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Human monocyte/macrophages are relevant targets for pro-inflammatory mediators: focus on Substance P (SP) and Macrophage Stimulating Protein (MSP)

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INTRODUCTION

1 - Monocyte/macrophages and their functions

Monocyte/macrophages are distributed throughout the body, in various fluids, tissues and organs. Monocytes arise in the bone marrow from a common progenitor, shared with neutrophils, and are released in peripheral blood. The common progenitor belongs to the stem cell lineage, which gives rise to the distinct lineages of tissue macrophages, myeloid dendritic cells and osteoclast (Volkman & Gowans, 1965). Monocytes remain in blood vessels for several days, before constitutively enter all tissue compartiment of the body. Mature circulating monocytes show heterogeneous morphology, such as different granularity, size and nuclear shape, and represent about 10% of peripheral blood leukocytes (Gordon & Taylor, 2005).

Monocytes are recruited from blood to peripheral sites by immune, metabolic and proinflammatory stimuli; the level where they leave blood flow, and where differentiation into macrophages is appreciable, defines their functions (Van Furth *et al*., 1973). High expression of CD14 surface marker, a part of LPS receptor, identifies the monocyte lineage. Following expression and identification of many other antigenic markers, peripheral blood monocytes show heterogeneity and physiological activity.

Adhesion molecules and chemoattractants, released by endothelial cells (EC) in inflammatory state, promotes monocyte recruitment. The presence of cytokines stimulates EC to produce molecules like ICAM-1 and vascular cell-adhesion molecule-1 (VCAM-1) (Albelda *et al*., 1994). EC also produce chemoattractant proteins, such as MCP-1 that recognizes and binds to the chemokine (C-C motif) receptor 2 (CCR2), expressed on monocytes. Interaction of MCP-1 with CCR2 leads to monocyte recruitment by stimulating their migration to the intima of the arterial wall (Gertszen *et al*., 1999).

After being distributed through the blood flow, monocytes constitutively enter all tissue compartments of the body. Subsequent differentiation into tissue-resident macrophages is dependent, at least in part, on signals received from the local microenvironment (Gordon, 2003). The high heterogeneity represents the high grade of specialization reached by macrophage populations that reside in different anatomical locations, and is therefore

dependent from surface and secretory cell products, i.e. β1 and β2 integrins, immunoglobulin-superfamily molecules and selectins (Stacey *et al*., 2000).

Macrophages are possibly equipped with all the innate immune recognition receptors thus far identified. These receptors collectively provide robust surveillance mechanisms responding to vascular infection as well as to oxidized Low-Density Lipoproteins (ox-LDL) and microbial stimuli. Stimulation by both endogenous and exogenous ligands of macrophage TLR2, TLR4 and CD-14/LPS binding proteins has been implicated in inflammation, foam cell formation and macrophage differentiation. Importantly, these receptors are essential to the molecular basis for macrophage- mediated crosstalk between innate and adaptive immunity (Gordon, 2002; Yan & Hansson, 2007). Stimulated macrophages produce a wide range of proinflammatory mediators, such as interferon α/β, Reactive Oxygen Species (ROS) and nitric oxide (NO).

IFN-γ produced by innate or adaptive immune cells, in combination with tumour necrosis-factor (TNF) triggers the so-called "classical activation" in macrophages, characterized by enhanced phagocytic and secretory activities, and high capacity to release cytokines, nitrogen radicals and superoxide anions (O'Shea & Murray, 2008).

Among the different mediators involved in monocyte recruitment, we focused our interest on Peroxisome Proliferator-Activated Receptors (PPARs), which were previously shown to have a role in monocyte recruitment and tissue retention (Marx *et al*., 1999), PPAR-γ being particularly active in modulating cell-to-tissue adhesion (Jung *et al*., 2008).

Moreover, PPAR-γ takes part in atherosclerosis. This disease is now considered as a type of inflammation where monocyte-derived macrophages interact with endothelium, platelets and smooth cell muscles (Glass & Witztum, 2001). PPAR-γ is implicated in inflammatory responses to lipids.

We also pointed our interest on a particular type of tissue-resident macrophages, the alveolar macrophages, which act an important role in the defence of the organism, due to their positioning at the barrier towards external environment and their action of clearing microorganisms, virus and exogenous particles in the lungs.

Alveolar macrophages (AM), or resident lung macrophages, have been reported to be derived from peripheral blood precursors and local proliferation of precursors. AM have a well distinct phenotype compared to the other resident macrophages: they show an important expression of scavenger receptors, G-protein coupled receptors, CD14, and toll-like receptors (TLRs). They encode for a large number of proinflammatory cytokines and can release

various immunosuppressive products. They are therefore the key feature in lung defence and modulate airway reactivity in immunity. Interaction between AM and pathogens leads to cell activation, phagocytosis and respiratory burst (Goldsmith *et al*., 1998).

Macrophages participate in adaptive immunity by developing antigen presentation and processing, through the major histocompatibility class II receptor (MHC II). As said before, it is possible to detect a broad number of receptors on macrophage surface, including macrophage scavenger receptors (MSR), G-protein coupled receptors, CD14, toll-like receptors (TLR), cytokine and chemokine receptors (Monick & Hunninghake, 2002). TLRs and CD14 are part of the LPS pathway (Mayeux, 1997); TLRs are capable to activate the transcription factor Nuclear Factor-κB (NF-κB) and promote cytokine release. Ryan *et al.* (2004) showed that LPS action is maximal with the combinate action of TLRs and CD14.

The so-called respiratory burst, which involves the production of reactive oxygen species (ROS), is one of the main killing processes of phagocytic cells.

ROS, like superoxide anion (O_2^-) and the hydroxyl radical OH• are highly reactive molecules, characterized by a chemical instability due to unpaired electrons. ROS oxidize DNA, proteins and lipids, causing direct damage or generating other noxious metabolites. OH• radical is the most reactive and the less stable (so its generation must occur in the proximity to DNA in order to have oxidating effects, Grisham, 1992), whereas hydrogen peroxide H_2O_2 is more stable and can diffuse through the cells (Klaunig & Kamendulis, 2004).

The respiratory burst takes part during activation of phagocytic leucocytes, such as macrophages, and results in the assembly and activation of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase. Activation causes dephosphorylation of p47 subunit of NADPH oxidase, the enzyme which uses cytosolic NADPH to reduce O_2 to O_2^-, with the oxidation of NADPH to $NADP^+ + 2H^+$. Superoxide dismutase enzyme (SOD) dismutates superoxide anion to obtain hydrogen peroxide and oxygen.

Observations from our group (Brunelleschi *et al*., 1990) showed that guinea pig AM produce relevant amount of O_2^- when challenged, in vitro, with tachykinins, substance P (SP) and Neurokinin A (NKA). More recently, we demonstrated that human AM constitutively express NK-1 receptors and produce O_2^- , as well as TNF-α and IL-1β (two well-known pro-inflammatory cytokines), when stimulated with SP (Bardelli *et al*., 2005).

Hydrogen peroxide is a highly oxidant molecule, but it is also more likely to be degraded by peroxidases to hydroxyl radical and serve as a precursor via a Fenton-like reaction, which utilizes ions Fe^{3+} and Fe^{2+} ions.

Apart from ROS, the respiratory burst generates other reactive molecules, such as hemoprotein myeloperoxidase (MPO), which promotes Cl^- oxidation to obtain the highly reactive hypoclorous acid HOCl (Klebanoff, 1992). Hypoclorous acid, which is about 1000 times more reactive and toxic than ROS, acts in a non-specific manner on a wide array of biological molecules, from DNA to fatty acids (Grisham, 1992).

Besides being two highly reactive molecules, O_2^- and H_2O_2 have received large interest for their role in signal transduction, as they can act as second messengers or primary activators (Iles & Forman, 2002).

The transcription factor NF-κB is one of the main pathways that can be activated by ROS; two decades ago, Toledano & Leonard (1991) reported that increased ROS production is detectable in response to agents that activates NF-κB.

NF-κB mainly controls the expression of genes involved in inflammation, and from its discovery, this transcription factor has been shown to play a key role in many different biologic functions, e.g. immune cell regulation, cytokine expression induction, immunity gene regulation, inflammation, cell response and cancer progression (Karin & Greten, 2005; Gilmore, 2006).

NF-κB is present in the cytoplasm of unstimuled cells in its inactive form, bound to a member of the IκB family of inhibitor proteins: IκBα, IκBβ or IκBε.

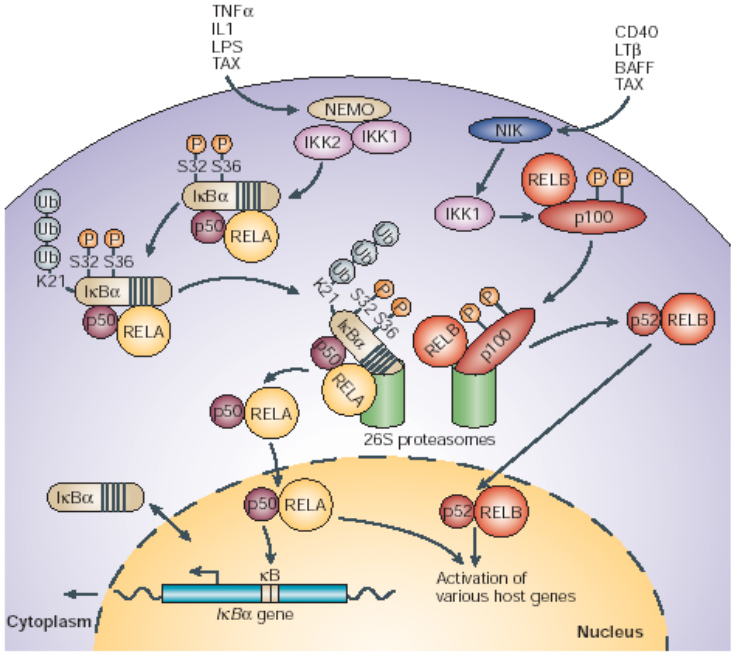

FIG. 1 Schematic representation of "classical" (left) and "non canonical" (right) NF-κB activation pathway. In the classical pathway IκBα is phosphorylated at Ser 32 and Ser 36 in an IKK2 –mediated process, triggering a rapid ubiquitynilation and degradation by 26S proteasome. IκB degradation liberates NF-κB, unmasking nuclear localization signal in RelA and inducing translocation of NF-κB complex into the nucleus. Non-canonical pathway starts from NF-κB inducing kinase (NIK) activation, which induces p100 phosphorylation and ubiquitynilation, followed by 26S processing. This pathway generates p52-RelB heterodimers, whereas the canonical generates p50-RelA. *From Chen & Greene, Nat Rev Mol Cell Biol. 2004 May;5(5):392-401.*

When cells are activated, IκBα, bound to the inactive NF-κB heterodimer, is phosphorylated by the kinase complex IKK, (that consists of IKK-α, IKK-β and NF-κB essential modifier, NEMO). Phosphorylated IκB-α is then ubiquitinylated and degraded by the 26S proteasome (Hayden & Ghosh, 2004): NF-κB dimers are free to be translocated in the nucleus.

It is thought that this process follows a kind of homeostatic process, in which the degradation of IκBα shifts the equilibrium of NF-κB/IκBα complex from cytoplasm to the nucleus (Nelson *et al*., 2004). It is also appreciated that the phosphorylation (through MAP kinase) of p65 subunit is required before gene transcription (Monick & Hunninghake, 2002).

NF-κB activation is reported in areas of strong oxidative stress, e.g. in many inflammatory lung diseases. In tobacco-induced lung inflammation, oxidative stress induces NF-κB dependent gene transcription, either activating IKK pathway or triggering transcriptional coactivators (Rajendrasozhan *et al*., 2008). ROS production is often a result of a decrease in the antioxidant/oxidant ratio towards the oxidants. Increased levels of ROS are involved in promoting inflammatory processes in the lungs, through the activation of NF-κB and AP-1.

ROS can activate either the classical NF-κB pathway *via* IKKβ, or the non-canonical pathway via NIK, IKKα and CREB binding protein (CBP), both leading to histone acetylation and increase in inflammatory genes transcription (Yao *et al*., 2007).

In 2001, our group (Brunelleschi *et al.*, 2001) originally demonstrated that human macrophages of different origin (alveolar, peritoneal and monocyte-derived macrophages) undergo a respiratory burst when challenged with Macrophage Stimulating Protein (MSP), a peptide discovered in 1976 and so named for its ability to stimulate chemotaxis in murine peritoneal resident macrophages (Leonard & Skeel, 1976).

2 - Macrophage Stimulating Protein (MSP)

Since its discovery in 1976, the spectrum of the actions evoked by MSP has been broadly investigated and widened. Originally, it was regarded as a serum protein capable to induce shape change, migration and phagocytosis of mouse peritoneal macrophages (Leonard & Skeel, 1976, 1978, 1979); after that, several investigations discovered that MSP modulates the activity of different cell types, such as megakaryocytes, erithroid cells, liver cells and fibroblasts (Leonard, 1997).

MSP is a 80-kDa serum growth factor which belongs to a family of proteins characterized by a kringle domain and an inactive serine-like protease domain (Yoshimura *et al*., 1993). Composed by a 53 KDa α-chain and a 30KDa β-chain linked by a disulphide bond, MSP is produced mainly in liver cells (Bezerra *et al*., 1993), and circulates in the blood in nanomolar concentrations as pro-MSP, a single chain precursor. Proteolitic conversion into active MSP happens during coagulation processes and local inflammation, by the action of kallikrein, factor XIa and XIIa (Wang *et al.*, 1994). Pro-MSP convertase, an enzyme which cleaves pro-MSP, has been found in wound fluid exudate and on human macrophage cell surface (Camp *et al*., 2005).

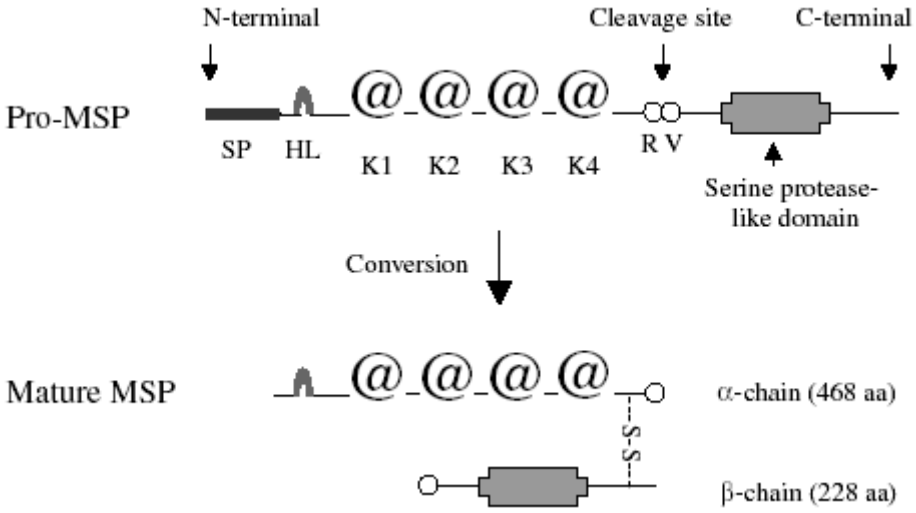

FIG. 2 Schematic representation of structure of Macrophage Stimulating Protein. MSP is synthesized by hepatocytes and circulates in blood flow as pro-MSP, inactive single-chain precursor. Mature MSP results from proteolytic cleavage at a specific site between Arg554 and Val 555. Mature MSP consists of a 53 kDa α chain and 30 kDa β chain. In the α chain a signal peptide (SP) and a hairpin loop (HL) are detectable. β-chain contains a serine-protease like domain without enzymatic activity, and the receptor binding site.
From Wang et al., Scand. J. Immunol. (2002) 56:545-553

The receptor for MSP is a tyrosine-kinase receptor, member of the Met protooncogene family, known as récepteur d'origine nantais (RON). RON is a disulfide-linked heterodimer composed by an extracellular 40 kDa α-chain and a transmembrane 150 kDa β-chain with intrinsic kinase activity (Gaudino *et al*., 1994; Morrison *et al*., 2004).

Stimulation of RON-expressing cells leads to activation of tyrosine-kinase activity of the receptor, and signalling occurs with the involvement of many effectors: phospholipase C-γ, phosphatidil inositole 3-kinase (PI 3-Kinase), c-Src, mitogen activated antigen kinase (MAP Kinase) and c-Jun amino terminal kinase (JNK). Signalling process starts from the phosphorylation of an unique multifunctional site, shared with all the members of the Met family (Danilkovitch-Miagkova *et al.,* 2000). In murine models, expression of RON homologue Stk has been regarded as a marker of differentiation of peritoneal macrophages (Iwama *et al*., 1995).

From its discovery, MSP spectrum of activity has widened, till becoming considered as a pleiotropic factor. In fact many studies demonstrated that MSP stimulates human and murine bone marrow megakaryocytopoiesis (Banu *et al*., 1996), induces proliferation and migration of murine keratinocytes (Wang *et al*., 1996), stimulates bone resorption in osteoclasts (Kurihara *et al*., 1996) and promotes tumor cell migration (Willett *et al*., 1998).

Moreover, MSP inhibits nitric oxide (NO) release induced by LPS and cytokines (Chen *et al*., 1998), and inducible nitric oxide synthase (iNOS) expression in murine peritoneal macrophages, negatively modulating NF-κB pathway (Liu *et al.*, 1999).

RON receptor is expressed in many cell types, and is involved in tumor progression and malignancy. Malignant neoplastic tissues of different origin show RON expression: recent data report RON presence in tumoral gastric tissues. Interestingly, protein expression is correlated to tumor invasivity, but not to tumor growth pattern (Zhou *et al*., 2008). In human epithelial cancers, RON activation can mediate adhesion, motility and invasion. Moreover, engineered mice overexpressing RON show a higher rate of pulmonary and mammary gland tumors development (Camp *et al.*, 2005; Wagh *et al*., 2008). In murine models of mammalian cells cancer, blockade of RON by selective antagonist reduces cell scattering and invasion caused by TGF-β lacking fibroblasts. Moreover, antibodies against RON inhibit Hepatocyte Growth Factor (HGF)-induced cell scattering correlated with reduced Stat3 and p42/44MAPK expression (Cheng *et al*., 2008). Recent research data (Zhang *et al*., 2008) identified an inhibitor of RON kinase activity, which acts in a concentration-dependent manner upon MSP-mediated signalling.

RON expression has been detected in lung cancer cell lines, together with high levels of its endogenous ligand, MSP (Willett *et al*., 1998).

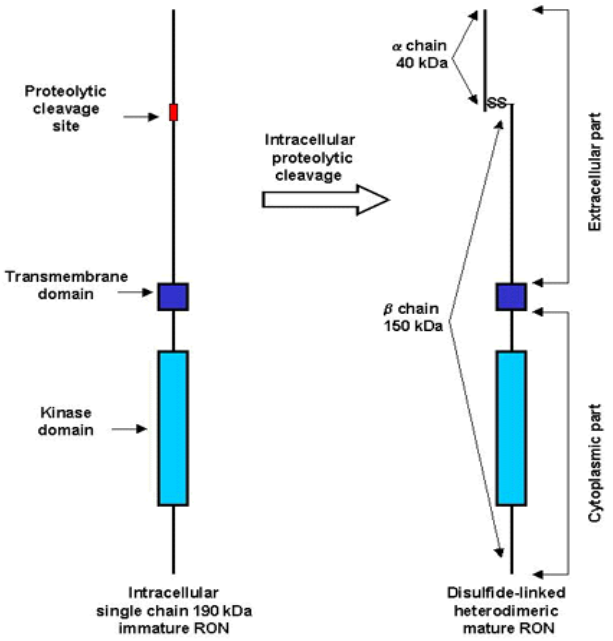

FIG. 3 Schematic representation of pro-RON (left) and mature (right) RON receptor. Pro-RON is synthesized as a single-chain protein; after protein cleavage onto a specific site (K^{305} –R-R-R-R ^{309}) mature RON is generated: it is composed by a 40 kDa αchain, totally extracellular, and a 150 kDa β-chain linked by a disulphide bond. β-chain cytoplasmic portion exerts intrinsic tyrosine kinase activity.
From Danilkovitch-Miagkova A, Leonard EJ., Academic Press cytokine reference, 2001

The system MSP/RON is also involved in inflammatory processes. In 2001 our group originally demonstrated that RON receptor is expressed in human monocyte-derived macrophages (MDM), peritoneal macrophages from ascitic fluid and alveolar macrophages, but not in human monocytes, and that MSP triggered superoxide anion production (Brunelleschi *et al*., 2001). In particular, MSP, utilized in the concentration range 1-600 ng/ml, dose-dependently evoked O_2^- production from MDM, peritoneal and alveolar macrophages but not in human monocytes: maximal production was about 40 nmol cytochrome C reduced/10^6 cells at 600 ng/ml.

This MSP action is similar to the one induced by phorbol 12-myristate 13-acetate (PMA), a well known proinflammatory stimulus, and more efficient than the one evoked by the chemotactic peptide FMLP (N-formyl methionyl leucyl phenylalanine).

MSP-induced respiratory burst is mediated by tyrosine kinase activity, but it seems independent from PI 3-kinase although this enzyme is implicated in MSP / RON signal transduction in other cell types (Brunelleschi *et al*., 2001).

The role of MSP/RON on alveolar macrophages was suggested by previous papers: Takano *et al*. (2000) reported MSP presence at about 8 ng/ml concentration in induced sputum from healthy subjects, and MSP was also found by Sakamoto *et al*. (1997) in broncho-alveolar lavage (BAL) samples from healthy non-smoker donors.

In murine models, the lack of the RON-encoding gene enhances susceptibility to LPS-induced septic shock (Correll *et al*., 1997). Recent literature demonstrates that RON regulates the production of, and the response to IFN-γ, enhancing the susceptibility to endotoxin challenge in double knockout mice (Wilson *et al*., 2008); moreover, the increased sensibility of RON is dependent on IFN-γ mediated signals.

In this PhD thesis (please, see Paper 1), I have evaluated the effects of MSP on human AM from smoker and non-smoker patients I have originally demonstrated that MSP, in a concentration-dependent manner, induces O_2^- production, cytokine release and NF-κB translocation, being particularly effective in healthy smokers and in patients with pulmonary fibrosis.

3 - Tachykinins

Tachykinins are a class of peptides with a common C-terminal pentapeptide sequence Phe-X-Gly-Leu-Met-NH_2, where X can be an aromatic or a hydrophobic aminoacid, essential for the tachykinin's receptor interations. In mammals five tachykinins have been identified: Substance P (SP), neurokinin A (NKA), neurokinin B (NKB), neuropeptide K and neuropeptide γ.

Effects on target cells are mediated by three types of receptors, NK -1R, NK-2R and NK-3R, members of the superfamily of guanine nucleotide-binding coupled receptors. Generally, SP activates NK-1 receptor, NKA activates NK-2, whereas NK-3 receptor is activated by NKB (O'Connor, 2004).

Substance P, identified at the beginning of the last century, is synthetized from preprotachykinin-A (PPT-A), a common gene which codifies for others tachykinins such as NKA, NP-K and NP-γ. By the action of convertase enzyme, SP leaves ribosomes and is localized on the assonic terminals, where it reaches its biologically active form (Brimjoin *et al*., 1980).

SP can be found in central and peripheral nervous system, where it participates in the regulation of respiratory, cardiovascular and behavioural functions.

A peculiar role for SP is the so-called "neurogenic inflammation", a process represented by inflammatory symptoms caused from the release of substances from primary sensory nerve terminals.

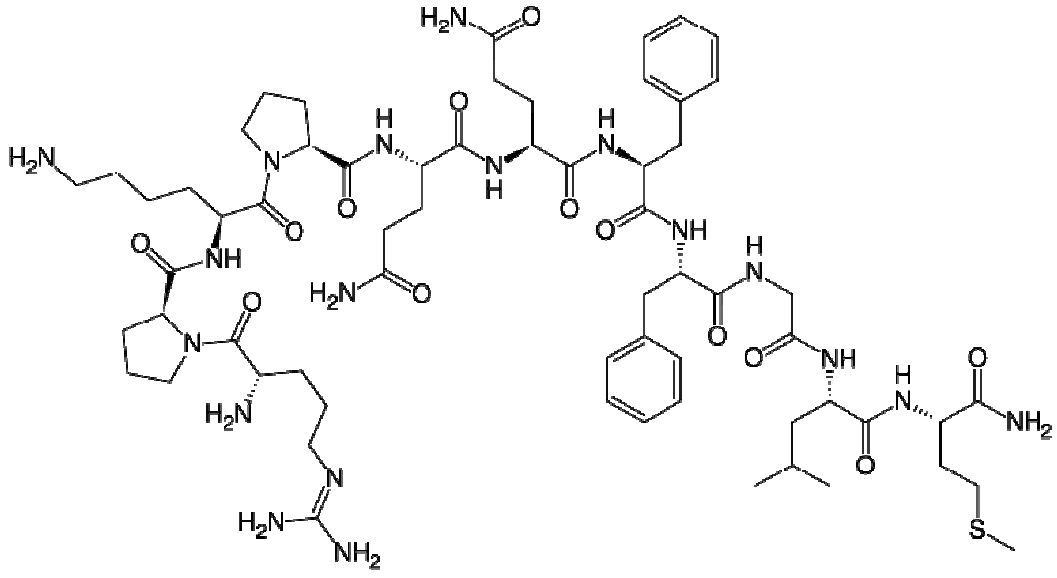

FIG. 4 Skeletal formula of Substance P .

Public domain image

When SP is released from small diameter sensory neurons, it leads to plasma extravasation (which causes swelling) venular permeability and vasodilatation (which cause redness and warmth) and leukocyte activation (Holzer, 1998; Richardson & Vasko, 2002).

SP is one of the main promoters of neurogenic inflammation, interacting with endothelial cells, mast cells, arterioles and immune cells; the same action can be reproduced by SP administration and reduced using anti-SP antibodies (Maggi *et al*., 1995).

Despite its neuronal origin, SP is reported to play an important role in inflammatory processes: several studies also report its production by inflammatory cells such as macrophages, lymphocytes and dendritic cells (Killingsworth *et al*., 1997; Joos & Pauwels, 2000). Immune cells represent an important source of tachykinins: it is known that SP is produced by eosinophils in mouse and man, monocytes and macrophages in rat and man, lymphocytes in man and dendritic cells in mouse (Maggi, 1997; 2000). Using LPS as an inflammatory stimulus, it is possible to up-regulate tachykinins' concentration in immune cells (Germonpre *et al.,* 1999; Lambrecht *et al*., 1999).

Immunomodulatory actions of SP are broad and can involve several body districts. In gastrointestinal tract, both NK-1R expression and SP production has been detected. Moreover, NK-2 receptors have been found in muscolaris mucosa, associated to pathological conditions (Warner *et al*., 2000) and it is responsible for circular muscle contraction (Cao *et al*., 2000).

Pathological stimuli in intestinal tract activate SP synthesis by both macrophages and sensory neurons: SP/NK-1R binding promotes PKC-δ phosphorylation, which activates NF-κB pathway leading to the synthesis of proinflammatory genes, like IL-8 (Koon *et al*., 2005).

Tachykinins exert several effects in the lung: SP and NKA mediate the excitatory part of the NANC nervous system which regulates the airway system. For these actions, both NK-1 and NK-2 receptors are involved: NK-1 mediates the proinflammatory effects of SP, NK-2 mediates part of the bronchoconstrictor action of tachykinins (Joos, 2001).

Substance P and NKA also induces mucous secretion: in asthma and chronic obstructive pulmonary disease (COPD), besides neurally released tachykinins, the action of inflammatory cell-secreted tachykinins can induce secretion by NK-1 receptor stimulation (Springer *et al.*, 2005).

In lung epithelium it has been reported a broad network of nervous sensorial fibers, most of which are rich in SP, and represent structural support for the local axonic reflex (Lamb & Sparrow, 2002). Neuropeptides can stimulate the release of cytokines in human bronchial epithelial cell lines: exposure to SP results in increase of intracellular calcium, followed by the release of the inflammatory cytokines TNF-α, IL-6 and IL-8 (Veronesi *et al*., 1999). In

2008, Sio *et al.* showed a relevant SP release after acute burn injury in lung murine models: this high response is observed along with increased TNF-α, IL-6 and IL-10 release.

In human monocytes and macrophages, SP can promote the secretion of cytokines such as TNF-α and IL-10 (Ho *et al*., 1996). In murine models, SP induces the release of arachidonic acid metabolites, prostaglandin E2 and ROS (Murris-Espin *et al*., 1995). NK-1R is basally expressed in the human macrophage cell line THP-1; the challenge with proinflammatory cytokines increases NK-1R gene expression at both mRNA and protein level. Moreover, NK-1 protein expression in THP-1 cells is strongly reduced after transfection with IκBα, showing the strong involvement of NF-κB in regulating NK-1R expression during inflammation (Simeonidis *et al*., 2003).

Recent observations by our group report the expression of NK-1 receptor protein in AM (Bardelli *et al*., 2005), extending previous data by other groups. Takeyama *et al.* (1996) detected important levels of SP in BAL from patients with chronic lung inflammation, e.g pulmonary fibrosis and sarcoidosis, and healthy smokers; other observations demonstrated the presence of SP in induced sputum samples from asthma or chronic bronchitis patients (Tomaki *et al*., 1995). In addition, a higher NK-2 gene expression was detected in several patients: a four fold increase in asthmatics, a three-fold in smokers and a two-fold increase in patients of chronic obstructive pulmonary disease (COPD), while an increased NK-1 gene expression was documented only in smokers (Bai *et al*., 1995).

Evaluation of tachykinin effects on AM has been a point of interest of our laboratory since 1990. SP, NKA, NKB and selective receptor agonists have been used to evaluate superoxide anion production in guinea pig AM. SP evoked O_2^- release in a concentration - dependent manner, with an ED_{50} of 0,7 nM; NKA and NKB were also able to produce O_2^-, NKA showing an ED_{50} of 0,1 nM (Brunelleschi *et al*., 1990).

Moreover, ovoalbumin- sensitized guinea pig AM showed an enhanced responsiveness to NK-2 receptor stimulation, NKA and the NK-2 agonist [β-Ala^8]-NKA(4-10) inducing significantly enhanced release of O_2^- and prostanoids (PGE_2 and TXB_2) (Brunelleschi *et al*., 1992).

Human AM, collected from healthy smokers or patients with sarcoidosis, also underwent a respiratory burst when challenged with tachykinins (Brunelleschi *et al.*, 1996). More recent studies (Brunelleschi *et al*., 2000) demonstrated that SP and NKA, as well as the selective NK-1 agonist [$Sar^9Met(O_2)^{11}$]SP and the NK-2 selective agonist [β- Ala^8] NKA (4–10), induce O_2^- production in monocytes obtained from human volunteers, healthy smokers or patients with interstitial lung diseases. All agonists acted dose-dependently: maximal effects

were observed at micromolar concentrations. We also demonstrated (Bardelli *et al*., 2005) that human AM constitutively express NK-1 receptors and release significant amounts of O_2^- and TNF-α when challenged in vitro with SP and NK-1 receptor agonists. In this thesis, (please see Paper 3) we describe a novel activity of SP, that is its ability to affect PPAR-γ expression in human monocyte/macrophages.

4 – Peroxisome Proliferator-Activated Receptors (PPARs)

Peroxisome Proliferator-Activated Receptors (PPARs) are transcription factors belonging to the hormonal nuclear receptor superfamily, that comprises receptors for estrogens, glucocorticoids, thyroid hormones, vitamin D3 and retinoic acid, too. PPARs are important regulators of lipid metabolism and energy homeostasis (Francis *et al*., 2003).

PPAR domain structure is similar for all the three known receptor isoforms. The N-terminal domain is highly variable and contains a transcriptional activator, AF-1, which phosphorylates PPAR. The structure with two zinc fingers of DNA-binding domain promotes the binding of PPAR to PPREs (PPAR response elements) to specific DNA regions of target genes (Duval *et al*., 2002). This domain is linked to ligand binding domain (LBD), responsible for ligand specificity and transcriptional activity (AF-2). The two latter domains ensure that the nuclear receptor acts as a part of a multiprotein complex, allowing the heterodimerisation of PPAR with retinoid X receptor (RXR) (Lee *et al*., 2006). There are three subtypes of these ligand-activated factors, known as PPAR-α, PPAR-δ (PPAR-β) and PPAR-γ. PPAR-δ is the least understood PPAR subtype because of its ubiquitous localization and few selective ligands. Nevertheless, it has recently been reported that PPAR-δ can regulate lipogenesis and ameliorate hepatic steatose in murine models (Qin *et al.*, 2008).

PPAR-α is expressed in skeletal muscle, liver and heart tissue, where it mediates metabolic homeostasis and fatty acids metabolization (Reddy & Hashimoto, 2001).

PPAR-γ is expressed in a large number of cell types: intestinal, endothelial, smooth muscle, skeletal cells and monocyte/macrophages (Tontonoz *et al*., 1998; Neve *et al*., 2000; Heikkinen *et al*., 2007). PPAR-γ gene contains three promoters for three PPAR-γ isoforms, whose expression is dependent from the tissue. PPAR-γ1 is expressed in a broad range of tissues, PPAR-γ2 is found in adipose tissue whereas PPAR-γ3 is abundant in macrophages, in large intestine and in white adipose tissue, specialized in lipid storage (Gerstzen *et al*., 1999; Xu *et al*., 2001).

It was originally cloned as a regulator gene, which acts as a key regulator of adipocyte function (Rosen & Spiegelman, 2001). The main physiological functions of PPAR-γ are in fact adipogenesis, glucose homeostasis and lipid metabolism. Adipogenesis is positively regulated by PPAR-γ, which can improve insulin sensitivity through its adipocyte-specific activities. Adipose tissue is known to have endocrine functions, because it secretes metabolically active hormones like lectins, cytokines (IL-6 and TNF-α) (Hotamisligil *et al*.,

1993; Fried *et al*., 1998), and monocyte chemoattractant protein (MCP-1) (Sartipy & Loskutoff., 2003). PPAR-γ is the molecular target of thiazolidinediones, a class of drugs largely used in type 2 diabetes therapy.

As a member of the nuclear receptor superfamily, PPAR-γ binds response elements, where it forms heterodimers with retinoid X receptor (RXR). The complex PPAR-γ /RXR recruits co-activator or co-repressor molecules, that contain PPAR-γ response elements, and activates gene expression or repression (Varga & Nagy, 2008). PPAR γ is involved in many cellular functions, like adipocyte differentiation (Spiegelman & Flier, 1996), glucose homeostasis (Deeb *et al*., 1998), inflammatory response (Jiang *et al*., 1998; Ricote *et al*., 1998) and apoptosis (Chinetti *et al*., 1998). Because of its expression in metabolic tissues and in inflammatory cells, PPAR-γ is a regulator which links metabolism to immunity.

PPAR-γ is activated by exogenous and endogenous ligands, including unsaturated fatty acids, prostaglandins, e.g. 15-deoxy-$\Delta^{12\text{-}14}$-prostaglandinJ_2 (15d-PGJ_2) and 15-Hydroxyeicosatetraenoic acid (15-HETE), and fatty-acid derived components of oxidized low-density lipoproteins, e.g. 9- and 13-HODE (9-hydroxy-10, 12-octadecadienoic acid) (Forman *et al*., 1995; Nagy *et al*., 1998; Huang *et al*., 1999). In the absence of ligands, the heterodimer is associated with the nuclear receptor corepressor complex; after activation by a ligand, the corepressor complex is replaced by coactivators, that lead to transcriptional initiation of PPAR-γ target genes (Ye *et al*., 2008).

Besides natural and synthetic ligands, gene transcription can be achieved by other factors, such as RXRs, PPAR-responding elements (PPREs) and cofactors. PPAR-γ has been also described to repress transcription of other transcription factors, such as NF-κB and AP-1, *via* the process of "transrepression" (Ricote & Glass, 2007).

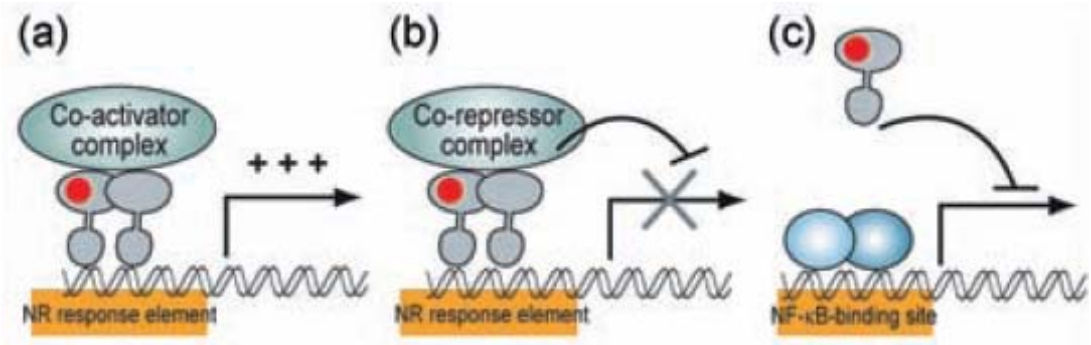

FIG. 5 Ligand-dependent transcriptional regulation by PPAR-γ. a)PPAR-γ bound to its ligands forms heterodimers with retinoid x receptor RXR, recruiting coactivators and promoting gene expression. b) a possible effect of PPAr-γ ligand binding can be the repression of target genes. c) transrepression: upon ligand binding PPAR-γ can induce the blockade of distinct transcription factors via protein-protein interactions.

From Varga T. and Nagy L, European Journal of Clinical Investigation 2008; 38(10): 695-707

Prostaglandin (PG)-related compounds, such as 15d-PGJ_2, were identified as potent PPAR-γ agonists. Experimental data suggest that 15d-PGJ_2 exerts anti-inflammatory effects (Scher & Pillinger, 2005): during inflammatory processes, PPAR ligands, most of all 15d-PGJ_2, interfere with the transcription factor NF-κB, either inhibiting its DNA binding, or promoting activation of IκB kinase (Straus *et al*., 2000). The class of thiazolidinediones (TZDs) is the most important among the exogenous PPAR-γ ligands: these drugs are used for improving insulin resistance and lowering blood glucose levels in type 2 diabetes patients (Lehmann *et al*., 1995). Several TZD-class drugs, such as rosiglitazone, ciglitazone and pioglitazone, are selective PPAR-γ agonists and show reduced affinity towards the other PPAR subtypes (Chen *et al*., 2005).

PPAR-γ expression is inhibited by inflammatory cytokines like TNF-α, thus contributing to several diseases, e.g atherosclerosis, cancer cachexia, inflammation. In particular, TNF-α inhibits PPAR-γ ligand-dependent transcriptional activity at mRNA level. In murine adipocytic cell lines TNF-α inhibits expression of CCAAT/enhancer binding protein δ (C/EBPδ), which activates PPAR-γ gene promoter: low levels of C/EBPδ expression induce suppression of PPAR-γ gene transcription (Kudo *et al*., 2004).

PPAR-γ acts a well-documented role in macrophages which, as known, represent the first line of defense of our organism.

Treatment of AMs with PPAR- γ agonists (TZDs or the endogenous ligand 15d-PGJ_2) resulted in suppression of LPS-induced TNF-α release, iNOS expression, and oxidative burst. Likewise, PPAR-γ has been shown to be strongly expressed in human AM, and incubation of these cells with PPAR-γ agonists significantly decreased the expression of TNF-α while enhancing the cell surface expression of CD36, a scavenger receptor that mediates the phagocytosis and clearance of apoptotic neutrophils within the airspace (Asada *et al*., 2004).

Pretreating macrophages with PPAR-γ ligands results in inhibition of transcriptional activation of inflammatory genes upon inflammatory stimulus (Welch *et al*., 2003). Antiinflammatory functions of PPAR-γ develop by transrepressing NF-κB and MAPK pathways, main regulators of inflammatory genes. General inhibition of NF-κB leads to the anti-inflammatory activity of PPAR-γ. PPAR-γ ligands also exert receptor-independent effects: as an example, natural and synthetic ligands can downregulate TNF-α and IL-6 encoding genes in PPAR-γ deficient murine models (Chawla *et al*., 2001).

In this PhD thesis, we provide a semi-quantitative evaluation of the amount of PPAR-γ protein in human monocytes and macrophages, and demonstrate, for the first time, that PPAR-γ expression is constitutively enhanced in healthy smokers (please, see Paper 2). We also provide evidence that, in vitro, nicotine directly affects PPAR-γ expression, and that SP and NK-1 agonists dose-dependently induce it (please, see Paper 3).

PAPER N°1

(In appendix)

Macrophage stimulating protein differently affects human alveolar macrophages from smoker and non-smoker patients: evaluation of respiratory burst, cytokine release and NF-κB pathway

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Macrophage activation is a common feature of several inflammatory reactions, such as bacterial inflammation, immune response and tissue damage. Previous observations from our group demonstrated that human macrophages from different origin express RON (recepteur d'origine nantais), the tyrosine-kinase MSP receptor, and that MSP induces superoxide anion production (Brunelleschi *et al.,* 2001).

Macrophage Stimulating Protein, MSP, had been identified in 1976 as a factor able to induce shape change, phagocytosis and migration of murine peritoneal macrophages.

In this paper we evaluate MSP effects on AM from healthy donors and patients with chronic interstitial lung diseases such as sarcoidosis and pulmonary fibrosis, either smokers or non-smokers. We have assessed MSP-induced proinflammatory cytokine release, superoxide anion production and NF-κB activation. MSP effects have been compared with those induced by well-known inflammatory stimuli, such as PMA, FMLP and lipopolisaccaride from bacterial cell wall (LPS).

Our results show that MSP activates human AM, inducing cytokine release, O_2^- production and NF-κB activation in a concentration-dependent manner. AM from smokers, either healthy or with pulmonary fibrosis, show an O_2^- production higher than the one from healthy, non-smoker subjects, thus suggesting an interaction MSP/tobacco smoke in pulmonary diseases.

MSP, used between 3 and 200 ng/ml, induces TNF-α release in AM from every subject evaluated, with a significantly higher production (more than 2-fold) in healthy smokers or patients with fibrosis.

MSP (but not PMA and fMLP), also releases small amounts of IL-10 (an anti-inflammatory cytokine) in AM from healthy non smokers. MSP stimulation enhances NF-κB activation, with an effect similar to the one evoked by LPS. MSP induces a 2,5-fold enhancement of NF-κB translocation in healthy donors and in pulmonary fibrosis patients, and is particularly effective on the p50 subunit. This effect is receptor-mediated, as it is prevented by a monoclonal anti-human MSP antibody.

Our observations demonstrate that MSP induces cytokine release, O_2^- production and NF-κB activation in monocyte and MDM from healthy subjects and patients with chronic pulmonary inflammatory diseases, smokers and non smokers.

This paper is the first to describe those features and to suggest that MSP can be a contributor of tobacco smoke toxicity.

Paper in appendix

PAPER N°2

(In appendix)

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

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It is known that monocyte/macrophages, which are the first line line of defence against several infective and inflammatory stimuli, express Peroxisome Proliferator-Activated Receptor gamma (PPAR-γ).

In this paper we intended to give a semiquantitative evaluation of PPAR-γ expression, both basal and ligand-induced, in monocytes and macrophages isolated from healthy donors, smokers and non smokers, and we originally demonstrate that nicotine affects PPAR-γ expression.

PPAR-γ expression has been evaluated in freshly isolated monocytes, in partially differentiated (cultured for 4 days) and fully differentiated macrophages (monocyte-derived macrophages, MDM). The expression has been evaluated by western blot, calculating the ratio between PPAR-γ and β-actin protein expression. Our data show a low basal expression in monocytes, which comes to a higher amount along with the differentiation process. Stimulation with specific ligands, either endogenous, e.g. 15d-PGJ_2, or synthetic, e.g. ciglitazone, enhances PPAR-γ expression: the highest expression has been obtained with 15d-PGJ_2 at micromolar concentrations.

The same molecules inhibit the release of proinflammatory cytokines, such as TNF-α and IL-6: in this case, ciglitazone shows the highest efficiency. We can see that ciglitazone, at the maximal concentration used (50 μM), inhibits TNF-α release by 90-95% in monocyte and macrophages from healthy non-smokers, whereas 15d-PGJ_2 inhibition was only 50-60%. A higher basal TNF-α release can be reported in monocyte and macrophages from smokers; ciglitazone-induced inhibition was higher than the 15d-PGJ_2 -induced one.

According to our data, ligand-induced PPAR-γ expression and cytokine inhibition are similar in healthy smokers and non smokers, whereas monocytes and MDM from healthy smokers show a 4-fold and 2-fold higher constitutive PPAR-γ expression, respectively.

This study also shows the possible direct effect of nicotine, which enhances, in a concentration-dependent manner, PPAR-γ expression, and inhibits proinflammatory cytokine release: (with no synergic effect between PPAR-γ agonists and nicotine). Nicotine action is receptor-mediated, since its effects are reversed by α-bungarotoxin, a well-known antagonist of α7 subunit of the nicotinic receptor, that is expressed in monocytes and MDM from healthy non-smokers.

In conclusion, PPAR-γ is constitutively expressed in higher amount in monocytes and MDM from healthy smokers, and this effect is somehow reproduced by nicotine in vitro.

Paper in appendix

PAPER N°3

(In appendix)

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

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Substance P, discovered in the first half of the last century, is a neuropeptide belonging to the tachykinin family, a class of neurotransmitters which share a common aminoacidic sequence, and is a key actor in the neurogenic inflammation (Holzer, 1998; Richardson & Vasko, 2002). Several studies, either in murine models or in human subjects, demonstrated SP release from inflammatory cells, e.g. macrophages, lymphocytes, eosinophils and dendritic cells (Maggi, 1997; Severini *et al*., 2002).

Observations from our group show that, in a concentration-dependent manner, SP and selective NK-1 agonists induce the release of proinflammatory cytokines, superoxide anion production and transcription factor NF-κB activation in human alveolar macrophages and MDM (Bardelli *et al*., 2005), as well as in human monocytes (Brunelleschi *et al*., 1998) and guinea-pig AM (Brunelleschi *et al*., 1990).

PPARs (Peroxisome Proliferator-Activated Receptors), are a class of transcription factors involved in nutrient metabolism regulation, energetic homeostasis and adipocyte differentiation.

PPAR-γ has been demonstrated in many cell types, including monocyte/macrophages (Tontonoz *et al*., 1998, Amoruso *et al*., 2007) and has been suggested as a relevant anti-inflammatory factor.

Both SP and PPAR-γ can act important roles in inflammatory processes and are expressed in human monocyte/macrophages, but it is not known if they can interact with each other. We originally observed that PPAR-γ expression is induced by SP and NK-1 agonists, and reverted by NK-1 antagonists; we also assessed the influence of SP and PPAR-γ ligands upon

inflammatory cytokine release. Our data show that SP, when used in the range 10^{-10}-10^{-6} M, stimulates PPAR-γ expression in monocytes and MDM, and that cells from healthy smokers are more sensitive to SP action, and present a constitutively increased expression of NK-1 receptors.

We have assessed the receptorial action of substance P-induced PPAR-γ expression: it was reproduced by [$Sar^{9}Met(O_2)^{11}$]SP, a selective NK-1 agonist, and reverted by the competitive NK-1 antagonist GR71251.The endogenous ligand 15d-PGJ_2 evoked effects similar to the maximal effects induced by SP, whereas the PPAR-γ antagonist GW9662 reduced them.

15d-PGJ_2 and the NK-1 antagonist GR71251 inhibited SP-induced TNF-α release from monocytes isolated from healthy smokers and non smokers.

The results of this study show a novel activity for SP: the SP-induced PPAR-γ enhanced protein expression in healthy smokers, compared with non-smokers, could have an importance in chronic inflammatory diseases.

Paper in appendix

CONCLUSIONS

Cells belonging to the mononuclear phagocyte system have marked phenotypic heterogeneity and immune functions, are distributed throughout the body and are characterized by varied responsiveness according to different stimuli. In this thesis we directed our interest to monocyte/macrophage behaviour in response to proinflammatory substances, such as Substance P and Macrophage Stimulating Protein, also in relation to tobacco smoking habit.

There is no doubt that cigarette smoke plays a pivotal role in many lung diseases, as well as in cardiovascular and tumour diseases (Conti-Fine *et al*., 2000). The chemical composition of tobacco smoke is highly complex; so, it is difficult to define the most dangerous component and/or the single direct responsible for a given disease. Generally, ROS and aromatic polycyclics hydrocarbons are the main responsibles for tobacco smoke toxicity, whereas nicotine is the leading molecule for tobacco addiction.

By itself, nicotine profoundly affects the immune system, some divergent effects being reported.

Transdermal nicotine inhibits cytokine secretion from monocytes (van Dijk *et al.*, 1998); in vitro, at a 200 ng/ml concentration, nicotine inhibits cytokine release by about 38% in human peripheral blood mononuclear cells (Ouyang *et al.,* 2000), and also affects cytokine secretion from human lymphocytes, (Madretsma *et al*., 1996).

In U937 cells, a monocyte/macrophages cell line, nicotine inhibits LPS-induced expression at the transcriptional level of IL-1, IL-8 and PGE_2 (Sugano *et al*., 1998).

By evaluating superoxide anion production, a dual effect has been described: low doses (0.5-5 nM) of nicotine enhance zymosan-induced O_2^- production in guinea pig AM, whereas high doses (50-500 nM) inhibit it (Ogunbiyi & Misra, 1989).

Recent data state that cigarette smoke activates extracellular signal-related kinase (ERK) and p38 mitogen-activated protein (MAP) kinase, enhances IL-8 production and inhibits phagocytosis in GM-CSF derived macrophages (Winkler *et al*., 2008). Interestingly, this phenotype has been largely described in AM from healthy smokers (Winkler *et al*., 2008).

Tobacco smoke significantly enhanced O_2^- production in murine AM; moreover, AM isolated from smoke-exposed mice, inhibited LPS-induced B lymphocyte proliferation (Ishida *et al*., 2009).

The data presented in this PhD thesis are in good agreement with these results, as we report a significantly enhanced O_2^- production and a more robust release of proinflammatory cytokines in monocyte/macrophages from healthy smokers and smoker patients.

In the first paper (Gunella *et al*., 2006) presented here, we evaluated the effect of MSP on human AM isolated from healthy donors or patients with interstitial lung diseases (smokers and non-smokers).

Previous experimental data by our group had originally assessed the presence of RON, the receptor for MSP, on human macrophages of different origin, but not on human monocytes (Brunelleschi *et al*., 2001).

In this paper, we confirm that MSP evokes, in a concentration-dependent manner, O_2^- production in human AM, maximal effects being observed at 300-500 ng/ml. Interestingly, MSP-induced O_2^- production is significantly enhanced in healthy smokers, as compared to non-smokers, and is particularly elevated in AM from pulmonary fibrosis patients.

MSP also triggers TNF-α and IL-1β release, a more than doubled secretion being documented in AM from smokers. It has to be noted that the basal release of pro-inflammatory cytokines from unstimulated AM was generally higher in smokers as compared to non-smokers, and that the highest basal secretion was found in patients with pulmonary fibrosis, so confirming previous data (Conron *et al*., 2001; Ziegenhagen & Muller-Quernheim, 2003).

We originally demonstrate that, at 100 ng/ml, MSP induces NF-κB translocation in AM and MDM from smokers and non-smokers. Here again, cells from smokers present constitutively higher degree of activation, and MSP is about as effective as LPS in inducing NF-κB translocation.

Moreover, MSP is particularly active on the p50 subunit of the transcription factor, which, according to previous results, (Carter *et al*., 1998, Bardelli *et al*., 2005) and here confirmed, is the most relevant in human AM.

In the second paper (Amoruso *et al*., 2007) we investigated PPAR-γ expression, (either basal or ligand-induced), in human monocyte/macrophages, and the possible influence of nicotine on this expression. We confirm that PPAR-γ is a relevant macrophage marker (Chinetti *et al*., 1998; Tontonoz *et al*., 1998, Chawla *et al*., 2001), its level of expression increasing along with differentiation of monocytes into macrophages, and point out originally that cells from healthy smokers have a constitutively higher PPAR-γ expression. Moreover, nicotine challenge dose-dependently increases PPAR-γ expression, with a maximum of about 2-fold in monocytes and 1.5- fold in macrophages.

Nicotine was used at concentrations similar to those measured in the blood (about 70 ng/ml, Russell *et al.*, 1980) and tissues (0.5-2.6 times serum levels, Benowitz, 1988) of smokers subjects.

We also confirm that nicotine effects in human monocytes/macrophages are receptor-mediated, as previously stated (Yoshikawa *et al*., 2006; Wang *et al.*, 2003). In fact, we present evidence that:

a) α7 subunit of the nicotinic receptor is expressed on both monocytes and macrophages and

b) α-bungarotoxin, a selective α7 antagonist, significantly reduces nicotine-induced PPAR-γ expression.

The third paper (Amoruso *et al*., 2008) demonstrates that Substance P (SP), a well-known pro-inflammatory mediator, is able to enhance in human monocyte/macrophages, the expression of PPAR-γ, a suggested anti-inflammatory receptor, expression being affected by tobacco smoke.

First of all, we demonstrate that monocytes and MDM from healthy smokers present a more than double NK-1 receptor expression, as compared to cells from healthy non-smokers.

SP stimulates PPAR-γ expression in a concentration-dependent manner (10^{-10} M-10^{-6} M) with maximal effects similar to those evoked by the endogenous PPAR-γ agonist 15d-PGJ_2. Moreover, SP-induced PPAR-γ expression is reproduced by selective NK-1 agonists and reverted by competitive NK-1 antagonist. Interestingly, GW9662, a PPAR-γ antagonist, significantly reduces SP- induced PPAR-γ expression.

SP promotes proinflammatory cytokine release (Delgado *et al*., 2003; Bardelli *et al*., 2005), while PPAR-γ agonists reduce it (Subbaramaiah *et al*., 2001). We report here that both the endogenous PPAR-γ ligand and ciglitazone dose-dependently inhibit SP-induced release of TNF-α and IL-6 by human monocytes and macrophages.

Monocytes and macrophages are extremely responsive to a variety of stimuli, which can markedly affect their phenotype and functional activities. In this PhD thesis we have provided some examples which can contribute to a more precise evaluation of their functioning.

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APPENDIX

Papers 1, 2, 3

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Macrophage-stimulating protein differently affects human alveolar macrophages from smoker and non-smoker patients: evaluation of respiratory burst, cytokine release and NF-κB pathway

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1 Macrophage activation is a key feature of inflammatory reactions occurring during bacterial infections, immune responses and tissue injury. We previously demonstrated that human macrophages of different origin express the tyrosine kinase receptor recepteur d'origine nantaise, the human receptor for MSP (RON) and produce superoxide anion (O_2^-) when challenged with macrophage-stimulating protein (MSP), the endogenous ligand for RON.

2 This study was aimed to evaluate the role of MSP in alveolar macrophages (AM) isolated from healthy volunteers and patients with interstitial lung diseases (sarcoidosis, idiopathic pulmonary fibrosis), either smokers or non-smokers, by evaluating the respiratory burst, cytokine release and nuclear factor-kappa B (NF-κB) activation. MSP effects were compared with those induced by known AM stimuli, for example, phorbol myristate acetate, *N*-formyl-methionyl-leucyl-phenylalanine, lipopolysaccharide.

3 MSP evokes O_2^- production, cytokine release and NF-κB activation in a concentration-dependent manner. By evaluating the respiratory burst, we demonstrate a significantly increased O_2^- production in AM from healthy smokers or smokers with pulmonary fibrosis, as compared to non-smokers, thus suggesting MSP as an enhancer of cigarette smoke toxicity.

4 Besides inducing interleukin-1 beta (IL-1β) and interleukin-10 (IL-10) production, MSP triggers an enhanced tumor necrosis factor-alpha release, especially in healthy and pulmonary fibrosis smokers. On the contrary, MSP-induced IL-10 release is higher in AM from healthy non-smokers.

5 MSP activates the transcription factor NF-κB; this effect is more potent in healthy and fibrosis smokers (2.5-fold increase in p50 subunit translocation). This effect is receptor-mediated, as it is prevented by a monoclonal anti-human MSP antibody.

6 The higher effectiveness of MSP in AM from healthy smokers and patients with pulmonary fibrosis is suggestive of its role in these clinical conditions.
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Abbreviations: AM, alveolar macrophages; BAL, broncho-alveolar lavage; FMLP, *N*-formyl-methionyl-leucyl-phenylalanine; IL-10, interleukin-10; IL-1β, interleukin-1 beta; IPF, idiopathic pulmonary fibrosis; LPS, lipopolysaccharide; MDM, monocyte-derived macrophages; MSP, macrophage-stimulating protein; NF-κB, nuclear factor-kappa B; O_2^-, superoxide anion; PMA, phorbol 12-myristate 13-acetate; RON, recepteur d'origine nantaise, the human receptor for MSP; STK, stem cell-derived tyrosine kinase, the murine receptor for MSP; TNF-α, tumor necrosis factor-alpha

Introduction

Macrophage-stimulating protein (MSP) is a 80-kDa serum protein that was identified about 30 years ago by its ability to stimulate shape change, migration and phagocytosis of murine resident peritoneal macrophages (Leonard & Skeel, 1976). MSP is synthesized in the liver and circulates in the blood at a serum concentration of 2–5 mM as an inactive precursor, pro-MSP. The bioactive MSP is produced by proteolytic conversion during blood coagulation and at sites of inflammation (Wang *et al*., 2002). MSP acts on target tissues by activating the tyrosine kinase receptors recepteur d'origine nantaise (RON), the human receptor for MSP, and stem cell-derived tyrosine kinase (STK), the murine receptor for MSP, the expression of STK being regarded as a marker of terminal differentiation of murine macrophages (Iwama *et al*., 1995). MSP has been shown to inhibit lipopolysaccharide (LPS)- and cytokine-induced nitric oxide (NO) production as well as inducible NO synthase (iNOS) and cyclooxygenase-2

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expression in mouse peritoneal macrophages (Chen *et al*., 1998; Wang *et al*., 2002; Zhou *et al*., 2002). Targeted deletion of STK resulted in enhanced NO production by murine macrophages (Correll *et al*., 1997) and the MSP/STK complex reduced the nuclear translocation of nuclear factor-kappa B (NF-κB) induced by LPS plus IFN-γ in STK-transfected RAW 264.7 cells (a murine macrophage cell line) (Liu *et al*., 1999). As known, human macrophages present marked differences from murine peritoneal macrophages and macrophage cell lines, especially regarding NO production: while murine macrophages rapidly produce large amounts of NO after challenge with inflammatory cytokines or LPS, human macrophages usually do not, although they express the iNOS gene (Albina, 1995).

The effects of MSP on human macrophages and its role in human pathophysiology have been poorly investigated. In 2001, we originally demonstrated that human macrophages of different origin (peritoneal macrophages isolated from ascitic fluid of cirrhotic patients, alveolar macrophages (AM) from eight patients with different lung diseases, as well as monocyte-derived macrophages from healthy volunteers), but not human monocytes, express authentic and functional RON receptors and undergo a respiratory burst upon challenge with MSP (Brunelleschi *et al*., 2001). MSP-evoked superoxide anion (O_2^-) production is mediated by tyrosine kinase activity, requires the activation of Src, but not of phosphatidyl-inositol 3-kinase (which is implicated in MSP/RON signal transduction in other cell types) and involves MAP kinase and p38 signalling pathways (Brunelleschi *et al*., 2001).

Other authors also reported that MSP is present, at biological significant concentrations, in the broncho-alveolar spaces, where AM are located, as well as in induced sputum from healthy subjects and patients with bronchiectasis (Sakamoto *et al*., 1997; Takano *et al*., 2000).

The present study was undertaken to explore the role of MSP in different lung diseases, by evaluating the respiratory burst, cytokine release and NF-κB signalling in AM isolated from healthy volunteers and patients with interstitial lung diseases, for example, sarcoidosis and idiopathic pulmonary fibrosis (IPF), either smokers or non-smokers.

We demonstrate that, in a concentration-dependent manner, MSP evokes O_2^- production and cytokine release, being more effective in healthy smokers and in patients with IPF. We also present direct evidence that MSP activates the transcription factor NF-κB, the p50 subunit being especially involved, so providing new insights on the possible mechanisms involved in the control of AM responsiveness.

Methods

Isolation of human AM from broncho-alveolar lavage

This study and the research protocol were approved by the local Ethical Committee. Broncho-alveolar lavage (BAL) was mainly performed for diagnostic purposes, to have a further validation/confirmation of the suspected disease. AM were isolated from BAL as described (Brunelleschi *et al*., 2001; Bardelli *et al*., 2005). After informed consent and pretreatment with parenteral atropine sulphate, airways were anaesthetized and a fiberoptic bronchoscope was advanced and wedged into the middle lobe under direct visualization. Lavage was carried out with 140–200 ml of prewarmed (37°C) sterile saline solution in 20-ml aliquots with immediate gentle vacuum (syringe) aspiration after each injection. The fluid obtained was filtered through two layers of sterile surgical gauze and centrifuged ($400 \times g$, 30 min). The whole BAL pellet was washed twice in phosphate-buffered salt solution (PBS), resuspended in RPMI 1640 medium supplemented with 5% fetal calf serum (FCS), 2 mM glutamine, 10 mM Hepes, 50 μg ml^{-1} streptomycin and 5 U ml^{-1} penicillin, and plated in six-well tissue culture plates (Costar, U.K.). After 2 h at 37°C in humidified 5% CO_2 atmosphere, nonadherent cells (mainly lymphocytes) were gently removed and AM were used for the experiments. Total cell count and viability evaluation (Trypan blue dye exclusion test, always $>98\%$) were performed on a Burker haemocytometer. Differential cell count was carried out on Diff-Quick (Don Baxter)-stained cytospin smears, counting at least 400 cells. The adherent cell population was $>99\%$ AM. Phenotypical analysis was carried out on cytocentrifuge (Cytospin, U.K.; 500 r.p.m., 10 min) slides by employing leukocyte-specific monoclonal antibodies for CD68, CD14 and HLA-DR (from Becton Dickinson, U.K.). In some cases, monocyte-derived macrophages (MDM) were prepared from circulating monocytes of single individual patients, cultured for 7–8 days in a CO_2 incubator at 37°C in RPMI 1640 medium containing 10% FCS, glutamine and antibiotics, as described (Brunelleschi *et al*., 2001). MDM were defined as macrophage-like cells also by evaluating the decrease in the surface monocyte marker CD14 (Brunelleschi *et al*., 2001).

Superoxide anion (O_2^-) production in AM

Adherent AM ($0.4–1 \times 10^6$ cells/plate) were washed twice with PBS, incubated in RPMI 1640 medium (without phenol red, no antibiotics and no FCS) and challenged with increasing concentrations of MSP (3–500 ng ml^{-1}) for 30 min. The effects of MSP were compared with those evoked by maximal effective concentrations of the protein kinase C activator, phorbol 12-myristate acetate (PMA; 10^{-7} M) and the bacterial peptide, *N*-formyl-methyl-leucyl-phenylalanine (FMLP; 10^{-6} M). O_2^- production, evaluated by the superoxide dismutase (SOD)-inhibitable cytochrome C reduction, was expressed as nmol cytochrome C reduced 10^6 cells^{-1} 30 min^{-1}, using an extinction coefficient of 21.1 mM (Brunelleschi *et al*., 2001). To avoid interference with spectrophotometrical recordings of O_2^- production, AM were incubated with RPMI 1640 without phenol red. Experiments were performed in duplicate; control values (e.g., basal O_2^- production in the absence of stimuli) were subtracted from all determinations.

Cytokine release in AM

Adherent AM were challenged with MSP (3–500 ng ml^{-1}) or the standard stimuli (PMA 10^{-7} M, FMLP 10^{-6} M, LPS 10 ng ml^{-1}) for 24 h at 37°C to ensure maximal cytokine release (Bardelli *et al*., 2005). Supernatants were collected and stored at −20°C. Tumor necrosis factor-alpha (TNF-α), interleukin 1 beta (IL-1β) and interleukin-10 (IL-10) (the latter was evaluated as the most important anti-inflammatory cytokine) in the samples were measured using enzyme-linked immunoassay kit (Pelikine Compact™ human ELISA kit). The

measurements were performed according to the manufacturer's instructions. The minimum detectable concentrations of TNF-α, IL-1β and IL-10 were 1.4, 1.5 and 1.3 pg ml^{-1}, respectively. No crossreactivity was observed with any other known cytokine. Control values (e.g., cytokine release from untreated, unstimulated AM) were subtracted from all determinations. Results are expressed in pg ml^{-1}.

Evaluation of NF-κB activation

The activation of NF-κB induced by MSP, PMA and LPS 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), as previously described (Bardelli *et al*., 2005). As EMSA assays require large numbers of cells (5–10 $\times$ 10^6 for each sample) to perform these experiments, we used MDM obtained from the same individuals who underwent BAL procedure. In EMSA assays, nuclear extracts (5 μg) from MDM were incubated with 2 μg poly (dI-dC) and [^{32}P]ATP-labelled oligonucleotide probe (100,000–150,000 c.p.m.; Promega, St Louis, U.S.A.) in binding buffer for 30 min at room temperature. The NF-κB consensus oligonucleotide (5′-AGTTGAGGGGACTTTCC CAGGC-3′) was from Promega. The nucleotide–protein complex was separated on a polyacrylamide gel, the gel was dried and radioactive bands were detected by autoradiography (Bardelli *et al*., 2005). Supershift assays were performed with commercial antibodies (anti-NF-κB p50: ab 7949 and anti-NF-κB p65: ab 7970) from Abcam (U.K.) at a final concentration of 1 μg ml^{-1}. AM nuclear extracts were prepared and evaluated for the presence of p50 and p65/RelA subunits using Trans AM™ 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. 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 1 luminometer and results are expressed as RLU (Relative Luminescence Unit), according to Bardelli *et al*. (2005).

Drugs and analytical reagents

Human recombinant MSP and anti-human MSP β-chain monoclonal antibody (MAB 735) were obtained from R&D Systems (Minneapolis, U.S.A.); FCS (Lot 40F-7234K) was from Gibco (Paisley, U.K.). PBS, RPMI 1640 (with or without phenol red), BSA, glutamine, Hepes, streptomycin, penicillin, LPS, PMA, SOD, cytochrome *c*, bromophenol blue, glycine, glycerol, methanol and Tween 20 were obtained from Sigma (Milwaukee, U.S.A.). Nitro-cellulose filters (Hybond) and poly(dI-dC) were from Amersham (Buckinghamshire, U.K.). All the reagents for EMSA assays were purchased from Promega Corporation (St Louis, U.S.A.). Tissue-culture plates were from Costar Ltd (Buckinghamshire, U.K.); all cell culture reagents, with the exception of FCS, were endotoxin-free according to details provided by the manufacturer. TNF-α, IL-1β and IL-10 immunoassay kits were obtained from CLB/Sanquin, Central Laboratory of the Netherlands Red Cross (Netherlands).

Data and statistical analysis

Data are mean $\pm$ s.e.m. of duplicate determinations of '*n*' independent experiments. Concentration–response curves for MSP were constructed and EC_{50} values were interpolated from curves of best-fit. When required, statistical evaluation was performed by unpaired, two-tailed Student's *t*-test.

Results

Study population, BAL and phenotype of AM

A total of 47 subjects, 25 male and 22 female subjects, aged between 45 and 68 years, 23 smokers and 24 non-smokers, were studied; 15 patients had pulmonary sarcoidosis, 14 patients had IPF and 18 individuals were classified as healthy subjects, that is, individuals with no history of cardiopulmonary disease or other chronic diseases, no diagnosed lung disease and no medication. In a few cases, the attribution of an 'healthy' subject to the category was done after the BAL procedure. The characteristics and smoking history of the study population are presented in Table 1.

Total and differential cell counts in BAL and phenotype of AM are presented in Table 2. As expected, a significant ($P<0.05$) increase in the total cell number was observed in all the smoker subjects as compared to non-smokers; patients with sarcoidosis (both smokers and non-smokers) presented an alveolar lymphocytosis (25$\pm$2 and 24$\pm$2%, respectively) and a reduction in AM percentage (74.2$\pm$1.4% in smokers and 74.6$\pm$3% in non-smokers) (Table 2). The great majority of AM (96$\pm$1%) in healthy smokers was CD68+ and a high percentage (84$\pm$1 and 68$\pm$3%, respectively) of AM expressed also HLA-DR and CD14. As known, CD68 expression is related to the presence of AM involved in the oxidative burst, CD14 expression is related to cytokine production by LPS, whereas HLA-DR is related to antigen presentation. The expression CD14 and CD68 was significantly ($P<0.05$) higher in AM collected from healthy smokers and smoker patients as compared to the respective non-smoker groups (Table 2). The non-smoker sarcoidosis group presented a very low (26$\pm$2.5%; $n=8$) CD14 expression as compared to sarcoidosis smokers (55$\pm$2%; $n=7$); similar results were obtained in the

Table 1 Study population

Subjects	*Sex (M/F)*	*Age (years)*	*Number of cigarettes day^{-1}*	*Years on smoke*
Healthy				
Smokers ($n=10$)	6/4	51.4$\pm$1.6	20.3$\pm$2	23.4$\pm$2.5
Non-smokers ($n=8$)	4/4	54.3$\pm$2.7	—	—
Sarcoidosis				
Smokers ($n=7$)	4/3	55.3$\pm$2.2	18.3$\pm$2.3	25.4$\pm$2.7
Non-smokers ($n=8$)	3/5	53.4$\pm$2.4	—	—
Pulmonary fibrosis				
Smokers ($n=6$)	3/3	52.8$\pm$2	19.7$\pm$3	25.3$\pm$3
Non-smokers ($n=8$)	5/3	53.8$\pm$1.5	—	—

Table 2 Total and differential cell count in BAL and AM phenotype

Subjects	*Total cell ml^{-1} BAL*	*AM (%)*	*Lympho (%)*	*PMN (%)*	*CD68+(%)*	*HLA-DR+(%)*	*CD14+(%)*
Healthy							
Smokers ($n=10$)	390.600±6.000	91.4±1.9	8.2±2	1±0.5	96±1	84±1	68±3
Non-smokers ($n=8$)	138.000±5.000*	90.6±1	8.6±1	0.2±0.1	83±1*	86±1	51±1*
Sarcoidosis							
Smokers ($n=7$)	348.000±6.200	74.2±1.4	25±2	1±0.5	91±0.4	77±3	55±2
Non-smokers ($n=8$)	170.000±6.900*	74.6±3	24±2	1±0.5	82±0.2*	72±4	26±2.5*
Pulmonary fibrosis							
Smokers ($n=6$)	380.500±6.000	86±3	12±4	1±0.3	93±0.8	82±1	58±3
Non-smokers ($n=8$)	202.000±5.500*	82±5	13±3	3±1.2	85±0.5*	71±2*	32±3*

Data are given as total cell number ml^{-1} BAL and percentage of total cell population (differential) in BAL. AM = alveolar macrophages; Lympho = alveolar lymphocytes; PMN = alveolar neutrophils. The AM phenotype was evaluated by measuring CD68, CD14 and HLA-DR; positive cells are expressed as percentage of total AM. *Denotes $P<0.05$ vs smokers of the corresponding group.

IPF group (Table 2). HLA-DR expression presented only minor variations among groups (values around 70–80% being always measured), with the only exception of the non-smoker IPF patients who presented a significant reduced HLA-DR expression (71±2; $n=8$) as compared to the IPF smokers (82±1%; $n=6$) (Table 2).

MSP evokes O_2^- production in human AM

Control, unstimulated human AM from healthy subjects (smokers and non-smokers) and patients with interstitial lung diseases (both smokers and non-smokers) spontaneously released substantial amounts of O_2^-, as reported in Table 3. These values were subtracted from those obtained after MSP, FMLP or PMA challenge to obtain the net O_2^- production. PMA, used at 10^{-7} M (a near maximal concentration), produced 23±2 ($n=10$) and 17±1.5 ($n=8$; $P<0.05$) nmol cytochrome *c* reduced per 10^6 AM in healthy smokers and non-smokers, respectively (Table 3) and even higher amounts in AM isolated from patients with interstitial lung diseases, the maximal effect (38±2.2 nmol cytochrome *c* reduced 10^6 AM^{-1}; $n=6$) being observed in smoker patients with IPF (Table 3). FMLP, used at the fully effective 10^{-6} M concentration, was less potent than PMA; in this case, too, maximal O_2^- production was observed in smoker patients with IPF (Table 3). On the contrary, LPS-evoked O_2^- production was minimal (data not shown). In the concentration range 3–500 ng ml^{-1}, MSP evoked O_2^- production in AM from both smokers and non-smokers, higher production being observed in smokers (Figure 1). As depicted in Figure 1, maximal activation by MSP was observed at 300–500 ng ml^{-1}, MSP being particularly effective in healthy smokers ($P<0.05$ vs non-smokers; Figure 1a) and in patients with IPF (Figure 1c). In AM from sarcoidosis patients, MSP, although effective, did not demonstrate significant differences between smokers and non-smokers (Figure 1b). In all cases, MSP-induced maximal O_2^- production was quantitatively similar to the PMA-evoked one (Figure 1 and Table 3). The EC_{50} values for MSP were: 55 ng ml^{-1} in healthy smokers and 103 ng ml^{-1} in healthy non-smokers, 100 and 118 ng ml^{-1} in sarcoidosis patients (smokers and non-smokers, respectively), 35 ng ml^{-1} in smokers with IPF and 65 ng ml^{-1} in non-smokers with IPF (Figure 1 a–c).

Table 3 Superoxide anion production from AM

Subjects	*O_2^- production, control*	*O_2^- production, PMA 10^{-7} M*	*O_2^- production, FMLP 10^{-6} M*
Healthy			
Smokers ($n=10$)	13.1±2	23±2	9.5±1.2
Non-smokers ($n=8$)	2.2±0.5**	17±1.5*	3.5±0.6*
Sarcoidosis			
Smokers ($n=7$)	9±1	30±2	8±2
Non-smokers ($n=8$)	4±0.8*	24±1.8*	7.2±1.8
Pulmonary Fibrosis			
Smokers ($n=6$)	8.2±1.2	38±2.2	18.2±3
Non-smokers ($n=8$)	4.4±1.8*	30±2	16±3

Data are means±s.e.m. of *n* patients. *$P<0.05$ vs smokers; **$P<0.001$ vs smokers. Spontaneous O_2^- production was subtracted from any determination with stimuli. O_2^- production is expressed as nmol cytochrome C reduced/10^6 AM.

MSP-evoked cytokine release in AM

We also evaluated the release of proinflammatory cytokines, namely TNF-α and IL-1β, as well as IL-10 release (the most relevant anti-inflammatory cytokine in AM), after challenge with MSP or the standard stimuli PMA, FMLP or LPS. Basal values (i.e. the release from control, unstimulated AM) were subtracted from all determinations and are listed in Table 4: TNF-α represents the most abundant cytokine in AM and is spontaneously released to significant higher amounts in healthy smokers ($P<0.05$ vs non-smokers) and patients with interstitial lung diseases.

As reported in Table 5, dealing with FMLP-, PMA- and LPS-evoked cytokine release, TNF-α is the cytokine released to significant higher amounts by all stimuli in all patients, LPS is the most effective AM stimulus for cytokine release from human AM, whereas PMA evoked no IL-10 release above baseline levels in all subjects (Table 4).

In the concentration range 3–500 ng ml^{-1}, MSP induced TNF-α release from AM and was more effective in AM from healthy smokers (Figure 2a; $P<0.05$) and smokers with IPF

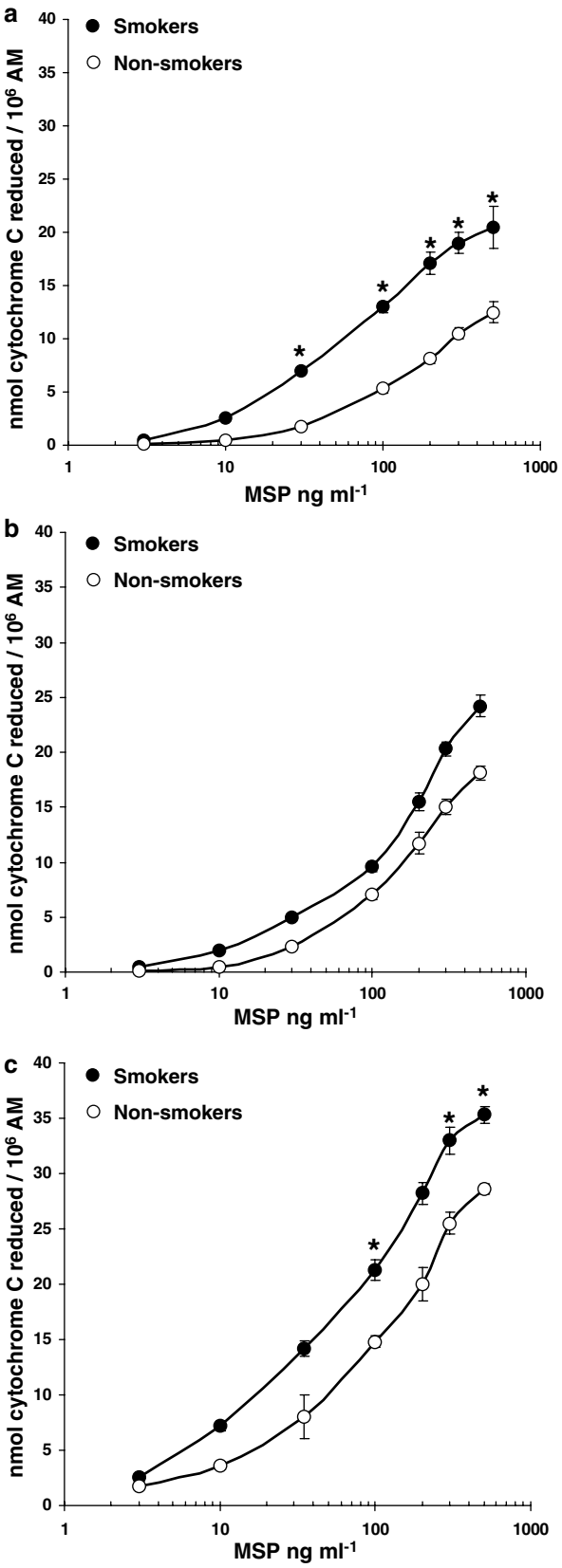

Figure 1 MSP evokes O_2^- production in AM. AM from healthy smokers and non-smokers (a), patients with sarcoidosis (b) and idiopathic pulmonary fibrosis (c) were challenged with increasing concentrations of MSP (3–500 ng ml^{-1}) for 30 min; ● = smokers, ○ = non-smokers. Results are means ± s.e.m. of six to 10 experiments in duplicate. *$P<0.05$ vs non-smokers.

Table 4 Basal release of cytokines in AM

Subjects	*TNF-α* (pg ml^{-1})	*IL-1β* (pg ml^{-1})	*IL-10* (pg ml^{-1})
Healthy			
Smokers ($n=5$)	63 ± 3	10 ± 0.5	30 ± 3
Non-smokers ($n=5$)	35 ± 2*	8 ± 2	49 ± 4
Sarcoidosis			
Smokers ($n=5$)	139 ± 10	39 ± 4	20 ± 11
Non-smokers ($n=5$)	137 ± 14	35 ± 2	10 ± 3
Pulmonary fibrosis			
Smokers ($n=5$)	146 ± 20	50 ± 6	38 ± 13
Non-smokers ($n=5$)	91 ± 13*	30 ± 8	36 ± 15

Values are means ± s.e.m. of experiments in duplicate. *$P<0.05$ vs smokers.

(Figure 2c; $P<0.05$) as compared to non-smokers (Figure 2). No major differences between smokers and non-smokers were observed in AM from sarcoidosis patients (Figure 2b). To assess the specificity of MSP response, we used a commercial monoclonal anti-human MSP β-chain antibody (R&D), which, at 2 μg ml^{-1}, inhibited 90% of the MSP-evoked TNF-α release in healthy smokers (data not shown). By evaluating IL-1β production from human AM, we observed that MSP acted in a concentration-dependent manner, maximal release being documented in smokers with IPF (Figure 3c). In healthy subjects and sarcoidosis patients, MSP-evoked IL-1β release was similar in smokers and non-smokers (Figure 3a and b).

In keeping with our previous observations (Bardelli *et al*., 2005), human AM released lower amounts of IL-10, as compared to other cytokines (Figure 4). MSP-induced IL-10 release in AM from healthy non-smokers was higher ($P<0.05$) than in healthy smokers (Figure 4a) and was quantitatively reduced in AM obtained from patients with sarcoidosis (Figure 4b) and IPF (Figure 4c). Similar results were observed by evaluating MSP-induced cytokine release in MDM obtained from both healthy smokers and non-smokers (data not shown).

MSP induces NF-κB activation

In EMSA studies, we recently reported that AM from healthy smokers present an enhanced nuclear translocation of the transcription factor NF-κB as compared to AM from healthy non-smokers, the p50 subunit of NF-κB being the predominant one (Bardelli *et al*., 2005). Others have reported that NF-κB activity is elevated in AM collected from patients with active sarcoidosis (Culver *et al*., 2004) or fibrosing alveolitis (Conron *et al*., 2002). As large numbers of cells are required in these studies, we used MDM from individual smokers and non-smokers for EMSA studies (5–10 $\times$ 10^6 cells per sample). We used AM (2–3 $\times$ 10^6 cells per sample) to measure the nuclear content of p50 and p65 subunits by an ELISA kit, to ensure a better quantitative evaluation. Although different NF-κB forms have been described, the p50–p65 heterodimer is the predominant species in many cell types (Baldwin, 1996).

Gel shift analysis demonstrates that MSP 100 ng ml^{-1} induced NF-κB activation in MDM from healthy non-smokers (Figure 5). As previously demonstrated (Wang *et al*., 1997),

Table 5 FMLP-, PMA- and LPS-evoked cytokine release in AM

Subjects	*TNF-α* (pg ml^{-1})			*IL-1β* (pg ml^{-1})			*IL-10* (pg ml^{-1})		
	FMLP 10^{-6} M	*PMA* 10^{-7} M	*LPS* 10 ng ml^{-1}	*FMLP* 10^{-6} M	*PMA* 10^{-7} M	*LPS* 10 ng ml^{-1}	*FMLP* 10^{-6} M	*PMA* 10^{-7} M	*LPS* 10 ng ml^{-1}
Healthy									
Smokers ($n=5$)	37±4	515±36	3230±80	55±10	120±30	475±40	35±6	12±4	385±11
Non-smokers ($n=5$)	11±5	112±30*	1703±70*	46±8	108±10	290±30	45±7	10±3	360±20
Sarcoidosis									
Smokers ($n=5$)	30±5	596±45	3330±80	60±15	80±10	320±30	25±5	15±7	232±35
Non-smokers ($n=5$)	24±3	242±40*	1780±80*	30±10	50±22	242±28	29±3	12±3	442±80
Pulmonary fibrosis									
Smokers ($n=5$)	309±40	560±50	4.685±100	115±12	179±15	1.580±70	15±7	11±6	520±25
Non-smokers ($n=5$)	65±20	240±30*	3.380±40*	70±8	80±8	890±20	20±5	10±4	816±40

Data are means±s.e.m. of n patients. AM were challenged for 24 h with FMLP 10^{-6} M, PMA 10^{-7} M or LPS 10 ng ml^{-1}. *$P<0.05$ vs smokers.

MSP binds to its receptor RON *via* the β-chain; so, to confirm the ligand specificity in the activation of NF-κB, we used a commercial monoclonal antibody against human MSP β-chain. In the presence of this antibody (2 μg ml^{-1}, preincubated for 45 min), the nuclear translocation induced by MSP was significantly reduced (Figure 5). MSP at two different concentrations, 50 and 100 ng ml^{-1}, induced NF-κB activation in MDM from both healthy smokers and non-smokers (Figure 6); in keeping with Bardelli *et al*. (2005), we detected a relevant spontaneous activation in MDM from healthy smokers (Figure 6b and d; control, lane 1), which is significantly higher than in non-smokers (Figure 6a and c; control, lane 1). The effect of MSP is concentration-dependent and, in smokers, even higher than the LSP-induced one (Figure 6b and d). Figure 6 also shows supershift assays for p65 in both non-smokers (Figure 6a) and smokers (Figure 6b), as well as for p50 (Figure 6c: non-smokers; Figure 6d: smokers). In any case, p65 supershift is weak, thereby suggesting that it is not the major component involved in the activation, whereas p50 is potently supershifted (Figure 6).

To ensure a better quantitative evaluation, we also assessed the translocation of p65 and p50 subunits in AM from healthy subjects (three smokers and three non-smokers), patients with sarcoidosis (three smokers and three non-smokers) or IPF (three smokers and three non-smokers), using a commercially available ELISA kit. First of all, we confirmed our previous observation (Bardelli *et al*., 2005) that the p50 subunit is the more abundant and/or more translocated one. In fact, RLU values for p50 are about two-fold higher than those measured for p65 in each group of patients (please, see, for a comparison, Figures 7 and 8). As depicted in Figure 7 (dealing with p65 subunit), MSP, LPS and PMA induced the nuclear translocation of this subunit. Interestingly, MSP induced an enhanced ($P<0.05$) nuclear translocation of p65 subunit in AM from healthy smokers (Figure 7a) and smokers with IPF (Figure 7c) as compared to non-smokers, so confirming what observed by measuring the respiratory burst (see Figure 1) and TNF-α release (see Figure 2). Moreover, in keeping with previous demonstrations (Culver *et al*., 2004), NF-κB activity in AM from non-smokers with sarcoidosis was upregulated, as revealed by the high amounts of translocated p65 subunit in unstimulated AM (Figure 7b). In this case, no significant differences were observed between smokers and non-smokers after MSP challenge (Figure 7b). By evaluating the nuclear translocation of the p50 subunit (Figure 8), MSP was particularly effective in AM from healthy smokers (Figure 8a) and IPF smokers (Figure 8c), but induced a similar effect in AM from sarcoidosis patients (Figure 8b), as already observed with p65. It is also worth noting that, in AM from IPF patients, MSP is the only stimulus which induced a more than doubled p50 nuclear translocation in smokers as compared to non-smokers ($P<0.01$), the amount of translocated p50 reaching about 5000 RLU (Figure 8c).

Discussion

Several observations indicate that growth factors and proinflammatory cytokines are exaggerated in fibrotic lung diseases (Krein & Winston, 2002; Ziegenhagen & Muller-Quernheim, 2003; Khalil & O'Connor, 2004). A growing body of evidence suggests that hepatocyte growth factor (HGF), which shares 45% homology with MSP and belongs to the same receptor family, could play an important role. In fact, enhanced HGF concentrations have been documented in sera from patients with IPF (Hojo *et al*., 1997; Yamanouchi *et al*., 1998), in BAL from sarcoidosis or IPF patients (Sakai *et al*., 1997), and a defective HGF secretion by lung fibroblasts has been related to IPF development (Marchand-Adam *et al*., 2003). As a member of the HGF family of growth factors, MSP has been evaluated for its effects in the lung. Currently, we recognize that the MSP/RON complex increases ciliary beat frequency of human nasal cilia (Sakamoto *et al*., 1997), is induced in early preneoplastic lung injury in hamster (Willett *et al*., 1997), is expressed in non-small-cell lung tumors (Willett *et al*., 1998) and stimulates oxy-radical production from human macrophages (Brunelleschi *et al*., 2001). Furthermore, MSP has been detected in induced sputum from normal subjects (about 8 ng ml^{-1}; Takano *et al*., 2000), as well as in BAL from four healthy non-smokers, at concentrations ranging from 1.3 to 5.8 ng ml^{-1} (Sakamoto *et al*., 1997).

We report here that MSP, in a concentration-dependent manner, induces significant respiratory burst and cytokine release in AM from patients with interstitial lung diseases and healthy volunteers, both smokers and non-smokers. This growth factor mainly acts at concentrations higher than those

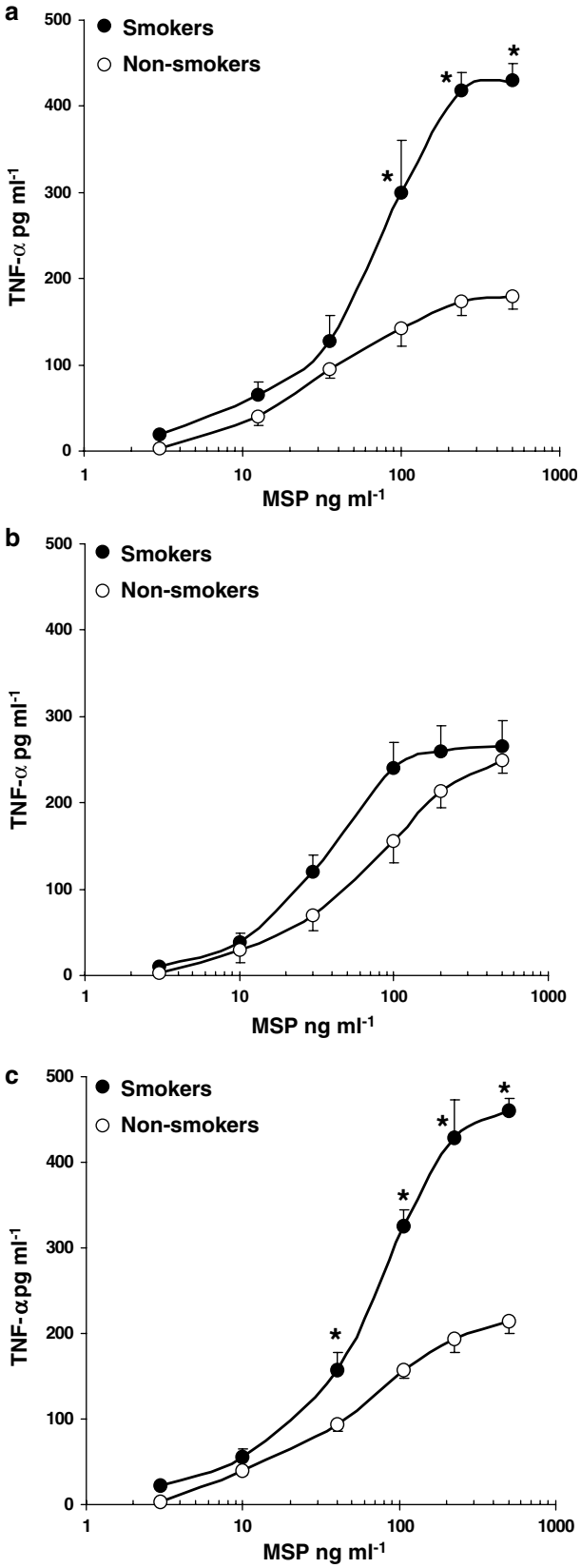

Figure 2 MSP evokes TNF-α release in AM. AM from healthy smokers and non-smokers (a), patients with sarcoidosis (b) and idiopathic pulmonary fibrosis (c) were challenged with increasing concentrations of MSP (3–500 ng/ml) for 24 h; ● = smokers, ○ = non-smokers. Results are means ± s.e.m. of five experiments in duplicate. *$P<0.05$ vs non-smokers.

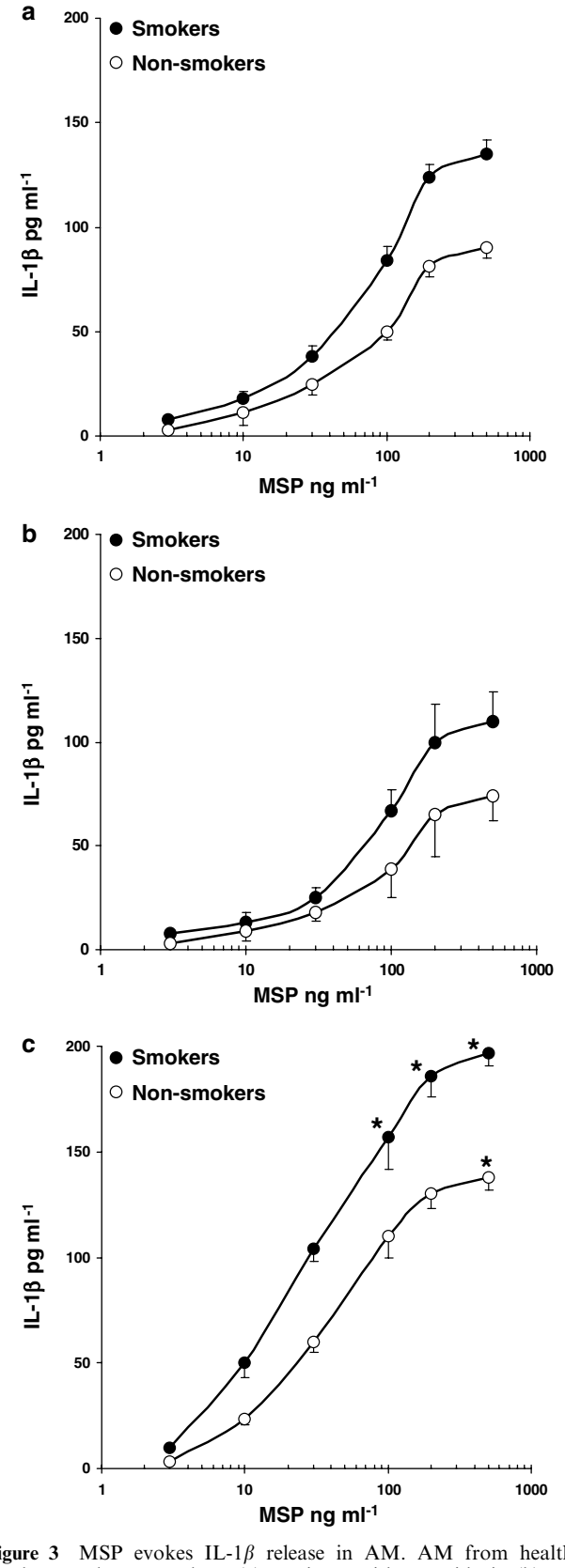

Figure 3 MSP evokes IL-1β release in AM. AM from healthy smokers and non-smokers (a), patients with sarcoidosis (b) and idiopathic pulmonary fibrosis (c) were challenged with increasing concentrations of MSP (3–500 ng/ml) for 24 h; ● = smokers, ○ = non-smokers. Results are means ± s.e.m. of five experiments in duplicate. *$P<0.05$ vs non-smokers.

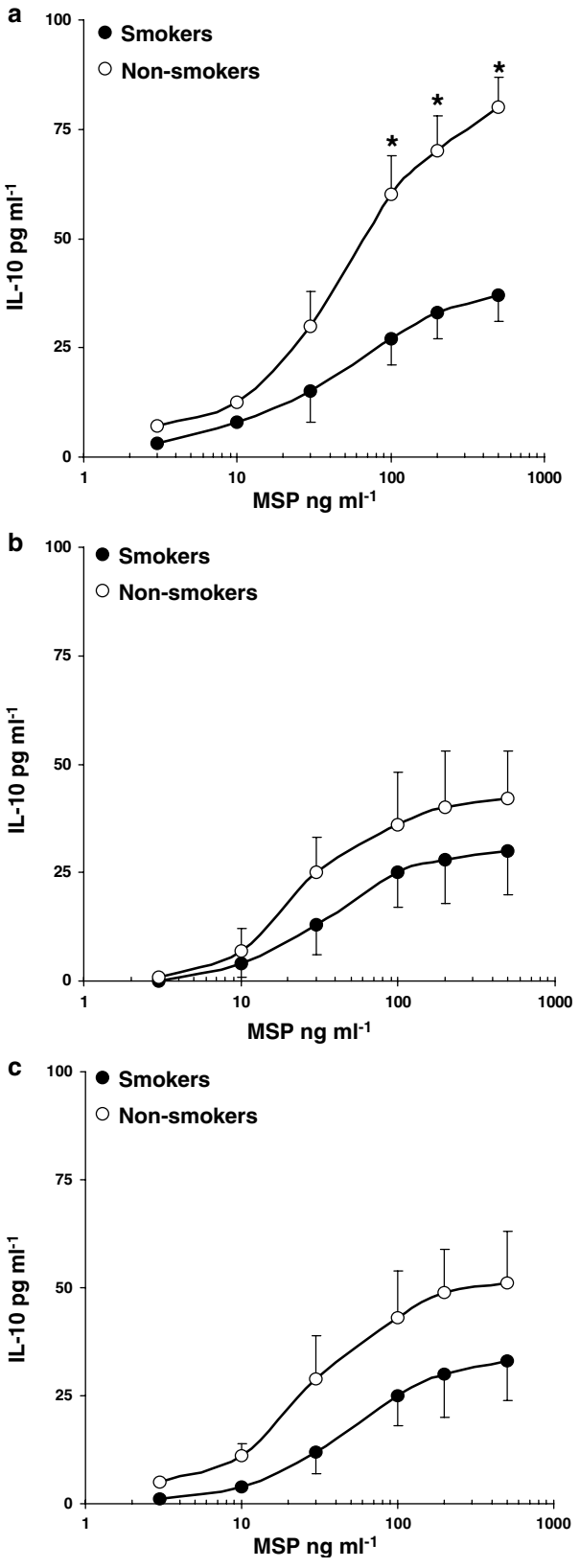

Figure 4 MSP evokes IL-10 release in AM from smokers and non-smokers. AM from healthy smokers and non-smokers (a), patients with sarcoidosis (b) and idiopathic pulmonary fibrosis (c) were challenged with increasing concentrations of MSP (3–500 ng/ml) for 24 h.; ● = smokers, ○ = non-smokers. Results are means ± s.e.m. of five experiments in duplicate. $^*P<0.05$ vs smokers.

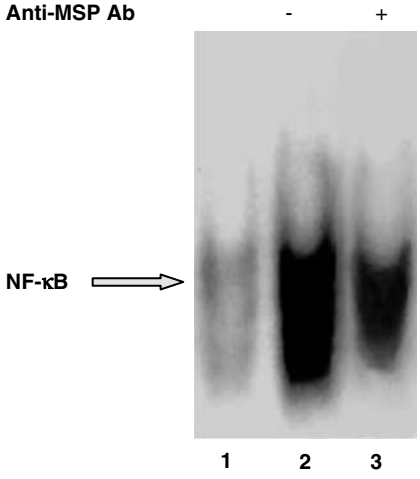

Figure 5 MSP evokes NF-κB activation in human MDM from healthy non-smokers. MDM from healthy non-smokers were challenged with MSP in the absence or presence of a monoclonal anti-MSP antibody ($2\,\mu g\,ml^{-1}$). Lane 1 = unstimulated, control MDM; lane 2 = MSP $100\,ng\,ml^{-1}$; lane 3: MSP + anti-MSP antibody. This experiment was performed three times with similar results.

measured in BAL, even if it is worth reminding that the absolute concentration of MSP in the broncho-alveolar spaces, where AM are located, should be higher (Sakamoto *et al*., 1997). We measured a significantly ($P<0.05$) increased O_2^- production when AM from healthy smokers or IPF smokers were challenged with MSP; on the contrary, no major differences were observed between smokers and non-smokers in the sarcoidosis group, despite the relevant respiratory burst induced by MSP. Regardless of the clinical condition evaluated, this growth factor is a potent AM stimulus, since MSP-induced respiratory burst is quantitatively similar to the PMA-evoked one and significantly higher than the FMLP-evoked one. These observations extend our previous data in human macrophages of different origin (Brunelleschi *et al*., 2001). Among the numerous cytokines involved in lung diseases, TNF-α has been appreciated as a crucial mediator for IPF and sarcoidosis (Ziegenhagen and Muller-Quernheim, 2003). In our experiments, MSP evoked the secretion of TNF-α in AM from all patients, a more than doubled release being observed, at the highest concentrations evaluated, in healthy smokers and IPF smokers as compared to the respective non-smoker groups. MSP also induced IL-1β release from AM, no significant differences being observed between smokers and non-smokers, except for the IPF group. In keeping with the fact (see below) that MSP activates NF-κB signalling, this result was somewhat unexpected. However, both PMA and LPS, although activating NF-κB and inducing cytokine secretion, did not release an enhanced amount of IL-1β in smokers (Bardelli *et al*., 2005; this paper). We have no conclusive explanation for this effect, but we remind that TNF-α and IL-1β production can be induced also through NF-κB-independent pathways (Bardelli *et al*., 2005). We and others (Conron *et al*., 2001; Ziegenhagen & Muller-Quernheim, 2003; Bardelli *et al*., 2005) observed that AM from smokers and sarcoidosis patients spontaneously produce a number of proinflammatory cytokines, including TNF-α, IL-1β and IL-6, but little of the immunoregulatory cytokine

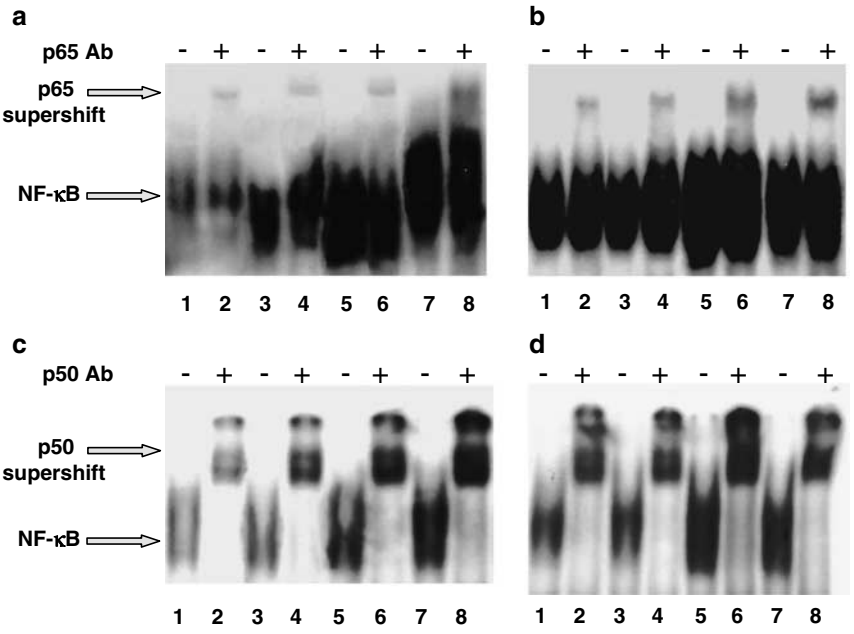

Figure 6 MSP evokes NF-κB activation in human MDM from healthy non-smokers and smokers: supershift assays. MDM from healthy non-smokers (a and c) and smokers (b and d) were challenged with MSP (50 and 100 ng ml^{-1}) or LPS 500 ng ml^{-1} for 2 h. Nuclear extracts (5 μg) were prepared and assayed for NF-κB activity by EMSA (see text for further details). In (a) and (b), supershifts with p65 antibody; in (c) and (d), supershift with p50 antibody. Lanes 1 and 2 = unstimulated, control MDM; lanes 3 and 4 = MSP 50 ng ml^{-1}; lanes 5 and 6 = MSP 100 ng ml^{-1}; lanes 7 and 8 = LPS 500 ng ml^{-1}. Experiments here depicted were performed with MDM from different individual donors: this fact could explain the different shape and intensity of the gel.

IL-10. Interestingly, MSP released higher amounts of IL-10 in AM from healthy non-smokers. This represents a peculiar feature of MSP: in fact, PMA- and FMLP-evoked release was negligible, whereas LPS released similar amounts in smokers and non-smokers of the three groups. As previously documented, IL-10 exerts anti-inflammatory effects and inhibits NF-κB activation in LPS-stimulated human AM (Raychaudhuri *et al*., 2000).

There is mounting evidence that NF-κB activation is important in the pathogenesis of different pulmonary diseases: elevated levels of NF-κB have been detected in AM obtained from patients with acute respiratory distress syndrome, sarcoidosis or IPF (Schwartz *et al*., 1996; Conron *et al*., 2001; Culver *et al*., 2004), but not in cells from healthy non-smokers (Farver *et al*., 1998). In the majority of unstimulated cells, NF-κB is located in the cytoplasm as a heterodimer or homodimer of protein components (p50 and p65, mainly) bound to an inhibitor IκB protein (Baldwin, 1996). Activation of this transcription factor involves sequential phosphorylation, ubiquitination and proteasome-mediated degradation of IκBα, resulting in the migration of the NF-κB complex to the nucleus and binding to promoter region of many cytokine and growth factor genes (Baldwin, 1996).

The presence of potential NF-κB sites in the STK/RON promoter was demonstrated some years ago (Waltz *et al*., 1998): however, only a few reports are available in the literature concerning MSP effects on NF-κB signalling.

We originally report here that, in human AM and MDM from both smokers and non-smokers, MSP efficiently activates NF-κB: at 100 ng ml^{-1}, a concentration which represents the EC_{50} value for the respiratory burst in healthy non-smokers, MSP is about as effective (or even more effective, see below) as PMA and LPS. As we reported previously, NF-κB is constitutively activated in healthy smokers (Bardelli *et al*., 2005); in this case, MSP-induced NF-κB nuclear translocation in MDM resulted in a two-fold increase over baseline and was more intense than the LPS-evoked one. Different NF-κB complexes are generated in AM from healthy volunteers; Carter *et al*. (1998) reported that specific NF-κB complexes are used for the transcription of various cytokine genes and that the p50 subunit binds the TNF-α sequence, mainly. A previous paper of our group demonstrated that the p50 subunit is the most abundant one in AM from both smokers and non-smokers, and is more efficiently translocated in smokers (Bardelli *et al*., 2005). We further confirm these observations by showing a weak p65 supershift but a very intense p50 supershift in MDM, and a doubled nuclear translocation of p50 (but about the same for p65) in unstimulated AM from healthy smokers as compared to non-smokers. In addition, there is a good correlation between the results of the p50 supershift assay and those obtained by the ELISA kit. When AM were challenged by PMA, LPS or MSP, a further enhanced nuclear translocation of NF-κB subunits was observed: all stimuli were particularly effective on p50 translocation (2.3–3-fold above baseline control values). Even in this case, MSP effects were significantly enhanced in AM from healthy smokers and smokers with IPF. However, no major differences were observed in smokers and non-smokers with sarcoidosis after challenge with MSP or other stimuli. We have no definite explanation for this fact, but we think it could rely, at least partially, on the documented upregulation of NF-κB in this disease (Culver *et al*., 2004). On the contrary, MSP was very effective (about three-fold increase) in inducing the translocation of the p50 subunit in AM from IPF smokers.

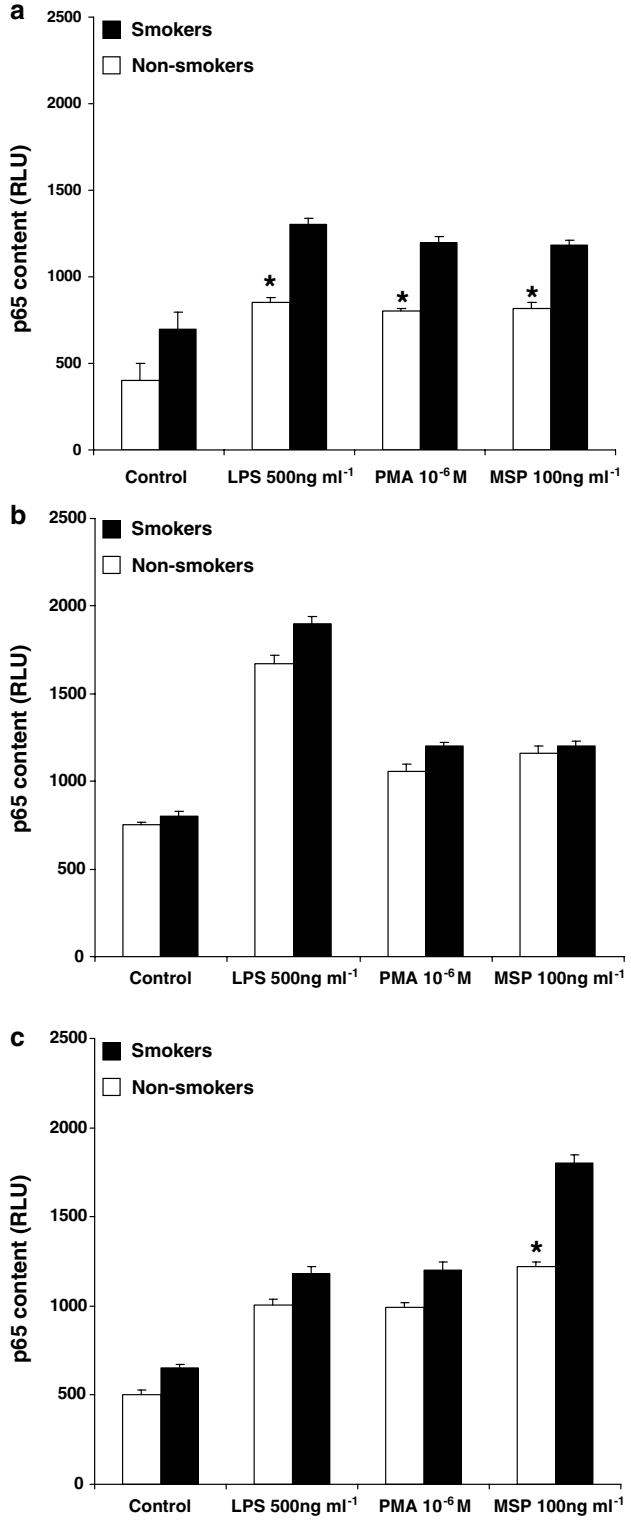

Figure 7 MSP induces the translocation of p65 subunit in AM. AM from healthy smokers and non-smokers (a), patients with sarcoidosis (b) and idiopathic pulmonary fibrosis (c) were challenged with MSP 100 ng ml^{-1}, PMA 10^{-6} M or LPS 500 ng ml^{-1} for 2 h. Nuclear extracts (1 μg) were prepared and evaluated for their content in p65 subunit. Results are expressed as relative luminescence units (RLU) and are means $\pm$ s.e.m. of three experiments in duplicate. *$P<0.05$ vs smokers.

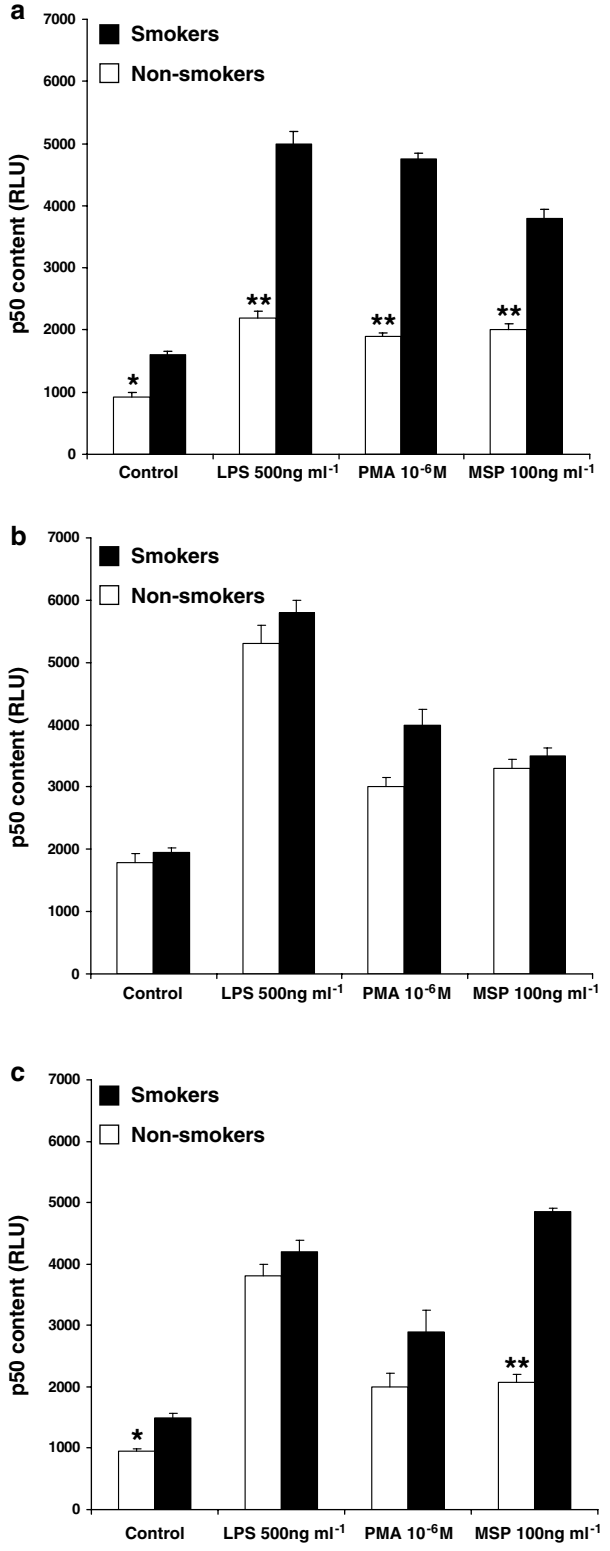

Figure 8 MSP induces the translocation of p50 subunit in AM. AM from healthy smokers and non-smokers (a), patients with sarcoidosis (b) and idiopathic pulmonary fibrosis (c) were challenged with MSP 100 ng ml^{-1}, PMA 10^{-6} M or LPS 500 ng ml^{-1} for 2 h. Nuclear extracts (1 μg) were prepared and evaluated for their content in p50 subunit. Results are expressed as relative luminescence units (RLU) and are means $\pm$ s.e.m. of three experiments in duplicate. *$P<0.05$ vs smokers; **$P<0.001$ vs smokers.

The clinical relevance of this finding remains to be ascertained, but, in our opinion, it could support an intriguing role for MSP in IPF development. The somewhat different effects evoked by MSP in AM collected from patients with interstitial lung diseases could also depend on both the peculiar type of disease and the more or less enhanced baseline of cytokines and oxy-radicals, given the fact that these mediators play a relevant role in sarcoidosis and IPF. As it is known that the activity of NF-κB is enhanced by free radicals and proinflammatory cytokines (Baldwin, 1996; Bowie & O'Neill, 2000), it is tempting to speculate that MSP-evoked respiratory burst, as well as TNF-α release, largely contribute to MSP ability in activating the transcription factor NF-κB.

Overall, these observations indicate that MSP triggers O_2^- production, cytokine release and NF-κB activation in AM from healthy volunteers and patients with sarcoidosis or IPF, both smokers and non-smokers. To our knowledge, this is the first paper that describes such effects and suggests MSP as a possible contributor for tobacco smoke toxicity.

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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

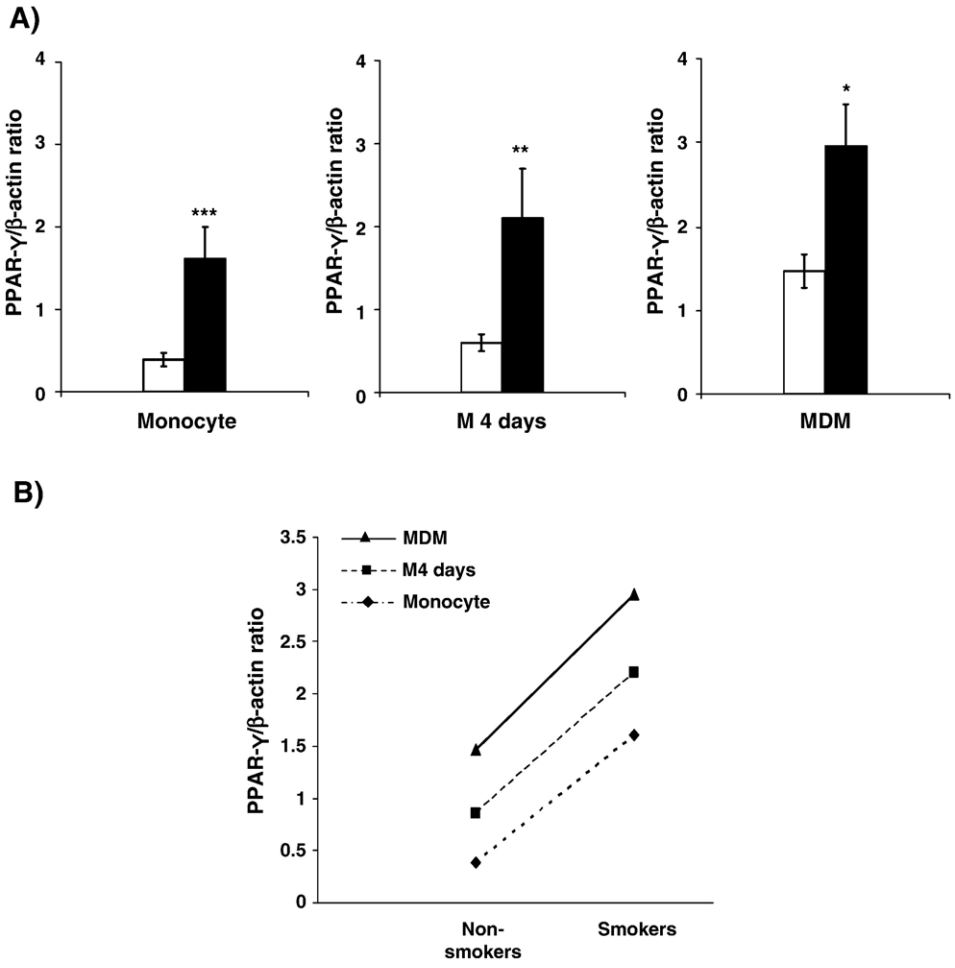

Fig. 1. Constitutive PPAR-γ protein expression in human monocyte/macrophages from healthy non-smokers (□) and smokers (■). In A: PPAR-γ/β-actin ratio in monocytes, partially differentiated macrophages (M 4 days) and fully differentiated macrophages (MDM) from twelve healthy smokers and twelve healthy 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

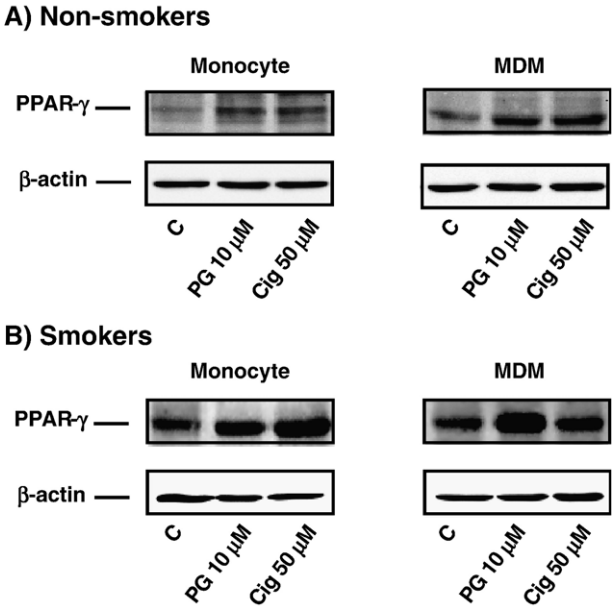

Fig. 2. Ligand-induced PPAR-γ expression in human monocyte/macrophages. A: Western blot of PPAR-γ and β-actin in monocyte/macrophages from non-smokers. B: Western blot of PPAR-γ and β-actin in monocyte/macrophages from smokers. Monocytes and fully differentiated macrophages (MDM) were challenged for 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

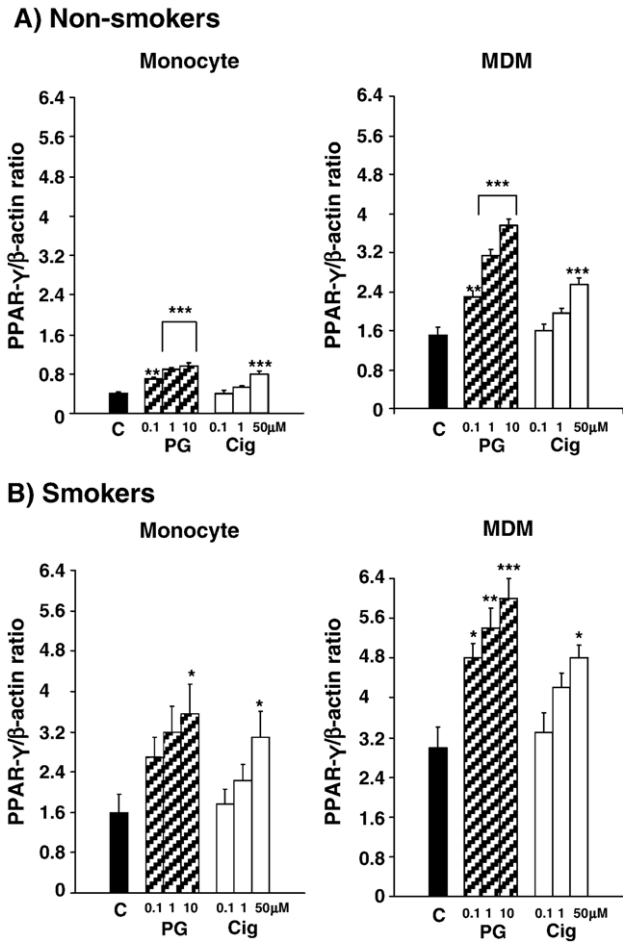

Fig. 3. Concentration-dependent effects of selective ligands on PPAR-γ expression in monocytes and MDM from healthy non-smokers (A) and healthy smokers (B). A: Cells from non-smokers were challenged for 6 h in the absence (C, control; ■) or presence of 15d-PGJ_2 (PG, 0.1–10 μM; ▨) or ciglitazone (Cig, 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).

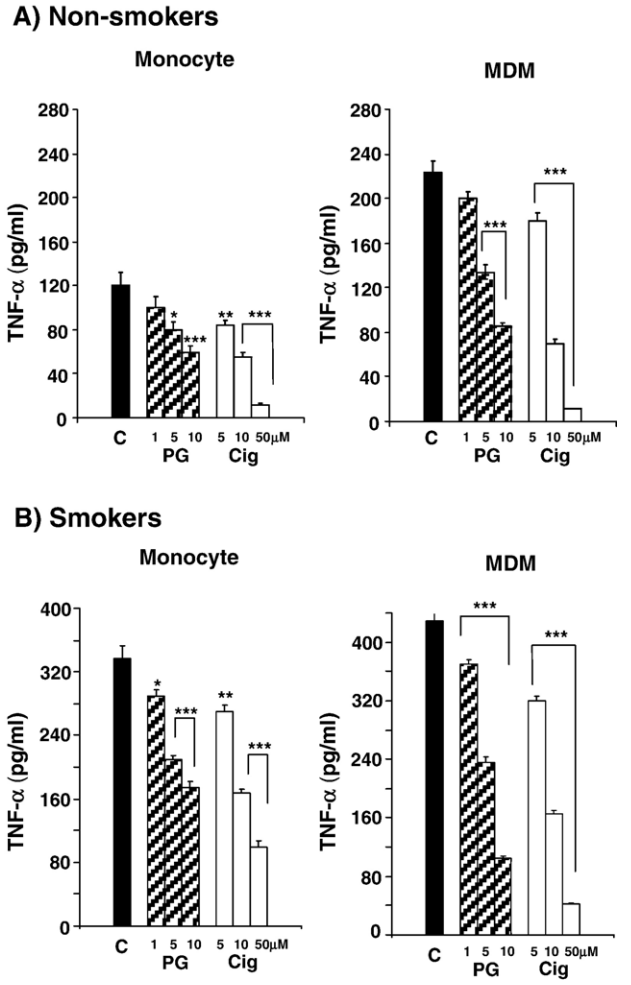

Fig. 4. Selective PPAR-γ agonists inhibit the spontaneous release of TNF-α in human monocytes and MDM from healthy non-smokers (A) and healthy smokers (B). Cells from non-smokers were challenged for 6 h in the absence (C, control; ■) or presence of 15d-PGJ_2 (PG, 1–10 μM; ▨) or ciglitazone (Cig, 5–50 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;

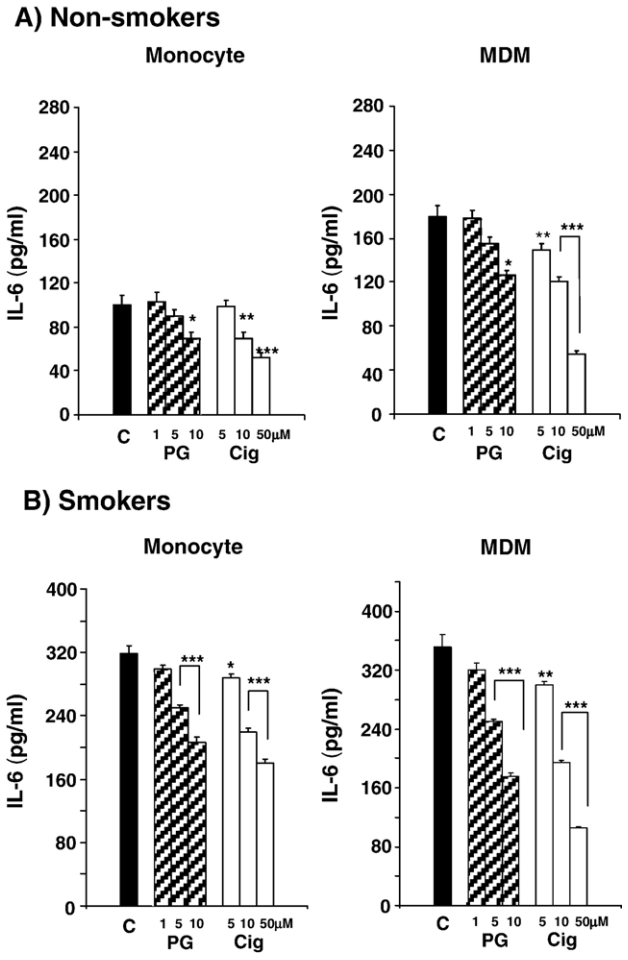

Fig. 5. Selective PPAR-γ agonists inhibit the spontaneous release of IL-6 in human monocytes and MDM from healthy non-smokers (A) and healthy smokers (B). Cells were challenged for 6 h in the absence (C, control; ■) or presence of 15d-PGJ_2 (PG, 1–10 μM; ▨) or ciglitazone (Cig, 5–50 μM; □). Cytokine 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).

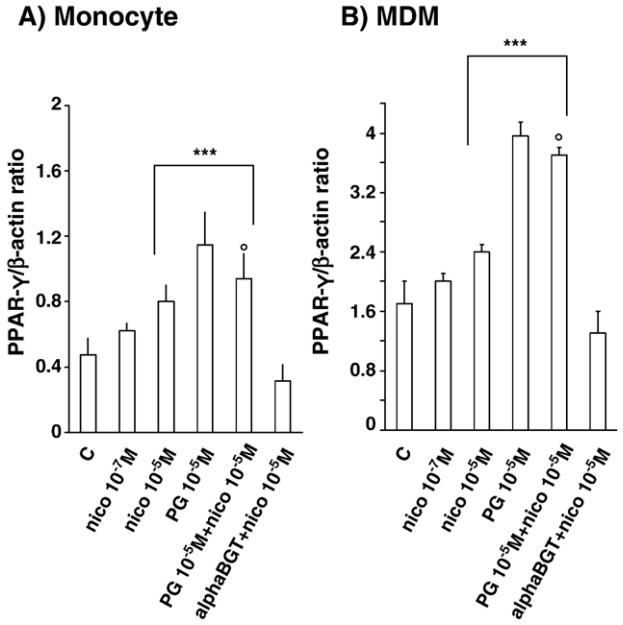

Fig. 6. Effects of nicotine and 15d-PGJ_2 on PPAR-γ expression in human monocytes (A) and MDM (B) from healthy non-smokers. Monocytes and MDM were treated with or without nicotine (nico, 0.1 μM or 10 μM), 15d-PGJ_2 (PG, 10 μM) or a combination of both for 6 h. α-bungarotoxin (alphaBGT, used at 100 ng/ml) 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.

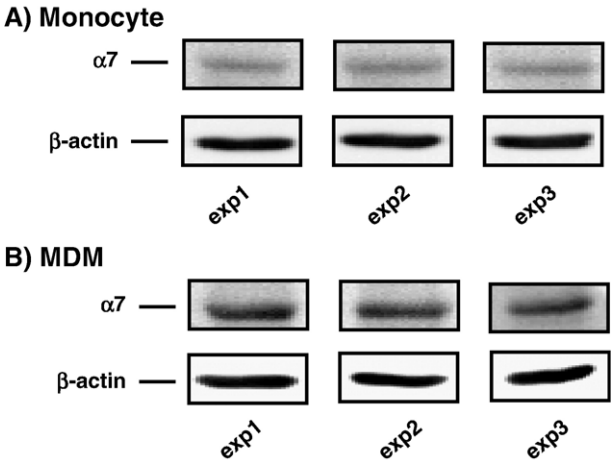

Fig. 7. Constitutive expression of nicotinic acetylcholine receptor α 7 (α7nAChR) protein in human monocytes (A) and MDM (B) from three healthy non-smokers. Immunoblots were performed by using a monoclonal antibody for the α 7 subunit; β-actin protein expression is shown for comparison (see text for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

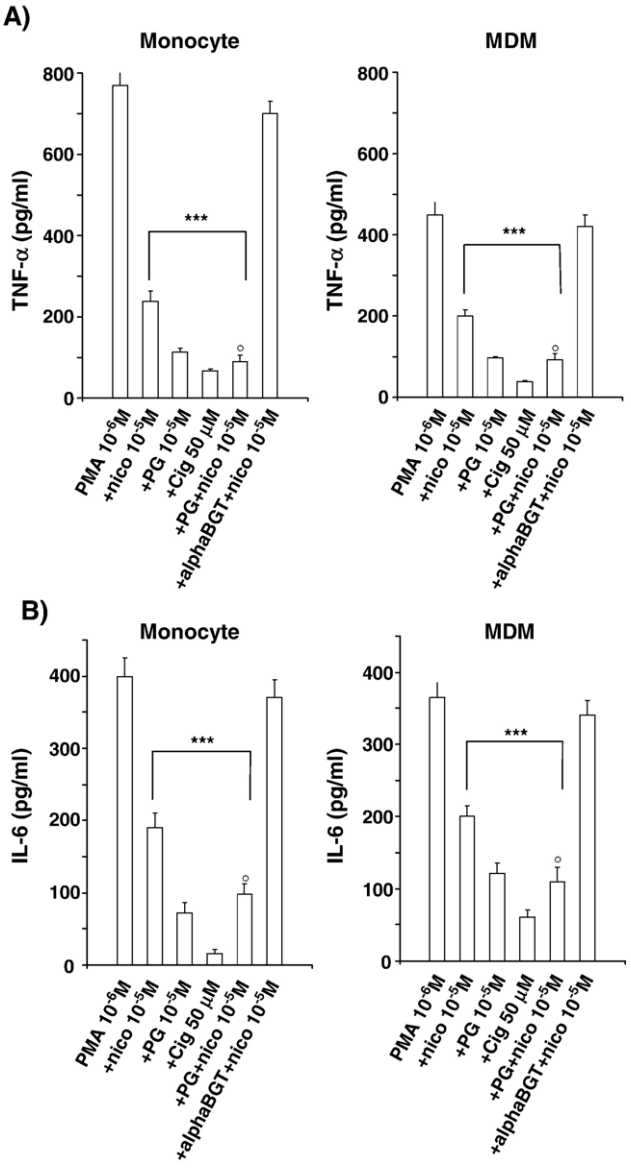

Fig. 8. Effects of nicotine and selective PPAR-γ ligands on PMA-induced cytokine secretion in monocytes and MDM from healthy non-smokers. In A: PMA-induced TNF-α release in monocytes and MDM; in B: PMA-induced IL-6 release in monocytes and MDM. Monocytes and MDM were challenged, in the absence or presence 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

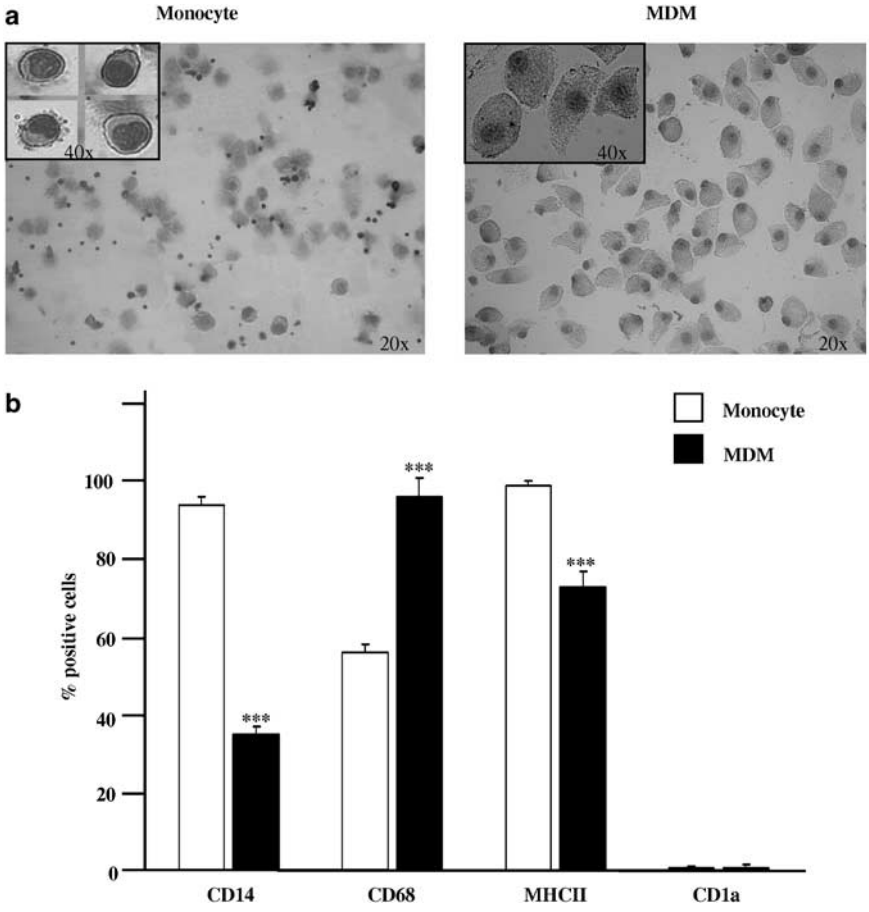

Figure 1 Morphology and phenotype of monocytes/macrophages from healthy non-smokers. In (**a**), May–Gruenewald–Giemsa stain of monocytes and monocyte-derived macrophages (MDMs) from healthy non-smokers. Magnification × 20 (magnification × 40 in the insets). In (**b**), surface marker expression in monocytes 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;

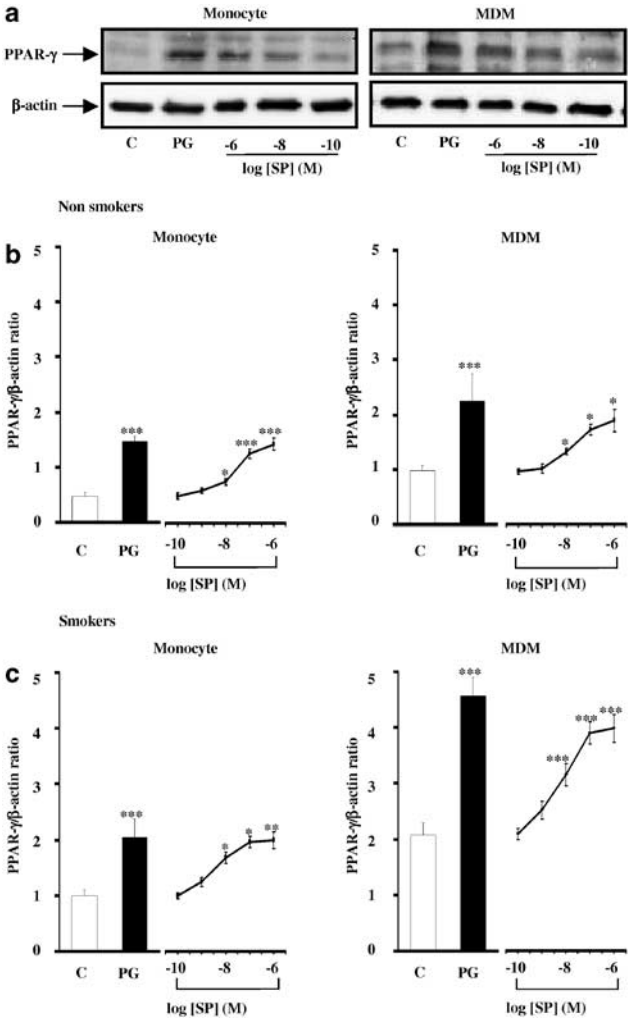

Figure 2 Concentration-dependent effects of substance P (SP) on peroxisome proliferator-activated receptor-γ (PPAR-γ) protein expression in human monocytes and monocyte-derived macrophages (MDMs) from healthy non-smokers. A representative western blot of PPAR-γ and β-actin in monocytes and MDMs from 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.

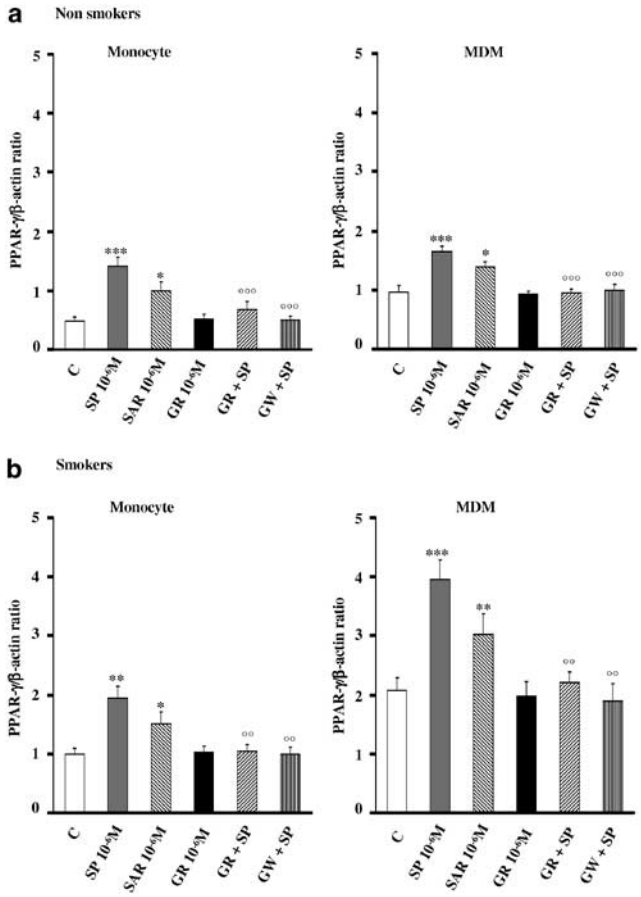

Figure 3 Effects of selective NK_1 receptor agonists and antagonists on peroxisome proliferator-activated receptor-γ (PPAR-γ) protein expression. Monocytes and monocyte-derived macrophages (MDMs) from non-smokers (**a**) and smokers (**b**) were challenged for 6 h with substance P (SP, 10^{-6} M), the 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

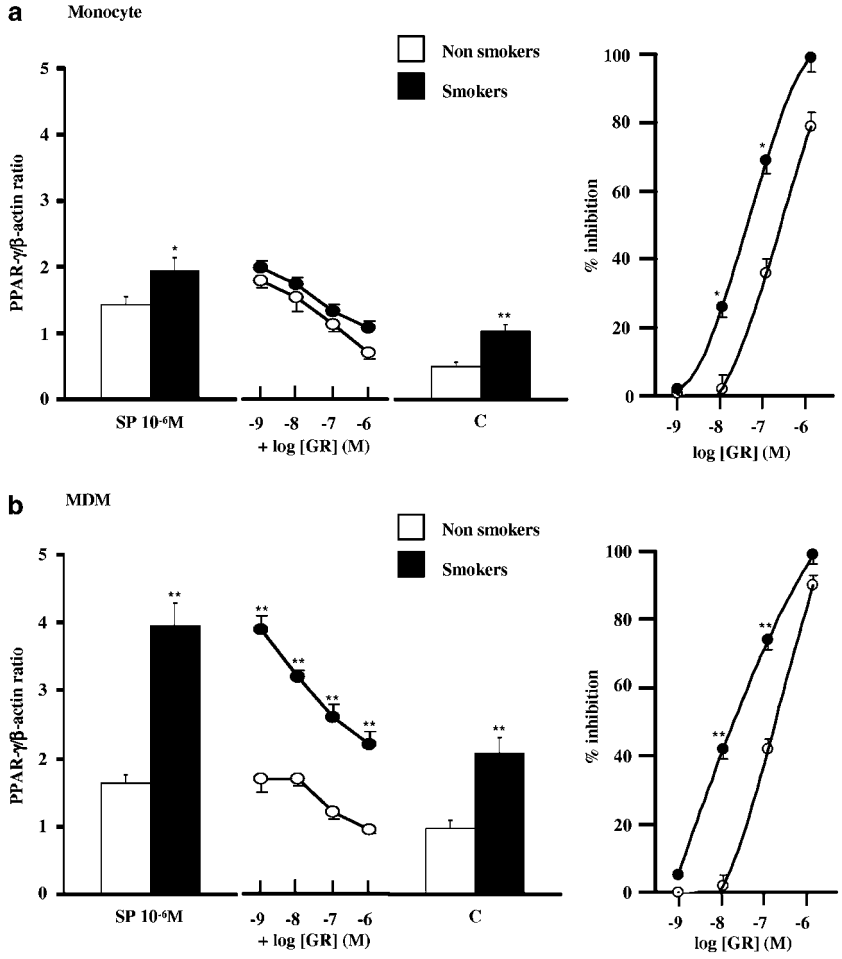

Figure 4 Substance P (SP)-induced peroxisome proliferator-activated receptor-γ (PPAR-γ) expression was mediated by NK_1 receptors: reversal by the NK_1 antagonist GR71251 ([D-Pro^9,(spiro-γ-lactam)Leu^{10},Trp^{11}]substance P). Monocytes (in **a**) and monocyte-derived macrophages (MDMs) (in **b**) 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*.,

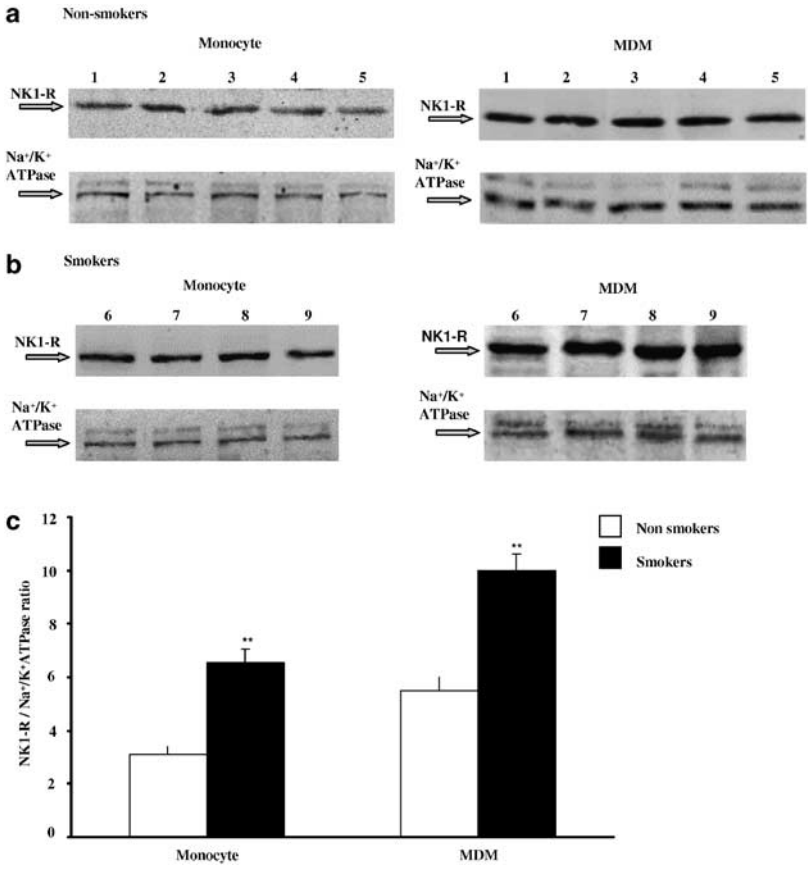

Figure 5 Western blots of NK_1 receptors (NK_1-R) and Na^+/K^+ ATPase. In (**a**), monocytes and monocyte-derived macrophages (MDMs) from non-smokers (lanes 1–5 = different healthy non-smokers). In (**b**), monocytes and MDMs from smokers (lanes 6–9 = different healthy smokers). In (**c**), NK_1 receptor 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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