ROLE OF BIOACTIVE FOOD COMPOUNDS IN ACUTE AND CHRONIC INFLAMMATION

Victor Pallarés López

Dipòsit Legal: T.1472-2012

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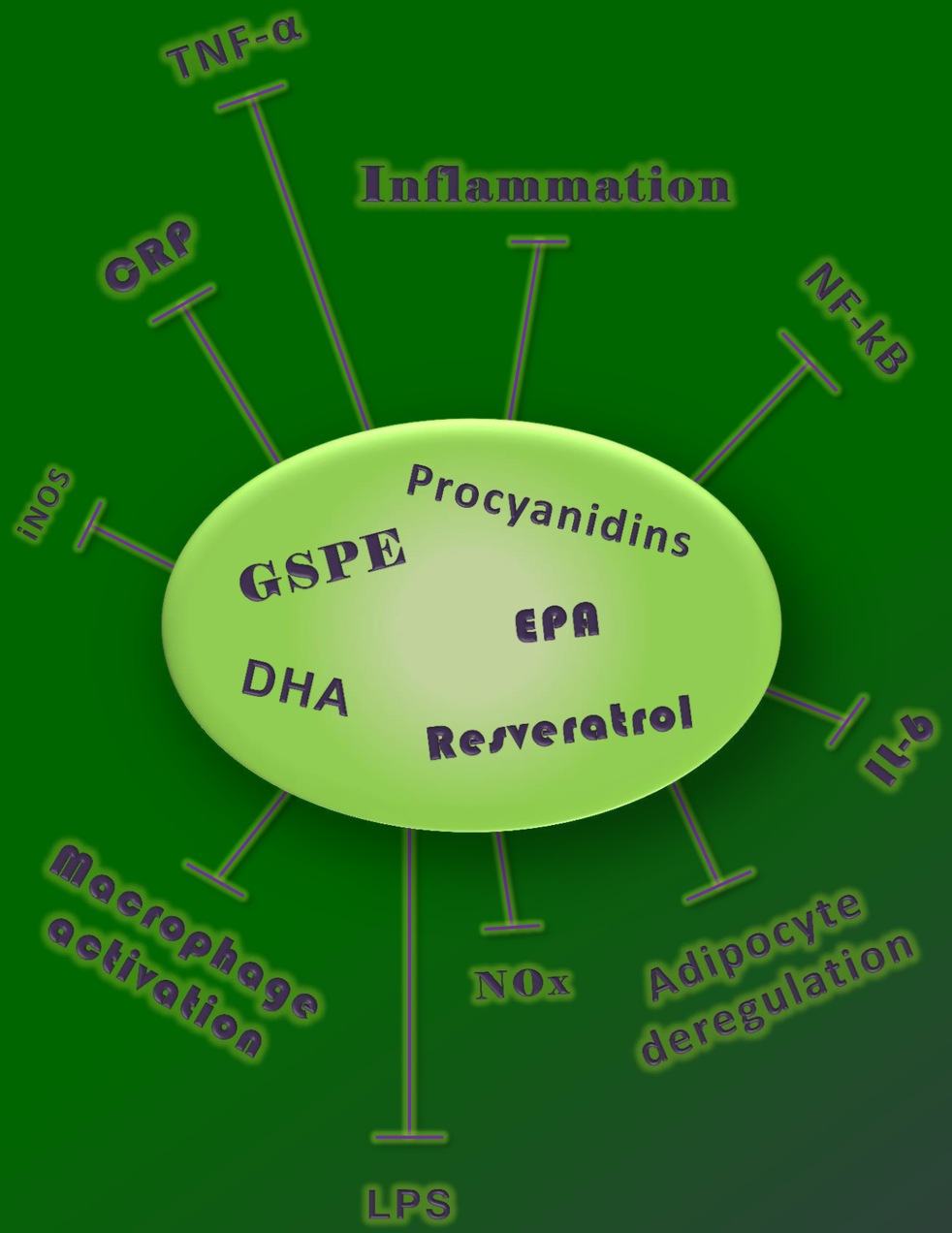

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Doctoral Thesis

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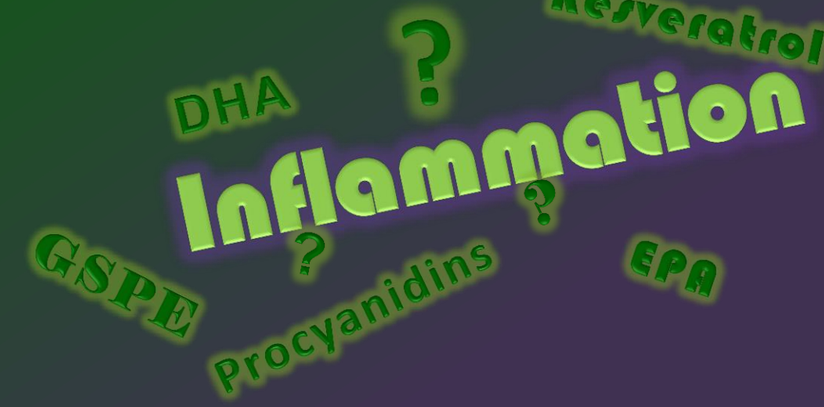

Victor Pallarés López

Universitat Rovira i Virgili

2012

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Role Of Bioactive Food Compounds In Acute And Chronic Inflammation

Ph. Doctoral Thesis

Directed by Dr. Maria Teresa Blay Olivé

Departament de Bioquímica i Biotecnologia

Tarragona

2012

UNIVERSITAT ROVIRA I VIRGILI

Departament de Bioquímica i Biotecnologia

C/ Marcel·lí Domigo s/n

Campus Sescelades

43007 Tarragona

Tel. +34 977 55 84 97

Fax. +34 977 55 82 32

FAIG CONSTAR que aquest treball, titulat “Role of bioactive food compounds in acute and chonic inflammation”, que presenta Victor Pallarés López per a la obtenció de títol de Doctor, ha estat realitzat sota la meva direcció al Departament de Bioquímica i Biotecnologia d’aquesta universitat i que acompleix els requeriments per poder optar a Menció Europea.

Tarragona, 5 de Novembre de 2012

La directora de la tesi doctoral

Dra. M. Teresa Blay Olivé

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A la meva família

"No sé com em deu veure el món, però al meu entendre, em sembla que he estat només com un nen que juga a la vora del mar, i que es diverteix buscant de tant en tant una pedra més polida i una petxina més bonica del normal, mentre que el gran oceà de la veritat s'exposava davant meu completament desconegut."

Sir Isaac Newton

Agraïments

Bé, començo aquesta part després de finalitzar totes les altres sabent que no és menys important que cap altra, ja que sense la vostra aportació, consells, ajuda, companyia, bon rotllo, i més i més i més, no s'hagués pogut dur a terme aquesta tesi. Després d'aquesta petita "intró", m'agradaria afegir que no em vull deixar a ningú, però tot pot passar, així que us demano disculpes a aquells que no estiguin entre aquests agraïments, però, si heu format part d'aquest període directa o indirectament, us en dono les gràcies també.

Primer de tot, voldria agrair a la Mayte, la meva tutora, el temps que m'ha brindat, així com l'orientació i els consells que m'ha anat donant des de fa 5 anys, durant el treball de fi de carrera, el màster i ara el doctorat. Gràcies per donar-me l'oportunitat d'endinsar-me en el món de la investigació i de confiar en els meus passos, que finalment també formen part dels teus. També vull agrair-te tot l'esforç realitzat al final del trajecte. A pesar de totes les inconveniències que se'ns han presentat, hem pogut tirar-ho endavant. Gràcies per tot.

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Ei!! I també la Mari!! Quines xerrades pel passadís... Gràcies pels teus "Bon dia" i el teu bon rotllo! També, als membres d'Enologia, amb els que sempre es pot comptar quan es necessita, en especial, al Braulio i la Marta. I pels seus "passadís-time"....

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I sobretot a la Lídia, que m'ha recolzat sempre, m'ha aguantat tot aquest temps, en els moments bons i dolents i segueix al peu del canó. Ets una part molt gran de tot això. Així que l'agraïment més gran és per tu, que comparteixes amb mi la teva vida i em dones la felicitat que necessito.

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Index

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Dipòsit Legal: T.1472-2012

1

ANNEX I

1.1 Summary

The study of how anti-inflammatory agents found in food can mediate in various diseases in which inflammation is involved has become relevant in recent years. Procyanidins present in fruits and vegetables and polyunsaturated fatty acids (PUFAs) present in fish and fish oils are compounds that have anti-inflammatory properties against several diseases such as obesity, diabetes and cardiovascular disease. The goal of this study was to investigate some aspects and mechanisms related to the reported efficacy of grape seed procyanidin extract (GSPE) against the inflammatory state in the genetically obese *Zucker fa/fa* rat model with low-chronic inflammation and a model of acute inflammation triggered by the injection of lipopolysaccharide (LPS) into *Wistar* rats. A moderate dose of 35 mg/kg*day of GSPE for 10 weeks had anti-inflammatory effects at the gene expression level in mesenteric adipose tissue in *Zucker fa/fa* rats. However, no significant effects on systemic inflammation were found. In contrast, GSPE had anti-inflammatory effects at the systemic and gene expression levels when administered for 15 days prior to LPS-induced inflammation in *Wistar* rats. The moderate-high dose of 75 mg/kg*day and the high dose of 200 mg/kg*day of GSPE were the most effective doses in preventing acute inflammation. In addition, several pure bioactive flavonoids such as resveratrol (RES), epigallocatechin gallate, B1-B4 dimeric procyanidins, and PUFAs such as EPA and DHA were administered in various combinations to assess any potential synergistic, additive or antagonistic anti-inflammatory effects that may exist between these flavonoids and PUFAs in LPS-stimulated murine macrophages. Importantly, the combinations of B3 plus EPA and RES plus EPA had strong synergistic anti-inflammatory effects manifested by a decrease in nitric oxide levels, a decrease in the expression of proinflammatory and oxidative stress-related genes and the modulation of the phosphorylation status of various proteins involved in the activation of the NF-κB and AP-1 proinflammatory pathways.

1.2 Abbreviation list

ADIPOR	Adiponectin receptor
ADIPQ	Adiponectin
ALA	α-linolenic acid
AMP	Adenine phosphoribosyltransferase
AMPK	Catalytic subunit of AMP-activated protein kinase
AP-1	Activator protein 1
APP	Acute-phase protein
ARA	Arachidonic acid
AT	Adipose tissue
CCL	Chemokine (C-C motif) ligand
CCR2	C-C chemokine receptor 2
COX2	Cyclooxygenase-2
CSF	Colony stimulating factor
CVD	Cardiovascular disease
DAMP	Damage-associated molecular pattern
DC	Dendritic cell
DHA	Docosahexaenoic acid
EGCG	Epigallocatechin gallate
EPA	Eicosapentaenoic acid
ERK	Extracellular signal-regulated kinase
FA	Fatty acid
FADD	Fas-associated death domain
FFA	Free fatty acid
GSPE	Grape seed procyanidin extract
IFN	Interferon
IKK	Inhibitor of nuclear factor kappa-B kinase
Iκ-B	Inhibitor of nuclear factor kappa-B
IL	Interleukin
IL-1RA	Interleukin 1 receptor antagonist
IL-6R	IL-6 receptor
iNOS	Inducible nitric oxide synthase
IRF3	Interferon regulatory factor 3
JAK	Janus kinase
JNK	c-JUN N-terminal kinase

JUN	Proto-oncogene c-Jun or AP-1
LCω-3PUFA	Long chain omega-3 polyunsaturated fatty acid
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
MAT	Mesenteric adipose tissue
MCP-1	Monocyte chemotactic protein-1
MIF	Macrophage migration inhibitory factor
MKK	Mitogen-activated protein kinase kinase
MUFA	Monounsaturated fatty acid
NEMO	Nuclear factor kappa-B essential modulator
NO	Nitric oxide
NOS	Nitric oxide synthase
NOx	Nitrate and nitrite oxides
NF-κB	Nuclear factor kappa B
NK	Natural killer
PA	Proanthocyanidin
PAI	Plasminogen activator-inhibitor
PAMP	Pathogen-associated molecular pattern
PPAR	Peroxisome proliferator-activated receptor
PUFA	Polyunsaturated fatty acid
REL	Proto-oncogene c-Rel
RES	Resveratrol
ROS	Reactive oxygen species
SAPK	Stress-activated protein kinase
SFA	Saturated fatty acid
SOCS	Suppressor of cytokine signaling
STAT	Signal transducer and activator of transcription
SVF	Stromal vascular fraction
TG	Triglyceride
TH	T helper cells
TIR	IL-1 receptor
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TNFR	Tumor necrosis factor receptor
TRADD	TNF receptor-associated death domain
TRAF2	TNF receptor-associated factor 2

TYK	Tyrosine kinase
WAT	White adipose tissue

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2

INTRODUCTION

2 Introduction

2.1 Inflammation

Inflammation is one of the physiological responses of organisms to harmful physical, chemical or biological stimuli (Monteiro & Azevedo 2010) and is considered to be a localized protective reaction of tissue to irritation, injury or infection (Khan & Khan 2010). Inflammatory responses are usually generated to promote the reestablishment of homeostasis, and these responses involve the coordinated action of many cell types and mediators whose role depends on the nature of the inflammatory insult.

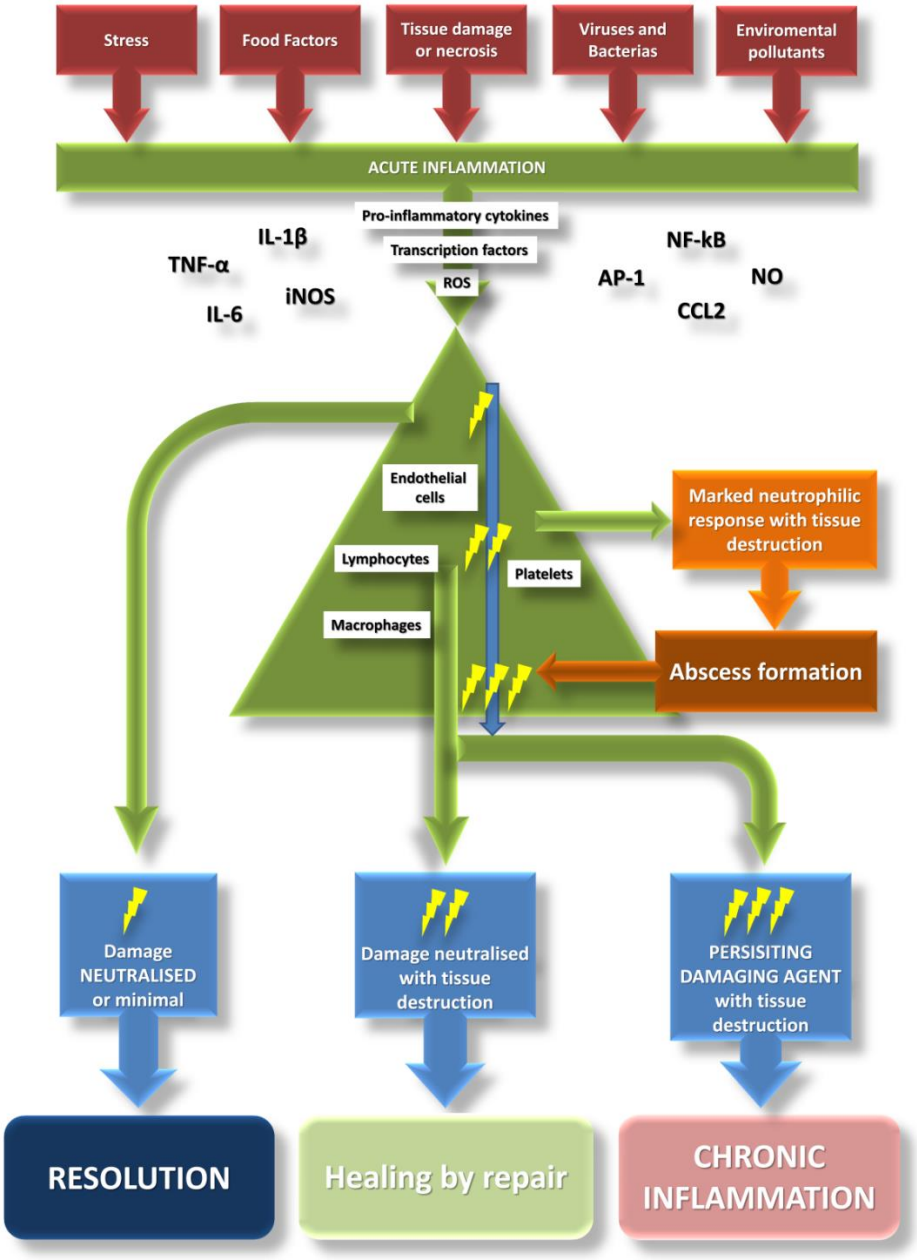


Figure 1. Schematic representation of the different inflammation processes.

The normal acute inflammatory response involves the delivery of plasma components and leucocytes to the site of insult and is initiated by tissue-resident macrophages and mast cells. This response leads to the activation in these cells of several transcription factors such as the mitogen-activated protein kinase (MAPK), the janus kinase/signal transducer and activator of transcription (JAK/STAT) and the nuclear factor kappa B (NF-κB). The production of different types of inflammatory mediators such as chemokines, cytokines, vasoactive amines and eicosanoids through these signaling pathways promotes the neutralization of the damage and leads to the resolution of inflammation (Monteiro & Azevedo 2010). However, if the immune cells cannot completely eliminate the agent responsible for the inflammation or if tissue damage is persistent, then the inflammation enters a chronic state (Fig. 1).

In the context of inflammation, there are several molecules in the typical human diet that can help to minimize, reduce, palliate or improve the proinflammatory states promoted by infections, stress, malnutrition, sedentary habits, smoking or other tissue damage that modify these signaling pathways and the expression and secretion of cytokines and other inflammatory mediators. This study focuses on some of these food compounds that have demonstrated bioactivity against the acute and chronic inflammatory states.

2.2 Bioactive food compounds

In the last few decades, substantial progress has been made to expand our knowledge of bioactive components found in foods and their various associations with human health. Some foods, primarily those of plant and marine origin, contain hundreds of compounds that are not considered typical nutrients but appear to play a role in the maintenance of health (M Blay et al. 2010). These observations suggest that food is a vehicle to provide the body with essential nutrients as well as various compounds that contribute to normal function and can help prevent/ameliorate disease (Fraga & Oteiza 2011). Previous research has promoted the understanding of how certain chemical species interact with cellular components to trigger molecular events that lead to well orchestrated cellular responses. These responses range from changes in cellular metabolism to altering major decisions regarding cell fate (Fraga & Oteiza 2011). This study focuses on the anti-inflammatory effects and the associated mechanisms of two types of molecules on effector cells and several diseases related to inflammation: flavonoids and polyunsaturated fatty acids (PUFAs).

2.2.1 Polyphenols

2.2.1.1 Classification and structure

The prefix “Phyto-“ from phytochemical is derived from the Greek word phyto, which means plant. Therefore, phytochemicals are plant chemicals. Phytochemicals can be classified as carotenoids, phenolics, alkaloids, nitrogen-containing compounds, and organosulfur compounds. The most studied of the phytochemicals are the phenolics and carotenoids (Liu 2004), and over 8000 phenolic compounds have been isolated from various natural products (Erdman et al. 2007).

Phenolics are compounds possessing one or more aromatic rings with one or more hydroxyl groups and are generally categorized as phenolic acids, flavonoids, stilbenes, coumarins, and tannins (Erdman et al. 2007)·(Shoji et al. 2006) (Fig. 2). Each group is further divided into subgroups on the basis of their chemical structure. In the flavonoid group, more than 5000 distinct molecules have been identified (Erdman et al. 2007) and divided into various subclasses among which are the flavones, flavonols, flavanols, flavanones, isoflavones and anthocyanidins (Ding et al. 2006) (Fig. 2).

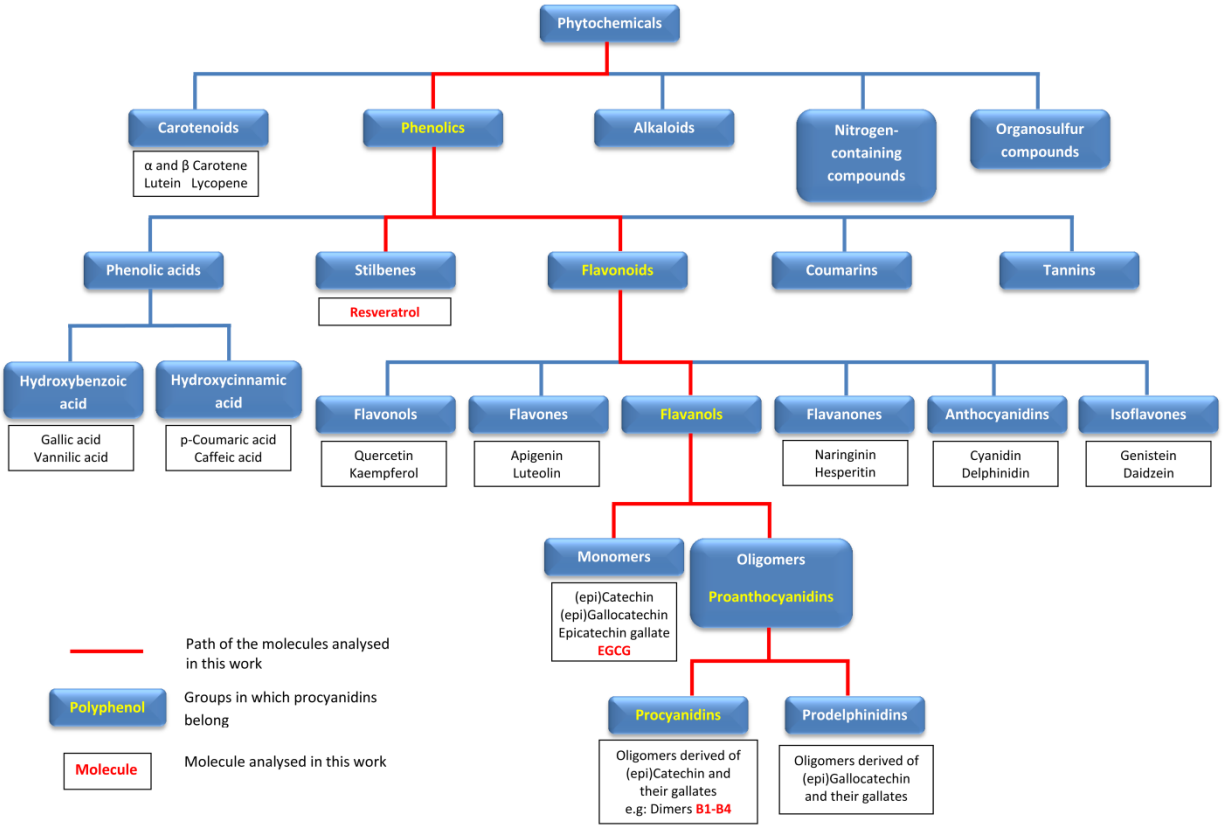
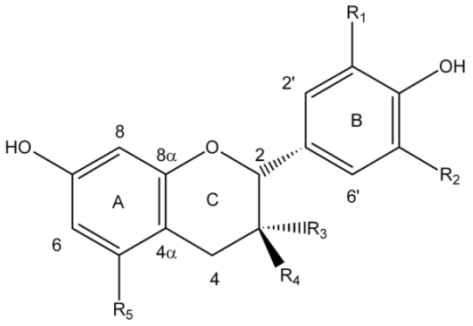


Figure 2. Classification of phytochemicals (based on (Liu 2004)·(Ding et al. 2006)).

Proanthocyanidins (PAs) belong to the flavanol group and are among the most abundant polyphenols (Shoji et al. 2006). Monomeric flavanols or catechins (flavan-3-ols) are biosynthetic precursors of PAs and are characterized by possessing a C6-C3-C6 skeleton with a hydroxyl group in position three of the C-ring (Fig. 3). Flavan-3-ols represent the largest class of monomeric C6-C3-C6 flavanols (de Pascual-Teresa et al. 2010).

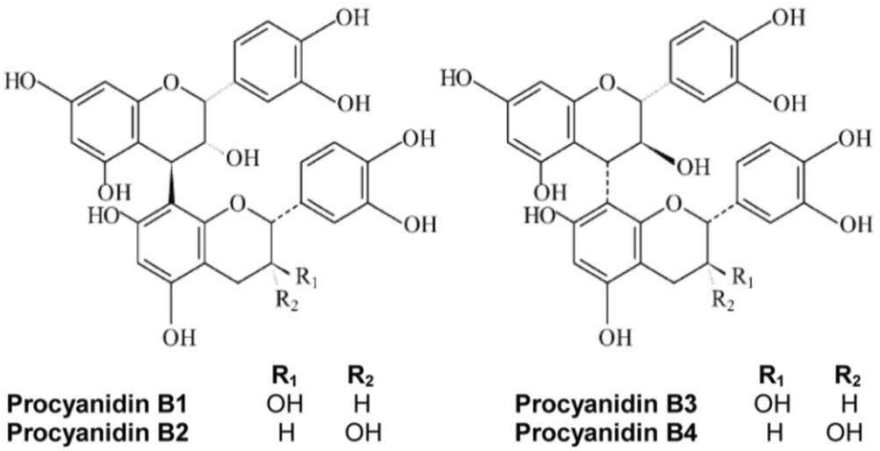
The two compounds catechin and epicatechin are among the most common flavonoids, but gallocatechin, epigallocatechin and their galloyl substituted derivatives epicatechin gallate and epigallocatechin gallate (EGCG) are also well known (Fig. 3). On the other hand, flavanols are commonly found in plant-derived food products in their polymerized forms as oligomers (dimers to pentamers) or polymers (six or more units), in which form these molecules are considered PAs. If the molecules are composed of catechin or epicatechin units, then they are named procyanidins, whereas if the molecules are composed of gallocatechin units, then they are named prodelphinidins.



2*R* Flavan-3-ol Monomers	R_1	R_2	R_3	R_4	R_5
(+)-afzelechin	H	H	H	OH	OH
(-)-epiafzelechin	H	H	OH	H	OH
(+)-catechin	H	OH	H	OH	OH
(-)-epicatechin	H	OH	OH	H	OH
(+)-gallocatechin	OH	OH	H	OH	OH
(-)-epigallocatechin	OH	OH	OH	H	OH
(+)-fisetinidol	H	OH	H	OH	H
(-)-epifisetinidol	H	OH	OH	H	H
(+)-robinetinidol	OH	OH	H	OH	H

Figure 3. Structure of flavan-3-ol monomers (Aron & Kennedy 2008).

In addition, there are other compounds that belong to the PAs; however, the most common oligomers are the B series (Fig. 4) B1 to B8, which are formed by two flavanol units, either catechin or epicatechin, joined by a C4-C8 linkage (B1 to B4) [4 → 8 bond (epicatechin-(4β → 8)-catechin); or C4–C6 linkage (B5 to B8) (de Pascual-Teresa et al. 2010).



	R_1	R_2
Procyanidin B1	OH	H
Procyanidin B2	H	OH

	R_1	R_2
Procyanidin B3	OH	H
Procyanidin B4	H	OH

Figure 4. Skeletal structure of dimeric procyanidins (B1, B2, B3 and B4) (Fine 2000).

Moreover, one of the most studied polyphenols is resveratrol (RES). RES (3,5,4'-trihydroxystilbene) belongs to the stilbenes group and is produced by plants such as grapevines, pines, and legumes (Burns et al. 2002).

Most of the beneficial properties that all of these polyphenols exert are produced by their structural conformation, having the capacity to affect signal transduction pathways, modulate endocrine systems, and alter the effect of hormones as a result of their binding to metal ions and enzyme cofactors (Afaq & Katiyar 2011; Perron & Brumaghim 2009; Vauzour et al. 2010; Kao et al. 2000).

2.2.1.2 Presence in food and consumption

The PAs form one of the most abundant subclasses of phenolic compounds found in the human diet (Tab. 1). Many regularly consumed foods and beverages contain high amounts of PAs such as apples, berries, nuts, chocolate, wine, and cranberry juice (Appeldoorn et al. 2009). Consumption of total catechins and dimeric procyanidins has been estimated to be between 18 and 50 mg per day in the Spanish population and 50 mg (s.d. 56 mg/day) of catechins per day in a nationwide dietary survey of 6,200 Dutch men and women aged 1–97 years. Catechin intake increased with age, and catechin intake was higher in women (60 mg/day) than in men (40 mg/day) (de Pascual-Teresa et al. 2010).

On the other hand, the major dietary sources of stilbenes include grapes, wine, soy, peanuts, and peanut products; however, stilbenes may also be ingested through herbal remedies (Burns et al. 2002). Thus, one of the many reasons that we chose to study these flavonoids was their availability in many foods, by increasing the consumption of a particular food to combat certain inflammatory diseases.

Concentration of PAs in common foods[1,2]

No.	Food	Monomers	Dimers	Trimers	4–6 mers	7–10 mers	>10 mers	Total PAs	Moisture %	Type
		mg/100 g (fresh weight foods) *mg/L* (beverages)								
	Fruits									
1a	Blueberries, cultivated highbush	4.0 ± 1.5	7.2 ± 1.8	5.4 ± 1.2	19.6 ± 3.4	14.5 ± 2.0	129.0 ± 47.3	179.8 ± 50.8	85.0	PC
1b	Blueberries, lowbush	3.4 ± 0.5	9.0 ± 0.5	6.8 ± 0.4	25.7 ± 1.2	27.8 ± 1.3	260.4 ± 11.7	331.9 ± 14.0	85.0	PC
2	Cranberries	7.3 ± 1.5	25.9 ± 6.1	18.9 ± 3.4	70.3 ± 13.1	62.9 ± 14.7	233.5 ± 49.1	418.8 ± 75.3	87.2	A, PC
3	Blackberries	3.7 ± 2.2	6.7 ± 2.9	3.6 ± 1.9	7.3 ± 5.0	4.2 ± 4.5	1.5 ± 0.0	27.0 ± 17.5	86.9	PC
4	Marion berries	0.9 ± 0.0	3.4 ± 0.1	2.4 ± 0.0	2.2 ± 0.0	ND	ND	8.9 ± 0.1	86.9	PC
5	Choke berries	5.2 ± 0.2	12.5 ± 0.4	10.3 ± 0.3	40.3 ± 0.8	52.9 ± 3.1	542.6 ± 42.9	663.7 ± 47.7	71.8	PC
6	Raspberries	4.4 ± 3.4	11.5 ± 9.8	5.7 ± 5.5	7.7 ± 5.2	0.9 ± 2.2	ND	30.2 ± 23.4	85.8	PP, PC
7	Strawberries	4.2 ± 0.7	6.5 ± 1.3	6.5 ± 1.2	28.1 ± 6.5	23.9 ± 3.5	75.8 ± 13.4	145.0 ± 24.9	91.1	PP, PC
8	Blackcurrants	0.9 ± 0.2	2.9 ± 0.4	3.0 ± 0.3	10.6 ± 1.7	9.9 ± 1.4	122.4 ± 28.0	147.8 ± 33.0	79.4	PC, PD
9	Cherries	4.2 ± 1.1	2.8 ± 0.7	2.8 ± 0.5	6.5 ± 0.8	1.9 ± 0.1	ND	8.2 ± 03.2	80.2	PC
10a	Green grapes	1.0 ± 0.1	2.3 ± 0.0	1.9 ± 0.1	8.4 ± 0.3	9.2 ± 0.6	58.9 ± 14.2	81.5 ± 15.0	80.7	PC, PD
10b	Red grapes	0.8 ± 0.2	2.0 ± 0.3	1.5 ± 0.2	6.1 ± 0.9	6.2 ± 1.1	44.6 ± 9.9	61.0 ± 12.3	80.4	PC, PD
10c	Grape seed (dry)	660.3 ± 8.3	417.3 ± 4.8	290.2 ± 4.5	664.0 ± 8.2	400.3 ± 31.3	1100.1 ± 86.3	3532.3 ± 105.8		PC
11a	Apple, red delicious, with peel	9.6 ± 0.9	13.8 ± 0.6	9.3 ± 0.4	30.2 ± 1.2	25.4 ± 1.2	37.6 ± 2.6	125.8 ± 6.8	86.1	PC
11b	Apple, red delicious without peel	6.8 ± 0.9	11.3 ± 1.6	7.2 ± 1.0	24.3 ± 3.4	20.3 ± 3.1	28.7 ± 7.1	98.7 ± 17.0	86.7	PC
11c	Apple, golden delicious, with peel	4.7 ± 0.2	10.2 ± 0.2	6.3 ± 0.1	22.8 ± 0.6	19.5 ± 0.7	27.7 ± 2.9	91.1 ± 4.7	87.0	PC
11d	Apple, golden delicious, without peel	4.1 ± 0.1	9.4 ± 0.5	5.8 ± 0.5	21.2 ± 2.6	17.5 ± 3.2	22.4 ± 5.7	80.4 ± 12.4	86.9	PC
11e	Apple, granny smith	7.5 ± 1.0	15.0 ± 2.3	9.1 ± 1.5	32.9 ± 5.9	30.1 ± 6.1	46.3 ± 9.5	141.0 ± 26.1	85.7	PC
11f	Apple, gala	5.9 ± 0.4	9.5 ± 0.3	6.2 ± 0.2	21.3 ± 1.5	18.7 ± 1.4	30.7 ± 5.6	92.4 ± 8.4	86.0	PC
11g	Apple, fuji	6.5 ± 1.7	9.9 ± 2.6	6.1 ± 1.4	19.1 ± 4.3	13.8 ± 2.8	14.2 ± 3.1	69.6 ± 15.8	84.2	PC
11h	Apple sauce	2.3 ± 0.0	6.0 ± 0.0	3.0 ± 0.0	10.7 ± 0.1	8.3 ± 0.1	16.9 ± 0.4	47.2 ± 0.6	88.3	PC
12a	Peaches	4.7 ± 1.4	7.0 ± 2.2	5.0 ± 1.4	17.7 ± 5.5	10.9 ± 3.7	22.0 ± 7.7	67.3 ± 20.9	88.3	PC
12b	Peach, canned heavy syrup	0.6 ± 0.1	2.3 ± 0.6	ND	ND	ND	ND	2.9 ± 0.6	79.3	PC
13a	Pears, green cultivars	2.0 ± 0.3	2.7 ± 0.4	2.0 ± 0.3	6.0 ± 1.1	5.4 ± 1.4	24.2 ± 15.3	42.3 ± 18.6	83.4	PC
13b	Pears	2.7 ± 1.5	2.8 ± 1.3	2.3 ± 0.9	6.5 ± 1.9	4.6 ± 1.0	13.1 ± 11.3	31.9 ± 7.8	83.4	PC
14	Nectarines	1.9 ± 1.2	2.3 ± 1.2	1.7 ± 0.8	6.0 ± 3.0	3.6 ± 1.9	7.3 ± 6.5	22.8 ± 14.6	89.0	PC
15a	Black plums	6.8 ± 0.1	16.0 ± 0.4	14.9 ± 0.3	49.9 ± 0.1	34.9 ± 0.2	115.3 ± 2.0	237.9 ± 3.1	87.9	A, PC
15b	Plums, black diamond	9.9 ± 0.6	23.4 ± 1.6	22.8 ± 1.4	64.7 ± 4.3	41.2 ± 2.1	94.6 ± 8.7	256.6 ± 18.7	87.9	A, PC
15c	Plums	11.4 ± 3.4	31.5 ± 7.4	23.9 ± 5.1	58.0 ± 12.5	33.8 ± 11.9	57.3 ± 24.4	215.9 ± 50.7	87.4	A, PC
16	Apricots	2.8 ± 0.0	3.1 ± 0.0	1.9 ± 0.0	4.9 ± 0.1	2.2 ± 0.0	0.8 ± 0.2	15.6 ± 0.4	86.3	PC
17a	Kiwis, gold	1.1 ± 0.1	1.6 ± 0.1	1.2 ± 0.0	5.0 ± 0.1	5.0 ± 0.2	ND	13.9 ± 0.4	85.0	PC
17b	Kiwis	0.6 ± 0.5	0.8 ± 0.1	0.7 ± 0.0	1.3 ± 0.8	0.2 ± 0.0	ND	3.7 ± 1.6	83.9	PC
18	Avocados	1.0 ± 0.8	1.5 ± 0.8	1.4 ± 0.4	3.2 ± 0.8	0.4 ± 0.7	ND	7.4 ± 4.3	72.0	A, PC
19	Mangos	2.3 ± 0.1	1.8 ± 0.0	1.4 ± 0.0	7.2 ± 0.5	ND	ND	12.8 ± 0.5	81.7	PC
20	Dates, Deglet Noor (fresh)	ND	1.8 ± 0.5	3.0 ± 0.5	5.9 ± 0.7	ND	ND	10.7 ± 1.6	22.5	PC
21	Bananas	0.2 ± 0.0	0.7 ± 0.1	0.8 ± 0.1	2.3 ± 0.4	ND	ND	4.0 ± 0.6	73.5	PC
	Vegetable									
22	Indian squash, raw	1.6 ± 0.2	2.0 ± 0.2	1.5 ± 0.1	4.6 ± 0.4	3.2 ± 0.3	3.5 ± 0.4	16.4 ± 1.6	93.3	PC
	Cereals and beans									
23a	Sorghum, sumac bran	27.8 ± 1.2	78.2 ± 3.4	99.2 ± 7.7	585.5 ± 50.0	734.3 ± 69.3	2440.4 ± 271.0	3965.4 ± 402.5	9.2	PC
23b	Sorghum, sumac whole grain	18.0 ± 0.1	35.4 ± 0.4	45.6 ± 0.7	224.0 ± 9.2	289.2 ± 5.9	1307.3 ± 34.6	1919.5 ± 45.5	9.2	PC
23c	Sorghum, hi-tannin whole grain	0.9 ± 0.2	8.0 ± 1.1	10.3 ± 1.1	85.1 ± 14.4	150.0 ± 26.2	1533.3 ± 395.01	787.6 ± 438.1	9.2	PC
23d	Sorghum, hi-tannin whole grain extrudate	7.6 ± 0.0	23.5 ± 0.1	21.2 ± 0.0	80.6 ± 0.3	76.1 ± 0.1	238.3 ± 2.1	447.3 ± 1.8	9.2	PC
24a	Pinto beans, raw	14.8 ± 0.9	32.0 ± 2.6	28.3 ± 2.1	125.9 ± 9.2	135.6 ± 10.4	459.6 ± 34.2	796.3 ± 58.7	11.0	PP, PC
24b	Pinto beans, simmered 2 h	1.7 ± 0.0	4.4 ± 0.4	3.9 ± 0.3	10.5 ± 5.5	4.3 ± 3.9	1.4 ± 0.6	26.3 ± 12.8	11.0	PP, PC
25	Small red beans	10.6 ± 0.0	19.4 ± 0.8	18.1 ± 0.6	80.0 ± 2.7	75.7 ± 2.4	252.9 ± 0.8	456.6 ± 7.5	12.0	PP, PC
26	Red kidney beans	21.9 ± 0.2	26.4 ± 0.7	29.1 ± 0.7	117.7 ± 2.8	105.3 ± 2.2	263.4 ± 4.1	563.8 ± 10.4	12.0	PP, PC
27	Barley	11.0 ± 0.3	21.4 ± 1.1	14.6 ± 1.0	27.2 ± 0.6	ND	ND	74.2 ± 3.0	9.4	PC
28	Black eye peas	14.0 ± 4.4	6.0 ± 0.2	6.1 ± 0.1	7.3 ± 0.3	ND	ND	33.3 ± 4.2	12.0	PC
29	Black beans	2.9 ± 0.0	5.2 ± 0.1	ND	ND	ND	ND	8.1 ± 0.1	11.0	PC
	Nuts									
30	Hazelnuts	9.8 ± 1.6	12.5 ± 3.8	13.6 ± 3.9	67.7 ± 20.3	74.6 ± 21.9	322.4 ± 102.5	500.7 ± 152.0	5.3	PC, PD
31	Pecans	17.2 ± 2.5	42.1 ± 5.4	26.0 ± 2.0	101.4 ± 10.4	84.2 ± 12.9	223.0 ± 59.1	494.1 ± 86.2	3.5	PC, PD
32	Pistachios	10.9 ± 4.3	13.3 ± 1.8	10.5 ± 1.2	42.2 ± 5.2	37.9 ± 4.9	122.5 ± 37.1	237.3 ± 52.0	4.0	PC, PD
33	Almonds	7.8 ± 0.9	9.5 ± 1.6	8.8 ± 1.7	40.0 ± 8.5	37.7 ± 8.4	80.3 ± 28.1	184.0 ± 48.2	5.2	PP, PC
34	Walnuts	6.9 ± 3.4	5.6 ± 0.9	7.2 ± 1.2	22.1 ± 3.3	5.4 ± 0.8	20.0 ± 9.3	67.3 ± 14.7	4.1	PC
35	Peanuts, roasted	5.1 ± 1.0	4.1 ± 0.7	3.7 ± 0.5	2.8 ± 0.2	ND	ND	15.6 ± 2.3	2.0	A, PC
35	Peanut butter	2.0 ± 0.9	3.0 ± 0.7	8.1 ± 3.5	ND	ND	ND	13.2 ± 5.2	1.3	A, PC
36	Cashews	6.7 ± 2.9	2.0 ± 0.4	ND	ND	ND	ND	8.7 ± 3.2	5.2	PC
	Beverages and snacks									
37	Baking chocolate, unsweetened	198.5 ± 3.0	206.5 ± 15.4	130.9 ± 15.0	332.6 ± 58.4	216.4 ± 56.0	551.0 ± 186.8	1635.9 ± 334.6	2.4	PC
37	Black chocolate	31.4 ± 0.2	31.2 ± 0.9	21.1 ± 0.8	55.5 ± 3.5	38.5 ± 3.0	68.2 ± 8.8	246.0 ± 0.3	1.3	PC
37	Milk chocolate	26.9 ± 3.0	26.2 ± 2.5	19.3 ± 2.6	51.4 ± 9.8	35.3 ± 7.2	32.8 ± 9.2	192.0 ± 28.8	1.3	PC
37	Chocolate milk	4 ± 1	22 ± 0	ND	ND	ND	ND	26 ± 2	82.3	PC
38	Red wine	20 ± 1	40 ± 1	27 ± 1	67 ± 2	50 ± 1	110 ± 2	313 ± 5	88.5	PC, PD
39	Beer	4 ± 0	11 ± 1	3 ± 0	4 ± 0	ND	ND	23 ± 2	95.2	PC, PD
2b	Cranberry juice cocktail	6 ± 0	29 ± 0	17 ± 0	49 ± 1	41 ± 1	89 ± 3	231 ± 2	85.5	A, PC
10d	Grape juice	18 ± 0	34 ± 0	19 ± 0	80 ± 0	69 ± 0	303 ± 2	524 ± 2	87.0	PC, PD
11i	Apple juice	1 ± 0	2 ± 0	1 ± 0	4 ± 0	1 ± 0		9 ± 0	87.9	PC
	Spices									
40	Cinnamon, ground	23.9 ± 1.3	256.3 ± 11.3	1252.2 ± 62.2	2608.6 ± 140.3	1458.3 ± 116.1	2508.8 ± 92.9	8108.2 ± 424.2	9.5	A, PP, PC
41	Curry powder	ND	9.5 ± 0.2	22.9 ± 0.5	41.8 ± 1.5	ND	ND	74.2 ± 2.2	9.5	A, PC

[1] Values are means ± SD, $n = 4–8$.
[2] Monomers, dimers, and trimers are listed separately. Tetramers through hexamers are pooled together as 4–6 mers. Polymers with DP > 10 are quantified collectively and listed as >10 mers. The moisture contents of the fresh fruits and the type of PAs are also presented, so that the PA contents can be converted to a dry weight basis.
Abbreviations and symbols: ND, not detected. The PP, PC, and PD are propelargonidins, procyanidins, and prodelphinidins, respectively. "A" indicates the existence of A-type PAs. The same number indicates the same kind of food, with different varieties within one kind of food labeled a, b, c, d, and so on.

Table 1. Concentrations of proanthocyanidins in food (Rasmussen et al. 2005)[,](Gu et al. 2004).

2.2.1.3 Polyphenols and inflammation

Phenolics are the products of secondary metabolism in plants serving essential roles in the reproduction and the growth of plants, acting as defense mechanisms against pathogens, parasites and predators, and contributing to the color of plants. In addition to their roles in plants, phenolic compounds in the human diet may provide health benefits associated with reduced risk of chronic diseases (Liu 2004) such as cancers, coronary artery disease, stroke, obesity and diabetes (Heiss et al. 2010).

The possibility that plant-derived compounds could interact with cells and affect cell signaling has received much attention. Flavonoids are one example of these plant compounds, and flavanols are a subgroup of flavonoids that have been associated with human health benefits (Fraga & Oteiza 2011).

For a long time, the biological activity of various plant polyphenols has been attributed to their antioxidant effects and/or their propensity to form precipitated complexes with proteins in a rather nonspecific manner. Today, there is compelling evidence that strongly suggests that the mechanisms by which plant polyphenols exert their protective actions are not exclusive to their redox properties, but rather to their ability to directly bind to target proteins (or peptides). Such a mode of action would result in the inhibition of key enzymes, the modulation of cell receptors or transcription factors, as well as the perturbation of protein (or peptide) aggregates. These effects could affect various signal transduction pathways, which could in turn regulate several cell functions including those involved in growth and proliferation, inflammation, apoptosis, angiogenesis, metastasis, and immune responses (Quideau et al. 2011).

The human diet contains a mixture of phenolic compounds and it is likely that the anti-inflammatory effects may be due not to one molecule but to the synergistic effects of the polyphenols (Terra et al. 2011). Therefore, plant or fruit extracts are used in studies *in vitro* and *in vivo* to determine the potential anti-inflammatory activity of their compounds in mixture, besides assessing their use as functional foods. In fact, several plant extracts derived from ginger, licorice, pomegranate or nutmeg have been exhibited anti-inflammatory activity by elevating anti-inflammatory IL-10 production, reducing pro-inflammatory IL-6 or TNF-α production, or reducing the expression of iNOS and COX-2 in several studies (Mueller et al. 2010). These effects may be due to the bioactive compounds present in the extracts working synergistically, enlarging the individual anti-inflammatory action of these molecules. In the recent years, different extracts from cocoa and grape seed containing large amounts of proanthocyanidins have been extensively used for their well-known antioxidant and anti-inflammatory bioactivities. These studies reported anti-inflammatory effects in macrophages

and adipocytes *in vitro* (Chacón et al. 2009; Ono et al. 2003; Ramiro et al. 2005), in various *in vivo* models of local inflammation such as arthritis or colitis (Andújar et al. 2011; Cho et al. 2009), as well as in other diseases in which inflammation has a role such as type II diabetes, CVD or Alzheimer (Kar et al. 2009; Mellor et al. 2010; Monagas et al. 2009; Wang et al. 2009). In these conditions, cocoa and grape seed extracts decrease several pro-inflammatory cytokines such as TNF-α, IL-1, IL-6, chemokines such as MCP-1 and other inflammatory mediators such as NO.

In the present work, we used a grape seed procyanidin extract (GSPE) composed by a mixture of flavonoids containing various polymeric types of procyanidins, predominantly dimeric fraction, and various monomers of flavanol (Tab. 2). This extract has also been shown to possess several bioactivities. GSPE improves antioxidant cell defenses and the plasma lipid profile, while limiting adipogenesis. Additionally, GSPE has been shown to function as an insulin-mimetic and an anti-inflammatory agent (Serra et al. 2010). In fact, this extract functions as powerful antioxidant and exert anti-inflammatory activities *in vitro* and *in vivo* (Terra et al. 2007; Terra, Montagut, et al. 2009; Terra, Fernández-Larrea, et al. 2009; Terra et al. 2011). The mechanisms of their anti-inflammatory action remain poorly understood; however, several studies suggest that is related to oxygen free-radical scavenging, antilipid peroxidation, and the inhibition of inflammatory cytokine secretion as well as alterations in cell membrane receptors, intracellular signaling pathway proteins and gene expression and enzyme activity (Kris-Etherton et al. 2004).

Flavanol	**mg/g extract (GSPE)**
Catechin	16.84
Epicatechin	15.09
Epicatechin gallate	39.37
Epigallocatechin gallate	0.64
Epigallocatechin	1.68
Dimer	144.63
Trimer	13.59
Tetramer	10.16
Pentamer	1.05

Table 2. Composition of grape seed extract (GSPE) used in this work (Serra et al. 2010).

In this context, the mechanisms of action of GSPE could be elucidated by assessing their anti-inflammatory effects in models of chronic and acute inflammation *in vivo*. Analyzing several markers in different target tissues can help to evince their anti-inflammatory role.

Despite working with extracts is useful to evaluate the synergistic effects of the compounds present in food, it is also important to identify which components of the extract exert these effects and their mechanisms of action. In this way, various studies have shown that dimeric procyanidins such as B1, B2, B3 and B4 have anti-inflammatory effects on murine macrophages (Montagut et al. 2009; Terra et al. 2007), Similarly, resveratrol (RES) exerts anti-inflammatory effects in several conditions and models, reducing the expression of proinflammatory markers and inflammatory signaling factors such as NF-κB (Sánchez-Fidalgo et al. 2010; de la Lastra & Villegas 2005; de la Lastra & Villegas 2007; Tsai et al. 1999). However, the mechanisms by which these molecules exert their effects, as well as the synergistic effects that they can exert in combination with other bioactive molecules are still unknown.

2.2.2 Polyunsaturated fatty acids (PUFAs)

2.2.2.1 Classification and structure

Fatty acids (FAs) are long aliphatic carbon chains that vary in their length, degree of unsaturation, and structure. PUFAs contain two or more double bonds that are classified as omega-3 (ω-3) and omega-6 (ω-6) based on the location of the final double bond relative to the terminal methyl end of the molecule (Wall et al. 2010) (Fig. 5 and 6). The human body can produce all but two of the FAs required for growth: linoleic acid (LA, 18:2ω-6) and α-linolenic acid (ALA, 18:3ω-3).

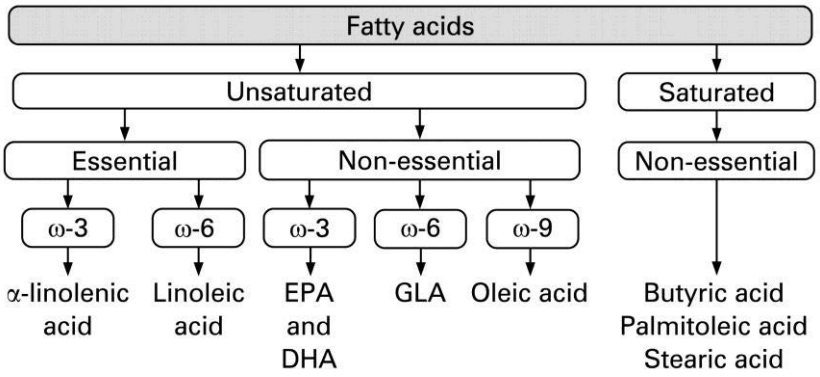


Figure 5. Simple classification of fatty acids (Yashodhara et al. 2009).

These essential plant-derived C18 PUFAs (18-carbon FAs with two or more double bonds) are precursors for the long chain (LC) PUFA of the ω-6 group arachidonic acid (ARA, 20:4ω-6) as well as for the LC PUFAs of the ω-3 group eicosapentaenoic acid (EPA, 20:5ω-3) and

docosahexaenoic acid (DHA, 22:6ω-3), which are synthesized via alternating desaturation and elongation steps (Khozin-Goldberg et al. 2011) (Fig. 7).

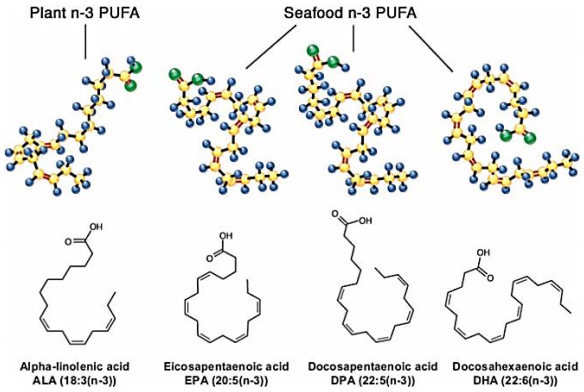


Figure 6. Skeletal structure and 3-dimensional configuration of major (n-3) PUFAs (Mozaffarian & Wu 2012)

In fact, the FAs EPA, DHA, dihomo-gamma linolenic acid (DGLA, 20:3ω-6), and ARA are the most metabolically significant (Ortega et al. 2012).

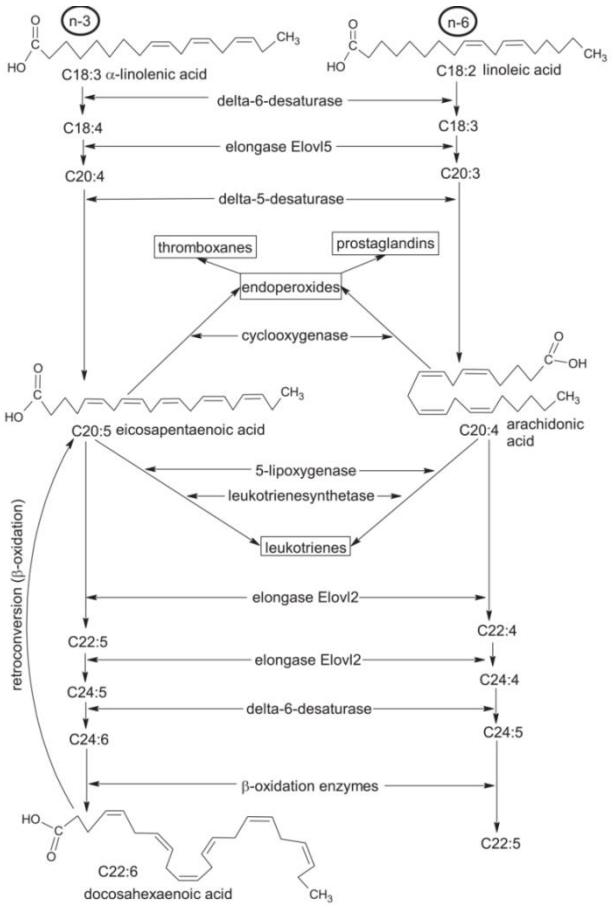


Figure 7. Metabolism of omega-3 (ω-3) and omega-6 (ω-6) fatty acids (Komprda 2012).

Intrinsically by their structure, PUFAs constitute an important component of all cell membranes and influence membrane fluidity and the behavior of membrane-bound enzymes and receptors (Wall et al. 2010). Thereby, PUFAs regulate a wide range of functions in the body.

2.2.2.2 PUFAs in food and consumption

The two main ω-3 PUFAs are EPA and DHA, and these FAs are found predominantly in oily fish such as salmon, mackerel and herring; however, they are also present in many other sources of seafood (Tab. 3).

Common Dietary Sources	EPA, mg/100 g	DPA, mg/100 g	DHA, mg/100 g	EPA+DHA, mg/100 g	Common Dietary Sources	ALA, g/100 g
Anchovy	763	41	1,292	2,055	Flaxseed (linseed) oil	53.3
Herring, Atlantic	909	71	1,105	2,014	Canola (rapeseed oil)	9.1
Salmon, farmed	862	393	1,104	1,966	Walnuts, English	9.1
Salmon, wild	411	368	1,429	1,840	Butternuts	8.7
Mackerel, Atlantic	504	106	699	1,203	Soybean oil, nonhydrogenated	6.8
Bluefish	323	79	665	988	Mustard oil	5.9
Sardines, Atlantic	473	0	509	982	Soybean oil, hydrogenated	2.6
Trout	259	235	677	936	Walnuts, black	2.0
Golden bass (tilefish)	172	143	733	905	Beechnuts	1.7
Swordfish	127	168	772	899	Pecans	1.0
Tuna, white (albacore)	233	18	629	862	Seaweed, Spirulina, dried	0.8
Mussels	276	44	506	782	Soybeans, boiled	0.6
Striped bass	169	0	585	754	Navy beans, boiled	0.2
Shark	258	89	431	689	Kale, raw	0.2
Pollock, Atlantic	91	28	451	542	Kidney beans, boiled	0.1
Oysters, wild	274	16	210	484		
King Mackerel	174	22	227	401		
Tuna, light (skipjack)	91	17	237	328		
Snapper	48	22	273	321		
Flounder and sole	168	34	132	300		
Clams	138	104	146	284		
Grouper	35	17	213	248		
Halibut	80	20	155	235		
Lobster	117	6	78	195		
Scallops	72	5	104	176		
Blue Crab	101	9	67	168		
Cod, Pacific	42	5	118	160		
Shrimp	50	5	52	102		
Catfish, farmed	20	18	69	89		
Eggs	0	7	58	58		
Chicken breast	10	10	20	30		
Beef	2	4	1	3		
Pork	0	10	2	2		

Data from the U.S. Department of Agriculture National Nutrition Database for Standard Reference Release 23, 2010 (274). These are average values that might vary due to methodological, geographic, temporal, and sample-to-sample differences.
ALA = alpha-linolenic acid; DHA = docosahexaenoic acid; DPA = docosapentaenoic acid; EPA = eicosapentaenoic acid; PUFA = polyunsaturated fatty acid.

Table 3. Food sources for major long chain n-3 PUFAs (Mozaffarian & Wu 2011).

The parent C18 ω-3 PUFA ALA is found in vegetable oil; however, the rate of conversion from ALA to EPA and DHA in humans is not very efficient, and humans have no other means of synthesizing ω-3 PUFAs. Therefore, EPA and DHA are classified as ‘essential’ nutrients (Kremmyda et al. 2011)[,](Hull 2011).

A general consensus exists on the recommendation of the consumption of 500 mg of LCω-3PUFA per day and a separate recommendation of 200 mg of DHA per day for pregnant and lactating women. Apart from the Inuit of Nunavik and the Japanese, the average human does not meet the recommended intake of fish and LCω-3PUFA for optimal health. Fish and other seafood consumption is the easiest way to meet these recommended intakes, but the appropriate intake of PUFAs can also be achieved by the consumption of foods enriched with LCω-3PUFA. In conclusion, most people do not consume enough LCω-3PUFA for optimal health (Meyer 2011).

Many results have shown that PUFA long-term supplementation is associated with a significant reduction in blood pressure. Additionally, PUFA consumption has beneficial effects on coronary heart disease, partially mediated by the positive effects on blood pressure and through the modulation of signaling pathways involved in lipid oxidation and synthesis. Weight loss programs promoting fatty fish intake have shown increased benefits on lipid profiles, blood pressure and insulin levels (Abete et al. 2011).

2.2.2.3 PUFAs and inflammation

As mentioned above, LC-PUFAs perform vital functions in the human organism. As essential nutrients, LC-PUFAs are of high physiological and therapeutic significance for human well-being (Wall et al. 2010). In fact, dietary consumption of fish oil is increasingly recognized to have beneficial health effects particularly for the prevention or treatment of specific diseases. For example, fish oil intake has been associated with decreased risk for coronary heart disease, and prescription fish oil supplements have been shown to lower serum triglycerides (TGs). There is also emerging evidence that fish oil has immunosuppressive properties, which may have clinical applications for the treatment of symptoms associated with autoimmune and inflammatory diseases (Shaikh 2012).

The consumption of monounsaturated FAs (MUFAs) and PUFAs in place of saturated FAs (SFAs) is considered one of the major worldwide nutritional policies effective for the prevention and control of chronic diseases. Most importantly, MUFA, PUFA, and SFA adjustment may not only influence present health but may also determine whether an individual will develop obesity, diabetes, hypertension, dyslipidemia, cardiovascular disease (CVD), stroke or various types of cancer later in life (Ortega et al. 2012).

In addition, ω-3 PUFA consumption may improve vascular and cardiac hemodynamics, TGs, and possibly endothelial function and inflammation (Mozaffarian & Wu 2011). In fact, PUFAs

appear to play a role in regulating inflammatory responses through the production of the eicosanoid inflammatory mediators. ARA is usually the major substrate for eicosanoid synthesis. However, increased consumption of long chain n-3 PUFAs such as EPA and DHA results in increased proportions of those fatty acids in inflammatory cell phospholipids. As a result of being less substrate available for synthesis of eicosanoids from ARA, the production of PGE2, TXB2, LTB4, 5-hydroxyeicosatetraenoic acid and LTE4 decreased in inflammatory cells (Calder 2008). In this context, EPA and DHA generate E- and D-series resolvins, protectins/neuroprotectins and maresins that have stereochemically defined structures acting via specific G-protein-coupled receptors (GPCRs). These receptors directly affect the expression levels of multiple enzymes, chemokines, cytokines and growth factors providing potent anti-inflammatory and pro-resolving actions (Serhan & Petasis 2011).

For instance, EPA and DHA have important anti-inflammatory properties that modulate adipose tissue (AT) inflammation via the GPR120-mediated suppression of macrophage proinflammatory resolvin cytokine secretion, as well as protectin-mediated resolution of inflammation (Kalupahana et al. 2011)'(González-Périz & Clària 2010). The GPR120 pathway is essential in other anti-inflammatory processes mediated by PUFAs in cells such as macrophages or endothelial cells (Oh et al. 2010; Ringseis & Eder 2010) (Fig. 8).

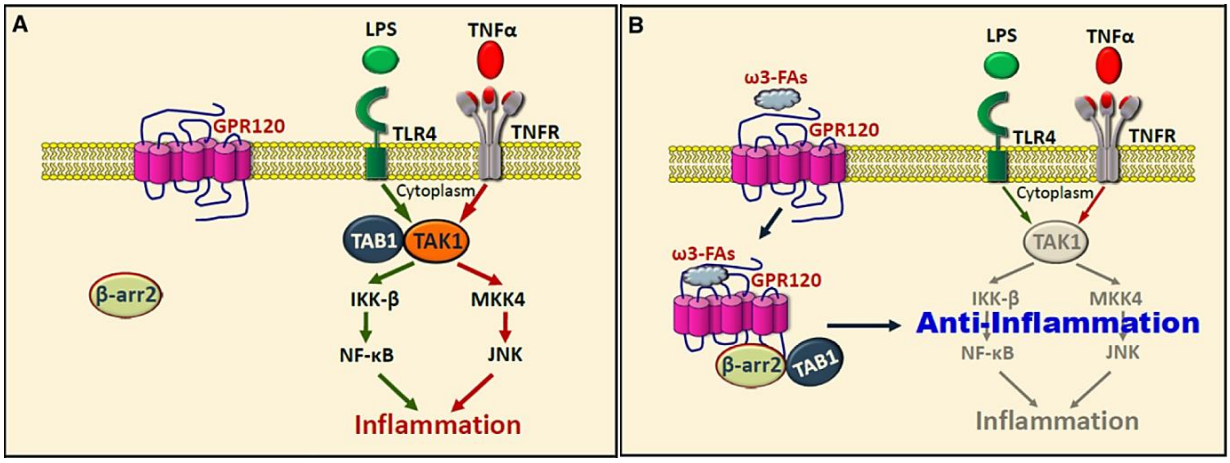


Figure 8. Omega-3 fatty acids and the GPR120 signaling pathway.

Another key anti-inflammatory effect of ω-3 PUFAs is manifested through the modulation of inflammatory gene expression, exerted through decreased activation of the proinflammatory transcription factor NF-κB and increased activation of anti-inflammatory peroxisome proliferator-activated receptor-gamma (PPAR-γ) (Wall et al. 2010). It is well-known that EPA and DHA are putative natural ligands for PPARs and several studies indicate that the anti-inflammatory effects of ω-3 PUFA are PPAR-γ dependent (Tai & Ding 2010). In this context,

the modification of the NF-κB and PPAR-γ transcription factors through ω-3 PUFAs promotes anti-inflammatory actions, including the decrease of leukocyte chemotaxis, adhesion molecule expression and inflammatory cytokine production (Fig. 9). Thus, n-3 PUFAs act via both lipid mediator-related and nonlipid mediator-related mechanisms (Calder 2008).

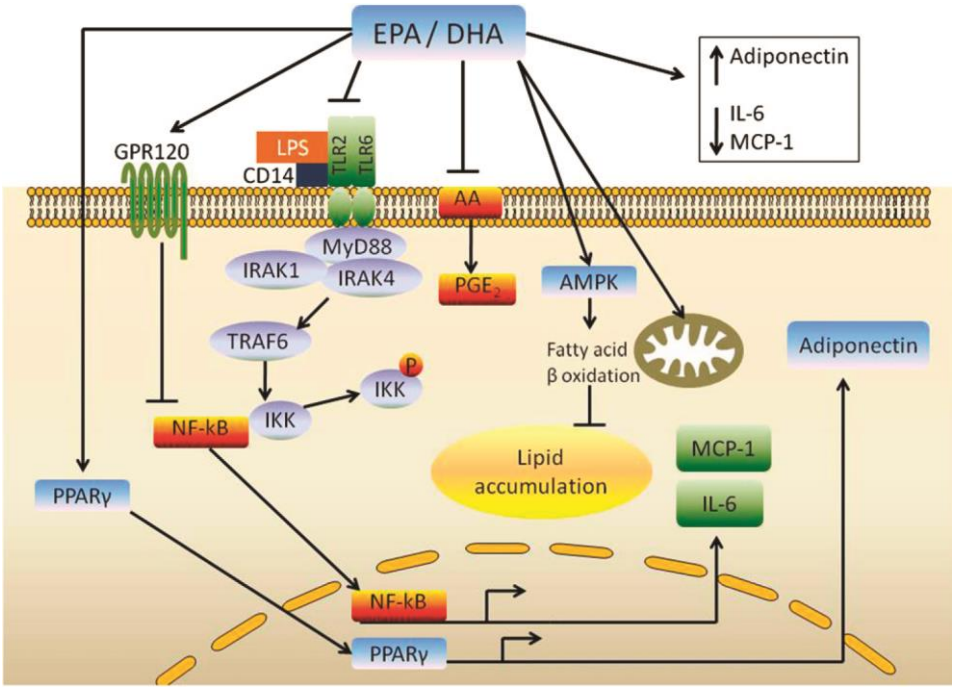


Figure 9. Mechanisms by which EPA and DHA modulate adipose tissue (AT) function (Kalupahana et al. 2011).

In human monocytes, supplemental EPA plus DHA or fish oil inhibit lipopolysaccharide (LPS)-induced cytokine expression (including IL-1β, IL-6 and TNF-α). Furthermore, adipocytes treated with DHA express more anti-inflammatory IL-10 when compared with untreated cells. *In vivo*, the consumption of oils containing ω-3 PUFA resulted in the decreased production of IL-1, IL-6 and TNF-α by murine peritoneal macrophages. In agreement with these animal experiments, fish oil supplementation in humans diminished the ability of peripheral blood monocytes to produce TNF-α, IL-1α and IL-1β (Akhtar Khan 2010). Finally, previous studies have shown that PUFAs have beneficial effects on low-chronic inflammation diseases such as obesity. The beneficial properties of ω-3 PUFAs partially result from the modulation of white adipose tissue (WAT) metabolism and the secretion of bioactive adipokines such as leptin, adiponectin (ADIPQ) and visfatin, which directly regulate nutrient metabolism and insulin sensitivity. In this context, ω-3 PUFAs have been shown to prevent and/or ameliorate inflammation in key metabolic organs including WAT, liver and muscle. Indeed, the ω-3 PUFAs EPA and DHA have been widely reported to have protective effects in a range of

chronic inflammatory conditions including obesity, insulin resistance and CVD (Moreno-Aliaga et al. 2010).

In contrast to this, previous studies have also shown that DHA and EPA enhance TNF-α and IL-6 secretion in macrophages and that dietary fish oil supplementation increases serum TNF-α concentration in response to endotoxin challenges, which supports a role for ω-3 PUFAs as proinflammatory factors (Tai & Ding 2010).

In spite of these controversial results, the interpretation of experimental outcomes can be confounded by the failure to consider the effects of the molecular form and the dose of the FAs used as well as the incorporation of the FAs into discrete intracellular domains (Kim et al. 2010). Nonetheless, when administered at the recommended dose, PUFAs have beneficial effects (Serini et al. 2011).

On the other hand, there are some differences between EPA and DHA in the context of inflammation. These PUFAs can be transformed by various enzymes (COX type 1 and 2 and lipoxygenases (LOXs) type 5, 8, 12 and 15) to final pre-resolving, anti-inflammatory and proinflammatory mediators such as resolvins, protectins, neuroprotectins, maresins and other products. In this context, whereas EPA can derive to some final proinflammatory mediator, DHA mainly results to final pro-resolving and neuroprotector products (Kremmyda et al. 2011). In fact, studies in murine and human macrophages *in vitro* showed that DHA has more potent anti-inflammatory effects than EPA, alleviating pro-inflammatory cytokine production, deactivating intracellular signaling, as well as downregulating proinflammatory genes (Rahman et al. 2008; Weldon et al. 2007). However, the authors indicate that further work is required to elucidate additional divergent mechanisms to account for apparent differences between EPA and DHA. Moreover, despite all of the studies performed with these PUFAs, the synergistic anti-inflammatory effects with other bioactive molecules from food remain still unknown, although it begins to be object of study (Medina et al. 2006; Saw et al. 2010).

2.3 Cytokines, adipokines and other mediators of inflammation

To determine the effects of the aforementioned bioactive molecules found in foods, several cytokines, adipokines and other inflammatory mediators have been assessed using different models *in vivo* and *in vitro* to identify the anti-inflammatory mechanisms through which the bioactive molecules modify the expression of these key markers of inflammation. Thus, the most important markers assessed in this study will be presented and defined.

2.3.1 Cytokines and adipokines in inflammation

Cytokines are soluble hormone-like proteins that belong to a large family of polypeptide signaling molecules and are released by various cells in response to an activating external stimulus (Tayal & Kalra 2008; Jaffer et al. 2010). The word cytokine is an umbrella term that encompasses lymphokines, monokines, interleukins, colony stimulating factors (CSFs), interferons (IFNs), TNFs and chemokines (Tayal & Kalra 2008), and hematopoietic and growth factors. Cytokines are small proteins of approximately 25 kDa in size and bind to specific receptors in an autocrine, paracrine and/or endocrine manner (Jaffer et al. 2010). Cytokines are involved in extensive networks that accommodate synergistic as well as antagonistic interactions and exhibit both negative and positive regulatory effects on various target cells (Feghali & Wright 1997). More than 100 inflammatory cytokines have been identified and shown to influence in growth, immunity, inflammation, apoptosis, and cell division. TNF-α, IL-1 and IL-6, which can initiate both acute and chronic inflammation, are controlled by three classes of proteins: soluble receptors that prevent binding to the cell surface, competitive binding proteins such as IL-1 receptor antagonist (IL-1RA), and anti-inflammatory cytokines such as IL-4, IL-10 and transforming growth factor beta (TGF-β), which decrease inflammatory cytokine production (Hansson 2005). *TNF-α* increases the production of itself and of IL-6, nerve growth factor, monocyte chemotactic protein-1 (MCP-1), resistin and visfatin. TNF-α also decreases ADIPQ and leptin concentrations and facilitates endothelial dysfunction and atherogenesis. Expression of the plasminogen activator-inhibitor (*PAI*) gene increases when insulin resistance develops, promotes thrombosis when the local concentration is increased particularly atheromas in type II diabetic patients, and has been posited to bear a relationship with ADIPQ with respect to CVD. Adipocyte differentiation increases as PAI decreases and ADIPQ and resistin increase (Wozniak et al. 2009). In summary, these proteins allow for communication between cells and the external environment in acute and chronic inflammatory processes (Tayal & Kalra 2008).

On the other hand, the adipokines are also very important in the study of inflammation. The term adipokine was originally proposed to describe proteins secreted specifically from adipocytes; however, as many cell types in AT have been found to secrete proteins, the term adipokine is now widely used to describe proteins secreted by AT (Tab. 4). Adipokines have been shown to influence a wide range of biological functions such as immunity, insulin sensitivity, inflammation, blood pressure, lipid metabolism, energy homeostasis and appetite. To date, over 100 adipokines have been cataloged, and more adipokines continue to be discovered using new technologies and innovative approaches (Stryjecki & Mutch 2011).

Adipokine	Cellular Origin		Reported Actions
	Adipocytes	Stromal Cells	
Leptin	++	—	Signals satiety in the hypothalamus; proinflammatory effects in peripheral tissues
TNF-α	+	++	Proinflammatory effects; insulin resistance
IL-6	+	++	Proinflammatory effects; insulin resistance
IL-10	(?)	+	Anti-inflammatory effects
MCP-1	+	++	Monocyte/macrophage recruitment; insulin resistance (?)
PAI-1	+	+	Thrombosis; insulin resistance (?)
MIP-1α	(?)	+	Monocyte/macrophage recruitment
Angiotensinogen	+	(?)	Cardiovascular function; mediator of chronic inflammation
Resistin	++	—	Proinflammatory effects (mouse); insulin resistance (mouse)
Visfatin	+	—	Proinflammatory effects (?); natural insulin mimetic (?)
Adiponectin	++	—	Anti-inflammatory effects; insulin-sensitizing effects
Chemerin	+	(?)	Regulates adipogenesis; anti-inflammatory actions (?)

Stromal cells include endothelial cells, macrophages, and preadipocytes. ++, High expression; +, clear evidence; (?), data not confirmed yet. MIP-1α: macrophage inflammatory protein 1α.

Table 4. List of selected adipokines secreted by AT (González-Périz & Clària 2010).

Adipokines engage in a wide variety of physiological and pathological processes such as immunity and inflammation through endocrine, paracrine, autocrine or juxtacrine mechanisms of action. Adipokines generally refer to biologically active substances secreted by the adipocytes of WAT; however, adipokines can be synthesized at other sites and can participate in functions unrelated to those associated with WAT. Adipokines include a variety of proinflammatory peptides such as TNF-α. Proinflammatory adipokines appear to contribute to the 'low-grade inflammatory state' of obese patients promoting several metabolic aberrations such as cardiovascular complications and inflammatory disease. Notably, adipokine production by WAT in the context of obesity is strongly influenced by the presence of infiltrating macrophages through mutual cross-talk mechanisms. Macrophages represent an additional source of soluble mediators and might contribute to and perpetuate local and systemic inflammation (Lago et al. 2007). Thus, the study of the various cytokines and/or adipokines as they relate to the inflammatory macrophages is important to better understand the mechanisms and pathways through which various bioactive molecules exert their anti-inflammatory effects. Appropriately, this study analyzes cytokines and adipokines based on their importance to various inflammatory pathways.

2.3.1.1 Tumor necrosis factor-alpha (TNF-α)

TNF-α is also known as cachectin and was identified in 1975 as an endotoxin-induced glycoprotein (Bradley 2008). This soluble protein is 17-kDa in size and exists as a trimer in the physiological state in mammals. TNF-α is one of the products of activated macrophages/monocytes, fibroblasts, smooth muscle cells, adipocytes, mast cells, and some T and natural killer (NK) cells (Feghali & Wright 1997; Jaffer et al. 2010; Popa et al. 2007). TNF-α is a member of a growing family of peptide mediators comprising at least 19 cytokines including lymphotoxin-α, Fas ligand, and CD40 ligand (TNF receptor superfamily, member 5).

Biological responses to TNF-α are mediated by ligand binding via two structurally distinct receptors: TNF receptor 1 and 2 (TNFR1 or p55 and TNFR2 or p75), which are present on the cell membrane of all cell types except erythrocytes (Popa et al. 2007). Upon stimulation, the intracellular domain of TNFR1 binds to the TNF receptor-associated death domain protein (TRADD), which can activate either the apoptotic pathway via the Fas-associated death domain protein (FADD) or the proinflammatory pathway via TNF receptor-associated factor 2 (TRAF2) and receptor-interacting protein culminating in the activation of NF-κB (Fig. 10). In contrast to TNFR1, TNFR2 transduces extracellular signals only through the TRAF2-associated pathway (Popa et al. 2007). TNFR1 is mainly responsible for mediating the inhibitory effects of TNF-α on the insulin receptor signaling pathway and the lipolytic effect of TNF-α on TGs. TNFR2 is likely to play an important role in human obesity by neutralizing the actions of TNF-α, which suggests that TNFR2 expression can be used as a predictor of adipose TNF-α activity in obese subjects.

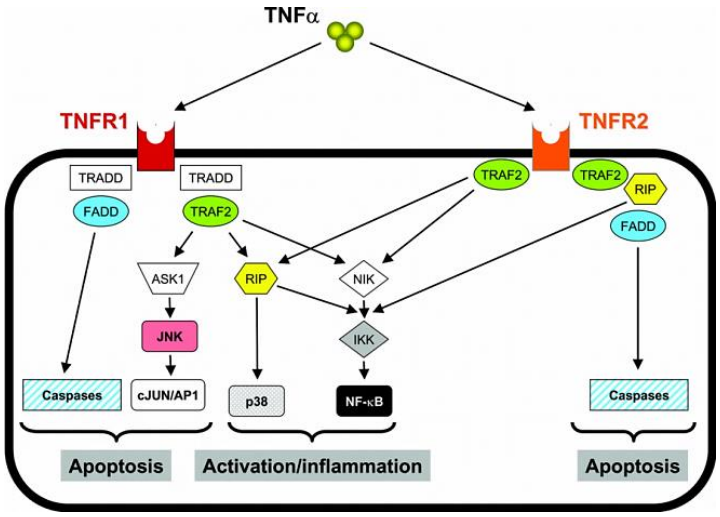


Figure 10. Main pathways by which TNF-α triggers inflammation (Russo & Polosa 2005).

TNF-α has important proinflammatory properties, which are important to the innate and adaptive immune responses, cell proliferation, and apoptotic processes. TNF-α is not usually detectable in healthy individuals, but elevated serum and tissue levels are found in inflammatory and infectious conditions, and serum levels correlate with the severity of infections (Bradley 2008). TNF-α is a proinflammatory cytokine whose expression and circulating levels increase with obesity and decrease with weight loss. TNF-α has numerous effects in AT including the regulation of apoptosis, adipogenesis, lipid metabolism and insulin signaling (Stryjecki & Mutch 2011). TNF-α is also expressed more highly in visceral than in subcutaneous fat and is more abundantly produced by the stromal vascular fraction (SVF; mainly macrophages) than adipocytes (Maury & Brichard 2010). Therefore, TNF-α is a reliable

marker for several conditions and models of acute or chronic inflammation. Appropriately, TNF- α was analyzed at the transcript and/or protein levels in all aspects of this study.

2.3.1.2 Interleukin 6 (IL-6)

IL-6 is a glycoprotein that ranges from 21 to 28 kDa depending on the degree of post-translational modification (Feghali & Wright 1997). IL-6 is produced in mammals by a variety of cells including fibroblasts, endothelial cells, monocytes, and adipocytes, and IL-6 mediates its effects on cells through a complex mechanism. IL-6 binds to IL-6 receptor (IL-6R) forming the IL-6/IL-6R complex, which in turn binds to gp130, a membrane-bound protein that is involved in non-ligand-binding signal transduction (Fig. 11). A soluble version of IL-6R also exists that can also bind IL6 and form a complex with gp130. Upon IL-6/IL-6R complex formation, the intracellular signaling pathway becomes activated, which involves the Janus kinases (JAKs) JAK1 and JAK2, tyrosine kinase 2 (TYK2), STAT1 and STAT3, tyrosine phosphatase SHP2 and NF-κB (Fonseca et al. 2009). Upon activation, STAT3 up-regulates the transcription of genes encoding the suppressor of cytokine signaling 3 (SOCS3) protein, which provides an intracellular negative-feedback system that inhibit the JAK/STAT pathway (Nishimoto & Kishimoto 2006). Thereupon, IL-6 possesses both proinflammatory and anti-inflammatory character.

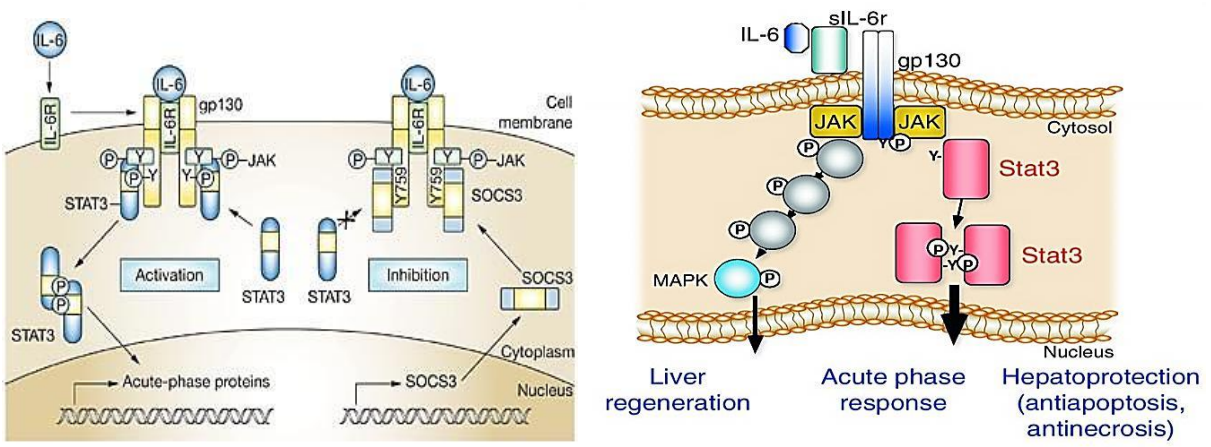


Figure 11. Main pathways by which IL-6 activates or inhibits inflammatory processes (Nishimoto & Kishimoto 2006)(Taub 2003).

In fact, IL-6 stimulates the production of acute-phase proteins (APPs) in the liver and induces leukocytosis, fever and angiogenesis and encourages chemotaxis via the stimulation of the TLR family (Jaffer et al. 2010). Additionally, IL-6 contributes to the transition into the chronic phase of inflammation by mononuclear cell accumulation at the site of injury through the stimulation of continuous MCP-1 secretion, angioproliferation and antiapoptotic functions on T

cells. IL-6 is a pivotal cytokine in acute and chronic inflammation but also imposes systemic repercussions by affecting APPs, the endocrine system, the central nervous and the cardiovascular system (Fonseca et al. 2009). However, some of the regulatory effects of IL-6 involve the inhibition of TNF-α production effectively providing negative feedback to limit the acute inflammatory response. Up-regulation of *IL-6* production has been observed in a variety of chronic inflammatory disorders such as type I diabetes and is also overexpressed in the AT of obese patients. However, the precise role of IL-6 in effecting the metabolic changes associated with obesity remains unclear. Whereas circulating levels and visceral AT production of IL-6 are increased in obese patients (Maury & Brichard 2010) suggesting the proinflammatory character of this cytokine, insulin action in skeletal muscle also increases circulating IL-6 levels dramatically suggesting a possible anti-inflammatory role for IL-6 in skeletal muscle (Rasouli & Kern 2008). Taken together, these results highlight the role of IL-6 as a cytokine that can amplify acute inflammation and promote the transition from acute to a chronic inflammatory state (Fonseca et al. 2009). Thus, IL-6 was considered in this study as a proinflammatory marker of chronic and acute inflammation.

2.3.1.3 Adiponectin (ADIPQ)

Adiponectin (ADIPQ), also known as GBP28, apM1, Acrp30, or AdipoQ, is a 244-residue protein (30-kDa) that is produced and expressed mainly in adipocytes in WAT in mammals in an inverse correlated relationship to fat mass (Conde et al. 2011) (Drouet et al. 2012). ADIPQ circulates in a trimeric, hexameric and higher order multimeric form in serum (Buechler et al. 2011), and these isoforms have specific effects on the organism (Neumeier et al. 2006; Schober et al. 2007; Wang et al. 2008). ADIPQ is induced by the activity of the nuclear PPAR-γ (Puglisi & Fernandez 2008) and acts via two receptors: one receptor (ADIPOR1) is expressed predominantly in skeletal muscle, and the other receptor (ADIPOR2)

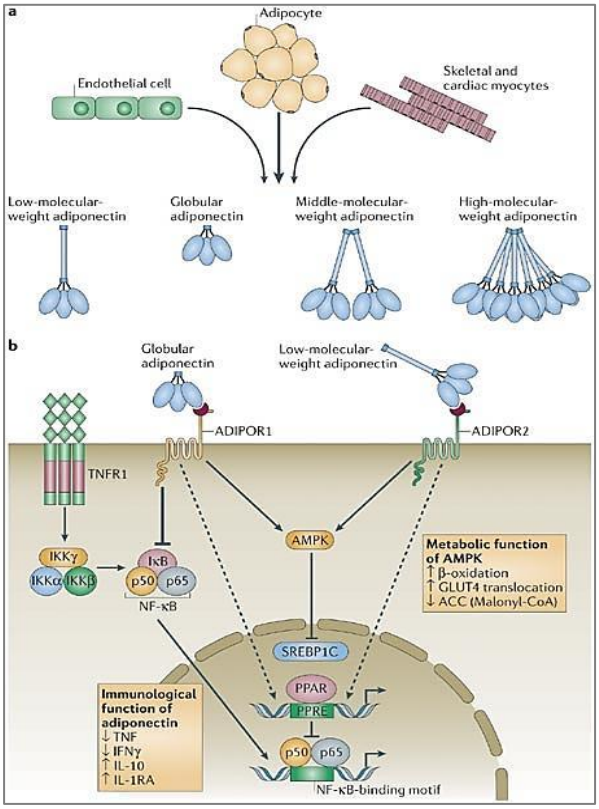


Figure 12. Signaling pathways of ADIPQ (Tilg & Moschen 2006).

is expressed mainly in the liver. Transduction of signals by ADIPQ through ADIPOR1 and ADIPOR2 involves the activation of the catalytic subunit of AMP-activated protein kinase (AMPK), PPAR-α, PPAR-γ, and other signaling molecules (Fig. 12). ADIPQ increases FA oxidation and reduces the synthesis of glucose in the liver (Conde et al. 2011) resulting in improved glycemic control and insulin sensitivity (Puglisi & Fernandez 2008). Previous reports have shown that ADIPQ also possesses anti-inflammatory properties at the cardiovascular level and confers a protective effect against atherosclerosis (Lau et al. 2005). ADIPQ inhibits IL-6 and TNF-α production by macrophages and increases the production of important anti-inflammatory factors such as IL-10 or IL-1RA by human monocytes, macrophages, and dendritic cells (DCs) (Conde et al. 2011)·(Drouet et al. 2012).

Moreover, large adipocytes found in obese subjects produce lower levels of ADIPQ but higher levels of proinflammatory adipokines such as TNF-α and IL-6, which in turn inhibit the production of ADIPQ in adipocytes. In contrast, ADIPQ can exert anti-inflammatory effects on adipocytes as well as in other cellular populations primarily via the inhibition of the NF-κB pathway (Karastergiou & Mohamed-Ali 2010). Furthermore, PPAR-γ agonists, which promote adipocyte differentiation, increase ADIPQ expression *in vitro* and *in vivo*. Other factors that negatively regulate ADIPQ expression include hypoxia and oxidative stress. In addition, low plasma ADIPQ levels are closely associated with obesity-linked complications including type 2 diabetes, coronary heart disease and hypertension (Puglisi & Fernandez 2008)·(Ouchi et al. 2012). Therefore, ADIPQ was used in this study as an anti-inflammatory marker in obesity models, and the expression of ADIPQ in mesenteric adipose tissue (MAT) and isolated adipocytes from MAT was analyzed.

2.3.1.4 Interleukin 10 (IL-10)

IL-10 is the most important anti-inflammatory cytokine associated with the human immune response (Opal & DePalo 2000). IL-10 is an 18 kDa anti-inflammatory cytokine produced in mammals by monocytes/macrophages, NK cells, DCs and lymphocytes such as CD4+ T cells, CD8+ T cells, and B cells (Jaffer et al. 2010)·(Opal & DePalo 2000)·(Mocellin et al. 2004). However, IL-10 has also been shown to be an anti-inflammatory adipokine released by adipocytes in the context of obesity (Fain 2010). IL-10 interacts with the receptor IL-10R, which engages JAK1 and TYK2 tyrosine kinases leading to tyrosine phosphorylation and subsequent activation of the transcription factors STAT1 and STAT3. SOCS1 and SOCS3 appear to be two of the genes targeted by IL-10-activated STAT proteins (Mocellin et al. 2004)

resulting in the blockade of the NF-κB and JAK/STAT pathways (Jaffer et al. 2010) (Fig 13). Thus, IL-10 is a potent inhibitor of cytokine secretion by several cells (T helper cells type 1 (TH1), macrophages, neutrophils, NK cells, etc.) down-regulating *TNF-α, IL-1, IL-2, IL-3, IL-6, IL-8, IL-12, IFN-γ* and *CSF2* expression, decreasing MHC class II expression and chemokine secretion and increasing B-cell survival time (Jaffer et al. 2010)·(Opal & DePalo 2000)·(Pestka et al. 2004)·(Lin & Karin 2007). Because IL-10 can be produce d by TH2 cells and inhibits TH1 function by preventing TH1 cytokine production (such as IFN-γ), IL-10 is considered a T cell cross-regulatory factor and has thus been referred to as an "anticytokine" (Feghali & Wright 1997). The anti-inflammatory effects of IL-10 have led to numerous *in vitro* and *in vivo* studies demonstrating beneficial effects of IL-10 in models of sepsis and endotoxemia (Groux & Cottrez 2003)·(Gotoh et al. 2012). Therefore, IL-10 represents a good marker for the study of several tissues involved in immune or inflammatory models such as adipocytes in the context of obesity or hepatocytes in the context of endotoxic shock.

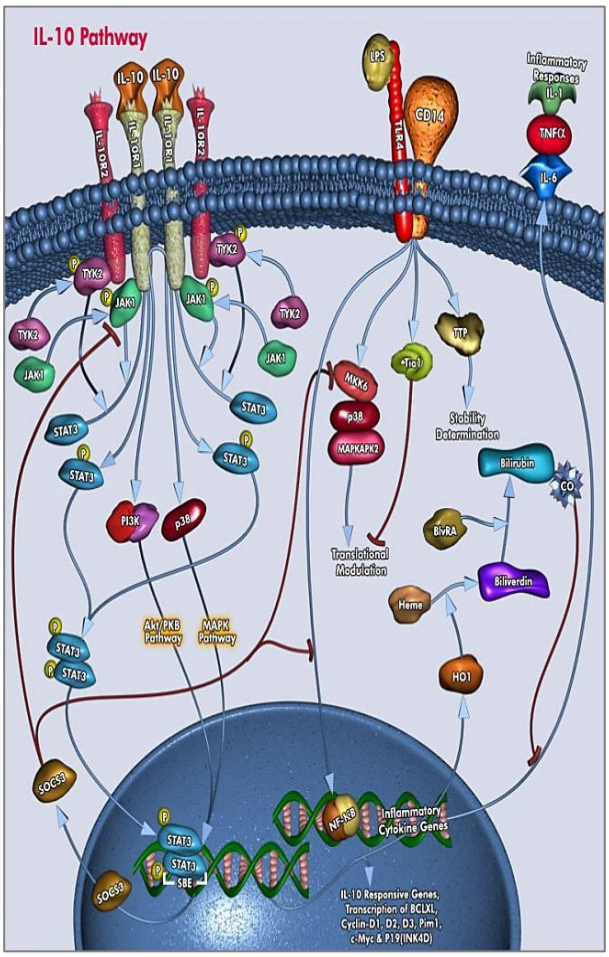


Figure 13. IL-10 anti-inflammatory pathway

2.3.2 Other mediators in inflammation

2.3.2.1 C-reactive protein (CRP)

CRP, named for its capacity to precipitate the somatic C-polysaccharide of *Streptococcus pneumonia*, is an α-globulin found in vertebrates (Tîrziu 2009). CRP was the first APP to be described and is an exquisitely sensitive systemic marker of inflammation and tissue damage (Pepys & Hirschfield 2003). CRP is a protein of ~115 kDa consisting of five identical subunits of ~23 kDa each. Previous studies have suggested that CRP is a marker of underlying infection or tissue injury, but has more recently been considered a marker for chronic systemic inflammation (Bucova et al. 2008). In response to an acute infection or inflammation, cytokines

such as IL-6, IL-1, and TNF-α are released into circulation, and these cytokines regulate the secretion of CRP in the liver. CRP concentration in the plasma can increase by as much as 1000-fold during injury and infection (Puglisi & Fernandez 2008). In the context of obesity, circulating IL-6 can synergize with secreted proinflammatory IL-1 and TNF-α to stimulate the secretion of CRP into the plasma. CRP levels directly correlate with the amount of body fat, visceral obesity and adipocyte hypertrophy (Lau et al. 2005). Recent evidence has also shown that circulating CRP concentrations are higher in adults with metabolic syndrome, and increased CRP is an independent risk factor for type II diabetes and CVD (de Ferranti & Mozaffarian 2008). In this study, CRP was used as a proinflammatory marker *in vivo* in different tissues such as MAT and liver as well as in the plasma.

2.3.2.2 Nitric oxide (NO)

NO, one of the few gaseous radical signaling molecules known, is synthesized by most human cells (Roszer s.d.)·(Guix et al. 2005)·(Conforti & Menichini 2011) and is a ubiquitous mediator of many different biological processes such as vasodilation, neurotransmission, macrophage-mediated cytotoxicity, gastrointestinal smooth muscle relaxation and bronchodilation through a variety of downstream pathways (Villanueva & Giulivi 2010). NO is produced by a group of enzymes that catalyzes the conversion of L-arginine into L-citrulline known as nitric oxide synthases (NOS): neuronal NOS (nNOS; NOS1), endothelial NOS (eNOS; NOS3), inducible NOS (iNOS; NOS2) and mitochondrial NOS (mtNOS). nNOS and eNOS are thought to be critical for normal physiology, whereas iNOS appears to be associated with injury or inflammation. iNOS is expressed in many cell types including macrophages, hepatocytes, neutrophils, DCs, endothelial cells, and epithelial cells raising the possibility that the effect of iNOS-derived NO may depend on the cell type (Guix et al. 2005)·(Kobayashi 2010)·(Murakami & Ohigashi 2007).

In the presence of infectious and proinflammatory stimuli, iNOS protein is highly induced to produce NO in the micromolar range, whereas NO generation from nNOS and eNOS enzymes remains constant and within the nanomolar range (Murakami & Ohigashi 2007). Overstimulation of individual NOS isoforms, especially nNOS and iNOS, plays a role in several disorders including septic shock, arthritis, diabetes, ischemia-reperfusion injury, pain and various neurodegenerative diseases (Joubert & Malan 2011). LPS, IL-1β, IL-6, TNF-α, macrophage migration inhibitory factor (MIF), and others (Fortin et al. 2010), whose production are triggered by multiple factors such as septic shock, various diseases and chronic inflammatory processes, regulate the transcription of iNOS through the JAK/STAT, MAPK and NF-κB pathways (Guix et al. 2005)·(Dyson et al. 2011) depending on the stimulus

and the cell type (Guix et al. 2005) (Fig. 14). The induction of iNOS produces large amounts of NO, which may be used as an indicator of a proinflammatory process in various models of inflammation (Cauwels 2007)·(Juan et al. 2008).

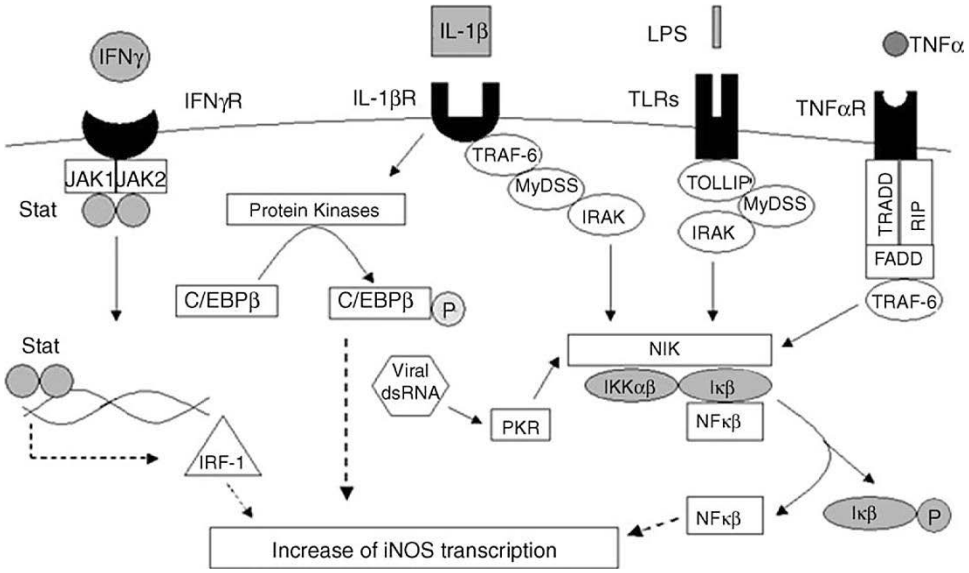


Figure 14. Activation of iNOS transcription by inflammatory stimulation (Guix et al. 2005).

In addition, NO can interact with superoxide (O_{2}^{-}) to produce peroxynitrite ($ONOO^{-}$), which may be generated in excess during the host response against viral and antibacterial infections and may contribute to the pathogenesis of inflammatory diseases by promoting oxidative stress, tissue injury and even cancer. Oxidative damage caused by action of free radicals may initiate and promote the progression of a number of chronic diseases including diabetes and inflammation (Conforti & Menichini 2011). However, NO possesses biphasic character due to the numerous molecular targets subject to NO regulation. NO controls servoregulatory functions such as neurotransmission and vascular tone, regulates gene transcription and mRNA translation and affects post-translational modifications of proteins (Förstermann & Sessa 2012). Thus, NO plays an immunoregulatory role in the induction and resolution of inflammation, which appears to be determined by the level of NO (Kobayashi 2010). NO appears to activate the NF-κB pathway at lower levels early during infections and deactivate the NF-κB at high levels much later during infections. Moreover, in the context of obesity, an increase in visceral adiposity and related risk factors are associated with a proinflammatory state that results in decreased NO availability and activity, which is responsible, at least in part, for endothelial injury and dysfunction (Esposito & Giugliano 2011). Despite the differential effects of NO, NO in nitrate and nitrite forms (NOx) are considered excellent markers of inflammation in diseases such as septic shock (Dyson et al. 2011). Therefore, NOx concentration in the liver, spleen and plasma was used as proinflammatory marker in our *in*

vivo model of acute inflammation. NO levels were also analyzed in our *in vitro* studies in macrophages as a proinflammatory marker following LPS treatment.

2.3.2.3 Eicosanoids and docosanoids

Eicosanoids and docosanoids are key mediators and regulators of inflammation and are generated from 20 and 22 carbon PUFAs, respectively. These molecules, which include resolvins (Rvs) prostaglandins (PGs), thromboxanes (TXs), leukotrienes (LTs) and other oxidized derivatives, are generated mainly from ARA, EPA and DHA by different metabolic processes, involving distinct enzymes such as COX2, cytochrome P450 and different lipoxygenases in these processes (Ji et al. 2011) (Fig. 15).

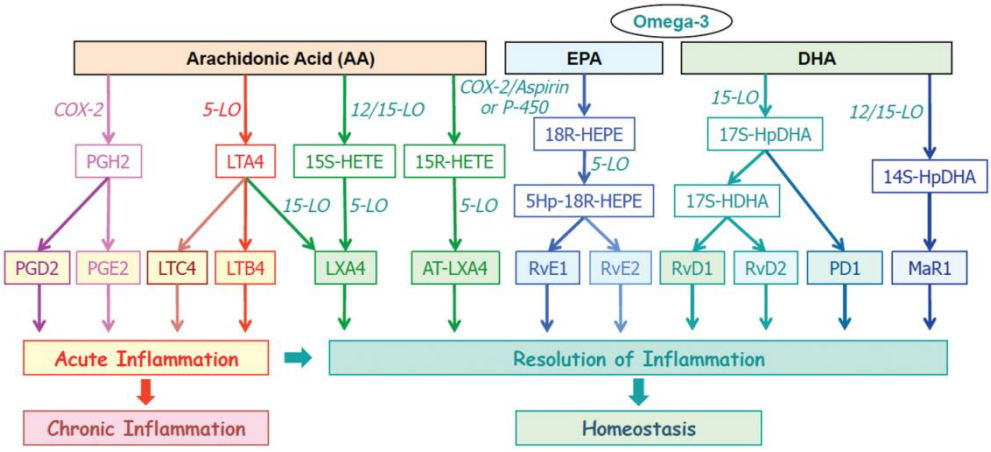


Figure 15. Biosynthetic cascades and actions of selected lipid mediators derived from arachidonic acid (ARA or AA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA)(Serhan & Petasis 2011).

Eicosanoids are involved in modulating the intensity and duration of the inflammatory response; they have cell- and stimulus-specific sources and frequently have opposing effects. Thus, the overall physiological (or pathophysiological) outcome will depend upon the cells present, the nature of the stimulus, the timing of eicosanoid generation, their concentration and the sensitivity of target cells and tissues to the eicosanoids generated. Long chain n-3 PUFAs decrease the production of inflammatory eicosanoids from ARA and promote the production of less inflammatory eicosanoids from EPA than those produced from ARA, as well as of anti-inflammatory resolvins and similar mediators from EPA and DHA. In this context, the E- and D-series resolvins, formed from EPA and DHA respectively, as well as the neuroprotectin D1 formed from DHA, appear to be the most common eicosanoids that exert potent anti-inflammatory actions (Calder 2008). Thus, the generation of resolvins derived from EPA and DHA are another mechanism of action of both ω3-PUFAs.

2.3.3 Main intracellular signaling pathways in inflammation

Pathogen-associated molecular patterns (PAMPs) such as microbial nucleic acids, lipoproteins, and carbohydrates, damage-associated molecular patterns (DAMPs) released from injured cells and cytokines such as interleukin 1 (IL-1) and tumor necrosis factor-alpha (TNF-α) are the main inflammatory stimuli, which act through the toll-like receptors (TLRs), the IL-1 receptor (TIR) family and the tumor necrosis factor (TNF) receptor family, respectively (Kaminska 2005)·(Newton & Dixit 2012) (Fig. 16).

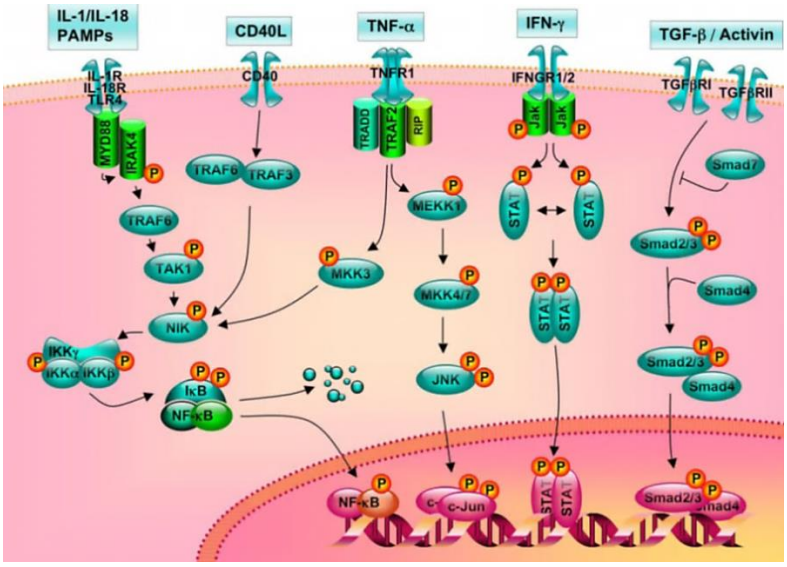


Figure 16. Principal signaling pathways in inflammation (Tedgui & Mallat 2006).

Depending on the primary stimulus, the NF-κB, the JAK/STAT and/or the MAPK pathways may become activated triggering an inflammatory state through the expression of proinflammatory and anti-inflammatory genes, which promotes the neutralization of the damage and leads to the resolution of inflammation.

Three major groups of distinctly regulated MAPK cascades have been identified in mammals that lead to altered gene expression: the extracellular signal-regulated kinase 1 and 2 (ERK 1/2), and the two SAPKs families, c-JUN N-terminal kinase (JNK) and p38 MAPK. ERK1/2 is activated by MAPK kinase (MKK) and MKK2, JNK is activated by MKK4 and MKK7, and p38 MAP kinase is activated by MKK3, MKK4, and MKK6. Negative feedback mechanisms including MAPK phosphatases (MKPs) exist to ensure that MAPK enzymes are not constitutively activated. Upon activation of the MAPKs, transcription factors present in the cytoplasm or nucleus are phosphorylated and activated leading to the expression of target genes and producing various biological responses such as the inflammatory response (Kaminska 2005; Thalhamer et al. 2008). Importantly, p38 MAPK is relevant in the activation

of inflammation. In this way, this MAP kinase positively regulates expression of many genes involved in inflammation, such as those coding for TNF-α, IL-1β, IL-6, IL-8, cyclooxygenase- 2, and collagenase-1, -3 and this regulation can occur at both transcriptional and post-transcriptional levels. p38 MAPK activation is also involved in the increase of iNOS expression, and the final production of NO in activated macrophages. In addition, p38 MAPK can increase the expression, or regulate by phosphorylation, several transcription factors, including activating transcription factor-2 (ATF-2 is a possible partner of c-Jun in the AP-1 complex), GADD153, myocyte enhancer factor 2C (MEF 2C), C/EBP homologous protein 1, and NF-κB. In fact, p38 MAPK is believed to regulate NF-κB-dependent transcription in part by modulating activation of basal transcription factors (Kaminska 2005).

On the other hand, the NF-κB pathway is one of the main signaling pathways activated in response to the proinflammatory cytokines TNF-α, IL-1, and IL-18 as well as following the activation of the TLRs that bind to their respective PAMPs. Activation of the NF-κB pathway plays a central role in inflammation through the regulation of genes encoding proinflammatory cytokines, adhesion molecules, chemokines, growth factors, and inducible enzymes such as cyclooxygenase-2 (COX2) and iNOS. NF-κB is a dimeric transcription factor formed by the hetero- or homodimerization of proteins of the REL family including p50 and p65. In its inactive form, NF-κB is bound to the inhibitor of κB (Iκ-B) in the cytoplasm. Proinflammatory cytokines and pathogens act through distinct signaling pathways that converge on the activation of an Iκ-B kinase (IKK) complex containing the two kinases IKK1/IKKα and IKK2/IKKβ and the regulatory protein NEMO (NF-κB essential modifier, also named IKK-γ). IKK activation initiates Iκ-B phosphorylation at specific NH_2-terminal serine residues, which leads to the ubiquitination and subsequent degradation of Iκ-B by the 26S proteasome. The degradation of IK-B releases NF-κB dimers from the cytoplasmic NF-κB/Iκ-B complex allowing NF-κB to translocate to the nucleus. Once in the nucleus, NF-κB binds to κB enhancer elements on specific genes to promote the transcription of NF-κB target genes including Iκ-B, the expression of which ensures that NF-κB is only transiently activated. This negative-feedback regulation produces the characteristic oscillations observed in NF-κB nuclear translocation (Tedgui & Mallat 2006). In addition, after NF-κB activation by external inflammatory signals such as bacterial endotoxin, it positively regulates the gene expression of several cytokines such as TNF-α and β, IL-1β, IL-2, IL-6, IL-8, MCP-1, G-CSF, GM-CSF and IFN-β, which in turn can produce a positive feedback and amplify the original inflammatory signal. Because of its potential ability to influence the production of an array of cytokines, NF-κB is an appealing target for therapeutic strategies designed to attenuate cytokine-mediated inflammation (Blackwell & Christman 1997).

Moreover, IFN-γ, one of the most potent endogenous macrophage-activating factors, signals through the JAK/STAT signaling pathway (Hu et al. 2007). The activation of JAKs after cytokine stimulation results in the phosphorylation of STATs, which then dimerize and translocate to the nucleus to activate gene transcription (Shuai & Liu 2003). There are seven mammalian STATs: STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B and STAT6 and each one regulates inflammation differently having distinct proinflammatory and anti-inflammatory effects depending on the particular STAT. For instance, STAT1 plays a major role in mediating immune responses and the proinflammatory activity of IFN-γ (Hu et al. 2002). However, when STAT3 becomes activated by IL-6, STAT3 causes the transcription of SOCS3, which can deactivate the NF-κB and JAK/STAT pathway (Nishimoto & Kishimoto 2006). In fact, the JAK/STAT pathway can be regulated by different ways, but the SOCS protein family is the most studied. Biochemical and genetic studies have clearly shown a crucial role for SOCS proteins in immune system. SOCS1, SOCS2, SOCS3 and CIS are generally expressed at low levels in unstimulated cells and become rapidly induced by cytokines, thereby inhibiting STATs signaling and forming a classic negative-feedback loop. On the other hand, several serine kinases have been implicated in the phosphorylation of STATs such as ERK, p38, JNK, and protein kinase Cδ (PKCδ), whereas NF-κB activation can mediate the transcription of SOCS3, inhibiting JAKs through binding to their receptor (Shuai & Liu 2003). Thus, signal transduction cross-talk is regulated in a dynamic manner and differs depending on the homeostatic or pathological context. The deregulation of signal transduction cross-talk could conceivably contribute to the pathogenesis of chronic inflammatory diseases (Hu et al. 2007) (Fig. 17).

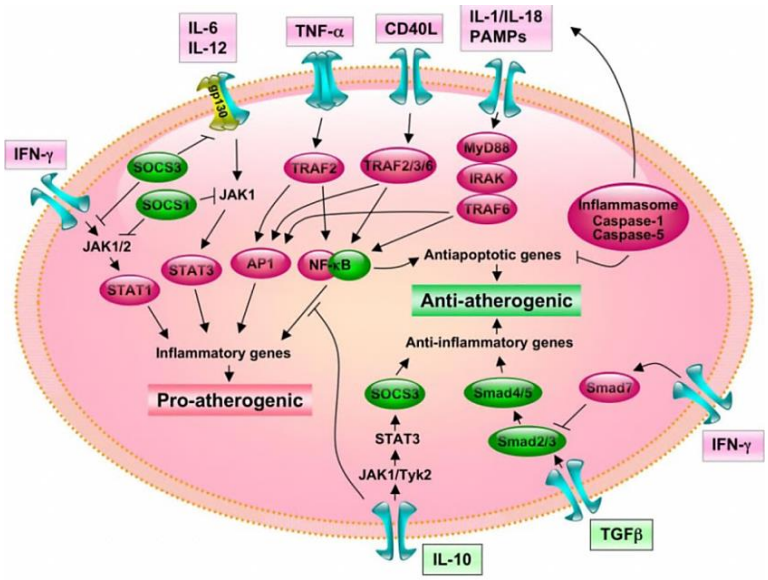


Figure 17. Cross-talk between proinflammatory and anti-inflammatory signal transduction pathways (Tedgui & Mallat 2006).

In addition, the role of cytokines in immunoregulation and inflammation is well established, and multiple genome-wide association studies have documented that polymorphisms and mutations of cytokine receptors and their downstream signaling components appear to contribute to autoimmune disorders (O'Shea & Murray 2008).

Thus, the study of cross-talk between the proinflammatory pathways and various cytokines or other factors that induce these states can help to provide an explanation of the effects of bioactive compounds.

2.4 Models of inflammation

2.4.1 Acute inflammation

2.4.1.1 Model of acute inflammation *in vitro*

2.4.1.1.1 Lipopolysaccharide (LPS): an in vitro inflammatory inducer

In this thesis, LPS was used as an inducer of inflammation *in vitro*. Although there are various molecules to induce inflammatory response such as IFN-γ, TNF-α, peptoglycan, CpG DNA, poli (I:C) and HSP60 and 70, LPS is the most used in research (Duque & Rojas 2007). LPS, also known as endotoxin, has a dominant role in Gram-negative bacteria. The outer membrane of Gram-negative bacteria is composed of a lipid bilayer that is separated from the plasma membrane by a relatively thin layer of peptidoglycan. The LPS molecule is embedded in the outer membrane and the lipid A portion of LPS serves to anchor LPS in the bacterial cell wall (Cohen 2002). The incubation of effector cells of inflammation such as macrophages with LPS induces the production of relevant levels of cytokines and other mediators of inflammation, detectable after few hours.

The principal mechanism by which LPS is detected by cells is via the LPS-binding protein (LBP)/LPS complex, which then signals through the TLR-4/CD14/MD-2 complex (Fig. 18). Once activated, the TLR-4/CD14/MD-2 complex triggers a phosphorylation cascade through the interferon regulatory factor 3 (IRF3), MAPK and the NF-κB signaling pathways, which results in changes in gene expression and in the increased production of inflammatory cytokines such as IFN-β, IFN-γ, IL-1β, IL-6, IL-12 and TNF-α and other proinflammatory mediators such as NO. The activation of these different pathways results in a succession of autocrine/paracrine feedback loops, which in turn modifies LPS-induced cytokine expression. Hence, the release of IFN-β interferon α receptor (IFNAR1) dependently induces the expression of anti-inflammatory IL-10, which in turn leads to the sustained activation of STAT3 and the expression of SOCS3 (Cohen 2002)' (Bode et al. 2012).

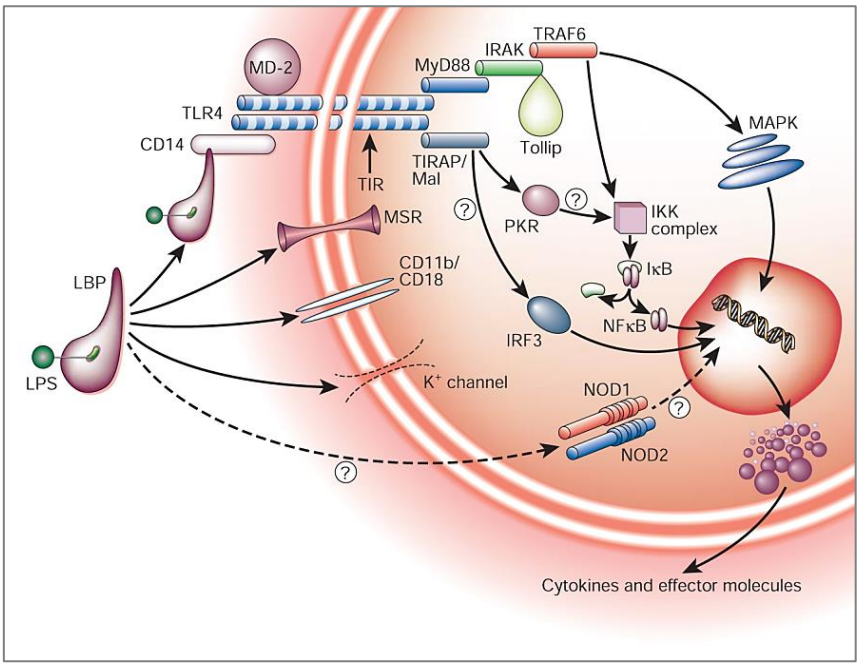


Figure 18. Cell-surface recognition of lipopolysaccharide (LPS) and their signaling pathways (Cohen 2002).

The interconnectivity of these pathways results in the resolution of inflammation; however, modifications within these pathways due to the intervention of bioactive molecules can alleviate the proinflammatory state and facilitate the resolution of inflammation.

2.4.1.1.2 Macrophages: effector cells of inflammation

Macrophages were analyzed in the studies in *vitro* of this thesis because they participate in multiple aspects of the inflammatory response. Upon activation by microbial components such as LPS, mainly through the TLR-4 macrophages release a diverse range of products (Tab. 5). Most of these products have multiple targets and produce their effects through several parallel mechanisms. For instance, NO has direct antimicrobial properties, can modify vascular tone (and hence tissue oxygenation), and can also cause macrophage apoptosis.

Furthermore, many macrophage products are involved in the regulation of each other. Thus, TNF-α up-regulates tissue factor and *NOS* expression, IL-18 induces IFN-γ, which in turn further activates macrophages, whereas IL-10 is a global suppressor of macrophage function. These highly complex and tightly regulated networks make it difficult to predict the consequences of blocking or inhibiting a single pathway (Cohen 2002).

Mediators	Typical effects
Cytokines	
Il-1, IL-6, IL-12, IL-15, IL-18, TNF-α, MIF, HMGB1, IL-10	Activate neutrophils, lymphocytes and vascular endothelium; upregulate cellular adhesion molecules; induce prostaglandins, nitric oxide synthase and acute-phase proteins; induce fever. Note that IL-10 is predominantly a negative regulator of these effects
Chemokines	
IL-8, MIP-1α, MIP-1β, MCP-1, MCP-3	Mobilize and activate inflammatory cells, especially neutrophils; activate macrophages
Lipid mediators	
Platelet-activating factor, prostaglandins, leukotrienes, thromboxane, tissue factor	Activate vascular endothelium; regulate vascular tone; activate extrinsic coagulation cascade
Oxygen radicals	
Superoxide and hydroxyl radicals, nitric oxide	Antimicrobial properties; regulation of vascular tone

Table 5. Macrophage products implicated in the pathogenesis of sepsis (Cohen 2002).

In this context, studies must take into account several different pathways to understand the complexity of the effects of bioactive molecules derived from food sources.

2.4.1.2 Models of acute inflammation *in vivo*

Inflammation is the major and complex reaction of the body against infection upon tissue injury. After gaining bodily access to the site of injury, the infectious microorganisms cause local inflammation. Many bacterial components and products such as peptidoglycans, lipoteichoic acid, exotoxins, lipoproteins and glycolipids can initiate the local inflammatory processes (Khan & Khan 2010). In this context, there are many experimental models that have been used in the recent years to mimic inflammatory processes at local level such as arthritis, asthma, inflammatory bowel disease, acute lung injury, colitis, angiogenesis, skin inflammation, joint and cartilage inflammation and multiple sclerosis, as well as at systemic level such as sepsis and the systemic inflammatory response syndrome (SIRS) (Morgan & Marshall 1999; Morán 2007). The models used to induce systemic inflammation include intravascular infusion of endotoxin or live bacteria, colon ascendens stent peritonitis (CASP), cecal ligation and puncture (CLP), soft tissue infection, pneumonia model and meningitis model (Garrido et al. 2004; Zanotti-Cavazzoni & Goldfarb 2009). All of these models have in common an activation of TLRs in macrophages and mast cells and their derivative cells, triggering protein cascades, synthesizing cytokines, growth factors and other pro-inflammatory mediators such as NO, and finally leading to sepsis or SIRS (Morán 2007). Several animals are used to this purpose such as rodents, rabbits, dogs, pigs, sheep and nonhuman primates. However, rats and mice are the most commonly used species in great part because of their

low cost, their ease to breed and house and their many biologic features common to mammals (Garrido et al. 2004; Zanotti-Cavazzoni & Goldfarb 2009).

2.4.1.2.1 Lipopolysaccharide (LPS): an inducer of *in vivo* acute inflammation

In vivo, LPS endotoxin can be administered through different routes such as intravenous, intraperitoneal and intratracheal. A bolus injection of endotoxin quickly causes an acute inflammatory response with a pronounced increase in cytokines and other proinflammatory mediators. Advantages of using endotoxin include technical ease to perform, avoiding surgical operations necessary in other models like CLP or CASP; homogeneous response to insult, which make it straightforward to replicate; and a quick pronounced increase of inflammatory markers with detectable levels from minutes to hours (Zanotti-Cavazzoni & Goldfarb 2009).

2.4.1.2.2 Acute inflammation in tissues

2.4.1.2.2.1 Liver

The liver is constantly exposed to antigenic loads. Therefore, pathogenic microorganisms must be efficiently eliminated while harmless antigens derived from the gastrointestinal tract must be tolerated by the liver. To achieve this delicate balance, the hepatic innate immune system is equipped with multiple cellular components such as monocytes, macrophages, granulocytes, NK cells and DCs, which coordinate to exert tolerogenic potential while maintaining their ability to detect, respond to and eliminate invading pathogens and transformed cells to establish an effective immune response (Liaskou et al. 2012) (Shoelson et al. 2007). Thereupon, the liver is a special organ that suffers constant changes during inflammatory states, and thus the liver is an important organ to include in studies regarding the effects of bioactive molecules.

During local liver injury (accumulation of lipid) or infection as well as the presence of LPS in the organism, resident Kupffer cells (KCs) and monocytes/macrophages initiate an immune response. Upon phagocytosis of the pathogenic material, phagocytes release a variety of chemical messengers such as TNF-α, IL-1 and IL-6 that initiate the acute-phase response and associated inflammation. APPs are a heterogeneous group of plasma proteins, which are mainly synthesized in the liver, but also in other cell types, and include pentraxins (CRP, serum amyloid P (SAP) and the long pentraxin 3 (PTX)), serum amyloid A (SAA), serum mannose-binding lectin, orosomucoid, inhibitors of proteases (α1-antitrypsin, α1-antichymotrypsin, α1-ACH, α2-macroglobulin), coagulation factors (fibrinogen, prothrombin, fVIII, plasminogen), transport proteins (haptoglobin, hemopexin, ferritin) and

complement components. Most of the secreted factors derived from resident hepatic macrophages can aggravate the systemic inflammatory state (Liaskou et al. 2012).

The liver is continuously exposed to food and microbial antigens absorbed through the intestines, and thus the liver functions as a barrier towards environmental antigens. In addition, as the primary metabolic organ, the liver produces a multitude of neo-antigens. Thus, the risk of immune activation by the liver is more prevalent than elsewhere in the body. The liver has in turn acquired specialized mechanisms of immune tolerance to avoid an over-activation of the innate and adaptive immune responses. KCs represent the largest group of tissue-resident macrophages in the body. KCs mediate host resistance to infection and release proinflammatory cytokines such as IL-1, IL-6, and TNF-α, which promote the infiltration of neutrophilic granulocytes to eliminate bacteria. However, following the initial activation to produce proinflammatory cytokines, KCs release IL-10, which attenuates the production of TNF-α, IL-6 and other cytokines (Tiegs & Lohse 2010). Therefore, we included the analysis of these cytokines in the liver in our *in vivo* acute inflammation model.

2.4.1.2.2.2 Spleen

The spleen is the largest lymphoid organ in the body and plays an important role in the host immune function and blood filtration via the removal and destruction of aged or damaged erythrocytes and other blood cells. Splenic gene expression of proinflammatory cytokines such as TNF-α and IL-6 is decreased in the context of obesity. In contrast, IL-10, which is synthesized by the cells of multiple organs including the spleen, is a potent anti-inflammatory cytokine that inhibits the synthesis of proinflammatory cytokines. Large amounts of IL-10 are produced by activated B-cells that mature in the marginal zone of the spleen. Recent studies have suggested that IL-10-producing B-cells play a regulatory role in suppressing harmful immune responses (Gotoh et al. 2012). However, in acute inflammation *in vivo* induced by LPS injection, the NOx levels become increased due to the activation of the iNOS pathway (Dyson et al. 2011). Thus, the levels of NOx released by the spleen were analyzed in our model to assess whether GSPE has anti-inflammatory effects on this organ.

2.4.2 Chronic inflammation

2.4.2.1 Models of chronic inflammation *in vivo*

Inflammation is considered to be a response of the body against an external infectious agent such as microbes or viruses, but also counteracts the harmful effects of tissue damage. If the neutralization and removal of the noxious stimuli or even if the clearance of apoptotic

inflammatory cells from the inflamed tissue fail, the inflammatory process persists and a state of chronic inflammation or autoimmunity may arise (Monteiro & Azevedo 2010). In this context, the obesity, characterized by adipose tissue dysregulation, can be used as a low-grade chronic inflammation model.

Nowadays, several animal models are available to study the effects of bioactive molecules against chronic inflammation related to obesity (Panchal & Brown 2011). Rodents are the most used animals for this purpose. There are several models of rodents with mutations in the leptin gene or receptor, that promote a dysregulation in food intake, presenting signs of obesity and inflammation at few weeks of age. These genetic models of obesity include *db/db* mice, *ob/ob* mice and Zucker fatty rats (*fa/fa*). Moreover, Otsuka Long-Evans Tokushima Fatty rats can also be used as an obesity model because these rats have absence of cholecystokinin (CCK-1) receptors, making them insensitive to the actions of CCK, which controls food intake. Thereby, these genetic models reach the obesity because the animals have an uncontrolled desire to feed.

On the other hand, the diet-induced obesity (DIO) models are also used in studies of chronic inflammation. Fructose, sucrose, high-fat and high-carbohydrate/high-fat diets are used to induce obesity and chronic inflammation in lean rodents after several weeks of treatment. The model that best reproduce the Western diet is the “cafeteria diet”. In this model the combination of high-carbohydrate and high-fat food, which is also highly palatable, administrated *ad libitum* promotes the voluntary hyperphagia and the development of obesity in rodents. This obesity induces a low-grade of chronic inflammation in target tissues such as adipose tissue and at systemic level. Although the DIO models mimic the human diet more closely, the genetic obese models apparently need less time to develop obesity and their collateral effects.

In recent studies, several high-fat and cafeteria diets were administrated in rats to induce obesity and low-chronic inflammation in order to assess the effects of the bioactive molecules presents in GSPE (Terra, Montagut, et al. 2009; Terra et al. 2011). The results showed that the consumption of these bioactive molecules has significant anti-inflammatory effects effectively decreasing the expression levels of several proinflammatory markers in these rats. Thus, it seems interesting to assess whether the effects of these bioactive molecules can reproduce in other more potent models of obesity such as the genetic obese *fa/fa Zucker* rats, which is the most well characterized and most widely used animal genetic model of obesity.

2.4.2.2.1 *Zucker fa/fa* rats as a low-chronic inflammation *in vivo* model

The *fa* mutation was discovered in 1961 by Lois Zucker in a cross between the Merck M-strain and Sherman rats. Animals homozygous for the *fa* allele, the *fa/fa Zucker* rats, better known as obese *Zucker* rats, become noticeably obese between the third and the fifth week of life. These animals harbor a mutation in the gene for the leptin receptor, which is the molecular basis for their characteristic phenotype. Leptin is produced by AT and plays an important role in the central regulation of energy balance. Leptin is released into the circulatory system by AT in proportion to the amount of stored lipids and acts in the brain on the leptin receptors to facilitate a decrease in food intake and an increase in energy expenditure. A consequence of a lack of leptin receptor-mediated counter-regulation is manifested in the obese *Zucker* rats, which display markedly elevated circulating leptin levels and a considerable increase in weight when compared with their lean counterparts (de Artiñano & Castro 2009).

The obesity observed in *fa/fa Zucker* rats is associated with hyperphagia, defective non-shivering thermogenesis and preferential deposition of energy into AT. In fact, by 14 weeks of age, the body composition of the obese *Zucker* rats is approximately 40% lipid by weight, which is accommodated by adipocyte hyperplasia and hypertrophy (de Artiñano & Castro 2009).

In addition, studies have shown that obese *Zucker* rats have increased levels of proinflammatory markers such as TNF-α, IL-6 and CRP at the systemic and local levels in several tissues including the AT and liver (Endo et al. 2010; Martín-Cordero et al. 2011; Picchi et al. 2006). Based on the increase in markers of inflammation, obese *Zucker* rats are a good model of low-chronic inflammation to assess the anti-inflammatory effects of bioactive molecules *in vivo*.

2.4.2.2 Obesity and inflammation

In obesity, the degree of inflammatory activation is modest, which is often referred to as “low-grade” chronic inflammation. Other researchers have attempted to label this inflammatory state “metaflammation”, which emphasizes the fact that the inflammation is metabolically triggered, as well as “parainflammation,” which describes the fact that this inflammatory state is an intermediate state between the basal and inflammatory states (Monteiro & Azevedo 2010). Over the past few years, evidence has accumulated supporting the idea that obesity-associated inflammation plays a crucial role in the development of insulin resistance, the accumulation of AT and the development of diseases such as CVD (Lolmède et al. 2011). In fact, obesity is characterized by the synthesis of proinflammatory cytokines such as TNF-α

and IL-6 from both adipocytes and immune cells (Fresno et al. 2011) (Tab. 6). In addition, NF-κB is an essential factor in acute and chronic inflammation, pathological states that are either causes or co-factors for a great variety of diseases (Schmid & Birbach 2008).

Factors	Metabolic regulation	Effects	Mouse model
TNF-α	↑ in obesity (S1, S2)[A]	Promotes insulin resistance (S1, S3)	LOF (S4), GOF (S5)
IL-6	↑ in obesity (S6, S7)	Promotes insulin resistance; central anti-obesity action (S8–S11)	LOF (S10), GOF (S11)
Leptin	↑ in obesity (S12)	Multiple effects on immune function; suppresses appetite; promotes FA oxidation (S12–S14)	LOF (S12)
Adiponectin	↓ in obesity (S15)	Antiinflammatory; promotes insulin sensitivity; stimulates FA oxidation (S15, S16)	LOF (S17), GOF (S18, S19)
Visfatin	↑ in obesity (S20)	Early B cell growth factor; insulin mimetic (S20, S21)	LOF (S20)
Resistin	Variable in obesity (S22, S23)	Induced in endotoxemia/inflammation; promotes insulin resistance; regulates fasting blood glucose level (S23–S26)	LOF (S24), GOF (S25, S27)
IL-1	↑ by hyperglycemia (S28)	Proinflammatory; regulates insulin secretion; involved in central leptin action (S29, S30)	LOF (S30)
IL-1Rα	↑ in obesity (S31)	Antiinflammatory; opposes leptin action (S29)	
IL-8	↑ in obesity (S32)	Proatherogenic (S33, S34)	LOF (S33)
IL-10	↑ in obesity; ↓ in metabolic syndrome (S35)	Antiinflammatory; promotes insulin sensitivity (S36)	
IL-18	↑ in obesity (S37, S38)	Proatherogenic (S39, S40)	LOF (S40) GOF (S39)
MCP-1	↑ in obesity (S41)	Proatherogenic; promotes insulin resistance (S34, S41, S42)	LOF (S42)
MIF	↑ in obesity (S43)	Inhibits macrophage migration	
M-CSF		Monocyte/macrophage differentiation; stimulates adipose growth (S44)	GOF (S44)
TGF-β	↑ in obesity (S45, S46)	Inhibits adipocyte differentiation and adipose tissue development; regulates atherosclerosis (S47–S49)	LOF (S50, S51), GOF (S49)
Soluble TNFR	↑ in obesity (S52–S54)	Proinflammatory	
C-reactive protein	↑ in obesity (S55, S56)	Proinflammatory; atherogenic; risk factor for diabetes (S55, S57–S59)	GOF (S57)
Haptoglobin	↑ in obesity (S60)	Proinflammatory	

↑, increase; ↓, decrease; FA, fatty acid; GOF, gain-of-function; IL-1Rα, IL-1 receptor α; LOF, loss-of-function; MCP-1, monocyte chemotactic protein-1; MIF, macrophage migration inhibitory factor; TNFR, TNF receptor. [A]See Supplemental References; supplemental material available online with this article; doi:10.1172/JCI200525102DS1.

Table 6. Factors that mediate the intersection of metabolism and immunity (Wellen & Hotamisligil 2005).

Obesity-induced inflammation is an important contributor to the development of insulin resistance. Recently, the cytokine IL-1β has emerged as a prominent instigator of the proinflammatory response in the context of obesity. Several studies over the last year have deciphered the molecular mechanisms responsible for IL-1β-mediated activation in AT, liver, and macrophages (Stienstra et al. 2012).

The most relevant of these pathways as they relate to obesity include the NF-κB pathway, in which free fatty acids (FFAs) activate the TLR-4 receptor in macrophages and adipocytes similarly to LPS. Other relevant pathways include the c-Jun N-terminal kinase/activator protein 1 (JNK/AP-1) pathway, in which insulin signaling is inhibited in the presence of TNF-α, the cyclic adenosine monophosphate responsive element binding protein H (CREB-H) pathway, which promotes the secretion of APPs such as CRP and promotes the generation of reactive

oxygen species (ROS), and the JAK/STAT pathway. All of these pathways become activated by excess FAs, ROS, endoplasmic reticulum stress, and lipid by-products such as diacylglycerol and ceramide, and these pathways become inhibited by ADIPQ and thiazolidinediones (Cusi 2012). In this context, our model of obesity is appropriate for the study of the anti-inflammatory effects of GSPE against chronic inflammation.

2.4.2.3 Target tissues and cells in chronic inflammation related to obesity

2.4.2.3.1 Adipose tissue (AT)

Currently, AT is becoming increasingly recognized as a highly active metabolic tissue and an endocrine organ because it secretes a wide variety of hormones, cytokines, chemokines and growth factors that influence metabolism, vascular and endothelial function, appetite and satiety, immunity, fertility, inflammation, tumor growth and many other bodily processes (Schäffler et al. 2007), (Peinado et al. 2010).

In the context of obesity, AT is subjected to increased infiltration of immune cells especially macrophages. The infiltration of macrophages *per se* can trigger increased local and systemic inflammation (Attie & Scherer 2009). However, adipocytes play an important role in inflammation, and abnormal levels of metabolites such as lipids, FAs and cytokines from AT can activate monocytes and increase the secretion of inflammatory cytokines.

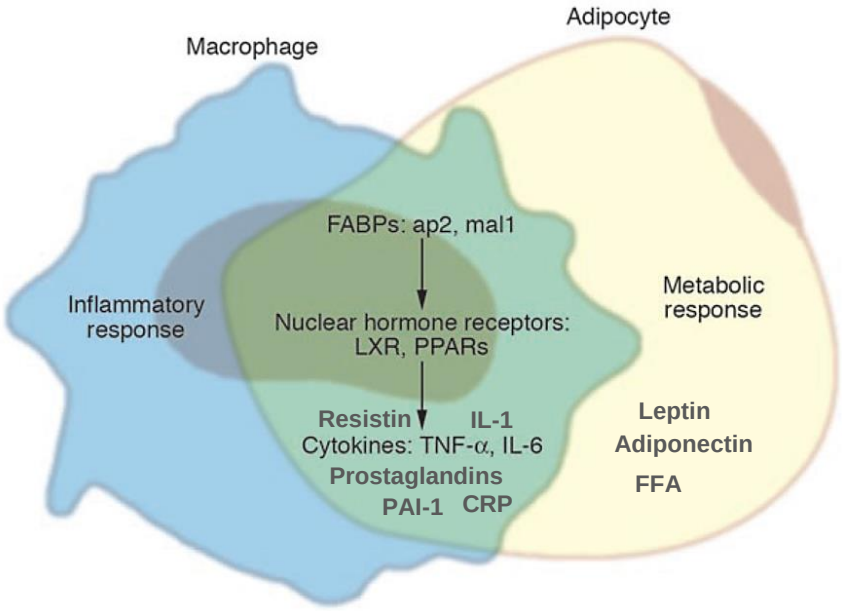


Figure 19. Lipids and inflammatory mediators: integration of metabolic and immune responses in adipocytes and macrophages through shared mechanisms in AT (Wellen & Hotamisligil 2005), (Wang & Nakayama 2010).

In fact, AT from obese individuals contains activated macrophages that together with adipocytes produce various cytokines (Fig. 19). These cytokines include inflammation-related adipokines such as leptin, ADIPQ, TNF-α, IL-1, IL-6, procoagulant substances such as PAI-1, vasoactive substances such as leptin, angiotensinogen and endothelin, and molecules that may contribute to insulin resistance such as FFA, TNF-α and resistin (Wang & Nakayama 2010). Based on their importance in metaflammation in obesity, AT cytokines are good candidates in the evaluation of the anti-inflammatory roles of bioactive compounds in models of chronic inflammation.

2.4.2.3.2 Adipocytes

Adipocytes are classically known as the storage depot of the body for excess energy. However, adipocytes are endocrine cells, as they serve multiple metabolic roles in the regulation of whole-body physiology. In the context of obesity, enlarged adipocytes have the capacity to recruit macrophages, release a range of adipokines and promote inflammation (Greenberg & Obin 2006). Adipocytes produce a wide range of hormones and cytokines involved in glucose metabolism (e.g., ADIPQ and resistin), lipid metabolism (e.g., cholesteryl ester transfer protein, CETP), inflammation (e.g., TNF-α and IL-6), acute phase reactants (CRP), coagulation (PAI-1), blood pressure (e.g., angiotensinogen and angiotensin II), and feeding behavior (leptin) thus affecting the metabolism and function of many organs and tissues including muscle, liver, vasculature, and brain (Attie & Scherer 2009) (Hajer et al. 2008) (Fig. 20).

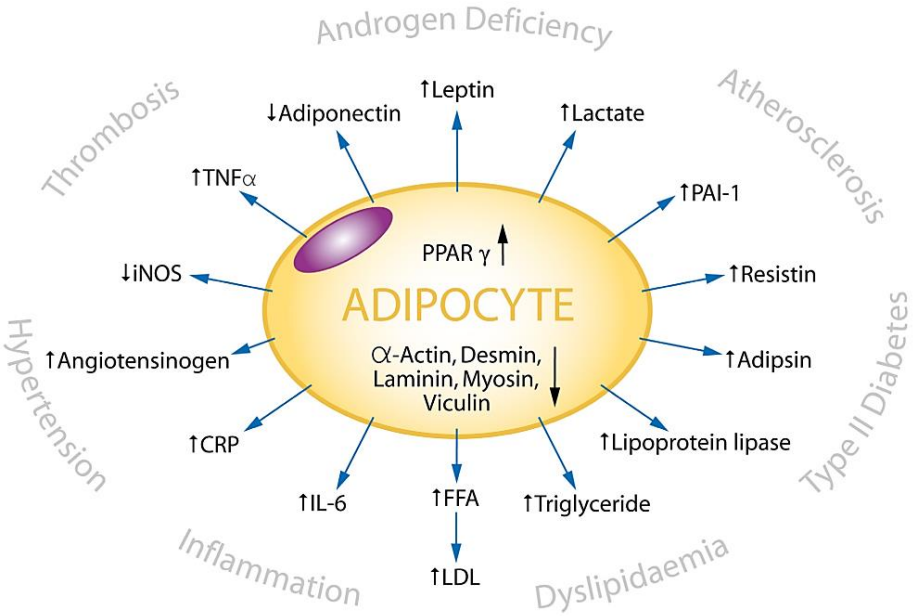


Figure 20. The metabolic and clinical effects of adipocyte activity (Carruthers et al. 2008).

Therefore, the adipocytes may have a relevant role in the AT by the expression of several adipokines and other proteins related to inflammation, being a suitable target of bioactive compounds that can exert anti-inflammatory effects in this tissue.

2.4.2.3.3 The stromal vascular fraction (SVF) and macrophages

Macrophages (M1) represent an important component of the SVF in AT, but this fraction is also known to contain mesenchymal stem cells (MSC), T regulatory cells, endothelial precursor cells, preadipocytes, and anti-inflammatory M2 macrophages (Riordan et al. 2009).

M1 macrophages or “classically activated” macrophages play a key role in humoral immunity and response to common pathogens. M1 macrophages secrete large amounts of proinflammatory cytokines such as TNF-α, iNOS, CC chemokine ligand 2 (CCL2 or MCP-1), and IL-12 and low levels of the anti-inflammatory cytokine IL-10. In contrast, M2 macrophages or “alternatively activated” M2-type macrophages play an anti-inflammatory role in AT. An increase in the number of M1 relative to M2 macrophages is characteristic of animals fed a high-fat diet and is observed in human obesity (Cusi 2012).

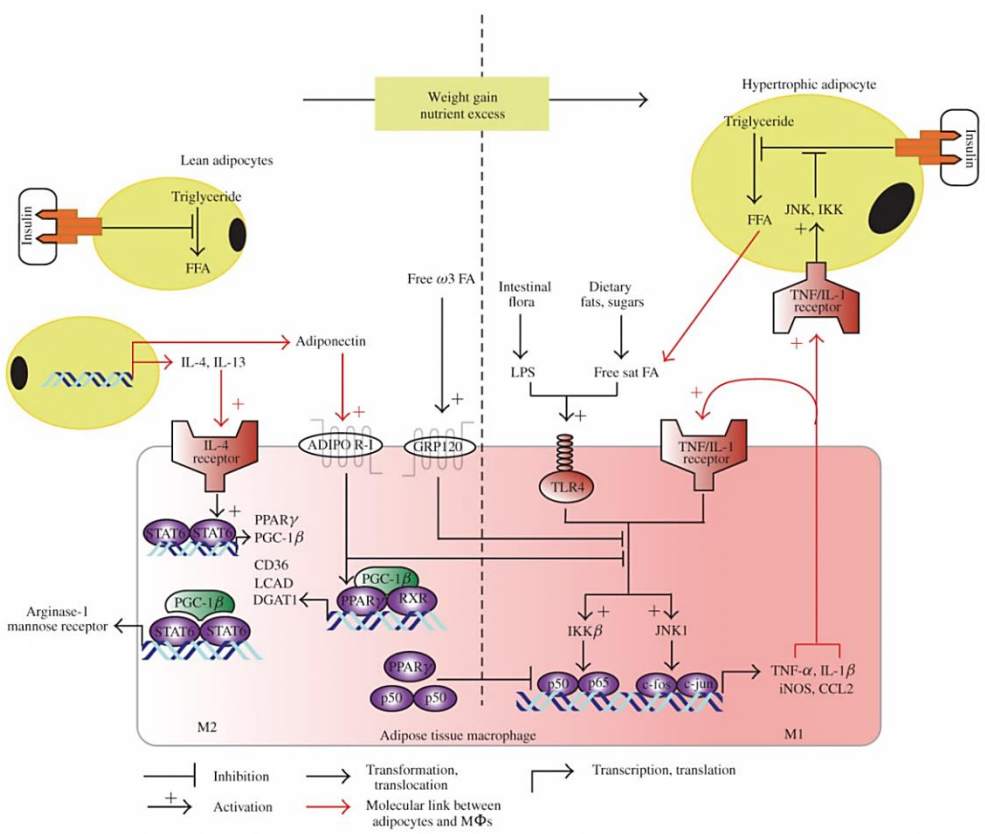


Figure 21. Interdependence of adipocyte and macrophage functional modes in the lean and obese states (Shapiro et al. 2011).

Macrophage infiltration occurs after the monocytes roll and attach to activated endothelial cells. These monocytes then extravasate through the endothelial cell layer and differentiate into macrophages (Neels & Olefsky 2006).

Cross-talk among adipocytes, macrophages, and endothelial cells may aggravate the inflammatory state resulting in the increased secretion of proinflammatory cytokines/chemokines, adipokines, and angiogenic factors (Neels & Olefsky 2006) (Fig. 21).

In fact, SVF cells contribute to the majority of the release of inflammatory mediators and interleukins such as TNF-α or IL-6. These and other adipose-derived factors participate in the induction and maintenance of the subacute proinflammatory state associated with obesity (Peinado et al. 2010), and can be included in studies of chronic inflammation as targets of bioactive compounds.

2.4.2.3.4 Liver

The liver represents one of the major metabolically active organs and is responsible for regulating ~80% of endogenous glucose production. The liver also plays other key roles in the regulation of lipid and protein metabolism, lipoprotein metabolism, and detoxification (Guri & Bassaganya-Riera 2011). The liver has the ability to control gluconeogenesis and glycogen storage as well as lipogenesis and cholesterol synthesis secretion and degradation (Gregor & Hotamisligil 2011). Hepatic lipid content and insulin resistance are associated with increased size and abundance of TG-rich very low-density lipoproteins, which contribute heavily to CVD and atherosclerosis. Both lipid accumulation and inflammation have been proposed to play significant roles in the dysfunction of the liver, which can induce significant systemic consequences because of the diverse array of metabolic activities associated with the liver (Guri & Bassaganya-Riera 2011).

One possibility is that chronic lipid infiltration induces stress kinase pathways and NF-κB-induced inflammation in hepatocytes, which over time can worsen and significantly impair hepatic function.

The accumulation of lipids in the liver often accompanies and parallels weight gain and obesity. Hepatic steatosis, the accumulation of lipid by the hepatocytes, has negative effects on liver function, which may also be manifested in the form of inflammation. For example, messenger RNA (mRNA) expression of proinflammatory cytokines IL-6, TNF-α, and IL-1 increases in the liver as hepatic adiposity increases. This suggests that steatosis might induce a subacute inflammatory response in the liver similar to that seen with the accumulation of lipid by adipocytes. As an alternative possibility, proinflammatory cytokines and lipids and

other substances produced by abdominal fat and carried to the liver through the portal circulation could contribute to hepatic inflammation. Proinflammatory substances activate the KCs, which are abundant in the liver and account for over 5% of the total hepatic cells. KCs become activated but do not increase in number in the context of obesity (Shoelson et al. 2007). Thus, several hepatic inflammatory proteins such as CRP and IL-6 can be suitable markers to assess the anti-inflammatory effects of bioactive compounds in this tissue.

2.4.2.3.5 Cross-talk between inflammatory mediators in tissues during obesity

A paracrine loop involving FFAs and inflammatory cytokines is thought to exist representing a vicious cycle between adipocytes and macrophages that accelerates inflammation (Nishimura et al. 2009). Obesity results in the *de novo* recruitment of monocytes and macrophages to AT, and this promotes AT inflammation and insulin resistance. In part, dietary SFAs activate TLR-2 and TLR-4 in AT macrophages resulting in the activation of inflammatory signaling cascades mediated by IRF3, JNK and NF-κB. These pathways induce the production of proinflammatory cytokines such as TNF-α. IL-1β is produced as a result of the activation of the NLRP3 (NOD-, LRR- and pyrin domain-containing protein 3) inflammasome potentially by ceramides, which are synthesized at higher levels during obesity. Moreover, proinflammatory cytokines inhibit the sensitivity of adipocytes to insulin. Once initiated, these proinflammatory cascades are perpetuated by cross-talk between the inflamed adipocytes and classically activated AT macrophages via the production of various factors (Chawla et al. 2011) (Fig. 22).

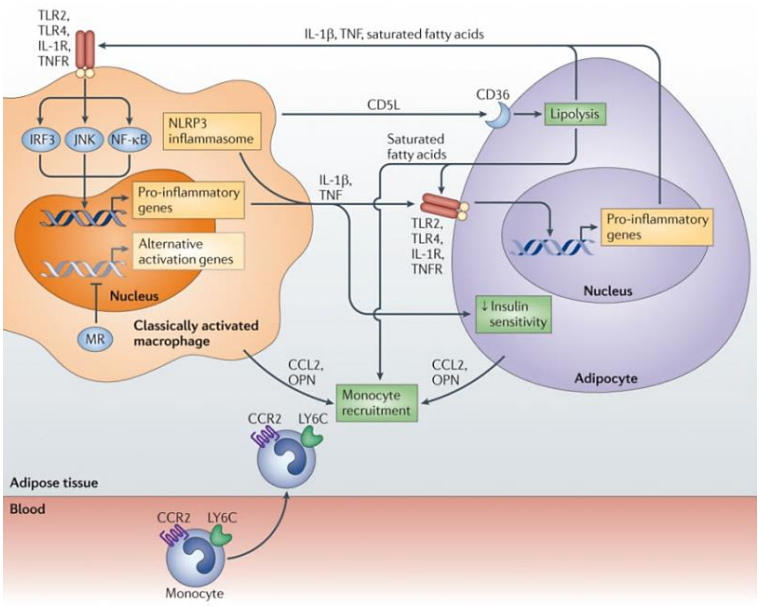


Figure 22. Classically activated macrophages contribute to AT inflammation and insulin resistance (Chawla et al. 2011).

Some of the identified chemotactic factors include CCL2 (or MCP-1) and osteopontin (OPN), the expression of which is induced in adipocytes and macrophages in the context of obesity. Importantly, CCL2 leads to the recruitment of LY6C+CCR2+ inflammatory monocytes, which differentiate into classically activated AT macrophages to enhance AT inflammation. In addition, AT macrophages release CD5−like antigen (CD5L), which promotes lipolysis in adipocytes after being taken up by adipocytes via CD36−mediated endocytosis. In a feed-forward loop, the released FAs induce the expression of chemokines leading to the recruitment of LY6C+CCR2+ inflammatory monocytes and macrophages into AT. Reciprocally, saturated FAs and proinflammatory cytokines (TNF-α and IL-1β) from adipocytes sustain the activation of AT macrophages. Mineralocorticoid receptor (MR) signaling also contributes to the classical activation of AT macrophages by inhibiting their alternative activation (Chawla et al. 2011). Therefore, the study of several proteins in adipocytes and macrophages in AT is essential to gain an understanding of the impact of obesity while understanding how bioactive molecules affect the body systemically.

On the other hand, one of the first organs that is affected when AT becomes dysfunctional and inflamed is the liver. In contrast to AT, the liver does not experience an infiltration of macrophages during the onset of obesity but instead undergoes the activation of inflammation within hepatic cells including the resident macrophage-like KCs (Gregor & Hotamisligil 2011). In addition, AT and the liver are in constant communication with each other via adipokines, lipid factors, and lipoprotein particles (Fig. 23).

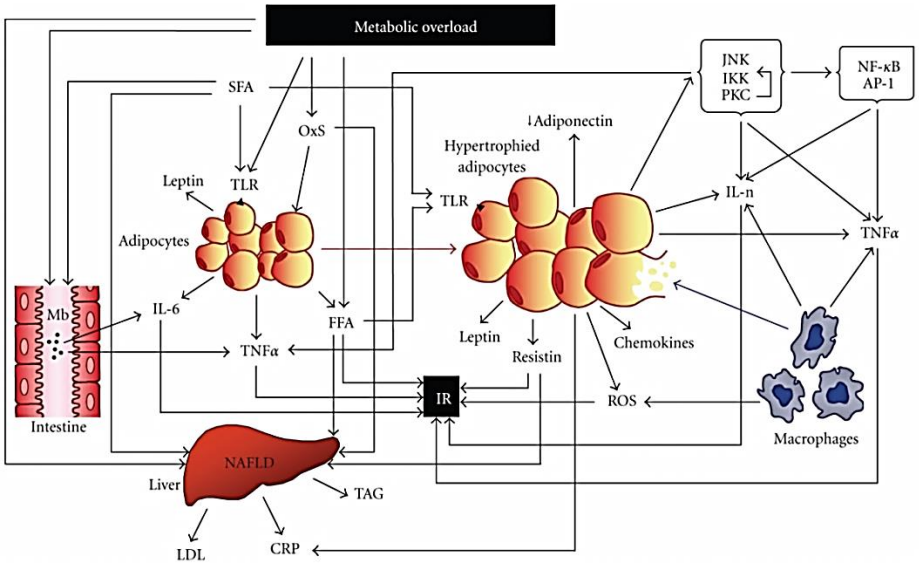


Figure 23. Overview of the complex interplay between obesity-related inflammation (Monteiro & Azevedo 2010).

In fact, the effects of the adipokines leptin and ADIPQ have been well characterized to have an effect on the liver. Leptin and ADIPQ stimulate hepatic FA oxidation through AMPK. IL-6 is also released into systemic circulation by AT during obesity and promotes the synthesis of the APP CRP in the liver further aggravating the inflammatory state in the context of obesity. Thus, the dysregulation of either AT or the liver is detrimental to the other and is ultimately detrimental for the entire system (Attie & Scherer 2009).

The study of the liver in obesity models is as important as the study of AT due to the complexity of metaflammation, in order to observe this complex interplay as well as any effects that bioactive molecules might exert on these tissues or on the phenotype of the rodent models.

2.5 References

Abete, I. et al., 2011. Obesity and metabolic syndrome: potential benefit from specific nutritional components. *Nutrition, metabolism, and cardiovascular diseases: NMCD*, 21 Suppl 2, p.B1–15.

Afaq, F. & Katiyar, S.K., 2011. Polyphenols: Skin Photoprotection and Inhibition of Photocarcinogenesis. *Mini Reviews in Medicinal Chemistry*, 11(14), p.1200–1215.

Akhtar Khan, N., 2010. Polyunsaturated fatty acids in the modulation of T-cell signalling. *Prostaglandins, leukotrienes, and essential fatty acids*, 82(4-6), p.179–187.

Andújar, I. et al., 2011. Inhibition of ulcerative colitis in mice after oral administration of a polyphenol-enriched cocoa extract is mediated by the inhibition of STAT1 and STAT3 phosphorylation in colon cells. *Journal of agricultural and food chemistry*, 59(12), p.6474–6483.

Appeldoorn, M.M. et al., 2009. Procyanidin dimers A1, A2, and B2 are absorbed without conjugation or methylation from the small intestine of rats. *The Journal of nutrition*, 139(8), p.1469–1473.

Aron, P.M. & Kennedy, J.A., 2008. Flavan-3-ols: nature, occurrence and biological activity. *Molecular nutrition & food research*, 52(1), p.79–104.

de Artiñano, A. & Castro, M., 2009. Experimental rat models to study the metabolic syndrome. *The British journal of nutrition*, 102(9), p.1246–1253.

Attie, A.D. & Scherer, P.E., 2009. Adipocyte metabolism and obesity. *Journal of lipid research*, 50 Suppl, p.S395–399.

Blackwell, T.S. & Christman, J.W., 1997. The Role of Nuclear Factor- κ B in Cytokine Gene Regulation. *American Journal of Respiratory Cell and Molecular Biology*, 17(1), p.3–9.

Blay, M et al., 2010. Isoflavone effect on gene expression profile and biomarkers of inflammation. *Journal of pharmaceutical and biomedical analysis*, 51(2), p.382–390.

Bode, J.G., Ehlting, C. & Häussinger, D., 2012. The macrophage response towards LPS and its control through the p38MAPK–STAT3 axis. *Cellular Signalling*, 24(6), p.1185–1194.

Bradley, J.R., 2008. TNF-mediated inflammatory disease. *The Journal of pathology*, 214(2), p.149–160.

Bucova, M., Bernadic, M. & Buckingham, T., 2008. C-reactive protein, cytokines and inflammation in cardiovascular diseases. *Bratislavské lekárske listy*, 109(8), p.333–340.

Buechler, C., Wanninger, J. & Neumeier, M., 2011. Adiponectin, a key adipokine in obesity related liver diseases. *World journal of gastroenterology: WJG*, 17(23), p.2801–2811.

Burns, J. et al., 2002. Plant foods and herbal sources of resveratrol. *Journal of agricultural and food chemistry*, 50(11), p.3337–3340.

Calder, P.C., 2008. Polyunsaturated fatty acids, inflammatory processes and inflammatory bowel diseases. *Molecular nutrition & food research*, 52(8), p.885–897.

Carruthers, M. et al., 2008. Are the adverse effects of glitazones linked to induced testosterone deficiency? *Cardiovascular diabetology*, 7, p.30.

Cauwels, A., 2007. Nitric oxide in shock. *Kidney international*, 72(5), p.557–565.

Chacón, M.R. et al., 2009. Grape-seed procyanidins modulate inflammation on human differentiated adipocytes in vitro. *Cytokine*, 47(2), p.137–142.

Chawla, A., Nguyen, K.D. & Goh, Y.P.S., 2011. Macrophage-mediated inflammation in metabolic disease. *Nature Reviews Immunology*, 11(11), p.738–749.

Cho, M.-L. et al., 2009. Grape seed proanthocyanidin extract (GSPE) attenuates collagen-induced arthritis. *Immunology letters*, 124(2), p.102–110.

Cohen, J., 2002. The immunopathogenesis of sepsis. *Nature*, 420(6917), p.885–891.

Conde, J. et al., 2011. Adipokines: biofactors from white adipose tissue. A complex hub among inflammation, metabolism, and immunity. *BioFactors (Oxford, England)*, 37(6), p.413–420.

Conforti, F. & Menichini, F., 2011. Phenolic compounds from plants as nitric oxide production inhibitors. *Current medicinal chemistry*, 18(8), p.1137–1145.

Cusi, K., 2012. Role of Obesity and Lipotoxicity in the Development of Nonalcoholic Steatohepatitis: Pathophysiology and Clinical Implications. *Gastroenterology*, 142(4), p.711–725.e6.

Ding, E.L. et al., 2006. Chocolate and Prevention of Cardiovascular Disease: A Systematic Review. *Nutrition & Metabolism*, 3(1), p.2.

Drouet, M. et al., 2012. Visceral fat and gut inflammation. *Nutrition (Burbank, Los Angeles County, Calif.)*, 28(2), p.113–117.

Duque Correa, M.A. & Rojas López, M., 2007. Activación alternativa del macrófago: La diversidad en las respuestas de una célula de la inmunidad innata ante la complejidad de los eventos de su ambiente. *Inmunología*, 26(2), p.73–86.

Dyson, A. et al., 2011. An integrated approach to assessing nitroso-redox balance in systemic inflammation. *Free radical biology & medicine*, 51(6), p.1137–1145.

Endo, Y. et al., 2010. Experimental periodontitis induces gene expression of proinflammatory cytokines in liver and white adipose tissues in obesity. *Journal of periodontology*, 81(4), p.520–526.

Erdman, J.W. et al., 2007. Flavonoids and Heart Health: Proceedings of the ILSI North America Flavonoids Workshop, May 31–June 1, 2005, Washington, DC. *The Journal of Nutrition*, 137(3), p.718S–737S.

Esposito, K. & Giugliano, D., 2011. Obesity, the metabolic syndrome, and sexual dysfunction in men. *Clinical pharmacology and therapeutics*, 90(1), p.169–173.

Fain, J.N., 2010. Release of inflammatory mediators by human adipose tissue is enhanced in obesity and primarily by the nonfat cells: a review. *Mediators of inflammation*, 2010, p.513948.

Feghali, C.A. & Wright, T.M., 1997. Cytokines in acute and chronic inflammation. *Frontiers in bioscience: a journal and virtual library*, 2, p.d12–26.

de Ferranti, S. & Mozaffarian, D., 2008. The perfect storm: obesity, adipocyte dysfunction, and metabolic consequences. *Clinical chemistry*, 54(6), p.945–955.

Fine, A.M., 2000. Oligomeric proanthocyanidin complexes: history, structure, and phytopharmaceutical applications. *Alternative medicine review: a journal of clinical therapeutic*, 5(2), p.144–151.

Fonseca, J.E. et al., 2009. Interleukin-6 as a key player in systemic inflammation and joint destruction. *Autoimmunity reviews*, 8(7), p.538–542.

Förstermann, U. & Sessa, W.C., 2012. Nitric oxide synthases: regulation and function. *European heart journal*, 33(7), p.829–837, 837a–837d.

Fortin, C.F. et al., 2010. Sepsis, leukocytes, and nitric oxide (NO): an intricate affair. *Shock (Augusta, Ga.)*, 33(4), p.344–352.

Fraga, C.G. & Oteiza, P.I., 2011. Dietary flavonoids: Role of (−)-epicatechin and related procyanidins in cell signaling. *Free Radical Biology and Medicine*, 51(4), p.813–823.

Fresno, M., Alvarez, R. & Cuesta, N., 2011. Toll-like receptors, inflammation, metabolism and obesity. *Archives of physiology and biochemistry*, 117(3), p.151–164.

Garrido, A.G., Figueiredo, L.F.P. de & Silva, M.R. e, 2004. Experimental models of sepsis and septic shock: an overview. *Acta Cirurgica Brasileira*, 19(2), p.82–88.

González-Périz, A. & Clària, J., 2010. Resolution of adipose tissue inflammation. *TheScientificWorldJournal*, 10, p.832–856.

Gotoh, K. et al., 2012. A Novel Anti-Inflammatory Role for Spleen-Derived Interleukin-10 in Obesity-Induced Inflammation in White Adipose Tissue and Liver. *Diabetes*. Available at: http://www.ncbi.nlm.nih.gov/pubmed/22648387 [Accedit juliol 5, 2012].

Greenberg, A.S. & Obin, M.S., 2006. Obesity and the role of adipose tissue in inflammation and metabolism. *The American Journal of Clinical Nutrition*, 83(2), p.461S–465S.

Gregor, M.F. & Hotamisligil, G.S., 2011. Inflammatory Mechanisms in Obesity. *Annual Review of Immunology*, 29(1), p.415–445.

Groux, H. & Cottrez, F., 2003. The complex role of interleukin-10 in autoimmunity. *Journal of autoimmunity*, 20(4), p.281–285.

Gu, L. et al., 2004. Concentrations of proanthocyanidins in common foods and estimations of normal consumption. *The Journal of nutrition*, 134(3), p.613–617.

Guix, F.X. et al., 2005. The physiology and pathophysiology of nitric oxide in the brain. *Progress in Neurobiology*, 76(2), p.126–152.

Guri, A.J. & Bassaganya-Riera, J., 2011. Systemic effects of white adipose tissue dysregulation and obesity-related inflammation. *Obesity (Silver Spring, Md.)*, 19(4), p.689–700.

Hajer, G.R., van Haeften, T.W. & Visseren, F.L.J., 2008. Adipose tissue dysfunction in obesity, diabetes, and vascular diseases. *European heart journal*, 29(24), p.2959–2971.

Hansson, G.K., 2005. Inflammation, atherosclerosis, and coronary artery disease. *The New England journal of medicine*, 352(16), p.1685–1695.

Heiss, C., Keen, Carl L. & Kelm, M., 2010. Flavanols and cardiovascular disease prevention. *European Heart Journal*. Available at: http://eurheartj.oxfordjournals.org/content/early/2010/09/18/eurheartj.ehq332 [Accedit juliol 11, 2012].

Hu, X. et al., 2007. Crosstalk among Jak-STAT, Toll-like receptor, and ITAM-dependent pathways in macrophage activation. *Journal of leukocyte biology*, 82(2), p.237–243.

Hu, X. et al., 2002. Sensitization of IFN-gamma Jak-STAT signaling during macrophage activation. *Nature immunology*, 3(9), p.859–866.

Hull, M.A., 2011. Omega-3 polyunsaturated fatty acids. *Best practice & research. Clinical gastroenterology*, 25(4-5), p.547–554.

Jaffer, U., Wade, R.G. & Gourlay, T., 2010. Cytokines in the systemic inflammatory response syndrome: a review. *HSR Proceedings in Intensive Care and Cardiovascular Anesthesia*, 2(3), p.161–175.

Ji, R.-R. et al., 2011. Emerging roles of resolvins in the resolution of inflammation and pain. *Trends in neurosciences*, 34(11), p.599–609.

Joubert, J. & Malan, S.F., 2011. Novel nitric oxide synthase inhibitors: a patent review. *Expert opinion on therapeutic patents*, 21(4), p.537–560.

Juan, C.-C. et al., 2008. Involvement of iNOS and NO in TNF-alpha-downregulated resistin gene expression in 3T3-L1 adipocytes. *Obesity (Silver Spring, Md.)*, 16(6), p.1219–1225.

Kalupahana, N.S., Claycombe, K.J. & Moustaid-Moussa, N., 2011. (n-3) Fatty acids alleviate adipose tissue inflammation and insulin resistance: mechanistic insights. *Advances in nutrition (Bethesda, Md.)*, 2(4), p.304–316.

Kaminska, B., 2005. MAPK signalling pathways as molecular targets for anti-inflammatory therapy—from molecular mechanisms to therapeutic benefits. *Biochimica et Biophysica Acta (BBA) - Proteins & Proteomics*, 1754(1–2), p.253–262.

Kao, Y.H., Hiipakka, R.A. & Liao, S., 2000. Modulation of endocrine systems and food intake by green tea epigallocatechin gallate. *Endocrinology*, 141(3), p.980–987.

Kar, P. et al., 2009. Effects of grape seed extract in Type 2 diabetic subjects at high cardiovascular risk: a double blind randomized placebo controlled trial examining metabolic markers, vascular tone, inflammation, oxidative stress and insulin sensitivity. *Diabetic medicine: a journal of the British Diabetic Association*, 26(5), p.526–531.

Karastergiou, K. & Mohamed-Ali, V., 2010. The autocrine and paracrine roles of adipokines. *Molecular and cellular endocrinology*, 318(1-2), p.69–78.

Khan, F.A. & Khan, M.F., 2010. Inflammation and acute phase response. *International Journal of Applied Biology and Pharmaceutical Technology*, 1(2), p.312–321.

Khozin-Goldberg, I., Iskandarov, U. & Cohen, Z., 2011. LC-PUFA from photosynthetic microalgae: occurrence, biosynthesis, and prospects in biotechnology. *Applied microbiology and biotechnology*, 91(4), p.905–915.

Kim, W., McMurray, D.N. & Chapkin, R.S., 2010. n-3 polyunsaturated fatty acids--physiological relevance of dose. *Prostaglandins, leukotrienes, and essential fatty acids*, 82(4-6), p.155–158.

Kobayashi, Y., 2010. The regulatory role of nitric oxide in proinflammatory cytokine expression during the induction and resolution of inflammation. *Journal of leukocyte biology*, 88(6), p.1157–1162.

Komprda, T., 2012. Eicosapentaenoic and docosahexaenoic acids as inflammation-modulating and lipid homeostasis influencing nutraceuticals: A review. *Journal of Functional Foods*, 4(1), p.25–38.

Kremmyda, L.S. et al., 2011. Fatty acids as biocompounds: their role in human metabolism, health and disease: a review. part 2: fatty acid physiological roles and applications in human health and disease. *Biomedical papers of the Medical Faculty of the University Palacký, Olomouc, Czechoslovakia*, 155(3), p.195–218.

Kris-Etherton, P.M. et al., 2004. Bioactive compounds in nutrition and health-research methodologies for establishing biological function: the antioxidant and anti-inflammatory effects of flavonoids on atherosclerosis. *Annual review of nutrition*, 24, p.511–538.

Lago, F. et al., 2007. Adipokines as emerging mediators of immune response and inflammation. *Nature clinical practice. Rheumatology*, 3(12), p.716–724.

de la Lastra, C Alarcón & Villegas, I, 2007. Resveratrol as an antioxidant and pro-oxidant agent: mechanisms and clinical implications. *Biochemical Society Transactions*, 35(Pt 5), p.1156–1160.

de la Lastra, Catalina Alarcón & Villegas, Isabel, 2005. Resveratrol as an anti-inflammatory and anti-aging agent: mechanisms and clinical implications. *Molecular Nutrition & Food Research*, 49(5), p.405–430.

Lau, D.C.W. et al., 2005. Adipokines: molecular links between obesity and atheroslcerosis. *American journal of physiology. Heart and circulatory physiology*, 288(5), p.H2031–2041.

Liaskou, E., Wilson, D.V. & Oo, Y.H., 2012. Innate immune cells in liver inflammation. *Mediators of inflammation*, 2012, p.949157.

Lin, W.-W. & Karin, M., 2007. A cytokine-mediated link between innate immunity, inflammation, and cancer. *Journal of Clinical Investigation*, 117(5), p.1175–1183.

Liu, R.H., 2004. Potential Synergy of Phytochemicals in Cancer Prevention: Mechanism of Action. *The Journal of Nutrition*, 134(12), p.3479S–3485S.

Lolmède, K. et al., 2011. Immune cells in adipose tissue: key players in metabolic disorders. *Diabetes & metabolism*, 37(4), p.283–290.

Martín-Cordero, L. et al., 2011. The interleukin-6 and noradrenaline mediated inflammation-stress feedback mechanism is dysregulated in metabolic syndrome: effect of exercise. *Cardiovascular diabetology*, 10, p.42.

Maury, E. & Brichard, S.M., 2010. Adipokine dysregulation, adipose tissue inflammation and metabolic syndrome. *Molecular and cellular endocrinology*, 314(1), p.1–16.

Medina, I. et al., 2006. Functional fatty fish supplemented with grape procyanidins. Antioxidant and proapoptotic properties on colon cell lines. *Journal of Agricultural and Food Chemistry*, 54(10), p.3598–3603.

Mellor, D.D. et al., 2010. High-cocoa polyphenol-rich chocolate improves HDL cholesterol in Type 2 diabetes patients. *Diabetic medicine: a journal of the British Diabetic Association*, 27(11), p.1318–1321.

Meyer, B.J., 2011. Are we consuming enough long chain omega-3 polyunsaturated fatty acids for optimal health? *Prostaglandins, leukotrienes, and essential fatty acids*, 85(5), p.275–280.

Mocellin, S. et al., 2004. The multifaceted relationship between IL-10 and adaptive immunity: putting together the pieces of a puzzle. *Cytokine & Growth Factor Reviews*, 15(1), p.61–76.

Monagas, M. et al., 2009. Effect of cocoa powder on the modulation of inflammatory biomarkers in patients at high risk of cardiovascular disease. *The American journal of clinical nutrition*, 90(5), p.1144–1150.

Montagut, G. et al., 2009. A trimer plus a dimer-gallate reproduce the bioactivity described for an extract of grape seed procyanidins. *Food Chemistry*, 116(1), p.265–270.

Monteiro, R. & Azevedo, I., 2010. Chronic Inflammation in Obesity and the Metabolic Syndrome. *Mediators of Inflammation*, 2010, p.1–10.

Morán Penco, J.M., 2007. [Animal models for the study of systemic inflammatory response and parenteral nutrition]. *Nutrición hospitalaria: organo oficial de la Sociedad Española de Nutrición Parenteral y Enteral*, 22(2), p.146–159.

Moreno-Aliaga, M.J., Lorente-Cebrián, S. & Martínez, J Alfredo, 2010. Regulation of adipokine secretion by n-3 fatty acids. *The Proceedings of the Nutrition Society*, 69(3), p.324–332.

Morgan, D.W. & Marshall, L.A. ed., 1999. *In Vivo Models of Inflammation*, Birkhäuser. Available at: http://www.springer.com/birkhauser/biosciences/book/978-3-7643-7757-1 [Accedit octubre 12, 2012].

Mozaffarian, D. & Wu, J.H.Y., 2012. (n-3) fatty acids and cardiovascular health: are effects of EPA and DHA shared or complementary? *The Journal of nutrition*, 142(3), p.614S–625S.

Mozaffarian, D. & Wu, J.H.Y., 2011. Omega-3 fatty acids and cardiovascular disease: effects on risk factors, molecular pathways, and clinical events. *Journal of the American College of Cardiology*, 58(20), p.2047–2067.

Mueller, M., Hobiger, S. & Jungbauer, A., 2010. Anti-inflammatory activity of extracts from fruits, herbs and spices. *Food Chemistry*, 122(4), p.987–996.

Murakami, A. & Ohigashi, H., 2007. Targeting NOX, INOS and COX-2 in inflammatory cells: chemoprevention using food phytochemicals. *International journal of cancer. Journal international du cancer*, 121(11), p.2357–2363.

Neels, J.G. & Olefsky, J.M., 2006. Inflamed fat: what starts the fire? *The Journal of clinical investigation*, 116(1), p.33–35.

Neumeier, M. et al., 2006. Different effects of adiponectin isoforms in human monocytic cells. *Journal of leukocyte biology*, 79(4), p.803–808.

Newton, K. & Dixit, V.M., 2012. Signaling in innate immunity and inflammation. *Cold Spring Harbor perspectives in biology*, 4(3). Available at: http://www.ncbi.nlm.nih.gov/pubmed/22296764 [Accedit juliol 2, 2012].

Nishimoto, N. & Kishimoto, T., 2006. Interleukin 6: from bench to bedside. *Nature clinical practice. Rheumatology*, 2(11), p.619–626.

Nishimura, S., Manabe, I. & Nagai, R., 2009. Adipose tissue inflammation in obesity and metabolic syndrome. *Discovery medicine*, 8(41), p.55–60.

O'Shea, J.J. & Murray, P.J., 2008. Cytokine signaling modules in inflammatory responses. *Immunity*, 28(4), p.477–487.

Oh, D.Y. et al., 2010. GPR120 is an omega-3 fatty acid receptor mediating potent anti-inflammatory and insulin-sensitizing effects. *Cell*, 142(5), p.687–698.

Ono, K. et al., 2003. Effects of an aqueous extract of cocoa on nitric oxide production of macrophages activated by lipopolysaccharide and interferon-gamma. *Nutrition (Burbank, Los Angeles County, Calif.)*, 19(7-8), p.681–685.

Opal, S.M. & DePalo, V.A., 2000. Anti-inflammatory cytokines. *Chest*, 117(4), p.1162–1172.

Ortega, A. et al., 2012. Dietary fatty acids linking postprandial metabolic response and chronic diseases. *Food & function*, 3(1), p.22–27.

Ouchi, N. et al., 2012. Adipocytokines and obesity-linked disorders. *Nagoya journal of medical science*, 74(1-2), p.19–30.

Panchal, S.K. & Brown, L., 2011. Rodent models for metabolic syndrome research. *Journal of biomedicine & biotechnology*, 2011, p.351982.

de Pascual-Teresa, S., Moreno, D.A. & García-Viguera, C., 2010. Flavanols and anthocyanins in cardiovascular health: a review of current evidence. *International journal of molecular sciences*, 11(4), p.1679–1703.

Peinado, J.R. et al., 2010. The stromal-vascular fraction of adipose tissue contributes to major differences between subcutaneous and visceral fat depots. *Proteomics*, 10(18), p.3356–3366.

Pepys, M.B. & Hirschfield, G.M., 2003. C-reactive protein: a critical update. *The Journal of clinical investigation*, 111(12), p.1805–1812.

Perron, N.R. & Brumaghim, J.L., 2009. A review of the antioxidant mechanisms of polyphenol compounds related to iron binding. *Cell biochemistry and biophysics*, 53(2), p.75–100.

Pestka, S. et al., 2004. Interleukin-10 and related cytokines and receptors. *Annual review of immunology*, 22, p.929–979.

Picchi, A. et al., 2006. Tumor necrosis factor-alpha induces endothelial dysfunction in the prediabetic metabolic syndrome. *Circulation research*, 99(1), p.69–77.

Popa, C. et al., 2007. The role of TNF-alpha in chronic inflammatory conditions, intermediary metabolism, and cardiovascular risk. *Journal of lipid research*, 48(4), p.751–762.

Puglisi, M.J. & Fernandez, M.L., 2008. Modulation of C-reactive protein, tumor necrosis factor-alpha, and adiponectin by diet, exercise, and weight loss. *The Journal of nutrition*, 138(12), p.2293–2296.

Quideau, S. et al., 2011. Plant polyphenols: chemical properties, biological activities, and synthesis. *Angewandte Chemie (International ed. in English)*, 50(3), p.586–621.

Rahman, M.M., Bhattacharya, A. & Fernandes, G., 2008. Docosahexaenoic acid is more potent inhibitor of osteoclast differentiation in RAW 264.7 cells than eicosapentaenoic acid. *Journal of cellular physiology*, 214(1), p.201–209.

Ramiro, E. et al., 2005. Flavonoids from Theobroma cacao down-regulate inflammatory mediators. *Journal of agricultural and food chemistry*, 53(22), p.8506–8511.

Rasmussen, S.E. et al., 2005. Dietary proanthocyanidins: occurrence, dietary intake, bioavailability, and protection against cardiovascular disease. *Molecular nutrition & food research*, 49(2), p.159–174.

Rasouli, N. & Kern, P.A., 2008. Adipocytokines and the metabolic complications of obesity. *The Journal of clinical endocrinology and metabolism*, 93(11 Suppl 1), p.S64–73.

Ringseis, R. & Eder, K., 2010. Fatty acids and signalling in endothelial cells. *Prostaglandins, Leukotrienes and Essential Fatty Acids*, 82(4–6), p.189–198.

Riordan, N.H. et al., 2009. Non-expanded adipose stromal vascular fraction cell therapy for multiple sclerosis. *Journal of translational medicine*, 7, p.29.

Roszer, T., *The Biology of Subcellular Nitric Oxide*, Available at: http://www.springer.com/biomed/book/978-94-007-2818-9 [Accedit juliol 6, 2012].

Russo, C. & Polosa, R., 2005. TNF-alpha as a promising therapeutic target in chronic asthma: a lesson from rheumatoid arthritis. *Clinical science (London, England: 1979)*, 109(2), p.135–142.

Sánchez-Fidalgo, S. et al., 2010. Dietary supplementation of resveratrol attenuates chronic colonic inflammation in mice. *European Journal of Pharmacology*, 633(1-3), p.78–84.

Saw, C.L.L., Huang, Y. & Kong, A.-N., 2010. Synergistic anti-inflammatory effects of low doses of curcumin in combination with polyunsaturated fatty acids: docosahexaenoic acid or eicosapentaenoic acid. *Biochemical Pharmacology*, 79(3), p.421–430.

Schäffler, A., Schölmerich, J. & Salzberger, B., 2007. Adipose tissue as an immunological organ: Toll-like receptors, C1q/TNFs and CTRPs. *Trends in Immunology*, 28(9), p.393–399.

Schmid, J.A. & Birbach, A., 2008. IkappaB kinase beta (IKKbeta/IKK2/IKBKB)--a key molecule in signaling to the transcription factor NF-kappaB. *Cytokine & growth factor reviews*, 19(2), p.157–165.

Schober, F. et al., 2007. Low molecular weight adiponectin negatively correlates with the waist circumference and monocytic IL-6 release. *Biochemical and biophysical research communications*, 361(4), p.968–973.

Serhan, C.N. & Petasis, N.A., 2011. Resolvins and protectins in inflammation resolution. *Chemical reviews*, 111(10), p.5922–5943.

Serini, S. et al., 2011. Dietary n-3 polyunsaturated fatty acids and the paradox of their health benefits and potential harmful effects. *Chemical research in toxicology*, 24(12), p.2093–2105.

Serra, A. et al., 2010. Bioavailability of procyanidin dimers and trimers and matrix food effects in in vitro and in vivo models. *The British journal of nutrition*, 103(7), p.944–952.

Shaikh, S.R., 2012. Biophysical and biochemical mechanisms by which dietary N-3 polyunsaturated fatty acids from fish oil disrupt membrane lipid rafts. *The Journal of nutritional biochemistry*, 23(2), p.101–105.

Shapiro, H., Lutaty, A. & Ariel, A., 2011. Macrophages, Meta-Inflammation, and Immuno-Metabolism. *The Scientific World Journal*, 11, p.2509–2529.

Shoelson, S.E., Herrero, L. & Naaz, A., 2007. Obesity, inflammation, and insulin resistance. *Gastroenterology*, 132(6), p.2169–2180.

Shoji, T. et al., 2006. Apple procyanidin oligomers absorption in rats after oral administration: analysis of procyanidins in plasma using the porter method and high-performance liquid chromatography/tandem mass spectrometry. *Journal of agricultural and food chemistry*, 54(3), p.884–892.

Shuai, K. & Liu, B., 2003. Regulation of JAK-STAT signalling in the immune system. *Nature reviews. Immunology*, 3(11), p.900–911.

Stienstra, R. et al., 2012. The inflammasome puts obesity in the danger zone. *Cell metabolism*, 15(1), p.10–18.

Stryjecki, C. & Mutch, D.M., 2011. Fatty acid-gene interactions, adipokines and obesity. *European journal of clinical nutrition*, 65(3), p.285–297.

Tai, C.C. & Ding, S.T., 2010. N-3 polyunsaturated fatty acids regulate lipid metabolism through several inflammation mediators: mechanisms and implications for obesity prevention. *The Journal of nutritional biochemistry*, 21(5), p.357–363.

Taub, R., 2003. Hepatoprotection via the IL-6/Stat3 pathway. *Journal of Clinical Investigation*, 112(7), p.978–980.

Tayal, V. & Kalra, B.S., 2008. Cytokines and anti-cytokines as therapeutics--an update. *European journal of pharmacology*, 579(1-3), p.1–12.

Tedgui, A. & Mallat, Z., 2006. Cytokines in Atherosclerosis: Pathogenic and Regulatory Pathways. *Physiological Reviews*, 86(2), p.515–581.

Terra, X. et al., 2007. Grape-seed procyanidins act as antiinflammatory agents in endotoxin-stimulated RAW 264.7 macrophages by inhibiting NFkB signaling pathway. *Journal of Agricultural and Food Chemistry*, 55(11), p.4357–4365.

Terra, X., Montagut, G., et al., 2009. Grape-seed procyanidins prevent low-grade inflammation by modulating cytokine expression in rats fed a high-fat diet. *The Journal of Nutritional Biochemistry*, 20(3), p.210–218.

Terra, X., Fernández-Larrea, J., et al., 2009. Inhibitory effects of grape seed procyanidins on foam cell formation in vitro. *Journal of Agricultural and Food Chemistry*, 57(6), p.2588–2594.

Terra, X. et al., 2011. Modulatory effect of grape-seed procyanidins on local and systemic inflammation in diet-induced obesity rats. *The Journal of nutritional biochemistry*, 22(4), p.380–387.

Thalhamer, T., McGrath, M.A. & Harnett, M.M., 2008. MAPKs and their relevance to arthritis and inflammation. *Rheumatology*, 47(4), p.409–414.

Tiegs, G. & Lohse, A.W., 2010. Immune tolerance: What is unique about the liver. *Journal of Autoimmunity*, 34(1), p.1–6.

Tilg, H. & Moschen, A.R., 2006. Adipocytokines: mediators linking adipose tissue, inflammation and immunity. *Nature reviews. Immunology*, 6(10), p.772–783.

Tîrziu, E., 2009. Acute-phase proteins in immune response. *Lucrari Stiintifice Medicina Veterinara*, 42(1).

Tsai, S.-H., Lin-Shiau, S.-Y. & Lin, J.-K., 1999. Suppression of nitric oxide synthase and the down-regulation of the activation of NFκB in macrophages by resveratrol. *British Journal of Pharmacology*, 126(3), p.673–680.

Vauzour, D. et al., 2010. Polyphenols and Human Health: Prevention of Disease and Mechanisms of Action. *Nutrients*, 2(11), p.1106–1131.

Villanueva, C. & Giulivi, C., 2010. Subcellular and cellular locations of nitric-oxide synthase isoforms as determinants of health and disease. *Free radical biology & medicine*, 49(3), p.307–316.

Wall, R. et al., 2010. Fatty acids from fish: the anti-inflammatory potential of long-chain omega-3 fatty acids. *Nutrition reviews*, 68(5), p.280–289.

Wang, Y. et al., 2008. Post-translational modifications of adiponectin: mechanisms and functional implications. *The Biochemical journal*, 409(3), p.623–633.

Wang, Y.-J. et al., 2009. Consumption of grape seed extract prevents amyloid-beta deposition and attenuates inflammation in brain of an Alzheimer's disease mouse. *Neurotoxicity research*, 15(1), p.3–14.

Wang, Z. & Nakayama, T., 2010. Inflammation, a Link between Obesity and Cardiovascular Disease. *Mediators of Inflammation*, 2010, p.1–17.

Weldon, S.M. et al., 2007. Docosahexaenoic acid induces an anti-inflammatory profile in lipopolysaccharide-stimulated human THP-1 macrophages more effectively than eicosapentaenoic acid. *The Journal of Nutritional Biochemistry*, 18(4), p.250–258.

Wellen, K.E. & Hotamisligil, G.S., 2005. Inflammation, stress, and diabetes. *The Journal of clinical investigation*, 115(5), p.1111–1119.

Wozniak, S.E. et al., 2009. Adipose tissue: the new endocrine organ? A review article. *Digestive diseases and sciences*, 54(9), p.1847–1856.

Yashodhara, B.M. et al., 2009. Omega-3 fatty acids: a comprehensive review of their role in health and disease. *Postgraduate medical journal*, 85(1000), p.84–90.

Zanotti-Cavazzoni, S.L. & Goldfarb, R.D., 2009. Animal Models of Sepsis. *Critical Care Clinics*, 25(4), p.703–719.

3 HYPOTHESES AND OBJECTIVES

3 Hypothesis and Objectives

In the past years, many of the beneficial effects of polyphenols like procyanidins have been discovered such as their role as an antioxidant and an anti-inflammatory. Our research group has recently illustrated these effects using different models of induced inflammation by analyzing the effects of procyanidins on activated macrophages *in vitro* and *in vivo* using models of chronic inflammation such as diet-induced obesity in rats fed a cafeteria diet or a high-fat diet.

The objectives of this thesis focused on expanding our knowledge regarding the anti-inflammatory properties of these molecules using other models not studied yet, which led to our three main hypotheses:

Hypothesis 1: Several polyphenols and PUFAs in their pure form, which are both well-known anti-inflammatory molecules, possess the capability to inhibit various proinflammatory signaling pathways such as NF-κB or AP-1. Those bioactive compounds have a synergistic anti-inflammatory effect in activated macrophages based on the overlap in their mechanisms of action.

Hypothesis 2: The procyanidins from grape seed procyanidin extract (GSPE) will have preventive anti-inflammatory effects in a rat model of acute inflammation. GSPE prevent the acute inflammatory response after a high dose of LPS in the rat.

Hypothesis 3: GSPE will have anti-inflammatory effects in an aggravated model of chronic inflammation *in vivo* such as a genetically obese rat model. Based on previous evidence supporting the idea that GSPE has anti-inflammatory effects in models of obesity induced by diet, we want to know if these molecules can promote similar responses in a genetic model of obesity.

To assess the validity of these hypotheses, the following objectives were proposed:

Objectives to test hypothesis 1:

- To determine the anti-inflammatory effects of several polyphenols such as the EGCG, the stilbene resveratrol, and the dimeric procyanidins B1, B2, B3 and B4, and PUFAs such as EPA and DHA against the inflammatory response in macrophages *in vitro*. RAW 264.7 murine macrophages were incubated with LPS in the presence of or without the bioactive molecules to mimic the proinflammatory response.
- To determine the synergistic, additive and antagonistic effects of the polyphenols when combined with PUFAs by assessing their ability to modulate NO levels.

- To determine the mechanisms that these molecules follow to exert their anti-inflammatory action by assessing the essential signaling pathways of inflammation such as NF-κB, p38, SAPK/JNK and STAT3.
- To determine the effect of these bioactive molecules on the expression of genes related to inflammation, oxidative stress and metabolism with low density arrays.

Objectives to test hypothesis 2:

- To ascertain the anti-inflammatory effects of GSPE in an acute inflammation model *in vivo*. LPS was administered intraperitoneally to mimic acute inflammation in *Wistar* rats. Several doses of GSPE were assessed to determine which dose is the most effective using moderate-high nutraceutical doses and pharmacological doses.
- To determine the anti-inflammatory effect of GSPE based on the ability of GSPE to modulate several proinflammatory and anti-inflammatory proteins at the systemic level in the plasma. To analyze the ability of GSPE to modulate NOx levels at both the systemic and local levels in key metabolic tissues as well as organs involved in the innate immune response such as the liver and the spleen.
- To determine the effects of GSPE on the expression of proinflammatory and anti-inflammatory genes in the liver.

Objectives to test hypothesis 3:

- To investigate the anti-inflammatory effects of GSPE using an *in vivo* model of chronic inflammation different than those previously studied. To this end, we used a genetic model of obesity represented by the *Zucker fa/fa* rats. The dose of GSPE administered orally (35 mg/kg*day of GSPE) is considered a moderate nutritional dose.
- To ascertain that the anti-inflammatory effects of GSPE modulate the proinflammatory proteins at both the systemic and local levels in the plasma and in various relevant tissues.
- To determine the effects of GSPE locally in isolated adipocytes and SVF from MAT and contrast these results with the effects of GSPE in whole MAT at the gene expression level focusing on the expression of proinflammatory and anti-inflammatory genes. The results of this study will offer evidence as to the identity of the target cells of procyanidins through which they promote their anti-inflammatory action in a key tissue of the obesity.

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ROLE OF BIOACTIVE FOOD COMPOUNDS IN ACUTE AND CHRONIC INFLAMMATION
Victor Pallarés López
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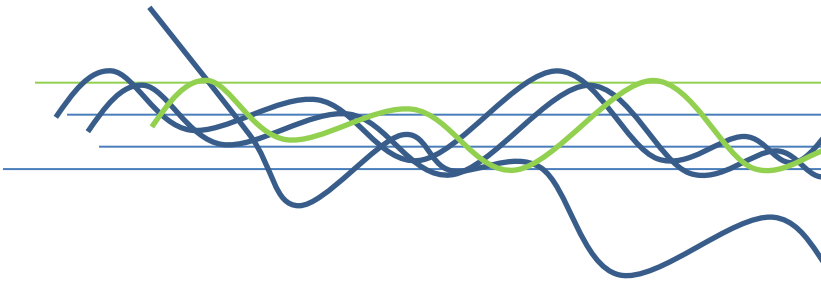
4

RESULTS



In vitro studies

The aim of this part was to evaluate the anti-inflammatory effects of several bioactive molecules present in food in activated macrophages. The dimeric procyanidins B1, B2, B3 and B4, resveratrol, and EGCG, which are polyphenolic compounds, and EPA and DHA as examples of PUFAs, were the molecules tested.

In recent *in vitro* studies, these molecules were shown to have anti-inflammatory effects in several models and cellular types. However, the mechanisms by which these compounds modulate the intracellular signaling and the transcription of genes related to inflammation, as well as the possible synergistic effects of these molecules in combination, have not been elucidated in murine macrophages activated by lipopolysaccharides.

4.1

Additive, antagonistic, and synergistic effects of procyanidins and polyunsaturated fatty acids over inflammation in RAW 264.7 macrophages activated by lipopolysaccharide.

Victor Pallarès M.Sc. [a], Damien Calay Ph.D. [b], Lídia Cedó M.Sc. [a], Anna Castell-Auví Ph.D. [a], Martine Raes Prof. [b], Montserrat Pinent Ph.D. [a], Anna Ardèvol Ph.D. [a], Lluís Arola Prof. [a], Mayte Blay Ph.D. [a], *

a Departament de Bioquímica i Biotecnologia, Universitat Rovira i Virgili, Tarragona, Spain

b Laboratory of Cellular Biochemistry and Biology, University of Namur (FUNDP-URBC), Namur, Belgium

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Basic nutritional investigation

Additive, antagonistic, and synergistic effects of procyanidins and polyunsaturated fatty acids over inflammation in RAW 264.7 macrophages activated by lipopolysaccharide

Victor Pallarès M.Sc. [a], Damien Calay Ph.D. [b], Lídia Cedó M.Sc. [a], Anna Castell-Auví M.Sc. [a], Martine Raes Ph.D. [b], Montserrat Pinent Ph.D. [a], Anna Ardévol Ph.D. [a], Lluís Arola Prof. [a], Mayte Blay Ph.D. [a,*]

[a] *Departament de Bioquímica i Biotecnologia, Universitat Rovira i Virgili, Tarragona, Spain*
[b] *Department of Biochemistry and Cellular Biology, University of Namur (FUNDP-URBC), Namur, Belgium*

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ABSTRACT

Objective: Macrophages play an important role in immunogenic challenges and can aggravate and propagate local inflammation. Nuclear factor-kappa B (NF-κB) and activator protein 1 pathways can regulate these inflammatory processes by modulating expression of proinflammatory genes. Bioactive molecules present in food, such as procyanidins and polyunsaturated fatty acids, possess antiinflammatory effects in vivo and in vitro. Our aim was to assess whether they have synergistic antiinflammatory effects in murine macrophages.
Methods: A nitric oxide production assay, a phosphoprotein assay, and a low-density array for 91-gene expression related to inflammation, oxidative stress, and metabolism were performed to assess the synergistic antiinflammatory effects of dimeric procyanidins (B1, B2, B3, B4) (5 μg/mL), and the polyunsaturated fatty acids, docosahexaenoic acid, and eicosapentaenoic acid (30 μM) coincubated with lipopolysaccharide for 19 h to mimic inflammation in RAW 264.7 macrophages (mouse leukaemic monocyte macrophage cell line).
Results: Adding eicosapentaenoic acid plus B3 had synergistic effects leading to decreased nitric oxide levels; the modulation of phosphoprotein levels, such as P-nuclear factor-[kappa] B p65 and P-stress-activated protein kinase/Jun-amino-terminal kinase; the down-regulation of proinflammatory genes, such as interleukins, chemokines, transcription factors; and up-regulation of antioxidant genes.
Conclusion: This combination has a stronger antiinflammatory effect than either of these molecules separately in RAW macrophages. These results could lead to in vivo studies that may yield novel preventive or palliative nutritional treatments for obesity, atherosclerosis, and cardiovascular diseases.

Introduction

Localized inflammation that is considered a protective reaction to tissue irritation, injury, or infection can also be involved in several pathologies [1–3]. Macrophages are considered to play a major role in the body's response to immunogenic challenges by reestablishing tissue homeostasis, producing large amounts of reactive oxygen species (ROS), nitric oxide (NO), and proinflammatory cytokines, which aggravate and propagate local inflammation and disrupt the normal function of target cells [4]. In fact, previous studies performed in RAW 264.7 (mouse leukaemic monocyte macrophage cell line) murine macrophage cells have shown the importance of macrophages in enhancing inflammation by secreting proinflammatory factors, such as tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) [5].

It is known that multiple mechanisms can propagate inflammation. Some pathways in the cell can modulate inflammation through phosphorylation cascades of proteins that finally translocate to the nucleus to induce the expression of inflammation genes. One of the more important cascades is the nuclear factor κB (NF-κB) pathway. The currently known subunit members of the NF-κB family in mammals are p50, p65 (RelA), c-Rel, p52, and RelB; moreover, Ik-B has multiple mammalian forms, such as Ik-Bα, β, γ (p105), δ (p100), and ε as well as Bcl-3. Canonical NF-κB, a dimeric protein (p50 and p65), exists in the cytoplasm in non-stimulated cells bound to an inhibitor protein, Ik-B. On stimulation with many NF-κB endogenous inducers,

* Corresponding author. Tel.: 0034 977558497; fax: 0034 977558232.
E-mail address: mteresa.blay@urv.cat (M. Blay).

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such as IL-1β and TNF-α, or potent exogenous inducers, such as lipopolysaccharide (LPS), Ik-Bα is rapidly phosphorylated by Ik-B kinases and is marked for ubiquitination and degradation in the cytoplasm. The released NF-κB dimer can be activated by p65 phosphorylation and then translocated to the nucleus where NF-κB will trigger transcription of target genes by binding with high affinity to κB elements in their promoters [6–8].

Other molecular partners are implicated in inflammatory cascade, including the transcription factor activator protein 1 (AP-1). When an endotoxin stimulus arrives at the cell membrane, specific toll-like receptors activate complex signaling cascades, including the three major mitogen-activated protein kinase (MAPK) signaling pathways, namely, the ERK, Jun-amino-terminal kinase/stress-activated protein kinase (JNK/SAPK), and p38 pathways. These MAPKs activate the transcriptional activity of AP-1 proteins, such as Fos and Jun, by phosphorylation and then translocate to the nucleus, induce the expression of several inflammation target genes, such as Tnf-α and Il-1β, and genes implicated in invasive cell growth, such as matrix metalloprotease [9,10].

In this context, several food compounds could help to decrease inflammation associated with disease states by modulating the expression of proteins related to these pathways. Polyphenols are an extensive molecule family that has well-known antioxidant, antiinflammatory, antitumor, and antiatherogenic abilities [11]. These effects are related to free radical scavenging, NO regulation, inhibition of inflammatory cytokine production, intracellular signaling pathway proteins, and modulation of gene expression and all of them may contribute to their potentially protective role in inflammatory and cardiovascular diseases [12–14].

One important kind of polyphenol is procyanidins, which are oligomers of catechins or epicatechins and belong to the flavan-3-ol group. Procyanidins are present in high concentrations in red wine, grapes, and grape seeds, cocoa, berries, and apples. Dimeric procyanidins, like B1, B2, B3 and B4 (Fig. 1), are involved in free radical scavenging and possess antioxidant activity in vivo and in vitro [15]. In the last few years, it has been demonstrated that procyanidins also have antiinflammatory effects in vivo [13] and in vitro [16,17]. This antiinflammatory activity implicates several mechanisms, such as the modulation of proteins in the NF-κB pathway, and/or in the regulation of proinflammatory genes, like IL-2, IL-6, IL-8, and IL-1β. This modulation decreases the activation of inflammation-related transcription factors [18,19].

Other bioactive compounds that can ameliorate inflammation are polyunsaturated fatty acids. Eicosapentaenoic acid (EPA) and the docosahexaenoic acid (DHA) (Fig. 1) are long-chain n3-polyunsaturated fatty acids (PUFAs) that are present mainly in marine oils, plant seed oils, like corn oil or soybean oil, or processed foods made up of these oils, like margarine. It has been demonstrated that these molecules possess several beneficial properties for human cardiovascular and inflammatory diseases. These biological effects have been characterized by their effects on lipoprotein metabolism, endothelial cell function, vascular reactivity, inflammatory markers, and cytokine production [20,21]. In addition, it is known that these molecules have antioxidant effects due to their ability to decrease ROS [22]. Some of these beneficial effects are due to the ability of these molecules to decrease the generation of proinflammatory cytokines, like TNF-α, IL-1β, and IL-6, leading to the deactivation of the NF-κB signaling cascade and the activation of the peroxisome proliferator-activated receptor-γ antiinflammatory pathway [23].

Thus, antiinflammatory properties have been well defined in both kinds of molecules separately, but not conjunctly. Some studies begin to assess the benefits of functional foods rich in both molecules [24], but they are not centered on understanding the mechanisms in cellular types such as macrophages, targets of inflammation. So our study assessed whether procyanidins (dimers B1, B2, B3 and B4) and PUFAs (DHA and EPA) could modulate the expression of inflammatory genes and proinflammatory proteins, also implicating synergistic antiinflammatory effects at different levels on murine macrophages stimulated with LPS to induce inflammation. Furthermore, this study also aimed to define the individual antiinflammatory role of these bioactive compounds.

First, we tested whether procyanidins and PUFAs alone and in combination can decrease NO production produced by the inducible nitric oxide synthase (iNOS) overtranscription.

Second, we evaluated the changes in pathways involved in inflammatory signaling and assessed the importance of these food-derived molecules and their combinations in the interaction with the phosphoproteins NF-κB, Ik-Bα, SAPK/JNK, and p38 related to the NF-κB and AP-1 pathways, and also signal transducer and activator of transcription 3 (STAT3), which can be phosphorylated in the cytoplasm because of IL-6 stimulation in the cell membrane [25].

Third, we evaluated the effects of these compounds on the expression of target genes associated with inflammation, oxidative stress, and metabolism, such as genes related to TNF family, interleukins, and their receptors (IL-1α and -β, IL-6, IL-10), chemokines and their receptors (Mcp-1), transcription factors and regulators (Nf-κB, Jun), MAPK signaling and related genes (Jnk or p38), oxidative stress response genes (Nos2, Hmox1), genes related directly to inflammation (Ptgs2, Cox2), or monocyte receptor genes (Emr1, Tlr4).

Materials and methods

Reagents and cells

The procyanidins B1 (Cat. No. 0983, ≥80%) and B2 (Cat. No. 0984, ≥90%) were provided by Extrasynthèse Chemical (Genay Cedex, France). Dimers B3 and B4 were provided by Apin Chemicals (Oxon, UK). The following chemicals were

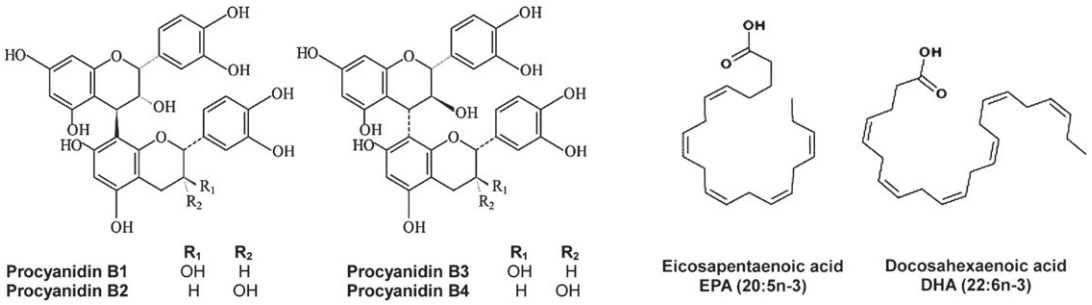

Fig. 1. Molecular structure of dimeric procyanidins and PUFAs.

purchased from Sigma-Aldrich (St. Louis, MO, USA): DHA (ref. D2534, ≥98%), EPA (ref. E2011, ≥99%), and LPS from *Escherichia coli* 0111:B4 (ref. L4391, Lot: 127K4037).

The murine macrophage cell line RAW 264.7 (European Tissue Culture Collection ECACC, ref. 91062702, London, UK) was cultured at 37°C under 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) fetal bovine serum (FBS), 2 mM D-glutamine, penicillin (100 U/mL), streptomycin (100 μg/mL), and 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES). The cells were between 10 and 16 passages when they were treated.

Experimental design

For the NO production experiment, 16 conditions were tested: C− (only medium, with vehicle), C+ (LPS 100 ng/mL), B1, B2, B3, B4, DHA, EPA, B1+DHA, B2+DHA, B3+DHA, B4+DHA, B1+EPA, B2+EPA, B3+EPA, B4+EPA. All of the conditions had coincubation with LPS at 100 ng/mL after the RAW 264.7 macrophage cells were cultured in 12-well plates. At 80-90% confluency, the medium was replaced with a colorless DMEM medium with antibiotics and without FBS, and then the cells were stimulated with LPS and coincubated with procyanidins and PUFAs with a final concentration for the following compounds: B1, B2, B3, and B4 (5 μg/mL or 8.64 μM), and DHA and EPA (30 μM). After 19 h of treatment, the supernatant was collected and stored at −20°C.

For the phosphoprotein immunoassay, RAW 264.7 cells were cultured in 12-well plates. At 80-90% confluency, the medium was replaced with a colorless DMEM medium with antibiotics and without FBS, and then the cells were treated with the following concentrations of compounds: LPS (100 ng/mL), B3 (5 μg/mL or 8.64 μM), and EPA (30 μM). The following conditions were used (three wells/condition): medium only (with vehicle), LPS, B3+LPS, EPA+LPS, and B3+EPA+LPS. After 30 min of coincubation, the medium was collected and stored at −80°C.

For the TaqMan Low Density Array (TLDAs) assay, RAW 264.7 cells were cultured in 12-well plates. At 80-90% confluency, the medium was replaced with a colorless DMEM medium with antibiotics and without FBS, and then the cells were treated with the following concentrations of compounds: LPS (100 ng/mL), B3 (5 μg/mL or 8.64 μM), EPA (30 μM). The following conditions were used (three wells/condition): medium only (with vehicle), LPS, B3+LPS, EPA+LPS, and B3+EPA+LPS. After 19 h of treatment, the supernatant was collected and stored at −80°C. The cells were counted with a Countess Automated Cell Counter (ref. C10227; Invitrogen, Paisley, UK) with a final average of 1.2×10^6 live cells/mL and a viability of 93%.

Testing the capacity of procyanidins and PUFAs alone and in combination for suppressing NO production in RAW 264.7 cells

To analyze the putative synergistic effect of procyanidins and PUFAs alone or in combination, NO production was determined by the Griess reagent. This reacted with NO_2^- ion from the medium collected after the treatments and produced a final stable purple product that can be quantified measuring the absorbance at 540 nm [16]. Nitrite production was normalized to protein content using 0.1 M NaOH for the cell lysate and the Bradford method (Sigma-Aldrich, ref. B6916, St. Louis, MO, USA). The assays were performed in technical and biological triplicate. The results are represented as the percentage of NO production versus that produced by the cells stimulated with just LPS (C+).

Immunoassay of phosphoproteins using the Pathscan ELISA kit

To assess the levels of several phosphoproteins that are able to activate inflammation in macrophages, a multitarget ELISA kit was used. The PathScan Inflammation Multi-Target Sandwich Enzyme-Linked Immunosorbent Assay (ELISA) Kit (ref. 7276) was provided by Cell Signaling (Danvers, MA, USA).

After treatment, the adherent monocytes were washed with ice-cold phosphate-buffered saline (PBS), and then the 1× Cell Lysis Buffer (ref. 9803; Cell Signaling) plus 1 mM phenylmethylsulfonyl fluoride was added. The plate was incubated for 5 min on ice. The cells were scraped and transferred to a tube, and each lysate was sonicated for 10 s using a Vibra-Cell VCX 750 Sonicator (Sonics and Materials, Inc., Newtown, CT, USA). The lysates were centrifuged at $11\,000 \times g$ for 10 min at 4°C, and the supernatant, containing the proteins of whole cells, was stored in a new tube at −80°C until assay day. The protein content of the lysates was determined by the Bradford Method (Sigma-Aldrich, ref. B6916) to readjust all of the samples to 5 mg/mL of protein. Phosphoproteins P-NFκB p65 (Ser536), P-SAPK/JNK (Thr13/Tyr185), P-p38 (Thr180/Tyr182), P-IκB-α (Ser32), and P-STAT3 (Tyr705) as well as NFκB p65 were semiquantified using the PathScan Inflammation Multi-Target Sandwich ELISA Kit. A colorimetric assay, reading the absorbance of treated samples at 450 nm in an ELISA spectrophotometer, Anthos Zenyth 200 st (Anthos Labtec Instruments, Salzburg, Austria), was performed. Sandwich ELISA protocol of the kit was used to prepare the samples and test the levels of these endogenous proteins. The assay was performed in technical duplicate. Each phosphoprotein absorbance was corrected by the negative control and normalized by its relevant NF-kB p65 absorbance. The results were expressed as the percentage of phosphoprotein absorbance versus that produced by cells just stimulated with LPS.

TaqMan low-density array gene expression analysis

To make a screening of numerous genes implicated in inflammation, oxidative stress, and other metabolic mechanisms, a TLDA was used. The RNeasy Mini Kit (ref. 74106, Qiagen, Barcelona, Spain) was used according the manufacturer's instructions to isolate RNA from pretreated RAW cells. After collecting the medium, ice-cold PBS was added to the adherent monocyte cells. Then, the PBS was removed, and lysis buffer was added to each well. After several centrifugations and washings, the RNA was diluted with 50 μL of RNase-free water. RNA purity and quantity were measured with the Nanodrop 1000 (Thermo Fisher Scientific, Waltham, MA, USA), and RNA integrity was checked with the Agilent 2100 Bioanalyzer (ref. G2938C) using the Agilent RNA 6000 Nano Kit (ref. 5067-1511; Agilent Technologies, Santa Clara, CA, USA). Finally, the RNA was stored at −80°C. Next, cDNA was synthesized from 2 μg of total RNA using the High-Capacity cDNA Reverse Transcription Kit (ref. 4368814; Applied Biosystems, Madrid, Spain), diluted to 100 ng/μL of cDNA to load into each reservoir of the Taqman Array Micro Fluidic Card (TLDA). Each TLDA plate (96a format), which contains sets of 96 mouse gene probes and mouse primers (91 genes + 5 controls), was analyzed using a real-time PCR amplification system with the Taqman Universal PCR Master Mix (Applied Biosystems). These genes are related to different biologic functions and were organized into the following 12 categories: 1) cytokines (30.2% of the total number of genes analyzed) including members of the TNF family (5.2%), interleukins and interleukin receptors (15.6%), and chemokines and chemokine receptors (8.3%); 2) transcription factors and regulators (16.7%); 3) MAPK signaling cascade proteins (6.3%); 4) oxidative stress components (10.4%); 5) scavengers receptors and cholesterol efflux channels (8.3%); 6) adhesion molecules (2.1%); 7) apoptosis proteins (2.1%); 8) proinflammatory protein (5.2%); 9) matrix degradation and regulation proteins (2.1%); 10) proteins involved in metabolism (5.2%); 11) monocyte markers (5.2%); and 12) binding proteins (1.0%). The 7900HT Fast Real-Time PCR with 384-Well Block Module and the 7900HT Sequence Detection System software (SDS 2.3) were used to perform real-time PCR and analyze the subsequent results by the $2^{-\Delta\Delta Ct}$ method.

Calculating the synergistic effects

Synergy, broadly defined, refers to the effects of the whole that exceed the additive effect, which is the sum of the effects of the individual parts alone [26,27]. Antagonism is present if the effects of the whole are less than the additive effect. Using these definitions, we determine if the results show additive, synergistic, or antagonistic effects.

To evaluate the synergistic effects of the compounds we considered the following:

Compounds	% of maximum inhibition of compounds alone in medium
X	% NOP in positive control − % NOP of compound "x" alone + SEM
Y	% NOP in positive control − % NOP of compound "y" alone + SEM
Compounds	**% of minimum inhibition of compounds together in medium**
(X + Y)	% NOP in positive control − % NOP of compounds "x" and "y" together − SEM

NOP, nitrite production

			Effect of compounds
X + Y	<	(X + Y)	Synergistic
	=		Additive
	>		Antagonistic

Both compounds had to have a significant NO production individually compared with the NO production when combined. If one of the compounds alone did not fulfill these criteria, we concluded the compounds would not have an effect when combined. These criteria were also used to evaluate the effects on phosphoprotein and gene expression levels.

Statistical analysis

The results are presented as the mean ± SEM. The data were analyzed by a one-way ANOVA (Tukey) or Student's *t* test to determine the statistical differences between groups using SPSS statistical software (version 17.0 for Windows). A $P < 0.05$ was considered statistically significant.

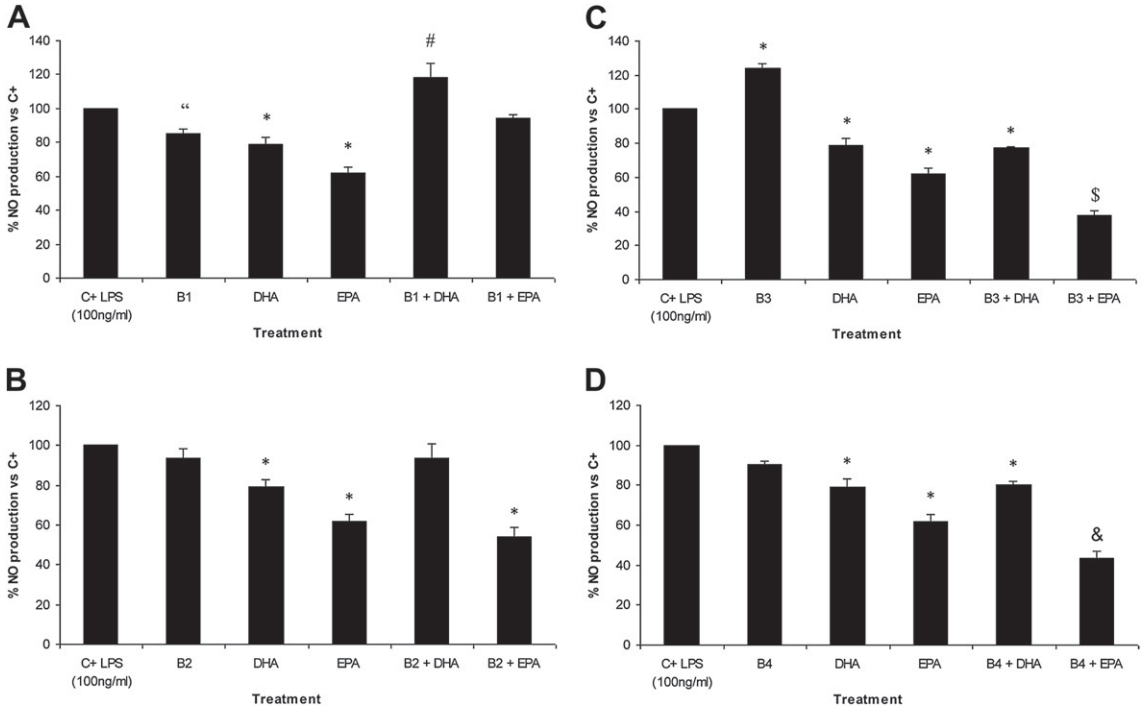

Fig. 2. The effects of procyanidins and PUFAs on NO production in RAW 264.7 macrophages stimulated with LPS. The results are presented as the percentage of NO production and normalized to the positive control (100%). Each value represents the mean ± SEM of biological and technical triplicates. The statistical significance versus C+ is represented by *, and versus C+ and groups of compounds alone are represented by #, &, and $, which signify antagonistic, additive, and synergistic effects, respectively. For all of the comparisons, a one-way ANOVA with Tukey's test was used, with $P < 0.05$ as the threshold for statistical significance. Finally, statistical significance versus C+ by Student's t test was represented by " with $P < 0.05$.

Results

Effects of procyanidins and PUFAs in suppressing NO production in stimulated RAW cells

LPS is a well-known trigger of the inflammatory response in macrophages, and several biomarkers, like NO, can be tested to measure this response. RAW 264.7 macrophages were stimulated with LPS for 19 h, and NO production was determined. Macrophages in the positive control medium had a mean NO production of 37.7 μM. To examine the synergistic effects in suppressing NO, procyanidins B1, B2, B3, and B4 were tested using 5 μg/mL (8.64 μM) doses and the PUFAs, DHA and EPA, were tested using 30 μM doses. These doses were chosen on the basis of previous experiments in RAW 264.7 macrophages stimulated with LPS as a positive control to check the inhibition of NO [28].

With these conditions, synergistic effects were found when B3 and EPA were combined (Fig. 2). EPA alone significantly inhibited NO production, and even though B3 significantly activated it, the compounds together had a synergistic effect on inhibition of NO production. Interestingly, the combination of B1 and DHA had an antagonistic effect. Separately, these compounds significantly inhibited NO production compared with the positive control, but they produced an antagonistic effect when used in combination. In addition, the combination of B4 and EPA had a significant additive effect compared with the positive control and the compounds when used separately.

Effect of B3 in combination with EPA in suppressing phosphoprotein levels in stimulated RAW macrophages

To measure the levels of several proteins involved in inflammatory pathways, PathScan Inflammation Multi-Target Sandwich ELISA was used. These proteins, which reside in the cytoplasm, are phosphorylated by other proteins in the phosphorylation cascade that is induced by an external inflammatory agent, like LPS, and lead to the activation of proteins that modulate gene transcription in the nucleus. The phosphorylated proteins, P-NFκB p65, P-SAPK/JNK, P-p38, P-IκB-α, and P-STAT3, were tested. RAW 264.7 macrophages were stimulated with LPS (100 ng/mL) for 30 min to detect phosphoprotein levels in these conditions [29–33]. At the same time, different concentrations of the following compounds were applied to different groups of cells: B3 (5 μg/mL or 8.64 μM), EPA (30 μM) and B3 + EPA (5 μg/mL or 8.64 μM, 30 μM). These assays revealed a synergistic effect (Fig. 3). The absorbance level of P-NFκB p65 in EPA-treated cells was elevated compared with the positive control. B3 had no effect on P-NFκB p65 levels; however, when the cells were treated with a combination of B3 and EPA, P-NFκB p65 was synergistically decreased. In addition, additive effects were found modulating P-SAPK/JNK levels when the compounds were combined. Although many treatments were statistically significant compared with the positive control, no combination of treatments affected P-p38, P-IκB-α, and P-STAT3 levels synergistically.

Effect of B3 in combination with EPA in modulating the expression of pro-inflammation target genes in stimulated RAW macrophages

RAW 264.7 macrophages were stimulated with LPS (100 ng/mL) for 19 h to trigger the expression of several target genes involved in inflammation, oxidative stress, and metabolism pathways. Cells were treated with LPS alone (positive control) or LPS and B3, EPA or B3 and EPA, to measure the inhibition of proinflammatory genes. The most important synergistic effects of B3 and EPA are showed in Table 1, whereas the rest of the

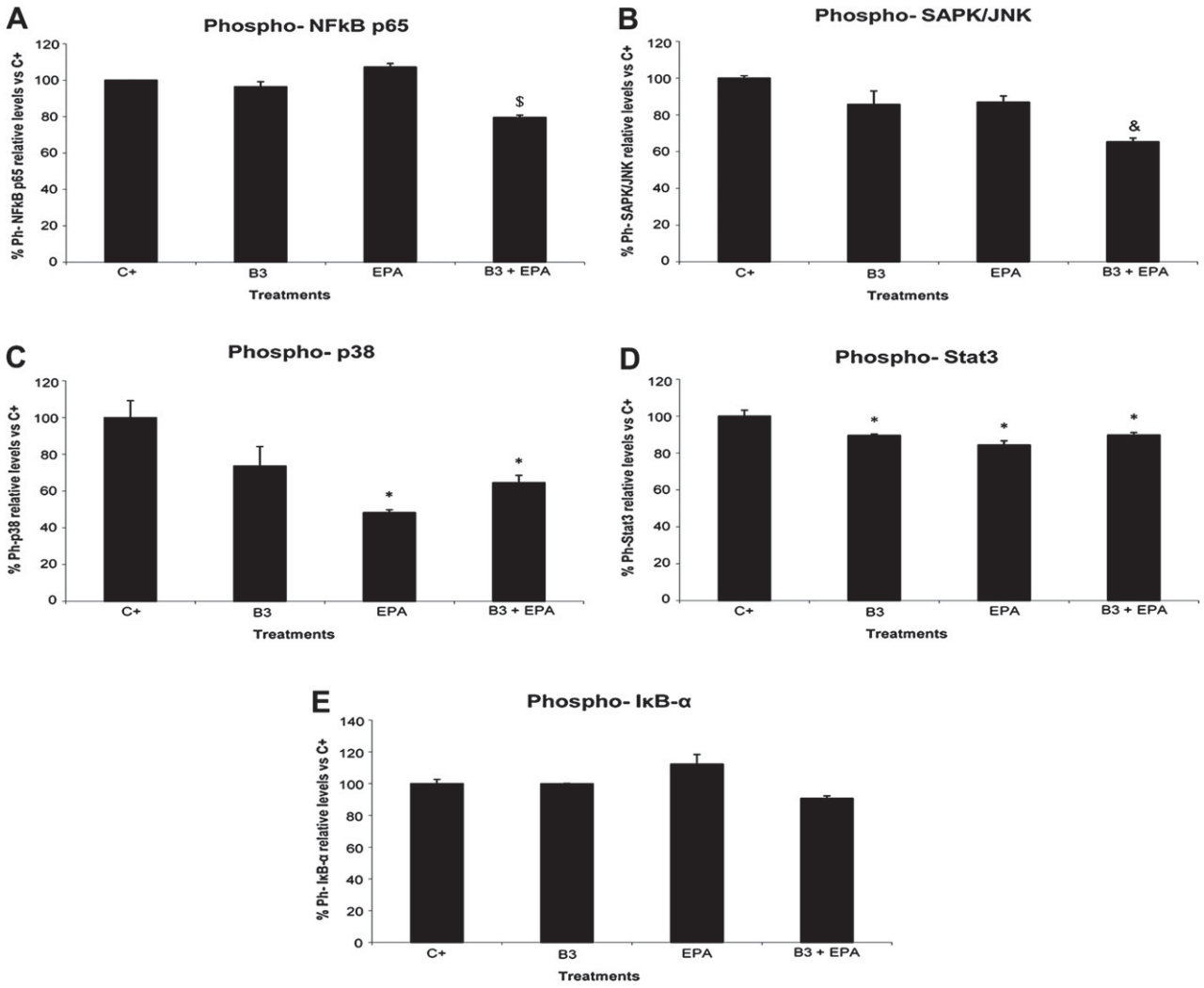

Fig. 3. The effects of procyanidins and PUFAs on levels of P-NFκB p65, P-SAPK/JNK, P-p38, P-IκB-α, and P-Stat3 in RAW 264.7 stimulated with LPS. The results are presented as the percentage of relative levels of phosphoproteins and are normalized to the positive control (100%). Each value represents the mean ± SEM of technical duplicates. The statistical significance versus C+ was represented by *, and versus C+ and groups of compounds alone are represented by & and $, which signify additive and synergistic effects, respectively. For all of the comparisons, a one-way ANOVA with Tukey's test was used, with $P < 0.05$ as the threshold for statistical significance.

results are shown in Table 2. We considered that genes were to be up-regulated or down-regulated if there were significant differences between the negative and positive control groups. The present result indicated that LPS treatment led to a greater than 100-fold increase in the expression of Il-1α, Il-1β, Il-6, Il-2rα, Ccl7, and Csf2 genes and more than a 10-fold increase in the expression of Tnfrsf1b, Il-10, Il-1rn, Il-12β, Ccl2, Ccl5, Timp1, Nos2, Ptgs2, Serpine1, Socs2, and Socs3 genes. Most of these genes have important proinflammatory and antiinflammatory functions. Thus, results indicated that LPS-mediated gene regulation seems to follow an inflammatory profile [34–36]. Treatment with B3 increased levels of Il-2 receptor, Il-2Rα, and decreased proinflammatory genes, such as Tnfrsf18, Fas, Il-12β, Ccl2, Map3k8, and Cd80. In contrast, treatment with EPA increased the proinflammatory Fas and Msr1 genes, the antiinflammatory Il1rn, Nfkbia, Socs1, and Socs3 genes, and the antioxidant Hmox1, Cat, and Gclm genes. In addition, EPA treatment decreased the proinflammatory Il-2rα, Il-12β, and Map3k8 genes. Treatment with B3 and EPA increased the gene expression of Msr1, Ager, Hmox1, Cat, and Gclm and decreased Il-1β, Il-2rα, Il-12β, Ccl2, Nfkb2, and Map3k8. In addition, our study indicated synergistic inhibition of the expression of proinflammatory genes, including Il-6, Csf2, Ccl5, Ccl7, and Serpine1, and, surprisingly, the antiinflammatory Il-10 gene.

Discussion

An interesting area in functional food science is discovering synergy between bioactive components to create foods that have health benefits. Such an investigation would require studies with in vitro and in vivo models. The present study is a first step in achieving these goals.

Macrophages, which play an important role in the inflammatory states of several tissues and various diseases, can help us understand how procyanidins and PUFAs modulate local inflammation at different biological levels. Thus, we stimulated murine RAW 264.7 macrophages with LPS to mimic this inflammatory state and study the synergistic effects of these natural compounds, going beyond our previous work, which demonstrated the antiinflammatory effects of a grape-seed extract containing a rich amount of dimeric and oligomeric procyanidins, by decreasing NO and prostaglandin E_2 levels, by

Table 1
The most important effects of B3 and EPA on the expression of 19 genes in RAW 264.7 macrophages stimulated with LPS

Gcne function	Gene symbol	Gene name	LPS		LPS + molecules					
			LPS		B3		EPA		B3 + EPA	
			Mean	SD	Mean	SD	Mean	SD	Mean	SD
TNF family	TNF	Tumor necrosis factor α	1.59	0.06	1.31=	0.18	1.36=	0.01	1.26=	0.16
	TNFRSF18	Tumor necrosis factor receptor superfamily, member 18	0.84=	0.05	0.14"	0.00	1.22=	0.02	0.96=	0.51
Interfeukins and receptors	IL1α	Interfeukin 1 α	315.29	8.42	301.39	1.24	289.44	18.91	270.15	35.37
	IL1β	Interfeukin 1 β	287.99	2.95	286.35	17.85	261.88	15.49	230.88"	3.53
	IL1RN	Interfeukin 1 receptor antagonist	13.70	0.17	12.99	0.62	18.11*	0.20	14.65	0.71
	IL6	Interfeukin 6	275.75	26.35	261.63	6.92	286.27	0.80	228.46#	4.28
	IL23A	Interfeukin 23. α subunit p 19	2.60	0.44	2.24	0.19	1.65=	0.19	1.53=	0.33
	IL10	Interfeukin 10, cytokine synthesis inhibitory factor	85.63	10.39	91.11	10.90	90.55	11.73	39.31#	0.47
Chemokines and receptors	CSF2	Granulocyte-macrophage colony stimulating factor	102.36	2.02	101.08	12.50	94.37	2.74	52.54#	9.32
	CCL5	Chemokine (C-C motif) ligand 5	27.60	1.24	26.51	1.33	31.62	0.36	21.12#	2.12
	CCL7	MCP-3. chemokine (C-C motif) ligand 7	127.04	2.01	133.24	2.15	138.66	18.43	91.90#	10.80
Transcription factors and related	SERPINE1	PAI-1. plasminogen activator inhibitor 1	39.72	2.39	39.42	0.75	36.09	0.05	28.14#	3.06
Oxidative stress	HMOX1	HO-1. Heme oxygenase (decycling) 1	9.88	0.52	8.61	0.10	18.12*	0.75	14.97*	0.86
	CAT	Catalase	0.79=	0.05	0.67	0.01	1.16"=	0.05	1.17"=	0.14
	GCLM	γ-Glutamylcysteine synthefase regulatory subunit	0.81=	0.04	0.72=	0.10	1.62"	0.15	1.38"=	0.26
Scavenger receptors	AGER	Receptor for advanced glycosylation end products	0.16=	0.02	0.55=	0.05	0.79=	0.64	1.62"=	0.21
	MSR1	Macrophage scavenger receptor 1	1.28	0.00	1.06=	0.04	1.84"	0.02	1.63"	0.06
Metabolism	HSPA5	BiP, lmmunoglobulin heavy chain-binding protein	3.85	0.05	3.17*	0.02	2.32*	0.13	1.87#	0.20
Monocyte markers and surface	CD80	T-lymphocyte activation antigen CD80	2.42	0.13	1.82"	0.12	1.93	0.11	1.85	0.20

The results are presented as the relative levels of gene expression versus cells without treatment (C−). We used the well-known mathematic equation δδCT to calculate these relative levels versus C−. The results are presented as the mean ± SEM of technical duplicates. The statistical significance versus C+ is represented by ", versus all of the treatments by *, versus C+ and groups of compounds alone by #, signify synergistic effect. Finally, non-statistical significance versus C− is represented by =. For all of the comparisons, a one-way ANOVA with Tukey's test was used, with $P < 0.05$ as the threshold for statistical significance

avoiding the translocation of NFκB p65 to the nucleus, and by down-regulating the expression of iNos and IκB-α in RAW 264.7 macrophages stimulated with LPS and interferon-γ [17].

Important synergistic antiinflammatory effects were found when procyanidin B3 at 5 μg/mL (8.64 μM) plus EPA at 30 μM were in combination. It has been reported that a study of 48 subjects consuming fish one to two times a month had a detectable plasma DHA level of about 60 μg/mL and EPA of about 10 μg/mL, which is equivalent to 182 and 33 μM, respectively [22]. Furthermore, several studies in human and rats are in controversy in respect to the bioavailability of dimeric procyandins in blood plasma. It can vary depending on the molecules they joined to, the source of food used (apples, cocoa, or grapes), and the doses administrated. In fact, it has been reported that the levels of pure procyandins in plasma, like B2, are between 0.041 and 0.4 μM, and the total dimeric procyandins are between 0.54 nM and 3 μg/mL [37–39]. Thus, our PUFAs tested concentrations close to the bioavailability levels found in plasma; in contrast, dimeric procyanidins concentrations could be approached by a supranutritional diet or a pharmacological intake.

First, we tested whether procyanidins and PUFAs can inhibit NO production, a clear marker of ROS and inflammation in cells. Results indicated that LPS stimulation at 100 ng/mL for 19 h can trigger NO production. This concentration is good because it is less aggressive than other treatments used previously, 1 μg/mL [30–33,40], and 10 μg/mL [22,41]. Also, this concentration is useful for testing whether procyanidins can exert an inhibitory effect, as seen in previous studies of inflammation [16]. In fact, the study indicated that B1, DHA, and EPA inhibited NO production by themselves and that B3 activated NO production after 19 h of coincubation with LPS (Fig. 2). When the compounds were combined, the inhibitory effect of B2 and EPA was not greater than the effect of EPA alone (Fig. 2B). This result suggests that EPA is solely responsible for the inhibition. Moreover, results indicated an additive effect with B4 and EPA, which suggests that the combination of their effects promotes greater inhibition (Fig. 2D). Finally, our study indicated a synergistic effect between B3 and EPA (Fig. 2C), which suggests that whereas B3 can activate NO production by itself, adding EPA can modulate some mechanisms that promote an even stronger inhibition of inflammation. An explanation for this result may be that EPA permits the action of B3 or that B3 affects the activity of EPA. Either way, these combinations seem to have a strong inhibitory effect on NO production in macrophages. Interestingly, the study indicated a clear antagonistic effect when B1 and DHA were combined (Fig. 2A). The two compounds alone had inhibitory effects on NO production, but when combined, this effect was reversed, suggesting that they trigger inflammation in macrophages by propagating the effect of LPS or activating more inflammatory pathways. However, this issue can be studied in future works.

Our next aim was to determine whether B3 and EPA in combination could modulate the levels of phosphoproteins implicated in inflammatory pathways, such as NF-κB and AP-1. Results indicated a synergistic effect of these compounds on P-NFκB p65 levels (Fig. 3A). This could be the key to the inhibition of the inflammation state because B3 and EPA by themselves have no inhibitor effect on NFκB p65 phosphorylation, whereas

Table 2
The effects of B3 and EPA on the expression of 56 genes in RAW 264.7 macrophages stimulated with LPS

Gene Function	Gene Symbol	Gene Name	LPS		LPS + Molecules					
			LPS		B3		EPA		B3 + EPA	
			Mean	SD	Mean	SD	Mean	SD	Mean	SD
TNF family	TNFRSF1B	Tumor necrosis factor receptor superfamily, member 1B	23.24	0.51	19.86	4.00	23.93	0.22	25.49	1.12
	FAS	TNF receptor superfamily, member 6	3.69	0.11	2.94*	0.13	4.47*	0.25	3.74	0.11
Interleukins and receptors	IL2RA	Interleukin 2 receptor, alpha	159.53	33.06	613.17*	10.00	0.86"=	0.04	70.34*	9.20
	IL12B	Interleukin 12, subunit beta	36.19	0.82	7.05*	0.11	0.86"=	0.04	13.33*	1.74
	IL18	Interleukin 18, IFN-gamma- inducing factor, IL1 gamma	0.66	0.00	0.58	0.03	0.80=	0.06	0.72=	0.04
Chemokines and receptors	CCL2	MCP-1, Monocyte chemo- attractant protein 1	63.74	0.60	54.37"	0.82	64.44	1.67	56.56"	2.03
	CCL3	MIP 1-alpha, Chemokine (C-C motif) ligand 3	3.09=	0.65	3.55=	0.64	5.02	1.30	2.98=	0.05
	CSF1	Macrophage colony stimulating factor	1.39=	0.61	1.22=	0.15	0.73=	0.26	1.21=	0.21
Transcription factors and related	JUN	AP-1 Transciption factor	1.63	0.18	1.30=	0.22	1.36	0.01	1.24=	0.01
	IKBKB	IKK-beta, Nuclear factor NF- kappa-B inhibitor kinase beta	0.42	0.02	0.38	0.04	0.48	0.03	0.45	0.05
	MYC	Proto-oncogene c-Myc, transcription factor p64	4.93	0.10	4.14	0.27	4.13	0.30	3.78	0.56
	NFKB1	Nuclear factor NF-kappa-B p50/p105 subunit	0.92=	0.04	0.74=	0.03	0.94=	0.06	0.75=	0.07
	NFKB2	Nuclear factor NF-kappa-B p49/p100 subunit	1.26	0.11	1.15=	0.03	1.21=	0.03	0.98"=	0.01
	NFKBIA	IkB-alpha, NF-kappa-B inhibitor alpha	3.41	0.01	3.18	0.17	3.89*	0.12	3.37	0.08
	NFKBIB	IkB-beta, NF-kappa-B inhibitor beta	1.95	0.21	1.66	0.06	2.16	0.12	1.93	0.02
	PPARG	Peroxisome proliferator- activated receptor gamma	0.09	0.00	0.12	0.00	0.07	0.05	0.09	0.02
	RELA	Nuclear factor NF-kappa-B p65 subunit	0.69	0.04	0.61	0.07	0.65	0.04	0.66	0.09
	SOCS1	Suppressor of cytokine signaling 1	2.82=	0.60	4.64	0.60	5.48"	0.78	3.23=	0.81
	SOCS2	Suppressor of cytokine signaling 2	11.50	0.65	8.69	0.14	11.40	0.57	10.67	1.40
	SOCS3	Suppressor of cytokine signaling 3	24.52	0.67	26.40	0.45	33.04*	0.79	24.91	0.36
	HDAC2	Histone deacetylase 2	0.45	0.02	0.44	0.02	0.53	0.03	0.39	0.03
	CHUK	IKK-alpha, Nuclear factor NF-kappa-B inhibitor kinase alpha	0.47	0.02	0.46	0.03	0.58	0.05	0.69	0.13
	SIRT1	Sirtuin type 1	0.28	0.03	0.25	0.01	0.29	0.02	0.27	0.02
MAPKs and related	MAP3K8	Mitogen-activated protein kinase kinase kinase 8	1.48	0.11	0.89"=	0.20	0.96"=	0.01	0.83"=	0.04
	MAP2K1	MAPK/ERK kinase 1, Mitogen-activated protein kinase kinase 1	1.80=	0.45	1.35=	0.49	1.15=	0.75	1.32=	0.32
	MAPK14	p38 MAP kinase, Mitogen- activated protein kinase 14	0.39	0.03	0.39	0.03	0.38	0.01	0.33	0.05
	MAPK3	ERK1, Mitogen-activated protein kinase 3	1.40	0.92	1.30	0.46	1.04	0.28	1.12	0.50
	MAPK8	JNK1, Stress-activated protein kinase	0.66	0.04	0.67	0.02	0.59	0.03	0.53	0.08
Oxidative stress	GPX1	GSHPX1, Glutathione peroxidase 1	0.50	0.01	0.50	0.03	0.61	0.11	0.55	0.09
	SOD1	Superoxide dismutase 1, soluble	0.93=	0.00	0.69=	0.18	1.26=	0.33	0.61=	0.01
	SOD2	Superoxide dismutase 2, mitochondrial	2.76	0.25	2.27=	0.31	3.34	0.53	2.54	0.40
	NFE2L2	NRF2, Nuclear factor (erythroid-derived 2)-like 2	0.47	0.04	0.39	0.05	0.54	0.07	0.43	0.05
	NQ01	NAD(P)H dehydrogenase, quinone 1	1.74=	0.23	0.99=	0.65	2.88=	0.47	3.24=	1.99
	NOS1	constitutive NOS , Nitric oxide synthase 1	0.10	0.07	0.10	0.07	0.16	0.15	0.09	0.00
	NOS2	iNOS, Nitric oxide synthase 2, inducible	17.69	0.69	17.07	2.22	16.26	0.15	17.73	1.19
Scavenger receptors and cholesterolefflux	CD36	Scavenger receptor class B, member 3, thrombospondin receptor	3.02=	1.95	3.58=	0.04	4.98	0.01	4.65	0.32
	SCARB1	Scavenger receptor class B, member 1	0.24	0.02	0.20	0.03	0.26	0.01	0.25	0.02
	ABCA1	ATP-binding cassette subfamily A member 1	0.17	0.01	0.15	0.07	0.13	0.05	0.13	0.04
	ABCG1	ATP-binding cassette subfamily G member 1	0.15	0.01	0.14	0.01	0.18	0.00	0.17	0.02
	CD68	Scavenger receptor class D, member 1	0.59	0.07	0.53	0.00	0.72	0.00	0.73	0.01
	ACAT1	Acetyl-Coenzyme A acetyltransferase 1	0.14	0.00	0.12	0.01	0.19	0.01	0.17	0.02
Adhesion molecules	ICAM1	Intercellular adhesion molecule 1	0.26	0.01	0.22	0.03	0.30	0.03	0.30	0.05
Apoptosis	BAX	BCL2-associated X protein, apoptosis regulator	0.69	0.02	0.60	0.02	0.75	0.04	0.64	0.04
	BCL2	B-cell CLL/lymphoma 2, apoptosis regulator	0.48	0.02	0.39	0.01	0.57	0.03	0.53	0.09
Inflammation	PTGS1	COX1, Prostaglandin- endoperoxide synthase 1	0.82=	0.09	0.66=	0.03	0.82=	0.13	0.74=	0.07
	PTGS2	COX2, Prostaglandin- endoperoxide synthase 2	83.90	6.02	73.05	0.26	82.20	7.77	88.09	7.41

(*continued*)

Table 2 (*continued*)

Gene Function	Gene Symbol	Gene Name	LPS		LPS + Molecules					
					B3		EPA		B3 + EPA	
			Mean	SD	Mean	SD	Mean	SD	Mean	SD
Matrix degradation	MMP9	Matrix metallopeptidase 9	3.95	0.13	3.19	0.51	4.82	0.50	3.61	0.36
	TIMP1	Tissue inhibitor of metalloproteinase 1	17.35	2.96	17.77	3.72	16.31	3.81	12.73=	4.19
Metabolism	H6PD	Hexose-6-phosphate dehydrogenase	0.14	0.02	0.13	0.02	0.16	0.00	0.18	0.01
	HMGCR	NADPH, 3-hydroxy-3- methylglutaryl CoA reductase	0.18	0.01	0.17	0.01	0.23	0.02	0.19	0.02
	ARG1	Arginase type 1	1.26=	0.73	1.79=	1.32	2.67=	1.36	0.08*	0.01
	CTSS	Cathepsin S	0.38	0.06	0.37	0.00	0.60*	0.06	0.32	0.05
Monocyte markers and surface	EMR1	EGF-like module-containing mucin-like hormone receptor-like 1	1.29=	0.18	1.01=	0.03	1.69=	0.06	1.03=	0.20
	TLR4	Toll-like receptor 4	0.22	0.02	0.20	0.02	0.28	0.03	0.30	0.01
	CD14	Myeloid cell-specific leucine- rich glycoprotein, monocyte differentiation antigen	1.40	0.03	1.30	0.10	1.43	0.02	1.25=	0.07
	B2M	Beta-2-microglobulin	0.73	0.08	0.67	0.01	0.82=	0.01	0.80=	0.09

The results are presented as the relative levels of gene expression versus cells without treatment (C−). We used the well-known mathematic equation δδCT to calculate these relative levels versus C−. The results are presented as the mean ± SEM of technical duplicates. The statistical significance versus C+ is represented by " and versus all of the treatments by *. Finally, non-statistical significance versus C− is represented by =. For all of the comparisons, a one-way ANOVA with Tukey's test was used, with $P < 0.05$ as the threshold for statistical significance

the combination does. P-NFκB p65 is crucial in the activation of inflammation target genes, because when NFκB p65 is phosphorylated, it is able to translocate to the nucleus and trigger the expression of inflammatory genes, such as Il-1β, Il-6, and Tnf-α. This expression can increase the secretion of these proteins and can promote a paracrine activation of inflammation that affects other macrophages and cell types.

Moreover, an additive effect on inhibition was found for P-SAPK/JNK (Fig. 3B). Similar to the previous experiment, neither B3 nor EPA could inhibit protein phosphorylation by itself. However, both showed a tendency to inhibit protein phosphorylation that was not statistically significant. In contrast, when the compounds were combined, this inhibition was significant. P-SAPK/JNK inhibition may lead to the activation of the transcription factor AP-1 and may decrease the expression of proinflammatory genes, such as Tnf-α and Il-1β. These molecules could block the inflammatory state in cells. In addition, the study indicated a decrease of the levels of phosphoproteins P-p38 and P-STAT3 with all treatments (Figs. 3C and 3D) with the EPA treatment having a greater effect. This result suggests that these molecules alone or in combination have the capacity to interact with macrophages to blunt these proinflammatory pathways. Finally, the combination of B3 and EPA had inhibitory effects on four of the five phosphoproteins assessed. This result suggests the combination has more antiinflammatory power than either molecule alone.

Our final aim was to assess whether the combination of B3 and EPA had effects on the expression of genes related to inflammation and oxidative stress. As seen in the regulation profiles of proinflammatory, antiinflammatory, and antioxidant genes (Figs. 4 and 5), results indicated many genes that were activated in LPS-stimulated macrophages; specifically, around 50-60% of these genes were up-regulated by treatment. This result suggests that stimulation with LPS promotes an inflammatory state that has been seen in other studies [34-36] by increasing the expression of proinflammatory target genes (Fig. 4). However, antiinflammatory and antioxidant genes are up-regulated as well (Fig. 5), possibly to counteract this induced proinflammatory state. Thus, the cell is in a struggle to return to fix damaged areas and return to normal. In this context, the study indicated molecules that reverse inflammation by down-regulating proinflammatory genes and upregulating antiinflammatory and antioxidant genes (Figs. 4 and 5). Specifically, EPA seems to have a greater capacity for upregulating antiinflammatory genes, such as Il-1rn, which is an antagonist of the receptor for the proinflammatory IL-1 and suggests an indirect decrease in IL-1 activity, IκB-α (Nfkbia), which is involved in sequestering NF-κB in the cytoplasm, or Socs3, and antioxidant genes, such as Hmox1, Cat, and Gclm, in charge of the destruction of ROS (Tables 1 and 2). In contrast, B3 has more effects in the down-regulation of proinflammatory genes, such as Tnfrsf18, Fas, Il-12β, Ccl2, Map3k8, and Cd80 (Tables 1 and 2). This result suggests that B3 helps decrease inflammation by down-regulating proinflammatory target genes (Fig. 4) but that EPA decreases inflammation by up-regulating antiinflammatory and antioxidant genes (Fig. 5). Our study indicated that the combination of B3 and EPA promotes a greater down-regulation of proinflammatory target genes (Fig. 4) as well as a remarkable up-regulation of antiinflammatory and antioxidant-related genes (Fig. 5). Importantly, combined treatment decreased proinflammatory Il-1α and -β compared with the positive control (Table 1). Il-6, which is another important proinflammatory cytokine, also displayed synergistic effects. In addition, the expression of Tnf-α, a molecule crucial in inflammation-related diseases and the activation of macrophages, had a tendency to decrease with the combination of

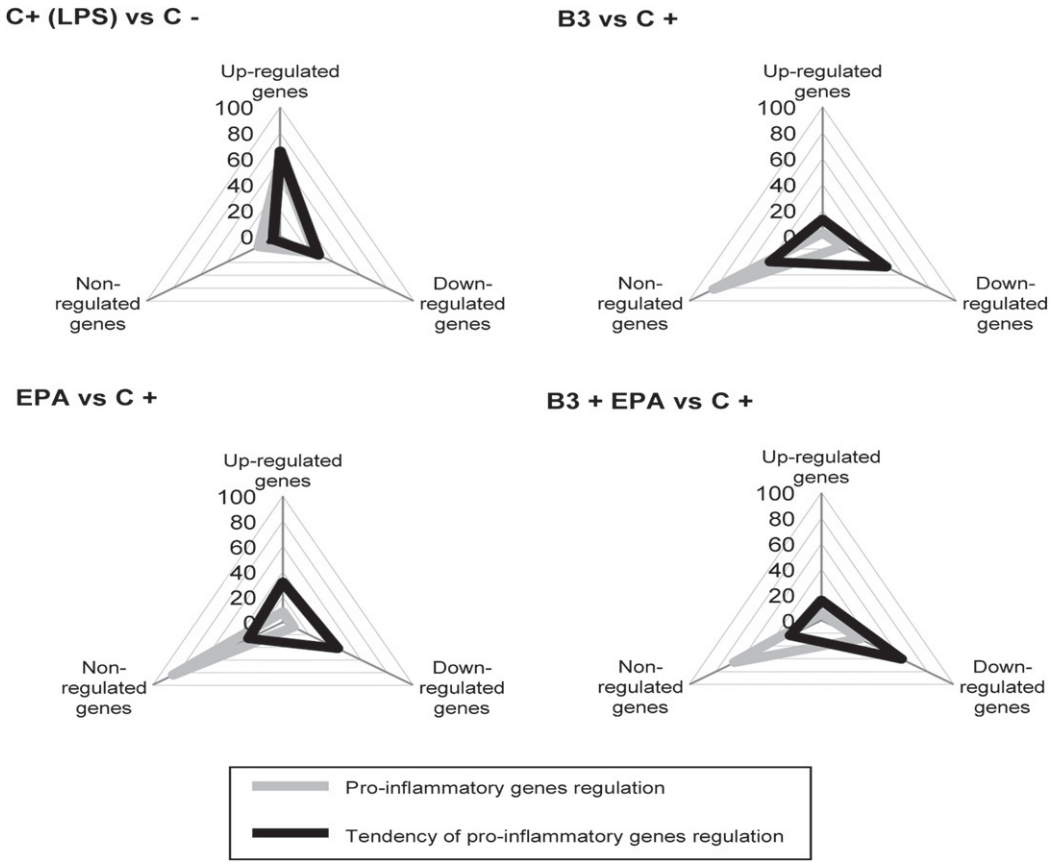

Fig. 4. The regulation of proinflammatory genes in RAW 264.7 macrophages treated with LPS (C+) alone or LPS and B3, EPA, or B3 plus EPA. The results are presented as the percentage of genes that were up-regulated, down-regulated, or not regulated with the statistical significance (gray line) and the tendency of their regulation (black line) versus the control group in each title. For all of the comparisons, a one-way ANOVA with Tukey's test was used, with $P < 0.05$ as the threshold for statistical significance. For the tendency, if the mean value + SEM of the control group was lower than the mean value − SEM of the treatment group, the gene was considered to be up-regulated. If the mean value − SEM of the control group was higher than the mean value + SEM of treatment group, this gene was considered to be down-regulated. If neither of these conditions were true, the gene was considered not to be regulated. There were 38 proinflammatory genes detected in this study.

compounds. In contrast, the expression of antiinflammatory Il-10 was decreased with the combination of compounds, suggesting that this combination could have inhibitory effects over the expression of antiinflammatory proteins. However, it could also suggest that the LPS-induced global inflammatory state was decreased by other pathways, doing unnecessary the expression of Il-10. On the other hand, proinflammatory chemokines, such as Ccl7, Ccl5, and Csf2, had synergistic effects, which could suggest that the cells return to the normal levels of differentiation and production after 19 h of treatment under the influence of the molecules. This down-regulation of several antiinflammatory and proinflammatory genes suggests that multiple antiinflammatory and proinflammatory pathways are affected. Therefore, the cells treated with B3 and EPA had decreased global inflammation. In oxidative stress-related genes, results indicated an increase in the expression of various antioxidant proteins, such as Hmox1, which catalyzes the degradation of heme, catalase (Cat), and γ-glutamylcysteine synthetase regulatory subunit (Gclm) and is the first rate-limiting enzyme of glutathione synthesis. These three proteins have been implicated in a ROS reduction pathway. However, these effects were not stronger than EPA up-regulation.

These results suggest that the synergistic combination of B3 and EPA has an antiinflammatory effect by modulating several inflammatory and oxidative stress-related genes. Furthermore, these inhibitory effects could result from the sum of the EPA-mediated up-regulation of antioxidant genes and B3-mediated down-regulation of proinflammatory genes.

Conclusion

Combining B3 (5 μg/mL or 8.64 μM) and EPA (30 μM) has synergistic effects on LPS-stimulated RAW 264.7 macrophages by decreasing the expression of proinflammatory and oxidative stress proteins and affecting the phosphorylation of proteins implicated in the activation of NF-κB and AP-1 proinflammatory pathways. These results could lead to in vivo studies that may yield novel preventive or palliative nutritional treatments for obesity, atherosclerosis, and cardiovascular diseases.

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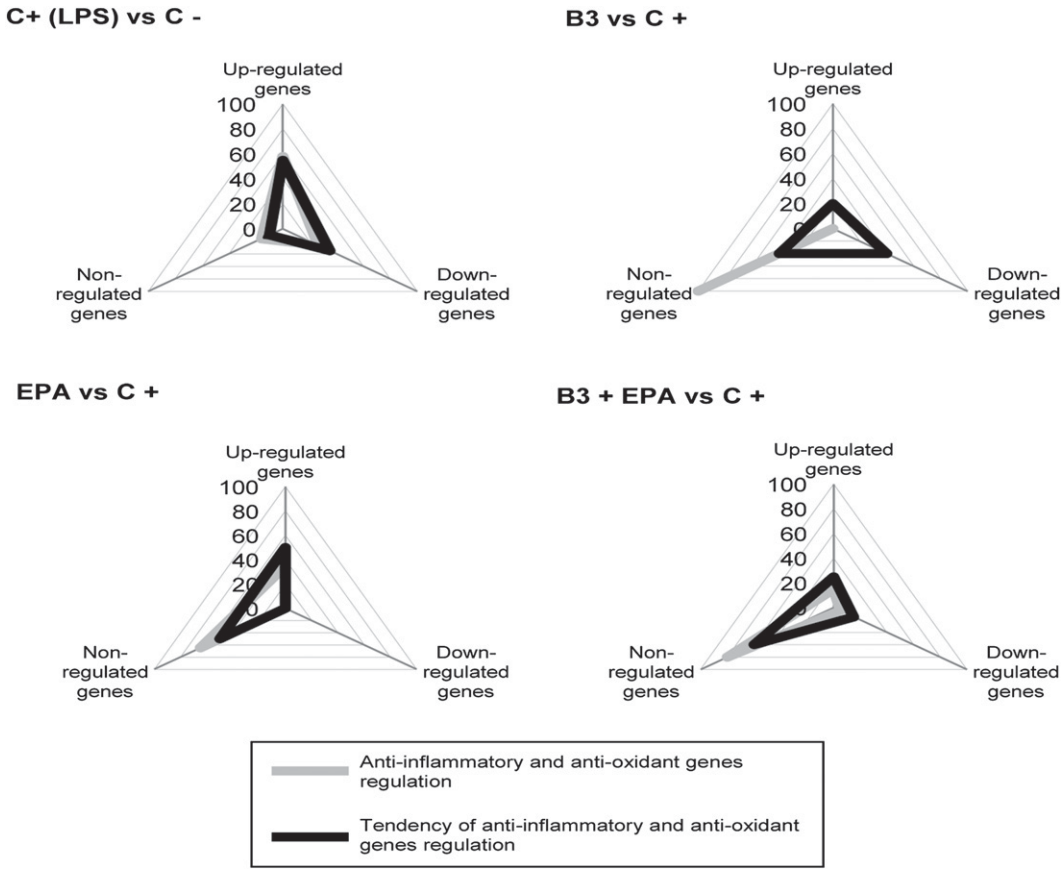

Fig. 5. The regulation of antiinflammatory and antioxidant genes in RAW 264.7 macrophages treated with LPS (C+) alone or LPS and B3, EPA, or B3 plus EPA. The results are presented as the percentage of genes that were up-regulated, down-regulated, or not regulated with the statistical significance (gray line) and the tendency of their regulation (black line) versus the control group in each title. For all of the comparisons, a one-way ANOVA with Tukey's test was used, with $P < 0.05$ as the threshold for statistical significance. For the tendency, if the mean value + SEM of the control group was lower than the mean value − SEM of the treatment group, the gene was considered to be up-regulated. If the mean value − SEM of the control group was higher than the mean value + SEM of treatment group, this gene was considered to be down-regulated. If neither of these conditions were true, the gene was considered not to be regulated. There were 12 antiinflammatory and 8 antioxidant genes detected in this study.

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References

[1] Ho S-C, Hwang LS, Shen Y-J, Lin C- C. Suppressive effect of a proanthocyanidin-rich extract from longan (Dimocarpus longan Lour.) flowers on nitric oxide production in LPS-stimulated macrophage cells. J Agric Food Chem 2007;55:10664–70.
[2] Maury E, Brichard SM. Adipokine dysregulation, adipose tissue inflammation and metabolic syndrome. Mol Cell Endocrinol 2010;314:1–16.
[3] Holvoet P. Relations between metabolic syndrome, oxidative stress and inflammation and cardiovascular disease. Verh K Acad Geneeskd Belg 2008;70:193–219.
[4] Oh MS, Park W, Yoon S-B, Lee Y-J, Park SK, Kim H-C, et al. Anti-inflammatory effects of Scutellaria baicalensis water extract on LPS-activated RAW 264.7 macrophages. J Ethnopharmacol 2009;125:286–90.
[5] Permana PA, Menge C, Reaven PD. Macrophage-secreted factors induce adipocyte inflammation and insulin resistance. Biochem Biophys Res Commun 2006;341:507–14.
[6] Bowie A, O'Neill LAJ. Oxidative stress and nuclear factor-[kappa]B activation: A reassessment of the evidence in the light of recent discoveries. Biochem Pharmacol 2000;59:13–23.
[7] Aktan F. iNOS-mediated nitric oxide production and its regulation. Life Sci 2004;75:639–53.
[8] Ghosh S, Karin M. missing pieces in the NF-[kappa]B puzzle. Cell 2002;109:S81–96.
[9] Kunz M, Ibrahim S, Koczan D, Thiesen J-J, Kohler HJ, Acker T, et al. Activation of c-Jun NH2-terminal kinase/stress-activated protein kinase (JNK/SAPK) Is critical for hypoxia-induced apoptosis of human malignant melanoma. Cell Growth Differ 2001;12:137–45.
[10] Hu X, Chen J, Wang L, Ivashkiv LB. Crosstalk among Jak-STAT, Toll-like receptor, and ITAM-dependent pathways in macrophage activation. J Leukoc Biol 2007;82:237–43.
[11] Rahman I, Biswas SK, Kirkham PA. Regulation of inflammation and redox signaling by dietary polyphenols. Biochem Pharmacol 2006;72:1439–52.
[12] Arts ICW, Hollman PCH. Polyphenols and disease risk in epidemiologic studies. Am J Clin Nutr 2005;81:317S–25S.
[13] Terra X, Montagut G, Bustos M, Llopiz N, Ardèvol A, Bladé C, et al. Grape-seed procyanidins prevent low-grade inflammation by modulating cytokine expression in rats fed a high-fat diet. J Nutr Biochem 2009;20:210–8.
[14] Nichols JA, Katiyar SK. Skin photoprotection by natural polyphenols: anti-inflammatory, antioxidant and DNA repair mechanisms. Arch Dermatol Res 2010;302:71–83.
[15] Fine AM. Oligomeric proanthocyanidin complexes: history, structure, and phytopharmaceutical applications. Altern Med Rev 2000;5:144–51.
[16] Terra X, Valls J, Vitrac X, Mérrillon J-M, Arola L, Ardèvol A, et al. Grape-seed procyanidins act as antiinflammatory agents in endotoxin-stimulated RAW 264.7 macrophages by inhibiting NFkB signaling pathway. J Agric Food Chem 2007;55:4357–65.
[17] Terra X, Fernández-Larrea J, Pujadas G, Ardèvol A, Bladé C, Salvadó J, et al. Inhibitory effects of grape seed procyanidins on foam cell formation in vitro. J Agric Food Chem 2009;57:2588–94.
[18] Mackenzie GG, Delfino JM, Keen CL, Fraga CG, Oteiza PI. Dimeric procyanidins are inhibitors of NF-kappaB-DNA binding. Biochem Pharmacol 2009;78:1252–62.
[19] Simón I, Vendrell J, Chacón MR, Ceperuelo-Mallafré V, Maymó-Masip E, Mateo-Sanz JM, et al. Grape-seed procyanidins modulate inflammation on human differentiated adipocytes in vitro. Cytokine 2009;47:137–42.
[20] Rahman MM, Bhattacharya A, Fernandes G. Docosahexaenoic acid is more potent inhibitor of osteoclast differentiation in RAW 264.7 cells than eicosapentaenoic acid. J Cell Physiol 2008;214:201–9.
[21] Calder CP. Polyunsaturated fatty acids and inflammation. Biochem Soc Trans 2005;33:423–7.

[22] Saw CLL, Huang Y, Kong A- N. Synergistic anti-inflammatory effects of low doses of curcumin in combination with polyunsaturated fatty acids: docosahexaenoic acid or eicosapentaenoic acid. Biochem Pharmacol 2010;79:421–30.
[23] Calder PC. n-3 polyunsaturated fatty acids, inflammation, and inflammatory diseases. Am J Clin Nutr 2006;83:1505S–19S.
[24] Medina I, Lois S, Lizárraga D, Pazos M, Touriño S, Cascante M, et al. Functional fatty fish supplemented with grape procyanidins. Antioxidant and proapoptotic properties on colon cell lines. J Agric Food Chem 2006; 54:3598–603.
[25] Levy DE, Lee C.-kuo. What does Stat3 do? J Clin Invest 2002;109:1143–8.
[26] Corning PA. "The synergism hypothesis": On the concept of synergy and its role in the evolution of complex systems. J Soc Evol Syst 1998;21:133–72.
[27] Chou T- C. Theoretical basis, experimental design, and computerized simulation of synergism and antagonism in drug combination studies. Pharmacol Rev 2006;58:621–81.
[28] Montagut G, Baiges I, Valls J, Terra X, del Bas JM, Vitrac X, et al. A trimer plus a dimer-gallate reproduce the bioactivity described for an extract of grape seed procyanidins. Food Chem 2009;116:265–70.
[29] Lee T-P, Leu S-JJ, Huang JC, Song Y-C, Jhou R-S, Tang S-J, et al. Anti-ribosomal phosphoprotein autoantibody triggers interleukin-10 overproduction via phosphatidylinositol 3-kinase-dependent signalling pathways in lipopolysaccharide-activated macrophages. Immunology 2009;127:91–102.
[30] Ci X, Ren R, Xu K, Li H, Yu Q, Song Y, et al. Schisantherin A exhibits anti-inflammatory properties by down-regulating NF-kappaB and MAPK signaling pathways in lipopolysaccharide-treated RAW 264.7 cells. Inflammation 2009;33:126–36.
[31] Lee HJ, Lim HJ, Lee DY, Jung H, Kim MR, Moon DC, et al. Carabrol suppresses LPS-induced nitric oxide synthase expression by inactivation of p38 and JNK via inhibition of I-kappaBalpha degradation in RAW 264.7 cells. Biochem Biophys Res Commun 2010;391:1400–4.
[32] Jeong Sung Mi, Davaatseren M, Kim W, Kwang Park Sung, Kim SH, Haeng Jeon Hur, et al. Vitisin A suppresses LPS-induced NO production by inhibiting ERK, p38, and NF-kappaB activation in RAW 264.7 cells. Int Immunopharmacol 2009;9:319–23.
[33] Huang J-M, Yu J-Y, Jang Y-O, Kim B-T, Hwang K-J, Jeon Y-M, et al. A phenolic acid phenethyl urea compound inhibits lipopolysaccharide-induced production of nitric oxide and pro-inflammatory cytokines in cell culture. Int Immunopharmacol 2010;10:526–32.
[34] Scott MG, Rosenberger CM, Gold MR, Finlay BB, Hancock RE. An alpha-helical cationic antimicrobial peptide selectively modulates macrophage responses to lipopolysaccharide and directly alters macrophage gene expression. J Immunol 2000;165:3358–65.
[35] Dos Santos S, Delattre A-I, De Longueville F, Bult H, Raes M. Gene expression profiling of LPS-stimulated murine macrophages and role of the NF-kappaB and PI3K/mTOR signaling pathways. Ann NY Acad Sci 2007; 1096:70–7.
[36] Aung HT, Schroder K, Himes SR, Brion K, van Zuylen W, Trieu A, et al. LPS regulates proinflammatory gene expression in macrophages by altering histone deacetylase expression. FASEB J 2006;20:1315–27.
[37] Serra A, Macià A, Romero M-P, Valls J, Bladé C, Arola L, et al. Bioavailability of procyanidin dimers and trimers and matrix food effects in in vitro and in vivo models. Br J Nutr 2010;103:944–52.
[38] Holt RR, Lazarus SA, Sullards MC, Zhu QY, Schramm DD, Hammerstone JF, et al. Procyanidin dimer B2 [epicatechin-(4beta-8)-epicatechin] in human plasma after the consumption of a flavanol-rich cocoa. Am J Clin Nutr 2002;76:798–804.
[39] Shoji T, Masumoto S, Moriichi N, Akiyama H, Kanda T, Ohtake Y, et al. Apple procyanidin oligomers absorption in rats after oral administration: analysis of procyanidins in plasma using the porter method and high-performance liquid chromatography/tandem mass spectrometry. J Agric Food Chem 2006;54:884–92.
[40] Blasi E, Ardizzoni A, Colombari B, Neglia R, Baschieri C, Peppoloni S, et al. NF-kB activation and p38 phosphorilation in microglial cells infected with Leptospira or exposed to partially purified leptospiral lipoproteins. Microb Pathog 2007;42:80–7.
[41] Harasstani OA, Moin S, Tham CL, Liew CY, Ismail N, Rajajendram R, et al. Flavonoid combinations cause synergistic inhibition of proinflammatory mediator secretion from lipopolysaccharide-induced RAW 264.7 cells. Inflamm Res 2010;59:711–21.

4.2

Enhanced anti-inflammatory effect of resveratrol and EPA in treated endotoxin-activated RAW 264.7 macrophages.

Victor Pallarès M.Sc. [a], Damien Calay Ph.D. [b], Lídia Cedó M.Sc. [a], Anna Castell-Auví Ph.D. [a], Martine Raes Prof. [b], Montserrat Pinent Ph.D. [a], Anna Ardèvol Ph.D. [a], Lluís Arola Prof. [a], Mayte Blay Ph.D. [a], *

a Departament de Bioquímica i Biotecnologia, Universitat Rovira i Virgili, Tarragona, Spain

b Laboratory of Cellular Biochemistry and Biology, University of Namur (FUNDP-URBC), Namur, Belgium

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Enhanced anti-inflammatory effect of resveratrol and EPA in treated endotoxin-activated RAW 264.7 macrophages

Victor Pallarès[1], Damien Calay[2], Lídia Cedó[1], Anna Castell-Auví[1], Martine Raes[2], Montserrat Pinent[1], Anna Ardévol[1], Lluís Arola[1] and Mayte Blay[1]*

[1]*Departament de Bioquímica i Biotecnologia, Universitat Rovira i Virgili, Marcel·lí Domingo, s/n 43007, Tarragona, Spain*
[2]*Department of Biochemistry and Cellular Biology, University of Namur (FUNDP-URBC), Namur, Belgium*

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Abstract

Macrophages play an important role in immunogenic challenges by producing reactive oxygen species, NO and proinflammatory cytokines that can aggravate and propagate local inflammation. Multiple mechanisms regulate these inflammatory processes. NF-κB and activator protein 1 pathways are crucial in the expression of proinflammatory genes, such as TNF-α, IL-1 (α or β) and -6. Some polyphenols, which are present in beverages, vegetables and fruits, and PUFA, which are present in marine oils and fish food, possess anti-inflammatory effects *in vivo* and *in vitro*. Our aim in the present study was to assess whether polyphenols and PUFA have synergistic anti-inflammatory effects in murine macrophages *in vitro*. Inflammation in RAW 264.7 macrophages was induced by lipopolysaccharide at 100 ng/ml. The treatments with molecules were performed by co-incubation for 19 h. A NO production assay by Griess reaction, a phosphoprotein assay by Pathscan ELISA kit and gene expression analysis using the TaqMan® Low-density Array for ninety-one genes related to inflammation, oxidative stress and metabolism were performed to assess the synergistic anti-inflammatory effects of polyphenols, epigallocatechin gallate and resveratrol (Res; 2·5 μg/ml), and the PUFA, DHA and EPA (30 μM). Adding Res + EPA had an enhanced anti-inflammatory effect, in comparison with EPA and Res alone, leading to decreased NO levels; modulating the phospho-stress activated protein kinase/Jun N-terminal kinase (P-SAPK/JNK) level; down-regulating proinflammatory genes, such as IL, chemokines, transcription factors; and up-regulating several antioxidant genes. Therefore, this combination has a stronger anti-inflammatory effect than either of these molecules separately in RAW macrophages.

Key words: Macrophages: Lipopolysaccharide: Resveratrol: PUFA

Localised inflammation that is considered a protective reaction to tissue irritation, injury or infection can also be involved in several pathologies[1–3]. Macrophages are considered to play a major role in the body's response to immunogenic challenges, by re-establishing tissue homeostasis, producing large amounts of reactive oxygen species, NO and proinflammatory cytokines, which aggravate and propagate local inflammation and disrupt the normal function of target cells[4]. In fact, previous studies performed in RAW 264.7 murine macrophage cells have shown the importance of macrophages in enhancing inflammation by secreting pro-inflammatory factors, such as TNF-α and IL-1β[5].

Multiple mechanisms can propagate inflammation. Some pathways in the cell can modulate inflammation through phosphorylation cascades of proteins that translocate to the nucleus to induce the expression of inflammation genes. One of the more important cascades is the NF-κB pathway. The currently known subunit members of the NF-κB family in mammals are p50, p65 (RelA), c-Rel, p52 and RelB. Moreover, NF-κB inhibitor (Iκ-B) has multiple mammalian forms, such as Iκ-Bα, β, γ (p105), δ (p100) and ε as well as Bcl-3. In unstimulated cells, canonic NF-κB dimeric protein (p50 and p65) is bound in the cytoplasm to Iκ-Bα, and upon stimulation with many NF-κB endogenous inducers, such as IL-1β and TNF-α, or potent exogenous inducers, such as lipopolysaccharide (LPS), Iκ-Bα is rapidly phosphorylated by Iκ-B kinases (IKK) and is marked for ubiquitination and degradation in the cytoplasm. The released NF-κB dimer can be activated by p65 phosphorylation and then translocated to the nucleus where NF-κB will trigger the transcription of target genes by binding with high affinity to κB elements in their promoters[6–8].

Abbreviations: AP-1, activator protein 1; EGCG, epigallocatechin gallate; Iκ-B, NF-κB inhibitor; IKK, Iκ-B kinases; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; Mmp, matrix metalloprotease; NOP, NO production; Res, resveratrol; P-SAPK/JNK, phospho-stress activated protein kinase/Jun N-terminal kinase.

* **Corresponding author:** M. Blay, fax +34 977558232, email mteresa.blay@urv.cat

British Journal of Nutrition

Other pathways are implicated in inflammation, and include molecular partners, such as the transcription factor activator protein 1 (AP-1). When an extracellular stimulus arrives at the cell membrane, specific toll-like receptors activate complex signalling cascades, including the three major mitogen-activated protein kinase (MAPK) signalling pathways, namely, the extracellular-signal-regulated kinase, the SAPK/JNK and p38 pathways. These MAPK activate the transcriptional activity of AP-1 proteins, such as FBJ murine osteosarcoma viral oncogene homologue (FOS) and V-junavian sarcoma virus 17 oncogene homologue (JUN), by phosphorylation and then, translocate to the nucleus, induce the expression of several inflammation target genes, such as *Tnf-α* and *Il-1β*, and genes implicated in matrix degradation, such as matrix metalloprotease (*Mmp*)[9,10].

In this context, several food compounds could help to decrease inflammation associated with disease states by modulating the expression of proteins related to these pathways. Polyphenols are an extensive molecule family that have well-known antioxidant properties and are widely distributed in foods, such as cereals, fruits, vegetables and beverages. In addition, these molecules have anti-inflammatory, antitumour and antiatherogenic abilities[11]. These effects are related to free-radical scavenging, NO regulation, leucocyte immobilisation, apoptosis induction, inhibition of cell proliferation and angiogenesis, antilipid peroxidation, inhibition of inflammatory cytokine production, alterations in cell membrane receptors, intracellular signalling pathway proteins and modulation of gene expression[12,13]. All of these effects may contribute to their potentially protective role in inflammatory and CVD[12,14].

One important polyphenol is epigallocatechin gallate (EGCG; Fig. 1), that belongs to the flavan-3-ol group, and is present mostly in tea leaves and in green tea. It has been reported that EGCG possesses the ability to inhibit the activation of transcription factors, such as NF-κB and AP-1[15], and mRNA expression levels of proinflammatory cytokines, such as TNF-α, in macrophage RAW 264.7 cells[16].

On the other hand, resveratrol (Res; *trans*-3,5,49-trihydroxystilbene, Fig. 1), that belongs to the stilbenes group, is present in dark-coloured grapes[14,17], mulberries, peanuts and red wines, and it has been known as an anti-inflammatory and antioxidant agent in studies performed *in vitro* and *in vivo*. In addition, Res is known to inhibit the transcription of proinflammatory genes and to modulate the action of transcription factors, such as NF-κB or AP-1, in mouse and human cells[18–21].

Other bioactive compounds that can ameliorate inflammation are PUFA. EPA and DHA (Fig. 1) are long-chain *n*-3 PUFA that are present mainly in fish oils and marine products, such as tuna, cod, sardine or salmon[22]. It has been demonstrated that these molecules possess several beneficial properties for human CVD and inflammatory disease. These biological effects are related to lipoprotein metabolism, endothelial cell function, vascular reactivity, inflammatory markers and cytokine production[23,24]. Most of these beneficial effects are due to their antioxidant and anti-inflammatory effects by decreasing reactive oxygen species[25] and by decreasing the generation of proinflammatory cytokines, like TNF-α, IL-1β and IL-6, leading to the deactivation of the NF-κB signalling cascade and the activation of the PPARγ anti-inflammatory pathway[26].

The present study assessed whether polyphenols EGCG and Res, and PUFA, DHA and EPA, can modulate the expression of inflammatory genes and proinflammatory proteins and whether polyphenols and PUFA have enhancer anti-inflammatory effects at different levels on murine macrophages stimulated with LPS to induce inflammation. Furthermore, this study aimed to define the individual anti-inflammatory role of these bioactive compounds.

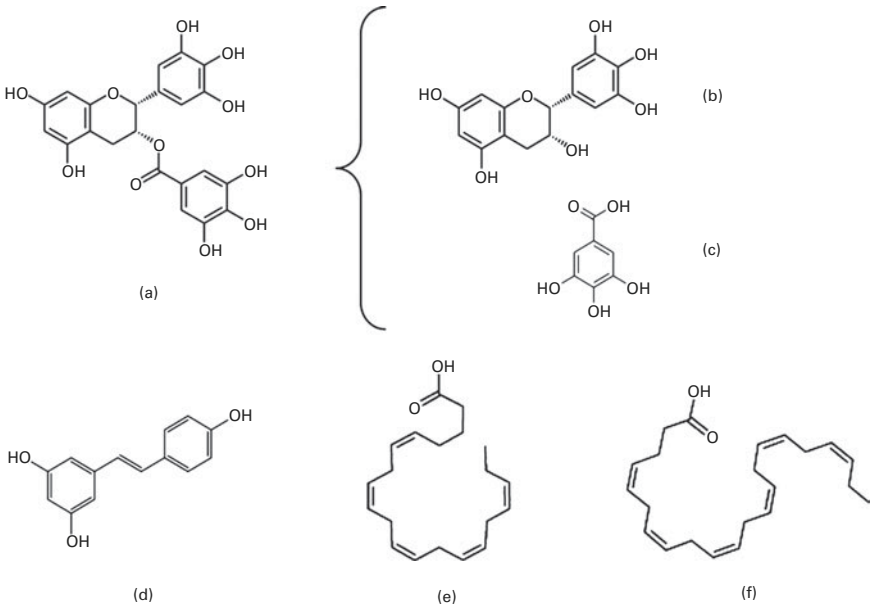

Fig. 1. Molecular structure of polyphenols ((a) epigallocatechin gallate, (b) epigallocatechin, (c) gallic acid and (d) resveratrol) and PUFA ((e) EPA (20 : 5*n*-3) and (f) DHA (22 : 6*n*-3)).

British Journal of Nutrition

British Journal of Nutrition

Experimental methods

Reagents and cells

The following chemicals were purchased from Sigma-Aldrich: EGCG (ref. E4143, ≥95 %), Res (ref. R5010, >99 %), DHA (ref. D2534, ≥98 %), EPA (ref. E2011, ≥99 %) and LPS from *Escherichia coli* 0111: B4 (ref. L4391, lot: 127K4037).

The murine macrophage cell line RAW 264.7 (ref. 91062702; European Tissue Culture Collection ECACC) was cultured at 37°C under 5 % CO_2 in Dulbecco's modified Eagle's medium containing 10 % (v/v) fetal bovine serum, 2 mM-D-glutamine, penicillin (100 U (62·7 μg)/ml), streptomycin (100 μg/ml) and 25 mM-HEPES. The cells were between ten and sixteen passages when they were treated.

Experimental design

For all the experiments, RAW 264.7 cells were cultured in twelve-well plates. At 80–90 % confluency, the medium was replaced with a colourless Dulbecco's modified Eagle's medium with antibiotics and without fetal bovine serum, and was then treated depending on the experiment. Previously, the administration in cells, EPA and DHA were diluted in colourless Dulbecco's modified Eagle's medium with bovine serum albumin NEFA with a ratio of 1:4 (PUFA:bovine serum albumin), and EGCG and Res in PBS. The final concentrations of compounds were the following in all the experiments: EGCG and Res (2·5 μg/ml), and DHA and EPA (30 μM).

For the NO production (NOP) experiment, a total of eight conditions were tested: C− (only vehicle, PBS + ethanol), C+ (LPS 100 ng/ml), EGCG, Res, DHA, EPA, EGCG + DHA, Res + DHA, EGCG + EPA, Res + EPA. Cells were stimulated with LPS (100 ng/ml) and co-incubated with polyphenols and PUFA and after 19 h of treatment, the medium was collected and stored at −20°C.

For the phosphoprotein immunoassay, the following conditions were used (three wells/condition): medium only with vehicle, LPS, Res + LPS, EPA + LPS, and Res + EPA + LPS. After 30 min of co-incubation, the medium was collected and stored at −80°C.

For the TaqMan® Low-density Array assay, the following conditions were used (three wells/condition): medium only with vehicle, LPS, Res + LPS, EPA + LPS and Res + EPA + LPS. After 19 h of treatment, the medium was collected and stored at −80°C. The cells were counted with a Countess® Automated Cell Counter (ref. C10227; Invitrogen) with a final average of $1{\cdot}2 \times 10^6$ live cells/ml, and a viability of 93 %.

Testing the capacity of polyphenols and PUFA alone and in combination for suppressing nitric oxide production in RAW 264.7 cells

To analyse the putative effect of polyphenols and PUFA alone or in combination, NOP was determined by the Griess reagent, which reacts with the NO_2^- ion to produce a final stable product that is purple and can be quantified using colorimetric or spectrophotometric techniques at 540 nm[17]. Nitrite production was normalised to protein content using 0·1 M-NaOH for the cell lysate and the Bradford method (ref. B6916; Sigma-Aldrich). The assays were performed in technical and biological triplicate. The results are represented as the percentage of NOP *v.* that produced by the cells stimulated with LPS (C+).

Immunoassay of phosphoproteins using Pathscan ELISA kit

To assess the levels of several phosphoproteins that are able to activate inflammation in macrophages, a multi-target ELISA kit was used. The PathScan® Inflammation Multi-Target Sandwich ELISA Kit (ref. 7276) was provided by Cell Signaling.

After treatment, the adherent monocytes were washed with ice-cold PBS, and then the 1X Cell Lysis Buffer (ref. 9803; Cell Signaling) + 1 mM-phenylmethylsulphonyl fluoride were added. The plate was incubated for 5 min on ice. The cells were scraped and transferred to a tube, and each lysate was sonicated for 10 s using a Vibra-Cell VCX 750 Sonicator (Sonics and Materials, Inc.). The lysates were centrifuged at 11 000 ***g*** for 10 min at 4°C, and the supernatant was stored in a new tube at −80°C until assay day. The protein content of the lysates was determined by the Bradford method (Sigma-Aldrich) to readjust all of the samples to 5 mg/ml of protein. Phosphoproteins P-NF-κB p65 (Ser536), P-SAPK/JNK (Thr13/Tyr185), P-p38 (Thr180/Tyr182), P-IκB-α (Ser32) and P-STAT3 (Tyr705; phospho-Signal transducer and activator of transcription 3) as well as NF-κB p65 were semi-quantified using the PathScan® Inflammation Multi-Target Sandwich ELISA Kit. Briefly, the lysates were diluted with a sample diluent and were added to the appropriate well that contained its relevant antibody adhered at the bottom. The plate was incubated overnight at 4°C, and the wells were washed with washing buffer four times. Then, detection or primary antibody was added to the appropriate well, and the plate was incubated for 1 h at 37°C. After a second washing of four times, the horseradish peroxidase (HRP)-linked secondary antibody was added, and the plate was incubated for 30 min at 37°C. The 3,3′,5,5′-tetramethylbenzidine (TMB) substrate was added to each well, and the plate was incubated for 30 min at room temperature. Finally, the reaction was stopped and the samples were read at an absorbance of 450 nm in an ELISA spectrophotometer (Anthos Zenyth 200 st; Anthos Labtec Instruments). The assay was performed in technical duplicate. Each phosphoprotein absorbance was corrected by the negative control and was normalised by its relevant NF-κB p65 absorbance. The results were expressed as the percentage of phosphoprotein absorbance *v.* that produced by cells just stimulated with LPS.

TaqMan® Low-density Array gene expression analysis

To make screening of numerous genes implicated in inflammation, oxidative stress and other metabolic mechanisms, a TaqMan® Low-density Array was used. The RNeasy Mini Kit (ref. 74 106; Qiagen) was used according to the manufacturer's instructions to isolate RNA from pretreated RAW cells. After collecting the medium, ice-cold PBS was added to the adherent monocyte cells. Then, the PBS was removed, and lysis buffer was added to each well. After several centrifugations

British Journal of Nutrition

and washings, the RNA was diluted with 50 μl of RNase-free water. RNA purity and quantity were measured with the Nanodrop 1000 (Thermo Fisher Scientific), and RNA integrity was checked with the Agilent 2100 Bioanalyzer (ref. G2938C) using the Agilent RNA 6000 Nano Kit (ref. 5067-1511; Agilent Technologies). Finally, the RNA was stored at −80°C. Next, complementary DNA was synthesised from 2 μg of total RNA using the High-Capacity cDNA Reverse Transcription Kit (ref. 4368814; Applied Biosystems), diluted to 100 ng/μl of complementary DNA to load into each reservoir of the TaqMan® Low-density Array (array on demand; Applied Biosystems). Each TaqMan® Low-density Array plate (96a format), which contains sets of ninety-six mouse gene probes and mouse primers (ninety genes + five controls), was analysed using a real-time PCR amplification system with the Taqman Universal PCR Master Mix (Applied Biosystems). These genes are related to different biological functions and were organised into twelve categories: (1) cytokines (30·2% of the total number of genes analysed) including members of the TNF family (5·2%), IL and IL receptors (15·6%), and chemokines and chemokine receptors (8·3%); (2) transcription factors and regulators (16·7%); (3) MAPK signalling cascade proteins (6·3%); (4) oxidative stress components (10·4%); (5) scavenger receptors and cholesterol efflux channels (8·3%); (6) adhesion molecules (2·1%); (7) apoptosis proteins (2·1%); (8) proinflammatory proteins (5·2%); (9) matrix degradation and regulatory proteins (2·1%); (10) proteins involved in metabolism (5·2%); (11) monocyte markers (5·2%); (12) binding proteins (1·0%). The 7900HT Fast Real-Time PCR with 384-Well Block Module and the 7900HT Sequence Detection System software (SDS 2.3) (Applied Biosystems) were used to perform real-time PCR and subsequent analysis of the results.

Calculating the potentiating effects of the molecules in combination

To evaluate the enhancer effects of the compounds in combination, we considered the following:

Percentage of maximum inhibition of compounds alone in medium

X: (% NOP in positive control
− % NOP of compound 'X' alone + SEM)

Y: (% NOP in positive control
− % NOP of compound 'Y' alone + SEM).

Percentage of minimum inhibition of compounds together in medium

(X + Y): (% NOP in positive control
− % NOP of compounds 'X' and 'Y' together − SEM).

If X + Y is less than or equal to (X + Y), there is an enhanced anti-inflammatory effect. If X + Y is greater than (X + Y), there is an enhanced proinflammatory effect.

Both compounds had to have a significant NOP individually compared with the NOP when combined. If one of the compounds alone did not fulfil these criteria, we concluded that the compounds would not have an effect when combined. These criteria were also used to evaluate the effects on phosphoprotein and gene expression levels.

We also used one of the most cited, recommended and accepted models for defining drug interactions in order to assess if our enhancer effects could be considered synergistic, additive or antagonistic: The Bliss independence model[27–29]. This model is also called effect multiplication or the fractional product, which has the form $E_{(12)} = E_{(1)} \times E_{(2)}$, where $E_{(12)}$ is the effect of the compounds in combination expressed as a fraction, and $E_{(1)}$ and $E_{(2)}$ are the effects for compound 1 and compound 2 expressed as fractions. Combination doses with effects less than that predicted are synergistic, with effects equal are additive and with effects greater are antagonistic. However, this consideration can only be claimed using the Loewe model[28,29], which refers to the combinatorial study of drugs performing isobolographic analysis; so that the interpretation of our results using the Bliss method must be qualified as a precursor of new studies that reassert these effects.

Statistical analysis

The results are presented as means with their standard errors. The data were analysed by one-way ANOVA to determine the statistical differences between groups using SPSS statistical software (version 17.0 for Windows; SPSS, Inc.). $P<0·05$ was considered statistically significant.

Results

Effects of polyphenols and PUFA suppressing nitric oxide production in stimulated RAW cells

LPS is a well-known trigger of the inflammation response in macrophages, and several biomarkers, like NO, can be tested to measure this response. RAW 264.7 macrophages were stimulated with LPS for 19 h, and NOP was determined. Macrophages in the positive control medium had a mean NOP of 37·7 μM. To examine the synergistic effects in suppressing the NO, polyphenols Res and EGCG were tested using 2·5 μg/ml doses and the PUFA, DHA and EPA, were tested using 30 μM doses. These doses and time were chosen on the basis of dose–response and time-course experiments in RAW 264.7 macrophages stimulated with LPS as a positive control to check the inhibition of NO at different concentrations of the compounds[21,25,30] (see supplementary Figs. S6 and S7 available online at http://www.journals.cambridge.org/bjn). The molecules are non-toxic in RAW 264.7 macrophages at least below the following concentrations: Res 22·82 μg/ml, EGCG 4·58 μg/ml, EPA 100 μM, DHA 100 μM[25,31,32].

In these conditions, an enhancer effect was found when Res was combined with EPA (Fig. 2(B)). The treatment with EPA significantly inhibited NOP *v.* the positive control (−45·0% of NOP, Fig. 2(B)), while Res did not inhibit it at the tested concentration (+8·5% of NOP, Fig. 2(B)). In spite of this, the combination of these compounds had a stronger inhibition than Res or EPA individually (−83·6% of NOP, Fig. 2(B)). Besides, using the Bliss model ($E_{(12)} = E_{(1)} \times E_{(2)}$), we found that $E_{(12)}$ was 0·164, less than $E_{(1)} \times E_{(2)}$ that was 0·597 (Res × EPA = 1·085 × 0·55), which signifies a synergistic

effect. In addition, another strong inhibitory effect was found when Res and DHA were combined (−33·7% of NOP; Fig. 2(B)), with their effect being significantly different compared to DHA (−20·6% of NOP, Fig. 2(B)) and Res (+8·5% of NOP, Fig. 2(B)) alone and classified as a synergistic effect using the Bliss model, with $E_{(12)} = 0{\cdot}663$ being less than $E_{(1)} \times E_{(2)} = 0{\cdot}861$ (Res × DHA = 1·085 × 0·794). In contrast, EGCG alone did not inhibit NOP significantly *v.* positive control (−14·3% of NOP; Fig. 2(A)), and the combination with EPA had the same effect than EPA alone (−45% of NOP; Fig. 2(A)).

Effect of resveratrol in combination with EPA in suppressing phosphoprotein levels in stimulated RAW macrophages

To measure the levels of several proteins involved in inflammatory pathways, PathScan® Inflammation Multi-Target Sandwich ELISA was used. These proteins, which reside in the cytoplasm, are phosphorylated by other proteins in the phosphorylation cascade that is induced by an external inflammatory agent, like LPS, and lead to the activation of proteins that modulate gene transcription in the nucleus. The phosphorylated proteins, P-NF-κB p65, P-SAPK/JNK, P-p38, P-IκB-α and P-STAT3, were tested. RAW 264.7 macrophages were stimulated with LPS (100 ng/ml) for 30 min to detect phosphoprotein levels in these conditions[33–37]. At the same time, different concentrations of the following compounds were applied to different groups of cells: Res (2·5 μg/ml), EPA (30 μM) and Res + EPA (2·5 μg/ml, 30 μM). These assays revealed an enhancer effect on P-SAPK/JNK protein (Fig. 3(B)). The absorbance level of P-SAPK/JNK in Res-treated and EPA-treated cells was decreased significantly compared with the positive control (−13% of P-SAPK/JNK level, Fig. 3(B)). In addition, when the cells were treated with a combination of Res and EPA, P-SAPK/JNK level had a stronger decrease than Res or EPA separately (−30·8% of P-SAPK/JNK level, Fig. 3(B)). Although many treatments were statistically significant compared with the positive control, no more enhancer effects were found in P-NF-κB p65 (Fig. 3(A)), P-p38 (Fig. 3(C)), P-STAT3 (Fig. 3(D)) and P-IκB-α levels (Fig. 3(E)).

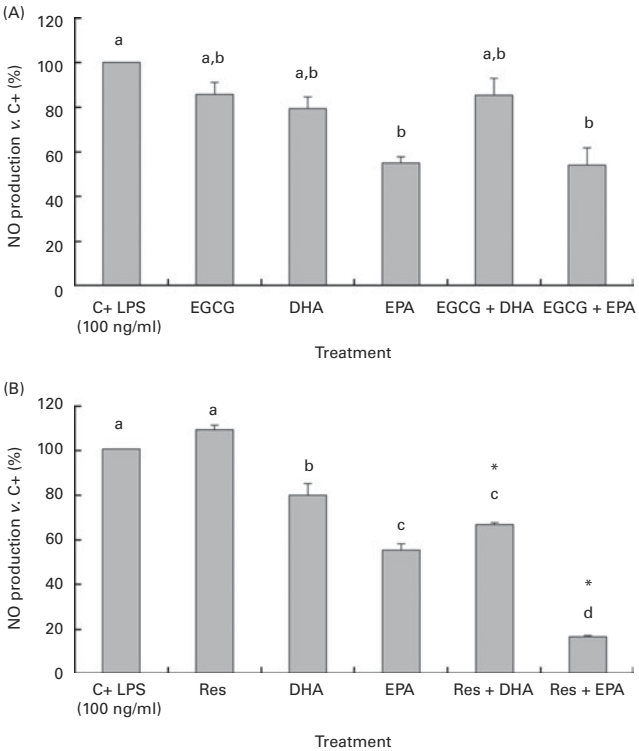

Fig. 2. The effects of polyphenols (epigallocatechin gallate (EGCG) and resveratrol (Res); 2·5 μg/ml) and PUFA (DHA and EPA; 30 μM) on nitric oxide production in RAW 264.7 macrophages stimulated with lipopolysaccharide (LPS; 100 ng/ml). The results are presented as the percentage of nitric oxide production and normalised to the positive control (100%). Values are means with their standard errors of biological and technical triplicates. [a,b,c,d]Mean values with unlike letters were significantly different and the symbol * signifies enhanced effect. For all of the comparisons, a one-way ANOVA with *post hoc* test was used, with $P < 0{\cdot}05$ as the threshold for statistical significance. The values of EPA and DHA represented in (A) and (B) are the same, but were included in both because they were necessary for the statistical analysis with the other groups.

Effect of resveratrol in combination with EPA in modulating the expression of pro-inflammation target genes in stimulated RAW macrophages

RAW 264.7 macrophages were stimulated with LPS (100 ng/ml) for 19 h to trigger the expression of several target genes involved in inflammation, oxidative stress and metabolism pathways. Cells were treated with LPS alone (positive control) or LPS and Res, EPA or Res and EPA to measure the inhibition of proinflammatory genes. The most important enhanced effects of Res and EPA are shown in Table 1, whereas the rest of the results are in Table S2 (available online at http://www.journals.cambridge.org/bjn). We considered that the genes were up-regulated or down-regulated if they had significant differences *v.* the negative and/or positive control groups. We found that LPS treatment led to a greater than 100-fold increase in the expression of *Il-1α, Il-1β, Il-6, Ccl7* and *Csf2* and more than a 10-fold increase in the expression of *Tnfrsf1b, Il-10, Il-1rn, Il-12β, Ccl2, Ccl5, Timp1, Nos2, Ptgs2, Serpine1, Socs2* and *Socs3* genes. Most of these genes have important proinflammatory and anti-inflammatory functions. Thus, we found LPS-mediated gene regulation that seems to follow an inflammatory profile[38–41]. Moreover, we found different profiles of the regulation of anti-inflammatory, antioxidant and proinflammatory genes depending on the treatment administrated (Figs. 4 and 5), showing that the treatment of Res + EPA had a stronger anti-inflammatory profile than Res or EPA individually. Specifically, treatment with Res increased significantly the levels of Il-2 receptor, *Il-2rα*, the kinase inhibitor of *Nf-κb, Ikk-β, Tnfrsf18* and *Socs3*, and decreased significantly proinflammatory genes, such as *Fas, Il-1β, Ccl2, Socs2*, or the proto-oncogene *Myc*. In addition, other proinflammatory genes, such as *Il-1α, Il-6, Csf2, Nos2, Ptgs2* and *Mmp9*, had a tendency to decrease their levels in comparison to the positive control. In contrast, treatment with EPA increased the proinflammatory *Fas, Ccl3, Tnfrsf18* and *Msr1* genes, the anti-inflammatory *Il-1rn, Nfkbia* and

British Journal of Nutrition

Socs3 genes, and the antioxidant *Hmox1, Cat* and *Gclm* genes. In addition, EPA treatment decreased the proinflammatory *Tnf-α, Il-2rα* and *Il-12β* genes. Treatment with Res and EPA increased the gene expression of *Il-12β, Hmox1* and *Cat*, and decreased *Il-1β, Il-6, Il-23a, Serpine1, Nos2, Il-2rα, Il-10* and *Nfkbia* in comparison to positive control. In addition, we found an enhancer effect in the inhibition of the expression of proinflammatory genes, including *Tnf-α, Tnfrsf18, Csf2, Ccl2, Ccl5, Nf-κb P65, Ikk-β, Map3k8* and *Mapk8*, and, surprisingly, the anti-inflammatory *Tnfrsf1b, Hdac2, Socs2* and *Socs3* genes. We also found a potentiating effect in the inhibition of the expression of a stress-inducible chaperone protein, *Hspa5*.

Discussion

An interesting area in functional food science is discovering the enhancer effects or synergies between bioactive components to create foods that have improved health benefits. Such an investigation would require studies with *in vitro* and *in vivo* models. Our work is the first step in achieving these goals.

Macrophages, which play an important role in the inflammatory states of several tissues and various diseases, can help us to understand how polyphenols and PUFA modulate local inflammation at different biological levels. Thus, we stimulated murine RAW 264.7 macrophages with LPS to mimic this inflammatory state and study the synergistic effects of these natural compounds.

Important enhanced anti-inflammatory effects were found when Res at 2·5 μg/ml + EPA at 30 μM were in combination. It has been reported that a study of forty-eight subjects consuming fish once or twice a month had a detectable plasma DHA level of about 60 μg/ml and EPA of about 10 μg/ml, which is equivalent to 182 and 33 μM, respectively[25]. Therefore, our tested concentrations of PUFA are close to the bioavailability levels found in plasma of these studies *in vivo*. In contrast, several studies in human subjects and rats are in controversy regarding the bioavailability of Res in blood plasma, with some studies reporting that this is between 1×10^{-3} and 9×10^{-3} μg/ml[42,43], while the others reporting that it is 1·2 μM (0·273 μg/ml)[44], being the free-Res form that is very low in plasma in comparison with their metabolised forms (derivated glucuronides and sulphates) generated in the intestine and the liver before being delivered to the systemic circulation[45]. However, pharmacokinetic studies in human subjects, following the oral administration of 2 g of Res twice daily, obtained a plasma concentration of 1274 (SEM 790) ng/ml of free-Res[46], which is not very far from our tested dose of 2·5 μg/ml Res. Despite this, our tested concentration is far from being physiological and the concentration might be adjusted to ng/ml to confirm, in new future experiments, the enhancer effect of Res in combination with PUFA at physiological levels. Also, combinatorial studies may be performed with these molecules in order to assess if these enhanced effects can be considered as synergistic.

First, we tested whether polyphenols and PUFA can inhibit NOP, a clear marker of inflammation in macrophages.

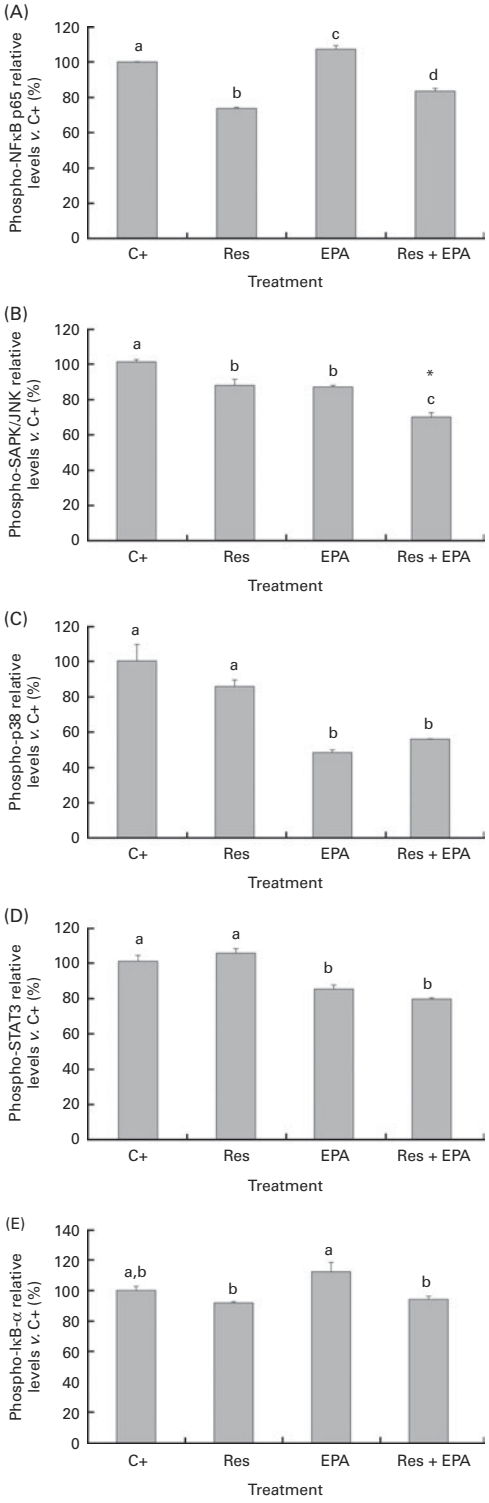

Fig. 3. The effects of resveratrol (Res; 2·5 μg/ml) and EPA (30 μM) on the levels of (A) phospho-NFκB p65, (B) phospho-stress activated protein kinase/Jun N-terminal kinase (phospho-SAPK/JNK), (C) phospho-p38, (D) phospho-STAT3 and (E) phospho-IκB-α in RAW 264.7 stimulated with lipopolysaccharide (100 ng/ml). The results are presented as the percentage of relative levels of phosphoproteins and are normalised to the positive control (100 %). Values are means with their standard errors of technical duplicates. [a,b,c,d] Mean values with unlike letters were significantly different and the symbol * signifies enhanced effect. For all of the comparisons, a one-way ANOVA with *post hoc* test was used, with $P<0·05$ as the threshold for statistical significance.

British Journal of Nutrition

Table 1. The most important effects of resveratrol (Res; 2·5 μg/ml) and EPA (30 μM) on the expression of twenty-seven genes in RAW 264.7 macrophages stimulated with lipopolysaccharide (LPS; 100 ng/ml)*

(Mean values and standard deviations)

					LPS + molecules					
			LPS		Res		EPA		Res + EPA	
Gene function	Gene symbol	Gene name	Mean	SD	Mean	SD	Mean	SD	Mean	SD
TNF family	*Tnf*	Tumour necrosis factor alpha	1·62	0·09	1·60	0·01	1·40†	0·01	1·15‡§	0·02
	Tnfrsf18	Tumour necrosis factor receptor superfamily, member 18	0·15	0·01	0·24†	0·02	0·30†	0·02	0·02‡	0·00
	Tnfrsf1b	Tumour necrosis factor receptor superfamily, member 1B	23·24	0·51	21·78	1·03	23·93	0·22	17·38‡	1·87
IL and receptors	*Il-1α*	Interleukin 1 alpha	315·29	8·42	281·59	3·64	289·44	18·91	252·41	33·92
	Il-1β	Interleukin 1 beta	280·73	1·58	191·80‖	2·16	254·74	15·96	216·50‖	0·66
	Il-1rn	Interleukin 1 receptor antagonist	13·70	0·17	14·02	0·58	18·11†	0·20	14·07	0·35
	Il-6	Interleukin 6	275·75	26·35	223·42	31·21	295·48	12·23	160·62‖	13·93
	Il-23a	Interleukin 23, alpha subunit p19	2·60	0·44	2·98	0·36	1·65§	0·19	1·42§‖	0·06
	Il-10	Interleukin 10, cytokine synthesis inhibitory factor	84·58	7·65	70·35	10·46	83·86	10·34	43·93‖	0·10
Chemokines and receptors	*Csf2*	Granulocyte-macrophage colony stimulating factor	104·43	0·08	89·04	6·02	99·07	2·29	66·34‡	9·41
	Ccl2	MCP-1, monocyte chemo-attractant protein 1	63·74	0·60	52·62†	2·32	64·44	1·67	41·36‡	3·30
	Ccl5	Chemokine (C–C motif) ligand 5	27·60	1·24	26·54	1·79	31·62	0·36	20·74‡	2·22
	Ccl7	MCP-3, chemokine (C–C motif) ligand 7	126·91	2·55	124·22	8·40	138·80	17·46	90·61	10·74
Transcription factors and related	*Ikbkb*	IKK-beta, nuclear factor NF-kappa-B inhibitor kinase beta	0·42	0·02	0·67†	0·05	0·48	0·04	0·33‡	0·02
	RelA	Nuclear factor NF-kappa-B p65 subunit	0·72§	0·04	0·78§	0·05	0·71§	0·00	0·53‡	0·00
	Hdac2	Histone deacetylase 2	0·45	0·02	0·47	0·02	0·52	0·03	0·34‡	0·01
	Socs2	Suppressor of cytokine signalling 2	13·48	0·77	7·49†	0·51	13·69	0·68	1·03‡§	0·02
	Socs3	Suppressor of cytokine signalling 3	24·52	0·67	29·59†	0·40	33·04†	0·79	14·38‡	0·72
	Serpine1	PAI-1, plasminogen activator inhibitor 1	39·57	2·39	34·53	0·75	35·94	0·05	28·87‖	3·06
MAPK and related	*Map3k8*	Mitogen-activated protein kinase kinase kinase 8	1·10§	0·06	1·19	0·08	1·15§	0·03	0·85‡§	0·01
	Mapk8	JNK1, stress-activated protein kinase	0·66	0·04	0·60	0·04	0·60	0·03	0·43‡	0·02
Oxidative stress	*Hmox1*	HO-1, heme oxygenase (decycling) 1	9·88	0·47	9·74	0·66	18·39†	0·50	15·02†	0·82
	Nos2	iNOS, nitric oxide synthase 2, inducible	17·69	0·69	14·57	1·78	16·26	0·15	12·15‖	2·06
	Gclm	Gamma-glutamylcysteine synthetase regulatory subunit	0·81§	0·04	0·86§	0·02	1·62†	0·15	1·20§	0·12
Scavenger receptors	*Msr1*	Macrophage scavenger receptor 1	1·28§	0·00	1·10§	0·15	1·85§	0·01	1·46§	0·22
Metabolism	*Hspa5*	BiP, immunoglobulin heavy chain-binding protein	3·85†	0·05	4·62†	0·00	2·32†	0·13	1·76‡	0·17
	Ctss	Cathepsin S	0·38	0·06	0·40	0·06	0·60†	0·06	0·29	0·01

MAPK, mitogen-activated protein kinase.

* The results are presented as the relative levels of gene expression *v.* cells without treatment (C−). We used the well-known mathematical equation δδCT to calculate these relative levels *v.* C−. The results are presented as the means and standard deviations of technical duplicates. The statistical significance *v.* C+ is represented by ‖, *v.* all of the treatments by †, *v.* C+ and groups of compounds alone by ‡, and signifies enhanced effect. Finally, no symbol signifies statistical significance *v.* C−, while the symbol § signifies non-statistical significance *v.* C−. For all of the comparisons, a one-way ANOVA with Tukey's test was used, with $P<0·05$ as the threshold for statistical significance.

We found that LPS stimulation at 100 ng/ml for 19 h can trigger NOP. This treatment is less aggressive than other treatments used previously; 1 μg/ml[34–37,47] and 10 μg/ml[25,48]. Also, this concentration is useful for testing whether polyphenols can exert an inhibitory effect, as seen in previous studies of inflammation[17]. In fact, we found that DHA and EPA inhibited significantly NOP *v.* the positive control as in previous studies[49]. But, in controversy with a work performed in RAW macrophages[50], we found that EPA had a stronger effect than DHA. It suggests that the effects of DHA and EPA depend on the doses and the time of administration. On the other hand, EGCG at 2·5 μg/ml (5·4 μM) had a tendency to inhibit NOP (−14·3 %) but not significantly (Fig. 2(A)), similar to a work which found an inhibition of NOP of 19 % in RAW macrophages activated with 5000 ng/ml of LPS with similar concentration of EGCG, 5 μM[51]. In contrast, Res had a similar effect to the positive control, suggesting that Res cannot inhibit NOP at 2·5 μg/ml (10·9 μM) (Fig. 2(B)). However, we found inhibition effects in NOP at doses 22, 44 and 66 μM (5, 10 and 15 μg/ml of Res in supplementary Fig. S6 (available online at http://www.journals.cambridge.org/bjn), as in other studies that used similar Res concentrations[21,31,52], suggesting that Res has intrinsic anti-inflammatory power in several conditions in RAW. When the compounds were combined, we found an enhancer effect with Res and EPA (Fig. 2(B)) and the Bliss model classified this combination as a synergy because the effect we found (0·164) was much lower than the predicted effect (0·597). These results suggest that Res and EPA could be investigated in future studies performing combination of doses between compounds and using the Loewe method to calculate synergistic effects. In this context, it suggests that while Res cannot inhibit NOP by itself, with the addition of EPA it can modulate some mechanisms that promote an even stronger inhibition of inflammation. An explanation for this result may be that EPA permits the action of Res or that Res affects the effect of EPA at cellular level, maybe because one of them could interact with the receptors of the other, increasing its effect over cells. It could also be that they use different signalling pathways leading to a final enhancer anti-inflammatory effect. In contrast, although the inhibitory effect of Res and DHA was greater than the effect of DHA and Res alone (Fig. 2(B)), using the Bliss model the effect we found (0·663) was not too far from the predicted effect (0·861), in comparison with Res and EPA results. So it suggests that the effect of this combination should be studied more to be claimed as a synergy and it must be considered as a potentiating effect. On the other hand, EGCG tended to decrease the NOP, but when combined with DHA and EPA, the inhibitory effect was not greater than the effect of the molecules alone, which suggests that EGCG cannot increase the anti-inflammatory effect of PUFA (Fig. 2(A)).

Our next aim was to determine whether Res and EPA in combination could modulate the levels of phosphoproteins implicated in inflammatory pathways, such as NF-κB and AP-1. We found an enhancer effect of these compounds on P-SAPK/JNK levels (Fig. 3(B)), having an inhibitor effect stronger than the molecules alone, as was seen in previous studies that reported the capacity of Res and EPA to interact with

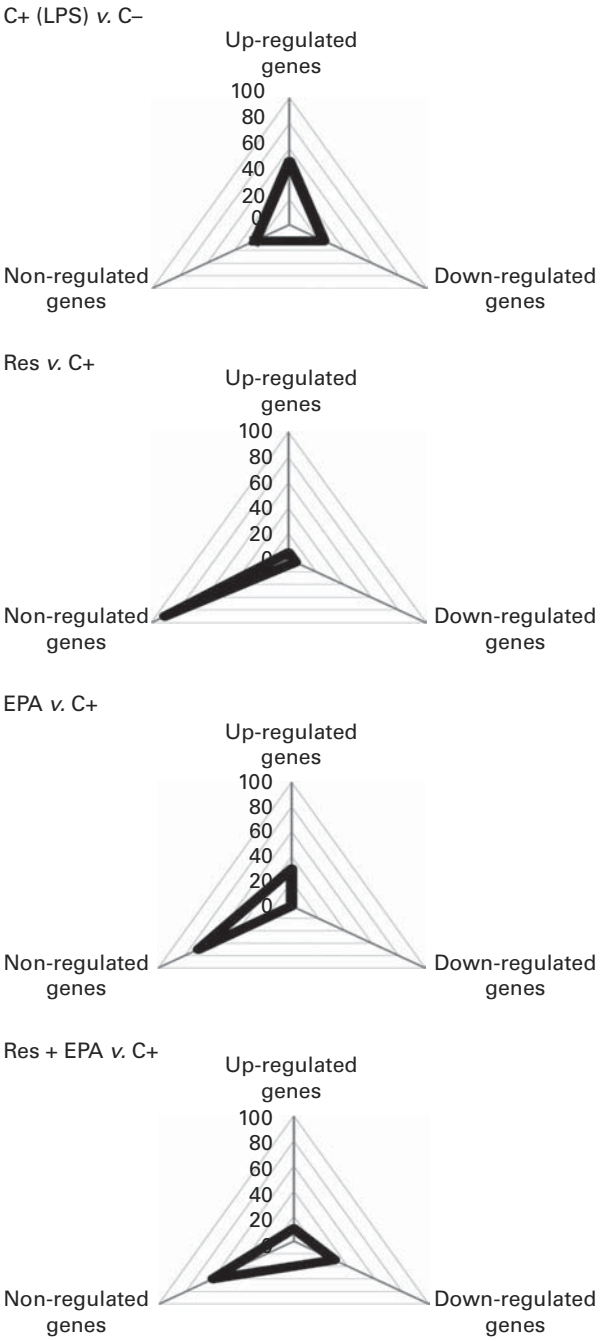

Fig. 4. The regulation (▬) of proinflammatory genes in RAW 264.7 macrophages treated with lipopolysaccharide (LPS) (C+, 100 ng/ml) alone or LPS and resveratrol (Res; 2·5 μg/ml), EPA (30 μM) or Res + EPA (2·5 μg/ml; 30 μM). The results are presented as the percentage of genes that were up-regulated, down-regulated or not regulated with the statistical significance *v.* the control group in each title. For all of the comparisons, a one-way ANOVA with *post hoc* test was used, with $P<0·05$ as the threshold for statistical significance. These values were extracted from *Effect of resveratrol in combination with EPA in modulating the expression of pro-inflammation target genes in stimulated RAW macrophages* of this article (see Table 1 and Supplementary Table S2 (available online at http://www.journals.cambridge.org/bjn) to find out which gene is up-, down- or non-regulated for each treatment). There were thirty-eight proinflammatory genes (*Tnf, Tnfrsf18, Fas, Il-1α, Il-1β, Il-6, Il-23a, Il-2ra, Il-12b, Il-18, Ccl2, Ccl3, Ccl5, Ccl7, Csf2, Serpine1, Jun, Ikbkb, Myc, Nfkb1, Nfkb2, RelA, Chuk, Map3k8, Map2k1, Mapk14, Mapk3, Mapk8, Nos2, Ager, Cd36, Msr1, Cd68, Acat1, Icam1, Ptgs2, Mmp9, Cd80*) detected in this study.

MAPK in macrophages[53,54]. P-SAPK/JNK inhibition may lead to the activation of the transcription factor AP-1 and may decrease the expression of proinflammatory genes, such as *Tnf-α* and *Il-1β*. Thus, these molecules could attenuate the inflammatory state in cells. In addition, we found a decrease of the levels of phosphoproteins NF-κB p65, P-p38 and STAT3 when the molecules were combined (Fig. 3(A), (C) and (D)). Moreover, we found a strong decrease of P-NF-κB p65 levels when the cells were treated with Res alone (Fig. 3(A)), suggesting the high power of this molecule to affect the NF-κB signal pathway by itself as in other studies performed in RAW macrophages reporting the reduction of NF-κB activity by Res[21,55]. On the other hand, the treatment with EPA alone not only produced a slight decrease of P-SAPK/JNK and P-STAT3 levels (Fig. 3(B) and (D)), but also produced a strong decrease of P-p38 levels (Fig. 3(C)). These results suggest that EPA can modulate the phosphorylation of proteins related to proinflammatory pathways in RAW macrophages, as has been reported before[23,54,56]. However, the combination of Res and EPA had inhibitory effects on four of the five phosphoproteins assessed (P-NF-κB p65, P-SAPK/JNK, P-p38 and P-STAT3) in comparison to the C+, while EPA had it in three (P-SAPK/JNK, P-p38 and P-STAT3) and Res just in two (P-NF-κB p65 and P-SAPK/JNK). Thus, this result suggests that the combination has more anti-inflammatory power than either molecule alone because it can modulate more pathways than Res or EPA separately.

Our final aim was assessing whether the combination of Res and EPA had effects on the expression of genes related to inflammation and oxidative stress. As seen in the regulation profiles of proinflammatory, anti-inflammatory and antioxidant genes (Figs. 4 and 5), we found many genes that were activated in LPS-stimulated macrophages; specifically, around 50–60 % of these genes were up-regulated by treatment. This result suggests that stimulation with LPS promotes an inflammatory state that has been seen in other studies[38–41] by increasing the expression of proinflammatory target genes (Fig. 4). However, small numbers of anti-inflammatory and antioxidant genes are up-regulated as well (Fig. 5), possibly to counteract this induced proinflammatory state. Thus, the cell is in a struggle to return to fix damaged areas and return to normal. In this context, we found molecules that reverse inflammation by down-regulating proinflammatory genes and up-regulating anti-inflammatory and antioxidant genes (Figs. 4 and 5). Specifically (Table 1 and Table S2; available online at http://www.journals.cambridge.org/bjn), EPA seems to have a greater capacity for up-regulating anti-inflammatory genes, such as *Il-1rn*, which is an antagonist of the receptor for the proinflammatory IL-1 and suggests an indirect decrease in IL-1 activity, IκB-α (*Nfkbia*), which is involved in sequestering NF-κB in the cytoplasm, or *Socs3*, and antioxidant genes, such as *Hmox1, Cat* and *Gclm*, in charge of the destruction of reactive oxygen species, demonstrating the antioxidant ability of this molecule, as has been shown in previous works[49]. In contrast, Res has more significant effects in the down-regulation of proinflammatory genes, such as *Il-1β, Ccl2* and *Fas*, and non-significant effects over genes, such as *Il-1α, Il-6, Csf2, Serpine1, Nos2, Jun* and

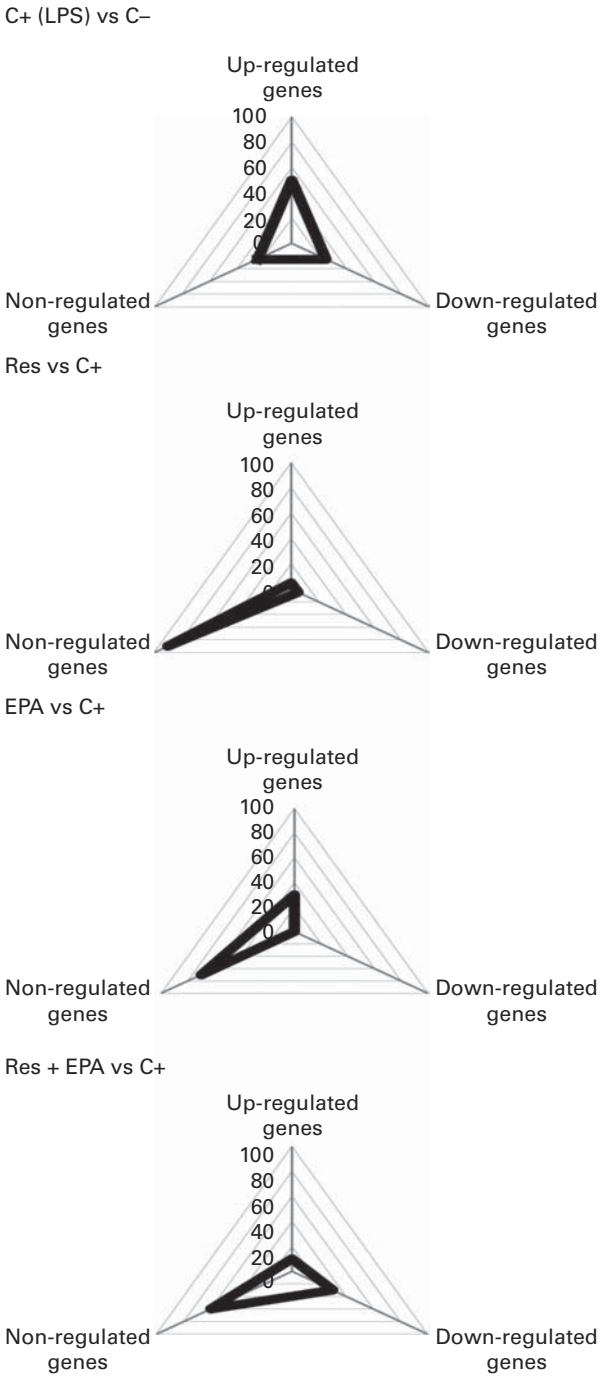

Fig. 5. The regulation (▬) of anti-inflammatory and antioxidant genes in RAW 264.7 macrophages treated with lipopolysaccharide (LPS) (C+, 100 ng/ml) alone or LPS and resveratrol (Res; 2·5 μg/ml), EPA (30 μM) or Res + EPA (2·5 μg/ml; 30 μM). The results are presented as the percentage of genes that were up-regulated, down-regulated or not regulated with the statistical significance *v.* the control group in each title. For all of the comparisons, a one-way ANOVA with *post hoc* test was used, with $P<0·05$ as the threshold for statistical significance. These values were extracted from *Effect of resveratrol in combination with EPA in modulating the expression of pro-inflammation target genes in stimulated RAW macrophages* of this article (see Table 1 and Supplementary Table S2 (available online at http://www.journals.cambridge.org/bjn) to find out which gene is up-, down- or non-regulated for each treatment). There were twelve anti-inflammatory (*Tnfrsf1b, Il-1rn, Il-10, Nfkbia, Nfkbib, Pparg, Socs1, Socs2, Socs3, Hdac2, Sirt1, Timp1*) and eight antioxidant (*Hmox1, Cat, Gpx1, Nfe2l2, Gclm, Sod1, Sod2, Nqo1*) genes detected in this study.

British Journal of Nutrition

British Journal of Nutrition

Ptgs2 (Tables 1 and Table S2; available online at http://www.journals.cambridge.org/bjn). In this context, there is controversy with the ability of Res to modulate proinflammatory genes, with some studies reporting that Res cannot regulate them[31,55] and the others supporting our results[21,57]. In overview, our results suggest that Res helps to decrease inflammation by down-regulating proinflammatory target genes (Fig. 4), but that EPA decreases inflammation by up-regulating anti-inflammatory and antioxidant genes (Fig. 5). We found that the combination of Res and EPA promotes a greater down-regulation of proinflammatory target genes (Fig. 4) as well as a surprisingly remarkable down-regulation of anti-inflammatory and antioxidant-related genes (Fig. 5). Importantly (Table 1), combined treatment decreased proinflammatory *Il-1α* and *β*, and *Il-6* compared with the positive control. In addition, *Tnf-α*, a molecule crucial in inflammation-related diseases and the activation of macrophages, was down-regulated, demonstrating a potentiated anti-inflammatory effect when the molecules were combined. These results may be due to the ability of Res and EPA to decrease the levels of P-SAPK/JNK (Fig. 3(B)), which is able to lead the expression of IL-1β and TNF-α, through the activation of AP-1. In contrast, the expression of anti-inflammatory *Il-10* was decreased with the combination of compounds, which suggests that this combination could have inhibitory effects over the expression of anti-inflammatory proteins or that the LPS-induced global inflammatory state was decreased by other pathways, rendering unnecessary its expression. On the other hand, proinflammatory chemokines, such as *Csf2, Ccl2* and *Ccl5*, were down-regulated, reasserting the enhancer effect of the molecules in combination, which could suggest that the cells return to their normal levels of differentiation and production after 19 h of treatment under the influence of the molecules. Expression of Serpine1 (*Pai-1*), which encodes the inhibitor of plasminogen activator, was decreased, suggesting that this treatment could ameliorate the coagulation of blood around tissues with macrophages. In addition, expression levels of transcription factors and proteins related to inflammation pathways, such as *NF-κB p65*, the inhibitor of NF-κB kinase *Ikk-β, Map3k8*, or *Jnk1* were decreased, most of them as a consequence of the enhancer effect of the molecules in combination, in comparison with the positive control. In contrast, the expression of anti-inflammatory genes, such as histone deacetylase 2 (*Hdac2*), and suppressors of cytokine signalling 2 and 3 (*Socs2* and *Socs3*), were potentially down-regulated, which suggests that cells return to the state of non-inflammation or non-activation and that they do not need the action of some anti-inflammatory mechanisms. In conclusion, the cells treated with Res and EPA had decreased global inflammation, affecting the gene expression of proteins related to NF-κB and AP-1 pathways, and important pro-inflammatory interleukins, chemokines and cytokines. In oxidative stress-related genes, we found an increase in the expression of various antioxidant proteins, such as haeme-oxygenase 1 (*Hmox1*), which catalyses the degradation of haeme, catalase (*Cat*) and γ-glutamylcysteine synthetase regulatory subunit (*Gclm*), which is the first rate-limiting enzyme of glutathione synthesis. These three proteins have been implicated in an reactive oxygen species-reduction pathway. However, these effects were not stronger than EPA up-regulation. In addition, *iNos* gene expression was down-regulated when the molecules were combined, suggesting a reduction of the pro-oxidative stress effects promoted by LPS.

These results suggest that the combination of Res and EPA has an enhancer anti-inflammatory effect by modulating several inflammatory and oxidative stress-related genes. Furthermore, these inhibitory effects could result from the sum of the EPA-mediated up-regulation of antioxidant genes and Res-mediated down-regulation of proinflammatory genes.

Conclusions

Combining Res and EPA has potentiating anti-inflammatory effects in LPS-stimulated RAW 264.7 macrophages by decreasing NO levels, decreasing the mRNA levels of proinflammatory and oxidative stress-related genes and affecting the phosphorylation of proteins implicated in the activation of NF-κB and AP-1 proinflammatory pathways. These results could lead to *in vivo* studies that may yield novel preventive or palliative nutritional treatments for obesity, atherosclerosis and CVD.

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References

1. Ho S-C, Hwang LS, Shen Y-J, *et al.* (2007) Suppressive effect of a proanthocyanidin-rich extract from longan (*Dimocarpus longan* Lour.) flowers on nitric oxide production in LPS-stimulated macrophage cells. *J Agric Food Chem* **55**, 10664–10670.
2. Maury E & Brichard SM (2010) Adipokine dysregulation, adipose tissue inflammation and metabolic syndrome. *Mol Cell Endocrinol* **314**, 1–16.
3. Holvoet P (2008) Relations between metabolic syndrome, oxidative stress and inflammation and cardiovascular disease. *Verh K Acad Geneeskd Belg* **70**, 193–219.
4. Fujihara M, Muroi M, Tanamoto K, *et al.* (2003) Molecular mechanisms of macrophage activation and deactivation by

lipopolysaccharide: roles of the receptor complex. *Pharmacol Ther* **100**, 171–194.
5. Permana PA, Menge C & Reaven PD (2006) Macrophage-secreted factors induce adipocyte inflammation and insulin resistance. *Biochem Biophys Res Commun* **341**, 507–514.
6. Bowie A & O'Neill LAJ (2000) Oxidative stress and nuclear factor-kappaB activation: a reassessment of the evidence in the light of recent discoveries. *Biochem Pharmacol* **59**, 13–23.
7. Aktan F (2004) iNOS-mediated nitric oxide production and its regulation. *Life Sci* **75**, 639–653.
8. Ghosh S & Karin M (2002) Missing pieces in the NF-kappaB puzzle. *Cell* **109**, S81–S96.
9. Kunz M, Ibrahim S, Koczan D, *et al.* (2001) Activation of c-Jun NH_2-terminal kinase/stress-activated protein kinase (JNK/SAPK) is critical for hypoxia-induced apoptosis of human malignant melanoma. *Cell Growth Differ* **12**, 137–145.
10. Hu X, Chen J, Wang L, *et al.* (2007) Crosstalk among Jak-STAT, toll-like receptor, and ITAM-dependent pathways in macrophage activation. *J Leukoc Biol* **82**, 237–243.
11. Rahman I, Biswas SK & Kirkham PA (2006) Regulation of inflammation and redox signaling by dietary polyphenols. *Biochem Pharmacol* **72**, 1439–1452.
12. Arts ICW & Hollman PCH (2005) Polyphenols and disease risk in epidemiologic studies. *Am J Clin Nutr* **81**, 317S–325S.
13. Terra X, Montagut G, Bustos M, *et al.* (2009) Grape-seed procyanidins prevent low-grade inflammation by modulating cytokine expression in rats fed a high-fat diet. *J Nutr Biochem* **20**, 210–218.
14. Nichols JA & Katiyar SK (2010) Skin photoprotection by natural polyphenols: anti-inflammatory, antioxidant and DNA repair mechanisms. *Arch Dermatol Res* **302**, 71–83.
15. Surh Y-J (2006) NF-κB and AP-1 as molecular targets for chemoprevention with EGCG, a review. *Environ Chem Lett* **4**, 137–141.
16. Na H-K & Surh Y-J (2006) Intracellular signaling network as a prime chemopreventive target of (−)-epigallocatechin gallate. *Mol Nutr Food Res* **50**, 152–159.
17. Terra X, Valls J, Vitrac X, *et al.* (2007) Grape-seed procyanidins act as antiinflammatory agents in endotoxin-stimulated RAW 264.7 macrophages by inhibiting NFkB signaling pathway. *J Agric Food Chem* **55**, 4357–4365.
18. de la Lastra CA & Villegas I (2005) Resveratrol as an anti-inflammatory and anti-aging agent: mechanisms and clinical implications. *Mol Nutr Food Res* **49**, 405–430.
19. Sánchez-Fidalgo S, Cárdeno A, Villegas I, *et al.* (2010) Dietary supplementation of resveratrol attenuates chronic colonic inflammation in mice. *Eur J Pharmacol* **633**, 78–84.
20. de la Lastra CA & Villegas I (2007) Resveratrol as an antioxidant and pro-oxidant agent: mechanisms and clinical implications. *Biochem Soc Trans* **35**, 1156–1160.
21. Tsai S-H, Lin-Shiau S-Y & Lin J-K (1999) Suppression of nitric oxide synthase and the down-regulation of the activation of NFκB in macrophages by resveratrol. *Br J Pharmacol* **126**, 673–680.
22. Gogus U & Smith C (2010) *n*-3 Omega fatty acids: a review of current knowledge. *Int J Food Sci Technol* **45**, 417–436.
23. Rahman MM, Bhattacharya A & Fernandes G (2008) Docosahexaenoic acid is more potent inhibitor of osteoclast differentiation in RAW 264.7 cells than eicosapentaenoic acid. *J Cell Physiol* **214**, 201–209.
24. Calder PC (2005) Polyunsaturated fatty acids and inflammation. *Biochem Soc Trans* **33**, 423–427.
25. Saw CLL, Huang Y & Kong A-N (2010) Synergistic anti-inflammatory effects of low doses of curcumin in combination with polyunsaturated fatty acids: docosahexaenoic acid or eicosapentaenoic acid. *Biochem Pharmacol* **79**, 421–430.
26. Calder PC (2006) *n*-3 Polyunsaturated fatty acids, inflammation, and inflammatory diseases. *Am J Clin Nutr* **83**, 1505S–1519S.
27. Corning PA (1998) "The synergism hypothesis": on the concept of synergy and its role in the evolution of complex systems. *J Soc Evol Syst* **21**, 133–172.
28. Chou T-C (2006) Theoretical basis, experimental design, and computerized simulation of synergism and antagonism in drug combination studies. *Pharmacol Rev* **58**, 621–681.
29. Lee JJ, Kong M, Ayers GD, *et al.* (2007) Interaction index and different methods for determining drug interaction in combination therapy. *J Biopharm Stat* **17**, 461–480.
30. Liang Y-C, Huang Y-T, Tsai S-H, *et al.* (1999) Suppression of inducible cyclooxygenase and inducible nitric oxide synthase by apigenin and related flavonoids in mouse macrophages. *Carcinogenesis* **20**, 1945–1952.
31. Wadsworth TL & Koop DR (1999) Effects of the wine polyphenolics quercetin and resveratrol on pro-inflammatory cytokine expression in RAW 264.7 macrophages. *Biochem Pharmacol* **57**, 941–949.
32. Elbling L, Weiss R-M, Teufelhofer O, *et al.* (2005) Green tea extract and (−)-epigallocatechin-3-gallate, the major tea catechin, exert oxidant but lack antioxidant activities. *FASEB J* **19**, 807–809.
33. Lee T-P, Leu S-JJ, Huang JC, *et al.* (2009) Anti-ribosomal phosphoprotein autoantibody triggers interleukin-10 overproduction via phosphatidylinositol 3-kinase-dependent signalling pathways in lipopolysaccharide-activated macrophages. *Immunology* **127**, 91–102.
34. Ci X, Ren R, Xu K, *et al.* (2009) Schisantherin A exhibits anti-inflammatory properties by down-regulating NF-kappaB and MAPK signaling pathways in lipopolysaccharide-treated RAW 264.7 cells. *Inflammation* **33**, 126–136.
35. Lee HJ, Lim HJ, Lee DY, *et al.* (2010) Carabrol suppresses LPS-induced nitric oxide synthase expression by inactivation of p38 and JNK via inhibition of I-kappaBalpha degradation in RAW 264.7 cells. *Biochem Biophys Res Commun* **391**, 1400–1404.
36. Jeong Sung Mi, Davaatseren M, Kim W, *et al.* (2009) Vitisin A suppresses LPS-induced NO production by inhibiting ERK, p38, and NF-kappaB activation in RAW 264.7 cells. *Int Immunopharmacol* **9**, 319–323.
37. Hwang J-M, Yu J-Y, Jang Y-O, *et al.* (2010) A phenolic acid phenethyl urea compound inhibits lipopolysaccharide-induced production of nitric oxide and pro-inflammatory cytokines in cell culture. *Int Immunopharmacol* **10**, 526–532.
38. Scott MG, Rosenberger CM, Gold MR, *et al.* (2000) An alpha-helical cationic antimicrobial peptide selectively modulates macrophage responses to lipopolysaccharide and directly alters macrophage gene expression. *J Immunol* **165**, 3358–3365.
39. Dos Santos S, Delattre A-I, De Longueville F, *et al.* (2007) Gene expression profiling of LPS-stimulated murine macrophages and role of the NF-kappaB and PI3K/mTOR signaling pathways. *Ann N Y Acad Sci* **1096**, 70–77.
40. Aung HT, Schroder K, Himes SR, *et al.* (2006) LPS regulates proinflammatory gene expression in macrophages by altering histone deacetylase expression. *FASEB J* **20**, 1315–1327.
41. Mendes SDS, Candi A, Vansteenbrugge M, *et al.* (2009) Microarray analyses of the effects of NF-kappaB or PI3K pathway inhibitors on the LPS-induced gene expression profile in RAW 264.7 cells: synergistic effects of rapamycin

British Journal of Nutrition

on LPS-induced MMP9-overexpression. *Cell Signal* **21**, 1109–1122.
42. Vitaglione P, Sforza S, Galaverna G, *et al.* (2005) Bioavailability of *trans*-resveratrol from red wine in humans. *Mol Nutr Food Res* **49**, 495–504.
43. Goldberg DM, Yan J & Soleas GJ (2003) Absorption of three wine-related polyphenols in three different matrices by healthy subjects. *Clin Biochem* **36**, 79–87.
44. Meng X, Maliakal P, Lu H, *et al.* (2004) Urinary and plasma levels of resveratrol and quercetin in humans, mice, and rats after ingestion of pure compounds and grape juice. *J Agric Food Chem* **52**, 935–942.
45. Santos AC, Veiga F & Ribeiro AJ (2011) New delivery systems to improve the bioavailability of resveratrol. *Expert Opin Drug Deliv* **8**, 973–990.
46. la Porte C, Voduc N, Zhang G, *et al.* (2010) Steady-state pharmacokinetics and tolerability of *trans*-resveratrol 2000 mg twice daily with food, quercetin and alcohol (ethanol) in healthy human subjects. *Clin Pharmacokinet* **49**, 449–454.
47. Blasi E, Ardizzoni A, Colombari B, *et al.* (2007) NF-κB activation and p38 phosphorilation in microglial cells infected with Leptospira or exposed to partially purified leptospiral lipoproteins. *Microb Pathog* **42**, 80–87.
48. Harasstani OA, Moin S, Tham CL, *et al.* (2010) Flavonoid combinations cause synergistic inhibition of proinflammatory mediator secretion from lipopolysaccharide-induced RAW 264.7 cells. *Inflamm Res* **59**, 711–721.
49. Kim YJ & Chung HY (2007) Antioxidative and anti-inflammatory actions of docosahexaenoic acid and eicosapentaenoic acid in renal epithelial cells and macrophages. *J Med Food* **10**, 225–231.
50. Komatsu W, Ishihara K, Murata M, *et al.* (2003) Docosahexaenoic acid suppresses nitric oxide production and inducible nitric oxide synthase expression in interferon-gamma plus lipopolysaccharide-stimulated murine macrophages by inhibiting the oxidative stress. *Free Radic Biol Med* **34**, 1006–1016.
51. Lin YL & Lin JK (1997) (−)-Epigallocatechin-3-gallate blocks the induction of nitric oxide synthase by down-regulating lipopolysaccharide-induced activity of transcription factor nuclear factor-kappaB. *Mol Pharmacol* **52**, 465–472.
52. Chan MM, Mattiacci JA, Hwang HS, *et al.* (2000) Synergy between ethanol and grape polyphenols, quercetin, and resveratrol, in the inhibition of the inducible nitric oxide synthase pathway. *Biochem Pharmacol* **60**, 1539–1548.
53. Huang Z, Wang C, Wei L, *et al.* (2008) Resveratrol inhibits EMMPRIN expression via P38 and ERK1/2 pathways in PMA-induced THP-1 cells. *Biochem Biophys Res Commun* **374**, 517–521.
54. Lo CJ, Chiu KC, Fu M, *et al.* (2000) Fish oil modulates macrophage P44/P42 mitogen-activated protein kinase activity induced by lipopolysaccharide. *JPEN J Parenter Enteral Nutr* **24**, 159–163.
55. Djoko B, Chiou RY-Y, Shee J-J, *et al.* (2007) Characterization of immunological activities of peanut stilbenoids, arachidin-1, piceatannol, and resveratrol on lipopolysaccharide-induced inflammation of RAW 264.7 macrophages. *J Agric Food Chem* **55**, 2376–2383.
56. Zhao Y, Joshi-Barve S, Barve S, *et al.* (2004) Eicosapentaenoic acid prevents LPS-induced TNF-alpha expression by preventing NF-kappaB activation. *J Am Coll Nutr* **23**, 71–78.
57. Chung EY, Kim BH, Hong J-T, *et al.* (2011) Resveratrol down-regulates interferon-γ-inducible inflammatory genes in macrophages: molecular mechanism via decreased STAT-1 activation. *J Nutr Biochem* **22**, 902–909.

British Journal of Nutrition

Supplementary Data

Supplementary Table 2

			LPS		LPS + Molecules					
					Res		EPA		Res + EPA	
Gene Function	Gene Symbol	Gene Name	Mean	SD	Mean	SD	Mean	SD	Mean	SD
TNF family	FAS	TNF receptor superfamily, member 6	3.69	0.11	3.16 *	0.25	4.47 *	0.25	3.57	0.02
Interleukins and receptors	IL2RA	Interleukin 2 receptor, alpha	7.62	0.61	12.14 *	0.82	0.86 "=	0.04	1.03 "=	0.02
	IL12B	Interleukin 12, subunit beta	14.24	0.78	13.57	0.88	0.86 *=	0.04	18.81 *	0.42
	IL18	Interleukin 18, IFN-gamma-inducing factor, IL1 gamma	0.68	0.02	0.53	0.07	0.82 =	0.04	0.59	0.06
Chemokines and receptors	CCL3	MIP 1-alpha, Chemokine (C-C motif) ligand 3	3.01 =	0.50	2.97 =	0.18	5.19 *	1.02	2.28 =	0.22
	CSF1	Macrophage colony stimulating factor	1.34 =	0.65	1.46 =	0.10	0.78 =	0.35	0.82 =	0.16
Transcription factors and related	JUN	AP-1 Transcription factor	1.65	0.16	1.34 =	0.09	1.37 =	0.01	1.14 =	0.19
	MYC	Proto-oncogene c-Myc, transcription factor p64	5.20	0.06	4.02 "	0.27	4.38	0.39	3.83 "	0.32
	NFKB1	Nuclear factor NF-kappa-B p50/p105 subunit	0.94 =	0.00	0.82 =	0.06	0.97 =	0.01	0.71 =	0.03
	NFKB2	Nuclear factor NF-kappa-B p49/p100 subunit	1.24 =	0.12	1.28 =	0.09	1.20 =	0.06	0.82 =	0.04
	NFKBIA	IkB-alpha, NF-kappa-B inhibitor alpha	3.45	0.03	3.05	0.21	4.06 *	0.05	2.83 "	0.17
	NFKBIB	IkB-beta, NF-kappa-B inhibitor beta	1.94	0.21	2.00	0.13	2.14	0.12	1.77	0.04
	PPARG	Peroxisome proliferator-activated receptor gamma	0.09	0.00	0.09	0.01	0.07	0.05	0.04	0.02
	SOCS1	Suppressor of cytokine signaling 1	2.82 =	0.60	4.37 =	1.22	5.48	0.78	2.37 =	1.36
	CHUK	IKK-alpha, Nuclear factor NF-kappa-B inhibitor kinase alpha,	0.47	0.02	0.54	0.04	0.58	0.05	0.52	0.06
	SIRT1	Sirtuin type 1	0.28	0.03	0.30	0.03	0.29	0.02	0.26	0.00
MAPKs and related	MAP2K1	MAPK/ERK kinase 1, Mitogen-activated protein kinase kinase 1	1.55 =	0.25	1.33 =	0.09	1.10 =	0.53	0.69 =	0.25
	MAPK14	p38 MAP kinase, Mitogen-activated protein kinase 14	0.40	0.02	0.41	0.03	0.46	0.01	0.35	0.04
	MAPK3	ERK1, Mitogen-activated protein kinase 3	1.24 =	0.20	1.06 =	0.07	1.36 =	0.10	1.10 =	0.02
Oxidative stress	CAT	Catalase	0.80 =	0.05	0.78 =	0.05	1.17 "=	0.05	1.06 "=	0.05
	GPX1	GSHPX1, Glutathione peroxidase 1	0.50	0.01	0.57	0.04	0.61	0.11	0.50	0.04
	SOD1	Superoxide dismutase 1, soluble	0.89 =	0.01	0.84 =	0.05	1.27	0.31	0.49 =	0.03
	SOD2	Superoxide dismutase 2, mitochondrial	2.76	0.25	1.89 =	0.03	3.34	0.53	2.48	0.08
	NFE2L2	NRF2, Nuclear factor (erythroid-derived 2)-like 2	0.46	0.04	0.45	0.02	0.54	0.07	0.47	0.03
	NQO1	NAD(P)H dehydrogenase, quinone 1	1.75 =	0.24	0.86 =	0.35	2.90 =	0.47	2.19 =	0.73
	NOS1	Nitric oxide synthase 1, constitutive NOS	0.10	0.07	0.14	0.03	0.16	0.15	0.11	0.00

Scavenger receptors and cholesterol efflux	AGER	Receptor for advanced glycosylation end products	0.16 =	0.02	1.04 =	1.08	0.79 =	0.64	0.75 =	0.60
	CD36	Scavenger receptor class B, member 3, thrombospondin receptor	3.02 =	1.95	3.77 =	0.11	4.98	0.01	4.11 =	0.03
	SCARB1	Scavenger receptor class B, member 1	0.24	0.02	0.17	0.02	0.26	0.01	0.22	0.00
	ABCA1	ATP-binding cassette sub-family A member 1	0.17	0.01	0.19	0.03	0.13	0.05	0.14	0.02
	ABCG1	ATP-binding cassette sub-family G member 1	0.15	0.01	0.14	0.01	0.18	0.00	0.14	0.01
	CD68	Scavenger receptor class D, member 1	0.59	0.07	0.60	0.08	0.73	0.01	0.63	0.01
	ACAT1	Acetyl-Coenzyme A acetyltransferase 1	0.14	0.00	0.12	0.00	0.19	0.01	0.16	0.01
Adhesion molecules	ICAM1	Intercellular adhesion molecule 1	0.26	0.01	0.20	0.00	0.30	0.03	0.24	0.01
Apoptosis	BAX	BCL2-associated X protein, apoptosis regulator	0.69	0.02	0.65	0.02	0.75	0.04	0.53	0.04
	BCL2	B-cell CLL/lymphoma 2, apoptosis regulator	0.48	0.02	0.51	0.06	0.59	0.04	0.50	0.01
Inflammation	PTGS1	COX1, Prostaglandin-endoperoxide synthase 1	0.83 =	0.07	0.71 =	0.08	0.84 =	0.11	0.58	0.02
	PTGS2	COX2, Prostaglandin-endoperoxide synthase 2	83.90	6.02	73.28	2.24	82.20	7.77	74.22	2.72
Matrix degradation	MMP9	Matrix metallopeptidase 9	3.96	0.20	3.32	0.28	4.80	0.49	2.74	0.42
	TIMP1	Tissue inhibitor of metalloproteinase 1	17.35	2.96	12.57	0.94	16.31	3.81	14.79	1.57
Metabolism	H6PD	Hexose-6-phosphate dehydrogenase	0.14	0.02	0.11	0.03	0.16	0.00	0.13	0.01
	HMGCR	NADPH, 3-hydroxy-3-methylglutaryl CoA reductase	0.18	0.01	0.23	0.02	0.23	0.02	0.17	0.00
	ARG1	Arginase type 1	1.26 =	0.73	1.40 =	1.16	2.67 =	1.36	0.86 =	0.05
Monocyte markers and surface	CD80	T-lymphocyte activation antigen CD80	2.42	0.13	1.94	0.35	1.93	0.11	1.77 =	0.14
	EMR1	EGF-like module-containing mucin-like hormone receptor-like 1	1.29 =	0.17	1.19 =	0.12	1.68 =	0.06	0.69 =	0.02
	TLR4	Toll-like receptor 4	0.22	0.02	0.25	0.00	0.28	0.03	0.22	0.01
	B2M	Beta-2-microglobulin	0.73	0.08	0.64	0.01	0.82 =	0.01	0.76	0.09
	CD14	Myeloid cell-specific leucine-rich glycoprotein, monocyte differentiation antigen	1.48	0.02	1.25 =	0.10	1.51	0.02	1.13 =	0.22

Table 2: The effects of Res (2.5 µg/ml) and EPA (30 µM) on the expression of 48 genes in RAW 264.7 macrophages stimulated with LPS (100 ng/ml). The results are presented as the relative levels of gene expression versus cells without treatment (C-). We used the well-known mathematic equation δδCT to calculate these relative levels vs. C-. The results are presented as the mean ± SEM of technical duplicates. The statistical significance vs. C+ is represented by ", vs. all of the treatments by *, vs. C+ and groups of compounds alone by #, and signifies enhanced effect. Finally, non-statistical significance vs. C- is represented by =. For all of the comparisons, a one-way ANOVA with Tukey's test was used, with $p<0.05$ as the threshold for statistical significance.

Figure 6

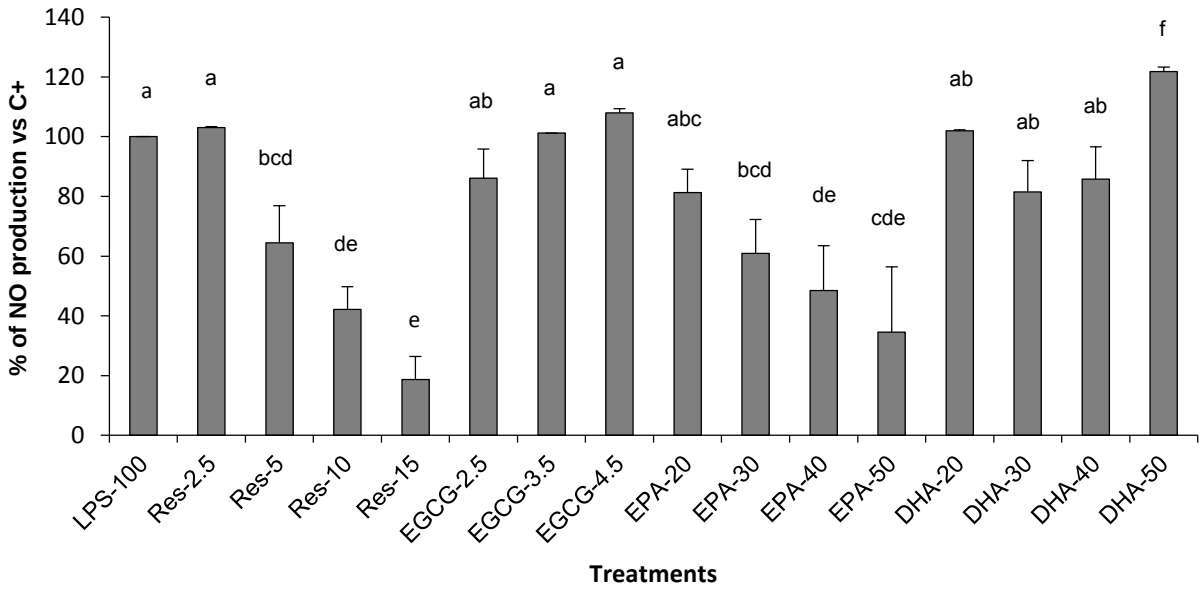
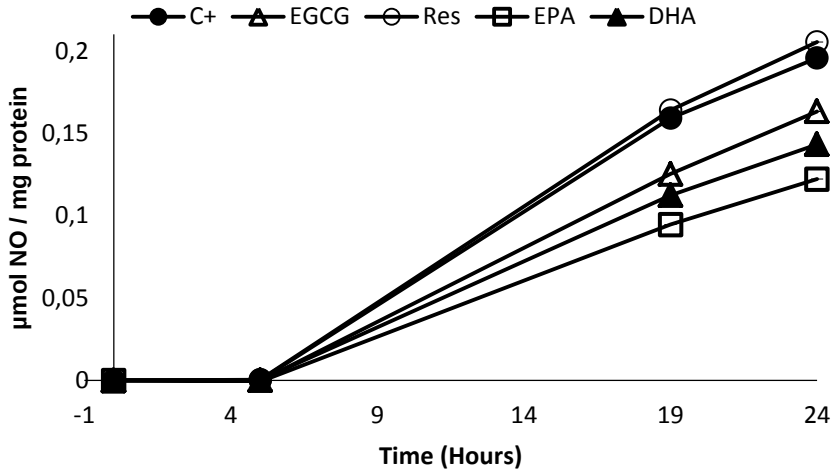


Figure 6: The effects of polyphenols (EGCG doses: 2.5, 3.5 and 4.5 µg/ml; and Res doses: 2.5, 5, 10 and 15 µg/ml) and PUFAs (DHA and EPA doses: 20, 30, 40, and 50 µM) on NO production in RAW 264.7 macrophages stimulated with LPS (100 ng/ml). The results are presented as the % of NO production and normalized to the positive control (100%). Each value represents the mean ± SEM of biological duplicate and technical triplicates. Different letters are representing significantly different groups. For all of the comparisons, a one-way ANOVA with post hoc test was used, with $p<0.05$ as the threshold for statistical significance.

Figure 7



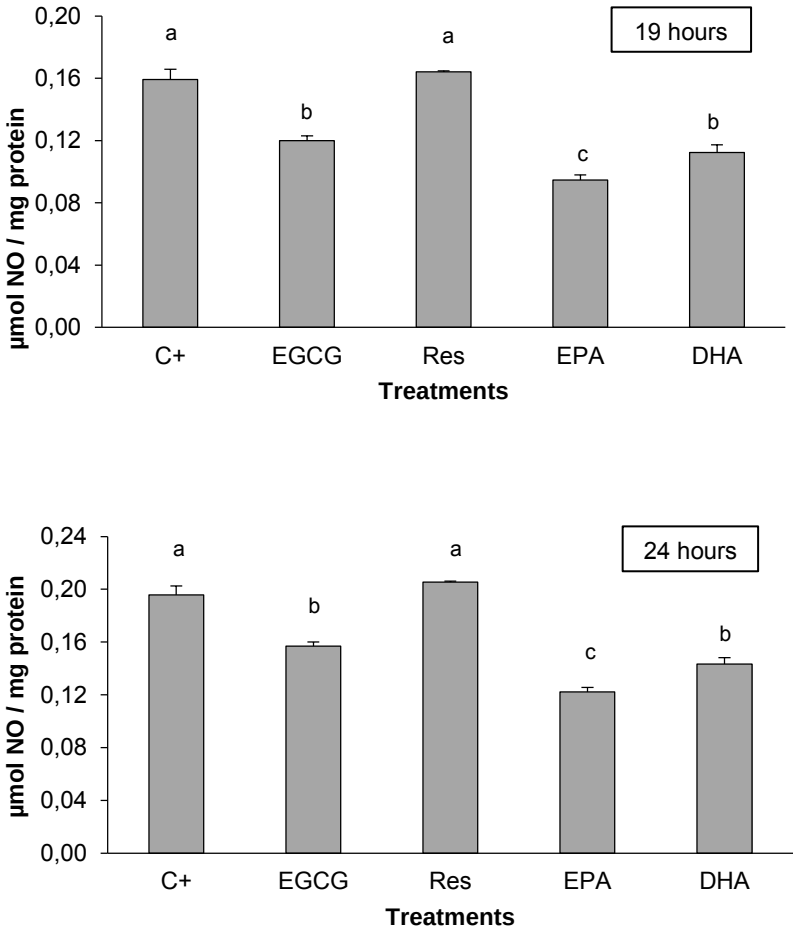


Figure 7: The effects of polyphenols (EGCG and Res; 2.5 µg/ml) and PUFAs (DHA and EPA; 30 µM) on NO production in RAW 264.7 macrophages stimulated with LPS (100 ng/ml) in 0, 5, 19 and 24 hours. The results are presented as the concentration of NO production in µmol NO/mg of protein. In the first figure it is presented the effect in NO production of each treatment in time (0, 5, 19 and 24 hours). The figures with bars represent the effect in NO production of each treatment in 19 and 24 hours. These values in bars are the same than in curves. Idem than Fig. 6 for the presentation of the results and the statistical methods used.

In vivo studies

The objective of this part was to investigate the anti-inflammatory effects of an extract of procyanidins from grape seed (GSPE) in models of acute and chronic inflammation. In recent years, GSPE has been shown to exert anti-inflammatory effects against the chronic inflammation present in various models of diet-induced obese rats.

Several animal models have been used to study obesity, both with genetically or diet-induced obesity. In the present work, a model of genetic obesity was chosen, in order to confirm the observed anti-inflammatory effects of procyanidins *in vivo*. In this way, a moderate dose of the extract was administrated in *Zucker* Fatty rats, which is comparable to a moderate consumption of procyanidins in the human diet.

On the other hand, the preventive effects of GSPE were studied *in vivo* in a model of acute inflammation. To induce the inflammatory response, lipopolysaccharides were administrated intraperitoneally to the rats previously treated with GSPE for 15 days at nutritional and pharmacological doses.

Both studies *in vivo* were focused on analyzing the levels of several proteins and mediators, closely related to chronic and acute inflammation, which can be modified by the GSPE treatment, at systemic level and in various target tissues of inflammation.

4.3

Grape seed procyanidin extract reduces the endotoxic effects induced by lipopolysaccharides in rats.

V. Pallarès et al.

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UNIVERSITAT ROVIRA I VIRGILI
ROLE OF BIOACTIVE FOOD COMPOUNDS IN ACUTE AND CHRONIC INFLAMMATION
Victor Pallarés López
Dipòsit Legal: T.1472-2012

Abstract

Acute inflammation is a response to injury, infection, tissue damage or shock. Bacterial lipopolysaccharide (LPS) is an endotoxin implicated in triggering sepsis and septic shock, and LPS promotes the inflammatory response resulting in the secretion of proinflammatory and anti-inflammatory cytokines such as the interleukins (IL-6, IL-1β and IL-10) and tumor necrosis factor-alpha by the immune cells. Furthermore, nitric oxide (NO) and reactive oxygen species levels increase rapidly, which is partially due to the activation of inducible nitric oxide synthase (iNOS) in several tissues in response to inflammatory stimuli.

Previous studies have shown that procyanidins, which are polyphenols present in food such as apples, grapes, cocoa and berries, have several beneficial properties against inflammation and oxidative stress using *in vitro* and *in vivo* several models. In the current study, the anti-inflammatory and antioxidant effects of two physiological doses and two pharmaceutical doses of grape seed procyanidin extract (GSPE) were analyzed using a rat model of septic shock by the intraperitoneal injection of LPS derived from *E. coli*.

The high nutritional (75 mg/kg*day) and the high pharmacological doses (200 mg/kg*day) of GSPE showed anti-inflammatory effects by decreasing the proinflammatory marker NOx in the plasma, red blood cells, spleen and liver. Moreover, the high pharmacological dose also down-regulated the genes *Il-6* and *iNos*; and the high nutritional dose increased the glutathione ratio (SGGS/Total glutathione) further illustrating the antioxidant capability of GSPE. In conclusion, several doses of GSPE can alleviate acute inflammation triggered by LPS in rats at the systemic and local levels when administered for as few as 15 days prior the injection of endotoxin.

Introduction

Acute inflammation is a response to mechanical injury, infection, chemical injury, burns, radiation and tissue injury or shock(He et al. 2007). Bacterial lipopolysaccharide (LPS) is a glycolipid component of the cell wall of Gram-negative bacteria and is an endotoxin implicated in triggering sepsis and septic shock (Freudenberg & Galanos 1990). LPS causes an acute inflammatory response, which evokes a state characterized by fever, hypotension and multi-organ system failure (Tracey et al. 1986). The inflammation associated with endotoxic shock promotes an early response that involves the secretion of cytokines, chemokines, and other mediators, which triggers a sequence of events such as blood vessel dilation, increased blood flow, leukocyte infiltration, release of proteases and the formation of oxygen free radicals (He et al. 2007).

In response to endotoxin, proinflammatory cytokines such as the interleukins (IL-6 and IL-1β) and tumor necrosis factor-alpha (TNF-α) and anti-inflammatory cytokines such as IL-10 are produced by inflammatory cells (Olinga et al. 2001). Furthermore, nitric oxide (NO) levels increase rapidly within minutes to hours (Rosengarten et al. 2009) in response to inflammatory stimuli. In fact, inducible nitric oxide synthase (iNOS), which is induced upon exposure to endotoxin (LPS) as well as by cytokines such as TNF-α and IL-1β, produces NO in large amounts, as iNOS is expressed by a variety of cells including macrophages, Kupffer cells, hepatocytes and glial cells (Olinga et al. 2001; Dyson et al. 2011; Aono et al. 1997). NO is a crucial factor during acute inflammation and sepsis and has been used as a marker of inflammation in many studies (Olinga et al. 2001; Dyson et al. 2011). Moreover, TNF-α IL-1β and NO levels are altered at the systemic level in blood cells such as the erythrocytes (Dyson et al. 2011) as well as in peripheral tissues such as the liver, spleen, brain, and adipose tissue. Therefore, many studies have focused on assessing how endotoxic shock increases the production of these proteins and molecules in these tissues as well as their relevance to the pathogenesis of endotoxemia (Kapur et al. 1999; Olinga et al. 2001; Dyson et al. 2011).

Previous studies have shown that molecules derived from food have anti-inflammatory effects in endotoxin-treated rats (Sebai et al. 2010; Sebai et al. 2009; Sebai et al. 2008; Singal et al. 2006). Several of these studies have shown that procyanidins, a class of polyphenols that belongs to the flavanol group and is present in food such as apples, grapes, cocoa and berries and in beverages such as wine or tea, have several beneficial properties against inflammation and oxidative stress in several models *in vitro* (Pallarès et al. 2012a; Pallarès et al. 2012b; Ximena Terra et al. 2007; X Terra et al. 2011) and *in vivo* (Mansouri et al. 2011; Ximena Terra et al. 2009; Ximena Terra et al. 2011).

In the current study, the anti-inflammatory and antioxidant properties of grape seed procyanidin extract (GSPE) were analyzed using a rat model of septic shock by the intraperitoneal injection of LPS derived from *E. coli*. Treatment with a moderate-high and a high nutritional doses (50 and 75 mg/kg*day of GSPE, respectively) and two high pharmaceutical doses (100 and 200 mg/kg*day of GSPE) were tested to assess the preventive action of GSPE against an acute inflammatory response in vivo.

Materials and methods

Reagents

GSPE was provided by Les Dérives Résiniques et Terpéniques (Dax, France). According to the manufacturer, the GSPE contained monomeric (16.6%), dimeric (18.8%), trimeric (16.0%), tetrameric (9.3%) and oligomeric (5–13 units) (35.7%) procyanidins and phenolic acids (4.2%). The vehicle of GSPE was sweetened condensed milk diluted in water in a 1:6 ratio. The milk composition per 100 g was as follows: 8.9 g protein, 0.4 g fat, 60.5 g carbohydrates and 281 kcal.

Lipopolysaccharide from *Escherichia coli* 0111:B4 (ref. L2630, lot no. 030M4114) was purchased from Sigma-Aldrich (St. Louis, MO, USA). LPS was diluted in salt serum and was administered to the animals intraperitoneally (i.p).

Animals and experimental protocol

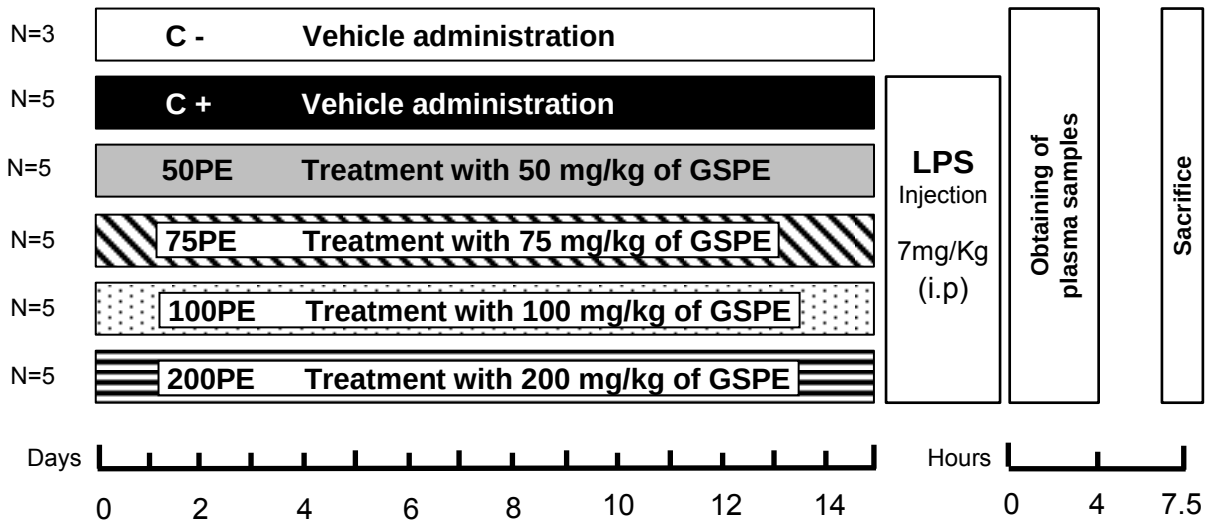
Wistar female rats (Charles River Laboratories, L'Arbresle Cedex, France) weighing between 200 and 225 grams at 11 weeks of age were housed in cages in pairs or trios at a constant temperature (22ºC). Rats were subjected to a standard 12-h light/dark cycle and were provided with water and feed *ad libitum*. Rats were allowed to adapt to their environment 1 week before the beginning of the experiments.

Rats were randomly distributed in groups according to the treatment they received: negative control (C-) (n=3) and positive control groups (C+) (n=5) were treated with vehicle. Four groups were treated with different doses of GSPE: 50 mg/kg*day of GSPE (50PE, n=5), 75 mg/kg*day of GSPE (75PE, n=5), 100 mg/kg*day of GSPE (100PE, n=5) and 200 mg/kg*day of GSPE (200PE, n=5) (Fig. 1). The treatment was previously to LPS administration.

All groups received treatment at 7 pm for 15 days by controlled oral intake using a syringe until a day before the animals were sacrificed. During the experimental period, the rats were provided with free access to water and feed immediately after the treatment until the morning to improve the absorption of GSPE with the diet. In the morning, the feed was removed from

the animal cages and was replenished after GSPE treatment. The food intake and body weight were measured weekly. LPS was injected (i.p.) after 15 days of treatment at 7 mg/kg into the C+, 50PE, 75PE, 100PE and 200PE groups on the day the animals were sacrificed (Fig. 1). After LPS injection, the rats were fasted until euthanasia, and during the fasting period, blood samples were taken from the tail at 0 and 4 hours. Seven-and-a-half hours later after the LPS injection, rats were anesthetized with 75 mg/kg of sodium pentobarbital (i.p.) and were sacrificed by exsanguination to obtain blood from the aorta and collect to pre-rinsed tubes with EDTA. The liver and spleen were surgically excised. Plasma was obtained by centrifuging the blood samples at 2000 x *g* for 10 min. All of the samples were stored at -80ºC.

Figure 1. Different groups and treatments applied during the experiment.



Timeline for the different groups and treatments applied in the experiment. GSPE; grape seed procyanidin extract. LPS; lipopolysaccharide.

Nitrate and nitrite (NOx) measurements in the plasma, RBC, liver and spleen

For the quantification of NOx levels, a colorimetric assay kit (ref. 780001) purchased from Cayman Chemical (Madrid, Spain) was used. The preparation of the samples was performed as follows: RBC samples were obtained from the blood after separating the plasma. RBC lysis buffer (0.154 M NH_4Cl, 9.988 mM $KHCO_3$, 0.126 mM EDTA; autoclaved) was added to the

blood, and then the samples were vortexed and centrifuged at 2000 x *g* for 5 minutes. The supernatants were collected and were stored at -80ºC. Liver and spleen samples were homogenized in autoclaved PBS, centrifuged at 10,000 x *g* for 20 minutes and were stored on ice until the deproteinizing treatment. Plasma, RBC, liver and spleen samples were deproteinized using pre-rinsed Amicon Ultra-0.5 centrifugal filter devices (ref. UFC501096; 10K) purchased from Millipore (Billerica, MA, USA). Then, the NOx levels were quantified using a colorimetric kit according to the manufacturer's instructions. Prior to deproteinization, protein concentrations in all samples were determined by using the Bradford assay (Bradford 1976) to normalize the results.

TNF-α and IL-10 measurements in plasma

For the TNF-α and the IL-10 assays, two ELISA kits were used (ref. KRC3011 and KRC0101, respectively). These kits were purchased from Invitrogen (Camarillo, CA, USA). TNF-α and IL-10 levels were quantified according to the manufacturer's instructions.

Semi-quantitative real-time PCR of proinflammatory and anti-inflammatory genes from the liver

RNA extraction, cDNA reverse transcription and quantitative RT-PCR amplification using the SYBR Green dye was performed as previously described (Ximena Terra et al. 2009) using 2 µg of total RNA. The relative expression levels of the proinflammatory genes *Crp, Il-6, iNos and Tnf-α* and the anti-inflammatory gene *Il-10* were assessed in the liver and were normalized to cyclophilin (*Ppia*) mRNA levels. The forward and reverse primers for the rat genes analyzed are shown in Table 1.

Table 1. Rat-specific primer sequences.

Gene	Primer sequence
Crp	Fw: 5' GGCTTTTGGTCATGAAGACATG 3'
	Rv: 5' TCTTGGTAGCGTAAGAGAAGA 3'
Tnf-α	Fw: 5' CCTCACACTCAGATCATCTTCTC 3'
	Rv: 5' TTGGTGGTTTGCTACGACGTG 3'
Il-6	Fw: 5' CTCTCCGCAAGAGACTTCC 3'
	Rv: 5' GCCATTGCACAACTCTTTTCTC 3'
Il-10	Fw: 5' GCAGGACTTTAAGGGTTACTTGG 3'
	Rv: 5' GGAGAAATCGATGACAGCGT 3'
iNos	Fw: 5' CACCCGAGATGGTCAGGG 3'
	Rv: 5' CCACTGACACTCCGCACAA 3'
Ppia	Fw: 5' CTTCGAGCTGTTTGCAGACAA 3'
	Rv: 5' AAGTCACCACCCTGGCACATG 3'

Rat-specific primer sequence. C-reactive protein (*Crp*), tumor necrosis factor-alpha (*Tnf-α*), interleukin-6 (*Il-6*), interleukin-10 (*Il-10*), Inducible nitric oxide synthase (*iNos*) and cyclophilin A (*Ppia*). FW; forward primer sequence. RV; reverse primer sequence.

Oxidative stress analysis by measuring the hepatic glutathione ratio (GSSG/Total glutathione)

For the reduced/oxidized glutathione analyses, we used the enzymatic method reported by Griffith was used (Griffith 1980). Briefly, this method is based on the reaction of the glutathione present in the sample with DTNB (5,5′-Dithiobis(2-nitrobenzoic acid) D8130, Sigma-Aldrich; St. Louis, MO, USA) to generate 2-nitro-5-thiobenzoic acid, a yellow compound that absorbs at 412 nm, and oxidized glutathione (GSSG). To determine total glutathione in the sample, reduced form is regenerated from oxidized form due to the presence of glutathione reductase (GR) in excess. On the other hand, the determination of GSSG exclusively could be measured

due to the GSH scavenge by 2-vinylpyridine, a thiol-scavenging reagent to form a pyridinium salt. For this assay, livers were homogenized and deproteinized with metaphosphoric acid (6%) and were centrifuged at 5000 x *g* for 15 min at 4ºC. The supernatants were used for the GSSG and the total glutathione analysis.

Statistical analysis

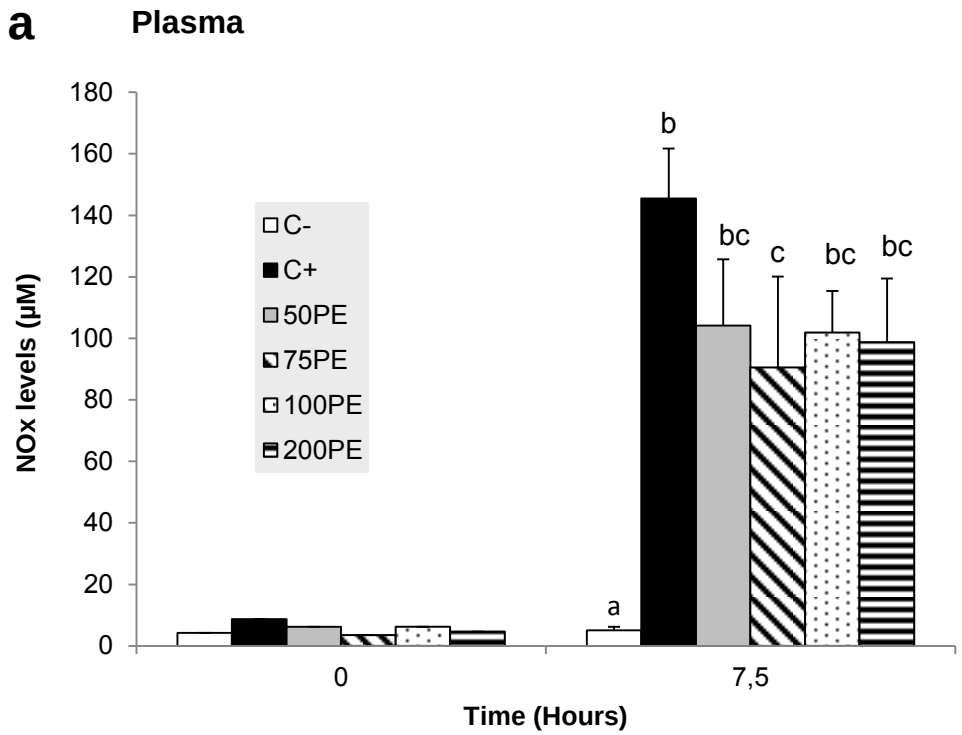
The results are presented as the mean with the associated standard error (SE). The data were analyzed using a one-way ANOVA test to determine the significant differences using IBM SPSS statistical software (version 19.0 for Windows; SPSS, Inc.). p-values<0.05 were considered statistically significant.

Results

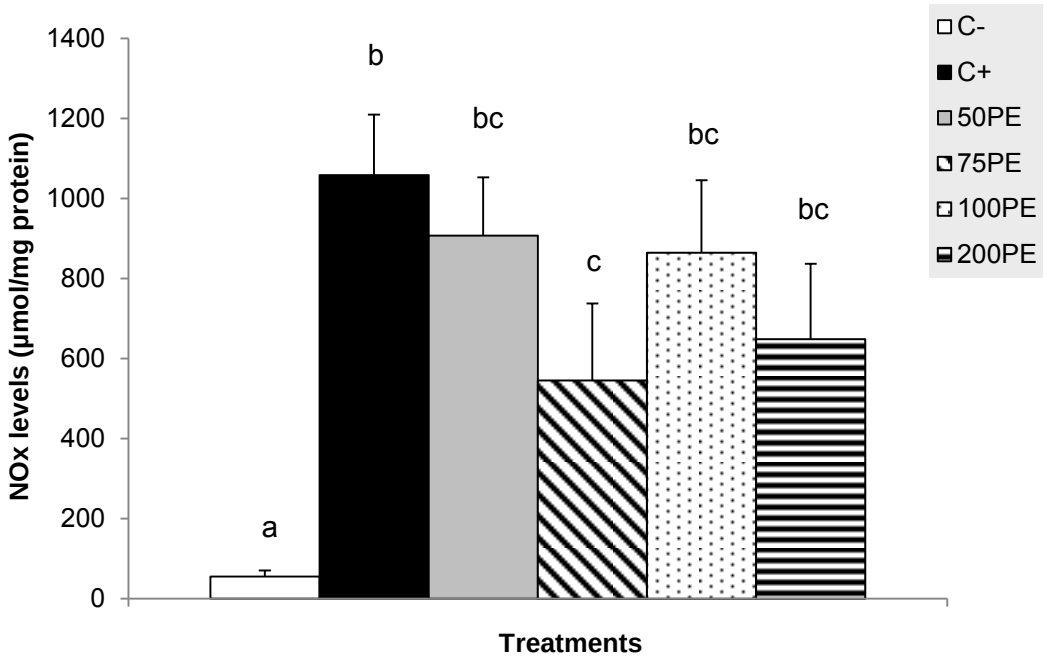
1. NOx levels are reduced in the blood and in tissues in GSPE groups

NOx levels were analyzed in the liver, spleen, plasma and RBCs. The NOx levels of the C+ group were increased relative to the C- group in all tissues analyzed (Fig. 2a, b, c, d). In the plasma (Fig. 2a) and RBCs (Fig. 2b), rats treated with 75 mg/kg of GSPE (75PE) showed significantly decreased levels of NOx when compared with the C+ group. Moreover, the hepatic NOx levels of the 75PE and 200PE groups decreased significantly when compared with the NOx levels of the C+ group (Fig. 2c), whereas the splenic levels of NOx in the 75PE, 100PE and 200PE groups were also decreased relative to the C+ group (Fig. 2d). Furthermore, all groups treated with GSPE showed decreased levels of NOx when compared to the C+ group; however, only the 75PE group reached statistical significance in all the locations assessed.

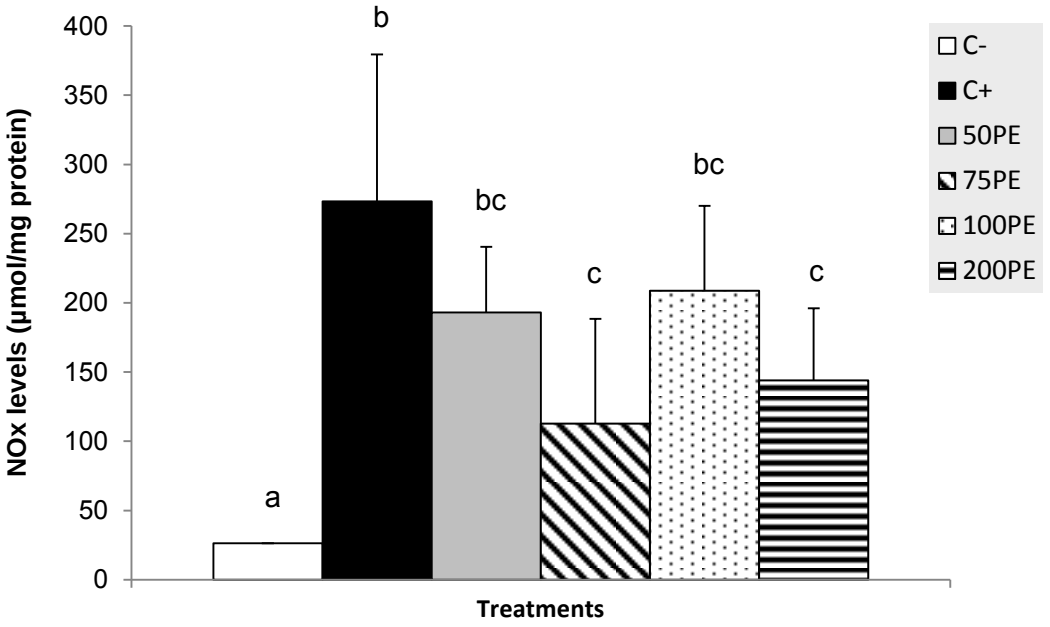
Figure 2. NOx levels in the plasma, red blood cells (RBCs), liver and spleen.

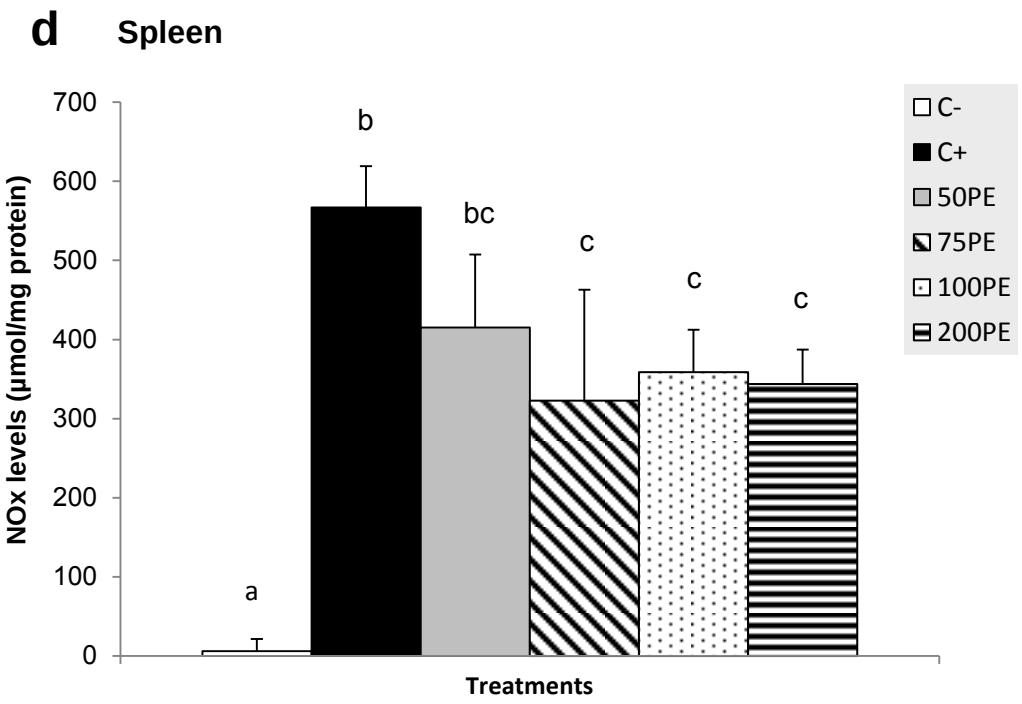


b **RBC (Red blood cells)**



c **Liver**



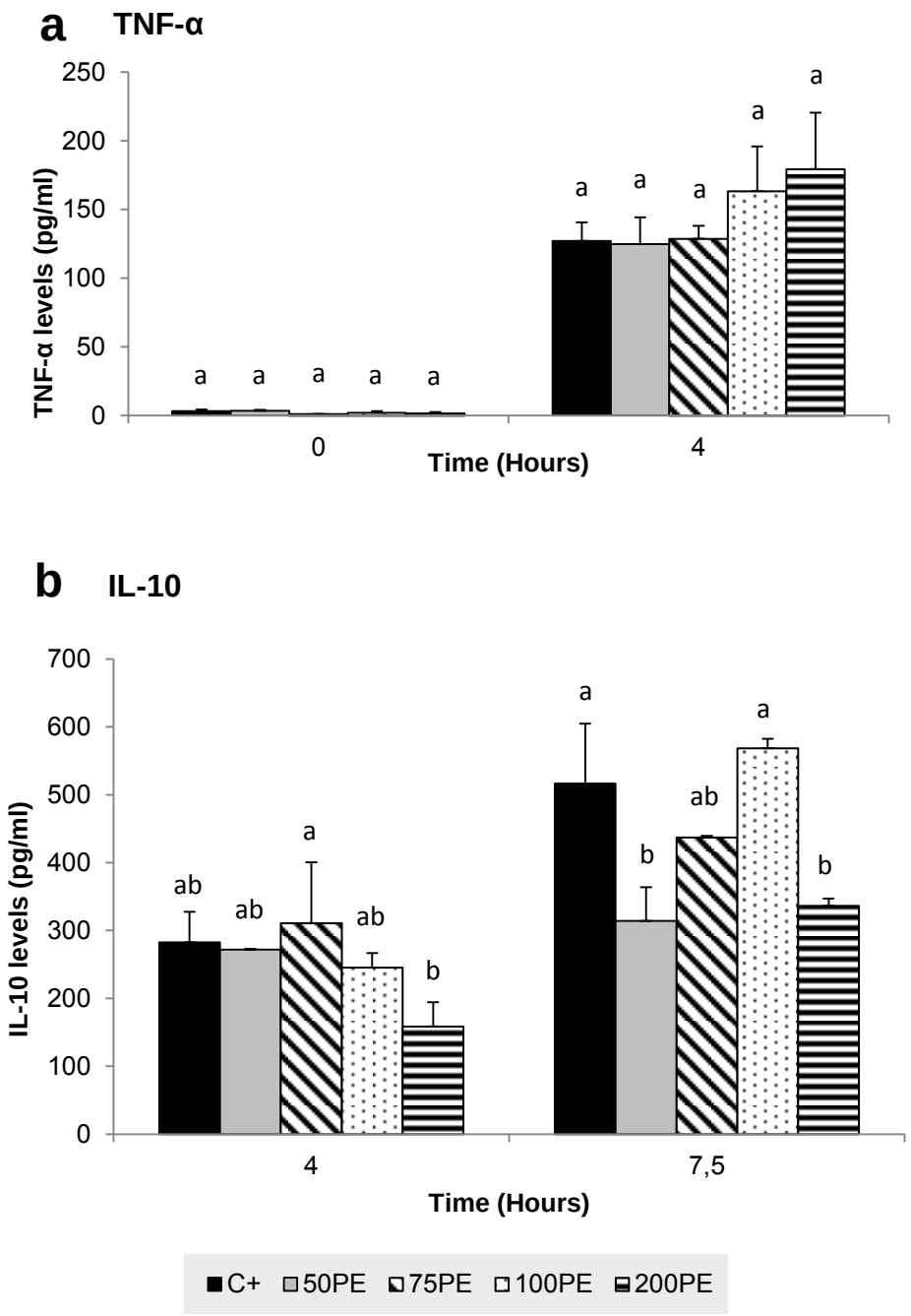


NOx (nitrate + nitrite) levels (µmol/mg protein) in a) plasma b) red blood cells (RBCs) c) liver and d) spleen. The results are presented as the mean ± standard error (SE) for each group (n=5) (C-; n=3): negative control group (C-), positive control group (C+) and groups treated with 50, 75, 100 and 200 mg/kg of GSPE (50PE, 75PE, 100PE and 200PE, respectively). Significant differences are indicated using different letters. Statistical significance was determined using a one-way ANOVA test, and p-values<0.05 were considered statistically significant. GSPE; grape seed procyanidin extract.

2. Plasma levels of IL-10 decrease upon GSPE treatment

The plasma levels of the proinflammatory molecule TNF-α and the anti-inflammatory molecule IL-10 were assessed after the injection of LPS. On one hand, TNF-α levels were increased in all of the groups 4 hours after LPS injection relative to the basal levels of TNF-α (Fig. 3a). On the other hand, IL-10 levels were elevated at 4 and 7.5 hours after LPS injection (Fig. 3b). Importantly, the IL-10 levels of the 50PE and 200PE groups were significantly decreased when compared with the C+ group 7.5 hours after LPS injection (Fig. 3b).

Figure 3. Plasma TNF-α and IL-10 levels.

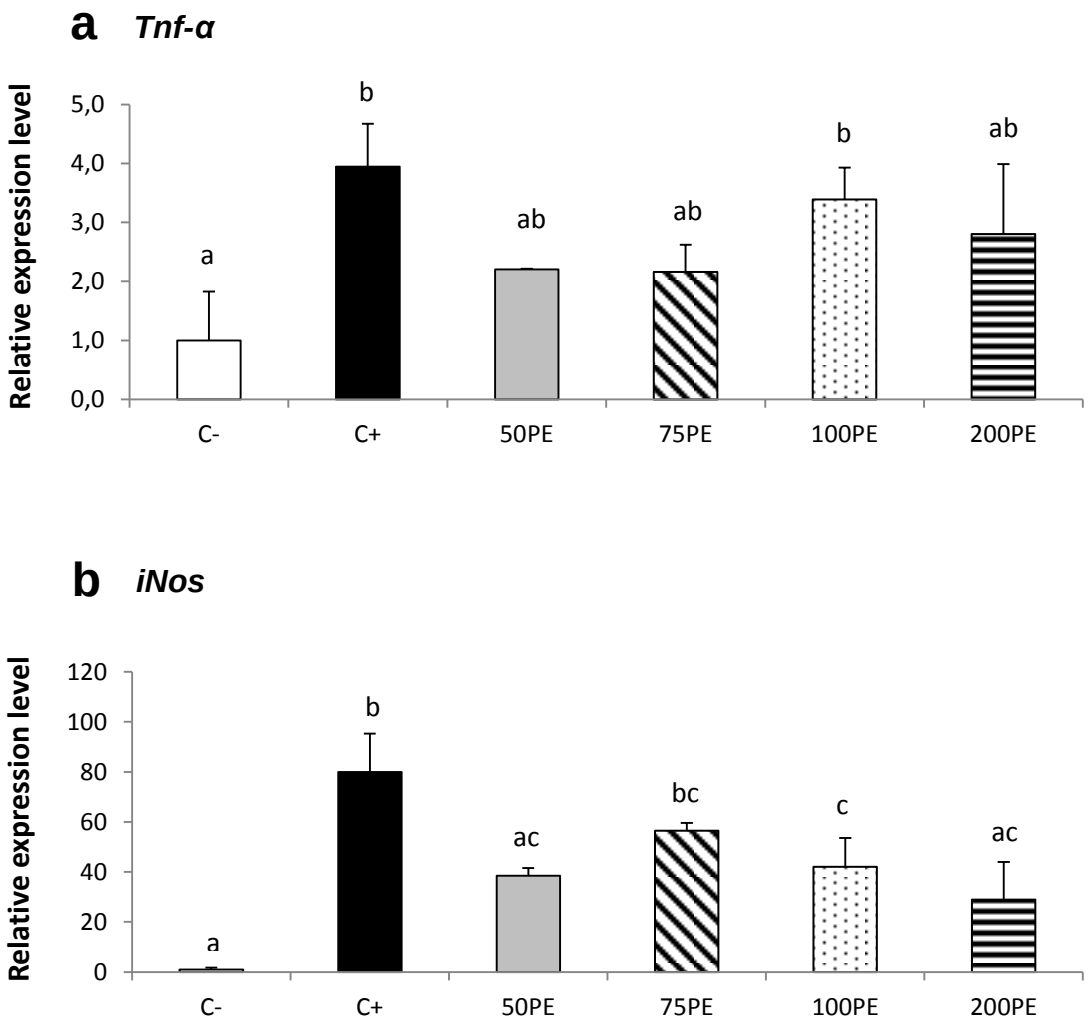


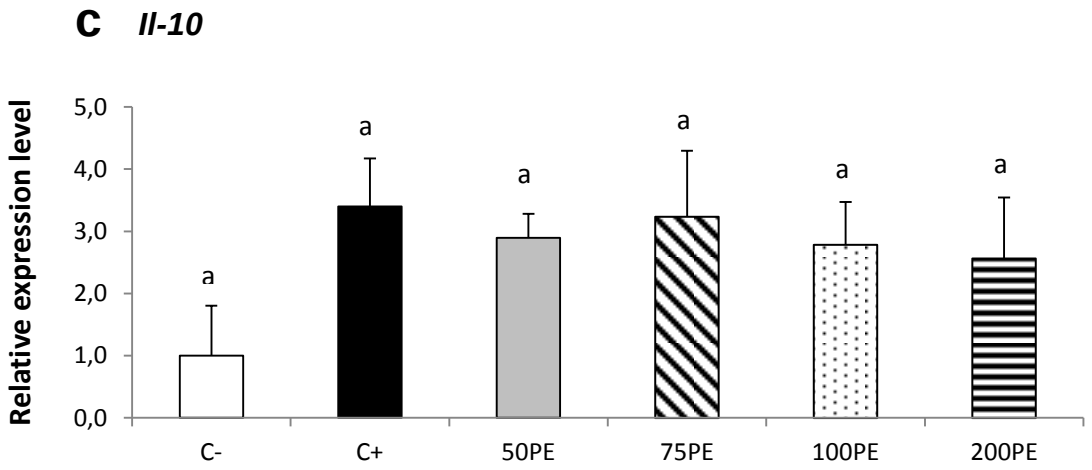
a) Tumor necrosis factor-alpha (TNF-α; pg/mL) and b) interleukin-10 (IL-10; pg/mL) levels in the plasma. The results are presented as the mean ± standard error (SE) for each group (n=5): positive control group (C+) and groups treated with 50, 75, 100 and 200 mg/kg of GSPE (50PE, 75PE, 100PE and 200PE, respectively). Significant differences are represented with different letters. Statistical significance was determined using the one-way ANOVA test, and p-values<0.05 were considered statistically significant. GSPE; grape seed procyanidin extract.

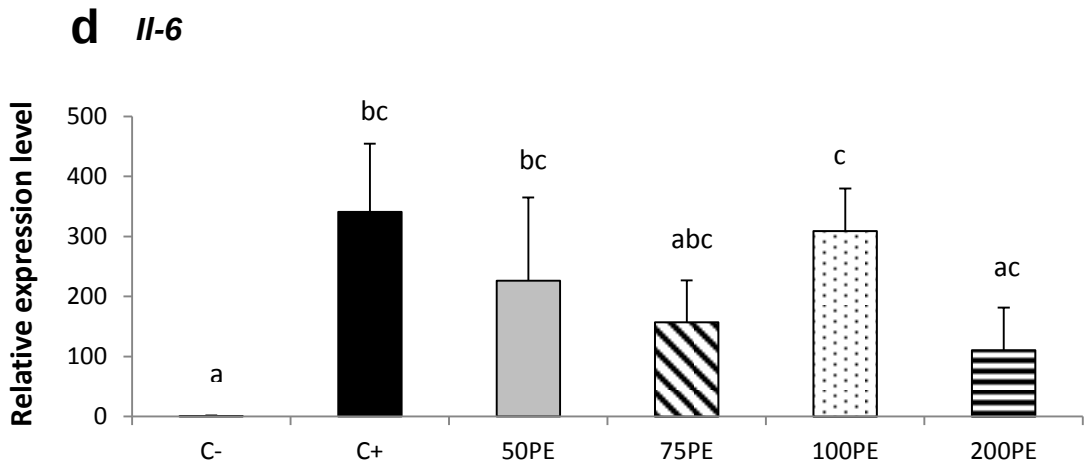
3. Down-regulation of proinflammatory genes in the liver by GSPE treatments

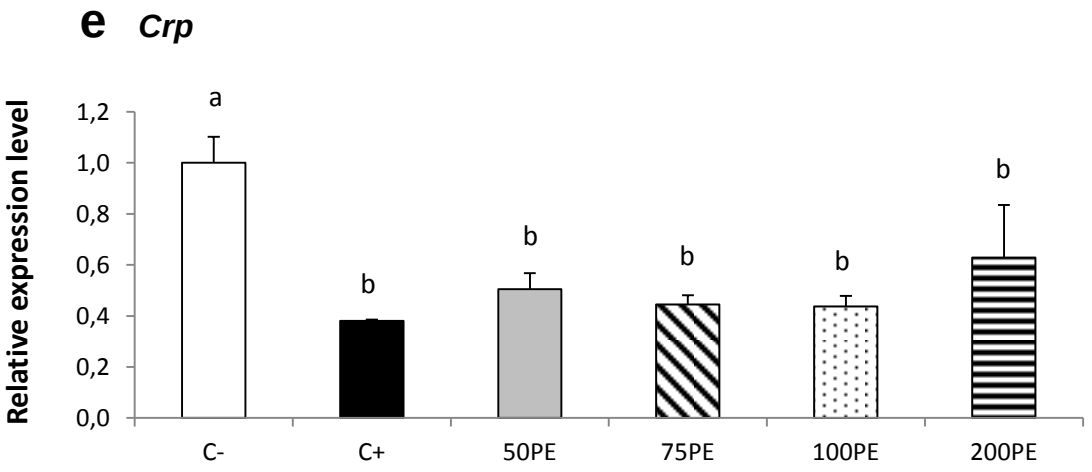
The relative hepatic mRNA expression levels of the proinflammatory genes *Tnf-α, iNos, Il-6* and *Crp* and the anti-inflammatory gene *Il-10* were analyzed. Expression of *Tnf-α, iNos, Il-6* and *Il-10* was up-regulated upon injection with LPS (C+) in contrast to the non-LPS group (C-) (Fig. 4a, b, c, d), whereas *Crp* gene expression was down-regulated (Fig. 4e). Expression of *iNos* was down-regulated by GSPE pre-treatment in the 50PE, 100PE and 200PE groups (Fig. 4b), whereas *Il-6* gene expression was down-regulated in the 200PE group relative to the C+ group (Fig. 4d). In contrast, *Il-10* and *Crp* gene expression was not transcriptionally regulated by any of the GSPE dosages relative to the C+ group (Fig. 4c and e).

Figure 4. Relative expression levels of proinflammatory and anti-inflammatory genes in the liver.









Relative hepatic mRNA expression levels of the genes a) *Tnf-α, b) iNos, c) Il-10, d) Il-6 and e) Crp*. To calculate the relative mRNA expression levels, the δδCT method was used. The positive control group (C+) and groups treated with 50, 75, 100 and 200 mg/kg of GSPE (50PE, 75PE, 100PE and 200PE, respectively) were normalized to the negative control group (C-). The results are presented as the mean ± standard error (SE). Significant differences

are indicated by different letters. Statistical significance was determined using a one-way ANOVA test, and p-values<0.05 were considered statistically significant. GSPE; grape seed procyanidin extract.

4. GSPE reverses hypotension upon injection of LPS

Treatment with GSPE for 15 days had no effect on the body weight (Tab. 2) or the amount of food ingested (data not shown). However, pretreatment with GSPE reversed the drop in systolic pressure (SYS), diastolic pressure (DIAS) and the SYS/DIAS ratio at the 7.5-hour time point after LPS injection (Tab. 2). In particular, the 200PE group reversed the drop in the DIAS and the SYS/DIAS ratio when compared with the C+ group, whereas the 75PE, 100PE and 200PE groups reversed the drop in the SYS, DIAS, and then SYS/DIAS ratio (Tab. 2).

Table 2. Body weight and blood pressure measures.

	C+		50PE		75PE		100PE		200PE	
Parameter	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Body weight (0 days)	203.00^{a}	2.28	202.60^{a}	3.90	205.40^{a}	4.03	206.00^{a}	3.53	206.00^{a}	3.70
Body weight (15 days)	234.00^{a}	2.83	224.80^{a}	2.68	243.10^{a}	2.89	234.60^{a}	3.69	241.12^{a}	4.57
SYS Drop	-49.65^{a}	6.36	-44.23^{ab}	6.67	-19.00^{bc}	10.63	-10.21^{c}	5.08	-7.23^{c}	6.40
DIAS Drop	-60.82^{a}	6.02	-31.15^{bc}	17.85	-43.56^{abc}	9.65	-49.51^{ab}	3.62	-20.66^{c}	4.97
SYS/DIAS Ratio Drop	-53.85^{a}	6.19	-34.30^{ab}	10.70	-38.08^{ab}	13.00	-43.20^{ab}	6.87	-21.57^{b}	4.58

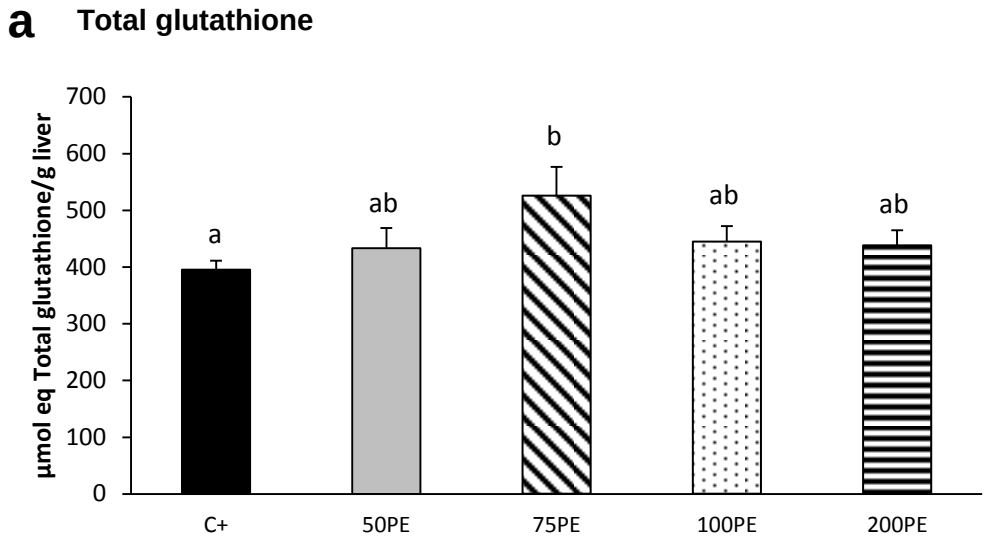
Body weight and blood pressure by groups: positive control group (C+) and groups treated with 50, 75, 100 and 200 mg/kg of GSPE (50PE, 75PE, 100PE and 200PE, respectively). Body weight was measured at the initiation of GSPE treatment and after 15 days of GSPE or vehicle treatment. To calculate the SYS and DIAS drop and

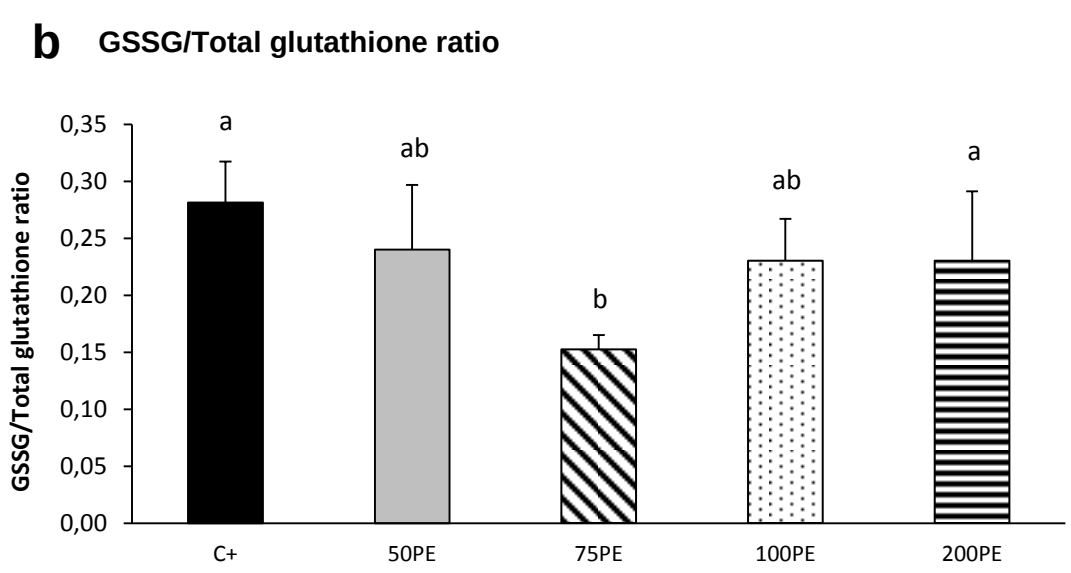
SYS/DIAS ratio drop, the mean value of the negative control group (C-) was subtracted from each group. The results are presented as the mean ± standard error (SE). Significant differences are indicated by different letters. Statistical significance was determined using a one-way ANOVA test, and p-values<0.05 were considered statistically significant. GSPE; grape seed procyanidin extract. SYS; systolic blood pressure. DIAS; diastolic blood pressure.

5. A high nutritional dose of GSPE decreases the GSSG/total glutathione ratio in the liver

When cells are exposed to increased levels of oxidative stress, the oxidized form of glutathione (GSSG) accumulates, and the GSSG/total glutathione ratio increases. The quantification of GSSG, total glutathione and the GSSG/Total glutathione ratio were measured as useful indicators of oxidative stress in the liver. The 75PE group showed significantly increased levels of total glutathione and decreased the GSSG/Total glutathione ratio at 7.5 h after LPS injection (Fig. 5a and b).

Figure 5. Total glutathione levels and the GSSG/total glutathione ratio in the liver.





a) Total glutathione (µmol/g liver) and b) the oxidized form of glutathione (GSSG) versus total glutathione (GSSG/Total glutathione ratio) levels in the liver. The results are presented as the mean ± standard error (SE) for each group (n=5): positive control group (C+) and groups treated with 50, 75, 100 and 200 mg/kg of GSPE (50PE, 75PE, 100PE and 200PE, respectively). Significant differences are indicated by different letters. Statistical significance was determined using a one-way ANOVA test, and p-values<0.05 were considered statistically significant. GSPE; grape seed procyanidin extract.

Discussion

In recent years, the study of the anti-inflammatory effects of bioactive molecules present in food has become more popular. How bioactive molecules influence the proinflammatory and anti-inflammatory effects triggered during acute inflammation remains to be fully understood.

To assess the preventative effects of GSPE on acute inflammation, our experiments focused in the NOx species. NOx species (nitrate and nitrite) have been widely tested in septic shock studies (Dyson et al. 2011; Rosengarten et al. 2009; Aono et al. 1997). NOx has been shown to be a relevant marker of inflammation in tissues such as the liver and spleen (Olinga et al. 2001; Bircan et al. 2011). In the liver, the inducible expression of *iNos,* the enzyme implicated in the synthesis of NO, appears to play an important role in the pathogenesis of endotoxemia (Olinga et al. 2001). Therefore, NOx as well as the classical proinflammatory cytokine TNF-α and anti-inflammatory cytokine IL-10 (Dyson et al. 2011) were used as markers to study the effects of GSPE in rats treated with LPS.

Rats treated with GSPE for 15 days did showed no differences in food intake or in weight (Tab. 2). Treatment with 7 mg/kg of LPS derived from *E. coli* (i.p.) caused acute inflammation as indicated by an increase in proinflammatory proteins such as TNF-α in the plasma at 4 h after LPS treatment (Fig. 3a). LPS treatment also caused an increase in NOx levels in the blood (Fig. 2a and b), in the liver (Fig. 2c) and in the spleen (Fig. 2d) at the 7.5-h time point after LPS treatment, and these results are similar to previous studies (Olinga et al. 2001; Prima et al. 2011; Dyson et al. 2011; Li et al. 2009; Hagiwara et al. 2009). In addition to the increase in expression of proinflammatory genes such as *Il-6*, *iNos* and *Tnf-α* and the anti-inflammatory gene *Il-10* at the transcript (Fig. 4) and protein level (Fig. 3b), these results indicate that the dose of LPS administered was optimal to induce the proinflammatory state without causing death from septic shock within the time frame of the experiment similarly to previous studies in rats (Guo et al. 2009; Huet et al. 2007; Tavares & Miñano 2010).

Additionally, the effects of GSPE on the levels of NOx in the RBCs, plasma, liver and spleen were analyzed. The liver was included because is one of the major endocrine organs involved

in the regulation of metabolism by synthesizing cytokines and acute phase proteins during inflammation, whereas the spleen was included because it is responsible for both specific as well as nonspecific host defense. Furthermore, the spleen harbors a high content of phagocytes and maintains a direct connection with the bloodstream, which illustrates the importance of the spleen in the clearance of circulating microorganisms, particles and some large molecules such as LPS (Romanovsky & Petersen 2003; Bircan et al. 2011). The level of NOx species correlates with the activity of agents that cause acute inflammation such as LPS. In a global overview, several doses of GSPE reduced the levels of NOx in the plasma and RBCs as well as in the liver and spleen. Interestingly, the nutritional high dose of 75 mg/kg*day of GSPE (75PE) decreased the levels of NOx in all locations assessed (Fig. 2; a, b, c, d). These results suggest that the 75 mg/kg*day dose has a stronger anti-inflammatory effect than the others effectively reducing the level of NOx induced by LPS in several peripheral organs as well as at the systemic level. Significantly, high doses of GSPE also appeared to reduce the level of NOx in the liver (200PE; Fig. 2c) and the spleen (100PE and 200PE; Fig. 2d), although decreases were not observed in the circulating levels of NOx (Fig. 2a and b). These results could suggest that highest doses may not be as effective at reducing the level of NOx in the various tissues affected by LPS and may ultimately contribute to the NOx circulating levels in the blood.

On the other hand, other parameters assessed in the plasma such as the anti-inflammatory IL-10 (Fig. 3b) decreased in rats treated with either the highest dose (200PE) and the lowest dose (50PE), suggesting that GSPE could inactivate the synthesis of IL-10 to counteract the action of this anti-inflammatory cytokine. In contrast, these results could also suggest that the dose of GSPE is sufficient to reduce inflammation to the extent that IL-10 synthesis is no longer necessary resulting in IL-10 down-regulation.

Although TNF-α plasma levels were slightly increased in the highest doses (100PE, 200PE; Fig. 3a), any of the GSPE dosages could not change significantly these levels in contrast to the positive control. It suggests that any dose could counteract the activation of TNF-α caused by exposure to a proinflammatory stimulus.

The expression of proinflammatory and anti-inflammatory genes in the liver was analyzed to assess the effects of GSPE on this organ. The results showed that almost all GSPE dosages down-regulated *iNos* expression significantly when compared with the C+ group (Fig. 4b). The group receiving 200 mg/kg*day of GSPE (200PE) showed the greatest down-regulation of *iNos* and *Il-6* expression (Fig. 4b and d). These data indicate that GSPE exerts its effects at the level of expression; however, high doses may be necessary to achieve the full regulatory potential of GSPE. Surprisingly, Crp, a gene that encodes a proinflammatory acute-phase protein, was down-regulated in all groups treated with LPS when compared with the C- group (Fig. 4e). On the other hand, the expression of *iNos* (Fig. 4b) correlates with NOx levels (Fig. 2c) in the liver. This result suggests that GSPE could have a direct negative transcriptional effect on the *iNos* gene resulting in lower levels of NO, which represents one of the key elements involved in liver pathology during endotoxic shock (Olinga et al. 2001).

Reduced glutathione (GSH) is the main non-enzymatic antioxidant defense within the cell, and GSH plays an important role in the protection against oxidative stress (Martín et al. 2010). Therefore, hepatic GSH levels were measured to determine whether GSPE treatments affect the level of oxidative stress related to acute inflammation induced by LPS. Treatment with the nutritional high dose of 75 mg/kg*day of GSPE (75PE) increased the total glutathione level and decreased the GSSG/Total glutathione ratio (Fig. 5a and b), suggesting that GSPE also acts as an antioxidant agent reducing the oxidative stress.

A recent study showed that the anti-inflammatory effects of proanthocyanidins could be due to the capacity of these molecules to bind to LPS and repress the binding to the toll-like receptor 4 (TLR-4) (Delehanty et al. 2007), which mediate the activation of the pro-inflammatory NF-κB pathway. Thus, the anti-inflammatory action of GSPE in this model could be explained to their capacity to bind to LPS, promoting a non-activation of pro-inflammatory pathways and decreasing the expected inflammatory response.

In conclusion, GSPE has anti-inflammatory and antioxidant effects at the systemic and gene expression levels when administered 15 days prior to LPS-induced acute inflammation. In

particular, the nutritional high dose (75 mg/kg*day) and the high dose (200 mg/kg*day) possess anti-inflammatory effects as shown by a decrease in the NOx proinflammatory marker in the plasma, RBC, spleen and liver. Furthermore, the highest dose (200 mg/kg*day) down-regulated the genes *Il-6* and *iNos*, and the nutritional high dose (75 mg/kg*day) decreased the GSSG/Total glutathione ratio, illustrating the antioxidant capacity of GSPE.

References

Aono, K. et al., 1997. In vitro and in vivo expression of inducible nitric oxide synthase during experimental endotoxemia: involvement of other cytokines. *Journal of cellular biochemistry*, 65(3), p.349–358.

Bircan, F.S. et al., 2011. Effects of taurine on nitric oxide and 3-nitrotyrosine levels in spleen during endotoxemia. *Neurochemical research*, 36(11), p.1978–1983.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical biochemistry*, 72, p.248–254.

Delehanty, J.B. et al., 2007. Binding and neutralization of lipopolysaccharides by plant proanthocyanidins. *Journal of natural products*, 70(11), p.1718–1724.

Dyson, A. et al., 2011. An integrated approach to assessing nitroso-redox balance in systemic inflammation. *Free radical biology & medicine*, 51(6), p.1137–1145.

Freudenberg, M.A. & Galanos, C., 1990. Bacterial lipopolysaccharides: structure, metabolism and mechanisms of action. *International reviews of immunology*, 6(4), p.207–221.

Griffith, O.W., 1980. Determination of glutathione and glutathione disulfide using glutathione reductase and 2-vinylpyridine. *Analytical biochemistry*, 106(1), p.207–212.

Guo, Z. et al., 2009. Soluble TNFR II/IgG1 Fc fusion protein treatment in the LPS-mediated septic shock of rats. *Biomedicine & pharmacotherapy = Biomédecine & pharmacothérapie*, 63(7), p.537–542.

Hagiwara, S. et al., 2009. Landiolol, an ultrashort-acting beta1-adrenoceptor antagonist, has protective effects in an LPS-induced systemic inflammation model. *Shock (Augusta, Ga.)*, 31(5), p.515–520.

He, M. et al., 2007. Chemokines in acute inflammation: regulation, function and therapeutic strategies. *International Journal of Integrative Biology*, 1(1), p.18.

Huet, O. et al., 2007. Induced mild hypothermia reduces mortality during acute inflammation in rats. *Acta anaesthesiologica Scandinavica*, 51(9), p.1211–1216.

Kapur, S., Marcotte, B. & Marette, A., 1999. Mechanism of adipose tissue iNOS induction in endotoxemia. *The American journal of physiology*, 276(4 Pt 1), p.E635–641.

Li, L. et al., 2009. GYY4137, a novel hydrogen sulfide-releasing molecule, protects against endotoxic shock in the rat. *Free radical biology & medicine*, 47(1), p.103–113.

Mansouri, E. et al., 2011. Effects of grape seed proanthocyanidin extract on oxidative stress induced by diabetes in rat kidney. *Iranian biomedical journal*, 15(3), p.100–106.

Martín, M.A. et al., 2010. Protection of human HepG2 cells against oxidative stress by the flavonoid epicatechin. *Phytotherapy research: PTR*, 24(4), p.503–509.

Olinga, P. et al., 2001. Rat liver slices as a tool to study LPS-induced inflammatory response in the liver. *Journal of hepatology*, 35(2), p.187–194.

Pallarès, V. et al., 2012a. Additive, antagonistic, and synergistic effects of procyanidins and polyunsaturated fatty acids over inflammation in RAW 264.7 macrophages activated by lipopolysaccharide. *Nutrition (Burbank, Los Angeles County, Calif.)*, 28(4), p.447–457.

Pallarès, V. et al., 2012b. Enhanced anti-inflammatory effect of resveratrol and EPA in treated endotoxin-activated RAW 264.7 macrophages. *The British journal of nutrition*, p.1–12.

Prima, V. et al., 2011. Inhibition of LPS toxicity by hepatic argininosuccinate synthase (ASS): novel roles for ASS in innate immune responses to bacterial infection. *International immunopharmacology*, 11(9), p.1180–1188.

Romanovsky, A.A. & Petersen, S.R., 2003. The spleen: another mystery about its function. *American journal of physiology. Regulatory, integrative and comparative physiology*, 284(6), p.R1378–1379.

Rosengarten, B. et al., 2009. Effects of inducible nitric oxide synthase inhibition or norepinephrine on the neurovascular coupling in an endotoxic rat shock model. *Critical care (London, England)*, 13(4), p.R139.

Sebai, H. et al., 2009. Protective effect of resveratrol in endotoxemia-induced acute phase response in rats. *Archives of toxicology*, 83(4), p.335–340.

Sebai, H. et al., 2008. Protective effect of resveratrol on acute endotoxemia-induced nephrotoxicity in rat through nitric oxide independent mechanism. *Free radical research*, 42(11-12), p.913–920.

Sebai, H. et al., 2010. Resveratrol, a red wine polyphenol, attenuates lipopolysaccharide-induced oxidative stress in rat liver. *Ecotoxicology and Environmental Safety*, 73(5), p.1078–1083.

Singal, A. et al., 2006. Green tea (Camellia sinensis) extract ameliorates endotoxin induced sickness behavior and liver damage in rats. *Phytotherapy research: PTR*, 20(2), p.125–129.

Tavares, E. & Miñano, F.J., 2010. Immunoneutralization of the aminoprocalcitonin peptide of procalcitonin protects rats from lethal endotoxaemia: neuroendocrine and systemic studies. *Clinical science (London, England: 1979)*, 119(12), p.519–534.

Terra, X et al., 2011. Procyanidin dimer B1 and trimer C1 impair inflammatory response signalling in human monocytes. *Free radical research*, 45(5), p.611–619.

Terra, Ximena et al., 2007. Grape-seed procyanidins act as antiinflammatory agents in endotoxin-stimulated RAW 264.7 macrophages by inhibiting NFkB signaling pathway. *Journal of agricultural and food chemistry*, 55(11), p.4357–4365.

Terra, Ximena et al., 2009. Grape-seed procyanidins prevent low-grade inflammation by modulating cytokine expression in rats fed a high-fat diet. *The Journal of nutritional biochemistry*, 20(3), p.210–218.

Terra, Ximena et al., 2011. Modulatory effect of grape-seed procyanidins on local and systemic inflammation in diet-induced obesity rats. *The Journal of nutritional biochemistry*, 22(4), p.380–387.

Tracey, K.J. et al., 1986. Shock and tissue injury induced by recombinant human cachectin. *Science (New York, N.Y.)*, 234(4775), p.470–474.

4.4

Effects of grape seed procyanidin extract (GSPE) over low-grade chronic inflammation of obese *Zucker fa/fa* rats.

V. Pallarès et al.

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UNIVERSITAT ROVIRA I VIRGILI
ROLE OF BIOACTIVE FOOD COMPOUNDS IN ACUTE AND CHRONIC INFLAMMATION
Víctor Pallarés López
Dipòsit Legal: T.1472-2012

Abstract

Obesity is defined as possessing abnormal or excessive fat accumulation and is a major risk factor for a number of chronic diseases. Obesity is associated with chronic inflammation and is related to the accumulation and release of several cytokines from adipose tissue (AT). Procyanidins are phenolic compounds widely distributed in food such as cereals, vegetables, and many fruits, and procyanidins are known to have anti-inflammatory and antioxidant effects and are capable of ameliorating obesity-related inflammation. In previous studies using low doses of grape seed procyanidin extract (GSPE) as a treatment in rats with diet-induced obesity, anti-inflammatory effects at the systemic and gene expression levels were reported, as GSPE reduced several proinflammatory markers such as tumor necrosis factor-alpha (TNF-α), interleukin 6 (IL-6) and C-reactive protein (CRP). Therefore, understanding the anti-inflammatory effects of GSPE using a model of genetic obesity would be interesting. In this study, several *Zucker fa/fa* rats were used as the obese control group (n=10) and received vehicle treatments, whereas other Zucker fa/fa rats (n=10) were treated with a moderate dose of 35 mg/kg*day of GSPE for a period of 10 weeks. Importantly, GSPE treatment significantly decreased the average level of CRP by 5 weeks. Moreover, although GSPE had no significant palliative effect on systemic inflammation, GSPE showed anti-inflammatory effects on mesenteric AT, as GSPE treatment down-regulated the expression of *Crp* and *Il-6* genes in the adipocytes.

Introduction

Obesity is defined as abnormal or excessive fat accumulation that presents a risk to one's health, and obesity is a major risk factor for a number of chronic diseases including diabetes, cardiovascular diseases and cancer (Anòn s.d.). Central obesity is a particularly prominent risk factor for the development of insulin resistance, which is characteristic of type 2 diabetes mellitus (T2DM), and other features of metabolic syndrome such as dyslipidemia and hypertension(Zeyda & Stulnig 2009). Excessive energy intake and decreased energy expenditure is one of the main causes of human obesity (Gregor & Hotamisligil 2011). Obesity has become epidemic in industrialized countries and is increasingly common in developing countries worldwide as a consequence of sustained overnutrition (Shoelson et al. 2007).

In recent years, an abundance of evidence has emerged supporting the fact that obesity is associated with inflammation. In obese patients, chronic low-grade inflammation occurs as indicated by increased plasma levels of C-reactive protein (CRP) and inflammatory cytokines such as tumor necrosis factor-alpha (TNF-α) and interleukin 6 (IL-6) (Zeyda & Stulnig 2009; Shoelson et al. 2007). Previous studies using animal models showed that the circulating levels of TNF-α, IL-6, CRP and interleukin 1-beta (IL-1β) proinflammatory proteins are significantly increased in obese *Zucker* rats when compared with the lean control group (Ruth et al. 2008; Takeda et al. 2005; Miranville et al. 2012; D. Sánchez et al. 2011; Martín-Cordero et al. 2011). In addition, white adipose tissue (WAT), is commonly defined as an active secretory organ of a variety of substances that help to regulate metabolic homeostasis. WAT secretes leptin, adiponectin (ADIPQ), resistin, monocyte chemotactic protein-1 (MCP-1), nitric oxide (NO), TNF-α and IL-6 in humans and rodents (Rivera et al. 2008; Shoelson et al. 2007). Moreover, adipose tissue in obese subjects is characterized by macrophage infiltration. Thus, the elevation in the secretion of proinflammatory signaling molecules appears to be produced mainly by adipocytes and macrophages; however, the relative amount of each proinflammatory molecule produced by the adipocyte versus the macrophages remains unclear (Shoelson et al. 2007). On the other hand, there are several other important tissues that participate in the production of obesity-related metaflammation (inflammation derived by

metabolic causes). The liver plays an important role in the activation of systemic inflammation by secreting proinflammatory CRP in response to IL-6. Adipose tissue could be the driver of CRP production by the liver, as AT secretes IL-6 (Berg & Scherer 2005). Therefore, to understand the effects of anti-inflammatory agents, one must study inflammation in several different tissues.

Previous studies have shown that several foods and food extracts that contain bioactive molecules have powerful anti-inflammatory effects and are able to reduce this obesity-related state of inflammation (Min et al. 2012; O. Y. Kim et al. 2012; Rivera et al. 2008; Terra et al. 2011; Belobrajdic et al. 2011). Procyanidins are phenolic compounds that belong to the flavonoid group, and procyanidins are widely distributed in food. Cereals, vegetables and many fruit such as tea leaves, berries, grapes, apples, cocoa, blue corn and beans, and beverages such as wine and tea infusions contain significant amounts of procyanidins. Procyanidins are most widely known to possess anti-inflammatory and antioxidant effects (Castrillejo et al. 2011; Quesada et al. 2009; Terra et al. 2009; Terra et al. 2011), as procyanidins have been shown to be involved in oxygen free-radical scavenging, antilipid peroxidation, inhibition of inflammatory cytokines, alterations in cell membrane receptors, modulation of intracellular signaling pathway proteins and modulation of gene expression (Terra et al. 2009).

In previous studies using low doses of grape seed procyanidin extract (GSPE) as a treatment in rats with diet-induced obesity, anti-inflammatory effects were found at the systemic level and the gene expression level (Terra et al. 2009; Terra et al. 2011). In many treatment regimens, circulating levels of CRP and TNF-α were decreased by GSPE (Terra et al. 2009; Terra et al. 2011). In adipose tissue the levels of CRP and TNF-α were decreased by GSPE (Terra et al. 2011) as well as the expression of the *Crp, Tnf-α* and *Il-6* proinflammatory genes, whereas the expression level of *ADIPQ* was up-regulated upon GSPE treatment (Terra et al. 2009; Terra et al. 2011). A similar effect was observed in the liver. The *Crp* and *Tnf-α* proinflammatory genes were down-regulated by GSPE, and nuclear factor-kappa B (NF-κB) activity was decreased when compare with the obese control group (Terra et al. 2011).

Therefore, to broaden our understanding of the effects of GSPE as anti-inflammatory agent, our study was performed using *Zucker fa/fa* genetically obese rats (Kurtz et al. 1989; Panchal & Brown 2011). This model allowed us to attain a more extreme degree of obesity relative to the diet-induced obese rats. The GSPE dose used in this experiment (35 mg/kg*day) was reasonably higher than in previous studies (Terra et al. 2011), the reasoning for which was to conclusively determine any relevant anti-inflammatory effects in this model.

Methods and procedures

Reagents

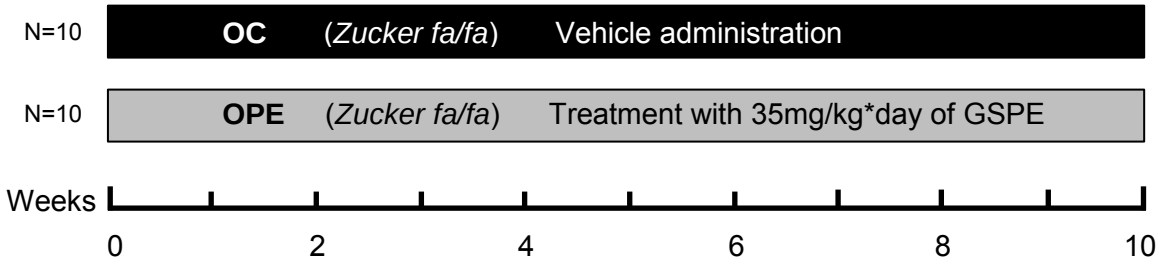
GSPE was provided by Les Dérives Résiniques et Terpéniques (Dax, France). According to the manufacturer, the GSPE contained monomeric (16.6%), dimeric (18.8%), trimeric (16.0%), tetrameric (9.3%) and oligomeric (5–13 units) (35.7%) procyanidins and phenolic acids (4.2%). The vehicle of GSPE was sweetened condensed milk diluted in water in a 1:6 ratio. The milk composition per 100 g was as follows: 8.9 g protein, 0.4 g fat, 60.5 g carbohydrates and 281 kcal.

Collagenase P from *Clostridium histolyticum* (ref. 11213865001) was purchased from Roche (Barcelona, Spain).

Animals and experimental protocol

Obese female *Zucker fa/fa* rats (n=20) (Charles River Laboratories, Barcelona, Spain) at 5 weeks of age were housed in cages by pairs at a constant temperature (22ºC), were subjected to a standard 12-h light/dark cycle and were given food and water *ad libitum*. Rats were allowed to adapt to their environment for 1 week before beginning experiments.

Figure 1. Different groups and treatments applied in the experiment across the time.



Different groups and treatments applied in the experiment across the time. GSPE; grape seed procyanidin extract. OC; obese control group. OPE; obese group treated with GSPE (35 mg/kg*day).

Obese rats were divided into two groups of 10 rats; one group served as the obese control group (OC), and the other group served as the group treated with GSPE (OPE). The OPE group received 35 mg/kg of GSPE mixed in the vehicle, while the OC group received the vehicle (Fig. 1). Treatments were administered once daily at 4 pm for 10 weeks by controlled oral intake using a syringe until one day before the animals were sacrificed. During the experimental period, rats were given free access to water and feed *ad libitum* immediately after the treatment until the next morning to improve the absorption of GSPE. In the morning (9 pm), food was removed from the animal cages until 1 hour after the next treatment (5 pm). The food intake and weight of the rats were measured every 2 weeks. At the end of the experimental period, the rats were fasted overnight and then were anesthetized with 75 mg/kg of sodium pentobarbital injected intraperitoneally (i.p.) and were sacrificed by exsanguination to obtain blood from the aorta and collect in heparinized tubes. The rats were 16 weeks of age when they were sacrificed. Mesenteric adipose tissue (MAT) and liver samples were excised. Plasma was obtained by centrifuging blood samples at 2000 x *g* for 10 min. All of the samples were stored at -80ºC until used.

Separation of adipocytes and the stromal vascular fraction (SVF) from MAT

MAT samples (3 g per rat) were surgically excised and were treated in 3 mL of incubation buffer (IB) with collagenase (0.5 mg/mL) for 30 min at 37ºC under agitation and then for 15 min at room temperature (RT). The IB is a modified Krebs-Henseleit buffer (150 mM NaCl, 6.17 mM KCl, 1.54 mM KH_2PO_4, 1.58 mM $MgSO_4$ and 25 mM $NaHCO_3$, pH 7.4) supplemented with 1 mM $CaCl_2$, 5 mM glucose and 3% (w/v) bovine serum albumin (BSA). Following the incubation, the mixture was pressure filtered using a piece of nylon and a syringe to filter out tissue that was not disaggregated. Then, 5 mL of washing buffer (WB) (IB but with 0.5 mM of $CaCl_2$) was added to the cell mixture. After centrifugation at 400 x *g* for 1 min and a subsequent incubation for 2 min to allow the phase to stabilize, the adipocytes, which settle near the surface of the solution, were collected. The pellet containing the SVF was re-suspended in 1 mL of IB and was stored at 80ºC until further analysis. The adipocyte phase was treated with 5 mL of WB and was centrifuged at 400 x *g* twice for 1 min each. After

the final incubation at room temperature to allow phase stabilization, the fat layer over the adipocytes was removed and the adipocyte phase was collected and diluted with 1 mL of IB. The cells were counted using a Neubauer chamber. The mean cell count of the samples was $4.1*10^5$ total adipocytes. The final cell suspension was stored at 80ºC until further analysis.

Plasma analytical procedures

CRP, TNF-α, MCP-1 and nitrate plus nitrite (NOx) levels were quantified using commercially available kits according to the manufacturer's instructions. For the CRP assay an ELISA kit was used (ref. E-25CRP, Immunology Consultants Laboratory, Inc. (ICL) (Newberg, OR, USA). For the TNF-α assay, a specific ELISA kit (ref. 88-7340) purchased from eBioscience (San Diego, CA, USA) was used. For the MCP-1 assay, a specific ELISA kit (ref. KRC1011) purchased from Invitrogen (Camarillo, CA, USA) was used. For the quantification of NOx levels, a colorimetric assay kit (ref. 780001) purchased from Cayman Chemical (Madrid, Spain) was used.

Measurements of CRP and TNF-α in MAT and liver

MAT and liver were homogenized in Triton X-100 cell lysis buffer (50 mM HEPES (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 1% Triton X-100, 10% glycerol) containing 1 mM phenylmethylsulfonyl fluoride, 2 µg/mL leupeptin, and 2 µg/mL aprotinin. Cells were incubated on ice in lysis buffer for 30 min. Lysates were centrifuged at 12000 x *g* for 10 min at 4°C. The cleared supernatant was assessed for CRP and TNF-α levels in the MAT and the liver. Protein concentration was measured based on the Bradford assay (Bradford 1976) after diluting the samples 1:5 with water to avoid lysis buffer interference. For the CRP assays, a specific enzyme immunoassay was used (ICL). For the TNF-α assays, a specific ELISA kit (ref. 88-7340) purchased from eBioscience (San Diego, CA, USA) was used. Both assays were performed according to the manufacturer's instructions.

Real-time PCR of proinflammatory and anti-inflammatory genes from MAT, liver, adipocytes and SVF

RNA extraction, reverse transcription and quantitative RT-PCR amplification using SYBR Green dye was performed as previously described (Terra et al. 2009) using 2 µg of total RNA as the template for the reverse transcription reaction. The relative mRNA levels of the proinflammatory genes *Crp, Il-6 and Tnf-α* were assessed in all tissues assayed. Relative mRNA expression levels of the target genes were normalized to cyclophilin (*Ppia*) mRNA levels. *Emr1* was included as the control in the adipocyte samples for the macrophage marker, and *ADIPQ* was included as the control in the SVF for the marker of mature adipocytes. The forward and reverse primers for the rat genes are shown in Table 1.

Table 1. Rat-specific primer sequences.

Gene	Primer sequence	
Crp	Fw:	5' GGCTTTTGGTCATGAAGACATG 3'
	Rv:	5' TCTTGGTAGCGTAAGAGAAGA 3'
Tnf-α	Fw:	5' CCTCACACTCAGATCATCTTCTC 3'
	Rv:	5' TTGGTGGTTTGCTACGACGTG 3'
Il-6	Fw:	5' CTCTCCGCAAGAGACTTCC 3'
	Rv:	5' GCCATTGCACAACTCTTTTCTC 3'
ADIPQ	Fw:	5' ACACCAAAAGTTCCAGGACTCA 3'
	Rv:	5' GACCAAGAACACCTGCGTCT 3'
Emr1	Fw:	5' ATGCATAATCGCTGCTGGCTGAA 3'
	Rv:	5' GAGGGCAGAGTTGATCGTGATGATC 3'
Ucp2	Fw:	5' CCTCTGGAAAGGGACCTC 3'
	Rv:	5' GAGGTCGTCTGTCATGAGG 3'
iNos	Fw:	5' CACCCGAGATGGTCAGGG 3'
	Rv:	5' CCACTGACACTCCGCACAA 3'
Ppia	Fw:	5' CTTCGAGCTGTTTGCAGACAA 3'
	Rv:	5' AAGTCACCACCCTGGCACATG 3'

Rat-specific primer sequences. C-reactive protein (*Crp*), inducible nitric oxide synthase (*iNos*), tumor necrosis factor-alpha (*Tnf-α*), interleukin 6 (*Il-6*), adiponectin (*ADIPQ*), uncoupling protein 2 (*Ucp2*), EGF-like module-containing mucin-like hormone receptor-like 1 (*Emr1*) and cyclophilin A (*Ppia*). FW; forward primer sequence. RV; reverse primer sequence.

Statistical analysis

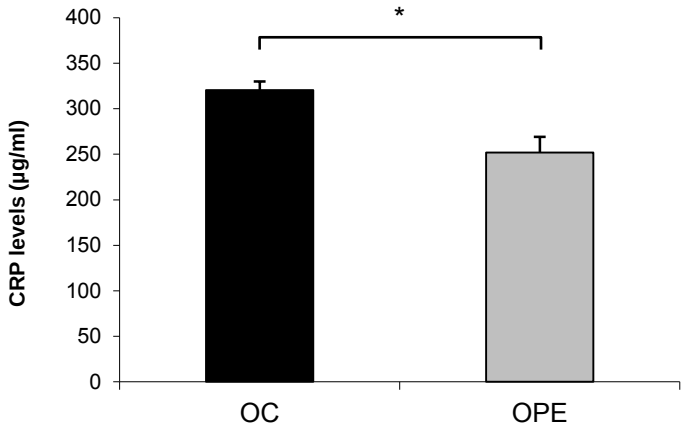
The results are presented as the mean with the associated standard error (SE). The data were analyzed by Student's T-test to determine the significant differences between the groups (OC and OPE) using IBM SPSS statistical software (version 19.0 for Windows; SPSS, Inc.). p-values<0.05 were considered statistically significant.

Results

1. GSPE decreased the level of serum CRP after 5 weeks treatment. However, GSPE had no significant effects on CRP, MCP-1, TNF-α and NOx levels in the plasma, liver and MAT

Plasma levels of the proinflammatory protein CRP were analyzed. Although treatment with GSPE significantly decreased CRP levels at 5 week of treatment (Fig. 2), CRP levels in the OPE group were similar to the OC at the end of the experiment (Tab. 2). In the liver and the MAT (Tab. 2), the CRP levels were not affected by the treatment.

Figure 2. Levels of CRP in the plasma at week 5 of treatment



CRP levels (µg/mL) in the plasma at week 5 of treatment. The results are presented as the mean ± standard error (SE) for each group (n=10): obese control group (OC) and obese group treated with GSPE (35 mg/kg) (OPE). Significant differences are represented by the symbol *. Student's T-test was used, and p-values<0.05 are considered statistically significant. GSPE; grape seed procyanidin extract.

The TNF-α levels in several tissues and the plasma were measured (Tab. 2). In the plasma, the levels of TNF-α were undetectable. In the MAT and in the liver, TNF-α levels did not change by treatment. Finally, plasma MCP-1 and NOx levels (Tab. 2) remained unchanged by treatment.

Table 2. Levels of CRP, MCP-1, TNF-α and NOx in the plasma and various tissues after 10 weeks of treatment.

	Experimental groups	OC		OPE	
		Mean	SE	Mean	SE
CRP	Plasma (µg/mL)	284.65	35.88	233.60	9.49
	Liver (ng/100 µg protein)	4.64	0.33	4.04	0.14
	MAT (ng/100 µg protein)	112.99	37.49	45.57	8.52
MCP-1	Plasma (pg/mL)	61.08	6.07	76.04	4.77
TNF-α	Plasma (pg/mL)	NA (< 16)		NA (< 16)	
	Liver (pg/100 µg protein)	59.46	4.98	56.68	0.82
	MAT (pg/100 µg protein)	0.50	0.17	0.22	0.03
NOx	Plasma (µM)	2.99	0.21	2.59	0.25

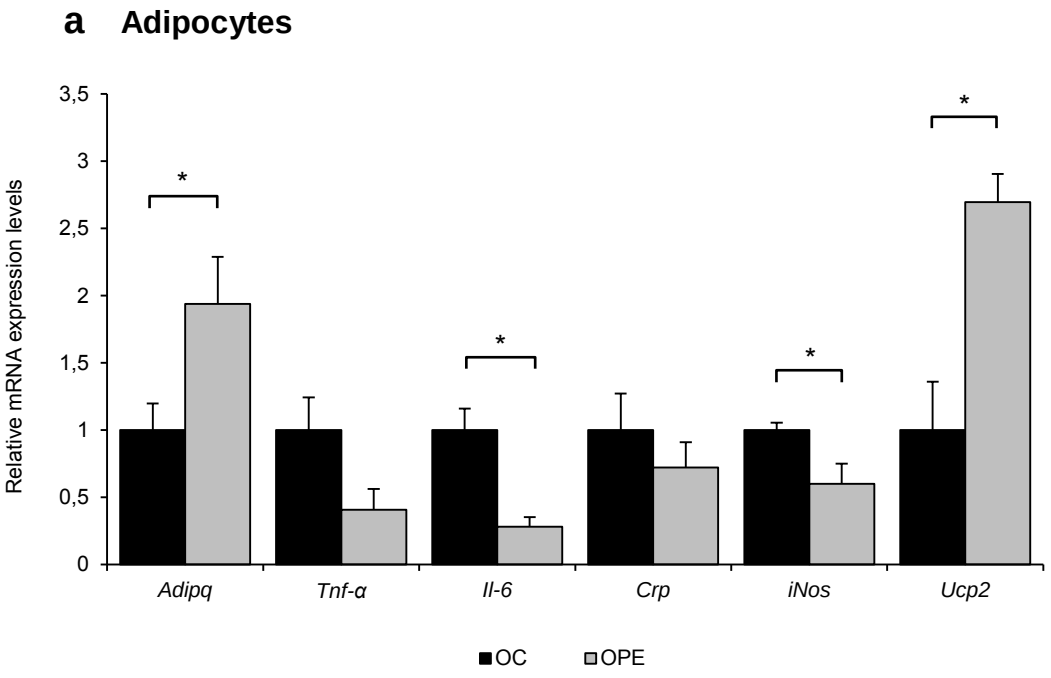
CRP, MCP-1, TNF-α and NOx levels in plasma and various tissues. The obese control group (OC, n=10) and the obese group treated with GSPE (35 mg/kg) (OPE, n=10) are presented in columns. The results are presented by the mean ± standard error (SE). Significant differences are indicated with the symbol *. Student's T-test was used, and p-values<0.05 were considered statistically significant. Units are presented in each measure. MAT; mesenteric adipose tissue. GSPE; grape seed procyanidin extract.

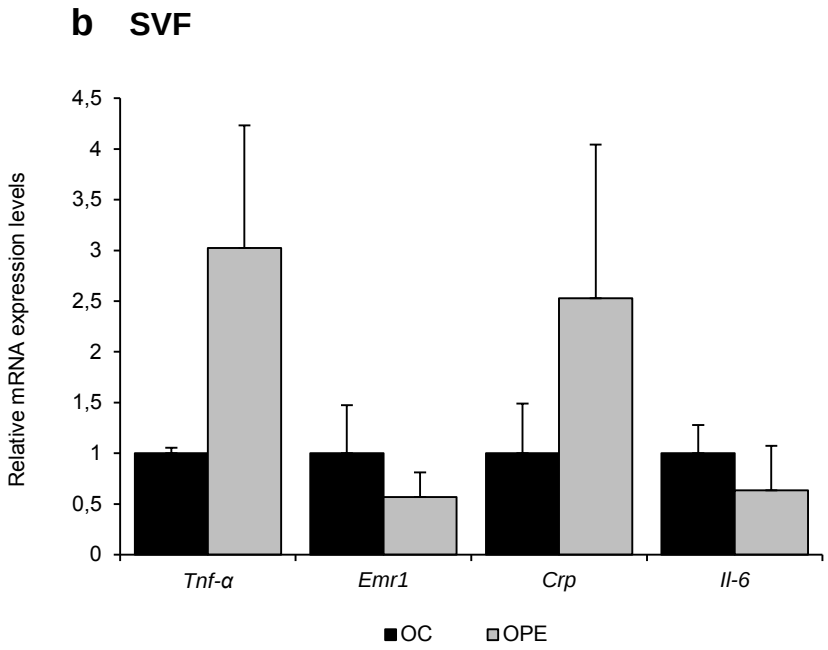
From a global perspective, these results suggest no significant differences between the OC and OPE groups (Tab. 2).

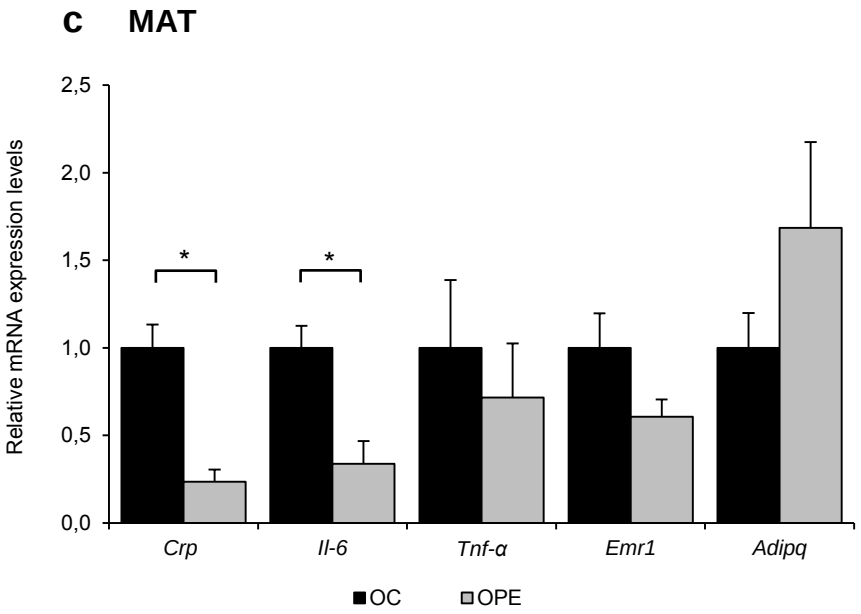
2. GSPE affected the relative expression levels of proinflammatory and anti-inflammatory genes in MAT

The relative gene expression levels of the proinflammatory genes *Tnf-α, Il-6, Crp, Emr1* and *iNos* and the anti-inflammatory genes *ADIPQ* and *Ucp2* were determined in adipocytes, SVF and in the whole MAT (Fig. 3).

Figure 3. Relative mRNA expression levels of proinflammatory and anti-inflammatory genes in MAT, MAT-adipocytes and MAT-SVF.







Relative mRNA expression levels of proinflammatory and anti-inflammatory genes in adipocytes (3A) and SVF (3B) from MAT and whole MAT (3C). To calculate the relative mRNA expression levels, the δδCT method was used. *Crp, Tnf-α, Il-6* and *iNos* were analyzed as proinflammatory genes, and *ADIPQ* expression was analyzed as an anti-inflammatory gene. *Ucp2* expression was analyzed as a metabolic gene in adipocytes (3A). *Crp, Tnf-α, Il-6* and *Emr1* were analyzed in SVF (3B), and *Crp, Tnf-α, Il-6, ADIPQ* and *Emr1* were analyzed in whole MAT (3C). The obese group treated with GSPE (35 mg/kg) (OPE, n=10) was normalized to the obese control group (OC, n=10) for adipocytes (3A), SVF (3B) and whole MAT (3C). The results are presented as the mean ± standard error (SE). Significantly different groups are represented with the symbol *. Student's T-test was used, and p-values<0.05 were considered statistically significant. GSPE; grape seed procyanidin extract. SVF; stromal vascular fraction. MAT; mesenteric adipose tissue.

In the adipocytes (Fig. 3A), a significant decrease was observed in the levels of *Il-6* and *iNos* expression in the OPE group relative to the OC group. In contrast, the expression levels of *ADIPQ* and *Ucp2* were significantly increased in the OPE group.

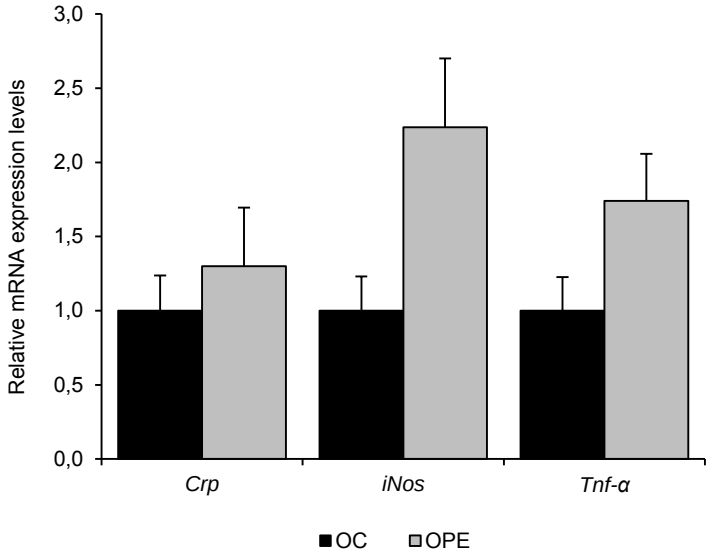
In the SVF (Fig. 3B), no changes by treatment in any gene expression level was observed.

Finally, analysis of the whole MAT samples (Fig. 3C) revealed that the expression levels of proinflammatory genes *Crp* and *Il-6* decreased in the OPE group when compared with the OC group.

3. GSPE did not affect the expression levels of proinflammatory genes in the liver

The expression levels of *Crp*, *Tnf-α* and *iNos* were assessed in the liver to test the anti-inflammatory action of GSPE in this tissue, however the mRNA levels were similar in both groups (OC and OPE, Fig. 4).

Figure 4. Relative mRNA expression levels of *Crp, iNos* and *Tnf-α* in the liver.



Relative mRNA expression levels of the proinflammatory genes *Crp, iNos and Tnf-α* in the liver. To calculate the relative mRNA expression levels, the δδCT method was used. The obese group treated with GSPE (35 mg/kg) (OPE, n=10) was normalized to the obese control treatment group (OC, n=10). The results are presented as the mean ± standard error (SE). Significant differences are represented by the symbol * in independent comparisons for each gene. Student's T-test was used to confirm statistical significance, and p-values<0.05 were considered statistically significant. GSPE; grape seed procyanidin extract.

Discussion

The study of the efficacy of nutritionally derived anti-inflammatory agents as a means to counteract various diseases has become more relevant in recent years. Procyanidins are present in several foods in the human diet and have been shown to act as anti-inflammatory agents at certain doses. In the current study, a moderate dose of GSPE was assessed as an anti-inflammatory agent using a model of chronic inflammation such as genetically obese rats. This model permits implementing a treatment with the anti-inflammatory extract in early state of chronic inflammation due to these rats are obese since they have 5 weeks of life.

Obesity is characterized by an excessive and uncontrolled increase of adipose tissue and is generally recognized as a low-chronic inflammatory disease (Lolmède et al. 2011). The deregulation of adipose tissue hypertrophy and hyperplasia occurs through the action of several molecules such as adipokines, chemokines and acute-phase proteins, which promote a chronic inflammatory state at the local and systemic levels (Attie & Scherer 2009). For instance, previous studies have shown that circulating levels of CRP (an acute-phase protein) and TNF-α increase in rats with obesity (Takeda et al. 2005; Martín-Cordero et al. 2011; Terra et al. 2011; Terra et al. 2009; Endo et al. 2010) as does the expression levels of the proinflammatory genes *Crp* and *Il-6* in MAT (Terra et al. 2009; Terra et al. 2011; Endo et al. 2010). In the current work, MCP-1 plasma levels were significantly increased in *fa/fa Zucker* rats in contrast to the lean group (data not shown), exhibiting a low-grade of chronic inflammation induced by genetically-mediated obesity. Based on the inflammatory environment in the context of obesity, the effects of GSPE on the expression and secretion of cytokines and adipokines during inflammation in several target tissues including the MAT and the liver were assessed.

First, the effects of GSPE on the CRP level in the plasma were tested. Treatment with GSPE (OPE group) decreased the serum levels of CRP when compared with the OC group as early as 5 weeks after treatment was initiated (Fig. 2). These results suggest that the OPE rats experience less inflammation than the OC rats. However, by the end of the experiment, the

GSPE-treated group did not show significantly lower levels of CRP in the plasma at the systemic level or at the local level (Tab. 2). These results suggest that the anti-inflammatory effects of GSPE may be most evident during an initial treatment (5 weeks in rats, fig. 2) but not at later time point when the chronic inflammation may become too great to overcome with the doses used.

Nevertheless, although GSPE treatment did not appear to be very effective at the systemic level, GSPE treatment had a significant impact on the gene expression level. GSPE treatment had a potent anti-inflammatory effect on expression of inflammatory genes in the MAT (Fig. 3C), as the relative expression levels of the proinflammatory genes *Crp* and *Il-6* were significantly down-regulated upon GSPE treatment (OPE, Fig 3C), whereas *ADIPQ* expression levels were up-regulated, albeit not significantly (Fig. 3C). In addition, the expression of *Tnf-α* and *Emr1* (Fig. 3C), which is a gene that encodes a transmembrane protein ubiquitously expressed by macrophages, were down-regulated upon GSPE treatment (OPE), but these decreases were not significant.

To further specify the differential effect of GSPE in MAT, mature adipocytes and the SVF, which contains various cell types such as macrophages, pre-adipocytes, and erythrocytes, were isolated to test the influence of GSPE on the expression of proinflammatory and anti-inflammatory genes within these fractions. Our results indicate that GSPE had pronounced anti-inflammatory effects in the adipocyte fraction but not in the SVF (Fig. 3A and 3B).

GSPE showed anti-inflammatory effects in the adipocytes by down-regulating the proinflammatory genes *Il-6* or *iNos* and up-regulating the expression of the anti-inflammatory *ADIPQ* gene (Fig. 3A). In addition, *Ucp2,* which encodes an abundant protein in WAT whose expression is controlled by the anti-inflammatory peroxisome proliferator-activated receptor-g (PPAR-g) (González-Yanes & Sánchez-Margalet 2003) and is involved in thermogenesis and apoptosis of adipocytes in rodents (Chen et al. 2009; Almanza-Perez et al. 2010; Sun & Zemel 2004; Tsuboyama-Kasaoka et al. 2000), was up-regulated upon GSPE treatment (Fig.

3A). Moreover, in the context of our results from the analysis of the SVF (Fig. 3B), GSPE appears to have more influence on the adipocyte fraction of MAT.

Ultimately, to assess the anti-inflammatory effect of GSPE in other major organs, the expression levels of several proinflammatory genes were analyzed in the liver. The levels of *Crp, iNos and Tnf-α* in the OPE group was similar to the one in the OC group (Fig. 4). These data suggest that GSPE has no significant effects in this model on proinflammatory gene expression in the liver. On the other hand, the *Il-6* expression level in the liver was undetectable in both groups, which suggests a relatively normal liver physiology.

In conclusion, treatment of genetically obese *Zucker* rats with a moderate dose of GSPE appears to have anti-inflammatory effects at the gene expression levels in MAT by targeting the adipocyte fraction, which represents one of the main producers of proinflammatory markers in obesity. In contrast, although most of the inflammatory markers assessed were slightly decreased by GSPE, GSPE treatment has no significant palliative effect on systemic inflammation in genetically obese rats at least for the treatment regimen used in this study. Longer treatments and/or higher doses should be tested in future studies to further investigate the validity of these results.

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Disclosure

All authors contributed to the development, writing and review of this paper. We declare no conflicts of interest associated with any of the contributing authors regarding the content of this paper.

Supplementary data

Table 3. Weight of rats during the treatment and weight of the liver and the MAT.

Experimental groups	OC		OPE	
	Mean	SE	Mean	SE
Body weight (g)				
At 5 weeks of treatment	298.70	7.35	325.00*	4.89
At 10 weeks of treatment	399.10	9.16	428.80*	10.05
Liver weight (% of body weight)	3.69	0.18	3.65	0.12
MAT weight (% of body weight)	1.80	0.09	2.10	0.14

Weight of rats at 5 and 10 weeks after the beginning of the treatment and the weight of the liver and the MAT at the end of the treatment. The weight of the liver and the MAT are presented as a percentage of the total body weight. The obese control group (OC, n=10) and the obese group treated with GSPE (35 mg/kg) (OPE, n=10) are presented in columns. The results are presented as the mean ± standard error (SE). Significant differences are represented by the symbol *. Student's T-test was used to determine statistical significance and p-values<0.05 were considered statistically significant. MAT; mesenteric adipose tissue. GSPE; grape seed procyanidin extract.

References

Almanza-Perez, J.C. et al., 2010. Glycine regulates inflammatory markers modifying the energetic balance through PPAR and UCP-2. *Biomedicine & pharmacotherapy = Biomédecine & pharmacothérapie*, 64(8), p.534–540.

Anòn, WHO | Obesity and overweight. *WHO*. Available at: http://www.who.int/mediacentre/factsheets/fs311/en/index.html [Accedit maig 24, 2012].

Attie, A.D. & Scherer, P.E., 2009. Adipocyte metabolism and obesity. *Journal of lipid research*, 50 Suppl, p.S395–399.

Belobrajdic, D.P. et al., 2011. Cereal based diets modulate some markers of oxidative stress and inflammation in lean and obese Zucker rats. *Nutrition & metabolism*, 8, p.27.

Berg, A.H. & Scherer, P.E., 2005. Adipose tissue, inflammation, and cardiovascular disease. *Circulation research*, 96(9), p.939–949.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical biochemistry*, 72, p.248–254.

Castrillejo, V.M. et al., 2011. Antioxidant effects of a grapeseed procyanidin extract and oleoyl-estrone in obese Zucker rats. *Nutrition (Burbank, Los Angeles County, Calif.)*, 27(11-12), p.1172–1176.

Chen, N. et al., 2009. Green tea, black tea, and epigallocatechin modify body composition, improve glucose tolerance, and differentially alter metabolic gene expression in rats fed a high-fat diet. *Nutrition Research*, 29(11), p.784–793.

Endo, Y. et al., 2010. Experimental periodontitis induces gene expression of proinflammatory cytokines in liver and white adipose tissues in obesity. *Journal of periodontology*, 81(4), p.520–526.

González-Yanes, C. & Sánchez-Margalet, V., 2003. Pancreastatin, a chromogranin A-derived peptide, inhibits leptin and enhances UCP-2 expression in isolated rat adipocytes. *Cellular and molecular life sciences: CMLS*, 60(12), p.2749–2756.

Gregor, M.F. & Hotamisligil, G.S., 2011. Inflammatory Mechanisms in Obesity. *Annual Review of Immunology*, 29(1), p.415–445.

Kim, O.Y. et al., 2012. Influence of quercetin-rich onion peel extracts on adipokine expression in the visceral adipose tissue of rats. *Phytotherapy research: PTR*, 26(3), p.432–437.

Kurtz, T.W., Morris, R.C. & Pershadsingh, H.A., 1989. The Zucker Fatty Rat as a Genetic Model of Obesity and Hypertension. *Hypertension*, 13(6), p.896–901.

Lolmède, K. et al., 2011. Immune cells in adipose tissue: key players in metabolic disorders. *Diabetes & metabolism*, 37(4), p.283–290.

Martín-Cordero, L. et al., 2011. The interleukin-6 and noradrenaline mediated inflammation-stress feedback mechanism is dysregulated in metabolic syndrome: effect of exercise. *Cardiovascular diabetology*, 10, p.42.

Min, S.Y. et al., 2012. Cocoa polyphenols suppress adipogenesis in vitro and obesity in vivo by targeting insulin receptor. *International journal of obesity (2005)*. Available at: http://www.ncbi.nlm.nih.gov/pubmed/22641065 [Accedit maig 31, 2012].

Miranville, A. et al., 2012. Differential Adipose Tissue Inflammatory State in Obese Nondiabetic Zucker Fatty Rats Compared to Obese Diabetic Zucker Diabetic Fatty Rats. *Hormone and Metabolic Research*, 44(04), p.273–278.

Panchal, S.K. & Brown, L., 2011. Rodent Models for Metabolic Syndrome Research. *Journal of Biomedicine and Biotechnology*, 2011.

Quesada, H. et al., 2009. Grape seed proanthocyanidins correct dyslipidemia associated with a high-fat diet in rats and repress genes controlling lipogenesis and VLDL assembling in liver. *International journal of obesity (2005)*, 33(9), p.1007–1012.

Rivera, L. et al., 2008. Quercetin ameliorates metabolic syndrome and improves the inflammatory status in obese Zucker rats. *Obesity (Silver Spring, Md.)*, 16(9), p.2081–2087.

Ruth, M.R. et al., 2008. Abnormal immune responses in fa/fa Zucker rats and effects of feeding conjugated linoleic acid. *Obesity (Silver Spring, Md.)*, 16(8), p.1770–1779.

Sánchez, D. et al., 2011. Soluble fiber-enriched diets improve inflammation and oxidative stress biomarkers in Zucker fatty rats. *Pharmacological research: the official journal of the Italian Pharmacological Society*, 64(1), p.31–35.

Shoelson, S.E., Herrero, L. & Naaz, A., 2007. Obesity, inflammation, and insulin resistance. *Gastroenterology*, 132(6), p.2169–2180.

Sun, X. & Zemel, M.B., 2004. Role of uncoupling protein 2 (UCP2) expression and 1alpha, 25-dihydroxyvitamin D3 in modulating adipocyte apoptosis. *FASEB journal: official publication of the Federation of American Societies for Experimental Biology*, 18(12), p.1430–1432.

Takeda, R. et al., 2005. Blockade of endogenous cytokines mitigates neointimal formation in obese Zucker rats. *Circulation*, 111(11), p.1398–1406.

Terra, X. et al., 2009. Grape-seed procyanidins prevent low-grade inflammation by modulating cytokine expression in rats fed a high-fat diet. *The Journal of Nutritional Biochemistry*, 20(3), p.210–218.

Terra, X. et al., 2011. Modulatory effect of grape-seed procyanidins on local and systemic inflammation in diet-induced obesity rats. *The Journal of nutritional biochemistry*, 22(4), p.380–387.

Tsuboyama-Kasaoka, N. et al., 2000. Conjugated linoleic acid supplementation reduces adipose tissue by apoptosis and develops lipodystrophy in mice. *Diabetes*, 49(9), p.1534–1542.

Zeyda, M. & Stulnig, T.M., 2009. Obesity, inflammation, and insulin resistance--a mini-review. *Gerontology*, 55(4), p.379–386.

UNIVERSITAT ROVIRA I VIRGILI
ROLE OF BIOACTIVE FOOD COMPOUNDS IN ACUTE AND CHRONIC INFLAMMATION
Victor Pallarés López
Dipòsit Legal: T.1472-2012

5

DISCUSSION

5 Discussion

Studies investigating the effects of bioactive molecules present in food have increased within the last few years. Various bioactive molecules have been used as a nutraceuticals at their recommended doses to achieve beneficial effects against diseases such as obesity, diabetes, cardiovascular diseases and cancer (Brower 2005; Pearson et al. 2007; Kris-Etherton et al. 2004; E. L. Ding et al. 2006; Hertog et al. 1993; Tomé-Carneiro et al. 2012; Aggarwal 2010). Therefore, an understanding of the mechanisms through which these bioactive molecules act as well as an understanding of the proper dosages and conditions under which to administer these agents is particularly important to maximize their anti-disease capacity. Our studies are based on the use of various flavonoids and polyunsaturated fatty acids (PUFAs) as bioactive molecules to palliate, alleviate or prevent chronic and acute inflammation using different models.

One of our hypotheses predicted a synergistic effect between several bioactive molecules from food such as wine, tea, and fruits such as grapes or apples when combined with other molecules present in fish and oils derived from fish when applied to an *in vitro* macrophage model. Inflammatory response in RAW 264.7 murine macrophages was triggered by LPS derived from *E. coli*. RAW 264.7 cells were co-incubated with LPS (100 ng/ml) and combinations of the bioactive molecules for 19 hours at the following concentrations: epigallocatechin gallate (EGCG; 2.5 µg/mL), resveratrol (RES; 2.5 µg/mL) and dimeric procyanidins B1-B4 (5 µg/ml) were used for the flavonoids and eicosapentaenoic acid (EPA; 30 µM) and docosahexaenoic acid (DHA; 30 µM) were used for the PUFAs. The flavonoids and PUFAs were added individually and in combination with each other, and a total of 12 combinations were analyzed. Each of these molecules have been shown to possess anti-inflammatory effects by reducing nitric oxide (NO) levels, by reducing cytokines such as TNF-α, or by affecting the proinflammatory signaling pathways such as nuclear factor kappa-B (NF-κB) or activator protein-1 (AP-1). Flavonoids and PUFAs have also been shown to down-regulate various genes induced by these pathways in activated RAW 264.7 macrophages such as *iNos* and *Tnf-α* (Y. L. Lin & J. K. Lin 1997; Park et al. 2000; Kim & Chung 2007; Komatsu et al. 2003; Wadsworth & Koop 1999; Tsai et al. 1999; Chan et al. 2000). Our results show that the molecules B1, EPA and DHA had inhibitory effects on inflammation, as they suppressed NO secretion when administered individually. The combinations that showed synergistic inhibitory effects were RES plus EPA and B3 plus EPA. Based on these two combinations, several studies were performed to determine which pathways became activated upon LPS treatment such as the NF-κB, mitogen-activated protein kinase (MAPK) or signal transducer and activator of transcription (STAT) pathways, and which pathways could be suppressed by the bioactive molecules. We also determined whether individual pathways were inhibited in combination with the molecules exerting their anti-inflammatory effects on

different nodes of the same pathway. To this end, several assays were performed analyzing several proteins from these pathways and the expression levels of genes related to these pathways to further elucidate the mechanisms of action for bioactive molecules. Importantly (Fig. 24), the combination of B3 plus EPA had a synergistic effect on the NF-κB and the stress-activated protein kinase/c-JUN N−terminal kinase (SAPK/JNK) pathways, whereas the combination of RES and EPA had a synergistic effect on the SAPK/JNK pathway. In addition, both combinations decreased the overall protein levels of all of the pathways analyzed when compared with cells treated with LPS (Fig. 24).

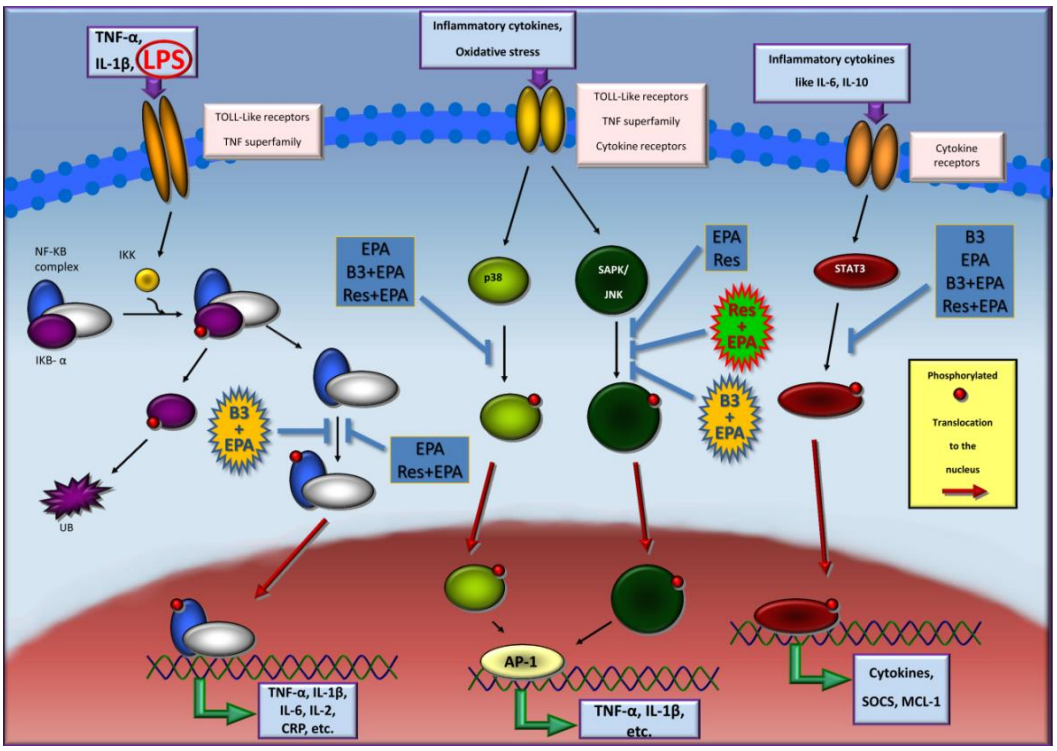


Figure 24. Main effects of the combinations B3 plus EPA and RES plus EPA on several inflammatory pathways in RAW 264.7 murine macrophages

Our results showed significant differences in the ability of various bioactive molecules to regulate several genes involved in the inflammatory response. The two synergistic combinations down-regulated many proinflammatory genes that encode cytokines such as *Tnf-α*, *Il-1β*, *Il-6* and plasminogen activator-inhibitor 1 (*Pai-1*), chemokines such as colony stimulating factor 2 (*Csf2*), chemokine (C-C motif) ligand 2 (*Ccl2*), *Ccl5* and *Ccl7*, and enzymes that produce oxygenated molecules such as *iNos*. However, these combinations could also up-regulate the expression of antioxidant genes such as heme oxygenase (decycling) 1 (*Hmox-1*), catalase (*Cat*) and glutamate-cysteine ligase regulatory subunit (*Gclm*). Collectively, these results suggest that some combinations of bioactive molecules have amplified anti-inflammatory effects because they act in concert on various pathways involved in inflammation. These effects promote the up-regulation or down-regulation of many

genes that influence inflammatory cells such as macrophages resulting in the amelioration of the proinflammatory state. However, more studies should be performed *in vitro* to corroborate these results. For instance, kinetic studies with the same doses could be done to test if these anti-inflammatory effects are time-dependent or, on the contrary, they are constant in time. In addition, studies with different concentrations and combinations implementing statistic models to test these interactions must be performed (Chou 2006), to ensure that the observed synergistic effects are dose-dependent. After confirming the results *in vitro, in vivo* studies could be performed in models of acute inflammation, administrating pure molecules such as RES and EPA, as well as extracts or oils with large amounts of these molecules such as GSPE or fish oil, to test the anti-inflammatory synergistic actions of these compounds.

Our second hypothesis predicted that GSPE possesses anti-inflammatory effects against an *in vivo* model of acute inflammation. To assess the effect of GSPE in the context of acute inflammation, we employed a lipopolysaccharide (LPS)-induced rat model. As shown in previous studies (Olinga et al. 2001; Prima et al. 2011; Dyson et al. 2011; Li et al. 2009; Hagiwara et al. 2009), this model permits the assessment of the proinflammatory response in a relative short period of time, as several parameters such as nitrate and nitrite species (NOx) and the cytokines TNF-α, IL-6 and IL-10 are rapidly affected. This model permitted us to apply a less invasive technique, and avoid the surgical operations that must be performed in other models of induction of acute inflammation such as CLP or CASP, that can produce the death of the rats through the handling process. On the other hand, this model is interesting because LPS acts through TLR-4, which is a classical and very important pathway activated in inflammation that trigger the canonical NF-κB pathway. In contrast, the dose of LPS used in this model cannot be extrapolated in humans due to the resistance of rodents to this endotoxin. Moreover, although the porcine model is the most used as a sepsis model and mimics better the inflammatory response of humans, we chose the rat model because rats are handier to treat and house, they have lower cost, and it is possible to use more animals doing the statistic more homogenous and robust. In addition, our goal was to test the anti-inflammatory actions of GSPE against an acute inflammatory response, thus this model fits our aims.

Several doses of GSPE were administered for 15 days prior to LPS injection to analyze the preventive effect of GSPE at nutritional (50 and 75 mg/kg of GSPE; 50PE and 75PE) and pharmacological doses (100 and 200 mg/kg GSPE; 100PE and 200PE). The results are shown in Figure 25. The highest nutritional dose (75PE) was the most effective, as this dosage was able to decrease the NOx levels in the liver and the spleen as well as at the circulating levels in the plasma and the erythrocytes (red blood cells; RBCs) (Fig. 25). This dose is high when it is extrapolated to the humans, thereby the dietary habits should be modified to achieve a diet rich in procyanidins and reach similar results. Additionally, more

experiments *in vivo* using CLP or CASP models should be performed to confirm the effective anti-inflammatory effects of GSPE in other models of acute inflammation.

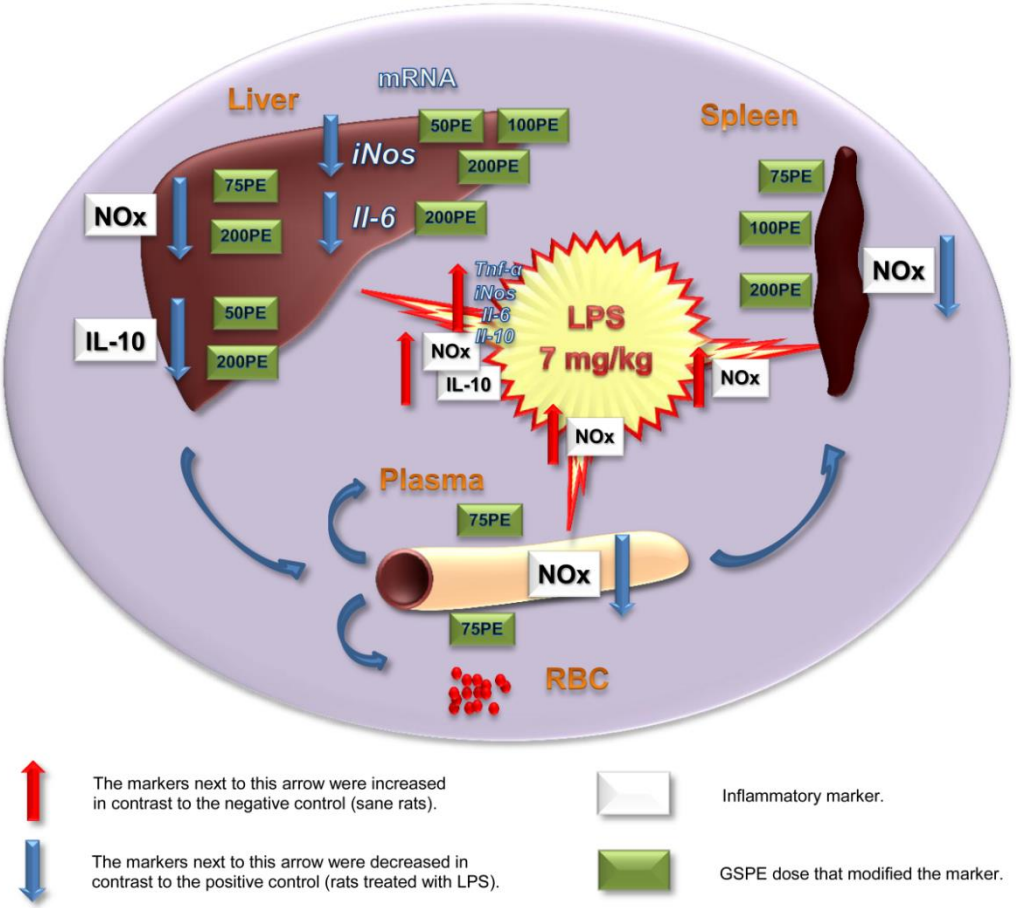


Figure 25. Effects of several doses of GSPE on NOx and IL-10 levels and the relative expression levels of proinflammatory genes in rats with acute inflammation induced by LPS injection.

On the other hand, the highest pharmacological dose (200PE) also had important anti-inflammatory effects, as this dose decreased the NOx levels in the liver and the spleen and down-regulated the proinflammatory genes *Il-6* and *iNos* in the liver (Fig. 25).

Thus, nutritional supplements rich in procyanidins should be implemented in diet to reach these effects in humans. From a global perspective, these data suggest that GSPE has powerful anti-inflammatory effects against acute inflammation induced by an external endotoxic agent such as LPS. Furthermore, GSPE appears to modulate the expression of proinflammatory genes in the liver and ultimately decreases the levels of proinflammatory markers at circulating levels. A recent study showed that the anti-inflammatory effects of proanthocyanidins could be due to the capacity of these molecules to bind to LPS and repress the binding to the complex CD14/MD2/TLR-4 (Delehanty et al. 2007), promoting a non-activation of the pro-inflammatory pathways such as NF-κB, and decreasing the expected inflammatory response.

Our third hypothesis predicted that the procyanidins present in grape seed possess anti-inflammatory effects in an aggravated model of chronic inflammation. The genetically obese *Zucker* rat model strain deficient in leptin receptor (*fa/fa*) was administered grape seed procyanidin extract (GSPE) to assess the efficacy of GSPE as a preventive treatment. Our results indicate that GSPE has anti-inflammatory effects, as GSPE down-regulated the expression of the proinflammatory genes C-reactive protein (*Crp)*, interleukin-6 (*Il-6)* and inducible nitric oxide synthase (*iNos*) and up-regulated the anti-inflammatory gene adiponectin (*Adipq*) in the mesenteric adipose tissue (MAT), which was similarly shown in previous studies using diet-induced rat models of obesity (Terra et al. 2009; Terra et al. 2011). However, we observed a more modest effect at the systemic level on all of the inflammatory markers analyzed. These results contrast with previous studies and suggest that procyanidins most evidently exert their systemic anti-inflammatory effects in rats in more modestly aggravated models of obesity such as the cafeteria diet model or the high-fat diet models (Terra et al. 2009; Terra et al. 2011). Our study shows that the effects of the procyanidins present in GSPE administrated at 35 mg/kg*day for 10 weeks is not sufficient to significantly counteract the proinflammatory state in the genetically obese *Zucker* rat model. Therefore, future studies should assess higher doses and/or durations of GSPE treatment to determine whether stronger anti-inflammatory effects may be observed in this model.

The effects of GSPE shown in the models of acute and chronic inflammation used in the present doctoral thesis can be partially explained by the xenohormesis hypothesis. This hypothesis suggests that the molecules from plants synthesized in situations of stress, could promote an activation of the defense pathways against the cellular stress in the organism when ingested by animals and humans (Howitz & Sinclair 2008). Therefore, procyanidins, which are stress-induced molecules, could activate these pathways in the rats pre-treated with GSPE, acquiring beneficial mechanisms against the inflammation, which will be reflected in the modulation of markers and mediators of inflammation.

In general, our studies show that procyanidins have powerful anti-inflammatory effects, which are manifested by down-regulating the proinflammatory genes *Il-6* and *iNos* in various tissues *in vivo* and in activated macrophages *in vitro*. Furthermore, our results indicate that the anti-inflammatory effects observed are dose-dependent, which suggests that the use of these bioactive molecules as anti-inflammatory agents against diseases related to chronic or acute inflammation may only be effective at the appropriate dose.

Taking all in consideration, the results of this thesis have provided preliminary studies of the anti-inflammatory effects of several bioactive molecules from food, and have shown how these compounds can exert synergistic effects when they are in combination *in vitro*. In addition, an extract of polyphenols such as GSPE have exhibited potent anti-inflammatory effects against

acute inflammation *in vivo* at several doses, whereas exerted slight effects against chronic inflammation. These studies are considered as preliminary because more experiments must be performed to extrapolate the doses of this extract and the pure molecules in humans, implementing nutritional diets rich in these compounds to reach similar anti-inflammatory effects against diseases related to inflammation.

5.1 References

Aggarwal, B.B., 2010. Targeting Inflammation-Induced Obesity and Metabolic Diseases by Curcumin and Other Nutraceuticals. *Annual review of nutrition*, 30, p.173–199.

Brower, V., 2005. A nutraceutical a day may keep the doctor away. *EMBO Reports*, 6(8), p.708–711.

Chan, M.M. et al., 2000. Synergy between ethanol and grape polyphenols, quercetin, and resveratrol, in the inhibition of the inducible nitric oxide synthase pathway. *Biochemical pharmacology*, 60(10), p.1539–1548.

Chou, T.-C., 2006. Theoretical basis, experimental design, and computerized simulation of synergism and antagonism in drug combination studies. *Pharmacological Reviews*, 58(3), p.621–681.

Delehanty, J.B. et al., 2007. Binding and neutralization of lipopolysaccharides by plant proanthocyanidins. *Journal of natural products*, 70(11), p.1718–1724.

Ding, E.L. et al., 2006. Chocolate and Prevention of Cardiovascular Disease: A Systematic Review. *Nutrition & Metabolism*, 3(1), p.2.

Dyson, A. et al., 2011. An integrated approach to assessing nitroso-redox balance in systemic inflammation. *Free radical biology & medicine*, 51(6), p.1137–1145.

Hagiwara, S. et al., 2009. Landiolol, an ultrashort-acting beta1-adrenoceptor antagonist, has protective effects in an LPS-induced systemic inflammation model. *Shock (Augusta, Ga.)*, 31(5), p.515–520.

Hertog, M.G.L., Hollman, P.C.H. & van de Putte, B., 1993. Content of potentially anticarcinogenic flavonoids of tea infusions, wines, and fruit juices. *Journal of Agricultural and Food Chemistry*, 41(8), p.1242–1246.

Howitz, K.T. & Sinclair, D.A., 2008. Xenohormesis: Sensing the Chemical Cues of Other Species. *Cell*, 133(3), p.387–391.

Kim, Y.J. & Chung, H.Y., 2007. Antioxidative and anti-inflammatory actions of docosahexaenoic acid and eicosapentaenoic acid in renal epithelial cells and macrophages. *Journal of medicinal food*, 10(2), p.225–231.

Komatsu, W. et al., 2003. Docosahexaenoic acid suppresses nitric oxide production and inducible nitric oxide synthase expression in interferon-γ plus lipopolysaccharide-stimulated murine macrophages by inhibiting the oxidative stress. *Free Radical Biology and Medicine*, 34(8), p.1006–1016.

Kris-Etherton, P.M. et al., 2004. Bioactive compounds in nutrition and health-research methodologies for establishing biological function: the antioxidant and anti-inflammatory effects of flavonoids on atherosclerosis. *Annual review of nutrition*, 24, p.511–538.

Li, L. et al., 2009. GYY4137, a novel hydrogen sulfide-releasing molecule, protects against endotoxic shock in the rat. *Free radical biology & medicine*, 47(1), p.103–113.

Lin, Y.L. & Lin, J.K., 1997. (-)-Epigallocatechin-3-gallate blocks the induction of nitric oxide synthase by down-regulating lipopolysaccharide-induced activity of transcription factor nuclear factor-kappaB. *Molecular pharmacology*, 52(3), p.465–472.

Olinga, P. et al., 2001. Rat liver slices as a tool to study LPS-induced inflammatory response in the liver. *Journal of hepatology*, 35(2), p.187–194.

Park, Y.C. et al., 2000. Activity of monomeric, dimeric, and trimeric flavonoids on NO production, TNF-alpha secretion, and NF-kappaB-dependent gene expression in RAW 264.7 macrophages. *FEBS letters*, 465(2-3), p.93–97.

Pearson, W. et al., 2007. Anti-inflammatory and chondroprotective effects of nutraceuticals from Sasha's Blend in a cartilage explant model of inflammation. *Molecular nutrition & food research*, 51(8), p.1020–1030.

Prima, V. et al., 2011. Inhibition of LPS toxicity by hepatic argininosuccinate synthase (ASS): novel roles for ASS in innate immune responses to bacterial infection. *International immunopharmacology*, 11(9), p.1180–1188.

Terra, X. et al., 2009. Grape-seed procyanidins prevent low-grade inflammation by modulating cytokine expression in rats fed a high-fat diet. *The Journal of Nutritional Biochemistry*, 20(3), p.210–218.

Terra, X. et al., 2011. Modulatory effect of grape-seed procyanidins on local and systemic inflammation in diet-induced obesity rats. *The Journal of nutritional biochemistry*, 22(4), p.380–387.

Tomé-Carneiro, J. et al., 2012. One-year consumption of a grape nutraceutical containing resveratrol improves the inflammatory and fibrinolytic status of patients in primary prevention of cardiovascular disease. *The American journal of cardiology*, 110(3), p.356–363.

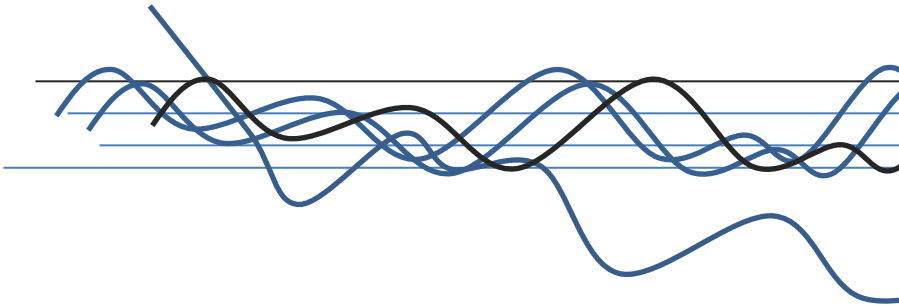
Tsai, S.H., Lin-Shiau, S.Y. & Lin, J.K., 1999. Suppression of nitric oxide synthase and the down-regulation of the activation of NFkappaB in macrophages by resveratrol. *British journal of pharmacology*, 126(3), p.673–680.

Wadsworth, T.L. & Koop, D.R., 1999. Effects of the wine polyphenolics quercetin and resveratrol on pro-inflammatory cytokine expression in RAW 264.7 macrophages. *Biochemical pharmacology*, 57(8), p.941–949.

UNIVERSITAT ROVIRA I VIRGILI
ROLE OF BIOACTIVE FOOD COMPOUNDS IN ACUTE AND CHRONIC INFLAMMATION
Victor Pallarés López
Dipòsit Legal: T.1472-2012

6

CONCLUSIONS



6 Conclusions

The main conclusions are presented after each objective:

Objective 1

Individual molecules B1 (5 μg/mL), EPA (30 μM) and DHA (30 μM) have anti-inflammatory effects, as they decrease NO levels in LPS-stimulated RAW 264.7 macrophages.

Combining B3 (5 μg/mL) and EPA (30 μM) appears to have a synergistic anti-inflammatory effect in activated RAW macrophages.

The combination of RES (2.5 μg/mL) and EPA (30 μM) has the most dramatic anti-inflammatory effect on LPS-stimulated macrophages of all combinations assessed.

The combinations of B3 plus EPA and RES plus EPA decrease NO levels, decrease the expression of proinflammatory and oxidative stress-related genes and affect the phosphorylation of NF-κB, p38, SAPK/JNK and STAT3, which are proteins implicated in the activation of the NF-κB and AP-1 proinflammatory pathways.

Objective 2

GSPE has anti-inflammatory effects at the systemic and gene expression levels when administered for 15 days prior to LPS-induced acute inflammation in a rat model.

The nutritional high dose of 75 mg/kg*day and the pharmacological high dose of 200 mg/kg*day of GSPE show the greatest anti-inflammatory impact when administered prior to LPS-induced acute inflammation.

The most effective doses have the capacity to decrease the levels of the proinflammatory marker NOx in the plasma, RBC, spleen and liver.

The highest dose down-regulates the proinflammatory genes *Il-6* and *iNos* in the liver.

Objective 3

Treatment with a moderate dose of 35 mg/kg*day of GSPE for 10 weeks has anti-inflammatory effects at the gene expression levels in the MAT of *Zucker fa/fa* rats.

GSPE down-regulates the expression of the proinflammatory genes *Crp* and *Il-6* in whole MAT. These results are consistent with the down-regulation of *Il-6* promoted by GSPE in the adipocyte fraction derived from the MAT.

GSPE has greater effects on the down-regulation of the proinflammatory genes *Il-6* and *iNos* and the up-regulation of the anti-inflammatory gene *Adipq* in the adipocyte fraction than the SVF.

GSPE has no significant effect on systemic inflammation in genetically obese rats; however, GSPE decreases the circulating levels of CRP after 5 weeks of treatment.

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7

ANNEX II

7.1 Resum

La inflamació és un procés fisiològic de l'organisme en front d'un estímul perjudicial, el qual pot ser físic, químic o biològic. La resposta que es genera normalment conclou amb la resolució de la inflamació i el restabliment de l'homeòstasi, i implica una acció coordinada de diferents mediadors i tipus cel·lulars com els macròfags o limfòcits, la qual depèn de la naturalesa de l'estímul inicial. Productes microbians com els lipopolisacàrids (LPS) i citoquines com la interleuquina 1 (IL-1) i el factor de necrosis tumoral alfa (TNF-α) activen aquesta resposta mitjançant els receptors de membrana TLRs (*Toll-like receptors*) o altres receptors específics (receptors de IL-1 (TIR) i família de receptors TNF), activant diferents vies senyalitzadores de la inflamació com el factor de transcripció nuclear kappa B (NF-κB), el Janus quinasa/Transductor de senyal i activador de la transcripció (JAK/STAT) o la proteïna quinasa mitogen-activada (MAPK) amb les cascades de fosforilació derivades ben conegudes com ERK1/2, JNK, i p38. Aquestes vies de senyalització regulen la transcripció de gens proinflamatoris i antiinflamatoris tals com la *Il-1,* la *Il-6,* el *Tnf-α* o l'òxid nítric sintasa induïble (*iNos)*, els quals provoquen un creuament de senyals entre aquestes vies claus en la resolució de la inflamació. Si aquestes vies es troben constantment regulades o aquest creuament esta desequilibrat pot provocar una acció autocrina i paracrina de la inflamació i acabar produint malalties cròniques derivades directament d'aquesta inflamació no resolta, com l'obesitat o l'aterosclerosi. Per tant, l'estudi d'aquestes vies i de les citoquines derivades o altres factors que indueixen a l'activació d'aquestes vies ens pot ajudar a comprendre els efectes de compostos bioactius en estudis *in vivo* o *in vitro*.

Existeixen diferents tipus de molècules bioactives presents en aliments comuns, els quals posseeixen propietats antiinflamatòries i antioxidants que, entenent-los com a elements nutracèutics implementats en la nostra dieta en dosis adients, poden ajudar-nos a prevenir o pal·liar aquests estats proinflamatoris que caracteritzen malalties com l'obesitat, o malalties cardiovasculars com l'aterosclerosi, a més de millorar la resolució de la inflamació en casos d'infecció o de danys tissulars. Diferents tipus de polifenols com els flavonoides (flavanols, procianidines, etc.), els estilbens (resveratrol; RES) o els àcids fenòlics (àcid gàl·lic), i els àcids grassos, en concret els poliinsaturats omega 3 o ω3-PUFAs (α-linolenic, ALA; eicosapentaenòic EPA; docosahexaenòic; DHA) en són un exemple. Les procianidines són un tipus de flavonoide que pertany al grup dels flavanols o flavan-3-ols, característics per ser oligòmers formats per unitats de catequina o epicatequina, ja estiguin gal·lades o no, i estan presents en fruites i vegetals tals com el raïm, les pomes, els fruits secs, les fruites vermelles, el cacau, o les begudes derivades d'aquestes com el vi o el most. El RES, una molècula molt estudiada actualment per les seves propietats antiinflamatòries i antioxidants, és un polifenol molt important en el grup dels estilbens. Aquestes molècules fenòliques tenen propietats antiinflamatòries i antioxidants conegudes i els mecanismes que fan servir per a realitzar

aquestes accions recauen en el segrest de radicals lliures, la inhibició de la peroxidació lipídica, la inhibició de la formació de citoquines inflamatòries, l'alteració de receptors cel·lulars de membrana, i la modulació de proteïnes de vies senyalitzadores intracel·lulars i de l'expressió gènica i activitat enzimàtica de proteïnes.

Per altra banda, els ω3-PUFAs també tenen propietats antiinflamatòries importants, com la d'alterar l'expressió gènica de proteïnes inflamatòries a través de la modulació de factors de transcripció, com són l'NF-κB i els receptors activats pel proliferador de peroxisoma (PPARs). En concret, es coneix que l'EPA i el DHA tenen importants propietats antiinflamatòries capaces de modular la secreció de citoquines proinflamatòries en els macròfags en el teixit adipós, així com d'inhibir l'expressió de gens proinflamatoris com *Il-1β, Il-6* i *Tnf-α* en monòcits animals i humans activats amb LPS. I per altra banda, també poden activar l'expressió del gen antiinflamatori *Il-10* en adipòcits.

En aquest context, el primer objectiu va ser conèixer els efectes antiinflamatoris sinèrgics, additius i antagònics de diversos polifenols com el flavanol epigal·locatequina gal·lat (EGCG), l'estilbè RES i les procianidines dimèriques B1, B2, B3 i B4 en combinació amb els PUFAs EPA i DHA, en front d'una resposta inflamatòria aguda en macròfags *in vitro*, cèl·lules efectores de la inflamació. Es va utilitzar l'LPS per a mimetitzar aquesta resposta en macròfags RAW 264.7 de ratolí. Per tal d'analitzar aquests efectes es van estudiar els nivells d'òxid nítric o nitrits (NO) en el medi després de realitzar la co-incubació de les cèl·lules amb les molècules estudiades i l'LPS. A més, per conèixer els mecanismes que utilitzen aquestes molècules per a realitzar aquests efectes sinèrgics es va realitzar un estudi de diferents vies de regulació claus de la inflamació com l'NF-κB, el p38, la proteïna quinasa activada per estrès/ quinasa c-JUN N-terminal (SAPK/JNK) i l'STAT3. Per acabar d'entendre aquests mecanismes es va realitzar una estada a la universitat URBC-FUNDP de Namur (Bèlgica) on es van analitzar els nivells de 96 gens relacionats amb la inflamació, estrès oxidatiu i el metabolisme utilitzant arrays de baixa densitat (TLDAs; TaqMan$^{(R)}$ Low Density Array). Els resultats van destacar que les molècules B1, EPA i DHA tenien efectes inhibidors sobre els NO per si soles. En canvi, les combinacions que van tenir un efecte inhibidor sinèrgic varen ser RES més EPA i B3 més EPA. Es va observar que la combinació de B3 més EPA tenia efectes sinèrgics sobre les vies de l'NF-κB i del SAPK/JNK, mentre que la combinació RES i EPA ho feia sobre la via del SAPK/JNK. A més, totes dues combinacions van poder inhibir totes les vies estudiades respecte el tractament amb LPS (control positiu). Aquest efecte s'acaba d'entendre millor quan s'observen els resultats en l'expressió gènica on les dues combinacions són capaces, en molts casos sinèrgicament, de reduir els nivells de molts gens proinflamatoris que codifiquen per citoquines com el *Tnf-α, la Il-1β, la Il-6* o l'inhibidor de l'activador del plasminogen (*Pai-1)* per quimioquines com el factor estimulador de colònies 2 (*Csf2),* el lligand de quimioquina *(motiu C-C) 2 (Ccl2), el Ccl5* i el *Ccl7*, o per enzims que

generen estrès oxidatiu com l'*iNos*, alhora que també són capaces d'augmentar els nivells d'expressió de gens antioxidants com l'hemo-oxigenasa 1 (*Hmox-1),* la catalasa *(Cat)* o la proteïna lligasa reguladora de la cisteïna-glutamat *(Gclm)*. Per tant, tot això suggereix que aquestes molècules en combinació poden realitzar un efecte antiinflamatori més potent ja que exerceixen un efecte sobre totes les vies estudiades i això fa que es redueixi l'activació de la transcripció de molts gens, que sintetitzats per aquests macròfags generarien accions paracrines i autocrines i promourien i augmentarien finalment aquest estat proinflamatori.

Per una altra banda, en els últims anys el nostre grup ha constatat els diversos efectes que tenen els compostos fenòlics (majoritàriament procianidines) presents en un extracte de pinyol de raïm (grape seed procyanidin extract; GSPE) en diversos models d'inflamació induïda, analitzant-los en macròfags activats *in vitro*, i també en models d'inflamació crònica com és l'obesitat induïda per dieta, tant en models de cafeteria com en dieta alta en greixos *in vivo*. Els nostres objectius en aquesta tesi eren ampliar els coneixements sobre els efectes antiinflamatoris d'aquestes molècules en altres models encara no estudiats.

Per tant, el nostre segon objectiu va ser conèixer els efectes antiinflamatoris del GSPE en un model d'inflamació aguda. Es va utilitzar LPS administrat intraperitonealment per a mimetitzar aquest efecte en rates sanes *Wistar*. Es van administrar diferents tractaments per analitzar quines dosis de GSPE podrien ser més efectives, utilitzant dosis nutricionals moderades-altes (50 i 75 mg/kg de GSPE; 50PE i 75PE) i dosis farmacològiques (100 i 200 mg/kg GSPE; 100PE i 200PE). Per tal d'analitzar aquests efectes antiinflamatoris, es van estudiar els nivells de proteïnes proinflamatòries i antiinflamatòries a nivell sistèmic en plasma, i sobre els nivells d'òxids de nitrat i nitrit (NOx) tant a nivell sistèmic com local, en teixits claus del metabolisme i la immunitat innata, el fetge i la melsa respectivament. Finalment, també es van realitzar estudis a nivell de regulació gènica sobre gens proinflamatoris i antiinflamatoris en el fetge. Els resultats van indicar que la dosis nutricional alta (75PE) va ser la més efectiva, disminuint els nivells de NOx a nivell local en el fetge i la melsa, i a nivell circular en el plasma i els eritròcits. Per altra banda, la dosi farmacològica més alta (200PE) també va aportar importants efectes antiinflamatoris disminuint els NOx al fetge i la melsa, i regulant a la baixa gens proinflamatoris clau com *Il-6* o *iNos* al fetge. En global, aquestes dades suggereixen que el GSPE té un efecte antiinflamatori molt poderós en front de la inflamació aguda induïda per un agent endotòxic extern com l'LPS, modificant la regulació de gens proinflamatoris en un òrgan endocrí tan important com és el fetge i, en definitiva, disminuint nivells de marcadors proinflamatoris a nivells circulars.

Per últim, es van voler conèixer els efectes antiinflamatoris del GSPE en un model d'inflamació crònica més agreujat que els estudiats anteriorment en el grup. Es va utilitzar un model d'obesitat genètica amb rates *Zucker fa/fa* amb una dosi nutricional moderada de

35 mg/kg de GSPE, en el qual es va estudiar l'efecte del GSPE sobre els nivells de proteïnes proinflamatòries tant a nivell sistèmic en plasma, com a nivell local en teixits com el fetge o el teixit adipós mesentèric (TAM). A més, es van analitzar els nivells d'expressió gènica de gens proinflamatoris i antiinflamatoris en aquests teixits, en adipòcits i en la fracció vascular estromal (SVF) aïllats ambdós del TAM, per tal de comprendre quines cèl·lules tenen el GSPE com a diana per a produir aquest efecte antiinflamatori en aquest model d'obesitat genètica. Els resultats van indicar que el GSPE tenia un efecte antiinflamatori clar, per una banda regulant a la baixa l'expressió gènica de gens proinflamatoris en el TAM tals com la proteïna C-reactiva (*Crp), Il-6* o *iNos,* i regulant a l'alça gens antiinflamatoris com l'adiponectina (*ADIPQ),* com es va veure en estudis previs amb obesitat induïda per la dieta; i per una altra banda, amb un efecte molt més lleu a nivell sistèmic, no podent modificar cap paràmetre estudiat com la CRP o el TNF-α ni a nivells circulars ni a nivell local en diferents teixits. Aquests resultats, contrastats amb estudis previs, ens suggereixen que encara que les procianidines tenen un efecte antiinflamatori en rates amb obesitat en altres models més lleus en relació amb la inflamació crònica com el de cafeteria o de dieta alta en greix, no és suficient per contrarestar significativament aquest estat proinflamatori en aquest model amb la dosis subministrada (35mg/kg) durant 10 setmanes. Per tant, caldria canviar la dosis o el temps de tractament per veure algun efecte més potent a nivell fisiològic.

En general, tots aquests estudis ens permeten evidenciar que les procianidines tenen un efecte antiinflamatori poderós, que podem veure en tots els estudis realitzats durant aquesta tesi, a l'hora de regular a la baixa gens proinflamatoris claus com *Il-6* o *iNos* en diferents teixits *in vivo*, i fins i tot en l'estudi *in vitro* en macròfags, on diferents molècules bioactives individualment i en combinació també podien modificar la regulació d'aquests dos gens. Per últim, també cal dir que aquests efectes estan lligats fortament i depenen de les dosis induïdes en aquests models tant *in vivo*, com *in vitro*, el que fa pensar que en futurs experiments s'hauria de tenir molt en compte usar les dosis més adequades per tal d'aconseguir els efectes antiinflamatoris pertinents en vers malalties relacionades amb la inflamació, ja sigui crònica o aguda.

7.2 Hipòtesis i objectius

En els últims anys s'han conegut els efectes beneficiosos que tenen diversos polifenols, entre ells les procianidines, evidenciant el seu rol com a antioxidants i antiinflamatoris. En el nostre grup hem vist aquests efectes en diversos models d'inflamació induïda, analitzant els efectes en macròfags activats *in vitro*, i també en models d'inflamació crònica com és l'obesitat induïda per dieta, tant en models de cafeteria com en dieta alta en greixos *in vivo*.

Els nostres objectius en aquesta tesi estan enfocats en ampliar els coneixements sobre els efectes antiinflamatoris d'aquestes molècules en altres models encara no estudiats, marcant 3 hipòtesis com a principals:

Hipòtesi 1 - Diversos polifenols i PUFAs en la seva forma pura, ben coneguts com molècules antiinflamatòries, tenen efectes sinèrgics antiinflamatoris en macròfags activats *in vitro,* solapant els diversos mecanismes que utilitzen aquestes molècules individualment, com la desactivació de vies inflamatòries com la de l'NF-κB o l'AP-1.

Hipòtesi 2 - Les procianidines presents en un extracte de pinyol de raïm (GSPE) tenen un efecte antiinflamatori de caràcter preventiu en un model d'inflamació aguda *in vivo*. El GSPE és capaç de prevenir una resposta inflamatòria aguda potent després d'injectar una dosi alta d'LPS en rates.

Hipòtesi 3 - El GSPE produeix efectes antiinflamatoris en un model més agreujat d'inflamació crònica *in vivo* com és el de la obesitat genètica. Amb l'evidència que el GSPE té efectes antiinflamatoris en vers l'obesitat induïda per la dieta, és d'esperar una resposta similar en un model més agreujat.

Per validar les nostres hipòtesis vàrem proposar els següents objectius:

Objectius per a resoldre la hipòtesi 1:

- Determinar els efectes antiinflamatoris de diversos polifenols com l'EGCG, l'estilbè RES i les procianidines dimèriques B1, B2, B3 i B4 i els PUFAs EPA i DHA, en una resposta inflamatòria aguda en macròfags *in vitro*, cèl·lules efectores de la inflamació. Es van incubar macròfags de ratolí RAW 264.7 amb l'endotoxina LPS, amb i sense la presencia de les molècules bioactives, per a mimetitzar aquesta resposta.
- Determinar els efectes antiinflamatoris sinèrgics, additius i antagònics que poden exercir els polifenols combinats amb els PUFAs, avaluant la capacitat que tenen per modificar els nivells de NO.

- Conèixer els mecanismes d'acció que utilitzen aquestes molècules per a realitzar aquests efectes antiinflamatoris analitzant vies claus de la inflamació com la de l'NF-κB, la del p38, la del SAPK/JNK i la de l'STAT3.
- Determinar els efectes d'aquestes molècules sobre l'expressió de gens relacionats amb la inflamació, l'estrès oxidatiu i el metabolisme, utilitzant arrays de baixa densitat.

Objectius per a resoldre la hipòtesi 2:

- Conèixer els efectes antiinflamatoris del GSPE en un model d'inflamació aguda *in vivo*. Es va utilitzar LPS administrat intraperitonealment per a mimetitzar aquest efecte en rates sanes *Wistar*. Es van realitzar diferents tractaments preventius amb GSPE, utilitzant dosis nutricionals moderades-altes i dosis farmacològiques, per determinar quines dosis podrien ser més efectives.
- Determinar l'efecte antiinflamatori del GSPE basat en la capacitat que té per modular els nivells de proteïnes proinflamatòries i antiinflamatòries a nivell sistèmic en plasma. Analitzar com el GSPE modula els nivells de NOx tant a nivell sistèmic com local, en teixits claus del metabolisme i la immunitat innata com són el fetge i la melsa.
- Conèixer els efectes del GSPE sobre l'expressió de gens proinflamatoris i antiinflamatoris en el fetge.

Objectius per a resoldre la hipòtesi 3:

- Determinar els efectes antiinflamatoris del GSPE en un model d'inflamació crònica més agreujat que els estudiats anteriorment en el grup. Per dur a terme aquest objectiu, es va emprar un model d'obesitat genètica utilitzant rates *Zucker fa/fa*. La dosi escollida va ser de 35 mg/kg de GSPE, considerada una dosis nutricional moderada.
- Comprovar l'efecte antiinflamatori del GSPE en els nivells de proteïnes proinflamatòries, tant a nivell sistèmic en plasma, com a nivell local en teixits com el fetge o el teixit adipós mesentèric (TAM).
- Constatar els efectes que té el GSPE en adipòcits i SVF aïllats del TAM i comparar-los amb l'efecte en el TAM, analitzant l'expressió de gens proinflamatoris i antiinflamatoris. Els resultats poden ajudar a comprendre quines cèl·lules diana té el GSPE per a produir aquest efecte antiinflamatori en aquest teixit clau de l'obesitat.

7.3 Conclusions

Les principals conclusions estan presentades segons l'objectiu plantejat:

Objectiu 1

Les molècules pures B1 (5 µg/mL), EPA (30 µM) i DHA (30 µM) tenen efectes antiinflamatoris, disminuint els nivells de NO en macròfags RAW 264.7 activats per LPS.

La combinació entre B3 (5 µg/mL) i EPA (30 µM) té efectes sinèrgics antiinflamatoris en macròfags RAW activats.

La combinació entre RES (2.5 µg/mL) i EPA (30 µM) té l'efecte sinèrgic antiinflamatori més potent de totes les combinacions avaluades en macròfags RAW activats.

Les combinacions entre B3 i EPA, i RES i EPA disminueixen els nivells de NOs, disminueixen l'expressió de gens proinflamatoris i d'altres relacionats amb l'estrès oxidatiu i modifiquen la fosforilació d'NF-κB, p38, SAPK/JNK i STAT3, les quals són proteïnes implicades en l'activació de les vies senyalitzadores proinflamatòries de l'NF-κB i de l'AP-1.

Objectiu 2

El GSPE té efectes antiinflamatoris a nivell sistèmic i a nivell d'expressió gènica, quan és administrat durant 15 dies prèviament a la inducció d'inflamació aguda per la injecció d'LPS en rates.

La dosi nutricional alta de 75 mg/kg*dia i la dosi farmacològica alta de 200 mg/kg*dia de GSPE mostren el major impacte antiinflamatori quan són administrades prèviament a la inducció d'inflamació aguda per la injecció d'LPS.

Les dosis de GSPE més efectives tenen la capacitat de disminuir els nivells de NOx, un marcador proinflamatori, en el plasma, els eritròcits (RBC), la melsa i el fetge.

La dosi més alta de GSPE regula a la baixa els gens proinflamatoris *Il-6* i *iNos* en el fetge.

Objectiu 3

El tractament amb una dosi moderada de 35 mg/kg*dia de GSPE durant 10 setmanes té efectes antiinflamatoris a nivell d'expressió gènica en el TAM de les rates *Zucker fa/fa*.

El GSPE regula a la baixa l'expressió dels gens proinflamatoris *Crp* i *Il-6* en el TAM. Aquests resultats es consoliden amb la regulació a la baixa del gen *Il-6,* promoguda per l'acció del GSPE en la fracció dels adipòcits derivada del TAM.

El GSPE té uns efectes més potents en la fracció dels adipòcits que en la fracció vascular estromal, regulant a la baixa l'expressió de gens proinflamatoris com *Il-6* i *iNos,* i regulant a l'alça l'expressió del gen antiinflamatori *Adipq.*

El GSPE no té efectes significatius sobre la inflamació sistèmica en rates genèticament obeses, tot i que el GSPE disminueix els nivells circulants de CRP després de 5 setmanes de tractament.