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Peroxisome Proliferator-Activated Receptor-γ: its role and regulation in human monocytes and monocyte-derived macrophages

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INTRODUCTION

Peroxisome Proliferator-Activated Receptors (PPARs)

Peroxisome Proliferator-Activated Receptors (PPARs) are transducer proteins belonging to the nuclear receptor superfamily that also includes the retinoic acid receptors (RARs), the thyroid hormone receptors (TRs) and the steroid receptors [1,2].

PPARs were identified in the 1990s and the term Peroxisome Proliferator-Activated Receptor derives from early observations in rodents that certain industrial compounds could induce peroxisomes, subcellular organelles involved in fatty acid β-oxidation and detoxification steps, to increase in size and number. To date, three major types of PPAR, encoded by separate genes, have been identified: PPAR-α (NR1C1), PPAR-β/δ (NR1C2) and PPAR-γ (NR1C3).

All three PPAR isoforms present similar structural and functional features. Principally, four functional domains have been identified, called A/B, C, D and E/F (Fig. 1). The N- terminal A/B domain contains a ligand-independent activation function 1 (AF-1) [3] responsible for the phosphorylation of PPAR. The DNA binding domain (DBD) or C domain promotes the binding of PPAR to the peroxisome proliferator response element (PPRE) in the promoter region of target genes [4]. The D site is a docking domain for co-factors. The E/F domain or ligand-binding domain (LBD) is responsible for ligand specificity, allowing the heterodimerisation of PPARs with the retinoid X receptor (RXR); the resultant complex subsequently binds to PPRE with the recruitment of co-factors, increasing the expression of targeted genes [5,6]. Recruitment of PPAR co-factors to assist the gene transcription processes is carried out by the ligand-dependent activation function 2 (AF-2), which is located in the E/F domain [5].

Fig. 1. Schematic representation of the functional domains of PPARs. PPARs are composed of four distinct functional regions. The A/B domain located at N-terminal with AF-1 is responsible for phosphorylation, the domain C is implicated in DNA binding, domain D is the docking region for cofactors and domain E/F is the ligand specific domain, containing AF-2, which promotes the recruitment of cofactors required for the gene transcription. (*B.P. Kota et al. / Pharmacological Research 51 (2005) 85–94*).

In addition to the activation of PPARs by natural and synthetic ligands, other factors such as RXR, PPREs and co-factors play a pivotal role in achieving the desired transcription. The mechanisms by which activated PPARs initiate gene transcription are illustrated in Fig. 2.

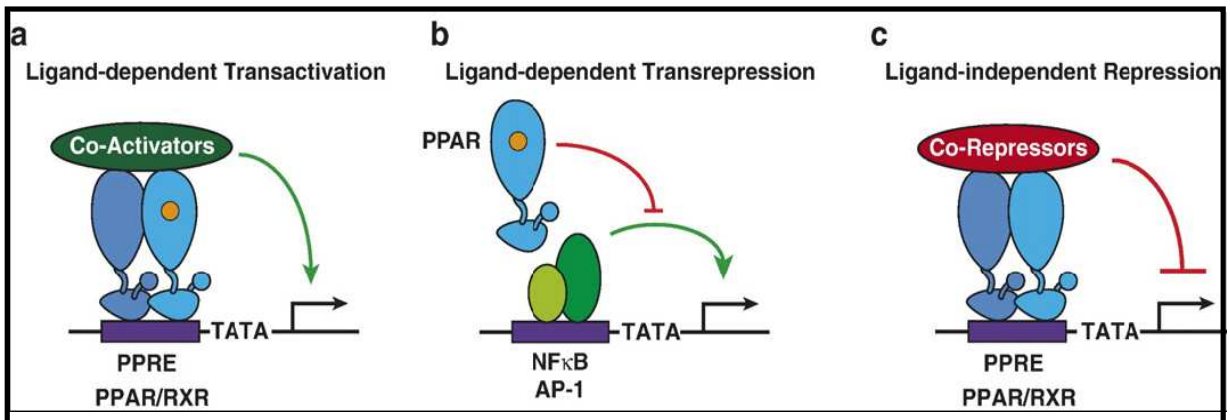


Fig. 2. Transcriptional activities of the peroxisome proliferator-activated receptors. PPARs can both activate and inhibit gene expression. (a)Ligand-dependent transactivation. PPARs activate transcription in a ligand-dependent manner by binding directly to specific PPAR-response elements (PPRE) in target genes as heterodimers with RXR. Binding of agonists ligand leads to the recruitment of coactivator complexes that modify chromatin structure and facilitate assembly of the general transcriptional machinery to the promoter. (b)Ligand-dependent transrepression. PPARs repress transcription in a ligand dependent manner by antagonizing the actions of other transcription factors, such as nuclear factor-κB (NF-κB) and activator protein-1 (AP-1). (c)Ligand independent repression. PPARs bind to response elements in the absence of ligand and recruit corepressor complexes that mediate active repression. This complex antagonizes the actions of coactivators and maintains genes in a repressed state in the absence of ligand. *(Ricote: BBA. 1771: 926–93. 2007)*

A major mechanism that underlies the capacity of PPARs to interfere with the activities of transcription factors, such as nuclear factor-κB (NF-κB), has been termed transrepression (fig2b). The process of transcription begins with the binding of ligands (endogenous or exogenous) to the PPAR-receptor. The heterodimer LBPPAR-RXR binds to the promoter region of PPRE, with the recruitment of co-activators or co-repressors (fig2a, 2c). Several proteins act as co-activators or co-repressors of the ability of nuclear receptors to initiate or suppress respectively the transcription process, interacting in a ligand-dependent manner [2]. In the absence of ligand, heterodimerised nuclear receptor associates with multicomponent co-repressors containing histone deacetylase activity, such as nuclear co-repressor receptor (NCoR) and the silencing mediator for retinoid and thyroid hormone receptor (SMRT) [7,8].

The deacetylated state of histone inhibits transcription [9]. Alternatively, coactivators such as steroid receptor co-activator (SRC)-1 and the PPAR binding protein (PBP) with histone acetylase activity [10,11] initiate a sequence of events which induces the gene transcription process upon ligand binding.

The PPAR-γ gene contains three promoters that yield three isoforms, namely, PPAR-γ1, PPAR-γ2 [12] and PPAR-γ3 [13]. PPAR-γ1 and -γ3 RNA transcripts translate into the identical PPAR-γ1 protein. PPAR expression is tissue-dependent. PPAR-γ1 is found in a broad range of tissues, whereas PPAR-γ2 is restricted to adipose tissue. PPAR-γ3 is abundant in macrophages, the large intestine and white adipose tissue [13-15].

Adipogenesis, glucose homeostasis and lipid metabolism are the major physiological functions modulated by PPAR-γ to improve insulin resistance [16,2].

Polyunsaturated fatty acids, such as linoleic acid, arachidonic acid and eicosapentaenoic acids, are endogenous ligands of PPAR-γ. Prostaglandins are autacoids synthesized from 20 carbon-containing polyunsaturated fatty acids, principally arachidonic acid (AA), derived from membrane phospholipids and from dietary sources [17]. Prostaglandin (PG)-related compounds such as 15-deoxy-Δ^{12-14}-PGJ_2 (15d-PGJ_2) were identified as potent PPAR-γ agonists and accumulating data suggest that 15d-PGJ_2 exerts anti-inflammatory effects [17].

Also oxidized lipids such as 9-hydroxy-10,12-octadecadienoic acid (9-HODE), 13-hydroxyoctadecadienoic acid (13-HODE) and 15-Hydroxyeicosatetraenoic acid (15-HETE) were found to be effective activators of PPAR-γ in primary human trophoblasts and monocytes [18].

Thiazolidinediones (TZDs) are the most notable synthetic drugs with PPAR-γ activation properties, through which they are able to improve insulin resistance and lower blood glucose levels in type 2 diabetes. Several TZDs (rosiglitazone, ciglitazone and pioglitazone) are PPAR-γ selective agonists and show reduced affinity towards PPAR-α or PPAR-β [19].

Novel PPAR-γ partial agonists and antagonists have been recently identified. Bisphenol diglycidyl ether (BADGE) and LG-100641 are recently recognized PPAR-γ antagonists [20,21]. Although these compounds have less clinical significance, they may be useful pharmacological tools in PPAR-γ physiology and in the identification of new ligands.

In addition to synthetic chemical methods, research in natural products has also yielded potent PPAR-γ agonists from several medicinal plants. Flavonoids, such as chrysin and phenolic compounds, have been recently identified as PPAR-γ agonists.

PPAR-α is the receptor for a structurally diverse class of compounds, including the hypolipidemic fibrates. In rodents and humans, PPAR-α is expressed in numerous tissues (including liver, kidney, heart, skeletal muscle and brown fat [22,23]) and is also present in

different cell types e.g., endothelial cells (EC) [24], vascular smooth muscle cells (VSMC) [25,26] and monocytes/macrophages [27].

The hypolipidemic effect of fibrates is well documented by the critical role of PPAR-α in the regulation of β-oxidation of fatty acid (FA) [28,29], in their ability to stimulate cellular uptake of FA by increasing the expression of the fatty acid transport protein (FATP) and fatty acid translocase (FAT) [30].

PPARs and cardiovascular disease

Each year cardiovascular diseases (CVD) cause over 4.3 million deaths (48%) in Europe and over 2.0 million deaths (42%) in the European Union (EU). In all Europe, CVD is the main cause of death in women in all countries and is the main cause of death in men except France, the Netherlands and Spain [31].

Development of atherosclerosis is a complex, ongoing process that occurs over several decades. The early stages of atherosclerosis may result from an insult to the endothelium (e.g., oxidized lipoproteins, cytokines) and/or from decreased levels of protective factors (e.g., nitric oxide, prostacyclin). These phenomena cause other functional changes, such as expression of adhesion molecules, reduction of barrier function and uptake of lipoproteins into the sub-endothelial matrix. One of the earliest events involved in the development of the atherosclerotic plaque is likely to be the adhesion of monocytes and lymphocytes to the "activated" endothelium and the migration of such cells into the sub-endothelial region (Figure 3), where they become important modulators of the atherogenic process. Monocytes differentiate into macrophages, cells capable of taking up modified lipoproteins, such as oxidized low-density lipoproteins (LDL). These changes in LDL result from entrapment of native LDL from the plasma compartment, and subsequent modification within the vessel wall.

In this way macrophages become engorged with lipid, especially cholesteryl esters, differentiating into foam cells (Figure 3).With time, some of these foam cells will die releasing cholesteryl esters, free cholesterol crystals, and lipoprotein-derived lipids and proteins. Under these conditions, macrophages also express large amounts of the pro-coagulant Tissue Factor (TF). Adhesion of platelets to the luminal side of the lesion results in the secretion of agents (such as Platelet-Derived Growth Factor or Thrombin), that stimulate smooth muscle cell (SMC) proliferation and migration. The ongoing recruitment of monocytes and lipoproteins into the plaque results in a gradual increase in lesion size. This can subsequently evolves in plaque rupture and/or acute occlusion, resulting in myocardial infarction and stroke.

During the last years important progresses have been made in the understanding of the control of macrophage functions by PPARs.

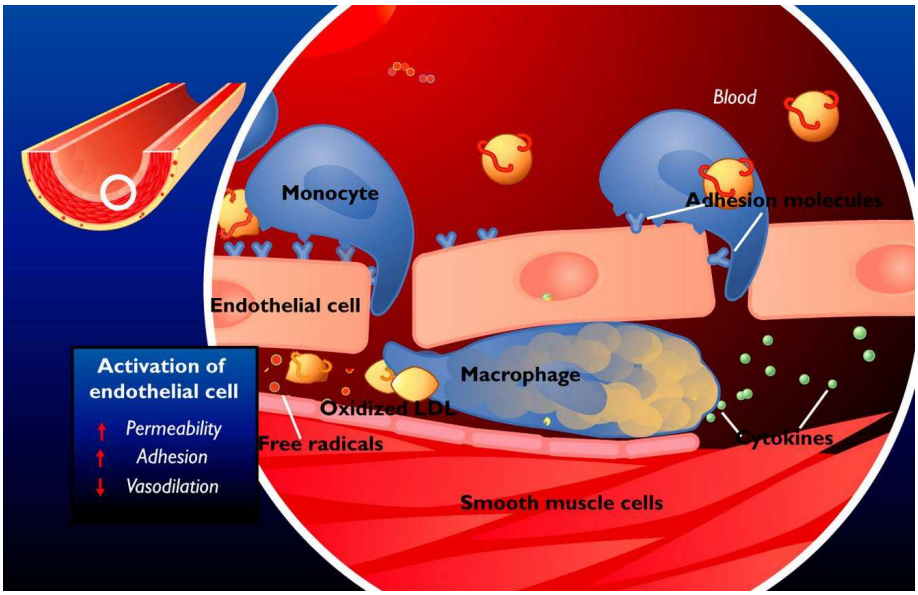


Fig. 3: early stages of plaque formation
(modified image from CD-Rom *Dyslipidemia and atherosclerosis* of Jean-Charles Fruchart)

Presently, a growing body of evidence from *in vitro* and *in vivo* studies in animals and, more importantly, in humans, indicates that PPAR agonists have beneficial effects in the modulation of macrophage lipid metabolism and inflammatory status, which may impact on atherosclerosis development.

Clinical trials using fibrates (fenofibrate, bezafibrate or gemfibrozil) and TZDs (rosiglitazone or pioglitazone) also provide indications regarding the clinical efficacy of PPAR agonists in the control of lipid and glucose metabolism and inflammation.

Fenofibrate administration lowers the plasma levels of inflammatory biomarkers, such as IL-6, fibrinogen and C-reactive proteins (CRP) in patients with established atherosclerosis [32,33] and significantly reduces plasma levels of interferon-γ (IFN-γ), Tumor Necrosis Factor-α (TNF–α), intercellular adhesion molecule-1 (ICAM-1), monocyte chemotactic protein-1 (MCP-1), α2-macroglobulin and plasminogen in patients with hyperlipoproteinaemia [33,34].

On the other side, TZDs modulate the expression of cardiovascular biomarkers. In fact, in type 2 diabetes patients, rosiglitazone administration rapidly reduces the levels of inflammatory biomarkers, such as CRP, matrix metalloproteinase-9 (MMP-9), MCP-1 or TNF-α [35]. In line with this hypothesis, results from a study with rosiglitazone in non-diabetic patients with symptomatic carotid artery stenosis showed reduced CRP and serum amyloid A (SAA) levels associated with a reduction of the expression of MMP-3, MMP-8 and MMP-9 in the plaque [36].

Thus, short-term rosiglitazone treatment significantly reduces vascular inflammation in non-diabetic subjects, leading to a more stable type of atherosclerotic lesion.

The influence of fibrates on cardiovascular morbidity and mortality was investigated in various cardiovascular prevention studies: Helsinki Heart Study [37], Fenofibrate Intervention and Event Lowering in Diabetes (FIELD) [38], Bezafibrate Infarction Prevention [39], and Veterans Affairs High-density Lipoprotein Cholesterol Intervention trial [40]. The results from these trials indicate that fibrate therapy reduces coronary heart disease (CHD) and is most effective in overweight individuals with insulin resistance and chronic inflammation.

Fibrates are generally considered as safe drugs with only few side effects [38]. However, a moderate and reversible increase in plasma creatinine and homocysteine levels in humans is a common side effect [41], whereas rabdomyolysis, a severe and life-dangerous undesired effect, is rare. Nevertheless, novel generation of highly active PPAR-α agonists should also be monitored for myopathy induction [41].

TZDs administration is associated with a number of adverse effects that have been categorized as either unique to individual glitazones or common to the class. For instance, hepatotoxicity is a side effect specifically associated with troglitazone treatment (for this reason, troglitazone has been withdrawn in many countries since 2000) [42].

Recently, three independent studies reported results from a meta-analysis suggesting that rosiglitazone administration may be associated with an increase of risk of Myocardial infarction (MI) [43, 44]. These studies raise questions on the cardiovascular safety of rosiglitazone in the treatment of type 2 diabetes. However, the increase in absolute cardiovascular risk after rosiglitazone treatment was very small in these studies on low-risk patients, such as DREAM and ADOPT [45,46]. Intermediary safety analysis of a trial assessing the cardiovascular effects of rosiglitazone combined with metformin or sulfonylurea, the Rosiglitazone Evaluated for Cardiac Outcomes and Regulation of Glycemia in Diabetes (RECORD) study, reported non-significant changes in cardiovascular morbidity and mortality [47]. These reports should be interpreted with caution and only the final outcome from the RECORD study will provide evidences on the long-term cardiovascular effects of rosiglitazone in patients with type 2 diabetes. Meantime, it remains puzzling why rosiglitazone, in contrast to pioglitazone, does not decrease the risk of CVD. Indeed, results from a meta-analysis on the risk of cardiovascular events after treatment with pioglitazone indicated that pioglitazone lowers the risk of death, MI or stroke in patients with diabetes, whereas, as expected, the risk of heart failure increases [48].

Despite the growing number of reports in the literature identifying sex-related differences in cardiac function in both rodents and humans, the underlying mechanisms have yet to be determined.

Variables of experimental studies, such as diet, animal models, and age, in addition to sex hormones and other factors, may play a role in sex-related variations in cardiac responses.

There are numerous health problems affected by gender. Women are more susceptible than men to depression, osteoporosis, asthma, lung cancer due to cigarette-smoking, and autoimmune disease [49]. Gender effects in disease are complex [50]. As an example, the lethality of acute MI is higher in women than men, moreover, interaction between diabetes and coronary artery disease (CAD) is stronger in women than in men [51]. Little is known about the basis for these differences in cardiovascular disease. Much focus has been placed on the potential cardio-protective role of estrogens; however, the finding that estrogens replacement therapy in postmenopausal women actually increased heart disease has changed this view [52].

Sex is an extremely potent modifier of the myocardium and it will be very interesting to identify which pathways have been implicated in some of these differences. Although the role of estrogens in providing cardio-protection is no longer so clear, this is not to say that sex hormones do not have an effect on the cardiovascular system.

Monocytes and Monocyte-Derived Macrophages

Monocytes originate in the bone marrow from a common myeloid progenitor and are then released into the peripheral blood, where they circulate for several days before entering tissues and replenishing the tissue macrophage populations [53]. Mature monocytes constitute about 5–10% of peripheral-blood leukocytes in humans and represent a heterogeneous population. In fact, they vary in size and have different degrees of granularity and varied nuclear morphology [54].

As long ago as 1939, Ebert and Florey [55] observed that monocytes migrate from blood vessels and develop into macrophages in the tissues. Pro-inflammatory, metabolic and immune stimuli all elicit increased recruitment of monocytes to peripheral sites [56], where differentiation into macrophages occurs, contributing to host defence, tissue remodelling and repair. Monocytes are identified by their expression of large amounts of co-receptor CD-14 (which is part of the receptor for lipopolysaccharide).

The leading conditions to the transmigration of circulating monocytes into the neointimal sub-endothelial space are the inflammatory state of endothelial cells (EC) and the presence of Ox-LDL in the injured vessel [10]. Adhesion molecules and chemoattractant factors released by EC promote monocyte recruitment. Thus, in the presence of Ox-LDL, EC express at their surface selectins, like P-selectin and E-selectin, which promote the adhesion and the 'rolling' of monocytes along the endothelium [11]. Further, the presence of cytokines stimulates EC to produce molecules like ICAM-1 and vascular cell-adhesion molecule-1 (VCAM-1) [12]. EC also produce specific chemoattractant proteins, such as MCP-1 that recognizes and binds to the chemokine (C-C motif) receptor 2 (CCR2), expressed on monocytes. When MCP-1 interacts with CCR2, this leads to monocyte recruitment by stimulating their migration to the intima of the arterial wall [13].

Experimental data provide evidences that the three PPAR isotypes modulate peripheral monocyte recruitment and retention, PPAR-γ being also involved in monocyte adhesion and transmigration [14, 15, 57].

Apart this role on monocytes migration in early stages of atherogenesis, PPAR-α and PPAR-γ control even later steps of atherosclerosis. Upon vascular injury, smooth muscle cells (SMC) migrate from the media to the neointima where they proliferate and synthesize proteoglycans thus leading to intima hyperplasia. In this context, PPAR-α inhibits SMC proliferation by blocking

G1⁄ S cell cycle transition (through the induction of the cyclin-dependent kinase inhibitor p16), and reducing neointima formation in a mouse model of carotid artery injury [58].

Similarly, PPAR-γ agonists decrease both SMC migration and proliferation [59,60].

In early atherosclerosis, one of the main functions of monocyte-derived macrophages (MDM) is to scavenge modified LDL. MDM captate infiltrated modified LDL in the intima because they express at their surface specific lipoprotein receptors, the scavenger receptors, whose expression is not under negative feedback control by cellular cholesterol content [61]. The major members of the scavenger receptor family are CD-36 [62] and scavenger receptor A (SR-A) [63, 64]. Macrophage accumulation of lipids, such as cholesterol and triglycerides (TG) originating from lipoproteins, leads to foam cell formation and drives lipid deposition in atherosclerotic plaques.

Besides lipid storage, macrophages are able to eliminate excess of cholesterol by specific efflux pathways. Effluxed cholesterol is then carried by high density lipoproteins (HDL) to the liver to be catabolized. When lipid uptake and storage are dominant over lipid efflux, lipid droplets enlarge and macrophages evolve to foam cells.

The modulation of the expression of genes involved in lipid uptake, metabolism and efflux, might be a tool to prevent atherosclerosis development. In fact PPAR-γ has a key role in adipocyte differentiation by inducing lipid uptake and storage [65]. Hence, PPAR-γ has been initially presented as an activator of the genes involved in cholesterol uptake by macrophages, such as CD-36, thus suggesting a promoting role of PPAR-γ in foam cell formation [66]. However, PPAR-γ activation also represses SR-A expression in macrophages [67]. Moreover, no difference was observed in term of cholesterol content in macrophages treated with PPAR-α or PPAR-γ agonists in the presence of acetylated LDL [68]. In addition, activated PPAR-α and PPAR-γ are potent suppressors of apoB-48 receptor expression in human macrophages and they have been shown to reduce triglyceride accumulation in macrophages incubated with triglyceride-rich lipoproteins [69]. Interestingly, cholesterol content is reduced in human macrophages treated with PPAR-α or PPAR-γ activators and incubated in medium supplemented with glycated LDL (gly-LDL), an abundant cholesterol carrier in diabetic patients [70]. Lipoprotein lipase (LPL) is required for binding and internalization of gly-LDL [71] and, through decreasing LPL secretion and activity, PPAR-α and PPAR-γ activation results in reduced cholesterol content in human macrophages [70]. Taken together, these data show that PPARs activation preferentially lowers lipid uptake and storage in macrophages.

Endothelial inflammation is one of the primary events in atherosclerotic plaque formation and leads to the recruitment of monocytes to the neointima. MDM release proinflammatory cytokines and chemoattractant molecules in the sub-endothelial space. Proinflammatory molecules, such as TNF-α, interleukin-6 (IL-6), IL-12 or IL-1β, are known to promote EC inflammation, monocyte differentiation into macrophages and SMC proliferation [72,73].

PPARs exert acute anti-inflammatory activities *via* multiple molecular mechanism.

Transrepression is a mechanism of negative interference of activated PPARs with proinflammatory signalling pathways, such as NF-κB and activator protein-1 (AP-1) [74], thus inhibiting the expression of proinflammatory genes, like MMP9, TNF-α or IL-6 [22,23]. On the other hand, PPAR-γ can also exert anti-inflammatory effects by inducing the expression of anti-inflammatory genes, such as the IL-1 receptor antagonist (IL-1Ra) [22,23]. Moreover, PPAR-γ enhances the alternative activation and differentiation of macrophages [25,27]. Such alternatively differentiated macrophages display a more pronounced anti-inflammatory phenotype (Fig. 4) [27].

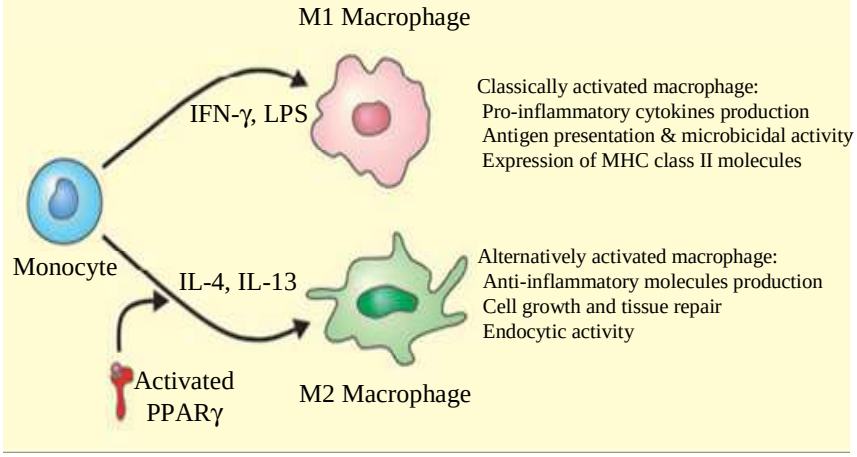


Fig. 4: pathways of classical and alternative macrophage differentiation from monocytes. (modified image from *Bouhel MA, Cell Metab 2007 6: 137-43*)

The activation state and functions of mononuclear phagocytes are profoundly affected by different cytokines and microbial products. While Th1 cytokines, (e.g., IFN-γ, IL-1β), and lipopolysaccharide (LPS), induce a ‘‘classical’’ activation profile (M1), Th2 cytokines, such as IL-4 and IL-13, induce an ‘‘alternative’’ activation program (M2) in macrophages. Moreover, macrophages are plastic cells because they can switch from an activated M1 state back to M2, and vice versa, upon specific signals [75]. M1 macrophages are potent effector cells that kill

microorganisms and produce primarily pro-inflammatory cytokines, such as TNF-α, IL-6, and IL-12 [76]. In contrast, M2 macrophages dampen these inflammatory and adaptive Th1 responses by producing anti-inflammatory factors (IL-10, transforming growth factor β [TGF-β], and IL-1Ra), scavenging debris, and promoting angiogenesis, tissue remodeling and repair [76,77].

IL-4, an anti-inflammatory cytokine and an activator of alternative differentiation of macrophages *in vitro*, also stimulates cellular generation of natural PPAR-γ ligands by the activation of the 12⁄15-lipoxygenase pathway in macrophages [27].

PPAR-α activation also inhibits various proinflammatory molecules. Shu et al. (2000) have shown that PPAR-α activation represses MMP-9 gene expression in macrophages [30] and inhibits osteopontin expression, a pro-inflammatory cytokine implicated in the chemo-attraction of monocytes [78].

Substance P (SP) and PPARs

Tachykinins (TKs) are members of a family of peptides which share the common C-terminal sequence Phe-Xaa-Gly-Leu-MetNH2 [79]: in the peripheral nervous system, two TKs have established role as neurotrasmitters/neuromodulators, substance P (SP) and neurokinin A (NKA) [80,81].

TK effects on target cells are mediated by at least three specific receptors, the neurokinin-1 receptor (NK-1R), NK-2R, and NK-3R. These receptors are members of the superfamily of guanine nucleotide binding-coupled receptors, which interact with G-proteins to promote high-affinity binding and signal transduction [82].

Each TK appears to preferentially activate a distinct NK-receptor, although at high concentrations, each one can activate all the NK-receptors: the NK-1R is activated preferentially by SP [83].

SP is widely distributed in the central and peripheral nervous system. In the central nervous system, SP participates in various behavioural responses and in regulating neuronal survival and degeneration; it also regulates cardiovascular and respiratory functions.

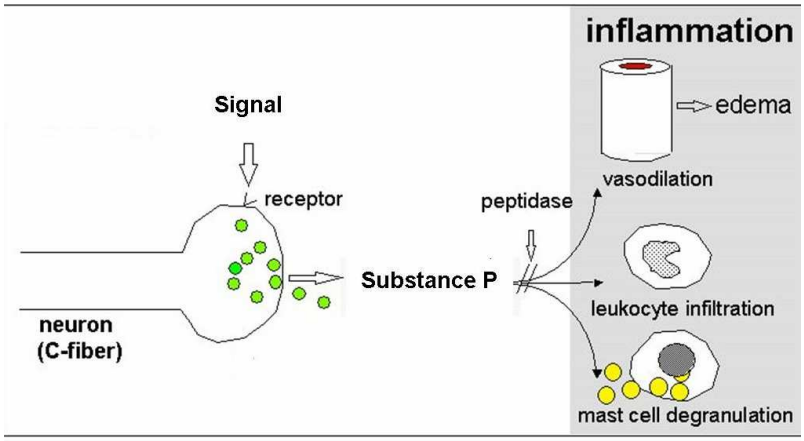


Fig. 5: Mechanism of Neurogenic Inflammation (modified image from www.egms.de)

SP is released from unmyelinated sensory nerve endings, thus evoking inflammatory peripheral effects such as vasodilatation, increased vascular permeability, plasma extravasation and leukocyte activation, which are collectively referred as “neurogenic inflammation” [84-90]. In addition, SP degranulates rat mast cells, stimulates DNA and protein synthesis from human T lymphocytes, evokes the release of inflammatory cytokines from human blood monocytes,

enhances the phagocytic activity of human neutrophils and rat peritoneal macrophages, promotes lysosomal enzyme release and oxy-radical production from human neutrophils [91-95].

SP is a chemoattractant for human monocytes [96]. SP chemotactic activity resides in its C-terminal amino acid sequence. SP can stimulate the secretion of cytokines such as IL-1, TNF-α and IL-6 from monocytes and macrophages [94,97,98]. Moreover, it induces oxy-radical production in guinea-pig and human alveolar macrophages [99-102]. The expression of SP and NK-1R in monocytes/macrophages is upregulated by endotoxin [103,104].

A previous paper of our group [105] demonstrated that human alveolar macrophages possess functional NK-1R on their surface and that the expression of this receptor is significantly increased in healthy smokers. Moreover, SP induces an enhanced nuclear translocation of the transcription factor NF-κB and an increased release of inflammatory cytokines and oxy-radicals, as compared to cells collected from healthy non-smokers.

NF-κB activates the expression of a wide variety of genes including cytokines, chemokines, adhesion molecules, and inducible effector enzymes such as iNOS and cycloxygenase-2 (COX-2), which are crucial in the development of the inflammatory process. Gallicchio et al. (2006) demonstrated that human umbilical vein endothelial cells (HUVEC) possess functional NK-1R and NK-2R, which mediate the ability of SP to induce COX-2 expression, showing a direct effect of SP in inflammation pathway [106].

The causal role of cigarette smoking in both heart and lung diseases is well established and tobacco has been shown to affect the responsiveness of monocyte/macrophages [102,107,108].

Since cigarette-smoke produces neurogenic inflammation in the airways by releasing endogenous TK [109,110,111], these neuropeptides are likely to play an important role in cigarette smoke-induced inflammation.

Our group has also demonstrated (see this thesis, paper 1) that PPAR-γ protein is constitutively present in human monocytes, its expression being upregulated along with differentiation to MDM, and that monocytes and MDM from healthy smokers present a significantly enhanced constitutive PPAR-γ expression, as compared with healthy non-smokers [112].

Despite the fact that both PPAR-γ and NK-1R are expressed at significant levels in human monocyte/macrophages, it is not known whether or not they interact with each other. Therefore, to evaluate such as possibility, we examined the ability of SP, as well as selective NK_1 agonists and antagonists, to modulate PPAR-γ expression in monocyte and MDM from healthy smokers and non-smokers. The results we obtained are presented in paper 2 of this thesis.

Olive oil and inflammation

Olive oil is an integral part of the "Mediterranean diet" which is associated with a sensible tasty and a more enjoyable eating. People who eat a "Mediterranean diet" have been shown to have a remarkable variety of health benefits. Olive oil can quickly satisfy hunger and lead to fewer total calories ingested at mealtime.

Several studies suggest a beneficial role for olive oil in cardiovascular disease and inflammation process [113]. However, it is unclear if any single component of olive oil or the combination of olive oil and a diet high in vegetables, fruit and fish, is responsible for these health benefits. Extra virgin olive oil is one of the few oils that can be eaten without chemical processing (nearly every other vegetable oil has not been detoxified and refined with steam and solvents). Fresh pressed olive oil can be eaten immediately, retains the natural flavours, vitamins, minerals, polyphenolic antioxidants of the ripe olive fruit, and has a balanced ratio of monounsaturated (MUFA) and polyunsaturated fatty acids (PUFA). The beneficial effects of olive oil on coronary heart disease (CHD) are now well recognized and often attributed to its high levels of MUFA [113].

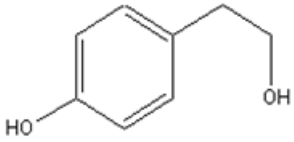
There are multiple mechanisms by which olive oil might impact the development of atherosclerosis: many studies indicate that, along with MUFA consumption, LDL-C levels are reduced and HDL-C levels are enhanced, as compared to PUFA consumption [114-116]. MUFA consumption also reduces oxidative stress, mainly due to its content in polyphenols, which are able to scavenge free radicals and protect LDL from oxidation [117-119]. In addition, olive oil components may interfere with the inflammatory response within atherosclerotic lesion, by inhibiting endothelial activation and macrophage production of inflammatory cytokines and matrix degrading enzymes, thus improving vascular stability [120-123].

For years, most of the attention has focused on the impact of the major dietary components, such as fats, proteins, carbohydrates and fibers, but now the interest for the role of minor components, in particularly Minor Polar Compounds (MPC), is rapidly growing.

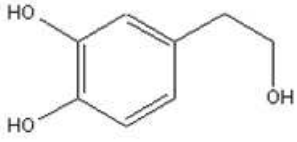
Hydroxytyrosol and oleuropein aglicon, two MPC particularly abundant in olive oil, are endowed with potent antioxidant and cardio-protective activities [124-127]. In LPS-stimulated murine macrophage cell line (J774 cells), hydroxytyrosol blocked the activation of NF-κB reducing iNOS and COX-2 gene expression, suggesting it may represent a non-toxic agent for the control of pro-inflammatory genes [123].

Minor Polar Compounds (MPC)

Simple phenols

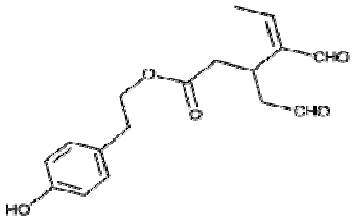


Tyrosol

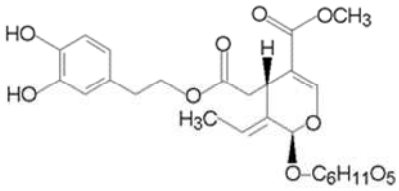


5 -hydroxytyrosol

Secoiridoids



Oleocanthal



Oleuropein

Fig. 6: Examples of Minor polar compounds of extra virgin oil extract.

As reported above, NF-κB regulates COX-2 expression and this pathway is an important key in inflammatory process. Beauchamp et al (2005), demonstrated that oleocanthal, another MPC of extra-virgin olive oil, has COX-inhibitory activity similar to anti-inflammatory drug ibuprofen [128].

Considering that PPARs and NF-κB have a key role in the inflammatory and atherosclerosis development, in order to identify the anti-inflammatory mechanism(s) for MPC, we decided to investigate its possible effects on PPAR-γ expression in human monocytes and MDM, as well as the possible crosslink between PPAR-γ and NF-κB pathways.

PAPER n.1

(in appendix)

Angela Amoruso, Claudio Bardelli, Gabriele Gunella, Luigia Grazia Fresu, Valeria Ferrero, Sandra Brunelleschi

"Quantification of PPAR-γprotein in monocyte/macrophages from healthy smokers and non-smokers: A possible direct effect of nicotine"

Life Sciences 2007; 81(11):906-915

Several studies have demonstrated that tobacco-smoke affects the responsiveness of monocyte/macrophages [102,107,108]. Our group previously reported that alveolar macrophages from healthy smokers present a constitutively enhanced nuclear translocation of the transcription factor NF-κB, spontaneously release higher amounts of pro-inflammatory cytokines and oxy-radicals, and present relevant increase in NK_1 receptor expression, as compared to cells collected from healthy non-smokers [105,102].

This paper was aimed to evaluate the constitutive expression of PPAR-γ protein in circulating monocytes and macrophages (evaluated as MDM) from healthy smokers and non-smokers and to assess the possible direct effect of nicotine. Since monocyte/macrophages spontaneously release inflammatory cytokines, we also evaluated the ability of PPAR-γ agonists (the endogenous and the synthetic ligand: 15d-PGJ_2 and ciglitazone) and nicotine to affect basal secretion in both cell types.

PPAR-γ protein was detected by Western blot and quantification was performed by calculating the ratio between PPAR-γ and β-actin (a house-keeping protein) protein expression. Cytokine release was measured with enzyme-linked immunoassay kits.

This paper confirms that PPAR-γ protein is detected in human monocytes and its expression is up-regulated along with differentiation to MDM. We originally demonstrate that cells from healthy smokers present a constitutively enhanced PPAR-γ expression, which is reproduced, to some extent, by *in vitro* nicotine.

Moreover, by quantifying PPAR-γ/β-actin ratio, we provide, for the first time, an idea on the possible physiological amounts/levels of PPAR-γ.

Epidemiological and clinical studies have clearly established the role of PPARs in the genesis of inflammation and cardiovascular disease, also suggesting the possibility of dimorphic expression for PPARs. Recent data in animal models have demonstrated that female are more susceptible to develop atherosclerosis, because they have a reduced PPAR-α expression as compared to male [129,130].

We have planned an observational pilot study to evaluate PPAR-γ and-α expression in monocyte/macrophages of CHD patients of both sexes, smokers and non-smokers, as well as possible gender-related differences in cytokine secretion. We have also tried to correlate these findings to clinically relevant parameters (HDL-C, LDL-C, total cholesterol, BMI, etc…) of CHD patients.

For this study, we have enrolled 40 CHD patients and 30 healthy controls of both sexes, smokers and non-smokers.

Our preliminary results (as reported below) demonstrate that: 1) PPAR-γ (but not PPAR-α) expression in cells from CHD patients is significantly higher than in healthy donors; 2) non-smoker CHD females show enhanced PPAR-γ expression and reduced release of pro-inflammatory cytokines as compared to male patients and to smoker CHD females. Such differences may modulate the response to PPAR-γ activators and should be considered when treating patients with these drugs.

All these data indicates that further studies are needed to define both the role of PPAR-γ in the complex inflammatory pathway and its anti-atherogenic or pro-atherogenic properties.

In our preliminary experiments we have recruited a study group of forty Caucasian patients with coronary heart disease (CHD) (20 men and 20 women) and a control group of 30 healthy individuals (15 men and 15 women)

In the patient group, 5 men and 5 women were heavy tobacco-smokers (20 cigarettes per day for more than 10 years), whereas in the control group, half of the individuals were smokers, and the other half had never smoked.

In the study group, CHD patients (with either stable or unstable angina), had angiographic evidence of significant coronary artery disease (diameter stenosis >70%) in at least one major epicardial coronary vessel, and required revascularization. Healthy subjects were age-matched to CHD patients, had no history of cardio-pulmonary or other chronic diseases, no diagnosed lung disease, and were drug-free at the time of the study.

We have evaluated the constitutive expression in PPAR-α and PPAR-γ proteins in monocytes and MDM from CHD patients and healthy donors.

As previously reported [112], PPAR-γ expression is confirmed to be up-regulated along with differentiation to MDM (Fig. 1). Furthermore, compared to healthy donors, monocytes, partially differentiated macrophages (M 4days) and fully differentiated macrophages (MDM) obtained from CHD patients present a significantly higher constitutive expression of PPAR-γ protein **(***P* < 0.001), with a four-to-fivefold increase in non-smokers and a threefold increase in smokers (Fig. 1).

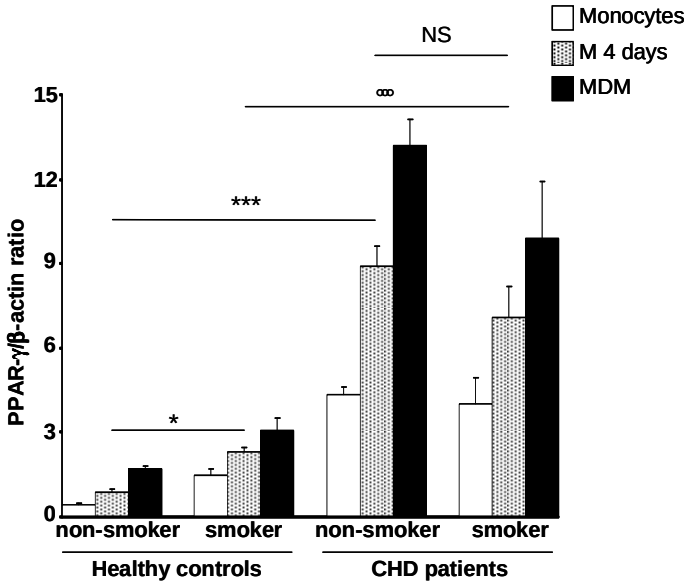


Figure 1. Constitutive PPAR-γ protein expression in monocytes (M), partially differentiated macrophages (M 4 days) and fully differentiated macrophages (MDM) isolated from CHD patients and healthy controls according to tobacco smoking habits. Results are means ± SE. ****P* **<0.05 healthy non-smokers *vs* healthy smokers; ******P* **<0.001 healthy non-smokers *vs* CHD non-smoker patients; °°°*P* < 0.001 healthy smokers *vs* CHD smoker patients.

The evaluation of PPAR-γ/β-actin ratio in monocyte/macrophages reveals that tobacco-smoke enhances PPAR-γ protein expression in cells from healthy donors ($P < 0.05$ *vs* non-smokers), but not in CHD patients (Fig. 1).

Analysis by gender in CHD patients and healthy volunteers reveal that monocytes and MDM from non-smoker CHD post-menopausal female patients express the highest levels of PPAR-γ protein (PPAR-γ/β-actin ratio= 5.8 ± 0.2 and 18.14 ± 3.8 in monocytes and MDM, respectively; $n = 15$; Fig. 2).

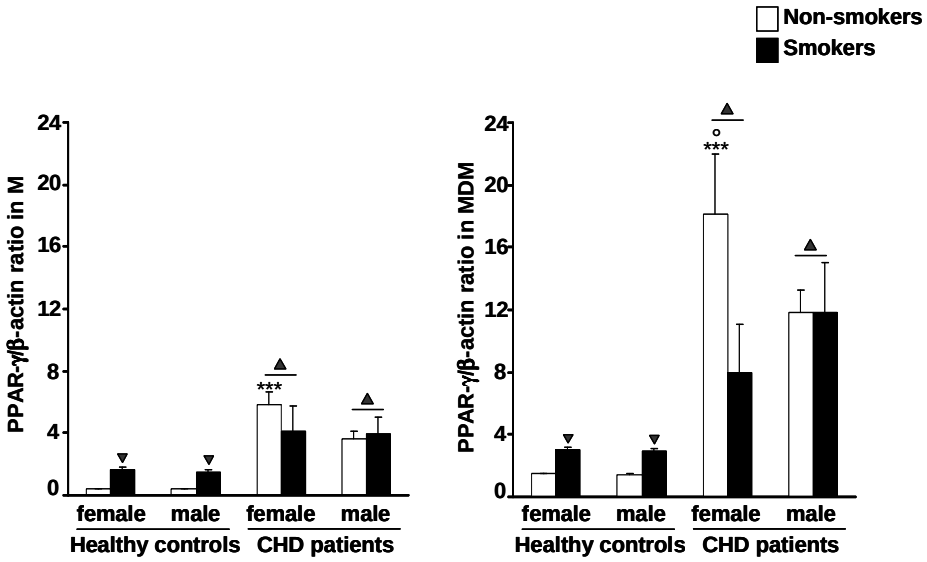


Figure 2. Constitutive PPAR-γ protein expression in monocytes (M) and macrophages (MDM) isolated from CHD patients and controls, according to gender and smoking habit. Results are means ± SE. ***P <0.005 non-smoker CHD females *vs* non-smoker CHD males; °P <0.05 non-smoker CHD females *vs* smoker CHD females; ▲P < 0.001 CHD patients *vs* healthy donors; ▼P < 0.05 healthy smokers *vs* healthy non-smokers.

These PPAR-γ protein levels are significantly higher than those measured in male non-smoker CHD patients ($n = 15$; $P < 0.005$) and female smoker CHD patients ($n= 5$; $P < 0.05$) (Fig. 2). On the contrary, no significant variations are detected either between female and male CHD smokers, or between male CHD non-smokers and smokers (Fig. 2).

By evaluating PPAR-α expression, no major differences are found between monocytes and MDM or between CHD patients and healthy donors (Fig. 3).

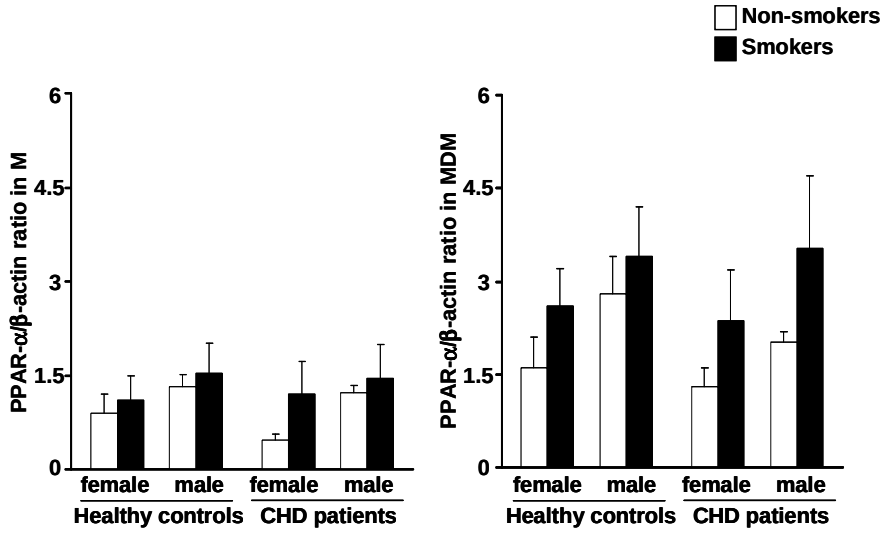


Figure 3. Constitutive PPAR-α protein expression in monocytes (M) and macrophages (MDM) isolated from CHD patients and controls, according to gender and smoking habit. Results are means ± SE and are expressed as PPAR-α/β-actin ratio. *P* <0.01 M *vs* MDM of male CHD smoker patients.

Since both PPAR-α and PPAR-γ are suggested to modulate the development of atherosclerosis and monocytes and MDM of CHD patients express more PPAR-γ than PPAR-α protein, to obtain a normalization parameter of this differential expression, we calculated the PPAR-γ/PPAR-α ratio for each patient and we used this ratio to analyze eventual correlations with clinical parameters.

Monocytes and MDM from female non-smoker CHD patients present an increased PPAR-γ/PPAR-α ratio as compared with male non-smoker CHD patients and female smoker CHD patients (Fig. 4), so confirming the results on PPAR-γ expression.

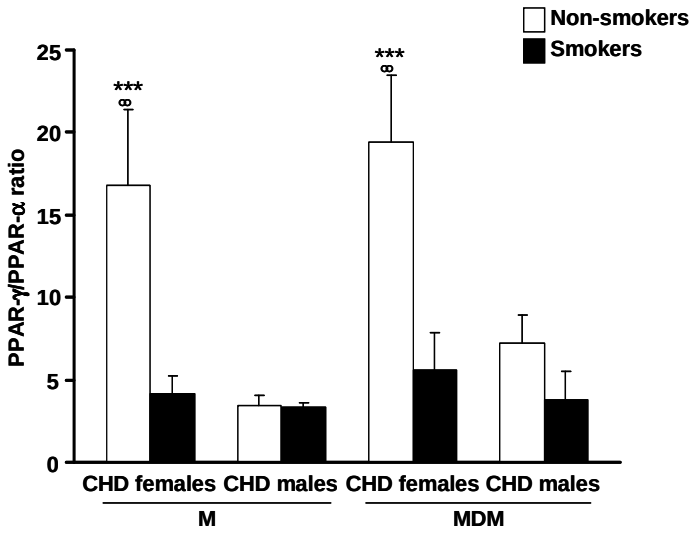


Figure 4. Ratio between the constitutive expression of PPAR-γ and PPAR-α proteins in monocytes (M) and macrophages (MDM) isolated CHD patients. Results are means ± SE and are expressed as PPAR-γ/PPAR-α ratio. * *P* <0.001 *vs* CHD males; °°*P* <0.01 *vs* female CHD smokers.**

As reported in Fig. 5, PPAR-γ/PPAR-α ratio in MDM from female, but not male, CHD patients is inversely related to LDL-C levels (r^2=0.373; *P*=0.004).

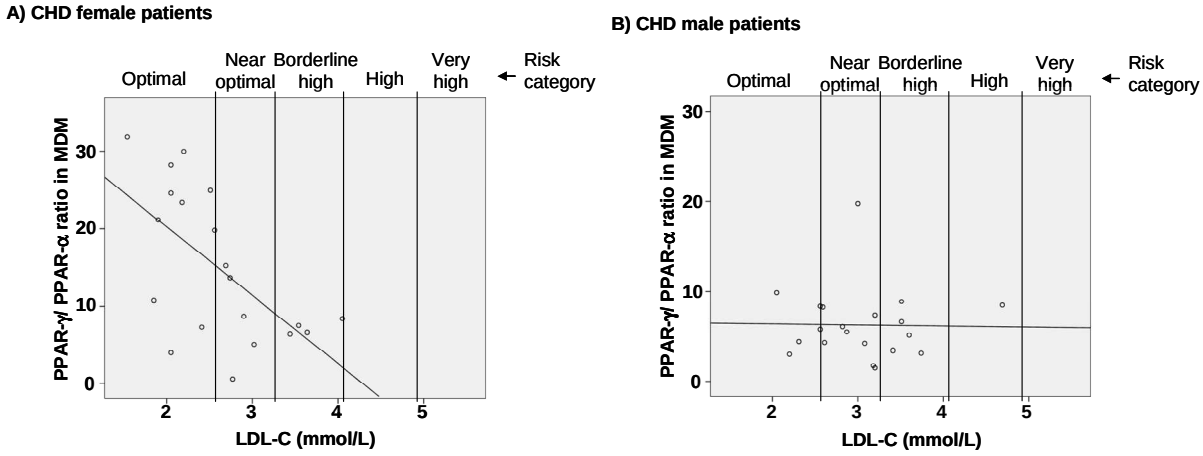


Figure 5. Relation between PPAR-γ/PPAR-α ratio in MDM isolated from CHD patients and measured LDL-C levels. (in CHD female: *P*=0.004; Pearson r^2=0.373; *n*=20).

On the contrary, no significant correlations are found between the PPAR-γ/PPAR-α ratio and BMI, glucose, triglycerides, total cholesterol and HDL-C (data not shown).

By evaluating PPAR ratio according to disease characteristics, we have documented intriguing gender differences. In fact, MDM from CHD females with unstable angina, multi-vessel disease, hypertension (*n*=15) and diabetes (*n*=7) have higher PPAR-γ/PPAR-α ratio than male patients (Fig. 6).

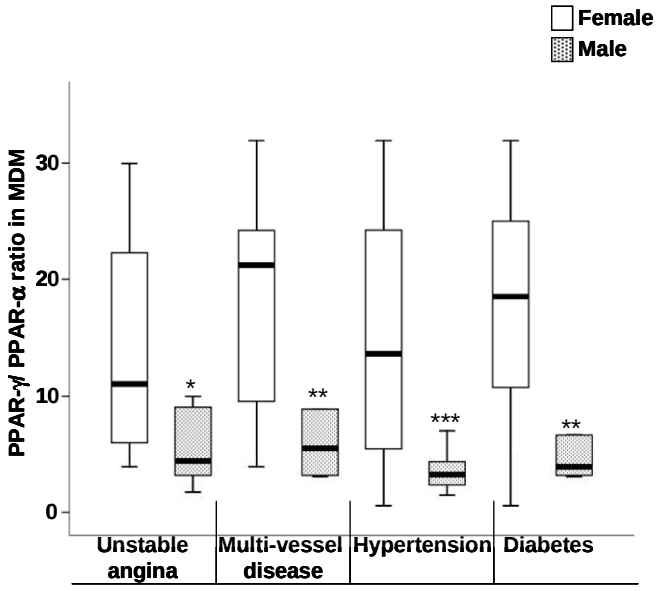


Figure 6. Gender difference in PPAR expression in CHD patients according to disease characteristics. * *P* < 0.05, ** *P* < 0.01 and * *P* < 0.001 *vs* corresponding CHD females.**

Since in our previous study we evidenced that monocytes and MDM from healthy smokers spontaneously release higher amounts of pro-inflammatory cytokines as compared to non-smokers and that this release is modulated by PPAR-γ [112], we evaluated in CHD patients the possible gender-related difference in cytokine secretion and its relationship with smoking habit.

As shown in Fig. 7, monocytes from CHD patients secrete more cytokines than MDM. Interestingly, monocytes and MDM from non-smoker CHD women spontaneously release less TNF-α than non-smoker CHD men ($P < 0.05$), whereas no major differences are found in smoker CHD patients (Fig. 7A). IL-6 release, although not reaching statistical significance, tends to be higher in cells from non-smoker CHD males as compared to females of the same study group (Fig. 7B). Conversely, no major difference occurs in IL-10 secretion by monocytes and MDM from CHD patients, irrespective of gender and smoking habits (Fig. 7C).

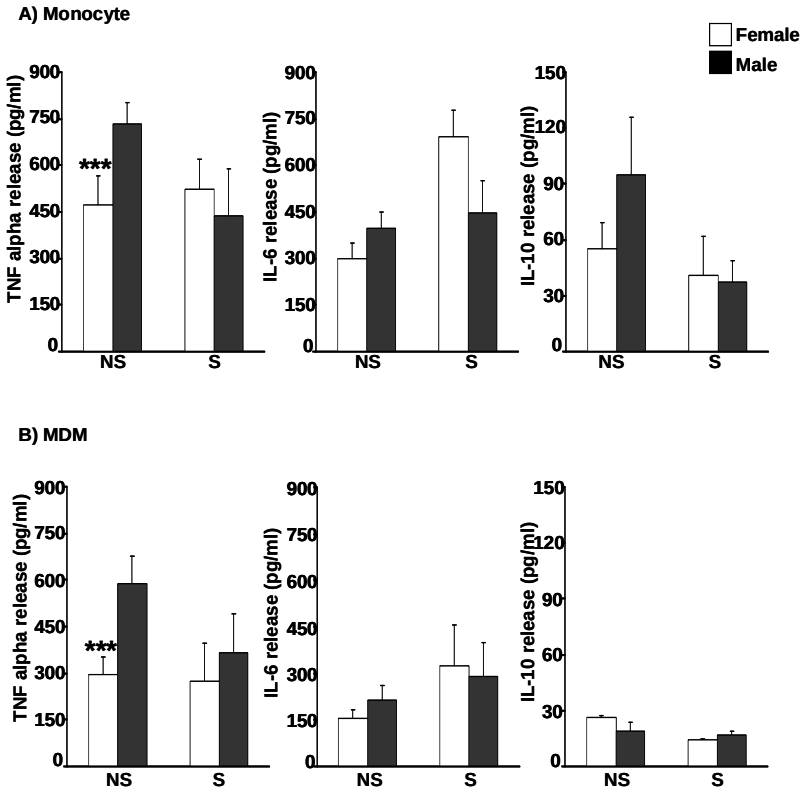


Figure 7. Basal release of TNF-α, IL-6 and IL-10 in monocytes (M) and macrophages (MDM) isolated from non-smoker (NS) and smoker (S) CHD patients of both sexes. Results are means ± SE and are expressed as pg/ml. ***$P < 0.01$ *vs* male cells.

Therefore, monocyte/macrophages from non-smoker CHD women have a higher PPAR-γ expression and less pro-inflammatory cytokine release than cells from non-smoker CHD men**.** These preliminary results document a real gender difference in PPAR-γ expression and suggest its possible involvement in the determination of cardiovascular risk in post-menopausal women.

PAPER n.2

(in appendix)

Angela Amoruso, Claudio Bardelli, Gabriele Gunella, Flavio Ribichini, Sandra Brunelleschi

"A novel activity for Substance P: stimulation of peroxisome proliferator-activated receptor-γprotein expression in human monocytes and macrophages"

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Since SP evokes vasodilatation, increases vascular permeability, plasma extravasation and leukocyte activation, collectively referred to as "neurogenic inflammation" [84-90], and tobacco smoke is suggested to produce neurogenic inflammation in the airways, TKs are likely to play an important role in this context.

Our group has previously demonstrated that human alveolar macrophages possess functional NK-1R on their surface, receptor expression being increased in healthy smokers [105] and that PPAR-γ protein is constitutively present in human monocytes/macrophages, its expression being significantly enhanced in healthy smokers [112].

However, despite the fact both PPAR-γ and NK-1R are expressed at significant levels in human monocyte/macrophages, it is not known whether or not they interact with each other.

Therefore, to evaluate such as possibility, we examined the ability of SP, as well as selective NK_1 agonists and antagonists, to modulate PPAR-γ expression in monocyte and MDM from healthy smokers and non-smokers. We report that, in a concentration dependent manner (10^{-10}-10^{-6}M), SP stimulates PPAR-γ protein expression in monocytes and MDM and that this effect is potently reduced by a PPAR-γ antagonist or NK_1 antagonist. SP and PPAR-γ ligands exerts divergent effects on TNF-α release, which is stimulated by SP and NK_1 agonists and inhibited by PPAR-γ agonists. The results presented in this paper show, originally, that SP, by activation of NK_1 receptors, an enhanced PPAR-γ protein expression in human monocytes/macrophages, suggesting the possibility of a physiologically relevant cross-talk between the two receptors and representing a novel activity for SP.

PAPER n.3

(in appendix)

Sandra Brunelleschi, Claudio Bardelli, Angela Amoruso, Gabriele Gunella, Francesca Ieri, Annalisa Romani, Walter Malorni, Flavia Franconi.

"Minor polar compounds extra-virgin olive oil extract (MPC-OOE) inhibits NF-kB translocation in human monocyte/macrophages "

Pharmacological Research 2007; 56(6):542-549

In the last years, interest has been focused on the anti-inflammatory and anti-oxidant role of the Minor Polar Compounds (MPC), especially simple and complex phenols, which are present in extra-virgin olive oils [127].

In vitro studies [117,131] show that some individual phenolic MPC prevent oxidation of human LDL, but few data are available on the anti-oxidant effect of whole oil extract.

Previous data demonstrated that a total extract from a Tuscan olive oil reduces the copper-induced LDL oxidation with an IC_{50} of 0.6±0.2μM [126], very similar to the concentration measured in human plasma after nutritional intake of virgin olive oil [132].

In collaboration with colleagues of other Universities, our group prepared a defatted extract from a Tuscan extra-virgin olive oil, particularly rich in MPC (minor polar compounds-olive oil extract: MPC-OOE): in fact, in this extract total polyphenols are about 40 mM. MPC-OOE was used for experiments and we evaluated its ability to modulate NF-κB activation and PPAR-γ expression in human monocytes and MDM.

Our results demonstrate that MPC-OOE does not affect PPAR-γ expression, but, in a concentration-dependent manner, potently inhibits p50 and p65 NF-κB translocation, in both monocytes and MDM. The inhibition of NF-κB activity is quantitatively similar to the one exerted by ciglitazone, a selective PPAR-γ ligand.

We suggest that this beneficial effect of MPC-OOE can have a therapeutically relevant anti-atherosclerotic role and could, therefore, largely contribute to the cardio-protective activity of virgin olive oil.

CONCLUSION

Experimental results *in vivo* and *in vitro* indicate that PPAR-α and PPAR-γ regulate inflammatory processes and are involved in cardiovascular disorders, including atherosclerosis [133-137,112].

In murine models of atherosclerosis, most studies demonstrated beneficial effects for PPAR-α agonists and suggested that PPAR-α expression exerts protective anti-atherogenic effects, by modulating cholesterol trafficking and inflammatory activity [138-140,130].

PPAR-γ ligands were shown to inhibit the development of atherosclerosis in LDL-deficient mice, anti-atherogenic effects correlating with improved insulin sensitivity and inhibition of TNF-α [141].

In clinical and epidemiological study, Taylor et al (1998) highlighted that smokers have increased risk to develop atherosclerosis and that tobacco-smoke accelerates the progression of this disease through different mechanisms [107].

In our first paper [112], we confirm PPAR-γ as a key regulator in the macrophage differentiation and demonstrate, for the first time, that healthy smokers present a constitutively increased PPAR-γ expression as compared to non-smokers (4-fold in monocytes and 2-fold in MDM). This effect is partly reproduced by *in vitro* challenge with physiologically relevant nicotine concentrations. We also confirm that PPAR-γ ligands reduce pro-inflammatory cytokines release.

Although the clinical relevance of these findings remains to be elucidated, in keeping with the suggested anti-inflammatory role for PPAR-γ, we hypothesize that the two observed phenomena, enhancement of PPAR-γ expression and inhibition of cytokine release, could represent a protective mechanism to counteract tobacco smoke toxicity.

Moreover, our preliminary results also demonstrated that monocyte/macrophages from non-smoker CHD women have a higher PPAR-γ expression and less pro-inflammatory cytokine release than cells from non-smoker CHD men. This confirms that PPAR-γ is a key regulator of inflammation and, for the first time, indicates its possible gender-related effects. Different clinical trials have demonstrated, but not fully investigated, important gender differences in the molecular patho-physiology of the most frequent cardiovascular diseases, and gender-specific effects of current cardiovascular drugs [51].

In the second paper [142], we demonstrate that monocytes and MDM from healthy smokers present an enhanced NK_1 receptor expression and that, in both cell types, SP stimulates PPAR-γ expression with a greater efficiency, compared with monocyte/macrophages from non-smokers.

We suggest that the increased expression of NK_1 receptors in the cell surface of monocyte/macrophages from healthy smokers is not only associated with, but could also be responsible for, the higher PPAR-γ expression induced by SP. In fact, SP-induced PPAR-γ expression was reverted by NK_1 antagonist or PPAR-γ antagonist.

These results represent a novel activity for SP, which could play a role in chronic inflammatory conditions, such as atherosclerosis, rheumatoid arthritis and inflammatory disease.

In addition to smoking, obesity and/or diabetes are relevant risk factor for the development cardiovascular disease. The Mediterranean diet, in which olive oil is the main source of fat, is largely recognised to prevent the onset and progression of CHD, metabolic disorders, and several types of cancer [143].

In the last paper [144], we demonstrate that in monocyte/macrophages of healthy individuals, MPC-OOE (a defatted extra-virgin olive oil extract, particularly rich in Minor Polar Compounds) potently inhibits NF-κB nuclear translocation as the PPAR-γ agonist, ciglitazone, does.

We hypothesized that olive oil could also affect PPAR-γ expression, but the results we obtained indicate that MPC-OOE does not modulate PPAR-γ expression.

Many studies have supported the hypothesis of the cardio-protective effect of olive oil enriched diets [145,127,146]: in this study we confirm the anti-inflammatory role of our MPC-OOE, and demonstrate that it is strongly corroborated by its ability to potently inhibit, at nutritional concentrations, the PMA-induced NF-κB activation.

As previously reported [132], a Mediterranean diet rich in olive oil supplies 10–20 mg of phenols per day and ensues a MPC plasma level of about 0.6 μM, that is well within the *in vitro* concentrations we used. Interestingly, at the highest concentration evaluated, MPC-OOE significantly reduces p50 translocation in un-stimulated monocytes, in agreement with recent observations [146]. Therefore, inhibition of NF-κB activation with MPC-OOE might represent a target for reducing the risk of CHD.

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APPENDIX

(Paper 1,2,3)

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Quantification of PPAR-γ protein in monocyte/macrophages from healthy smokers and non-smokers: A possible direct effect of nicotine

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Abstract

Previous observations demonstrated that Peroxisome Proliferator-Activated Receptor-gamma (PPAR-γ), a key regulator of adipocyte differentiation, is expressed in a large variety of cells, including cells of the monocyte/macrophage lineage. This study was aimed to quantify both the constitutive and ligand-induced PPAR-γ expression in monocytes and monocyte-derived macrophages (MDM) isolated from healthy smokers and non-smokers, and to evaluate the possible direct effect of nicotine. PPAR-γ protein was detected by Western blot and quantification was performed by calculating the ratio between PPAR-γ and β-actin protein expression. Cytokine release was measured with enzyme-linked immunoassay kits. Constitutive PPAR-γ protein was detected in human monocytes and its expression was up-regulated along with differentiation to MDM. The endogenous ligand 15-deoxy-delta^{12,14}-prostaglandin J_2 and the synthetic agonist ciglitazone enhanced PPAR-γ expression, the former being effective also at low micromolar concentrations. Both agonists significantly inhibited the basal secretion of pro-inflammatory cytokines (e.g., TNF-α, IL-6), ciglitazone being more potent. Monocytes and MDM from healthy smokers presented a significantly enhanced (4-fold and 2.5-fold, respectively) constitutive PPAR-γ expression, as compared to those from healthy non-smokers. However, ligand-induced PPAR-γ expression and inhibition of cytokine secretion were similar in healthy smokers and non-smokers. Nicotine dose-dependently enhanced PPAR-γ expression with a maximum at 10 μM, and inhibited release of pro-inflammatory cytokines; these effects were reversed by α-bungarotoxin. Nicotine and PPAR-γ agonists did not exert synergistic effects. In conclusion, monocytes and MDM from healthy smokers present a constitutively enhanced PPAR-γ expression; this effect is reproduced, to some extent, by nicotine *in vitro*.

Keywords: Peroxisome Proliferator-Activated Receptor-gamma; Monocytes; Monocyte-derived macrophages; Tobacco smoke; Nicotine; Tumour Necrosis Factor-alpha; Interleukin-6; Ciglitazone; PPAR-γ ligands

Introduction

The peroxisome proliferator-activated receptors (PPAR) are lipid-activated transcription factors that act as important regulators of lipid and glucose metabolism, adipocyte differentiation and energy balance. Three subtypes, PPAR-α, PPAR-β (also known as PPAR-δ) and PPAR-γ, have been described so far; they have different tissue distribution and different (although overlapping) ligand specificity (Berger et al., 2005). Besides being expressed at high levels in white adipose tissue, PPAR-γ has been demonstrated in a large variety of cells, including intestinal, endothelial and smooth muscle cells, as well as cells of the monocyte/macrophage lineage (Neve et al., 2000; Ricote et al., 1998a,b; Tontonoz et al., 1998). PPAR-γ can be activated by naturally occurring ligands, including 15-deoxy-delta^{12,14}-prostaglandin J_2 (15d-PGJ_2), a major metabolite of PGD_2, 15-hydroxyeicosatetraenoic acid (15-HETE), 13-hydroxyoctadecadienoic acid (HODE) and oxidized low-density lipoproteins (ox-LDL), as well as by synthetic agents, such as the thiazolidinedione class of anti-diabetic drugs (i.e.,

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rosiglitazone, ciglitazone) and some selected non-steroidal anti-inflammatory drugs (NSAIDs; i.e., indomethacin, ibuprofen) (Forman et al., 1995; Jiang et al., 1998; Lehmann et al., 1995; Tontonoz et al., 1998). The observation that PPAR-γ is activated by arachidonic acid metabolites and some NSAIDs suggests that this nuclear receptor plays a role in the control of inflammation. Indeed, selective PPAR-γ ligands reduce the expression of genes for TNF-α, IL-6, IL-1β, inducible NO synthase (iNOS), gelatinase B, scavenger receptor A and COX-2 in activated macrophages, in part by antagonizing the activities of the transcription factors AP-1, STAT and NF-κB (Jiang et al., 1998; Ricote et al., 1998a,b; Subbaramaiah et al., 2001). Jiang et al. (1998) also demonstrated that 15d-PGJ_2 and synthetic ligands inhibit the release of pro-inflammatory cytokines, namely TNF-α and IL-6, from PMA-challenged human monocytes, but not LPS-stimulated monocytes. No suppression of cytokine secretion was observed in thiazolidinedione-treated mice after *in vivo* challenge with LPS (Thieringer et al., 2000). It was therefore suggested that some of the anti-inflammatory effects of PPAR-γ ligands are independent of PPAR-γ expression (Chawla et al., 2001a; Moore et al., 2001).

Moreover, PPAR-γ is largely suggested as a key modulator of macrophage differentiation, despite some controversial results obtained in different animal species and macrophage-like cell lines (Chawla et al., 2001a; Chinetti et al., 1998; Moore et al., 2001; Ricote et al., 1998a; Tontonoz et al., 1998).

The causal role of cigarette smoking in both heart and lung diseases is well established and tobacco has been shown to affect the responsiveness of monocyte/macrophages (Brunelleschi et al., 1996; Taylor et al., 1998; Vayssier et al., 1998). We previously reported that alveolar macrophages from healthy smokers present a constitutively enhanced nuclear translocation of the transcription factor NF-κB and spontaneously release higher amounts of inflammatory cytokines and oxy-radicals, as compared to cells collected from healthy non-smokers (Bardelli et al., 2005; Brunelleschi et al., 1996; Gunella et al., 2006).

The present study was undertaken to quantify the constitutive expression of PPAR-γ protein in circulating monocytes and macrophages (evaluated as monocyte-derived macrophages, MDM) from healthy smokers and non-smokers and to assess the possible direct effect of nicotine.

We confirm that PPAR-γ protein is present in human monocytes and MDM, its expression increasing along with differentiation into macrophages. We also present direct evidence that monocytes isolated from healthy smokers present a constitutive four-fold enhanced PPAR-γ expression, as compared to cells collected from healthy non-smokers, and that this effect is reproduced, at least partially, by *in vitro* challenge with nicotine. Although the clinical relevance of these findings remains to be ascertained, this is the first paper that, to our knowledge, indicates an enhanced PPAR-γ expression in monocytes and MDM from healthy smokers.

Materials and methods

Study population

This study and the research protocol were approved by the local Ethical Committee; informed written consent was obtained from all participants. A total of 24 healthy subjects, 14 males and 10 females, between 20 and 51 years (mean age = 33.9 ± 2 years; mean age of male and female subjects: 32.8 ± 2.5 and 35.5 ± 3.5 years, respectively, $p = 0.07$), was evaluated. Eight males and four females were heavy smokers (number of cigarettes per day = 20.7 ± 1.5; years of smoking: 12.9 ± 2; means + s.e.m.; $n = 12$) whereas six males and six females were non-smokers. Mean age of smokers (33.08 ± 2.8 years; $n = 12$) and non-smokers (34.75 ± 3 years; $n = 12$) was very similar. Healthy subjects had no history of cardiopulmonary or other chronic diseases, no diagnosed lung disease and no medication at the time of the study; they were all blood donors at the Transfusion Service of Borgomanero (Novara, Italy). Blood was withdrawn between 8.00 and 9.00 a.m.; smokers refrained from smoking at least one hour before phlebotomy.

Preparation of human monocytes and monocyte-derived macrophages (MDM)

Human monocytes were isolated from fresh buffy-coat preparations of whole human blood, collected from healthy smokers and non-smokers attending the Transfusion Service of Borgomanero (Novara, Italy). Experiments were initiated on the day of blood collection; all manipulations were carried out under endotoxin-free conditions. The mononuclear cell fraction was diluted with an equal volume of phosphate-buffered saline (PBS, pH 7.4) at room temperature, layered over a Histopaque (density = 1.077 g/cm^3) gradient solution, centrifuged (400 ×*g*, 30 min, room temperature) and recovered by thin suction at the interface. The mononuclear cell layer was transferred to another tube, mixed with PBS and centrifuged for 10 min at 400 ×*g*. The supernatant was removed; cells were then washed twice with PBS and resuspended in RPMI 1640 medium supplemented with 5% heat-inactivated fetal bovine serum (FBS), 2 mM glutamine, 50 μg/ml streptomycin, 5 U/ml penicillin and 2.5 μg/ml amphotericin B (Brunelleschi et al., 1998). Purified monocyte populations were obtained by adhesion (90 min, 37 °C, 5% CO_2), non adherent cells (mainly lymphocytes) being removed by three gentle washing with PBS; cell viability (trypan blue dye exclusion) was usually >98% (Brunelleschi et al., 1998). Expression of surface markers was analyzed by flow cytometry: purified monocyte populations routinely consisted of >90% $CD14^+$, <2% $CD3^+$ and 99% $MHCII^+$ cells. Monocyte-derived macrophages (MDM) were prepared from monocytes, as described (Brunelleschi et al., 2001). Briefly, monocytes were cultured for 8–10 days in a 5% CO_2 incubator at 37 °C in RPMI 1640 medium containing 20% FBS, 2 mM glutamine, 10 mM HEPES and antibiotics; medium was exchanged every 2–3 days (Brunelleschi et al., 2001). MDM were defined as macrophage-like cells, according to Gantner et al. (1997), by evaluating surface markers CD14, MHCII, CD1a and CD68. Briefly, adherent cells were detached by gentle scraping with a plastic scraper. After three washings with sterile PBS, cells were resuspended at the final concentration of 1×10^5 cells/ml and fluorescent dye-labelled antibodies against the different surface markers (anti-CD14 from Becton Dickinson, Oxford, UK; anti-CD68 and anti-MHCII from Dako,

Milan, Italy; anti-CD1a from eBioscence, San Diego, CA, USA) were added for 30 min on ice. Incubation was performed in the dark and expression of surface markers was analyzed by flow cytometry.

PPAR-γ protein expression and quantification

Cells from healthy smokers and non-smokers were evaluated either as they were (i.e., "basal, constitutive PPAR-γ expression") or after challenge (6 h, 37 °C, 5% CO_2) with the PPAR-γ ligands 15d-PGJ_2 (used at 0.1–10 μM) and ciglitazone (used at 0.1–50 μM). Monocytes and MDM from non-smokers were also challenged with nicotine (0.1–10 μM; 6 h) to evaluate its possible effects on PPAR-γ expression. Cells (2×10^6), seeded in six-well plates, were washed twice with ice-cold PBS and scraped off the wells in lysis buffer containing 3% SDS, 0.25 M Tris and 1 mM phenyl–methyl–sulfonyl fluoride (PMSF) and lysed by sonication; when necessary, cell lysates were stored at −80 °C. The determination of protein concentration was done with a Bradford-based assay. Protein samples (20 μg) were analyzed by SDS-PAGE (10% acrylamide) and electro-blotted on nitrocellulose membrane (Protran, Perkin Elmer Life Sciences, Boston, MA, USA). Immunoblots were performed according to standard methods using the following antibodies: monoclonal mouse anti-human PPAR-γ (E-8; Santa Cruz, CA, USA; 1:1000 in TBS-T 5% milk) and monoclonal mouse anti-human β-actin (Sigma, St. Louis, MO, USA; 1:5000 in TBS-T 3% BSA). Anti-mouse secondary antibody was coupled to horseradish peroxidase (Amersham Biosciences, Buckinghamshire, UK). Proteins were visualized with an enzyme-linked chemiluminescence detection kit according to the manufacturer's (Perkin Elmer) instructions. Chemiluminescence signals were analyzed under non-saturating conditions with an image densitometer (Versadoc, Bio-Rad, Hercules, CA, USA). Quantification of PPAR-γ protein was performed by calculating the ratio between PPAR-γ and β-actin protein expression; the latter was selected as reference house-keeping gene.

Nicotinic acetylcholine receptor α 7(α7nAChR) protein expression

The constitutive expression of α7nAChR protein was evaluated in monocytes and MDM from healthy non-smokers. Immunoblots were performed as described above, by using a monoclonal anti-human nicotinic acetylcholine receptor, alpha 7 subunit, antibody (clone mAb 306; Sigma, Milwaukee, WI, USA); β-actin protein expression is shown for comparison.

Cytokine release

Cells (1×10^6) were treated in the absence or presence of PPAR-γ agonists (15d-PGJ_2, used at 1–10 μM; ciglitazone, used at 5–50 μM) for 6 h; supernatants were collected and stored at −20 °C. In some cases, cells from non-smokers were also challenged, in the presence or absence of PPAR-γ agonists or nicotine, with phorbol 12-myristate 13-acetate (PMA) 1 μM for 24 h. This 24-h stimulation time was chosen to ensure maximal cytokine release, as observed previously (Bardelli et al., 2005; Gunella et al., 2006). TNF-α, IL-6 and IL-10 (the latter was evaluated as the most important anti-inflammatory cytokine) in the samples were estimated by ELISA (Pelikine Compact™ human ELISA kit) following the manufacturer's instructions (CLB/Sanquin, Netherlands). Results are expressed in pg/ml.

Drugs and analytical reagents

FBS (Lot 40F-7234K) was from Gibco (Paisley, UK). PBS, Hystopaque, RPMI 1640 (with or without phenol red), glutamine, HEPES, streptomycin, penicillin, amphotericin B, PMA, nicotine hydrogen tartrate salt, α-bungarotoxin, bromophenol blue, glycine, glycerol, methanol and Tween 20 were obtained from Sigma (Milwaukee, WI, USA). The PPAR-γ agonists, 15-deoxy-delta^{12,14}-prostaglandin J_2 and ciglitazone, were from Biomol (Plymouth Meeting, PA, USA). Nitro-cellulose filters (Protran) were from Perkin Elmer Life Sciences (Boston, MA, USA). Tissue-culture plates were from Nunc Ltd (Denmark); all cell culture reagents, with the exception of FBS, were endotoxin-free according to details provided by the manufacturer. TNF-α, IL-6 and IL-10 immunoassay kits were obtained from CLB/Sanquin, Central Laboratory of the Netherlands Red Cross (The Netherlands).

Data and statistical analysis

Data are mean±s.e.m. of "*n*" independent experiments; cytokine determinations were performed in duplicate. Statistical evaluation was performed by ANOVA analysis and Bonferroni correction. For studying the main effects and interaction between tobacco smoke and differentiation we used two-way ANOVA analysis.

Results

Expression of PPAR-γ in monocytes and macrophages (MDM) from healthy smokers and non-smokers

Before performing any biochemical assay, we verified the purity of our MDM preparation by morphological and phenotypical examinations (data not shown). During the 8–10 days of culture, the morphology of peripheral blood monocytes changed consistently, acquiring a macrophage-like profile. The pattern of surface marker expression was also modified, an increase in $CD68^+$ cells and a reduction of $CD14^+$ cells being observed in MDM (data not shown). Moreover, the absence of CD1a expression demonstrated that no differentiation towards dendritic cells occurred in our MDM preparations (data not shown).

To quantify PPAR-γ protein expression in both monocytes and MDM from healthy smokers and non-smokers, we calculated the ratio between PPAR-γ and β-actin protein expression; in our experiments, β-actin levels were constant and stable in each cell type and were neither induced nor inhibited by the different 6-hour *in vitro* treatments.

As depicted in Fig. 1, constitutive PPAR-γ protein was detected in monocytes, partially differentiated (M 4 days) and

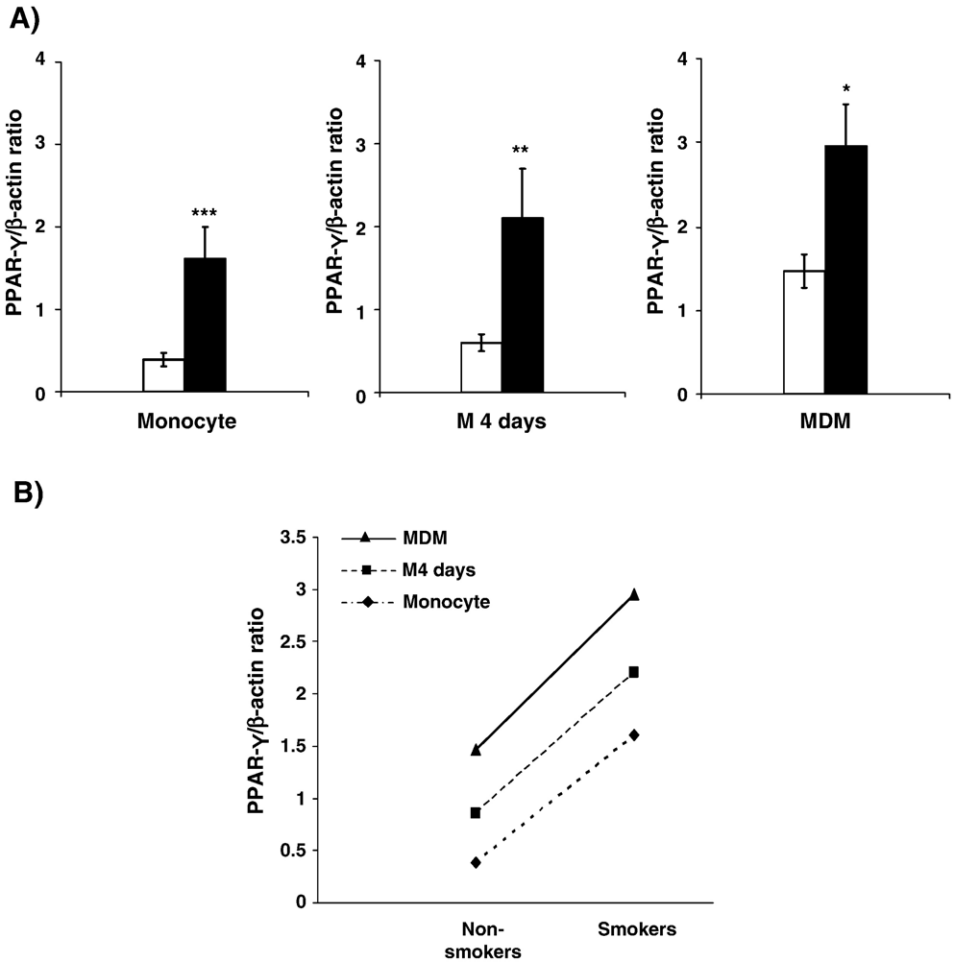

Fig. 1. Constitutive PPAR-γ protein expression in human monocyte/macrophages from healthy non-smokers (□) and smokers (■). In A: PPAR-γ/β-actin ratio in monocytes, partially differentiated macrophages (M 4 days) and fully differentiated macrophages (MDM) from twelve healthy smokers and twelve healthy non-smokers. Data are means±s.e.m.. ***$p<0.001$, **$p<0.01$ and *$p<0.05$ vs non-smokers (Student's t test). In B: These data were analyzed by two-way ANOVA, one variable being tobacco smoke (smokers and non-smokers) and the other being the differentiation level of PPAR-γ/β-actin ratios (monocytes, M 4 days and MDM). The PPAR-γ/β-actin ratios were significantly greater for smokers than for non-smokers ($p<0.00001$) and increased along with differentiation ($p<0.005$). The interaction effect was non-significant, $F=0.06$, $p>0.05$ (see text for further details).

fully differentiated macrophages (MDM) and its expression was up-regulated along with differentiation. Our results show for the first time that PPAR-γ protein expression was significantly enhanced in healthy smokers as compared to non-smokers: in monocytes from healthy smokers and non-smokers, PPAR-γ/β-actin ratios were 1.61±0.38 ($n=12$) and 0.38±0.08 ($n=12$), respectively (Fig. 1A). The ratios measured in MDM from healthy smokers and non-smokers were 2.95±0.6 ($n=12$) and 1.47±0.2 ($n=12$), respectively and similar results were observed also in partially differentiated (M 4 days) macrophages (Fig. 1A). In non-smokers, PPAR-γ protein expression was about 4-fold higher in MDM than in monocytes; in smokers, PPAR-γ protein expression in MDM was about 2-fold, as compared to monocytes (Fig. 1A). These data were subjected to a two-way analysis of variance, one variable being tobacco smoke (smokers and non-smokers) and the other being the differentiation level (monocytes, M 4 days and MDM) of PPAR-γ/β-actin ratios. The main effect of smoking habit yielded an F ratio=22.37, $p<0.00001$, indicating that the PPAR-γ/β-actin ratios were significantly greater for smokers than for non-smokers. The main effect of differentiation level yielded an F ratio=6.04, $p<0.005$, indicating that the PPAR-γ/β-actin ratios were significantly increased along with differentiation. The interaction effect was not significant, $F=0.06$, $p>0.05$ (Fig. 1B).

Ligand-induced PPAR-γ expression in monocytes and macrophages (MDM) from healthy smokers and non-smokers

In keeping with previous observations, a 6-hour challenge with the endogenous ligand 15d-PGJ_2 (used at 10 μM) or the synthetic ligand ciglitazone (used at 50 μM) enhanced PPAR-γ expression in monocytes and MDM from healthy non-smokers (Fig. 2A) and healthy smokers (Fig. 2B). Fig. 2 deals with representative Western blot of monocyte/macrophages from smokers and non-smokers; in any case 15d-PGJ_2 was more potent than ciglitazone.

To ensure a better evaluation of ligand-induced PPAR-γ expression, we also performed concentration-response studies. As shown in Fig. 3A, dealing with cells from healthy non-smokers, both ligands increased PPAR-γ expression in a concentration-dependent manner. At the maximal concentration evaluated

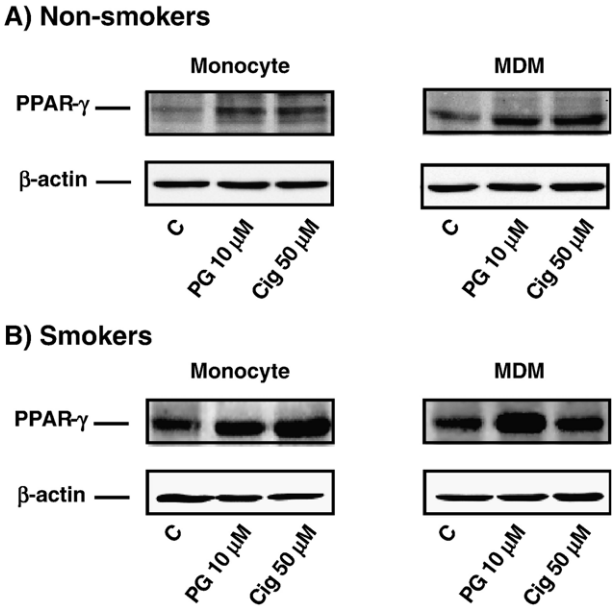

Fig. 2. Ligand-induced PPAR-γ expression in human monocyte/macrophages. A: Western blot of PPAR-γ and β-actin in monocyte/macrophages from non-smokers. B: Western blot of PPAR-γ and β-actin in monocyte/macrophages from smokers. Monocytes and fully differentiated macrophages (MDM) were challenged for 6 h in the absence (C, control) or presence of 15d-PGJ_2 (PG, 10 μM) or ciglitazone (Cig, 50 μM). Each blot is representative of five others.

(10 μM), 15d-PGJ_2 increased PPAR-γ expression about 2.4-fold and 2.5-fold in monocytes and MDM, respectively, and was effective also at the low 0.1 μM concentration (1.7-fold in monocytes and 1.5-fold in MDM) (Fig. 3A). Ciglitazone was inactive at low micromolar concentrations and, at the maximal concentration evaluated (50 μM), increased PPAR-γ expression about 2-fold in monocytes and 1.7-fold in MDM (Fig. 3A). Similar results were obtained by evaluating the ability of both ligands to enhance PPAR-γ expression in partially differentiated macrophages (data not shown). Ciglitazone and 15d-PGJ_2 dose-dependently up-regulated PPAR-γ expression also in monocytes and MDM from healthy smokers, 15d-PGJ_2 being more potent than ciglitazone (Fig. 3B). The endogenous ligand significantly increased PPAR-γ expression also at 0.1 μM (1.7-fold in monocytes and 1.6-fold in MDM), whereas ciglitazone was effective at higher concentrations only (Fig. 3B).

Effects of PPAR-γ ligands on cytokine release in monocytes and MDM from healthy smokers and non-smokers

Since monocyte/macrophages spontaneously release significant amounts of inflammatory cytokines, we evaluated the ability of PPAR-γ agonists to affect basal secretion. Monocytes and MDM were treated with or without relatively high concentrations of PPAR-γ agonists (ciglitazone: 5–50 μM; 15d-PGJ_2: 1–10 μM) for 6 h, the same time used in Western blot experiments.

By evaluating the spontaneous TNF-α release in monocyte/macrophages from smokers and non-smokers (Fig. 4), we observed that ciglitazone was overall more effective than 15d-PGJ_2. At the maximal 50 μM concentration, ciglitazone inhibited TNF-α release by 90–95% in monocytes and macrophages from healthy non-smokers, whereas only a 50–60% inhibition was afforded by the maximal 15d-PGJ_2 concentration (Fig. 4A). Both PPAR-γ ligands dose-dependently inhibited basal TNF-α secretion in monocytes and MDM from healthy smokers; again, ciglitazone 50 μM was more effective than 15d-PGJ_2 10 μM (Fig. 4B). No major variations were observed for both PPAR-γ ligands in the amount of inhibition in monocytes and MDM from smokers and non-smokers (Fig. 4). Interestingly, cells from healthy smokers displayed a significantly enhanced TNF-α secretion as compared to those from non-smokers (Fig. 4). As depicted in Fig. 5, PPAR-γ ligands inhibited the spontaneous IL-6 release. Again, ciglitazone 50 μM proved itself more effective than 15d-PGJ_2 10 μM in both non-smokers ($n=6$; Fig. 5A) and smokers ($n=4$; Fig. 5B) and was somewhat more potent in MDM than monocytes. Basal IL-6 release was higher in cells from healthy smokers than in

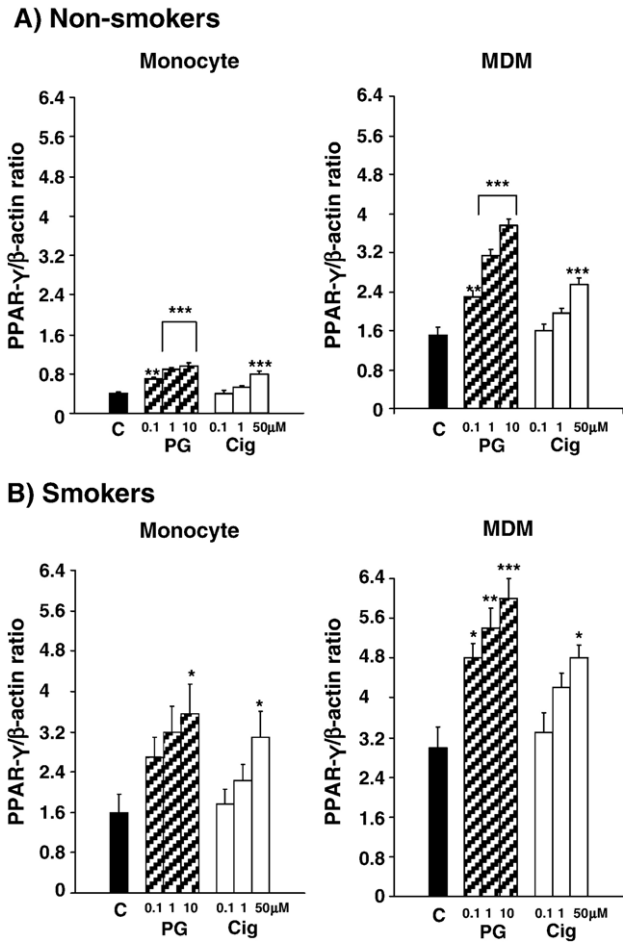

Fig. 3. Concentration-dependent effects of selective ligands on PPAR-γ expression in monocytes and MDM from healthy non-smokers (A) and healthy smokers (B). A: Cells from non-smokers were challenged for 6 h in the absence (C, control; ■) or presence of 15d-PGJ_2 (PG, 0.1–10 μM; ▨) or ciglitazone (Cig, 0.1–50 μM; □). Results are expressed as PPAR-γ/β-actin ratio (see text for further details). Means± s.e.m.; $n=6$. $p<0.0001$ (ANOVA); ***$p<0.001$, **$p<0.01$ (Bonferroni correction). B: Monocytes and MDM from healthy smokers were challenged for 6 h in the absence (C, control; ■) or presence of 15d-PGJ_2 (PG, 0.1–10 μM; ▨) or ciglitazone (Cig, 0.1–50 μM; □). Means±s.e.m.; $n=5$. For monocytes: $p<0.05$ (ANOVA); *$p<0.05$ (Bonferroni correction). For MDM: $p<0.01$ (ANOVA); ***$p<0.001$, **$p<0.01$, *$p<0.05$ (Bonferroni correction).

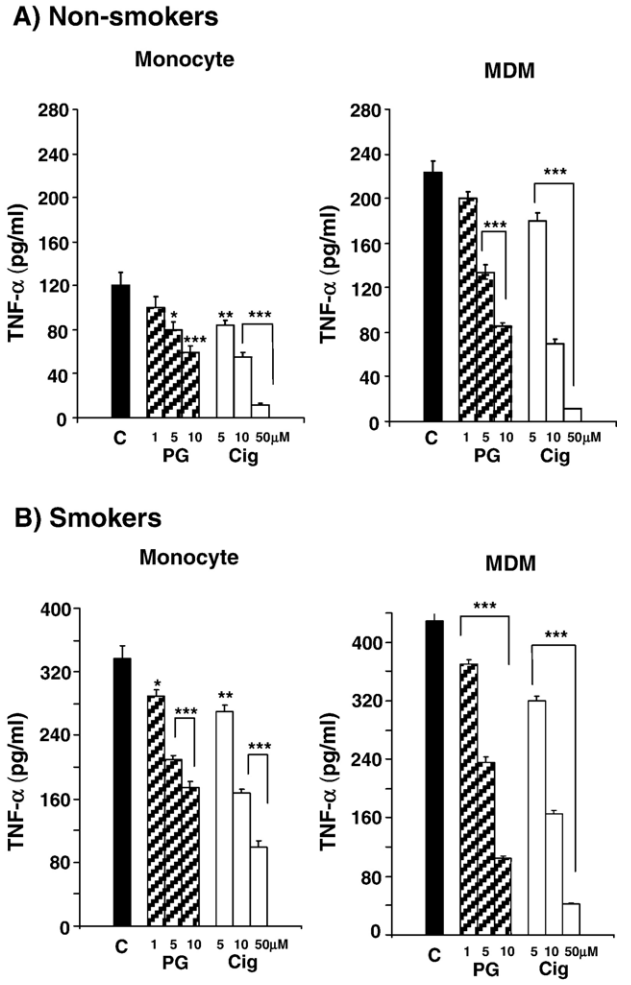

Fig. 4. Selective PPAR-γ agonists inhibit the spontaneous release of TNF-α in human monocytes and MDM from healthy non-smokers (A) and healthy smokers (B). Cells from non-smokers were challenged for 6 h in the absence (C, control; ■) or presence of 15d-PGJ_2 (PG, 1–10 μM; ▨) or ciglitazone (Cig, 5–50 μM; □). Cytokine release is expressed in pg/ml (please, note the different scale). Data are means±s.e.m. In A: healthy non-smokers; $n=6$; $p<0.0001$ (ANOVA) for each group, ***$p<0.001$, **$p<0.01$, *$p<0.05$ (Bonferroni correction). In B: healthy smokers; $n=4$; $p<0.0001$ (ANOVA); ***$p<0.001$, **$p<0.01$, *$p<0.05$ (Bonferroni correction).

those from non-smokers (Fig. 5). Interestingly, ciglitazone and 15d-PGJ_2 did not affect basal IL-10 release from both monocytes and macrophages isolated from healthy smokers and non-smokers (data not shown). As known, IL-10 is the major anti-inflammatory cytokine and was spontaneously released in modest amounts in both cell types (data not shown).

Effects of nicotine on PPAR-γ expression in monocytes and MDM from healthy non-smokers

To verify whether or not the enhanced PPAR-γ protein expression we measured in cells from healthy smokers could rely, at least partially, on a direct effect of nicotine, we evaluated the ability of nicotine to affect, *in vitro*, PPAR-γ expression. Cells from healthy non-smokers were treated with nicotine 10 μM or 0.1 μM for 6 h, 15d-PGJ_2 being used as a positive control. As depicted in Fig. 6, nicotine dose-dependently enhanced PPAR-γ expression, with a maximum increase of about 1.7-fold in monocytes ($n=5$; Fig. 6A) and 1.5-fold in MDM ($n=5$; Fig. 6B) at 10 μM. No additive or synergistic effect between nicotine and 15d-PGJ_2 was demonstrated (Fig. 6). Nicotine's effects were reverted in the presence of α-bungarotoxin (evaluated at 100 ng/ml), the selective antagonist of the α7nAChR (Fig. 6). As reported in Fig. 7, Western blot experiments documented the presence of α7nAChR protein in monocytes and MDM of healthy non-smokers, a similar expression being observed in both cells.

Effects of nicotine on cytokine release in monocytes and MDM from healthy non-smokers

Nicotine per se inconsistently affected basal cytokine release (data not shown), but potently inhibited the PMA-induced TNF-α and IL-6 release, as seen with ciglitazone and 15d-PGJ_2;

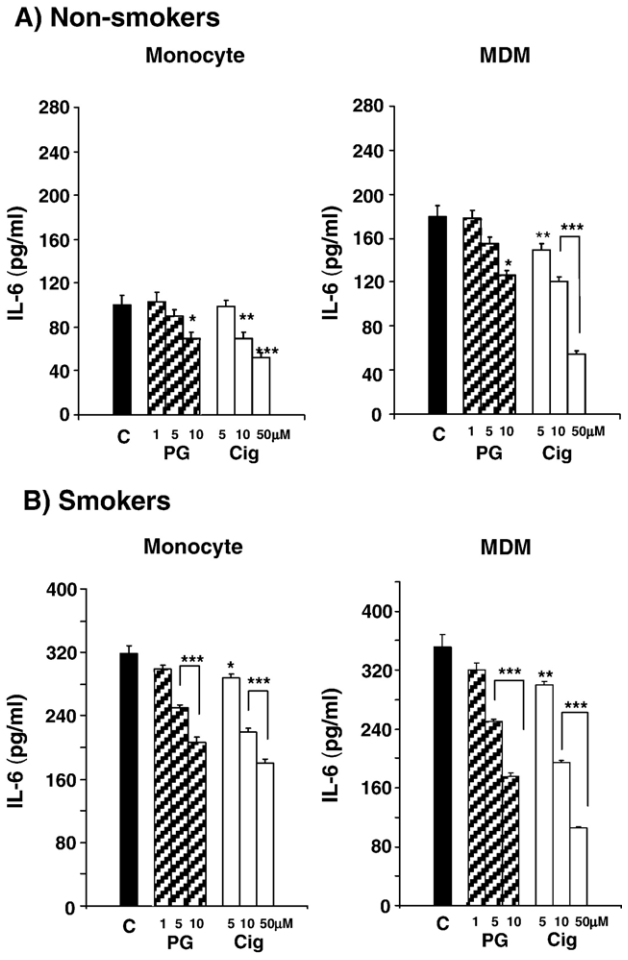

Fig. 5. Selective PPAR-γ agonists inhibit the spontaneous release of IL-6 in human monocytes and MDM from healthy non-smokers (A) and healthy smokers (B). Cells were challenged for 6 h in the absence (C, control; ■) or presence of 15d-PGJ_2 (PG, 1–10 μM; ▨) or ciglitazone (Cig, 5–50 μM; □). Cytokine release is expressed in pg/ml (please, note the different scale). Data are means±s.e.m. In A: healthy non-smokers; $n=6$; $p<0.0001$ (ANOVA) for ciglitazone and $p<0.01$ (ANOVA) for 15d-PGJ_2, ***$p<0.001$, **$p<0.01$, *$p<0.05$ (Bonferroni correction). In B: healthy smokers; $n=4$; $p<0.0001$ (ANOVA); ***$p<0.001$, **$p<0.01$, *$p<0.05$ (Bonferroni correction).

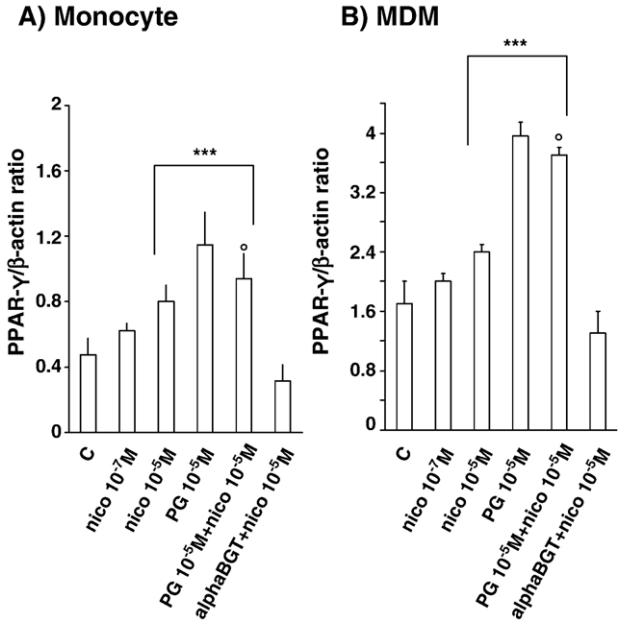

Fig. 6. Effects of nicotine and 15d-PGJ_2 on PPAR-γ expression in human monocytes (A) and MDM (B) from healthy non-smokers. Monocytes and MDM were treated with or without nicotine (nico, 0.1 μM or 10 μM), 15d-PGJ_2 (PG, 10 μM) or a combination of both for 6 h. α-bungarotoxin (alphaBGT, used at 100 ng/ml) prevented nicotine-induced PPAR-γ expression. Results are expressed as PPAR-γ/β-actin ratio. Means ± s.e.m; $n=5$. $p<0.0001$ (ANOVA); ***$p<0.001$, °not significant vs 15d-PGJ_2 (Bonferroni correction).

again, no additive effect between nicotine and 15d-PGJ_2 was observed (Fig. 8). In addition, neither nicotine nor PPAR-γ agonists inhibited IL-10 release (data not shown).

Discussion

This study confirms PPAR-γ as a key regulator of macrophage differentiation and demonstrates for the first time that: a) monocytes and MDM from healthy smokers present a constitutively enhanced PPAR-γ protein expression as compared to non-smokers, b) nicotine significantly increases PPAR-γ expression in human monocyte/macrophages.

To avoid possible confounding elements in the determination of the constitutive and ligand-induced PPAR-γ expression in monocyte/macrophages, we differentiated monocytes into mature macrophages using RPMI 1640 medium supplemented with 20% FBS, as previously described (Brunelleschi et al., 2001). We did not use M-CSF or GM-CSF (as required in many protocols for macrophage differentiation) because they are reported to induce and/or up-regulate PPAR-γ expression in murine macrophages (Ricote et al., 1998a; Huang et al., 1999; Ditiatkovski et al., 2006) and macrophage cell lines (Chawla et al., 2001b). Interestingly, Chinetti et al. (1998) used a similar procedure (e.g., monocyte culture in the presence of human serum) to demonstrate, for the first time, the PPAR-γ expression in human macrophages. To quantify PPAR-γ protein expression in both monocytes and MDM from healthy smokers and non-smokers, we calculated the ratio between PPAR-γ and β-actin protein expression. Although some variations in the amount of β-actin mRNA have been reported in the literature (Bas et al., 2004; Selvey et al., 2001), β-actin is largely regarded as reference house-keeping gene.

We confirm that PPAR-γ protein, constitutively present at low levels in human monocytes, is up-regulated along with differentiation into mature macrophages, as previously suggested (Chawla et al., 2001a; Chinetti et al., 1998; Ricote et al., 1998b; Tontonoz et al., 1998). Moreover, we report here for the first time that monocytes and MDM isolated from healthy smokers present a significantly higher constitutive expression of PPAR-γ protein, as compared to those from non-smokers (4-fold in monocytes and 2-fold in MDM). By using a two-way ANOVA analysis, we demonstrate that both smoking habit and cell differentiation significantly increase PPAR-γ protein expression. However, the interaction effect is non-significant, differentiation and smoking habit being two independent variables. Consistently, the two PPAR-γ ligands we used, the endogenous 15d-PGJ_2 and the synthetic ciglitazone, dose-dependently enhance PPAR-γ protein expression in monocytes and MDM, no major differences in fold-increase being observed between the two cell types and/or the smoking habit. Interestingly, 15d-PGJ_2, but not ciglitazone, significantly induces PPAR-γ expression also at the low 0.1 μM concentration, supporting the major potency of the endogenous ligand in this regard.

Notably, PPAR-γ ligands have been demonstrated to exert anti-inflammatory effects, which are generally observed at concentrations 2–3 orders of magnitude greater than those required for insulin-sensitizing actions and PPAR-γ stimulation. For example, ciglitazone bound the PPAR-γ ligand-binding domain with a 3 μM EC_{50} (Lehmann et al., 1995), whereas 15d-PGJ_2 activated PPAR-γ with an EC_{50} of 2 μM in a murine chimera system (Forman et al., 1995). In keeping with Hinz et al. (2003), the highest concentrations of PPAR-γ ligands we used are 10 μM for 15d-PGJ_2 and 50 μM for ciglitazone.

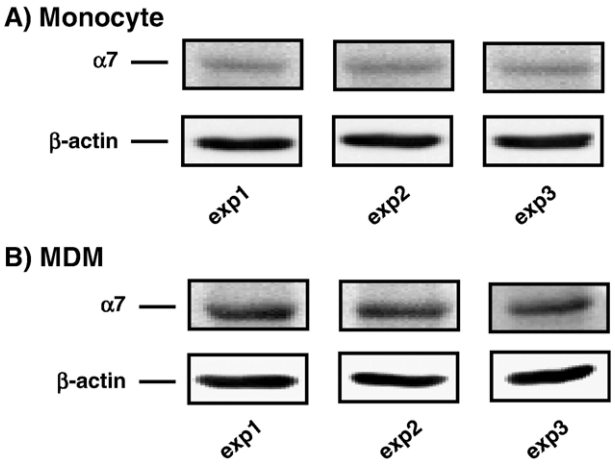

Fig. 7. Constitutive expression of nicotinic acetylcholine receptor α 7 (α7nAChR) protein in human monocytes (A) and MDM (B) from three healthy non-smokers. Immunoblots were performed by using a monoclonal antibody for the α 7 subunit; β-actin protein expression is shown for comparison (see text for further details).

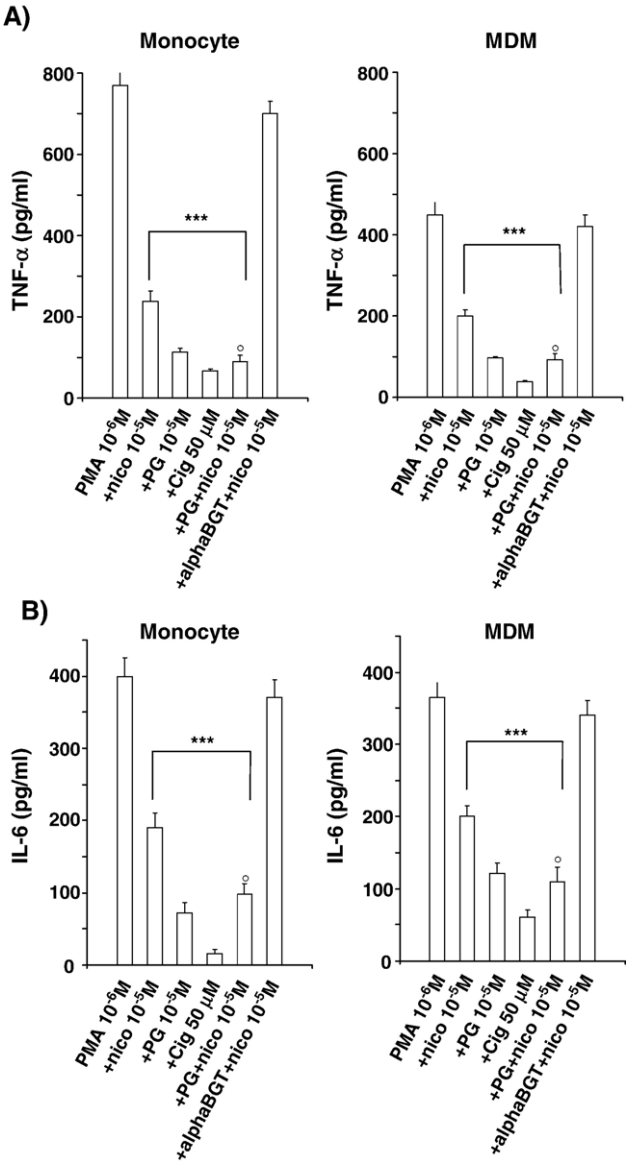

Fig. 8. Effects of nicotine and selective PPAR-γ ligands on PMA-induced cytokine secretion in monocytes and MDM from healthy non-smokers. In A: PMA-induced TNF-α release in monocytes and MDM; in B: PMA-induced IL-6 release in monocytes and MDM. Monocytes and MDM were challenged, in the absence or presence of nicotine, 15d-PGJ_2, ciglitazone or combination, with PMA 1 μM for 24 h. α-bungarotoxin (alphaBGT, used at 100 ng/ml) reduced the nicotine-induced inhibition. Cytokine release is expressed in pg/ml. Means ± s.e.m.; $n=5$. $p<0.0001$ (ANOVA); ***$p<0.001$, °not significant vs 15d-PGJ_2 (Bonferroni correction).

Previous reports indicated that PPAR-γ agonists inhibit the secretion of pro-inflammatory cytokines in cells of the monocyte/macrophage lineage, despite some controversial results due to the stimulant used (LPS or PMA) and the single cell type (Alleva et al., 2002; Hinz et al., 2003; Hong et al., 2003; Jiang et al., 1998; Thieringer et al., 2000). In our hands, both agonists inhibited the spontaneous and PMA-induced cytokine release in monocytes and MDM acting in a narrow concentration range (1–10 μM for the endogenous ligand; 5–50 μM for the synthetic ligand). Ciglitazone resulted more effective than 15d-PGJ_2: at the maximal 50 μM concentration, it inhibited by about 90% the spontaneous TNF-α release from monocytes as compared to the 50% inhibition afforded by 15d-PGJ_2 10 μM.

By comparing these results with those observed in ligand-induced PPAR-γ expression, it appears that ciglitazone is more active on cytokine release inhibition, whereas 15d-PGJ_2 is more potent in up-regulating PPAR-γ expression. In our opinion, and in keeping with previous reports (Chawla et al., 2001a; Moore et al., 2001; Hinz et al., 2003), the different profile of the two PPAR-γ agonists further suggests that their ability to inhibit cytokine release is partly independent of PPAR-γ expression. Indeed, no major differences were observed in the ability of both ligands to inhibit cytokine release in cells from smokers and non-smokers.

As largely established, smokers are at increased risk for developing atherosclerosis and tobacco smoke has been demonstrated to accelerate the progression of this disease through different mechanisms (Taylor et al., 1998). Tobacco smoke also affects cytokine expression; however, divergent effects, either stimulatory or inhibitory, have been observed, depending on the cell type and the period of exposure (Ouyang et al., 2000; Ryder et al., 2002).

Tobacco smoke is a complex mixture which contains approximately 5000 compounds (Stedman, 1968), including nicotine, nitrosamine, polycyclic aromatic hydrocarbons, aromatic amines, unsaturated aldehydes, phenolic compounds, and it is incorrect and even hazardous to award a single component the total effect induced by tobacco smoke.

We focused our attention on nicotine, since it represents the addictive component of cigarette smoke and affects the responsiveness of both neuronal and non-neuronal cells (Conti-Fine et al., 2000). Contradictory effects of nicotine are reported in the literature, since some authors demonstrated detrimental activities while others suggested a beneficial effect.

Lau et al. (2006) documented a 2.5-fold larger atherosclerotic lesion in nicotine-treated than placebo-treated mice, and nicotine was demonstrated to enhance adhesion molecule expression in human endothelial cells through macrophages releasing TNF-α and IL-1β (Wang et al., 2004). In immortalized cell lines, nicotine-induced apoptosis, increased oxidative stress and activated NF-κB (Crowley-Weber et al., 2003; Wu et al., 2002). Conversely, nicotine was reported to significantly reduce the secretion of inflammatory mediators in human monocytes and macrophage cell lines, by inhibiting NF-κB activation (Sugano et al., 1998; Vayssier et al., 1998). More recent studies suggested nicotine as a key regulator of monocyte/macrophages, recognizing its role in the cholinergic anti-inflammatory pathway (Borovikowa et al., 2000; De Jonge et al., 2005; Wang et al., 2003; Yoshikawa et al., 2006). Indeed, functional nicotinic acetylcholine receptors α-7 are present in human monocytes (Yoshikawa et al., 2006) and MDM (Wang et al., 2003): activation of these receptors by nicotine resulted in a dose-dependent inhibition of LPS-induced TNF-α and IL-6 release, but not IL-10 release (Wang et al., 2003; Yoshikawa et al., 2006). In human monocytes, nicotine inhibited the phosphorylation of I-κBα and suppressed the transcriptional activity of NF-κB (Yoshikawa et al., 2006). By interacting with

α7nAChR, nicotine also activated the transcription factor STAT3, a negative regulator of inflammatory responses, in mouse peritoneal macrophages (De Jonge et al., 2005).

We provide evidence that α7nAChR are present in human monocytes and MDM, a similar expression being documented in cells from three healthy non-smokers.

We also demonstrate that nicotine inconsistently affected basal cytokine release from human monocytes and MDM, but potently reduced PMA-evoked TNF-α and IL-6 release, but not IL-10 release. PMA was chosen as the monocyte/macrophage stimulus since Jiang et al. (1998) first demonstrated that PPAR-γ ligands inhibited PMA-evoked cytokine release, but not that evoked by LPS. The lack of inhibitory actions on IL-10 (the major anti-inflammatory cytokine, which is largely regulated by STAT3) can contribute to the anti-inflammatory effects of nicotine, as previously suggested (De Jonge et al., 2005). In addition, we show that nicotine enhances PPAR-γ expression in human monocytes and MDM: this effect is mediated by a nicotinic α7nACh receptor, since it is prevented by the selective antagonist, α-bungarotoxin.

Stimulation of PPAR-γ protein expression in human monocytes and MDM represents a novel activity for nicotine, which could contribute to the cholinergic anti-inflammatory pathway. In our hands, nicotine does not exert additive or synergistic effects with PPAR-γ ligands: we can hypothesize a scenario involving different signal transduction pathways but, at this stage, such a careful evaluation is beyond the aim of the paper.

It is important to underline that the nicotine concentrations we used in our *in vitro* experiments are in the same range as those measured in the blood (around 70 ng/ml; Russel et al., 1980) and tissues (0.5–2.6 times serum levels; Benowitz, 1988) of smokers. Therefore, in spite of the continuous exposure to a number of bioactive compounds and the resultant inflammatory state which, in our opinion, largely underlies the enhanced PPAR-γ protein expression in healthy smokers, it is conceivable to suggest a relevant role for nicotine, too. To our knowledge, only one recent paper (Lee et al., 2006) evaluated the potential interplay between PPAR-γ and tobacco smoke: in NCI-H292 cells (a human airway epithelial cell line) rosiglitazone inhibited smoke-induced TNF-α and mucin production and up-regulated PTEN (phosphatase and tensin homolog deleted on chromosome 10), which was suggested as a mechanism for PPAR-γ anti-inflammatory activity (Lee et al., 2006).

Conclusion

We have demonstrated a constitutively enhanced PPAR-γ expression in monocytes and, to a lesser extent, in MDM from healthy smokers. This effect is partly reproduced by *in vitro* challenge with physiologically relevant concentrations of nicotine. Although the clinical relevance of these findings remains to be elucidated, in keeping with the suggested anti-inflammatory role for PPAR-γ, we hypothesize that the two observed phenomena, enhancement of PPAR-γ expression and inhibition of cytokine release, could represent a protective mechanism to counteract tobacco smoke toxicity.

Acknowledgements

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RESEARCH PAPER

A novel activity for substance P: stimulation of peroxisome proliferator-activated receptor-γ protein expression in human monocytes and macrophages

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Background and purpose: Substance P (SP) and peroxisome proliferator-activated receptor-γ (PPAR-γ) play important roles in different inflammatory conditions and are both expressed in human monocytes and macrophages. However, it is not known whether or not they interact. This study was undertaken to evaluate the effects of SP on PPAR-γ protein expression in monocytes and macrophages (MDMs: monocyte-derived macrophages) from healthy smokers and non-smokers.
Experimental approach: PPAR-γ protein was detected by western blot and quantified by calculating the ratio between PPAR-γ and β-actin protein expression. Constitutive tachykinin NK_1 receptor expression in monocytes and MDMs from healthy smokers and non-smokers was evaluated by western blot. Cytokine release was evaluated by ELISA.
Key results: In the concentration range 10^{-10}–10^{-6} M, SP stimulated PPAR-γ protein expression in monocytes and MDMs, being more effective in cells from healthy smokers. Moreover, in these cells there was a constitutively increased expression of NK_1 receptors. SP-induced expression of the PPAR-γ protein was receptor-mediated, as it was reproduced by the NK_1 selective agonist [$Sar^9Met(O_2)^{11}$]SP and reversed by the competitive NK_1 antagonist GR71251. SP-induced maximal effects were similar to those evoked by 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2; an endogenous PPAR-γ agonist, and were significantly reduced by a PPAR-γ antagonist. NK_1 and PPAR-γ agonists exerted opposite effects on TNF-α release from monocytes and MDMs.
Conclusions and implications: Enhancement of PPAR-γ protein expression represents a novel activity for SP, which could contribute to a range of chronic inflammatory disorders.
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Keywords: substance P; PPAR-γ; monocytes; macrophages; NK_1 receptors; GR 71251; PGJ_2; TNF-α; tobacco smoke

Abbreviations: GR71251, [D-Pro^9,(spiro-γ-lactam)Leu^{10},Trp^{11}]substance P; GW9662, 2-chloro-5-nitrobenzanilide; MDM, monocyte-derived macrophage; 15d-PGJ_2, 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2; PPAR-γ, peroxisome proliferator-activated receptor-γ; SP, Substance P

Introduction

Although substance P (SP) was originally described as a peptide of neuronal origin, studies in rodents and humans demonstrated its production by inflammatory cells (for example, macrophages, eosinophils, lymphocytes and dendritic cells) and suggested that this neuropeptide could be an autocrine, paracrine or endocrine regulator (Maggi, 1997; Severini *et al*., 2002; O'Connor *et al*., 2004). In monocyte/macrophages, SP stimulates the release of both arachidonic acid metabolites and proinflammatory cytokines, induces the respiratory burst and acts as a potent chemoattractant (Lotz *et al*., 1988; Brunelleschi *et al*., 1990, 1998; O'Connor *et al*., 2004; Bardelli *et al*., 2005), most of the proinflammatory effects of SP being mediated by NK_1 receptors. We previously reported that SP and selective NK_1 agonists induce superoxide anion production, tumour-necrosis factor (TNF)-α release (as well as an enhanced TNF-α mRNA expression) and triggers activation of nuclear factor-κB in human monocytes and alveolar macrophages (Brunelleschi *et al*., 1998; Bardelli *et al*., 2005). Interestingly, very relevant increases in NK_1 receptor expression (>threefold), TNF-α release (about fourfold) and nuclear factor-κB nuclear translocation (threefold) were documented in alveolar macrophages from healthy smokers as compared with non-smokers (Bardelli *et al*., 2005).

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The peroxisome proliferator-activated receptor-γ (PPAR-γ), a key regulator of adipocyte differentiation, lipid storage and glucose metabolism, is expressed in a wide variety of cells, including monocytes, macrophages and foam cells (Ricote *et al*., 1998b; Tontonoz *et al*., 1998; Amoruso *et al*., 2007). Even if a recent meta-analysis raised some concerns about the serious cardiovascular effects of rosiglitazone treatment in type II diabetes patients (Nissen and Wolski, 2007), PPAR-γ agonists have been proposed as possible anti-inflammatory drugs.

We previously showed that PPAR-γ protein is constitutively present in human monocytes and that its expression is upregulated along with differentiation to monocyte-derived macrophages (MDMs); moreover, monocytes and MDMs from healthy smokers presented a significantly enhanced constitutive PPAR-γ expression, as compared with healthy non-smokers (Amoruso *et al*., 2007).

Despite the fact that both PPAR-γ and NK_1 receptors are expressed at significant levels in human monocyte/macrophages, it is not known whether or not they interact with each other.

Therefore, to evaluate such a possibility, we examined the ability of SP, as well as the selective NK_1 agonist [Sar^9 $Met(O_2)^{11}$]SP and the NK_1 antagonist GR71251, to modulate PPAR-γ protein expression in human monocytes and MDMs from healthy smokers and non-smokers. We demonstrated that, in a concentration-dependent manner, SP stimulated PPAR-γ protein expression in both cell types and that this effect was potently reduced by a PPAR-γ antagonist or an NK_1 antagonist. We also report here that SP and PPAR-γ ligands exerted divergent effects on TNF-α release, which was stimulated by SP and NK_1 agonists and inhibited by PPAR-γ agonists. However, the evidence that a PPAR-γ antagonist enhances SP-induced cytokine release further supports the possibility of cross-talk between the two receptors.

Methods

Preparation of human monocytes and monocyte-derived macrophages

This study and the research protocol were approved by the Local Ethical Committee; informed written consent was obtained by all participants. Human monocytes were isolated from fresh buffy-coat preparations of whole human blood, collected from healthy non-smokers and smokers of both sexes, as described (Amoruso *et al*., 2007). Briefly, the mononuclear cell fraction was diluted with phosphate-buffered saline (PBS, pH 7.4), layered over a Histopaque (density = 1.077 g cm^{-3}) gradient solution, centrifuged (400 *g*, 30 min, room temperature) and recovered by thin suction at the interface. The mononuclear cell layer was mixed with PBS and centrifuged for 10 min; cells were then resuspended in RPMI 1640 medium, supplemented with 5% heat-inactivated fetal bovine serum, 2 mM glutamine, 10 mM Hepes, 50 μg ml^{-1} streptomycin, 5 U ml^{-1} penicillin and 2.5 μg ml^{-1} amphotericin B. Purified monocytes were obtained by adhesion (90 min, 37 °C, 5% CO_2), non-adherent cells (mainly lymphocytes) being removed by three gentle washes with PBS; cell viability (Trypan blue dye exclusion) was usually >98% (Brunelleschi *et al*., 1998; Amoruso *et al*., 2007). Monocyte-derived macrophages (MDMs) were prepared from monocytes cultured for 8–10 days in a 5% CO_2 incubator at 37 °C in RPMI 1640 medium containing 20% fetal bovine serum, 2 mM glutamine, 10 mM HEPES and antibiotics; medium was changed every 2–3 days (Amoruso *et al*., 2007). MDMs were defined as macrophage-like cells, by evaluating surface markers CD14, MHCII, CD1a and CD68. Briefly, adherent cells were detached by gentle scraping with a plastic scraper. After three washings with sterile PBS, cells were resuspended at the final concentration of 1×10^5 cells ml^{-1} and fluorescent dye-labelled antibodies against the different surface markers (anti-CD14 from Becton Dickinson, Oxford, UK; anti-CD68 and anti-MHCII from Dako, Milan, Italy; anti-CD1a from eBioscence, San Diego, CA, USA) were added for 30 min on ice. Incubation was performed in the dark and expression of surface markers was analysed by flow cytometry.

TNF-α release in monocytes and MDMs

Cells (1×10^6) were treated in the absence or presence of the PPAR-γ agonist 15d-PGJ_2, (used at 1–10 μM) for 30 min and then challenged with SP (10^{-8}–10^{-6} M) for 24 h; supernatants were collected and stored at −20 °C. This 24-h stimulation time was chosen to ensure maximal cytokine release, as observed previously (Bardelli *et al*., 2005; Gunella *et al*., 2006). In some cases, cells were pretreated for 30 min with the NK_1 antagonist GR71251 (10^{-8}–10^{-6} M) or the PPAR-γ antagonist GW9662 (2-chloro-5-nitrobenzanilide; 10^{-6} M) and then stimulated by SP (10^{-6} M). TNF-α in the samples was estimated by ELISA (Pelikine Compact human ELISA kit) following the manufacturer's instructions (CLB/Sanquin, Amsterdam, The Netherlands). No cross-reactivity was observed with any other known cytokine; results are expressed in pg ml^{-1}.

PPAR-γ protein expression in monocytes and MDMs

Cells from healthy smokers and non-smokers were evaluated either as untreated (that is, 'basal, constitutive PPAR-γ expression') or after challenge (6 h, 37 °C, 5% CO_2) with SP (concentration range: 10^{-10}–10^{-6} M); the PPAR-γ ligand 15d-PGJ_2 (10 μM) was used for comparison. To confirm that enhancement of PPAR-γ expression is a receptor-mediated effect, cells were also challenged with the selective NK_1 agonist [$Sar^9Met(O_2)^{11}$]SP, or were pretreated for 30 min with the NK_1 antagonist GR71251 (10^{-9}–10^{-6} M) and then challenged with SP. In some experiments, cells were pretreated for 30 min with the PPAR-γ antagonist GW9662 (used at 10^{-6} M) and then stimulated by SP.

Cells (2×10^6), seeded in six-well plates, were washed twice with ice-cold PBS and scraped in lysis buffer containing 3% SDS, 0.25 M Tris and 1 mM phenylmethylsulphonyl fluoride and lysed by sonication; when necessary, cell lysates were stored at −80 °C. The determination of protein concentration was done with a Bradford-based assay. Protein samples (20 μg) were analysed by SDS-polyacrylamide gel electrophoresis (10% acrylamide) and electroblotted on nitrocellulose membrane (Protran; PerkinElmer Life Sciences,

Boston, MA, USA). Immunoblots were performed as described (Amoruso *et al*., 2007) using the following antibodies: monoclonal mouse anti-human PPAR-γ (E-8; Santa Cruz Biotechnology, Santa Cruz, CA, USA; 1:1000 in TBS-T 5% milk) and monoclonal mouse anti-human β-actin (Sigma, St Louis, MO, USA; 1:5000 in TBS-T 3% BSA). Anti-mouse secondary antibody was coupled to horseradish peroxidase (Amersham Biosciences, Buckinghamshire, UK). Proteins were visualized with an enzyme-linked chemiluminescence detection kit according to the manufacturer's (PerkinElmer) instructions. Chemiluminescence signals were analysed under non-saturating conditions with an image densitometer (Versadoc; Bio-Rad, Hercules, CA, USA). Quantification of PPAR-γ protein was performed by calculating the ratio between PPAR-γ and β-actin protein expression; the latter was selected as reference housekeeping protein.

Western blotting for NK_1 receptors in monocyte and MDM membranes

Cells (3×10^6), seeded in six-well plates, were washed twice with ice-cold PBS and scraped in ice-cold PBS containing protease inhibitors (10 μg ml^{-1} aprotinin, 10 μg ml^{-1} pepstatin, 50 μg ml^{-1} leupeptin, 1 mM phenylmethylsulphonyl fluoride) and centrifuged (14 000 *g*; 30 s, 4 °C). The pellet was resuspended in 10 mM Tris-HCl, containing 1 mM EDTA and protease inhibitors, and subjected to three cycles of rapid freezing and thawing. The lysate was centrifuged (14 000 *g*; 15 min, 4 °C); the pellet (membranes) so obtained was suspended in 10 mM Tris-HCl, 10% sucrose and protease inhibitors, and used for the western blot experiments. The determination of protein concentration was done with a Bradford-based assay. Protein samples (20 μg) were analysed by SDS-polyacrylamide gel electrophoresis (10% acrylamide) and electroblotted on nitrocellulose membrane (Protran; PerkinElmer Life Sciences). Immunoblots were performed as described (Bardelli *et al*., 2005) using a rabbit polyclonal NK_1 receptor antibody (ab466; Abcam, Cambridge, UK; 1:1000 in TBS-T 5% milk) specific for human NK_1 receptors, and a monoclonal anti-Na^+/K^+ ATPase (α-subunit) antibody (clone M7-PB-E9; Sigma, St Louis, MO, USA; 1:250 in TBS-T 5% milk;). Proteins were visualized by using ECL western blotting detection reagents (PerkinElmer); Na^+/K^+ ATPase was selected as reference housekeeping membrane enzyme. Quantification of western blots was performed by densitometry using 'Quantity One, 1-D Analysis' software (Bio-Rad) and expressed as the ratio between NK_1 receptor and Na^+/K^+ ATPase protein expression.

Data and statistical analyses

Data are mean ± s.e.mean of *n* independent experiments. Concentration–effect curves for SP and for the NK_1 antagonist GR71251 were constructed; EC_{50} values (for SP) and IC_{50} values (for GR71251) were interpolated from curves of best fit. Statistical evaluation was performed by one-way or two-way ANOVA and Bonferroni post-test correction.

Drugs and analytical reagents

Fetal bovine serum was from Gibco (Paisley, UK). PBS, Histopaque, RPMI 1640, glutamine, HEPES, streptomycin, penicillin, amphotericin B, protease inhibitors, monoclonal anti-Na^+/K^+ ATPase (α-subunit) and monoclonal mouse anti-human β-actin antibodies were obtained from Sigma (Milwaukee, WI, USA). The rabbit polyclonal NK_1 receptor antibody (ab466; specific for human NK_1 receptors) was from Abcam; the monoclonal mouse anti-human PPAR-γ (E-8) antibody was from Santa Cruz. The PPAR-γ agonist 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 was from Biomol (Plymouth Meeting, PA, USA) and the PPAR-γ antagonist GW9662 was from Cayman Chemical (Ann Arbor, MI, USA). SP, [Sar^9 $Met(O_2)^{11}$]SP and GR71251 ([D-Pro^9,(spiro-γ-lactam)-Leu^{10},Trp^{11}]substance P) were from Neosystem (Strasbourg, France). Tissue-culture plates were from Nunc Ltd (Roskilde, Denmark); all cell culture reagents, with the exception of fetal bovine serum, were endotoxin free according to details provided by the manufacturer.

Results

Characterization of human monocyte/macrophage preparations

As shown in Figure 1, monocytes cultured for 8–10 days in RPMI medium supplemented with 20% fetal calf serum acquired a macrophage-like profile, which was accompanied by an increase in $CD68^+$ cells and a decrease of $CD14^+$ cells as compared with monocytes. Moreover, the absence of CD1a expression demonstrated that no differentiation towards dendritic cells occurred in our MDM preparations, as previously documented (Amoruso *et al*., 2007).

SP stimulates PPAR-γ protein expression in human monocytes and MDMs

To quantify PPAR-γ protein expression in both monocytes and MDMs from healthy smokers and non-smokers, we calculated the ratio between PPAR-γ and β-actin protein expression; in our experiments, β-actin levels were constant and stable in each cell type and were neither induced nor inhibited by the different 6-h *in vitro* treatments.

As illustrated by Figure 2a (a representative western blot of monocyte/macrophages from healthy non-smokers), constitutive PPAR-γ protein was detected in monocytes and MDMs and its expression was upregulated along with differentiation into mature macrophages. A 6-h challenge with the endogenous PPAR-γ ligand 15d-PGJ_2 (used at 10 μM) or with SP enhanced PPAR-γ expression in monocytes and MDMs from healthy non-smokers (Figure 2a). To ensure a better evaluation of SP-induced PPAR-γ expression, we performed concentration–response curves. As shown in Figure 2b, dealing with cells from five healthy non-smokers, SP, in the concentration range 10^{-10}–10^{-6} M, stimulated PPAR-γ expression in human monocytes and MDMs. Maximal effect (about twofold increase) was observed with SP 10^{-6} M and was quantitatively similar to that induced by the endogenous PPAR-γ agonist 15d-PGJ_2 (Figure 2b). The calculated EC_{50} values are similar in both cell types: 19 nM in monocytes and 17 nM in MDMs (Figure 2b). By evaluating SP-induced effects in cells obtained from four healthy smokers, we confirmed our previous observation (Amoruso *et al*., 2007) that exposure to tobacco smoke *in vivo* greatly affects PPAR-γ

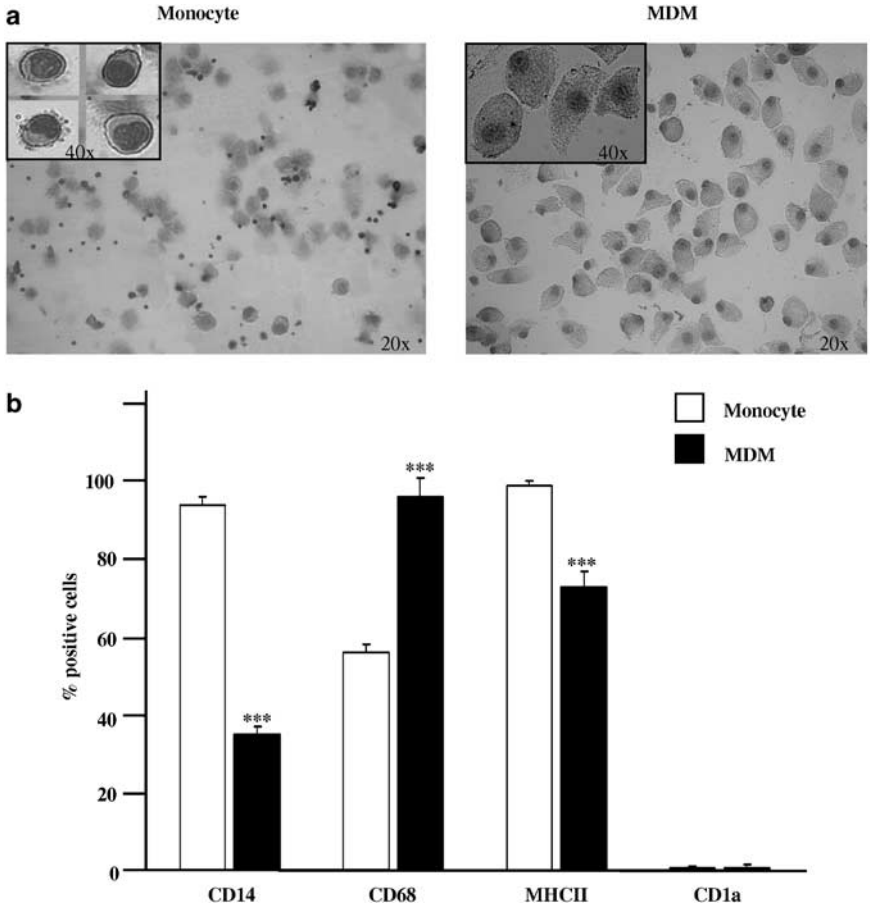

Figure 1 Morphology and phenotype of monocytes/macrophages from healthy non-smokers. In (**a**), May–Gruenewald–Giemsa stain of monocytes and monocyte-derived macrophages (MDMs) from healthy non-smokers. Magnification $\times 20$ (magnification $\times 40$ in the insets). In (**b**), surface marker expression in monocytes and MDMs. Data are means ± s.e.mean; $n=4$; ***$P<0.001$ vs monocytes.

expression. Monocytes and MDMs from healthy smokers exhibited a significantly enhanced constitutive PPAR-γ protein expression as compared with non-smokers (Figure 2c). In this case, too, SP dose dependently stimulated PPAR-γ expression, with EC_{50} values (6 nM in monocytes, 4 nM in MDMs) lower than those measured in cells from non-smokers (Figure 2c). Also in cells from healthy smokers, the maximal SP-induced effect was observed at 10^{-6} M and was quantitatively similar to that for 15d-PGJ_2 (Figure 2c).

Stimulation of PPAR-γ induced by SP was mediated by NK_1 receptors

To demonstrate that SP-induced PPAR-γ protein expression is a receptor-mediated activity, we evaluated the effects of selective NK_1 receptor agonists and antagonists. As shown in Figure 3, SP effects were reproduced, although to a minor extent, by the NK_1 selective agonist [$Sar^9Met(O_2)^{11}$]SP in both monocytes and MDMs from healthy non-smokers ($n=5$; Figure 3a) and healthy smokers ($n=4$; Figure 3b). At the highest concentration evaluated, 10^{-6} M, the NK_1 antagonist GR71251, which had no effect by itself, completely reversed the SP-induced effects (Figure 3). Interestingly, expression of PPAR-γ protein induced by SP was largely inhibited when cells were pretreated for 30 min with GW9662, a PPAR-γ antagonist, used at 10^{-6} M (Figure 3). GW9662, given alone, did not modify constitutive PPAR-γ protein expression (data not shown).

Over a concentration range (10^{-9}–10^{-6} M), the NK_1 receptor antagonist, GR71251, was more effective in reversing SP-induced PPAR-γ protein expression in cells from healthy smokers (Figure 4). At the highest concentration tested, this antagonist reversed almost completely this effect of SP, yielding a level of PPAR-γ protein very similar to that in untreated cells that is, basal, constitutive levels. These data were obtained by subtracting the value of basal constitutive PPAR-γ expression (in monocytes and MDMs, non-smokers and smokers) from all the determinations with SP. The calculated IC_{50} values were 84 and 38 nM in monocytes from non-smokers and smokers, respectively. In MDM, the IC_{50} values for GR71251 were 77 nM (non-smokers) and 19 nM (smokers) (Figure 4).

SP-induced cytokine release and modulation by PPAR-γ ligands

Previous reports from our and other laboratories indicated that PPAR-γ agonists inhibited the release of proinflammatory cytokines in monocyte/macrophages (Jiang *et al.*, 1998;

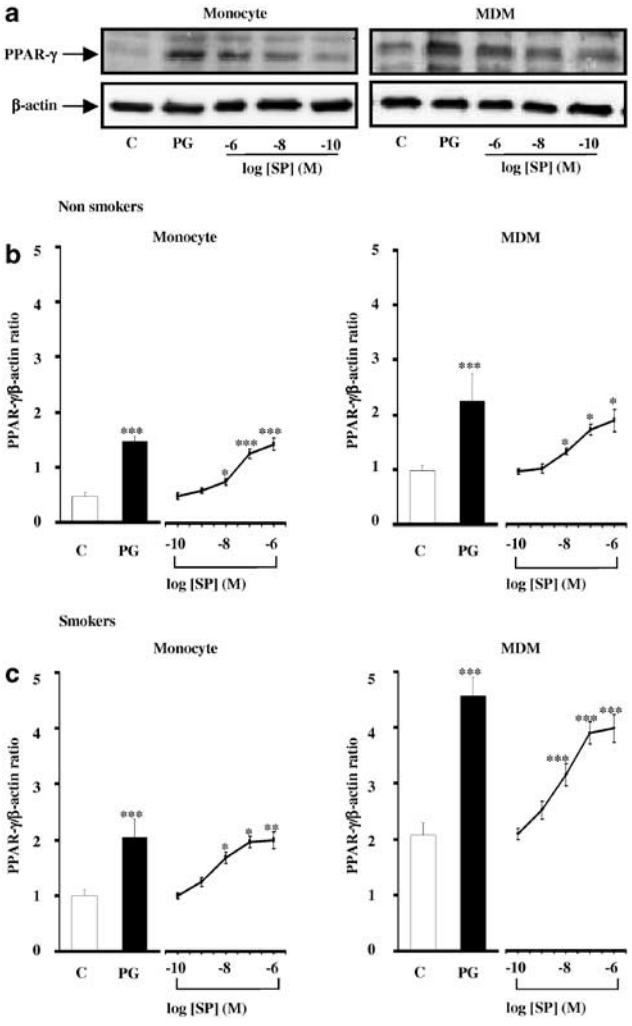

Figure 2 Concentration-dependent effects of substance P (SP) on peroxisome proliferator-activated receptor-γ (PPAR-γ) protein expression in human monocytes and monocyte-derived macrophages (MDMs) from healthy non-smokers. A representative western blot of PPAR-γ and β-actin in monocytes and MDMs from a non-smoker male volunteer (in **a**); SP-induced PPAR-γ expression in human monocytes and MDMs from five healthy non-smokers (in **b**) and four healthy smokers (in **c**). Cells were challenged for 6 h in the absence (C, control) or presence of 15d-PGJ_2 (15-deoxy-$\Delta^{12,14}$-prostaglandin J_2; PG, 10 μM; shown for comparison) and SP (10^{-10}–10^{-6} M). Results are expressed as PPAR-γ/β-actin ratio. Means ± s.e.mean; $n=4$–5. ***$P<0.0001$, **$P<0.001$, *$P<0.05$ vs control.

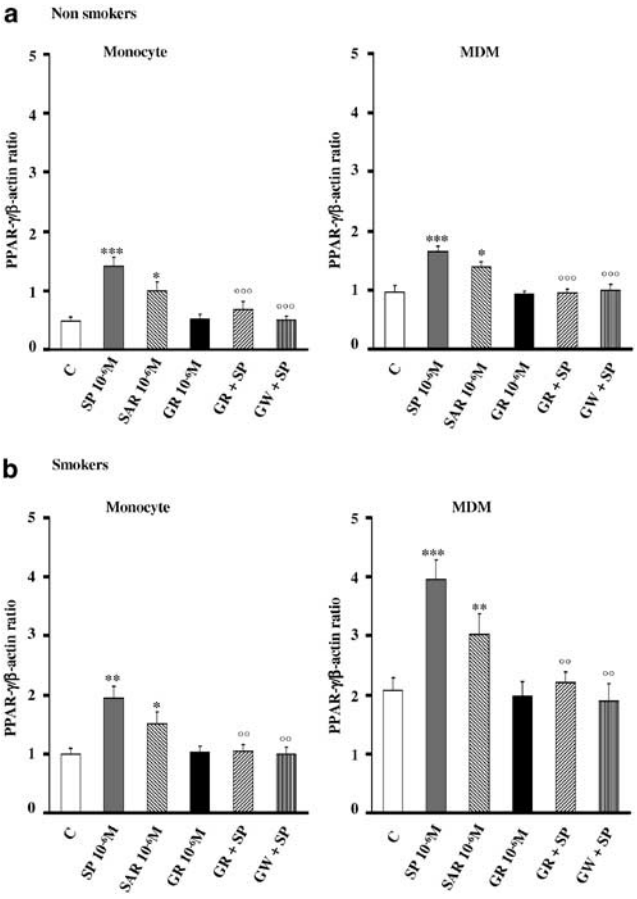

Figure 3 Effects of selective NK_1 receptor agonists and antagonists on peroxisome proliferator-activated receptor-γ (PPAR-γ) protein expression. Monocytes and monocyte-derived macrophages (MDMs) from non-smokers (**a**) and smokers (**b**) were challenged for 6 h with substance P (SP, 10^{-6} M), the NK_1 selective agonist [$Sar^9Met(O_2)^{11}$]SP (SAR, 10^{-6} M), the NK_1 antagonist GR71251 ([D-Pro^9,(spiro-γ-lactam)Leu^{10},Trp^{11}]substance P; GR, 10^{-6} M), a combination of SP + GR71251, or SP + PPAR-γ antagonist GW9662 (2-chloro-5-nitrobenzanilide; GW, 10^{-6} M). Results are expressed as PPAR-γ/β-actin ratio. Means ± s.e.mean; $n=4$–5. ***$P<0.0001$, **$P<0.001$, *$P<0.05$ vs control; °°°$P<0.0001$, °°$P<0.001$ vs SP.

Ricote *et al*., 1998b; Amoruso *et al*., 2007), whereas SP and NK_1 agonists induced the release of TNF-α, IL-1β and IL-6 (Lotz *et al*., 1988; Brunelleschi *et al*., 1998; Bardelli *et al*., 2005). Besides confirming these data, we now provide evidence of the interaction between SP and PPAR-γ.

As shown in Table 1, SP-induced TNF-α release from monocytes isolated from healthy smokers and non-smokers was inhibited, in a concentration-dependent manner, by both the PPAR-γ agonist 15d-PGJ_2 and the NK_1 receptor antagonist GR71251. When cells were pretreated for 30 min with the PPAR-γ antagonist GW9662, used at 10^{-6} M, and then challenged by SP, an enhanced cytokine release ($P<0.05$; $n=4$) was observed. In keeping with our previous data (Bardelli *et al*., 2005; Gunella *et al*., 2006; Amoruso *et al*., 2007), cells from smokers released higher amounts of TNF-α, compared with non-smokers (Table 1). Similar results were also obtained in MDMs (data not shown).

NK_1 receptor expression in monocytes and MDMs from healthy smokers and non-smokers

Our previous observations demonstrated the presence of authentic NK_1 receptor in human alveolar macrophages, a threefold enhanced expression being observed in healthy smokers (Bardelli *et al*., 2005). We now confirmed these observations in peripheral monocytes and MDMs, too. The western blot experiments performed in cells from four healthy smokers and five healthy non-smokers (Figure 5) clearly indicated that MDMs have a higher membrane expression of NK_1 receptors than monocytes, and that cells from smokers (Figure 5b) have higher NK_1 receptor content

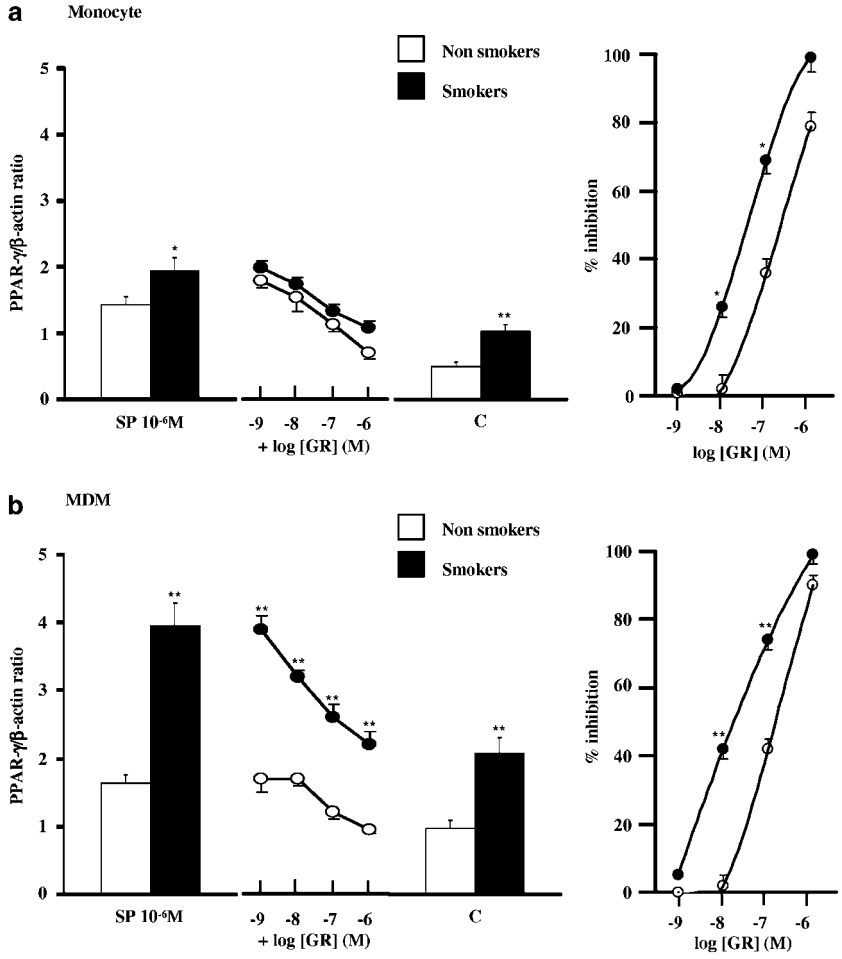

Figure 4 Substance P (SP)-induced peroxisome proliferator-activated receptor-γ (PPAR-γ) expression was mediated by NK_1 receptors: reversal by the NK_1 antagonist GR71251 ([D-Pro^9,(spiro-γ-lactam)Leu^{10},Trp^{11}]substance P). Monocytes (in **a**) and monocyte-derived macrophages (MDMs) (in **b**) from non-smokers and smokers were challenged for 30 min with increasing concentrations of GR71251 (10^{-9}–10^{-6} M) and then exposed to SP 10^{-6} M. Control, unstimulated (C) cells and SP-stimulated cells are shown for comparison. Results are expressed as PPAR-γ/β-actin ratio (on the left) and as % inhibition of SP-induced effect (on the right). Means ± s.e.mean; $n = 4$. $^{**}P<0.001$, $^{*}P<0.05$ vs non-smokers. See text for further details.

Table 1 TNF-α release (pg ml^{-1}) in monocytes isolated from healthy smokers and non-smokers

	Non-smokers (n = 4)	*Smokers* (n = 4)
Control	110 ± 10	280 ± 15
Control + 15d-PGJ$_2$ (10^{-6} M)	90 ± 10	268 ± 10
Control + 15d-PGJ$_2$ (10^{-5} M)	63 ± 8**	170 ± 15**
SP (10^{-8} M)	200 ± 20*	420 ± 12*
SP (10^{-6} M)	270 ± 15**	600 ± 15**
GR (10^{-8} M) + SP (10^{-6} M)	200 ± 10	520 ± 10
GR (10^{-6} M) + SP (10^{-6} M)	125 ± 15°	290 ± 12°°
GW (10^{-6} M) + SP (10^{-6} M)	340 ± 10°°	740 ± 15°°
15d-PGJ$_2$ (10^{-6} M) + SP (10^{-6} M)	238 ± 12	560 ± 8
15d-PGJ$_2$ (10^{-5} M) + SP (10^{-6} M)	140 ± 10°°	310 ± 10°°

GR, GR71251 ([D-Pro^9,(spiro-γ-lactam)Leu^{10},Trp^{11}]substance P), NK_1 antagonist; GW, GW9662 (2-chloro-5-nitrobenzanilide), PPAR-γ antagonist; 15d-PGJ$_2$, 15-deoxy-$\Delta^{12,14}$-prostaglandin J$_2$; PPAR-γ, peroxisome proliferator-activated receptor-γ; SP, substance P; TNF-α, tumour-necrosis factor-α.
Data are means ± s.e.mean. $^{*}P<0.05$, $^{**}P<0.01$ vs control, unstimulated monocytes; $^{\circ}P<0.05$, $^{\circ\circ}P<0.01$ vs SP (10^{-6} M).

than monocytes/macrophages from non-smokers (Figure 5a). The calculated NK_1 receptor: Na^+/K^+ ATPase protein ratios in Figure 5c summarize the results from the western blots.

Discussion

The results presented in this paper show, for the first time, that SP, by activation of NK_1 receptors, enhanced PPAR-γ protein expression in human monocytes/macrophages, suggesting the possibility of a physiologically relevant cross-talk between the two receptors.

PPAR-γ is expressed in a wide variety of cells, including human monocytes and macrophages, its expression being stimulated by endogenous (for example, 15d-PGJ$_2$, oxidized low-density lipoproteins, advanced glycation end products) or exogenous ligands, mainly thiazolidinedione antidiabetic drugs (Nagy *et al*., 1998; Ricote *et al*., 1998a, 1999; Tontonoz *et al*., 1998; Scher and Pillinger, 2005; Amoruso *et al*., 2007). Despite a number of diverging reports (Nagy *et al*., 1998; Chinetti *et al*., 2000; Desmet *et al*., 2005), most experimental data indicated that the anti-inflammatory potential of PPAR-γ mainly resides in the ability of PPAR-γ agonists to inhibit monocyte/macrophage activation and expression of inflammatory molecules, that is, TNF-α, IL-6, IL-1β, inducible nitric oxide synthase, gelatinase B and COX-2 (Chinetti *et al*., 1998; Jiang *et al*., 1998; Ricote *et al*.,

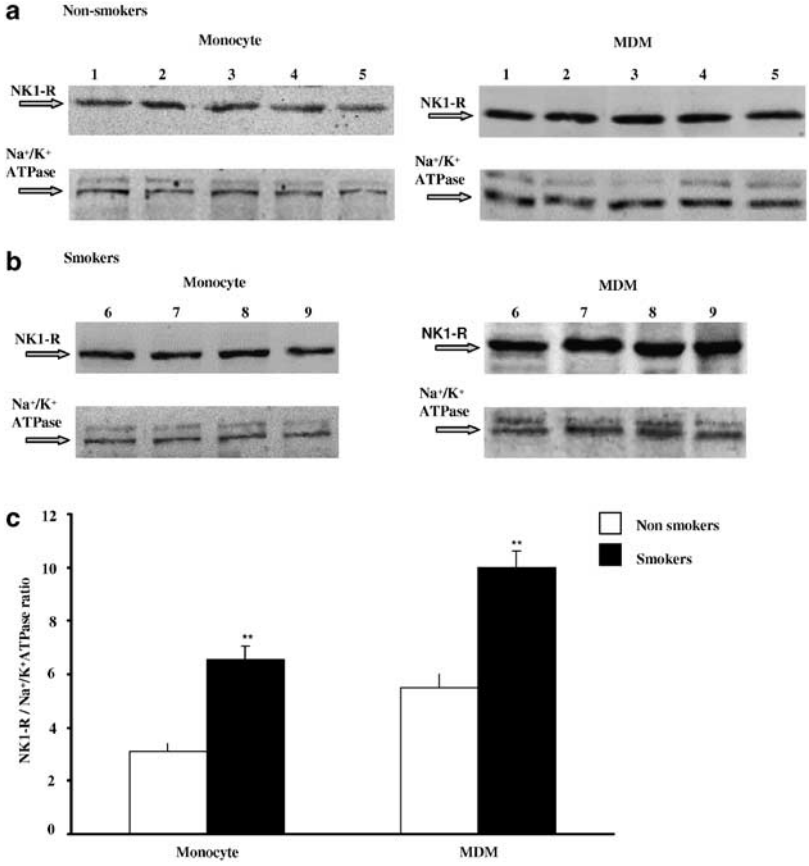

Figure 5 Western blots of NK_1 receptors (NK_1-R) and Na^+/K^+ ATPase. In (**a**), monocytes and monocyte-derived macrophages (MDMs) from non-smokers (lanes 1–5 = different healthy non-smokers). In (**b**), monocytes and MDMs from smokers (lanes 6–9 = different healthy smokers). In (**c**), NK_1 receptor and Na^+/K^+ ATPase ratio in monocytes and MDMs from non-smokers ($n=5$) and smokers ($n=4$). Means ± s.e.mean; $^{**}P<0.001$ vs non-smokers. See text for further details.

1998a, b, 1999; Subbaramaiah *et al*., 2001; Amoruso *et al*., 2007).

Interestingly, the anti-inflammatory effects of PPAR-γ have been demonstrated in animal models of arthritis, ischaemia reperfusion, inflammatory bowel diseases and chronic airway inflammation (see Daynes and Jones, 2002; Scher and Pillinger, 2005 and Belvisi *et al*., 2006), that is, pathological conditions in which a role for SP has long been established (Mantyh *et al*., 1995; Maggi, 1997; Keeble and Brain, 2004; O'Connor *et al*., 2004; Keeble *et al*., 2005; Reed *et al*., 2005). Moreover, previous studies indicate that NK_1 receptors are upregulated at inflamed sites in many tissues, including joints and intestine (Mantyh *et al*., 1995; Keeble and Brain, 2004; Keeble *et al*., 2005; Reed *et al*., 2005). By using a rabbit polyclonal anti-human NK_1 receptor antibody, we first demonstrated that NK_1 receptor expression was potently upregulated in alveolar macrophages from healthy smokers as compared with non-smokers (Bardelli *et al*., 2005). At variance from those experiments, in which we used total cell extracts (Bardelli *et al*., 2005), we have now employed membrane extracts to evaluate NK_1 receptor expression in monocytes and MDMs. The use of membrane extracts allows us to evaluate only those NK_1 receptors that are present and functionally active (see also below, cytokine experiments) on cell membranes. Consistent with our previous observations (Bardelli *et al*., 2005), we report here that cells from healthy smokers have more than double the NK_1 receptor expression of cells from non-smokers and that MDMs have significantly higher NK_1 receptor content than monocytes.

Therefore, we are now suggesting that the increased expression of NK_1 receptor in the cell surface of monocyte/macrophages from healthy smokers is not only associated with, but could also be responsible for, the higher PPAR-γ expression induced by SP in smokers. This suggestion mainly comes from the following experimental results we obtained.

First, SP, at physiological concentrations and in a concentration-dependent manner, stimulated PPAR-γ protein expression in human monocytes and MDMs, with a maximal effect similar to the endogenous PPAR-γ agonist 15d-PGJ_2 and a greater efficiency in cells from healthy smokers (as demonstrated by the lower EC_{50} values). The EC_{50} values we calculated for SP-induced PPAR-γ expression in monocytes and MDMs from non-smokers (19 and 17 nM, respectively) are identical to the K_D value (19 nM) reported by Hartung *et al*. (1986) for SP-binding studies in guinea-pig macrophages, whereas lower EC_{50} values (4 and 6 nM, in MDMs and monocytes, respectively) were documented in cells from smokers.

Then, we have also demonstrated that SP-induced PPAR-γ protein expression was a receptor-mediated effect, as it was

reproduced by the NK_1 selective agonist $[Sar^9Met(O_2)^{11}]SP$ and reversed by the competitive NK_1 antagonist GR71251. Interestingly, GR71251 is more potent in cells from healthy smokers than non-smokers; IC_{50} values were 84 and 77 nM (monocytes and MDMs, respectively) in non-smokers and 38 and 19 nM (monocytes and MDMs, respectively) in smokers. In isolated spinal cord preparations of neonatal rats, GR71251 was demonstrated to cause a rightward shift of the concentration–response curve for SP with a pA2 value of 6.14 (Guo *et al*., 1993). The IC_{50} values we measured are far below the previously reported pA2; however, it must be noted that, apart from the different experimental models and the possible variations in affinity due to the different species (human and rat), in human monocytes/macrophages, SP acts at concentrations lower than those used by Guo *et al*. (1993).

Finally, there are two major experimental results that, in our opinion, indicate the relevance of cross-talk between SP and PPAR-γ: the ability of a PPAR-γ antagonist to potently reduce SP-induced PPAR-γ expression, as well as the ability of PPAR-γ ligands to affect SP-induced TNF-α release. As is well-known, SP stimulates proinflammatory cytokine release (Lotz *et al*., 1988; Lee *et al*., 1994; Delgado *et al*., 2003; Bardelli *et al*., 2005), whereas PPAR-γ agonists reduce it (Chinetti *et al*., 1998; Jiang *et al*., 1998; Ricote *et al*., 1998a, b, 1999; Subbaramaiah *et al*., 2001; Amoruso *et al*., 2007). In our experiments, SP-induced TNF-α release was inhibited, in a concentration-dependent manner, by the endogenous PPAR-γ ligand 15d-PGJ_2 and increased in the presence of GW9662, a PPAR-γ antagonist.

Cytokine release is a complex phenomenon, which involves several signal pathways and it is tightly regulated, often in a stimulus- and cell-specific manner (Bondeson *et al*., 1999; Andreakos *et al*., 2004). We can postulate a scheme involving different signal transduction pathways but, at this stage, such evaluation is beyond the scope of the paper. It is nevertheless tempting to speculate that the ability of 15d-PGJ_2 to inhibit SP-induced TNF-α release *in vitro* could similarly affect cytokine release *in vivo*. Interestingly, human monocytes and macrophage cell lines have been shown to express higher levels of NK_1 receptors in response to TNF-α and other cytokines (Ho *et al*., 1997; Marriott and Bost, 2000; Simeonidis *et al*., 2003; Arsenescu *et al*., 2005), and SP has been shown to participate in positive feedback loops, in which it enhances the production of cytokines that, on their own, increase SP secretion and/or NK_1 receptor stimulation (Reinke and Fabry, 2006). So, the documented level of expression of a given receptor in a given condition is the resultant of the interplay between various factors. We have disclosed the existence of such interplay by demonstrating that SP, a well-known proinflammatory mediator, is able to enhance the expression of PPAR-γ, a suggested anti-inflammatory receptor. Although the clinical relevance of these results remains to be elucidated, it is worth reminding that tobacco smoke potently affects both PPAR-γ expression and SP/NK_1 receptor function. A number of experimental observations have described the acceleration, by tobacco smoke, of the progression of atherosclerosis through different mechanisms, and epidemiological and clinical findings indicate that smokers have an increased risk to develop atherosclerosis (Taylor *et al*., 1998).

The results of this study demonstrate that monocytes and MDMs from healthy smokers present an enhanced NK_1 receptor expression and that, in both cell types, SP stimulates PPAR-γ expression with a greater efficiency, compared with monocytes/macrophages from non-smokers. This represents a novel activity for SP, which could play a role in chronic inflammatory conditions, such as atherosclerosis, rheumatoid arthritis and inflammatory bowel diseases.

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Conflict of interest

The authors state no conflict of interest.

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Minor polar compounds extra-virgin olive oil extract (MPC-OOE) inhibits NF-κB translocation in human monocyte/macrophages

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Abstract

Epidemiological studies demonstrate that the Mediterranean diet, in which olive oil is the major source of fat, reduces the risk of coronary heart disease and cancer. It has been proposed that the beneficial effects of olive oil not only depend on oleic acid, but are also associated with minor polar compounds (MPC). A positive correlation between inflammation and cardiovascular diseases has long been described, monocyte/macrophages and NF-κB playing a pivotal role. The aim of this work was to investigate the effects of an extra-virgin olive oil extract (MPC-OOE), particularly rich in MPC and prepared by some of us, on NF-κB translocation in monocytes and monocyte-derived macrophages (MDM) isolated from healthy volunteers. In a concentration-dependent manner, MPC-OOE inhibited p50 and p65 NF-κB translocation in both un-stimulated and phorbol-myristate acetate (PMA)-challenged cells, being particularly effective on the p50 subunit. Interestingly, this effect occurred at concentrations found in human plasma after nutritional ingestion of virgin olive oil and was quantitatively similar to the effect exerted by ciglitazone, a PPAR-γ ligand. However, MPC-OOE did not affect PPAR-γ expression in monocytes and MDM. These data provide further evidence of the beneficial effects of extra-virgin olive oil by indicating its ability to inhibit NF-κB activation in human monocyte/macrophages.

Keywords: Olive oil; Minor polar compounds; NF-κB; p50 subunit; p65 subunit; PPAR-γ; Human monocyte/macrophages

1. Introduction

As early as the XV century, Michele Savonarola (1384–1468), professor at the Universities of Padua and Ferrara, recognized virgin olive oil as the best and most wholesome among the condiments and recommended to Borso d'Este, the Duke of Ferrara, to always dress his food with oil obtained from the juice of sour olives [1].

Nowadays, extensive scientific evidence shows that the Mediterranean diet, which is rich in fruits, vegetables and olive oil, prevents the onset and progression of coronary heart disease (CHD), metabolic disorders and some types of cancer, and indicates a remarkably low cardiovascular mortality in the Mediterranean area [2–8].

Research on the impact of olive oil consumption on CHD and mortality has expanded over the last decades, but interest has progressively moved from the role of the major component, i.e. the monounsaturated fatty acid oleic acid [4], to that of the minor polar compounds (MPC), especially those with potent antioxidant properties, e.g., simple and complex phenols, which are present in appreciable amounts in extra-virgin olive oils [9,10]. Franconi et al. [11] previously demonstrated that a total extract from a Tuscan olive oil reduces the copper-induced LDL oxidation with an IC_{50} of $0.6 \pm 0.2\,\mu M$, very similar to the concentration measured in human plasma after nutritional intake of virgin olive oil [12].

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Single MPC, such as hydroxytyrosol and oleuropein aglicon, particularly abundant in olive oil, are endowed with potent antioxidant and cardio-protective activities [10,13]. In both *in vitro* [14,15] and animal models [16], olive oil phenolic compounds inhibit LDL oxidation, in a concentration-dependent manner. In LPS-stimulated J774 cells (a murine macrophage cell line), hydroxytyrosol also reduces iNOS and COX-2 gene expression by preventing the activation of transcription factors NF-κB, STAT-1α and IRF-1 [17]. Both monocyte/macrophages and NF-κB are known to play a pivotal role in atherosclerosis [18–21]. The redox-sensitive transcription factor NF-κB regulates many inflammatory genes and can be activated by various stimuli, including reactive oxygen species, hypoxia/anoxia, cytokines, bacterial and viral products [19–22]. *Ex vivo* observations in healthy volunteers showed that, in contrast to butter- and walnut-rich meals, consumption of an olive oil-rich meal does not induce the post-prandial activation of NF-κB pathway in monocytes [23], thus suggesting a possible anti-inflammatory effect.

Therefore, we prepared a defatted extract from a Tuscan extra-virgin olive oil, particularly rich in MPC, and therefore identified as MPC-OOE (Minor Polar Compounds-Olive Oil Extract), quantified the MPC herein and evaluated its ability to affect NF-κB activation in human monocytes and monocyte-derived macrophages (MDM) isolated from healthy donors.

2. Materials and methods

2.1. Preparation, characterization and quantification of MPC-OOE

The extra-virgin olive oil was kindly supplied by a Tuscan enterprise (Italy). Sample preparation and extraction, as well as identification, characterization and quantification of single polar compounds were carried out as previously reported [24]. To completely remove the lipid fraction, 5 mL of hydro-alcoholic extract (EtOH/H_2O 7/3 (v/v)) was defatted with cyclohexane (five times with 5 mL (v/v)) and *n*-hexane (five times with 5 mL). The extract was concentrated under reduced pressure to dryness, dissolved with 5 mL of hydro-alcoholic extract (EtOH/H_2O 5/5 (v/v)) and defatted with cyclohexane (three times with 5 mL) and *n*-hexane (three times with 5 mL). The extract was concentrated under reduced pressure to dryness, dissolved with 2.5 mL of hydro-alcoholic extract (EtOH/H_2O 5/5 (v/v)) and analysed by HPLC using an HP-1100 liquid chromatograph equipped with a DAD detector and an HP 1100 MSD API-electrospray (Agilent Technologies, Palo Alto, CA, USA). The MPC were identified based on their retention times, and spectroscopic and spectrometric data, using 5-hydroxytyrosol, tyrosol, luteolin and oloeuropein as reference compounds. Lignan was identified and analysed as described in Mulinacci et al. [25]. Oleocanthal was identified according to Beauchamp et al. [26]. The single minor compounds were quantified with HPLC/DAD using a four-point regression curve constructed with the available standards. Calibration curves with a $r^2 \geq 0.9998$ were used. In all cases actual concentrations of derivatives were calculated after applying corrections for changes in molecular weight: knowing the molecular weight of each compound (PM_x), their actual concentration was obtained by applying a multiplication factor of PM_x/PM_y, where PM_y is the molecular weight of the specific reference compound. The same extract analysed by HPLC was used for experiments in human monocytes and MDM.

2.2. Isolation of human monocytes and monocyte-derived macrophages (MDM)

Human monocytes were isolated from heparinized venous blood of healthy non-smokers by standard techniques of dextran sedimentation and Ficoll–Paque gradient centrifugation and recovered by thin suction at the interface [27]. Cells were then washed twice with phosphate-buffered saline (PBS) and resuspended in RPMI 1640 medium, supplemented with 5% heat-inactivated foetal calf serum (FCS), 2 mM glutamine, 50 μg/ml streptomycin and 5 U/ml penicillin; purified monocyte populations were obtained by adhesion (90 min, 37 °C, 5% CO_2), non-adherent cells (mainly lymphocytes) being removed by three gentle washings with PBS [27]. Cell viability (trypan blue dye exclusion) was usually >98%.

Experiments were initiated on the day of blood collection; all manipulations were carried out under endotoxin-free conditions. Expression of surface markers was analysed by flow cytometry; purified monocyte populations routinely consisted of >90% $CD14^+$, <2% $CD3^+$ and 99% $MHCII^+$ cells [27]. Monocyte-derived macrophages (MDM) were prepared from monocytes as described [27]. Briefly, monocytes were cultured for 8–10 days in a 5% CO_2 incubator at 37° C in RPMI 1640 medium containing 20% FCS, 2 mM glutamine, 10 mM HEPES and antibiotics; the medium was changed every 2–3 days [27]. This procedure enables monocytes to acquire a morphological macrophage-like profile, which is accompanied by an increase in $CD68^+$ cells and a decrease of $CD14^+$ cells as compared to monocytes [27].

Cells were challenged with MPC-OOE (1 nM to 10 μM) or the PPAR-γ agonist ciglitazone (which is known to inhibit NF-κB activation and was used at 50 μM, as a positive control) for 3 h and then challenged by PMA 10^{-6} M for 1 h.

2.3. Preparation of nuclear and cytosolic fractions

Nuclear and cytosolic fractions from human monocytes and MDM (about 5×10^6 cells) were performed by using a Nuclear Extract Kit (Active Motif Europe, Belgium), according to the manufacturer's instructions. The supernatant was aliquoted and stored at −80° C until use for p50/p65 assays. Protein concentration was determined by using a protein assay (Pierce, USA).

2.4. Evaluation of NF-κB activity

The activation of NF-κB was evaluated by measuring the nuclear migration (by electrophoretic mobility shift assay; EMSA) as well as the nuclear content of p50 and p65 subunits (by ELISA).

EMSA assays were performed as previously reported [28]. Briefly, nuclear extracts (5 μg) were incubated with 2 μg poly (d*I*–d*C*) and the γ[^{32}P]ATP-labelled oligonucleotide probe (100,000–150,000 cpm; Promega) in binding buffer (50% glycerol, 10 mM Tris–HCl, pH 7.6, 500 mM KCl, 10 mM EDTA, 1 mM dithiothreitol) in a final volume of 20 μl for 30 min at room temperature. The NF-κB consensus oligonucleotide was obtained from Promega. The nucleotide–protein complex was separated on a 5% polyacrylamide gel in 0.5× TBE buffer (100 mM Tris–HCl, 100 μM boric acid, 2 mM EDTA) at 150 V on ice. The gel was dried and radioactive bands were detected by autoradiography.

To have a better quantitative evaluation of NF-κB activation, we also used commercially available ELISA kits for p50 and p65 subunits. Nuclear and cytosolic extracts were prepared as described above and evaluated for the presence of p50 and p65/RelA subunits using Trans AM^{TM} NF-κB p50 Chemi and NF-κB p65 Chemi Transcription Factor Assay kits (Active Motif Europe, Belgium), according to the manufacturer's instructions. An equal amount (1 μg) of lysate was used for each sample [28]. These assay kits specifically detected bound NF-κB p65 or p50 subunits in human extracts; activities of p50 and p65 were measured by a Rosys Anthos Lucy luminometer and expressed as RLU (Relative Luminescence Unit). The amount of translocated p50 and p65 subunits is evaluated as the nuclear/cytoplasm (N/C) ratio [28].

2.5. PPAR-γ protein expression

In order to identify the anti-inflammatory mechanism(s) for MPC-OOE, we also evaluated its ability to affect PPAR-γ protein expression in human monocytes and MDM. Cells were challenged (6 h, 37 °C, 5% CO_2) with the PPAR-γ agonist ciglitazone (50 μM) as a positive control or increasing concentrations (1 nM to 10 μM) of MPC-OOE. Experiments were performed according to Amoruso et al. [29]. Briefly, cells (2×10^6), seeded in six-well plates, were washed twice with ice-cold PBS and scraped off the wells in lysis buffer containing 3% SDS, 0.25 M Tris and 1 mM phenyl-methyl-sulfonyl fluoride (PMSF) and lysed by sonication; when necessary, cell lysates were stored at −80 °C. Protein samples (20 μg) were analysed by SDS-PAGE (10% acrylamide) and electro-blotted on nitrocellulose membrane (Protran, Perkin-Elmer Life Sciences, Boston, MA, USA). Immunoblots were performed as described [29] using the following antibodies: monoclonal mouse anti-human PPAR-γ (E-8; 1:1000 in TBS-T 5% milk) and monoclonal mouse anti-human β-actin (Sigma; 1:5000 in TBS-T 3% BSA). Anti-mouse secondary antibody was coupled to horseradish peroxidase (Amersham Biosciences, Buckinghamshire, UK). Proteins were visualized with an enzyme-linked chemiluminescence detection kit according to the manufacturer's (Perkin-Elmer) instructions. Chemiluminescence signals were analysed under non-saturating conditions with an image densitometer (Versadoc, Bio-Rad, Hercules, CA, USA). Quantification of PPAR-γ protein was performed by calculating the ratio between PPAR-γ and β-actin protein expression; the latter was selected as reference house-keeping protein.

2.6. Statistical analysis

Statistical significance of data was checked by analysis of variance (ANOVA), followed by the Bonferroni test. A corresponding probability (p) value of <0.05 was considered to be significant.

2.7. Drugs and analytical reagents

Tyrosol, luteolin and oleuropein were obtained from Extrasynthèse (Genay, France). 5-Hydroxytyrosol was purchased from Cayman Chemical (SPI-BIO, Montigny le Bretonneux, France). Solvents for the HPLC/DAD analyses were of analytical grade and were purchased from Carlo Erba (Milan, Italy). Other reagents were of analytical grade and were purchased from Sigma (St. Louis, MO, USA); PBS, RPMI 1640, glutamine, Hepes, streptomycin, penicillin and PMA were also obtained from Sigma (St. Louis, MO, USA). Nuclear Extract Kit and Trans AM^{TM} NF-κB p50 Chemi and NF-κB p65 Chemi Transcription Factor Assay kits were obtained from Active Motif Europe (Belgium). Monoclonal mouse anti-human PPAR-γ antibody (E-8) was from Santa Cruz (CA, USA); monoclonal mouse anti-human β-actin antibody was from Sigma (St. Louis, MO, USA). Tissue-culture plates were purchased from Costar Ltd. (Buckinghamshire, UK).

All cell culture reagents, with the exception of FCS, were endotoxin-free according to details provided by the manufacturer. FCS was from Life Technologies Inc. (Rockville, USA).

3. Results

3.1. Characterization of MPC-OOE

As shown in Table 1 and Fig. 1, the MPC identified and quantified in MPC-OOE belong to four classes: simple phenols (tyrosol and 5-hydroxytyrosol), secoiridoids (oleuropein aglycones, deacetoxy-oleuropein aglycone, oleocanthal and secoiridoids derivatives), lignan derivatives (acetoxypinoresinol) and flavones (luteolin). Oleocanthal, deacetoxy-oleuropein aglycone and tyrosol are the main components (all >6 mM),

Table 1
Composition of MPC-OOE (extra-virgin olive oil extract, particularly rich in MPC)

Compounds	g/L	Mm
5-Hydroxytyrosol	0.928	4.41
Tyrosol	0.608	6.03
Elenolic acid	1.195	4.94
Elenolic acid derivatives	0.634	2.62
Deacetoxy-oleuropein aglycone	1.933	6.04
Oleocanthal	1.855	6.10
Secoiridoid derivatives	2.261	5.98
Lignan derivatives[a]	1.104	2.65
Oleuropein aglycones	0.486	1.29
Luteolin	0.012	0.04
Total polyphenols	11.015	40.09

Data reported are the mean of three determinations, each performed in triplicate; S.E.M. was in the range 1–3%.
[a] Mainly acetoxypinoresinol.

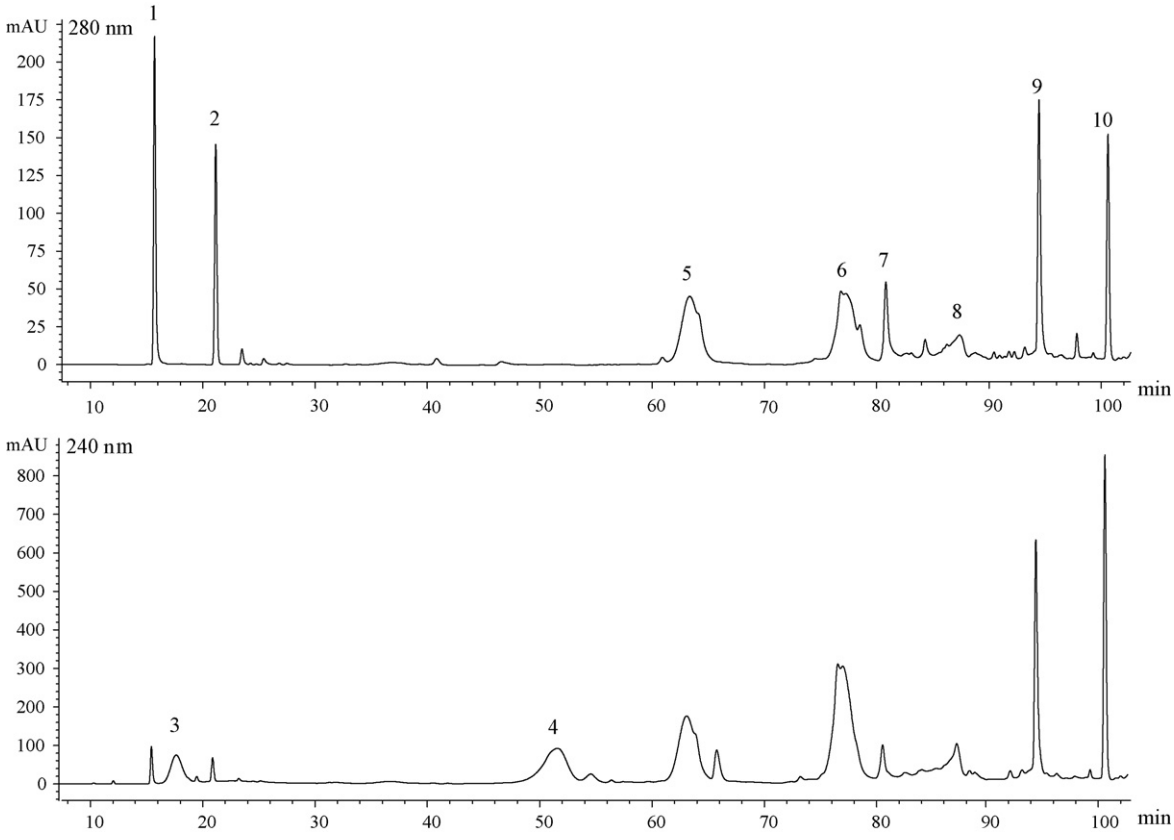

Fig. 1. HPLC/DAD profiles acquired at 280 and 240 nm for MPC-OOE. Identified compounds: 1, 5-hydroxytyrosol; 2, tyrosol; 3, elenoic acid derivatives; 4, elenolic acid; 5, deacetoxy-oleuropein aglycone; 6, oleocanthal; 7, acetoxypinoresinol; 8, oleuropein aglycone; 9–10, secoiridoids.

followed by secoiridoid derivatives (5.98 mM), elenolic acid (4.94 mM) and hydroxytyrosol (4.41 mM). In the examined MPC-OOE, total polyphenols are about 40 mM (Table 1). The same extract analysed by HPLC was used for experiments in human monocytes and MDM.

3.2. MPC-OOE inhibits NF-κB activation

We first evaluated MPC-OOE effects on the DNA binding activity of NF-κB by EMSA. As previously reported [28] and further shown in Fig. 2, NF-κB is constitutively low activated in both monocytes (Fig. 2A, lane 4) and MDM (Fig. 2B, lane 4) and is present as p50/p65 heterodimer or p50/p50 homodimer. At 10^{-6} M, PMA potently stimulates NF-κB nuclear translocation (lane 1: total effect, not supershifted; lane 2: p65 supershift; lane 3: p50 supershift. Please, note that the p50 antibody also reveals the p50/p65 heterodimer); the PPAR-γ agonist ciglitazone (lane 5), a known inhibitor of NF-κB activation [30], has been used as positive control. In both cell types, MPC-OOE (evaluated at 10 μM) effectively inhibits NF-κB nuclear translocation in un-stimulated (lane 6) and PMA-stimulated (lane 7) cells. For the purpose of clarity and brevity, Fig. 2 deals with supershifts only, except for PMA.

To ensure a better quantitative evaluation, we also assessed the translocation of p65 and p50 subunits in monocytes and MDM, by using a commercially available ELISA kit (Fig. 3). In both un-stimulated monocytes and MDM, a low basal activation of NF-κB is detected; conversely, PMA at 10^{-6} M potently stimulates p50 ($p < 0.01$ vs. control monocytes, $p < 0.05$ vs. control MDM; Fig. 3A) and p65 nuclear translocation ($p < 0.05$ vs. control monocytes, $p < 0.001$ vs. control MDM; Fig. 3B).

MPC-OOE inhibits, in a concentration-dependent manner (1 nM to 10 μM), the nuclear translocation of the NF-κB p50 subunit: at the highest 10 μM concentration, PMA-induced p50 translocation is inhibited by about 70% in both monocytes ($p < 0.001$) and MDM ($p < 0.01$) (Fig. 3A). MPC-OOE is about as effective as the PPAR-γ agonist ciglitazone, which has been used as a positive control (Fig. 3A). Interestingly, at the highest concentration evaluated, MPC-OOE also significantly reduces p50 translocation in un-stimulated monocytes ($p < 0.05$ vs. control; Fig. 3A).

As depicted in Fig. 3B, MPC-OOE does not significantly affect p65 translocation in un-stimulated cells, but it dose-dependently inhibits the PMA-induced one. At the maximum 10 μM concentration, MPC-OOE is even more effective than ciglitazone. In keeping with a previous paper by some of us [28], the p50 subunit is the most abundant and efficiently translocated in both monocytes and MDM (Fig. 3).

3.3. Effects of MPC-OOE on PPAR-γ expression in monocytes and MDM

In order to identify the anti-inflammatory mechanism(s) for MPC-OOE, we also evaluated its ability to affect PPAR-γ pro-

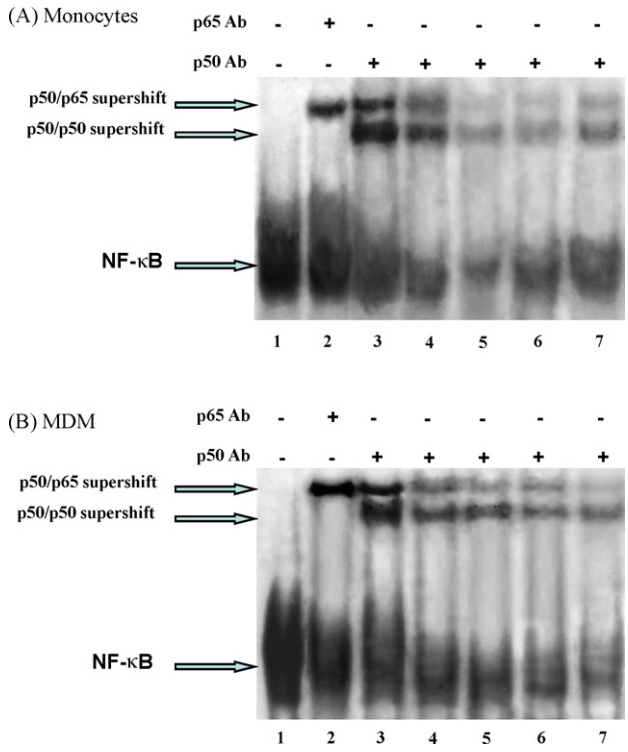

Fig. 2. NF-κB activation, p50 and p65 supershifts in human monocytes and MDM. Nuclear extracts were prepared and assayed for NF-κB activity by EMSA and supershift assays were performed by using specific antibodies (see text for further details). For clarity and brevity, in all cases except PMA, only supershifts are demonstrated. In A: human monocytes; in B: MDM. Lane 1: PMA 10^{-6} M, total effect, not supershifted; lane 2: PMA, supershift with p65 antibody; lane 3: PMA, supershift with p50 antibody (which also reveals the p50/p65 heterodimer); lane 4: control, un-stimulated cells, supershift; lane 5: ciglitazone 50 μM, supershift; lane 6: MPC-OOE 10 μM, supershift; lane 7: PMA 10^{-6} M + MPC-OOE 10 μM, supershift. This experiment was performed three times with similar results.

tein expression in human monocytes and MDM. As reported in Fig. 4, MPC-OOE does not affect PPAR-γ expression in both monocytes and MDM, whereas the PPAR-γ ligand ciglitazone (shown for comparison) enhances it about twofold.

4. Discussion

Olive-oil composition depends on many factors, such as olive cultivar, climate, ripeness of the olives at harvesting, agronomic and technological aspects of production [10,11]. The MPC-OOE we used in this study presents a higher amount of total polyphenols than others previously evaluated [11] and is particularly rich in the anti-inflammatory component oleocanthal, which has a chemical structure similar to ibuprofen and inhibits prostaglandin biosynthesis pathway [26]. This extract was obtained from a Tuscan extra-virgin olive oil rich in antioxidant compounds; in particular, MPC-OOE is an extract abundant in MPC and deprived in other active compounds such as fatty acids, tocopherol and other lipophilic components.

Our study demonstrates that MPC-OOE potently inhibits NF-κB nuclear translocation in monocyte/macrophages, as the PPAR-γ agonist ciglitazone does. As known, NF-κB is a redox-sensitive transcription factor that comprises RelA (p65), NF-κB1 (p50 and p105), NF-κB2 (p52 and p100), c-Rel and RelB. In resting cells, NF-κB is retained in the cytoplasm through an association with inhibitory proteins of the IκB family [21,22]. Different stimuli, including cytokines, bacterial and viral products, hypoxia/anoxia and reactive oxygen species, activate NF-κB through the phosphorylation of IκB and its subsequent release from the complex; this results in the translocation of NF-κB subunits from the cytoplasm into the nucleus, where they bind to target genes involved in the inflammatory and immune response and induce their transcription [21,22]. Although different homo- and heterodimeric forms of this factor have been described, NF-κB is usually composed of the p50/p65 heterodimer [21,22], p50 homodimers being demonstrated as transcriptional activators of the anti-inflammatory cytokine IL-10, at least in murine macrophages [31].

In our study, we used cells from healthy non-smoking volunteers and not monocyte/macrophage cell lines as most authors did [17,32]. This strengthens the relevance and the potential clinical impact of our results since monocyte/macrophages have long been described as key cells for atherosclerosis [18].

Olive oil polyphenols (600 ppm) added to virgin olive oil were demonstrated to exert protective effects in inflammation models *in vivo* [33]. Even more relevant are the results of a recent randomized, cross-over, controlled trial, conducted in male healthy volunteers, who were administered low-, medium-, and high-polyphenol olive oils [34]: olive oils with greater polyphenol content increased high-density lipoprotein (HDL) cholesterol levels and decreased serum markers of oxidation [34]. *In vitro* experiments also indicated that single antioxidant polyphenols inhibit LPS-induced NF-κB activation in endothelial cells: oleuropein aglycone was the most active compound and, at 15 μmol/L, decreased NF-κB activation by about 70% [35]. Moreover, a recent study on healthy volunteers submitted, in a randomized cross-over design, to three diet intervention periods of 4 weeks duration, clearly indicates that 1 month consumption of a Mediterranean diet enriched in olive oil reduces NF-κB activation in monocytes and VCAM-1 plasma concentrations [36]. These protective effects of olive oil on NF-κB activity, partly attributed to its antioxidant compounds, have been suggested for other popular beverages, such as red wine and green tea, tea polyphenols being evaluated also as proteasome inhibitors [37].

The anti-inflammatory potential of our MPC-OOE is strongly corroborated by its ability to potently inhibit, at nutritional concentrations, PMA-induced NF-κB activation in monocytes and MDM from healthy volunteers, thus extending the idea of the cardio-protective effect of olive oil-enriched diets [23,34,36].

As previously reported [12], a Mediterranean diet rich in olive oil supplies 10–20 mg of phenols per day and ensues a MPC plasma level of about 0.6 μM, that is well within the *in vitro* concentrations we used. Interestingly, at the highest concentration evaluated, MPC-OOE also significantly reduces p50 translocation in un-stimulated monocytes, in good agreement with recent *ex vivo* observations [36]. Perez-Martinez et al. [36] evaluated NF-κB activity by EMSA in monocytes only; on the contrary, we use both monocytes and MDM and provide a more careful

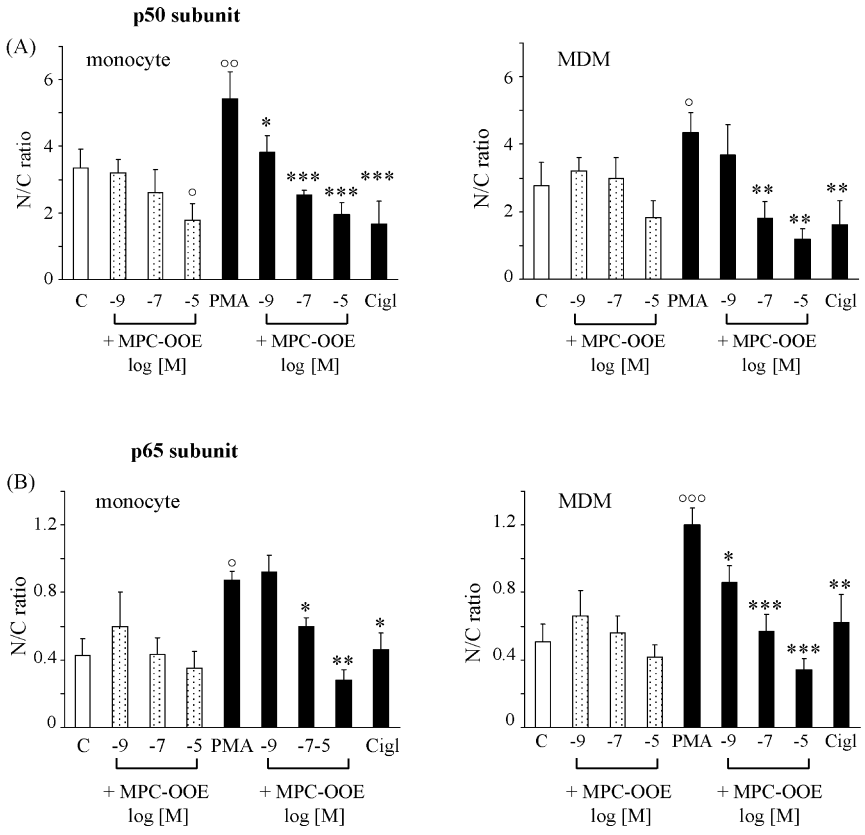

Fig. 3. MPC-OOE inhibits NF-κB translocation in human monocytes and MDM. MPC-OOE inhibits, in a concentration-dependent manner, the nuclear translocation of activated p50 subunit (A) and p65 subunit (B) in cells stimulated by PMA 10^{-6} M, but has minor effects in un-stimulated (C, control) cells. The effects of ciglitazone (Cigl; 50 μM) are demonstrated for comparison. Results are expressed as nuclear/cytoplasmic (N/C) ratio. Data are the mean ± S.E.M.; $n = 5$. $^{\circ}p < 0.05$, $^{\circ\circ}p < 0.01$, $^{\circ\circ\circ}p < 0.001$ vs. control cells; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. PMA-stimulated cells (ANOVA).

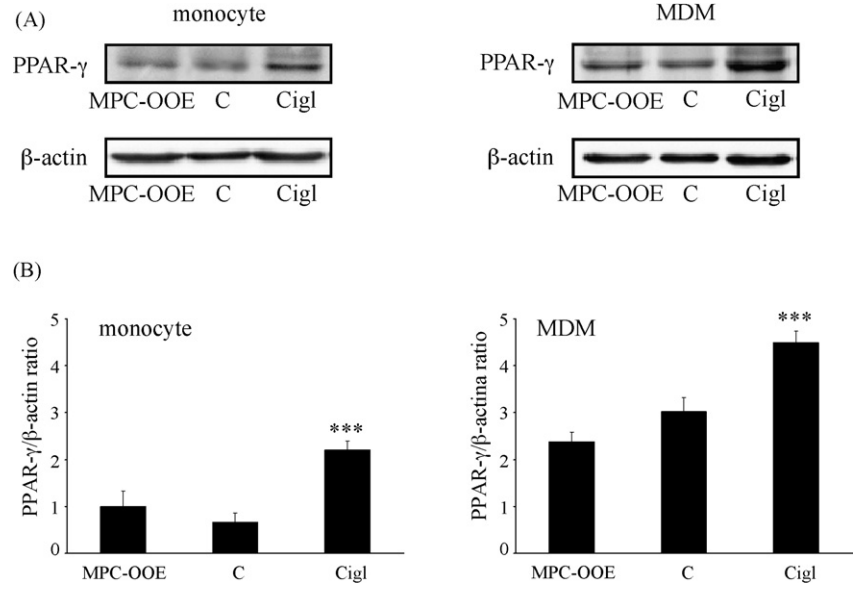

Fig. 4. MPC-OOE does not affect PPAR-γ expression in human monocytes and MDM. In A: Western blot of PPAR-γ and β-actin in monocytes and MDM from healthy non-smokers. Cells were challenged for 6 h in the absence (C, control) or presence of MPC-OOE 10 μM. The effects of ciglitazone (Cigl, 50 μM) are demonstrated for comparison. Each blot is representative of two others. In B: results are expressed as PPAR-γ/β-actin ratio (see text for further details). Means ± S.E.M.; $n = 3$; $^{***}p < 0.001$ vs. control.

evaluation of NF-κB activation (EMSA assays plus ELISA kits for p50 and p65 subunits).

We also demonstrate that, in human monocytes and MDM, p50 protein is more abundant than p65: we found about sixfold more p50 protein than p65 protein. As previously reported, p50 homodimers lack the transactivation domain, but they still bind to NF-κB consensus sites in DNA; therefore, they can function as transcriptional repressors [22] and have been demonstrated as transcriptional activators of IL-10 [31].

Interestingly, the p50 subunit has been shown to play a crucial role in atherosclerosis [38–40]. In human hepatoma cells, over-expression of p50 protein induces the transcription of C-reactive protein (CRP, a major marker of cardiovascular inflammation), whereas p65 over-expression inhibits it [38]. Kanters et al. [39] reported that p50-deficient mice present a 40% lower rate of atherosclerosis than control mice. They also demonstrated that macrophages lacking p50 showed an altered cytokine secretion *in vitro* and a reduced uptake of oxidized low-density lipoprotein (LDL) [39]. Recently, mice with a targeted deletion of the p50 NF-κB subunit have been demonstrated to undergo a reduced early mortality after myocardial infarction (as compared to wild-type), which is associated with lower collagen content and matrix metalloproteinase-9 expression [40]. Thus, we suggest that MPC-OOE ability to inhibit, in a concentration-dependent manner, the translocation of p50 protein may have a therapeutically relevant anti-atherosclerotic role and could, therefore, largely contribute to the cardio-protective activity of virgin olive oil.

In this regard, a recent study [41] demonstrates that monocytes isolated from patients with unstable angina and elevated levels of CRP present a persistent spontaneous activation of NF-κB and that these patients undergo recurrence of coronary events over a 1-year follow-up period.

Therefore, inhibition of NF-κB activation (as documented in our *in vitro* experiments with MPC-OOE) might represent a useful target for reducing the risk of coronary heart diseases.

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