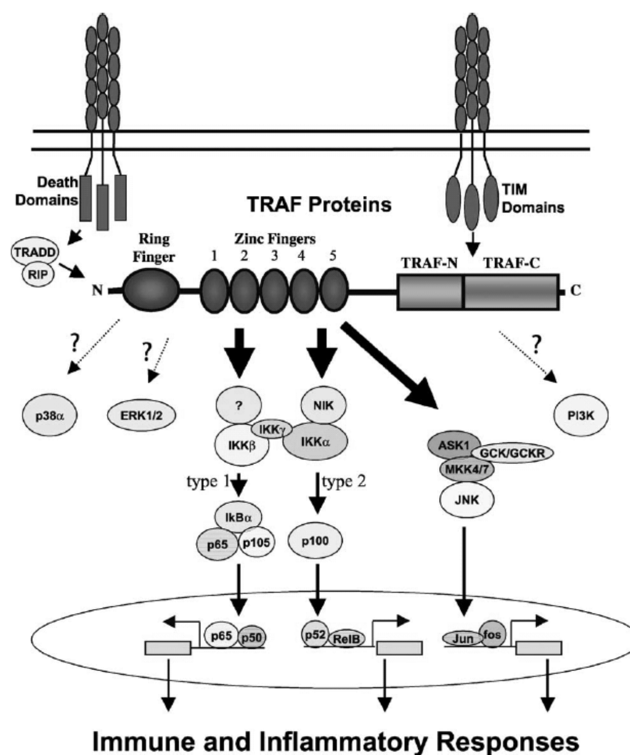


Figure 13. Signaling pathways activated by TRAFs (adapted from Dempsey P.W. et al, 2003)

TRAF proteins are characterized by a conserved C-terminal domain, known as TRAF domain, which is responsible for their multimerization with their respective partner molecules as well as for interaction with the surface receptors (Kaufman D. et al, 1999; Park Y.C. et al, 1999; Ye H. et al, 1999). Except TRAF1, all other TRAF members contain a N-terminal ring finger domain, indispensable for downstream effector responses (Lee N.K. et al, 2002). Six mammalian TRAF were identified so far of which TRAF2 seems to be the most relevant in the transmission of TNFR-1 signals (MacEwan DJ, 2002). TRAF2 can associate with TRAF1. This complex is required for the antiapoptotic molecules c-IAP1 and c-IAP2 to bind to TNFR-1, inhibiting caspase-8 activation (Rothe M. et al, 1995; Lee N.K. et al, 2002). Moreover, TRAF2 also plays an essential role in the activation of both NF-κB and JNK signaling transduction pathways induced by TNFR-1 (Chung J.Y. et al, 2002; MacEwan DJ, 2002).

The main adaptor protein that links death receptors to initiator caspases is the Fas Associated Death Domain (FADD) (Chinnaiyan A.M. et al, 1995, 1996; Boldin M.P. et al, 1995; Wajant H. et al, 2000; Werner M.H. et al, 2006). Upon cytokine stimulation, FADD is engaged to the death receptor complex by DD interactions. The Death Effector Domain (DED) motif in the N-terminal portion of the protein allows FADD to interact with the pro-form of caspase-8 promoting its recruitment to the Death Inducing Signaling Complex (DISC), formed by the receptor and the adaptor molecules engaged (Lawrence CP. et al, 2005) (Figure 14).

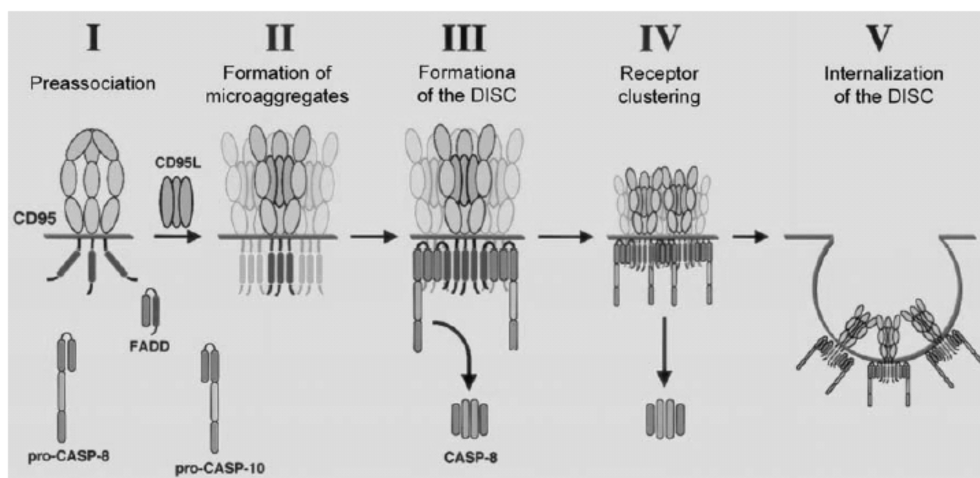


Figure 14. The five steps of Fas (CD95)-induced DISC formation (adapted from Algesiras-Schimnich A. et al, 2002).

Upon DISC formation, the high local concentration of pro-caspase-8 promotes its self-activation and the assembly of the mature enzyme. This event promotes the activation of downstream effector caspases, i.e. caspase-3 and -7. Once activated,

caspase-3 can cleave pro-caspase 8 thereby amplifying the death process (Yakovlev AG, 2004).

The formation of DISC-complex is essential for death receptors-mediated apoptosis (Walczak H. et al, 2001). Intriguingly, the apoptotic signaling induced by TNFR-1 requires distinct death-complex formations to transduce the signal (Micheau O. et al, 2003). Even though both FADD and caspase-8 recruitments are essential for TNFR-1-mediated apoptosis, activation of this initiator caspase occurs through a different mechanism. Once TNFα binds TNFR-1, the rapid engagement of adaptor proteins to form TNFR-1 signaling complex does not include either FADD or caspase-8. These two molecules are recruited only after internalization and formation of a second complex which lacks TNFR-1, but induces caspase-8 activation. Contrarily to Fas and TRAIL-mediated cell death, TNFR-1 internalization is essential for its cytotoxic activity, but not for activation of NF-κB and JNK signaling transduction pathways (Harper N. et al, 2003).

4.3.5. TNFR-1 apoptotic pathway

Unlike other death receptors, TNFR-1-induced apoptosis requires the formation of two distinct signaling complexes (Figure 15). The decision to commit cell death is not made at the level of plasma membrane-formed complex I, but depends on the formation of a complex that dissociates from TNFR-1, i.e. complex II, which is mostly localized in the cytoplasm (Micheau O. et al, 2003).

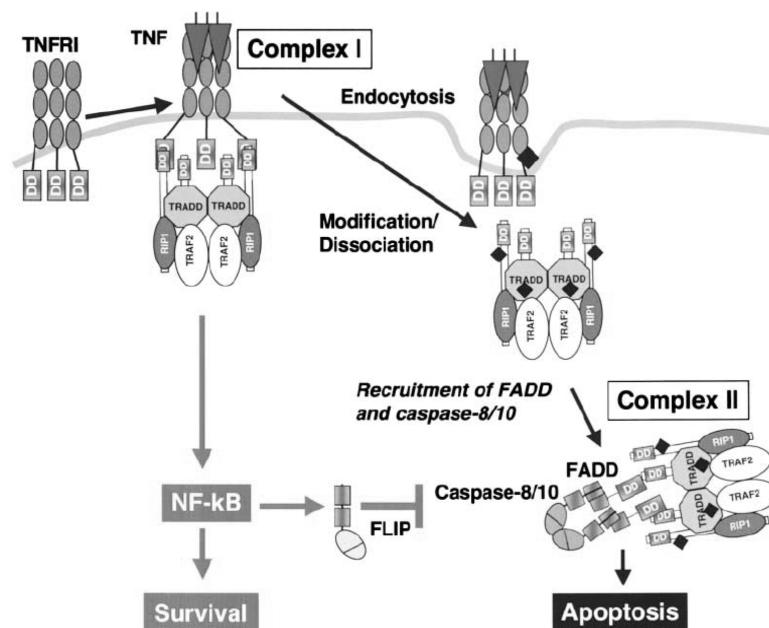


Figure 15. TNF-R1-mediated apoptosis (adapted from Micheau O. and Tschopp J., 2003).

The complex I is originated immediately upon TNFα binding to TNFR-1 and takes place in the plasma membrane. It is composed by TNFR1, TRADD, RIP, TRAF2 and c-IAP1, and its main function is to trigger cell survival as well as inflammation via the expression of NF-κB dependent pro-inflammatory genes (Micheau O. et al, 2003).

Under homeostatic conditions, a balance between cell survival and cell death is maintained upon TNFR-1 engagement. However, when this equilibrium is shifted versus cell death, i.e. when NF-κB fails to promote the transcription of anti-apoptotic genes, binding of TNFα to TNFR-1 induces the formation of another complex in the cytoplasm, the complex II. Complex II lacks TNFR-1 but is composed by FADD and pro-caspase 8 and -10. The formation of the complex II is the essential step to start the apoptotic program which leads to the activation of caspase-3 (Micheau O. et al, 2003).

Although intrinsic and extrinsic apoptotic signals follow different pathways depending on different stimuli, a crosstalk between them is observed (Li et al, 1998; Luo et al, 1998; Yin et al, 1999; Gross et al, 1999a; Cory et al, 2003).

4.3.6. *Crosstalk between extrinsic and intrinsic apoptotic pathways*

When the amount of caspase-8 activated by TNFR-1 is not sufficient to induce the direct cleavage of caspase-3, an amplification of the apoptotic cascade mediated by the mitochondria occurs (Bratton SB et al, 2003). This death receptor induced cell death is called type II, in order to differentiate it from type I in which the target of the caspase-8 is directly the executor caspase-3 (Figure 16) (Scaffidi et al, 1998; Cory et al, 2003).

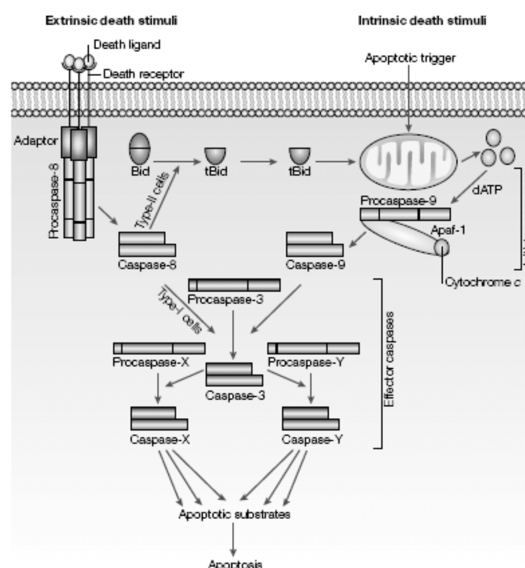


Figure 16. In type I cells, the level of caspase-8 is sufficient to directly induce the cleavage of effector caspase-3. In type II cells the low level of caspase-8 activated needs a mitochondrial amplification to induce cell death (adapted from Jesemberg V. et al, 2002).

The main link between the intrinsic and the extrinsic apoptotic pathway is identified in the pro-apoptotic protein Bid.

4.3.7. *Bid*

Bid is a BH3 only protein, normally present in the cytosol. Activated caspase-8 cleaves Bid into a truncated form, tBid, corresponding to the C-terminal portion of the protein. tBid translocates from the cytosol to the outer mitochondrial membrane, where it induces Bak oligomerization (Ruffolo S.C. et al, 2003). Moreover, tBid heterodimerization with Bax promotes Bax insertion in the mitochondrial membrane, where functional Bax oligomers are detected (Figure 17) (Krajewska M. et al, 2002). Bak and Bax activation results in the destabilization of the mitochondrial membrane potential and the release of important molecules which lead to the proceeding of the apoptotic cascade (Wei M.C. et al, 2000; Ruffolo S.C. et al, 2003).

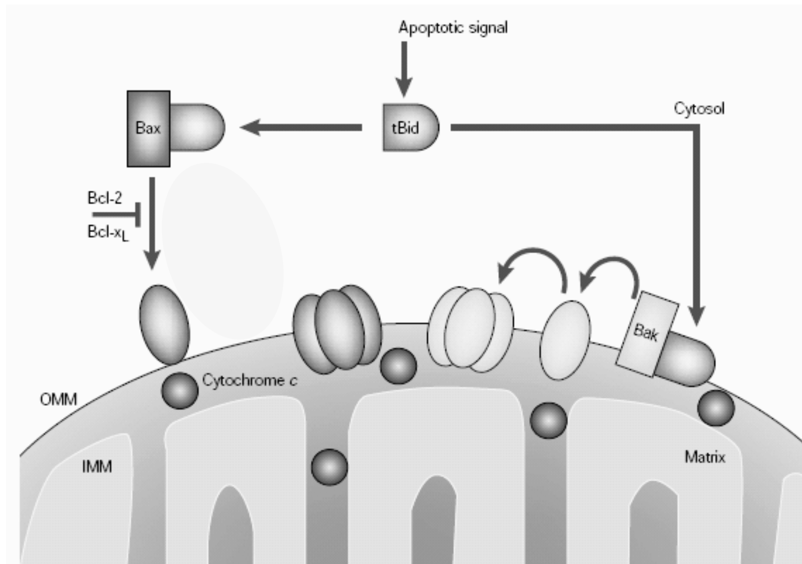


Figure 17. BH3-only protein Bid activates Bax and Bak (modified from Martinou J.C. et al, 2001)

4.3.8. *ROS generation and JNK signaling transduction pathway activation*

An important example of crosstalk between intrinsic and extrinsic apoptotic pathways is given by the accumulation of radical oxygen species (ROS) which promotes JNK activation (Kamata H. et al, 2005; Shen H.M. et al, 2006). Oxidative stress is a cellular condition in which the endogeous antioxidant enzymes can not cope with the excess of free radicals. This intracellular state is normally originated by an increase in ROS generation which results in the disruption of the intracellular redox equilibrium, as shown in figure 18 (Scandalios J.G. et al, 2005).

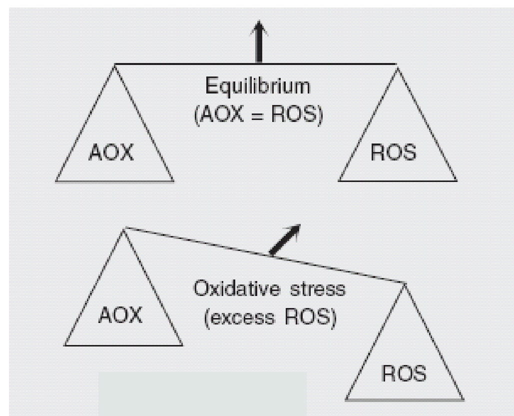


Figure 18. Oxidative stress results from the imbalance between the levels of antioxidant enzymes (AOX) and the production of reactive oxygen species (ROS) (modified from Scandalios J.G., 2005).

Low levels of ROS play an important role as second messenger in mediating different signaling pathways, among which particular relevant are the regulation of cell adhesion (Albelda S.M. et al, 1994), of vascular functions (Suzuki Y.J. et al, 1999; Griendling K.K. et al, 2000), the amplification of the immune response (Hehner S.P. et al, 2000) and the promotion of anti-oxidant gene transcription (Droge W. et al, 2002; Martindale J.L. et al, 2002; Shen H.M. et al, 2006). Nevertheless, the induction of intracellular high ROS expression is deleterious for the cells as they can react with and damage DNA, proteins and lipids, causing cell death (Figure 19).

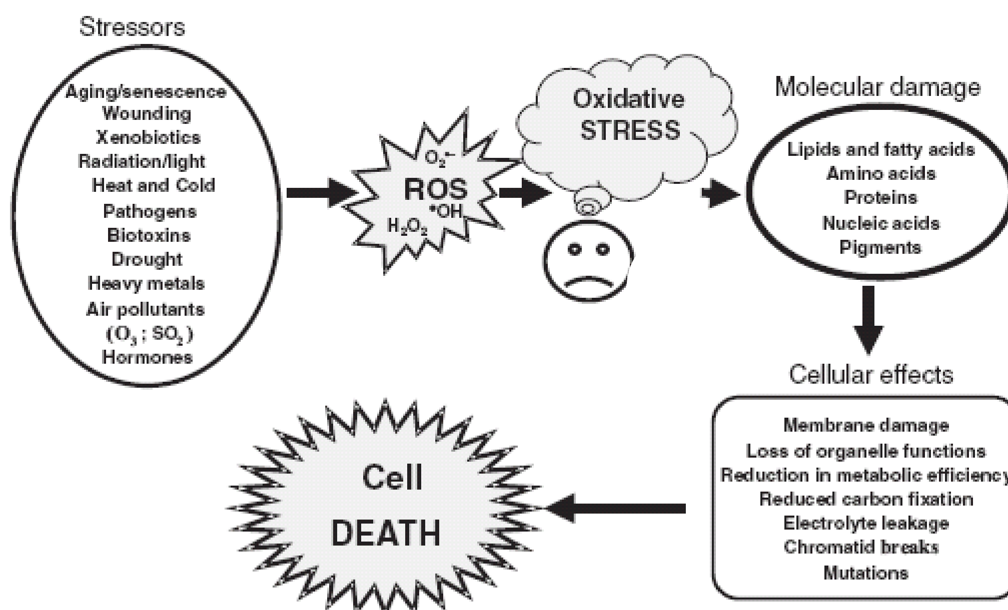


Figure 19. ROS inducers and the biological effects that lead to cells death (modified from Scandalios J.G., 2005)

TNFα-mediated high intracellular ROS accumulation promotes apoptosis. TNFα-induced ROS takes place in the mitochondrial electron transport chain, which is considered the main source of oxygen radical production in eukariots (Papa S. et al, 2005; Kamata H. et al, 2005).

The toxicity induced by ROS is mediated by the activation of a group of kinases belonging to the mitogen activated protein kinases, i.e. JNK. These kinases can be associated with both cell survival and death, depending on their short or prolonged activation respectively. In the case of TNFα-mediated ROS generation, the reported sustained JNK activation promotes cell death. This identifies ROS production as a potent activator of this pathway (Takeda K. et al, 2003; Torres M. et al, 2003; Shen H.M. et al, 2006). Moreover, a positive feedback loop was described between ROS and JNK: whereas JNK activation contributes to ROS production, high intracellular levels of ROS promote sustained JNK activation. This is due to high TNFα-induced ROS generation which suppresses the function of MAPK phosphatase (MKP) by oxidation of a cystein residue required for its catalytic activity. The inhibition of MKP promotes persistent JNK activation that triggers cell death (Figure 20) (Kamata H. et al, 2005).

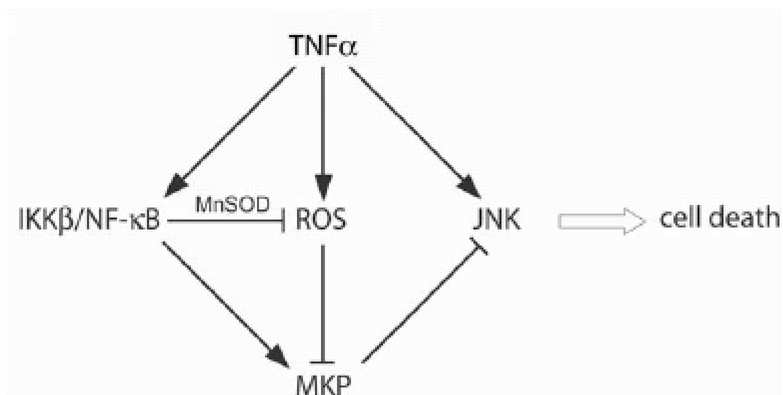


Figure 20. NF-κB modulates TNFα-induced death response through the control of ROS accumulation and MKP activity. TNFα induces NF-κB and JNK activation, as well as ROS production. NF-κB prevents ROS accumulation in part through induction of MnSOD. This preserves MKP activity, ensuring transient JNK activation and cell survival. Whether NF-κB is inhibited, ROS accumulates and MKPs function is suppressed, promoting sustained JNK activation which leads to cell death (adapted from Kamata H. et al, 2005).

JNK refer to a group of three isoforms, JNK-1, -2 and -3, which are products of individual genes. While JNK1 and JNK2 are ubiquitinously expressed, JNK3 is neural specific. Jnk1/Jnk3-deficient mice as well as Jnk2/Jnk3 $^{-/-}$ are viable, whereas Jnk1/Jnk2-null animals presents embryonic lethality suggesting some overlapping functions between JNK1 and JNK2 during the development (Kuan C.Y. et al, 1999; Sabapathy K. et al, 1999; Dietrich N. et al, 2004). Particularly relevant to the nervous

system is JNK3, as JNK3-deficient animals show resistance to the excytotoxic stimuli induced by glutamate. Moreover and from a clinical point of view, JNK3 mutation causes severe neurological deficits in children, indicating its essential requirement for a correct development of human brain (Gelderblom M. et al, 2004).

The JNK family is activated by the MAPK kinases (MAPKK, e.g. MKK4 and MKK7) which in turn are activated by MAPK kinase kinases (MAPKKK, e.g. MLKs or ASK). ASK1 is a conserved serine-threonine kinase responsible for MAPKs activation in *Chaenorhabditis elegans*, *Drosophila melanogaster* and mammalian cells where it activates MKK4/MKK7 in response to different stimuli including death receptors (Ichijo H. et al, 1997; Tobiume K. et al, 1997; Sagasti A. et al, 2001; Kuranaga E. et al, 2002; Matzukawa J. et al, 2004). MKK7 and MKK4 phosphorilates JNK at distinct sites, threonine 180 for MKK7 and tyrosine 182 for MKK4 (Davis R.J., 2000; Herdegen T. et al, 2001; Rincon M. et al, 2001; Waetzig V. et al, 2004). Overexpression of TRAF2 induces a strong ASK1 activation, revealing the importance of ASK1/TRAF2 interaction in the activation of MAPK cascade (Nishitoh H. et al, 1998; Zhao Y. et al, 2007)

JNK promotes the transcription of apoptotic as well as proinflammatory genes by the phosphorylation of c-Jun and ATF-2 transcription factors which are involved in cellular processes e.g. proliferation, differentiation, inflammation, and reclutation of apoptosis (Manning A.M. et al, 2003; Gelderblom M et al, 2004; Karin M. et al, 2005; Lu Z. et al, 2006).

Apoptosis induced by JNK activation is dependent on the mitochondria. The sustained activation of JNK by TNFR-1 promotes Bid cleavage in a different way than that mediated by caspase-8. This new product, known as jBid, translocates to the mitochondria where it induces the preferential and selective release of the proapoptotic factor SMAC/DIABLO. Once in the cytosol, SMAC/DIABLO migrates to TNFR-1-death receptor complex where binds and antagonize c-IAP1. This removes caspase-8 from the inhibitory effect of c-IAP1 and the TRAF2 complex, leading to its activation (Figure 21) (Deng et al, 2003; Muppidi JR et al., 2004).

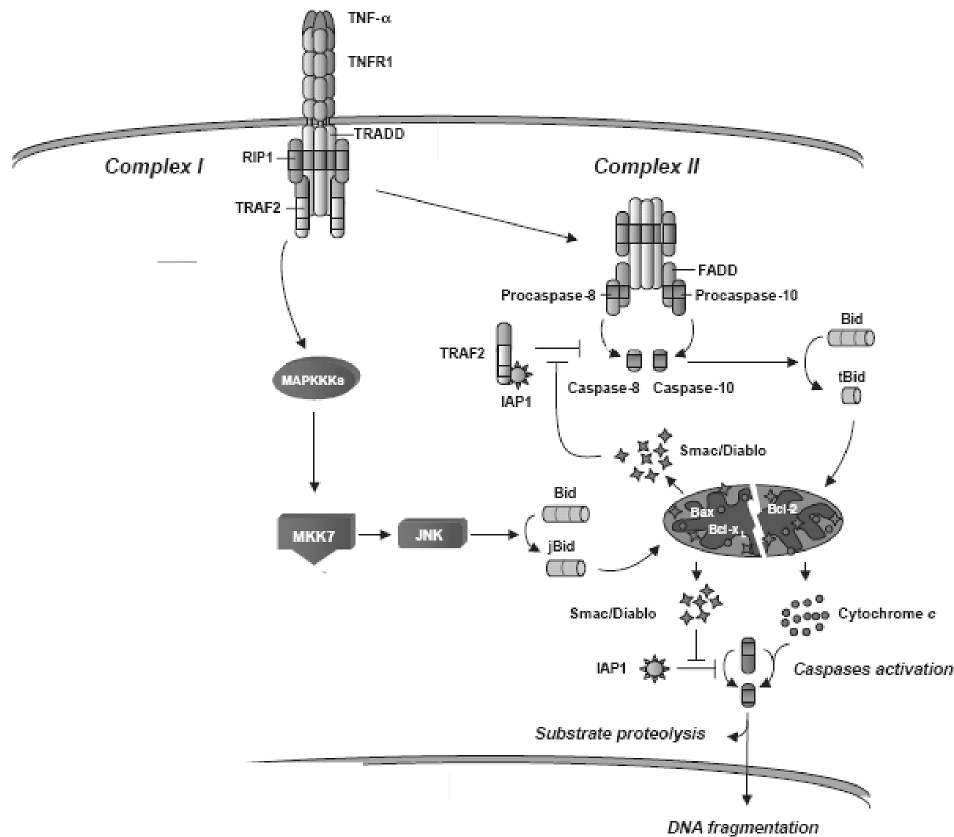


Figure 21. TNFR1-complex I leads to the formation of the complex II and to JNK activation which induces Bid cleavage. jBid translocates to the mitochondria where it promotes the selective release of SMAC/DIABLO. SMAC/DIABLO inhibits TRAF2-IAP1 complex which results in caspase-8 activation (modified from Papa S. et al, 2004).

4.4. *ANTI-APOPTOTIC SIGNALING PATHWAYS*

4.4.1. *NF-κB signaling transduction pathway*

NF-κB is an evolutionary and conserved signaling pathway that plays a crucial role in many biological processes (Hayden M. et al, 2004). Although it is required for immune and inflammatory response, NF-κB constitutive activation is associated with cancer and other diseases e.g. cardiovascular disease, diabets, chronic inflammation and CNS-related pathology (Brown M.A. et al, 2004; Ditsworth D. et al, 2004; Huang Y. et al, 2005; Lgssiar A. et al, 2004; Luo J.L. et al, 2004; Sathe S.S. et al, 2004; Xiao W. et al, 2004). NF-κB consists of homo or heterodimers of different subunits, members of a family of structurally related proteins named Rel/NF-κB proteins. In mammalian cells, five different proteins were identified: NF-κB1/p50, constitutively processed from its

precursor p105 by proteolysis, NF-κB2/p52, inducibly processed from its precursor p100, c-Rel, RelA/p65 and RelB. The predominant form is the heterodimer of NF-κB1/p50 and RelA/p65. The NF-κB/Rel proteins are characterized by the presence in the N-terminal region of a conserved 300 amino acids Rel homology domain (RHD), which is responsible for dimerization, DNA binding, nuclear localization and interaction with members of the I-κB protein family – the inhibitors of the NF-κB/Rel proteins (Hayden M. et al, 2004).

In the absence of the stimuli, the inhibitors I-κB bind NF-κB dimers, maintaining the transcription factor in an inactive state in the cytoplasm. Seven IkBs were identified I-κBα, I-κBβ, I-κBγ, I-κBε, Bcl-3 and the precursors of NF-κB1 and NF-κB2, p105 and p100, respectively. All the IκBs contain motifs known as ankyrin repeats, which are responsible for the association between the IκB and NF-κB dimers. IκBs interact with a region within the RHD of the NF-κB proteins, masking their Nuclear Localization Signal (NLS) and preventing their nuclear localization (Figure 22) (Wajant H. et al, 2003; Xiao W. et al, 2004).

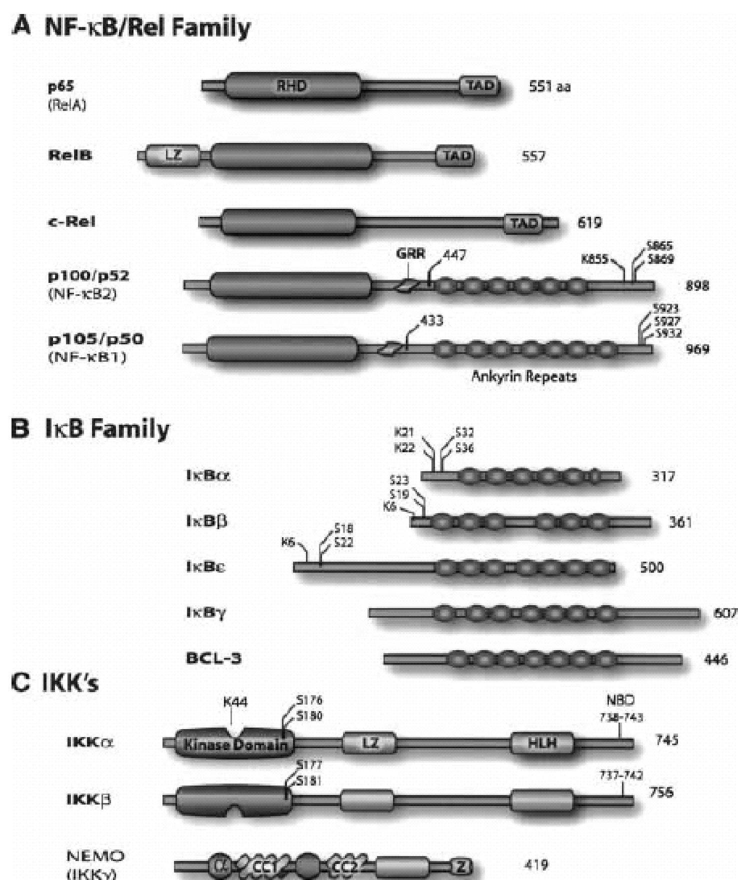


Figure 22. NF-κB, IκB and IKK protein family members. For each protein the number of amino acids is indicated on the right. Moreover, it is shown the presumed site of cleavage for p100 (amino acids 447) and

p105 (amino acid 433); phosphorylation and ubiquitination sites on p100, p105 and IκB proteins; Rel Homology Domain (RHD); Transactivation Domain (TAD) Leucine Zipper (LZ) domain on IKKα/β and Rel-B; Glycin Rich Region (GRR); Helix-loop-Helix (HLH) domain; Zing (Z) finger domain; Coiled-coil domains (CC1/2); Nemo-binding domain (NBD); α-helical domain (α) (adapted from Hayden M. S. et al, 2004).

NF-κB can be activated according to two different pathways, of which the most common is the so-called "classical pathway" (Figure 23). Upon stimulation by proinflammatory cytokines, i.e. TNFα, NF-κB activation is mediated by a complex of kinases (IKK), formed by two catalytic subunits, IKKα and IKKβ (or IKK1 and IKK2), and a regulatory one, IKKγ or NEMO. In mammalian cells, the predominant form is the IKKα:IKKβ heterodimer associated with IKKγ. Phosphorylation of IKKβ is an essential step for the activation of IKK by TNFα, as IKKβ and not IKKα is the target for TNFα (Delhase M. et al, 1999; Gupta 2002).

IKK phosphorilate IκB proteins on two N-terminal serine residues (serine 32 and serine 36). This leads to IκBs ubiquitination and degradation by 26S proteasome subunit. Therefore, NF-κB dimers are released from IκB molecules and free to translocate from the cytoplasm to the nucleus, where they promote gene transcription upon binding to κB consensus sequence present in NF-κB target genes promoter (Verna I.M. et al, 1995; Baeuerle P.A. et al, 1996; Li Q. et al, 2002; Hayden M.S. et al, 2004).

The other NF-κB activation pathway, known as "alternative pathway" takes place when upstream NF-κB–inducing kinase (NIK) is activated. The function of NIK is to activate IKKα homodimers, independently of either IKKβ or IKKγ. This leads to the phosphorylation of p100 and its consequently inducible processing to p52. This pathway is completely independent of I-κB degradation (Karin M. et al, 2005).

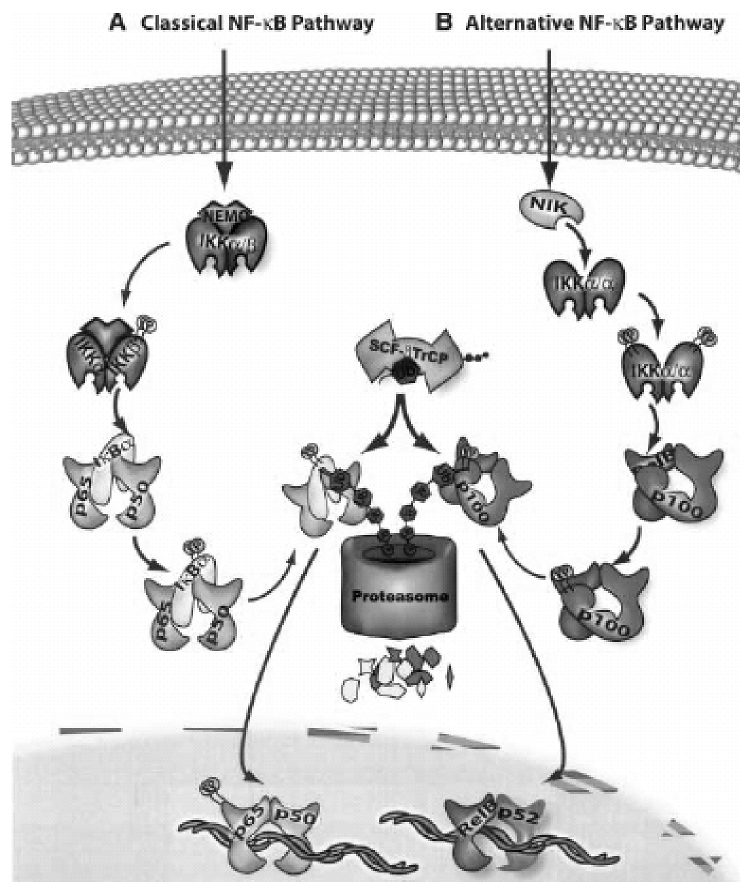


Figure 23. NF-κB activation pathways. The classical pathway (A) is mediated by IKKβ and leads to IκBα phosphorylation. In the alternative pathway (B) NIK activates IKKα which leads to the phosphorylation and the processing of p100 (adapted from Hayden M.S. et al, 2004).

To address the importance of NF-κB signaling transduction pathway, mammalian Rel and IκB family members were deleted in mice by homologous recombination. In particular, $p65^{-/-}$ mice are embryonic lethal due to liver degeneration mediated by TNFα signaling. This phenotype can be rescued crossing $p65^{-/-}$ with either $TNF\alpha^{-/-}$ or $TNFR1^{-/-}$ (Beg et al, 1995, Gupta, 2002).

The cell survival promoted by NF-κB is mediated by the transcription of anti-apoptotic genes, e.g. c-FLIP, IAPs, A20 and the anti-apoptotic Bcl-2 family members e.g. Bcl-2, Bcl-x_L, Bcl-w, Mcl-1 and A1 which confer protection against the apoptotic stimuli (Tian B. et al, 2005). Moreover, NF-κB signaling transduction pathway plays an important role in mediating resistance against the damage induced by high levels of ROS, through the transcription of MnSOD and H-Ferritin (Papa S. et al, 2005).

4.4.2. *Erk signaling*

ERK signaling pathway is activated in response to several distinct stimuli, e.g. cytokines, growth factors, oxidative stress, etc., and is involved in regulation of cell proliferation, differentiation and survival (Zhang W. et al, 2002; Grethe S. et al, 2006; Mehta S. et al, 2007). ERK activation is mediated by the upstream kinase MEK1, which can be activated by Raf-1, whose activity in turn is promoted by Ras (Kinoshita T. et al, 1997; Bonni A. et al., 1999).

TNFα triggers ERK activation via RIP-related serine/threonine kinase RIP2 (Navas T.A. et al, 1999). Moreover, TRAF-2, TRAF-5 and TRAF-6 are involved in TNFα-promoted ERK signaling (Sakon S. et al, 2003). It was observed that FLIP-s enhances ERK phosphorylation upon TNFα stimulation through direct interaction with Raf-1, thereby recruiting upstream kinase of the ERK pathway into the death receptor complex (Kataoka T. et al, 2000; Lüschen S. et al, 2005).

It is known that ERK activation mediates important survival signals through the interaction and inactivation of pro-apoptotic Bcl-2 family members, e.g. Bad and Bim as well as the inhibition of caspase-8 activation (Tran S.E. et al, 2001; Eisenmann K.M. et al, 2003; Zhang B. et al, 2003; Ley R. et al, 2004).

ERK activation plays an important role in promoting survival of post-mitotic cells within the central and peripheral nervous system, where the two ERK isoforms, ERK1 and ERK2, are highly expressed. Interestringly, the increased ERK1/ERK2 phosphorylation of neurons upon ischemic injury, suggests that the activation of this pathway confers neuronal protection against transient ischemia (Irving E.A. et al, 2000). Even though the protective effect renders ERK-mediated signaling object of study for the development of therapeutical inhibitors for cancer treatment, in certain contexts, i.e. cellular models of excitotoxic injury, prolonged ERK activation can lead to neuronal death (Roberts P.J. et al, 2007). This demonstrates the protecting or degenerating action induced by this pathway depending on the time of activation (Irving E.A. et al, 2001).

4.4.3. *Akt signaling*

TNFα stimulates the phosphorylation of Akt, a critical serine/threonine kinase whose activation leads to the induction of phosphatidylinositol-3 kinase (PI3K) signaling. PI3K/Akt signaling pathway is involved in the regulation of cell proliferation (Amaravadi R. et al, 2005). It is essential to promote cell survival, upon death receptor stimulation, in different cell types including neurons (Brunet A. et al, 2001; Vantler M. et

al, 2005). Activation of PI3K leads to the generation of phosphatidylinositide 3,4,5-triphosphate (PIP_3), responsible for Akt recruitment to the inner portion of the plasma membrane (Vivanco I. et al, 2002). As a consequence, confomational changes are induced in this kinase, which results in the exposure of its phosphorylation residues. Akt activation is mediated by the PIP_3-triggered recruitment of phosphoinositide-dependent kinase-1 (PDK1) and by a kinase activity referred to PDK2 (Partovian C. et al, 2004; Amaravadi R. et al, 2005). Possible candidates involved in PDK2 activation include integrin-linked kinase (ILK), DNA-dependent protein kinase and PKCα (Fukuda T. et al, 2003; Feng J. et al, 2004; Partovian C. et al, 2004; Hannigan G. et al, 2005).

Activation of Akt signaling pathway confers resistance to cell death by inactivation, stabilization and repression of different substrates (Datta S.R. et al 1999). Among the proteins, whose Akt-mediated inactivation promotes cell survival, are Bad, caspase-9 and the forkhead transcription factor, FKHRL1 (Brunet A. et al, 2001; Amaravadi R. et al, 2005). Moreover, stabilization of the anti-apoptotic XIAP protein as well as the regulation of NF-κB activation by TNFR-1 confers protection against the apoptotic stimuli (Datta S.R. et al, 1997; Cardone M.H. et al, 1998; Kane L.P. et al, 2002). In addition, the repression of p53 activity and its consequent Bax and Noxa-induced expressions promotes survival in hippocampal neurons (Miyashita T. et al, 1995; Oda K. et al, 2000;Yamaguchi A. et al, 2001)

According to its protective role, using a model of retinal ischemia-reperfusion it was demonstrated that Akt phosphorylation and activation is regulated by TNFR-2, as mice deficient for this receptor present an exacerbation of cell death in retinal layers upon TNFα stimulation (Fontaine V. et al, 2002). Therefore, activation of Akt survival pathway is suggested to be crucial in protecting neurons from reperfusion injury mediated by TNFα, since a decrease in its activity is associated with ischemic-induced cell death (Kawano T. et al, 2002; Fukunaga K. et al, 2003).

4.5. *ANTI- APOPTOTIC PROTEINS*

Apoptosis can be inhibited at different levels by the presence of proteins normally expressed in cells, some of them regulated by NF-κB transcription factor (Antwerp D.J.V. et al, 1996; Beg A.A. et al, 1996; Barkett M. et al, 1999; Baldwin A.S., 2001; Micheau O. et al, 2001; Hu X. et al, 2003).

4.5.1. *A20*

A20 is a cytoplasmatic protein whose transcription is mediated by NF-κB signaling transduction pathway (Lee E.G. et al, 2000). This protein presents a dual function, both as an anti-apoptotic protein and as an inhibitor of NF-κB activity (Opipari A.W. et al, 1992; Cooper J.T. et al, 1996; Beyaert R. et al, 2000; Miao H. et al, 2005). A20 possesses an ubiquitin ligase activity which is mediated by its zinc finger domain present in the C-terminal portion of the protein. This induces a negative feed-back regulation on NF-κB pathway, which results in the termination of the NF-κB transmitted signal. The principal target of this enzymatic activity is RIP, a molecule essentially required for TNFα-mediated NF-κB activation. The ovarian tumor domain (OUT) identified in the N-terminal motif of A20 inactivates RIP by removing Lys63-linked polyubiquitin, whereas the ubiquitin ligase domain of the C-terminal portion of A20 coordinates the addition of Lys48-linked polyubiquitin. As a consequence RIP is targeted by the proteasome, which promotes the degradation of the protein suppressing NF-κB activation signal (Figure 24) (Wertz I.E. et al, 2004; Evans P.C. et al, 2005; Heyninck K. et al, 2005).

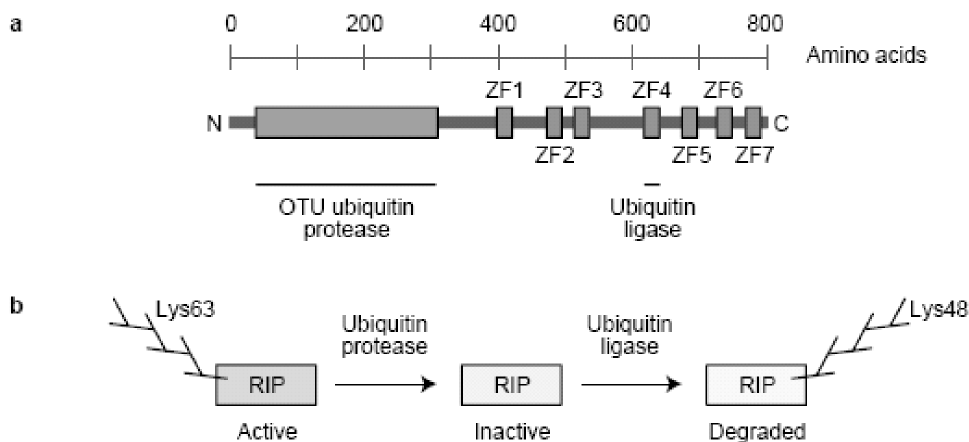


Figure 24. a) A20 structure. In the N-terminal part A20 present a ovarian tumor domain (OTU). The C-terminal portion contains seven zinc finger motifs (ZFs). b) A20 modulates RIP activation and stability (adapted from Evans P.C., 2005).

A20-deficient mice develop multi-organ inflammation and are extremely sensitive to TNFα-induced apoptosis (Lee E.G. et al, 2000). It was demonstrated that A20 overexpression reduces the infart volume and improves the neurological deficit in ischemic rats (Yu L. et al, 2006). Moreover, A20 upregulation confers resistance against TNFα-triggered cell death (Beyaert R. et al, 2000).

4.5.2. *Inhibitors of Apoptosis Proteins (IAPs)*

IAPs molecules were identified first in baculovirus as proteins with the ability to confer resistance to virus-induced apoptosis (Crook N.E. et al, 1993). To date eight mammalian IAPs were identified, among which the most characterized are are X-linked inhibitor of apoptosis (XIAP, hILP), c-IAP1 (HIAP2), c-IAP2 (HIAP1), neuronal IAP (NAIP), and survivin (Roy et al., 1997; Deveraux et al, 1997; 1998; Liston et al, 1996; Uren et al, 1996; Tamm et al, 1998; Ambrosini et al, 1997; Zimmermann K. et al, 2001).

IAPs inhibit caspase activation via a conserved sequence of aminoacids named baculoviral IAP repeats (BIR) domain (Figure 25). All mammalian IAPs have three BIR domains, except survivin, which has only one (Takahashi et al, 1998; Zimmermann K. et al, 2001). Another important region of these proteins is the RING domain, which acts as a ubiquitin ligase to promote caspase degradation (Yang et al, 2000; Zimmermann K. et al, 2001). Besides the RING finger motif, both c-IAP1 and c-IAP2 possess a CARD domain, suggesting that these IAPs might directly or indirectly regulate the processing of caspases (Zimmermann K. et al, 2001).

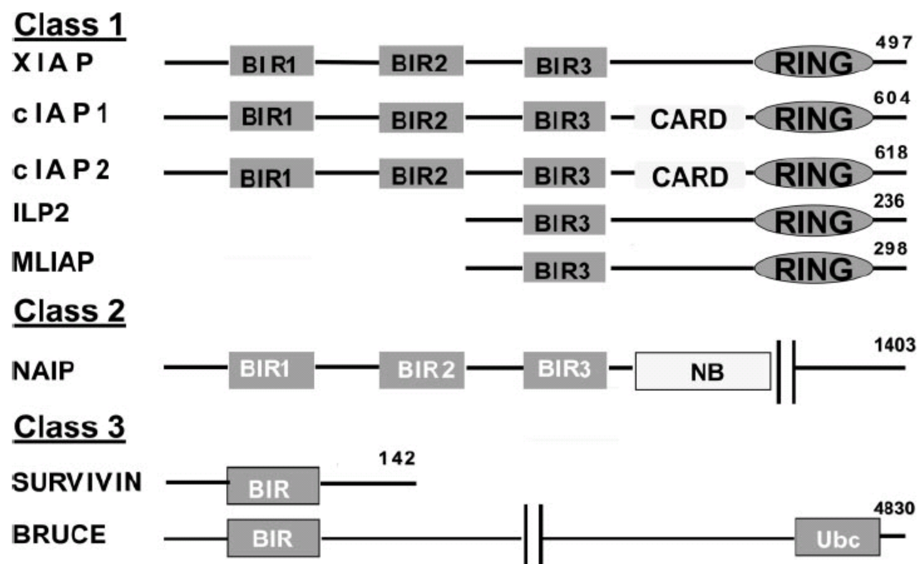


Figure 25. Human IAPs family proteins. In addition to at least one baculoviral IAP repeat (BIR) domain, they also contain a CARD domain and a RING finger motif. Bruce has an ubiquitin conjugation (Ubc) domain, whereas NAIP presents a nucleotide-binding domain (NB) (adapted from Schimmer A.D. et al, 2004).

The IAP family members are classified in three different groups based on their structural homology (Verhagen A.M. et al, 2001).

The first class is composed of XIAP, c-IAP1, c-IAP2 ILP-2, ML-IAP proteins.

XIAP is widely expressed in adult tissue (Duckett C.S. et al, 1996). Its anti-apoptotic activity is mediated by its BIRs motifs. BIR3 XIAP domain inhibits caspase-9, whereas BIR2 caspase-3 and -7. (Deveraux Q.L. et al, 1997). Moreover, the E3

ubiquitin ligase activity present in the RING sequence is essential for the induction of caspase proteasomal degradation (Yang Y. et al, 2000; Suzuki Y. et al, 2001; Dean E.J. et al, 2007).

c-IAP1 is highly expressed in thymus, testis and ovary, whereas c-IAP2 is expressed in the spleen and thymus. Human c-IAP1 and c-IAP2 inactivate caspase-8, -3, -7, and -9 (Deveraux Q.L. et al, 1997; 1999; Roy et al, 1997; Zimmermann K. et al, 2001; Deng Y. et al, 2003; Dean E.J. et al, 2007), although considered less potent inhibitors when compared with its homologous XIAP. c-IAP1 and c-IAP2 full length fail in conferring protection against apoptotic stimuli e.g. Bax overexpression, virus infection and TNFα treatment. In contrast, their C-terminal truncation increases the anti-apoptotic activity of these molecules, identifying this sequence as a negative regulator motif of cIAPs (Clem R.J. et al, 2001).

ML-IAP and ILP-2 are normally expressed in adult testis, fetal thymus, liver and kidney, as well as cancer cell lines (Kasof G.M. et al, 2001; Richter B.W.M. et al, 2001). These anti-apoptotic proteins bind and inhibit caspase-3, -7 and -9 (Vucic D. et al 2000; Dean E.J. et al, 2007).

The second group of IAP family members includes NAIP, expressed in adult liver, central nervous system and placenta. NAIP inactivates caspase-3 and -7 (Maier J.K. et al, 2002).

The third group is composed by survivin and BRUCE proteins.

Expression of survivin is cell cycle dependent, meaning that it is present just in those tissue in which cells are proliferating, e.g. embryonic liver, kidney, lung and gastrointestinal tract, and almost absent in normal adult tissue (Ambrosini G. et al, 1997; Dean E.J. et al, 2007). The inhibitory action of this molecule is on caspase-3, -7 and -9 (Conway E.M. et al, 2000).

BRUCE is upregulated in brain and ovarian cancer cells (Chen Z. et al, 1999; Dean E.J. et al, 2007) and possesses a C-terminus E2 ubiquitin-conjugation motif which allows this molecule to target proteins mediating their ubiquitin degradation (Hauser H.P. et al, 1998; Hao Y. et al, 2004).

4.5.3. *FLICE-like Inhibitory Protein (FLIP)*

Other proteins that inhibit death receptor induced apoptotis include a family of components of γ-herpesviruses, i.e. Kaposi's sarcoma associated herpesvirus, named viral–FLICE–inhibitory proteins (v-FLIP). It was observed that the two DEDs identified in v-FLIP molecular sequence bind other DED containing proteins, i.e. FADD and

caspase-8 and -10. Therefore, this avoids the association between FADD and caspase-8/-10 which results in the inhibition of caspase activation and apoptosis (Djerbi M. et al, 1999).

Homologoues of v-FLIP exist in mammalian cells and are known as cellular–FLICE-inhibitory protein (cFLIP) (Figure 26) (Thome M. et al, 1997).

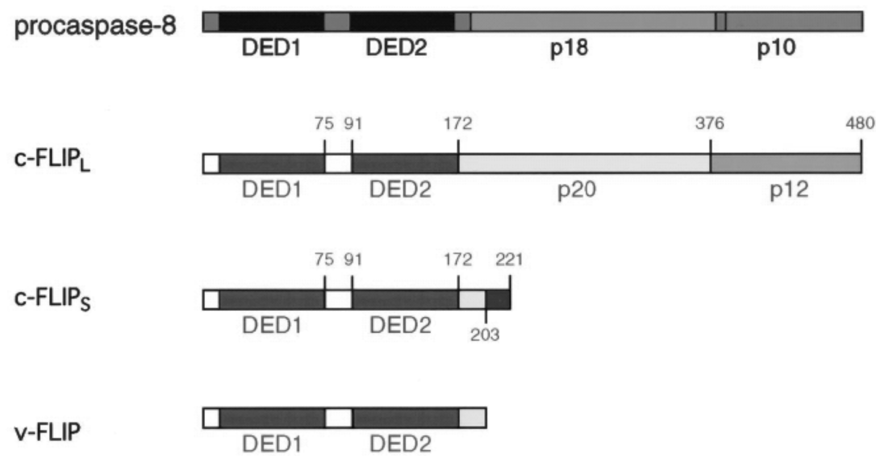


Figure 26. Structural similarity between caspase-8 and FLIP (adapted from Krueger A. et al, 2001).

c-FLIP exists as a multiple splice variants both at mRNA as well as protein level. Three splicing variants of the ubiquitinously c-FLIP were identified i.e. $FLIP_L$, $FLIP_S$ and the recently discovered $FLIP_R$ (Figure 27). All the isoforms possess a conserved and catalitically inactive DED, which confers the ability to function as caspase-8 inhibitor (Golks A. et al, 2005).

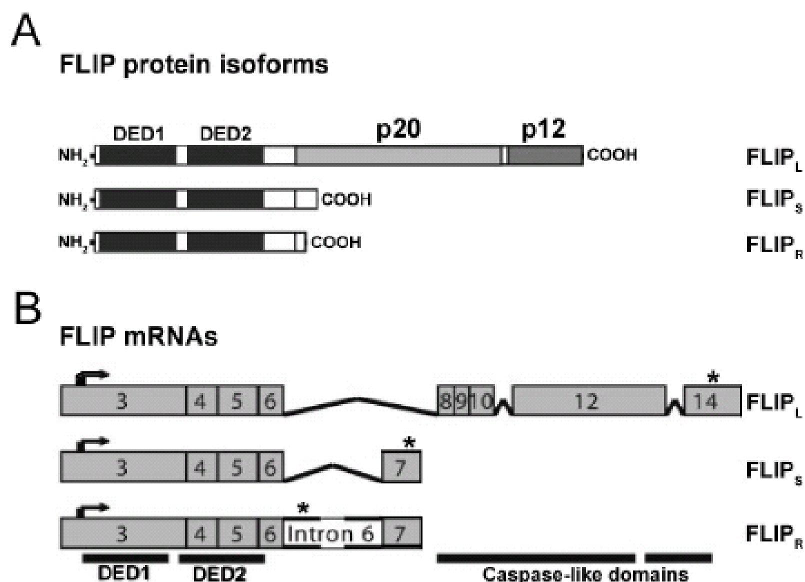


Figure 27. Exon-intron organization of protein encoding c-FLIP mRNAs. **A.** The three described c-FLIP proteins contain two death effector domains (DEDs) at their N-terminal (*dark bars*). In addition, $FLIP_L$

contains a large (p20) and a small (p12) caspase-like domain without catalytic activity. **B.** All the three described c-FLIP isoforms contain exons 3–6 encoding two DEDs. Start codons are marked by *arrows*, whereas stop codons by *asterisks* (adapted from Golks A. et al, 2005).

c-$FLIP_S$ presents in its sequence only two DEDs and a short C-terminal portion which is structurally different to the one of c-$FLIP_L$ (Micheau O. et al, 2001).

c-$FLIP_L$ exhibits high homology to caspase-8 since it contains two DEDs and a caspase-like domain, even though it lacks the cystein of the catalytic center which confers the activity to the protease (Yeh W.C. et al, 2000).

The molecular structure of c-$FLIP_S$ and c-$FLIP_R$ are very similar, as both isoforms contain two DEDs in the N-terminal portion of the protein. However, they differ in the C-terminal part, shorter in $FLIP_R$ (Golks A. et al, 2005). Moreover and regarding the functional property, both c-$FLIP_S$ and c-$FLIP_R$ can be recruited to the death complex, where they interact with FADD and form heterodimers with caspase-8, avoiding its processing and consequent activation. Therefore, c-$FLIP_S$/c-$FLIP_R$ are proteins that inhibit the extrinsic apoptotic pathway (Golks A. et al, 2005).

A different antiapoptotic mechanism was proposed for $FLIP_L$. Once within the death complex, $FLIP_L$ competes with caspase-8 for the recruitment to the DISC. $FLIP_L$ is cleaved into a p43 subunit that contains the DED motifs and localizes at DISC level. The other p12 subunit is released (Figure 28) (Krueger A. et al, 2001). Therefore, in presence of $FLIP_L$ caspase-8 undergoes an incomplete cleavage which prevents its full activation and the proceeding of the apoptotic cascade (Krueger A. et al, 2001).

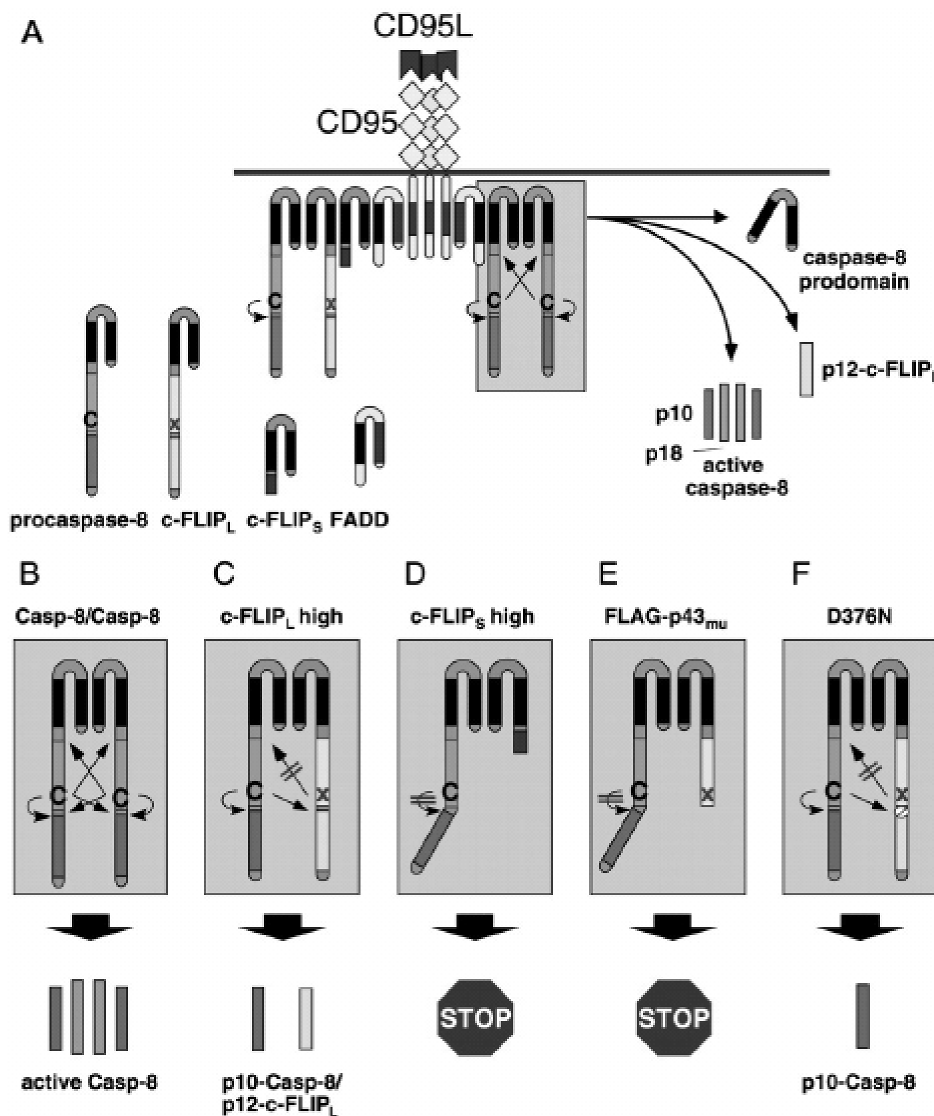


Figure 28. Model for c-FLIP mediated inhibition of procaspase-8. **A**. Representation of the DISC assembly, which results in caspase-8 activation. **B**. Low amounts of c-FLIP proteins allow caspase-8 activation in the active heterotetramer of p18 and p10 subunits. **C**. In the presence of high amounts of c-FLIP$_L$, procaspase-8 is recruited into the DISC, and cleavage is inhibited after the generation of the p43 cleavage products of both caspase-8 and c-FLIP$_L$. **D**. In the presence of high amount of c-FLIP$_S$, procaspase-8 is recruited into the DISC but remains unprocessed. **E**. High amounts of a mutated form of truncated c-FLIP$_L$-p43 prevent completely procaspase-8 activation. **F**. Expression of an unprocessable form of c-FLIP$_L$, c-FLIP$_L$-D376N allows initial cleavage of procaspase-8 but prevents further processing, leading to accumulation of p43/41-caspase-8 in the DISC (adapted from Krueger A. et al, 2001).

Caspase-8 activation is prevented by NF-κB-promoted transcription of c-FLIP. Therefore, depending on cell types and death stimuli, a decrease in c-FLIP expression triggers cell death. Recently, it was demonstrated that JNK activation promotes the proteasomal degradation of c-FLIP$_L$ through the activation of the ubiquitin ligase ITCH, whose catalytic activity is mediated by JNK1-phosphorylation (Figure 29) (Chang L. et al, 2006). ITCH is an E3 ubiquitin ligase which controls apoptosis via ubiquitin-

degradation of anti-apoptotic proteins. Due to the structural differences between c-FLIP isoforms, ITCH interacts specifically with c-$FLIP_L$ but not with $FLIP_S$ or $FLIP_R$. The biologic relevance of this protein was also demonstrated by the use of ITCH-deficient animals which, as $FLIP_L$ can not be degraded, are resistant to TNFα cytotoxic effect (Maeda S. et al, 2003; Chang L. et al, 2006).

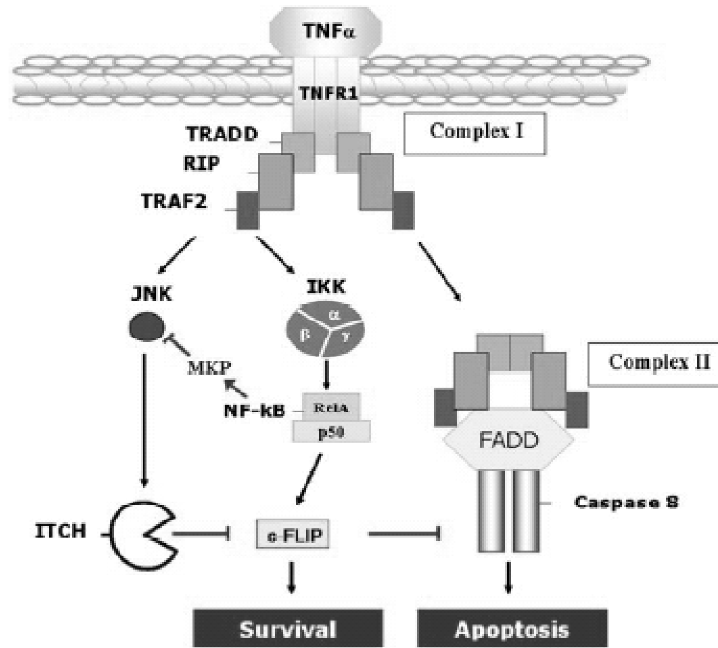


Figure 29. TNFα-induced apoptosis depends on ITCH activation by JNK1 and c-$FLIP_L$ degradation (adapted from Chang L. et al, 2006)

In addition to its antiapoptotic function, c-FLIP plays an important role in NF-κB signaling transduction pathway. $FLIP_L$-p43 cleaved form interacts and binds TRAF2 and RIP, resulting in an efficient NF-κB activation which protects cell from the apoptosis induced by death receptors (Kataoka T. et al, 2000; Hur G.M. et al, 2003; Liu Z.G., 2005; Golks A. et al, 2006). According to this, a small upregulation of c-FLIP renders cells resistant to TNFα, FasL and/or TRAIL induced apoptosis, reason for which its high expression level is associated with cancer disease (Djerbi M. et al, 1999; Alizadeh A.A. et al, 2000; Sun Q. et al, 2002).

4.5.4. *Anti-apoptotic Bcl-2 family members*

Based on its ability to inhibit the apoptotic process, Bcl-2 protein was first identified as an oncogene in the follicular lymphoma (McDonnell T.J. et al, 1989). Further studies revealed the existence of a family of related genes (Figure 30) (Harris M.H. et al, 2000; Fleisher A. et al, 2006).

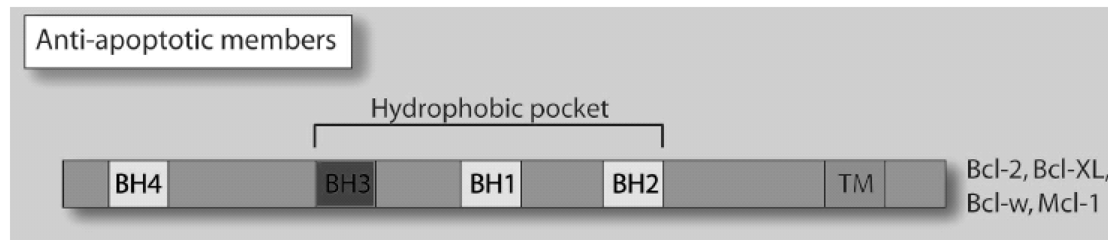


Figure 30. Anti-apoptotic Bcl-2 family members. Bcl-2 homology regions (BH1-BH4) are indicated as well as the transmembrane domain (TM) (modified from Brass B. et al, 2005).

Bcl-2 family proteins are among the most relevant and potent suppressors of the apoptotic cell death and include Bcl-2, Bcl-x_L and Bcl-w, A1 and Mcl-1.The anti-apoptotic Bcl-2 family members avoid matrix swelling and ROS damage. Moreover, they preserve mitochondrial membrane integrity by preventing both translocation and activation of Bax-like proteins, suppressing cytochrome c release and the proceeding of the apoptotic cascade (Gross A. et al, 1999; Harris M.H. et al, 2000; Thomenius M.J. et al, 2003).

Bcl-2 is the prototypical member of the Bcl-2 family. Bcl-2 is highly expressed in the developing nervous system, although its protein level decreases in postnatal brain in almost all neuronal populations. In the absence of stimuli, Bcl-2 is localized in the outer mitochondrial membrane, endoplasmic reticulum and nuclear envelop (Monaghan et al, 1992; Krajewski et al, 1993; Lithgow et al, 1994, Cory et al, 2003).
Bcl-2 deficient mice are viable and show a normal nervous system development at the embryonic stage, whereas they present an increase pheripheal neuronal apoptosis during postnatal life (Akhtar R.S. et al, 2004).

Bcl-x_L share 45% sequence homology with Bcl-2, supporting the similar anti-apoptotic functions of these two proteins (Tomita Y. et al, 2006). However, biological differences were detected between these two homologues. Bcl-x_L, which localizes in the outer mitochondrial membrane together with Bcl-2, is expressed at high levels in both embryonic and mature neuronal cells (O'Reilly et al, 2001; Kaufmann et al, 2003; Wilson-Annan et al, 2003; Cory et al, 2003). To better address the biologic relevance of Bcl-x_L anti-apoptotic role in the nervous system, Bcl-x_L-deficient mice phenotype was analyzed. In contrast to Bcl-2, mice lacking *bcl-x* show embryonic lethality due to apoptosis of immature neurons during the development of the nervous system (Yakovlev AG, 2004; Akhtar R.S. et al, 2004). This neuronal cell death involves the activation of the intrinsic apoptotic pathway. Simultaneous deficiency of Bcl-x_L and either caspase-9 or -3 abrogate immature neuronal apoptosis and rescue mice from lethality. This observation suggests that Bcl-x_L is the responsible for post-mitotic

immature neurons survival, as caspase activation is inhibited by endogenous expression of Bcl-x_L (Fujita E. et al, 2000).

In addition to their anti-apoptotic effects, Bcl-2 and Bcl-x_L also inhibit cell proliferation through a delay of the quiescent G0-phase cells into the G1-phase of the cell cycle (Krajewska M. et al, 2002; Zinkel S., et al 2006). Overexpression of Bcl-2 is associated with cellular senescence, due to a G1-phase cell cicle arrest (Chen Q.M. et al, 2000; Sasaki M. et al, 2001; Schmitt C.A. 2003), mediated by an inhibition of the cyclin dependent kinase, Cdk2.

Mcl-1 is another important member of Bcl-2 family. Mcl-1 was first identified as a gene early up-regulated during the differentiation of human myeloid leukemia cell line (Kozopas K.M. et al, 1993). Subsequently and because of its homology with Bcl-2, Mcl-1 was found to protect cells from several different stimuli, including death receptors, growth factor deprivation, chemotherapeutic agents and UV irradiation (Yang T. et al, 1995; Zhou P. et al, 1998; Nijhawan D. et al, 2003). As for Bcl-x_L, there are two alternative Mcl-1splicing isoforms, a long and short form. The short form of Mcl-1 is a BH3-only protein whose pro-apoptotic effect is counterbalanced by the presence of the anti-apoptotic Mcl-1-L (Bae J. et al, 2000). In eukariotyc cells, Mcl-1 interacts with Bcl-2 pro-apoptotic members and antagonizes their functions. Via a mechanism not yet elucidated Mcl-1 regulates Bax translocation, Bax and Bak oligomerization, cytochrome c release and caspase activation. Therefore, Mcl-1 is a crucial protein which avoids the activation of the intrinsic apoptotic pathway induced by genotoxic damage e.g. UV irradiation or drugs as etoposide (Letai A. et al, 2002; Nijhawan D. et al, 2003).

4.5.5. ***Fas-apoptosis inhibitor molecule (FAIM)***

Fas-apoptosis inhibitor molecule (FAIM) is a death receptor antagonist originally identified in B cells which affords protection against Fas-mediated cell death. Homologoues of FAIM were identified in different species, from *C. elegans* to humans, suggesting that this gene is evolutionary conserved (Schneider T.J. et al, 1999). Two splicing isoforms, a long ($FAIM_L$) and a short form ($FAIM_S$), were identified (Rothstein T.L. et al, 2000). Contrarily to $FAIM_S$ which is widely expressed, $FAIM_L$ is predominantly present in the nervous system (Zhong X. et al, 2001; Sole C. et al, 2004).

$FAIM_L$ is upregulated during neuronal differentiation, both *in vivo* and *in vitro*, achiving its maximal expression during embryonic or early post-natal stages. Even though the mechanism is not totally elucidated, the main regulator of this high

expression seems to be ERK signaling pathway, as motifs for ERK-regulated transcription factors were identified in *Faim* promoter. $FAIM_L$ overexpression confers resistance to Fas and TNFα-induced apoptosis through the inhibition of caspase-8 activation, demonstrating that $FAIM_L$ intereferes with a step prior the engagement of the initiator caspase. On the other hand, endogenous decrease of $FAIM_L$ renders primary cortical neurons and motoneurons susceptible to the cytotoxic effect of the cytokines. Therefore, $FAIM_L$ is perceived as a sort of modulator of death receptors-mediated apoptosis during the development of different embryonic neuronal population of the CNS (Segura M.F. et al, article in press).

Contrasting with the anti-apoptotic role of $FAIM_L$ the short form, i.e. $FAIM_S$, is required during developing nervous system for neurite out-growth induced by neurothrophic factors, a process mediated by NF-κB and ERK signaling pathways (Sole C. et al, 2004). Therefore, the neuronal resistance to death stimuli as well as the survival/differentiation exerted by the two FAIM isoforms suggests the presence of a balance which contribute to the correct functional development of central nervous system.

4.5.6. *Life-guard (LFG)*

The anti-apoptotic gene Life-guard (LFG) is regulated throughout development of a variety of neuronal cell types. It is highly expressed in post-natal brain and spinal cord, whereas low or undetectable LFG levels are observed in other tissues (Schweitzer B. et al, 1998; 2002). LFG expression is promoted by the activation of PI3K/Akt signaling pathway, as the overexpression of a dominant negative Akt mutant as well as the chemical inhibition of PI3K completely suppresses LFG activity (Beier C.P. et al, 2005).

LFG was discovered as an anti-apoptotic molecule involved in Fas-mediated cell death. It binds directly to the receptor, without interacting with DISC formation. Overexpression of LFG renders cells resistant to Fas-mediated apoptosis (Somia N.V. et al, 1999). The cell death induced by the cytokine is inhibited by the presence of LFG at the level or upstream caspase-8 activation. Moreover, as LFG is mainly detectable in the raft fraction of the plasma membrane, its interaction with Fas receptor occurs only upon the death receptor activation and consequent localization in the rafts (Segura M.F. et al, unpublished data). On the other hand, the absence of LFG renders neurons susceptible to Fas-induced apoptosis (Beier C.P. et al, 2005). Interestingly, LFG does

not confer protection against TNFα stimulus, probably due to the different proteins engaged in the formation of the death complex (Somia N.V. et al, 1999).

In neurodegenerative disorders, e.g. ischemia, Parkinson's disease and multiple sclerosis, low levels of LFG are detected, which means a higher sensitivity to Fas-mediated cell death. This suggests that activation of PI3K/Akt-promoted LFG expression could represent an important therapeutical approach for the treatment of these neurological diseases (Beier C.P. et al, 2005).

4.6. *ROS INVOLVEMENT IN TNFα-MEDIATED CELL DEATH*

TNFα-mediated ROS generation is involved in promoting cell death through the activation of the JNK signaling transduction pathway (Shen H.M. et al, 2006). A positive feed-back loop exists between ROS and JNK, according to which ROS causes a sustained JNK activation that is subsequently responsible for increase ROS production (Ventura J.J. et al, 2004; Kamata H. et al, 2005). ROS-mediated JNK activation seems to involve the ubiquitinously expressed MAPKKK ASK1 (Wang X.S. et al, 1996; Ichijo H. 1997). To support the essential role played by JNK in the generation of oxidative stress, it was observed that TNFα-induced ROS is abrogated in *jnk* $^{-/-}$ cells (Ventura J.J. et al, 2004).

The suppression of intracellular ROS formation in response to TNFα is mediated via NF-κB activation and subsequent transcription of anti-oxidant enzymes, i.e. MnSOD and H-Ferritin (Shen H.M. et al, 2006). Sustained TNFα-mediated increase of ROS levels occurs when NF-κB signaling transduction pathway is inhibited, e.g. in TRAF2-TRAF5 double deficient mice as well as in p65-null animals (Sakon S. et al, 2003; Shen H.M. et al, 2003).

4.6.1. *MnSOD and H-Ferritin*

Important genes which confer resistance against ROS-induced damage are promoted by NF-κB transcription factor (Figure 31). These enzymes, i.e. MnSOD and H-Ferritin, afford protection against oxidative stress-mediated apoptosis avoiding ROS-mediated JNK-dependent cell death (Shen H.M. et al, 2006).

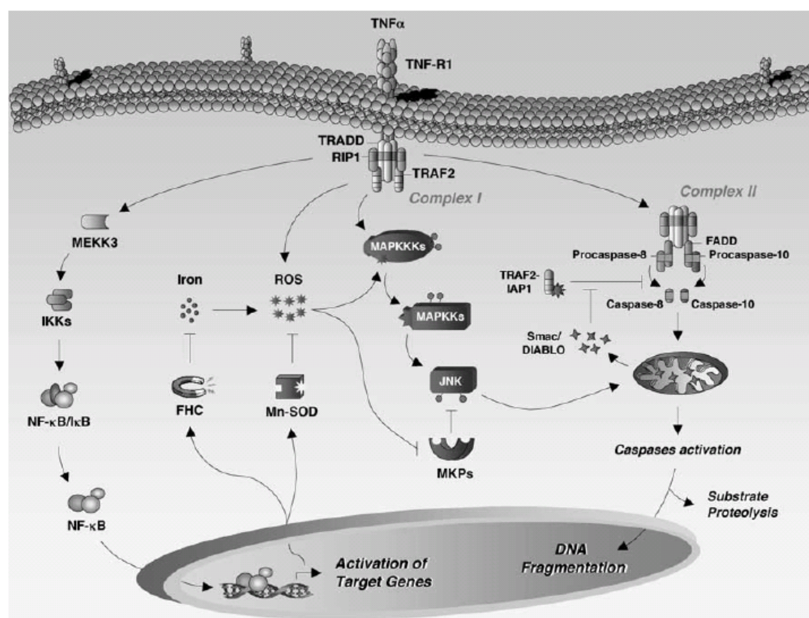


Figure 31. TNFα engagement of TNFR1 promotes the accumulation of ROS. ROS-mediated cell death occurs via at least two mechanisms: inactivation of MKPs and activation of MAPKKKs. Death signaling promoted by ROS is inhibited by NF-κB by the expression of protective genes i.e. FHC and MnSOD (adapted from Papa S. et al, 2005).

MnSOD reduces the accumulation of ROS originated in the mitochondria, catalyzing the dismutation of superoxide anion into hydrogen peroxide (Delhalle S. et al, 2002; Shen H.M. et al, 2006).

TNFα stimulation induces an exacerbated ROS accumulation in NF-κB-deficient cells (Pham C.G. et al, 2004; Papa S. et al, 2005). Under such conditions, MnSOD overexpression is not sufficient to rescue from ROS generation and to afford protection against TNFα-mediated apoptosis (Sakon S et al, 2003; Papa S. et al, 2005). To obtain complete resistance against ROS-induced damage, MnSOD must cooperate with others NF-κB target genes, i.e. H-Ferritin (Bubici C. et al, 2006).

Ferritin heavy chain (FHC) is one of the two subunit of Ferritin, a multiprotein complex which includes also the light chain (FLC) (Torti and Torti, 2002; Wagener F. et al, 2003). Unlike FLC, FHC possesses a ferroxidase activity which is required for iron sequestration (Arosio and Levi, 2002; Pharm C.G. et al, 2004). FHC is the main iron storage device in cells. The control of iron levels, promoted by H-Ferritin, prevents the formation of highly reactive hydroxyl radicals through the Fenton reaction (Pham C.G. et al, 2004; Papa S. et al, 2005).

4.6.2. *Heme oxygenase-1*

Heme Oxygenase I is another important gene upregulated in stressful conditions, e.g. TNFα-mediated oxidative stress. Heme Oxygenase I (HO-1) is the inducible form of the heme oxygenase family members (Lang D. et al, 2004). In mammalian cells three known isoforms are expressed, i.e. HO-1, HO-2 and HO-3. HO-1 and HO-2 are catalitically active, whereas HO-3, which derives from a retrotransposition of HO-2 gene, is catalitically inactive and its function remains unclear (Jin Y. et al, 2005). Of the two functional isoforms, HO-1 plays an essential role in preventing cell integrity as it is potently induced by several different stimuli, among which inflammatory cytokines, free heme, heavy metals, UV irradiation, etc (Immenschuh S. et al, 2000). Contrarily to MnSOD and H-Ferritin, its transcription is not promoted by NF-κB but its expression is regulated by Nrf2 transcription factor, a labile protein rapidly degraded by the proteasome. Under physiological conditions, Nrf2 is retained in the cytoplasm by its inhibitor Keap I. Upon stimuli Keap I undergoes conformational changes which render the protein unable to bind Nrf2, promoting its nuclear translocation and the consequent induction of gene transcription, among which HO-1 (Figure 32) (Leung L. et al, 2003; Lee J.S. et al, 2005).

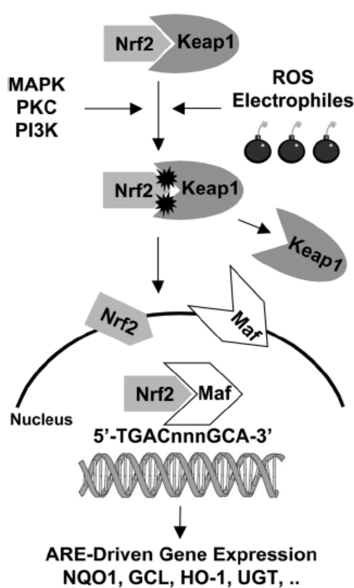


Figure 32. The anti-oxidant response promotes the dissociation of Nrf2-Keap1 complex, which allows the transcription factor Nrf2 to translocate to the nucleus, where upon its binding to anti-oxidant response element, drive anti-oxidant gene expression (adapted from Lee J.M. et al, 2004).

The function of HO-1 is to open the protoporphyrin ring of heme, converting this lipophilic molecule into biliverdin, free ferrous iron and carbon monoxide. In turn, biliverdin reductase converts biliverdin into the bile pigment bilirubin, both with potent

anti-oxidant effects, whereas free ferrous iron released from the heme molecule promotes its sequestration by H-Ferritin (Figure 33) (Otterbein L.E. et al, 2003). Therefore, HO-1 induction is correlated with an increase in H-Ferritin expression via a mechanism that is iron-dependent. Moreover, it was observed that CO is a potent anti-inflammatory, anti-apoptotic and vasodilatatory gaseous product, being highly protective in several different disease models, e.g. multiple sclerosis, cerebral malaria, atherosclerosis, etc. (Bach F.H. et al, 1997; Liu Y. et al, 2001; Nathan C., 2002; Otterbein L.E. et al, 2003; Chora A. et, 2007, Pamplona A. et al, 2007).

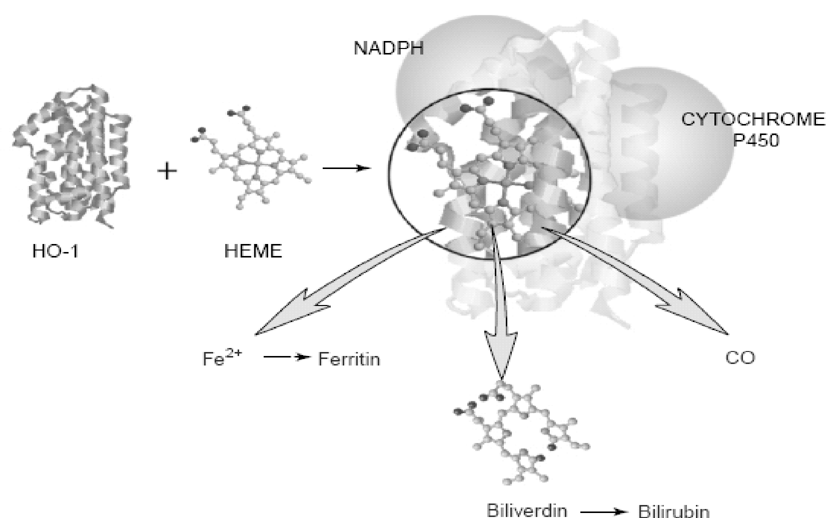


Figure 33. Heme oxygenase I (HO-1) catalyzes heme degradation, which results in free ferrous iron (Fe^{2+}), carbon monoxide (CO) and biliverdin products. Fe^{2+} induces the upregulation of H-Ferritin which is responsible for its storage, whereas biliverdin is rapidly converted to bilirubin by biliverdin reductase (adapeted from Otterbein L.E. et al, 2003).

*4.*7. DEATH RECEPTORS IMPLICATION IN NEUROPATHOLOGY

4.7.1. I*schemia*

With the term ischemia we refer to a time deprivation of oxygen and nutrients, i.e. glucose, which induces damage and malfunctioning of the affected tissue. The insufficient blood supply promotes hypoxia and consequently triggers cell death. When this disorder occurs in the brain, the result is reflected in a high percentage of neurons death in the affected area and of non-functional ones in the surrounded zone, preventing the normal neurological function (Darwin M. et al, 1995).

The ischemia disease can be induced by several distinct stimuli, among which the injury mediated by glutamate excitotoxicity and free radicals. Excessive release of glutamate occurs during cerebral ischemia. N-methyl-d-aspartate (NMDA) antagonist receptors fail to protect from the axonal damage observed. This suggests that the

activation of other cell death pathways is involved in mediating brain injury (Yam P.S. et al, 2000; MacDonald J.F. et al, 2007).

Among the distinct factors contributing to ischemic brain injury, there is the expression of proinflammatory cytokines, i.e. TNFα, promoted by activated microglial cells and occurring during the first hours after ischemia induction (Clark W.M. et al, 2001). The protection afforded by the pharmacological inhibition of TNFα biologic activity results in the decrease of the oxidative stress-induced by the cytokine as well as the reduction of the infarct size observed in the affected area, showing that TNFα-promoted stress increases brain ischemic damage (Caso J.R. et al, 2006).

Activation of JNK signaling transduction pathway, by TNFα and TNFα-mediated ROS production, plays an important role in cerebral ischemia-induced apoptosis (Brecht S. et al, 2005). Chemical JNK inhibitor rescues neurons from ischemia-promoted cell death. JNK3 is the critical component of the JNK-mediated death signal leading to neuron degeneration (Ferrer I. et al, 2003; Guan Q.H. et al, 2006). Moreover, genetic deletion of JNK3 renders mice resistant against the brain injury observed after cerebral ischemia induction (Guan Q.H. et al, 2006)

Although caspase-independent cell death seems to be the main pathway which causes neurotoxicity during ischemic brain injury, mitochondria and death receptor-mediated apoptosis are involved in the pathogenesis of this disease (Zheng Z. et al, 2003; Guan Q.H. et al, 2006). The activated apoptotic program results in an increase of caspase-3 activity in the hippocampal region. The inhibition of this executor protease protects against the neuronal apoptosis reducing the volume of brain infarct area (Guan Q.H. et al, 2006). A similar decrease in brain injury affected zone was observed in Bid-deficient animals (Ferrer I. et al, 2003).

However, in short ischemic events TNFα upregulation affords neuronal resistance against the subsequent severe ischemic injury (Kirino T. et al, 2002). During this process, known as ischemic preconditioning (IPC) an increase TNFR-1 expression was observed. Intracerebral downregulation of TNFR1 inhibits TNFα-mediated IPC (Ginis I. et al, 2002; Pradillo J.M. et al, 2005). Even though the mechanism is still largely unknown, it seems that TNFα-IPC involves the activation of NF-κB signaling transduction pathway (Ginis I. et al, 2002; Hung L.M. et al, 2004).

4.7.2 *Parkinson's disease*

Parkinson's disease is associated with the loss of dopaminergic neurons in the substancia nigra where activated microglial cells are also detected. The toxic substances released by glial cells contributes to the propagation and progression of the neuronal degeneration observed during the pathology. TNFα is upregulated in CSF as well as in brain parenchima of Parkinson patients (Boka G. et al, 1994; Sriram K. et al, 2002; Nagatsu T. et al, 2005)

To better understand the role of TNFα in this neurodegenerative disorder, the effects of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), a dopaminergic neurotoxin which causes Parkinson-like features was analyzed. In this model, enhanced expression of TNFα, associated with TNFR-1 induction in nigrostriatal dopaminergical neurons, correlates with a strong caspase-8 and -3 activation (McGuire S.O. et al, 2001; Hirsch E.C. et al, 2003). However, chemical inhibition of caspase cascade does not avoid the progression of the disease, suggesting that the selective neutralization of one specific pathway is not sufficient to promote PD recovery (Hirsch E.C. et al, 2003). Wessig J. and collaborators demonstrated that TNFR-1 promotes activation of the JNK/c-Jun signaling transduction pathway, which contributes to neuronal degeneration in PD animal models (Wessig J. et al, 2005). Inhibition of JNK activity through mice genetic inactivation of c-Jun or by JNK-deficient animals confers protection against the neurotoxic damage (Brecht S. et al, 2005)

TNFα released by activated microglial cells also induces the production of nitric oxide, which was detected in nigrostriatal region and basal ganglia of post-mortem PD affected brains (Eve D.J. et al, 1998). NOS inhibitor and NOS gene deficiency confer protection against MPTP-induced dopaminergic neurotoxicity (Schulz J.B. et al, 1995; Hantraye P. et al, 1996; Ebadi M. et al, 2003; Kavya R. et al, 2006). Furthermore, inhibition of TNFα/TNFR1 abrogates microglial glutamate release, suggesting also the involvement of NMDA in TNFα-mediated cytotoxicity (Hunot S. et al, 1999; Hirsch E.C. et al, 2003; Takeuchi H. et al, 2006).

Therefore, as TNFα is the main responsible for the detected neurotoxicity *in vivo*, the neutralization of its biological activity renders resistant the animal model of Parkinson's disease. A recent compound acting as a dominant-negative of TNFα, which show the ability to inhibit cytokine-mediated signaling, provides neuroprotection attenuating loss of dopaminergic neurons (McCoy M.K. et al, 2006). Moreover, TNFα-deficient mice and TNFR-1 $^{-/-}$ are completely resistant against MPTP-induced neurotoxicity (Sriram K et al, 2002).

4.7.3. *Alzheimer's disease*

Alzheimer's disease is a form of dementia characterized by a progressive loss of memory in later life (Culpan D. et al, 2003). This feature is associated with synaptic degeneration, resulting in neuronal death in the limbic portions of the brain (Fleischer A. et al, 2006). Typical of AD is the extracellular accumulation of peptide aggregates, derived by the processing of the Amyloid Precursor Protein (APP) in brain areas involved in cognitive functions (Del Villar K. et al, 2004).

Deposition of Amyloid-β leads to a chronic inflammatory response mediated by activated microglial cells and associated with the generation of proinflammatory cytokines, including TNFα. Increase in TNFα expression both in the cerebrospinal fluid (CSF) and serum of AD patients correlates with clinical deterioration, whereas upregulation of TNFR1 is detected only in AD brain (Tarkowski E. et al, 2003; Zuliani G. et al, 2007). Increased TNFα production is required for the neurotoxicity, which characterizes the disease (Rosenberg P.B., 2006; Lio D. et al, 2006).

Even though the mechanisms by which TNFα mediates neuronal death and dysfunction during AD are not totally clear, it was observed that enhanced glutamate-induced excytotocixity promoted by this cytokine plays an important role. TNFα potentiates glutamate-induced neuronal damage as demonstrated in experimental models using β-amyloid stimulated microglia cells (Chao C.C. et al, 1995; Floden A.M. et al, 2005; Zou J.Y. et al, 2005).

TNFR1-mediated apoptosis is dramatically induced in Alzheimer patients and it is correlated with the progression of the disease. As a consequence, an increase of 10-fold in the levels of active caspase-3 was detected in AD cases when compared with controls (Zhao M. et al, 2003). Furthermore, the apoptotic cascade can also be activated by the oxidative damage to which neurons undergo during the AD progression and which exerts an important role in cognitive impairment (Madeiros R. et al, 2007). In particular, increased levels of oxidative markers, i.e. inducible nitric oxide synthase (iNOS), were reported in AD patients. Supporting this theory, it was shown that selective inhibitors of iNOS protects against the neuronal death induced by β-amyloid peptide-stimulated microglial cells (Combs C.K. et al, 2001). Recently, it was demonstrated that the activation of JNK and NF-κB signaling transduction pathways mediate the crosstalk between TNF and iNOS. Moreover, genetic deletion of TNFR1 as well as iNOS significantly reduces the cognitive deficits observed during this disease, protecting mice from β-amyloid toxicity (Del Villar K. et al, 2004; Madeiros R. et al, 2007).

In conclusion, the inhibition of the neurotoxic effect of TNFα represents a possible therapeutic approach for the treatment of Alzheimer's disease. A drug able to bind specifically TNFα and suppress its interaction with TNFR-1 is already available for human use and considered promising for a better stand-life of AD patients (Tobinick E. et al, 2006).

4.7.4. *Multiple Sclerosis*

Multiple Sclerosis (MS) is a demyelinating neurodegenerative disease. It is characterized by a T cell mediated response directed against antigens in the CNS. This leads to the development of a deleterious neuroinflammation which triggers myelin destruction and phagocytosis by activated microglia and macrophages. The result is apoptotic and necrotic cell death of oligodendrocytes-producing myeling as well as neurons (Butovsky O. et al, 2006; Fleischer A. et al, 2006).

The animal model used for the study of the Multiple Sclerosis, is the Experimental Autoimmune Encephalomyelitis (EAE), which can be induced by immunizing animals with myelin or myelin components, e.g. Myelin Basic Protein (MBP) or Myelin Oligodendrocyte Protein (MOG) (Probert L. et al, 2000).

TNFα concentration overproduced in serum and cerebrospinal fluid of MS patients promotes the invasion in the CNS of activated lymphocytes (Pouly S. et al, 1999; Gimenez M.A. et al, 2006). TNFα and TNFR-1 upregulation correlate with disease severity. Therefore, inhibition of TNF signaling pathway improves clinical disease (Ruddle N.H. et al, 1990; Baker D. et al, 1994; Buntinx M. et al, 2004).

Even though TNFα contributes to CNS injury at distinct levels, during EAE it appears to play a critical role in the early inflammatory process regulating the initial leucocytes infiltration within the CNS, through the induction of adhesion molecules and chemokines (Körner H. et al, 1997; Probert L. et al, 2000).

TNFR-1 signaling pathway promotes oligodendrocytes apoptosis, myelin vacuolization and macrophages-mediated demyelination. According to this, the MOG-induced demyelination is significantly reduced in TNFR1-deficient mice, suggesting that TNFα/TNFR1 pathway is also important in the late phases of EAE. Moreover TNFR1^{-}/$^{-}$ also presents a delay in the onset of the pathology. The involvement of TNFR1 in the process of demyelination reflects the toxic effect induced by TNFα on oligodendrocytes (Eugester H.P. et al, 1999).

Nevertheless, the partial protection afforded by TNFR1-deficient animals indicates that alternative pathways mediate EAE progression. Upregulation of Fas and

its receptor was detected by histological studies in MS white matter brain lesions (Dowling P. et al, 1996). Fas or FasL mice expressing mutations are relatively resistant to EAE induction (Waldner H. et al, 1997; Sabelko K.A. et al, 1997; Okuda Y. et al, 2000). Interestingly, mutant mice for both TNFα and FasL present a significant delay in the onset of disease as well as a reduction in demyelination. However, deletion by homologous recombination of both TNFR-1 and Fas receptors prevents oligodendrocytes death and confers almost complete resistance to EAE induction. This suggests that TNFα and Fas signaling pathway are the main mechanisms involved in EAE progression (Hövelmeyer N. et al, 2005).

4.7.5. *Huntington's disease*

Huntington disease (HD) is a hereditary neurodegenerative disorder characterized by movement dysfunction, dementia, and change in personalities which lead to death of the individual, with a mean survival following onset of 15–20 years (Pattinson L.R. et al, 2006). The neuronal degeneration, which characterizes the disease, is the responsible for a decrease in the striatal volume of more than 60% (Sipione S. et al, 2001).

HD patients present a mutation which includes an expanded trinucleotide repeat (CAG encoding glutamine) in the huntingtin gene, resulting in expansion of glutamines in the N-terminus of huntingtin protein (Htt). The length of the CAG/polyglutamine repeat is inversely correlated with the age of disease onset. Cleavage of this mutated Htt (mHtt) by caspases, calpains and endopeptidases is reported (Lunkes A. et al, 2002; Gafni J. et al, 2004). Selective deletion of caspase-6 cleavage site in mHtt confers protection against the excytotoxic neuronal cell death occurring during this pathology (Graham R.K. et al, 2006). Therefore, the formation of N-terminal Htt fragments, containing expanded CAG repeats, can be considered the responsible for the progressive and massive loss of striatal and cortical neurons associated with the development of HD (Albin R.L, et al, 1992; Gutekunst C.A. et al, 1999; Kim Y.J. et al, 2001).

The function of Htt is not completely elucidated although it is reported to play an essential role for the increase transcription of brain derived neurotrophic factor (BDNF), responsible for the neuronal maintenance and differentiation (Zuccato C. et al, 2001; Hermel E. et al, 2004). Moreover, Htt-deficient mice show embryonic lethality, demonstrating the relevance of this protein during the development stage (Duyao M.P., et a, 1995). Furthermore, the observation that endogenous Htt decrease in post-natal

mouse brain leads to neurodegeneration confers Htt an important function in neuronal survival in mature brain (Dragatsis I. et al, 2000).

The presence of dying neurons and mutated Htt triggers microglial activation which promotes the induction of cytotoxic TNFα (Kim S.U. et al, 2005; Tai Y.F. et al, 2005, 2007). The loss of normal Htt function leads TNFα to activate the caspase cascade, therefore causing neurotoxicity (Wang X. et al, 2005; Zhang Y. et al, 2006). Moreover, expression of a dominant-negative mutant of capase-1 prolongs mean survival in HD mice model. The inhibition of caspase-1 activity also delays the appearance of neuronal inclusions, alterations of neurotransmitter receptors and onset of symptoms, revealing the importance of caspase-1 in the pathogenesis of the disease. Furthermore, the intracerebroventricular administration of a caspase inhibitor delays the progression of HD (Ona V.O. et al, 1999). Therefore, the inhibition of the caspase cascade can be converted in one of the possible target for the therapeutical approach (Wellington C.L. et al, 2002; Graham R.K. et al, 2006).

4.7.6. *Transmissible Spongiform Encephalopathies*

Transmissible Spongiform Encephalopathies is a pathological disease which affects brain and nervous system of both animals and humans. This neurodegenerative disease is characterized by amyloid plaque deposition, astrocytosis, neuronal loss and spongiform changes (Thackray A.M. et al, 2004).

The responsible for TSE transmission is the pathological prion protein (PrP^{sc}), which accumulates in brains of infected animals (Prusiner SB, 1998). PrP^{sc} proteins are mutated, insoluble and protease resistant isoforms of normal cellular prion (PrP^{C}). PrP^{sc} accumulation as well as PrP^{C} deficiency contributes to the neuronal loss occurring during the disease (Yokoyama T. et al, 2001; Sakudo A. et al, 2003). Intracellular formation of compartimentalized cytosolic PrP^{SC} aggregates is associated with caspases activation, revealing the involvement of the apoptotic pathway in TSE neurodegeration (Kristiansen M. et al, 2005).

Activated microglial cells, which appear in the early prion response, are detected in brain of Creutzfeldt-Jacob disease patients, the human form of TSE, as well as of TSE infected animals (Betmouni S. et al, 1996; Manuelidis L. et al, 1997; Baker C.A., et al, 1999). Therefore, exacerbated cytokine production, including TNFα, triggered by the inflammatory process precedes hippocampal neurons apoptosis of prion-infected mice (Pahan K. et al, 2000). Inhibition of TNFR1 signaling pathway promotes significant delay in the onset of the pathology as well as a reduced early

accumulation of disease-specific prions in the spleen (Mabott N.A. et al, 2002). PrP^C overexpression confers resistance against TNFα-mediated cell death interfering with Bax-mediated release of cytochrome c from mitochondrial compartment (Diarra-Mehrpour M. et al, 2004).

Further studies with deficient animals reveal that hippocampal neurons derived from PrP^C-deficient mice are more susceptible to apoptotic stimuli. This susceptibility is due to a decrease of Bcl-2 and Bcl-x_L endogenous expression detected in infected mice (Park S.K. et al, 2000; Sakudo A. et al, 2003; Garcia-Crespo D. et al, 2006). Accordingly, Bcl-2 and Bcl-x_L ectopic expression attenuate neuronal loss caused by PrP^C deficiency. Therefore, pharmacological induction of anti-apoptotic Bcl-2 family proteins would represent a therapeutical approach in the treatment of prion disease (Sakudo A. et al, 2003).

Objectives

TNFα induces a wide range of biological responses, including apoptosis. Even though TNFR-1 is expressed on a variety of different cell types, TNFR-1-expressing cells are normally resistant to TNFα cytotoxic effect. Treatment with RNA transcription or protein syntheis inhibitor, i.e. Actinomycin D (ActD) and cycloheximide (CHX) respectively, sensitizes to TNFα-triggered apoptosis (Fulda S. et al, 2000; Mielke K. et al, 2002). Therefore, the purpose of this study was to investigate in the nervous system which is/are the key element(s) responsible in regulating the resistance to TNFα-mediated apoptosis.

1. Since maintaining a state of resistance to apoptosis often requires de novo proteins or RNA synthesis, the first objective of this study was to assess whether the treatment with RNA transcription inhibitor, Actinomicyne D (ActD), would sensitize PC12 cells to TNFα-mediated apoptosis.

2. ActD stimulation promotes the decrease of short half-life proteins expression. According to this, our purpose was to address at which molecular level ActD interferes in TNFα-induced apoptosis.

3. A misbalance between Bcl-2 pro- and anti-apoptotic proteins is required by death stimuli to promote apoptosis. Therefore, our aim was to address the involvement of mitochondrial Bcl-2 family members in TNFα-mediated cytotoxic effect. Moreover, as Bcl-2 proteins are targets of NF-κB transcription factor, we wanted to assess whether mitochondrial damage would affect the survival pathway promoted by NF-κB.

4. As heme is a potent inducer of the death stimuli, we wanted to assess whether it would sensitize to TNFα-mediated apoptosis.

Materials and Methods

6.1. *CELL CULTURE*

6.1.1. *Cell lines and culture conditions*

PC12 rat pheocromocytoma cell line was grown in Dulbecco's modified Eagle medium (DMEM, GIBCO Invitrogen) supplemented with 6% fetal bovine serum (FBS) and 6% heat-inactivated horse serum (HS) (GIBCO Invitrogen), 10mM HEPES, 20Units/ml penicillin and 20Units/streptomycin. HeLa cell line, derived from human cancerous cervical tissue, was maintained in DMEM (GIBCO Invitrogen) supplemented with 20Units/ml penicillin, 20Units/streptomycin, 10% FBS and 1% of non-essential aminoacids (GIBCO Invitrogen). Hepa cell line, a mouse hepatoma cell line, was cultured in DMEM supplemented by 10% fetal calf serum, 20Units/ml penicillin and 20Units/streptomycin.

All cell lines were grown at 37ºC in a humidified atmosphere of 5% CO_2.

6.1.2. *Establishment of Primary Mouse Cortical Neurons*

Primary cortical neurons were obtained using the minimum numbers of animals according with the scientific objectives of the research. Ethical procedures according to national and European guidelines were followed to sacrifice the animals. At 15 days gestation (E15), pregnant females were killed and cultures of cortical neurons were established from each embryo. The dissected cerebral cortices were collected in ice-cold HBSS (GIBCO Invitrogen), trypsinized (8 minutes at 37ºC) and dissociated with a fire-polished Pasteur pipette. The resulting cells were suspended in DMEM (GIBCO Invitrogen) supplemented with 5% fetal bovine serum, 20units/ml penicillin and 20 µg/ml streptomycin and then plated in 10µg/ml poly-L-lysin-coated culture dishes. 4h later, the medium was replaced for serum-free DMEM medium supplemented with B27 and N2 (GIBCO Invitrogen). After 2 days in culture, non-neuronal cell division was inhibited by the addition of 10µM cytosine arabinoside, maintained in the culture medium also during the stimulation treatment. Primary cortical neuron cells were kept at 37ºC in humidified 5% CO_2-containing atmosphere. Experiments were performed after 6 days.

6.2. *REAGENTS*

All the biochemicals utilized for this study were dissolved to a stock concentration according the manufacturer data sheets. Subsequently, the reagents were diluted in the cell culture medium to reach a final working concentration(s). Cytokines, antibiotics, caspase fluorogenic substrates, caspases inhibitors as well as NF-κB and JNK inhibitors used in this study are described in the following table.

Name	Description	Working dilution	Provider	Nº Catalogue
TNFα	Cytokine	100 ng/ml	Sigma	T-6674
ActD	RNA transcription inhibitor	1 nM	Sigma	A-9415
IETD-fmk	Caspase 8 inhibitor	100 μM	MP Biochemicals	FK012
zVAD-fmk	Pan Caspase inhibitor	50 μM	MP Biochemicals	FK009
IETD-afc	Caspase 8 fluorogenic substrate	250 μM	Calbiochem	368059
DEVD-afc	Caspases fluorogenic substrate	250 μM	Calbiochem	264151
SP600125	JNK inhibitor	10 μM	Biomol	129-56-6
SN50	NF-κB p50 inhibitor	50 μM	Calbiochem	481480
SN50M	Mutated form of NF-κB p50 inhibitor	50 μM	Calbiochem	481486

6.3. *SURVIVAL ASSAYS*

6.3.1. *MTT Reduction*

MTT is a water-soluble tetrazolium salt which is reduced by metabolically viable cells to a coloured, water-insoluble formazan salt, allowing cell viability measurements. Cells were seeded in a 96-multiwell plate. The day after, cells were treated and, according to the time stimulation, MTT assay was performed. MTT, in a final concentration of 0.5mg/ml, was added to the cell culture medium. The plates were incubated at 37ºC for about 30 minutes and the assay was stopped by replacement of MTT-containing medium with 100μl DMSO. Formazan salts were allowed to dissolve in DMSO with gentle shaking (10 minutes at room temperature) and the assay was quantified by means of an ELISA plate reader. Each experiment was repeated at least three times. The final values, resulting by subtracting 620 nm from 590 nm lectures, were loaded into a Microsoft Excel data sheet and the mean ± SEM calculated. Final

graphic and statistical results were obtained by processing all data through the software mentioned above.

6.3.2. *Trypan Blue Exclusion*

Trypan blue is a vital diazo dye, used to selectively stain coloured death cells in blue. Live cells with intact cell membranes are not coloured as the colorant, contrarily to death cells, does not pass the membrane.

Cells were seeded in a 24-multiwell plate at $5x10^4$ cells/well, cultured for 24 hours with complete growth medium and then treated adequately for the indicated times. Cells were gently harvested and resuspended in 100μl of PBS solution. 20μl of trypan blue dye (0.08% final working concentration) (Sigma) was added to the cell suspension. Cells were counted using a hemocytometer. Each experiment was performed in triplicate. The value defined as 100% survival corresponds to unstimulated cells. Mean ± SEM values were finally calculated.

6.3.3. *Crystal Violet staining*

Crystal violet staining is based on a colorimetric assay and it is used to know the relative density of adhering cells to multi-well plates. The crystal violet dye stains DNA. After solubilization, the amount of dye is quantified using a spectrophotometer.

Cells were seeded in a 96-multiwell at $8x10^3$ cells/well. 48h later the treatment was performed and after stimulation, the medium was removed carefully and cells were washed once with PBS. Crystal violet solution was obtained by dissolving the crystal violet powder in ethanol 70% to a final concentration of 0.05%. Cells were incubated with this solution (10 minutes), washed by immersion in a tap water and dried at room temperature. Samples were solubilized with acetic acid (50%) and read at λ=595nm at the spectrophotometer. Each experiment was performed in sixplicate. The values defined both as 100% of survival or 0% of cell death in untreated cells were analyzed with the Excel program through which means and the respective SEM were calculated. The final graphs and the statistic analisis were performed using the same programme.

6.4. *CELL DEATH ASSAYS*

6.4.1. *Hoechst 33258 Chromatine staining*

Hoechst 33258 is a fluorescent dye used to label DNA.

It is known as bisBenzimide ([2'-(4-hydroxyphenyl)-5-(4-methyl-1-piperazinil)-2,5'-bi-1H-benzimidazole trihydrochloride]). Hoechst chromatine staining can be used in both live and dead cells, as the ethyl group present in its molecular structure renders the molecule lipophilic, allowing it to cross through intact cell membrane. Hoechst DNA binding properties were well-characterized by Churchill and Suzuki in 1989 (Churchill M.E. et al, 1989). For the assessment of apoptotic nuclear morphology, cells were fixed with 4% paraphormaldheyde and stained with 0.05µg/ml Hoechst 33258 (30 minutes at room temperature). Cells were counted using an Olympus fluorescence microscope equipped with UV illumination. Apoptotic cell death was expressed as percentage of apoptotic versus viable cells. Mean ± SEM values were finally calculated.

6.4.2. *TUNEL staining*

Terminal Transferase dUTP Nick End Labeling (TUNEL) is commonly used to detect DNA fragmentation of apoptotic cells. The assay is based on the presence of nicks in the DNA which are identified by Terminal Transferase, the enzyme that catalyzes the addition of dUTPs which are subsequently labelled by the marker. To perform the assay cells were fixed with 4% paraphormaldheyde/PBS solution (60 minutes at room temperature) and permeabilized with 0.1% Triton X-100 in 0.1% Sodium Citrate (1 hour at 4ºC). Cells were subsequently processed with the *In situ Cell Death Detection Kit* according with the instructions of the provider. In the final step, 0.05 µg/ml Hoechst 33258 was added to the cell culture dish and cell death was quantified using an Olympus microscope equipped with epifluorescence optics.

6.5. *CASPASE ACTIVITY ASSAY*

DEVD/IETD -directed Caspase Activity

To quantify either IETD or DEVD-directed caspase activity, cells were grown in a 35-mm cell culture dishes. After the indicated treatment, cells were rinsed once with cold PBS and resuspended in lysis buffer containing 25mM Hepes/NaOH (pH 7.2), 5mM $MgCl_2$, 5mM EDTA, 1% PMSF, 10mM DTT for the DEVD-directed activities (caspase-3-like) or containing 20mM Hepes/NaOH (pH 7.2), 150mM NaCl, 10mM DTT and 1mM PMSF for IETD-like activities (caspase-8-like). Both buffers were supplemented with 1% TRITON X-100. After rotational shaking (20 minutes at 4ºC), lysates were clared by centrifugation at 16.000xg (5 minutes at 4ºC). Supernatants were quantified using Bradford method. The assay was performed with 25µg of protein for caspase-3-like activity and 100µg for caspase-8-like activity in the specific lysis buffer supplemented with 10% saccharose and 0.1% CHAPS plus 50µM of the fluuorogenic substrate Ac-DEVD-afc or 150µM Ac-IETD-afc.

The plate was read in a Bio-Tek FL 600 fluorimeter using a 360nm (40nm bandwidth) excitation filter and 530nm (25 nm bandwidth) emission filter.

6.6. *PROTEIN EXTRACTION AND WESTERN BLOT ASSAY*

6.6.1. *Triton X-100 Protein extraction*

Cells were grown in a 35-mm cell culture dish. After the indicated treatment, cells were rinsed once with cold PBS and resuspended in lysis buffer containing 50mM Tris-HCl (pH 6.8), 150mM NaCl, 1mM EDTA, and 1% Triton X-100. Pellets were gently disrupted by pipetting and left on ice (10 minutes) to perform protein extraction. Nuclear and mitochondrial materials were pelletted by centrifugation at 16.000xg (15 minutes at 4ºC) and clear supernatants were obtained. These supernatants were subjected to determination of total protein concentration by means of Lowry Assay, Bio-Rad DC Protein Assay (Bio-rad, Hercules, CA). Equal amounts of proteins were loaded into SDS-polyacrylamide gel electrophoresis.

6.6.2. *Total Protein Extraction*

Cells were washed once with cold PBS and SDS lysis buffer containing 2% SDS and 125mM Tris-HCl (pH 6.8), previously heated at 95ºC, was added to the cell culture dish. Samples were collected in 1.5ml microcentrifuge tube and boiled at 95ºC to allow protein denaturalization. Protein concentration was quantified when viscosity disappeared by Lowry Assay, Bio-Rad DC Protein Assay (Bio-rad, Hercules, CA).

6.6.3. *Western blot*

25µg of cellular extracts were resolved in SDS-polyacrylamide gels. According to the manufacturer instructions, proteins were elecrophoresed and electrotransferred from the gel to a Polyvinylidene difluoride (PVDF) Immobilon-P transfer membrane (Millipore, Bedford, MA) using a semidry Trans-Blot apparatus for 1-1.5 hour (Hoefer, Amersham Pharmacia Biotech). The membrane was blocked with Tris-buffered saline with Tween 20 (20mM Tris-HCl pH 7.4, 150mM NaCl and 0.05% Tween 20) containing 5% non fat dry milk (1 hour at room temperature) and probed with the appropriate primary antibody according to the specific requirements indicated by each provider. After 1 hour incubation with the specific peroxidase-coniugated secondary antibody (Sigma, Jackson Immunoresearch or Cell Signaling), the membrane was finally developed with EZ-ECL (Biological Industries, Kibbutz Beit Haemek, Israel) or SuperSignal chemiluminescent detection kit (Amersham Pharmacia Biotech, Pierce, USA).

The antibodies used in this study are described in the following table.

Antibody	**MW antigen**	**Source**	**Dilution**	**Supplier**	**Nº Catalog**
α-**Bcl-2**	26 kDa	Mouse	1 : 1.000	DAKO	M0877
α-**Bcl-X$_L$**	29 kDa	Rabbit	1 : 3.000	BD Transduction Laboratories	610211
α-**Bcl-w**	21 kDa	Rat	1 : 1.000	Calbiochem	AM54
α-**Mcl-1**	40-42 kDa	Rabbit	1 : 10.000	Sigma	M8434
α-**Bak**	30 kDa	Rabbit	1 : 5.000	Biocarta	12-01-16348
α-**Bax**	21 kDa	Rabbit	1 : 3.000	Cell Signaling	#2772

α-**BID**	15 kDa	Goat	1 : 1.000	R & D System	AF860
α-**BIM/BOD**	33-25-15	Rabbit	1 : 1.000	Stressgen	AAP-330
α-**HrK (M-20)**	20 kDa	Goat	1 : 300	Santa Cruz	sc-6973
α-**cIAP 1 (H83)**	68 kDa	Rabbit	1 : 300	Santa Cruz	sc-7943
α-**cIAP 2**	65 kDa	Rabbit	1 : 300	Santa Cruz	sc-7944
α-**XIAP**	54 kDa	Mouse	1 : 1.000	Stressgen	AAM-0560
α-**cFLIP**	28-55 kDa	Rabbit	1 : 1.000	Stressgen	AAP-440
	55 kDa	Rat	1 : 1.000	Alexis	ALX-804-127
α-**TNFα**	32-17 kDa	Rabbit	1 : 1.000	Cell Signaling	#3707
α-**TNF-R1 (H-5)**	55 kDa	Rabbit	1 : 1.000	Santa Cruz	sc-8436
α-**TNF-R2 (D-2)**	75 kDa	Rabbit	1 : 1.000	Santa Cruz	sc-8041
α-**TRADD (3E11)**	34 kDa	Rabbit	1 : 1.000	Upstate	05-473
α-**TRAF 2 (H10)**	52 kDa	Mouse	1 : 1.000	Santa Cruz	sc-7346
α-**TRAF 6 (H274)**	60 kDa	Rabbit	1 : 1.000	Santa Cruz	sc-7221
α-**RIP**	74 kDa	Mouse	1 : 1.000	BD Transduction Laboratories	610458
α-**NF-κB p65 (C-20)**	65 kDa	Rabbit	1 : 1.000	Santa Cruz	sc-372
α-**Iκ-Bα (C-21)**	37 kDa	Rabbit	1 : 1.000	Santa Cruz	sc-371
α-**FADD**	27 kDa	Mouse	1 : 2.000	BD Pharmingen	556402
α-**Caspase 8**	50 kDa	Rabbit	1 : 300	Santa Cruz	sc-7890
α-**Caspase 9**	51-37 kDa	Mouse	1 : 1.000	Cell Signaling	#9508
α-**Cleaved Caspase 9**	17-38 kDa	Rabbit	1 : 2.000	Cell Signaling	#9507
α-**Caspase 3**	35-19-17 KDa	Rabbit	1 : 4.000	Cell Signaling	#9665

α-**Cleaved Caspase 3**	17-19 kDa	Rabbit	1 : 2.500	Cell Signaling	#9661
α-**SAPK/JNK**	46-54 kDa	Rabbit	1 : 1.000	Cell Signaling	#9252
α-**pSAPK/JNK (Thr183/Tyr185)**	46-54 kDa	Mouse	1 : 750	Cell Signaling	#9255
α-**pSAPK/JNK (Thr183/Tyr185 Thr221/Tyr223)**	46-54 kDa	Rabbit	1 : 150	Upstate	#07-175
α-**Cytochrome c**	15 kDa	Mouse	1 : 1.000	BD Pharmingen	556433
α-**SMAC/DIABLO**	25 kDa	Rabbit	1 : 1.000	ProSci	2409
α-**panERK**	54-44-42 kDa	Mouse	1 : 5.000	BD Transduction Laboratories	612641
α-**Tubulin**	50 kDa	Mouse	1 : 40.000	Sigma	T5168

In some instances, membranes were alternatively stripped with 100mM β-mercaptoethanol and 62.5mM Tris-HCl (pH 6.8) (30 minutes) at 65-70ºC to be reprobed with other primary antibodies.

6.7. *CELL TRANSFECTION*

6.7.1. *Standard Electroporation* (BioRad).

Cells were harvested by centrifugation at 1500xg (5 minutes at room temperature). Pellets were resuspended in 750μl of ice cold PBS solution. 10μg of DNA plasmid were added to each sample and incubated on ice (10 minutes). Cell suspensions were transferred to a pre-cooled 0.4cm electroporation cuvette (BioRad) and subjected to a shock at 286V and 750μF in a Gene Pulser II System (BioRad). After the electroporation, cell suspensions were immediately transferred to the cell culture dishes, previously coated with poly-D-lysin 10μg/ml, and maintained in complete medium. The plates were then incubated at 37ºC in a humidified atmosphere of 5% CO_2 until assayed for gene expression.

6.7.2. *Calcium Phosphate cell transfection*

To obtain calcium phosphate–DNA co-precipitates solutions were prepared in two distinct 1.5 ml eppendorfs:

A. 250mM $CaCl_2$, H_2O and DNA

B. 280mM NaCl, 50mM HEPES and Na_2HPO_4, which are the components of the so-called HBS buffer.

The formation of DNA insoluble aggregates were obtained vortexing solution A to which solution B was added drop by drop. The mixed solution was incubated at room temperature for 30 minutes. The pH solution should be around 7. Lower pH decreases the efficiency of transfection, whereas higher pH promotes toxicity.

DNA–calcium phosphate cell transfection solution was added to the cell culture dish drop by drop. Cells were incubated at 37ºC in a humidified atmosphere of 5% CO_2.

6.7.3. ***Polyethilenimine (PEI) cell transfection***

Polyethilenimine (PEI) cell transfection was performed in medium without serum and antibiotics. DNA was prepared in sterile NaCl150 mM, as well as PEI solution, which is diluted to a final concentration of 10μM. PEI was directly added to DNA solution. After shaking (1 minute), PEI-DNA complexes were obtained incubating the mix at room temperature (10 minutes). The resulting cell transfection solution was added drop by drop to the cell culture dish. Cell culture medium was changed after 3 hours to avoid toxicity.

6.7.4. *Lipofectamine^{TM}2000 cell transfection*

The relation between Lipofectamine^{TM} 2000 and DNA should be 1μl:1μg. Cells were seeded in a 35-mm cell culture dish. 5μg of DNA and 5μl of Lipofectamine^{TM} 2000 reagent, in different tubes, were diluted in the cell culture medium without serum and antibiotics. Lipofectamine^{TM} 2000 reagent should be incubated for 5 minutes at room temperature. The two solutions were finally mixed and incubated at room temperature (20–30 minutes) to allow the formation of DNA-Lipofectamine^{TM} 2000 reagent complexes. The resulting cell transfection solution was added drop by drop to the cell culture dish. Cells were then incubated at 37ºC in a CO_2 incubator until assayed for gene expression.

6.7.5. *Oligofectamine^{TM}2000 cell transfection*

Cells were seeded in a 35-mm cell culture dish. Medium withouth serum shoud be replaced during the transfection. 10µl of the 20µM oligonucleotide stock were diluted in 175µl of medium without serum and the mix was shacked gently. Moreover, 4µl of the Oligofectamine ^{TM}2000 reagent were diluted in medium without serum to obtain a final volume of 15µl, shacked gently and incubated at room temperature (10 minutes). The final combination of the mixed solutions was incubated at room temperature (20 minutes). Oligofectamine cell transfection solution was finally added drop by drop. Four hours later, serum was added to the cell culture medium to a final concentration of 10%. Cells were then incubated at 37ºC in a CO_2 incubator for 72 hours before assessing gene expression.

To obtain a stable cell line expressing standard electroporation and Lipofectamine^{TM} 2000 kit were used to transfect cells.

Stable cell lines were obtained incubating the plate with the antibiotic to which the transfected plasmid is resistant. Cells which incorporated the plasmid in their genetic material are resistant to the antibiotic selection. Stable transfected cells were obtained in 1-2 week(s). Antibiotic final concentrations are: geneticin 500 µg/ml (G-418; GIBCO), puromycin 1 µg/ml (Sigma) and hygromycin 300 µg/ml (Sigma). Cell culture medium should be changed twice a week. A pool population was obtained through the collection of all the cells presents in the plate, otherwise subclonal population could be selected.

6.8. *PLASMID CONSTRUCTS*

6.8.1. *Plasmid expression vector constructs for mammalian cells*

Bcl-x_L (Yuste VJ. Et al, 2001), XIAP and FLIP proteins were expressed under the control of Citomegalovirus promoter (CMV) in pcDNA3 vector (Invitrogen Corporation, Carisbad, CA; USA). For transient overexpression, human Bcl-x_L cDNA was extracted from the pcDNA3 expression vector (Invitrogen) and subcloned into pWPI vector (Naldini et al., 1996; Zufferey et al., 1998).

For experiments performed with silence interference RNA (siRNA), specific oligonucleotide sequences were introduced in pSUPER.retro.puro vector (OligoENgine,

Seatle, WA; USA) following the recommendend ligation and transformation protocol.

The sequences for each genes are reported in the following table.

Gene	Type	Spec.	Sequence
Bcl-x$_L$	**Fwd**	**Rat**	gatccccGATTGCAAGTTGGATGGCCttcaagagaGGCCATCCAACTTGCAATCttttt
	Rev	**Rat**	agctaaaaaGATTGCAAGTTGGATGGCCtctcttgaaGGCCATCCAACTTGCAATCggg
FLIP	**Fwd-1**	**Rat**	gatccccGGTTCCGATCAGTTGAATTTTcaagagaAATTCAACTGATCGGAACCttttt
	Rev-1	**Rat**	agctaaaaaGGTTCCGATCAGTTGAATTTctcttgaaAATTCAACTGATCGGAACCggg
	Fwd-2	**Rat**	gatccccCACCTTGTTTCCGATTATATTcaagagaTATAATCGGAAACAAGGTGttttt
	Rev-2	**Rat**	agctaaaaaCACCTTGTTTCCGATTATATctcttgaaTATAATCGGAAACAAGGTGggg
SMAC	**Fwd-1**	**Rat**	gatccccGCCAGAGTTGAGATGACTTttcaagagaAAGTCATCTCAACTCTGGCttttt
	Rev-1	**Rat**	agctaaaaaGCCAGAGTTGAGATGACTTtctcttgaaAAGTCATCTCAACTCTGGCggg
	Fwd-2	**Rat**	gatccccTGATAAGACCATGGCACAAttcaagagaTTGTGCCATGGTCTTATCAttttt
	Rev-2	**Rat**	agctaaaaaTGATAAGACCATGGCACAAtctcttgaaTTGTGCCATGGTCTTATCAggg
c-IAP1	**Fwd-1**	**Rat**	gatccccTACAGTATGTGGCCATTAAttcaagagaTTAATGGCCACATACTGTAttttt
	Rev-1	**Rat**	agctaaaaaTACAGTATGTGGCCATTAAtctcttgaaTTAATGGCCACATACTGTAggg
	Fwd-2	**Rat**	gatccccGAACAAGGTGGCATTCATTttcaagagaAATGAATGCCACCTTGTTCttttt
	Rev-2	**Rat**	agctaaaaaGAACAAGGTGGCATTCATTtctcttgaaAATGAATGCCACCTTGTTCggg
c-IAP2	**Fwd-1**	**Rat**	gatccccCACGGAGAAGGCCAGATTAttcaagagaTAATCTGGCCTTCTCCGTGttttt
	Rev-1	**Rat**	agctaaaaaCACGGAGAAGGCCAGATTAtctcttgaaTAATCTGGCCTTCTCCGTGCggg
	Fwd-2	**Rat**	gatccccGTTCGTTGGCCAAGTTCAAttcaagagaTTGAACTTGGCCAACGAACttttt
	Rev-2	**Rat**	agctaaaaaGTTCGTTGGCCAAGTTCAAtctcttgaaTTGAACTTGGCCAACGAACggg

Oligonucleotides used to construct the plasmids were purchased from Sigma Aldrich and were cloned between Bgl II/Hind III sites of pSUPER.retro.puro plasmid as specified by OligoEngine procedures.

To obtain the highest efficiency of trasfection, Lentiviral constructs were performed. The constructs were achieved by digesting EcoR1-Cla1 sites from pSUPER-sh to replace H1 promoter with H1-shRNA cassette in pLVTHM.

6.8.2. *Lentiviral constructs*

pEIGW, pLVTHM, pSPAX2 and pM2G vectors were kindly given by Dr. Trono of the Laboratory of Virology and Genetics from the School Life of Science of Swiss Institute of Technology of Lausanne (Switzerland). FLIP-$_L$ and Bcl-x$_L$ genes were subcloned from pcDNA3 to pWPI vector originating FLIP-$_L$-pWPI and Bcl-x$_L$-pWPI constructs respectively. siRNA lentiviral constructs were obtained through a direct subclonation of the entire promoter H1, flanqued by the target restriction sites of Eco RI and Cla I, from pSUPER.retro.puro vector to pLVTHM vector.

6.8.3. *Lentiviral production*

In 2000 Dr. Trono and colleagues developed 3 generations of vectors used to product Lentivirus (Trono D., 2000). To perform this study Lentiviruses of second generation were used with a sufficient biosecurity level to what concerns genetic transference *in vitro*. Lentivirus production protocol was described by Naldini L. and collaborators in 1996 (Naldini L. et al, 1996). The plasmids used for Lentivirus production that belong to the second generation are the following:

1) Vectors pEIGW/pLVTHM were used to overexpress or inhibit gene expression, respectively. The vector itself is the only genetic material transferred to the target cells, as they lost the transcriptional capacity of the viral long terminal repeat (LTR). The vector sequence includes the trangene cassette flanked by cis-acting elements required for its encapsidation, reverse transcription and integration in the genome. In order to produce lentiviral siRNA construct, pLVTH was designed in such way that H1 Pol III promoter can be easily replaced by H1-siRNA cassette from pSUPER.retro.puro using EcoRI – Cla I.

2) psPAX2 vector codifies for viral packaging proteins. psPAX2 contains a very efficient promoter (CAG), which allows the expression of viral packaging compounds, among which TAT protein, DNA polimerase and Reverse Transcriptase are the most relevant. The CAG promoter includes CMV enhancer, chicken beta-actin promoter and intron.

3) pM2G vector codifies for virus envelope. The viral protein comes from Vescicular stomatitis Virus (VSV) and trigger to a wide range of tissues and cell lines infection.

For Lentiviral production constructs, HEK293T cells were seeded at a density of $2{,}5x10^6$ cells in 0.1% gelatin-coated 100-mm cell culture dishes. The following day, cells were transfected with:

Vector pWPI / pLVTHM	20µg
pSPAX2	13µg
pM2G	7µg

The transfection was routinely performed by the PEI transfection method. Cells were allowed to produce Lentiviruses for 48 hours. Then the medium was centrifuged at 4000xg (5 minutes) to collect and eliminate died cells present in the supernatant,

and subsequently clarified using a filter of 45μm. Lentiviruses were concentrated by centrifugation at 50.000xg (90 minutes), thereby preventing that components of cell culture medium could affect the experiments. To obtain high infection efficiency, lentiviruses were finally resuspended in a solution containing a “carrier” protein which preserves their conservation at -80ºC without affecting the infectivity capacity, i.e. 1% BSA in PBS solution. The biological titer of the viral preparation was expressed as a number of transducing units per millilitre (TU/ml) and was determined by transducing HEK293T cells in limiting dilutions. After 48 hours incubation, the percentage of GFP-positive cells was counted and viruses at $5 \times 10^8 - 1 \times 10^9$ TU/ml were used in the experiments.

6.8.4. *Cell transduction*

For lentiviral transduction, cells were seeded in a 24-multiwell plate and 2μl of the concentrated lentivirus were added to the medium. 4 hours later, the medium was changed and the efficiency of the infection was continuously monitored by direct counting of GFP-positive cells. The percentage of the infection was always comprised between 90% in cortical neurons and 99% for cell lines. A time course was always performed in order to assess protein overexpression or downregulation. In a transient infection, the protein expression level was tested by Western Blot 3 days post-infection. Pools of infected cells were obtained in 1–2 week(s) and were performed by adding geneticine to the cell culture medium.

6.8.5. *RT-PCR analysis*

mRNA was isolated from cells with the RNeasy® Mini kit according to the manifactured instruction (Qiagen, Valencia). 1μg of isolated mRNA was reverse transcribed by Taqman® Reverse Transcription Reagents (Applied Biosystem) according to the protocol provided by the manufacturer.

Approximately, 10ng of each cDNA obtained was amplified by polymerase chain reaction (PCR) in a PerkinElmer thermal cycler 2400 with 200nM each primer using as PCR conditions, 94ºC for 30s, 60ºC for 30s and 72ºC for 40s for 28 cycles.

6.8.6. *General techniques for gene cloning*

Plasmid DNA from *E. Coli* was extracted using Qiagen plasmid Max Kit or QIAprep Spin Miniprep Kit (Qiagen, Valencia) according to the instructions provided by

the manufacturer. The concentration of nucleic acid was determined in a UV-visible Recording Spectrophotometer (NanoDrop).

Digestion of DNA by restriction endonucleases and DNA ligation were performed according with the protocols of the providers.

DNA purification was performed using either QIAquick Gel Extraction Kit (Qiagen, Valencia) or High Pure PCR Product Purification Kit (Qiagen, Valencia) according with the protocols included in the commercial kits.

Antartic Phosphatase (BioLabs, New England) was used to dephosphorylate the DNA fragment. DNA transformation to *E.Coli* DH5α was carried out.

6.8.7. *E. Coli DNA transformation*

Compentent cells were prepared from an overnight growth of DH5α strain in LB medium at 37ºC. A dilution of the starter culture was newly incubated at 37ºC till reach a optical density of 0.4 (λ = 600nm). Therefore, cells were mantained on ice (10 minutes) and subsequently collected by centrifugation at 6000xg (5 minutes at 4ºC). Cells were resuspended in 50ml of 60mM $CaCl_2$, 15% glycerol and 10mM Hepes pH 7.0 resuspension buffer and incubate on ice (30 minutes). After centrifugation at 6000xg (5 minutes at 4ºC), cells were finally resuspended in the decribed buffer. Aliquots are kept at -80ºC.

Competent cells were placed on ice until thawed. About 50ng DNA was added to 200µl of competent cells whose transformation efficiency was measured in $1x10^6$. Cells suspension was maintained on ice (30 minutes) and consequently subjected heat–shock (1 minute at 42ºC) in a water bath. Cells were cooled down on ice (2 minutes) and 800µl of LB medium without antibiotics is added. Transformation reactions were finally incubated 1 hour at 37ºC, before being plated on a LB agar plate with the specific antibiotic. The plates should be incubated at 37ºC for 12–14 hours before performing the DNA extraction.

6.9. *IMMUNOFLUORESCENCE*

6.9.1. *Active Caspase-3 Immunofluorescence*

Cells were initially seeded in a pre-coated polylisine (10 µg/ml, diluted in water) and collagen (100 µg/ml, diluted in acetic acid 0.02 M) 35-mm cell culture dish. After the treatment cells were rinsed with cold PBS and fixed in 4% paraformaldheyde/PBS

for 30 minutes at room temperature. Then they were washed twice with PSB and subsequently permeabilized and blocked with 3% fetal bovine serum and 0.1% Triton X-100 in PBS for 60 minutes. Cells were incubated overnight at 4ºC with a PBS solution of the primary antibody, the ploycional anti-Caspase 3 (Cell Signaling) diluted 1:150. Subsequentely cells were rinsed three times with PBS and incubated with the secondary anti-rabbit antibody conjugated with Alexa Fluor 594 (Molecular Probes, Eugene, OR) diluted 1:250 for one hour at room temperature protected from light. Finally cells were rinsed three times with PBS and stained with 0.5 µg/ml Hoechst 33258 for 30 minutes. The observation was carried out using an Olympus microscope equipped with epifluorescence optics.

6.9.2. *RelA/p65 Nuclear Translocation Immunofluorescence*

Cells were seeded in a pre-coated polylisine (10µg/ml, diluted in water) and collagen (100 µg/ml, diluted in acetic acid 0.02 M) 35-mm culture dish. After TNFα stimulation, cells were rinsed with cold PBS and fixed in methanol 100% (5 minutes at room temperature). After three washes with PSB, cells were incubated (1 hour at room temperature) with a PBS solution of the primary antibody, the polyclonal anti-p65 (C-20 sc-372) (Santa Cruz Biotechnology) diluted 1:300. Subsequentely, cells were rinsed twice with PBS and incubated with the secondary anti-rabbit antibody conjugated with Alexa Fluor 488 (Molecular Probes, Eugene, OR) diluted 1:1000 (1 hour at room temperature) protected from light. Finally cells were rinsed three times with PBS and stained with 0.05µg/ml Hoechst 33258 (30 minutes). The observation was carried out using an Olympus microscope equipped with epifluorescence optics.

6.10. *LUCIFERASE REPORTER ASSAY*

The vector used for the Luciferase reporter assay was pGL3-basic (Promega).

Luciferase Assay is used as a repoter providing a sensitive instrument to better study cellular events which are potentially able to affect gene expression. The reporter activity is available immediately upon translation since the protein does not require post-translational processing. The vector contains a modified region of the luciferase protein of the Luciernaga, which was optimized in order to monitor the transcriptional activity in transfected eucariotic cells. Light is produced by converting the chemical energy of luciferin oxidation through an electron transition, forming the oxyluciferin.

Cells were treated with TNFα and after 6 hours, cell culture dishes were washed once with PBS. Cells were collected with 100µl of the assay lysis buffer, previously diluted in water as reported in the protocol of Promega Luciferase Assay Kit. Following the instruction provided by the commercial manufacturer (Promega, Madison WI, USA), light emission was quantified with a luminometer. The values obtained were analyzed with the Excel program and normalized in relation to protein concentration (RLU/µg protein). Means and respective SEM were calculated. The final graph and the statistical analisis were performed using the same informatic tool.

6.11. *OXIDATIVE STRESS MEASUREMENT*

6.11.1. *ROS Production*

ROS production was evaluated by FACS using CM-H_2DCFDA, the reactive oxygen species cell-permeable indicator (Molecular Probes, Invitrogen). Cells were seeded in 24-multiwell plate. Cells were treated and collected by centrifugation at 1000xg (3 minutes at 4ºC). Cells were rinsed once with PBS solution and resuspended in PBS supplemented with 10µM of the fluorogenic probe. Samples were incubated at 37ºC (15 minutes) in a humidified atmosphere of 5% CO_2. After one wash with PBS, cells were resuspended again in PBS solution and samples were immediately read by FACS.

6.11.2. *Lipid Peroxidation Measurement (OXI-TEC TBARS)*

Measurement of Thiobarbituric Acid Reactive Substances (TBARS) is a sensitive technique to value the induction of oxidative stress-mediated damage i.e. lipid peroxidation. The assay is based on the reaction between malondyaldehyde with thiobarbituric acid in a relation 1:2. To perform the assay, cells were collected and resuspended in PBS solution. 100µl of SDS and 2.5ml TBA/Buffer Reagent were added to each sample which was subsequently boiled at 95ºC (1 hour). Cells were then cooled down on ice (10 minutes) and centrifuged at 5000xg (15 minutes). The absorbance of the supernatant was read at λ=532nm at the spectrophotometer. Each experiment was performed in duplicate. The values were analyzed comparing untreated and treated cells by means of the Excel Program. Means and the respective SEM were calculated. The final graphs and the statistical analysis were performed using the same program.

6.12. *SUBCELLULAR FRACTIONING*

Cells were seeded in a 35-mm cell culture dish. After the indicated treatment, cells were collected in a 1.5ml eppendorf and centrifuged at 500xg (5 minutes at 4ºC). Cells were rinsed once with PBS solution and resuspended in 5mM HEPES (pH 7.2/KOH), 10mM KCl, 5mM EGTA, 2mM $MgCl_2$, 220mM Mannitol, 70mM Sucrose lysis buffer supplemented with protein inhibitor cocktail. Samples were incubated on ice (30 minutes) to perform the lysis. The supernatant, i.e. the cytosolic fraction, was collected by centrifugation at 16000xg (10 minutes at 4ºC) whereas the mitochondrial fraction was resuspended in the described buffer supplied with 1% Triton X-100 and incubated on ice (15 minutes). After centrifugation at 16000xg (10 minutes at 4ºC), mitochondrial solution was collected. Both fractions were quantified using the Lowry Assay, Bio-Rad DC Protein Assay (Bio-Rad, Hercules, CA) and loaded in a electrophoresis gel.

Results

7.1. OBJECTIVE Nº1

Since maintaining a state of resistance to apoptosis often requires de novo proteins or RNA synthesis, the first objective of this study was to assess whether the treatment with RNA transcription inhibitor, Actinomicyne D (ActD), would sensitize PC12 cells to TNFα-mediated apoptosis.

7.1.1. *INHIBITION OF TRANSCRIPTION RENDERS PC12 CELLS SENSITIVE TO TNFα CYTOTOXIC EFFECT*

Although TNFR-1 and adaptor proteins required for the induction of the death machinery are expressed in a variety of cell types, TNFR-1-expressing cells are resistant to TNFα-mediated apoptosis. These cells become sensitive to TNFα treatment when RNA transcription or protein synthesis is inhibited (Fulda et al, 2000; Mielke et al, 2002). Therefore, ActD, a RNA transcription inhibitor, or CHX, a protein synthesis inhibitor, sensitizes cells, i.e. PC12 cells, to TNFα-mediated apoptosis.

As shown in figure 34, addition of 100ng/ml TNFα to PC12 cells did not affect their viability or proliferation.

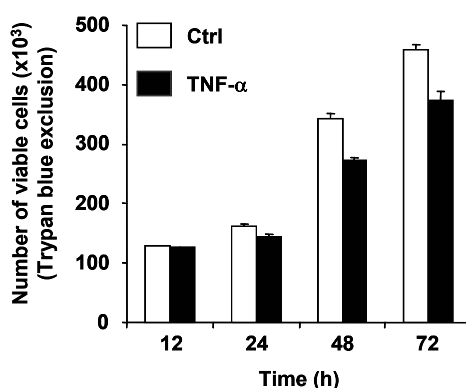


Figure 34. PC12 cells were incubated with TNFα (black columns) or untreated (white columns) for the indicated times. Cell viability was measured by cell counting using Trypan Blue Exclusion assay.

To sensitize PC12 cells to TNFα-promoted cell death, cells were treated with the cytokine in presence of 1nM ActD for a time course of 5 days and as illustrated in figure 35, after 24 hours 20% of cell death was obtained. It is interesting to point out that ActD treatment impares PC12 proliferation although the compound has no effect on cell viability.

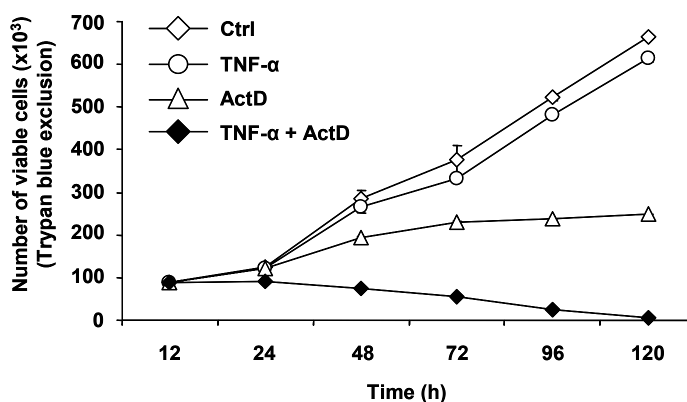


Figure 35. PC12 cells were maintained in culture for the indicated time and experimental conditions. Proliferation was estimated by Trypan Blue exclusion counting.

To assess whether TNFα/ActD co-treatment induced apoptotic cell death, experiments with Hoechst and TUNEL staining assay were performed. Due to the ability to label the DNA, these techniques demonstrated that upon ActD sensitization TNFα induced the typical apoptotic nuclear morphology characterized by chromatin condensation and nucleus shrinkage. Representative images of Hoechst and TUNEL staining after 24 hours of TNFα and ActD treatment are shown in figure 36.

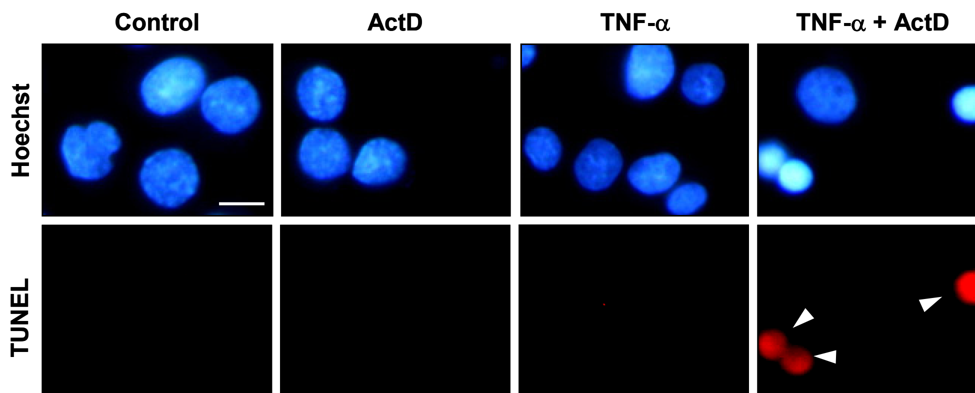


Figure 36. Fluorescent images showing nuclei stained with Hoechst 33258 or with the TUNEL assay.

To confirm that TNFα/ActD triggered the activation of the apoptotic program, the percentage of cell death obtained by Trypan Blue assay, Hoechst and TUNEL staining was compared (Figure 37).

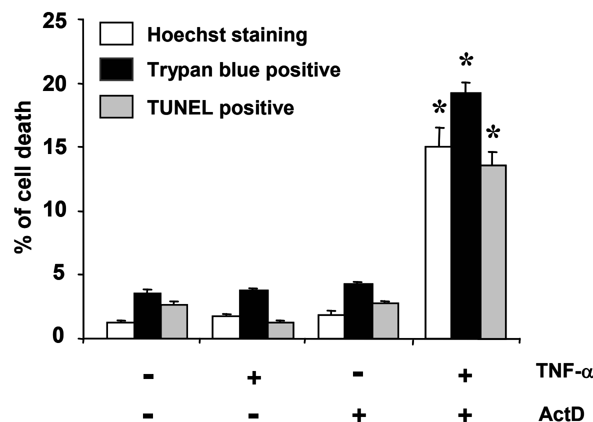


Figure 37. Quantification of cell death by Trypan Blue exclusion assay, Hoechst and TUNEL staining. * means $p < 0.01$.

These results show that the addition of TNFα in the cell culture media does not affect neither viability nor growth. Only when transcription is inhibited TNFα renders PC12 cell sensitive to its cytotoxic effect.

7.1.2. *INHIBITION OF TRANSCRIPTION ALLOWS TNF-α TO ACTIVATE THE INTRACELLULAR APOPTOTIC PATHWAY*

To better understand the molecular mechanism of the cytotoxic effect mediated by TNFα stimulation upon ActD sensitization, caspases activation was analyzed. As shown in figure 38, 8 hours TNFα/ActD co-treatment induced a near 3-fold increase in caspase-8-like activity, when compared with non-treated PC12 cells or PC12 cells cultured in the presence of ActD or TNFα alone. To verify the specific caspase-8 involvement, the experiment was carried out using caspase-8 inhibitor, z-IETD-fmk. When 50 μM z-IETD-fmk was added to the cell culture medium, caspase-8-like activation was abrogated.

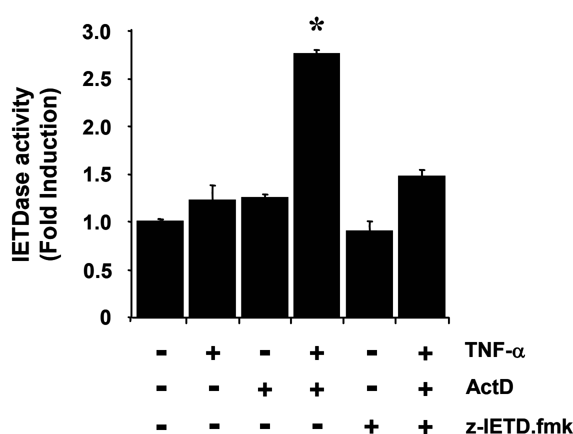


Figure 38. Caspase-8 activity was measured with the fluorogenic Ac-IETD-afc reagent. * means $p < 0.01$.

Processing of Bid into its caspase-8-specific truncated form, tBid, only occurs when cells were co-treated with TNFα and ActD (Figure 39).

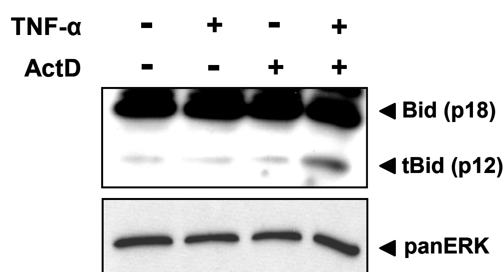


Figure 39. Total lysates form cells treated for 24 hours were analyzed by Western Blot to detect caspase-8 specific processing of Bid through the appearance of the truncated fragment tBID p12.

Since caspase-8 cleaved tBid translocates to the mitochondria acting as an amplifier of TNFα apoptotic signal (Chou J.J. et al, 1999), we further studied the implication of the intrinsic pathway in the execution of cell death. Figure 40 demonstrates that TNFα/ActD co-treatment promoted the processing of pro-caspase-9

into its p38 and p17 active fragments, and pro-caspase-3 into its p17 large active form. These fragments are considered good indicators of their activation (Johnson and Jarvis, 2004).

Figure 40. Caspase-9 and -3 activations were determined by the Western Blot detection of their respective active fragments.

Activation of caspase-3 upon 24 hours TNF/ActD stimulation is also demonstrated staining PC12 cells with an antibody raised against the active form of caspase-3 activation (Figure 41).

Figure 41. Immunofluoscent detection of caspase-3 activation in PC12 cells treated as indicated, using a specific antibody (red and arrows), merged with Hoechst 33258 nuclear staining.

Moreover, caspase-3 activation was shown by measuring caspase-3-like enzymatic activity using a specific fluorogenic substrate (DEVD) (Figure 42).

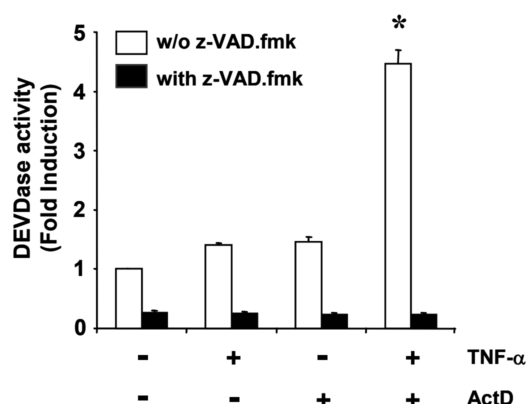


Figure 42. Caspase-3-like activity was measured using the fluorogenic Ac-DEVD-afc reagent after 8 hours of the indicated treatments. * means p< 0.01.

Taken together the results obtained from these experiments clearly indicate that caspase-3 activity could only be detected in cells co-treated with TNFα and ActD. Therefore, apoptosis and caspase-3 activation promoted by TNFα and ActD co-treatment can be fully inhibited by 50μM of broad caspase inhibitor z-VAD-fmk (Figure 43).

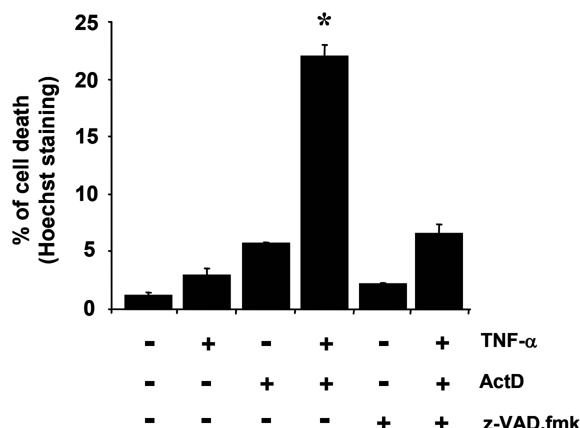


Figure 43. The inhibition of TNFα and ActD induced cell death by the caspase inhibitor, z-VAD-fmk, was measured by counting apoptotic nuclei stained with Hoechst 33258. * means p< 0.01.

7.1.3. *PRIMARY NEURONS ALSO REQUIRE INHIBITION OF TRANSCRIPTION TO BECOME SENSITIVE TO TNF-α- INDUCED APOPTOSIS*

Both naïve and differentiated PC12 are commonly used as experimental models of neural cells. Therefore, PC12 cells allow hypothesizing the molecular mechanism occurring in primary neurons upon stimulation. Therefore, TNFα and ActD treatment was analyzed in primary mouse cortical neurons.

Cortical neurons were isolated from E15 mouse embryos, maintained in culture with basal medium supplemented with N2 and B27 for 6 days and then treated for 24 hours with TNFα and ActD. PC12 cells and cortical neurons were resistant to TNFα-induced apoptosis (24 hours). However, the co-treatment with TNFα and ActD induced a significant increase in cell death, whereas ActD alone did not modify cell viability. As indicated in figure 44, TNFα/ActD-mediated apoptosis was associated to the processing of pro-caspase-9 and pro-caspase-3 in their respective active fragments.

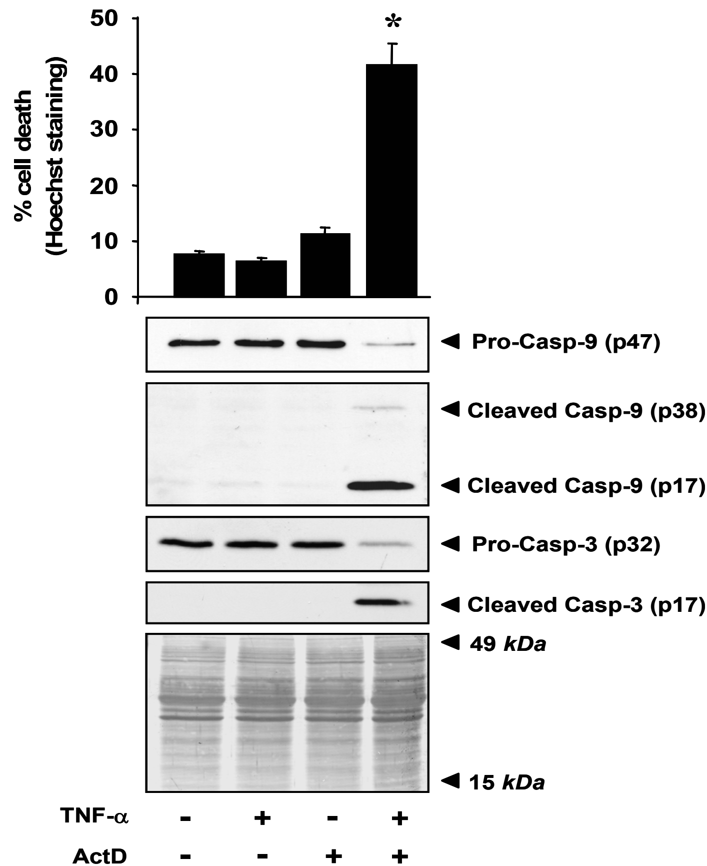


Figure 44. Percentage of cell death was quantified by counting apoptotic nuclei stained with Hoechst 33258. * means p< 0.01. The processing of pro-caspase-9 and pro-caspase-3 were analyzed by Western Blot in the indicated condition and using Naphtol Blue (NB) as control membrane protein content.

Caspase-3 activation after 24 hours TNFα/ActD co-treatment was observed also by neurons immunofluorescence staining (Figure 45).

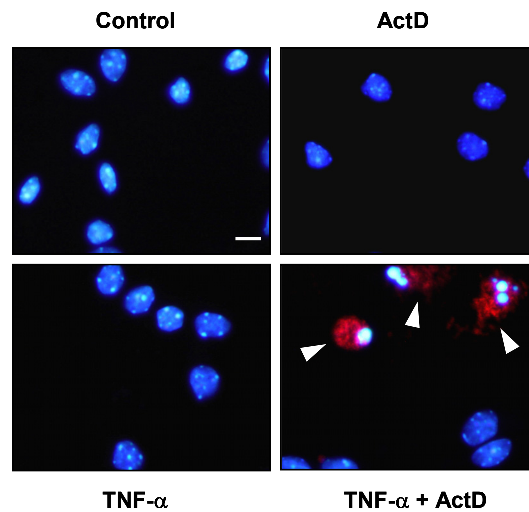


Figure 45. Representative pictures of the indicated 24 hours treatment show active caspase-3 immunofluorescence (red and arrows) merged with Hoechst 33258 nuclear staining.

To further assess caspases involvement, caspase-8-like and caspase-3-like activities were analyzed upon TNFα and ActD co-treatment by measuring their enzymatic activities using fluorogenic substrates IETD and DEVD, respectively (Figure 46).

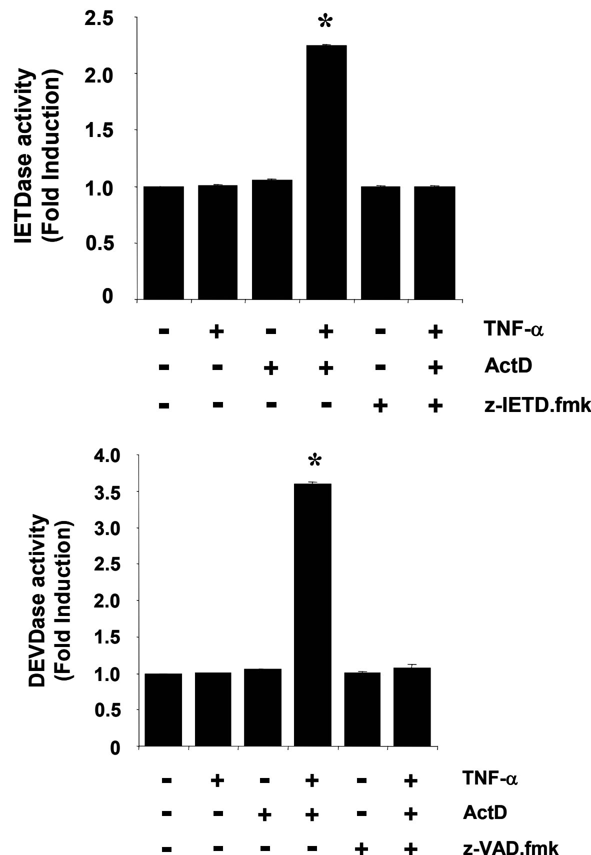


Figure 46. Caspase-8- and -3-like activity were measured after 8 hours of the indicated treatments using fluorogenic Ac-IETD-afc and Ac-DEVD-afc reagents, respectively. * means p< 0.01.

7.1.4. *INHIBITION OF TRANSCRIPTION DOES NOT MODIFY THE LEVELS OF THE DISC COMPONENTS*

The biologic effects promoted by TNFα are mediated by binding to TNFR-1 and TNFR-2 (van Horssen et al, 2006). The survival pathway mediated by TNFα/TNFR-1 requires the recruitment of the adaptor protein TRADD to TNFR-1, which acts as a platform allowing the engagement of TRAF-2 and RIP. Formation of this complex, known as "complex I", activates both MAPK and NF-κB signaling transduction pathways. TRADD can also trigger the activation of caspase-8 which occurs after the formation of a second complex which lacks the receptor. The DD of TRADD can recruit by protein interaction FADD molecule, which in turn engages the recruitment and the activation of caspase-8.

Since ActD downregulates the expression of short half-lifes proteins, it is reasonable to think that the sensitization to TNFα-induced cell death could be the consequence of a misbalanced between "survival complex" (integrated by TNFα, TNFR-1, TRADD, TRAF-2 and RIP) and "death complex" (formed by TRADD, FADD and caspase-8) (van Horssen et al, 2006). In order to assess this hypothesis, Western Blots against TNFR-1, and the adaptor molecules TRADD, FADD, TRAF-2 and RIP were performed. As show in figure 47, PC12 cells and cortical neurons were treated as indicated for 24 hours and total cell lysates were analyzed.

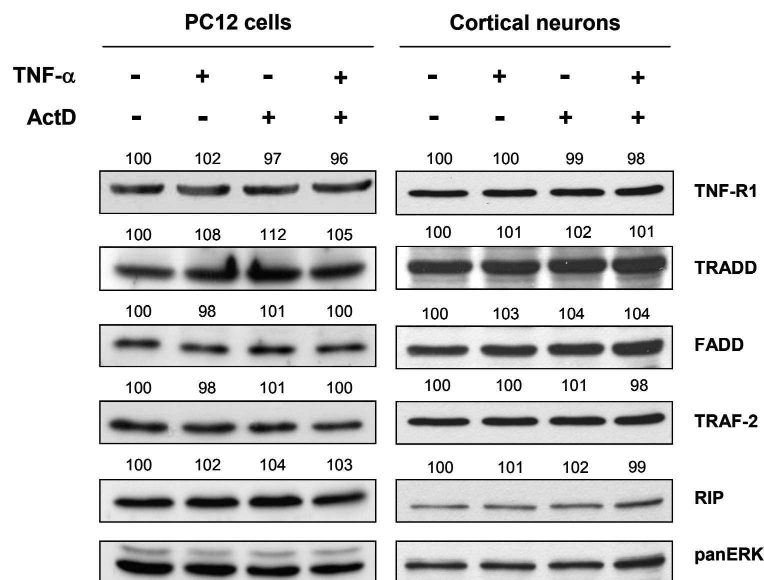


Figure 47. Total lysates of PC12 cells and primary cortical neurons were analyzed by Western Blot using specific antibody. Loading control was determined by probing the membrane with an anti-panERK antibody. The values indicated in the upper part of the images represent the ratio of the mean of three experiments calculated respect to the total protein content.

The expression of TNFR-2 and caspase-8 was analyzed by semi-quantitative RT-PCR. As shown in figure 48, the levels of these proteins remained unchanged after treatment with TNFα, ActD or both. While TNFR-1 was present in PC12 cells, we could not detect TNFR-2. This is in agreement with data published by other authors who demonstrated that PC12 cells do not express TNFR-2 (Mielke and Herdegen, 2002).

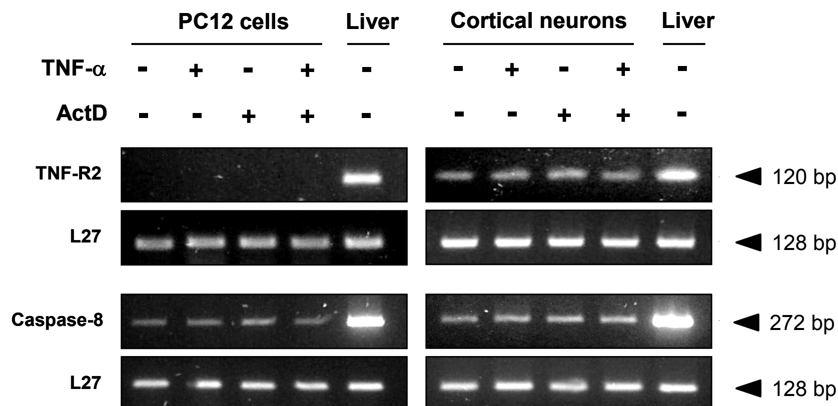


Figure 48. Total mRNA was extracted from PC12 cells and primary cortical neurons after 6 DIV and treated as described. Semi-quantitative RT-PCR of TNFR-2, caspase-8 and L27 (used as an internal control) transcripts were analyzed. As positive control of amplification, total mRNA isolated from post-natal liver was used.

According to these results the sensitization induced by ActD to TNFα-mediated apoptosis occurs below the assembly of the death complex promoted by the binding of the cytokine to TNFR-1.

7.2. OBJECTIVE Nº2

ActD stimulation promotes the decrease of short half-life proteins expression. According to this, our purpose was to address at which molecular level ActD interferes in TNFα-induced apoptosis.

7.2.1. *FLIP$_L$ AND IAPs PROTEINS ARE NOT INVOLVED IN THE SENSITIZATION INDUCED BY ActD TO TNF-α-MEDIATED CELL DEATH*

It is reported that FLIP$_L$ and IAPs play an essential role in affording protection against TNFα-induced apoptosis. Moreover, it is also described that the transcription of these proteins is enhanced by TNFα (Wullaert et al, 2006; Chang et al, 2006; Furusu et al, 2007). As shown in figure 49, FLIP$_L$ expression level remained unchanged during TNFα, ActD or both time course.

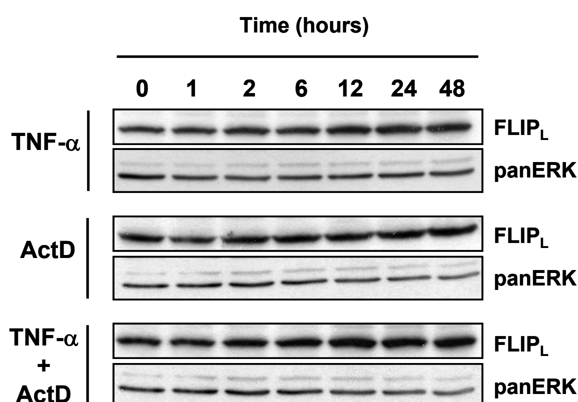


Figure 49. Cells were treated with 100ng/ml TNFα and/or 1nM ActD for the indicated time course. Western Blot analysis was performed using specific antibody in order to detect FLIP$_L$ level expression during the different treatments. Loading control was determined by probing the membrane with an anti-panERK antibody.

To assess whether FLIP$_L$ could afford protection against TNFα and ActD mediated cell death, PC12 cells were transfected with a eukaryotic expression plasmid carrying the open reading frame of rat/mouse *FLIP* gene. As shown in figure 50, PC12-FLIP pool of cells presents almost three fold protein expression when compared with pool of cells carrying the empty plasmid, PC12-Neo.

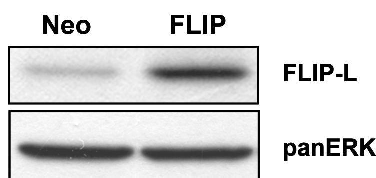


Figure 50. Pools of PC12 cells stably transfected with the empty vector (Neo) and with *Flip* gene were analyzed by Western Blot using a specific anti-FLIP$_L$ antibody. Loading control was determined by probing the membrane with an anti-panERK antibody.

As shown in figure 51, the stable expression of c-FLIP conferred resistance to TNFα and ActD co-stimulation, demonstrating that the cell death mediated by TNFα is caspase-8 dependent.

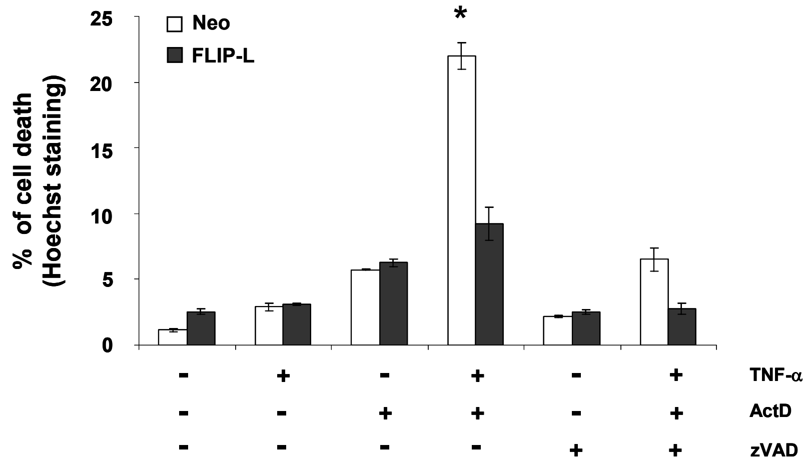


Figure 51. Cells were treated as indicated for24 hours. Percentage of cell death was assessed by counting stained nuclei with Hoechst 33258. * means p< 0.01.

Furthermore, in order to know whether ActD treatment can modulate the expression of cIAPs proteins, a time course analysis upon TNFα, ActD and both was performed. The result show that cIAP1 and cIAP2 levels increased during TNFα time course, even though ActD treatment did not affect significantly their expressions, neither alone or in the presence of TNFα (Figure 52).

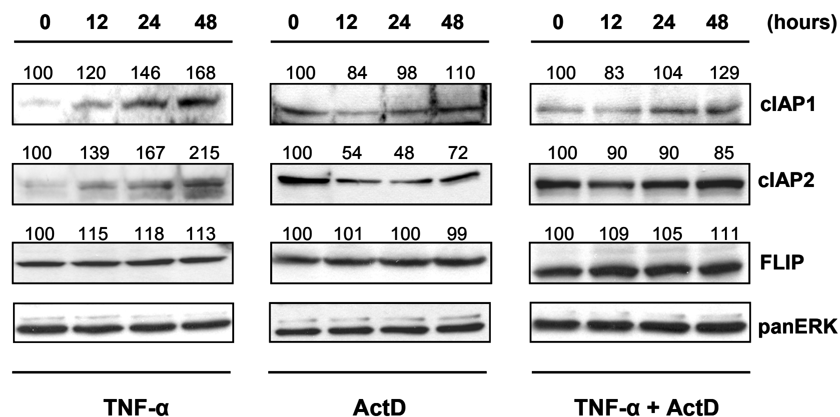


Figure 52. Cells were treated with 100ng/ml TNFα, 1nM ActD or both for the indicated time course. Western Blots against cIAP1 and cIAP2 were performed using specific antibodied. Loading control was determined by probing the membrane with an anti-panERK antibody. The values indicated in the upper part of the images represent the ratio of the mean of three experiments calculated respect to the total protein content.

As it is described that XIAP is one of the most relevant IAPs-anti-apoptotic molecules which affords protection against TNFα-mediated cell death, a time course was performed to assess its protein expression level upon TNFα, ActD and both. When PC12 cells were treated with 1nM ActD a decrease in XIAP expression was detected. The same result was obtained after TNFα and ActD co-treatment (Figure 53).

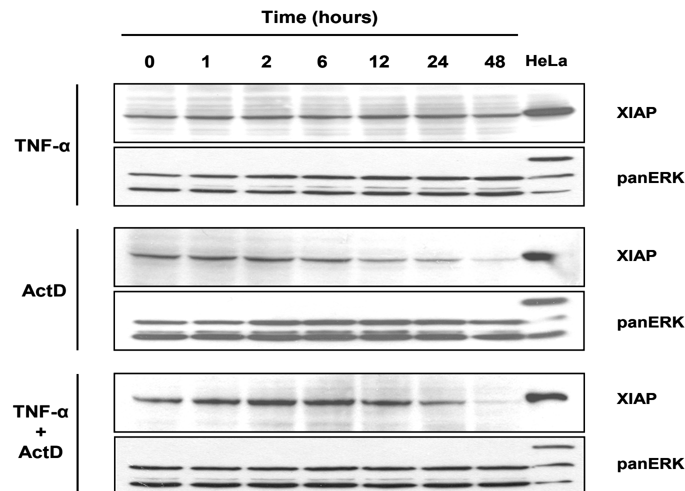


Figure 53. Cells were treated with 100ng/ml TNFα and/or 1nM ActD for the indicated time course. Western Blot analysis was performed using specific antibody in order to detect XIAP level expression during the different treatment. Loading control was determined by probing the membrane with an anti-panERK antibody.

To assess whether XIAP could confer resistance to TNFα and ActD treatment, we transfected PC12 cells with a eukaryotic expression plasmid carrying the construct that overexpress rat/mouse *XIAP*. As shown in figure 54, PC12-XIAP pool of cells presents almost two fold protein expression when compared with PC12-Neo.

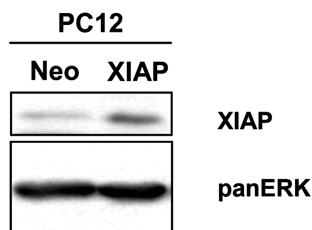


Figure 54. Pools of PC12 cells stably transfected with the empty vector (Neo) and with *xiap* gene were analyzed by Western Blot using a specific anti-XIAP antibody. Loading control was determined by probing the membrane with an anti-panERK antibody.

The graph in figure 55 shows that XIAP overexpression made cells partially resistant to TNFα and ActD co-treatment.

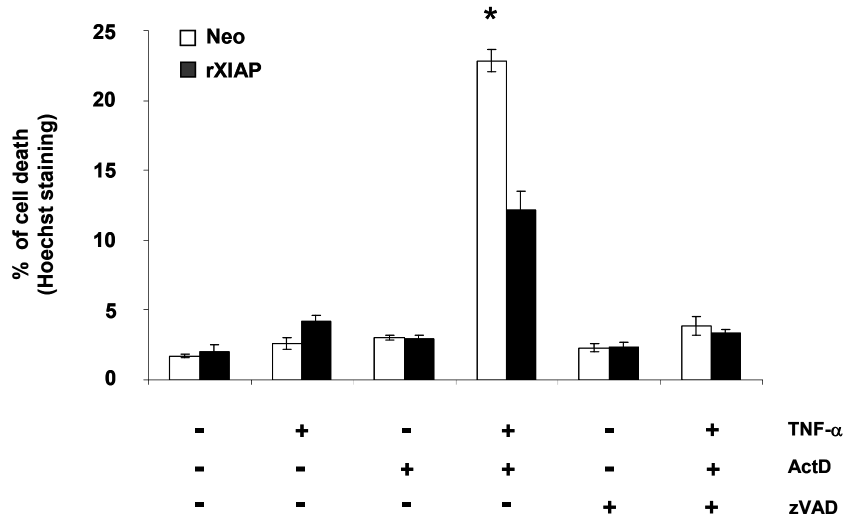


Figure 55. Cells were treated as indicated. Percentage of cell death was assessed by counting stained nuclei with Hoechst 33258. * means p< 0.1.

To demonstrate the relevance of these anti-apoptotic proteins, we wanted to assess whether their endogenous decrease would sensitize PC12 cells to TNFα cytotoxic effect. Therefore, as shown in figure 56 the singular downregulation of either c-FLIP, cIAP2 or XIAP was carried out. c-FLIP, cIAP2 or XIAP endogenous decrease did not affect the viability of cells in response to 24 hours treatment with TNFα (same result was observed with c-IAP1 endogenous decrease, data not shown).

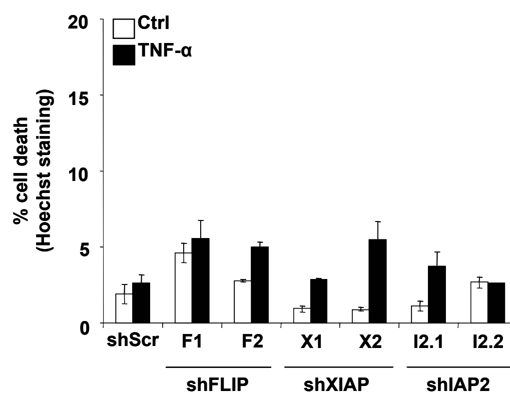


Figure 56. Cells were treated as indicated for 24 hours. Percentage of cell death was assessed by counting stained nuclei with Hoechst 33258.

Taken together, these data demonstrate that even though the overexpression of $FLIP_L$ and XIAP proteins confer resistance to TNFα and ActD co-treatment, their singular specific endogenous decrease indicate that neither the absence of c-FLIP nor IAPs sensitizes to TNFα-mediated apoptotis. Therefore, other mechanisms should exist to explain ActD sensitization to TNFα treatment.

7.3. OBJECTIVE Nº 3

A misbalance between pro- and anti-apoptotic proteins is required by death stimuli to promote apoptosis. Therefore, our aim was to address the involvement of mitochondrial Bcl-2 family members on TNFα-mediated cytotoxic effect. Moreover, as Bcl-2 proteins are targets of NF-κB transcription factor, we wanted to assess whether mitochondrial damage would affect the survival pathway promoted by NF-κB upon TNFα stimulation.

7.3.1. *INHIBITION OF TRASNCRIPTION INDUCES A DECREASE IN Bcl-x_L PROTEIN LEVELS*

As a misbalance between pro- and anti-apoptotic proteins is required by death stimuli to promote apoptosis, we decided to focus our efforts in studying the involvement of the mitochondria in ActD sensitization to TNFα-induced cell death (Deng et al, 2003). From one side, the pro-apoptotic members of the Bcl-2 family are responsible for mitochondrial changes which trigger the death signal induced by TNFα. On the other hand, the anti-apoptotic members belonging to the same family are among the most relevant proteins affording protection against TNFα-mediated cell death.

Therefore, we decided to assess whether ActD would affect the expression of the anti-apoptotic Bcl-x_L or pro-apoptotic molecules Bax and Bak in PC12 cells. PC12 cells do not express Bcl-2 (Vaux et al, 1988; Nunez et al, 1990). PC12 cells treated with 100ng/ml TNFα for 12 or 24 hours presented unchanged expression levels of Bcl-x_L, Bax or Bak protein. However, the stimulation with 1 nM ActD downregulated Bcl-x_L, without any modification of Bax or Bak protein levels (Figure 57).

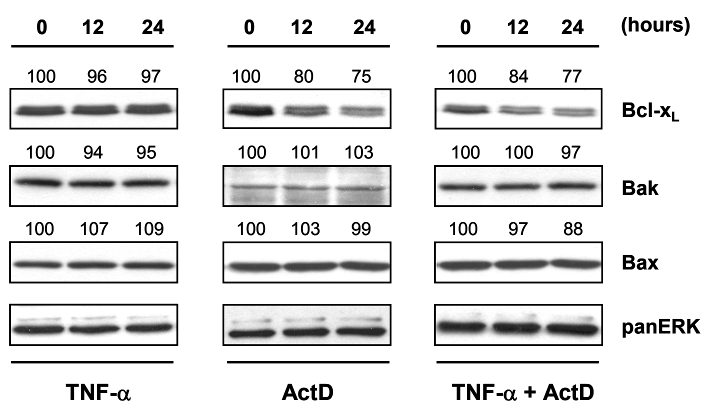


Figure 57. Total lysates were obtained after TNFα and/or ActD time course treatment and were analyzed by Western Blot with the indicated antibodies. The values in the upper part of the images represent the ratio of the mean of three experiments calculated respect to the total protein content.

The same result was obtained in primary mouse cortical neurons, where ActD selectively decreased Bcl-x_L expression, whereas Bax or Bak protein levels remained unchanged. Contrarily to PC12 cells, cortical neurons express Bcl-2. However, Bcl-2 was not modified by ActD treatment. This underlies the relevance of Bcl-x_L, and not of Bcl-2, in neuronal resistance to TNFα-induced cell death (Figure 58).

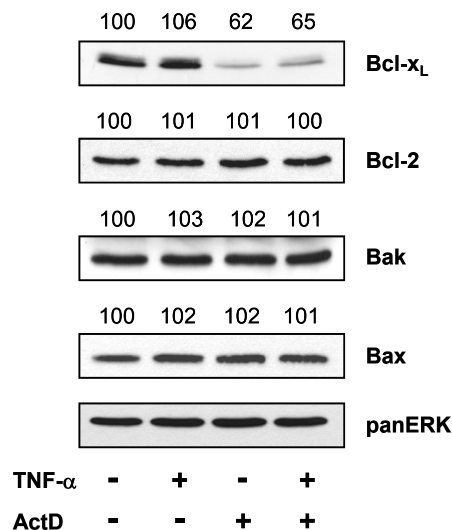


Figure 58. The expression of multidomain Bcl-2 family members (Bcl-x_L, Bcl-2, Bax and Bak) was analyzed by Western Blot in total lysates of neurons cultured for 6 DIV. The values indicated in the upper part of the images represent the ratio of the mean of three experiments calculated respect to the total protein content.

In a time course, it was observed that in PC12 cells Bcl-x_L levels diminished between 6 and 12 hours after ActD treatment reaching the maximum downregulation at 48 hours. At this time point Bcl-x_L expression decreased of about 40% when compared with untreated cells (Figure 59).

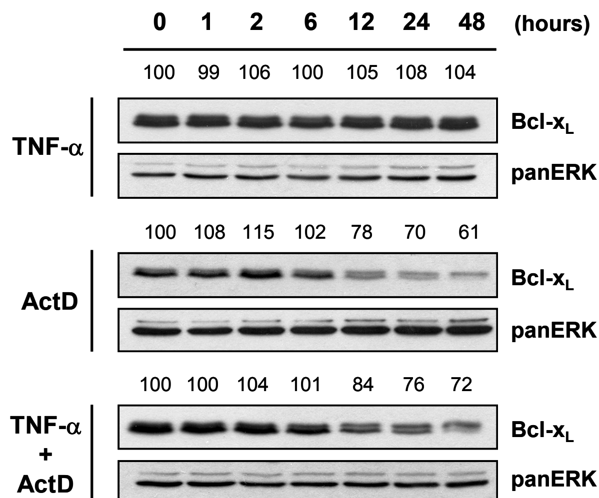


Figure 59. Cells were treated as indicated and a time course analysis of Bcl-x_L expression was performed. Equivalent loading controls between the lanes was determined by probing the membrane with an anti-panERK antibody. The values indicated in the upper part of the images represent the ratio of the mean of three experiments calculated respect to the total protein content.

Inhibition of transcription promoted by ActD treatment induces a decrease in Bcl-x_L protein levels which preceedes and is required for the sensitization to TNFα-induced cell death. The cytotoxic effect induced by TNFα and ActD co-treatement was

analyzed in a 48 hours time course and quantified by counting apoptotic nuclei stained with Hoechst 33258. It is interesting to point out that cell death occurs just after 24 hours stimulation, confirming that Bcl-x_L downregulation precedes TNFα/ActD-induced apoptosis (Figure 60).

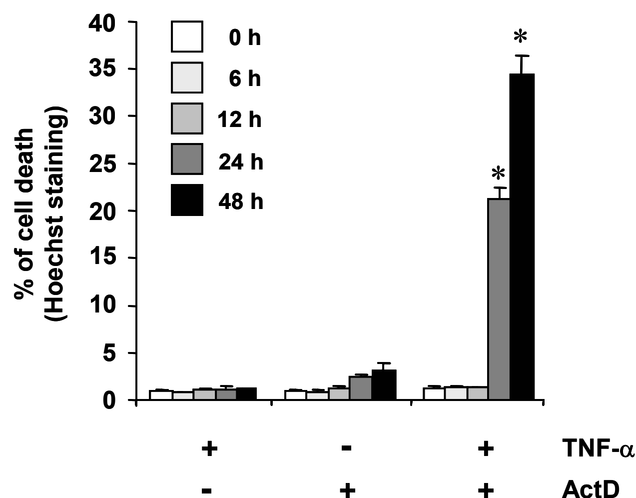


Figure 60. Cells were treated as indicated. Percentage of cell death was quantified by counting apoptotic nuclei stained with Hoechst 33258. * means $p < 0.01$.

7.3.2. *OVEREXPRESSION OF Bcl-x_L INHIBITS TNF-α/ActD-INDUCED CELL DEATH IN PC12 CELLS AND CORTICAL NEURONS*

To assess that the anti-apoptotic protein Bcl-x_L confers resistance to TNFα-induced cell death upon ActD sensitization, PC12 cells were infected with a lentivirus carrying the eukaryotic expression construct coding for the human *bcl-x_L* gene (PC12-Bcl-x_L). 72 hours later, cells were treated with ActD and Bcl-x_L expression level was analyzed. As shown in figure 61, PC12 cells infected with Bcl-x_L presented almost two-fold increase in the expression of Bcl-x_L in comparison with PC12 cells infected with an empty construct (Empty). ActD stimulation induced Bcl-x_L downregulation in both Empty- and Bcl-x_L-infected cells. However, even after 24 hours ActD treatment, PC12-Bcl-x_L cells maintained a high Bcl-x_L expression level when compared with Empty-PC12 cells.

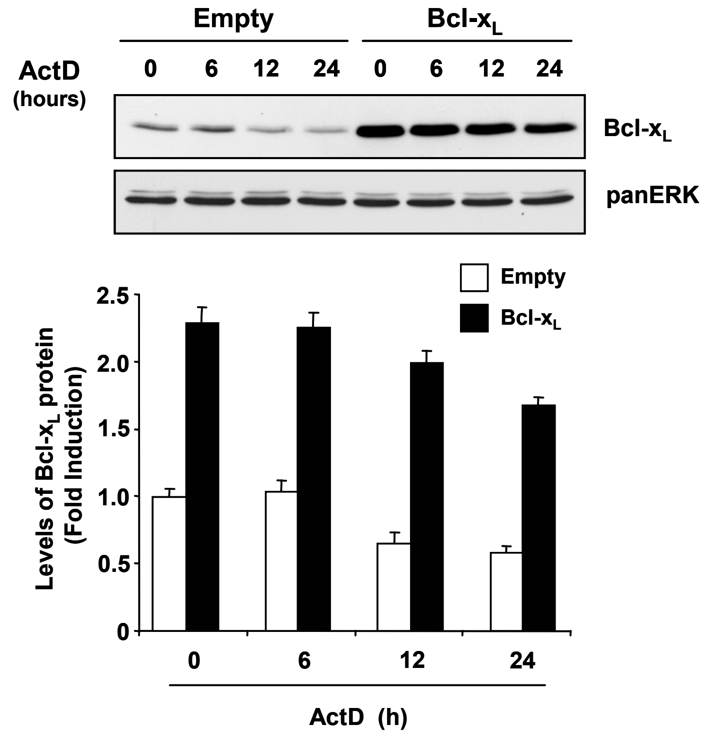


Figure 61. PC12 cells infected with the lentiviruses containing a Bcl-x_L or an empty construct were treated with ActD for the indicated times. Bcl-x_L expression was analyzed in total cell lysates by Western Blot using an anti-Bcl-x_L antibody. Total panERK was used as a loading control. The quantitative analysis of Bcl-x_L expression level in three independent experiments is shown in the figure.

Therefore, transient Bcl-x_L overexpression prevented PC12 cells from the apoptotic cell death induced by TNFα and ActD co-treatement. The same protection was afforded by caspases inhibitors e.g. z-IETD-fmk and z-VAD-fmk. In figure 62, cells were left untreated or treated for 24 hours with TNFα and/or ActD. The treatment was maintained also in presence of 50µM z-IETD-fmk or 50µM z-VAD-fmk.

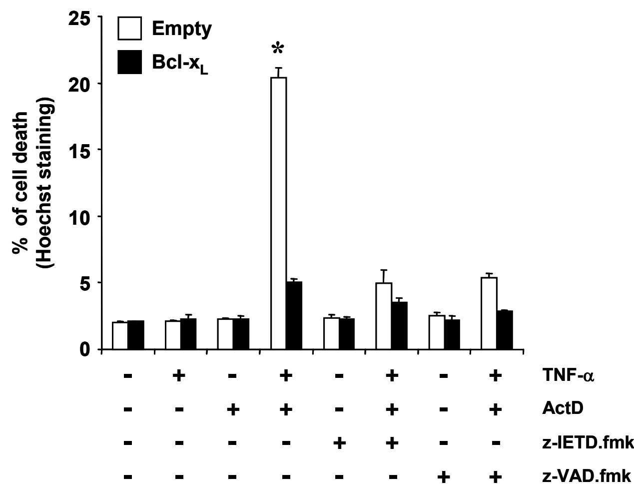


Figure 62. Cell death was quantified by counting apoptotic nuclei stained with Hoechst 33258. * means $p < 0.01$.

Bcl-x_L overexpression promoted resistance to TNFα/ActD-mediating cell death inhibiting caspase-3 and caspase-9 activation, as indicated in figure 63.

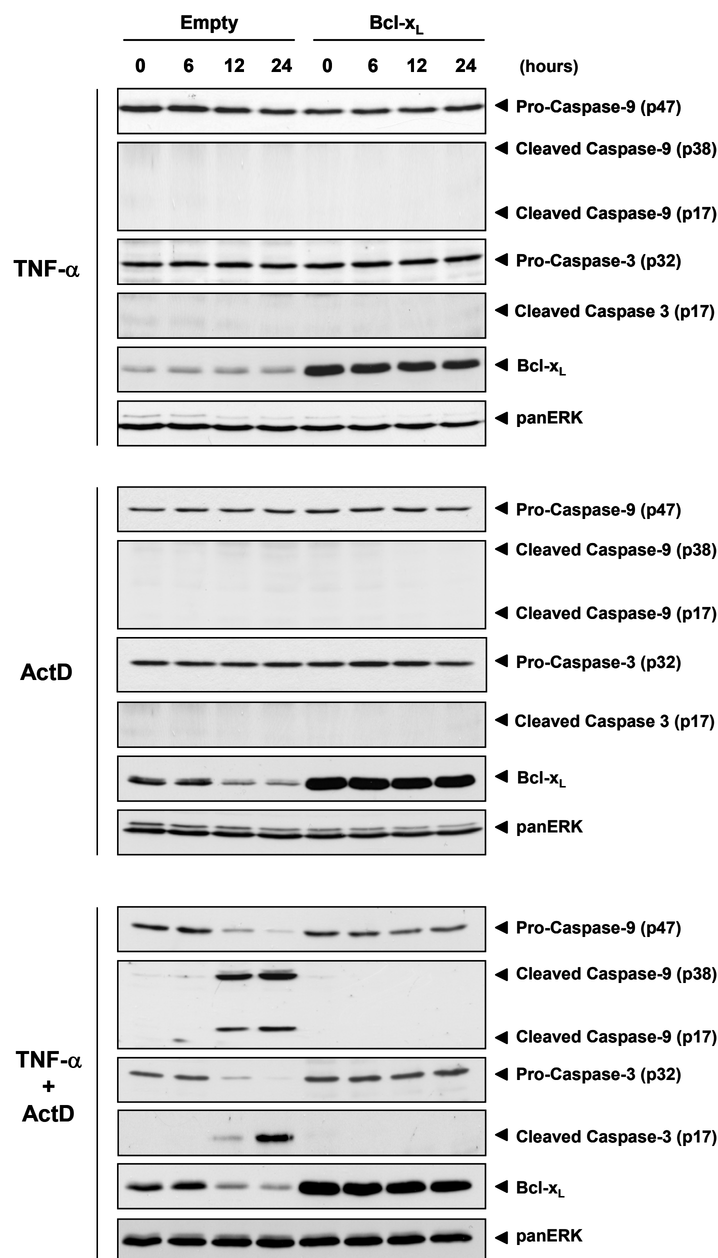


Figure 63. Pro-caspase-9 and pro-caspase-3 processing is analyzed by Western Blot. Equivalent loading controls were determined by probing the membrane with anti-panERK antibody.

The protection afforded by Bcl-x_L also occurred in primary cortical neurons which were infected with lentivirus carrying a construct that overexpresses human Bcl-x_L. The cell culture was maintained for 6 days *in vitro* and subsequently treated with TNFα and ActD. 24 hours later, the cell death induced by the treatment was assessed by counting apoptotic nuclei stained with Hoechst 33258. As shown in figure 64, Bcl-x_L

transient overexpression abrogated cell death induced by TNFα and ActD co-treatment.

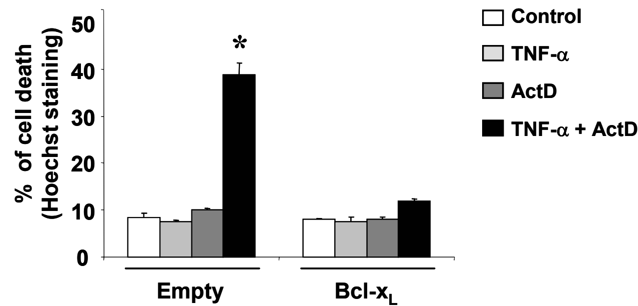


Figure 64. Cell death was assessed by counting apoptotic nuclei stained with Hoechst 33258. * means $p < 0.01$.

As occurred in PC12 cells, Bcl-x_L transient overexpression prevented caspases activation induced by TNFα and ActD co-treatment in primary cortical neurons. The formation of caspases active fragments was detected just in those cortical neurons infected with the Empty vector and co-stimulated with TNFα and ActD (Figure 65).

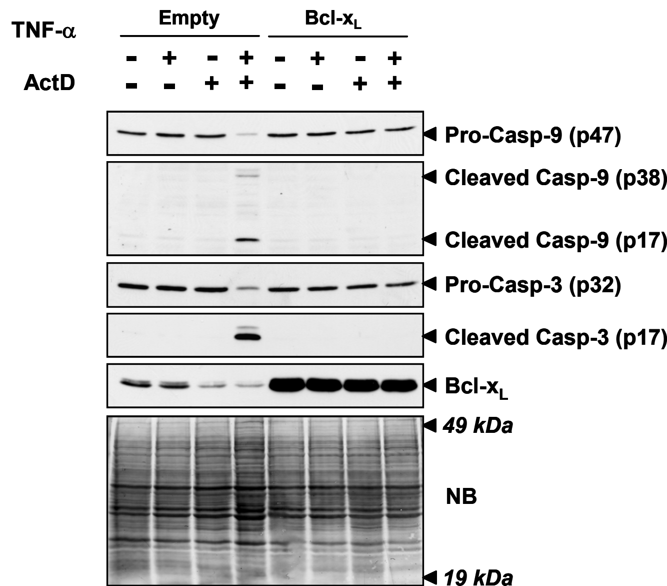


Figure 65. Caspase-9 and -3 activation was analyzed by Western Blot. Bcl-x_L expression was demonstrated by reprobing the membrane with the specific antibody. Loading control was performed by staining the membrane with naphtol blue (NB).

The protection conferred by Bcl-x_L was also analyzed by measuring both caspase-8 like (IETDase) and caspase-3 like (DEVDase) activities, as assessed in the following graphs (Figure 66).

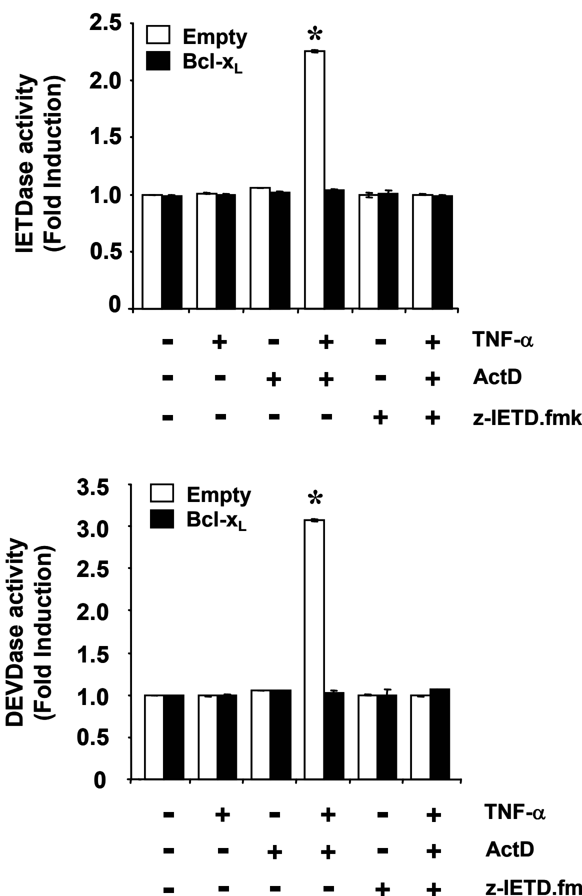


Figure 66. Caspase-8- and -3-like activity was measured after 8 hours of the indicated treatments using the fluorogenic Ac-IETD-afc and Ac-DEVD-afc reagents, respectively. * means $p < 0.01$.

Taken together, these results clearly show that increased Bcl-x_L levels protect neurons from apoptosis induced by TNFα and ActD co-treatment.

7.3.3. *DECREASE OF ENDOGENOUS Bcl-x_L LEVELS RENDERS PC12 CELLS AND CORTICAL NEURONS SENSITIVE TO TNF-α-DRIVEN APOPTOSIS*

To confirm that Bcl-x_L is the protein which mediates resistance against TNFα-induced cell death, the endogenous decrease of Bcl-x_L was performed. As shown in figure 67, PC12 cells infected with lentiviruses carrying a short hairpin sequence against rat/mouse Bcl-x_L (shBcl-x_L) exhibit a strong reduction in Bcl-x_L protein levels three days post-infection when compared with the lentiviruses carrying a scrambled sequence (shScr).

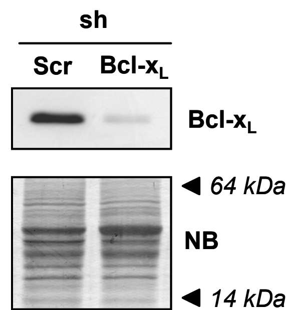


Figure 67. Bcl-x_L was analyzed in total lysates by Western Blot using anti-Bcl-x_L antibody. The membrane was stained with Naphtol Blue (NB) to assess equivalent loading.

PC12 cells were infected with shScr or shBcl-x_L and after three days they were treated with TNFα, ActD, or both for 24 hours. In agreement with previous results, shScr-PC12 cells only died upon 24 hours TNFα/ActD co-stimulation. On the contrary, shBcl-x_L-PC12 cells were sensitive to the TNFα alone. The co-treatment with ActD did not induce an increase in the percentage of cell death promoted by TNFα treatment (Figure 68).

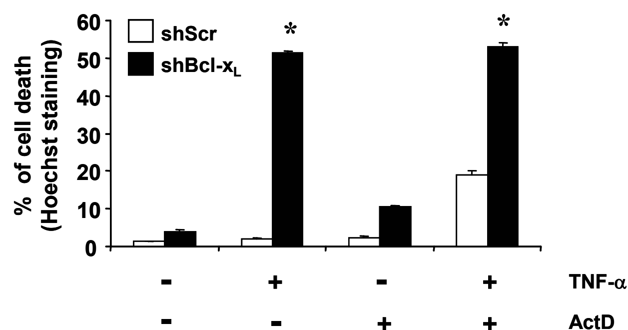


Figure 68. Cell death was assessed by counting apoptotic nuclei stained with Hoechst 33258. * means p< 0.01.

Endogenous decrease of Bcl-x_L protein level sensitized PC12 cells to TNFα-mediated apoptosis by promoting pro-caspase-9 and pro-caspase-3 processing and activation. As shown in figure 69 and contrarily to shBcl-x_L PC12 cells, TNFα treatment did not activate pro-caspase-9 and -3 in shScr-infected PC12.

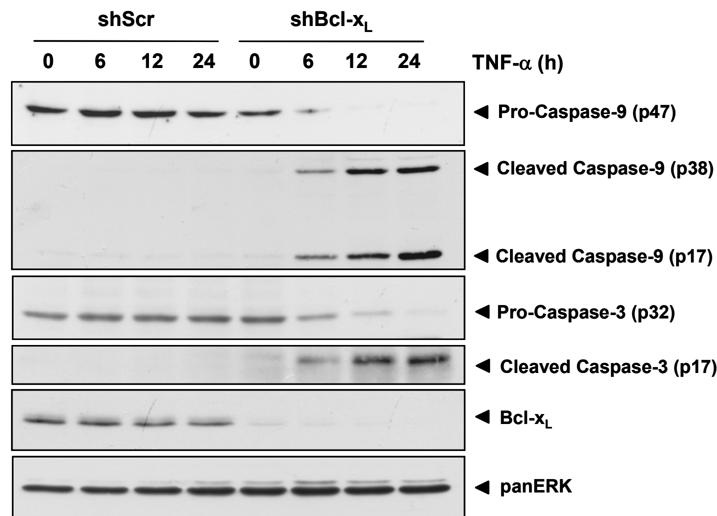


Figure 69. The processing of pro-caspase-9 and pro-caspase-3 is analyzed by Western Blot as indicated. Equivalent loading controls were determined by probing the membrane with anti panERK antibody.

Caspase activation was also assessed by measuring DEVDase activity upon TNFα treatment in shBcl-x_L-PC12 cells, which was completly inhibited by the use of caspase-8 inhibitor z-IETD-fmk or pan-caspases-inhibitor z-VAD-fmk (Figure 70).

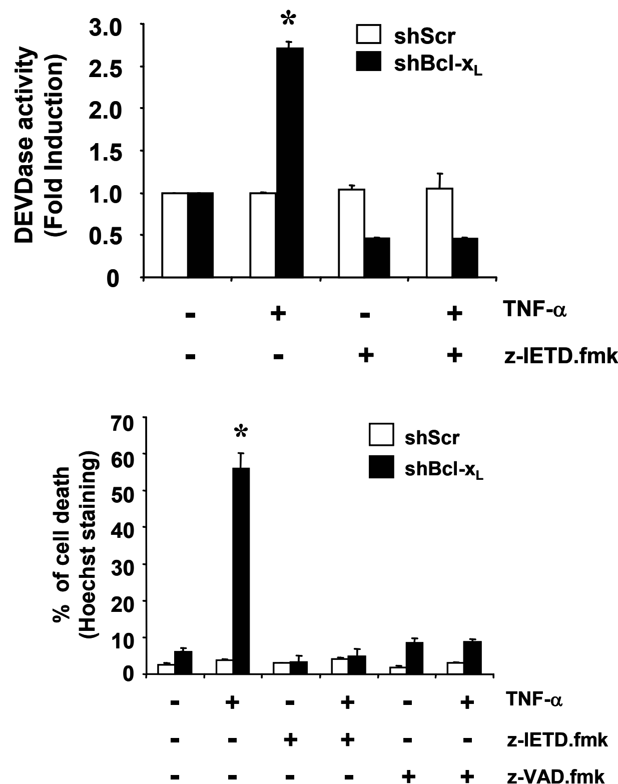


Figure 70 Caspase-3-like activity was measured after 8 hours of the indicated treatments using the fluorogenic Ac-DEVD-afc reagent, respectively. Percentage of cell death was assessed by counting the apoptotic nuclei after Hoechst 33258 staining. * means $p < 0.01$.

In order to assess the specificity of Bcl-x_L endogenous decrease and to discard the possibility that non-controlled off-targets could be the real responsible for Bcl-x_L-mediated sensitization to TNFα-induced cell death, complementation experiments were performed. In agreement with the high specifity of this technology, shBcl-x_L was designed against rat/mouse bcl-x which differs of 3 nucleotide from the sequence of the human orthologue (GeneBank™ entry NM_138578) (Figure 71).

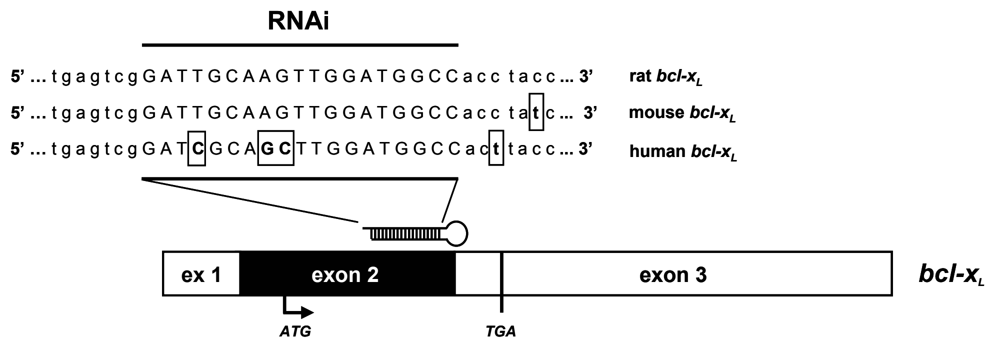


Figure 71. Coding sequence of rat, mouse and human bcl-x_L. RNAi was designed against the mouse/rat sequence pictured as underlined and in capital letters. Differences in sequence are indicated by bold and boxed letters.

Therefore, to affirm Bcl-x_L RNAi specificity, western blots and semiquantitative RT-PCR were carried out. By western blot, it was assessed that the lentivirus efficiently downregulated Bcl-x_L levels in PC12-transfected with an empty (Neo) vector but not in PC12-hBcl-x_L (Figure 72).

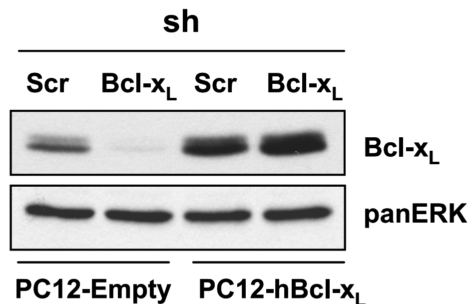


Figure 72. Protein lysates derived from untreated PC12-Neo or PC12-shBcl-x_L cells that were infected for 3 days with shScr or shBcl-x_L viruses. Total Bcl-x_L levels were analyzed by Western Blot confirming Bcl-x_L knockdown in PC12-Neo cells.

Moreover, as indicated in figure 73 the semiquantitative RT-PCR shows that shBcl-x_L decreased the levels of rat bcl-x mRNA, but not those of human bcl-x mRNA.

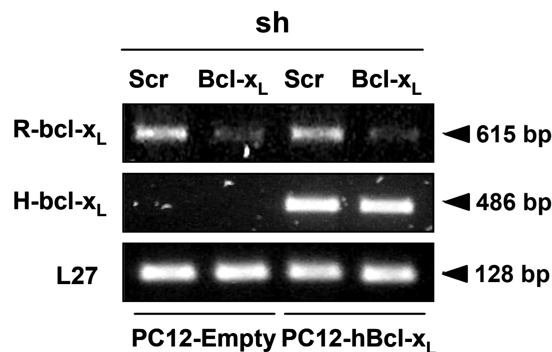


Figure 73. Total mRNAs were extracted from untreated PC12-Neo or PC12-shBcl-xL cells that were infected for 3 days with shScr or shBcl-xL viruses. Specific RT-PCR for rat bcl-x_L (R-bcl-x_L, 615 bp amplification band), human bcl-x_L (H-bcl-x_L, 486 bp amplification band) or the housekeeping L27 mRNA (as a control of mRNA loading and amplification, 128 bp band) was performed. shBcl-x_L efficiently and specifically reduces rat bcl-x_L but not human overexpressed bcl-x_L.

Therefore, as shown in figure 74, PC12 cells that stably express a human Bcl-x_L construct (PC12-hBcl-x_L) were infected with shBcl-x_L. In these cells endogenous decrease of rat/mouse Bcl-x_L did not sensitize to TNFα-induced apoptosis.

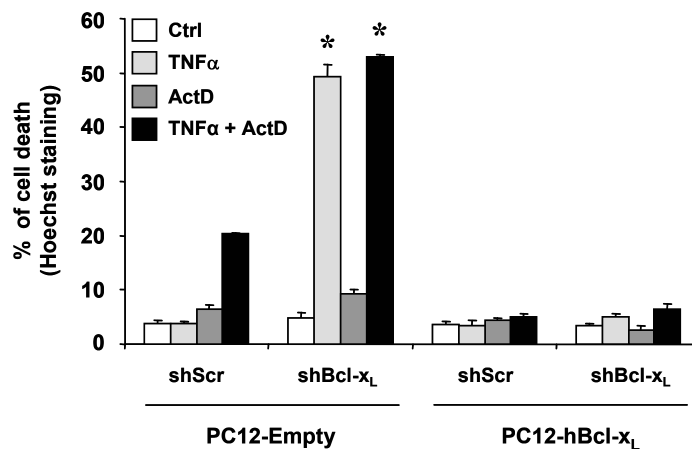


Figure 74. Cell death was measured by counting apoptotic nuclei after Hoechst 33258 staining. * means $p < 0.01$.

To confirm the results, primary cortical neurons were infected with shBcl-x_L lentiviral construct. In figure 75, it is shown that shBcl-x_L-cortical neurons died upon TNFα alone, presenting a five fold increase in the number of apoptotic cells compared with shScr infected neurons. Moreover, TNFα-mediated cell death in shBcl-x_L-cortical neurons was suppressed by caspase-8 inhibition achieved by the use z-IETD-fmk.

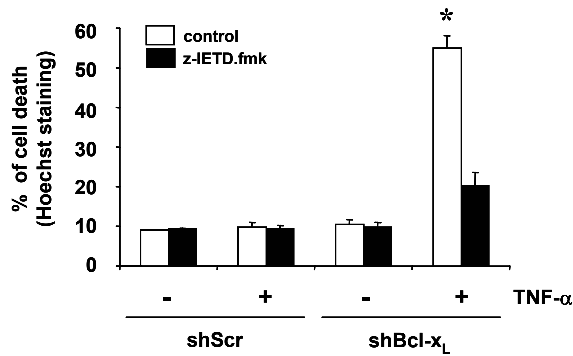


Figure 75. Percentage of cell death was measured by counting apoptotic nuclei after Hoechst 33258 staining. * means p< 0.01.

Caspase-9 and caspase-3 involvement were assessed using specific antibodies (Figure 76).

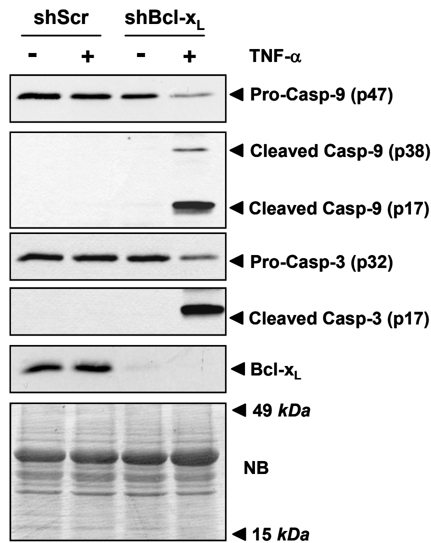


Figure 76. The processing of pro-caspase-9 and pro-caspase-3 is analyzed by Western Blot. Loading control was determined by probing the membrane with anti panERK antibody.

Caspase-3 activation was also assessed by measuring the DEVase activity and by neurons caspase-3 immunofluorescent staining (Figure 78; Figure 79).

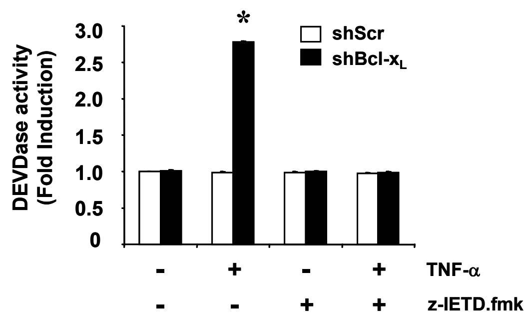


Figure 78. Caspase-3-like activity was measured after 8 hours of the indicated treatments using the fluorogenic Ac-DEVD-afc reagent. * means p< 0.01.

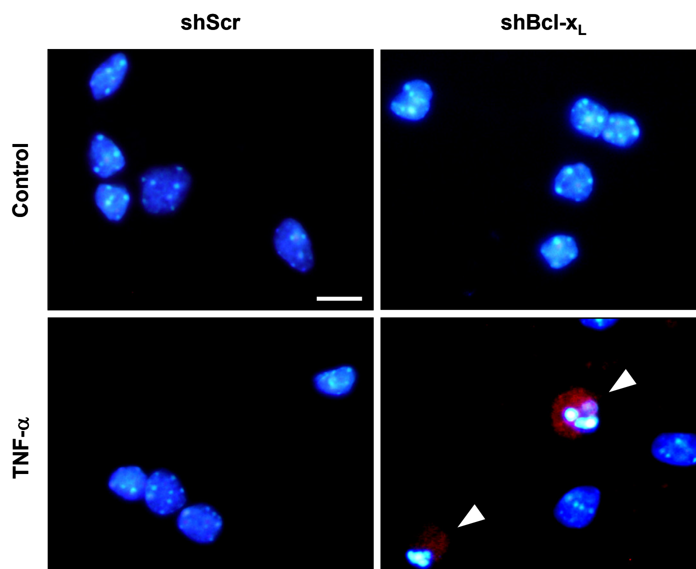


Figure 79. Representative pictures of the indicated 24 hours treatment show active caspase-3 immunofluorescence (red and arrows) merged with Hoechst 33258 nuclear staining.

Taken together, the results identify Bcl-x$_L$ as the anti-apoptotic molecule whose decrease expression level promotes TNFα cytotoxic effect in both PC12 cells and mouse primary cortical neurons.

7.3.4. *Bcl-x$_L$ IS THE Bcl-2 ANTI-APOPTOTIC PROTEIN TO MAINLY SENSITIZE TO TNF-α CYTOTOXIC EFFECT*

To extend the results obtained in PC12 cells and primary cortical neurons, we decided to perform additional experiments in HeLa cell line, in which, contrarily to PC12 cells, other Bcl-2 family members are expressed.

Therefore, to assess whether inhibition of transcription promoted by ActD would affect the expression of anti-apoptotic proteins, e.g. Bcl-2, Bcl-x$_L$, Mcl-1, Bcl-w and render cells susceptible to TNFα cytotoxic effect, HeLa cells were treated with 20nM of ActD during a 24 hours time course. Protein levels of the Bcl-2, Bcl-x$_L$, Mcl-1 and Bcl-w were detected by Western Blots using specific antibodies. Figure 80 shows how ActD specifically decreased Bcl-x$_L$ expression without altering any other proteins.

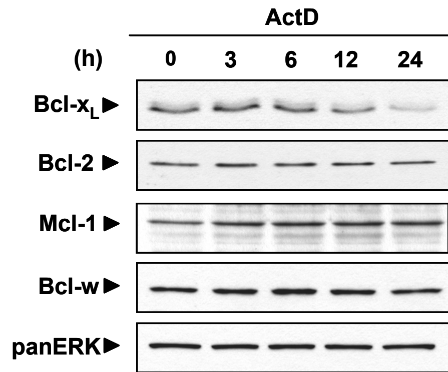


Figure 80. Cell were treated with 20nM ActD for the indicated time course. Western Blot analysis was perfomed using specific antibodies. Loading control was determined by probing the membrane with anti-panERK antibody.

To assess TNFα-mediated cytotoxicity upon ActD sensitization, percentage of cell death obtained by counting blue died cells with Trypan Blue Exclusion Assay and stained nuclei with Hoechst 33258 was measured. The results revealed that TNFα/ActD co-treatment induced cytotoxicity after 24 hours treatment (Figure 81).

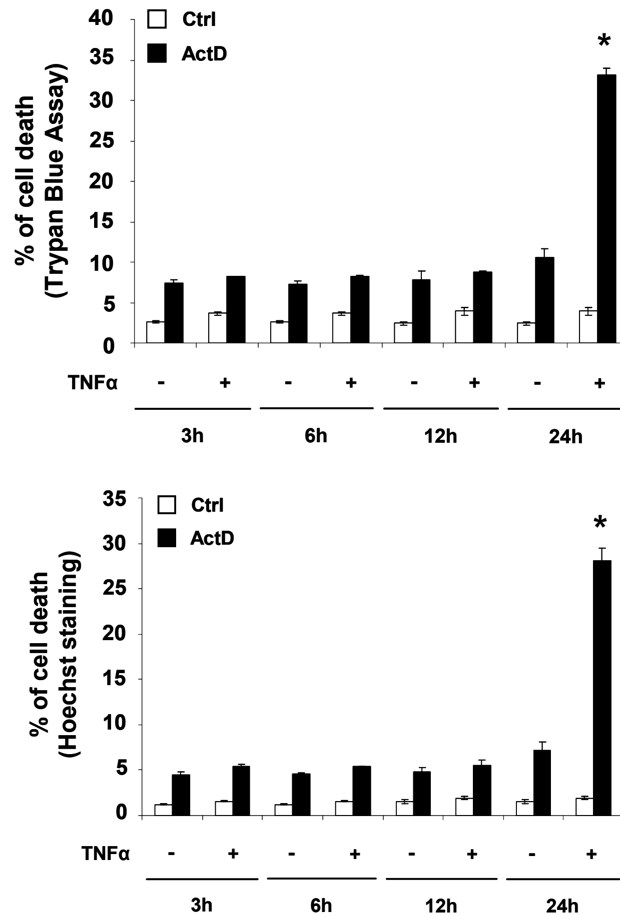


Figure 81. Cells were treated as indicated and cell death was assessed by Trypan Blue Exclusion Assay and Hoechst 33258 counting. * means $p < 0.01$.

To further confirm that Bcl-x_L affords protection against TNFα-induced cell death, knockdown experiments were performed. By short interfering RNA sequences (siRNA), Bcl-x_L endogenous expression was forcedly decreased. HeLa cells were transfected with Bcl-x_L-siRNA and Bcl-x_L levels were analyzed three days after. As expected, cells exhibited a strong reduction in Bcl-x_L when compared with cells transfected with a scrambled sequence (Scr). The specificity of the technique is demonstrated by the unaltered expression of other Bcl-2 family members expressed in HeLa cell line and analyzed in the following Western Blot (Figure 82).

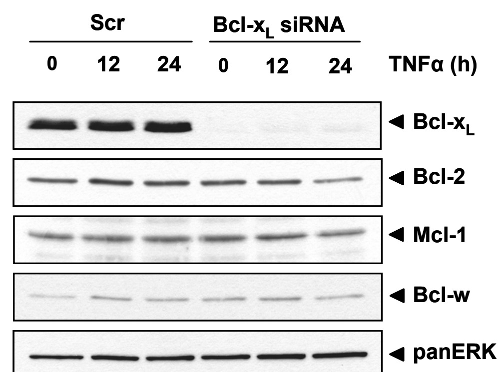
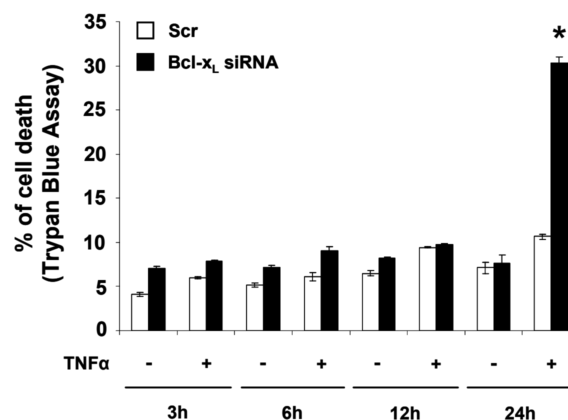


Figure 82. Cell were treated with 30ng/ml TNFα for the indicated time course. Western Blot analysys was perfomed using specific antibody to detect the protein indicated. Loading control was determined by probing the membrane with anti panERK antibody.

Three days post-transfection, Bcl-x_L-siRNA cells were treated with 30ng/ml TNFα for a 24 hour time course. In agreement with the results obtained in PC12 cells and primary cortical neurons, Bcl-x_L-siRNA HeLa cells showed sensibility to TNFα-mediated cell death, as demonstrating in figure 83 in which the cytotoxic effect induced by the cytokine was assessed by counting cells with Trypan Blue Exclusion Assay and Hoechst 33258 staining.



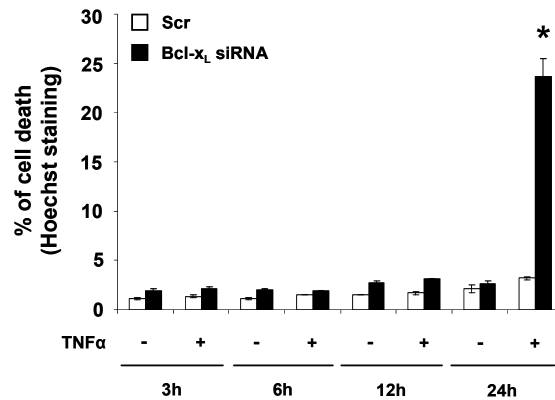


Figure 83. Cells were treated as indicated and percentage of cell death was assessed by Trypan Blue exclusion and Hoechst 33258 counting. * means p< 0.01.

To assess whether the main anti-apoptotic Bcl-2 family members would confer protection against TNFα cytotoxic effect, further knockdown experiments were performed. Cells were transfected with siBcl-x_L-, siBcl-2-, siMcl-1- and siBcl-w-RNA respectively. Three days post-transfection proteins expression levels were analyzed by Western Blot using specific antibodies (Figure 84).

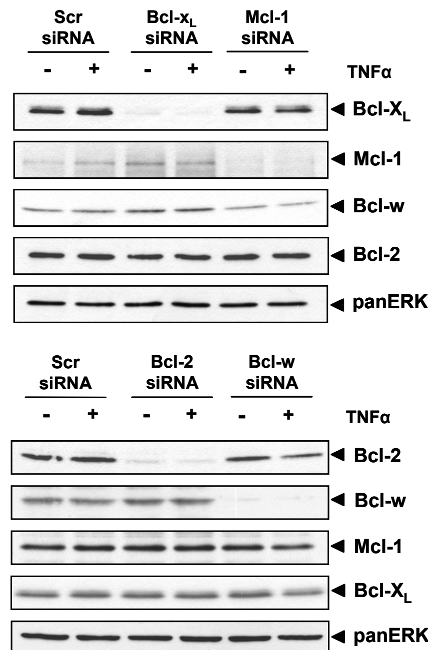


Figure 84. Cells were transfected and treated as indicated. Western Blot analysys was perfomed using specific antibodies. Loading control was determined by probing the membrane with anti panERK antibody.

Nijhawan and collegues demonstrated the relevance of Mcl-1 in preventing the activation of the intrinsic apoptotic pathway induced by the DNA-damaging reagents e.g. ultraviolet (UV) light and genotoxic chemotherapeutical agents as. etoposide

(Nijhawan D. et al, 2003). These stimuli induce a reduction in Mcl-1 expression level which is required to promote cell death. Therefore, to have a positive control of siRNA functioning, we reproduced in HeLa cell line the experiment performed by Nijhawan. Three days post-transfection, HeLa cells were treated with 30μM Etoposide and 24 hours later the percentage of cell death was assessed. Figure 85 shows that Mcl-1 and Bcl-x_L endogenous decrease rendered HeLa cells sensitive to etoposide-mediated apoptosis.

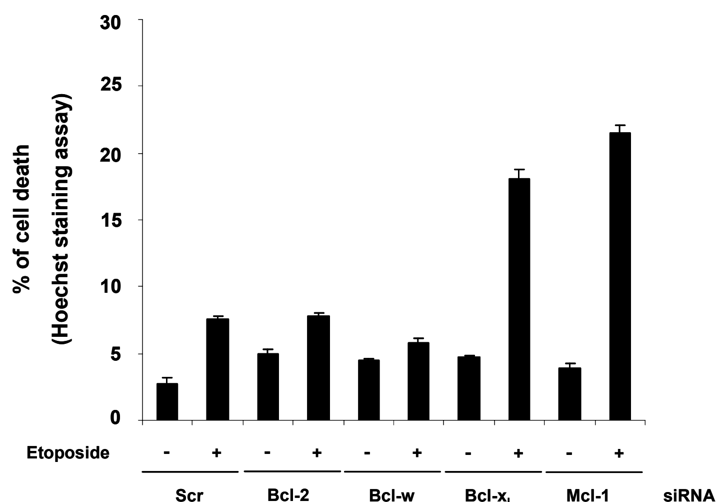


Figure 85. Cells were treated with 30μM etoposide for 24hours. Percentage of cell death was assessed by counting stained nuclei with Hoechst.

Therefore with the aim to assess whether endogenous downregulation of the main Bcl-2 anti-apoptotic proteins would sensitize to TNFα cytotoxic effect, cells were transfected with siBcl-x_L-, siBcl-2-, siMcl-1- and siBcl-w-RNA respectively. Three days post-transfection, the cell death induced by 24 hours of 30ng/ml TNFα was measured and compared by counting blue died cells with Trypan Blue Exclusion Assay and stained nuclei with Hoechst 33258. ActD and TNFα co-treatment was used a positive control of apoptosis.

As shown in figure 86, the downregulation of the tested Bcl-2 anti-apoptotic family members sensitized cells to TNFα-mediated apoptotis, although the effect could not be compared with Bcl-x_L endogenous decrease/TNFα stimulation. Although the presence in HeLa cell line of Bcl-2, Bcl-w and Mcl-1, no one of these proteins cope with TNFα cytotoxic effect induced when Bcl-x_L endogenous level is decreased. This suggests that the modulation of Bcl-x_L expression is the key event in mediating TNFα apoptosis.

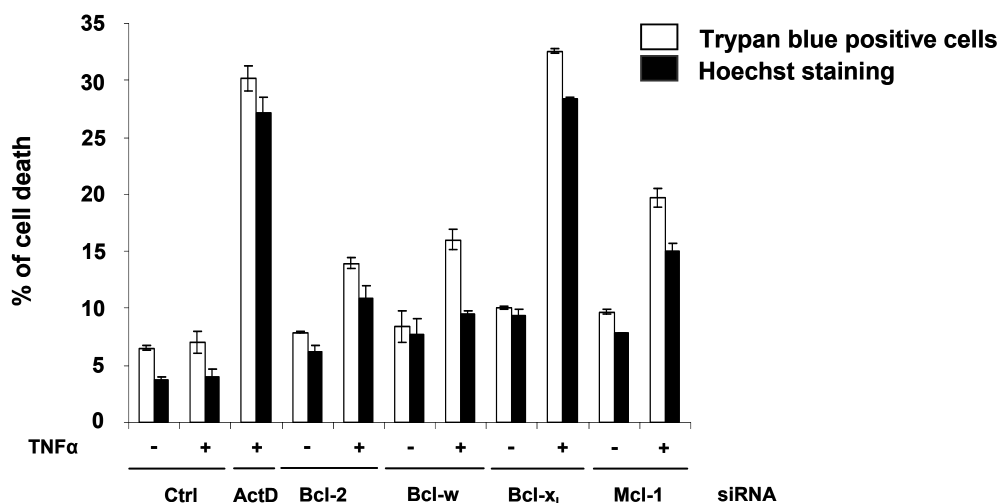


Figure 86. Cell were tranfected and three days later treated with 30ng/ml TNFα for 24 hours. Cell death was assessed by Trypan Blue Exclusion Assay and Hoechst 33258 staining.

Therefore, endogenous decrease of Bcl-x_L shifts the balance from cell survival to cell death upon TNFα stimulus, demonstrating the relevance of this protein in deciding cell faith. Apoptosis induced by siBcl-x_L-RNA/TNFα is comparable with the percentage of cell death obtained after TNFα and ActD co-treatment in non-transfected HeLa cells.

7.3.5. *Bcl-x_L DECREASE DOES NOT IMPARE TNF-α-MEDIATED NF-κB ACTIVATION AND PROMOTES JNK-DEPENDENT CELL DEATH*

When TNFα binds TNFR-1, it can activate both survival and cell death. Cell survival is triggered by the transcription of anti-apoptotic molecules mainly promoted by NF-κB signaling transduction pathway. Therefore, a failure in NF-κB activation leads to TNFα-mediated cytotoxicity.

As Bcl-x_L endogenous decrease sensitized to TNFα-promoted apoptosis, we wanted to assess whether this effect was due to a disruption of NF-κB pathway.

Figure 87 shows that Bcl-x_L knockdown did not affect TNFα-mediated nuclear RelA/p65 translocation analyzed in PC12 cells. PC12 cells were transduced with shScr and shBcl-x_L lentiviruses and three days post-infection were left untreated or treated with 100ng/ml TNFα for 30 minutes. These data suggest that the downregulation of Bcl-x_L sensitized cells to TNFα-mediated apoptosis without imparing NF-κB activity.

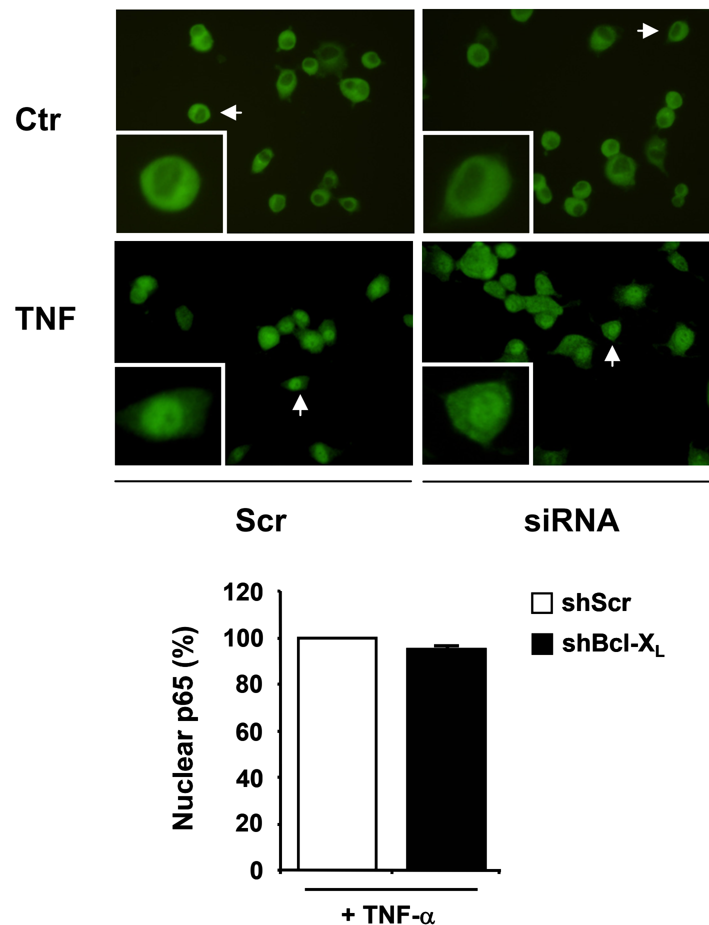


Figure 87. Cells were treated or not with 100ng/ml TNFα for 6 hours. Immunocytochemistry was performed to detect p65 subunit of NF-κB dimer. Representative low and high magnification photomicrographs and the respective quantification are shown in the figure.

Moreover, Luciferase reporter assay demonstrated that TNFα-induced NF-κB activity remained unaltered in cells infected with shBcl-x$_L$ when compared with cells infected with the scrambled sequence. It is known that cIAP1 is one of the targets whose transcription is promoted by NF-κB in response to TNFα stimulation. Therefore, cIAP1 increased expression revealed that Bcl-x$_L$ endogenous decrease did not impair NF-κB transcriptional activity (Figure 88 and 89).

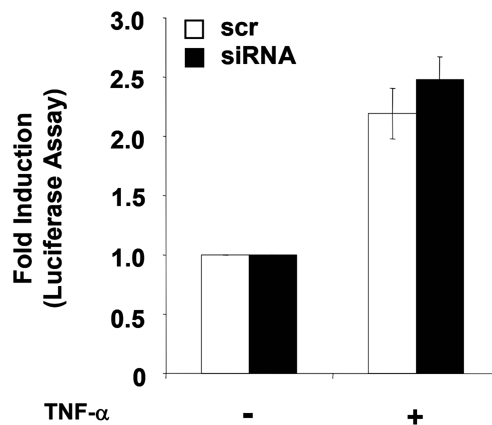


Figure 88. PC12 cells were transduced with shScr and shBcl-x_L lentiviruses and after three days they were transiently transfected with an NF-κB dependent reporter vector. After 24 hours, cells were treated with 100ng/ml TNFα for 6 hours. Luciferase Activity was measured by using the Luciferase Assay System.

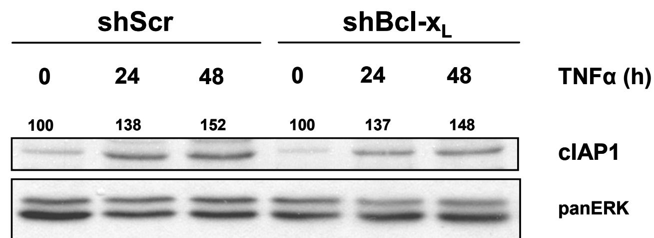


Figure 89. PC12 cells were transduced with shScr and shBcl-x_L lentiviruses and three days post-infection they were stimulated with TNFα for the indicated time course. Western Blot was performed in order to detect cIAP1 protein expression level using a specific antibody. The values indicated in the upper part of the images represent the ratio of the mean of three experiments calculated respect to the total protein content.

A mutant and undegradable form of I-κBα, in which serine 32 and 36 are alanine-substituted (SR-IκBα) promotes the inhibition of NF-κB signaling transduction pathway. Therefore, PC12 cells transfected with SR-IκBα (SR-IκBα-PC12) are sensitive to TNFα cytotoxic effect, dying by apoptosis upon 24 hours TNFα treatment (Figure 90 and 91).

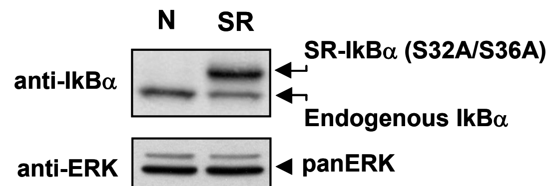


Figure 90. PC12 cells were stably transfected with the empty vector (N) or with SR-IκBα (SR). Total cell lysates were analyzed by Western Blot using an antibody against IκBα. Membrane was reprobed with anti-panERK antibody to normalize the loading content per lane.

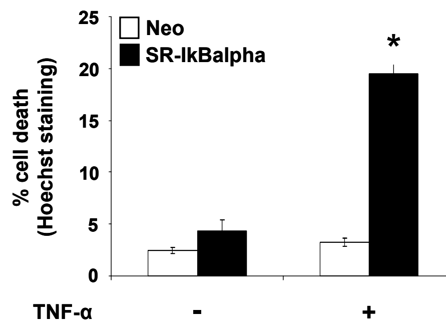


Figure 91. PC12 cells stably transfected with the Empty vector (Neo) and with the mutated form of IκBα (SR-IκBα) were treated for 24 hours with 100ng/ml TNFα. Percentage of cell death was assessed by counting stained nuclei with Hoechst 33258. * means p< 0.01.

Although SR-IκBα-PC12 cells underwent apoptosis upon TNFα stimulation, figure 92 shows that this effect was not due to a decrease in Bcl-x_L expression as the mutated form of IκBα did not alter the levels of this protein either in untreated cells or upon TNFα treatment.

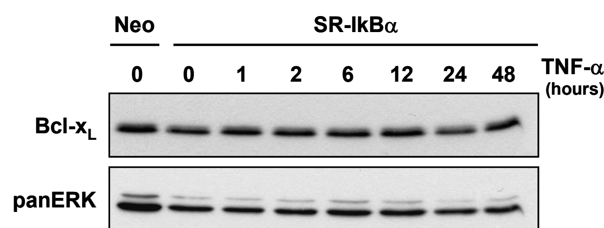


Figure 92. PC12 cells were stably transfected with the empty vector (Neo) or with SR-IκBα (SR-IκBα). Total cell lysates were analyzed by Western Blot using an antibody against Bcl-x_L. Membrane was reprobed with anti-panERK antibody to normalize the loading content per lane.

NF-κB protects cells from apoptosis also by inhibiting the activation of JNK signaling transduction pathway. In SR-IκBα-PC12 cells, TNFα induced a robust JNK activation already after 1 hour TNFα treatment that was maintained during the time course. This leaded to JNK modulation of pro-apoptotic gene expression, e.g. Bim and Hrk (Figure 93).

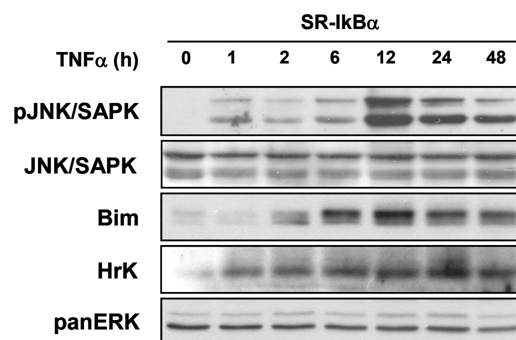


Figure 93. Cells were treated with TNFα for the indicated time course. Western Blots are performed using specific antibodies. Membranes were reprobed with anti-panERK antibody to normalize the loading content per lane.

The prolonged activation of JNK is associated with TNFα-mediated caspase activation (Tezel G et al, 2005; Tang et al, 2002). Figure 94 shows that TNFα-induced cell death in SR-IκBα-PC12 cells was completely abrogated by the chemical JNK inhibitor, revealing the importance of this pathway in promoting apoptosis when NF-κB is inhibited (Figure 94).

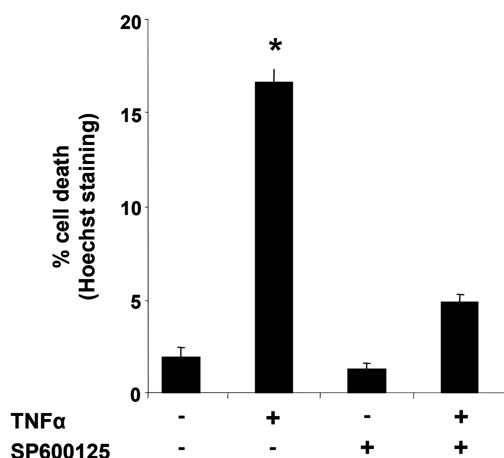


Figure 94. Cells were treated for 24 hours as indicated. Percentage of cell death was assessed by counting nuclei stained with Hoechst 33258. * means p< 0.01.

Although Bcl-x_L endogenous decrease did not impare NF-κB activity, its downregulation was sufficient to induce TNFα-dependent JNK-mediated apoptosis. As shown in figure 95, the cell death promoted by the cytokine was abrogated by JNK chemical inhibitor, i.e. SP600125.

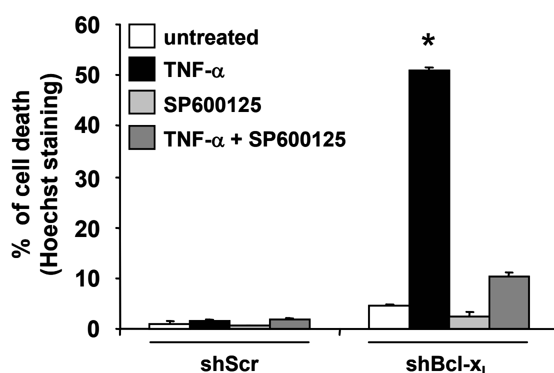


Figure 95. Cells were treated for 24 hours and percentage of cell death was assessed by counting stained nuclei with Hoechst 33258. * means p< 0.01.

It was demonstrated that JNK-mediated apoptosis upon TNFα stimulation occurs through JNK-induced Bid cleavage, which, once translocated from the cytosol to the mitochondria, promoted the selective release of SMAC/DIABLO. SMAC function is to disrupt the complex between cIAP1 and TRAF2, leading to the release of caspase-8

inhibition and its consequent activation. Infecting shBcl-x_L transducing cells with lentiviruses carrying a short hairpin sequence against rat/mouse SMAC/DIABLO (shSMAC) we observed that the reduction in SMAC/DIABLO expression level prevented completely the cell death induced by TNFα. This suggests the relevance of SMAC/DIABLO to promote JNK-dependent caspase-mediated apoptosis (Figure 96).

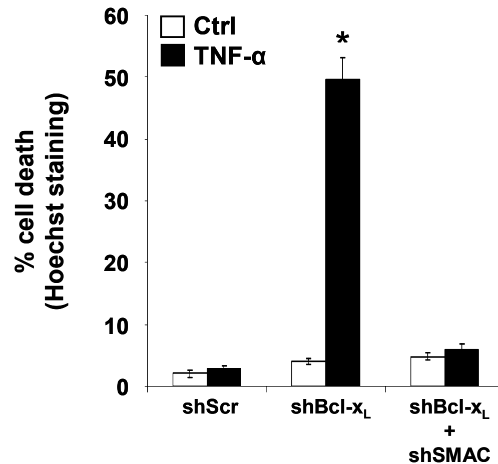


Figure 96. Cells were treated for 24 hours as indicated. Percentage of cell death was assessed by counting stained nuclei with Hoechst 33258. * means $p< 0.01$.

In conclusion, the data presented demonstrated that Bcl-x_L endogenous decrease shifts the balance from survival to cell death, besides the activation of NF-κB signaling transduction pathway. However, under these conditions it seems that NF-κB can not avoid TNFα-induced JNK activation as well as the resulting caspase-dependent apoptosis.

7.4. OBJECTIVE Nº 4

As it heme is a potent inducer of the death stimuli, we wanted to assess whether it would sensitize to TNFα-mediated apoptosis (performed in Instituto Gulbenkian de Ciência de Oeiras, Portugal).

7.4.1. *FREE HEME ACCUMULATION SENSITIZES TO TNFα-INDUCED APOPTOSIS*

Heme is an essential requirement for several protein functionalities. It acts as prosthetic group of different important molecules, known as hemoproteins, e.g. hemoglobin, cytochrome P450, catalases, peroxidases, tryptophan pyrrolases, etc. Heme is involved in many cellular mechanisms e.g. gene transcription, cell differentiation, proliferation, etc. (Ponka et al, 1999; Wagener F. A. et al, 2003).

However, it was demonstrated that heme is a potent inducer of the death stimuli. Balla G. and collaborators showed that endothelial cells treated with hemin, a heme analogue, are more susceptible to oxidative stress, i.e. H_20_2 (Balla G. et al, 1991; Balla G. et al 1992). In agreement with this data, we wanted to assess whether hemin, an analog heme substrate, would sensitize cells also to TNFα-induced apoptosis. Figure 96 shows that neither hemin nor TNFα stimulation affects the viability of Hepa cell line. However, hemin exposure sensitizes cells to TNFα cytotoxic effect. The percentage of cell death correlates with the increased hemin concentrations.

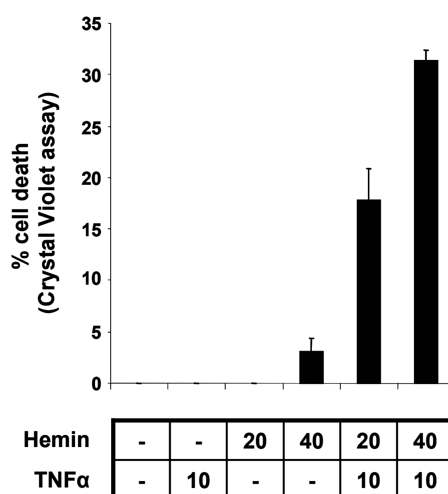


Figure 96. Hepa cell line were incubated 1 hour with 20 and 40 μM Hemin and then treated for 6 hours with 10ng/ml TNFα. Percentage of cell death was assessed by Crystal Violet assay and referred to the control.

Representative images of Hoechst 33252 nuclei staining reveal that, after 6 hours treatment, hemin sensitization to TNFα cytotoxic effect promotes the chromatine condensation and the nuclear shrinkage typical of the apoptotic process (Figure 97).

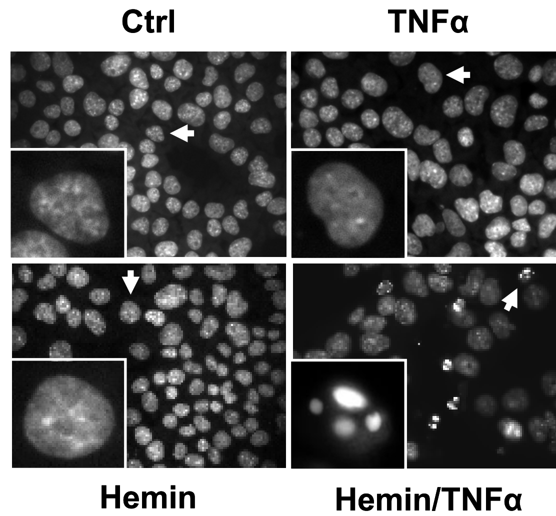


Figure 97. Fluorescent images showing stained nuclei with Hoechst 33252 of Hepa cells treated as indicated. TNFα is used at a concentration of 50ng/ml whereas Hemin at 40μM.

Apoptosis induced by Hemin and TNFα treatment was confirmed analyzing pro-caspase-3 processing into its p17 large active form. Figure 98 shows that caspase-3 is cleaved only when cells are treated with TNFα upon hemin sensitization.

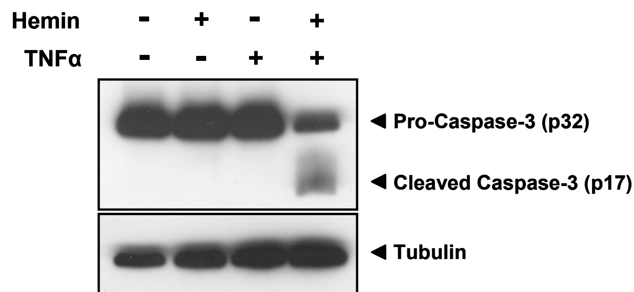


Figure 98. Caspase-3 activation was determined by Western Blot using specific antibody against its respective active fragment. Loading control was determined by reprobing the membrane with anti-tubulin antibody.

TNFα dose response reveals that hemin is a potent inducer of the death stimuli. Hemin sensitizes to TNFα cell death also when the cytokine is used at very low concentration, as shows figure 99. Moreover, the apoptosis promoted by hemin/TNFα is inhibited by 50μM of the broad caspases inhibitor z-VAD-fmk.

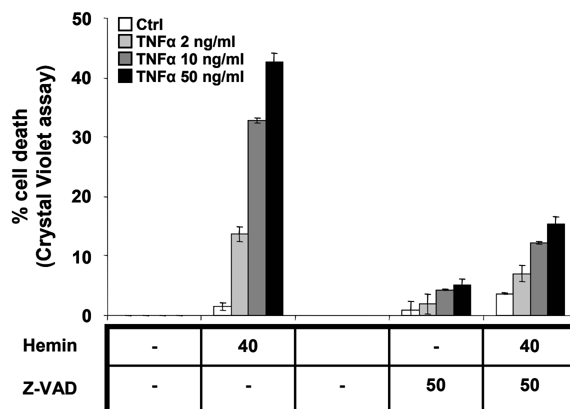


Figure 99. Cells were presensitized with hemin and then stimulated 6 hours with the indicated doses of TNFα. 50μM of the broad caspase inhibitor z-VAD was maintained in the cell culture medium during the treatement.

It is known that heme catalyzes the formation of reactive oxygen species (ROS) (Jeney et al, 2002; Wagener F.A. et al, 2003), whose levels in healthy cells are controlled by the presence of anti-oxidant enzymes. Therefore, we assessed whether the induction of hemin/TNFα-mediated apoptosis was due to oxidative stress. Experiments were performed using a cell permeable fluorogenic indicator which detects and measures ROS production. As shown in figure 100, a significant increase in ROS generation was obtained only when cells were pre-sensitize with hemin and then treated with TNFα, but not when exposed to TNFα or hemin alone.

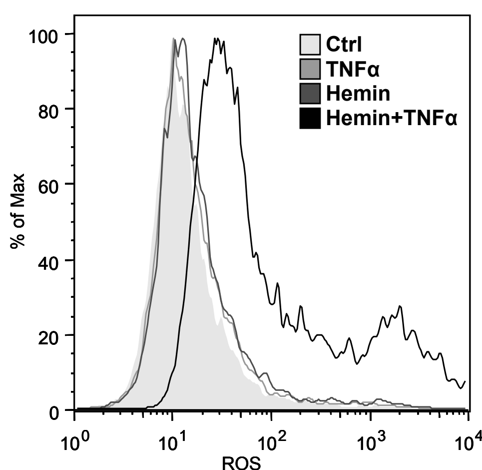


Figure 100. Cells were sensitized 1 hour with 40μM Hemin and treated 6 hours with 50ng/ml TNFα. ROS production was measured by FACS analysis using a specific probe.

It is described that ROS induction is deleterious for cells, as it can react with and damage lipid, DNA and proteins. Therefore, generation of ROS is also associated with an increase in lipid peroxidation, considered as a good parameter to measure oxydative stress (Guha M. et al, 2006). As shown in figure 101, the lipid peroxidation

induced by the treatment correlates with increasing TNFα concentrations, whereas neither the cytokine nor hemin treatment alone promotes significant lipid damage (Figure 101).

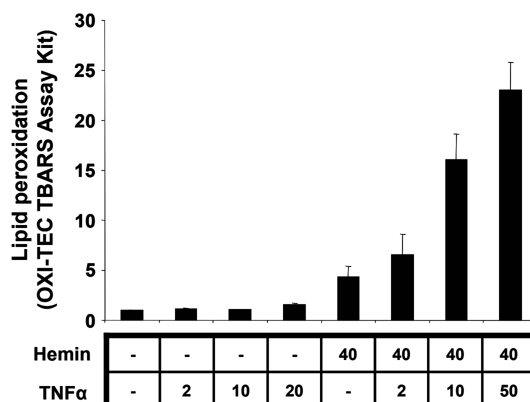


Figure 101. Cells were presensitized with hemin and treated 6 hours with the indicated concentration of TNFα. Lipid peroxidation is measured with OXI-TEC TBARS Assay kit as described in Matherial and Method session.

Furthermore, the use of scavengers, i.e. N-acetyl-cystein (NAC), suppresses hemin sensitization to TNFα-mediated apoptosis. The result shown in figure 102 indicates that NAC treatment confers protection against the stimuli, inhibiting significantly the percentage of cell death induced by hemin/TNFα treatment. This confirms the relevance of oxidative stress involvement in TNFα-mediated cytotoxic effect.

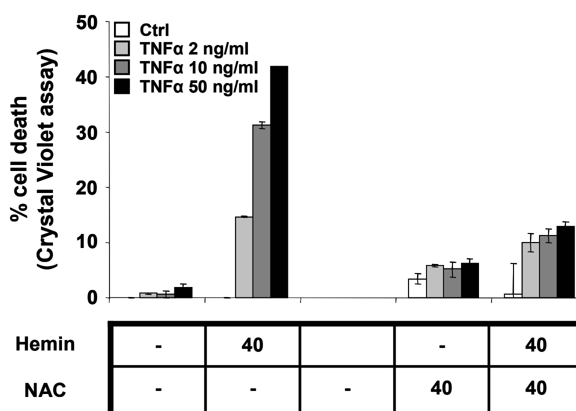


Figure 102. Cells were presensitized with 40µM hemin and treated 6 hours at the indicated concentration of TNFα. 40mM NAC treatment is maintained during all the stimulation. Percentage of cell death was assessed by Crystal Violet assay.

The toxicity mediated by ROS is also due to the inhibition of MKP, which results in a sustained and persistent JNK activation (Kamata H. et al, 2005). A time course of hemin/TNFα treatment revealed that this pathway is activated after 10 minutes stimulation and that this activation is maintained during all the stimulation (data not

shown). Moreover, to confirm that JNK signaling transduction pathway promotes hemin/TNFα-apoptosis, experiment with JNK chemical inhibitor was performed. SP600125 inhibits JNK-dependent mediated cell death induced by hemin/TNFα dose response. (Figure 103).

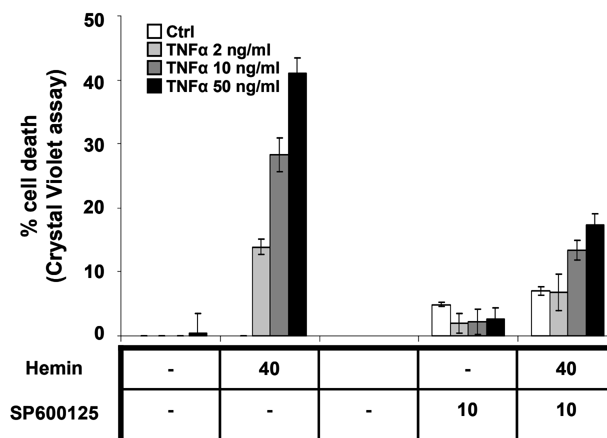
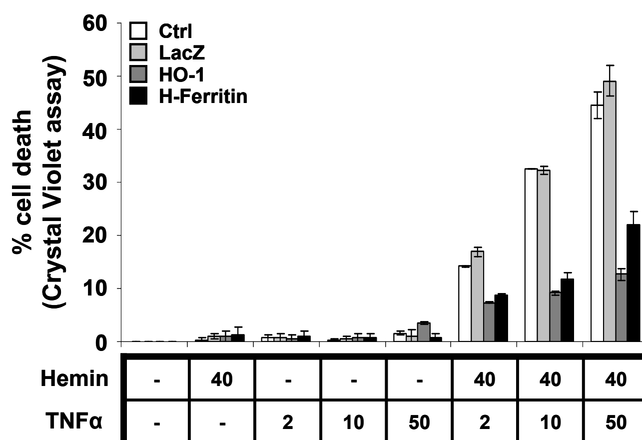


Figure 103. Cells were presensitized with hemin and treated 6 hours with the indicated concentration of TNFα. 10μM SP600125 treatment is maintained during all the stimulation. Percentage of cell death was assessed by Crystal Violet assay.

7.4.2. *HO-1 AND H-FERRITIN OVEREXPRESSION AFFORD PROTECTION AGAINST HEME/TNFα-PROMOTED APOPTOSIS*

The enzyme responsible for heme degradation is heme oxygenase I (HO-1), which is known to be upregulated in stressful conditions, e.g. TNFα treatment. Its function is to convert heme molecule into biliverdin, CO, both products with cytoprotective effects, and free ferrous iron. Subsequently, H-Ferritin devices free ferrous iron into cell storages preventing the formation of highly reactive hydroxyl radicals which are deleterious for cells.

In Hepa cell line, we wanted to assess whether singular HO-1 and H-Ferritin overexpression would afford protection against hemin sensitization to TNFα-mediated cell death. Cells were infected with the adenoviruses based sequence of HO-1 and H-Ferritin overexpression. LacZ adenovirus was used as infection control. 48 hours later, cells were sensitized with hemin and treated 6 hours with TNFα dose response. As indicated in figure 104, the singular upregulation of HO-1 and H-Ferritin confer resistance to TNFα cytotoxic effect (Figure 104).



Hemin	-	40	-	-	-	40	40	40
TNFα	-	-	2	10	50	2	10	50

Figure 104. Cells were infected with adenovirus based sequences of HO-1 and H-Ferritin overexpression. LacZ adenovirus was used as infection control. After 48 hours, cells were sensitized 1 hour with hemin and then treated 6 hours with the indicated TNFα concentrations. Cells death was assessed by Crystal Violet assay and the results referred to the control represented by non infected cells.

The scavenger properties of the enzymes suggest that HO-1 and H-Ferritin ectopic expression confer protection against TNFα cytotoxic effect by inhibiting ROS production. To confirm this theory, Hepa cells were infected with HO-1 and H-Ferritin overexpression adenoviral constructs. 48 hours later, cells were presensitized with hemin and then treated with TNFα. The result shows that the upregulation of HO-1 suppresses completely ROS induction promoted by hemin and TNFα treatment, whereas H-Ferritin avoids partially ROS generation (Figure 105).

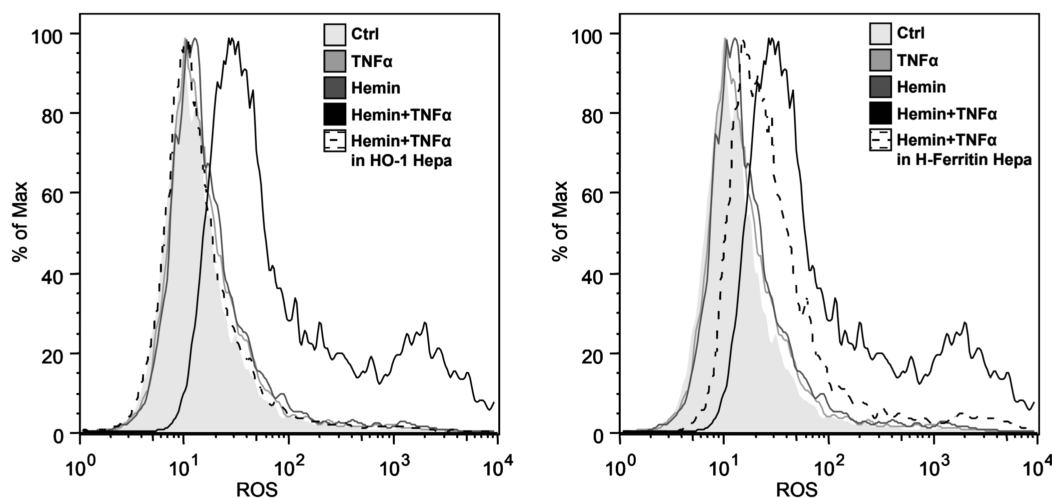
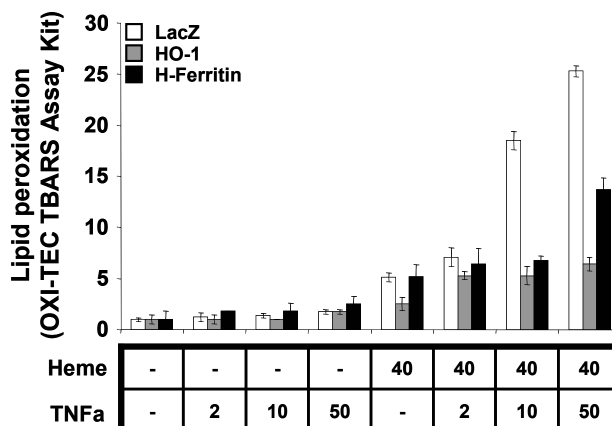


Figure 105. Cells were infected with HO-1 and H-Ferritin adenovirus and 48 hours later were sensitized one hour with 40μM Hemin and treated 6 hours with 50ng/ml TNFα. ROS production was measured by FACS analysis using a specific probe.

Furthermore, HO-1 overexpression avoids ROS-mediated damage induced by hemin and TNFα treatement, whereas the upregulation of H-Ferritin only promotes a

partial reduction (Figure 106). As H-Ferritin needs the collaboration of HO-1 to sequester and drive iron in cell storages, the partial protection afforded by the enzyme against hemin/TNFα treatment could be explained by a non sufficient endogenous HO-1 upregulation to assure a complete H-Ferritin mediated resistance to the stimulation.



Heme	-	-	-	-	40	40	40	40
TNFa	-	2	10	50	-	2	10	50

Figure 106. Cells were infected with adenovirus based sequences of HO-1 and H-Ferritin overexpression. LacZ adenovirus was used as infection control. After 48 hours, cells were presensitized with hemin and treated 6 hours with the indicated concentration of TNFα. Lipid peroxidation was measured with OXI-TEC TBARS Assay kit as described in Matherial and Method session.

Taken together these data indicate that heme is a potent inducer of the death stimuli, i.e. TNFα-triggered cell death. It sensitizes to TNFα-mediated apoptosis by boosting ROS production, which results in the activation of JNK signaling transduction pathway and pro-caspase-3. Moreover, singular HO-1 and H-Ferritin overexpression afford protection against hemin/TNFα cytotoxic effect avoiding the oxidative stress-mediated damage induced by the treatment.

Discussion

RNA TRANSCRIPTION INHIBITOR, ACTINOMICYNE D (ActD), SENSITIZES PC12 CELLS AND PRIMARY MOUSE CORTICAL NEURONS TO TNFα-MEDIATED APOPTOSIS

Apoptosis is a physiological mechanism essential to maintain tissue homeostasis. This programmed cell death can be induced by different stimuli, among which the activation of surface death receptors.

Many cell types are resistant to the apoptosis mediated by death receptors, i.e. TNFR-1. It is known that TNFR-1-mediated cell death usually requires the simultaneous inhibition of RNA transcription or protein synthesis. When metabolic inhibitors, e.g. CHX or ActD, are added to the cell culture medium in combination with TNFα the apoptotic process is induced (Kroemer and Martinez, 1994; Fulda et al, 2000). This suggests that the resistance to apoptosis could be mediated by intracellular signaling events, which are repressed under CHX or ActD stimulation (Okano H. et al, 2003).

ActD is a potent inducer of TNFα-mediated apoptosis both *in vitro* and *in vivo*. ActD binds the DNA and inhibits RNA transcription of short half-life proteins, promoting TNFα-induced cell death (Kleeff J. et al, 2000). We observed that whereas TNFα does not affect cell viability, ActD renders PC12 cells and primary mouse cortical neurons susceptible to the cytotoxic effect of the cytokine, suggesting that a first step of sensitization is required to promote apoptosis. TNFα and ActD co-treatment activates the initiator caspase-8. We demonstrate that the processing of Bid into its caspase-8-specific truncated form, tBid, only occurs when cells are co-treated with TNFα and ActD. tBid translocates to the mitochondria acting as an amplifier of TNFα apoptotic signal, which results in the processing of pro-caspase-9 and pro-caspase-3 into their respective active fragments.

ActD STIMULATION DOES NOT IMPAIR TNFα-DEATH COMPLEX PROTEINS

In order to assess at which molecular levels ActD inteferes and whether the sensitization promoted could be due to a misbalance between the “survival” and the “death complex” formed upon TNFα binding to TNFR-1, the expression of proteins recluted by TNFR-1 was analyzed. Our results demonstrate that TNFα and ActD co-treatment does not impair the levels of TNFRs receptors as well as of adaptor molecules e.g. TRADD, RIP, TRAF2 and FADD. Moreover, the expression of caspase-

8 was not modified by the experimental treatment, suggesting that ActD is acting below DISC assembly.

According to this, we can hypothesize that ActD treatment is sensitizing PC12 cells and primary cortical neurons to TNFα apoptosis by inhibiting the synthesis of medium/short-life proteins, which are essential to protect cells against TNFα cytotoxic effect. Therefore, our next aim was to identify those pro-survival molecules required for cell resistance to TNFα-induced apoptosis.

ActD SENSITIZES TO TNF-α CYTOTOXIC EFFECT BY INHIBITING MEDIUM/SHORT HALF-LIFE PROTEINS

The apoptosis promoted by TNFα can be inhibited at different levels by anti-apoptotic proteins, e.g. c-FLIP, IAPs and Bcl-2 family members.

c-FLIP inhibits TNFα-mediated cell death avoiding the functioning of TNFR-1-formed complex II. When c-FLIP is engaged to the DISC, by a FADD-mediated interaction, prevents the recruitment and activation of caspase-8. In distinct cellular models, it is described that the sensitization induced by ActD to TNFα-mediated apoptosis is due to a time dependent degradation of c-FLIP (Fulda S. et al, 2000). However, when we analyzed the level of c-FLIP in PC12 cells, we observed that the protein expression remained unchanged upon TNFα, ActD or TNFα and ActD co-treatment.

It is known that increased c-FLIP expression confers protection against death receptor-mediated apoptosis. At this purpose, it was shown that its marked up-regulation contributes to cancer development and the formation of malignant tumor (Djerbi M. et al, 1999; Alizadeh A.A. et al, 2000; Sun Q. et al, 2002). Moreover, recently the relevance of FLIP was observed also in ischemia-induced cell death. In this injury model, transgenic mice carrying $FLIP_L$ overexpression show a significant reduction in brain lesions development after permanent middle cerebral artery occlusion (Taoufik E. et al, 2007). In our model c-FLIP overexpression prevents the apoptosis induced by TNFα and ActD co-treatment. According to published data, we can suggest that acting at DISC level FLIP up-regulation inhibits caspase-8 recruitment and activation (Chang D.W et al, 2002; Micheau O. et al, 2002; Boatright K.M. et al, 2004). Cells infected with a lentivirus carrying a short hairping sequence against rat/mouse FLIP are no sensitive to TNFα-mediated apoptosis.

However, FLIP-deficient animals show embryonic lethality due to defects in hearth development and functioning. In FLIP-null mice, a quickly processing of procaspase-8 and procaspase-3 is induced in response to TNFα stimuli. This suggests that FLIP plays an important role in preventing inappropriate activation of the caspase cascade (Yeh W.C. et al, 2000). Moreover, fibroblasts derived from FLIP-deficient embryos are highly sensitive to Fas and TNFα-mediated apoptosis (Yeh W.C. et al, 2000). As deletion of FLIP demonstrates that this protein is an important regulator for the apoptotic signaling induced by death receptors, our results could appear contradictory as the endogenous FLIP decrease does not render PC12 cells sensitive to TNFα-mediated apoptosis.

However, other candidates, i.e. cIAPs family members, are reported to play an important role in the intrinsic cells resistance to TNFα-induced apoptosis, preventing caspase-8 activation (Fotin-Mleczek M. et al, 2002; Deng Y. et al, 2003; McDonald R.E. et al, 2004). Therefore, we can suggest that the action of cIAP proteins is complementary to the cytoprotective role exerted by FLIP. This means that, in our model, either in absence of FLIP, caspase-8 inhibition mediated by cIAPs would be sufficient to avoid the proceeding of the apoptotic cascade upon TNFα treatment.

Therefore, our aim was to assess whether cIAPs could regulate TNFα-induced apoptosis. Some reports suggest that the increased IAPs expression promoted by TNFα is the molecular mechanism through which endothelial cells are protected by the cytotoxic effect of the cytokine (Deng et al, 2003; Furusu et al, 2007). The function of ActD is to avoid new TNFα-mediated proteins transcription, which could result in an inhibition of IAPs synthesis. This means that cells could undergo apoptosis whether ActD and TNFα co-treatment decreases the expression of IAPs proteins to levels which are not sufficient to inhibit cell death. In PC12 cells, TNFα promotes IAPs transcription increasing significantly both cIAP1 and cIAP2 expressions. Therefore, the rapid induction of cIAPs in response to the cytokine stimulus suggests that these anti-apoptotic proteins could be crucial components in maintaining resistance against death receptor-induced cell death. On the other hand, ActD slightly downregulates cIAP1 and cIAP2 expression levels. However, when cIAP1 and cIAP2 were analyzed upon TNFα and ActD co-treatment, we were unable to demonstrate differences in the expression levels of these proteins, when compared with the untreated control cells. Therefore, our results show that ActD was able to avoid the increased expression of both cIAP1 and cIAP2 proteins that TNFα alone provokes in the cell culture medium. To discard that the levels afforded in TNFα/ActD condition could not be sufficient to prevent the

apoptotic cell death induced by the treatment, cIAP1 and cIAP2 RNAi experiments were performed to assess whether singular IAP-mediated caspase-8 inhibition renders PC12 cells susceptible to TNFα-mediated apoptosis. We observed that neither cIAP1 nor cIAP2 single specific endogenous decrease sensitizes cells to TNFα cytotoxic effect. Therefore, these data suggest that the singular decrease of endogenous cIAP1 and cIAP2 is not sufficient to activate the initiator caspase-8 after TNFα treatment.

IAPs-deficient animals show that in the absence of one of IAPs proteins, the remaining IAPs family members upregulate in order to compensate the loss of their homologoues (Conte D. et al, 2006). In agreement with this and analyzing the results obtained in PC12 cells, in the absence of cIAP1 the expression level of cIAP2 could be sufficient high to mediate caspase-8 inhibition. Therefore, it would be interesting to assess whether cIAP1 and cIAP2 double deficiency would trigger that sensitization which is essential for the induction of TNFα cytotoxic effect. However, besides the removal of cIAPs mediated caspase-8 inhibition, the presence of c-FLIP could interfere in the activation of the extrinsic pathway, inhibiting apoptosis at the beginning of the process.

The other IAPs family member we have analyzed is XIAP, whose relevance in conferring protection against death receptors and TNFα-mediated cell death is well characterized (Stehlik C. et al, 1998; Tang G. et al, 2001; Braeuer S.J. et al, 2006; Bubici C. et al, 2006; Lee T.J., et al, 2006; Rigaud S. et al, 2006; Qin Y. et al, 2007). The transcription of this anti-apoptotic gene, as well as other IAP proteins, is promoted by TNFα-mediated NF-κB activation (Mattson M.P. et al, 2006; Furusu A. et al, 2007). In endothelial cells, the induction of XIAP prevents lipopolysaccharide-induced apoptosis during inflammation conditions (Stehlik C. et al, 1998). In embryonic kidney 293T cells, the expression of XIAP protects from Bax-triggered apoptosis (Stehlik C. et al, 1998). Moreover, the increase of XIAP expression plays an important role also in neurodegenerative disorders. XIAP overexpression attenuates the damage induced by transient forebrain ischemic injury in rat hippocampus and avoids dopaminergic neuron cell death in the animal models of Parkinson’s disease (Eberhardt O. et al, 2000; Siegelin M.D. et al, 2005). In neuronal pathologies, e.g. excitotoxic injury induced by kainic acid, mice model of amyotrophic lateral sclerosis and Huntington’s disease was observed a progressive XIAP degradation suggesting that this protein may be part of a survival pathway by which neurons counteract degeneration. Therefore, preventing apoptotic cell death by inhibitiing caspase-9 and -3, XIAP represents a potential pharmacological target whose function could amiliorate neurodegenerative diseases

(Korhonen L. et al, 2001; Guegan C. et al, 2001; Goffredo D. et al, 2005; Siegelin M.D. et al, 2005).

When we analyze XIAP expression in PC12 cells, we observed a decrease in its protein level during ActD time course, which is not recovered upon TNFα co-treatment. However, XIAP overexpression confers resistance to TNFα and ActD co-stimulation.

As demonstrated that XIAP downregulation is associated with a higher susceptibility to TNFα-mediated apoptosis (Mendoza-Milla C. et al, 2005), and in order to assess whether endogenous XIAP decrease would promote TNFα sensitization, XIAP RNAi experiments were performed. We observed that XIAP endogenous decrease does not sensitize cells to TNFα cytotoxic effect.

XIAP-deficient mice develop normally as do not express any physical or histological abnormalities. However, a substantial cIAP1 and cIAP2 upregulation was observed in XIAP-null animals, suggesting that the lack of phenotype presented could be due to compensation by other IAPs family members (Harlin H. et al, 2001; Cummins J.M. et al, 2004). Although XIAP protein inhibits efficiently caspase-9 and caspase-3, there are no data in the literature which report that this molecule can also interact with and consequently avoid caspase-8 activation. Therefore, besides the removal of XIAP-promoted caspase inhibition, our results could be explained considering that the inhibition of caspase-8 activation promoted by cIAPs and FLIP protein is sufficient to render PC12 cells resistant to TNFα cytotoxic effect.

Taking together, the results show that besides their anti-apoptotic relevance, neither FLIP nor IAPs proteins can be singularly considered as key molecules able to regulate the sensitization step required for TNFα-mediated cell death.

IMPLICATION OF THE MITOCHONDRIA IN TNF-α-MEDIATED CELL DEATH

As it is known that mitochondria plays an essential role in TNFα transduction signaling, we decided to focus our attention on mitochondrial anti-apoptotic proteins belonging to Bcl-2 family. In response to cytotoxic stimuli, the balance existing between mitochondrial pro- and anti-apoptotic Bcl-2 family members is shifted versus pro-apoptotic proteins, which activate the intrinsic apoptotic pathway (van Delft M.F. et al, 2006; Uren R.T. et al, 2007; Willis S.N. et al, 2007). Therefore, a malfunctioning in preventing the mitochondrial integrity is the crucial event which promotes caspase activation. As TNFα-induced apoptosis demands an active contribution of the

mitochondria, this organelle can be identified as the key keeper between cell survival and cell death. (Deng Y. et al, 2002, 2003).

In order to assess whether ActD would affect the levels of the main pro- and anti-apoptotic proteins, the expression of molecules e.g. Bax, Bak, Bcl-2 and Bcl-x_L, was analyzed. Our results demonstrated that, Bax and Bak expressions remained unchanged during ActD treatment, whereas, in agreement with other reports, we failed to detect Bcl-2 which is not expressed in PC12 cells. On the other hand, when we analyzed the level of Bcl-x_L, we detected that ActD treatment affected the expression of this protein in a time-dependent manner. Bcl-x_L decreases upon 12 hours ActD treatment in both PC12 cells and primary mouse cortical neurons. Interestingly in primary cortical neurons Bcl-2 protein expression remains unchanged upon ActD stimulation. This observation suggests that, even though the function of Bcl-2 family members looks similar, the pro-survival activities of Bcl-2 and Bcl-x_L are differentially regulated (Berardo M.D. et al, 1998; Athanassiadou P. et al, 1998). Our data show that Bcl-x_L and not Bcl-2 needs to be degraded to render cells sensitive to TNFα-mediated cell death.

Apoptosis mediated by death receptors can be prevented by Bcl-x_L overexpression, as observed in neuroblastoma-derived and human prostate carcinoma cell lines (Hinz S. et al, 2000; Song J.J., et al, 2007). In agreement with this knowledge, we observed that the upregulation of Bcl-x_L protects both PC12 cells and primary mouse cortical neurons against the apoptosis promoted by TNFα and ActD co-treatment. We show that Bcl-x_L overexpression avoids the loss of mitochondrial integrity which is responsible for the cell death induced by caspase activation. When Bcl-x_L protein expression level is upregulated in both PC12 cells and primary cortical neurons caspase activation is inhibited.

The increased levels of Bcl-2 and Bcl-x_L are involved in cancer development and progression (Olopade O.I. et al, 1997; Berardo M.D. et al, 1998; Athanassiadou P. et al, 1998). On the other hand, it was demonstrated that Bcl-x_L decrease level induces susceptibility to several chemotherapeutic drugs. Endogenous Bcl-x_L downregulation enhances colorectal cancer cells to 5-Fluorouracil-mediated apoptosis (Hayward R.L. et al, 2003). Furthermore, Bcl-x_L decrease promotes TNFα and TRAIL mediated apoptosis in pancreatic cancer cell line (Bai J. et al, 2005).

In agreement with these reports, we assessed whether a lentiviral-based construct carrying a short hairping sequence against rat/mouse Bcl-x_L would sensitize cells to the apoptotic effect mediated by TNFα. When we analyzed the cytotoxic effect

of TNFα in cells infected with the shRNA against Bcl-x_L, we observed that the strong reduction of Bcl-x_L protein expression was sufficient to sensitize both PC12 cells and primary cortical neurons to TNFα-induced apoptosis. Furthermore, when shBcl-x_L-cells were co-treated with TNFα and ActD, the presence of the RNA transcription inhibitor does not increase the percentage of cell death when compared with the apoptosis achieved with TNFα alone. Therefore, this result confirms that in our model the role played by ActD is to decrease Bcl-x_L expression to a protein level sufficient to induce sensitization to TNFα apoptotic cell death.

Taking together, these data suggest that Bcl-x_L is the crucial anti-apoptotic protein which regulates cell faith, as its overexpression confers resistance against TNFα and ActD co-treatment, whereas its endogenous downregulation induces cell death upon stimulation with the cytokine alone.

The physiological relevance of Bcl-x_L in the nervous system was demonstrated by the generation of mice carrying the homozygous mutation of *bcl-x*. Bcl-x_L-deficient mice are the only Bcl-2 family members-null animals to show neuronal phenotype. Bcl-x_L $^{-/-}$ embryos die around day 13–13.5 due to an extensive apoptosis of post-mitotic immature neurons of developing brain, spinal cord, and dorsal root ganglia, which already starts at day 11.5 (Motoyama N. et al, 1995). Bcl-x_L can be considered as the essential requirement for the correct maintenance of immature cells viability during the embryonic development of the nervous system. On the other hand, in the central nervous system no evident alterations were identified in Bcl-2-deficient mice. Moreover, Bcl-x_L protein level is maintained during the development and its expression remains high in the adult nervous system, whereas Bcl-2 decreases quickly (Gonzalez-Garcia M. et al, 1994). The high expression of Bcl-x_L in aged brain suggests a potential role of this protein not only in neuronal development but also in aging. As free radicals-mediated damage is one of the main causes of aging, it seems that Bcl-x_L can be involved in the anti-oxidant mechanism which represents a brain defensive response to increase exposure to oxidative stress (Ames B.N. et al, 1993; Drache B. et al 1997). Moreover, the importance of Bcl-x_L was also demonstrated in neurodegenerative pathologies, e.g. Alzheimer's disease and multiple sclerosis, in which its neuronal decrease enhances cell death promoted by activated microglia. On the contrary, the high Bcl-x_L protein expression detected in reactive microglia cells abrogates their clearance, leading to a more severe clinical profile (Drache B. et al, 1997; Waiczies S. et al 2002; Sharief M.K. et al 2003).

Discussion

The attenuation of ischemic-induced damage after brief episodes of sublethal ischemia, in which an upregulation of Bcl-2 and Bcl-x_L is observed, suggests the relevance these anti-apoptotic Bcl-2 family members present during ischemic preconditioning (Wu C. et al, 2003). Moreover, the overexpression of Bcl-x_L confers neuronal protection against ischemic-like stress, e.g. oxygen and glucose withdrawal, and prolonged hypoglycemia (Panickar K.S. et al, 2005).

The early downregulation of Bcl-x_L occurring after spinal cord injury is responsible for the neuronal loss which contributes to the functional deficits observed in the rat model. On the other hand, either a single injection of Bcl-x_L fusion protein into the contused spinal cord site is sufficient to improve the sensory and motor recovery, preserving the locomotor function after the spinal trauma (Nesic-Taylor O. et al, 2005). Furthermore, after 30 days of nerve trauma, Bcl-x_L expression was completely lost in axotomized neurons of the spinal ganglia (Chelyshev Y.A. et al, 2005). As resident glias that become activated in the brain tissue after different pathological stimuli promote a dramatic increase in TNFα expression levels, we could hypothesize that neurons having lost their Bcl-x_L expression result more sensitive to the cytotoxic action of glial-derived TNFα (Perry V.H. et al, 1995; Chelyshev Y.A. et al, 2005).

All these data suggest that the preservation of mitochondrial function is the crucial event for the inhibition of neurodegeneration.

To sort out that Bcl-x_L is the anti-apoptotic protein whose levels can decide about cell faith in TNFα-induced signaling pathway, we decided to performe further experiments in a cellular model in which, contrarily to PC12 cells, the main anti-apoptotic Bcl-2 family molecules could be detected. HeLa cell line expresses the most relevant anti-apoptotic members belonging to Bcl-2 family, e.g. Bcl-2, Bcl-x_L, Bcl-w, and Mcl-1 (Willis S.N. et al, 2005), providing an interesting tool to better analyze the implication of the anti-apoptotic mitochondrial proteins in TNFα-mediating cell death. The relevance of Bcl-x_L in this model was demonstrated by experiments in which Bcl-2, Bcl-x_L, Bcl-w and Mcl-1 protein expressions were individually and forcedly decreased. The endogenous decrease of either Bcl-2 or Bcl-w protein expression in HeLa cell line slightly sensitizes to TNFα-mediated apoptosis. However, the endogenous expression of Bcl-2 and Bcl-w can not compensate the loss of Bcl-x_L to prevent the apoptotic cell death promoted by TNFα treatment.

Mcl-1 is essential in promoting survival of malignant cells and a reduction in its expression triggers the apoptotic process (Derenne et al, 2002; Michels J. et al, 2005). In several cellular models, Mcl-1 expression is donwregulated during the apoptosis

induced by ultraviolet irradiation, adenovirus infection (Nijhawan D. et al, 2003; Cuconati A. et al, 2003; Weng C. et al, 2005) and TRAIL-stimulation (Weng C. et al, 2005). Consistent with this, we hypothesized that a reduction in its molecular levels can sensitize cells to TNFα-mediated death. According to the expectations, a decrease of Mcl-1 expression sensitizes HeLa cell line to TNFα-induced apoptosis although the percentage of cell death achieved was lower when compared with Bcl-x_L siRNA infected cells treated with TNFα.

INVOLVEMENT OF NF-κB AND JNK SIGNALING TRANSDUCTION PATHWAYS IN TNF-α-MEDIATED CELL DEATH

The binding of TNFα to TNFR-1 leads to survival or cell death. The survival pathway is mediated by the activation of NF-κB transcription factor (Wullaert et al, 2006). NF-κB promotes the transcription of anti-apoptotic proteins which inhibit TNFα-mediated cytotoxic effect.

It is known that the inhibition of NF-κB transcriptional activity induces cell death upon TNFα stimulation (Beg and Baltimore, 1996; Van Antwerp et al, 1996; Wang et al, 1996). The overexpression of a mutated serine-to-alanine undegradable form of IκBα (SR-IκBα), which avoids NF-κB activation, renders cells sensitive to TNFα-induced apoptosis. In these cells, the ectopic expression of Bcl-x_L suppresses the cytotoxic effect of TNFα (data not shown).

Data in the literature reported that Bcl-x_L is a transcriptional target of NF-κB (Chen C et al, 2000; Glasgow J.N. et al, 2000). Two specific NF-κB binding sites sequence were identified within the murine *bcl-x* promoter region (Glasgow J.N. et al, 2000). Moreover, the mapping of a functional κB DNA site in the human *bcl-x* promoter region confirmed its Rel-dependent transcriptional response (Chen C. et al, 2000). However, hypothesizing that, when NF-κB is inhibited the apoptosis promoted by TNFα is due to a decrease in Bcl-x_L, the expression level of this protein was analyzed. No differences in Bcl-x_L protein levels were found in both SR-IκBα- and Neo-PC12 cells. This result suggests the existance of two different pro-survival pathways, one NF-κB-dependent and the other NF-κB-independent but dependent on Bcl-x_L. Moreover, as the mutated form of IκBα failed to reveal alterations in Bcl-x_L levels, we can hypothesize the existence of other transcription factors which in conjunction cooperate to regulate its expression.

Furthermore, we assessed that NF-κB activation in shBcl-x_L-PC12 cells was comparable with wild-type cells after TNFα stimulation. This was demonstrated by the unaltered p65 nuclear translocation, Luciferase activity and the promotion of cIAP-1 NF-κB driven gene transcription upon TNFα stimulation. Therefore, we have provided evidence that in the absence of Bcl-x_L, NF-κB signaling transduction pathway is completely functional upon TNFα treatment, supporting our hypothesis of two distinct survival pathways. Morever, we suggest that the transcriptional gene activity promoted by NF-κB signaling is not sufficient to render cells resistance to the cytotoxic effect induced by TNFα when Bcl-x_L endogenous expression is forcedly decreased.

The binding of TNFα to TNFR-1 also promotes the induction of different mitogen-activated protein kinase (MAPK) cascade which results in JNK activation. When NF-κB signaling transduction pathway is inhibited, TNFα induces apoptosis through a prolonged and substained JNK activation (Wullaert et al, 2006).

Our experiments show that, in the absence of Bcl-x_L, TNFα induces persistant JNK activation which is inhibited by JNK chemical antagonist. It is known that JNK-mediated Bid cleavage product, jBid, translocates to the mitochondria and promotes the selective release of SMAC/DIABLO, but not of cytochrome c. The function of SMAC at the DISC level is to remove IAP-mediated inhibition of caspase-8, leading to its activation and to the proceeding of the apoptotic cascade (Deng Y. et al, 2003).

To assess whether endogenous Bcl-x_L protein decrease would regulate TNFα-mediated activation of JNK-dependent cell death, PC12 cells were simultaneously transduced with both shBcl-x_L and shSMAC. As demonstrated, Bcl-x_L endogenous downregulation sensitizes cells to TNFα-mediated apoptosis, however the simultaneously decrease of SMAC/DIABLO inhibits the death signal promoted by the treatment conditions. Therefore, SMAC/DIABLO can be identified as a regulator of the apoptosis induced by TNFα-dependent JNK activation in the absence of Bcl-x_L. We can suggest that the mitochondrial release of SMAC/DIABLO upon TNFα stimuli is a crucial event regulated by Bcl-x_L expression.

SMAC-deficient animals show a complete activation of both caspase-9 and caspase-3 (Okada H. et al, 2002; Rajalingam K. et al, 2007). As SMAC-null mice do not show higher sensitivity to death receptor induced apoptosis, other proteins are suggested to cope with the loss of SMAC or that SMAC participates only in regulating cell death induced by specific stimuli (Okada H. et al, 2002). According to this hypothesis, we suggest that Bcl-x_L expression levels would modulate SMAC release,

which converges in caspase-8 activation. In the absence of Bcl-x_L, we observed a small increase in caspase-8 activity when compared with the control cells (data not shown), although it results sufficient to trigger cell death induced by TNFα. Therefore, the absence of Bcl-x_L overcomes the inhibitory effect of FLIP and cIAPs on caspase-8 processing upon TNFα stimulation.

As described that JNK mediates caspase-8 activation, we suggest that in our model the endogeneous decrease of SMAC affords protection avoiding caspase-8 activation in response to TNFα/Bcl-x_L decrease-induced cell death. Further studies involving caspase-8 inhibition/activation processes could be interesting to better elucidate the mechanism by which cells inhibit death receptors-induced apoptosis.

In conclusion, this study shows that Bcl-x_L is the endogenous regulator of neuronal sensitivity to TNFα-induced apoptosis despite the simultaneous activation of NF-κB signaling transduction pathway. Bcl-x_L is the anti-apoptotic protein responsible for cell resistance to TNFα cytototoxic effect, as its decrease expression promotes caspases activation, leading to apoptotic cell death. Our experiments demonstrate that Bcl-x_L, and not proteins e.g. FLIP, IAPs, Bcl-2, Bcl-w and Mcl-1, is the key molecule mediating cell resistance against the apoptosis triggered by TNFα. This provides an important mechanism of crosstalk between the intrinsic and extrinsic apoptotic pathway.

Moreover, the delivery of therapeutical proteins, i.e. Bcl-x_L, into brain tissue was shown to be protective against brain injury (Kilic E. et al, 2002; Cao G. et al, 2002). As this application is based on intravenous infusion, it represents an open perspective for neurodegenerative disease treatment.

FREE HEME ACCUMULATION PROMOTES TNFα-MEDIATED APOPTOSIS

Heme molecule plays an essential role in regulating the biologic activity of Hemoproteins, in which it acts as a prosthetic group. Among them, the most relevant are hemoglobin and myoglobin, in which heme is used for oxygen transport and storage, cytochromes, where it is involved in electron transport and energy generation, tryptophan pyrrolase, in which it catalizes the oxidation of tryptophan, etc (Maines M.D. et al, 1997). Moreover, heme modulates differentiation and proliferation of various cell types, e.g. neuronal differentiation of mouse neuroblastoma cells and erythroid differentiation of erythroid leukaemia cells. Furthermore, it induces cell growth of

cultured fibroblasts (Granick J.L. et al, 1978; Ishii D.N. et al, 1978; Benz E.J. et al, 1980; Verger C. et al, 1983; Wagener F. et al, 2003).

However, as highly lipophilic, heme can intercalate in cellular membranes, destabilizing the cytoskeleton and the normal function of organelles, i.e. mytochondria (Balla G. et al, 1991; Wagener F. et al, 2003). Balla G. and collaborators showed that short exposure to free heme sensitizes endothelial cells to oxidant treatments, i.e. H_2O_2, enhancing the cytotoxicity promoted by the death stimuli (Balla G. et al, 1992).

With the aim to investigate whether heme accumulation would represent a potent inducer of death receptor-mediated cell death, we analyzed the sensitization induced by heme in TNFα-mediated apoptosis. In Hepa cell line, we demonstrated that heme is as a potent inducer of TNFα-cytotoxic effect. We assessed that whereas TNFα or heme treatement does not affect the viability of the cells, heme sensitization dramatically enhances the apoptosis promoted by TNFα. Moreover, we demonstrated that caspase-3 becomes activated only when cells are sensitized with heme and then treated with TNFα. In the same way, Hoechst staining reveals that upon heme/TNFα treatment dying cells display the typical apoptotic nuclear morphology characterized by high chromatine condensation and nucleus shrinkage.

The binding of TNFα to TNFR-1 promotes ROS generation, whose levels in healthy cells are regulated by the presence of anti-oxidant enzymes. As heme catalyzes the formation of radical oxygen species (Vercellotti et al, 1994; Jeney et al, 2002), we observed that heme sensitizes to TNFα-apoptosis through an exacerbation of the oxidative stress. We observed that whereas heme and TNFα alone promotes ROS generation which is comparable to untreated cells, a significant increase occurs when cells are sensitized with heme and then exposed to TNFα. A boost of ROS is deleterious for cells as they react with and damage DNA, proteins and lipids, promoting cell death. To detect the consequence induced by oxidative stress in Hepa cell line, lipid peroxidation was analyzed as a marker of ROS-mediated damage. The extent of lipid peroxidation positively correlates with increasing TNFα concentrations upon heme pre-sensitization. Once generated, ROS lead to apoptosis promoting the activation of pro-caspase-3 into its active fragment. Scavengers treatment, i.e. NAC, confers resistance to cell death promoted by heme and TNFα stimulation. This suggests that the oxidative stress induced by heme/TNFα treatment is required for the cytotoxic effect exerted by the cytokine.

The production of ROS is involved in JNK-dependent cell death, as ROS is one of the main activator of this pathway (Ventura J.J. et al, 2004). It was demonstrated

that ROS accumulation inactivated MAPK phosphatases (MKPs) by oxidation of their catalytic cystein residue, which as a consequence prolongs JNK activation potentiating JNK-dependent cell death (Kamata H. et al, 2005). When we examined the effect of cell exposure to heme and TNFα stimulation, a sustained and persistent JNK activation was detected during all the treatment (data not shown). Moreover, the inhibition of JNK by its chemical antagonist, affords protection against the cell death induced by heme/TNFα treatment, demonstrating the involvement of this pathway upon this stimulus.

HO-1 AND H-FERRITIN OVEREXPRESSION CONFER RESISTANCE TO TNFα-MEDIATED CELL DEATH

When exposed to free heme, cells upregulate the expression of heme oxygenase I, HO-1, which catalyzes heme degradation into carbon monoxide, biliverdin, both with antinfammatory properties, and free ferrous iron (Wagener F.A. et al, 2003).

In our model, the overexpression of HO-1 completely inhibits cell death induced by heme/TNFα treatment. We demonstrated that the protective effect of HO-1 is associated with the inhibition of ROS generation as well as the suppression of ROS-mediated damage. HO-1 avoids the lipid peroxidation promoted by heme/TNFα treatment, demonstrating its anti-oxidant role which prevents the apoptotic cell death.

The induction of HO-1 is associated with an up-regulation of H-Ferritin, as the free ferrous iron released by heme degradation rapidly promotes its protein expression (Balla G. et al, 1992; Bubici C. et al, 2006). Binding to free ferrous iron, H-Ferritin sequesters this metal and drives its device into cell storages in order to prevent the formation of highly reactive hydroxyl radicals through the Fenton reaction. In Hepa cell line, the overexpression of H-Ferritin partially inhibits ROS generation and ROS-mediated cell damage. To explain that increased levels of H-Ferritin confer partial resistance to TNFα-induced cell death upon heme sensitization when compared with HO-1 cytoprotection, we have to analyze its mechanism of activation. To device free ferrous iron originated by heme degradation and therefore to prevent cell damage, H-Ferritin requires the opening of the porfirin ring by the action of HO-1. HO-1 is upregulated in stressful conditions, e.g. heme and TNFα stimulation (Immenschuh S. et al, 2000). However, the increased endogenous expression of HO-1 could not achieve

those protein levels required by H-Ferritin overexpression in order to confer the total protection against TNFα cytotoxicty.

Taking together, the results obtained demonstrate that TNFα exerts its cytotoxic effect upon heme pre-sensitization through ROS-mediated JNK-dependent cell death, which leads to caspase activation. Moreover, our data show that anti-oxidant enzymes, i.e. HO-1 and H-Ferritin, afford protection against heme/TNFα-mediated apoptosis preventing the induction of ROS-promoted damage.

This mechanism may have important implications in diseases in which free heme accumulation is reported i.e. during hemolysis processes in which hemoproteins disruption occurrs. The release of free heme promotes an increase in vascular permeability, leading to leukocytes infiltration into the affected tissue, which results in oxidative stress-mediated inflammation (Wagener F.A. et al, 2001).

A disease associated with severe hemolysis is cerebral malaria, in which the blood-brain barrier disruption is cause of free heme release and its consequent plasma accumulation. It was demonstrated that HO-1 as well as the administration of exogenous CO counteracts completely the severity of the pathology, preventing the neuroinflammation induced by free heme accumulation. (Pamplona A. et al, 2007).

The anti-inflammatory property of HO-1 was recently demonstrated also in multiple sclerosis, in which this protein prevents mortality avoiding the demyelination and paralysis associated with the disease (Chora A. et al, 2007).

On the other hand, H-Ferritin significantly contributes to the cytoprotective effect of HO-1 against the apoptotic stimuli, as in endothelial cells it affords protection in a dose-dependent manner against TNFα, etoposide, serum deprivation, etc. Moreover, in the liver H-Ferritin prevents cellular damage upon transplantation, rescuing rats hepatocytes from ischemic/reperfusion injury (Berberat P.O. et al, 2003).

Therefore, the cooperative functions mediated by HO-1 and H-Ferritin indicate the relevance of these proteins in preventing the cytotoxic damage induced by the apoptotic stimuli.

Conclusions

First. TNFα treatment does not affect the viability of PC12 cells and primary cortical neurons.

Second. ActD renders PC12 cells and primary cortical neurons sensitive to TNFα cytotoxic effect.

Third. Cells treated with ActD become susceptible to TNFα-induced cell death through the activation of caspase-8, generation of tBid and activation of caspase-9 and -3.

Fourth. The sensitization promoted by ActD to TNFα-induced apoptotic cell death does not affect the proteins involved in TNFR-1 signaling complexes.

Fifth. FLIP and XIAP overexpression prevent the apoptosis promoted by TNFα/ActD co-treatment. However, FLIP and IAPs endogenous decrease do not sensitize PC12 cells to the cytotoxic effect of TNFα.

Sixth. Bcl-x_L expression level is reduced by ActD or TNFα/ActD co-treatment.

Seventh. Bcl-x_L overexpression fully protects PC12 cells and primary mouse cortical neurons against TNFα/ActD-induced cell death.

Eighth. Bcl-x_L endogenous decrease sensitizes PC12 cells and primary cortical neurons to TNFα-mediated apoptosis through caspase activation, withouth the requirement of ActD.

Ninth. Bcl-x_L endogenous decrease sensitizes HeLa cell line to TNFα cytotoxic effect.

Tenth. Bcl-x_L downregulation does not affect TNFα-induced NF-κB activation. Moreover, Bcl-x_L is not a major target of this transcription factor as Bcl-x_L expression is not affected by the inhibition of NF-κB signaling transduction pathway.

Eleventh. Bcl-x_L endogenous decrease promotes TNFα-mediated JNK-dependent cell death, which is completely prevented by SMAC/DIABLO downregulation.

Twelfth. Free heme accumulation sensitizes Hepa cell line to TNFα-mediated apoptosis.

Thirteenth. Heme sensitization induces TNFα-mediated ROS production, which activates JNK signaling transduction pathway.

Conclusions

Fourteenth. Singular HO-1 and H-Ferritin overexpression avoid ROS generation and afford protection against heme/TNFα-mediated cell death.

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Pubblications and congress partecipations

PUBBLICATIONS RELATED TO THE THESIS WORK:

Authors: **Gozzelino R.**, Solé C., Llecha N., Segura M.F., Moubarak R.S., Perez-Garcia M.J., Iglesias V., Trullas R., Yuste V.J. and Comella J.X.
Title: Endogenous levels of Bcl-x_L determine the biological response to TNFα in PC12 cells and cortical neurons independently of $FLIP_L$ protein levels and NF-kB activation status.

Manuscript in preparation.

PUBBLICATIONS NON-RELATED TO THE THESIS WORK:

Authors: Solé C., Dolcet X., Segura M.F., Gutierrez H., Diaz-Meco M.T., **Gozzelino R.**, Sanchis D., Bayascas J.R., Gallego C., Moscat J., Davies A.M., Comella J.X.
Title: FAIM, a death receptor antagonist, promotes neurite outgrowth by mechanism that depends on activation of ERK and NF-kB.
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Title: The long form of Fas apoptotic inhibitory molecule ($FAIM_L$) is specifically expressed in neurons and protects them against death receptor-triggered apoptosis.
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CONGRESS PARTECIPATION:

Authors: **Gozzelino R.**, Segura M.F., Solé C., Comella J.X.
Title: Dual mechanism of cell death after TNFα treatment in PC12 cells.
Congress: Red de grupos de muerte neuronal en modelos animales y patología humana. Sant Feliu de Guixols. October 22th-24th, 2003.

Authors: **Gozzelino R.**, Segura M.F., Solé C., Comella J.X.
Title: TNF alpha signaling mechanism inducing cell death.
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Congress: II Reunion anual de la RED del FIS sobre Muerte Neuronal. Madrid. November 8th-9th, 2004.

Authors: **Gozzelino R.**, Solé C., Segura M.F., and Comella J.X.
Title: Regulación de las vías de supervivencia y apoptosis por TNF alpha en sistema nervioso
Congress: ApoRed, Red Española de Apoptosis. Miraflores de la Sierra (Madrid). May 28th- June 1th, 2005.

Authors: **Gozzelino R.**, Solé C., Llecha N., Segura M.F., Trullas R. and Comella J.X.
Title: Carcterización del mecanismo de muerte inducido por TNF alpha en el sistema nervioso.
Congress: Sociedad Española de Bioquimica y Biologia Molecular. Zaragoza. September 12th–15th, 2005.