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XIMENA TERRA BARBADORA

Grape-Seed Procyanidins as Anti-inflammatory Agents

Ph Doctoral Thesis

Directed by Dr. Maria Teresa Blay Olivé

Departament de Bioquímica i Biotecnologia

UNIVERSITAT ROVIRA I VIRGILI

Tarragona
2009

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Maria Teresa Blay Olivé, professora titular del Departament de Bioquímica i Biotecnologia de la Universitat Rovira i Virgili,

CERTIFICO:

Que aquest treball, titulat “Grape-seed procyanidins as anti-inflammatory agents”, que presenta Ximena Terra Barbadora per a l’obtenció del 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, 7 d’abril de 2009

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Bueno, llegó la hora…parece mentira lo rápido que pasa el tiempo…y la cantidad de personas a las que me gustaría agradecer su contribución a que esta tesis haya concluido.

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A mis padres

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ABBREVIATION LIST

ABCA-1	ATP-binding cassette A1
ACTH	Adrenocorticotropic hormone
AP-1	Activator protein 1
ATM	Adipose tissue macrophages
BAT	Brown adipose tissue
BMI	Body mass index
C/EBP	CCAAT enhancer-binding protein
CD36	Scavenger receptor CD36
CD40	Scavenger receptor CD40
COX-2	Cyclooxygenase 2
CREB	cAMP responsive element binding protein
CRH	Corticotropine release hormone
CRP	C-reative protein
CVD	Cardiovascular disease
EDRF	Endothelium derived-relaxing factor
EG	Epigallocatechin
EGCG	Epigallocatechin gallate
Emr1	EGF-like module-containing, mucin-like hormone receptor 1
eNOS	Endothelial nitric oxide synthase
ER	Endoplasmic reticulum
ERK1/2	Extracellular Signal-regulated protein kinase
FFA	Free fatty acids
GSPE	Grape seed procyanidin extract
HDL	High density lipoproteins
HIF-1	Hypoxia-Inducible factor 1
HSL	Hormone sensitive lipase
ICAM-1	Intracellular adhesion molecule-1
IFN-γ	Interferon-γ
IkB	Nuclear factor kappa-b inhibitor
IKK	IkB kinase
IL	Interleukin
iNOS	Inducible nitric oxide synthase
IR	Insulin resistance

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IRAK	Interleukin receptor-associated kinase
IRS-1	Insulin receptor substrate
JAK	Cytokine receptor-mediated intracellular signal transduction Janus kinase
JNK	c-Jun N-terminal kinase
LDL	Low density lipoproteins
LPL	Lipoprotein lipase
LPS	Lipopolysaccharide
LT	Leukotriene
LXR	Liver X receptor
MAPK	Mitogen-activated Protein Kinase
MCP-1	Macrophage chemo attractant protein-1
MCSF	Macrophage colony stimulating factor
MEK	MAP/ERK kinase kinase 1
MHC	Major histocompatibility complex
MIF	Macrophage migration inhibitory factor
MIP-1α	Macrophage inflammatory protein 1α
MMP	Matrix metalloproteinases
MS	Metabolic syndrome
MyD88	Myeloid differenciation primary response gene 88
NF-kB	Nucear factor kappa beta
NIK	NF-kB inducing kinase
nNOS	Neuronal nitric oxide synthase
NO	Nitric oxide
oxLDL	Oxidized low density lipoproteins
PAI-1	Plasminogen activator inhibitor
PDK	Phosphoinositide-dependent kinase
PE	Procyanidin extract
PGE-2	Prostaglandin E2
PI3K	Phosphatidylinositol-3-kinase
PKA	Protein kinase A
PPARγ	Peroxisome-proliferator–activated receptor γ
RNS	Reactive nitrogen species
ROS	Reactive oxigen species
SOCS	Suppressor of cytokine signaling
SOD	Superoxide dismutase

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STAT	Signal Transducers and activators of the transcription
T2D	Tipe 2 diabetes mellitus
TG	Triglycerides
TGF-b1	Transforming growth factor b1
TLR	Toll-like receptor
TNFR	Tumor necrosis factor receptor
TNF-α	Tumor necrosis factor-α
TRAF	TNF-α receptor-associated factor
TZD	Thiazolidinedione
VAT	Visceral adipose tissue
VCAM-1	Vascular adhesion molecule-1
VEGF	Vascular endothelial growth factor
VLDL	Very low density lopoprotein
WAT	White adipose tissue

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I. SUMMARY

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Obesity is the result of numerous, interacting behavioural, physiological, and biochemical alterations. It is associated with reduced life expectancy and is an independent risk-factor for cardiovascular diseases (CVD), the leading cause of death in the world. In addition, obese patients, especially those with abdominal obesity due to visceral adipose tissue accumulation, are at higher risk from impaired glucose tolerance and impaired fasting glucose that frequently evolve into insulin resistance (IR) and type 2 diabetes mellitus (T2D). Obesity and IR are frequently associated with hypertension, proatherogenic dyslipidemia, chronic inflammation, and prothrombotic state. These metabolic disorders, together with obesity, are clustered together in the so-called metabolic syndrome. There is now growing consensus that obesity is an inflammatory condition leading to chronic activation of the innate immune system response. Although the inflammatory responses are different in various diseases, they can be characterised by a common spectrum of genes and endogenous mediators, including growth factors, inflammatory cytokines such as interleukin 1 β (IL-1β), tumour necrosis factor (TNF-α), interleukin-6 (IL-6), chemokines (MCP-1, IL-8), matrix metalloproteinases, and toxic molecules such as RNS or ROS. It is generally assumed that cytokines produced and released by white adipose tissue (WAT) are responsible for the obesity-associated inflammatory state. Adipocytes and adipose tissue produce a wide range of hormones and cytokines involved in glucose metabolism (e.g. adiponectin, resistin), inflammation (e.g. TNF-α, IL-6), coagulation (PAI-1), blood pressure (e.g. angiotensinogen, angiotensin II), feeding behaviour (leptin), and proteins involved in lipid metabolism (e.g. cholesteryl ester transfer protein, CETP) thus affecting the metabolism and the function of many organs and tissues including muscle, liver, vasculature, and brain. Plasma adipokine levels rise with an increase in adipose tissue and adipocyte volume, except for plasma adiponectin which is lower in obesity. In tissues, the activity of proinflammatory transcription factors such as NF-kB, are also augmented in obese subjects.

WAT is now recognized as an endocrine organ, containing adipocytes and their precursors, pre-adipocytes, as well as connective tissue matrix, nerve tissue and immune cells. Obesity has been linked to an increased macrophage infiltration into visceral adipose tissue. Macrophages are more prevalent in the adipose tissue of obese subjects than in that of lean subjects and the macrophage quantity correlates with measures of insulin resistance. Both macrophages and adipocytes are capable of accumulating lipids and secreting cytokines. Interplay between macrophages and adipocytes through paracrine effects are presumably central in initiating and maintaining adipocyte dysfunction.

Some dietary patterns and specific food components have been associated with a lower prevalence of CVD in the population. For example, the traditional Mediterranean diet, characterized by a high fibre content, low-glycemic index carbohydrates, unsaturated fats, vitamins and antioxidant polyphenols has been linked to a lower incidence of CVD, obesity and T2D. MS patients following a Mediterranean diet, showed significant reductions in total weight, IR, and inflammation markers, and amelioration of endothelial function. Likewise, the French population presents a very low prevalence of CVD induced death in spite of consuming a diet rich in saturated fats and cholesterol. This phenomenon, known as "the French Paradox", has been ascribed to the moderate consumption of red wine, and specifically to the content of red wine in the polyphenols resveratrol and procyanidins. Procyanidins, which comprise the oligomeric forms of (+)-catechin and (-)-epicatechin, are also found in grapes, berries, apples, nuts and chocolate. Numerous population and intervention studies have demonstrated that procyanidin-rich diets reduce the risk of CVD and studies with animal models have shown the benefits of procyanidins in preventing and ameliorating obesity, diabetes, atherosclerosis, and hypertension. Since oxidative stress and concomitant inflammation is a common backstage to these diseases, the beneficial effects of procyanidins have been largely attributed to their well known antioxidant activity. Procyanidins have also been shown to interact with the plasma membrane and

nuclear receptors, serving as signalling agents and eventually modulating gene expression and cell metabolism.

Due to the central role of macrophages in obesity induced low-grade inflammation, we first analyzed procyanidin effects in an *in vitro* model of inflammation. We evaluated the PE effect on RAW 264.7 macrophages stimulated with lipopolysaccharide plus interferon-γ that showed a rapid enhanced production of prostaglandin E2 (PGE-2) and nitric oxide (NO). Our results demonstrated that PE significantly inhibited the overproduction of NO, dose and time dependently. PE caused a marked inhibition of PGE2 synthesis when administered during activation. Moreover, PE pretreatment diminished the iNOS mRNA and protein amount dose dependently (10-65 μg/mL). PE (65 μg/mL) pretreatment inhibited NF-kB (p65) translocation to nucleus by nearly 40%. Trimeric and longer oligomeric-rich procyanidin fractions from PE (5-30 μg/mL) inhibited iNOS expression but not the monomeric forms catechin and epicatechin. Thus, we show that the degree of polymerization is important in determining procyanidin effects. PE was a considerably more effective inhibitor of NO biosynthesis (IC50= 50 μg/mL) compared to other anti-inflammatories, such as aspirin (3 mM), indomethacin (20 μM), and dexamethasone (9 nM). In conclusion we showed that, PE modulates the inflammatory response in activated macrophages by inhibiting NO and PGE2 production, suppressing iNOS expression, and the translocation of NF-kB (p65) to the nucleus. These results demonstrate that grape seed procyanidins have an immunomodulatory role thus potential health benefits for inflammatory conditions.

The composition of PE has been investigated extensively, and is known to consist largely of gallic acid, catechin, epicatechin and procyanidin dimers and trimers composed of flavan-3-ol units with C4-C8 or C4-C6 interflavan linkages. After determining the effect of the whole extract and some fractions on NO and PGE-2 production, we analyzed the effect of some pure molecules present in the extract, in order to evaluate its individual bioactivity. We showed that some pure molecules caused a marked inhibition of PGE2 and NO synthesis when administered during

activation with LPS and IFN-γ. Interestingly, our results also showed that GSPE and some pure oligomeric procyanidins reduced intracellular ROS production induced by LPS and H_2O_2 in the human macrophage cell line THP-1. The trimer C1 and the dimer B1 were the most effective ones, followed by EGCG. We then concluded that monomeric structures and some dimers were effective as anti-inflammatory agents and that trimeric procyanidin C1 together with the monomer EGCG were the most active pure compounds tested.

The inflammatory condition characterized by enhanced production of pro-inflammatory adipokines is involved in the pathogenesis of IR and atherosclerosis. Excessive supply and storage of fat eventually leads to a disturbed capacity for the uptake of free fatty acids (FFA) and to necrosis, which contributes to the attraction and activation of macrophages and eventually to chronic inflammation. Activated macrophages interact with vascular epithelial cells and cause local inflammation contributing to the development of atherosclerosis. Despite significant progress in the management of atherosclerosis and its complications, CVD resulting from atherosclerosis remains the major cause of death in the Western world. Human and animal studies have demonstrated that procyanidin-rich diets reduce the risk of cardiovascular diseases and atherosclerosis. Some beneficial effects have been attributed to the well-known antioxidant activity of procyanidins. In our second study, we investigated another potential role of procyanidins in lipid accumulation and inflammation in macrophage-derived foam cells. We cultured RAW 264.7 macrophages with moderately oxidized LDL, minimally oxidized LDL or with LPS and oxLDL to induce foam cell. Cells were then treated with procyanidins derived from grape seed (PE, 45 μg/mL) for the last 12 h of incubation with the different lipoproteins (25 μg/mL). After lipid extraction, we determined that the accumulation of total and esterified cholesterol and triglyceride in foam cells was increased by lipoprotein treatment but reduced by PE incubation.

In order to asses the effect of PE on gene expression we determined the relative mRNA levels of CD36, ABCA1, iNOS, COX-2 and IkBα. We show that PE reduced the

oxLDL scavenger receptor expression (CD36), and enhanced ABCA1 (ATP-binding cassette A1) expression, a key regulator of macrophage cholesterol efflux. PE also downregulated inflammatory related genes such as iNOS and IkBα without modifying COX-2 expression. In conclusion, we provided evidence that procyanidins may attenuate the development of foam cell formation by reducing cholesterol accumulation and modulating the expression of key genes in cholesterol flux and inflammation.

By using rat models of obesity, we also assessed procyanidin anti-inflammatory effects *in vivo*. The main objective of the third study was to evaluate the effect of procyanidin intake on the level of inflammatory mediators in rats fed a hyperlipidic diet, which are a model of low-grade inflammation as they show an altered cytokine production. Male Zucker Fa/fa rats were randomly grouped to receive a low-fat (LF), high-fat (HF) or high-fat diet supplemented with procyanidins from grape seed (HFPE) (0.345 g/Kg feed) for 19 weeks. We assessed CRP, IL-6, TNF-α and adiponectin gene expression in liver and white adipose tissue (WAT). As expected, rats fed the HF diet showed enhanced CRP plasma levels. Our results demonstrate that HFPE diet decreases rat plasma CRP levels, but not IL-6 ones. Plasma CRP decrease in HFPE rats was related to a down regulation of CRP mRNA expression in the liver and mesenteric WAT. We have also shown a decrease in the expression of the pro-inflammatory cytokines TNF-α and IL-6 in mesenteric WAT. In contrast, adiponectin mRNA was increased in this tissue due to the procyanidin treatment. These results suggested a beneficial effect of PE on low-grade inflammatory diseases that may be associated with the inhibition of the pro-inflammatory molecules CRP, IL-6 and TNF-α, and the enhanced production of the anti-inflammatory cytokine, adiponectin.

As chronic low-grade inflammation characterized by adipose tissue macrophage accumulation and abnormal cytokine production is a key feature of obesity and type 2 diabetes, in our last study we looked in depth at the mechanisms by which PE could modulate local and systemic inflammation in two models of diet-induced obese rats. In the first one, we analyzed the preventive effects of procyanidins (11 mg/day) on rats

fed a diet with 60% of energy as fat. In the second, we induced obesity by feeding the animals with a cafeteria diet and we analyzed the corrective effects of two PE doses (10 and 20 mg/ day) as well as two periods of treatment (10 and 30 days). We showed that in the preventive model, PE reduced the body weight, which was accompanied by a decrease in the systemic inflammation markers, TNF-α and CRP in plasma. PE had little corrective effects on the body weight, but in the short treatment, the high dose of PE reduced both TNF-α and CRP plasma levels. The PE preventive treatment significantly increased adiponectin expression, and decreased TNF-α, IL-6 and CRP expression in white adipose tissue (WAT) as we previously reported. PE also reduced NF-kB activity in liver, which explains the low expression rates of hepatic inflammatory markers (TNF-α and CRP) found in rats treated with procyanidins. PE also decreased TNF-α expression in muscle. Finally, dietary supplementation with PE reduced macrophage infiltration of WAT, this being detected by the reduced expression of Emr1, the gene that encodes the specific marker of macrophage F4/80. We therefore concluded that orally ingested PE prevents the cytokine imbalanced production associated with obesity and type 2 diabetes, although its corrective effects need to be further investigated. The dietary intake of food or drinks containing procyanidins might be beneficial in preventing low grade inflammatory related diseases such as metabolic syndrome.

To sum up, our results showed that procyanidins act as anti-inflammatory agents both *in vitro*, by modulating the macrophage inflammatory response, and *in vivo*, by preventing both local and systemic inflammation. These findings confirm that procyanidins are good candidates for dietary supplementation and should be considered as functional food components.

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II. INTRODUCTION

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1. METABOLIC SYNDROME

The clustering of cardiovascular risk factors associated with abdominal obesity is well established. Although currently lacking a universal definition, the term metabolic syndrome (also called syndrome X or insulin resistance syndrome) describes a constellation of metabolic abnormalities, including abdominal obesity, and was originally introduced to characterize people at high cardiovascular risk [1-3]. In 1988, Reaven proposed that insulin resistance was a fundamental "disorder" associated with a set of metabolic abnormalities which not only increased the risk of type 2 diabetes but also contributed to the development of cardiovascular disease (CVD) before the appearance of hyperglycaemia [4]. Since the introduction of the syndrome X concept, many studies have assessed insulin resistance using various methods and have shown that it is indeed a key factor associated with clustering atherogenic abnormalities which include a typical prothrombotic profile, a state of inflammation and a state of atherogenic dyslipidemia (i.e. high triglyceride and apolipoprotein B concentrations, an increased proportion of small dense LDL particles, a reduced concentration of HDL-cholesterol and smaller HDL particles) [5]. Hyperinsulinemia and insulin resistance, defined as a failure of target organs to respond normally to insulin, may be important in the pathogenesis of, and often coexist with, hypertension, obesity, and diabetes. In general, new definitions have been presented to provide clinicians with a better tool for correctly identifying individuals with a higher cardiovascular risk as a result of the aggregation of multiple interrelated abnormalities which in turn are caused by reduced sensitivity to insulin. However, in almost all cases, the new emerging criteria have not displaced the older ones, meaning that almost all of the different criteria proposed are still used by various organizations and scientific societies in their operative definitions [6]. By most criteria, the definitions of MS used for its diagnosis, include the presence of three out of six altered metabolic parameters (Table 1).

Table 1: Different criteria proposed for clinical diagnosis of MS in human

Clinical measure	WHO (1998)	EGIR (1999)	AACE (2003)	ATP III (2004)	IDF (2005)	AHA/NHLBI (2005)
Ins-R	IGT, IFG, T2DM, or lowered insulin sensitivity [a] plus any 2 of the following:	Plasma insulin >75th percentile plus any 2 of the following:	IGT or IFG plus any of the following based on clinical judgment:	None, but any 3 of the following 5 features:	None	None, but any 3 of following 5 features:
Body weight	Waist-to-hip ratio N0.90 and/or BMI >30 kg/m2	WC ≥94 cm	BMI ≥25 kg/m2	WC ≥102 cm	Increased WC >94 cm plus any 2 of the following:	WC ≥102 cm
Lipids	TG ≥ 1.7 mM and/or HDL-C <0.91 mM	TG ≥2.0 mM and/or HDL-C <1.01 mM or treated for dyslipidemia	TG ≥1.69 mM and HDL-C <1.03 mM	TG ≥1.69 mM, HDL-C <1.03 mM	TG ≥1.7 mM or on TG Rx, HDL-C <1.03 mM or on HDL-C Rx	TG ≥1.69 mM or on TG Rx, HDL-C <1.03 mM or on HDL-C Rx
BP	≥160/90 mm Hg	≥140/90 mm Hg or on hypertension Rx	≥130/85 mm Hg	≥130/85 mm Hg	≥130 mm Hg systolic or ≥85 mm Hg diastolic or on hypertension Rx	≥130 mm Hg systolic or ≥85 mm Hg diastolic or on hypertension Rx
Glucose	IGT, IFG, or T2DM	IGT or IFG (but not diabetes)	IGT or IFG (but not diabetes)	>5.6 mM (includes diabetes)	≥5.6 mM (includes diabetes)	≥ 5.6 mM or on hypoglycemic
Other	Other mALB		Other features of Ins-R [b]			

The diagnostic criteria proposed by the World Health Organization (WHO) (1998); European Group for the Study of Insulin Resistance (EGIR) (1999); Adult Treatment Panel III (ATP III) (2001); American Association of Clinical Endocrinologists (AACE) (2003); ATP III (2004); International Diabetes Federation (IDF) (2005); and American Heart Association/National Heart, Lung, and Blood Institute (AHA/NHLBI) (2005). WC, waist circumference; TG, triglycerides; mALB, microalbuminuria; BMI, body mass index; IGT, impaired glucose tolerance; IFG, impaired fasting glucose.
a Insulin sensitivity measured under hyperinsulinemic euglycemic conditions; glucose uptake below lowest quartile for background population under investigation. **b** Includes family history of type 2 diabetes mellitus, sedentary lifestyle, advancing age, and ethnic groups susceptible to type 2 diabetes mellitus. Adapted from P. Strazzullo et al. (2008).

Potential causes of this growing epidemic include changes in dietary patterns and physical inactivity, although this may also include as yet unidentified genetic and environmental determinants. In this regard, experimental data provide evidence for a direct link between obesity and subclinical inflammation and support the concept that metabolic syndrome and type 2 diabetes are, at least in part, inflammatory conditions [7-9]. Furthermore, elevated levels of inflammatory biomarkers are not only associated with the development of diabetes [10] but also with cardiovascular disease [11]. These findings suggest that subclinical inflammation may be a contributing factor not only to the etiology of these metabolic disorders but also to their cardiovascular complications.

The pathogenesis of obesity associated metabolic syndrome is mediated by the disturbed production and release of biologically active molecules by fat cells and other

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cells infiltrating fat tissue (Figure 1) [12]. In obese subjects, synthesis of several bioactive compounds (adipokines), by either adipocytes or adipose tissue infiltrated macrophages, is dysregulated. These bioactive molecules participate in regulating the appetite, energy homeostasis, lipid metabolism (tumour necrosis factor α - TNF-α), insulin sensitivity (TNF-α, adiponectin, resistin, visfatin, immunity monocyte chemoattractant protein-1 – MCP-1, TNF-α, IL-6), angiogenesis, blood pressure and hemostasis (plasminogen activator inhibitor – PAI-1). Obesity enhances the expression, synthesis and release of pro-inflammatory adipokines (TNF-α, IL-6, haptoglobin leptin, resistin) but decreases expression, synthesis and release of anti-inflammatory adipokines such as adiponectin [13, 14].

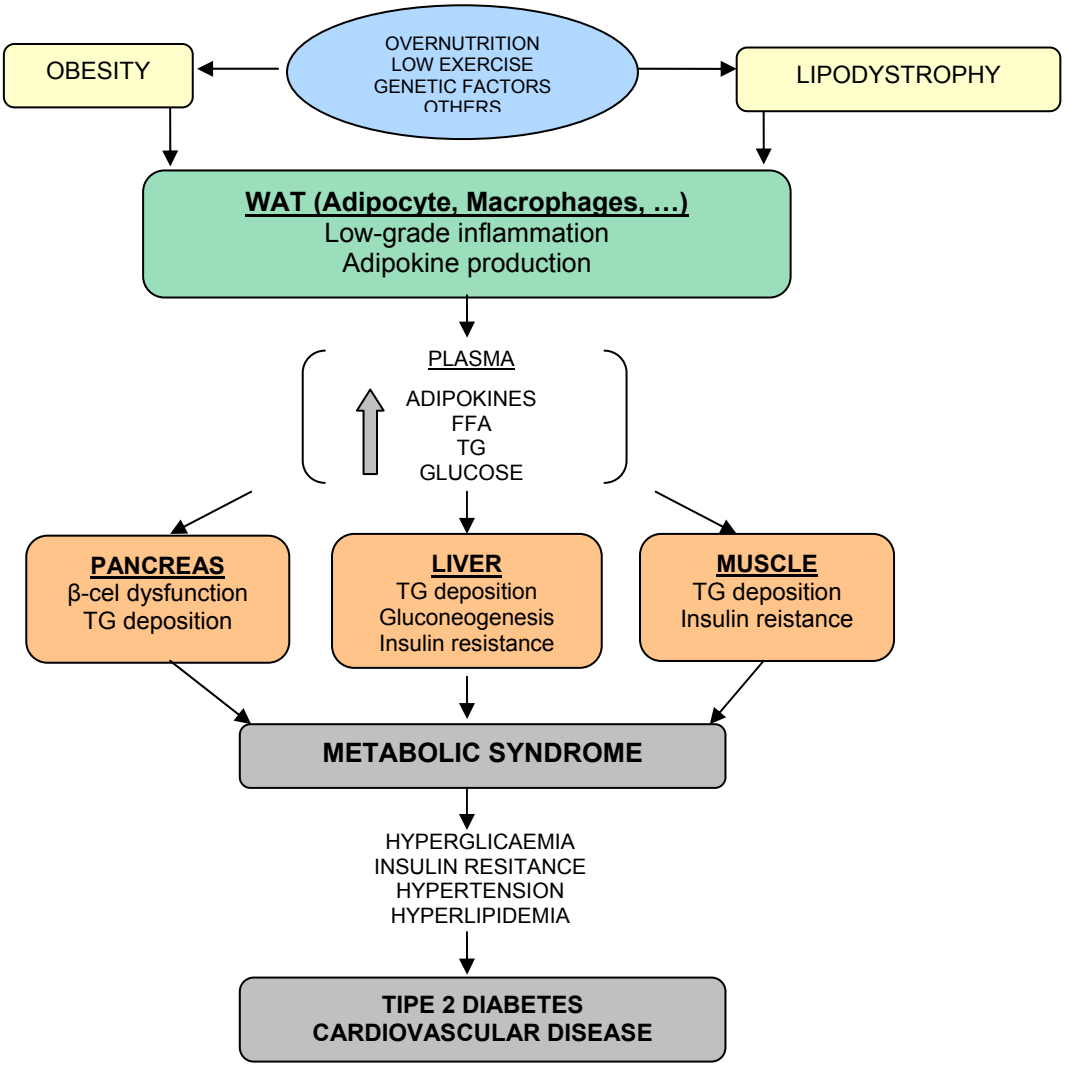

Figure 1: Simplified model illustrating the progression from obesity to T2D and CVD

There is a causal relation between obesity-related disorders such as cardiovascular disease (CVD) and diabetes, which means that the risk of mortality is significantly increased by the presence of systemic low-level inflammation, defined as 2-4 fold increases in the circulating levels of inflammatory mediators [15]. It has been suggested that metabolic syndrome involves inflammation with pathogenic mechanisms that influence its development. It has also been reported that the syndrome involves elevated CRP, IL-6 and TNF-α, associated with visceral adiposity. Adipocytes constitutively express the pro-inflammatory TNF-α, which decreases after weight loss. Further work in this area has confirmed increased plasma concentrations of CRP, IL-6, and plasminogen activator inhibitor-1 (PAI-1). Therefore, a complex interplay may exist between inflammatory responses and general metabolism in atherosclerosis, cardiovascular disease and metabolic syndrome.

2. RAT MODELS OF THE METABOLIC SYNDROME

Cardiovascular disease, the leading cause of death in much of the modern world, is the common symptomatic end stage of a number of distinct diseases and, therefore, is multifactor and polygenetic in character [16]. The two major underlying causes are disorders of lipid metabolism and metabolic syndrome. The ability to develop preventative and ameliorative treatments will depend on animal models that mimic human disease processes. The most useful and valid species/strains for the study of cardiovascular disease and metabolic syndrome appear to be small rodents, rats, and mice [17]. The development and the exploitation of animal models for the study of these phenomena are still at an early stage but offer the prospect of fundamental insights and novel clinical approaches to underlying aspects of metabolic syndrome. Rats are a social, highly adaptive, omnivorous species that resemble to some extend to humans in their metabolism and physiology [18]. The normal rat is typically resistant to the development of atherosclerosis, and this has led to the notion that the rat is not a

suitable subject for cardiovascular research. This idea might be correct in respect to the “normal” rat but is not necessarily correct in the case of genetic models of CVD that have been developed over recent years. With the recent sequencing of the rat genome, there is a renaissance of interest in the rat as a model organism for biomedical research because the development of new strains continues and will be accelerated by the perfection of techniques for direct genetic modification that have previously been restricted to the mouse.

2.1 NUTRITIONAL MODELS

HIGH-FAT DIETS

Researchers have been using fat-enriched, so-called high-fat (HF) diets, to generate obese rodent models. The studies have revealed that HF diets promote hyperglycaemia and whole body IR, and numerous researchers have examined their effects on muscle and liver physiology and on insulin signal transduction. Based on this experience, it is generally accepted that HF diets can be used to generate a valid rodent model for the metabolic syndrome with IR and compromised β-cell function [19]. An important downside, however, is the definition of the term “high fat diet” itself. A multitude of different semipurifyed chows have been used with relative fat fractions between 20% and 60% energy as fat, (a 10% energy as fat is used in low-fat control diets) and the basic fat component varies between animal-derived fats, and this has led to a considerable variability in the results reported. Prolonged feeding with fat-enriched diets induces an increase in body weight in susceptible rats in the range of 10% to 20% over standard chow-fed controls. Obesity induction is most effective when the diet is started at a young age and continues for several weeks. Body weight gain during the feeding period is gradual. Although an increase in body weight can be appreciated after as little as 2 weeks, the diet-induced phenotype becomes most apparent after more than 4 weeks of HF feeding [19]. The data presented about general HF diet effects seems justifiable to conclude that HF feeding for several weeks leads to obesity,

(moderate) hyperglycemia and hyperinsulinemia, hypertriglyceridemia, and also changes in the plasma adipokine pattern resembling human obesity and IR in susceptible animals. Wistar and Sprague-Dawley rats are the strains used most frequently for this model.

The Cafeteria (CAF) diet fed rats is another diet induced obesity model. The CAF diet is a palatable hypercaloric and hyperlipidic diet that induces voluntary hyperphagia and fast body weight gain, that represent a useful model for human obesity studies [20]. The CAF diet offers the animal a wide variety of items of different composition and taste. Most of the items chosen (by the researcher) are high in fat and sugar, as well as hyper-caloric, compared to chow. Usual components of this diet are cookies, pastry, nuts, chocolate, candy, bacon and other meat products, cheese, milk, water and also nonpurified rat diet pellets, although the number of items presented varies between 2 and 40. It is also essential that this diet contains a mineral and vitamin complement to avoid interference from deficiencies [21]. The duration of feeding is an important and variable factor in experimental design. CAF diet has been reported to induce increased body weight and increased adipose mass in rats even after a short period of time. Commonly, between 3 and 8 weeks of feeding is used, although durations of up to 5 months have been reported. Of the various procedures currently available to promote obesity in rats, the CAF diet model has the advantage that it is similar to some human cases in that obesity is induced by voluntary hyperphagia of energy-rich food [20].

The leading causes of obesity, among others, are nutritional overload, physical inactivity, and genetic and enviromental determinants. The yet presented characteristics of the nutritional model validate it as an appropriate model for the metabolic syndrome study. For this reason to asses the procyandin effect *in vivo,* we used this model, where rats reached an obesity state due to the HF diet consumption. To further resemble the human common intake of procyanidins we administered them orally.

2.2 GENETIC MODELS

FATTY ZUCKER RATS

The fatty Zucker strain incorporates a spontaneous mutant gene (*fa* or fatty) that affects the action of the adipocyte peptide hormone leptin, a key element in the regulation of food intake through the inhibition of the release of hypothalamic neuropeptide Y. *fa* mutation was much later shown to cause a glycine-to-proline substitution at position 269 of the leptin receptor (ObR), resulting in a 10-fold reduction in binding affinity for leptin [22]. When homozygous for the *fa* gene (*fa/fa*), the Zucker rat develops a variant of metabolic syndrome, becoming obese, moderately insulin resistant, and hypertriglyceridemic, but with no progression to diabetes or cardiovascular complications. Heterozygous animals or those homozygous normal (Fa/*fa* or Fa/Fa) animals are lean and metabolically normal.

ZUCKER DIABETIC FATTY (ZDF) RATS

The ZDF was developed from the original Zucker colony. Obese male rats of a colony were found to have become diabetic, and were selectively bred for this trait to create a stable new strain, the ZDF. The onset of diabetes in this strain appears to be related to the loss of GLUT-2 glucose transporters in pancreatic β cells and to concomitant loss of muscle GLUT-4 transporters. Thus, diabetes in this strain is associated with impaired insulin secretion and peripheral glucose transporter function [23].

SHROB RATS

In 1973, Koletsky isolated a new mutation in a rat line originating from a cross between Sprague–Dawley rats and spontaneously hypertensive rats (SHR). Animals of this strain were hypertensive and became highly obese, hyperlipidemic, hyperinsulinemic and developed CVD with atherosclerosis [24]. The mutation resulted in a Tyr763Stop nonsense codon leading to the absence of the transmembrane portion of the ObR and, thus, of the activity of all isoforms of the ObR. Initially the mutation was designed as *f*,

later as *cp* (corpulent), and as fa^k, and recently as $Lepr^{cp}$. Rats of strains that are homozygous for the autosomal recessive *cp* gene (*cp/cp*) develop obesity, hyperlipidemia, and insulin resistance, with resultant hyperinsulinemia. Animals that are heterozygous (*cp/+*) or homozygous normal (+/+) are indistinguishable, lean, and metabolically normal [17].

ZSF1 RATS

The ZSF1 rat is the most recently produced rat strain for the metabolic syndrome. It is generated by crossing a female heterozygous lean ZDF rat with a male heterozygous lean SHHF/Mcc-fa^{cp} rat. Obese ZSF1 are *fa*/fa^{cp} at the leptin receptor gene locus, and lean ZSF1 are either +/*fa*, +/fa^{cp} or +/+ at the leptin-receptor gene locus. Recent research using this strain has focused on hypertension, Type 2 diabetes, hyperlipidemia, nephropathy, and metabolic syndrome [25].

JCR RATS

The JCR:LA-cp strain has been maintained as a closed outbred colony from the early stages of development. This has maintained the highly insulin resistant status and the atherosclerosis-prone trait that have been lost in the SHROB, SHR/N-cp, and LA/N-cp strains. IR develops rapidly in young male *cp/cp* rats of the JCR:LA-cp strain and is highly correlated with the development of CVD and is prone to stress-induced myocardial infarcts. The lesions resemble the intimal atherosclerosis commonly seen in human coronary arteries [17, 18].

OBESE PRONE RATS

Developed from a line of Crl:CD(SD) rats. Two lines were developed from this outbred colony, the OP-CD (Obese Prone) and OR-CD (Obese Resistant). This model becomes obese when fed HF diets. Obesity develops despite having a fully functional leptin receptor. This strain is used for the study of obesity, and metabolic syndrome [26].

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3. THE INFLAMMATORY RESPONSE

The inflammatory response is activated in response to cell injury caused by trauma or infection and constitutes a complex network of molecular and cellular interactions directed at facilitating a return to physiological homeostasis and tissue repair. The response is composed of both local events and a systemic activation mediated by cytokines. Although the inflammatory responses are different in various diseases, they can be characterised by a common spectrum of the genes and endogenous mediators involved, including growth factors, inflammatory cytokines such as interleukin 1 (IL-1), tumour necrosis factor-α (TNF-α), interleukin-6 (IL-6), chemokines (MCP-1, IL-8), matrix metalloproteinases, and toxic molecules such as RNS and ROS [27]. If tissue health is not restored or if there is stable low grade irritation, inflammation becomes a chronic condition that continually damages the surrounding tissues. The collateral damage caused by this type of inflammation usually accumulates slowly, sometimes asymptomatically, for years but can eventually lead to severe tissue deterioration [28].

Cytokines are secreted molecules that act on the surrounding microenvironment by providing cell to cell signalling. Cytokines are components of a large, complex signalling network. The effects of cytokines on target cells may be inhibited or enhanced by other cytokines, hormones, and cytokine-receptor antagonists and circulating receptors. Tumor necrosis factor α (TNF-α), Interleukin-1 (IL-1) and IL-6 are pro-inflammatory cytokines [29]. Their ability to activate both local and systemic effects is well established. Locally, they contribute to the activation of the inflammatory cells and together with chemokines, which induce the expression of adhesion molecules, cause their local recruitment. When the causes of the inflammatory reaction are of a high intensity, the production of cytokines is increased and they are released into the circulation, thus provoking the "acute phase response". On the other hand, "inhibitory" cytokines such as IL-10 damp down the activation of some effector functions of T

lymphocytes and mononuclear phagocytes by inhibiting the release of pro-inflammatory cytokines and therefore turning off the inflammatory processes [30].

The acute phase response includes leukocytosis, fever, somnolence, anorexia and the acute phase synthesis of proteins such as C reactive protein (CRP) in the liver. It is a highly conserved inflammatory response which is rapidly activated by infections or trauma via pattern recognition molecules. Acute phase protein concentration rapidly increases after its induction, and the production of these proteins is controlled primarily by IL-6- and IL-1-type cytokines. The acute phase proteins provide enhanced protection against microorganisms and modify inflammatory responses by affecting cell trafficking and mediator release [31]. The more traditional view of acute phase proteins has extended beyond their role in the opsonization of microorganisms. Some acute phase proteins have anti-inflammatory effects while others have important effects on leukocyte activation and trafficking.

4. ADIPOCYTES, MACROPHAGES AND OBESITY

4.1 THE ADIPOSE ORGAN

Mammals are provided with an organ that has been neglected by scientists in the past, that is, the adipose organ. This organ is formed by a series of well-defined depots mainly located at two corporal levels: superficial (subcutaneous depots) and deep (visceral depots) (Figure 2). In adult rodents, two principal subcutaneous depots are the anterior and posterior depots. The first consists of a central body located in the area between the scapulae and several elongated projections abutting toward the cervical region and the axillae. The second extends from the dorsolumbar area to the gluteal, with an intermediate region located in the inguinal area [12].

The main visceral depots are tightly connected with viscera. In adult rodents, the main visceral depots are perirenal, perigonadal, mesenteric, and retroperitoneal. The weight of the adipose organ is about 20% of the body weight and therefore it is one of the

biggest organs in the body. Its colour is mainly white but in some areas it is brown. In young-adult rodents kept under standard conditions, the interscapular region and parts of the cervical and axillary projections of the anterior subcutaneous depot are brown, as are parts of the perirenal depots. There are two types of adipose tissue depending on its cell structure, location, colour, vascularization and function: white adipose tissue (WAT) and brown adipose tissue (BAT). WAT is the primary site for storing energy in the form of TGs in the lipid droplets of the adipocytes, whereas BAT contains multilocular adipocytes or cells with various lipid droplets. It has a large number of mitochondria and specializes in heat production and, therefore, energy expenditure. Nevertheless, in humans, BAT is only present in newborns for regulating thermogenic processes [32]. The mass of human adipose tissue changes dramatically. In fact, no other tissue can vary in the way that adipose tissue does, when it regulates the delicate equilibrium between the fat depot and fat utilization. Obesity is defined as an excessive growth of adipose tissue mass. The relative amount of the two tissues varies with age, strain, environmental and metabolic conditions, and consequently, the distribution of the two colours also varies and indicates the reversible transdifferentiation of the two types of adipocyte [33].

Adipose tissue contains adipocytes and a vascular-stromal fraction in which macrophages, fibroblasts, endothelial cells and preadipocytes are present [34]. Pre-adipocytes originate from a multipotent stem cell of mesodermal origin and the potential to generate new fat cells persists throughout the lifetime of the individual. Adipose tissue can be classified according to its function. The three primary and classic roles of adipose tissue are to insulate and cushion the body, to store free fatty acids (FFAs) after food intake and to release FFAs during the fasting state to ensure sufficient energy status.

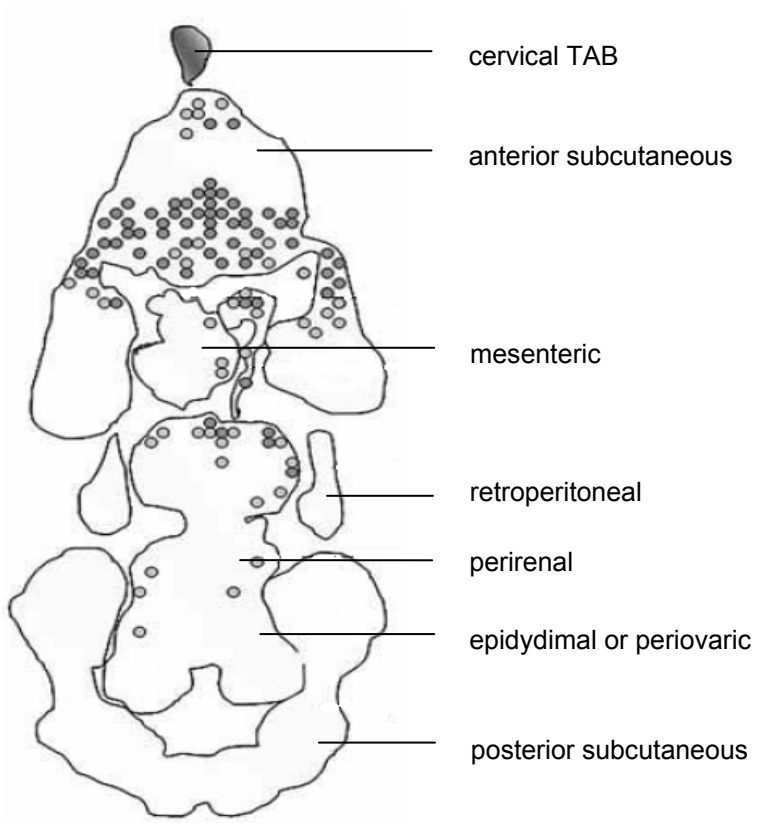

Figure 2: Gross anatomy of the adipose organ. White and brown areas are indicated in the scheme. Circles indicate brown areas (from Fantuzzi et al., 2007).

During the postprandial phase FFAs are taken from the blood in adipose tissue after lipoprotein lipase (LPL) has caused the triglycerides (TG) to be hydrolyzed from triglyceride-rich lipoproteins (i.e. from very low-density lipoprotein-cholesterol (VLDL-c), chylomicrons and their remnants). Mobilization of this reserve occurs by hydrolysis of adipocyte TG by hormone sensitive lipase (HSL). Insulin is the main regulator of adipocyte fat content, because it is both a potent inhibitor of HSL and an important activator of LPL, thus enhancing FFA uptake and triglyceride synthesis in adipocytes [35]. Finally, adipocytes secrete adipokines, which include hormones, cytokines and other proteins with specific biological functions. For these reasons, adipose tissue has an important influence on physiological processes such as the development and growth of the adipocyte and energy homeostasis. In addition, adipocytes and adipose tissue are actively involved in metabolic processes such as angiogenesis,

adipogenesis, extracellular matrix dissolution and reformation, steroid metabolism, immune response and hemostasis [36]. Therefore, it is clearly important that adipose tissue maintains this functionality; however, this can be highly affected by obesity.

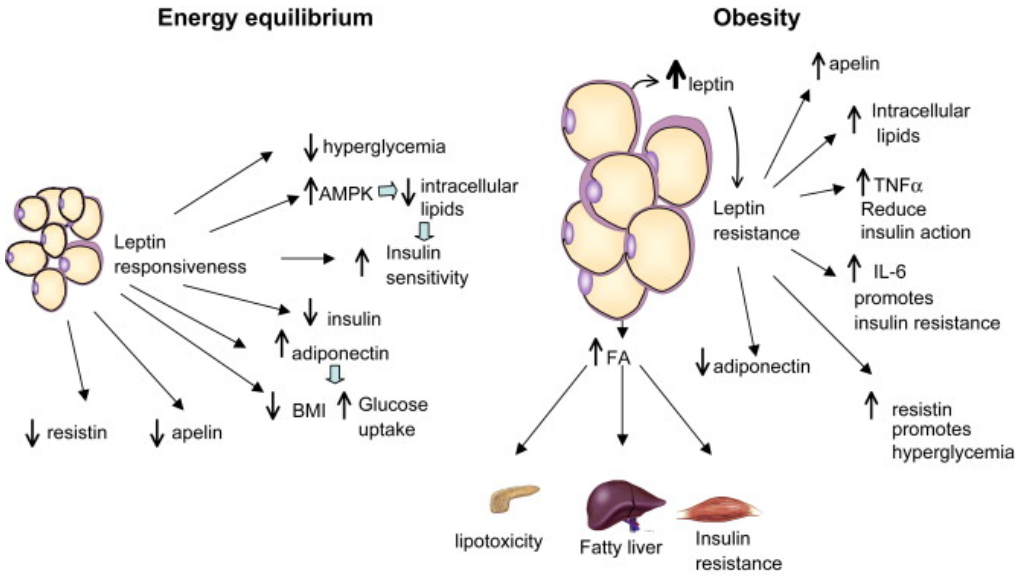

Figure 3: Obesity and adipocyte response. Adipocytes during energy equilibrium are leptin responsive and nonhypertrophic and nonadipose tissues are leptin and insulin sensitive. Under this condition adipocytes secrete adipokines to stimulate insulin sensitivity and fatty acid (FA) oxidation. Adipocytes during obesity are hypertrophic and nonadipose tissues are resistant to leptin and insulin action. Adipocytes secrete high amounts of FAs as well as adipokines that promote insulin resistance resulting in the ectopic accumulation of lipids in the pancreas, liver and skeletal muscle (from Vázquez-Vela et al., 2008).

It is becoming clear that the increased adipose tissue is an endocrine organ and not simply a reservoir for excess nutrients. It is an active and dynamic organ capable of expressing several cytokines and other fat-derived peptides (Figure 3). This regulation is achieved through endocrine, paracrine and autocrine signals that allow the adipocyte to regulate the metabolism of other fat cells or cells located in the brain, liver, muscle and pancreas.

A positive correlation was found between the body mass index and the percentage of resident macrophages, suggesting that fat tissue growth is associated with a recruitment of blood monocytes, which are responsible for cytokine production. This infiltration seems to be an important cause of the inflammatory state associated with obesity [13].

Visceral vs. subcutaneous adipose tissue

Subcutaneous and visceral adipose tissues have functional differences. For example, genes for angiotensinogen (blood pressure regulation), complement factors and fatty acid-binding protein 4 (involved in fatty acid trapping in adipocytes) are expressed at higher levels in visceral adipose tissue than in subcutaneous fat [37]. Leptin, however, is mainly produced by human subcutaneous adipose tissue whereas TNF-a is equally produced by both fat depots [29], although others have reported different findings from *in vitro* studies [35].

In contrast to subcutaneous adipose tissue, abdominal adipose tissue drains directly into the portal circulation. Although it is known that macrophages infiltrate adipose tissue during the development of obesity, it now appears that the infiltration rate of monocytes into visceral adipose tissue is higher than into subcutaneous adipose tissue [35, 38]. A study on extremely obese patients indicates that visceral adipose tissue is the main contributor of plasma IL-6 concentration [39]. It is therefore conceivable that viscerally produced adipokines directly influence liver function because IL-6 is an inducer of liver C-reactive protein (CRP) production and of the proteins involved in hemostasis (PAI-1, fibrinogen, tissue plasminogen activator). IL-6 also increases dyslipidaemia via the disinhibition of the microsomal TG transfer protein which controls the hepatic assembly of apolipoprotein B (ApoB)-containing lipoproteins *in vitro*. Centrally obese women have lower adiponectin levels than women with peripheral obesity. *In vitro*, human visceral fat cells secrete more adiponectin than subcutaneous cells and this secretion ratio between compartments declines as the body mass index (BMI) increases. Both visceral and subcutaneous adipose tissues are innervated by the autonomic nervous system, with different motor neurons for each depot and controlled by neuro-endocrine feedback. Stimulation of the parasympathetic nervous system leads to an anabolic state with decreased lipolysis, whereas stimulation of the sympathetic nervous system leads to a catabolic state with reduced adipogenesis and

stimulated lipolysis. However, at present it is unclear whether and how these different modes of neural innervation lead to functional differences in adipose tissue [12].

4.2 MONOCYTE/MACROPHAGE PHYSIOLOGY

Macrophages are mononuclear phagocytes found in almost all tissues. They are derived from a common pool of circulating monocytes. Although different macrophage populations possess distinct tissue-specific morphologies and phenotypes, they also share common functions that are required to maintain tissue homeostasis. As "professional phagocytes," macrophages clear apoptotic and necrotic cells, cellular debris, and foreign pathogens [40]. They are the primary coordinators of the innate immune response and act as immune sentries that are often the first to recognize exogenous pathogens. Through a series of pattern recognition receptors, including Toll-like and complement receptors, nucleotide-binding oligomerization domain proteins and mannose binding proteins, macrophages are able to identify almost all common microbial pathogens and orchestrate the initial inflammatory response. Macrophages are also responsible for scavenging endogenous debris, including cellular products of abnormal metabolism (e.g. ferrous sulphate in iron-overloaded states), and apoptotic and necrotic cells. Through the use of scavenger receptors, macrophages recognize and rapidly clear both cellular debris and dead cells [41, 42]. Two populations of macrophages exist within tissues, resident and elicited macrophages. Resident macrophages are cells found in tissues in the absence of pathological stimuli. They represent a stable population of macrophages that turnover at a basal rate. Elicited macrophages arrive in tissues in response to stimuli that have classically been defined as immunological [12]. However, the accumulation and maturation of macrophages in atherosclerotic lesions is an example of a population of elicited macrophages that are recruited in response to a stimulus that can more accurately be described as metabolic [43, 44]. It seems reasonable to hypothesize that with the onset of obesity the

accumulation and maturation of macrophages occurs through a process that entails the same basic steps of recruitment, differentiation, and activation that are characteristic of macrophage populations elicited by classic immunological provocations.

The initial step in the accumulation of elicited macrophages is the recruitment of monocytes (Figure 4). Monocytes and other circulating leukocytes have two classes of molecules on their cell surface, integrins and mucins, which are critical for their attachment to endothelial cells and transmigration through vessel walls. Endothelial cells express the counter-receptor selectins that bind mucins and receptors for integrins. In response to underlying activation signals, such as injury, microbial infection, or lipid accumulation, endothelial cells become “activated” [45]. Upon activation, endothelial cells release the contents of Weibel-Palade granules that contain P-selectin and upregulate the expression of E-selectin, and they also upregulate the expression of the integrin receptors, including intercellular adhesion molecule (ICAM)-1 and vascular cell adhesion molecule (VCAM)-1. The selectins are deposited on the surface of endothelial cells and allow initial attachment and rolling of monocytes along the endothelium. Firm adherence and efficient extravasation require the binding of integrins on monocytes by ICAM-1 or VCAM-1. For example, during the early evolution of an atherosclerotic plaque, endothelial cells overlying fatty streaks become activated and increase P- and E-selectin and ICAM-1 expression. Genetic deletion of either selectins or ICAM-1 markedly attenuates the development of atherosclerotic lesions in mouse models of atherosclerosis [45].

After attachment and extravasation, the migration of monocytes into and through tissues is dependent on MCPs [46]. These chemokines are released from activated endothelia, macrophages, and mesenchymal cells in response to pathological stimuli. MCP-1 (also known as chemokine C-C motif ligand 2 [CCL2]) is the best characterized of the MCPs and binds to chemokine C-C motif receptor 2 (CCR2) [47]. In mice, genetic deletion of CCL2 or CCR2 impairs accumulation of elicited macrophage

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populations, decreases the clearance of microbial pathogens, and markedly attenuates disease in mouse models of atherosclerosis [48].

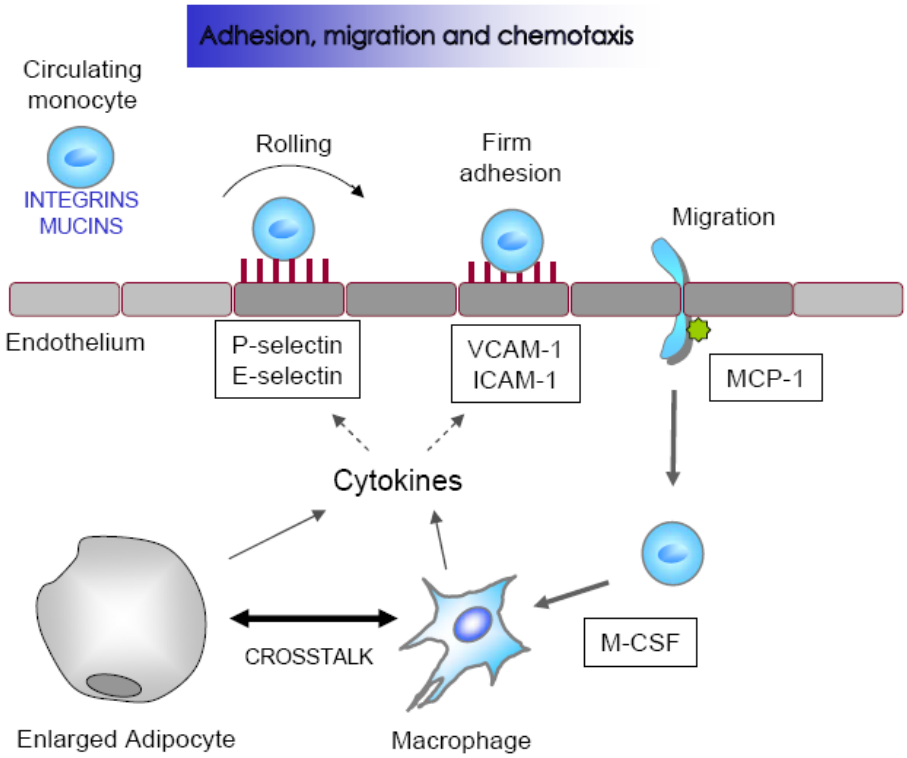

Figure 4: Recruitment of monocytes into adipose tissue.

Once monocytes have been recruited and enter a tissue, their differentiation into macrophages depends upon local signals. Colony-stimulating factors (CSFs) regulate the proliferation, differentiation, and survival of hematopoietically derived cells. M-CSF, also known as CSF-1, is the primary regulator of monocyte and macrophage differentiation [49]. In the absence of M-CSF, other CSFs, including GM-CSF and IL-3, can partially compensate and support monocyte differentiation [50]. Resident macrophage populations in *Csf1 op/op* mice or in mice that carry a targeted mutation in the M-CSF receptor (*Csf1r –/–*) are reduced, some by as much as 80% [51]. In the absence of M-CSF, elicited macrophage populations are markedly reduced, so that *Csf1 op/op* mice that are also deficient in *Apoe* are completely resistant to developing atherosclerosis, despite having circulating cholesterol levels 10 times higher than those of *Csf1* +/+ *Apoe* –/– atherogenic mice.

Macrophages can exist in quiescent or activated states. As sentries monitoring the presence of exogenous or endogenous pathogens, quiescent macrophages produce few inflammatory signals. When their pattern recognition receptors, scavenger receptors and cytokine receptors are engaged macrophages become activated [52]. The varied activation phenotypes of macrophages are signal-dependent but are broadly characterized by the expression and secretion of proinflammatory molecules, increased motility, and phagocytosis. For example, interferon-γ and lipopolysaccharide (LPS) from Gram negative bacteria together stimulate monocyte differentiation and the production of TNF-α, IL-6, IL-1, and nitric oxide, the so-called “classic activation response”. Stimulation of macrophages by IL-4 or IL-13 leads to upregulation of MHC and production of MIP1 and transforming growth factor (TGF)-α, in a response known as the “alternative activation response.” As the effects of various stimuli have been characterized, it has become apparent that there are not a few discrete activation states but rather a broad and varied spectrum of responses that are stereotypical for individual stimuli and contexts. Although there are clearly many different activation states, NF-kB activation is a common feature of many. For example, TNF- α, ER stress, and Toll-like receptor activation each activate NF-kB [53-55].

4.3 MACROPHAGES ARE IMPLICATED IN OBESITY-INDUCED ADIPOSE TISSUE INFLAMMATION

Studies of obesity-induced inflammation have until recently focused on the effects of specific inflammatory molecules on metabolically important cell types, including hepatocytes, adipocytes, myocytes, and hypothalamic neurons. Several recent analyses of adipocyte and nonadipocyte cell populations within adipose tissue demonstrate that much adipose tissue inflammatory gene expression is derived from the nonadipocyte, stromal vascular cells [56]. In the original model proposed by Hotamisligil et al. [57], obesity-induced increases in lipid content of adipocytes activate an inflammatory pathway in adipocytes. In this model, the resulting proinflammatory

cytokines act in autocrine fashion to impair adipocyte metabolic function, in a paracrine fashion to alter preadipocyte differentiation, and in a systemic fashion to reduce insulin sensitivity.

The role of classic inflammatory and immune cells has not been clearly delineated in adipose tissue physiology or obesity-associated phenotypes. However, sporadic reports have noted a correlation between adiposity and circulating leukocyte numbers, and decreases in leukocyte counts when weight is lost [58, 59]. Although these leukocyte studies primarily focused on how adiposity affects classical immune functions (i.e., responses to infections) they also suggested that obesity-induced inflammation is attributable to immune cells.

Some studies have revealed that macrophages accumulate in the adipose tissue of obese mice and humans [12], and contribute significantly to obesity-induced inflammation. In mice, the percentage of macrophages contained within each depot correlates strongly to adipocyte size [12]. In lean animals, approx 5% of the cells in adipose tissue were macrophages, but in severely obese, leptin-deficient mice, 50% or more of the cells in mesenteric, perigonadal, and perirenal adipose tissue depots are macrophages. In these morbidly obese mice the macrophage content of subcutaneous adipose tissue is markedly increased by about 30%, although it is slightly lower than that observed for visceral depots. However, the average adipocyte size is also lower in the subcutaneous depots than it is in the visceral depots of leptin-deficient mice [60]. Multinucleated giant cells formed from macrophages are characteristic of chronic inflammatory conditions, such as Wegener's granulomatosis and mycobacterial infections. In the adipose tissue of obese rodents and humans multinucleated giant cells are common [61]. Preadipocytes have a significant phagocytic capacity, and previous reports had suggested that preadipocytes may transdifferentiate into authentic macrophages. However, lethally irradiated mice with donor-tagged bone marrow demonstrated that *in vivo* in obese mice at least 85% of adipose tissue macrophages

are bone marrow-derived [62], suggesting that the vast majority of cells identified as adipose tissue macrophages (ATMs) are of bone marrow origin and do not arise from resident preadipocytes. Consistent with studies of isolated stromal vascular cell populations, expression studies of macrophages isolated from the stromal vascular fraction (SVC) of adipose tissue reveal that ATMs are responsible for a large portion of the proinflammatory gene expression that increases with the onset of obesity [28, 62, 63]. Secreted factors produced by adipocytes can induce the expression of TNF-α and other proinflammatory proteins by macrophages, this being consistent with the effects of obesity on adipose tissue gene expression [64]. An important caveat to these ex vivo and *in vitro* studies is that separation of adipose tissue cell populations or manipulation of cell lines may induce inflammatory changes that are not physiological. Indeed, standard techniques to isolate adipocytes and stromal vascular cells do induce, at least transiently, the expression of proinflammatory genes. Nonetheless, data support a model in which adipocytes can activate ATMs, thereby contributing to obesity-induced adipose tissue inflammation.

Adiposity also regulates the macrophage content of human adipose tissue depots. Body mass index (BMI) and adipocyte size are strong predictors of the macrophage content of both subcutaneous and visceral adipose tissue depots [29, 62]. Several studies have now shown that, similar to findings in rodents, ATM content is dynamically regulated. Weight loss and thiazolidinedione treatment reduce the macrophage content of adipose tissue in humans [65]. However, Di Gregorio and colleagues suggest that insulin sensitivity may be a better predictor of ATM content than adiposity is. In human subcutaneous adipose tissue they did not detect a significant (p = 0.18) correlation between BMI and the expression of the macrophage-expressed gene *Cd68*, but they did observe a significant negative correlation of *Cd68* expression with insulin sensitivity (p = 0.02). Larger studies will be required to determine which metabolic characteristics independently predict ATM content.

4.4 RECRUITMENT OF MONOCYTES TO ADIPOSE TISSUE IN OBESITY

Although recent studies have established that obesity is associated with the accumulation of macrophages in the adipose tissue of rodents and humans, the mechanisms that regulate this process are only now being studied. The first step in the recruitment of monocytes to a tissue is the adhesion to endothelial cells. In human studies, obesity and impaired insulin sensitivity are associated with elevated circulating concentrations of cellular adhesion molecules, including ICAM-1, VCAM-1, and E-selectin [44, 66, 67].

In vitro adipocyte-conditioned medium can directly up regulate the expression of ICAM-1 and the platelet-endothelial cell adhesion molecule (PECAM), and increase adhesion, migration, and chemotaxis of monocytes [45]. Recently leptin and adiponectin have been shown to have opposing effects on endothelial cells: leptin increases monocyte adhesion to endothelial cells, and adiponectin reduces expression of adhesion molecules and other proinflammatory molecules by endothelium [14, 68].

Several lines of evidence have also implicated MCPs, in particular CCL2 (MCP-1) and CCL7 (MCP-3), in the recruitment and chemotaxis of monocytes in adipose tissue. In both rodents and humans, adipose tissue expression of CCL2 and CCL7 are increased by obesity and reduced following weight loss or thiazolidinedione treatment [46]. CCR2 is a high-affinity receptor for CCL2 and CCL7, and is expressed in hematopoietic cells, including circulating monocytes. Genetic deficiency of CCR2 in mice with a C57BL/6J background partially protects them from obesity when fed a high-fat diet [69]. Furthermore, *Ccr2*-deficiency reduces the macrophage content of adipose tissue. This reduction of macrophages is associated with a reduction in proinflammatory gene expression and a coordinate upregulation of metabolically important genes in adipose tissue. Of particular importance is the fact that adiponectin adipose tissue expression and circulating adiponectin concentrations were increased in obese *Ccr2–/–* mice compared with wild-type obese animals. Glucose tolerance and insulin sensitivity were

improved, and hepatosteatosis was reduced in obese mice deficient in *Ccr2*. Short-term pharmacological antagonism of CCR2 also reduced the macrophage content of adipose tissue and improved glucose tolerance and insulin sensitivity in obese mice [69]. In contrast, deficiency in *Ccr2* in mice with a DBA/J background had no discernable effect on the inflammatory character of adipose tissue or systemic metabolic parameters when these mice were fed a high-fat diet [70]. The genotype-dependent differences in these two strains of *Ccr2–/–* mice may derive from inherent differences in these strains when they develop obesity-induced inflammation or from differences in the manner in which the experiments were carried out. These differences may, therefore, provide important clues as to the genetic and environmental modifiers of obesity-induced inflammation and complications.

An important unanswered question is what are the primary events that induce monocyte recruitment and macrophage differentiation in the adipose tissue of obese individuals? One hypothesis is that obesity accelerates adipocyte death and turnover, and that, in keeping with the role of macrophages in other tissue, ATMs serve to clear dead cells. In genetically engineered mice where apoptosis is induced specifically in adipocytes by treatment with an exogenous drug, it has been demonstrated that massive adipocyte apoptosis does indeed induce macrophage recruitment to adipose tissue. Cinti and colleagues [61] have suggested that obesity induces adipocyte necrosis and that electron micrographic data suggest that macrophages form multinucleated giant cells specifically in response to adipocyte necrosis. They argue that ultrastructure analysis is not consistent with apoptosis but is distinctively characteristic of necrosis. Further studies are needed to clarify whether the apoptosis or necrosis are in fact mechanistically involved in monocyte/macrophage recruitment to adipose tissue in the setting of obesity.

Another compelling hypothesis suggests that obesity induces metabolic derangements in adipocytes that lead to the production of factors that activate endothelial cells, direct

chemotaxis and induce differentiation. Interplay between macrophages and adipocytes by paracrine effects are presumably central in initiating and maintaining adipocyte dysfunction. Adipocytes become enlarged as a consequence of hyperalimentation. Large adipocytes release more (saturated) FFAs which can bind to macrophage toll-like receptor-4 (TLR-4), resulting in NF-kB activation and ultimately leading to augmented TNF-α production. In turn, macrophage-derived TNF-α activates human adipocytes, thereby further inducing lipolysis and enhancing the expression of intracellular adhesion molecule-1 (ICAM-1), IL-6 and macrophage chemo attractant protein-1 (MCP-1). The diapedesis of monocytes from the blood to adipose tissue and the differentiation into macrophages is further facilitated by MCP-1 and ICAM-1. This local paracrine loop involving adipocyte-derived FFAs and macrophage-derived TNF-α establishes a gradual vicious cycle that presumably leads to a pro-inflammatory state of both macrophages and adipocytes (Figure 5).

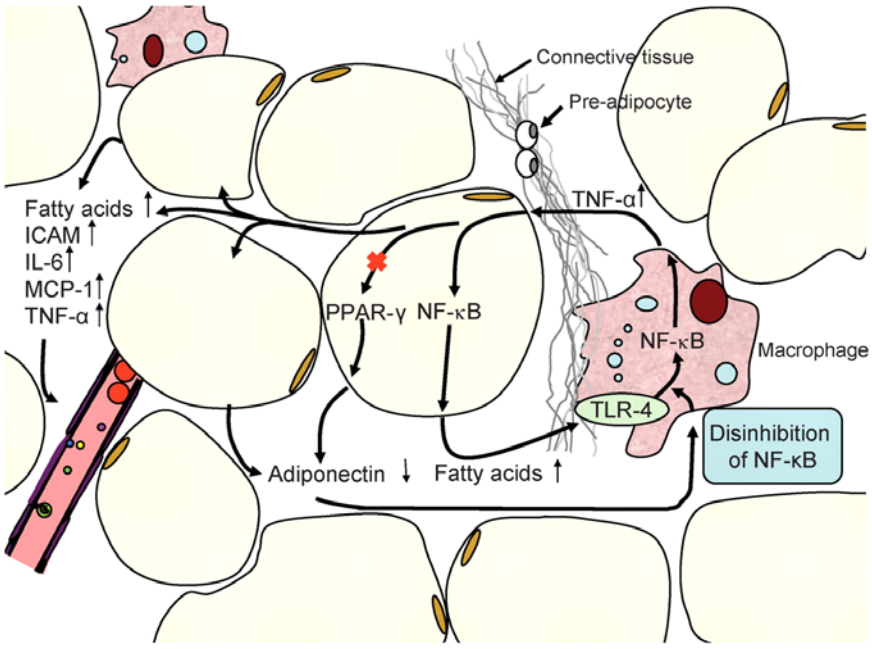

Figure 5: Adipocyte–macrophage interaction leading to dysfunction (from Hajer et al., 2008)

Of particular note is the fact that large adipocytes produce less adiponectin. Since adiponectin normally inhibits TLR-activated NF-kB activity, it is assumed that low adiponectin levels re-enforce the previously described loop [35].

Another possibility that has been recently suggested [71] is that hypoxia may occur in adipocytes distant from the vasculature as adipose tissue mass expands and that this underlies the inflammatory response exhibited by the tissue. Importantly, the expression and secretion of several adipokines, including FIAF/ angiopoietin-like protein 4, IL-6, leptin, macrophage migration inhibitory factor (MIF) and vascular endothelial growth factor (VEGF), is stimulated by hypoxia in human adipocytes, while adiponectin is inhibited [72]. These findings indicate that a number of genes are hypoxia-sensitive in human adipocytes and suggest that hypoxia-inducible factor 1 (HIF-1) is involved in the transmission of the response to low O_2 tension in these, as in other, cell types. The hypoxia-sensitive genes in adipocytes include those involved in basic metabolic processes, such as glucose transport, as well as those linked to energy balance, inflammation and angiogenesis. The stimulation of IL-6, leptin, MIF, PAI-1 and VEGF expression, together with the downregulation of adiponectin synthesis, is consistent with this hypothesis where hypoxia may underlie the inflammatory response in adipose tissue in obesity and play a causal role in the development of obesity-associated diseases. There was, however, no effect of hypoxia on the expression of TNFα, a key pro-inflammatory cytokine. The hypoxia-induced changes in IL-6 and adiponectin, with its anti-inflammatory action, in particular, might be expected to impact on insulin sensitivity and the macrophage content of the adipose tissue [72].

5. INFLAMMATORY MEDIATORS

5.1 CYTOKINES

TNF-α: The human protein is synthesized as a 26 kD membrane-bound protein (pro-TNF) that is cleaved by processing enzymes to release a soluble 17 kD TNF-α molecule. The soluble molecule can then bind to its main receptors TNFR1 and TNFR2. Adipocytes and macrophages are both the source and the target of TNF-α, a

multifunctional regulatory cytokine that is implicated in chronic inflammation contributing to atherosclerosis, the production of additional inflammatory cytokines, and induction of insulin resistance. TNF-α activates NF-kB, which initiates and organizes inflammatory changes in vascular tissue [10]. These vascular inflammatory changes produce endothelial dysfunction through expression of adhesion molecules in endothelial cells and vascular smooth muscle cells. In addition, increased TNF-α secretion from visceral fat appears to be a strong inhibitor of adiponectin promoter activity, which may partially explain the negative relationship between visceral adiposity and adiponectin levels [73]. In addition to its proinflammatory effects, TNF-α has been considered an important link to obesity and insulin resistance. Local elevations of TNF-α directly interfere with insulin signalling, which ultimately leads to insulin resistance. TNF-α expression and production are also increased in the adipocytes of obese individuals. In fact, levels of TNF-α mRNA are reported to be positively correlated to body fat [74]. Obesity markedly elevates the total release of TNF-α, IL-6, and IL-8 by adipose tissue but only that of TNF-α is enhanced in adipocytes. However, the vast majority of the TNF- α comes from the elicited macrophages in the adipose tissue [56, 75].

Interleukin-1: The IL-1α and IL-1β proteins are synthesized by a variety of cell types, including activated macrophages, stimulated B lymphocytes, and fibroblasts. IL-1α and IL-1β both belong to the same gene family of *Interleukin-1* and in humans are translated as precursor proteins with a molecular weight of 31 kDa. The processing of the isoforms of proIL-1α and proIL-1β by cellular proteases results in a mature form of the protein of approximately 17 kDa. They share only 26% homology at the protein level and 45% homology at the nucleic acid level. IL-1α and IL-1β are among the first identified cytokines and exert strong pro-inflammatory functions. IL-1α$^{-/-}$ mice have lower fasting glucose and insulin levels and improved insulin sensitivity, as determined by insulin tolerance testing, compared with wild-type controls. IL-1β and IL-6

concentrations together predict risk for T2D in humans better than either cytokine alone. IL-1β is able to reduce IRS-1 expression at a transcriptional level through a mechanism that is ERK dependent and at a posttranscriptional level it is independent of ERK activation. By targeting IRS-1, IL-1β is capable of impairing insulin signalling and action and could thus participate, in concert with other cytokines, in the development of IR [56, 76].

IL-6: In humans, it is a 184-amino acid protein with a calculated molecular mass of 20.9 kDa. IL-6 is a stress induced inflammatory cytokine that exerts pleiotropoic effects on a variety of tissues. Approximately 30% of circulating IL-6 is from WAT, with visceral WAT producing higher levels of IL-6 than subcutaneous WAT. Adipocytes and macrophages both contribute to WAT-derived IL-6, although the ultimate stimulus for IL-6 production in the presence of excess adiposity is currently unknown.

Increased serum IL-6 is a predictor of future cardiovascular problems. High levels of IL-6 are thought to be responsible for the increase in acute-phase proteins seen in obese patients, in particular CRP. This mechanism of inflammation has been proposed as having the same effect as low density lipoprotein cholesterol in its contribution to atherosclerosis. Similarly, IL-6 has been significantly associated with body mass index, waist circumference, and visceral adiposity in obese subjects. IL-6 tend to have a broad range of systemic effects on peripheral tissues such as muscle and liver, in addition to its effects on the endothelium [74].

MCP-1: The precursor form of monocyte chemotactic protein (MCP-1) consists of 99 amino acids with a signal peptide sequence consisting of 23 amino-terminal amino acids. The mature form of MCP-1 has 4 cysteine residues. The first two cysteine residues are in an adjacent position C-C, which characterizes MCP-1 as a member of the chemokine b subfamily. Human MCP-1 is a non-glycosylated protein consisting of 76 amino acids with a molecular weight of 8.7 KDa. MCP-1 mRNA expression can be

induced in adipocytes, monocytes/macrophages, B lymphocytes, endothelial cells and astrocytoma cells. The MCP-1 gene contains potential binding sites for several transcription factors, including AP-1, AP-2, NF-kB, and NF-IL6 [77].

Adipocytes secrete various chemoattractants that attract monocytes [77]. It has been demonstrated that obese adipose tissue exhibits increased expression of MCP-1 (or CCL2), a key factor in the recruitment of macrophages [65, 70]. It has been shown that MCP-$1^{-/-}$ mice exhibited reduced macrophage infiltration in the adipose tissue and reduced IR. Conversely, an increase in macrophage infiltration was observed when MCP-1 was overexpressed [69].

Another study also revealed reduced macrophage infiltration in the adipose tissue and decreased IR in MCP-$1^{-/-}$ mice [78]. Co-culture experiments with 3T3-L1 adipocytes and macrophages showed induction of MCP-1. The cross-talk between macrophages and adipocytes in cell culture seems to be attributable to the release of TNF-α by the macrophages and free fatty acids by the adipocytes. Activation of the TLR4 by fatty acids induces inflammatory changes in both macrophages and adipocytes by activating NF-kB. In addition, both TNF-α and IL-6 increase the expression of MCP-1 during differentiation of 3T3-L1 preadipocytes [79].

5.2 ADIPOKINES

Adiponectin: This adipokine exists both as a full-length protein as well as a proteolytic cleavage fragment, also known as globular adiponectin. Globular adiponectin can trimerize after cleavage, but cannot oligomerize further. Full-length adiponectin exists in serum as a trimer, a hexamer, and a high–molecular weight form, including 12–18 subunits. Adiponectin is mainly synthesized by adipocytes and to a lesser degree by other cells. Adiponectin circulates at high concentrations in human serum (5 to 10 μg/mL) and has a wide spectrum of biological activities. Serum levels of adiponectin are reduced in individuals with visceral obesity and states of IR such as non-alcoholic

fatty liver disease, atherosclerosis and T2D, and adiponectin levels correlate inversely with IR. TNF-α suppresses the transcription of adiponectin in adipocytes, which might explain the lower adiponectin levels in serum in individuals who are obese. Expression of adiponectin is also regulated by other pro-inflammatory mediators such as IL-6, which suppresses adiponectin transcription and translation in an adipocyte cell line. Weight loss induces adiponectin synthesis through the activation of peroxisome-proliferator–activated receptor γ (PPARγ) by its ligands thiazolidinediones (TZDs), which are used in the treatment of T2D [73, 80].

Initial studies suggested that adiponectin exerted anti-inflammatory effects on endothelial cells by inhibiting TNF-α induced adhesion-molecule expression and NF-κB activation. In obese animals, treatment with adiponectin decreases hyperglycaemia and levels of FFAs in the plasma and improves insulin sensitivity. Furthermore, adiponectin-deficient mice develop diet-induced IR on a high-fat, high-sucrose diet. In other studies with adiponectin-deficient mice, however, these animals either developed insulin resistance only if fed a high-fat diet or failed to develop insulin resistance even when fed a high-fat diet [81].

Two receptors for adiponectin have been identified: adipoR1 and adipoR2. Disruption of both receptors abolished adiponectin binding and actions, resulting in increased triglyceride content, inflammation, and oxidative stress, thus leading to IR and marked glucose intolerance.

Leptin: Human leptin is a 16 kDa protein that plays a critical role in the regulation of body weight by inhibiting food intake and stimulating energy expenditure. The discovery of leptin and the leptin receptor, the latter having both a long, full-length form and a short, truncated form, led researchers to hope that they had identified a highly effective molecule and/or pathway that could be targeted in the treatment of obesity. However, it soon became evident that obesity (a situation associated with high circulating leptin levels) resulted in leptin resistance in the central nervous system

(CNS) where endogenous leptin was no longer effective (leptin resistance) [28]. This phenomenon, although not completely understood, has been linked to a decreased uptake of leptin by the CNS. Another potential mechanism causing this resistance is an elevated expression of suppressor of cytokine signalling protein (SOCS), which occurs in both obese humans and rodents [36].

In addition to its well-defined role in energy balance, leptin has important effects on glucose homeostasis. First, leptin is able to reverse hyperglycaemia in *ob/ob* mice before body weight is corrected. It also improves glucose homeostasis in lipodystrophic mice and in humans with lipodystrophy or congenital leptin deficiency. Importantly, however, leptin fails to correct hyperglycaemia in patients with obesity, further supporting the idea that these patients have "leptin resistance". Leptin also improves insulin sensitivity in the liver. In this organ, leptin decreases intracellular hepatic triacylglycerol levels, as it does in muscle. There might also be a direct interaction with insulin metabolism, as leptin inhibits insulin release. Altogether, the data clearly support a role for leptin in the regulation of glucose homeostasis [82].

Resistin: The molecular weight of this protein in humans is approximately 12.5 kDa. Crystal structures of resistin reveal an unusual composition of several subunits that are held together by non-covalent interactions which make up its structure. Alpha-helical segments associate to form three-stranded coiled coils, and surface-exposed interchain disulfide linkages mediate the formation of tail-to-tail hexamers.

Resistin has been implicated in the pathogenesis of obesity-associated insulin resistance and T2D in mouse models, but it is still open to debate whether resistin has such a role in humans. Despite this, its pro-inflammatory properties indicate a role in inflammatory processes. Resistin and adiponectin have reciprocal effects on vascular endothelial cells: resistin induces the expression of VCAM1, ICAM1, and pentraxin 3, whereas adiponectin downregulates the expression of these molecules. Muse et al. [83] showed that hypothalmic resistin results in increased hepatic IR, which was

associated with increased expression of TNF-α, IL-6, and SOCS-3 in the liver. This observation not only provides new information as to how resistin might affect IR but also again demonstrates the role of the CNS in these metabolic processes [82].

Table 2: Adipose Tissue Biomolecules implicated in the Metabolic Syndrome

Molecule	Full name	Effects on
Leptin	Leptin	Food intake, fat mass
Adiponectin	Adiponectin	Insulin resistance, inflammation
Resistin	Resistin	Insulin resistance, inflammation
Visfatin	Visfatin	Insulin resistance
Omentin	Omentin	Insulin resistance
Vaspin	Visceral adipose tissue-derived serpin	Insulin resistance
Apelin	Apelin	Vasodilatation
CETP	Cholesteryl ester transfer protein	Lipid metabolism
LPL	Lipoprotein lipase	Lipid metabolism
HSL	Hormone sensitive lipase	Lipid metabolism
A-FABP 4 (aP2)	Adipocyte fatty acid-binding protein 4	Lipid metabolism
Perilipin	Perilipin	Lipid metabolism
RBP4	Renitol-binding protein4	Lipid metabolism
ASP	Acylation stimulating protein	Lipid metabolism
AT II	Angiotensin II	Blood pressure
ACE	Angiotensin converting enzyme	Blood pressure
AGT	Angiotensinogen	Blood pressure
TNF-α	Tumour necrosis factor-α	Inflammation
IL-6	Interleukin-6	Inflammation
CRP	C-reactive protein	Inflammation
Adipsin	Adipocyte trypsin/complement factor D	Inflammation
MCP-1	Macrophage chemo attractant protein-1	Macrophage attraction
ICAM-1	Intercellular adhesion molecule-1	Macrophage activation
PAI-1	Plasminogen activator inhibitor-1	Fibrinolysis

5.3 ACUTE-PHASE PROTEINS

C-REACTIVE PROTEIN

CRP was the first acute-phase protein to be described, and is an exquisitely sensitive systemic marker of inflammation and tissue damage [31]. While phosphocholine was the first defined ligand for CRP, a number of other ligands have been identified since then. In addition to interacting with various ligands, CRP can activate the classical

complement pathway, stimulate phagocytosis, and bind to immunoglobulin receptors. It is a member of the pentraxin family of plasma proteins, which are part of the lectin fold superfamily of calcium-dependent ligand-binding and lectin (carbohydrate-binding) proteins. CRP consists of five identical, non-covalently associated 23 kDa protomers arranged symmetrically around a central pole.

The CRP human gene, located on the short arm of chromosome 1, contains only one intron, which separates the region encoding the signal peptide from that encoding the mature protein. Induction of CRP in hepatocytes is principally regulated at the transcriptional level by the cytokine interleukin-6 (IL-6), an effect which can be enhanced by interleukin-1β (IL-1). Both IL-6 and IL-1β control expression of many acute phase protein genes through activation of the transcription factors STAT3, C/EBP family members, and Rel proteins (NF-kB). The unique regulation of each acute phase gene is due to cytokine-induced specific interactions of these and other transcription factors on their promoters. Thus, for the fibrinogen genes, STAT3 is the major factor, for the serum amyloid A genes, NF-kB is essential, and for CRP, the C/EBP family members C/EBPβ and C/EBPδ are critical for induction. In addition to C/EBP binding sites, the proximal promoter region of the CRP gene contains binding sites for STAT3 and Rel proteins. Interactions among these factors that result in enhanced stable DNA binding of C/EBP family members result in maximum induction of the gene [31].

In healthy blood donors, the median concentration of CRP is 0.8 mg/l, but following an acute-phase stimulus, values may increase 10000-fold. This value is the result of an increase in the plasma concentration of IL-6, which is produced predominantly by macrophages as well as adipocytes. In an apparently healthy population the median baseline value is slightly higher and tends to increase with age, with females showing slightly higher circulating concentrations. In most, but not all diseases, the circulating value of CRP reflects on-going inflammation much more accurately than do other

biochemical parameters of inflammation, such as plasma viscosity or the erythrocyte sedimentation rate [84].

Excess adiposity is associated with increased serum IL-6 and CRP and higher concentrations correlate to adipocyte hypertrophy. CRP is released by both the liver and adipose tissue. Circulating CRP concentrations are also higher in adults with metabolic syndrome, and increased CRP is an independent risk factor for type 2 diabetes and CVD [85]. An association between adiposity and CRP is also seen in children at the age of 10 to 11 years, suggesting that this relationship is one of the earliest steps in the path to chronic disease [7].

5.4 OXIDATIVE STRESS MEDIATORS

FREE RADICALS

During the last few decades, research data have prompted a passionate debate as to whether oxidation, or specifically, oxidative stress mediated by free radicals/reactive oxygen species (ROS)/reactive nitrogen species (RNS), is a primary or secondary cause of many chronic diseases. As a result, scientific resources have been focused to a large extent on the role that antioxidants could play in delaying or preventing oxidative stress and consequently the incidence of chronic disorders such as IR and atherosclerosis.

A free radical is any chemical species (capable of independent existence) possessing one or more unpaired electrons. Free radicals are formed from molecules by the breakage of a chemical bond so that each fragment keeps one electron (free radicals may also be formed by a collision of the non radical species through a reaction between a radical and a molecule -which must then result in a radical since the total number of electrons is odd), by the cleavage of a radical to give another radical and, finally by redox reactions. Radicals are generally less stable than non-radical species, although their reactivity varies [86].

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Important free radicals and reactive oxygen/nitrogen species in living organisms include hydroxyl (OH.), superoxide (O_2.-), nitric oxide (NO.), nitrogen dioxide (NO_2.) and peroxyl (ROO.). Peroxynitrite (OONO-), hypochlorous acid (HOCl), hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$), ozone (O_3), nitrous acid (HNO_2) and dinitrogen trioxide (N_2O_3) are not free radicals but can easily lead to free radical reactions in living organisms. 'Reactive oxygen species' (ROS) and 'reactive nitrogen species' (RNS) are collective terms that include not only radicals but also non-radicals. Oxidative stress is the term referring to the imbalance between the generation of reactive oxygen species and the activity of the antioxidant defences [87].

NITRIC OXIDE

Nitric oxide is formed from the oxidation of L-arginine by nitric oxide synthase (NOS), of which three isoforms are known, nNOS, eNOS and iNOS.

Excess NO is cytotoxic, both directly (e.g. by combining with tyrosine) and indirectly (by forming peroxynitrite (ONOO-)) [88]. NO relaxes smooth muscle in blood vessel walls resulting in lower blood pressure, therefore O_2.- could be a vasoconstrictor because it removes NO, and excess vascular O_2.- production could contribute to hypertension and vasospasm. ONOO- formed in blood vessel walls may aggravate atherosclerosis by depleting antioxidants and causing peroxidation of LDL. Furthermore, nitration of tyrosine by ONOO- may interfere with cell signal transduction [88]. A role for NO has also been demonstrated in such human diseases as cardiovascular disease, acute inflammation, and diabetes. Moreover NO has been implicated in adult septic shock, hypertension, and thrombosis.

Nitric oxide is produced by virtually all mammalian cells, including the endothelium lining the vasculature, the neurons of the central and enteric nervous system, and the cells of the immune system. Nitric oxide is constitutively produced by two distinct enzymes: an enzyme normally present primarily in the endothelium lining the vasculature (eNOS), and a neurally–associated NO synthase found in neurons of the

brain and enteric nervous system (nNOS). Activation of macrophages and many other cells with proinflammatory cytokines or endotoxin results in the expression of the inducible isoform of NOS (iNOS), the activity of which is functionally independent of changes in intracellular calcium [89]. The deletion of Inducible nitric oxide synthase (iNOS) is associated with an improvement in high-fat diet–induced IR [90]. Recently it has been demonstrated that blocking iNOS with *N*(G)-nitro-L-arginine methyl ester (L-NAME) improved high-fat diet– induced obesity and glucose intolerance. These effects were accompanied by reduced inflammation in the adipose tissue and improved signalling in skeletal muscle. During the course of an inflammatory response, large amounts of NO formed by iNOS surpass the physiological amounts of NO, which are usually made by nNOS or eNOS. The functions of iNOS-derived NO are also different. Produced by immunologically or chemically activated macrophages, NO kills microorganisms and nitrosylate macromolecules.

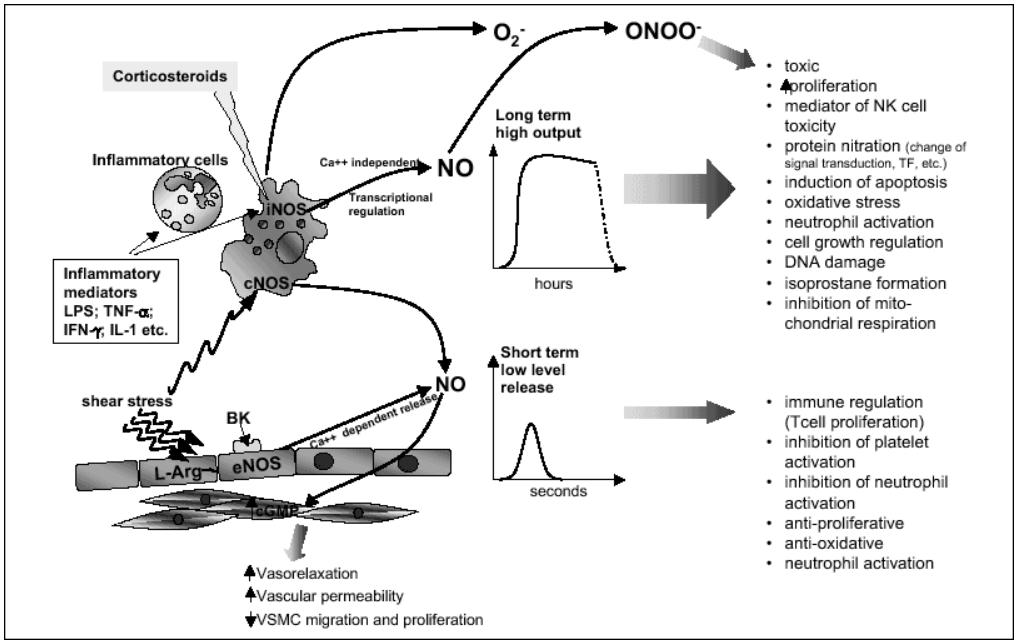

Figure 6: Kinetics and effects of nitric oxide produced by constitutive or inducible nitric oxide synthases (from Guzik et al., 2003)

Eventually, within a few seconds, NO is oxidized into nitrites or nitrates. Large amounts of "inflammatory NO" from myeloid cells are usually generated side by side with large amounts of superoxide anion (O_2-). These two can form peroxynitrite, which mediates

the cytotoxic effects of NO, such as DNA damage, LDL oxidation, isoprostane formation, tyrosine nitration, inhibition of aconitase and mitochondrial respiration [91]. The toxic properties of nitric oxide play a key role in the pathogenesis of septic shock. It is been claimed that over-production of NO by iNOS during septicaemia is responsible for irreversible arterial hypotension, vasoplegia (loss of responses to noradrenaline), lactic acidosis, necrosis and apoptosis. However, it is important to remember that NO made by iNOS is of benefit to the host defence reaction because it contributes to microbial killing.

Large amounts of NO and ONOO- may target numerous proteins and enzymes critical for cell survival and signalling. These include signalling molecules involved in cytokine signalling such as JAK or STAT proteins, the NF-kB/IkB pathway, MAPK, some G proteins and transcription factors. Nitration of cysteines in these proteins may lead to their activation or inactivation [92]. iNOS also represents another molecular mechanism linking cytokine-mediated inflammation to insulin resistance. Proinflammatory cytokines (TNF-α, IL-1 and interferon-γ) and endotoxins synergistically increased nitric oxide production by increasing the expression of inducible nitric oxide synthase (iNOS) in rat skeletal muscle and cultured myocytes and adipocytes. iNOS was increased in muscle and adipose tissue in genetic and dietary models of obesity [93, 94]. In addition, iNOS-deficient mice developed high-fat diet-induced obesity but had improved glucose tolerance, normal insulin sensitivity and normal insulin-stimulated glucose uptake in muscles [95]. Finally, treatment with iNOS inhibitor reversed fasting hyperglycaemia and hyperinsulinaemia and improved insulin sensitivity in *ob*/*ob* mice. iNOS inhibitor also increased the protein expression of IRS-1 and IRS-2, and enhanced IRS-1 and IRS-2-mediated insulin signalling in the liver of *ob*/*ob* mice [96].

5.5 PROSTAGLANDINS AND CYCLOOXYGENASES

Inflammation is characterized symptomatically by pain, redness, and swelling; disordered or excessive inflammation also entails loss of function. This clinical pathology is caused by the release of inflammatory mediators, predominantly from activated leukocytes and monocytes that migrate into the target area. Among the key inflammatory mediators are the n26 eicosanoids, prostaglandin E2 (PGE2) and leukotriene B4 (LTB4), which are derived from the n26 polyunsaturated fatty acid (PUFA) and arachidonic acid (AA; 20:4n26) [97].

Cyclooxygenase (PGH2 synthase, COX) donates 2 oxygen molecules to arachidonic acid to form PGG2 by peroxidation, which in turn is reduced to PGH2. This leads to the formation of PGE2, via concerted activation of PGE synthase (PGES). COX is the molecular target for the analgesic and anti-inflammatory remedies that have been used for hundreds of years. Three primary COX isoenzymes are distinguished: COX-1 (constitutive), COX-2 (inducible), and COX-3, which is detected mainly in the central nervous system. The expression of COX-1 is constitutive in many normal tissues at relatively stable levels and is believed to have some housekeeping functions, such as producing PG precursors for thromboxane in platelets, which is vital for regulating blood flow. In contrast to COX-1, COX-2 protein is only slightly expressed in most normal mammalian tissues when subjected to physical, chemical and biological stimuli, including UV light exposure, dioxin and LPS insult [98].

COX-2 mRNA expression is regulated during different stages in a complex manner. The earliest occurring induction mechanism is related to the finding that COX-2 mRNA contains an AU-rich element (ARE) in its 30-untranslated region, and this has some critical roles in the stability of its mRNA. Several reports of different cell types have shown that the activation of p38 MAPK leads to the stabilization of COX-2 mRNA. Next, a substrate for p38 MAPK, that is, MAPK-activated protein kinase 2 (MAPKAPK-2), induces the phosphorylation of certain candidate proteins, such as HSP27,

heterogeneous nuclear ribonucleoprotein A0, and Hu antigen R, which bind to AREs, thereby contributing to a rapid synthesis of COX-2 protein. In macrophages, LPS triggered induction and activation of signal transduction pathways lead to transcriptional activation of the COX-2 gene [99].

Members of the toll-like receptor (TLR) family, particularly TLR4, are now recognized as central receptors for LPS. TLR4 has several associated proteins, such as MD2 and Myd88, which are required for full activation, after which it stimulates various protein kinases, including PI3K and phosphoinositide-dependent kinase (PDK). The phosphorylated protein kinase B (Akt) in turn activates IkappaB (IkB) kinase (IKK), which causes NF-kB-induced COX-2 expression and PGE2 production [100].

Several NF-kB-targeted genes play major roles in the full induction of COX-2. Two of these genes are CCAAT enhancer-binding protein beta (C/EBPb) and delta (C/EBPd), which form b/b homodimer and b/d heterodimer, both of which have crucial parts in late and continuous COX-2 expression. In parallel, LPS insult of macrophages leads to the activation of the protein kinase A (PKA) pathway, in which PKA targets the cAMP-responsive element-binding protein (CREB), another major transcription factor for the COX-2 gene. It is also interesting to note that COX-2-generated PGE2 binds to its receptors (EP1-EP4) when activating adenylyl cyclase and thereby increases the level of intracellular cAMP. As mentioned above, this leads to the activation of PKA and, thus, CREB transcriptional activity. Collectively, these form a positive feedback loop in PKA-mediated COX-2 expression [99].

6. SIGNALLING PATHWAYS INVOLVED IN PRO-INFLAMMATORY RESPONSES

6.1 IKKβ/NF-κB pathway

The nuclear factor NF-kB is an exceptionally important transcription factor due to its pleiotropic effects, its inducible patterns, its unique regulatory mechanisms, its large number of activating signalling pathways and the number of genes that it controls

PROINFLAMMATORY CYTOKINES

Tumor necrosis factor α
Interleukin-1 β
Interleukin-2
Interleukin-6
Granulocyte colony-stimulating factor
Macrophage colony-stimulating factor

CHEMOKINES

Interleukin-8
Macrophage inflammatory protein 1 α
Macrophage chemotactic protein 1
Eotaxin

INFLAMMATORY ENZYMES

Inducible nitric oxide synthase
Inducible cyclooxygenase-2
5-lipooxygenase
Cytosolic phospholipase A2

ADHESION MOLECULES

Intracellular adhesion molecule 1
Vascular cell adhesion molecule 1
E-selectin

RECEPTORS

Interleukin-2 receptor (α chain)
T-cell receptor (β chain)

Figure 7: Genes regulated by NF-kB activation

(Figure 7) [101]. NF-kB factors are expressed in essentially all mammalian cells and can be activated by a wide variety of stimuli. Although NF-kB plays an essential beneficial role in normal physiology for immune and inflammation responses, constitutive activation of NF-kB has also been found in association with most cancers and other diseases such as cardiovascular disease, diabetes, chronic inflammation and CNS-related disease [102].

There are two NF-kB signalling pathways, classical (or canonical) (Figure 8) and alternative (or non-canonical), which lead to the activation of NF-kB. The distinguishing features of these two pathways are the members of IKK. These are involved in mediating the degradation of selective IκB, subsequently leading to the release of different dimers of NF-κB and the activation of the selective subset of genes and their responsible biological functions. The cytoplasm contains inactive NF-kB. Inhibitor kappa B alpha (IκBα) is a small inhibitory molecule that binds to NF-kB, retaining it in cytoplasm in the inactive form. Cellular stimulation by inflammatory cytokines activates the cytoplasmic enzyme, IκB kinase (IKKB), that phosphorylates IκBα, predominantly acting through IKKB in an IKKγ-dependent manner. The phosphorylated IκBα can no longer bind to (and inactivate) NF-kB and is released from the complex and

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metabolized. The free NF-kB can then translocate into the nucleus and activate the transcription of genes involved in inflammation. The activation of p50:p60 heterodimer has been found in association with an increased transcription of genes encoding chemokines, cytokines, adhesion molecules, enzymes that produce secondary inflammatory mediators and inhibitors of apoptosis [103].

This classic NF-kB pathway has been implicated as an important pathway in coordinating the expression of multiple inflammatory and innate immune genes by maintaining the survival of professional immune cells during bacterial infections or acute inflammatory stimuli.

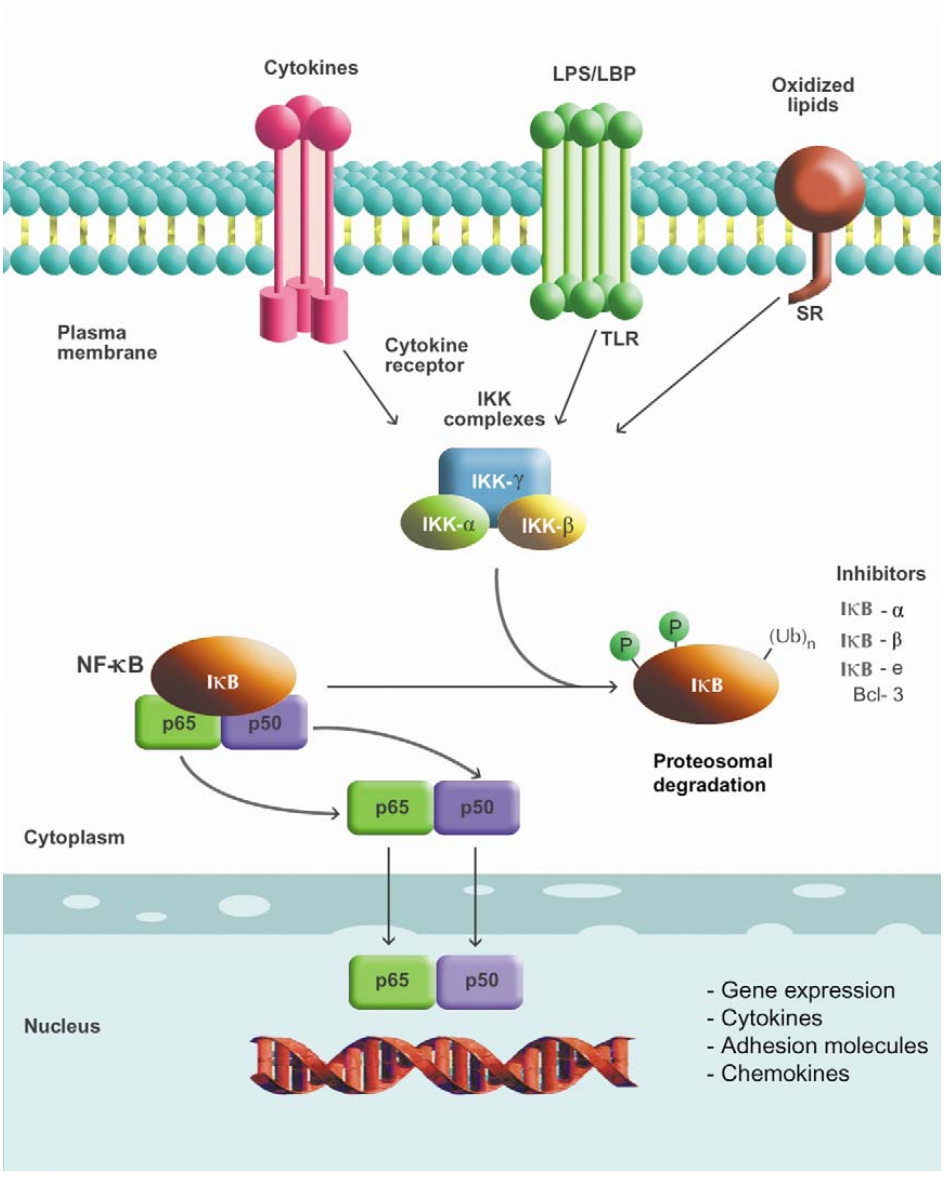

Figure 8: Nuclear factor-kB (NF-kB) activation pathways

The alternative pathway (or non-canonical pathway), in contrast to the classical NF-κB pathway, is mainly stimulated by ligation of LTβR, BAFFR and CD40R, mediated by NIK and strictly dependent on IKKα homodimer, but independent of IKKB and IKKγ. The target for IKKα homodimers is NF-kB p52/p100 protein. C-terminal phosphorylation is essential for p100 processing to p52, which is also dependent on polyubiquitination and proteasomal degradation. However, the phosphorylation dependent ubiquitination of p100 results in the degradation of C-terminal only, and leaves the N-terminal portion intact, thus producing the p52 polypeptide. As p100 is most commonly associated with RelB, activation of this 'alternative' pathway results in the nuclear translocation of the p52-RelB dimers and has been suggested as playing a central role in the expression of genes involved in developing and maintaining secondary lymphoid organs [104].

6.1.1 Nuclear factor-kB (NF-kB) activation by lipopolysaccharide

LPS is the principal component of the outer membrane Gram-negative bacteria. Humans have evolved to detect low levels of LPS to combat infection. Monocytes orchestrate the innate immunity response to LPS by expressing a variety of inflammatory cytokines such as tumor necrosis factor-alpha (TNF-α). However, over-reaction to the presence of LPS can lead to sepsis, septic shock, or systemic inflammatory response syndrome. Many studies have demonstrated that LPS activates NF-kB in human monocytes and monocytic cell lines [105]. However, until recently the intracellular signalling molecules that mediate this activation had not been identified. For instance, LPS activation of NF-kB in human THP-1 monocytic cells is inhibited by dominant-negative mutants of MyD88, IL-1 receptor-associated kinase (IRAK) and TNF-α receptor-associated factor (TRAF6) but not by dominant-negative TRAF2 [106]. In addition, IRAK does not associate with MyD88 in LPS-tolerant THP-1 cells. Genetic evidence confirms that MyD88 and TRAF6 have a role in LPS signalling. MyD88-deficient mice lack the ability to respond to LPS, this being measured by the secretion of cytokines by macrophages. Similarly, TRAF6-deficient mice were hyporesponsive to

LPS. Taken together, these data indicate that MyD88, IRAK, and TRAF6 play a role in LPS signalling in monocytes and macrophages. It is shown that LPS activates the IKKs in human monocytes, THP-1 monocytic cells, and mouse RAW 264.7 cells [107]. The kinetics of LPS activation of the IKKs was relatively slow (peaking at about 30 min) compared with TNF-α activation of the IKKs (peaking at about 5 min), which suggested that different signalling pathways were involved in the activation by these two agonists. Furthermore, a dominant-negative mutant of IKKb inhibited LPS induction of NF-kB-dependent gene expression and TNF-α promoter activity, whereas a dominant-negative mutant of IKKα had no effect. More recently, knocking out the IKKα and IKKB genes yielded remarkably different phenotypes. Disruption of the IKKB gene results in embryonic lethality caused by severe liver degeneration during mid-gestation, a phenotype consistent with excess TNF-α toxicity, whereas disruption of the IKKα gene results in abnormal morphogenesis. The phenotype of IKK-deficient embryos is remarkably similar to that of p65-deficient embryos. It was concluded from these results and from studies using IKKα- and IKKB-deficient fibroblasts that IKKB, but not IKKα, was required for TNF-α and IL-1 signalling. These studies support our conclusion that IKKB is required for LPS activation of NF-kB in THP-1 monocytic cells [108].

6.2 MAPK family

Innate immune cells respond swiftly to the presence of specific ligands that are signalling infectious danger, which they recognize through pattern recognition receptors. Activation of TLR, for example, by LPS of Gram-negative bacteria, lipopeptides derived from Gram-positive bacteria or immunostimulatory CpG DNA, induces the expression of cytokines, chemokines, and other inflammatory mediators in less than 1 hour. Two major signalling pathways are activated downstream of the TLR-induced Myd88-IL-1R-associated kinase-TNFR-associated factor 6 complex. These pathways are essential for such a rapid response and both involve activating the latent

transcription factors as follows: 1) they activate the IkB kinase complex, thus degrading IkB and leading to nuclear translocation of active NF-kB; and 2) they use the MAPK pathway, a cascade of phosphorylation events, to generate the posttranslational activation of transcription factors such as CREB and AP-1 [109]. Both pathways synergize in inflammatory gene expression through the coordinate binding of the transcription factors to the kB and AP-1 sites found together in the promoters of, for example, IL-6, TNF-α, and many other genes that are upregulated in response to TLR ligation. The three major subfamilies of MAPK that are expressed in the immune system are p38, ERK, and JNK. All three MAPK are phosphorylated on the threonine and tyrosine residues of the shared TxY motif minutes after TLR stimulation of the macrophages and dendritic cells [110]. Rapid transduction of the signal from TNFR-associated factor 6 to the MAPK is achieved through the sequential activation of upstream MAP3K and MAPK kinases [111]., A large number of substrates that are serine/threonine-phosphorylated have been defined downstream from the MAPK, including transcription factors of the ATF/CREB and AP-1 family, kinases such as Mapkapk2/MK2 and ribosomal protein kinase (RSK), and proteins controlling mRNA stability and translation. Although there is some overlap in the target proteins of MAPK, prototypic-specific downstream mediators have also been defined using specific pharmacological inhibitors. These studies demonstrated the importance of MAPK activation in cytokine and chemokine gene expression in general, and provided many specific examples of genes that are regulated preferentially by one or the other MAPK [112]. For example, IL-10 production was inhibited by the Map2k1/MEK1 inhibitor U0126, whereas IL-12 expression was suppressed by inhibiting p38 with SB203580. Furthermore, higher IL-12 production in dendritic cells than in macrophages after stimulation was inversely correlated to differences in the amount of ERK activation between the cell types. The concept that the pattern of MAPK activation may determine the type of cytokine output was further supported by investigations into the activation of

MAPK by different TLR ligands, inducing reciprocal patterns in the secretion of the immunosuppressive cytokine IL-10 and Th1-driving IL-12. High levels of IL-10 along with low IL-12 production in response to TLR2 stimulation were shown to correlate to strong ERK activation, whereas TLR4, TLR5, or TLR9 ligands preferentially activated p38 and induced more IL-12. The use of ERK1- and Fos-deficient macrophages delineated a pathway that controls the ratio of IL-10 versus IL-12 production. This pathway involves strong ERK activity that stabilizes and enhances the transcription of Fos which in turn supports IL-10 production and inhibits IL-12. Similar conclusions could be drawn from studies showing Th-2 type adjuvant activity in TLR2 ligands *in vivo* and the IL-10-promoting effects of ERK-activation by Leishmania phosphoglycans. Thus, the MAPK pathway is used in innate immunity not only to fast-track the alarm signals from TLR to the nucleus, but also to provide a means of translating the nature of the stimulus into appropriate responses by balancing the strength of individual MAPK signals.

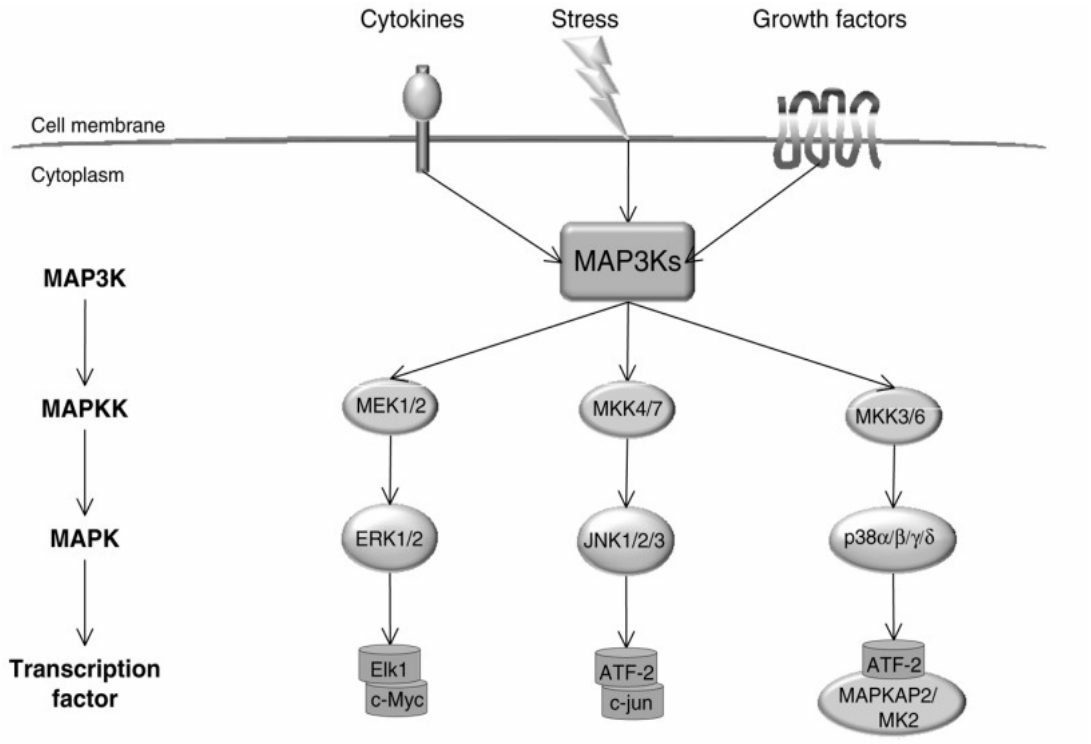

Figure 9: The mitogen-activated protein kinase (MAPK) signaling cascade.

7. SYSTEMIC INFLAMMATION IN METABOLIC SYNDROME

Persistent low-grade inflammation, as indicated by higher circulating levels of inflammatory mediators such as C-reactive protein, interleukin-6 and tumour necrosis factor-α, is a strong risk factor for several chronic diseases. Given the widespread deleterious health effects of an augmented inflammatory state, identification of therapies that reduce inflammation is critical. There are data indicating that decreasing energy intake and increasing physical activity may be effective therapies for reducing overall inflammation. Evidence is strong that circulating levels of inflammatory markers are elevated with total and abdominal obesity, owing to a higher secretion rate of cytokines by adipose tissue in obese people [1].

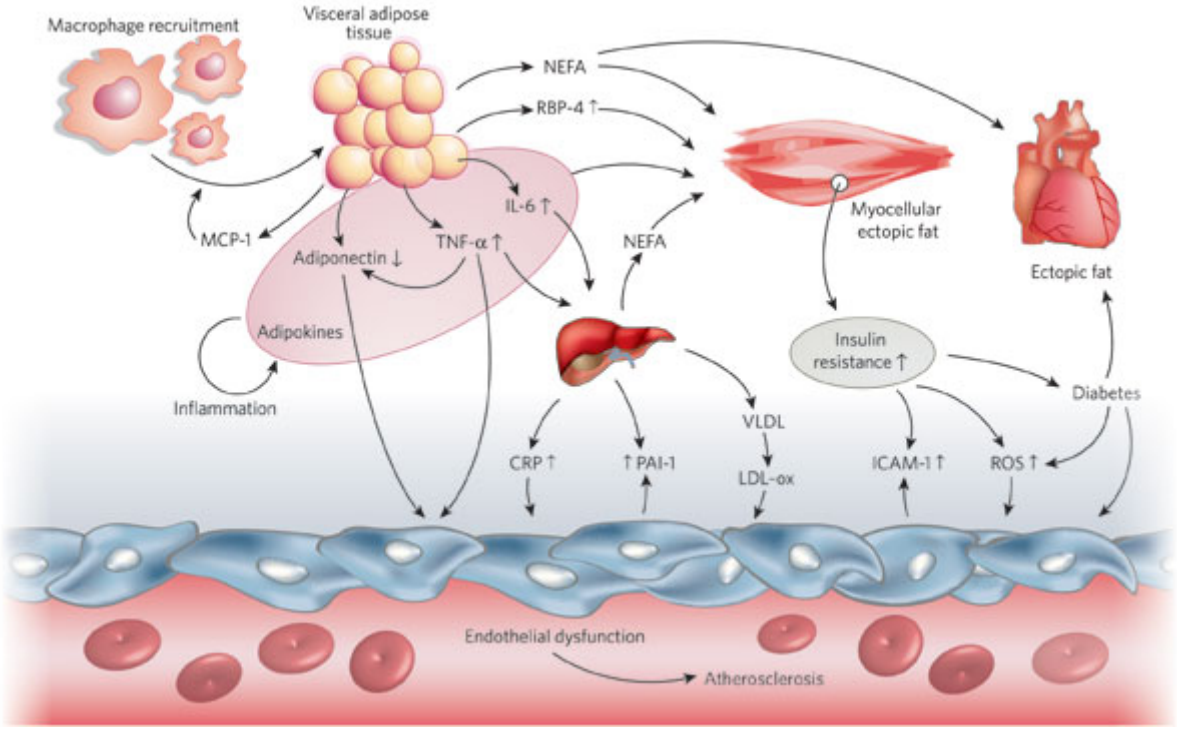

Figure 10: Inflammatory mediators in systemic inflammation (from Van Gaal et al., 2006)

As described in Figure 10, visceral fat in particular contributes to endothelial dysfunction through the direct effect of adipokines, mainly adiponectin and TNF-α, which are secreted by adipose tissue after macrophage recruitment through monocyte

chemoattractant protein-1 (MCP-1). Indirect effects of TNF-α and IL-6 might influence inflammation (CRP) and endothelial dysfunction [113]. Insulin resistance induced by cytokines (IL-6, TNF-α and adiponectin), NEFA and retinol-binding protein 4 (RBP-4) may induce oxidative stress and subsequent endothelial dysfunction (PAI-1 and ICAM-1). Fat accumulation, insulin resistance, liver-induced inflammation and dyslipidaemic features may all lead to premature CVD [114].

7.1 INFLAMMATION LINKED TO IR AND OBESITY IN METABOLIC SYNDROME

Insulin resistance is due to the reduced ability of peripheral tissues to properly respond to insulin induced activation. It is a key feature in the pathogenesis of type II diabetes and this condition may precede the onset of hyperglycaemia and the clinical manifestation of the disease by 10–20 years [35]. Data from the Insulin Resistance Atherosclerosis Study showed that insulin resistance, as assessed by frequently sampled glucose tolerance tests, correlated with high blood levels of CRP, fibrinogen and PAI-1, and levels of these inflammatory factors were predictors of type II diabetes development. Increased blood concentrations of TNF-α and IL-6 were associated with obesity and type II diabetes. Results from the population study from the European Prospective Investigation into Cancer and Nutrition-Potsdam indicated a significant interaction between plasma IL-1β, IL-6 and type II diabetes development. In fact subjects with detectable IL-1β levels and increased levels of IL-6 showed an independently elevated risk of developing the disease [66].

Inflammation may predispose the individual to a pre-diabetic state by increasing insulin resistance, since pre-diabetic subjects showed increased plasma levels of inflammatory proteins without the primary defects of beta cell functions. Sub-clinical inflammation was found to be significantly related to insulin resistance in a high risk group for diabetes, that is, in subjects with a positive family history of diabetes, obesity and hyper or dyslipoproteinemia. Recent results from the INCHIANTI population study

showed that subjects in the upper tertile of insulin resistance had increased serum levels of TNF-α, IL-1R antagonist and IL-6 [10].

As discussed by Dandona et al. [1], two mechanisms might be involved in the pathogenesis of inflammation. Glucose and macronutrient intake causes oxidative stress and inflammatory changes. Chronic overnutrition in obesity might thus be a pro-inflammatory state with oxidative stress [13, 29, 115]. The increased concentrations of TNF-α and IL-6, associated with obesity and type 2 diabetes, might interfere with insulin action by suppressing insulin signal transduction, which in turn might promote inflammation. In fact, insulin reduces ROS generation by mononuclear cells, suppresses NADPH oxidase expression and intranuclear NF-kB binding, induces IkB expression and suppresses some inflammatory molecules [115].

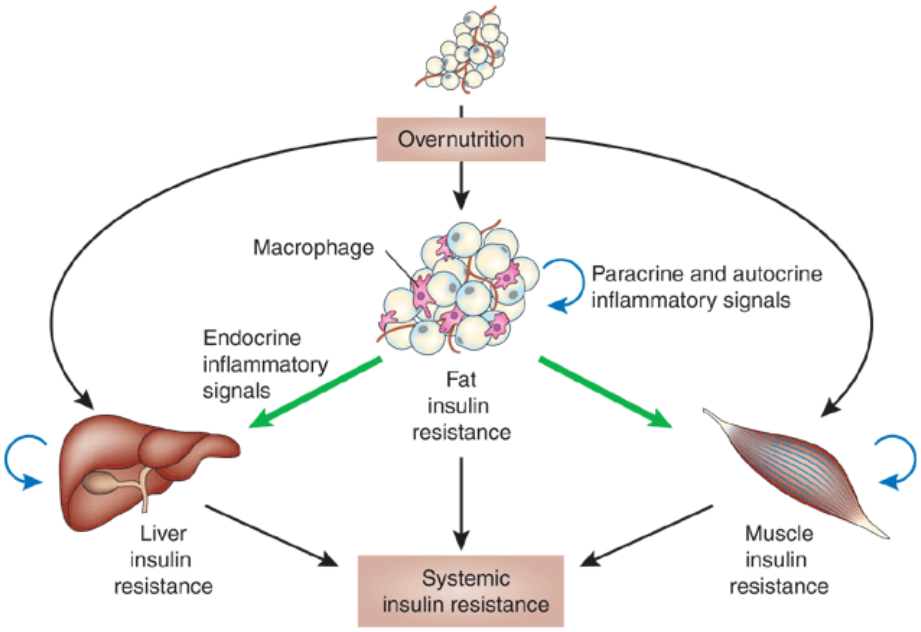

Figure 11: The development of systemic insulin resistance in obesity-induced inflammation and stress (from de Luca et al., 2006).

In searching for the mechanisms involved in inflammation-induced insulin resistance, Cai and co-workers identified the inhibitor of the nuclear factor-kB (NF-kB) kinase-B (IKKB) pathway of NF-kB activation as a mediator of TNF-induced insulin resistance [54]. They showed that overexpression of IKKB in a human embryonic kidney cell line

(the HEK293 cell line) attenuated insulin signalling, and that ob/ob mice expressing only one copy of the gene encoding IKKB (Ikbkb) were protected against the development of insulin resistance [116]. By studying mice with conditional deletion of Ikbkb in either hepatocytes or myeloid cells, it has been shown that mice lacking IKKB in hepatocytes retain insulin responsiveness in the liver but develop insulin resistance in muscle and fat in response to a high-fat diet, obesity or aging [117]. By contrast, mice lacking IKKB in myeloid cells retain global insulin sensitivity and are protected from insulin resistance. Therefore, this study indicates that myeloid cells, probably macrophages, regulate systemic insulin sensitivity and are involved in inflammation-associated insulin resistance, whereas hepatic IKKB expression is required for insulin resistance in the liver. Selective activation of NF-kB causes continuous low-level expression of IKKB in hepatocytes from a transgenic mouse model and leads to moderate systemic insulin resistance.

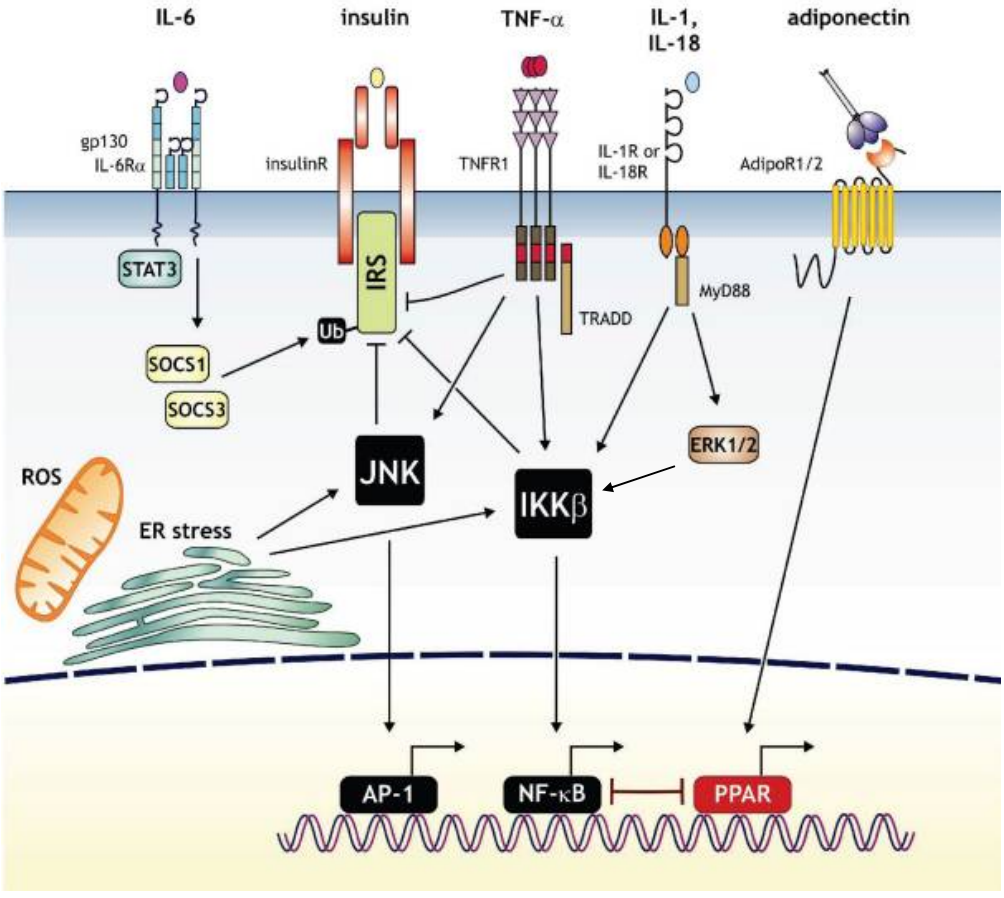

Figure 12: Regulation of IR: involved mediators and pathways (from Tilg et al., 2008).

In this study, insulin resistance was decreased by systemic neutralization of IL-6. Therefore, the regulation of insulin resistance involves not only a complex network of mediators, but also various cell types in addition to adipocytes, including hepatocytes, macrophages and muscle cells [115].

The JUN N-terminal kinase (JNK) family of serine/ threonine protein kinases are activated by many inflammatory stimuli, including TNF-α and ligation of Toll-like receptors (TLRs), and are also important regulators of insulin resistance in mouse models of obesity [111]. In both genetic and dietary animal models of obesity, JNK activity is increased in the liver, muscle and adipose tissue, and loss of JNK prevents insulin resistance [118]. TNF-α inhibits auto-phosphorylation of the tyrosine residues in the insulin receptor and induces serine phosphorylation of the insulin receptor substrate-1, which in turn causes serine phosphorylation of the insulin receptor (IRS-1) in adipocytes and inhibits tyrosine phosphorylation. Because IRS-1 is a direct substrate for IKKβ, it can inactivate IRS-1 through serine phosphorylation, a process which can be caused by TNF-α, and this may have contributed to the insulin resistance mediated by this inflammatory pathway [10]. Moreover, TNF-α antagonizes adiponectin, an important insulin-sensitizing adipokine that signals via adiporeceptors. IL-1 and IL-18 are also able to induce IR. IL-1 has been shown to reduce IRS-1 expression via ERK1/2 and can activate the IKKβ/NF-kB pathway. A role for IL-18 in the regulation of IR has recently been demonstrated in IL-$18^{-/-}$ and IL-$18R^{-/-}$ mice [119].

Several pro-inflammatory cytokines, SOCS proteins, ER stress, the IKKB pathway of NF-kB activation and JNK signalling pathways are all associated with the development of insulin resistance, indicating that various pro-inflammatory mediators released by adipocytes, in addition to the initially described proinflammatory cytokine TNF-α, link the immune system with obesity-related insulin resistance [66].

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7.2 INFLAMMATION LINKED TO VASCULAR DISEASE IN METABOLIC SYNDROME

With the recent trend for individuals to be more obese, a large increase in the prevalence of CVD in westernized is expected. Metabolic syndrome and IR, together with the associated hyperinsulinemia and hyperglycaemia, and the presence of pro-inflammatory mediators might lead to a state of vascular endothelial dysfunction, an abnormal lipid profile, hypertension and vascular inflammation, all of which promote the development atherosclerotic cardiovascular disease.

Atherosclerosis can be considered as an inflammatory disease [120]. One of the principal risk factors for atherosclerosis are the high plasma concentrations of cholesterol, in particular the concentrations of low-density lipoprotein (LDL) cholesterol, and because of this many researchers believe that the process of atherogenesis consists largely of the accumulation of lipids within the artery wall. However, it is much more than that. Despite changes in lifestyle and the use of new pharmacologic approaches to lower plasma cholesterol concentrations, cardiovascular disease continues to be the principal cause of death in the United States, Europe, and much of Asia. In fact, the lesions of atherosclerosis represent a series of highly specific cellular and molecular responses that can best be described as an inflammatory disease. The lesions of atherosclerosis occur principally in large and medium-sized elastic and muscular arteries and can lead to ischemia of the heart, brain, or extremities, resulting in infarction. They may be present throughout a person's lifetime. In fact, the earliest type of lesion, the so-called fatty streak, which is common in infants and young children, is a pure inflammatory lesion, consisting only of monocyte-derived macrophages and T lymphocytes. In persons with hypercholesterolemia, the influx of these cells is preceded by the extracellular deposition of amorphous and membranous lipids [43]. By asking questions about arterial inflammation, we may be able to gain insight into the process of atherogenesis. Figure 13 schematizes the steps in the recruitment of mononuclear phagocytes to the nascent atherosclerotic plaque and

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some of the functions of these cells in the mature atheroma. A normal arterial endothelium resists prolonged contact with leukocytes including the blood monocyte. When endothelial cells undergo inflammatory activation, they increase their expression of various leukocyte adhesion molecules. Vascular cell adhesion molecule-1 (VCAM-1) seems to have a major role in the monocyte recruitment to the atheroma. Once it has adhered to the activated endothelial layer, the monocyte diapedeses between intact endothelial cells to penetrate into the tunica intima, or innermost layer of the arterial wall. This directed migration requires a chemoattractant gradient.

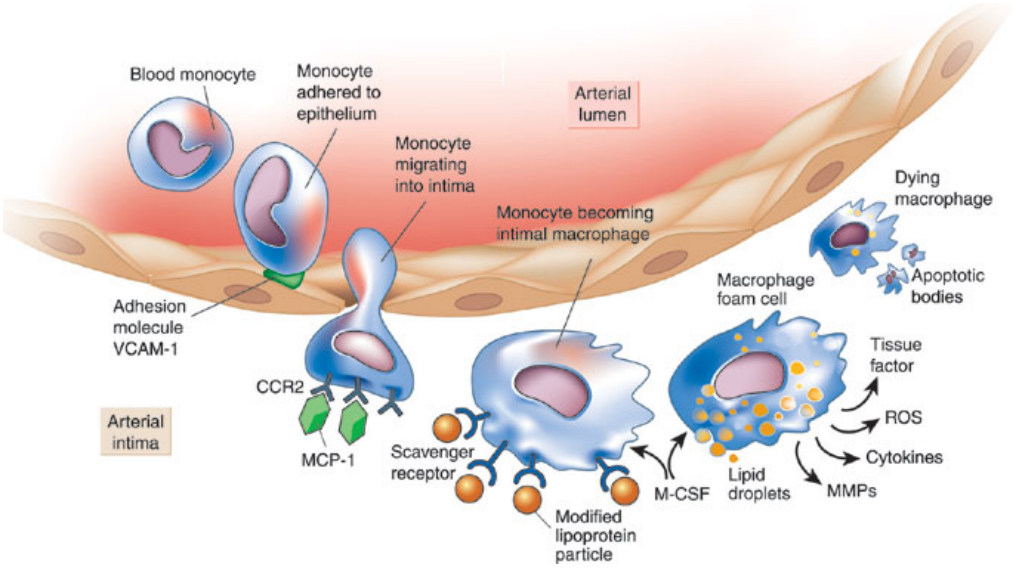

Figure 13: Mononuclear phagocytes in atherogenesis (from Libby et al., 2002).

Various chemokines seem to participate in this process, particularly in the interaction between MCP-1 and its receptor CCR2. Once resident in the intima the monocyte acquires the characteristics of the tissue macrophage. In the atheroma in particular, the macrophage expresses scavenger receptors that bind internalized lipoprotein particles modified, for example, by oxidation or glycation. These processes give rise to the arterial foam cell, a hallmark of the arterial lesion, so named because of its foamy appearance under the microscope, which is the result of accumulation of lipid droplets within the cytoplasm. Within the arterial intima, the macrophage serves many functions related to atherosclerosis and its complications. Notably, the foam cell secretes pro-

inflammatory cytokines and ROS that amplify the local inflammatory response in the lesion. The activated mononuclear phagocyte has a key role in the thrombotic complications of atherosclerosis because it produces matrix metalloproteinases (MMPs) that can degrade the extracellular matrix that lends strength to the plaque's fibrous cap. When the plaque ruptures as a consequence, it permits the blood to contact another macrophage product, the potent pro-coagulant protein tissue factor. Eventually the macrophages congregate in a central core, forming typical atherosclerotic plaque. Macrophages can die in this location, some by apoptosis, thus producing the so-called 'necrotic core' of the atherosclerotic lesion [43-45].

Increasing evidence suggests that an insult or injury to the vascular endothelium is needed for the process of atherogenesis to begin [121]. Triggering factors such as oxidized-lipoproteins, infections, shear stress forces, angiotensin II, increased oxidative stress and cytokine release [121] lead to endothelial activation and dysfunction and increased expression of proinflammatory cytokines such as macrophage colony stimulating factor (MCSF) and IL-1β, which induce monocyte/macrophage activation [50]. MCSF plays a central role in this transformation by increasing the expression of scavenger receptors on macrophages and by further triggering these cells to produce MCSF [50]. MCSF also promotes the release of other cytokines such as IL-1β and IL-6 by vascular cells, which lead to hepatic CRP production [122]. On the other hand, other cytokines may inhibit atherogenesis. Transforming growth factor b1 (TGF-b1) is a growth factor with anti-inflammatory/atheroprotective action. TGF-b1 production is mainly induced by lipid-laden foam cells and stimulates collagen production thus contributing directly to strengthening of the fibrous cap over the atheromatic plaque. The MCSF and CD40 ligands stimulate circulating monocytes, vascular endothelial cells, resident macrophages and smooth muscle cells to release tissue factor (TF), which induces thrombosis [44]. MCSF also induces increased release of IL-6 levels and MCP-1, which further promote TF expression.

CRP is released in the systemic circulation and acts as the final end product of various converging inflammatory pathways in atherosclerosis and acute coronary syndromes [123]. CRP may also have a direct proinflammatory effect on the endothelium, this effect being related to the enhancement of the proinflammatory iNOS and the inhibition of the atheroprotective eNOS [124]. CRP may significantly contribute to plaque rupture by inducing apoptosis of vascular smooth muscle cells. In summary, various inflammatory factors including MCSF, TGF-b1, IL-6, and CRP are currently believed to be actively involved in atherogenesis and to contribute to plaque instability, thrombosis and consequently the genesis of acute coronary syndromes [11].

Brand et al. provided the first evidence to suggest that the physiological and pathological activation of NF-kB might have implications in the pathogenesis of atherosclerosis [125]. Using immunofluorescence and immunohistochemical techniques, they were able to detect the activation of NF-kB in macrophages, endothelial cells and smooth muscle cells located in the media and intima of thickened fibrous atherosclerotic regions of the vessel wall. By contrast, no or little activation of NF-kB was detected in otherwise healthy parts of the arteries. This finding was further supported by other studies [55, 126, 127] which found NF-kB in the intimal layers of the coronary arteries in hypercholesterolemic diet-fed pigs and in human coronary artery plaques. NF-kB plays a crucial role in atherosclerosis because it is activated by a wide variety of stimulants and it regulates a wide range of genes, including those which encode cytokines, adhesion molecules, MCP-1 and IL-8. The products of these last two are thought to play particular roles in the initiation of atherosclerotic lesions [127]. Understanding the NF-kB-dependent mechanisms involved in the initiation phase of the development of atherosclerotic lesions could potentially provide us with valuable data for reducing and, more importantly, preventing lesion development in many cases of atherosclerosis.

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8. BIOACTIVE FOOD COMPOUNDS: PROCYANIDINS

8.1 INTRODUCTION

The "French paradox" has lead to increasing interest in phenolics. This paradox refers to the correlation between a high-fat and high-cholesterol diet and the lower incidence of coronary heart disease found in Mediterranean cultures, in contrast to the higher incidence of coronary heart disease among most Western cultures. It has been asserted that the French paradox may be attributable to regular consumption of red wine and that the unique anti-atherogenic effects of red wine reside in the action of polyphenols. Phenolic compounds or polyphenols constitute one of the most numerous and ubiquitously distributed groups of plant secondary metabolites, with more than 8000 phenolic structures currently known. Plant phenols can be subdivided in two families: flavonoids and non-flavonoids. Favonoids are classified in the following classes: flavanones, flavones, isoflavones, anthocyanidins, flavonols and flavanols (flavan-3-ols and proanthocyanidins or condensed tannins) (Figure 14). For flavonoids, more than 5000 different kinds have been described [128].

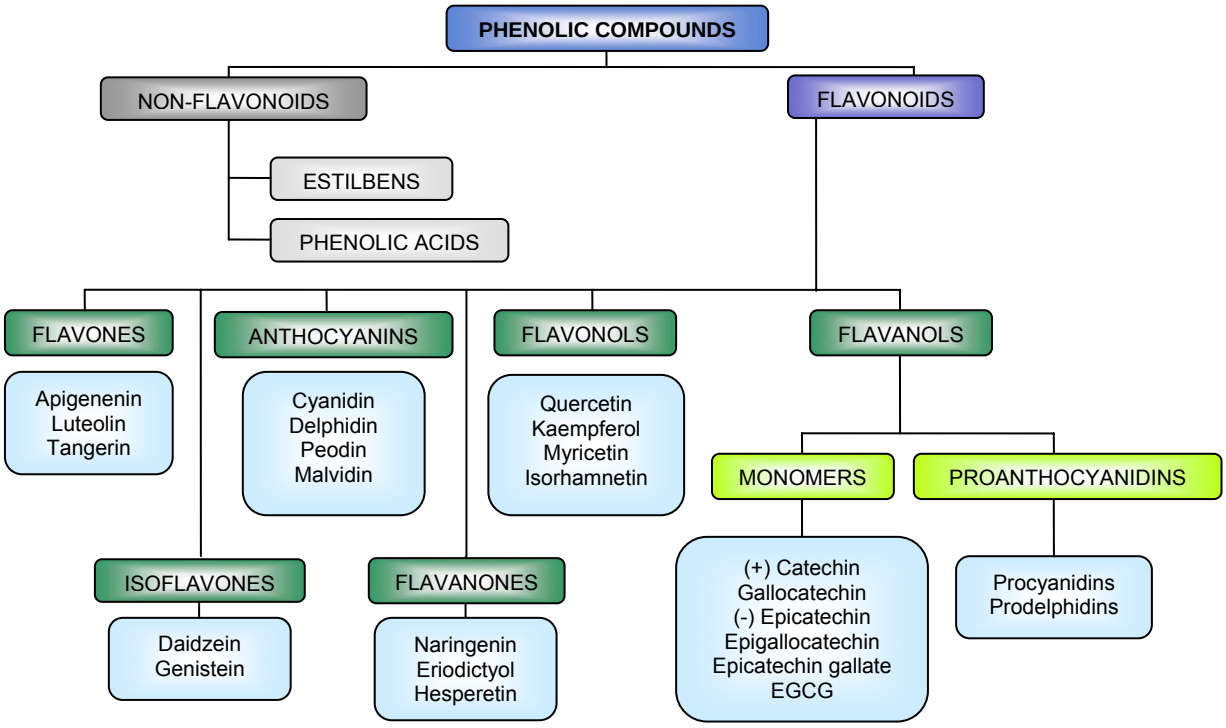

Figure 14: Main groups of phenolic compounds. EGCG: epigallocatechin gallate

Phenolics are therefore an integral part of the diet, with significant amounts being reported in vegetables, fruits, teas and plants. Although the dietary intake of phenolics varies considerably across geographic regions, it is estimated that daily intake range from about 20 mg to 1 g, which is higher than daily vitamin E intake [129]. Epidemiological evidence indicates that consuming fruit, vegetables and teas may reduce the risk of cardiovascular disease and it is increasingly suggested that this may be due to the antioxidants they contain, such as ß-carotene, vitamin C, vitamin E and polyphenolics. Dietary antioxidant phenolics may quench reactive oxygen and nitrogen species and thus potentially modify pathogenic mechanisms involved in cardiovascular disease [130]. The effectiveness of a dietary antioxidant will depend on a number of factors such as which ROS or RNS is being scavenged, how and where they are being generated and the accessibility of the antioxidant to possible sites of damage [130]. Many phenolic compounds have been shown to carry out antioxidant activity *in vitro* and several observational studies support their role in potentially protecting against cardiovascular disease [128, 131].

8.2 CHEMICAL STRUCTURE OF PROANTHOCYANIDINS

Proanthocyanidins are oligomers or polymers of flavan-3-ols and these units are linked mainly through the C4-C8 bond, although the C4-C6 linkage also exists. These linkages are both called B-type linkages. An additional ether bond between C2-C7 results in a doubly linkage of the flavan-3-ol units and is called an A-type linkage. Proanthocyanidins consist of different flavan- 3-ol subunits, also called proanthocyanidin monomers or catechins. The most common types are shown in Figure 15. The proanthocyanidins that exclusively consist of (epi)catechin units are called procyanidins and these are the most abundant type of proanthocyanidins in plants. The less common proanthocyanidins containing (epi)afzelechin or (epi)gallocatechin subunits are called propelargonidin or prodelphinidin, respectively

[132]. The flavan-3-ol subunits may carry acyl or glycosyl substituents. The most common acyl substituent is gallic acid, which is bound as an ester with the hydroxyl in the C3 position as in tea or wine. Several glycosylated proanthocyanidin oligomers have been identified, the most common glycosylation being the sugar linked to the C3 or the C5 position [132]. Procyanidins have a high structural diversity that is based on the four possible monomer units, catechin, and epicatechin, with their different configurations. Regarding the stereochemistry of their C2-C3 bond, they exhibit cis or trans configuration.

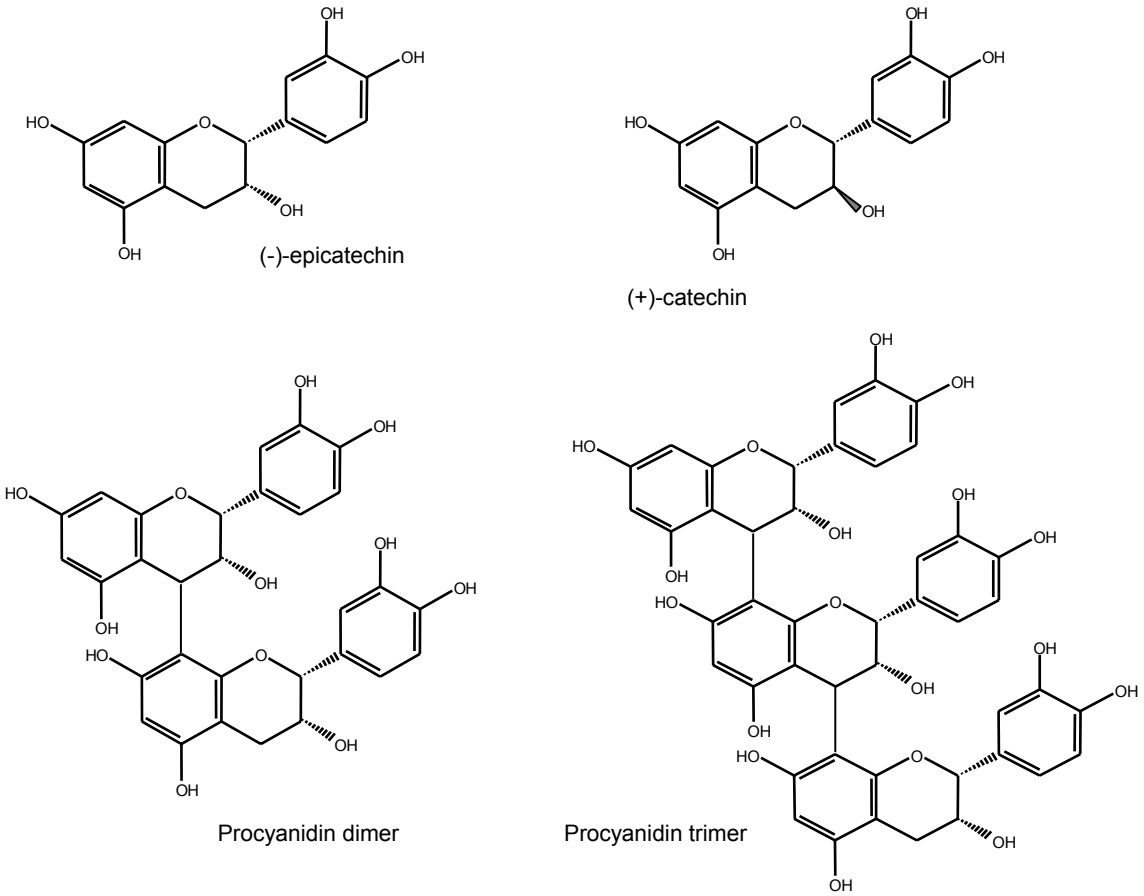

Figure 15: Structures of flavan-3-ols and procyanidin dimers and trimers.

8.3 INTAKE OF PROANTHOCYANIDINS

Gu et al. [133] estimated the daily average intake of proanthocyanidins in the USA and found that in total it is about 53.6 mg/day excluding the monomers, and 57.7 mg/day including the monomers. The distribution between intake of monomers, dimers, and trimers was found to be almost equal, with an intake of 4.1–6.4 mg/day of each group, whereas the intake of oligomers (4–10-mers) and polymers (>10-mers) was around 20 mg/day for both. Therefore, about 73% of the estimated total daily intake of proanthocyanidins had a DP > 3.

Other estimations of average proanthocyanidin intake ranged from tens to several hundreds of milligrams of proanthocyanidins per day. Proanthocyanidins are complex flavonoid polymers naturally present in fruits, legume seeds, cereal grains, and different beverages (wine, tea, cocoa/chocolate, cider). They are, therefore, an integral part of the human diet. In Gonthier et al. [134] work, it was estimated that the average of daily intake of proanthocyanidins might well represent over 50% of the 1 g of dietary polyphenols consumed every day.

Detailed quantitative information on the procyanidin profiles present in many food products is lacking. The compositional data are important for the initial understanding of which foods contribute most to the dietary intake of proanthocyanidins and may be used to compile a database necessary to infer epidemiological relationships to health and disease. The main sources of proanthocyanidins were identified as apples, pears, grapes, and red wine. In an analytical study by Hammerstone et al., the procyanidin content of red wine, chocolate, cranberry juice and four varieties of apples was determined. On average, chocolate and apples contained the largest procyanidin content per serving (164.7 and 147.1 mg, respectively) compared with red wine and cranberry juice (22.0 and 31.9 mg, respectively). However, the procyanidin content varied greatly between apple samples (12.3–252.4 mg/serving) with the highest amounts on average observed for the Red Delicious (207.7 mg/serving) and Granny

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Table 3: Content of Proanthocyanidins in common foods (from Rasmussen et al., 2005)

Food	Monomers	Dimers	Trimers	4–6-mers	7–10-mers	>10-mers	Total	Type
			(mg/100 g fresh weight foods or mg/L beverages)					
Blueberries	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	PC
Black-currants	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	PC, PD
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	A, PC
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	PP, PC
Apples	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	PC
Apple juice	1 ± 0	2 ± 0	1 ± 0	4 ± 0	1 ± 0	ND	9 ± 0	PC
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	PC
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	A, PC
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	PC
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	A, PC
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	PC
Barley	11.0 ± 0.3	21.4 ± 1.1	14.6 ± 1.0 27.	2 ± 0.6	ND	ND	74.2 ± 3.0	PC
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	PP, PC
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	PP, PC
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	PC, PD
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	PC, PD
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	PP, PC
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	PC
Peanuts	5.1 ± 1.0	4.1 ± 0.7	3.7 ± 0.5	2.8 ± 0.2	ND	ND	15.6 ± 2.3	A, PC
Peanut butter	2.0 ± 0.9	3.0 ± 0.7	8.1 ± 3.5	ND	ND	ND	13.2 ± 5.2	A, PC
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	PC
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	PC
Beer	4 ± 0	11 ± 1	3 ± 0	4 ± 0	ND	ND	23 ± 2	PC, PD
White wine	15.1	ND	ND			–	15.1	–
Rosé wine	17.1	ND	ND			–	17.1	–
Red wine (a)	190.0	274.3	93.4			–	557.7	–
Red wine	20 ± 1	40 ± 1	27 ± 1	67 ± 2	50 ± 1	110 ± 2	313 ± 5	PC, PD
Grape juice	18 ± 0	34 ± 0	19 ± 0	80 ± 0	69 ± 0	303 ± 2	524 ± 2	PC, PD
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

(a) Mean of 95 different French wines. Only monomers (catechin and epicatechin) up to trimers were measured. The total given is the sum of monomers to trimers.

Smith (183.3 mg/serving) varieties and the lowest amounts in the Golden Delicious (92.5 mg/serving) and McIntosh (105.0 mg/serving) varieties [135].

The quantity, structure, and degree of polymerization of grape proanthocyanidins differ, depending on their localization in the grape tissues. The seeds contain higher concentrations of monomeric, oligomeric, and polymeric flavan-3-ols than the skins. While seed tannins are oligomers and polymers composed of the monomeric flavan-3-ols (+)-catechin, (-)-epicatechin, and (-)-epicatechin gallate linked by C4-C8 and/or C4-C6 bonds (B type), skin tannins also contain (-)-epigallocatechin and trace amounts of (+)-gallocatechin and (-)-epigallocatechin gallate. Therefore, wine contains both procyanidins and prodelphinidins. It has been shown that red wine contributes significant amounts of proanthocyanidins compared to other beverages, and that, therefore, proanthocyanidin intake will be substantially higher in regular drinkers of red wine. Also Gu et al. determined that regular fruit eaters will easily have a daily intake higher than the average 57.7 mg/day. Proanthocyanidin intake from fruit and red wine is therefore substantial and adds significantly to the total daily intake of flavonoids, especially when compared to the mean intake of other flavonoid groups such as flavonols, flavones and flavanones which has been estimated to be about 24.2– 28.6 mg/day in the populations of Finland, Denmark and the Netherlands respectively [136].

8.4 BIOAVAILABILITY AND METABOLISM

It is important to realize that the commonest polyphenols in the human diet are not necessarily the most active within the body, either because they have a lower intrinsic activity or because they are poorly absorbed from the intestine, highly metabolized, or rapidly eliminated. In addition, the metabolites that are found in blood and target organs and that result from digestive or hepatic activity may differ from the native substances in terms of biological activity. Extensive knowledge of the bioavailability of polyphenols is essential if their health effects are to be understood [136].

Procyanidins are an important contributor to the potential health effects of dietary flavonoids [128], but to produce a biological effect *in vivo*, it is essential that they reach the target tissues. Until very recently the fate of proanthocyanidins in the human body was unknown. However, new analytical techniques have enabled researchers to study the uptake and metabolism of the complex mixtures of proanthocyanidins present in our food. The proanthocyanidins found in food cover a wide range of DP. As Table 3 shows, the concentration of higher polymerized proanthocyanidins in foods far exceeds that of monomers, dimers, and trimers. Recent studies suggest, however, that only the low-molecular-weight oligomers (DP < 3) are absorbed intact in the gastrointestinal tract. Deprez et al. [137] demonstrated that (+)-catechin and proanthocyanidin dimers and trimers were permeable through the Caco-2 human intestinal cell line. The permeability of a proanthocyanidin polymer with a mean DP of 6 was 10 times lower, suggesting that only monomers, dimers, and trimers are absorbed and that polymers are not. The data based on the absorption and degradation of proanthocyanidins are, however, somewhat conflicting. Spencer et al. [138] used *in vitro* experiments to demonstrate the possibility that, under *in vitro* conditions that simulate gastric juice (37ºC, pH 2.0, 1–4 h, no digestive enzymes), procyanidin oligomers are degraded to mixtures of epicatechin monomers and dimers that can then be absorbed in the small intestine. However, a later study by Gonthier et al. [134] showed that both purified procyanidin dimer B3 and grape seed proanthocyanidins with a higher degree of polymerization were not degraded into monomers in rats, and that the dimer was detected in plasma or urine. Further research investigated the stability of proanthocyanidins in humans by regularly analyzing gastric juice sampled with a gastric probe after ingestion of a proanthocyanidin rich beverage. This study suggested that proanthocyanidins are not degraded under the acidic conditions of the stomach *in vivo*. Once proanthocyanidins or their fermentation products have crossed the intestinal barrier, they reach the liver through the portal vein, where they are further metabolized.

In vitro studies have shown that methylated derivatives can be formed from the dimer B3 in fresh liver homogenate obtained from a human biopsy. There are only a few studies that have reported the methylation of proanthocyanidins, but it is very likely that these compounds are being extensively metabolized, hydroxylated, methylated or conjugated to sulfate esters or glucuronides in the same way that has been demonstrated for other flavonoids [128].

Another point to take into account when evaluating the bioavailability of polyphenols is the level of absorption in the tissues. This is conditioned by their hydrophilic nature because they are water soluble due to the glucuronide and sulfate moieties, which makes it difficult for them to pass through membranes; however, several studies have found these molecules in such tissues as the liver, kidneys, lungs, brain, pancreas and bladder [139].

The effects of the food matrix on the bioavailability of polyphenols have not been examined in much detail. Direct interactions between polyphenols and some components of food, such as binding to proteins and polysaccharides, can occur, and these interactions may affect absorption [140]. Furthermore, more indirect effects of the diet on various parameters of gut physiology (pH, intestinal fermentations, biliary excretion, transit time, etc) may have consequences on the absorption of polyphenols. Enzymes and carriers involved in polyphenol absorption and metabolism may also be induced or inhibited by the presence of some micronutrients or xenobiotics [136].

In conclusion, the notion of bioavailability contains many variables, such as the food matrix, intestinal absorption, excretion of glucuronides toward the intestinal lumen, metabolism by the microflora, intestinal and hepatic metabolism, plasma kinetics, the nature of circulating metabolites, albumin binding, cellular uptake, intracellular metabolism, accumulation in tissues, and biliary and urinary excretion. The difficulty lies in integrating all the information and relating the variables to health effects at the organ level. These tasks are made all the more difficult because the relative weight of

each variable may depend on the polyphenol considered. Some polyphenols may be less efficiently absorbed than are others but nevertheless reach equivalent plasma concentrations because of lower secretion toward the intestinal lumen and lower metabolism and elimination.

8.5 EFFECTS OF PROANTHOCYANIDINS ON HEALTH

Proanthocyanidins are defined as bioactive compounds because they influence physiological or cellular activities and, as a consequence, have a beneficial effect on health. Among other effects, they have been considered to be cardioprotective, anti-inflammatory, anti-carcinogenic and anti-mutagenic. These protective effects are related to their capacity to: (a) act as free radical scavengers; (b) chelate metals; (c) activate antioxidant enzymes; (d) reduce α-tocopherol radicals; and (e) inhibit oxidases in biological systems [136, 141].

8.5.1 Antioxidant activity

Highly reactive oxygen species, such as singlet oxygen, the superoxide anion radical O_2–, the hydroperoxyl radical OH, the nitrogenoxide radical NO, and alkyl peroxyl free radicals, are regularly produced in our body [142]. They cause damage to lipids, proteins, and DNA and participate in pathogenesis and ageing. Humans possess a wide array of endogenous antioxidant defences, which scavenge radicals and repair oxidative damage. A diet rich in antioxidants such as polyphenols is thought to further contribute to this defence. Polyphenols are able to scavenge reactive oxygen species through electron-donating properties, generating a relatively stable phenoxyl radical [142]. For most proanthocyanidins with an o-dihydroxyphenyl group in the B-ring and a fully saturated C-ring, the radical site is at the B-ring, and the substitution of the A-ring has only a limited influence on the reduction potentials of the semiquinone radical formed. These semiquinone radicals are quite stable, and proanthocyanidins may therefore protect the body against oxidation and thus limit the risk of developing

cardiovascular disease [143]. Several *in vitro* studies have been conducted on inhibiting the oxidation of LDL or membrane systems with purified proanthocyanidins. Studies on the antioxidant activity and membrane effects of proanthocyanidins have been performed on liposomes [144]. These studies conclude that the oligomer chain length influences the antioxidant activity so that increasing protection against oxidation correlates with increasing chain length in the proanthocyanidins. In another study on cranberry proanthocyanidins, it was likewise observed that only the fractions containing trimers to heptamers or pentamers to nonamers were able to increase the lag time of the Cu2+ induced oxidation of LDL [145]. Another study also found increased protection of LDL as the chain length of the proanthocyanidins increased [146]. *In vitro* and *in vivo* studies have also been conducted which used food products containing proanthocyanidins, such as red wine, cranberry products, grape skin extracts, cocoa, and chocolate, to protect against LDL oxidation. Most of these studies show some protection against LDL oxidation [147]. The effects seen in these studies may, however, be due to the presence of monomeric or dimeric proanthocyanidins in the food products.

Our research group has reported that flavonoids in red wine increase the enzymatic activity in the anti-oxidant enzyme system in rat hepatocytes (Fao cells) both *in vivo* and *in vitro*. They showed that the antigenotoxic effect of GSPE on Fao cells was submitted to oxidative stress [148, 149]. Furthermore, it has been reported that GSPE affects the activity and mRNA expression of anti-oxidant enzymes (GPx, glutathione peroxidase; GR, glutathione reductase; GST, glutathione *S*-transferase) in human hepatocarcinoma HepG2 cells. GSPE also affects these anti-oxidant enzymes in cells submitted to H_2O_2-induced oxidative stress [87]. These experiments confirm that GSPE not only acts as an antioxidant but also affects anti-oxidant enzyme gene expression.

8.5.2 Effects on atherosclerotic vascular diseases

In the past decade there has been a major shift in our understanding of the pathogenesis of atherosclerosis. It is now generally accepted that inflammatory mechanisms play a central role in mediating all phases of the development of atherosclerosis [79]. Since several of the potentially anti-atherogenic compounds in our diet have anti-inflammatory properties, they may function by inhibiting or blocking the inflammatory processes of atherosclerosis.

Proanthocyanidins have been shown to mediate several anti-inflammatory mechanisms involved in the development of cardiovascular disease [150]. Studies indicate that they are implicated in the modulation of the monocyte adhesion in the inflammatory process of atherosclerosis. Recent papers studied the effects of wine polyphenols on the adhesion of monocytes to endothelial cells [151, 152]. In these studies, monocytes were isolated in healthy men after 28 days of consumption of either red wine or gin. The TNF-α -induced adhesion of the isolated monocytes to an endothelial cell line was then studied, and it was found that the red wine consumption virtually abolished the adhesion, whereas it was only partially reduced after gin consumption [153]. Another recent human study confirms the ability of red wine to affect inflammatory markers of atherosclerosis [154].

The effect of oral doses of proanthocyanidins on atherosclerosis as end-point has been investigated, these studies being limited to grape proanthocyanidins provided in either wine, grape juice, or a grape/wine extract [155, 156]. A few studies have been conducted in hamsters fed an atherogenic diet rich in saturated fat and cholesterol, resulting in hypercholesterolemic hamsters with a similar lipid profile to hypercholesterolemic humans [157]. Auger et al. [158] used this model to investigate the early effects on atherosclerosis of a red wine phenolic extract containing in total 471 mg/g phenols. This study observed two groups, one receiving the phenolic extract with ethanol and one receiving the phenolic extract without ethanol, and revealed that

after 8 weeks, both groups had lowered plasma cholesterol and triglycerides, and the aortic fatty streak area was reduced by 32% and 29%, respectively, in comparison with their respective controls. Grape juice was calculated to be much more effective than red wine or dealcoholized red wine with the same polyphenol dose in inhibiting atherosclerosis and improving lipids and antioxidant parameters. This study thus suggests that grape juice or non-alcoholic red wine, are excellent alternatives to red wine in this model of atherosclerosis. A grape seed proanthocyanidin-rich extract has also been investigated in the hamster model for its effects on atherosclerosis. Two groups were dosed for 10 weeks, one with 50 mg/kg grape seed proanthocyanidin-rich extract and the other 100 mg/kg, and it was found that the atherosclerosis was reduced by 50% and 63% respectively. The effect of purified proanthocyanidins on the development of atherosclerosis has been recently investigated using the same animal model [159]. These findings demonstrate that catechin, quercetin, and resveratrol at nutritional doses prevent the development of atherosclerosis through several indirect mechanisms [160].

The catechins are the monomeric forms of proanthocyanidins and some studies suggest that the oligomers can be degraded into the monomeric forms, whereas others do not report this finding [132, 134]. Nevertheless, the monomeric catechin content in proanthocyanidin-rich foods is substantial, so the beneficial effects of these foods may also be due to the high catechin content. Studies on pure catechins' ability to prevent atherosclerosis are limited, but one study administered apolipoprotein E-deficient mice with catechins using an extract from green tea containing primarily epigallocatechin gallate and epicatechin [161]. This study showed that the catechins had a protective effect. The plasma lipid peroxides were reduced in the treated group at week 8, suggesting that the *in vivo* oxidative state was improved. Atheromatous areas in the aorta were also significantly attenuated in the treated group compared with the control group, and aortic cholesterol and triglyceride contents were 27% and 50% lower,

respectively, in the treated group. *In vitro* experiments [162] using macrophage-like cell lines showed a significant accumulation of ECG in the cells. They also demonstrated that ECG could suppress the gene expression of the scavenger receptor CD36, a key molecule for foam cell formation, in macrophage cells. These data suggest that catechins may be important contributors to the antiatherogenic effects observed in proanthocyanidin-rich foods, because of the concomitant content of catechins in these products [128].

8.6 EFFECTS OF FLAVONOIDS ON MARKERS OF INFLAMMATION

Recent evidence has indicated that cytokines and chemokines, as well as their receptors, are involved in the pathophysiology of many inflammatory diseases [28]. These pathological states seem to be linked to an imbalance of the cytokine network and to the excessive recruitment of leukocytes and macrophages to the inflammatory sites. Because of this, the cytokine system constitutes a very interesting and promising target for the development of clinically relevant anti-inflammatory drugs. In fact, some specific cytokine modulators have been introduced into clinical practice during the last few years. Despite the effectiveness described for these drugs, they have been associated with some side effects and, more importantly, are expensive to use. In this context, the identification of small molecule plant-derived compounds able to selectively interfere with the production and/or function of cytokines would constitute an important alternative for treating many inflammatory diseases.

A substantial body of evidence obtained from both *in vivo* and *in vitro* studies supports the concept that various plant-derived compounds with anti-inflammatory properties exert their effects by modulating the cytokine system. Flavonoids possess interesting anti-inflammatory actions. Xagorari et al. [163] have reported that luteolin (IC50 < 1 μM), quercetin (IC50 1 μM), luteolin 7-glucoside (approximate IC50 50 μM) and the isoflavonoid genistein (IC50 5 μM) inhibited LPS-stimulated TNF-α and interleukin-6 release in RAW 264.7 macrophages.

Baicalin, baicalein and wogonin are flavonoids present in Scutellaria baicalensis Georgi (Lamiaceae), a plant used in the treatment of a variety of inflammatory diseases such as bronchitis, nephritis, hepatitis, asthma, and atopic dermatitis. The anti-inflammatory activities of these flavonoids have been attributed to their antioxidant properties and to their ability to inhibit LPS-induced NO production and iNOS gene expression, as well as the increase in TNF-α levels by RAW 264.7 cells [164, 165].

Krakauer et al. [166] have shown that baicalin (IC50 values ranging from 3 to 50 μg/mL) inhibited the expression and production of IL-1β, IL-6, TNF-α, IFNγ, MIP-1α/β in human peripheral blood mononuclear cells under stimulation with superantigenic staphylococcal exotoxins. More recently, Shen et al. reported that baicalein and baicalin were able to decrease PMA-induced accumulation of reactive oxygen intermediates in human neutrophils and monocytes (IC50 values ranging from 1.5 to 64.5 μM). Baicalein and wogonin (approximate IC50 values of 1 to 40 μM) were also effective in blocking the production and expression of IL-6 and IL-8 in a human retinal pigment epithelial cell line [167].

It has been recently reported that standardised extracts of Ginkgo biloba L. and its flavonoid component quercetin inhibited TNF-α secretion in LPS-stimulated RAW 264.7 macrophages [168] by interfering with the phosphorylation and activation of JNK/SAPK and its downstream substrate ERK1/2 [169]. In addition, Wadsworth et al. have shown that Ginkgo biloba extract and quercetin suppress the activation of the transcription factor AP-1 [170].

Some authors reported that (-)-epigallocatechin gallate (EGCG, the main constituent of green tea polyphenols), tannins (geraniin and corilagin) and the aqueous extract of leaves of Acer nikoense Maxim (a Japanese herbal medicine used for eye and liver diseases) effectively prevented TNF-α release in BALB/3T3 cells stimulated with okadaic acid [171]. In addition, treatment of animals with geraniin and EGCG reduced the percentage of tumour-bearing mice from 80 to 40 % and 73 to 0 %, and the

average number of tumours from 3.8 to 1.1 and 4.2 to 0, following topical application of 7,12-dimethylbenz[a]anthracene (DMBA) plus okadaic acid, respectively.

Pycnogenol, a compound isolated from the bark of Pinus maritime Mill exhibited a marked scavenger activity when evaluated in the murine macrophage cell lines RAW 264.7, IC-21 stimulated with H_2O_2 and IC-21 stimulated with PMA [172]. Furthermore, pycnogenol was found to be effective in reducing both the production of IL-1β and the expression of IL-1β mRNA in LPS-stimulated RAW 264.7 cells, an effect dependent on interference with the transcription factors NF-kB and AP-1 [173].

Tsuda et al. reported that oral administration of a typical anthocyanin, cyanidin 3-O-β-D-glucoside, suppressed the inflammatory response in animal models. Treatment of cyanidin 3-O-β-D-glucoside reduced the elevation of RNS, TNF-α, IL-1β, IL-6 and CINC-1 concentrations [174, 175].

It has been recently demonstrated that phloroglucinol derivatives, are effective in inhibiting the mRNA expression and production of TNF-α and IL-6 in RAW 264.7 cells (IC50 values ranging between 0.7 and 30 μM). These compounds also reduced the formation of TNF-α and IL-6 by human blood monocytes activated with LPS [176]. The effects described for these compounds seem to depend on blocking NF-kB activation, but might also include the inhibition of other pro-inflammatory pathways [176].

Among the polyphenols, the compound obtained from the rhizome of Curcuma longa L., curcumin, is of great interest to researchers developing new clinically relevant anti-inflammatory drugs. This compound significantly blocked IL-12 mediated T cell proliferation and Th1 differentiation, an action that implies that it reduces the production of pro-inflammatory cytokines [177]. Curcumin was found to significantly downregulate the TNF-α-induced increase in MMP-13 mRNA and protein expression in primary human chondrocytes by a mechanism involving the inhibition of NF-kB, c-jun and JNK [178]. Moreover, curcumin significantly inhibited the increase of both IL-1β and TNF-α in a chronic model of inflammation in rats [179].

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III. Hypothesis and Objectives

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Obesity, which is characterised by a major expansion in adipose tissue mass, is associated with a state of chronic mild inflammation, there being increased circulating levels of inflammatory markers such as C-reactive protein, IL-6, IL-18, haptoglobin, MIF and PAI-1. Inflammation in WAT is considered to be causal in the development of type 2 diabetes and the metabolic syndrome linked to obesity.

There is persuasive epidemiological and experimental evidence that dietary polyphenols have anti-inflammatory activity. Since the use of conventional therapeutic drugs has been associated with some side effects and surgical approaches have not been able to fully control the incidence and outcome of many inflammatory diseases, there is an urgent need to find safer compounds and to develop mechanism-based approaches for the management of inflammatory diseases.

Polyphenols have been found to inhibit the inflammatory process in experimental animals. In addition, epidemiological studies have indicated that populations who consume foods rich in specific polyphenols have lower incidences of inflammatory disease. Although the detailed mechanism by which each polyphenol exert anti-inflammatory actions remain to be elucidated, they are relevant to oxygen free radical scavenging, anti-lipid peroxidation, alterations in cell membranes receptors, intracellular signalling pathway proteins and modulation of gene expression. Previous studies from our research group have demonstrated the effects of red wine on obesity *in vivo* where rats that consumed red wine showed lower weight gain rates. Furthermore, along with its antioxidant properties, procyanidins have shown other beneficial effects related with the ability of this phenolic compounds to interact with different signalling pathways, thus modulating gene expression.

The research work carried out in this Ph. D. Thesis is part of a more general research project developed by the Nutrigenomics Research Group of the University Rovira i Virgili, which deals with the potential beficial effects of dietary procyanidins in preventing and ameliorating the metabolic disorders associated with the so-called Metabolic Syndrome.

Once established that obesity is an inflammatory condition leading to chronic activation of the innate immune system response, the aim of this thesis has been to characterize and understand if dietary procyanidins could modulate the low grade inflammatory response related to obesity and the Metabolic Syndrome.

The objectives proposed to reach this aim are described above.

1. Because the control of macrophage overproduction of inflammatory mediators such as PGE2 and NO should greatly facilitate the treatment of inflammatory diseases we wanted to examine **the putative anti-inflammatory effect of procyanidins in macrophages** *in vitro*. For this purpose we stimulated murine macrophages with LPS and IFN-γ to mimic inflammation and specifically assess the procyanidin effect in this model.

2. Once we determined that the procyanidin extract was an effective anti-inflammatory *in vitro*, and because it is a mixture of different procyanidns, **we aimed to identify the pure structure(s) responsible for the effects described.**

3. After confirming the anti-inflammatory effects of procyanidns *in vitro,* we wanted to investigate its molecular mechanism. For this purpose we evaluated if **the anti-inflammatory effect of procyanidins could be the result of inhibiting the MAPK and NF-kB signalling pathways**, which are the most important pro-inflammatory pathways that regulate the production of many pro-inflammatory mediators.

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4. Since disturbances of glucose regulation and low-grade inflammation, as part of metabolic syndrome, are important risk factors for atherosclerosis, we wanted to investigate the procyanidn effect on this CVD where macrophages and inflammation are tightly implicated. Then, our forth objective was to evaluate **the PE effect on the foam cell formation** by assessing its effects on lipid accumulation and inflammation *in vitro*. We tested the PE effect in three foam cell models. Cells were treated with moderately oxidized lipoprotein, minimally oxidized lipoprotein or pretreated with endotoxin and incubated with oxLDL.

5. Our final objective was to assess if **procyanidins could modulate the obesity induced low grade inflammation in animal models of diet induced metabolic syndrome.** In order to study the effectiveness of procyanidins in different experimental rat models of obesity, we focused on examining the putative modulatory effects of procyanidins on cytokine production and expression in white adipose tissue, muscle and liver to gain insight on the mechanisms that underlie the anti-inflammatory effects ascribed to procyanidins.

The research work carried out in this Ph. D Thesis has been supported by a grant from the Rovira I Virgili University and performed mainly in the Nutrigenomics group from the same University. One international stage has been done in the Instituto di Patologia Generale in the Sacro Cuore University in Rome. It was supported by a grant from the European COST organization.

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IV. RESULTS

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I. Grape-Seed Procyanidins Act as Antiinflammatory Agents in Endotoxin-Stimulated RAW 264.7 Macrophages by Inhibiting NFkB Signaling Pathway

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Grape-Seed Procyanidins Act as Antiinflammatory Agents in Endotoxin-Stimulated RAW 264.7 Macrophages by Inhibiting NFkB Signaling Pathway

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Procyanindin extract (PE) is a mixture of polyphenols, mainly procyanidins, obtained from grape seed with putative antiinflammatory activity. We evaluated the PE effect on RAW 264.7 macrophages stimulated with lipopolysaccharide plus interferon-γ that show a rapid enhanced production of prostaglandin E_2 (PGE_2) and nitric oxide (NO). Our results demonstrated that PE significantly inhibited the overproduction of NO, dose and time dependently. PE caused a marked inhibition of PGE_2 synthesis when administered during activation. Moreover, PE pretreatment diminished iNOS mRNA and protein amount dose dependently (10−65 μg/mL). PE (65 μg/mL) pretreatment inhibited NFκB (p65) translocation to nucleus by nearly 40%. Trimeric and longer oligomeric-rich procyanidin fractions from PE (5−30 μg/mL) inhibited iNOS expression but not the monomeric forms catechin and epicatechin. Thus, we show that the degree of polymerization is important in determining procyanidin effects. PE was considerably a more effective inhibitor of NO biosynthesis (IC_{50} = 50 μg/mL) in comparison to other antiinflammatories, such as aspirin (3 mM), indomethacin (20 μM), and dexamethasone (9 nM). In conclusion, PE modulates inflammatory response in activated macrophages by the inhibition of NO and PGE_2 production, suppression of iNOS expression, and NFkB translocation. These results demonstrate an immunomodulatory role of grape seed procyanidins and thus a potential health-benefit in inflammatory conditions that exert an overproduction of NO and PGE_2.

KEYWORDS: Procyanidins; dimers; trimers; lipopolysaccharide; INF-γ; RAW 264.7; NO; iNOS; PGE_2; NFκB; p65

INTRODUCTION

Procyanidins are phenolic compounds from the flavonoid group that are widely found in cereals, vegetables, and fruits such as grapes, berries, cocoa, and apples. They are high molecular weight polymers of flavan-3-ol units (catechin, epichatechin) (**Figure 1A**).

They have a wide range of biological activities (*1*−*4*). They function as powerful antioxidants and could exert antiinflammatory activities (*5*). There is growing interest in the utilization of procyanidins for their dietary and pharmacological properties. The few *in vitro* studies into the effects of procyanidins on inflammation have shown different results (*6*). This is probably due to the different structural characteristics of the molecules tested and the different experimental designs used. Recent studies have revealed potent antiinflammatory properties of procyanidins on experimental inflammation in rats and mice (*7*, *8*). Their antiinflammatory mechanisms are still poorly understood (*9*). They are related to oxygen free radical scavenging, inhibition of the formation of inflammatory cytokines, and anti-lipid peroxidation (*9*, *10*).

Macrophage activation is crucial in the progression of multiple inflammatory diseases via the release of inflammatory mediators such as cytokines, nitric oxide, and prostaglandins (*11*). In macrophages, cytotoxicity and inflammation can all be promoted through cellular response to nitric oxide (*12*, *13*), a gaseous free radical and intercellular messenger that mediates a variety of biological functions. NO production is mediated by the inducible macrophage isoform iNOS, type II, which is a member of the nitric oxide synthase (NOS) family. iNOS catalyzes the oxidization of L-arginine to produce L-citrulline and NO, and it is expressed in macrophages upon stimulation by interferon-γ

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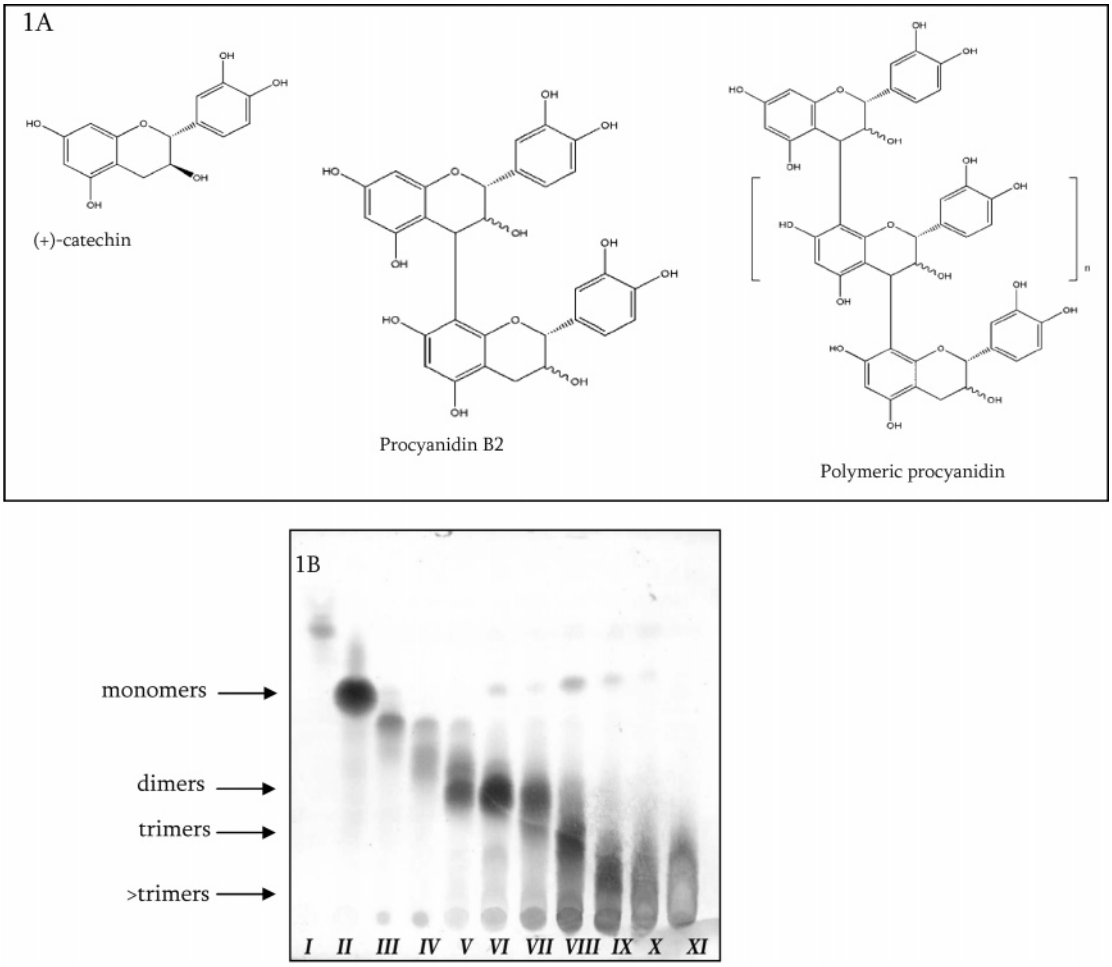

Figure 1. (**A**) Chemical structure of flavonoids. Procyanidins include from dimers to oligomers of (+)-catechin, (–)-epicatechin, and (–)-epicatechin gallate up to 10 units; further polymerized structures are classified as condensed tannins. (**B**) Polymerization degree of grape-seed procyanidin extract confirmed by TLC analysis.

(IFN-γ), tumor necrosis factor-α (TNF-α), interleukin-1 and -6, or lipopolysaccaride (LPS) causing an increase in iNOS mRNA, protein, and activity levels that is followed by a significant increase in NO production over a long period of time. iNOS activation is regulated mainly at the transcriptional level but also at the translational and post-translational levels through effects on protein stability, dimerization, phosphorylation, cofactor binding, and availability of substrates (*14*, *15*).

The control of macrophage overproduction of inflammatory mediators such as PGE_2 and NO should greatly facilitate the treatment of inflammatory diseases. PGE_2 synthesis is catalyzed by cyclooxygenase-2 (COX-2), which is induced in macrophages by the same stimulus as iNOS (*14*, *16*).

Antiinflammatory molecules, such as aspirin and its derivatives (and other NSAIDs) at low therapeutic doses, irreversibly inhibit the activity of cyclooxygenases (COX-1 and COX-2) and the subsequent formation of prostaglandins, mainly PGE_2 (*17*). Other antiinflammatory drugs, such as indomethacin and dexamethasone, have been shown to inhibit NO and PGE_2 synthesis at different levels (*17*, *18*). The expression of iNOS and COX-2 is closely related to the up-regulation of nuclear factor kappa B (NFκB). NFκB, inducible transcription factor, is activated in response to various extracellular stimuli, including INF-γ, LPS, and oxidative stress. NFκB sites have been identified in the promoter region of the iNOS gene and the COX-2 gene. NFκB is present in the cytosol as an inactive complex IkB-NFκB. The IkB complex includes IkBα, -β, and -γ forms. The IkB−NFκB complex is phosphorylated by IkB kinase (IKK), which facilitate the translocation of free NFκB from cytosol to the nucleus and the induction of iNOS and COX-2 gene expression (*19*). The combination of LPS with IFN-γ induces iNOS and COX-2 expression synergistically. The regulation of iNOS and COX-2 via the NFκB pathway is an important mechanism in inflammatory processes and a potential site for intervention in inflammatory diseases (*13*, *20*).

The aim of this study was to examine the putative antiinflammatory effect of procyanidins in macrophages. We used RAW 264.7 cells stimulated with LPS and IFN-γ to mimic inflammation and specifically assessed the procyanidin effect in this model. Their effect on NO and PGE_2 production as well as their mechanism of action in these cells was studied.

MATERIALS AND METHODS

Chemicals. Grape-seed procyanidin extracts (PE) were provided by Les Dérives Résiniques et Terpéniques (Dax, France). According to the manufacturer, the procyanidin extract contained essentially monomeric (21.3%), dimeric (17.4%), trimeric (16.3%), tetrameric (13.3%), and oligomeric (5−13 units) (31.7%) procyanidins and (4.7%) phenolic acids. Catechin, epicatechin, sulfanilamide, naphthylethylenediamide dihydrocloride, aspirin, indomethacin, dexamethasone, and LPS (*Escherichia coli* 0111:B4) were purchased from Sigma-Aldrich, Inc. rmINF-γ was supplied by ProSpec-Tany TechnoGene Ltd. Anti-iNOS antibody and anti-p65 antibody were purchased from SantaCruz Biotechnology, Inc. Anti-beta actin antibody was purchased from Abcam. Monoclonal anti-rabbit IgG was purchased from Sigma-Aldrich, Inc. and ECL Advanced Western Blotting Detection Kit from Amersham Biosciences.

Chromatographic Separation of Procyanidin Extract. PE (0.5 g) was subjected to normal-column chromatography (35−70 mesh, Interchim, Monluçon, France) preconditioned with solvent A (acetone:hexane 65:35) as follows. PE components were separated according to size using an increasing gradient of solvent B (acetone:hexane 80:20). First, low molecular weight compounds were eluted with solvent A. Then, the proportion of solvent B was gradually increased until reaching 100% after 1 h. Then, further volume of solvent B was added, and fractions of 10 mL were collected using a fraction collector. Monitoring of the collected fractions was achieved by TLC on PolyGram silica gel 0.2 mm with fluorescent indicator UV_{254} (Macherey-Nagel, Hoerdt, France), in the mixture toluene:acetone:acetic acid (3:3:1, v/v). Revelation of TLC plates was done by spraying anisaldehyde reagent. Eleven major fractions with increasing degree of polymerization were identified, according to their R_f (**Figure 1B**). These fractions were lyophilized and kept at −20 °C until their utilization for the biological studies.

Mean degree of polymerization (mDP) for the fractions of study was determined by thiolysis according to the method described by Kennedy and Jones (*21*). Five representative fractions with different polymerization degree were selected. These fractions contained mainly monomers (fraction II mDP = 1.01 ± 0.02), dimers (fraction VI mDP = 2.13 ± 0.17), trimers (fraction VIII mDP = 2.71 ± 0.09 and IX mDP = 2.44 ± 0.02) and oligomeric procyanidins longer than trimers (fraction XI mDP = 2.98 ± 0.19).

Cell Culture. Murine macrophage cell line RAW 264.7 (European Tissue Culture Collection ECACC, ref 91062702, London, UK) was cultured in DMEM with or without phenol red containing 10% (v/v) fetal bovine serum, 2 mM D-glutamine, penicillin (100 U/mL), streptomycin (100 μg/mL), and 25 mM HEPES. Cells were grown at 37 °C and with 5% CO_2 in fully humidified air and used for experiments between passages 5−14. At 80% of confluence, cells were stimulated with rIFN-γ (100 U/mL) and LPS (1 μg/mL).

Measurement of Cell Viability. Uptake of the neutral red dye was used as a measure of cell viability in response to procyanidin treatment. Assays were performed in triplicates.

Cell Treatment. At 80% of confluence, adherent monocyte-RAW 264.7 cells were incubated with different PE concentrations (0−85 μg/mL), catechin (0−65 μg/mL) or epichatechin (0−65 μg/mL), and PE fractions of monomers (fraction II), dimers (fraction VI), trimers (fraction VIII and IX) and longer than trimers (fraction XI) at 5−30 μg/mL and with different antiinflammatories such as aspirin (3 mM), indomethacin (5−20 μM), or dexamethasone (9 nM).

Three different treatments were performed: (A) Cells were incubated for 4 h with different compounds, and the culture medium was removed and then activated with 1 μg/mL LPS and 100 U/mL rmIFN-γ for 19 h. (B) Cells were incubated with different compounds (PE, aspirin, indomethacin, or dexamethasone) and with 1 μg/mL LPS and 100 U/mL rmIFN-γ simultaneously for 19 h. (C) Cells were activated with 1 μg/mL LPS and 100 U/mL rmIFN-γ for 15 h, and the different compounds tested were then added for 4 h. The culture medium for control and treated cells was collected and tested.

Measurement of NO Production in RAW 264.7 Cells. The nitrite concentration in the culture medium was measured as an indicator of NO production according to the Griess reaction (*22*). A standard procedure using Griess reagent [1% (w/v) sulfanilamide, 12.5 mM naphthylenediamide, and 6.5 M HCl] was used. Optical density was measured with a microplate reader at 540 nm (Anthos 2000, Pierce Laboratories). Nitrite production was normalized to protein content measured by Bradford method (Bio-Rad). Results were expressed as the percentage of NO production versus that produced by stimulated cells.

Measurement of PGE_2 Concentration. The level of PGE_2 released into culture medium was quantified using a competitive specific enzyme immunoassay (EIA) according to the manufacturer's instructions (Amersham Biosciences). PGE_2 production was normalized to protein content measured by Bradford method (Bio-Rad). Results were expressed as the percentage of PGE_2 production versus that produced by stimulated cells.

iNOS and IkBα mRNA Analysis by RT-PCR Real Time. RNA from treated cells was isolated with High Pure RNA Isolation Kit from Roche. cDNA was synthesized from 1 μg of total RNA using oligo-dT and Superscript II Rnase Reverse Trancriptase (Life Technologies). A 20 ng amount of cDNA was subjected to Quantitative RT-PCR amplification using Sybr Green Master Mix (Applied Biosystems). The forward and reverse primers for iNOS were 5′ GGATCTTCCCAGGCAACCA 3′ and 5′ AATCCACAACTCGCTCCAAGATT 3′, respectively. The forward and reverse primers for IkBα were 5′ CTTGGTGACTTTGGGTGCTGAT 3′ and 5′ GCGAAACCAGGTCAGGATTC 3′, respectively. The forward and reverse primers for G3PDH were 5′ CATGGCCTTCCGTGTTCCT 3′ and 5′ CCTGCTTCACCACCTTCTTGA 3′, respectively. Reactions were run on a quantitative PCR System, and the thermal profile settings were 50 °C for 2 min, 95 °C for 2 min, and then 40 cycles at 95 °C for 15 s and 60 °C for 2 min.

Immunoblotting Analysis of iNOS Enzyme. The cells were lysed with buffer L (0.01 M NaOH, 0.01% SDS, 0.14 M NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, and 8 mM $NaHPO_4{\cdot}12H_2O$). Equal amounts of total cellular protein (20 μg) were resolved by SDS-PAGE 7% under reducing conditions (90 V, 2 h) and transferred to nitrocellulose membranes (400 A, 2.5 h) in buffer T consisting of 20% (v/v) methanol, 200 mM Gly, 25 mM Tris, pH 8.3. The membrane was blocked for 1 h at RT and then incubated with anti-iNOS rabbit polyclonal antibody (1:500) or with anti-actin rabbit polyclonal antibody (1:10000) for 1 h at RT. Horseradish peroxidase-conjugated anti-rabbit antibody was used and incubated for 1 h. Immunodetection was performed using an ECL Advanced Western Blotting Detection Kit chemiluminescence system (Amersham Biosciences). The autoradiograms were quantified using scanning densitometry (Quantity One, Bio-Rad).

Cell Fractionation. Nuclear extracts were prepared as described (*23*, *24*). Briefly, after cell activation and treatment for the times indicated, 4×10^6 RAW 264.7 macrophages were washed twice with ice-cold PBS. A 100 μL of ice-cold hypotonic buffer (20 mM HEPES, pH 7.9, 10 mM EDTA, 10 mM KCl, 1 mM DTT, 0.5% (p/v) Nonidet p40 and 1% (v/v) protease inhibitors (Sigma)) was added and left at 4 °C for 10 min on a rocking platform. Cells were scraped from the plates, and the suspension was centrifuged at 14000*g* for 3 min at 4 °C. Aliquots of the supernatant (cytoplasmatic fraction) were collected and stored at −80 °C. Sedimented nuclei were gently resuspended in 20 μL of ice-cold saline buffer (20 mM HEPES, pH 7.9, 1 mM EDTA, 200 mM NaCl, 10% (p/v) glycerol, 1 mM DTT, and 1% (v/v) protease inhibitors), left on ice for 30 min, vortexed, and centrifuged at 14000*g* for 5 min at 4 °C. The supernatant was collected (nuclear fraction) and stored at −80 °C. Protein was determined by the Bradford method (Bio-Rad Protein Reagent).

Western Blotting Analysis of NFκB (p65) Translocation after Procyanidin Treatment. We assayed three different times 0, 30, and 60 min after cell stimulation. A 40 μg amount of cytosolic and a 20 μg amount of nuclear extracts from RAW 264.7 cells were electrophoresed in 12% SDS-PAGE under reducing conditions, transferred to nitrocellulose membranes and blocked with NFDM 3% in PBS. Membranes were incubated with polyclonal antibodies to p65 (1/500) or anti-actin from rabbit. Secondary anti-rabbit peroxidase bound antibody was used (Sigma-Aldrich). The immunodetection was performed using an ECL Advanced Western Blotting Detection Kit chemiluminescence system. The autoradiograms were quantified by densitometry.

Calculations and Statistical Analysis. Results are expressed as mean value ± SEM. Effects were assessed using ANOVA or Student's *t* test. We used Tukey's test of honestly significant differences to make pairwise comparisons. All calculations were performed using SPSS software.

RESULTS

Cell Viability. To assess the potential antiinflammatory effect of procyanidins, we worked on cell line RAW 264.7 of mouse monocytes/macrophages. First we checked the cytotoxicity of procyanidin extract administration to cells by the neutral red assay. We found a range of non-cytotoxic concentration of 0−85 μg/mL of PE extract. Cell viability was >98% at the concentrations treated, which confirms that the observed effects were not

Table 1. Neutral Red Assay in RAW 264.7 Macrophages after Procyanidin Treatment

procyanidin treatment	PE μg/mL	% neutral red in lysosomes[a]
4 h	control	100.00 ± 1.36
	0.86	102.29 ± 10.94
	8.6	98.79 ± 9.72
	46	112.67 ± 9.89
	85	117.85 ± 2.90
	430	89.51 ± 3.07*
	850	65.62 ± 1.36*
19 h	control	100.00 ± 1.25
	45	111.03 ± 3.99
	65	102.21 ± 4.02
19 h + LPS+IFN-γ	0	95.51 ± 4.77
	45	95.12 ± 1.37
	65	98.15 ± 3.00

[a] Cells were plated in 12-well plates and cultured in medium containing 40 μg/mL of neutral red. They were then incubated at 37 °C with PE (0–850 μg/mL) or vehicle for 4 h, PE (0–65 μg/mL) or vehicle for 19 h, or PE (0–65 μg/mL) or vehicle for 19 h with LPS+IFN-γ (1 μg/mL; 100 U/mL, respectively). The medium was removed and neutral red was extracted with 50% (v/v) ethanol, 49% water, 1% glacial acetic acid. The absorbance was measured at 540 nm. All values were taken from three experiments performed in triplicate. Data are mean ± SEM (n = 3). *, significant difference from control (PE = 0 μg/mL) in each treatment; $p < 0.05$.

due to cell death by treatment. Higher incubation periods (19 h) were also non-cytotoxic at the concentration tested (45−65 μg/mL). We also checked the cytotoxicity of LPS+IFN-γ (19 h) to cells by the neutral red assay, and we found non-cytotoxic effects (**Table 1**). In the case of pure compounds, the nontoxic range in this cell line was obtained from the literature (*25*).

Procyanidin Extract Inhibits NO Production in RAW 264.7 Macrophages Induced by LPS and IFN-γ. The PE induced an inhibition of NO production in RAW 264.7 cell line that was dose- and time-dependent. A 4 h preincubation with PE at 25 μg/mL reduced 20% of NO that was present in media compared with a reduction of 55% when pretreatment was performed during 10 h with the same PE concentration. When 4 h preincubation with PE was performed, NO production was significantly inhibited after induction with LPS+INF-γ at all tested concentrations (10−85 μg/mL) (**Figure 2A**).

When PE was administered for 19h of stimulation period, NO was strongly inhibited (only 40% of NO was present in media at 10 μg/mL compared with 90% when pretreatment was performed) **(Figure 2B)**. When PE was administered at the final stage of stimulation for 4 h, the inhibition of NO was significant beginning from 45 μg/mL **(Figure 2C)**.

As PE contains several compounds that could explain this inhibitory effect, we tested some pure compounds available in the extract at the concentrations expected to be in. Monomeric procyanidins epicatechin and catechin (5−65 μg/mL) did not exert any significant inhibition of NO synthesis in these cells after preincubation for 4 h (See **Figure 3**).

Procyanidin Extract Inhibits PGE_2 Production in RAW 264.7 Macrophages Induced by LPS and IFN-γ. The PE inhibited PGE_2 production in the RAW 264.7 cell line when procyanidins were coincubated with LPS+INF-γ for 19 h **(Figure 4B)**. When they were incubated during the last 4 h of stimulation, 45 μg/mL dose of PE significantly inhibited PGE_2 production (**Figure 4C**). A 4 h preincubation period with PE did not inhibit PGE_2 cell production (**Figure 4A**).

Procyanidin Extract Inhibition of NO and PGE_2 Production Is Comparable to Indomethacin, Aspirin, and Dexamethasone. We then compared the capacities of procyanidins

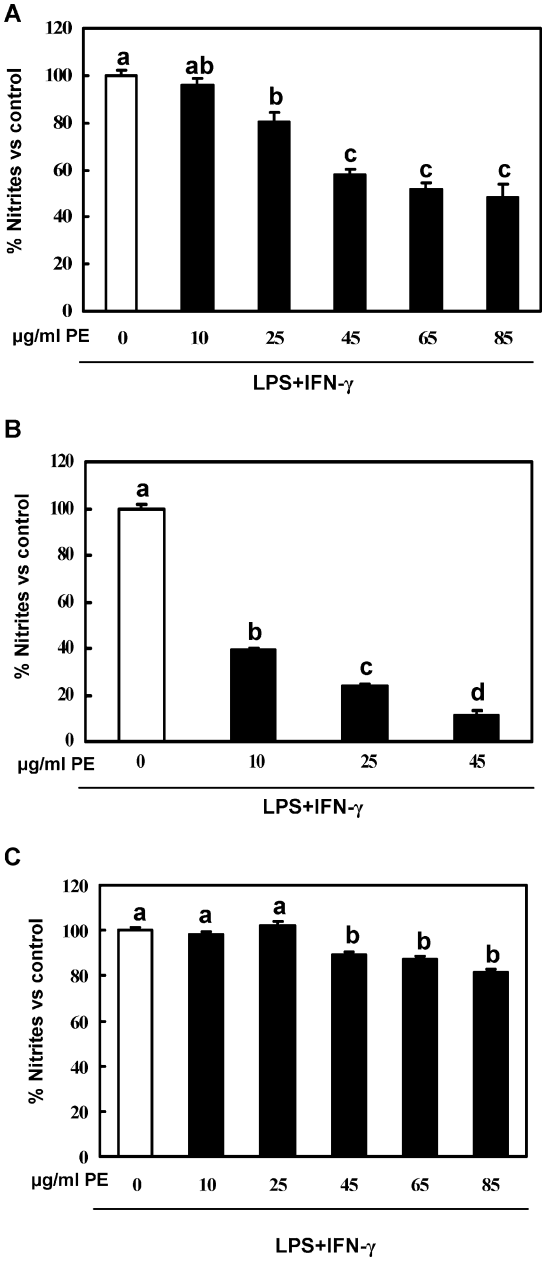

Figure 2. PE inhibition of NO production in LPS+IFN-γ induced RAW 264.7 macrophages. RAW 264.7 macrophages were preincubated for 4 h (**A**), coincubated for 19 h (**B**) or incubated for the last 4 h of activation (**C**) with different PE concentrations. Cells were stimulated with LPS+INF-γ. Results were normalized to control levels (100%). Each value represents mean ± SEM of three experiments performed in triplicate.

and the selected drugs to inhibit NO and PGE_2 production by iNOS and COX-2 in RAW 264.7 cells exposed to LPS+IFN-γ, as shown in **Tables 2** and **3**. We observed that a 4 h preincubation with PE, aspirin, and indomethacin (only at 20 μM) caused a decrease in NO when compared to stimulated control. Aspirin (3 mM), indomethacin (20 μM), and PE (65 μg/mL) inhibited NO production by about 19, 38, and 42%, respectively ($p < 0.001$) when compared to stimulated control. In contrast, dexamethasone (9 nM) did not exert any inhibition on NO production. All treatments showed similar results in NO inhibition by antiinflammatories (see **Table 2**). The most effective inhibition of NO with PE was observed in the coincubation treatment. We also tested lower doses of aspirin

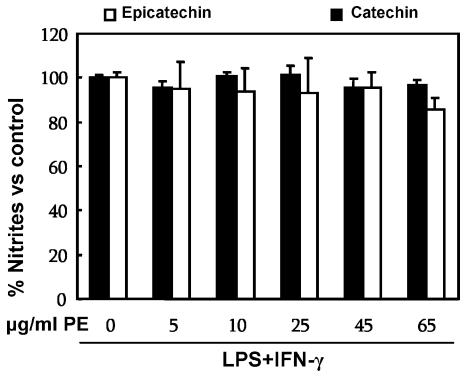

Figure 3. Monomeric procyanidins effect on NO production in LPS+IFN-γ induced RAW 264.7 macrophages. NO production after 4 h preincubation and subsequent stimulation with LPS+INF-γ for 19 h was measured. Each value represents mean ± SEM.

(0.015−1.5 mM), and we found the same inhibition as the high dose tested (3 mM).

We found inhibitory effects on PGE_2 production with all the selected antiinflamatories, except dexamethasone, in all the treatments performed. In contrast, PGE_2 production was inhibited when incubation with PE was performed during or at the end of the activation period but not by preincubation with PE (see **Table 3**). Although PE is an effective inhibitor of NO synthesis at tested concentrations, with respect to PGE_2 production it is not as effective as the drugs tested, which are more effective as PGE_2 inhibitors than are the NO inhibitors.

Modulation of iNOS Expression by Procyanidins. Several studies have shown that the induction of iNOS produces large amounts of NO during endotoxemia and under inflammatory conditions. Therefore, compounds that inhibit iNOS expression and/or enzyme activity decreased NO generation and may be beneficial in treating diseases caused by an overproduction of NO. In view of the involvement of iNOS in the inflammatory process, we monitored iNOS gene expression in macrophages exposed to PE.

We measured mRNA levels by real-time RT-PCR analysis. The expression of iNOS mRNA was hardly detectable in unstimulated cells. However, RAW 264.7 cells expressed high levels of iNOS mRNA when stimulated with LPS+IFN-γ for 19 h. Moreover, PE inhibited this LPS-stimulated iNOS mRNA production in a dose-dependent manner (**Figure 5A**). To determine the inhibitory mechanism of NO production from LPS-activated RAW 264.7 cells by PE, we checked the decrease in iNOS enzyme amount using the Western blotting technique. As **Figure 5B** shows, iNOS protein is barely detectable in unstimulated cells but it increased markedly 19 h after LPS (1 μg/mL) and IFN-γ (100 U/mL) treatment. Treatment with PE showed a concentration-dependent decrease in iNOS protein levels in LPS-stimulated RAW 264.7 cells that was statistically significant at the 45−85 μg/mL interval compared to control (**Figure 5**, table, lines 4−6).

Modulation of IkB-α mRNA Expression by Procyanidins. To assess the effect of PE on other NFκB target genes, other than iNOS, IkBα levels were measured by real-time RT-PCR analysis. The expression of IkBα mRNA was low in unstimulated cells. However, RAW 264.7 cells expressed high levels when stimulated with LPS+IFN-γ for 19 h. PE inhibited IkBα mRNA production in a dose-dependent manner (**Figure 5A**).

Differential Inhibitory Effects on NO Production and iNOS mRNA Expression Depending on the Procyanidin Fraction. The tested fractions induced an inhibition of NO production in RAW 264.7 cell line that was dose dependent.

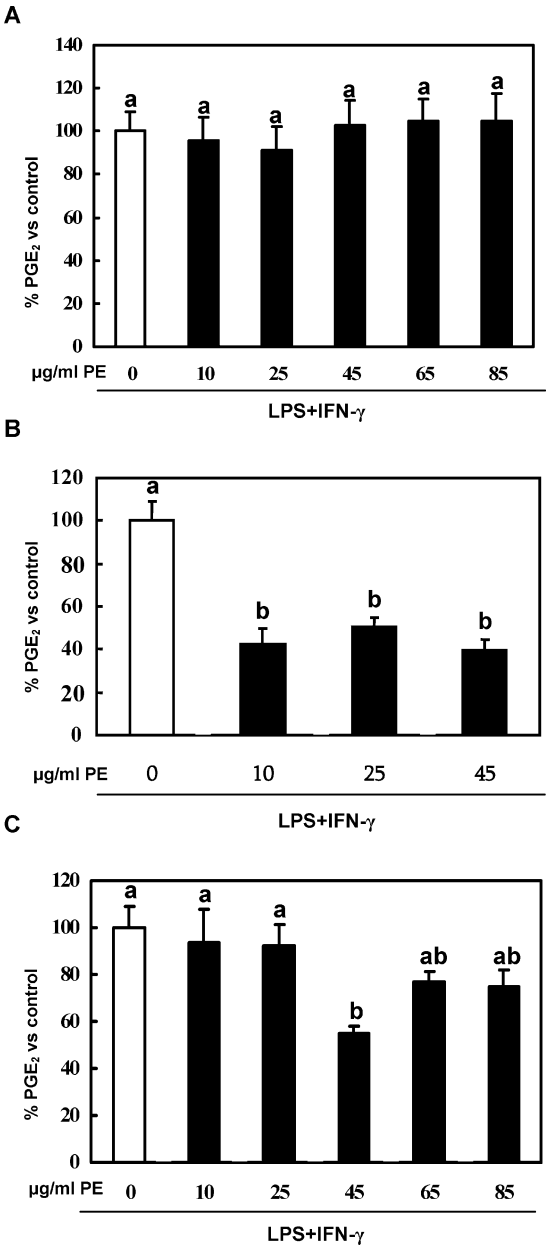

Figure 4. PE inhibition of PGE_2 production in LPS+IFN-γ induced RAW 264.7 macrophages. RAW 264.7 macrophages were preincubated for 4 h (**A**), coincubated for 19 h (**B**) or incubated for the last 4 h of activation (**C**) with different PE concentrations. Cells were stimulated with LPS+INF-γ and PGE_2 was measured after these treatments. Results were normalized to control levels (100%). Each value represents mean ± SEM of three experiments performed in triplicate.

The monomeric fractions did not exert any inhibitory effect on NO production coinciding with the results obtained with pure catechin and epicatechin.

When 4 h preincubation with the different fractions was performed, NO production was significantly inhibited after induction with LPS+INF-γ at all tested concentrations (5−30 μg/mL). This inhibitory effect increased with the polymerization degree of the procyanidins up to trimer and was dose dependent (**Figure 6A**).

To assess the effect of the procyanidin fractions on iNOS mRNA levels, we measured mRNA levels by real-time RT-PCR analysis. Our results show that trimeric and longer

Table 2. Effect of Procyanidins on NO Production in LPS+INF-γ Activated Macrophages Compared to Other Antiinflammatories

	% NO versus control		
treatment[a]	before activation	coincubation	4 h after activation
control	100.00 ± 6.70	100.00 ± 1.07	100.00 ± 1.41
3 mM aspirin	81.07 ± 4.87*	87.48 ± 6.00*	91.27 ± 3.07
5 μM indomethacin	85.89 ± 6.47	96.27 ± 4.04	89.36 ± 9.35
20 μM indomethacin	62.69 ± 2.79*	86.49 ± 2.58*	95.74 ± 1.16
9 nM dexamethasone	91.16 ± 4.93	100.02 ± 9.17	89.65 ± 2.12
65 μg/mL PE	38.64 ± 6.30*	11.05 ± 2.16*[b]	84.63 ± 1.79*

[a] RAW 264.7 macrophages were preincubated for 4 h, coincubated for 19 h, or incubated for the last 4 h of activation with PE 65 μg/mL, 3 mM aspirin, 5 and 20 μM indomethacin, or 9 nM dexamethasone. Cells were stimulated with LPS+INF-γ and NO production was measured after these treatments. Results were normalized to control levels (100%). Each value represents mean ± SEM of three experiments performed in triplicate. *, $p < 0.05$ as compared to control. [b] 45 μg/mL PE.

Table 3. Effect of Procyanidins on PGE_2 Production in LPS+IFN-γ-Activated Macrophages Compared to Other Antiinflammatories

	% PGE_2 versus control		
treatment[a]	before activation	coincubation	4 h after activation
control	100.00 ± 13.18	100.00 ± 6.78	100.00 ± 8.18
3 mM aspirin	13.67 ± 1.98*	1.20 ± 0.20*	31.41 ± 5.24*
5 μM indomethacin	10.33 ± 2.67*	0.43 ± 0.10*	10.11 ± 1.84*
20 μM indomethacin	9.74 ± 4.66*	0.47 ± 0.16*	11.64 ± 1.81*
9 nM dexamethasone	68.22 ± 1.77*	0.68 ± 0.16*	98.09 ± 7.53
65 μg/mL PE	96.75 ± 2.00	39.64 ± 5.31*[b]	83.54 ± 3.77*

[a] RAW 264.7 macrophages were preincubated for 4 h, coincubated for 19 h, or incubated for the last 4 h of activation with PE 65 μg/mL, 3 mM aspirin, 5 and 20 μM indomethacin, or 9 nM dexamethasone. Cells were stimulated with LPS+INF-γ, and PGE_2 was measured after these treatments. Results were normalized to control levels (100%). Each value represents mean ± SEM of three experiments performed in triplicate. *, $p < 0.05$ as compared to control. [b] 45 μg/mL PE.

oligomeric fractions inhibited this LPS-stimulated iNOS mRNA production in a dose-dependent manner (**Figure 6B**).

Procyanidin Extract Modulates NFκB Translocation to Nucleus. Because NFκB plays a key role in the induction of iNOS by LPS, we examined the effect of PE on this transcription factor. We observed that the incubation of cells with LPS+INF-γ produced an increase in NFkB translocation (p65 subunit) to the nuclear compartment that was evident at 30 min of incubation time. **Figure 7** shows that this translocation process is inhibited in cells pretreated with PE (65 μg/mL) before stimulation, which is demonstrated by the immunoblotting of the p65 protein in nuclear extracts. Maximal inhibition was at 60 min.

DISCUSSION

The results in the literature about the effect of procyanidins on NO metabolism are controversial (*25*−*28*). In agreement with our results, previous studies have shown that pine bark procyanidin extract (Pycnogenol) inhibited NO generation in RAW 264.7 macrophages (*26*). On the other hand, other authors reported a proinflammatory effect of trimer C2 and Pycnogenol in IFN-γ stimulated RAW 264.7 cells and antiinflammatory effect of monomers and dimers (*26*, *29*).

In this study, we show that PE acts as an antiinflammatory substance *in vitro*. We demonstrate that procyanidins inhibit PGE_2 production and regulate, at the transcriptional level, the amount of iNOS mRNA and protein and, consequently, NO production. We administered the bioactive substances before, during, and in the last period of cell stimulation. We found that PE protected cells from overproduction of inflammatory mediators, mainly NO and PGE_2 generation. PE inhibition of NO production was more effective when treatment with PE was performed during cell stimulation and for a longer period of time (*17*). Nevertheless, it was also observed in the other treatments. As far as we know, the effects of procyanidins on PGE_2 production have not been studied before in this cell model

Treatment	Relative iNOS mRNA	iNOS protein	Relative IKBα mRNA
Control	1.07 ± 0.12	100.00 ± 1.87	1.01 ± 0.04
PE µg/mL 10	0.58 ± 0.05 **	90.12 ± 7.09	0.81 ± 0.07*
25	0.45 ± 0.03 **	45.92 ± 7.29*	0.66 ± 0.05*
45	0.33 ± 0.05 **	18.63 ± 1.43*	0.56 ± 0.02*
65	0.16 ± 0.05 **	9.03 ± 1.45*	0.43 ± 0.01*
85	0.06 ± 0.01 **		0.51 ± 0.03*

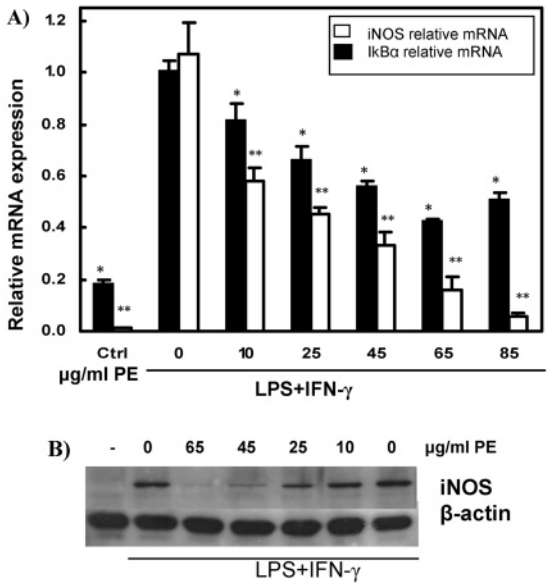

Figure 5. PE pretreatment of RAW 264.7 cells inhibits iNOS and IKBα gene expression and iNOS protein levels. Cells were preincubated with different PE concentrations for 4 h and then stimulated with LPS+INF-γ (1 μg/mL; 100 U/mL, respectively) for 19 h. After incubation, mRNA was extracted, cDNA was synthesized and measured by real-time RT-PCR (**A**). iNOS protein levels were determined by Western blotting in RAW 264.7 cells (**B**). Each value represents mean ± SEM of three experiments performed in triplicate. ∗, indicates significant differences from control group ($p < 0.05$). ∗∗, indicates significant differences from control group ($p < 0.0001$).

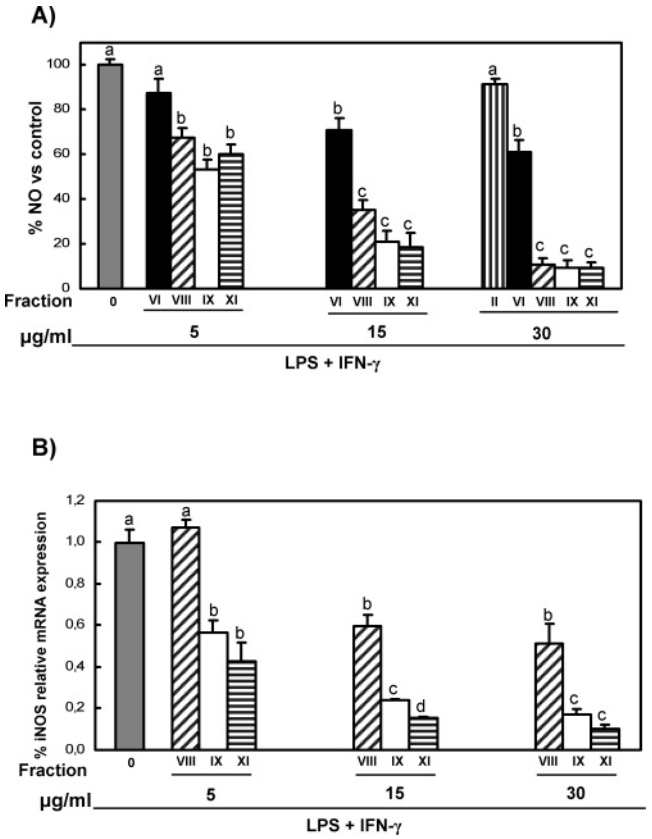

Figure 6. Effect of the procyanidin polymerization degree on NO production and iNOS mRNA expression. Cells were preincubated with the different fractions (5–30 μg/mL) for 4 h and then stimulated with LPS+INF-γ (1 μg/mL; 100 U/mL, respectively) for 19 h. Fractions tested were: II (monomers), VI (dimers), VIII (trimers), IX (trimers), and XI (oligomers longer than trimers). Results were normalized to control levels. Each value represents mean ± SEM of three experiments performed in triplicate. Effects were assessed using ANOVA ($p < 0.05$).

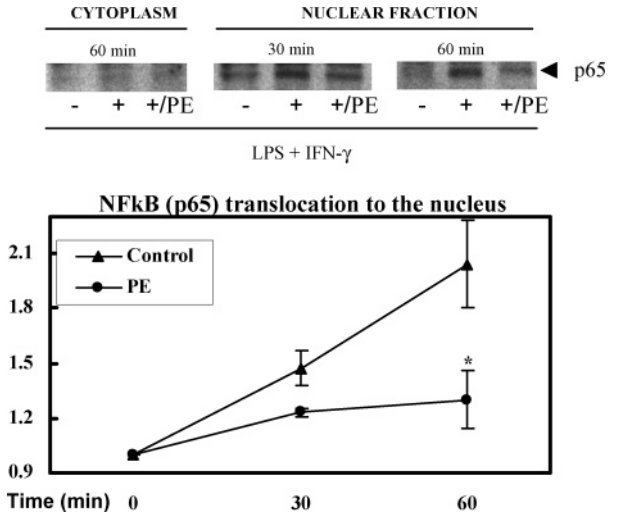

Figure 7. Effect of PE on p65 translocation. Cells were preincubated with PE (65 μg/mL) for 4 h and then stimulated with LPS+INF-γ (1 μg/mL; 100 U/mL, respectively) for 30 and 60 min. After cell fractionation, 20 μg of nuclear and 40 μg of cytosolic fractions were used for p65 Western blotting. Each value represents mean ± SEM of three experiments performed in triplicate.

although an inhibition of COX-2 expression by procyanidin dimer B2 has been recently described in human monocytes (*29*). We found that PE causes PGE_2 inhibition when it is administered during or at the end of the cell-stimulation period at concentrations of between 10 and 45 μg/mL but not with preincubation.

Procyanidins produced greater effect on NO production than on PGE_2 production in this cell model. In fact, the expression of COX-2 mRNA is regulated by several transcription factors including the cyclic-AMP response element binding protein (CREB), NFκB, AP-1, and the CCAAT-enhancer binding protein (C/EBP). COX-2 is also affected post-transcriptionaly, at the level of mRNA stability. Moreover, COX-2 can be affected directly at its enzymatic activity by nitric oxide. Then, each step of COX-2 regulation can be used as potential regulatory target (*30*). Despite our results showing an inhibitory effect of PE on NFκB translocation, there are many other regulatory targets for COX-2 expression and consequently, PGE_2 production, while, NFκB is the main regulatory step for iNOS expression and therefore NO production (*14*).

In this work, we compared the ability to inhibit NO and PGE_2 production in endotoxin-stimulated macrophages by PE (10−85 μg/mL), the classical NSAIDs (indomethacin, aspirin), and the glucocorticoid dexamethasone. Interestingly, the PE (65 μg/mL) inhibitory effect on NO production was substantially higher than the effect of indomethacin (5 and 20 μM), dexamethasone (9 nM), or aspirin (3 mM), which is at the upper range of plasma salicylate levels in individuals who are routinely taking high-dose aspirin. The mode of action of NSAIDs and dexamethasone is not yet fully understood (*31*) but their dose- and time-dependent inhibitory effect on iNOS and COX-2 activities has been established (*17*, *32*). Some studies indicate that the aspirin dose tested is probably not sufficient to block the transcription of iNOS gene (*17*). Furthermore, the NFκB-dependent transcription can be suppressed by NSAIDs at very high doses, which block IkB kinase activation (*33*).

Since iNOS is mainly regulated on the level of expression, the inhibitory effects of PE on NFκB translocation might be responsible at least in part of the inhibition of NO production. The concentrations tested of NSAIDs and dexamethasone might be ineffective on NFκB translocation and responsible for the difference found between commonly used antiinflammatories and PE on NO production.

On the contrary, NSAIDs exert a higher inhibition of PGE_2 production than PE. NSAIDs are traditional COX-2 activity inhibitors at pharmacological concentrations as shown in our results. Some authors have evaluated the role of NO on the activity of COX-2, and their data suggested that NO directly interacts with COX-2 to cause an increase in the enzymatic activity (*30*). Then, the PE inhibitory effect on PGE_2 production could be due to the diminished NO release, and consequently COX-2 activity is reduced. Moreover, this PE inhibitory effect of PGE_2 production was only shown when NO production was strongly inhibited in the coincubation treatment. Therefore, this may be one of the mechanisms of PE to reduce PGE_2 production. Further studies are needed to clarify the differences shown between PE and NSAIDs on NFkB, iNOS, and COX-2 regulation (*34*).

Since procyanidin extract is a complex mixture of different-sized monomers and polymers as well as phenolic acids, we determined which fraction in the extract is responsible for its effects. When trimeric or fractions of higher degree of polymerization were tested, we proved that dimers, trimers, and longer oligomers exerted an increasing degree of inhibition on NO production. We also tested some of the pure components of PE, including catechin and epicatechin, without any effect on NO synthesis.

To elucidate the mechanism by which PE inhibits NO production, we analyzed iNOS mRNA and total amount of iNOS enzyme. We found that PE strongly inhibited iNOS mRNA enzyme expression, which resulted in a decrease in iNOS protein. The iNOS enzyme inhibition mechanism was directly related to the reduction in the amount of NFκB(p65) in the cell nucleus. This resembles the mechanism described for other antioxidants such as anthocyanins and selenium, which are reported to suppress NO production in macrophages by mechanisms based on their ability to inhibit the activation of NFκB by reducing the intracellular redox state. This activation is critical in the induction of iNOS and is required to induce the expression of COX-2 in LPS-stimulated RAW 264.7 cells (*35*). Although no common second messenger has been identified, most NFκB activating signals can be inhibited by antioxidants because critical steps such as protein phosphorylation and binding of transcription factors to consensus sites on DNA are regulated by the intracellular redox status (*36*, *37*).

In RAW 264.7 macrophages, we propose procyanidins to act both by scavenging NO radicals (*38*) and by competition for TLR-4 (Toll Like Receptor-4) with LPS or by direct binding to LPS. This is reinforced by results shown in **Figure 2**, where coexistence of LPS and PE exert a higher inhibition of NO than that found when preincubation with PE alone was performed.

Procyanidins have been described to act by direct inhibiting NFκB target gene expression, although other sites of regulation of PE cannot be ignored. Our findings suggest that PE may exert antiinflammatory effect by inhibiting iNOS expression at transcriptional level by suppression of the NFκB signaling pathway.

Data presented herein show that PE treatment significantly inhibits NFκB translocation to the nucleus. These results suggest a possible inhibition of the degradation of IkB by regulation of IkB upstream proteins as IKK or 26S proteasome (*39*, *40*), but PE inhibition of NFκB DNA binding activity cannot be ruled out and has been described by others (*41*). We have found a down-regulation of IkBα that is a target gene of NFκB, thus reinforcing that PE could reduce NFκB activity.

The ability of procyanidins to ameliorate *in vivo* inflammation has been also reported in different animal models (*34*). The most of *in vivo* antiinflammatory effects are observed by intraperitoneal administration. Although, bioavailability of procyanidins *in vivo* is a matter of investigation, several studies indicate that some procyanidin dimers and trimers can be absorbed intact in rats and humans, but a possible oral effect is not characterized yet (*42*). Further studies are needed to confirm the role and pathophysiological implications of procyanidins *in vivo* in animal models of inflammation and in human disease.

ABBREVIATIONS USED

LPS, lipopolysaccharide; PE, procyanidin extract; NO, nitric oxide; iNOS, inducible nitric oxide synthase; INF-γ, interferon-γ; PGE_2, prostaglandin E_2; NFκB, nuclear factor-κB; COX-2, cyclooxygenase-2; NSAIDs, nonsteroidal antiinflammatory drugs; IkBα, alpha inhibitor of kappa beta.

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II. Oligomeric procyanidins modulate LPS-induced inflammatory response by inhibiting intracellular ROS production, ERK1/2 activation and IKK activity in human macrophages

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OLIGOMERIC PROCYANIDINS MODULATE LPS-INDUCED INFLAMMATORY RESPONSE BY INHIBITING INTRACELLULAR ROS PRODUCTION, ERK1/2 ACTIVATION AND IKK ACTIVITY IN HUMAN MACROPHAGES

Abstract

While there is substantial evidence to support the positive role of flavonoids to health, how specific flavonoids exert these benefits is under intensive investigation. For this reason, the capacity of monomeric, dimeric and trimeric pure procyanidins to modulate LPS-induced ROS production in the human cell line THP-1 was investigated. We also analyzed the effects of procyandins on two major signal transduction pathways, redox regulated, associated with inflammation: NF-kB and the mitogen-activated protein kinase, ERK1/2. The classic LPS-triggered increase of cell oxidants was prevented when cells were preincubated for 4 h with a procyanidin extract (PE), EGCG, EG, the dimer B1 and the trimeric procyandin C1, but not when incubated with the dimer B2 or epicatechin. LPS induced the activation of ERK1/2 through phosphorylation, and such activation was inhibited by all the compounds tested being the most active EG, followed by EGCG and C1. We then assayed the *in vitro* modulation of IkB kinase (IKK) activity driven by procyanidins. We identified that the inhibition of IKK activity was an important target in the anti-inflammatory action of procyanidins. C1 and PE exerted the maximal inhibition of IKK activation, followed by EGCG. Also the dimer B1 produced a significant reduction, in contrast to B3. Among the monomers tested catechin exerted a slight but significant inhibition differing from epicatechin that was ineffective. We conclude that some pure oligomeric procyanidins reduce the LPS-induced production of ROS, and that at least in part they exert their anti-inflammatory effects by inhibiting IKK and ERK1/2 activation.

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Introduction

The major outer membrane component of gram-negative bacteria, lipopolysaccharide (LPS) or endotoxin, is a potent activator of monocyte or macrophage function leading to responses that are both protective and injurious to the host [1]. LPS induces the expression of the procoagulant molecular tissue factor and inflammatory cytokines such as tumor necrosis factor alpha (TNF-α). LPS binds to LPS-binding protein (LBP) in serum, followed by the binding of the LBP/LPS complex to CD14, which activates signal transduction pathways and transcription factors and induces gene expression [2]. LPS has been shown to activate members of the mitogen-activated protein kinase (MAPK) family, including extracellular signal-regulated kinases (ERK)1/2, c-Jun amino terminal kinases (JNKs), and p38. Inhibition of MAPK kinase (MEK) in monocytes by a specific inhibitor U0126 reduced LPS induction of several inflammatory cytokines, including interleukin-1, interleukin-8, and TNF-α as well as prostaglandin E2, indicating a role for the ERK1/2 pathway in LPS signalling independent of the JNK and p38 pathways [3]. LPS stimulation of monocytes activates many transcription factors, including the nuclear factor NF-kB/Rel family, which induce genes encoding various inflammatory mediators. In unstimulated monocytes the NF-kB/Rel proteins are retained in the cytoplasm by their interaction with the inhibitors IkBs. LPS stimulation of monocytes leads to the phosphorylation of IkBs by IkB kinases (IKKs), leading to the rapid translocation of NF-kB/Rel proteins to the nucleus. It has been demonstrated that LPS activation of IKK is required for kB-dependent transcription and TNF-α expression in human monocytes and THP-1 cells [2].

The reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2), superoxide anion ($O_2^{\bullet -}$) and hydroxyl radical ($OH^{\bullet}$) are generated through multiple sources in the cell, such as the electron transport chain in mitochondria, ionizing radiations and through enzymes producing superoxide anion such as phagocytic and non-phagocytic NADPH oxidases and, lipoxygenases and cycloxygenases. When cellular production of ROS overwhelms its antioxidant capacity, a state of oxidative stress is reached leading to serious cellular injuries and contributing to the pathogenesis of several diseases [4, 5]. Nevertheless, if not generated in too high concentration, ROS act as second messengers in signal transduction and gene regulation in a variety of cell types and under several biological conditions such as cytokine, growth factor and hormone treatments, ion transport, transcription, neuromodulation and apoptosis. It is now well established that H_2O_2 is the main ROS mediating cellular signalling because of its capacity to inhibit tyrosine phosphatases through oxidation of cysteine residues in their catalytic domain, which in turn activates tyrosine kinases and downstream signalling. Depending on the level of ROS, different redox-sensitive transcription factors are

activated and coordinate distinct biological responses. A low oxidative stress induces Nrf2, a transcription factor implicated in the transactivation of gene coding for antioxidant enzymes. An intermediate amount of ROS triggers an inflammatory response through the activation of NF-κB and AP-1, and a high level of oxidative stress induces perturbation of the mitochondrial PT pore and disruption of the electron transfer, thereby resulting in apoptosis or necrosis. NF-κB was the first transcription factor shown to be redox-regulated.

The recent emphasis on the role played by RNS and ROS in pathological conditions has led to the discovery of new therapeutic agents. Antioxidants such as (−)-epigallocatechin-3-gallate (EGCG), resveratrol and naturally occurring flavonoids including apigenin and kaempferol have been reported to suppress RNS and ROS production. Epidemiologic studies have linked flavonoid-rich foods with a reduced risk of cancer and cardiovascular disease. Several food and drinks of plant origin such as grapes, cocoas and chocolate can be flavonoid-rich, composed of primarily the monomeric flavanols (−)-epicatechin and (+)-catechin, and oligomeric procyanidins formed from these monomeric units. Structurally, they consist of one or more aromatic rings with one or more hydroxyl groups, which can readily combine with free radicals to form resonance-stabilized phenoxyl radicals. Moreover, the catechol structure of procyanidins enables them to chelate transition metals such as copper and iron, which play an important role in oxygen metabolism. This structure confers strong antioxidant properties; therefore, procyanidins exhibit antioxidant activity and can thus help to prevent peroxidation and cellular oxidant damage. Recently, it has been shown that procyanidins modulate the activity of such regulatory enzymes as cyclooxygenase, lipoxygenase, and an angiotensin-converting enzyme, hyaluronidase, to limit free radical production. EGCG, the potent well known antioxidant [6-8], has been particularly well studied and shown to inhibit lipopolysaccharide (LPS)-induced TNF-α production, induction of inducible nitric-oxide synthase in mouse macrophages and IL-8 production by human keratinocytes or endothelial cells. Several studies have focused on the potential mechanisms of EGCG's anti-inflammatory and anticancer effects. One mechanism of action is inhibition of the activation of NF-kB which regulates the expression of a variety of genes critical for the induction of inflammatory cytokines and immune responses, and/or anti-apoptosis.

In the current study we compared the well established effect of EGCG reducing oxidative stress and inflammation with some pure oligomeric procyanidins and a grape-seed procyanidin extract.

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Materials and methods

Reagents:

Grape seed procyanidin extracts (PE) were provided by Les Dérives Résiniques et Terpéniques (Dax, France). Acording to the manufacturer the procyanidin extract contained 76% procyanidin of which 1.63% are phenolic acids (mainly gallic acid), 20.92% of monomers (mainly catechin + epicatechin), 20.71% dimers + EGCG, 17.33% trimers and 39.41% of oligomeric forms of 4 units or more. The pure molecules as the procyanidins B1 and B2, EG and EGCG were purchased from Sigma-Aldrich, Inc. The procyanidin extract or pure molecules were prepared in absolute ethanol. Appropriate dilutions were made to have a 0.01% (v/v) ethanol concentration in all control and treated wells.

Cell culture:

THP-1 (American Type Culture Collection, Rockville, MD) were grown in RPMI Dutch Modified (Sigma, Milan, Italy) supplemented with 10% fetal calf serum, nonessential amino acids, 2 mM glutamine, penicillin (100 U/mL), streptomycin (100 μg/mL) and 1 mM sodium pyruvate. Cells were maintained in log phase by seeding twice a week at density of 3×10^5 cells/ml at 37°C under 5% CO_2/air atmosphere. Experiments were routinely carried out on triplicate cultures. The trypan blue dye exclusion method was used to evaluate the percentage of viable cells.

DCF assay:

The cell-permeant dye 6-carboxy-2′,7′-dichlorodihydrofluorescein diacetate (DCF) is oxidized by hydrogen peroxide, peroxinitrite (ONOO_), and hydroxyl radicals (OH) to yield the fluorescent molecule 2′,7′-dichlorofluorescein. Thus, dye oxidation is an indirect measure of the presence of these reactive oxygen intermediates, calculated by dividing the mean channel fluorescence of a treated sample by that of the untreated one and multiplying by 100 to obtain the relative change, expressed as a percentage.

$2x10^6$ cells were cotreated with 0.1 μg/ml of LPS and 40 μg/ml of PE for 24 hours or preteated with PE (40 μg/ml) and the pure compounds (10 μg/ml) for 4 h and then stimulated with 1 μg/ml for 30 min. We also determined the LPS dose dependent production of ROS adding 1, 10 and 20 μg/ml of LPS. Following LPS stimulation, cells were collected and washed with PBS to eliminate the amount of the procyanidins not cell associated. Cells were incubated with 2.5 mM DCF (Molecular Probes, Inc., Eugene, OR) for 30 min at 37°C and 5% CO2. A change in fluorescence was assessed with a Cytofluor 2300/2350 Fluorescence Measurement System (Millipore Corp., Bedford, MA).

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Western blot analysis of pERK1/2 and total ERK1/2 expression:

Cells (10×10^6) were pretreated with the different procyanidins (PE, 40 µg/ml; pure compounds, 10 µg/ml)) for 4 hours and then stimulated with LPS (1 µg/ml) for 30 min. Cells were then harvested, washed once with ice-cold PBS, and gently lysed for 30 min in ice-cold lysis buffer (1 mM MgCl2, 350 mM NaCl, 20 mM Hepes, 0.5 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 1 mM Na4P2O7, 1 mM PMSF, 1 mM aprotinin, 1.5 mM leupeptin, 1 mM Na3VO4, 20% glycerol, 1% NP40). Cell lysates were centrifuged for 10 min at 4°C (10,000g) to obtain the supernatants, which were used for Western blot analysis. The anti-ERK1/2 (clone K-23, Cat. No. SC-94) and anti p-ERK1/2 (clone E-4, Cat. No. SC-7383) monoclonal antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The blots were washed and exposed to horseradish peroxidase-labeled secondary antibodies (Amersham Pharmacia Biotech, Arlington Heights, IL) for 1 hour at room temperature. The immunocomplexes were visualized by the enhanced chemiluminescence detection system and quantified by densitometric scanning.

IKK-2 activity assay

We assesed the effects of various procyanidins on the activation of IKK-2 using an in vitro assay following the manufacturers instructions (Cat. No. CBA044, Calbichem). It is based on a colorimetric detection of the phosphorilated substrate IkBa.

Calculations and statistical analysis:

Results are expressed as mean value ± S.E.M. Effects were assessed using ANOVA or Student's t-test. We used Tukey's Test of honestly significant differences to make pair wise comparisons. All calculations were performed using SPSS 15.0 software.

Results:

Viability assay

The typan blue exclusion method was used to evaluate the percentage of viable cells after the treatments with the different procyanidins and LPS in THP-1 cell line (Table 1). We only showed a decrease in THP-1 viability when cells were exposed to LPS (200 ng/mL) for 24h. In this condition, the cotreatment with PE (40 µg/mL) reduced significantly the viability differences with the non-treated cells. This LPS concentration was not used for the experiments.

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ROS production

The incubation of THP-1 cells with LPS at 100 ng/mL for 24h induced a significant increase of intracellular ROS production, as shown by the increased fluorescence obtained by DCF with respect to internal control (macrophages incubated in the presence of the vehicle alone) indicating the presence of intracellular H_2O_2, $ONOO^-$, or OH (Fig. 2). However, the combined addition of PE (40 µg/mL) to cells treated with LPS, prevented intracellular ROS production after 24 h of treatment. On the other hand, cells treated with PE alone also showed a significant decrease in DCF fluorescence.

THP-1 macrophages were also treated with H2O2 alone or in combination with LPS. ROS production H2O2-induced was higher than that induced by LPS alone, however the combination of both inducers produced the maximal response (Fig. 2). In all the treatments PE was able to strongly reduce the intracellular ROS production.

To further investigate the PE action over ROS production we tested some pure compounds that are present in the extract. Firstly we tested the LPS dose-dependent production of ROS (Fig. 3A) and we selected the minimal significant dose of LPS that produced an increase on ROS production after 30 min of incubation (10 µg/mL).

In these conditions, we showed that the PE produced the maximal reduction of ROS which inhibited approximately 60% of ROS compared to LPS, followed by the trimer C1 and the dimer B1. Surprisingly, dimer B2 did not produce any significant reduction (Fig. 3B).

Procyanidins reduce ERK1/2 phosphorylation

We also examined the expression of the ERK1/2 MAP kinases (Fig. 4-5). This kinase has been reported to be activated by various stress stimuli, including an overproduction of ROS. LPS induced a remarkable increase in the level of the phosphorylated form ERK1/2 (p-ERK12) at 30 min and 3h of incubation with LPS (Fig 4). Such increase was prevented by the pre-treatment of the cells with the different compounds, being EGC the most inhibitory one (Fig. 5).

Procyanidins inhibit IKK-2 activity in vitro

IkB kinases, are related kinases that play a major role in the activation and regulation of the transcription factor, NF-kB. In this work we assessed the inhibitory effect of some procyanidins on IKK-2 activity. We showed that the PE and the trimer C1 were the most inhibitory compounds followed by EGCG. The dimer B3 and epicatechin monomer did not affect IKK-2 activity (Fig. 6).

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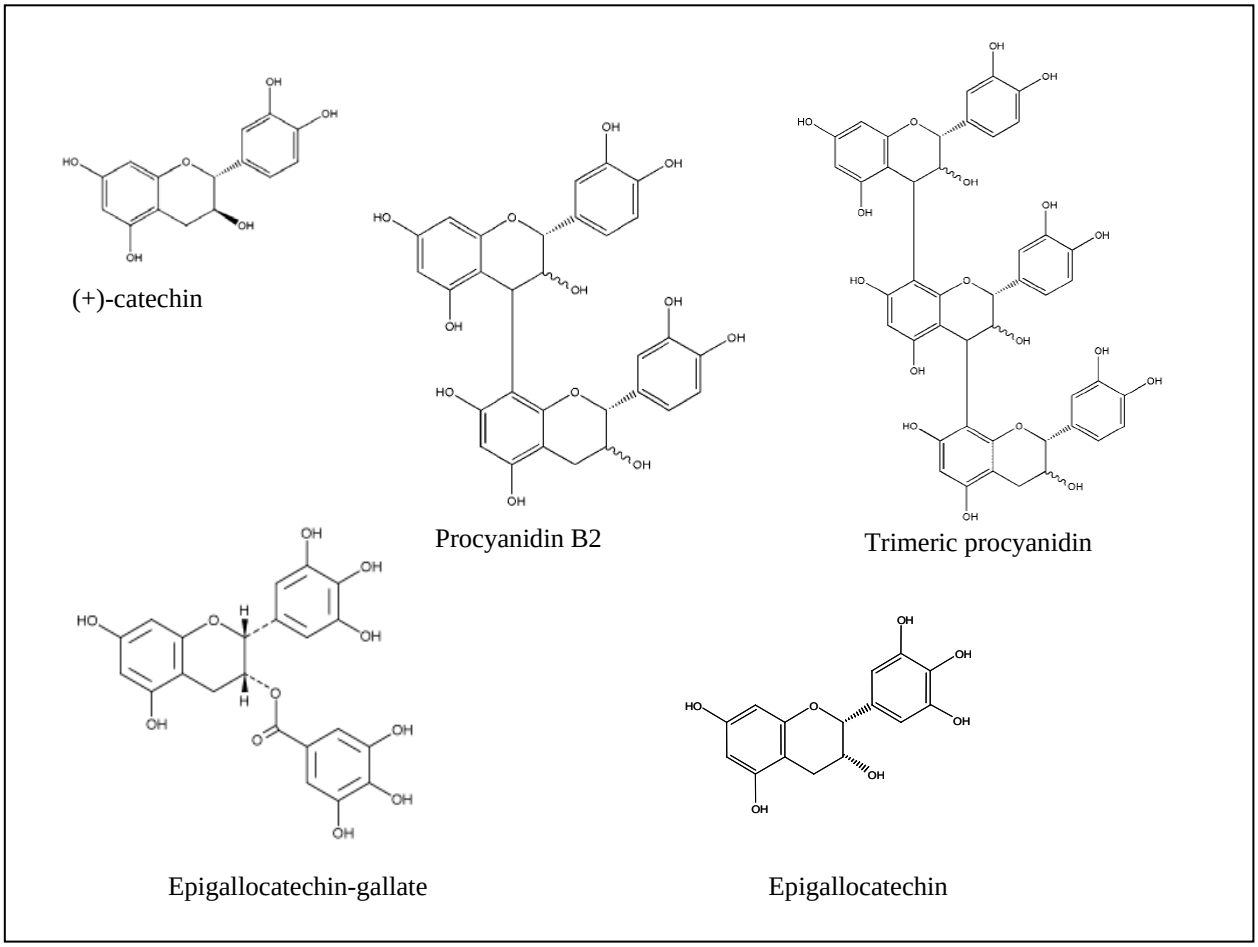

FIGURE 1. Chemical structure of flavonoids. Procyanidins include from dimers to oligomers of (+)-catechin and (-)-epicatechin up to 10 units; further polymerised structures are classified as condensed tannins.

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Table 1. Trypan blue staining of macrophages after treatments.

Treatment	LPS ug/ml	Compound	% living cells	% dead cells
coincubation 24h	0.1	control	98.6 ± 1.1	1.4 ± 1.1
		PE	98.9 ± 1.1	1.1 ± 1.1
		LPS	96.9 ± 2.6	3.1 ± 2.6
		PE+LPS	97.6 ± 0.6	2.4 ± 0.6
	0.2	control	99.6 ± 0.4	0.4 ± 0.4
		LPS	92.2 ± 2.7	7.8 ± 2.7
		PE+LPS	95.5 ± 1.1	4.5 ± 1.1
coincubation 5h	1	control	99.8 ± 0.1	0.2 ± 0.1
		LPS	94.7 ± 3.1	5.3 ± 3.1
		PE+LPS	98.9 ± 0.9	1.1 ± 0.9
4h pretreatment + 30' LPS	10	control	93.2 ± 0.2	6.8 ± 0.2
		LPS	95.0 ± 1.5	5.0 ± 1.5
		PE+LPS	93.7 ± 1.4	6.3 ± 1.4
		C1+LPS	94.7 ± 0.5	5.3 ± 0.5
		B1+LPS	94.2 ± 0.9	5.8 ± 0.9
		B2+LPS	94.6 ± 0.5	5.4 ± 0.5
		EGCG+LPS	95.9 ± 1.2	4.1 ± 1.2
		EG+LPS	94.0 ± 0.1	6.0 ± 0.1

PE (40 µg/ml), procyanidin extract; Pure compounds (10 µg/ml): C1, trimer C1; B1, dimer B1; B2, dimer B2; EGCG, epigallocatechin gallate; EG, epigallocatechin. Values are expressed as mean±S.E.M. The significance of difference among the treatments was analyzed by ANOVA. No significant diferences in cell viability were found.

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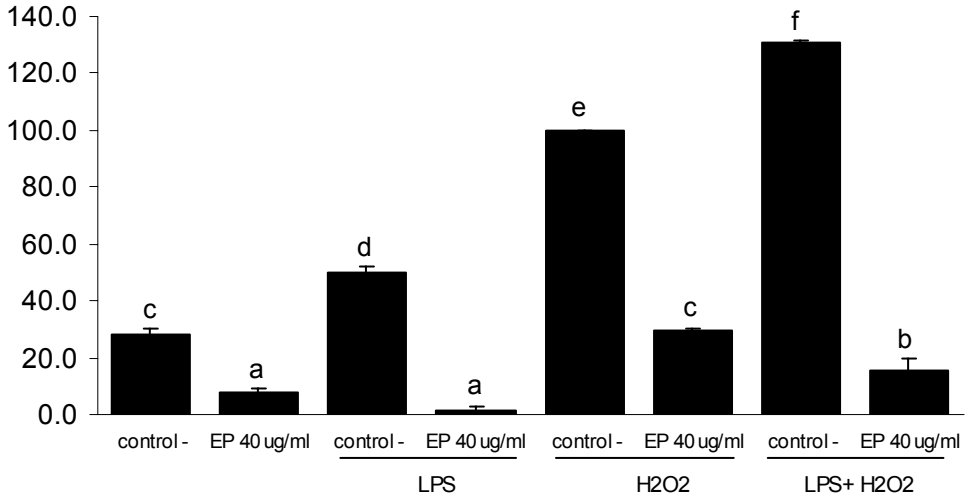
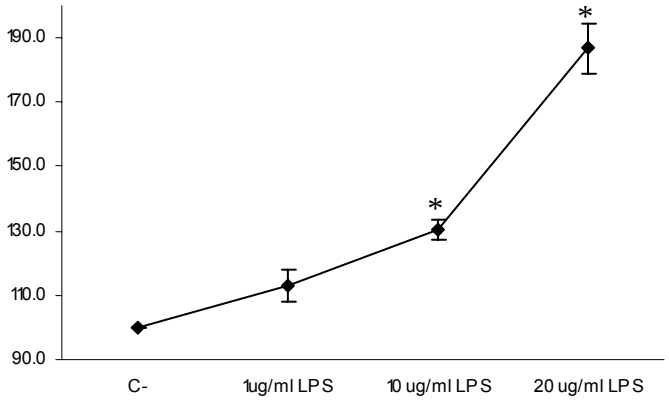


FIGURE 2. EP reduces ROS production in THP-1. Cells were coincubated for 24h with PE (40 µg/ml) and LPS (100 ng/mL). After the treatment, DCF was added and the fluorescence measured. The incubation with H2O2 (15 min) was performed after the DCF was added. Values are relative change expressed as a percentage respect the maximum value. Values are expressed as mean±S.E.M. The significance of difference among the groups was analyzed by ANOVA. Bars showing a superscript letter are significantly different (P>.05).

A.



B.

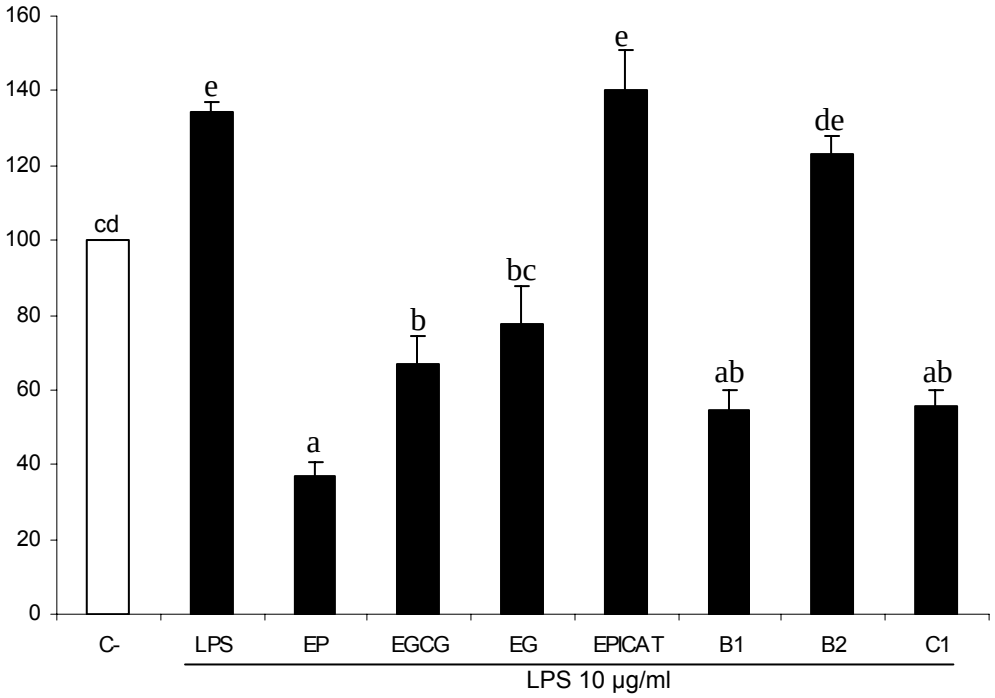
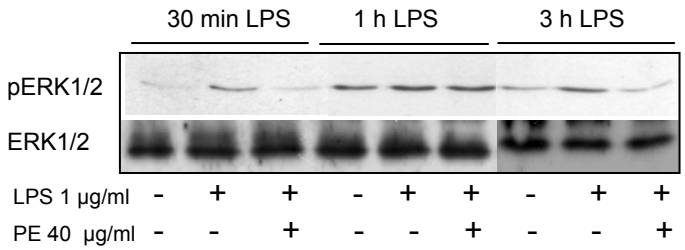


FIGURE 3. (A) LPS elicits a dose dependent oxidative burst. Cells were stimulated for 30 min with 1, 10 or 20 µg/ml of LPS. (B) Procyanidins reduced ROS production. Cells were preincubated with the different procyanidins for 4 hours and then stimulated for 30 min with 10 µg/ml of LPS. PE (40 µg/ml), procyanidin extract; Pure compounds (10 µg/ml): C1, trimer C1; B1, dimer B1; B2, dimer B2; EGCG, epigallocatechin gallate; EG, epigallocatechin. Values are expressed as mean±S.E.M. The significance of difference among the groups was analyzed by ANOVA. Bars showing a superscript letter are significantly different (P>.05).

A.

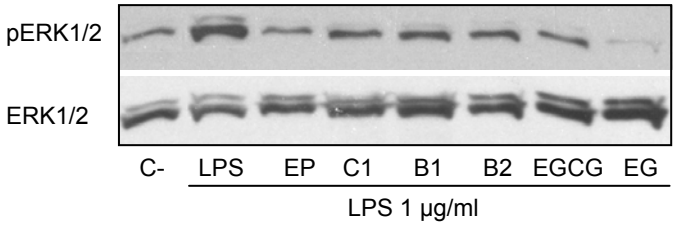


B.

Sample	30 min	1 hour	3 hour
Control	52.7+2.9[a]	79.9+2.2[a]	48.1+2.4[a]
LPS	100.0+0.2[b]	100.0+0.3[a]	100.0+0.1[b]
LPS+PE	33.9+4.3[a]	95.8+1.2[a]	34.6+1.4[a]

FIGURE 4. Time dependent effects of PE on ERK1/2 in THP-1 macrophages. Cells were pretreated with EP (40 µg/ml) for 4 hours and then stimulated with 1 µg/ml of LPS for the indicated times. (A) Representative western blot analysis. (B) Densitometric analyses of the inmunoblots. Values are expressed as mean±S.E.M. The significance of difference among the samples was analyzed by ANOVA. Values showing a superscript letter are significantly different (P>.05).

A.



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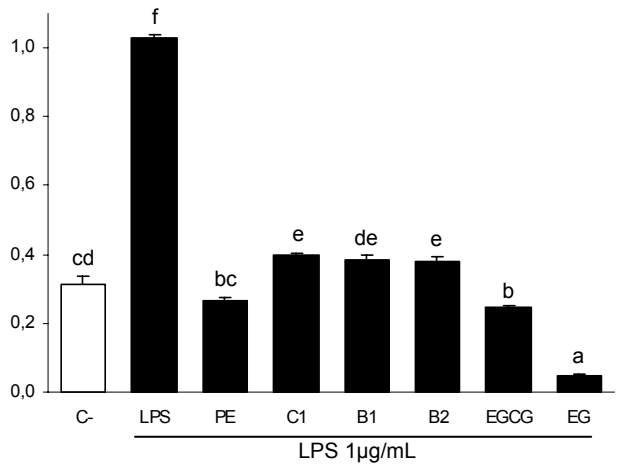

FIGURE 5. Effects of different procyanidins on ERK1/2 in THP-1 macrophages. Cells were pretreated with EP (40 µg/ml), C1, B1, B2, EGCG or EG (10 µg/ml) for 4 hours and then stimulated with 1 µg/ml of LPS for 30 min. (A) Representative western blot analysis. (B) Densitometric analyses of the inmunoblots. Values are expressed as mean±S.E.M. The significance of difference among the samples was analyzed by ANOVA. Bars showing a superscript letter are significantly different (P>.05).

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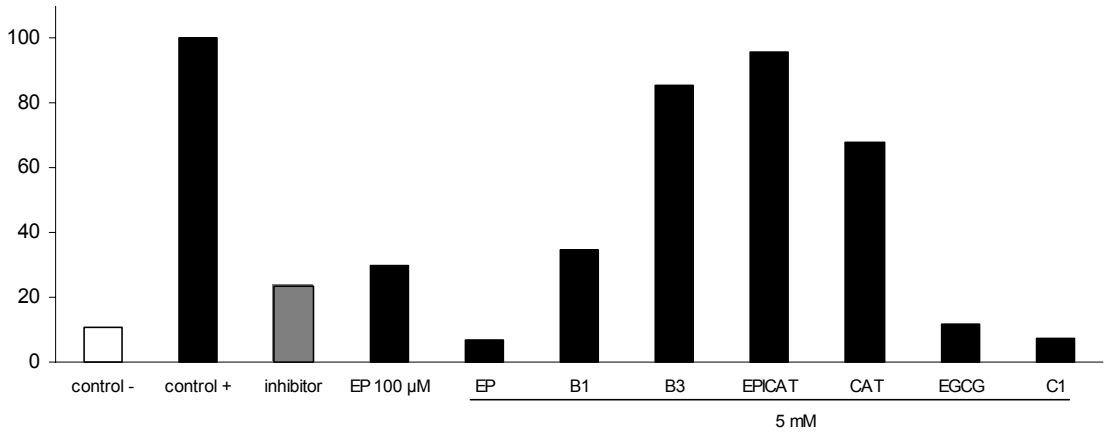

Figure 6. Inhibition of human IKK-B activity by various monomeric and oligomeric procyanidins. Kinase activity of human recombinant IKK-B was measured following the manufacturers instructions. EP 100 µM ~ 86 µg/ml. EP; procyanidin extract, B1; dimer B1, B3; dimer B3, EPICAT; epicatechin, CAT; catechin, EGCG; epigallocatechin gallate, C1; trimer C1.

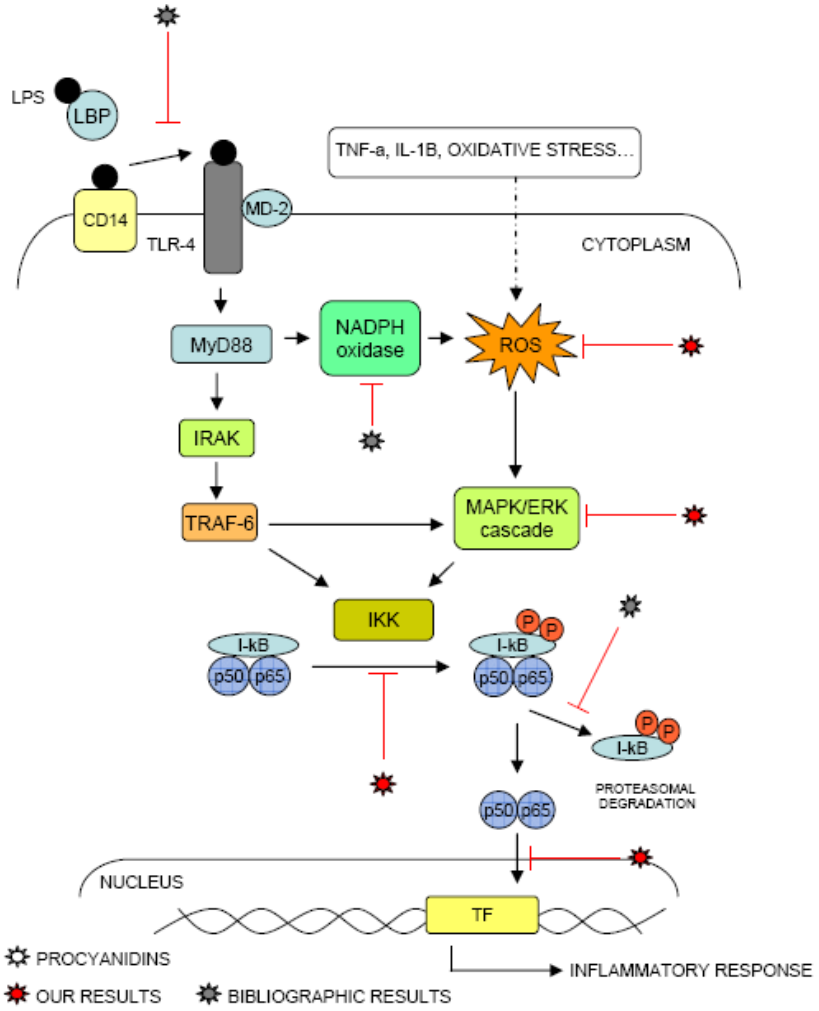

Figure 7. LPS signal transduction and activation of ROS production and proinflammatory transcription factors (TF). Procyanidin proposed model for its mechanism of action.

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Discussion

While there is a substantial body of scientific literature which supports a positive role of flavonoids to health, how specific flavonoids exert these benefits is under intense investigation. Moderate wine consumption has been inversely associated with the development and progression of cardiovascular diseases [9-11]. The underlying mechanisms responsible for the protective effects of procyanidins involve the antioxidant, and anti-inflammatory properties of polyphenols, of which (−)-epigallocatechin gallate (EGCG) is known as the most active catechin derivative. EGCG has the greatest antioxidative activity, inhibitory activity towards NF-KB and IkB degradation and it also inhibits MAPkinase pathway [12-14]. EGCG health benefits have been extensively studied. For this reason EGCG is an excellent natural compound to compare its effects with other oligomeric procyanidins. In the present study we investigated the anti-inflammatory effects of various oligomeric procyanidins in a human monocytic cell line activated with LPS.

The PE composition represents the ingestion of procyanidns in a Mediterranean diet. It is a mixture of varios monomeric and oligomeric procyanidins and phenolic acids from grape-seed which reduces intracellular ROS production induced by LPS and H2O2. We also demonstrate for the first time the same effects on LPS-induced ROS production with some pure oligomeric procyanidins, being the trimer C1 and the dimer B1 the most effective ones, followed by EGCG. Houde et al [15] and Roychowdhury et al. [16] have previously reported the effect of EGCG and a GSPE on ROS production in RAW 264.7 macrophages activated with LPS. Some authors have attributed anti-inflammatory properties to the dimer B2 [17], but in contrast to B1, in our conditions it did not show any inhibition of the ROS production. Then, the structural differences between both dimers must be the responsible for the differences in their effectiveness.

It has been reported that MAPKs play an important role in the expression of various genes because these kinases regulate some proinflammatory transcription factors. To further investigate the mechanism of action of procyanidins we assessed its modulation of ERK1/2 phosphorylation. We demonstrate that all the oligomeric procyanidins tested and the PE, inhibited such phosphorylation and as a consequence ERK1/2 activation. Interestingly, EG showed the highest inhibition values. It decreased ERK1/2 phosphorylation in approximately 90%. EGCG showed an inhibitory effect of the 70% followed by EP. However, these results are controversial. Ichikawa et al. [12], observed that EGCG and EG produced an increase in ERK1/2 phosphorylation after 24h of treatment. These differences might be due to the duration of the treatment, because it is well established that antioxidant effects of flavonoids are time and dose dependent

[4]. Dimers B1 and B2, and the trimer C1 were equally effective (60 %) reducing ROS production.

Some authors have reported that EGCG and EG inhibited LPS-induced NF-kB activation by inhibiting IkB degradation. We have also demonstrated that the PE is able to inhibit p65 translocation to the nucleus, iNOS expression and NO production in RAW 264.7 [10]. However procyanidin interaction with IKK has not yet been studied extensively. In this work we assessed the in vitro modulation of IKKb activation by procyanidins. We identified that the inhibition of IKK activity was an important target in the anti-inflammatory action of procyanidins. C1 and PE exerted the maximal inhibition of IKK activation, followed by EGCG. Also the dimer B1 produced a significant reduction, in contrast to B3. Among the monomers tested catechin exerted a slight but significant inhibition (35%) differing from epicatechin that was ineffective.

LPS induces oxidative stress and produces NO and ROS in macrophages [18]. It has long been known that ROS play a role in intracellular signalling [19], but the various mechanism by wich the cellular redox state can influence signalling pathways are extremely complex. We hypothetizice (Fig. 7) that procyanidins, as a potent antioxidants, prevent the production of reactive oxygen species (ROS), which have been suggested to be involved in the activation of the NF-κB signaling system [20, 21] and MAPK signal pathway [5]. Furthermore, accumulating evidence indicates that there is a crosstalk between ERK and NF-kB pathways, where ERK seams to activate NF-KB [22-24]. This crosstalk might explain how procyanidins could inhibit both pathways.

ROS are important intracellular second messengers generated in response to proinflammatory agents including TNF-α [25], IL-1 and LPS. Although the precise intracellular source from which LPS induces ROS generation remains to be clarified, it has been reported that TLR-4 directly interacts with NADPH oxidase, which is required for LPS-induced H2O2 generation in HEK 293 cells [26]. A similar mechanism has been suggested for ROS generation by LPS in THP-1 cells [18, 27, 28]. Also, in monocytic cells, the main source of ROS in IL-1b-induced NF-kB activation was shown to be the NADPH oxidase complex [26, 28]. Then the inhibition by procyanidins of NADPH oxidase, that has been extensively reported [29], might be responsible for their effects, although this point has not been revised in this work. Another possible mechanism of action of procyanidins has been proposed recently. Some authors [6, 30] have proposed that procyanidins from different sources inhibit LPS-response by direct neutralization and binding to LPS molecule. All these conjectures are represented in fig. 9 and need to be further investigated.

In conclusion we demonstrated that the oligomeric procyanidins B1 and C1 and the whole PE modulate the inflammatory response in human monocytes, and that its effects are comparable to those exerted by the well known EGCG.

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III. Inhibitory Effects of Grape Seed Procyanidins on Foam Cell Formation *in vitro*

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Inhibitory Effects of Grape Seed Procyanidins on Foam Cell Formation in Vitro

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Human and animal studies have demonstrated that procyanidin-rich diets reduce the risk of cardiovascular diseases and atherosclerosis. Some beneficial effects have been attributed to the well-known antioxidant activity of procyanidins. This study investigated another potential corrective role of procyanidins in cholesterol flux and inflammation in macrophage-derived foam cells. RAW 264.7 macrophages were cultured with moderately oxidized LDL (oxLDL), minimally oxidized LDL (moxLDL), or LPS (0.5 μg/mL) and oxLDL (LPS + oxLDL) to induce foam cells. Then, cells were treated with procyanidins derived from grape seed (PE, 45 μg/mL) for the last 12 h of incubation with the different lipoproteins (25 μg/mL). After lipid extraction, it was determined that total and esterified cholesterol and triglyceride accumulations in foam cells were increased by lipoprotein treatment but reduced by PE incubation. To asses the effect of PE on gene expression, the relative mRNA levels of CD36, ABCA1, iNOS, COX-2, and IκBα were determined by RT-PCR. It was shown that PE reduced the oxLDL scavenger receptor expression (CD36) and enhanced ATP-binding cassette A1 (ABCA1) expression, a key regulator of macrophage cholesterol efflux. PE also down-regulated inflammatory-related genes such as inducible nitric oxide synthase (iNOS) and kappa beta inhibitor-alpha (IκBα) without modifying COX-2 expression. In conclusion, evidence is provided that procyanidins may attenuate the development of foam cell formation by reducing cholesterol accumulation and modulating the expression of key genes in cholesterol flux and inflammation.

KEYWORDS: Procyanidins; atherogenesis; CD36; ABCA1; cholesterol; oxidized lipoproteins

INTRODUCTION

Procyanidins are natural phenolic compounds from the flavonoid class that comprise the oligomeric forms of monomeric catechins and epicatechins (see **Figure 1**). They are fairly abundant in several foods and drinks of plant origin such as grapes, cocoa, various berries, apples, nuts, red wine, chocolate, and tea. The beneficial effects of procyanidins have been largely attributed to their well-known antioxidant activity. Yet, their bioactivity is not limited to their antioxidant actions. We have previously reported that procyanidins can reduce the inflammatory response in LPS-activated macrophages by inhibiting the NF-κB pathway (*1*). Some recent human trials have demonstrated that procyanidins are cardioprotective, decreasing total cholesterol, LDL levels, and oxidized LDL (*2*), reversing the endothelial vasomotor dysfunction in patients with proven coronary artery disease (*3*), and reducing lipid peroxides in human plasma (*4*). Strong in vivo and in vitro evidence now exists to indicate that procyanidins could reduce cardiovascular disease resulting from atherosclerosis, which is the leading cause of morbidity and mortality in Westernized countries (*2, 3, 5*). Accumulation of modified LDL, such as oxidized LDL (oxLDL), and the recruitment of monocytes in the arterial subendothelial spaces are early events in atherogenesis. Macrophages take up oxLDL through the scavenger receptor pathways and become foam cells. Foam cells are well-known to play an important role in the development and progression of atherosclerosis, through the production of various bioactive molecules, such as growth factors and cytokines (*6*).

The primary cellular event that drives early atherosclerotic lesion formation is the unregulated accumulation of cholesterol ester by intimal-associated macrophages (*7*). As CD36 and other scavenger receptors are not subjected to negative regulation by high levels of intracellular cholesterol, massive accumulation of cholesterol esters can occur in macrophages, resulting in foam cell formation (*8*). It has been demonstrated that exposure to oxLDL resulted in a marked induction of CD36 mRNA expression, implying that CD36 mRNA was transcriptionally regulated by lipoproteins. Induction of CD36 mRNA expression was accompanied by an increase in CD36 protein (*9*). Thus,

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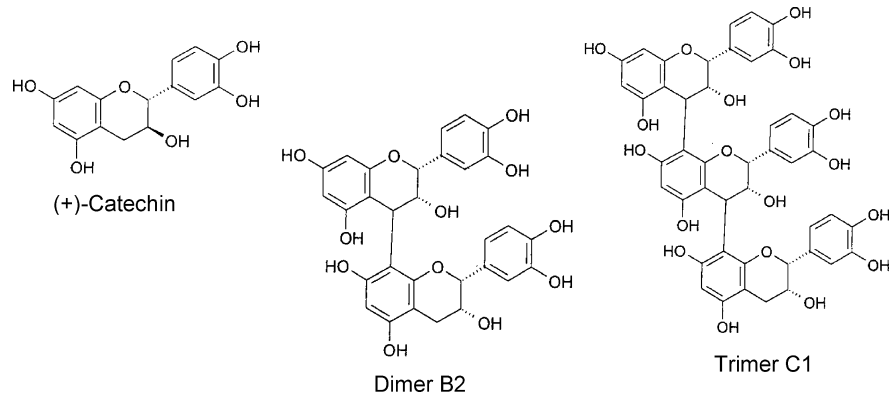

Figure 1. Structures of procyanidins derived from the three phenolic ring-basic structure of flavonoids. Procyanidins are highly hydroxylated polymers, composed of the monomeric structure flavanol (+)-catechin, (−)-epicatechin, and (−)-epicatechin gallate.

macrophage expression of CD36 and foam cell formation may be perpetuated by a cycle in which oxLDL drives its own uptake.

Although there are multiple mechanisms involved in the efflux of cellular cholesterol, the first step in the reverse cholesterol transport is linked to ABCA1 (*10*), a transmembrane protein mediating lipid efflux from cells to HDL, which then facilitates direct and indirect transport to the liver for biliary excretion (*11*). It has been concluded that ABCA1 plays an important role in cholesterol homeostasis and atherogenesis (*12*).

Several lines of evidence strongly support the hypothesis that oxidation of LDL is an essential step in its conversion to an atherogenic particle in animals (*13*). Although the precise mechanisms responsible for LDL oxidation are not known with certainty, lipooxygenases, myeloperoxidase, inducible nitric oxide synthase, and NADPH oxidases have been proposed as possible contributing enzymes because they can lead to LDL oxidation in vitro and they are expressed in human atherosclerotic lesions, where macrophages express each of these enzymes. Although macrophages may not be required to initiate LDL oxidation, they are likely to amplify oxidative reactions in macrophage-rich areas of atherosclerotic lesions (*14*). Activated macrophages also produce arachidonate metabolites that are synthesized by the cyclooxygenase (COX) enzyme. COX-2 is the inducible form of the enzyme that is rapidly induced by various proinflammatory stimuli to result in prostaglandin synthesis associated with inflammation, atherogenesis, and carcinogenesis (*15*).

Broadly speaking, atherosclerosis can be considered as a form of chronic inflammation resulting from interaction between modified lipoproteins, monocyte-derived macrophages, T cells, and the normal cellular elements of the arterial wall (*16*). The transcription factor NF-κB is a central mediator of gene expression induced by proinflammatory and proatherogenic stimuli, including inflammatory cytokines, oxidative stress, LPS, and bacterial products. Toll-like and interleukin receptor families deliver signals from a wide spectrum of ligands through downstream signaling to activate NF-κB translocation, inducing the coordinated expression of specific genes (*6, 17*).

Among the many genetic and environmental risk factors that have been identified by epidemiologic studies, infectious agents are recognized as being involved in the development of atherosclerosis. Recent studies have demonstrated that lipopolysaccharide (LPS) induces immune, proinflammatory, and other as yet undetermined mechanisms that may be important in triggering atherogenesis (*18*). Specialized functions of macrophages have evolved to protect the body from infection. However, the same mechanisms that enable the phagocytosis of pathogens and activation of leukocytes also permit the uptake of lipoproteins and release of reactive oxygen species and immune mediators that collectively contribute to atherosclerosis.

Most human trials and in vitro studies are focused on the preventive effects of procyanidins driven by its antioxidant capacity. Now, we want to investigate the corrective effects of procyanidins in cholesterol flux and inflammation in three models of macrophage-derived foam cells. New approaches to inhibiting lipid accumulation in macrophage foam cells and reducing inflammatory responses may be of therapeutic value in preventing coronary artery disease.

MATERIALS AND METHODS

Chemicals. According to the manufacturer, the grape seed procyanidin extract (PE) contained essentially monomeric (21.3%), dimeric (17.4%), trimeric (16.3%), tetrameric (13.3%), and oligomeric (5−13 units) (31.7%) procyanidins and (4.7%) phenolic acids. LPS (*Escherichia coli* 0111:B4) was purchased from Sigma-Aldrich, Inc. Human LDL were purchased from Calbiochem, U.K.

Oxidation of Lipoproteins. LDL was oxidized using 200 μg/mL in sterile PBS containing 10 μM $CuSO_4$ and incubated at 37 °C for 24 h (oxLDL; moderate oxidation) or 5 h (moxLDL; minimal oxidation) with shaking. Oxidation was arrested with 150 mM NaCl and 0.01% EDTA, pH 7.4, and the modified LDL was then washed and concentrated to about 1 mg/mL using Amicon Centriplus ultrafilters (Millipore). The extent of oxidation was determined by measuring the level of thiobarbituric acid-reactive substances (TBARs). Concentration of LDL protein was determined according to the Bradford method. All lipoproteins were used within 24 h of preparation.

Cell Culture Conditions and Treatments. Murine macrophage cell line RAW 264.7 (European Tissue Culture Collection ECACC, ref 91062702, London, U.K.) was cultured in DMEM containing 10%(v/v) fetal bovine serum, 2 mM D-glutamine, penicillin (100 U/mL), streptomycin (100 μg/mL), and 25 mM HEPES. Cells were grown at 37 °C and with 5% CO_2 in fully humidified air and used for experiments between passages 5 and 14. Uptake of the neutral red dye was used as a measure of cell viability in response to procyanidin treatment (*1*). Nontoxic concentration of oxidized LDL was obtained from the bibliography (*19, 20*). We used fully supplemented medium with 10% of FBS to perform the experiments. Four different models were assayed: (1) Cells were incubated with native LDL (nLDL, 25 μg/mL) for 24 h. (2) Cells were incubated with moderately oxidized LDL (oxLDL, 25 μg/mL) for 24 h. (3) Cells were activated with 0.5 μg/mL LPS for 12 h and then incubated with oxLDL (25 μg/mL) for 24 h in fresh medium. (4) Cells were incubated with minimally oxidized LDL (moxLDL, 25 μg/mL) for 24 h. The PE treatment was performed during the last 12 h of incubation with the different lipoproteins. We used nontreated macrophages as a control group. The culture medium for control and treated cells was collected and tested. TBARs of the culture medium after the treatments were measured using the TBARs assay. It was normalized to the total protein content.

Table 1. TC, TG, and TBARs Contents of nLDL, oxLDL, and moxLDL[a]

parameter	nLDL	oxLDL	moxLDL
TC (μg/μg of protein)	2.12 ± 0.11	2.45 ± 0.21	2.24 ± 0.23
TG (μg/μg of protein)	0.25 ± 0.01	0.28 ± 0.02	0.26 ± 0.01
TG/TC ratio	0.12 ± 0.01	0.11 ± 0.01	0.11 ± 0.02
TBARs (pmol/μg of protein)	0.75 ± 0.15	4.11 ± 0.38*	1.84 ± 0.28*

[a] oxLDL and moxLDL were prepared and oxidized by exposure to $CuSO_4$ (10 μM), and the total cholesterol (TC), triglyceride (TG), and thiobarbituric acid-reactive substances (TBARs) levels were determined. nLDL, native LDL; oxLDL, moderately oxidized LDL; moxLDL, minimally oxidized LDL. Data shown are the mean ± SEM of three independent preparations.

Quantification of Lipid Accumulation. The extent of foam cell formation was determined by monitoring the cell's lipid uptake using two different methods: oil red O staining and measurement of the internalized cholesterol and triglycerides. After the treatments, cells were washed twice with PBS, and total cellular lipids were extracted by incubating them for 30 min with hexane/2-propanol (3:2, v/v). The lipid extracts were dried under nitrogen and redissolved in 200 μL of 2-propanol. Total (TC) and free cholesterol (FC) and triglyceride (TG) contents of the extracts were measured using enzymatic colorimetric tests, following the manufacturers' instructions (QCA, Barcelona, Spain; and Wako Clinical Diagnostics Reagents, respectively). Cholesterol ester (CE) content was calculated as the difference between total and free cholesterol. All results were normalized to total protein content measured using the Bradford method.

CD36, ABCA1, iNOS, COX-2, and IκBα mRNA Analysis by RT-PCR Real Time. RNA from treated cells was isolated with a High Pure RNA Isolation Kit from Roche. cDNA was synthesized from 1 μg of total RNA using oligo-dT and Superscript II Rnase Reverse Transcriptase (Life Technologies). Twenty nanograms of cDNA was subjected to quantitative RT-PCR amplification using Sybr Green Master Mix (Applied Biosystems). The forward and reverse primers are as follows: CD36, F, 5′ TCGGAACTGTGGGCTCATTG 3′, R, 5′ CCTCGGGGTCCTGAGTTATATTTTC 3′; ABCA1, F, 5′ CAACTACAAAGCCCTCTTTG 3′, R, 5′ CTTGGCTGTTCTCCATGAAG 3′; iNOS, F, 5′ GGATCTTCCCAGGCAACCA 3′, R, 5′ AATCCACAACTCGCTCCAAGATT 3′; IκBα, F: 5′ CTTGGTGACTTTGGGTGCTGAT 3′, R, 5′ GCGAAACCAGGTCAGGATTC 3′; COX-2, F, 5′ GGAGAGACTATCAAGATAGT 3′, R, 5′ ATGGTCAGTAGACTTTTACA 3′; GAPDH, F, 5′ CATGGCCTTCCGTGTTCCT 3′, R, 5′ CCTGCTTCACCACCTTCTTGA 3′. Reactions were run on a quantitative PCR system, the thermal profile settings were 50 °C for 2 min, 95 °C for 2 min, and then 40 cycles at 95 °C for 15 s and 60 °C for 2 min. All results were normalized to the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression.

Calculations and Statistical Analysis. Results are expressed as mean value ± SEM. Effects were assessed using ANOVA or Student *t* test. We used Tukey's test of honestly significant differences to make pairwise comparisons. All calculations were performed using SPSS 14.0 software.

RESULTS

Characteristics of Native and Oxidized LDL. Total cholesterol and triglyceride contents were not significantly changed after oxidation of the lipoprotein particles. The extent of oxidation of the lipoprotein, as measured by the levels of TBARs present, was markedly increased after exposure to $CuSO_4$ (10 μM) by approximately 5.6-fold for oxLDL and 2-fold for moxLDL. The total cholesterol and triglyceride contents of nLDL, oxLDL, and moxLDL are given in **Table 1**.

Estimation of Lipid Peroxidation after PE Treatment. Concentrations of TBARs are an index of lipid peroxidation and oxidative stress. In the present study, malonaldehyde, a final product of the oxidative degradation of polyunsaturated fatty acids, was detected in the culture medium after treatment with different oxidized lipoproteins using the TBARs assay. As shown in **Table 2**, lipid peroxidation after oxLDL incubation increased 2-fold and in the LPS + oxLDL model increased 3-fold compared to the control and nLDL media. Treatment with moxLDL slightly increased lipid peroxidation. At the concentrations tested, we did not show any effect of PE (45 μg/mL) on lipid peroxidation in oxLDL, moxLDL, or LPS + oxLDL models.

Table 2. Estimation of Lipid Peroxidation in the Culture Medium after PE Treatment[a]

treatment	TBARs (pmol/μg of protein)
none	25.4 ± 3.8 (1.00)
nLDL	23.2 ± 1.1 (0.91)
oxLDL	48.5 ± 7.1* (1.91)
oxLDL + PE	46.4 ± 6.3* (1.83)
LPS + oxLDL	67.1 ± 3.7* (2.80)
LPS + oxLDL + PE	76.2 ± 3.7* (3.00)
moxLDL	37.9 ± 4.5* (1.49)
moxLDL + PE	36.2 ± 6.9* (1.42)

[a] Cells were incubated for 24 h with native LDL (nLDL; 25 μg/mL), moderately oxidized LDL (oxLDL; 25 μg/mL), pretreated for 12 h with LPS (500 ng/mL), and then incubated with oxLDL (LPS + oxLDL; 25 μg/mL) or incubated with minimally oxidized LDL (moxLDL; 25 μg/mL), in the absence or presence of the procyanidin extract (PE; 45 μg/mL) for 12 h. The extent of culture medium lipid peroxidation was determined by TBARs assay. Data are the mean ± SEM from three separate experiments. *, $P < 0.05$ compared to untreated macrophages. Values in parentheses are percent with respect to the culture medium of the untreated cells.

Procyanidin Extract Reduces Lipid Accumulation in Foam Cells. First, the effects of PE on lipid accumulation in RAW 264.7 macrophages were assessed by staining with oil red O. Qualitative determination of the extent of staining showed that exposure of macrophages to oxLDL, moxLDL, or LPS + oxLDL for 24 h led to an increase of lipid accumulation in cells, whereas nLDL had little effect. Furthermore, PE treatment reduced total lipid accumulation in all treatments (**Figure 2**).

When we measured the lipid content of the cells, we obtained results consistent with those obtained from oil red O staining (**Table 3**). No increase in lipid content was observed with nLDL. A marked rise of TC and CE was found after incubation with oxLDL, where PE was able to reduce both ester and total cholesterol contents. Although TG content was not increased, PE provoked a reduction of nearly 40% of its content. Incubation with LPS + oxLDL induced lipid accumulation, thus increasing TC, CE, and TG contents. After PE incubation, these parameters were significantly reduced. Addition of moxLDL increased CE and TG contents. In this treatment PE again reduced lipid accumulation. As shown in **Table 3**, any LDLox treatment or PE produced variations in FC levels.

Procyanidin Extract Modulates the Expression of Cholesterol Flux Related Genes. To further investigate PE activity, we assessed its effect on the expression of genes implicated in atherosclerosis development. CD36 expression levels are of particular importance for cholesterol influx as CD36 is implicated in oxidized lipid uptake. As shown in **Figure 3A**, CD36 mRNA was increased when macrophages were incubated with oxidized lipoproteins, up to 3-fold in moxLDL treatment. Moreover, PE reduced CD36 expression in all of the foam cell models tested. ABCA1 is characterized as the rate-limiting unidirectional cellular cholesterol exporter. Interestingly, PE was able to increase ABCA1 expression (**Figure 3B**).

PE Down-regulates Inflammatory-Related Genes as iNOS and IκBα. The inflammatory response production of NO by iNOS contributes to LDL oxidation in vivo. We show that iNOS

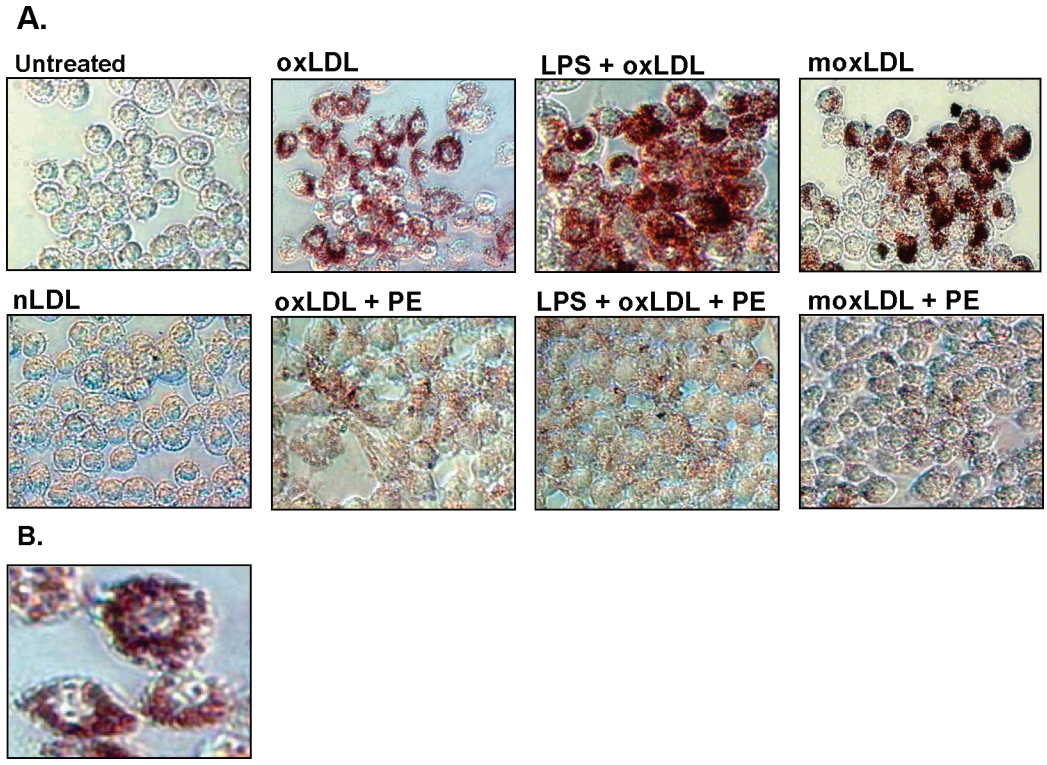

Figure 2. (**A**) Foam cell formation assayed by oil red O staining. RAW 264.7 macrophages were exposed to 25 μg/mL nLDL, oxLDL, LPS + oxLDL, or moxLDL for 24 h in the presence or absence of PE, fixed and stained with oil red O. Foam cell formation in the oxidized LDL-exposed cells is indicated by the punctate oil red O staining of lipid droplets. (**B**) Representative enlargement of RAW 264.7 macrophages oil red O staining.

expression was markedly modified as expected by LPS + oxLDL treatment, which is reflected in the increase of lipid peroxidation in this model. In addition, PE could reduce iNOS mRNA relative expression in all of the treatments performed (**Figure 3C**). Neither treatment with nLDL nor treatment with moxLDL modified the level of IκBα expression. In contrast, oxLDL and LPS + oxLDL increased the transcription of IκBα, and only in oxLDL treatment did PE reduce IκBα expression (**Figure 3D**).

We also determined COX-2 mRNA levels and showed that COX-2 expression was induced by only LPS + oxLDL treatment and that PE could not modulate this induction (LPS + oxLDL, 1.02 ± 0.02; LPS + oxLDL + PE, 0.938 ± 0.05).

CD36 Expression Positively Correlates with Total Cholesterol Accumulation. To test possible associations between mRNA levels of CD36, the total cholesterol accumulation and the effect of PE on these parameters, Spearman's rank correlation test was performed analyzing the data from the three independent experiments. We show a significant positive correlation between CD36 expression and total cholesterol levels ($\rho = 0.738$, $p < 0.05$).

DISCUSSION

Procyanidins are biochemically active compounds with anti-inflammatory and free radical-scavenging properties (*1*). In this study we assessed the corrective effect in vitro of a procyanidin extract (PE) on foam cell formation. For this reason we tested the PE effect in three foam cell models. Cells were treated with moderately oxidized lipoprotein (oxLDL) or minimally oxidized lipoprotein (moxLDL) or pretreated with endotoxin and incubated with oxLDL (LPS + oxLDL) to mimic the inflammatory state of atherosclerosis.

The bioavailability of procyanidins is a matter of intensive study. To better reflect the situation in vivo, we carefully studied the dosage used for the experiment. We used a dose of procyanidins of 45 μg/mL (~50 μM) that contains approximately 9.6 μg/mL of monomers (~26 μM), 7.8 μg/mL of dimers (~13.5 μM), 7.3 μg/mL of trimers (~8.4 μM), 6 μg/mL of tetramers (~11.5 μM), and 14.3 μg/mL of further polymerizated procyanidins (5−10 units). It is widely accepted that monomeric forms are absorbed in humans, reaching concentrations that range from 0.1 to 13 μM (*21, 22*). Further polymerized procyanidins have been detected in their native form in rat plasma (dimers to tetramers) and reached concentrations of ~3 μg/mL for dimers and of ~7.5 μg/mL for each trimeric and tetrameric form (*23*). Within these data, we selected a concentration that contained approximately the concentrations found in plasma of each component of the extract.

We first evaluated the PE effect on lipid accumulation in response to different degrees of lipoprotein oxidation. In agreement with the previously reported effects, after assessing the total cholesterol and triglyceride contents, we showed that native LDL had not caused any change in lipid accumulation in the cells because high cellular cholesterol levels down-regulate native LDL receptor gene transcription (*14, 20*). In contrast, total cholesterol increased because of incubation with the different oxidized lipoproteins. Free cholesterol is toxic for the cell and, as we show in our results, its levels must remain unchanged (*14*), whereas cholesterol esters were increased after the incubation with the oxLDLs. Once we confirmed the accumulation of lipids in the foam cells, we assessed the PE effect on the lipid content and observed that PE decreased the total cholesterol accumulation because of a decrease of approximately 30% in the content of cholesterol esters. PE also reduced TG accumulation in all treatments performed.

Experimental data suggest there is a role for oxLDL in the pathogenesis of atherosclerosis (*24*). Dietary antioxidants attenuate the cytotoxic effects of oxidatively modified LDL and restore endothelial function in patients with coronary artery

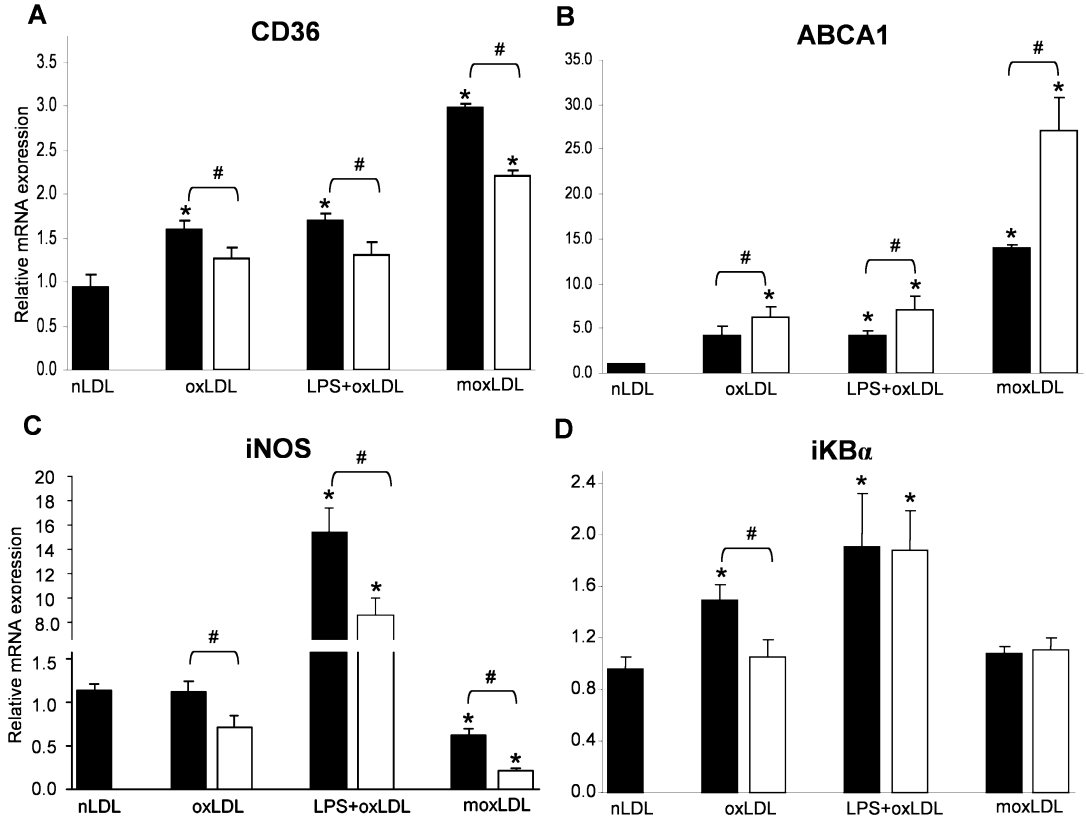

Figure 3. PE effect on CD36, ABCA1, iNOS, and iKBαa relative mRNA expression. Open bars indicates PE treatment. Cells were incubated for 24 h with native LDL (nLDL; 25 μg/mL), moderately oxidized LDL (oxLDL; 25 μg/mL), pretreated for 12 h with LPS (500 ng/mL), and then incubated with moderately oxidized LDL (LPS + oxLDL; 25 μg/mL) or incubated with minimally oxidized LDL (moxLDL; 25 μg/mL), in the absence or presence of the procyanidin extract (PE; 45 μg/mL). Values are means ± SEM of three independent experiments performed in triplicate. *, $P < 0.05$ compared to nLDL; #, $P < 0.05$ compared in each treatment in the absence or presence of PE.

Table 3. Effect of PE Treatment on the TC, CE, FC, and TG Contents[a]

treatment	TC	CE	FC	TG
none (μg/μg of protein)	0.54 ± 0.06	0.31 ± 0.05	0.23 ± 0.04	0.79 ± 0.14
none	100.0 ± 11.1	100.0 ± 16.9	100.0 ± 22.5	100.0 ± 17.6
nLDL	91.8 ± 20.6	81.2 ± 30.4	88.4 ± 14.5	122.3 ± 11.1
oxLDL	202.0 ± 10.2*	162.0 ± 22.0*	96.6 ± 12.0	102.4 ± 12.9
oxLDL + PE	147.8 ± 14.9*#	111.1 ± 13.2#	93.6 ± 13.9	60.9 ± 13.9*#
LPS + oxLDL	270.6 ± 12.4*	254.5 ± 33.0*	110.3 ± 5.1	178.3 ± 26.0*
LPS + oxLDL + PE	204.0 ± 24.2*	174.2 ± 46.8*#	117.7 ± 13.9	134.6 ± 17.8*#
moxLDL	137.7 ± 17.6	145.4 ± 13.0*	99.2 ± 13.0	141.2 ± 9.8*
moxLDL + PE	98.9 ± 11.1#	103.4 ± 14.8*#	102.6 ± 9.9	102.4 ± 5.1#

[a] Cells were incubated for 24 h with native LDL (nLDL; 25 μg/mL), moderately oxidized LDL (oxLDL; 25 μg/mL), pretreated for 12 h with LPS (500 ng/mL), and then incubated with oxLDL (LPS + oxLDL; 25 μg/mL) or incubated with minimally oxidized LDL (moxLDL; 25 μg/mL), in the absence or presence of the procyanidin extract (PE; 45 μg/mL). The total cholesterol (TC), cholesterol ester (CE), free cholesterol (FC), and triglyceride (TG) contents of the cells were determined. Lipid concentrations were normalized to the total cell protein content. Data are expressed as a percentage of the value found in the untreated macrophages and are the mean ± SEM from three separate experiments. *, $P < 0.05$ compared to untreated macrophages; #, $P < 0.05$ compared in each treatment in the absence or presence of PE.

disease (*25*). Some authors have described how flavonoids prevent and protect LDL from lipid peroxidation in vivo and in vitro (*2, 3, 5*). To further investigate the way PE works, we assessed the potential corrective effect of procyanidins after lipoprotein oxidation. We showed that the LPS + oxLDL model produced higher levels of lipid peroxidation than the oxLDL model. These results reveal that the LPS activation of the macrophage oxidases amplifies the lipid peroxidation of LDL. Many human trials have demonstrated a preventive role of natural antioxidants on LDL oxidation (*26, 27*), but with our results we should rule out a corrective effect of procyanidins because PE treatment could not return lipoproteins to their nonoxidized state at the concentration tested. These results indicate that PE is inhibiting different mechanisms, other than lipid peroxidation, that are able to reduce lipid accumulation in these foam cell models.

Because fatty streaks of atherosclerosis contain large numbers of macrophage derived foam cells that express class B scavenger receptor CD36 as well as other scavenger receptors, we investigated the PE effect on CD36 expression. OxLDL is not a single, well-defined entity, but has structural and physical properties that vary according to the degree of oxidation. The

extent of LDL lipid oxidation affects the induction of CD36 and influences its effect on cell function. As other authors have reported (*28*), in this work there appears to be greater induction of CD36 by moxLDL compared with oxLDL, suggesting that early products from moxLDL (such as lipid hydroperoxides) are involved in the transcriptional regulation of CD36 and that they are gradually degraded in oxLDL (*10*). Our results also show that PE modulates the expression of CD36. PE is able to reduce CD36 expression to basal levels in both oxLDL and LPS + oxLDL models and to inhibit CD36 expression significantly in the moxLDL model. As a result of this inhibiting effect, PE could reduce the cholesterol influx from oxidized lipoproteins.

Previously reported works showed a differential expression profile of ABCA1 gene in response to the treatment with different lipoproteins (*29, 30*). We show an enhanced expression of ABCA1 after the treatments with oxidized lipoproteins, where the highest induction resulted in moxLDL model. In this study, we observed for the first time how treating cholesterol-loaded macrophages with PE significantly induced ABCA1 expression in all of the treatments. PE then may exert antiatherogenic effects by facilitating the removal of cholesterol from macrophages via the cholesterol transporter protein ABCA1, but further studies must be performed to confirm such hypothesis.

Both clinical and experimental data suggest that atherosclerosis is a chronic inflammatory disorder associated with endothelial and cholesterol balance dysfunction (*17, 18*). Members of the NF-κB family coordinately regulate gene clusters that control inflammatory responses and have been involved in the development of atherosclerosis (*31*). The development of NF-κB inhibitors will be of interest because of their potential antiatherogenic properties (*32*). We have previously reported that PE inhibits the NF-κB pathway in LPS-activated macrophages (*1*). In the present study we investigated whether PE could inhibit the inflammatory response in a macrophage-derived foam cell model. For this reason we studied IκBα expression, which is an early target gene of NF-κB, the expression of which positively correlates with NF-κB activation (*33*). As expected, the LPS + oxLDL model produced a 2-fold induction of its expression. It is also important to appreciate that incubation with oxLDL also activated NF-κB, as other authors have reported (*31*), which could add to local inflammation when occurring in macrophages in atherosclerotic plaques. Interestingly, PE reduced IκBα expression when cells were treated with oxLDL, restoring its levels to the basal state.

Nitric oxide (NO) is a potent oxidant produced by both endothelial cells and macrophages. NO produced via the much higher capacity iNOS in macrophages can amplify the oxidative reactions in the foam cell. iNOS expression is mainly transcriptionally regulated through the activation of the NF-κB pathway. Evidence that inducible nitric oxide synthase contributes to LDL oxidation in vivo has recently been provided by studies demonstrating that apo E-deficient mice which lack iNOS develop less atherosclerosis and that inhibitors of iNOS decrease atherosclerosis in rabbits (*34*). We have shown that the extent of lipid peroxidation was higher in the LPS + oxLDL model, probably due to the induced activity of iNOS and other macrophage oxidases, as myeloperoxidase and NADPH, that contribute to LDL oxidation. In our study, iNOS expression was also induced in oxLDL treatment. Furthermore, in this work we have shown that iNOS expression is reduced by PE treatment, probably as a result of the inhibition of NF-κB activity. We also investigated the role of PE in the regulation of COX-2 expression, an inducible isoform responsible for high levels of prostaglandin production during inflammation and immune responses. As expected, we showed that COX-2 expression was induced by LPS + oxLDL treatment, although PE could not reduce this induction.

The current study reveals that foam cell formation was markedly induced after incubation with different oxidized lipoproteins. We have demonstrated that the endotoxin-induced model showed the highest lipid accumulation and peroxidation levels, probably because of the enhanced inflammatory response. However, the most important gene induction of CD36 and ABCA1 resulted in the moxLDL model. It has been suggested that there is a link between lipid metabolism and inflammatory pathways driven through the cross-talk between PPARs and NF-κB (*17*). CD36 and ABCA1 expressions are mostly regulated by PPARα and PPARγ nuclear receptors, which have been described as anti-inflamatory factors through inhibition of the NF-κB activation pathway. In this work we show that the lowest levels of iNOS and IκBα correspond to the highest induction of CD36 and ABCA1 in the moxLDL model, which can indicate a reduction in NF-κB activity driven by PE and the subsequent activation of PPARs.

In conclusion, we found that procyanidins have the ability to regulate the expression of genes causing the imbalance between lipid influx and efflux. These findings also show a down-regulation of the inflammatory response mediated by PE through the inhibition of iNOS and IκBα expression. These in vitro data help to interpret the beneficial effects of procyanidins in reducing the risk of atherosclerosis after the administration of flavonoid-rich foods or supplements. The differentially expressed genes modulated by procyanidins help to uncover novel targets and may help to target disease interactions in atherosclerosis in the future. Further studies utilizing atherosclerosis-prone animal models are needed to clarify our findings.

ABBREVIATIONS USED

ABCA1, ATP binding cassette A1; IκBα, alpha inhibitor of kappa beta; LPS, lipopolysaccharide; LDL, low-density lipoprotein; NO, nitric oxide; iNOS, inducible nitric oxide synthase; COX-2, cyclooxygenase 2; NF-κB, nuclear factor-kappaB; PE, procyanidin extract; PPARγ, peroxisome proliferator-activated receptor-gamma.

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IV. Grape-seed procyanidins prevent low-grade inflammation by modulating cytokine expression in rats fed a high-fat diet

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Grape-seed procyanidins prevent low-grade inflammation by modulating cytokine expression in rats fed a high-fat diet

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Abstract

Objective: The main objective of this study was to evaluate the effect of procyanidin intake on the level of inflammatory mediators in rats fed a hyperlipidic diet, which are a model of low-grade inflammation as they show an altered cytokine production.
Design: Male Zucker *Fa/fa* rats were randomly grouped to receive a low-fat (LF) diet, a high-fat (HF) diet or a high-fat diet supplemented with procyanidins from grape seed (HFPE) (345 mg/kg feed) for 19 weeks and were then euthanized. We determined biochemical parameters, C-reactive protein (CRP) and IL-6 levels in plasma. Adipose tissue depots and body weight were also determined. We assessed CRP, IL-6, TNF-α and adiponectin gene expression in liver and white adipose tissue (WAT).
Results: As expected, rats fed the HF diet show an enhanced production of CRP. Our results demonstrate that the HFPE diet decreases rat plasma CRP levels but not IL-6 levels. The decrease in plasma CRP in HFPE rats is related to a down-regulation of CRP mRNA expression in the liver and mesenteric WAT. We have also shown a decrease in the expression of the proinflammatory cytokines TNF-α and IL-6 in the mesenteric WAT. In contrast, adiponectin mRNA is increased in this tissue due to the procyanidin treatment.

As previously reported, CRP plasma levels correlate positively with its expression in the mesenteric WAT, suggesting that procyanidin extract (PE) modulates CRP at the synthesis level. CRP plasma levels also correlate positively with body weight. As expected, body weight is associated with the adiposity index. Also, TNF-α expression and IL-6 expression have a strong positive correlation. In contrast, the expression of the anti-inflammatory cytokine adiponectin correlates negatively with the expression of TNF-α and IL-6 in the mesenteric WAT.
Conclusion: These results suggest a beneficial effect of PE on low-grade inflammatory diseases, which may be associated with the inhibition of the proinflammatory molecules CRP, IL-6 and TNF-α and the enhanced production of the anti-inflammatory cytokine adiponectin. These findings provide a strong impetus to explore the effects of dietary polyphenols in reducing obesity-related adipokine dysregulation to manage cardiovascular and metabolic risk factors.

Keywords: Procyanidins; IL-6; CRP; TNF-α; Adiponectin; Low-grade inflammation

1. Introduction

Procyanidins are phenolic compounds from the flavonoids group that are widely found in cereals, vegetables and fruits like grapes, berries, cocoa and apples. They have a broad range of biological activities [1]. They function as powerful antioxidants and exert anti-inflammatory activities in vitro. Recent studies have shown potent anti-inflammatory properties of procyanidins on experimental inflammation in rats and mice [2,3]. Its mechanisms of anti-inflammatory action remain poorly understood and are relevant to oxygen free radical scavenging, antilipid peroxidation, inhibition of the formation of inflammatory cytokines, alterations in cell membranes receptors, intracellular signaling pathway proteins and modulation of gene expression [4].

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Obesity is associated with a state of chronic inflammation characterized by macrophage infiltration of muscle and adipose tissue and abnormal production of proinflammatory mediators. In addition to adipocytes, adipose tissue contains fibroblasts, preadipocytes, tissue-resident macrophages and vascular constituents. Macrophages are known to be crucial contributors to inflammation, but more recently, it has been recognized that adipocytes demonstrate significant intrinsic inflammatory properties as well. Like macrophages, the adipocyte is exquisitely sensitive to infectious disease agents and cytokine-mediated inflammatory signals. In turn, these stimuli induce the expression of inflammatory mediators such as IL-6, TNF-α and SAA. Although many of these activities are restricted to autocrine and paracrine effects, some of these cytokines that are secreted from adipocytes and adipose-resident macrophages make significant contributions to systemic inflammation [5].

Adipose tissue is not usually thought of as an immune or inflammatory organ. However, the discovery of elevated secretion of these factors from obese adipose tissue provided the first evidence of a direct connection between obesity and systemic inflammation [5].

The altered production of proinflammatory molecules (so-called "adipokines") by adipose tissue has been implicated in the metabolic complications of obesity [6]. Compared with adipose tissue of lean individuals, adipose tissue of obese individuals expresses increased amounts of proinflammatory proteins such as TNF-α, IL-6, inducible nitric oxide synthase, C-reactive protein (CRP), soluble ICAM and monocyte chemotactic protein-1, as well as reduced adiponectin expression [7].

CRP is an acute-phase protein that binds specifically to phosphorylcholine as a component of microbial capsular polysaccharide and participates in the innate immune response against microorganisms. CRP is the most extensively studied marker of systemic inflammation in humans. A large number of studies have further strengthened the association of elevated CRP levels with nearly all the important cardiovascular risk factors, including insulin resistance, diabetes, metabolic syndrome, hypertension, smoking and dyslipidemia. The regulation of this protein in the liver is believed to be driven by IL-6, IL-1 and TNF-α [8] from visceral adipose tissue draining directly into the portal system that causes the obesity-associated rise of CRP production. Furthermore, in addition to liver-derived CRP, newer data show that adipose tissue itself may contribute to obesity-associated increased CRP levels [9,10].

Adiponectin, the most abundantly secreted adipocytokine from differentiated adipocytes, has potent vasculoprotective, angiogenic, anti-inflammatory and antiatherogenic properties. High adiponectin levels are associated with a reduced risk of myocardial infraction in men, while low serum adiponectin levels are reported in obese individuals and in those with hypertension, coronary artery disease and type 2 diabetes [11]. Adiponectin has inflammatory-modulating activities demonstrated in clinical studies showing inverse associations between adiponectin levels and serum markers of inflammation [12]. Although it is not clear how or whether adiponectin itself has anti-inflammatory properties, it is clear that adiponectin production by adipose can be inhibited by systemic inflammation and confers protection against the metabolic syndrome and diabetes [13,14].

TNF-α, a proinflammatory cytokine originally defined by its antitumor activity, has a strong link with obesity. Some authors have reported that adipocytes directly express TNF-α in rodents and led to the concept of a role for inflammation in obesity. These observations were paralleled by human studies showing increased TNF-α expression in the adipose tissue of individuals who were obese and decreased TNF-α expression after weight loss. Evidence supporting a key role for TNF-α in obesity-related insulin resistance came from studies showing that *ob/ob* mice (leptin-deficient mice with evidence of insulin resistance) that were also deficient for TNF-α or TNF receptors (TNFRs) had improved insulin sensitivity in diet-induced obesity compared with TNF-α- and TNFR-sufficient *ob/ob* mice [15].

IL-6, a stress-induced inflammatory cytokine, is directly implicated in atherogenesis. High levels of IL-6 are thought to be responsible for the increase in acute-phase proteins seen in obese patients, in particular, CRP [11]. Obesity-associated induction of adipose IL-6 production induces CRP secretion, and there are data that suggest that IL-6 decreases lipoprotein lipase activity, which results in increased macrophage uptake of lipids [16]. In addition, IL-6 was significantly associated with body mass index, waist circumference and visceral adiposity in obese subjects. Adipocytes and macrophages both contribute to white adipose tissue (WAT)-derived IL-6, although the ultimate stimulus for IL-6 production in the presence of high adiposity is currently unknown.

Understanding the mechanisms that lead from obesity to inflammation will have important implications for the design of the new therapies to reduce the morbidity and mortality of obesity. The main objective of the present study was to examine the putative modulatory effects of procyanidin extract (PE) on cytokine expression and CRP and IL-6 release in rats fed the high-fat (HF) diet to gain insight on the mechanisms that underlie the anti-inflammatory effects ascribed to procyanidins.

2. Materials and methods

2.1. Chemicals

Grape seed PE was provided by Les Dérives Résiniques et Terpéniques (Dax, France). According to the manufacturer, the PE contained essentially monomeric (21.3%), dimeric (17.4%), trimeric (16.3%), tetrameric (13.3%) and oligomeric (5–13 units) (31.7%) procyanidins and phenolic acids (4.7%).

2.2. Diets

Semipurified diets were obtained from Research Diets (USA). Briefly, three diets were used (Table 1). The low-fat

Table 1
Composition of the LF, HF and HFPE test diets

	Test diets		
	LF (g/kg diet)	HF (g/kg diet)	HFPE (g/kg diet)
Ingredients			
Casein	190	190	190
DL-Methionine	3	3	3
Cornstarch	498.5	215	215
Maltodextrin	35	75	75
Sucrose	290	290	290
Cellulose	30	30	30
Butter fat	14.7	44.2	44.2
Corn oil	39.3	118	118
Mineral mixture	40	40	40
Vitamin mixture	11	11	11
PE	0	0	0.32
Energy (kcal/g)	3.9	4.41	4.41
Protein (% energy)	16.8	16.8	16.8
Carbohydrate (% energy)	72.6	51.4	51.4
Fat (% energy)	10.6	31.8	31.8

(LF) diet, the hyperlipidic (HF) diet and the hyperlipidic with PE (HFPE) diet had equal protein percentage. The standard control diet was the LF diet. The HFPE diet differs from the HF diet in PE content, which was 0.32 mg of PE per gram of feed. The procyanidin dose used corresponds to the estimated amount of procyanidins that humans consume daily.

2.3. Experimental design and euthanasia

Male Zucker *Fa/fa* rats (Charles River Laboratories, Spain) were used in all studies (n=30). Rats were left 1 week in quarantine. At ~15 weeks of age, rats were randomly assigned to receive the LF (n=10), HF (n=10) or HFPE (n=10) diet ad libitum. Rats were housed in cages by pairs and subjected to a standard 12-h light:12-h dark cycle. The experimental period lasted 19 weeks. After rats were weighed, they were anesthetized by sodium pentobarbital (100 mg/kg ip) and euthanized by exsanguination after 6 h of fasting. Blood was obtained from abdominal aorta. The entire liver and adipose tissues were dissected out, weighed and snap frozen in liquid N_2 and stored at −80°C. All the procedures were performed with the approval of the ethics committee of our center and followed the laws concerning animal experimentation of the Government of Catalonia.

2.4. Measurement of adiposity, food intake and body weight gain

Body weight changes and caloric ingestion were monitored weekly during the whole experiment. Adipose tissue fat pads (mesenteric, retroperitoneal and epididymal) were excised separately and weighed. Adiposity index was calculated as total adipose tissue weight versus total body weight.

2.5. Measurement of biochemical parameters

After sacrifice, blood was collected and heparinized plasma was obtained by centrifugation. Total cholesterol levels and total plasma glucose levels were measured by enzymatic colorimetric methods (QCA S.L.). Determination of the GSH/GSSG ratio was assessed by colorimetric assay from Oxford Biomedical Research according to the manufacturer's instructions.

2.6. Measurement of CRP levels

Plasma CRP levels were quantified using a specific enzyme immunoassay (EIA) according to the manufacturer's instructions (Helica Biosystems). The assay is a double polyclonal antibody sandwich EIA.

2.7. Measurement of IL-6 and adiponectin plasma levels

Plasma IL-6 and adiponectin levels were quantified using specific EIAs according to the manufacturer's instructions (Biosource International, Inc.). The assays are based on a sandwich EIA.

2.8. mRNA analysis of CRP, IL-6, TNF-α and adiponectin genes by real-time RT-PCR

RNA from liver tissue was isolated with High Pure RNA Isolation Kit from Roche. RNA from adipose tissue was isolated using Trizol reagent (Invitrogen) following the manufacturer's instructions. cDNA was synthesized from 1 μg of total RNA using oligo-dT and Superscript II Reverse Transcriptase (Life Technologies). cDNA (20 ng) was subjected to quantitative RT-PCR amplification using SYBR Green Master Mix (Applied Biosystems). The forward and reverse primers for rat genes are shown in Table 2. Reactions were run on a quantitative Real-Time PCR System (Applied Biosystems); the thermal profile settings were 50°C for 2 min and 95°C for 2 min and then 40 cycles at 95°C for 15 s and 60°C for 2 min. Relative expression levels of the mRNA of the target genes were normalized to GAPDH mRNA levels.

2.9. Calculations and statistical analysis

Results are expressed as mean±S.E.M. Effects were assessed using ANOVA or Student's t test. We used Tukey's test for honestly significant differences to make pairwise comparisons. Spearman's rank correlation test between the

Table 2
Rat-specific primer sequences

Gene	Primer sequence
CRP	F: 5′ TTTGTGCTATCTCCAGAACAGATCA 3′
(NM 017096)	R: 5′ GCCCGCCAGTTCAAAACAT 3′
IL-6	F: 5′ CCCAACTTCCAATGCTCTCCTAATG 3′
(NM 012589)	R: 5′ GCACACTGAGTTTGCCGAATAGACC 3′
Adiponectin	F: 5′ GGCCGTTCTCTTCACCTACG 3′
(NM 144744)	R: 5′ GGCTCCATGCTCCTCCATCT 3′
TNF-α	F: 5′ CGTCAGCCGATTTGCCATTTC 3′
(NM 12675)	R: 5′ TGGGCTCATACCAGGGCTTGAG 3′
GAPDH	F: 5′ CAT GGC CTT CCG TGT TCC T 3′
(NM 023964)	R: 5′ CCT GCT TCA CCA CCT TCT TGA 3′

Table 3
Body weight gain, food and fat intake and adipose weight of Zucker rats fed LF, HF and HFPE diets for 19 weeks

	Test diets		
	LF (n=10)	HF (n=10)	HFPE (n=10)
Body weight gain (%)	177.55±3.78[a]	188.66±2.27[b]	181.58±2.44[a,b] (n=9)
Fat intake (kcal)	211.81±4.07[a]	662.07±19.59[b]	664.18±18.08[b]
Total energy intake (kcal)	1998.19±38.37	2082.64±61.61	2088.64±56.85
Tissue weight (g)			
Epididymal fat	8.27±0.16[a]	8.45±0.52[a,b]	9.04±0.46[b]
Mesenteric fat	6.16±0.70	7.00±0.66	6.80±0.27
Retroperitoneal fat	9.04±0.56[a]	11.13±0.71[b]	10.6±0.55[a,b]
Adiposity index	4.43±0.20	5.06±0.14	5.07±0.19

Values are expressed as mean±S.E.M. The significance of difference among the three groups was analyzed by ANOVA. Values not showing a superscript letter among the three diet groups are not significantly different (P<05).

three experimental groups was assessed. All calculations were performed using SPSS 14.0 software.

3. Results

3.1. Food intake, body weight and adipose tissue weight

Zucker *Fa/fa* rats fed the HF diet had significantly higher body weights than control rats fed the LF diet (P<.05; Table 3). In spite of fat intake being significantly increased, the total energy intakes of the three groups of rats were comparable (P>.05), indicating that higher body weight gains in the HF group may be related to higher fat intake but not to higher energy intake.

The epididymal and retroperitoneal fat pad weights of rats fed the HF diet were higher than the weights of those fed the LF diet (P<05; Table 3), although mesenteric weight remained unchanged. Adiposity index in the three groups (LF, HF and HFPE) was unchanged.

A positive correlation was found between adiposity index and body weight (ρ=.408, P<05) (Table 6).

3.2. Diet effect on metabolic variables and GSH/GSSG ratio

The total plasma cholesterol levels of Zucker rats fed the HFPE, HF or LF diet did not change significantly, neither by diet nor by procyanidin ingestion. Glucose plasma levels were not significantly increased by the HF diet compared to the LF diet, but the HFPE diet reduced glucose levels significantly (Table 4).

In plasma analysis, rats fed the HF and HFPE diets showed a reduced GSH/GSSG ratio compared to those fed the LF diet, whereas no significant difference was found by PE treatment (Table 4).

3.3. PE modulates CRP and adiponectin plasma levels in rats fed the hyperlipidic diet without modifying IL-6 levels

CRP plasma levels were increased in HF rats, thus indicating a low-grade inflammation similar to that found in overweight/obese individuals. Moreover, HFPE administration to rats, that is, a daily ingestion per animal of nearly 7 mg of PE during 19 weeks of treatment, resulted in an important decrease in CRP that is in the range found in rats fed a standard diet (LF). In contrast to most adipocyte hormones, the anti-inflammatory cytokine adiponectin is decreased in obesity and increased in response to weight reduction. In this work, we found a decrease in adiponectin plasma levels in HF rats. Furthermore, adiponectin plasma levels were increased significantly in rats fed the HFPE diet (Table 4). We then measured IL-6 levels in the plasma of animals and found no difference in IL-6 levels between the

Table 4
Plasma analysis of markers of oxidative stress, inflammation and metabolic variables

	Group		
	LF	HF	HFPE
Oxidative stress			
GSH/GSSG	133.63±31.81 (n=6)	6.67±2.39 * (n=8)	11.96±1.84 * (n=8)
Inflammation			
CPR (μg/ml)	203.08±73.63 (n=7)	472.19±149.47 * (n=8)	109.48±32.32 ** (n=8)
IL-6 (pg/ml)	85.07±3.11 (n=8)	93.75±1.35 * (n=9)	96.27±3.13 * (n=7)
Adiponectin (μg/ml)	3.21±0.24 (n=9)	2.63±0.12 * (n=8)	3.37±0.42 ** (n=10)
Metabolic variables			
Total cholesterol (mg/ml)	1.29±0.05 (n=10)	1.22±0.08 (n=9)	1.12±0.05 (n=9)
Glucose (mg/dl)	186.35±11.36 (n=9)	207.09±10.46 (n=9)	177.46±7.96 ** (n=9)

Student's t test was used. Values are expressed as mean±S.E.M.
* P<05 compared to LF.
** P<05 compared to HF.

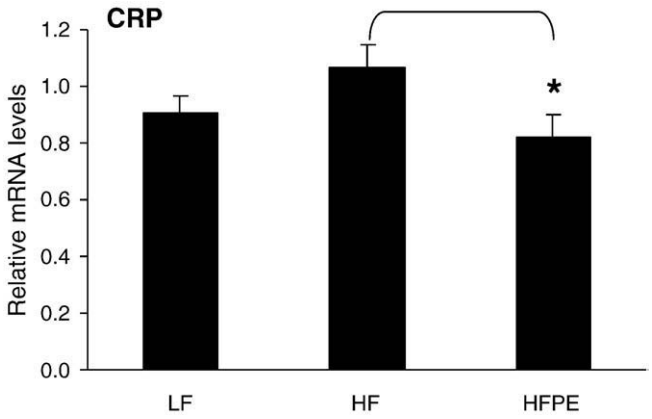

Fig. 1. Diet effect on CRP expression in liver. Liver mRNA was extracted, corresponding cDNA was synthesized and CRP gene expression was measured by quantitative real-time RT-PCR. Student's *t* test was used to evaluate significance between groups ($P<.05$).

HF group and the HFPE group, but both groups had higher IL-6 levels than rats receiving the standard LF diet.

3.4. PE acts by down-regulating mRNA CRP levels in the liver and mesenteric adipose tissue

Real-time PCR analysis of CRP in the liver showed differences between HF and HFPE rats. As expected, procyanidins caused a decrease in the synthesis of CRP mRNA in the liver with respect to rats receiving the same diet without procyanidins (Fig. 1).

Because CRP from adipose tissue is also an important source of this pentraxin in obese rats, we determined the CRP levels in adipose tissue of different origins: mesenteric, epididymal and retroperitoneal.

In mesenteric adipose tissue, we found that HF rats had higher CRP mRNA levels than control LF rats and HFPE rats. Thus, the HF diet increased CRP levels that were diminished by procyanidin treatment. In retroperitoneal and epididymal adipose tissue, nonsignificant differences were found between procyanidin treatment and diet (Table 5).

3.5. PE modulates gene expression in the mesenteric adipose tissue

Quantitative RT-PCR analysis of the mRNAs for CRP, IL-6, TNF-α and adiponectin genes in the mesenteric adipose tissue of rats fed the standard LF diet, HF diet or HFPE diet was performed. As we have previously described, procyanidin treatment modified not only CRP levels in mesenteric adipose tissue (Fig. 2A) but also IL-6, TNF-α and adiponectin gene expression. Our results show that IL-6 gene expression level was up-regulated by the HF diet and reduced significantly by procyanidin treatment (Fig. 2B). TNF-α gene expression level was also reduced significantly by procyanidins (Fig. 2C). On the contrary, the anti-inflammatory cytokine adiponectin was increased by PE treatment (Fig. 2D).

3.6. Correlations of CRP, IL-6, adiponectin and TNF-α expression in mesenteric adipose tissue and CRP and IL-6 plasma levels

To test possible associations between mRNA levels of CRP, IL-6, adiponectin and TNF-α in mesenteric adipose tissue; CRP and IL-6 plasma levels; body weight; and adiposity index, we performed Spearman's rank correlation test, analyzing the data from the three experimental groups. As shown in Table 6, a significant positive correlation between CRP expression and CRP plasma levels ($\rho=.623$, $P<001$) was found as expected. Furthermore, TNF-α expression and IL-6 expression correlated positively ($\rho=.745$, $P<.001$).

There were significant negative correlations between IL-6 and adiponectin expression ($\rho=-.436$, $P<.05$) and between TNF-α and adiponectin expression ($\rho=-.457$ $P<.05$). Finally, body weight correlated positively with CRP plasma levels ($\rho=.475$, $P<.05$).

There was no significant correlation of IL-6 plasma levels with any of the parameters measured. Liver CRP expression level was also examined, but no significant correlation between any of the parameters measured was found (data not shown).

4. Discussion

The primary function of adipose tissue is to store energy in the form of triglycerides during periods of energy excess and to release energy during fasting or starvation as free fatty acids and glycerol. Adipose tissue secretes a variety of peptides called adipokines including leptin, adiponectin, TNF-α, IL-6 and resistin, which have endocrine, autocrine and paracrine effects on the brain, liver and skeletal muscles [17]. Dysfunction of adipose tissue can result in insulin resistance and obesity-linked metabolic and vascular diseases. Obesity is associated with a chronic inflammatory response, which is characterized by abnormal cytokine production, increased synthesis of acute-phase reactants, such as CRP, and the activation of proinflammatory signaling

Table 5
Effect of the HFPE diet on CRP relative expression in adipose tissue

Group	Adipose tissue		
	Mesenteric	Retroperitoneal	Epididymal
LF	1.048±0.152[a]	1.049±1.152	1.022±0.274
HF	1.835±0.191[b]	1.166±0.124	1.345±0.270
HFPE	0.963±0.117[a]	1.173±0.100	1.398±0.145

Adipose tissue mRNA was extracted, corresponding cDNA was synthesized and CRP gene expression was measured by quantitative real-time RT-PCR. Results are expressed as relative expression levels normalized to the expression of the control group (LF). Values are expressed as mean±S.E.M. The significance of difference among the three groups was analyzed by ANOVA. Values not showing a superscript letter among the three groups are not significantly different ($P>.05$).

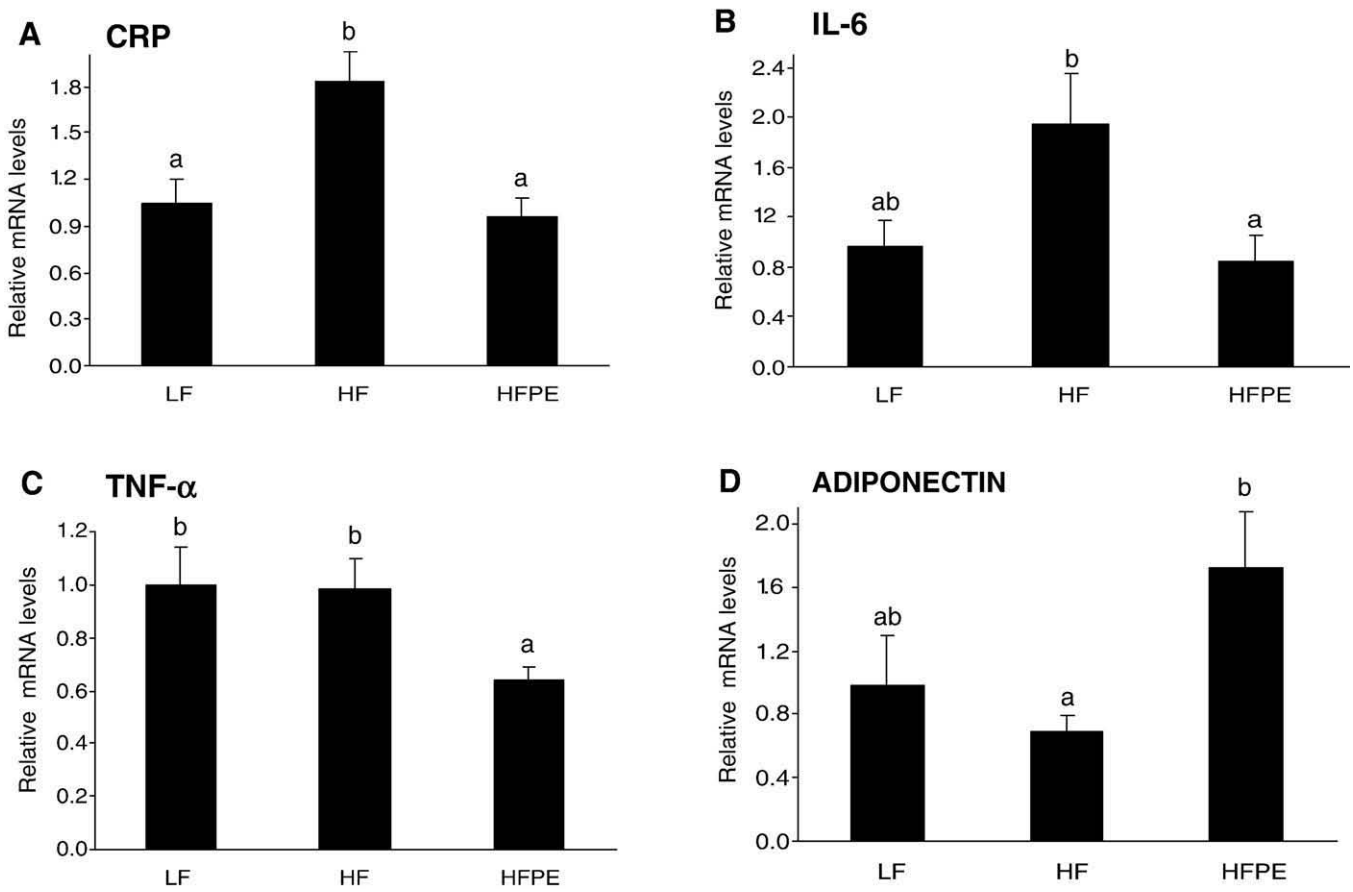

Fig. 2. Diet effect on gene expression in mesenteric adipose tissue. mRNA was extracted, corresponding cDNA was synthesized and CRP, IL-6, adiponectin and TNF-α gene expression was measured by quantitative real-time RT-PCR. ANOVA test was used to evaluate significance between groups ($P<.05$).

pathways [15]. It remains highly likely that adipokines contribute to obesity-associated systemic inflammation and remain potentially important targets for prevention of inflammation-induced insulin resistance or vasculopathy.

Procyanidins have been postulated to possess anti-inflammatory and immunomodulatory activities in vitro and in vivo [18]. In this work, we show that PE acts as an anti-inflammatory substance in vivo. To assess the effect of procyanidins, we compared the ability of PE to modify inflammatory parameters in Zucker *Fa/fa* rats after 19 weeks on a non-hyperlipidic diet, a hyperlipidic diet or a hyperlipidic diet with PE.

We found that feeding rats with the hyperlipidic diet resulted in a moderate increase in body weight as expected, as well as a less pronounced increase in rats receiving procyanidins, as we demonstrated before [19]. Body weight was positively related with adiposity index, comparing all the experimental groups. Moreover, biochemical parameters measured in plasma indicate that the HF diet produced a marked increase in oxidative stress, although it was not attenuated by procyanidin treatment. In contrast, total cholesterol was unchanged and glucose levels were reduced in HFPE-fed rats.

Previous studies indicate that a chronic low-grade inflammation is involved in the pathogenesis of atherosclerosis, and an elevated, highly sensitive CRP level is a risk factor for coronary artery disease. CRP is also a well-known systemic marker for inflammation in human and rats [20]. Plasma CRP levels were also strongly associated with obesity and obesity-related diseases, including insulin resistance, diabetes mellitus and hyperlipidemia. In this work, we found that CRP plasma levels were increased because of the HF diet up to 472 μg/ml, which is at the upper range in normal laboratory healthy rats [21]. We demonstrate that ingestion of procyanidins diminishes CRP levels, thus reducing the diet-induced low-grade inflammation. In addition, plasma CRP levels were positively associated with total body fat mass and CRP expression levels in mesenteric adipose tissue. We examined CRP expression in liver where procyanidins reduced its mRNA. Some authors have recently reported the same properties of red wine phenolics that reduced CRP expression in the human hepatic cell line Hep3B [22].

In the mesenteric adipose tissue, CRP was also down-regulated by the HFPE diet, while in retroperitoneal and epididymal adipose tissue, it was unchanged by either the HF

Table 6
Spearman's correlation coefficients (ρ) of CRP, IL-6, adiponectin and TNF-α expression in mesenteric adipose tissue, plasma levels of CRP and IL-6 and mass parameters

	mRNA levels in mesenteric adipose tissue			Plasma levels		Mass parameters	
	IL-6	Adiponectin	TNF-α	CRP	IL-6	Body weight	Adiposity
CRP mRNA							
ρ	.233	.216	−.096	.623**	.0409	.357	.178
P	.336	.348	.686	.008	.092	.102	.428
n	19	21	20	17	18	22	22
IL-6 mRNA							
ρ		−.436*	.745**	.256	.360	.209	.065
P		.033	.000	.277	.131	.337	.780
n		24	23	20	19	23	21
Adiponectin mRNA							
ρ			−.457*	−.118	.225	−.065	.148
P			.022	.610	.327	.756	.500
n			25	21	21	25	23
TNF-α mRNA							
ρ				−.054	.105	−.071	.014
P				.821	.660	.737	.950
n				20	20	25	23
CRP plasma							
ρ					.014	.475*	.081
P					.954	.029	.743
n					19	21	19
IL-6 plasma							
ρ						.092	.297
P						.683	.191
n						22	21
Body weight							
ρ							.408*
P							.039
N							18

* $P<05$.
** $P<01$.

diet or procyanidins. These variations found in CRP expression between the adipose tissues examined might be due to the different degree of macrophage infiltration in the WATs and the resultant different pattern of cytokine release [7,23], although the reason for this difference is unclear.

Taken together, the increased CRP expression in mesenteric adipose tissue may partially account for the elevation of plasma CRP and the effect of procyanidins on the adipose tissue may be responsible for the reduction of CRP protein and expression levels shown with the HFPE diet.

On the contrary, we have not detected changes in IL-6 plasma levels due to procyanidin treatment, although IL-6 levels were increased by the HF diet. There were no positive correlations between IL-6/CRP levels as could be expected [24].

It has been recently reported that adiponectin-deficient mice exhibit severe diet-induced insulin resistance and enhanced neointimal thickening after vascular injury [10]. These findings suggest that adiponectin has anti-inflammatory properties and acts as an endogenous modulator of obesity-related diseases. Furthermore, administration of adiponectin to obese or diabetic mice causes weight loss and also enhances insulin sensitivity and reduces the plasma glucose level by suppressing hepatic glucose production [25]. Previous studies in our group [26] demonstrate that PE acts as an enhancer of the glucose uptake in 3T3-L1 adipocytes and show that an acute gavage of PE (250 mg PE/kg body weight) significantly reduced blood glucose levels in streptozotocin-induced diabetic rats. In this work, adiponectin plasma levels and adiponectin expression in mesenteric adipose tissue of HFPE rats were highly increased compared with HF rats. Then, the reduction of glucose plasma levels may be driven as a consequence of the enhanced adiponectin expression that PE produces. These findings suggest that procyanidins act as anti-inflammatory molecules in vivo by increasing adiponectin expression. In agreement with our results, the monomeric procyanidin, catechin, has been recently described as an inducer of adiponectin expression in the adipocyte cell line 3T3-L1 [27].

In the current study, procyanidin treatment decreased IL-6 mRNA levels in the mesenteric WAT. In addition, IL-6 expression was negatively correlated with adiponectin expression, suggesting that the expression of IL-6 was negatively regulated by adiponectin in adipose tissue, as shown by other investigators [10].

Our results show that TNF-α expression in adipose tissue was also reduced by the HFPE diet and that this mRNA expression had a strong negative association with adiponectin expression. TNF-α suppresses the transcription of adiponectin in an adipocyte cell line, which might explain the lower levels of serum adiponectin in individuals who are obese [28].

The mechanisms regulating CRP synthesis at extrahepatic sites are unknown. CRP induction in hepatocytes is principally regulated at the transcriptional level by the cytokine IL-6. This cytokine controls expression of many acute-phase protein genes through activation of the transcription factors STAT3, C/EBP family members and Rel proteins (NF-kB) [29]. In searching for the mechanisms involved in inflammation-associated diseases, we have previously demonstrated that PE inhibits NF-kB activation in vitro [18].

We propose that the inhibition of the NF-kB pathway produced by procyanidins down-regulates TNF-α and IL-6 expression, which may explain the increase of adiponectin expression and the indirect reduction of CRP plasma and mRNA levels.

In summary, we have shown for the first time that procyanidins prevent low-grade inflammation in vivo, by adjusting adipose tissue cytokine imbalance, enhancing anti-inflammatory molecules and diminishing proinflammatory ones. Further studies are needed to elucidate the mechanism by which procyanidins may act as anti-inflammatory agents in obese humans.

Acknowledgments

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V. Modulatory Effect of Grape-Seed Procyanidins on Local and Systemic Inflammation in Diet-Induced-Obesity Rats

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ABSTRACT

Chronic low-grade inflammation characterized by adipose tissue macrophage accumulation and abnormal cytokine production is a key feature of obesity and type 2 diabetes. We tested the hypothesis that grape seed procyanidin extract (PE), which is known to exert potent antiinflammatory and antioxidant effects, would improve local and systemic inflammation in two models of diet induced obesity. In the first, we analyzed the preventive effects of procyanidins (11 mg/day) on rats fed a 60% fat synthetic diet. In the second, we induced obesity by feeding the animals with a cafeteria diet and analyzed the corrective effects of two PE doses (10 and 20 mg/ day) and two periods of treatment (10 and 30 days). We showed that in the preventive model, PE not only reduced body weight, but also the systemic markers of inflammation TNF-α and CRP in plasma. PE had little corrective effect on body weight but, in the short treatment, the high dose of PE reduced both TNF-α and CRP plasma levels.

The PE preventive treatment significantly increased adiponectin expression, and decreased TNF-α, IL-6 and CRP expression in white adipose tissue (WAT). PE also reduced NF-kB activity in liver, which explains the low expression rates of hepatic inflammatory markers (TNF-α and CRP) found in rats treated with procyanidins. PE also decreased TNF-α expression in muscle. Finally, dietary supplementation with PE reduced macrophage infiltration of WAT, detected by the reduced expression of Emr1, the gene that encodes the specific marker of macrophage F4/80.

We therefore conclude that orally ingested PE prevents imbalanced cytokine production associated with obesity and type 2 diabetes, but its corrective effects need to be further investigated. The dietary intake of food or drinks containing procyanidins might help to prevent such low grade inflammatory related diseases.

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Introduction

Metabolic syndrome comprises a cluster of risk factors for cardiovascular disease and type 2 diabetes mellitus [1, 2]. Although the specific etiology for this increasingly important proinflammatory condition is not known, obesity and insulin resistance are generally present. Because of the escalating levels of obesity, diabetes, and cardiovascular disease in today's society, metabolic syndrome is receiving considerable attention. This high prevalence has led to the development of new strategies that can reverse its detrimental physiological alterations. Greater insight into the mechanisms behind the syndrome may improve our understanding of how to prevent and best manage this complex condition.

It has been demonstrated that adipocyte dysfunction plays an important role in the development of obesity and insulin resistance [3]. The adipocyte is the primary site of energy storage and accumulates triacylglycerol during nutritional excess. Adipocytes also synthesize and secrete biologically active molecules called adipokines. The altered production of proinflammatory molecules by adipose tissue has been implicated in the metabolic complications of obesity. Compared with the adipose tissue of lean individuals, the adipose tissue of obese individuals expresses increased amounts of such proinflammatory proteins as tumor necrosis factor (TNF-α), interleukin-6 (IL-6), inducible nitric oxide synthase (iNOS), C-reactive protein (CRP), intracellular adhesion molecule-1 (ICAM-1), monocyte chemotactic protein-1 (MCP-1), and reduced adiponectin expression [4].

It has now been firmly established that obesity is associated with a low-grade proinflammatory state, characterized by macrophage infiltration of muscle and adipose tissue, adipocyte dysfunction and the abnormal production of proinflammatory mediators [5, 6]. In addition to adipocytes, adipose tissue contains fibroblasts,

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preadipocytes, tissue-resident macrophages and vascular constituents. Macrophages are known to be crucial contributors to inflammation but it has recently been recognized that adipocytes also have significant intrinsic inflammatory properties. Like macrophages, adipocytes are exquisitely sensitive to infectious disease agents and cytokine-mediated inflammatory signals. In turn, these stimuli induce the expression of inflammatory mediators such as IL-6, TNF-α and CRP. Although many of these activities are restricted to autocrine and paracrine effects, some of the cytokines that are secreted from adipocytes and adipose-resident macrophages make significant contributions to systemic inflammation. Interplay between macrophages and adipocytes by paracrine effects are presumably central in initiating and maintaining adipocyte dysfunction. Adipocytes increase in size as a consequence of hyperalimentation and large adipocytes release more (saturated) FFAs, which bind to macrophage toll-like receptor-4 (TLR-4). This results in NF-kB activation and, ultimately, to increased TNF-α production. In turn, macrophage-derived TNF-α activates human adipocytes, thereby further inducing lipolysis and enhancing the expression of ICAM-1, IL-6 and MCP-1. The diapedesis of monocytes from the blood to adipose tissue and differentiation into macrophages is further facilitated by MCP-1 and ICAM-1. This local paracrine loop involving adipocyte-derived FFAs and macrophage-derived TNF-α establishes a gradual vicious cycle that presumably leads to a pro-inflammatory state of both macrophages and adipocytes. It should be noted that large adipocytes produce less adiponectin. Since adiponectin normally inhibits TLR-activated NF-kB activity, it is assumed that low adiponectin levels re-enforce the previously described loop [6].

Procyanidins are phenolic compounds from the flavonoid group that are widely distributed in the human diet and which are particularly rich in such fruits as berries, blue corn and beans, and in many fruits and vegetables. This suggests that we ingest

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significant amounts of procyanidins from plant-based daily diets like the Mediterranean diet [7]. Of all the various classes of flavonoids, procyanidins are the ones that will potentially be most consumed in the diet. Not only does the concentration of procyanidins in food vary, but also the specific procyanidin present [8]. Because the human diet contains a mixture of procyanidins and it is likely that the effects are due not to one molecule but to the synergic effects of the flavonoids [9, 10], we assessed the potential health benefits of a grape seed procyanidin extract on the obesity-derived proinflammatory state.

Flavonoids function as powerful antioxidants and have anti-inflammatory activities in vitro [11] and in vivo [12]. They have proved to have antioxidant/free radical scavenging abilities; they can chelate minerals, modulate mammalian enzyme activity, enhance intracellular signalling, strengthen membranes, and bind to receptor sites, among other things. Apart from its antioxidant capacity, anti-inflammatory procyanidins are widely sought to provide wide-ranging health protection and bolster the human immune system, because inflammation is such a prominent early symptom of so many chronic human illnesses and injuries [13].

In our preliminary study [14], we showed that procyanidins prevent low-grade inflammation in vivo by adjusting adipose tissue cytokine imbalance, enhancing anti-inflammatory molecules and diminishing proinflammatory ones. In the present study, we evaluated the effectiveness of procyanidins in two experimental models of obesity, and when they were administered as a corrective or preventive treatment. We focused on the putative modulatory effects of procyanidins on cytokine expression in white adipose tissue, muscle and liver to gain insight into the mechanisms that underlie the anti-inflammatory effects ascribed to procyanidins.

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MATERIALS AND METHODS

Chemicals

Grape seed procyanidin extract (PE) contained essentially monomeric (21.3%), dimeric (17.4%), trimeric (16.3%), tetrameric (13.3%) and oligomeric (5-13 units) (31.7%) procyanidins and phenolic acids (4.7 %) according to HPLC analysis.

Animal experimental procedures

Wistar female rats, weighing between 160-175g, were purchased from Charles River Laboratories (Barcelona, Spain), housed in cages and subjected to a standard 12 h light: 12 h dark cycle. After one week in quarantine the animals were divided into the different experimental groups. The schematic diagram of procyanidin treatments are presented in **Figure 1.**

Preventive treatment

Semi purified diets were obtained from Research Diets (USA). Briefly, three diets were used (**Table 1**): a low-fat diet (LF), a high-fat diet (HF) and a high-fat diet supplemented with procyanidin extract (HFPE). The standard control diet was LF. The HFPE differs from HF in the PE content which was 1 mg of PE/g of feed (approximately 11 mg/animal*day). At ~ 15 weeks of age, the rats were randomly assigned to be fed *ad libitum* LF ($n = 6$), HF ($n = 6$) or HFPE (n= 6) diets. The experimental period lasted 19 weeks. Rats were weighed and sacrificed by beheading after a three-hour fast.

Corrective treatment

After the quarantine, 46 rats were divided into two groups: a control group (12 animals) fed with a standard diet (Panlab A-03, Barcelona, Spain) and another group (36 animals) fed with a cafeteria diet consisting of bacon, sweets, biscuits with pâté, cheese, muffins, carrots, milk with sugar and water plus the standard diet [15]. Animals were

fed *ad libitum* and the food was renewed daily. The animals were fed the cafeteria diet for 13 weeks. They were divided into three subgroups (12 animals/group): rats treated with vehicle (CAF), rats treated with 10mg of PE/animal*day (CAFPE10) or rats treated with 20mg of GSPE/animal*day (CAFPE20) in sweetened condensed milk. After 10 days of GSPE treatment, 6 animals of each group were sacrificed (short treatment) and the other animals were fed for another 20 days (long treatment). At 9 a.m. on the last experimental day, rats were treated with PE or vehicle and the food was renewed. At 11 a.m. food was withdrawn and after a three-hour fast the animals were beheaded.

In both treatments, blood was collected using heparin as anticoagulant. Plasma was obtained by centrifugation and animal tissues were excised, frozen immediately in liquid nitrogen and stored at -80°C until analysis. All the procedures were approved by the Experimental Animals Ethics Committee of the Rovira i Virgili University.

Measurement of food intake and body weight gain

Body weight changes and caloric ingestion were monitored weekly throughout the experiment. Liver and adipose tissue fat pads (mesenteric, retroperitoneal and periovaric) were excised separately and weighed. The adiposity index was calculated as the total adipose tissue weight versus total body weight.

Measurement of C-reactive protein and TNF-α levels

C-reactive protein and TNF-α levels were measured in plasma, liver and mesenteric adipose tissue. Tissues were homogenized on ice in a Triton X-100 cell lysis buffer [16]. After centrifugation for 20 minutes at 4ºC, the supernatant was used for the assay.

C-reactive protein levels were quantified using a specific enzyme immunoassay (EIA) according to the manufacturer's instructions (Immunology Consultants Laboratory, Inc., USA). The assay is a double polyclonal antibody sandwich enzyme immunoassay. TNF-

α levels were quantified using a quantitative enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions (Invitrogen, S.A. Barcelona).

NF-kB activity assay

Nuclear protein extracts were prepared from the liver tissue of LF, HF and HFPE rats. Tissue was homogenized in 0.25 M sucrose buffer. After centrifugation at 800*g* for 10 minutes, the resulting pellet was centrifuged at 800g for 5 minutes in hypotonic buffer to provide nuclei. Nuclear extract was obtained by incubation with lysis buffer following the manufacturer's instructions (TransAM NF-kB p65 kit; Active Motif, UK). Protein concentrations were then quantified using a Bradford assay, and equal amounts of protein were used in a colorimetric NF-kB assay specific for the activated form of the p65 subunit of NF-kB (TransAM NF-kB p65 kit; Active Motif, UK).

Quantitative real-time PCR

RNA extraction and real-time PCR analysis were performed, as previously described, with a SYBR Green dye [14]. The forward and reverse primers for rat genes are: CRP, 5' TTTGTGCTATCTCCAGAACAGATCA 3', 5' GCCCGCCAGTTCAAAACAT 3'; IL-6, 5' CCCAACTTCCAATGCTCTCCTAATG 3', 5' GCACACTGAGTTTGCCGAATAGACC 3'; Adiponectin, 5' GGCCGTTCTCTTCACCTACG 3', 5' GGCTCCATGCTCCTCCATCT 3', TNF-α, 5' CGTCAGCCGATTTGCCATTTC 3', 5' TGGGCTCATACCAGGGCTTGAG 3'; Emr1, 5' CATGGCCTTCCGTGTTCCT 3', 5' CCTGCTTCACCACCTTCTTGA 3' and GAPDH, 5' CAT GGC CTT CCG TGT TCC T 3', 5' CCT GCT TCA CCA CCT TCT TGA 3'.

Calculations and statistical analysis

Results are expressed as mean values ± S.E.M. Effects were assessed using ANOVA or Student's t-test. We used Tukey's Test of Honestly Significant Differences to make pairwise comparisons. All calculations were performed using SPSS 15.0 software.

RESULTS

The effect of procyanidins on body weight

Wistar female rats fed both hyperlipidic diets showed higher body weights than control rats (**Figure 2**), which indicated that both experimental models induced a highly significant obesity.

Preventive treatment

The PE preventive treatment produced significant body weigt loss and also diminished body weight gain (**Figure 2**). This treatment with PE also reduced liver weight, but no changes were found in adiposity or the weight of fat depots (**Table 2**).

Corrective treatment

The most significant differences in body weight were found in the corrective model, where the animals that were fed the cafeteria diet increased their body weight by 40%. Also, the fat pad weights of rats fed both diets were higher than those of the LF diet (Table 2). The corrective treatment with PE did not produce any significant reduction in body weight, although rats treated with procyanidins tended to weigh less. Some doses of PE showed tissue weight reductions compared with its obese controls, as shown with the highest dose of PE that was able to reduce the mesenteric fat in the long corrective model.

PE treatment modulates adipose, hepatic, muscular and systemic inflammation

Given our previous results [14], we went on to investigate the modulatory effect of procyanidins on both local and systemic manifestations of inflammation.

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Visceral adipose tissue

We analyzed the effect of preventive procyanidin treatment on the expression of genes known to be closely related to the inflammatory process in the visceral adipose tissue of rats fed a standard LF diet, HF diet or HFPE (**Figure 3**). We found that HF rats had higher CRP, TNF-α and IL-6 mRNA levels than control LF rats and HFPE rats. Furthermore, CRP and TNF-α protein levels were reduced in HFPE (**Table 3**) which is consistent with its gene expression. Thus, the cytokine levels increased by the HF diet were diminished by procyanidin treatment in this tissue. PE also stimulated the expression of the anti-inflammatory cytokine adiponectin. Emr1, which encodes the expression of F4/80, a surface marker of mature macrophages, increased in the HF group but decreased in the HFPE group.

Muscle

We determined the effect of PE on local inflammation by Real-time PCR analysis of gene expression in muscle from the preventive model rats. As shown in Figure 4, the analysis of TNF-α expression in muscle revealed a marked increase in HF rats and a significant reduction in TNF-α expression in rats fed the HFPE diet. We also assessed the expression of Emr1. Although its expression was higher in HF rats than in HFPE rats, this difference was not significant in muscle.

Liver

We also analyzed CRP and TNF-α hepatic gene expression in this model. Both parameters were increased by the HF diet and treatment with PE significantly decreased CRP and TNF-α expression (Figure 3). The hepatic analysis of CRP protein expression revealed that the HF diet significantly increased this parameter, but procyanidin treatment was not able to reduce it. Moreover, using a specific assay for NF-kB p65

subunit activity, we determined that there was significantly less p65 activity in liver nuclear extracts derived from HFPE group than in HF rat livers (Figure 4).

Systemic inflammation

To further investigate the effect of procyanidins on inflammation, we analysed CRP and TNF-α plasma levels in both models of obesity.

Preventive treatment

CRP plasma levels were increased in HF rats, thus indicating low grade inflammation induced by a high-fat diet, similar to that found in humans with overweight/obesity (**Table 3**). Moreover, the administration of HFPE to rats—that is to say, a daily ingestion per animal of nearly 11 mg of PE for 19 weeks—resulted in such a decrease in CRP that it fell to the range of levels found in rats fed the control diet (LF).

We also investigated the possibility that procyanidins could reduce systemic inflammation by modulating TNF-α. Interestingly, in the preventive model, TNF-α plasma levels increased in HF rats and decreased in PE rats.

Corrective treatment

The cafeteria diet increased CRP plasma levels in the short and long treatment in comparison to rats fed the standard diet. In this model, however, only the high dose of PE (20 mg/day*animal) reduced CRP plasma levels in the short treatment (Table 3).

Rats fed the cafeteria diet (CAF) had higher levels of TNF-α than CAFPE10 and CAFPE20 groups in the short treatment, although no changes in TNF-α plasma levels were detected between groups from the long corrective treatment (Table 3).

Discussion

Obesity is the key player in metabolic syndrome. Improving adipocyte dysfunction is one of the crucial ways of preventing this syndrome. In this study we have tried to increase our understanding of how procyanidins regulate the pathways responsible for

preventing obesity and modulating the associated low-grade inflammation. In our previous investigations we demonstrated that procyanidins were able to modulate adipokine expression in rats fed a 30% fat diet [14]. Now we wanted to evaluate the effect of procyanidins at different times and doses, and in different models of obesity. To prove how effective they are at preventing or treating diseases, our experimental design included several treatment conditions. We analyzed two different diets as models of a disturbed metabolic state. In the first, we analyzed the preventive effects of procyanidins on rats fed a 60% fat diet. In the second, we induced obesity by feeding the rats with a cafeteria diet, which is a rapid inducer of obesity. We analyzed the corrective effects of two PE doses (10 and 20 mg/ day) over two periods of time (short and long treatments). These two models gave us a greater scope for analyzing the duration of the treatment, and the preventive and curative effects of procyanidins.

Firstly, we analyzed the effects of PE on body weight. In the preventive model, we found that feeding rats with the hyperlipidic diet resulted in an increase of body weight as expected [17], but also a less pronounced increase in rats receiving procyanidins [14]. Moreover, rats fed the cafeteria diet increased in weight considerably more than the control group but procyanidin treatment had little effect on this parameter. The reason for these differences between models is unclear but they might be explained by the different degree of obesity in each model. In our previous studies we also fed rats diets with a lower percentage of fat. This suggested that procyanidins administered after obesity has been established cannot compensate for the high body weight caused by the cafeteria diet.

To further investigate the effects of procyanidins on systemic manifestations of inflammation we analyzed CRP and TNF-α plasma levels, which are markers of systemic inflammation, in both models of obesity [18, 19]. In the preventive model,

procyanidins diminished the diet-induced systemic inflammation by reducing the TNF-α and CRP plasma levels. On the other hand, the short corrective treatment with a high dose of procyanidins lowered CRP plasma levels. These results indicate that when the overproduction of CRP in obese rats is not extremely high a short curative dose of procyanidins might effectively diminish CRP plasma levels. TNF-α levels were also reduced by both doses of procyanidins in the short treatment. However in the long corrective treatment no changes in CRP or TNF-α plasma levels were detected.

We have focused on the cytokine expression profile in mesenteric adipose tissue as a representative of visceral adipose tissue, not only because it appears to be an important point in the development of low grade inflammation [20] but also because we have previously shown that adipose tissue can be an important target for procyanidins [21]. In this study, we have shown that IL-6, CRP, and TNF-α high-fat diet induced gene expression was reduced by the adding PE to the diet, as we have previously reported [14]. Also the gene expression of the anti-inflammatory molecule adiponectin was enhanced. Furthermore, procyanidin treatment reduced TNF-α and CRP protein levels in mesenteric adipose tissue, indicating that local inflammation in this tissue was prevented.

Only relatively recently have obese individuals been described to have increased macrophage infiltration of adipose tissue, but it now appears that the infiltration rate of monocytes into visceral adipose tissue is higher than into subcutaneous adipose tissue [6]. Macrophages have been identified as the primary source of many of the circulating inflammatory molecules that are detected in the obese state [22]. Weisberg et al. [5] demonstrated that adipose tissue macrophages are responsible for almost all adipose tissue TNF-α expression and significant amounts of IL-6 expression in mice. Furthermore, the preponderance of evidence supports the hypothesis that the amounts of

TNF-α mRNA and TNF-α released by adipose tissue are enhanced in human obesity and that the vast majority of TNF-α releases by adipose tissue come from the non-fat cells in this tissue [23]. Because obesity is associated with macrophage accumulation in adipose tissue [24, 25] we wished to examine the effects of procyanidins on macrophage infiltration by measuring the expression levels of Emr1, the gene encoding a specific marker for mature macrophages (F4/80) in the mesenteric adipose tissue. We found that macrophage presence increased significantly more than in control rats and, interestingly, procyanidins reduced this level. So, the inhibition of the cytokine expression in this tissue might be due to a decrease in the number of macrophages, but procyanidins might also directly affect the proinflammatory pathways in the adipocyte and the macrophage. The stimulus for macrophage influx into fat is largely unknown and probably involves a complex pathophysiology that includes physical and chemical adipocyte injury and cytokine cross-talk with increased local expression of TNF-α, MCP-1 and I-CAMs [26]. We found that procyanidin treatment decreased NF-kB activity in liver, which is directly associated with the decreased hepatic expression of such inflammatory molecules as TNF-α and CRP, as we found in our study. We hypothesise that procyanidins also reduce MCP-1 secretion in adipocytes partially due to the diminished TNF-α levels that we have detected in adipose tissue, muscle and liver, which might be a consequence of the inhibitory effects of procyanidins on NF-kB activation [13, 27]. As we have previously proposed, the local enhancement in adiponectin expression produced by PE might also be partly responsible for the reduced cytokine expression levels. On the other hand, TNF-α has been shown to induce the expression of adhesion molecules (I-CAM) and chemokines in human endothelial cells. So, another mechanism for procyanidin effects might be the result of diminishing I-CAM expression, which activates macrophages [6]. Some authors have recently reported similar results in

murine obesity models and in vitro experiments with such other flavonoids as curcumin, resveratrol and a GSPE [25, 28, 29].

We found reduced cytokine expression in liver, muscle and mesenteric adipose tissue, which lowers local inflammation and possibly also systemic inflammation [30, 31]. In any case, this is an interesting finding that demonstrates the potential effects of procyanidins on such low-grade inflammation related diseases as obesity.

In summary, we have shown that procyanidins modulate tissue and plasma cytokine levels partially by reducing macrophage infiltration in adipose tissue and NF-kB activity in liver. However, although procyanidins are effective at preventing the onset of inflammation, it is unclear whether procyanidins exert anti-inflammatory effects once inflammation is established and whether they can help to resolve inflammation.

In conclusion, our studies revealed that daily consumption of procyanidins prevent both systemic and local low-grade inflammation in adipose tissue, muscle and liver, which might improve obesity-induced insulin resistance in these tissues. Given these interesting findings, further investigation of the effects of procyanidins in human patients is required.

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Table 1. Composition of the LF, HF and HFPE test diets

Test diets	LF (g/kg diet)	HF (g/kg diet)	HFPE (g/kg diet)
Ingredients			
Corn Starch	545.0	0.0	0.0
Maltodextrine	118.5	121.1	121.2
Sucrose	0.0	129.1	129.2
Lard	19.0	316.3	316.6
Soy bean oil	23.7	32.3	32.3
Procyanidin extract	0.0	0.0	1.0
Energy (kcal/g)	3.85	5.24	5.24
Protein (% energy)	20.0	20.0	20.0
Carbohydrate (% energy)	70.0	20.0	20.0
Fat (% energy)	10.00	59.90	59.90

LF, low-fat diet; HF, high-fat diet; HFPE, high-fat diet supplemented with procyanidins from grape seed. Vitamin and mineral mixture was also added to all the diets.

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Table 2. Tissue weight of rats fed the different diets.

Preventive Treatment			
	LF	**HF**	**HFPE**
Total energy intake (kcal)	1075.4 ± 54.4^{a}	1410.3 ± 21.7^{b}	1306.0 ± 46.3^{b}
Tissue weight (g)			
Liver	10.1± 0.4^{ab}	11.1 ± 0.4^{b}	9.6 ± 0.5^{a}
Periovaric+retroperitoneal fat	19.9± 1.5^{a}	24.4 ± 1.3^{b}	27.5 ± 2.6^{b}
Mesenteric fat	5.7 ± 1.1^{a}	8.2 ± 0.5^{b}	8.7 ± 2.0^{b}
Adiposity index	7.8 ± 0.9^{a}	9.1 ± 0.4^{a}	10.4 ± 1.5^{a}
Corrective Treatment			
Short (10 days)	**LF**	**CAF**	**CAFPE10**	**CAFPE20**
Tissue weight (g)				
Periovaric+retroperitoneal fat	15.4± 2.5^{a}	46.3 ± 4.8^{b}	37.7 ± 5.8^{b}	37.9 ± 3.7^{b}
Mesenteric fat	4.7 ± 0.5^{a}	15.7 ± 3.1^{b}	18.3 ± 0.6^{b}	13.7 ± 2.1^{b}
Adiposity index (%)	7.0 ± 0.9^{a}	14.5 ± 0.9^{b}	13.9 ± 1.0^{b}	14.2 ± 1.0^{b}
Long (30 days)				
Tissue weight (g)				
Periovaric+retroperitoneal fat	12.7 ± 0.7^{a}	47.7 ± 2.9^{b}	46.5 ± 3.1^{b}	48.9 ± 3.6^{b}
Mesenteric fat	3.9 ± 0.4^{a}	20.2 ± 2.6^{b}	20.3 ± 2.3^{b}	13.0 ± 1.9^{c}
Adiposity index (%)	6.4 ± 0.4^{a}	15.7 ± 0.7^{bc}	13.6 ± 0.3^{c}	15.9 ± 0.7^{b}

LF, low-fat diet; HF, high-fat diet; HFPE, high-fat diet supplemented with procyanidins from grape seed; CAF, cafeteria diet; CAFPE10, cafeteria diet diet supplemented with procyanidins from grape seed 10 mg/day*animal; CAFPE20, cafeteria diet supplemented with procyanidins from grape seed 20 mg/day*animal. Values are mean ± S.E.M (n=6 per group). The significance of difference among the groups was analyzed by ANOVA (p< 0 .05).

Table 3. Cytokine levels in plasma and various tissues.

Preventive Treatment			
	LF	**HF**	**HFPE**
CRP			
Plasma (µg/mL)	387.4 ± 28.2^{ab}	658.1 ± 147.5^{b}	313.7 ± 21.7^{a}
Adipose tissue (ng/100 µg protein)	33.6 ± 1.3^{a}	74.22 ± 2.9^{b}	34.6 ± 3.4^{a}
Liver (ng/100 µg protein)	71.5 ± 4.2^{a}	139.1 ± 2.3^{b}	139.4 ± 3.2^{b}
TNF-a			
Plasma (pg/mL)	NA (< 4)	41.7 ± 9.3^{a}	15.2 ± 3.7^{b}
Adipose tissue (pg/100 µg protein)	NA (< 4)	76.9 ± 5.6^{a}	60.7 ± 4.3^{b}
Corrective Treatment			
	CAF	**CAFPE10**	**CAFPE20**
CRP			
Plasma Short T. (µg/mL)	798.4 ± 27.7^{a}	871.1 ± 110.6^{a}	430.4 ± 44.1^{b}
Plasma Long T. (µg/mL)	1050.1± 230.0^{a}	1230.7 ± 267.3^{a}	1029.1 ± 276.2^{a}
TNF-a			
Plasma Short T. (pg/mL)	21.3 ± 3.8^{a}	4.0 ± 0.6^{b}	5.4 ± 0.1^{b}
Plasma Long T. (pg/mL)	15.8 ± 1.3^{a}	11.2 ± 1.8^{a}	11.6 ± 3.7^{a}

LF, low-fat diet; HF, high-fat diet; HFPE, high-fat diet supplemented with procyanidins from grape seed; CAF, cafeteria diet; CAFPE10, cafeteria diet diet supplemented with procyanidins from grape seed 10 mg/day*animal; CAFPE20, cafeteria diet supplemented with procyanidins from grape seed 20 mg/day*animal. Values are mean ± S.E.M (n=6 per group). The significance of difference among the groups was analyzed by ANOVA (p< 0 .05).

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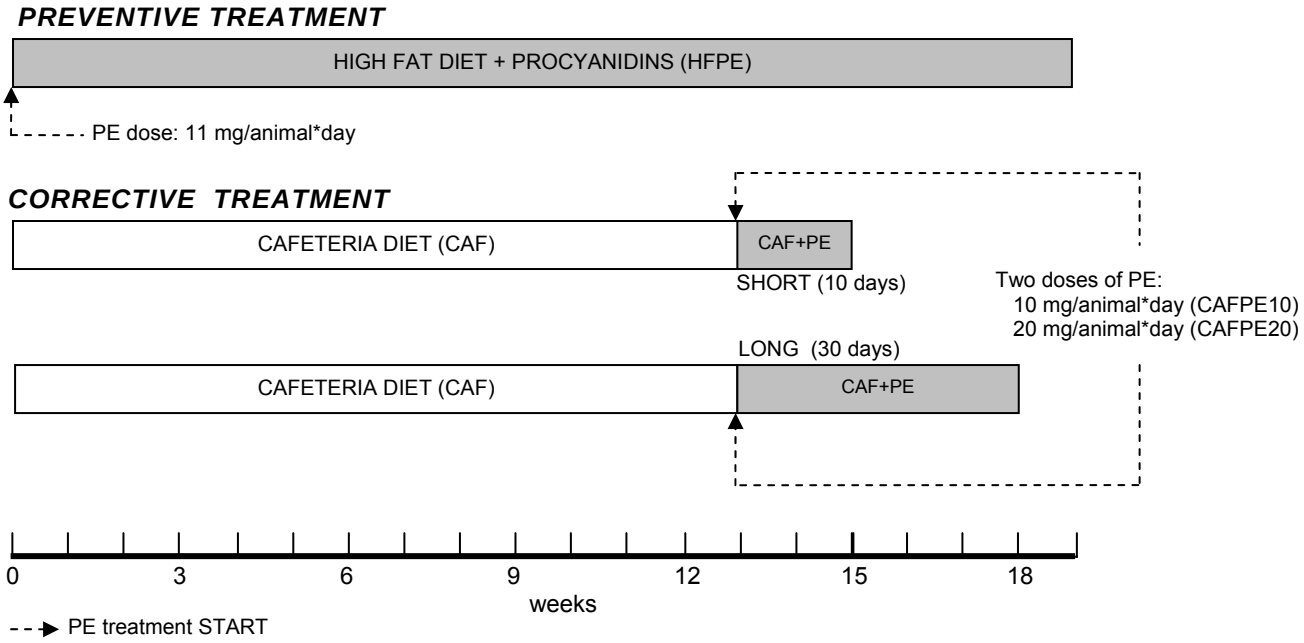

Figure 1. Schematic diagram of procyanidin treatments.

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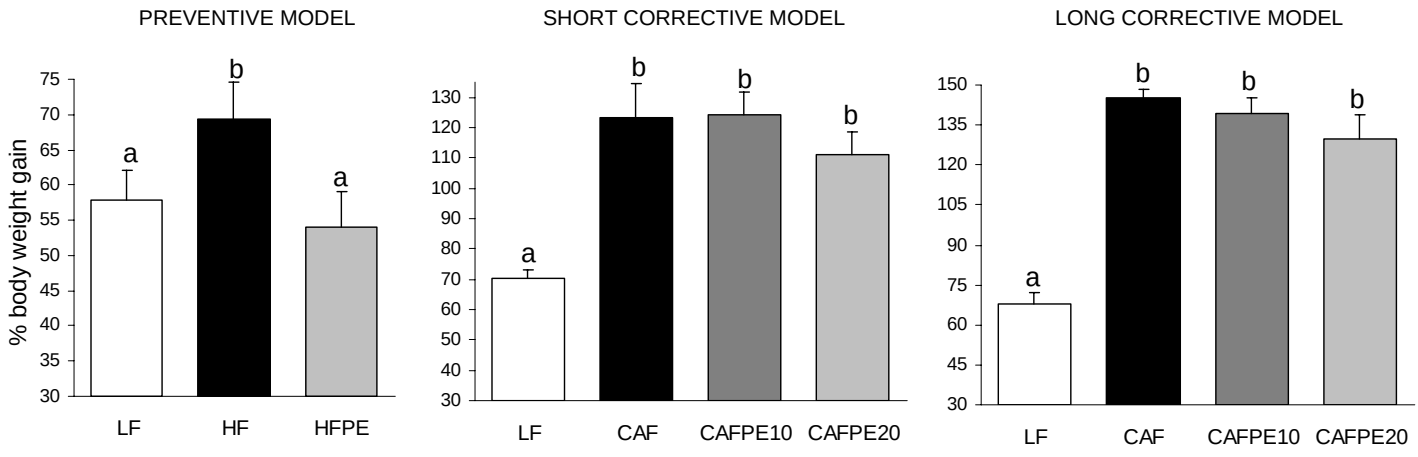

Figure 2. Effect of procyanidins on the body weigh gain of the different obesity models. LF, low-fat diet; HF, high-fat diet; HFPE, high-fat diet supplemented with procyanidins from grape seed; CAF, cafeteria diet; CAFPE10, cafeteria diet diet supplemented with procyanidins from grape seed 10 mg/day*animal; CAFPE20, cafeteria diet supplemented with procyanidins from grape seed 20 mg/day*animal. Values are mean ± S.E.M (n=6). The significance of difference among the groups was analyzed by ANOVA ($p< 0.05$).

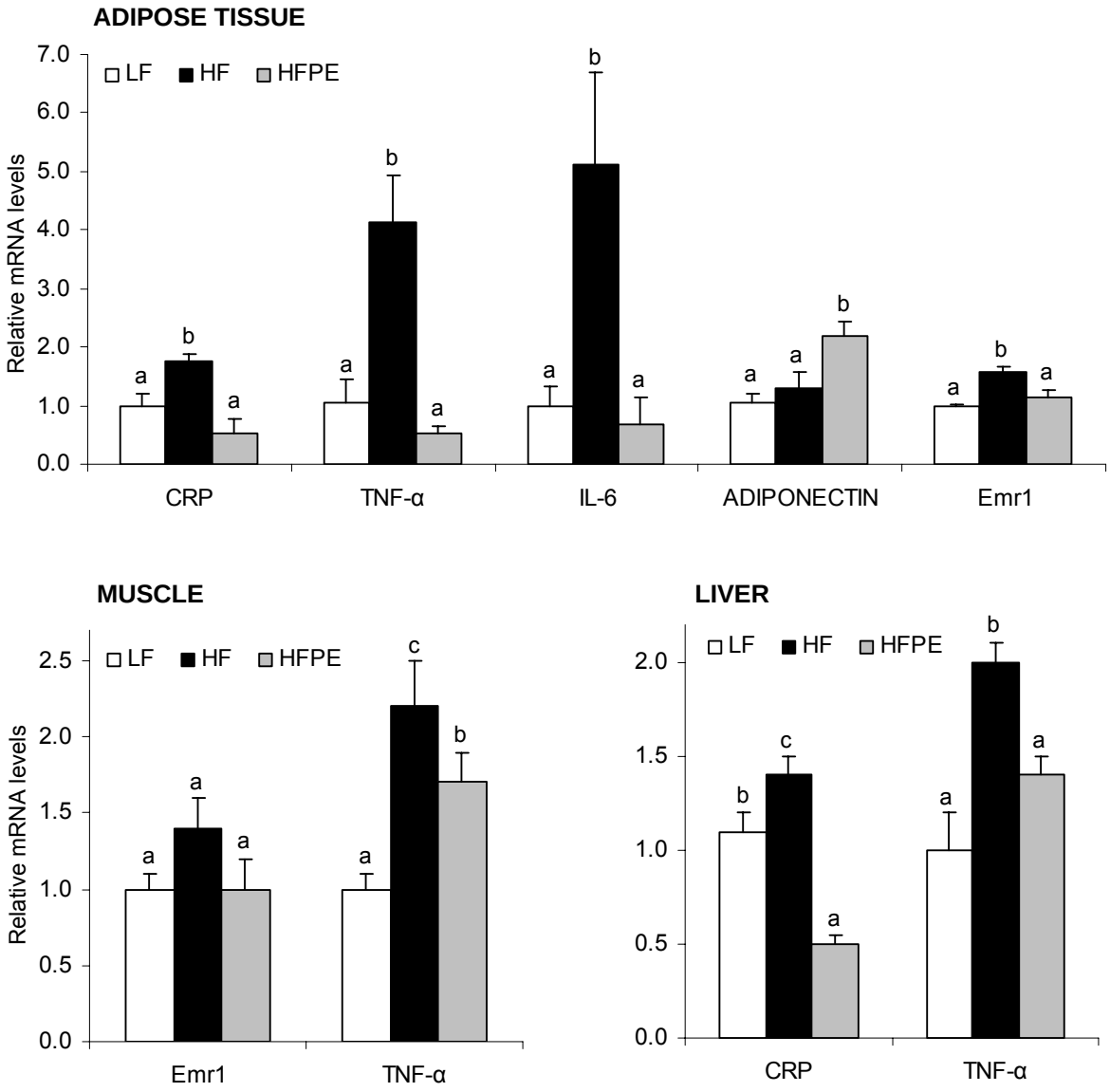

Figure 3. Procyanidin effect on gene expression in mesenteric adipose tissue, muscle and liver from the preventive treatment rats. mRNA was extracted, corresponding cDNA was synthesized and CRP, IL-6, adiponectin, TNF-α and Emr1 gene expression was measured by quantitative real-time RT-PCR. LF, low-fat diet; HF, high-fat diet; HFPE, high-fat diet supplemented with procyanidins from grape seed. ANOVA test was used to evaluate significance between groups ($p< 0.05$).

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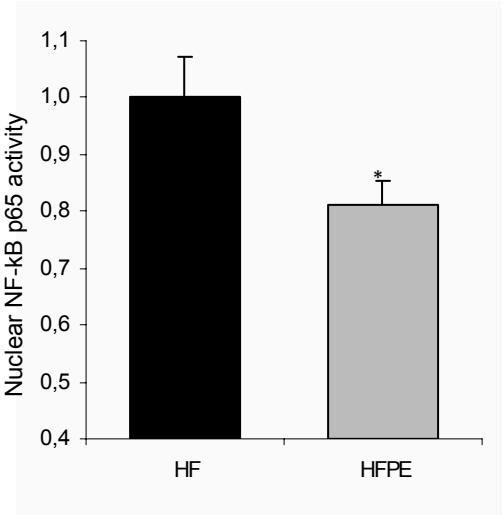

Figure 4. Procyanidin effect on NF-kB activity in liver from the preventive treatment rats. PE significantly decreases hepatic NF-kB activity after 19 weeks (n=6 per group). Student's t test was used to evaluate significance between groups ($p< 0.05$).

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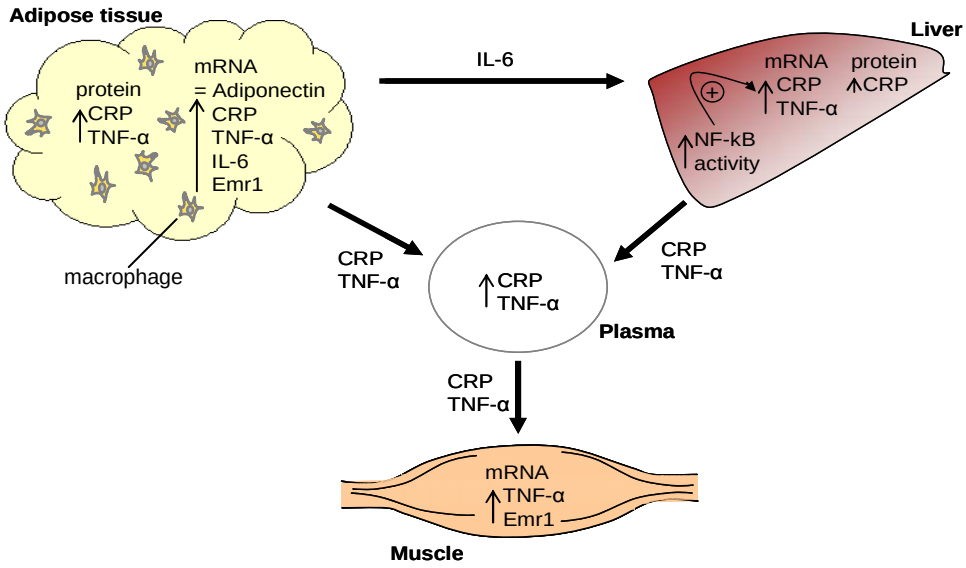
A. Inflammatory response in obese rats.



B. Antiinflammatory effects of procyanidins.

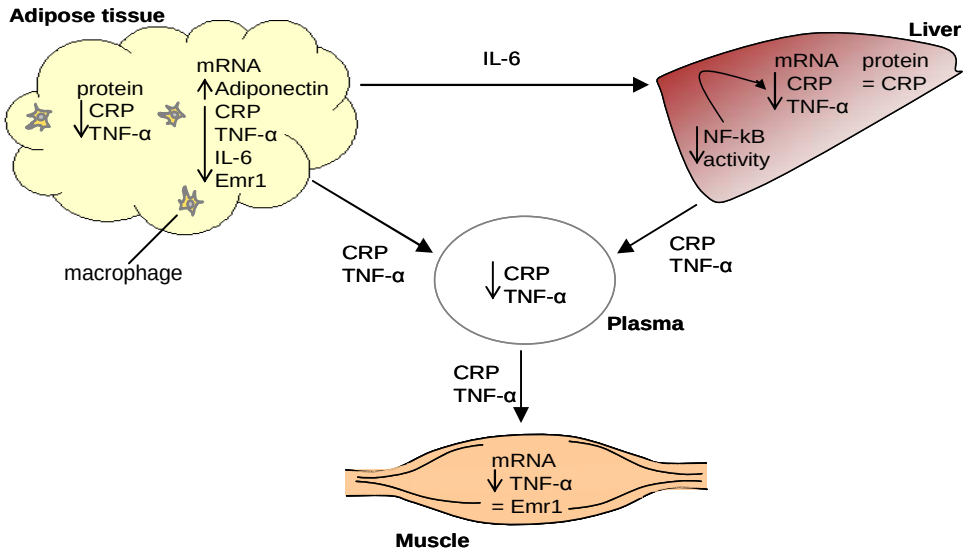


Figure 5. Schematic diagram of procyanidin effects in the preventive model rats.

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V. SUPPLEMENTARY RESULTS

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This work is the result of the collaboration between researchers in different groups. My personal contribution to this research article involved carrying out the assays to determine the production of NO and PGE-2 in the RAW 264.7 cell line and I included them in the discussion of this PhD Thesis.

A trimer plus a dimer -gallate reproduce the bioactivity described for an extract of grape seed procyanidins

Authors: Gemma Montagut, Isabel Baiges, Josep Valls, Ximena Terra, Josep M. del Bas, Xavier Vitrac, Tristan Richard, Jean-Michel Mérillon, Lluís Arola, Mayte Blay, Cinta Bladé, Juan Fernández-Larrea, Gerard Pujadas, Josepa Salvadó, and Anna Ardévol

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A trimer plus a dimer-gallate reproduce the bioactivity described for an extract of grape seed procyanidins

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ABSTRACT

The relationship between grape seed-derived procyanidin extract components and their bioactivity was explored. The monomeric and dimeric structures only acted as anti-inflammatory agents. Similarly, pure C1 trimer was highly effective on LPS-activated macrophages. To reproduce all of the bioactivities of the total extract, a fraction enriched with trimeric structures was needed. This trimeric-enriched fraction was divided into subfractions, the most bioactive of which contained two compounds with a molecular weight equal to a trimer (865) and a dimer-gallate (729), according to spectrometric analysis. Thus, it may be concluded that a mixture of both molecules reproduces the bioactivity in glucose metabolism (3T3-L1), lipid metabolism (HepG2) and macrophage functionality (RAW 264.6).

1. Introduction

The "French paradox" has attracted the attention of researchers worldwide for more than a decade (Renaud & De Lorgeril, 1992). Many studies have proved several positive and healthy effects of grape seed-derived procyanidins (GSPE) (Aron & Kennedy, 2008; Rasmussen, Frederiksen, Struntze Krogholm, & Poulsen, 2005). Most of them, however, have examined phenolic extracts derived from grape seed, because they are waste products of the winery and grape juice industry—a rich source of polyphenols—and because it is difficult to find individual compounds as pure structures in these extracts.

The composition of GSPE is known to consist largely of gallic acid, catechin, epicatechin and procyanidin dimers and trimers composed of flavan-3-ol units with C4–C8 or C4–C6 interflavan linkages (Agarwal et al., 2007). These compounds are also present as esters linked to gallic acid in the aliphatic 3-hydroxyl group in the C ring. Some attempts have been made to evaluate how effective the different components of these extracts are at improving some of the well-described properties of the whole extract, mainly growth inhibition and apoptotic death (Agarwal et al., 2007; Faria, Calhau, deFreitas, & Mateus, 2006; Lizarraga et al., 2007). Guo et al. (2007) proved that oligomeric and polymeric grape seed procyanidins are effective at protecting and treating ailments in the central nervous system induced by alcohol abuse.

Grape seed-derived procyanidin extracts have several healthful properties: for example, they act as antioxidants (Puiggròs et al., 2005), they improve lipid metabolism (del Bas et al., 2005), they limit adipogenesis (Pinent et al., 2005), and they function as insulinomimetic agents (Pinent et al., 2004) and anti-inflammatory agents (Terra et al., 2007). This study aims to identify the structure(s) responsible for these healthful effects. This was achieved by two fractionation steps of the initial extract according to its

Abbreviations: GSPE, grape seed-derived procyanidin extract; GA, gallic acid; EGCG, epigallocatechin-gallate; NO, nitric oxide; PGE2, prostaglandin E2; LPS, lipopolysaccharide; PBS, phosphate buffered saline; BSA, bovine seric albumin; EC, epicatechin; TAG, triacylglycerol; DMEM, Dulbecco's modified Eagle medium; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid; HPLC-ESI-MS, high performance liquid chromatography-electrospray ionisation-mass spectrometry; MALDI-TOF, matrix-assisted laser-desorption ionisation-time-of-flight mass spectrometer.

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effectiveness at improving several functions in three different cell lines. Once a highly effective fraction had been identified, its structures were characterised using HPLC-ESI-MS and the molecular weights of individual peaks were confirmed using MALDI-TOF.

2. Materials and methods

2.1. Cells, reagents and materials

The procyanidin extract contained 76% procyanidin with the following composition: 1.63% phenolic acids (mainly gallic acid), 20.92% monomers (mainly catechin + epicatechin), 20.71% dimers + epigallocatechin-gallate (EGCG), 17.33% trimers and 39.41% oligomeric forms of four units or more. Pure molecules were mostly obtained from SIGMA (Madrid, Spain). These were hippuric acid, ferulic acid, 3-hydroxybenzoic acid, 3,4-dihydroxyphenylacetic acid, *p*-hydroxyphenylacetic acid, vanillic acid, 3-hydroxyphenylacetic acid, *p*-coumaric acid, epigallocatechin, catechin gallate, epicatechin, gallocatechin gallate, epicatechin gallate, catechin hydrate and epigallocatechin gallate. Procyanidin B1–B4 came from APIN Chemicals (Abingdon, Oxon, UK). All procyanidin extracts, fractions, subfractions and pure molecules were prepared in absolute ethanol. Appropriate dilutions were made in order to obtain a 0.1% (v/v) ethanol concentration in all control and treated wells.

Cell culture reagents were obtained from BioWhittaker (Verviers, Belgium). Insulin (Actrapid®) was from Novo Nordisk Pharma SA (Madrid, Spain). Bradford protein reagent was from Bio-Rad Laboratories (Life Science Group, Hercules, CA, USA), 2-deoxy-[1-^{3}H]-glucose and ^{14}C-acetate was from Amersham Biosciences (Buckinghamshire, England).

2.2. Chromatographic separation of procyanidin extract

GSPE (0.5 g) was subjected to normal-phase chromatography column (35-70 mesh, Interchim, Montluçon, France) preconditioned with solvent A (acetone/hexane, 65:35) as follows: GSPE components were separated according to size using an increasing gradient of solvent B (acetone/hexane, 80:20). First, low molecular weight compounds were eluted with solvent A, then the proportion of solvent B was gradually increased until it reached 100% after 1 h. Finally, an additional volume of solvent B was added, and 10 mL fractions were collected using a fraction collector. The fractions collected were monitored using TLC on PolyGram silica gel 0.2 mm with fluorescent indicator UV_{254} (Macherey-Nagel, Hoerdt, France), with the mixture toluene/acetone/acetic acid (3:3:1, v/v/v). The TLC plates were visualised following spraying with anisaldehyde reagent. Eleven major fractions with increasing degrees of polymerisation were identified, according to their retention time, R_f (Terra et al., 2007). These fractions were vacuum-dried and kept at −20 °C for subsequent use in the biological studies.

The most effective fraction (VIII) was further subfractionated by semipreparative HPLC (Varian, Model 210 Walnut Creek, CA, USA) with a 4 × 250 mm Ultrasep RP18 column (4 μm) (Bischoff, Leonberg, Germany) at room temperature using the following solvents: water/formic acid (95.5:4.5, v/v) (A) and acetonitrile/solvent A (10:90, v/v) (B) with the following gradient system: 0–40% B (0–10 min), 40–60% B (10–35 min), 60–100% B (35–50 min) and 100% B (50–60 min). Detection was carried out at 286 and 306 nm, with a UV–vis detector (Varian, Model 345, Walnut Creek, CA, USA).

An initial approach for determining molecular weight was to use HPLC-ESI-MS. A Platform II (Micromass, Manchester, UK) with electrospray injection (ESI) was used, coupled to the LC apparatus (reversed phase LC on a Waters TM system 600 E, Saint-Quentin, France). Procyanidins can easily shed a proton, generating intense negative ions $[M–H]^-$, so detection was performed in the negative ion mode. A low voltage was used to avoid fragmentation; the products were identified by their molecular peaks.

The chromatogram peaks isolated by semipreparative HPLC were also characterised by MALDI-TOF. MALDI-MS spectra were obtained using a matrix-assisted laser-desorption ionisation-time-of-flight mass spectrometer (TofSpec MALDI-TOF) from Micromass (Manchester, UK). This instrument has a pulsed nitrogen laser (337 nm, 4 ns pulse width) and a time-delayed extracted ion source. Spectra were recorded in the positive-ion mode using the reflectron and a 20 kV accelerating voltage.

2.3. Cell culture and measurements

3T3-L1 preadipocytes were cultured and induced to differentiate as previously described (Ardévol, Bladé, Salvadó, & Arola, 2000). Ten days after differentiation, fully differentiated adipocytes were washed twice with phosphate buffered saline (PBS) and incubated at 37 °C with serum-free supplemented Dulbecco's modified Eagle medium (DMEM) containing 0.2% bovine serum albumin (BSA) (depletion medium) for 2 h. During the last 30 min of this depletion treatment, the cells were treated with GSPE or insulin. Afterwards, glucose transport was determined by measuring the uptake of 2-deoxy-D-[^{3}H] glucose, as previously described by Pinent et al. (2004). Each condition was run in triplicate.

Table 1
Summary of the bioactivity of the monomeric pure forms. Values in italics mean a statistically significant positive effect ($p < 0.05$).

Monomeric structures	Stimulation of glucose uptake (3T3-L1 adipocytes) 150 mg/L compound (stimulation vs insulin effect 1.04 ± 0.023)	PGE-2 production (RAW macrophages) 5 mg/L compound (% of inhibition vs LPS stimulation 100.0 ± 1.2)	NO production RAW macrophages 5 mg/L compound (% of inhibition vs LPS stimulation 100.0 ± 6.2)
Vanilic acid	0.055 ± 0.018	*60.63 ± 9.61*	*69.69 ± 0.56*
Epicatechin	0.024 ± 0.014	*46.73 ± 0.73*	*64.77 ± 0.70*
Epicatechin gallate	NA	*48.18 ± 6.66*	*32.87 ± 8.72*
EGCG	NA	*53.72 ± 5.63*	*6.67 ± 3.56*
Catechin	0.051 ± 0.018	57.98 ± 5.99	103.71 ± 1.35
Gallic acid	0.048 ± 0.010	*46.63 ± 2.51*	93.4 ± 0.8
p-Hydroxiphenylacetic acid	0.054 ± 0.014	*55.31 ± 4.00*	111.79 ± 5.28
p-Coumaric	0.058 ± 0.020	NA	NA
3-Hydroxybenzoic acid	0.058 ± 0.008	NA	NA
Protocatequic acid	0.073 ± 0.049	NA	NA
Ferulic acid	0.092 ± 0.068	NA	NA
Hypuric acid	0.085 ± 0.047	NA	NA

Table 2
Summary of the bioactivity of the oligomeric pure compounds. Values in italics mean a statistically significant positive effect ($p < 0.05$).

Oligomeric pure structures	Stimulation of glucose uptake 150 mg/L compound (stimulation vs insulin effect 1.037 ± 0.023)	PGE-2 production 5 mg/L compound (% of inhibition vs LPS stimulation 100.0 ± 1.2)	NO production 5 mg/L compound (% of inhibition vs LPS stimulation 100.0 ± 6.2)	Total cholesterol secretion 25 mg/L compound (fold change vs control 1.00 ± 0.02)	Triacylglycerol secretion 25 mg/L compound (fold change vs control 1.01 ± 0.01)
B3	0.09 ± 0.02	69.92 ± 11.14	83.98 ± 4.56	1.26 ± 0.16	1.18 ± 0.11
B1	0.08 ± 0.07	70.78 ± 15.85	42.56 ± 3.52	1.29 ± 0.12	1.05 ± 0.11
B2	0.09 ± 0.05	79.86 ± 0.17	60.12 ± 7.49	1.15 ± 0.11	1.07 ± 0.09
B4	0.07 ± 0.03	*68.92 ± 0.04*	*46.58 ± 3.90*	1.22 ± 0.13	1.20 ± 0.16
C1	*0.13 ± 0.00 100 mg/L*	*44.38 ± 1.42*	*20.96 ± 5.85*	1.05 ± 0.11	*0.82 ± 0.04*

Table 3
Summary of the bioactivity of the fractions obtained from the grape seed-derived extract. Values in italics mean a statistically significant positive effect ($p < 0.05$). In the first column, the intensity of the shade of grey correlates to the increasing degree of polymerisation.

GSPE fractions (amount obtained)	Stimulation glucose uptake 150 mg/L compound (vs insulin effect 1.04 ± 0.02)	PGE-2 production 5 mg/L compound (% of inhibition vs LPS stimulation 100.0 ± 1.2)	NO production 5 mg/L compound (% of inhibition vs LPS stimulation 100.0 ± 6.2)	Triacylglycerol secretion 25 mg/L compound (fold change vs control 1.01 ± 0.01)	Total cholesterol secretion 25 mg/L compound (fold change vs control 1.0 ± 0.02)	ApoB protein secretion 10 mg/L compound vs control (100.3 ± 0.3)
I (25 mg)	NA	NA	NA	1.07 ± 0.07	0.96 ± 0.06	NA
II (66 mg)	−0.04 ± 0.01	NA	NA	1.01 ± 0.12	1.03 ± 0.10	80.0 ± 12.8
III (6 mg)	−0.05 ± 0.01	NA	NA	0.95 ± 0.09	0.87 ± 0.08	*81.0 ± 3.9*
IV (4.5 mg)	−0.04 ± 0.02	NA	NA	0.85 ± 0.07	0.82 ± 0.05	NA
V (11.5 mg)	−0.09 ± 0.09	*51.91 ± 17.74*	*51.24 ± 14.63*	0.96 ± 0.06	1.03 ± 0.12	*78.5 ± 1.4*
VI (26 mg)	−0.02 ± 0.09	NA	*52.56 ± 9.67*	0.83 ± 0.05	0.97 ± 0.11	79.3 ± 9.1
VII (18 mg)	0.11 ± 0.03	*63.35 ± 14.12*	*23.03 ± 9.05*	*0.43 ± 0.08*	0.71 ± 0.18	NA
VIII (33 mg)	*0.40 ± 0.03*	NA	*44.60 ± 8.39*	*0.36 ± 0.03*	*0.59 ± 0.05*	*46.0 ± 4.9*
IX (23 mg)	*0.49 ± 0.02*	NA	*20.52 ± 5.85*	*0.32 ± 0.01*	*0.58 ± 0.06*	NA
X (16 mg)	*0.35 ± 0.04*	*63.34 ± 11.59*	*23.22 ± 5.76*	*0.41 ± 0.05*	*0.77 ± 0.01*	NA
XI (15 mg)	*0.40 ± 0.04*	NA	48.73 ± 0.77	*0.36 ± 0.03*	*0.81 ± 0.03*	NA
XII (75.4 mg)	NA	NA	*22.48 ± 5.67*	*0.45 ± 0.00*	*0.72 ± 0.03*	NA
XIII (81.6 mg)	NA	*53.85 ± 10.57*	*22.58 ± 3.96*	*0.34 ± 0.01*	*0.68 ± 0.06*	NA
XIV (70.5 mg)	NA	NA	49.44 ± 1.90	*0.45 ± 0.03*	0.77 ± 0.06	NA
XV (33.5 mg)	NA	NA	*26.58 ± 4.13*	0.78 ± 0.09	0.98 ± 0.07	NA

HepG2 cells (ATCC code HB-8065, Manassas, VA, USA) were propagated in DMEM and cultured as previously described (Puiggròs et al., 2005). The only modification was the addition of 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (HEPES) (SIGMA, Madrid, Spain) to the culture media. For the experiments, HepG2 cells were seeded at 750,000 cell/well in 12-well-plates and left to grow for two days (80% confluence) in a propagation medium. The medium was replaced 16 h before treatment. Then, lipid synthesis was measured. Procyanidins and ^{14}C-acetate (0.6 μCi/mL) were added to the cell media and 6 h after treatment the media and cells were collected and the lipids were extracted using 3 volumes and 0.5 ml of hexane/isopropanol (3:2, v/v) respectively. Thin layer chromatography was performed with petroleum either: diethyl ether: NH_3 (40:10:0.1) and an additional separation using a hexane/methyl tert-butyl ether (MTBE)/NH_4OH (30:20:01, v/v/v) solvent to obtain the free cholesterol, cholesterol esther and triacylglycerol (TAG) fractions. Each fraction was scraped and determined by scintillation counting. Values were corrected per milligram of protein, determined using the Bradford methodology (Bradford, 1976). The medium was collected after 24 h treatment and apolipoprotein B was detected as described in del Bas et al. (2008).

Murine macrophage cell line RAW 264.7 (European Tissue Culture Collection, ECACC, Ref. 91062702, London, UK) was cultured as previously described (Terra et al., 2007) and used for experiments between passages 5 and 14. At 80% of confluence, adherent monocyte-RAW 264.7 cells were incubated with different compounds and with 1 μg/mL LPS simultaneously for 19 h. The culture medium for control and treated cells was collected and tested for nitric oxide (NO) and prostaglandin E_2 (PGE_2) production. The nitrite concentration in the culture medium was measured as an indicator of NO production according to the Griess reaction (Terra et al., 2007). The level of PGE_2 released into culture medium was quantified and normalised as previously described (Terra et al., 2007).

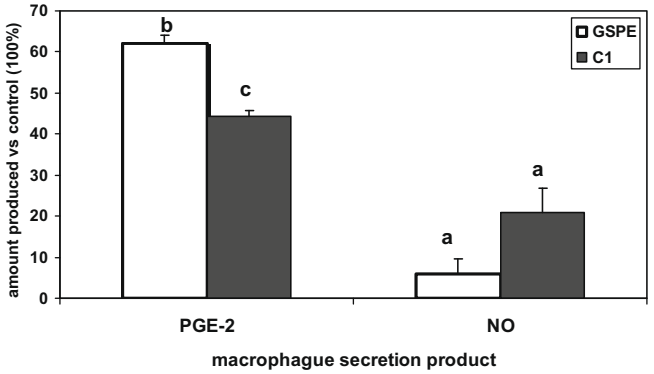

Fig. 1. Inhibition of PGE_2 and NO production by C1 in LPS induced RAW 264.7 macrophages. RAW 264.7 macrophages were simultaneously stimulated with LPS for 19 h and incubated with C1 (trimeric procyanidin) at 5 mg/L and GSPE (grape seed procyanidin extract) at 45 mg/L as reference. PGE_2 and NO production were measured after treatment. Results were normalised to control levels (100.0% ± 6.2). Each bar represents mean ± SEM of nine biological experiments. Different letters mean $p < 0.05$ as compared to GSPE treatment.

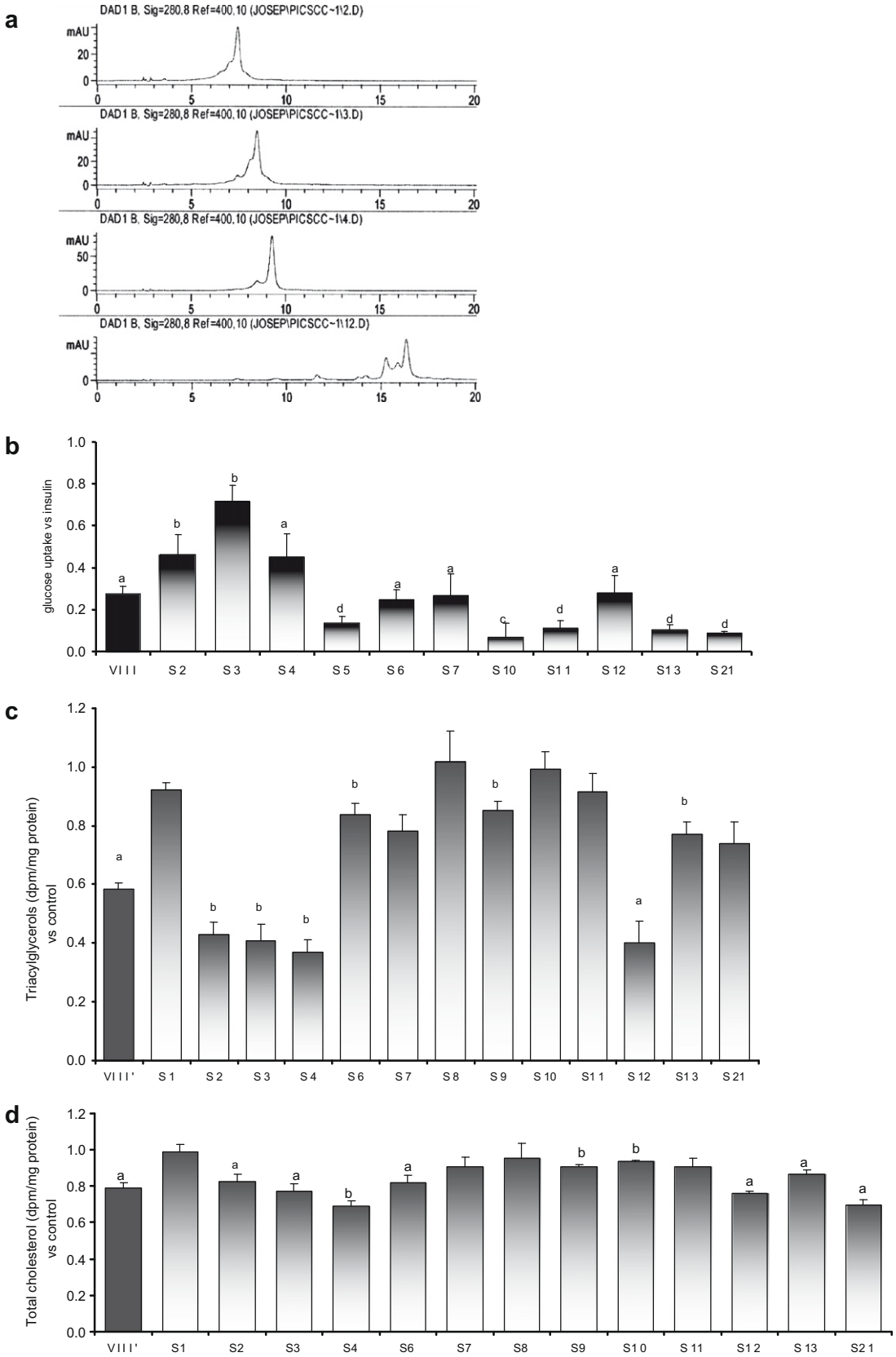

Fig. 2. Results from the second fractionation step. (a) Chromatograms of the most active subfractions. In correlative order: S2, S3, S4 and S12. (b) Stimulation of glucose uptake done by each of the subfractions obtained. After 2 h of depletion, differentiated 3T3-L1 adipocytes were treated for 30 min with 100 mg/L of total fraction VIII or each subfraction. Afterwards, glucose uptake was measured. Results are related to the maximum stimulation achieved by insulin (1.037 ± 0.023). (c and d) Effect of fraction VIII and its subfractions in *de novo* synthesised triacylglycerols (c) and total cholesterol (d) secretion to the extracellular media. HepG2 cultures were incubated for 6 h with fraction VIII or its subfractions at 25 mg/L and ^{14}C-acetate. Afterwards, lipid fractions secreted to the cell culture media were quantified. Media lipid fraction levels (dpm/mg protein) were normalised to the control levels set at one. Each bar represents mean ± SEM. Letters above the bars mean $p < 0.05$ as compared to control. Different letters mean $p < 0.05$ as compared to fraction VIII treatment.

2.4. Calculations and statistical analysis

Results are expressed as the mean ± SEM. Effects were assessed using Student's *t*-test. All calculations were performed using SPSS software.

3. Results and discussion

3.1. Monomeric and dimeric components of grape seed-derived extract act mainly as anti-inflammatory agents

The main objective of this work was to identify the molecule(s) responsible for the bioactivity of a grape seed procyanidin extract that act in vivo as (a) an antihyperglycemic (Pinent et al., 2004), (b) an antiatherogenic (del Bas et al., 2005, 2008) and (c) an anti-inflammatory (Terra et al., 2008) product. To meet this objective, three cell lines in which GSPE has been identified as being highly active, namely adipocytes (Pinent et al., 2005; Pinent, Bladé, Salvadó, Arola, & Ardévol, 2005a, 2005b), hepatocytes (del Bas et al., 2005; Puiggròs et al., 2005), and macrophage cells (Terra et al., 2007) were used. The effects that pure compounds have on these cells and the two fractionation steps have on the whole extract were evaluated.

The monomeric components of the extract have clearly been proven to reach body fluids and some of them have been modified in the body (Manach, Williamson, Morand, Scalbert, & Remesy, 2005). Thus, we hypothesise that these molecules or their modified forms are the main cause of the effects described. However, as Table 1 summarises, none of these pure monomeric compounds stimulated glucose uptake in the adipocytes, and HepG2 hepatocytes had no effect on TAG or total cholesterol secreted to the cell culture media while 25 mg/L GSPE inhibited TAG and TC secretion 45% and 25% respectively (results not shown). Neither did pure dimeric compounds act on the functions tested (Table 2). Both results agree with those found in the first fractionation step (Terra et al., 2007). Table 3 indicates that the fractions enriched with monomeric and dimeric compounds did not seem to affect adipocytes or hepatocytes. Monomeric components were previously reported not to induce lipolysis in adipocytes (Ardévol et al., 2000) or to protect against oxidative stress in Fao hepatocytes (Roig, Cascón, Arola, Bladé, & Salvadó, 2002). In both situations the total extract was highly effective. Only fractions III and V showed a slight but significant decrease in the amount of ApoB secreted to the cell culture media of HepG2 cells. Both fractions, together with fraction IV, share a peak at the end of the chromatogram that could be epicatechin gallate. In this respect, Yee et al. (2002) also found that, unlike EC, EGCG is a potent inhibitor of ApoB secretion, suggesting that the gallate moiety has a beneficial effect on the catechin molecule and that this is beneficial for lipid metabolism in terms of ApoB secretion.

Also, a common trait in the different approaches was that almost all monomeric (Table 1) and dimeric (Table 2) structures showed anti-inflammatory properties equal to the total grape seed procyanidin extract (Fig. 1) and in some cases had a stronger effect (i.e., EGCG limited NO production). These results agree with previously published works showing the anti-inflammatory effect of procyanidin B2 (Chen, Cai, Kwik-Uribe, Zeng, & Zhu, 2006; Zhang et al., 2006).

3.2. Oligomeric fractions of the extract justify its complete bioactivity

Park, Rimbach, Saliou, Valacchi, and Packer (2000) have shown that trimeric procyanidin C2 do not act as an anti-inflammatory compound. The trimer C1 has now been examined and it has been shown not only to have a considerable anti-inflammatory effect (Fig. 1) but also to be active on hepatocytes and adipocytes (Table 2). C1 reproduces most of the bioactive effects of the total extract. We evaluated it as a pure compound at the same concentration of the total extract and found a much lower effect (GSPE inhibited 45% TAG secretion in HepG2). However, this is the first time a C1 procyanidin has been described as having a bioactivity that is different from its antioxidant activity, which was previously shown to be higher than the antioxidant activity of other smaller oligomeric structures (da Silva Porto, Laranjinha, & de Freitas, 2003).

Examination of the trimeric-enriched fractions in the extract examined here showed that fractions VIII–XIII positively activate all the biological functions (see Table 3). Fraction VIII almost completely lacks monomers and dimers, but has the greatest bioactivity in the extract tested. Another fractionation step was carried out on fraction VIII to reach the objective. Fig. 2a shows a chromatogram of those subfractions whose bioactivity was closer to the total of fraction VIII (S2, S3, S4 and S12) for both cell lines. Fig. 2b–d summarises all the results obtained with these subfractions. Several bioactive subfractions (S2, S3, S4, S6, S12 and S13) can be found effective for both cell lines. However, it should be taken into account that the bioactivity of each one of these subfractions was evaluated at the same concentration as the total initial extract in each cell line. This facilitates the comparison of the effects between subfractions, but also distorts the truth because the amount of each subfraction obtained was in the order of S13 (3.6 mg) > S12 > S11 > S10 > S7 > S6 > S8 > S4 > S5–S9 > S2 > S3 (0.5 mg). S12 was the biggest subfraction and it had the greatest effect, so it was selected for further analysis. Two complementary analyses were carried out: the fraction VIII was analysed with HPLC-ESI-MS and the isolated S12 subfraction with MALDI-TOF analysis. The results showed that S12 has two molecular weight components: a dimer-gallate (729) and a trimer (865), the two peaks found in the HPLC chromatogram (Fig. 2a). The trimer component has been assigned to C1 procyanidin by comparing retention times and because it is the most abundant form in grape seed (de Pascual-Teresa, Santos-Buelga, & Rivas-Gonzalo, 2000). However, the dimer-gallate is probably not the B2-gallate, which was identified at S13, a fraction that did not share a single peak with S12 and which was barely active on the three assayed functions. Similarly, Schäfer and Högger (2007) have demonstrated that the inhibitory action of Pycnogenol© on α-glucosidase, in vitro, was stronger in extract fractions with higher procyanidin oligomers. Working with apple procyanidins, Sugiyama et al. (2007) also described an inhibitory effect on pancreatic lipase activity which depended on size. Mao, Van De Water, Keen, Schmitz, and Gershwin (2003) found that the effects on cytokine expression in peripheral blood mononuclear cells depended on the cocoa procyanidin fraction evaluated. Faria et al. (2006) showed that the simpler procyanidin structures, including catechin, have a higher antioxidant activity, which correlates with their antiproliferative effect on the cell lines of breast cancer. Agarwal et al. (2007) identified B2-3,3′-di-*O*-gallate as a major active constituent against growth inhibition and apoptosis, relaying most of its power to its galloyl group because B2 was barely active. Similarly, Lizarraga et al. (2007) observed that the fractions that were most efficient at inhibiting cell proliferation, arresting the cell cycle in the G(2) phase and inducing apoptosis, were the grape fractions with the highest percentage of galloylation and mean degree of polymerisation.

All these results together will be very helpful for understanding some of the differential and/or sometimes contradictory effects described for complete extracts of natural origin. Simple procyanidin structures have higher antioxidant properties. Short structures with higher galloylation seem to be more active as antiproliferatives. At least one trimer and one dimer-gallate are needed to have metabolic effects.

4. Conclusions

Monomeric structures and dimers (mainly fraction VI) of grape seed extract were the only effective as anti-inflammatory agents. Procyanidin C1 was also very active as an anti-inflammatory compound. Subfraction 12 was the most effective in all the parameters examined. As HPLC-ESI-MS and MALDI-TOF analysis showed, this fraction contains a trimer (865) and a dimer-gallate (729). Therefore, a mix of both molecules reproduces the bioactivity in glucose metabolism, lipid metabolism and macrophage functionality which has previously been described for the total grape seed extract.

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VI. GENERAL DISCUSSION

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Evidence has accumulated indicating that obesity is associated with a state of chronic, low-grade inflammation, suggesting that inflammation may be a potential mechanism whereby obesity leads to insulin resistance [1, 2]. The triggers and mechanisms leading to inflammation may vary between clinical conditions but they share many common mediators, including specific patterns of eicosanoid and cytokine production [3].

The study of bioactive food components that provide disease-preventing properties is becoming more important. In this context, we must continue to study food in our daily intake that is rich in beneficial and protective elements [4]. The Mediterranean diet is rich in flavonoids and the most abundant of these flavonoids are procyanidins. The protective action of procyanidins could extend to metabolic anomalies that collectively are termed metabolic syndrome [5]. Experimental evidence in our group demonstrated that procyanidins modulate the hepatic lipoprotein metabolism [6, 7], have an anti-hyperglycemic effect *in vivo* [8] and that can reduce the production and effect of free radical by enhancing the activity of anti-oxidant enzymes [9, 10]. Certain grape seed procyanidns have been found in model systems, to possess potential anti-inflammatory activity relevant to cardiovascular health [4, 5, 11-13]. All together, the above evidence supports the fact that procyanidins are able to act on several key points in the development of metabolic syndrome. Despite an increasing number of publications concerning the free radical-scavenging and hydrogen-donating activities of procyanidins [14, 15], little is known about their biological activity as anti-inflammatories in terms of cell signaling and gene expression.

In this context, the objectives of this thesis were on one hand to study the possible anti-inflammatory effect of procyanidins and its molecular mechanisms on *in vitro* models of inflammation. Secondly, we wanted to gain more insight into the procyanidin effect on *in vivo* models in a low grade inflammatory state derived from high-fat induced obesity.

Molecular mechanisms of *in vitro* anti-inflammatory effects

In our first study we demonstrated that procyanidins exert potent anti-inflammatory actions in an endotoxin induced model of *in vitro* inflammation. Our results showed that procyanidins reduce NO and PGE-2 production. PE caused a dose-dependent inhibition of iNOS at both mRNA and protein levels with the attendant release of NO. This inhibition was related to a decrease in NF-kB activation. Interestingly, the effects on NF-kB activation persist beyond the period during which cells are directly exposed to procyanidins.

Although the precise intracellular mechanism by which procyanidins modulate the inflammatory response remains to be clarified, it has been reported that procyanidins might directly interact with early events in the NF-kB and MAPK signaling pathways. Many studies have demonstrated that LPS activates both pathways and it has been reported that procyanidins may partially abrogate LPS interaction with its receptor, CD14/TLR-4/MD2, thus truncating the initiation of the LPS pro-inflammatory signal [16].

Reactive oxygen and nitrogen species control the action of several signalling pathways, including mitogen-activated protein (MAP) kinases, and triggers NF-kB activation signals [17, 18]. Thus, a constitutive high oxidant production could be a contributing factor to MAPK and NF-kB activation. Although the underlying mechanisms were not characterized, the binding of several cytokines to their receptors is associated with increased oxidant production [19]. Our results showed that PE was able to reduce the LPS-induced intracellular ROS production. The antioxidant action of procyanidins could thus be partly involved in the inhibition of these pro-inflammatory pathways [20, 21]. In this sense, our results show that PE is able to inhibit the LPS induced phosphorylation of extracellular signal-regulated kinases (ERK)1/2 from the MAPK family. Furthermore, accumulating evidence indicates that there is a crosstalk between ERK and NF-kB pathways, where ERK seams to activate NF-KB. This crosstalk might explain how procyanidins could inhibit both pathways.

Furthermore, we are currently investigating the interactions between IKK and procyanidins. Our preliminary results show that some pure procyanidins inhibit the activity of IKKB.

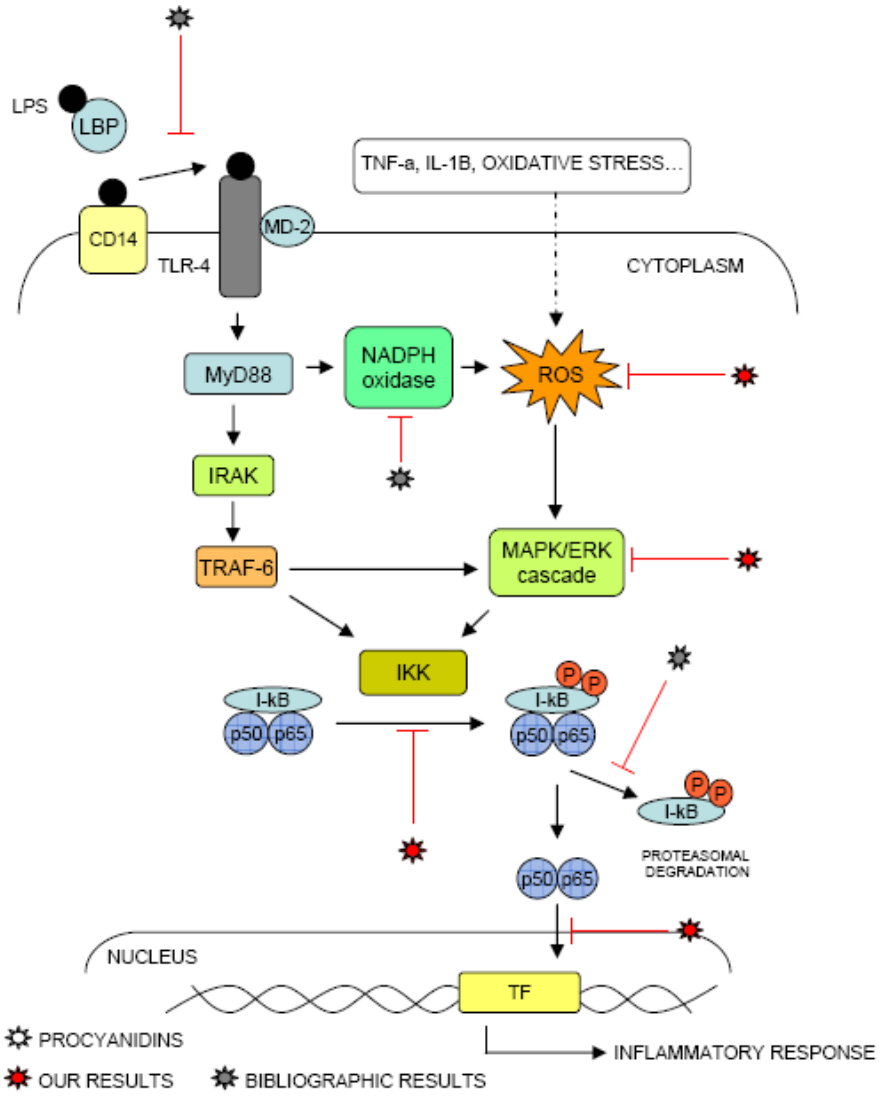

Figure 16: Procyanidin proposed model for its mechanism of action.

The NF-kB pathway has several points of regulation between the activation of IKK and the expression of its target genes, which are potential sites for procyanidin actions. Some authors have reported that the action of resveratrol could be mediated by an inhibition of the nuclear factor-kB (NF-kB) activation, mainly because the degradation of kB inhibitor (IkBα) is inhibited [22]. Our results showed that procyanidins reduced NF-kB translocation to the nucleus, which implies a reduction in the activation of this pro-

inflammatory pathway. Procyanidin B2 has been shown to selectively inhibit NF-kB in the nucleus by preventing the active NF-kB from binding to the DNA kB sites [20]. Furthermore, our results showed that PE inhibits iNOS and iKB mRNA expression. These are NF-kB early target genes whose inhibition also indicates a down-regulation of this pro-inflammatory pathway driven by PE. All together, the above evidence suggests that procyanidins are able to inhibit both early and late events in the NF-kB activation pathway.

In our third work we tried to gain insight into the molecular mechanism that underlies PE anti-inflammatory effects, with other *in vitro* model of inflammation. We used the foam cell model to study the anti-atherogenic effects ascribed to procyanidins. This study revealed that lipid accumulation in foam cells was increased by lipoprotein treatment but reduced by PE incubation. We have demonstrated that endotoxin induced models showed the highest lipid accumulation and peroxidation levels, probably because of the enhanced inflammatory response. However, the most important gene induction of CD36 and ABCA1 came from the moxLDL model. It has been suggested that there is a link between lipid metabolism and inflammatory pathways driven through the crosstalk between PPARs and NF-kB [23]. CD36 and ABCA1 expression are mostly regulated by PPARα and PPARγ nuclear receptors, which have been described as anti-inflammatory factors because they inhibit the NF-kB activation pathway. In this work we show that the lowest levels of iNOS and IkBα correspond to the highest induction of CD36 and ABCA1 in the moxLDL model, which may indicate a reduction in NF-kB activity caused by PE and the subsequent activation of PPARs. Some reports support such hypothesis and show that procyanidins activate PPARs in human endothelial cells [12].

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Analysis of pure compounds and oligomeric fractions that exert anti-inflammatory effects

The composition of GSPE has been investigated extensively and is known to consist largely of gallic acid, catechin, epicatechin and procyanidin dimers and trimers (Figure 14) composed of flavan-3-ol units with C4-C8 or C4-C6 interflavan linkages [24]. Once we determined that the procyanidin extract was an effective anti-inflammatory *in vitro*, we aimed to identify the structure(s) responsible for the effects described.

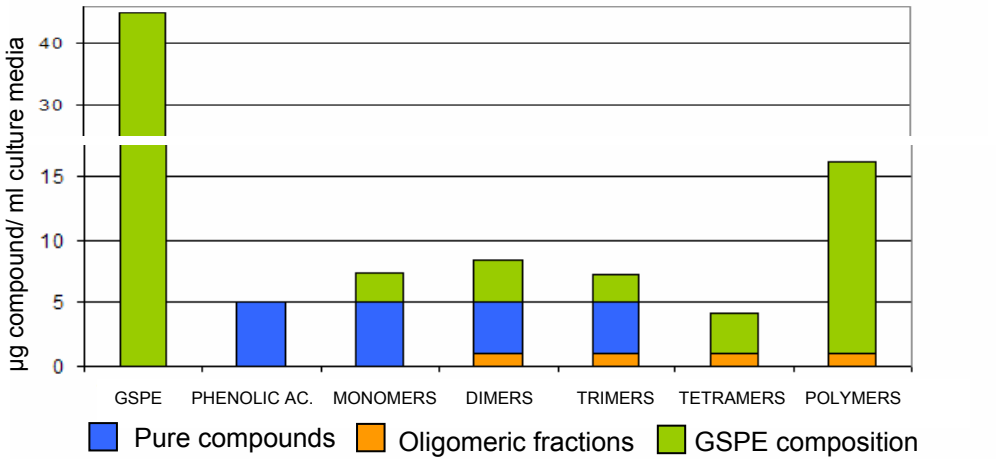

Figure 17: Comparison between grape-seed procyanidin extract (GSPE) composition and, the pure compounds and oligomeric fractions used in our experiments.

Our results showed that there is a relation between the effectiveness and structure of pure procyanidins. After studying the structure-activity relationship using four kinds of procyanidin monomers, we showed that the galloyl moiety of procyanidins appeared to be important to their inhibitory actions. The production of NO was inhibited by the epicatechin monomer, which has no galloyl moiety; however this monomer's effects increased with the galloyl moiety of the molecule, EGCG with two galloyl moieties showed the strongest inhibition (epicatechin<epicatechin gallate<epigallocatechin gallate). Recent studies revealed that the presence of galloyl moiety in catechins led to them having a high affinity for lipid bilayers. Galloylated catechins showed higher phospholipid/water partition coefficients, and were immersed in the phospholipid

palisade intercalating within the hydrocarbon chains. In contrast, non-galloylated catechins presented a shallow location close to the phospholipid/water interface [25]. Moreover, the interaction between epigallocatechin gallate and lipid bilayers was directly demonstrated by solid-state nuclear magnetic resonance [26]. Thus, polyphenols with galloyl moiety can effectively affect cells because of the formation of membrane structures. In keeping with this hypothesis, our results showed that the PE inhibited NF-kB activation even when it was removed from the media before LPS-stimulation, indicating that a part of the procyanidins remained bound to the membrane or were internalized by the cells.

In addition, the present findings suggest that the degree of polymerization of the procyanidins seems to be important in determining the mechanism(s) by which procyanidins may exert their immunomodulatory and anti-inflammatory activity. Our results showed that dimeric procyanidins were less effective than the trimeric fraction and the pure trimer C1. Interestingly, our results showed that GSPE reduces intracellular ROS production induced by LPS and H_2O_2 in human macrophages. We also demonstrate for the first time the same effects on LPS-induced ROS production with some pure oligomeric procyanidins, being the trimer C1 and the dimer B1 the most effective ones, followed by EGCG. Comparing the effect of pure molecules we showed that the antioxidant activity augmented again with the increase of the galloyl moiety, from epicatechin to EGCG. These results are consistent with the findings of Plumb et al. [27], who studied the antioxidant properties of catechin, epicatechin as well as structure-related dimeric and trimeric flavonoids similar to those used in our studies.

The production of PGE-2 was also modulated by pure procyanidins. Most of the molecules we tested limited PGE-2 production to about 30-40% and it is difficult to establish a structure-activity relationship, although the trimer C1 was more active than the dimers.

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In vivo anti-inflammatory effects of procyanidins

Evidence linking obesity with inflammation dates back at least to the 1960s when population studies found that obesity increases the circulating concentration of fibrinogen and other acute phase factors [28, 29]. The circulating concentration of more than a dozen of pro-inflammatory cytokines is now known to be increased by obesity [30, 31]. In addition, the circulating concentration of at least one protein with strong anti-inflammatory properties, adiponectin, is inversely related to adiposity and insulin sensitivity [32, 33]. That inflammation seems to have a mechanistic role in the development of obesity-induced complications in humans is demonstrated by the presence in the circulation of certain pro-inflammatory factors (e.g., IL-6, adiponectin, CRP) which predict the development of obesity-associated complications in studies on humans [34], and by the fact that mice are protected from obesity-associated complications if they are genetically modified to reduce pro-inflammatory signaling molecules (e.g. TNF-α, inducible nitric oxide synthase; iNOS, inhibitor of kB kinase B; IKK) [35, 36]. Our *in vivo* experiments focused on investigating how procyanidin modulates obesity related to low grade inflammation. We wanted to evaluate the procyanidin effect for different times, and doses. Our experimental design included several treatment conditions to test for effectiveness in disease prevention or treatment. We analyzed two different diets as models of disturbed metabolic states. In the first diet, we analyzed the preventive effects of procyanidins on rats fed a 30% or 60% fat synthetic diet. In the second, we induced obesity by feeding the animals with a cafeteria diet which is a rapid inducer of obesity. We analyzed the corrective effects of two PE doses (10 and 20 mg/ day) and the effects of short and long treatments. These two models gave us a wider range when analyzing the duration of the treatment, and the preventive and curative effects of procyanidins.

To summarize, we found a reduced cytokine expression in liver, muscle and mesenteric adipose tissue, which is involved in decreasing local inflammation and

possibly also systemic inflammation [37, 38]. We have shown that procyanidins modulate tissue and plasma cytokine levels partially because there is a reduction in macrophage infiltration in adipose tissue and diminished NF-kB activity in the liver.

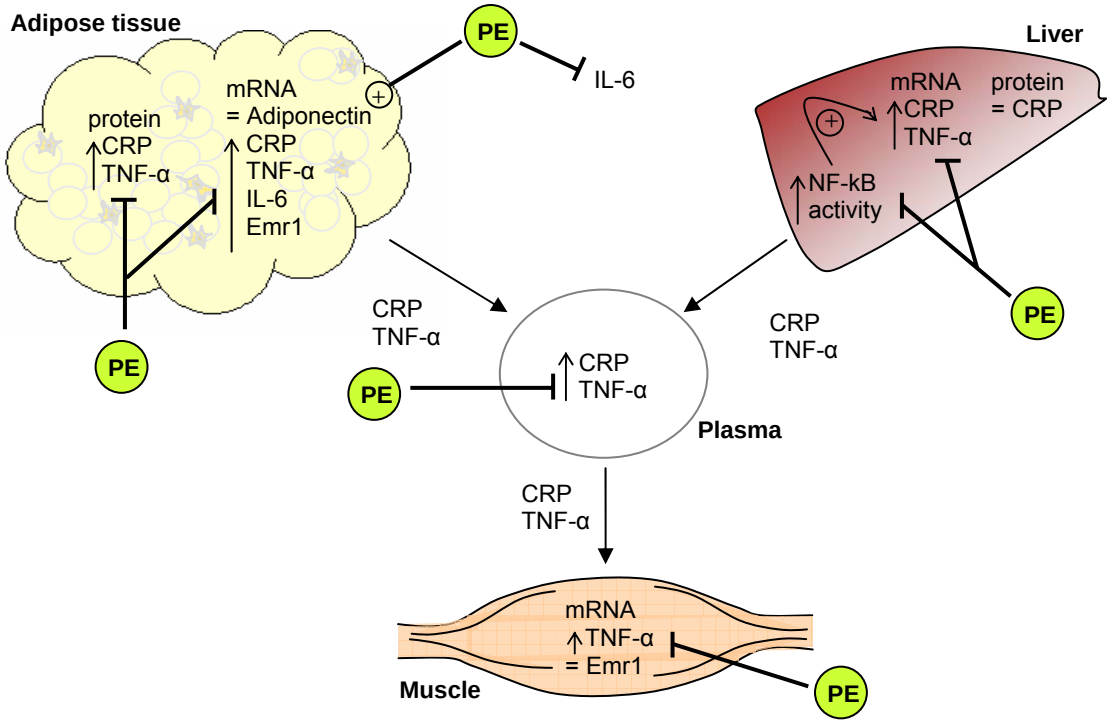

Figure 18: Schematic diagram of procyanidin effects in the preventive model rats.

Only relatively recently have obese individuals been described as having increased macrophage infiltration of adipose tissue, and it now appears that the infiltration rate of monocytes into visceral adipose tissue is higher than into subcutaneous adipose tissue [2]. Macrophages have been identified as the primary source of many of the circulating inflammatory molecules that are detected in the obese state [39]. Weisberg et al. [40] demonstrated that adipose tissue macrophages are responsible for almost all adipose tissue TNF-α expression and significant amounts of IL-6 expression in mice. Furthermore, most evidence supports the hypothesis that the amounts of TNF-α mRNA and TNF-α released by adipose tissue are enhanced in human obesity and that the vast majority of TNF-α releases by adipose tissue come from the non-fat cells in this tissue [41]. Because obesity is associated with macrophage accumulation in

adipose tissue [42, 43] we wished to examine the effects of procyanidins on macrophage infiltration by measuring the expression levels of Emr1, which is the gene that encodes a specific marker for mature macrophages (F4/80) in the mesenteric adipose tissue. We found that the presence of macrophage significantly increased and was much higher than that of the control rats and, interestingly, the procyanidins reduced this level. Therefore, the inhibition of the cytokine expression in this tissue might be due to a decrease in the number of macrophages, although procyanidins might also directly affect the pro-inflammatory pathways in the adipocyte and the macrophage.

What stimulates macrophage influx into fat is largely unknown and probably involves a complex pathophysiology that includes physical and chemical adipocyte injury and cytokine cross-talk with increased local expression of TNF-α, MCP-1 and I-CAMs [44]. We found that procyanidin treatment decreased NF-kB activity in liver, which is directly associated with the decreased hepatic expression of such inflammatory molecules as TNF-α and CRP, as we found in our study. We hypothesize that procyanidins also reduce MCP-1 secretion in adipocytes partially because of the diminished TNF-α levels that we have detected in adipose tissue, muscle and liver, which might be a consequence of the inhibitory effects of procyanidins on NF-kB activation [45, 46]. The local enhancement in adiponectin expression produced by PE might also be partly responsible for the reduced cytokine expression levels. On the other hand, TNF-α has been shown to induce the expression of adhesion molecules (I-CAM) and chemokines in human endothelial cells. One of the procyanidins' mechanisms might be to diminish I-CAM expression, which in turn would diminish the migration of macrophages [47]. Some authors have recently reported similar results in murine obesity models and *in vitro* experiments [43, 48, 49].

Although we demonstrated that procyanidins are effective at preventing the onset of inflammation, it is unclear whether procyanidins exert anti-inflammatory effects once

inflammation is established and whether they can promote the resolution of inflammation. In any case, our findings demonstrate the potential procyanidin effects on low-grade inflammation related diseases such as obesity.

The structure-activity relationship found *in vitro* raises the question of the bioavailability of procyanidins *in vivo*. The bioavailability of procyanidins is a matter of intense study [50]. It is widely accepted that monomeric forms are absorbed in humans and reach concentrations that range from 0.1 to 13 μM [51, 52]. Further polymerized procyanidins have been detected in their native form in rat plasma (dimers to tetramers) and reached concentrations of ~3 μg/ml for dimers and of ~7.5 μg/ml for each trimeric, and tetrameric form [53]. Scalbert et al. [54] reported that (+)-catechin, procyanidin dimer B3, and procyanidin trimer C2 passed through the human intestinal epithelial Caco-2 cell monolayer, whereas polymers with an average degree of polymerization of 7 did not. Lambert J. D. et al [55] reported that in mice the concentration of EGCG in the liver showed a linear relationship to the dose, reaching a maximum of 18.3 μg/g. By contrast, levels of EGCG in the small intestine and colon plateaued at high doses reaching a maximum concentration of 162.9 μg/g and 23.4 μg/g respectively. The discrepancies between results are probably due to the insufficient purity of the proanthocyanidins used in the assays, which in turn is caused by the complexity of their chemical structures, the difficulties involved in fractionating proanthocyanidins, the sensitivity and specificity of the measurements used to detect them in plasma and urine, and differences between the animals used in the assays. We carefully studied the dosage used for the experiments to better reflect the situation *in vivo* and because the absorption of procyanidins is highly dependent on the degree of polymerization.

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VII. CONCLUDING REMARKS

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The main conclusions obtained from this PhD Thesis are:

1. Procyanidins are anti-inflammatories in a macrophage-like model of *in vitro* LPS-induced inflammation.

Procyanidins inhibit ROS intracellular production and the production of PGE-2 and NO. The modulation of NO production is dose dependent, it also depends on the galloyl moiety of the molecule and on the degree of polymerization. These anti-inflammatory effects result from the modulation of ERK1/2 and NF-kB pro-inflammatory signalling pathways driven by procyanidins.

2. Procyanidins modulate foam cell formation *in vitro.*

GSPE reduces lipid accumulation in foam cells. One of the mechanisms that contribute to procyanidin actions is the reduction of CD36 and the induction of ABCA1 expression. Also GSPE is able to reduce the inflammatory response in some foam cell models by inhibiting iNOS and iKB expression.

3. GSPE act as anti-inflammatory agent *in vivo* preventing obesity related low grade inflammation

Procyanidns reduce both local and systemic inflammation by reducing CRP plasma levels and CRP hepatic and mesenteric adipose tissue expression. Interestingly, we found no changes of CRP expression in other visceral WATs. Procyanidins also modulate TNF-α, IL-6 and adiponectin expression in mesenteric adipose tissue, inhibiting pro-inflammatory cytokines and inducing the anti-inflammatory cytokine adiponectin. These effects are in part the result of the inhibition of NF-kB activity in liver and the reduction of macrophage infiltration in mesenteric adipose tissue driven by procyanidins.

In conclusion, the use of procyandins will represent an additional nutritional approach that, in association with lifestyle interventions, would improve the obesity induced inflammation and as a consequence ameliorate the metabolic syndrome.

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