Università del Piemonte Orientale "A. Avogadro"

PhD thesis in Molecular Medicine

XIX cycle (2003-2007)

Monocyte/macrophages in chronic inflammatory diseases: a role for oxy-radicals and NF-κB pathway

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CONTENTS

INTRODUCTION

Beginning with its discovery in 1986 and continuing throughout the present, the transcription factor Nuclear Factor-Kappa B (NF-κB) has attracted widespread interest based on its unusual regulation, the variety of stimuli that activate it, the diverse genes and biological responses that it controls, the striking evolutionary conservation of structure and function among family members, and its apparent involvement in a variety of human diseases (e.g. arthritis, asthma, atherosclerosis, diabetes, cancer, inflammatory bowel disease, AIDS, stroke).

NF-κB is activated by many environmental stimuli, leading to the coordinated expression of the inflammatory response. NF-κB plays a key regulatory role in host defence and chronic inflammatory diseases and is the target of several anti-inflammatory and anti-cancer drugs.

THE TRANSCRIPTION FACTOR NF-κB AND ITS REGULATION

Discovered by David Baltimore's group and first identified as a factor regulating the expression of κ light chains in mouse B lymphocytes in 1986 (Sen and Baltimore, 1986), NF-κB has subsequently been identified in most cell types, even in species as far back as insects.

NF-κB pathway is one of the most important cellular signalling transduction pathways involved in both physiological processes and disease conditions. It plays important roles in the control of immune function, differentiation, inflammation, stress response, apoptosis, cell survival (Chen et al.,2001; Lenardo and Baltimore, 1989), and is critically involved in the development and progression of cancers (Karin, 2006 a, b).

The NF-κB family is composed of five proteins: RelA (p65), RelB, c-Rel, NF-κB1(p50), and NF-κB2(p52), each of which may form homo- or hetero-dimers. Although many dimeric forms of NF-κB have been detected, the classic form of NF-κB is the hetero-dimer p65/RelA and p50 subunits.

In resting cells, NF-κB is sequestered in the cytoplasm through tight association with its inhibitors: IkBα (which acts as a NF-κB inhibitor) and p100 protein (which serves as both an inhibitor and precursor of NF-κB DNA-binding subunits).

The activation of NF-κB occurs through site-specific phosphorylation of IkBα by IKKβ and/or phosphorylation of p100 by IKKα, leading to degradation of IkBα by the 26S proteasome and/or the processing of p100 into smaller forms (p52).

This process allows two forms of NF-κB (p50-p65 and p52-RelB) to become free, resulting in the translocation of active NF-κB into the nucleus for binding to NF-κB-specific DNA-binding sites. In this way, it regulates the transcription of target genes (Ghosh et al., 1995; Karin and Greten, 2005).

The cloning of IκBα was facilitated by the observed homology with the COOH terminal region of the p105 of NF-κB (Baldwin, 1996). Other forms of IκB have been identified, including IκBβ and IκBε (Ghosh et al., 1998). IκBβ interacts with similar NF-κB subunits, but its degradation is specifically associated with persistent NF-κB activation (Ghosh et al., 1998).

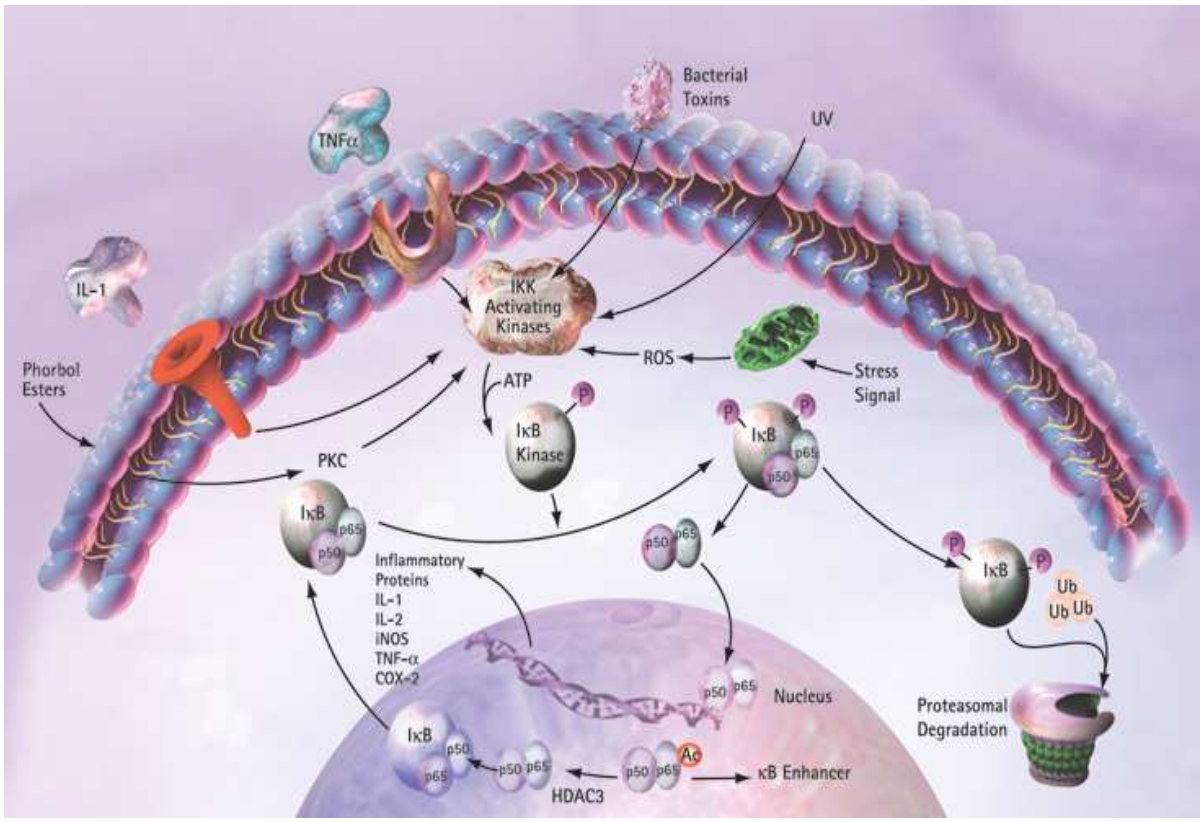

Fig. 1) NF-κB activation pathway

From: http://www.merckbiosciences.co.uk/html/CBC/other_inhibitors_NF-kB_activation.htm

Genes regulated by NF-κB include those encoding IL-2, IL-6, IL-8, the IL-2 receptor, the IL-12 p40 subunit, VCAM-1, ICAM-1, TNF-α, IFN-γ, c-Myc etc. (Baldwin, 1996; Ghosh et al., 1998; Barkett and Gilmore, 1999; Pahl, 1999).

Consistent with the regulation of genes involved in the immune and inflammatory response, mice null for several of the NF-κB subunits show defects in clearing bacterial infection along with defects in B- and T-cell functions (Ghosh et al., 1998). Surprisingly, the knockout mice for the p65/RelA subunit dies at day 16 of development from extensive liver apoptosis,

revealing a role for NF-κB in controlling cell death (Beg et al., 1995). It can be assumed that there is redundancy in the NF-κB system, since the combined p50 and p52 knockout animals show osteopetrosis with a block in osteoclast differentiation, whereas the individual p50 or p52 knockouts show no such defect (Franzoso et al., 1997; Iotsova et al., 1997).

The ability of NF-κB to be activated by inflammatory cytokines and to regulate genes involved in inflammatory response raised the question of whether NF-κB deregulation would be associated with inflammatory diseases.

A POSSIBLE ROLE FOR NF-κB IN CHRONIC INFLAMMATORY DISEASES (e.g., RHEUMATOID ARTHRITIS AND CHRONIC LUNG DISEASES)

Although NF-κB plays an essential beneficial role in physiology, inappropriate regulation of NF-κB activity has been implicated in the pathogenesis of several inflammatory diseases, including rheumatoid arthritis (RA), osteoarthritis (OA), atherosclerosis, asthma, inflammatory bowel disease, multiple sclerosis etc.

1) Rheumatoid Arthritis

RA, a common systemic disease of unknown origin which affects approximately 1% of the population (Levine et al., 1984; Devillier et al., 1986; Brunelleschi 1999), is characterized by a chronic inflammation of the synovial joints with infiltration and accumulation of activated blood cells, mainly memory T-cells, monocytes/macrophages and plasma cells. This leads to the progressive destruction of cartilage and bone (largely mediated by cytokines, secreted by T-lymphocytes and monocytes), the development of joint deformities, abnormal cellular and humoral immune responses (e.g., accumulation of T lymphocytes in the synovium, auto-antibodies, rheumatoid factors and antibodies against collagen type II, synovial hyperplasia characterized by proliferating synoviocytes and large numbers of infiltrating macrophages) (Gay et al., 1993; Feldmann et al., 1996a).

As demonstrated by electromobility shift assays, NF-κB binding is significantly higher in RA synovium compared with osteoarthritis synovium (Han et al. 1998; Handel et al., 1995). Immuno-histochemistry studies identify nuclear p50 and p65 translocation in the synovial lining and mononuclear cells of the sub-lining. *In vitro* studies confirm a role for NF-κB in the production of cytokines by macrophages, as well as elevated constitutive production of IL-6 by RA synoviocytes (Miyazawa et al., 1998). Moreover, animal models of RA also support the notion that NF-κB participates in synovitis. Time-course studies in both collagen-

induced arthritis and adjuvant-induced arthritis demonstrate NF-κB activation prior to the appearance of clinical disease (Tsao et al., 1997). Selective activation of IKK-2 by intra-articular gene transfer leads to arthritis in rats, thus confirming that IKK activation is sufficient to initiate synovitis (Tak et al., 2001).

The expression of NF-κB proteins can provide specificity in response to a particular stimulus. For instance, IL-1-induced collagenase expression in synoviocytes is primarily activated by p50 homodimers, that bind to a critical NF-κB–like binding site (Vincenti et al., 1998). Both p50 and p65 play a role in constitutive IL-6 production in RA synovial fibroblasts (Miyazawa et al., 1998), whereas p65 activation by thrombin regulates ICAM-1 expression in endothelial cells (Rahman et al., 1999). In human monocytes, p50-p65 heterodimers are intimately involved in activation of inflammatory genes by IL-1 or TNF-α, and these effects are blocked by the anti-inflammatory cytokine IL-10 (Schottelius et al., 1999).

Cytokines stimulated by NF-κB, such as IL-1β and TNF-α, can also directly activate the NF-κB pathway, thus establishing a positive auto-regulatory loop that can amplify the inflammatory response and leads to chronic inflammation. The fact that NF-κB regulates TNF-α expression and is a key effector of this cytokine, is consistent with the development of drugs aimed at blocking TNF-α (TNF-α antagonists such as etanercept, and chimeric anti-TNF-α monoclonal antibodies such as infliximab) as a therapy for RA (Jones and Moreland, 1999; Maini et al., 1999).

Therefore, it is evident that NF-κB is a valid candidate target for new anti-inflammatory treatments. Several commonly used therapeutic agents, most notably, corticosteroids, can suppress NF-κB, but more selective inhibitors are clearly desirable in order to minimize NF-κB–independent toxicity.

2) Chronic lung diseases

NF-κB is also activated in inflammatory airway diseases. In fact mice lacking the NF-κB subunits p50 or c-Rel develop less airway inflammation upon antigen challenge, demonstrating the causal role of NF-κB in allergic airway disease (Yang et al., 1998). Activation of NF-κB has also been demonstrated specifically within airway epithelium in animal models of allergic airway inflammation (Poynter et al., 2002; Bureau et al., 2000) and in patients with asthma (Hamilton et al., 2001; Zhao et al., 2001; Stacey et al., 1997; Vignola et al., 2001).

In vitro and *in vivo* studies have shown that NF-κB regulates gene expression of cytokines (TNF-α, IL-1β), chemokines, and adhesion molecules (ICAM-1, E-selectin), all these factors

playing an important role in lung inflammatory injury (Blackwell et al., 1997; Cohen et al., 1998; Manning et al., 1995). *In vivo* studies demonstrated that lung NF-κB activation is suppressed by antioxidants (Blackwell et al., 1996, Liu et al., 1976; Liu et al., 1999a; Liu et al., 1999b) or anti-inflammatory cytokines (Lentsch et al., 1997), resulting in decreased pro-inflammatory mediator expression and reduced inflammatory injury. Thus, it appears that activation of NF-κB is central to the development of pulmonary inflammation and acute lung injury.

Strategically situated at the air-tissue interface in the alveoli and alveolar ducts, alveolar macrophages (AM) are the first cells encountered by inhaled organisms and antigens in the lower respiratory tract. Upon appropriate simulation, AM can release a wide variety of biologically active products and thereby play a key role. Therefore, AM activation generally represents an initial event in the pathogenesis of lung inflammatory reactions.

In a rat model of injury induced by intrapulmonary deposition of IgG immune complexes, Lentsch et al. (1997) showed that early AM activation occurred in an NF-κB-dependent manner. Furthermore, depletion of AM attenuated NF-κB activation in whole lung tissues and decreased the broncho-alveolar lavage (BAL) fluid content in pro-inflammatory mediators. In addition, lung instillation of TNF-α in AM-depleted rats induced NF-κB activation in whole lungs (Lentsch et al., 1999). These results indicate that the products of activated AM, such as TNF-α, are essential in stimulating nuclear translocation of NF-κB in other lung cell types. Several other animal models have been used to evaluate the role of NF-κB in the production of inflammatory events in AM. All support the concept that regulating NF-κB activation in AM can significantly inhibit inflammatory events. Although the importance of NF-κB in cytokine transcription has been established in animal models, only a few published studies have evaluated NF-κB and cytokine release in human AM. Schwartz et al. (1996) reported that, in AM from patients with ARDS, NF-κB is activated to a significantly higher degree than in AM from critically ill patients with other diseases. In contrast, basal activation of NF-κB in AM from normal volunteers appeared to be minimal (Farver et al., 1998).

OXY-RADICAL PRODUCTION AND NF-κB REGULATION

Biological systems are continuously exposed to oxidants, either generated endogenously by metabolic reactions (e.g. from mitochondrial electron transport during respiration or during activation of phagocytes) or exogenously, e.g. by air pollutants or cigarette smoke.

A delicate balance exists between the toxicity of oxidants and the protective effects of intra- and extra-cellular antioxidant defence systems, which is critically important for the maintenance of normal pulmonary cellular functions. A shift of the oxidant/antioxidant balance in favour of oxidants has been termed as "oxidative stress". The oxidant burden in lungs may be further enhanced in smokers by the increased numbers of inflammatory leukocytes (by 10-fold) and AM (by two- to fourfold) (Kilburn and McKenzie, 1975; Hunninghake and Crystal, 1983) in both the circulation and the alveolar space. *In vitro* studies have shown that alveolar leukocytes and macrophages from cigarette smokers spontaneously release increased amounts of oxidants, such as superoxide anions (O_2^-) and H_2O_2 compared to those from non-smokers (Schaberg et al., 1992; Richards et al., 1989; Davis et al., 1988; Ludwig and Hoidal, 1982; Van Antwerpen et al., 1985). It has also been demonstrated that cigarette smokers have increased numbers of neutrophils sequestered in the pulmonary microcirculation with the potential to release oxidants (MacNee et al., 1989; Drost et al., 1992; Brown et al., 1993).

Reactive oxygen species (ROS), such as the O_2^- and the hydroxyl radical ($OH^\bullet$), are highly unstable species with unpaired electrons, capable of initiating oxidation. ROS causes oxidation of proteins, DNA and lipids, which may cause direct cell injury or induce a variety of cellular responses, through the generation of secondary metabolic reactive species. ROS may alter remodelling of extra-cellular matrix (ECM) and blood vessels, stimulate mucus secretion, cause apoptosis and regulate cell proliferation (Rahman, 2005). Alveolar repair responses and immune modulation in the lung may also be influenced by ROS (Rahman and MacNee, 1998).

The most likely sources of these oxidizing agents are the phagocytic leukocytes (e.g. neutrophils, monocytes, macrophages, and eosinophils) that infiltrate the tissue. Activation of phagocytes, *via* the interaction with certain pro-inflammatory mediators or bacterial products with specific receptors on the leukocyte plasma membrane, results in the assembly of the multicomponent flavoprotein nicotinamide adenine dinucleotide phosphate (NADPH) oxidase. This enzyme catalyzes the production of large amounts of O_2^- (Klebanoff, 1992):

$$NADPH + O_2 \longrightarrow NADP^+ + O_2^-$$

Superoxide anions are relatively un-reactive toward most biological substrates, as, in the presence of superoxide dismutase (SOD) they are rapidly dismutated to yield hydrogen peroxide (H_2O_2) and oxygen (O_2):

$$O_2^- + O_2^- + 2H^+ \longrightarrow H_2O_2 + O_2$$

Although H_2O_2 is a more powerful oxidant than is O_2^-, it is relatively un-reactive toward most biologic substrates, unless present in high concentrations, as it can be detoxified by peroxidases.

O_2^- and H_2O_2 may interact with low molecular weight iron (Fe) chelates to yield the highly reactive hydroxyl radical ($OH^\bullet$) via the superoxide-driven Fenton reaction:

$$O_2^- + Fe^{3+} \longrightarrow O_2 + Fe^{2+}$$
$$H_2O_2 + Fe^{2+} \longrightarrow OH^- + OH^\bullet + Fe^{3+}$$

Hydroxyl radical is an extremely reactive species, reacting immediately with virtually all known biomolecules. Thus, it is very short lived and will react at the site where it is formed (i.e. in a site-specific fashion). Hydroxyl radicals have been shown to peroxidize lipids, oxidize proteins, and promote DNA strand scission (Grisham, 1992). In fact, many of the H_2O_2-induced alterations in DNA and other organic substrates have been suggested to be due to the site specific metal catalyzed production of $OH^\bullet$ *via* the Fenton reaction as described above. In addition to these classical ROS, activated neutrophils and monocytes secrete the hemoprotein myeloperoxidase (MPO) into the extracellular medium, where it catalyzes the oxidation of Cl^- by H_2O_2 to yield hypochlorous acid ($^{\cdot}HOCl$) (Klebanoff, 1992):

$$H_2O_2 + Cl^- + H^+ \longrightarrow HOCl + H_2O$$

Hypochlorous acid possesses the two oxidizing equivalents of H_2O_2 and is approximately 100-1000 times more toxic than either O_2^- or H_2O_2. HOCl is a non-specific oxidizing and chlorinating agent that reacts rapidly with a variety of biological compounds, including sulphydryls, polyunsaturated fatty acids, DNA, pyridine nucleotides, aliphatic and aromatic amino acids, and nitrogen-containing compounds (Grisham, 1992).

As previously reported, neutrophils and macrophages, which are recruited into the lungs of cigarette smokers, produce very relevant amounts of ROS (Rahman and MacNee, 1996).

NF-κB is critically involved in the processes of oxidative stress response. Direct addition of H_2O_2 to cell culture medium can activate NF-κB in several cell lines (Bowie and O'Neill, 2000) and increased ROS production is documented in response to agents that also activate NF-κB (Toledano & Leonard, 1991). When ROS are formed intra-cellularly, they have the capacity to induce DNA damage and alter cellular signal transduction pathways, among which NF-κB signalling is the most important.

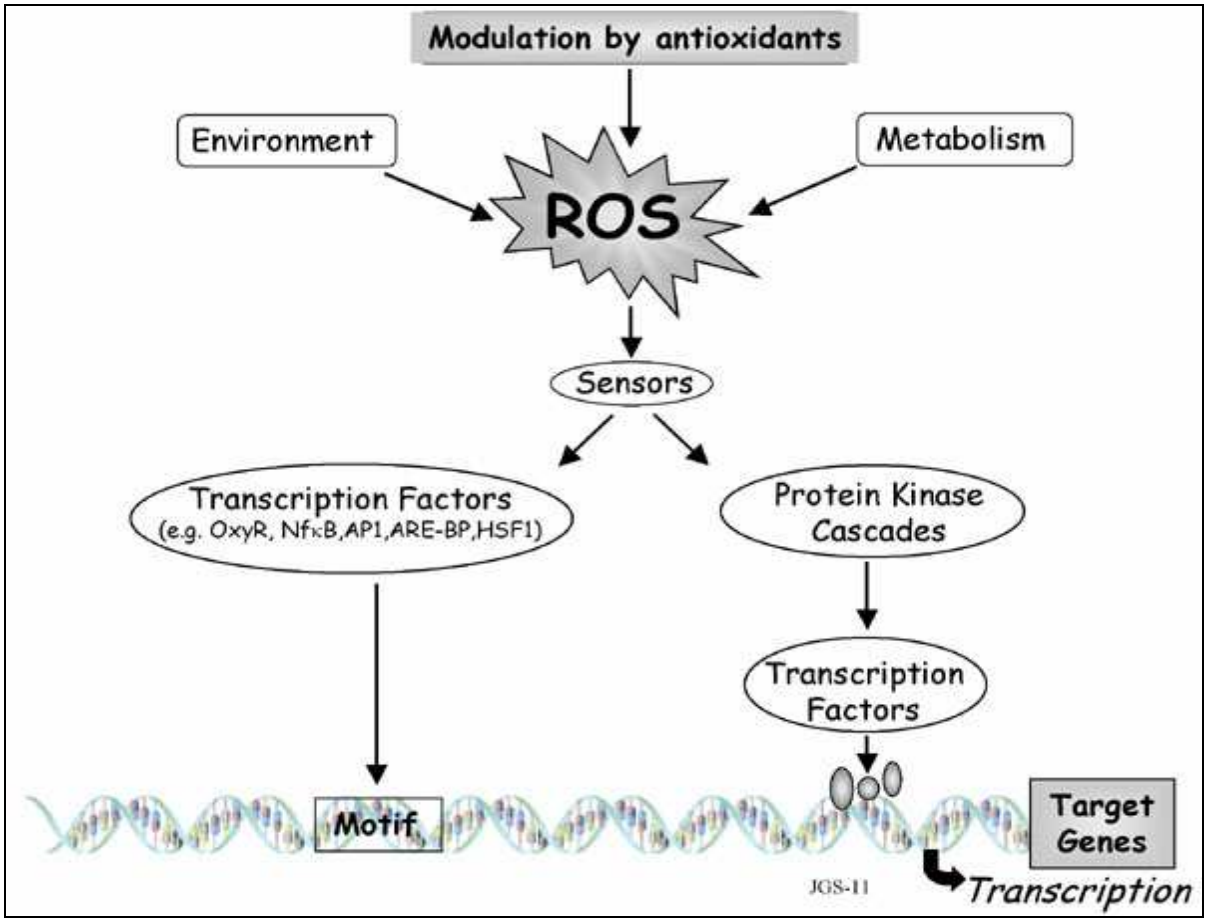

Fig.2) The major signaling pathways activated in response to oxidative stress. Reactive oxygen species (ROS) originating from environmental signals or from metabolic activity are modulated by antioxidants to non toxic levels, at which point they serve as signaling molecules. ROS can activate gene transcription in two ways: a) via transcription factors, such as NF-κB, AP-1, and ARE-binding proteins (ARE-BP) that can interact directly with specific DNA motifs on promoters of target genes, or b) via activation of MAPK cascades, which in turn activate transcription factors that trigger target gene transcription. The degree to which a given pathway is activated depends on the nature and duration of the stress, as well as on cell type and developmental stage. NF-κB = nuclear factor κB; AP-1 = activator protein-1; ARE-BP = antioxidant-responsive element binding proteins; HSF1 = heat shock transcription factor 1.

Several mechanisms have been proposed for oxidative stress-induced NF-κB activation. Redox stress (H_2O_2) has been proposed to directly enhance the activity of IKKs through an effect on phosphorylation of Ser180 of IKK1 and/or Ser 181 of IKK2 in HeLa cells (Kamata et al., 2002; Jasper et al., 2001). In some cell types, oxidative stress can cause rapid ubiquitination and phosphorylation of the IκB complex with subsequent degradation (Bowie et al., 1997; Ginn-Pease and Whisler, 1996).

Therefore, it is possible that oxidative stress and/or an imbalance in GSH redox status may directly stimulate the activity of IKK. Alternatively, oxidative stress may affect the proteosome enzymatic activity that leads to the activation of NF-κB (Jasper et al., 2001; Ukada et al., 1999). Overall, oxidative stress favours the activation and translocation of NF-κB to the nucleus, and nuclear GSH (reducing environment) facilitates the binding of NF-κB to DNA. New data also reported IkB-independent mechanisms of activation of NF-κB; phosphorylation of p65 NF-κB by various kinases had an effect on the transactivation activity of NF-κB, independently of nuclear translocation, possibly through activation of co-activators. Although not explored yet, these new pathways may be critical to the H_2O_2-induced activation of NF-κB. The profound effect of antioxidants on NF-κB activity resulted in the adoption of a model of NF-κB as a redox-sensitive transcription factor (Barnes et al., 2005; Di Stefano et al., 2002).

Relevant stimuli to the development of chronic obstructive pulmonary disease, such as particulate air pollution, have potent oxidant properties and activate NF-κB in alveolar epithelial cells (Jimenez et al., 2000). *In vitro* thiol antioxidants, such as N-acetyl-L-cysteine, have been shown to block the release of inflammatory mediators from epithelial cells and macrophages, by increasing intracellular glutathione and decreasing NF-κB activation (Antonicelli et al., 2000).

TACHYKININS IN LUNG PATHOPHYSIOLOGY

Tachykinins are a family of peptides which share the characteristic C-terminal pentapeptide Phe-X-Gly-Leu-Met-NH_2; amidation at the C terminus is crucial for biological activity, as de-amidated peptides are virtually inactive (Erspamer, 1994).

The mammalian tachykinins Substance P (SP), neurokinin A (NKA) and neurokinin B (NKB) are widely distributed throughout the central and peripheral nervous system, where they act as neurotransmitters or neuromodulators.

Tachykinins are derived from pre-pro-tachykinins (PPT; there are at least three different genes, all coding for SP) and are subjected to enzymatic degradation by angiotensin converting enzyme (ACE) and neutral endopeptidase (NEP), mainly. They have been implicated in a large array of biological actions, e.g., pain transmission, neurogenic inflammation, smooth muscle contraction, vasodilatation and activation of the immune system.

Their effects are largely mediated via specific G-protein-coupled receptors; at least three subtypes of tachykinin receptors, namely NK_1, NK_2 and NK_3, have been characterized by the rank order of potency of agonists, by using selective antagonists and by molecular cloning (Regoli et al., 1994; Barnes et al., 1998).

It has become clear from animal and human studies that immune cells may represent an additional source of tachykinins (Maggi, 1997; Joos and Pauwels, 2000). Evidence of the production of SP by eosinophils (mouse, man), monocytes and macrophages (rat, man), lymphocytes (man) and dendritic cells (mice) has been reported (Maggi, 1997; Maggi, 2000). Inflammatory stimuli such as LPS can up-regulate the concentration of tachykinins in these cells (Germonpré et al., 1999; Lambrecht et al., 1999). These data are strengthened by the finding of the presence of tachykinin receptors in nerves (NK_1) and in inflammatory cells (NK_2) such as T-lymphocytes, mast cells and macrophages (Mapp et al., 2000; Brunelleschi et al., 1996). Therefore tachykinins exert modulatory effects on cells involved in inflammatory and immune processes (Barnes et al., 1991). Besides representing the key mediator of the so-called “neurogenic inflammation”, SP degranulates mast cells, stimulates both DNA and protein synthesis from human T lymphocytes, exerts priming effects on neutrophils, induces oxy-radical production and TNF-α mRNA expression in human monocytes (Payan et al., 1983; Brunelleschi et al., 1991, 1998).

Moreover, a novel activity for SP has been recently identified by us (Amoruso et al., 2008): in the concentration range 10^{-10} – 10^{-6} M, SP stimulates PPAR-γ protein expression in monocytes and MDM, showing a greater efficiency in cells from healthy smokers. Interestingly, this effect is paralleled by a constitutively increased expression of NK_1 receptors in cells isolated from smoker volunteers. SP-induced PPAR-γ protein expression is receptor-mediated, as it is reproduced by the NK_1 selective agonist $[Sar^{9}Met(O_2)^{11}]SP$ and reverted by the competitive NK_1 antagonist GR71251 (Amoruso et al., 2008).

SP and NKA, which are co-localized in the sensory afferent C-fibers and released locally by an axon reflex, are deeply involved in lung pathophysiology (Barnes et al., 1991). Relationship between these nervous fibers and tachykinin levels within lung tissues is

underlined by the lack of tachykinin immunoreactive nerves in transplanted lungs and by the reduction of SP levels in BAL fluid from transplanted lungs (Springall et al., 1990; Fluge et al., 1994). Different experimental and clinical evidences indicated that tachykinins (and especially NKA) induce broncho-constriction in man and guinea-pigs (Joos et al., 1996; Lagente and Advenier, 1998). NK_1 and NK_2 antagonists potently reverted these effects in animals but were poorly effective in humans with asthma (Joos et al., 1996; Ichinose et al., 1996; Kudlacz et al., 1998; Lagente and Advenier, 1998; Joos and Pauwels, 2001).

Higher levels of substance P (SP) have been detected in lavage fluid from patients with idiopathic pulmonary fibrosis, sarcoidosis and healthy smokers (Takeyama et al., 1996) as well as in induced sputum in patients with asthma or chronic bronchitis (Tomaki et al., 1995). Nieber et al. (1992) found a significantly higher amount of SP in BAL of atopic patients compared to nonallergic subjects. In asthmatics, NKA content in BAL was increased 4 h after challenge with house dust mite (Heaney et al., 1998). In addition, an enhanced expression of NK_2 receptor gene was detected in asthmatics (four-fold increase), smokers (three-fold increase) and patients with chronic obstructive pulmonary disease (COPD; two-fold increase), while NK_1 receptor expression is increased in smokers only (Bai et al., 1995). Other observations indicate that the expression of SP, as well as of the NK_1 receptor, was significantly higher in the epithelium of asthmatic subjects (Chu et al., 2000). On surgical specimens, Mapp et al. (2000) found NK_1 and NK_2 receptors in bronchial glands, bronchial vessels and bronchial smooth muscle, but not in the epithelium; NK_1 receptors were occasionally found in nerves, whereas NK_2 receptors were found occasionally on macrophages, T lymphocytes and mast cells (Mapp et al., 2000).

Our laboratory has long been interested in the evaluation of tachykinins' effects in AM. We previously demonstrated that guinea-pig AM possess NK_2 and NK_1 receptors, their activation leading to superoxide anion production and prostanoid release, and that AM isolated from ovalbumin-sensitized animals presented an enhanced responsiveness to NKA and NK_2 selective agonists (Brunelleschi et al., 1990, 1992). We also demonstrated that mammalian tachykinins, SP and NKA, as well as the selective NK_1 agonist [$Sar^9Met(O_2)^{11}$]SP and the selective NK_2 agonist [β-Ala^8]-NKA(4-10) induce O_2^- production in circulating monocytes and alveolar macrophages from healthy smokers or patients with sarcoidosis and idiopathic pulmonary fibrosis (Brunelleschi et al., 1996, 2000). All agonists act dose-dependently and maximal effects are observed at micromolar concentrations but the respiratory burst is already detectable at picomolar concentrations. Selective NK_1 and NK_2 antagonists competitively

antagonise tachykinin-evoked O_2^- production from monocytes, confirming the involvement of NK_1 and NK_2 receptors (Brunelleschi et al., 2000).

The presence of NK_1 receptor (NK-1R) in monocyte/macrophages has been demonstrated by evaluating the effect of selective agonists and antagonists on functional parameters, mainly (Brunelleschi et al., 1990, 1992, 1998). Some years ago Marriott & Bost (2000) and Simeonidis et al. (2003) demonstrated the presence of NK-1R protein in murine peritoneal macrophages and THP-1 cells. Recent evidence indicates that NK-1R gene expression in THP-1 cells is increased after exposure to IL-1β and TNF-α: this effect is mediated by the transcription factor NF-κB, which binds to the promoter region of the NK-1R gene and so regulates its expression (Simeonidis *et al*., 2003).

MACROPHAGE STIMULATING PROTEIN IN THE LUNG

The regulation of macrophage activation represents a crucial step for the control of inflammatory and immune processes. Among the different factors which have been claimed to be involved, we pointed our attention on Macrophage Stimulating Protein (MSP)/Ron complex.

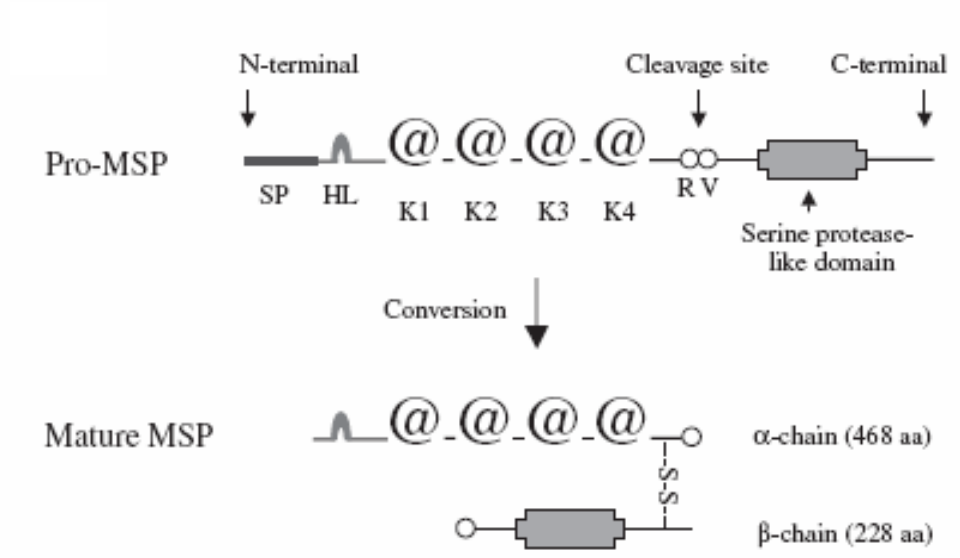

Fig 3) Schematic representation of the structures and activation mechanisms of human macrophage-stimulating protein (MSP) and RON (recepteur d'origine nantais). (A) MSP is synthesized by hepatocytes and circulates in the blood as a biologically inactive single-chain precursor (pro-MSP). Proteolytic cleavage at a specific dibasic arginine–valine (R^{554}–V^{555}) site results in the formation of biologically active MSP. MSP is a heterodimer consisting of a 60 kDa α-chain and 30 kDa β-chain, linked by a disulphide bond. The α-chain contains a signal peptide (SP) and a hairpin loop (HL), followed by four kringle domains. The β-chain contains a serine protease-like domain without enzymatic activity, owing to the replacement of three amino acids in the catalytic site. The receptor-binding site of MSP is located in the MSP β-chain

From "Scand J Immunol (2002) 56, 545-553"

MSP, also known as Hepatocyte Growth Factor-like protein (Han et al., 1991), was originally discovered by Leonard in 1976 as a serum protein that stimulates shape change, migration and phagocytosis of mouse peritoneal resident macrophages (Leonard et al., 1976; Leonard et al., 1978). Since then, the MSP spectrum of activity has widened and this protein is now regarded as a pleiotrophic factor, modulating the activity of megakaryocytes, erithroid cells, keratinocytes, liver cells and fibroblasts (Leonard, 1997; Brunelleschi et al., 2001).

MSP is a 80 kDa heterodimer composed of a 60 kDa α-chain and a 30 kDa β-chain; both chains are derived from a single-chain precursor and are linked by a disulphide bond (Skeel et al., 1991).

Liver cells are the major source of MSP (Bezerra et al., 1993), that circulates in the blood as a biologically inactive single-chain precursor (pro-MSP) (Wang et al., 1996a). Conversion into two-chain MSP is required for receptor binding and biological activities (Wang et al., 1997; Wang et al., 1994a). Proteolitic conversion of pro-MSP into biological active MSP occurs during blood coagulation and local inflammation (Wang et al., 1994b). Three enzymes, kallikrein, factor XIa and XIIa, have the ability to cleave pro-MSP (Wang et al., 1994b).

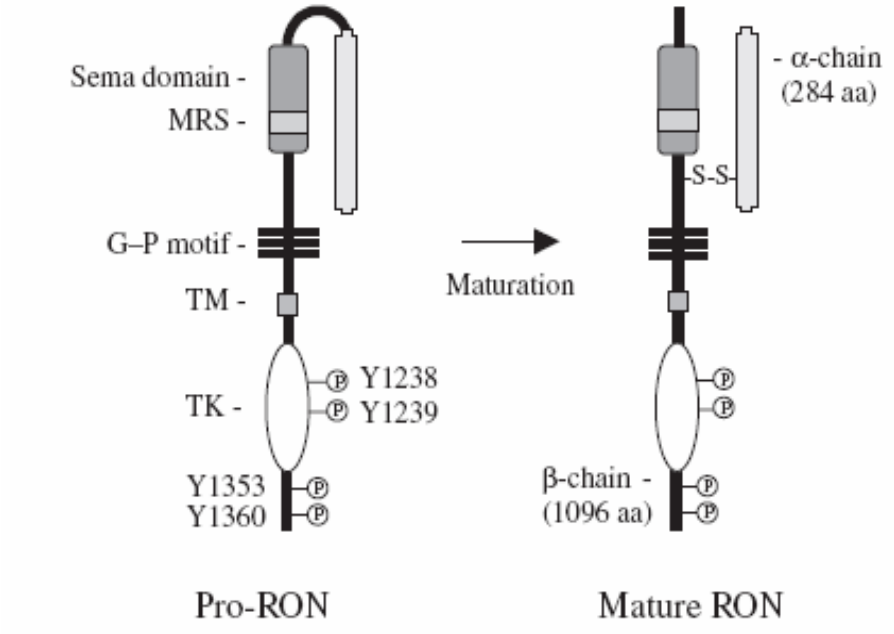

Fig. 4) RON is first synthesized as a single-chain pro-RON. Proteolytic conversion at a specific site (K^{305}–R–R–R–R^{309}) generates a mature RON with a 40 kDa α-chain and 150 kDa β-chain, linked by a disulphide bond. The α-chain is completely extracellular. The β-chain comprises an extracellular domain, a short transmembrane segment and a large cytoplasmic portion with intrinsic tyrosine kinase activity. The extracellular regions of the RON-β-chain contain several important structure domains such as Sema domain. The binding of MSP to RON causes phosphorylation of Y1238 and Y1239 within the catalytic site, leading to increased kinase activity. Phosphorylation of C-terminal Y1353 and Y1360 results in the formation of a so-called docking site [34], which is important for interaction with signal transducers. MRS, MET-related sequences; G-P motif, glycine-proline-rich repeat; TM, transmembrane segment; TK, tyrosine kinase domain; P, phosphotyrosine.

From "Scand J Immunol (2002) 56, 545-553"

The specific receptor for MSP is Ron (Récepteur d'origine nantais), a tyrosine kinase receptor, which belongs to the MET proto-oncogene family. Ron is a 180 kDa heterodimer composed of a 40 kDa α-chain and a 150 kDa β-chain with intrinsic tyrosine kinase activity (Ronsin et al., 1993; Iwama et al., 1994). MSP is the only protein identified as specific ligand for Ron. Ron expression is restricted to certain types of tissue macrophages, including those derived from the peritoneal cavity, skin, liver and bone (Iwama et al., 1995, Nanney et al., 1998; Kurihara et al., 1996; Liu et al., 1999), whereas murine macrophages from the lung and spleen do not express it (Iwama et al., 1995).

In 2001, our group originally demonstrated that the tyrosine kinase receptor Ron is expressed in human monocyte-derived macrophages, alveolar macrophages and peritoneal macrophages (isolated from ascitic fluid of cirrhotic patients), but not in human monocytes, and that MSP induces a dose-dependent respiratory burst (Brunelleschi et al., 2001). The stimulatory effect of MSP on AM was comparable to that evoked by the tumour promoter phorbol 12-myristate 13-acetate (PMA), and higher than the chemotactic peptide N-formylmethionyl-leucyl-phenylalanine (FMLP)-induced one (Brunelleschi et al., 2001). MSP-evoked superoxide production is mediated by tyrosine kinase activity and requires the activation of Src, but not of phosphatidylinositol 3-kinase (PI 3-kinase), although this enzyme has been implicated in MSP/Ron signal transduction in other cell types (Brunelleschi et al. 2001). Moreover, also MAP kinase and p38 signalling pathways are involved. (Brunelleschi et al. 2001).

Significant MSP amounts (ranging from 1.3 to 5.8 ng/ml) were detected in BAL fluids obtained from healthy non smokers, but the concentration of MSP in the bronchoalveolar space, where AM are located, was possibly higher (Sakamoto et al., 1997). In addition, MSP levels were increased in induced sputum from patients with bronchiectasis as compared with normal controls (Takano et al., 2000).

Moreover in animal models, MSP was demonstrated to inhibit nitric oxide (NO) production and iNOS expression by murine macrophages stimulated with endotoxin or cytokines such as IFN-γ and TNF-α (Wang et al.,1994; Chen et al., 1998).

Paper 1:

European Journal of Pharmacology (2004) 501, 199–208

Anti-inflammatory drugs and tumor necrosis factor-α production from monocytes: role of transcription factor NF-κB and implication for rheumatoid arthritis therapy

Luisa Lavagno, Gabriele Gunella, Claudio Bardelli, Simona Spina, Luigia Grazia Fresu, Ilario Viano, Sandra Brunelleschi

As previously demonstrated (Brunelleschi et al., 1998) monocytes isolated from RA patients present a greater sensitivity to tachykinins and NK-2R stimulation triggers an enhanced respiratory burst (Brunelleschi et al., 1998). Moreover, human monocytes are known to release TNF-α and other inflammatory cytokines, when challenged *in vitro* with nanomolar concentrations of SP and NKA (Lotz et al., 1988). TNF-α, a pleiotropic cytokine that plays a key role in RA, is at the top of the proinflammatory cytokine cascade and also represents a good therapeutic target. Since TNF-α blocking reduces the effects of many other pro-inflammatory mediators (Feldmann et al., 1996a,b), TNF-α antagonists (e.g. etanercept) and chimeric anti-TNF-α monoclonal antibodies (e.g. infliximab) are recognized as effective agents for short-term therapeutic use in RA (Jones and Moreland, 1999; Maini et al., 1999). Moreover, in monocytes isolated from RA patients treated with Cyclosporin A (Sandimmun^R Neoral^R) 2.5 mg/kg/die, our group measured a significant time-dependent reduction in TNF-α secretion (Lavagno et al., 2001).

Although it is generally approved that anti-inflammatory drugs regulate inflammation *via* the inhibition of cyclo-oxygenase (both COX-1 and COX-2), some novel mechanisms of action that concern regulation of cytokine secretion and NF-κB activation should be considered.

In this paper we investigated the ability of dexamethasone (a corticosteroid which is known to inhibit NF-κB activation), indomethacin (a non selective COX inhibitor, also suggested as a possible PPAR-γ agonist) and rofecoxib (a selective COX-2 inhibitor) to modulate NF-κB activation and TNF-α release from human monocytes challenged with LPS or PMA.

Dexamethasone potently inhibited TNF-α release, indomethacin inhibited only PMA- evoked release, while rofecoxib had no effect.

Whereas dexamethasone and rofecoxib potently inhibited NF-κB nuclear translocation, indomethacin exerted a modest inhibition only on PMA-stimulated monocytes.

These results indicate that anti-inflammatory drugs differ largely in their ability to inhibit NF-κB activity and /or TNF-α release from human monocytes and we suggest these effects to be relevant to rheumatoid arthritis therapy.

Short after the publication of this paper, rofecoxib was withdrawn from the market, owed to its increased cardiovascular risk.

Paper in appendix

Paper 2:

British Journal of Pharmacology (2005) 145, 385–396

Expression of functional NK_1 receptors in human alveolar macrophages: superoxide anion production, cytokine release and involvement of NF-κB pathway

Claudio Bardelli, Gabriele Gunella, Federica Varsaldi, Pietro Balbo, Elisa Del Boca, Ilaria Seren Bernardone, Angela Amoruso & Sandra Brunelleschi

In keeping with the role played by SP in the respiratory system and its involvement in pulmonary diseases, we have evalued the SP-induced effects in human AMs isolated from healthy smokers and non smokers. By using natural tachykinins and selective agonists and antagonists, it was previously demonstrated that guinea-pig AMs possess NK_1 receptors and NK_2 receptors, their stimulation leading to O_2^- production and eicosanoid release, and that ovalbumin-sensitized AMs demonstrate an enhanced responsiveness to NK_2 receptor stimulation (Brunelleschi et al., 1990; 1992). The biological responses to SP are mediated by the G protein-coupled tachykinin NK_1 receptor, although SP can also bind, with lower affinity, NK_2 and NK_3 tachykinin receptors (Severini et al., 2002; Pennefather et al., 2004). The presence of NK_1 receptors on monocyte/macrophages has been demonstrated by evaluating the effects of selective agonists and antagonists on functional parameters (O_2^- production and cytokine release) (Brunelleschi et al.,1998), and *in situ* hybridization has been used to identify NK_1 receptor mRNA expression in monocytes and macrophages (Ho et al.,1997; Germonpre et al., 1999), but little is known about NK_1 receptor expression at the protein level. Marriott & Bost (2000) and Simeonidis et al. (2003) demonstrated the presence of NK_1 receptors protein in murine peritoneal macrophages and THP-1 cells, respectively but, to our knowledge, nobody had investigated its expression in human AMs.

In the present study we demonstrate, by Western blot analysis and immunofluorescence, that authentic NK_1 receptor are present on human AMs, a three-fold enhanced expression being observed in healthy smokers versus healthy non-smokers. These NK_1 receptor are functional, as SP and NK_1 agonists (e.g [Sar9Met(O2)11]SP) dose-dependently induce O_2^- production and cytokine release. In AMs from healthy smokers, SP evokes an enhanced respiratory burst and a significantly increased release of TNF-α as compared to healthy non-smokers, but has

inconsistent effects on IL-10 release. The NK_1 selective antagonist CP 96,345 competitively antagonized SP-induced effects.

We have also investigated NF-κB (p50 and p65 subunits) activation in AMs from healthy smokers and non-smokers. We have detected that NF-κB nuclear translocation is more than doubled in unstimulated AMs from healthy smokers *versus* non-smokers. In AMs stimulated by NK_1 agonists or PMA, NF-κB nuclear translocation is further enhanced about three-fold. The effect is receptor-mediated, since it is reverted by the selective antagonist CP 96,345.

These results clearly indicate that human AMs express functional NK_1 receptors on their surface, which are upregulated in healthy smokers, providing new insights on the mechanisms involved in tobacco smoke toxicity.

Paper in appendix

Paper 3:

British Journal of Pharmacology (2006) 148, 478–489

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

Gabriele Gunella, Claudio Bardelli, Angela Amoruso, Ilario Viano, Piero Balbo & Sandra Brunelleschi

The effects of MSP on human macrophages and its role in human pathophysiology have been poorly investigated. Our group previously demonstrated that human macrophages of different origin (peritoneal macrophages isolated from ascitic fluid of cirrhotic patients, AM from patients with different lung diseases, as well as monocyte-derived macrophages from healthy volunteers), but not human monocytes, express the human receptor for MSP (RON) and produce O_2^- when challenged with MSP (Brunelleschi et al., 2001).

The experiments reported in this paper were undertaken to explore the role of MSP in AM isolated from healthy volunteers and patients with interstitial lung diseases (sarcoidosis and idiopathic pulmonary fibrosis, IPF) either smokers or non-smokers, by evaluating cytokine release, respiratory burst and NF-κB activation. MSP effects were compared with those induced by known AM stimuli: PMA, FMLP and LPS.

We report that MSP, in a concentration-dependent manner, induces significant O_2^- production and cytokine release in AM from patients with interstitial lung diseases and healthy volunteers, both smokers and non-smokers. We demonstrate a significantly increased O_2^- production in AM from healthy smokers or smokers with IPF, as compared to non-smokers, thus suggesting MSP as an enhancer of cigarette smoke toxicity. 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. MSP-induced respiratory burst is quantitatively similar to the PMA-evoked one and significantly higher than the FMLP-evoked one.

MSP induces TNF-α release in AMs from all patients; MSP-induced TNF-α release in healthy smokers and IPF smokers is more than doubled 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. On the contrary, MSP-induced IL-10 release is higher in AM from healthy non-smokers.

Moreover we confirm that NF-κB is costitutively activated in healthy smokers, as previously reported (Bardelli et al., 2005) and also demonstrate that MSP is able to induce NF-kB nuclear translocation.

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.

MSP-induced NF-κB activation is receptor-mediated, as it is prevented by a monoclonal anti-human MSP antibody.

Paper in appendix

CONCLUSIONS

Inflammation is a complex biological response of tissues to harmful stimuli. An initial response consist in the increased movement of plasma and leukocytes from the blood to the injured tissues. A cascade of biochemical events propagates and matures the inflammatory response, involving the local vascular system, the immune system, and various cells within the injured tissue.

In this condition, mediators such as cytokines, NO, O_2^-, tachykinins, prostaglandins, play an important role in the control of vital functions (e.g. regulation of blood pressure, platelet aggregation, body temperature, pain) and lead to a progressive leukocyte infiltration on the site of inflammation and simultaneous destruction and healing of the tissue from the inflammatory process.

Therefore the ability to selectively inhibit the expression of these (and other) inflammatory mediators remains one of the most hopeful strategies for the effective treatment of inflammation (Vane et al., 1990; Rink et al., 1996; Dinarello, 1997; Liaudet et al., 2000; Funk, 2001).

In the present Ph.D thesis, I have evaluated the role of monocyte/macrophages in the inflammatory response, regarding NF-κB signalling pathway, cytokine release and respiratory burst.

In the first paper of this thesis I have contributed to demonstrate the different effects of selected anti-inflammatory drugs on TNF-α release and NF-κB activation in PMA- or LPS-stimulated monocytes.

In human monocytes challenged with PMA or LPS, TNF-α release is potently inhibited by dexamethasone confirming previous observations (Adcock et al., 1995; Steer et al., 2000). Although TNF-α production is largely regulated by the transcription factor NF-κB, evidence exists that TNF-α and other cytokines can also be induced through NF-κB independent pathways. LPS-induced TNF-α production in human macrophages is largely controlled by NF-κB, instead for the PMA-induced activation of monocytic cells, the results are controversial: some studies indicate that NF-κB is involved (Bondeson et al., 1999; Kaufman et al., 1992) and others do not (Makarov et al., 1997; Tran-Thi et al., 1995).

Cytokine secretion by LPS- or PMA-stimulated monocytes can be regulated, cooperatively or not, by different signalling pathways [e.g., extracellular signal-regulated kinase (ERK)-1,

ERK-2, p38 mitogen-activated protein (MAP) kinase, MAP kinase kinase (MEK), c-jun N-terminal kinase (JNK), and phosphatidylinositol 3 kinase] independently of NF-κB activation (Meja et al., 2000).

In our experiments, LPS and PMA were both able to activate NF-κB and to induce TNF-α secretion from monocytes. In good agreement with previous reports (Adcock et al., 1995; Steer et al., 2000) dexamethasone potently and dose-dependently inhibited the translocation of p65 subunit, maximal inhibition being achieved at 10 μM and reaching about 80%, with no significant variation between PMA- or LPS-stimulated monocytes. P50 translocation was equally inhibited; so we can exclude a dexamethasone selective effect on p65 in human monocytes. Moreover, dexamethasone inhibited IKK kinase activity (as evaluated by measuring the amount of phosphorylated IkBa in cytosol) in both PMA- or LPS-stimulated monocytes.

Therefore glucocorticoids can suppress NF-κB-transactivation through a direct physical association of ligand-activated glucocorticoid receptors with p65 (Ray and Prefontaine, 1994, Scheinman et al., 1995) and enhancing expression of I-kBα (Auphan et al., 1995; Scheinman et al., 1995; Unlap and Jope, 1997).

Additionally dexamethesone, as all glucocorticoid drugs, suppresses TNF-α release by reducing NF-κB binding at TNF-α promoter sites.

On the contrary, the nonselective cyclooxygenase inhibitor indomethacin inhibited PMA-evoked TNF-α release, but failed to affect the LPS-induced one. Indomethacin has PPAR-γ agonistic activity at high concentrations and could, for this reason, block the production of inflammatory cytokines (Jiang et al., 1998; Lehmann et al., 1997).

In human monocytes and other cell types, activation of PPAR-γ receptors has been repetitively demonstrated to inhibit the production of inflammatory mediators (including TNF-α, COX-2, NO, IL-1, IL-2, and IL-6), transrepression of the transcription factors NF-κB and AP-1 being involved (Oates et al., 2002). In this study indomethacin had no effect on LPS stimulation, but it dose-dependently inhibited TNF-α release in PMA-stimulated human monocytes, in keeping with observations by Jiang et al. (1998). Maximal inhibition was approximately 80% and the IC50 value (4.6 μM) measured in this condition could be of clinical relevance, as a high-dose therapy is expected to produce an indomethacin plasma concentration of about 10 μM (Jiang et al., 1998).

In contrast to indomethacin, the selective COX-2 inhibitor, rofecoxib, inhibited PMA- or LPS-induced NF-κB activation in human monocytes, but had no effect on PMA- or LPS-

induced release of TNF-α. Rofecoxib inhibits the LPS-dependent activation of MAPKs. The phosphorylation of p44 ERK and p38 is completely abolished after treatment with 10 μM rofecoxib, and the same occurs for JNK (Callejas et al., 2003).

The mechanisms by which rofecoxib affects NF-κB activation in human monocytes are currently under investigation. Our results indicate that at 10 μM, a concentration sufficient to completely block COX-2 (Chan et al., 1999), rofecoxib inhibits more than 60% NF-κB activity in PMA-stimulated monocytes, as evaluated by EMSA. Our results indicate that rofecoxib dose-dependently reduces, although to a lesser extent, LPS-induced NF-κB activity. To our knowledge, this is the first demonstration of such an effect for rofecoxib on human monocytes and represents a somewhat expected result, since COX-2 activity is largely regulated by the transcription factor NF-κB.

Our observation that, in cytosolic extracts, rofecoxib strongly inhibits the phosphorylation of IkBα evoked by LPS or PMA indirectly confirms the results by Callejas et al. (2003) and further indicates IKK inhibition as an important mechanism for rofecoxib-mediated anti-inflammatory effects.

Therefore, this study demonstrates that anti-inflammatory drugs differ largely in their ability to inhibit NF-κB activity and TNF-α release from monocytes.

In the second paper, I have demonstrated that SP and the selective NK_1 agonist $[Sar^9Met(O_2)^{11}]SP$ induce O_2^- production, cytokine release and NF-κB activation in AMs isolated from BAL of healthy smokers and non smokers. All agonists act dose-dependently and maximal effects are observed at micromolar concentrations, the respiratory burst being already detectable at low picomolar concentrations.

CP 96,345, a selective NK_1 antagonist, competitively inhibits tachykinin-evoked O_2^- production, cytokine release and NF-κB nuclear translocation from AMs, confirming the involvement of NK_1 receptor.

NK-1R expression has been evaluated by different authors in different cell types, by using RT–PCR technology, mainly. By this approach, human monocytes and monocyte-derived macrophages were shown to express SP and NK-1R (Ho et al., 1997). NK_1 receptor presents many isoforms: in the rat, full-length and truncated receptors have molecular weights of about 80 and 50 kDa respectively, and deglycosylated forms 46 kDa and 37 kDa respectively (Li et al., 1997). In THP-1 cells, Marriott and Bost (2000) and Simeonidis et al. (2003) demonstrated a 42 kDa isoform of NK-1R.

In human AMs we detected three prominent bands of 68 (as indicated by the manufacturer), 53 and 42 kDa, in accordance to what observed by others in cells of the monocyte/ macrophage lineage.

In human AMs, NK_1 receptors are functional, since SP and [$Sar^9Met(O_2)^{11}$]SP induce O_2^- production, cytokine release and NF-κB activation. The increased O_2^- production, TNF-α release and NF-κB activation in AMs from healthy smokers suggest SP as a potent contributor for tobacco smoke toxicity.

We also demonstrate that SP and [$Sar^9Met(O_2)^{11}$]SP are particularly effective on p50 translocation (about three-fold) and their effects are significantly enhanced in AMs from smokers. On the contrary, SP and [$Sar^9Met(O_2)^{11}$]SP also stimulate p65 nuclear translocation, but, in this case, no significant variations are observed between smokers and non-smokers. These data are in good agreement with the data demonstrating p50 as the major NF-κB subunit for the transcription of TNF-α gene (Carter et al., 1998).

In the third paper we report 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, smokers and non-smokers.

We and others (Conron et al., 2001; Ziegenhagen and Muller-Quernheim, 2003; Bardelli et al., 2005) have previously observed that AMs from smokers and sarcoidosis patients spontaneously produce large amounts of proinflammatory cytokines, such as TNF-α, IL-1β and IL-6, but little of the anti-inflammatory cytokine IL-10. An interesting feature of this article is that MSP releases higher amounts of IL-10 in AMs from healthy non-smokers.

Moreover, the presence of potential NF-κB sites in STK/RON promoter (Waltz et al., 1998) and MSP ability to induce NF-κB activation support an intriguing role for this mediator in pulmonary diseases.

Since 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.

This paper 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, and suggests MSP as a possible contributor for tobacco smoke toxicity.

REFERENCES

Adcock, I.M. et al., Am. J. Physiol. 268: C331– C338 (1995)

Amoruso, A. et al., *Br J Pharmacol* Feb 18 [Epub ahead of print] (2008)

Antonicelli, F. et al., *Am. J. Respir. Crit. Care Med.* 161: A738 (2000)

Auphan, N. et al., *Science* 270(5234): 286-90 (1995)

Bai, TR. et al., *Am J Physiol.* 269(3 Pt 1): L309-17 (1995)

Baldwin A.S. *Annu.Rev. Immunol.* 14, 649-681 (1996)

Bardelli, C. et al., *Br. J. Pharmacol.* 145: 385–396 (2005)

Barkett and Gilmore *Oncogene* 18, 6910- 6924 (1999)

Barnes PJ. et al., *Eur Respir J* 25: 552–563 (2005)

Barnes PJ. et al., *Pharmacol Rev* 50: 515-596 (1998)

Barnes PJ. et al., *Pharmacol Rev* 50: 515-596 (1998)

Barnes, PJ. *Am Rev Respir Dis.* 143(3 Pt 2): S28-32 (1991)

Barnes, PJ. et al., *Am Rev Respir Dis*. 144(5): 1187-98 (1991)

Beg A.A. et al., *Nature* 376, 167–170 (1995)

Bezerra, JA. et al., *Hepatology* 8: 394-399 (1993)

Blackwell TS and Christman JW., *Am J Respir Cell Mol Biol.* 17(1):3-9 (1997)

Blackwell TS. et al., *J Immunol* 157: 1630–1637 (1996).

Bowie AG. et al., *J Biol Chem*; 272: 25941–25950 (1997)

Bowie, A. and O'Neill, L.A. *Biochem. Pharmacol.* 59 : 13–23 (2000)

Brown D. et al., *Am. Rev. Respir. Dis.* 144: 668 (1993)

Brunelleschi S. *Exp Opin Invest Drugs* 8: 1873-1891 (1999)

Brunelleschi S. et al., *Neuropeptides* 32: 215-223 (1998)

Brunelleschi, S. et al ., *Life Sci.* 48: PL1-PL5 (1991)

Brunelleschi, S. et al. *Neuropeptides* 30: 456-464 (1996)

Brunelleschi, S. et al., *Br J Pharmacol* 100: 417-420 (1990)

Brunelleschi, S. et al., *Br J Pharmacol* 107: 964-969 (1992)

Brunelleschi, S. et al., *Br J Pharmacol* 134:1285-1295 (2001)

Brunelleschi, S. et al., *Neuropeptides* 34(1): 45-50 (2000)

Bureau F. et al., *Am J Respir Crit Care Med.* 161(4 Pt 1): 1314-21 (2000)

Callejas, N.A. *Mol. Pharmacol.* 63: 671– 677 (2003)

Carter, A.B. et al., *Am. J. Respir. Cell. Mol. Biol.* 18: 384–391 (1998)

Chan, C.C. *J. Pharmacol. Exp. Ther.* 290: 551–560. (1999)

Chen F., V.Castranova & X.Shi; *Am J Pathol* 159, 387-397 (2001)

Chen, Y.Q. et al. *J. Immunol*. 161, 4950–4959 (1998)

Chu HW. et al., *J Allergy Clin Immunol* 106(4): 713-722 (2000)

Conron, M., et al., *Am. J. Resp. Cell Mol. Biol.* 25: 141–149 (2001)

Das, J. et al., *Nat. Immunol.* 2:45 (2001)

Davis W. et al., J. Lab. Clin. Med. 111 : 293-298 (1988)

Devillier P. et al., *N Engl J Med* 314: 1323 (1986)

Devillier P. et al., *N Engl J Med* 314 : 1323 (1986)

Di Stefano A. et al., *Eur Respir J* 20: 556–563 (2002)

Dinarello, CA. *Chest* 118: 503-508 (2000)

Drost, E.M. et al. *Am. J. Respir. Cell. Mol. Biol.* 6: 287-295 (1992)

Erspamer V. Bioactive secretions of the integument, in Amphibian Biology. I. The Integuments (Heatwole H and Barthalmus GT eds), Surrey Beatty & Sons, Chipping Norton, NSW, Australia.; 178-350 (1994)

Farver CF. et al., *Cytokine* 10: 868–871 (1998)

Feldmann M. et al., *Cell* 85: 307-310 (1996a)

Feldmann, M. et al., *Annu Rev Immunol* 14: 397-440 (1996b)

Franzoso G., et al.. *Genes Dev.* 11:3482–3496 (1997)

Funk, CD. et al., *Science* 294: 1871-1875 (2001)

Gay S. et al., *Ann Rheum Dis* 52: S39-S47 (1993)

Germonpré PR. et al., *Eur Respir J* 14: 776-782 (1999)

Germonpre, PR. et al. *Eur. Respir. J.* 14, 776–782 (1999)

Ghosh G., G.van Duyne, S.Ghosh & P.B.Sigler; *Nature* 373, 303-310 (1995)

Ghosh S. et al., *Annu. Rev. Immunol.* 16: 225–26 (1998)

Ginn-Pease ME and Whisler RL. *Biochem Biophys Res Commun* 226: 695–702 (1996)

Grisham, MB. Reactive oxygen metabolism. In: Grisham MB, ed. Reactive metabolites of oxygen and nitrogen in biology and medicine. Austin: RG Landers Company, 1992:39

Hamilton, L. et al., *Monaldi. Arch. Chest Dis.* 56:48 (2001)

Han, S. et al., *Biochemistry* 30 : 9768-80 (1991)

Han, Z.N. et al., *Autoimmunity* 28, 197–208 (1998)

Handel ML et al., *Arthritis Rheum.* 38(12): 1762-70 (1995)

Heaney, LG. et al., *Thorax* 53: 357-362 (1998)

Ho, W.Z. et al., J. *Immunol.* 159, 5654–5660 (1997)

Hunninghake G.W. and Grystal R.G. *Am Rev. Respir. Dis.* 128: 833-838 (1983)

Ichinose M. et al., *Am J Respir Crit Care Med* 153(3): 936-941 (1996)

Iotsova V., et al.. *Nat. Med.* 3: 1285–1289 (1997)

Iwama, A. et al., *Blood* 86: 3394-3403 (1995)

Iwama, A. et al., *Blood* 86: 3394-3403 (1995)

Jaspers I. et al., *Am J Respir Cell Mol Biol* 24: 769–777 (2001)

Jiang, C. et al. *Nature* 391: 82–86 (1998)

Jimenez, L.A. et al., *Toxicol. Appl. Pharmacol.* 166: 101–110 (2000)

Jones RE and Moreland LW, *Bull Rheum Dis.* 48 (3): 1-4 (1999)

Jones, RE. and Moreland, LW. *Bull Rheum Dis* 48: 1-4 (1999)

Joos,GF. and Pauwels RA. *Trends Pharmacol Sci* 21: 131-133 (2000)

Joos, GF. and Pauwels RA. *Curr Opin Pharmacol* ; 1(3):235-41 (2001)

Joos, GF. et al., *Am J Respir Crit Care Med* 153(6 Pt 1): 1781-1784 (1996)

Kamata, H. et al., *FEBS Lett* 519: 231–237 (2002)

Karin, M (a); *Nature* 441, 431-436 (2006)

Karin, M (b); *Mol Carcinog* 45, 355-361 (2006)

Karin, M. and F.R.Greten; *Nat Rev Immunol* 5, 749-759 (2005)

Kilburn, K. and McKenzie, W. *Science* 189 : 634-637 (1975)

Klebanoff S. Phagocytic cells: Products of oxygen metabolism. In: Gallen JI, Goldstein IM, Synderman R, eds. Injammation-Basic principles and clinical correlates. New York: Raven Press, 1992:541

Kudlacz, E. et al., *Int Arch Allergy Immunol* 115(2): 169-78 (1998)

Kurihara, N. et al., *Blood* 87: 3704-3710 (1996)

Lagente, V. and Advenier, C. *Pulm Pharmacol Ther* 11(5-6): 331-340 (1998)

Lambrecht, BN. et al., *Eur J Immunol* 29: 3815-3825 (1999)

Lavagno, L. et al., *Neuropeptides* 35 : 92–99 (2001)

Lehmann, J.M. *J. Biol. Chem.* 272: 3406–3410. (1997)

Lenardo, M.J. & D.Baltimore; *Cell* 58, 227-229 (1989)

Lentsch, AB. et al., *Am J Respir Cell Mol Biol* 20: 692–698 (1999)

Lentsch ,AB. et al., *J Clin Invest* 100: 2443–2448 (1997)

Leonard, EJ. and Skeel, A. *Exp Cell Res* 102: 434-438 (1976)

Leonard, EJ. and Skeel, A. *Exp Cell Res* 114: 117-126 (1978)

Leonard, EJ. Biological aspects of macrophage-stimulating protein (MSP) and its receptor. *Ciba Found Symp* 212: 183-191; discussion 192-197 (1997)

Levine, JD et al., *Science* 226: 547-549 (1984)

Li, H. et al., *PNAS* 94: 9475-9480 (1997)

Liaudet, L. et al., *Crit Care Med* 28, 37-52 (2000)

Liu, SF. et al., *Circulation* 100: 1330–1337 (1999a)

Liu, SF. et al., *Mol Pharmacol* 55: 658–667 (1999b)

Liu, Q-P. et al., *J Immunol* 163: 6606-6613 (1999)

Lotz, M. et al., *Science* 241: 1218-1221 (1988)

Ludwig, P.W. and Hoidal J.R. *Am. Rev. Respir. Dis.* 126: 977-980 (1982)

MacNee, W. et al., *N.Engl. J. Med*. 321 : 924-928 (1989)

Maggi CA. *Regul Pept* 70: 75-90 (1997)

Maggi CA. *Regul Pept* 70: 75-90 (1997)

Maggi CA. *Trends Pharmacol Sci* 21: 173-175 (2000)

Maggi CA. *Trends Pharmacol Sci* 21: 173-175 (2000)

Maini R. et al., *Lancet* 354: 1932-1939 (1999)

Maini, R. et al., *Lancet* 354: 1932-1939 (1999)

Makarov, S.S. et al., *Gene Ther*. 4, 846– 852 (1997)

Manning, AM. et al., *J Inflamm*. 45(4):283-96 (1995)

Mapp, CE. et al., *Am J Respir Crit Care Med* 161: 207-215 (2000)

Marriott, I. and Bost, KL. *J Immunol.* 165(1): 182-91 (2000)

Meja, K.K. et al., *Br. J. Pharmacol.* 131: 1143– 1153 (2000)

Miyazawa, K. et al., *J Biochem.* 124(6):1130-7 (1998)

Nanney, LB. J Invest *Dermatol* 111: 573-581 (1998)

Nieber, K. et al., *J Allergy Clin Immunol* 90: 646-652 (1992)

Oates, J.C. *Arthritis Rheum.* 46: 598–605 (2002)

Pahl, H.L. *Oncogene* 18, 6853–6866 (1999)

Payan, DG. et al., *J Immunol* 131(4): 1613-1615 (1983)

Pennefather, J.N., *Life Sci.* 74, 1445–1463 (2004)

Poynter, ME. et al., *Am J Pathol.* 160(4): 1325-34 (2002)

Rahman, A. et al., *J Immunol.* 162(9): 5466-76 (1999)

Rahman, I and MacNee W. *Free Rad Biol Med* 21: 669–681 (1996)

Rahman, I and MacNee W. *Thorax* 53: 601–612 (1998)

Rahman, I. *Treat Respir Med* 4: 175–200 (2005)

Ray, A. and Prefontaine, KE. *PNAS*. 91(2): 752-6. (1994)

Regoli, D. et al., *Pharmacol Rev* 46: 551-599 (1994)

Richards, G. et al., am Rev. Respir. Dis. 139 : 181-187 (1989)

Rink, L. et al., *Int Arch Allergy Immunol* 111: 199-209 (1996)

Sakamoto, O. et al., *J Clin Invest* 99: 701-709 (1997)

Schaberg, T. et al., *Eur. Respir. J.* 5 : 387-393 (1992)

Scheinman, RI. et al., Science. 270(5234): 283-6 (1995)

Schottelius, AJ. et al., *J Biol Chem.* 274 (45): 31868-74 (1999)

Schwartz, MD. et al., Crit Care Med 24: 1285–1292 (1996)

Sen, R. and Baltimore, D. *Cell.* 47, 921–928 (1986)

Severini, C. et al., *Pharmacol Reviews* 54: 285-322 (2002)

Simeonidis, S. et al., *PNAS* 100: 2957-2962 (2003)

Skeel , A. et al., *J Exp Med* 173: 1227-1234 (1991)

Springall, DR. et al., *Am Rev Respir Dis.* 141(6): 1538-46 (1990)

Springall, DR. et al., *Environ Health Perspect.* 85: 151-7 (1990)

Stacey, MA. et al., *Biochem Biophys Res Commun.* 236(2): 522-6 (1997)

Steer, J.H. et al., J. Biol. Chem. 275: 18432– 18440 (2000)

Steer, J.H. et al., *J. Biol. Chem.* 275: 18432– 18440 (2000)

Tak, P.P. et al., *J. Clin. Invest.* 107 : 7– 11 (2001)

Takano, Y. et al., *Resp. Med.* 94, 784–790 (2000)

Takeyama, M, et al., *Sarcoidosis Vasc Diffuse Lung Dis* 13(1): 33-37 (1996)

Tomaki M. et al., *Am J Respir Crit Care Med* 151: 613-617 (1995)

Tran-Thi, T.A. et al., *Hepatology* 22: 613– 619 (1995)

Tsao, PW. et al., *Clin Immunol Immunopathol.* 83(2):173-8 (1997)

Ukada, K. et al., *J Biol Chem* 274: 23787–23793 (1999)

Unlap, MT. and Jope, RS. *Brain Res Mol Brain Res.* 45(1): 83-9. (1997)

Van Antwerpen, V.L. et al., *Free Radic. Biol. Med.* 18: 935-943 (1995)

Vane, JR. et al., *Stroke* 21: 12-23 (1990)

Vignola, AM., et al., *Curr Allergy Asthma Rep.* 1(2): 108-15 (2001)

Vincenti, MP. et al., *Arthritis Rheum.* 41(11): 1987-94 (1998)

Walsh, DA. et al., *J Pharmacol Exp Ther* 267(2): 9519-9560 (1993)

Waltz, S.E., et al., *Oncogene* 16: 27–42 (1998)

Wang, M-H. et al., *J Clin Invest* 97: 721-727 (1996a)

Wang, MH. et al., *Oncogene* 13(10): 2167-2175 (1996b)

Wang, W. et al., *Immunology* 83(4): 601-610 (1994)

Yang, et al., *J Exp Med* 188: 1739-50 (1998)

Zhao, S. et al., *Chin Med J (Engl)* 114(9):909-11 (2001)

Ziegenhagen, MW. and Müller-Quernheim, J. *J Intern Med.* 253(1): 18-30 (2003)

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European Journal of Pharmacology 501 (2004) 199–208

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Anti-inflammatory drugs and tumor necrosis factor-α production from monocytes: role of transcription factor NF-κB and implication for rheumatoid arthritis therapy

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Received 15 March 2004; received in revised form 19 May 2004; accepted 23 July 2004
Available online 18 September 2004

Abstract

Inhibition of tumor necrosis factor-α (TNF-α) represents a relevant target in rheumatoid arthritis therapy. Besides inhibiting cyclooxygenase, anti-inflammatory drugs can affect the activation of transcription factors. We investigated the ability of dexamethasone, indomethacin, and rofecoxib to modulate nuclear factor-κB (NF-κB) activation and TNF-α release from human monocytes challenged with lipopolysaccharide (LPS) or phorbol 12-myristate 13-acetate (PMA). Both stimuli induced NF-κB nuclear translocation and TNF-α secretion. Dexamethasone potently inhibited TNF-α release, indomethacin inhibited only PMA-evoked release, while rofecoxib had no effect. In the electrophoretic mobility shift assay, dexamethasone and rofecoxib dose-dependently inhibited the DNA binding activity of NF-κB in stimulated monocytes, whereas indomethacin failed to inhibit the LPS-evoked one. These results were further confirmed by evaluating the drugs' ability to reduce nuclear NF-κB subunits, as well as the amount of phosphorylated IκBα in cytosolic fractions. In conclusion, these results indicate that anti-inflammatory drugs differ largely in their ability to inhibit NF-κB activity and/or TNF-α release from human monocytes. These effects can be relevant to rheumatoid arthritis therapy.

Keywords: Human monocyte; Tumor necrosis factor-α; Nuclear factor-κB; Rofecoxib; Indomethacin; Dexamethasone

1. Introduction

Rheumatoid arthritis is a common autoimmune disease of unknown aetiology characterized by symmetric erosive synovitis and infiltration of immunocompetent cells (Feldmann et al., 1996). Evidence suggests that there is an imbalance between pro-inflammatory and anti-inflammatory mediators, and much effort has been focused on the identification of cytokines that play a crucial role in joint damage (Feldmann et al., 1996; Choy and Panayi, 2001). Tumor necrosis factor-α (TNF-α) and interleukin-1 have been shown to have a pivotal role in the pathogenesis and development of rheumatoid arthritis, with high synovial and serum concentrations for both cytokines being detected in patients (Choy and Panayi, 2001). TNF-α stimulates the production of other inflammatory cytokines, promotes cartilage degradation and bone resorption in vitro, induces prostaglandin E_2 and collagenase release from synovial cells, and up-regulates the expression of vascular adhesion molecules, leading to tissue infiltration by neutrophils (Choy and Panayi, 2001). TNF-α can be produced by several cell types relevant to the rheumatoid joint, monocytes being the most important, and can be targeted to cure

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0014-2999/$ - see front matter © 2004 Published by Elsevier B.V.
doi:10.1016/j.ejphar.2004.07.101

disease, as demonstrated in recent years with the clinical application of infliximab and etanercept (American College of Rheumatology Subcommittee, 2002).

It is generally accepted that some anti-inflammatory drugs regulate inflammation via the inhibition of the enzyme cyclooxygenase-2 (COX-2), but some novel mechanisms of action, notably activation of peroxisome proliferator-activated receptor-γ (PPAR-γ) and inhibition of transcription factors such as nuclear factor-κB (NF-κB) and/or activator protein-1 (AP-1), have gained considerable attention (Jiang et al., 1998; Tegeder et al., 2001). The transcription factor NF-κB regulates the expression of many pro-inflammatory genes, including that of TNF-α, and, in turn, inflammatory cytokines, such as TNF-α and interleukin-1, are potent inducers of NF-κB activation (Baldwin, 1996; Tak and Firestein, 2001). This raises the question of whether NF-κB activation could play a pathogenic role in rheumatoid arthritis; this would be supported by the observation that NF-κB is overexpressed in the inflamed synovium of patients and experimental models of arthritis (Han et al., 1998; Tak and Firestein, 2001). Five related mammalian gene products participate in NF-κB functions (p50/NF-κB1, p52/NF-κB2, p65/Rel A, c-Rel, and RelB); although different homodimeric and heterodimeric forms of this factor have been described, the predominant species in many cell types is a p50–p65 heterodimer. In resting cells, NF-κB is retained in the cytoplasm through an association with inhibitory proteins of the IκB family (including the best characterized IκBα and IκBβ), which masks the nuclear localization signal (Baldwin, 1996; Tak and Firestein, 2001). IκBα is a key molecular target involved in the regulation of NF-κB activity during the inflammatory process. Upon stimulation, IκBα is phosphorylated by IκB kinases (IKKs), ubiquitinylated, and degraded by a proteasome complex, thus allowing NF-κB to translocate to the nucleus and bind DNA. Once NF-κB enters the nucleus, it binds to the promoter region of various genes involved in the inflammatory and immune response [e.g., COX-2, TNF-α, and inducible nitric oxide synthase (iNOS)] and induces their transcription. It also causes the transcriptional activation of the IκBα gene, thus allowing a negative feedback mechanism (Baldwin, 1996; Ghosh et al., 1998). Two IKKs, namely IKK-1 (IKKα) and IKK-2 (IKKβ), have been cloned, which mediate transient or persistent NF-κB activation in response to stimuli, respectively (Baldwin, 1996).

Several anti-inflammatory drugs have been shown to inhibit NF-κB activation at different stages: aspirin and salicylate specifically inhibit IKK-2 activity (Yin et al., 1998), while corticosteroids can directly interact with and inhibit NF-κB subunits and/or up-regulate the expression of the inhibitor IκBα, in addition to inhibiting cAMP response element-binding protein (CREB) and AP-1 (Almawi and Melemedjian, 2002; De Bosscher et al., 2003).

The initial pharmacological treatment of rheumatoid arthritis usually involves the use of low-dose oral glucocorticoids, nonsteroidal anti-inflammatory drugs, or a selective COX-2 inhibitor to reduce joint pain and swelling and to improve joint function (American College of Rheumatology Subcommittee, 2002). Therefore, we decided to evaluate the ability of indomethacin (a nonselective cyclooxygenase inhibitor), rofecoxib (a selective COX-2 inhibitor), and dexamethasone (as an example of glucocorticoid drug and also as a positive control, due to its described effects on transcription factors) to modulate NF-κB activation and/or TNF-α release from human monocytes.

2. Materials and methods

2.1. Isolation of peripheral blood monocytes

Peripheral monocytes were isolated from heparinized venous blood (30–40 ml) of healthy donors (aged 22–55 years) by standard techniques of dextran sedimentation and Ficoll-Paque (density=1.077 g/cm^3) gradient centrifugation (400×*g*, 30 min, room temperature), and recovered by thin suction at the interface (Brunelleschi et al., 2001). Cells were washed twice with phosphate-buffered saline (PBS) and resuspended at 1–2×10^7 cells/ml in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 2 mM glutamine, 10 mM HEPES, 50 μg/ml streptomycin, and 5 U/ml penicillin (Brunelleschi et al., 2001). Cell viability, as assessed by Trypan blue dye exclusion, was >98%. Cell suspensions (100 μl) were plated in six-well tissue culture plates (35 mm diameter; Costar, UK) and allowed to adhere for 90 min at 37 °C in a humidified atmosphere containing 5% CO_2 to isolate purified monocytes. Nonadherent cells (mainly lymphocytes) were discarded. The purity of adherent monocytes was assessed by flow cytometry with monoclonal antibodies, anti-CD14 (a marker for the LPS receptor) and anti-CD3, with monocyte populations routinely consisting of >90% CD14$^+$ cells and <3% CD3$^+$ cells, as described (Brunelleschi et al., 2001).

2.2. Stimulation of monocytes

Monocytes (0.5–1×10^6 cells) were challenged, in the absence or presence of anti-inflammatory drugs (see below), with appropriate concentrations of phorbol 12-myristate 13-acetate (PMA) or lipopolysaccharide (LPS; from *Salmonella typhimurium*) to evaluate TNF-α release and NF-κB activation. To verify the effects of anti-inflammatory drugs on TNF-α release, monocytes were preincubated with dexamethasone (10^{-11}–10^{-5} M) for 24 h according to Van der Goes et al. (2000), indomethacin (10^{-7}–10^{-4} M) for 2 h according to Jiang et al. (1998), rofecoxib (10^{-7}–10^{-5} M) for 2 h according to Chan et al. (1999), or solvent, and then challenged with 10^{-7} M PMA or 10 ng/ml LPS. Preliminary dose–response curves (10^{-9}–10^{-6} M PMA; 0.1–1000 ng/ml LPS) and time course experiments (30 min–48 h) demonstrated that these concentrations, combined with a 24-h

stimulation period, produced the maximum cytokine production, although TNF-α release was observed also at shorter or longer stimulation times. Supernatants were harvested and stored at −20 °C until assay. Dexamethasone and indomethacin were dissolved in ethanol, and rofecoxib was dissolved in dimethyl sulfoxide (DMSO); no major effect of solvents was observed under our conditions. To evaluate NF-κB activation, monocytes were challenged with anti-inflammatory drugs as above and then stimulated by 10^{-6} M PMA or 500 ng/ml LPS for different periods (see Results). For these experiments, a greater amount of cells was needed, approximately $5–10\times10^6$ monocytes. Electrophoretic mobility shift assay (EMSA; see below) and quantitative ELISA assays for translocated p50 and p65/RelA subunits or cytosolic phosho-IκBα were performed with 5 μg of nuclear extract or 100 μg of cytosolic extract, respectively.

2.3. Measurement of TNF-α release

TNF-α in the samples was measured using enzyme-linked immunoassay kit (Pelikine Compact™ human TNF-α ELISA kit), according to the manufacturer's protocol. The minimum detectable concentration of human TNF-α was <1.4 pg/ml. No cross-reactivity was observed with any other known cytokine. Control values (e.g., cytokine release from untreated, unstimulated cells) were subtracted from all determinations. Results are mean±S.E.M. of duplicate determinations of *n* independent experiments and are expressed in picograms per milliliter.

2.4. Preparations of nuclear and cytosolic cellular fractions

After treatment, cells were washed with ice-cold PBS, scraped, and centrifuged at 1000×*g* for 5 min at 4 °C. The cell pellet was resuspended in 300 μl of lysis buffer (10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM phenylmethylsulfonyl fluoride, and 0.5 mM dithiothreitol) and incubated on ice for 15 min. At the end of this incubation, 20 μl of 10% NP-40 was added and the tube was vortexed for 10 s. After centrifugation at 13,000×*g* for 1 min at 4 °C, supernatants (cytosolic fractions) were collected and stored at −80 °C, whereas the pellets were further processed to obtain nuclear extracts. The pellets were resuspended in extraction buffer (5 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 0.5 mM phenylmethylsulfonyl fluoride, 0.2 mM EDTA, 0.5 mM dithiothreitol, and glycerol 25% vol/vol) and incubated for 30 min at 4 °C. Nuclear proteins were isolated by centrifugation at 13,000×*g* for 15 min. The supernatant was aliquoted and stored at −80 °C until used for EMSA or p50/p60 ELISA assays. Protein concentrations were determined by using a protein assay (Bio-Rad, USA).

2.5. EMSA

Nuclear extracts (5 μg) were incubated with 2 μg of poly(dI–dC) and the γ-[^{32}P]ATP-labeled oligonucleotide probe (100,000–150,000 cpm; Promega, St. Louis, CA, USA) in binding buffer (50% glycerol, 10 mM Tris–HCl, pH 7.6, 500 mM KCl, 10 mM EDTA, and 1 mM dithiothreitol) in a final volume of 20 μl for 30 min at room temperature. The NF-κB consensus oligonucleotide (5′ -AGTTGAGGGGACTTTCCCAGGC-3′) was obtained from Promega. The nucleotide–protein complex was separated on a 5% polyacrylamide gel in 0.5× TBE buffer (100 mM Tris–HCl, 100 μM boric acid, and 2 mM EDTA) at 150 V on ice. The gel was dried and radioactive bands were detected by autoradiography.

2.6. p50 and p65/RelA assays

Nuclear extracts were prepared as described above and evaluated for the presence of p50 and p65/RelA subunits using Trans-AM™ NF-κB p50 and NF-κB p65 Transcription Factor Assay kits (Active Motif Europe, Belgium), according to the manufacturer's instructions. Briefly, an equal amount (5 μg) of nuclear lysate was added to incubation wells precoated with an oligonucleotide containing the NF-κB consensus site (5′ -GGGACTTTCC-3′) sequence, the active form of NF-κB contained in the cell extract specifically binding to this oligonucleotide. These assay kits specifically detected bound NF-κB p65 or p50 subunits in human extracts; activities of p50 and p65 were measured using a microplate spectrophotometer at 450 nm, and results are expressed as OD.

2.7. Assay of phospho-IκBα in cytosolic extracts

Cytosolic extracts (100 μg) were prepared as described above and evaluated for the presence of phosphorylated IκBα using Phospho-IκBα-ActivELISA™ kit (Alexis, Switzerland), according to manufacturer's protocol. This procedure allows quantitative measurement of IKK-induced phosphorylation of IκBα in response to external stimuli.

2.8. Data and statistical analysis

Data are expressed as mean±S.E.M. of *n* independent determinations. Concentration–response curves for anti-inflammatory drugs were constructed and logarithmically transformed. IC_{50} values were interpolated from curves of best fit using Graph-Pad Software (Graph-Pad, San Diego, CA, USA). When required, statistical analysis was performed by Student's *t* test for paired or unpaired samples. In all cases, significance was set at a P value <0.05.

2.9. Reagents

Dextran T-500, Ficoll-Paque, and poly(dI–dC) were obtained from Pharmacia (Uppsala, Sweden). Fluorochrome-conjugated anti-CD14 and anti-CD3 were purchased from Becton Dickinson (UK). PBS, RPMI 1640, L-glutamine, HEPES, streptomycin, penicillin, PMA, LPS from *S.*

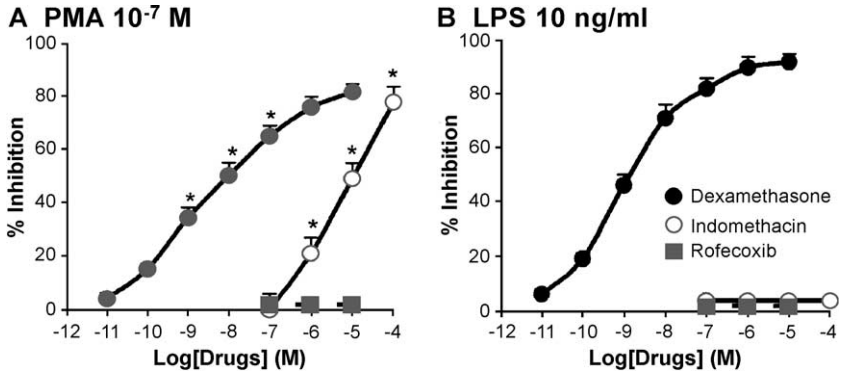

Fig. 1. Effects of anti-inflammatory drugs on TNF-α release from PMA- or LPS-stimulated monocytes. Adherent monocytes ($0.5–1\times10^6$ cells/plate) were preincubated at 37 °C in the absence or presence of dexamethasone (●; 24 h), indomethacin (○; 2 h), or rofecoxib (■; 2 h) and then treated with PMA 10^{-7} M (A) or 10 ng/ml LPS (B) for a further 24 h. The cytokine release was measured by ELISA. Results are mean±S.E.M. of four to six experiments in duplicate. Asterisk denotes a statistically significant difference ($P<0.05$) in the inhibitory effect of PMA-stimulated cells vs. LPS-stimulated cells.

typhimurium, DMSO, indomethacin, and ethanol were from Sigma (St. Louis, MO, USA). All cell culture reagents, with the exception of fetal bovine serum, were endotoxin-free according to details provided by the manufacturer. Fetal bovine serum (lot 40G3410K, containing <10 EU/ml) was from Life Technologies (Rockville, MD, USA). Dexamethasone was purchased from Alexis. Rofecoxib was a kind gift from Merck and Co. (Rahway, NJ, USA). TNF-α immunoassay kit was obtained from CBL, Central Laboratory of The Netherlands Red Cross (The Netherlands). Gel shift assay core system and all the reagents for NF-κB EMSA were from Promega. Trans-AM™ NF-κB p65 and NF-κB p50 Transcription Factor Assay kits were from Active Motif Europe, and Phospho-IκBα-ActivELISA™ kit was from Alexis.

3. Results

3.1. TNF-α release from human monocytes

Isolated adherent monocytes released TNF-α spontaneously or upon challenge with LPS or PMA. Basal TNF-α release in monocytes from healthy donors was low yet detectable (7.6±3 pg/ml; $n=10$). When challenged with stimuli at concentrations affording maximal effects, monocytes released 1039±160 pg/ml TNF-α ($n=10$; 10^6 monocytes/plate) after 10^{-7} M PMA, and 1367±178 pg/ml ($n=10$; not significant vs. PMA) after 10 ng/ml LPS. TNF-α release was unaffected by ethanol or DMSO, the solvents in which anti-inflammatory drugs were dissolved: a slight, but insignificant, increase was observed only at the highest (0.1% vol/vol) DMSO concentration (data not shown).

3.2. Effects of anti-inflammatory drugs on TNF-α release

In the concentration range 10^{-11}–10^{-5} M, dexamethasone inhibited TNF-α release from monocytes, the maximal effects being observed at 1–10 μM and the IC_{50} values being 3.8 nM and 1.2 nM following PMA or LPS challenge, respectively ($n=6$; Fig. 1A and B).

At intermediate concentrations (10^{-10}–10^{-7} M), the inhibition afforded by dexamethasone was higher ($P<0.05$) in LPS-challenged monocytes as compared to PMA-stimulated ones (Fig. 1). The nonselective cyclo-

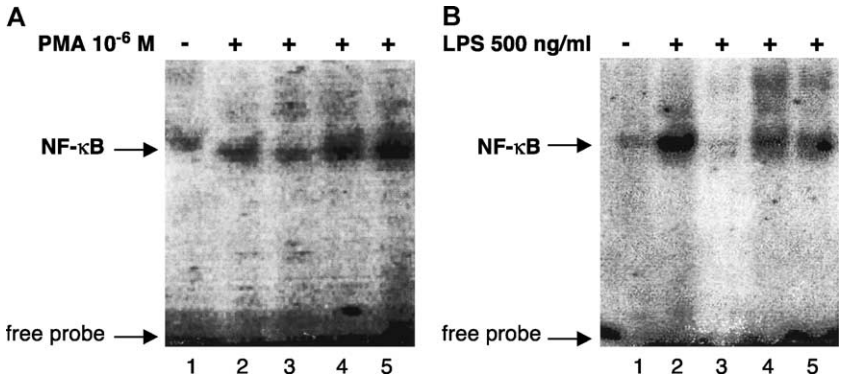

Fig. 2. PMA and LPS induce NF-κB activation in human monocyte in a time-dependent manner. Human monocytes were stimulated by 10^{-6} M PMA (A) or 500 ng/ml LPS (B) for different periods; then nuclear extracts (5 μg) were prepared and assayed for NF-κB activity by EMSA (see Materials and methods for further details). (A) PMA-induced NF-κB nuclear translocation. Unstimulated cells (lane 1) and PMA-stimulated cells (lanes 2–5); monocytes were challenged with PMA for 0.5 h (lane 2), 1 h (lane 3), 2 h (lane 4), or 4 h (lane 5). (B) LPS-induced NF-κB nuclear translocation. Unstimulated cells (lane 1) and LPS-stimulated cells (lanes 2–5); monocytes were challenged with 500 ng/ml LPS for 1 h (lane 2), 2 h (lane 3), 6 h (lane 4), or 8 h (lane 5).

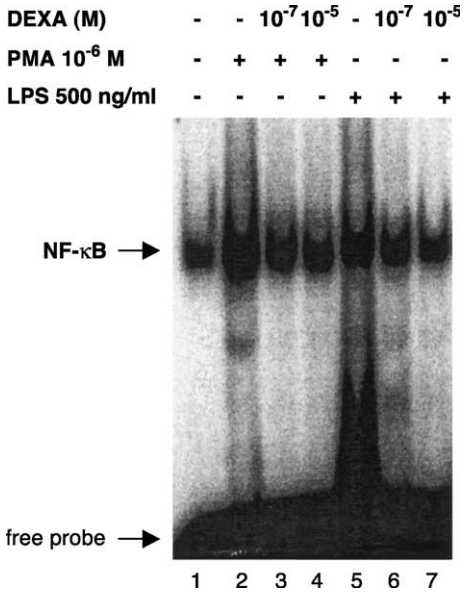

Fig. 3. Effects of dexamethasone on NF-κB activity in human monocytes. Cells were incubated in the absence (lanes 1, 2, and 5) or presence of dexamethasone, 10^{-7} M (lanes 3 and 6) or 10^{-5} M (lanes 4 and 7), for 24 h and then stimulated by 10^{-6} M PMA (lanes 2– 4) for 4 h or 500 ng/ml LPS (lanes 5–7) for 1 h. Nuclear extracts (5 μg) were subjected to EMSA as described (see text for further details). A representative blot of three independent experiments is shown.

oxygenase inhibitor, indomethacin, inhibited PMA-evoked TNF-α release in a concentration-dependent (10^{-7}–10^{-4} M) manner, the maximal effect being 78±3% inhibition (n=5; IC_{50}=4.6 μM), but it did not affect LPS-evoked TNF-α release (Fig. 1A and B). Unlike the two other drugs, the COX-2-selective inhibitor rofecoxib (10^{-7}–10^{-5} M) had no effect on either LPS- or PMA-induced TNF-α release from monocytes (Fig. 1).

3.3. LPS- and PMA-induced NF-κB activation

In unstimulated control monocytes, DNA binding of NF-κB was minimal, although detectable, whereas treatment with PMA or LPS considerably increased the DNA binding activity. The specificity of the NF-κB DNA binding was confirmed by the reversal of the binding by a 100-fold molar excess of unlabeled probe (data not shown). Nuclear translocation of the transcription factor was maximal (the intensity of the complex was threefold above the basal level) when monocytes were stimulated by PMA 10^{-6} M for 4 h, with some activation being present also at shorter time points (0.5–4 h; Fig. 2A). LPS enhanced NF-κB nuclear migration in a time-dependent manner, with maximal activation when monocytes were challenged with 500 ng/ml LPS for 1 h (Fig. 2B). LPS-induced nuclear translocation was negligible at 2 h, but present again when monocytes were stimulated for longer periods (6–8 h; Fig. 2B). Therefore, in the experiments aimed to evaluate the effects of anti-inflammatory drugs on NF-κB activation, monocytes were challenged with LPS for 1 h or PMA for 4 h.

3.4. Effects of anti-inflammatory drugs on NF-κB activation

We first assessed the effects of anti-inflammatory drugs on the DNA binding activity of NF-κB by EMSA. None of the drugs used here affected per se DNA binding activity (not shown). Dexamethasone, evaluated at two different concentrations of 10^{-7} M (Fig. 3, lanes 3 and 6) and 10^{-5} M (Fig. 3, lanes 4 and 7), strongly inhibited NF-κB nuclear translocation and DNA binding activity in both LPS- and PMA-challenged monocytes: maximal inhibition was observed at 10^{-5} M dexamethasone and reached approximately 50% for both stimuli (Fig. 3).

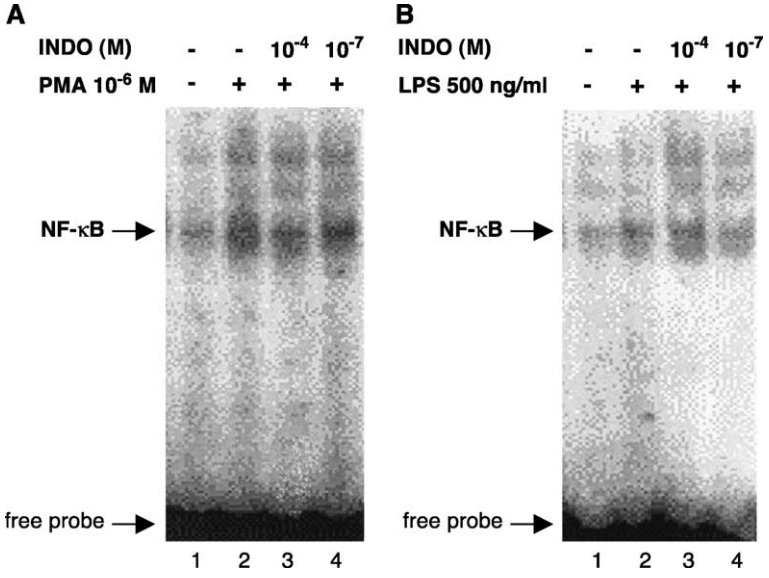

Fig. 4. Effects of indomethacin on NF-κB activity in human monocytes. Cells were incubated in the absence (lanes 1 and 2) or presence of indomethacin, 10^{-7} M (lane 4) or 10^{-4} M (lane 3), for 2 h and then stimulated by 10^{-6} M PMA (A; lanes 2–4) for 4 h or 500 ng/ml LPS (B; lanes 2–4) for 1 h. Nuclear extracts (5 μg) were subjected to EMSA as described (see text for further details). A representative blot of three independent experiments is shown.

As reported in Fig. 4, indomethacin did not affect LPS-induced NF-κB nuclear translocation (Fig. 4B), whilst causing minimal inhibition (about 15%, as evaluated by laser scanning densitometry) of PMA-induced translocation at the high 10^{-4} M concentration only (Fig. 4A, lane 4). The selective COX-2 inhibitor, rofecoxib, while devoid of effects on TNF-α production, inhibited NF-κB DNA binding activity in nuclear extracts (Fig. 5). At 10^{-5} M (Fig. 5A, lane 3), rofecoxib potently blocked PMA-induced NF-κB nuclear translocation (densitometric analysis revealed that the decrease was >60%) and exerted a 35% inhibition at 10^{-7} M (Fig. 5A, lane 4). When monocytes were stimulated by LPS, rofecoxib still inhibited NF-κB activation, although to a lesser extent (40% inhibition at 10^{-5} M and about 10% inhibition at 10^{-7} M) (Fig. 5B).

To ensure a better quantitative evaluation of the drugs' effect on NF-κB activation, we assessed the translocation of p50 and p65 subunits in nuclear extracts from PMA- or LPS-stimulated monocytes by using a commercially available ELISA kit. A significant increase (threefold to fivefold over unstimulated monocytes) in p65 and p50 content was detected in LPS- or PMA-challenged cells, with binding of p50 to DNA being higher in LPS-treated monocytes as compared to PMA-challenged cells (Table 1). Dexamethasone and rofecoxib inhibited the nuclear translocation of p50 and p65 subunits, in a concentration-dependent manner, in both PMA- or LPS-challenged monocytes, whereas indomethacin exerted a modest inhibition in PMA-stimulated monocytes only (Table 1), largely supporting the results obtained in EMSA assays. By subtracting the OD value of unstimulated control monocytes from all determinations, the inhibition afforded by dexamethasone was around 50% at 0.1 μM and about 80% at 10 μM in both PMA- or LPS-stimulated human monocytes (Table 1).

Table 1
Effect of anti-inflammatory drugs on the translocation of p50 and p65 subunits in PMA- or LPS-stimulated monocytes

Drug		% Inhibition			
		PMA (10^{-6} M)		LPS (500 ng/ml)	
		p50	p65	p50	p65
DEXA	10^{-7}	51±3	44±3	49±2	50±2
	10^{-5}	71±4	90±2	77±3	75±3
ROFE	10^{-7}	45±4	31±5	32±4	25±1
	10^{-5}	80±4	51±7	54±5	50±6
INDO	10^{-7}	13±4	7±4	7±5	12±7
	10^{-4}	25±2	35±4	7±5	18±8

Nuclear extracts were prepared as described in Materials and methods. The OD values of control monocytes (0.4±0.02 for p50; 0.5±0.03 for p65) were subtracted from all determinations. PMA (10^{-6} M) induces a threefold increase over control for p50 (OD=1.32±0.2) and p65 (OD=1.2±0.05). LPS (500 ng/ml) induces a fivefold increase over control for p50 (OD=1.8±0.1) and a threefold increase for p65 (OD=1.28±0.03). Values are mean±S.E.M. of four determinations.

Rofecoxib, too, dose-dependently inhibited p50 and p65 nuclear translocation and was particularly effective in inhibiting p50 subunit in PMA-challenged monocytes, whereas indomethacin had no effect in LPS-stimulated monocytes (Table 1). As previously demonstrated, activation of NF-κB requires phosphorylation by IKK of IκB proteins at specific protein residues (Ser 32 and Ser 36). We used a commercially available ELISA kit to measure the amount of phoshorylated IκBα in cytosolic extracts, and so indirectly evaluated whether or not anti-inflammatory drugs could affect PMA- or LPS-induced activation of IKK. In the experiments reported in Table 2, monocytes were stimulated in the absence or presence of anti-inflammatory drugs, with 500 ng/ml LPS or 10^{-6} M PMA, for 1 and 4 h, respectively, as for EMSA studies. These stimulation times were selected

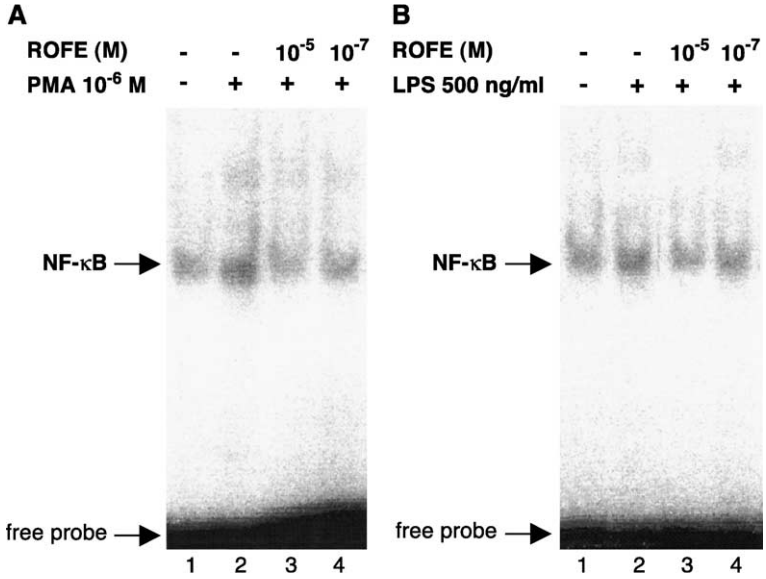

Fig. 5. Effects of rofecoxib on NF-κB activity in human monocytes. Cells were incubated in the absence (lanes 1 and 2) or presence of rofecoxib, 10^{-5} M (lane 3) or 10^{-7} M (lane 4), for 2 h and then stimulated by 10^{-6} M PMA (A; lanes 2–4) for 4 h or 500 ng/ml LPS (B; lanes 2–4) for 1 h. Nuclear extracts (5 μg) were subjected to EMSA as described (see text for further details). A representative blot of three independent experiments is shown.

Table 2
Effect of anti-inflammatory drugs on cytosolic phosphorylation IκBa in PMA- or LPS-stimulated monocytes

Drug		% Inhibition	
		PMA (10^{-6} M)	LPS (500 ng/ml)
DEXA	10^{-7}	34±8	31±6
	10^{-5}	62±4	56±6
ROFE	10^{-7}	21±9	28±4
	10^{-5}	55±7	71±10
INDO	10^{-7}	4±2	0
	10^{-4}	31±2	0

Cytosolic extract (100 μg) was prepared as described in Materials and methods. Values are mean±S.E.M. of four determinations (see Materials and methods for further details).

to give a snapshot of the moment in which DNA binding activity of NF-κB is maximal. In our hands, 10^{-6} M PMA induced the phoshorylation of IκBα (1092±220 RLU—relative chemiluminescence units; mean±S.E.M; n=4), a threefold increase over control unstimulated monocytes (323±72 RLU) being observed after 4 h and a fourfold increase after 1-h challenge (not shown). The amount of cytosolic phosphorylated IκBα was more than doubled after a 1-h challenge with 500 ng/ml LPS (806±180 RLU; mean±S.E.M; n=4) and fourfold higher when monocytes where challenged with LPS for 30 min. Dexamethasone and rofecoxib reduced LPS- or PMA-induced IKK activity, as shown by the reduced amount of phosphorylated IκBα in the cytosolic fractions, whereas indomethacin exerted some inhibition at the highest 10^{-4} M concentration only on PMA-challenged monocytes (Table 2).

4. Discussion

In this study, representative anti-inflammatory drugs differed markedly in their ability to affect TNF-α release from PMA- or LPS-stimulated monocytes. Dexamethasone, which was selected as an example of glucocorticoid drug and also as a positive control, due to its well-known ability to affect cytokine production in various cell types (Almawi and Melemedjian, 2002), potently inhibited TNF-α release from human monocytes challenged with both stimuli, thus confirming different previous observations (Adcock et al., 1995; Steer et al., 2000). The nonselective cyclooxygenase inhibitor indomethacin inhibited PMA-evoked TNF-α release, but failed to affect the LPS-induced one, whereas the selective COX-2 inhibitor, rofecoxib, was devoid of effect on TNF-α release from monocytes. Inhibition of cytokine release does not represent a common feature of nonsteroidal anti-inflammatory drugs and is regarded as an "extra" effect independent of cyclooxygenase inhibition. In this regard, indomethacin, ibuprofen, and fenoprofen have been described to possess PPAR-γ agonistic activity at high concentrations and, for this reason, to block the production of inflammatory cytokines (Jiang et al., 1998; Lehmann et al., 1997). Moreover, in human monocytes, LPS-induced cytokine secretion is largely refractory to the effects of PPAR-γ agonists, whereas PMA- and okadaic acid-induced cytokine release is susceptible to their action (Jiang et al., 1998). Although TNF-α production is largely regulated by the transcription factor NF-κB, evidence exists that TNF-α and other cytokines can also be induced through NF-κB-independent pathways. For example, LPS-induced TNFα production in human macrophages is largely controlled by NF-κB, whereas the zymosan-induced production is largely NF-κB-independent (Bondeson et al., 1999). For the PMA-induced activation of monocytic cells, the results are controversial: some studies indicate that NF-κB is involved (Bondeson et al., 1999; Kaufman et al., 1992) and others do not (Makarov et al., 1997; Tran-Thi et al., 1995). In THP-1 cells modified through stable retroviral gene transfer of IκB, PMA-induced cytokine release was independent of NF-κB activation (Makarov et al., 1997). Thus, the ability of a single gene to be controlled by several signal transduction pathways implies a level of complexity that allows its varied expression, depending either on the nature, duration, and intensity of the external stimuli and/or the particular cell type. Interestingly, a recent paper indicated the existence of two distinct pathways of LPS-induced NF-κB activation and cytokine production in human cells, with regulation being cell type-specific (Andreakos et al., 2004). In fact, myeloid differentiation protein 88 (MyD88), MYD88 adaptor-like/TIR domain-containing adaptor protein (Mal/TIRAP), and IKK2 were essential for LPS-induced IκBα phosphorylation, NF-κB activation, and cytokine production in human synovial fibroblasts and endothelial cells; on the contrary, neither MyD88, Mal/TIRAP, nor IKK2 was required for NF-κB activation or TNF-α secretion in human macrophages (Andreakos et al., 2004). So, the cytokine secretion by LPS- or PMA-stimulated monocytes could be regulated, cooperatively or not, by different signalling pathways [e.g., extracellular signal-regulated kinase (ERK)-1, ERK-2, p38 mitogen-activated protein (MAP) kinase, MAP kinase kinase (MEK), c-jun N-terminal kinase (JNK), and phosphatidylinositol 3 kinase] independently of NF-κB activation. This was the case for GM-CSF production in LPS-challenged human monocytes, which was dependent on both p38 MAP kinase and MEK, but not on NF-κB (Meja et al., 2000).

In our experiments, LPS and PMA were both able to activate NF-κB and to induce TNF-α secretion from monocytes (a more detailed evaluation of TNF-α/NF-κB mutual interactions being beyond the scope of this article).

Consistent with previous reports in which quiescent cells of the monocyte lineage were used (Frankenberger et al., 1994; Meja et al., 2000), a constitutive expression of NF-κB in the nucleus of unstimulated monocytes was always observed, probably due to the adhesion procedure. As previously demonstrated in a number of cells, nuclear NF-κB, besides inducing the transcription of different pro-inflammatory genes, also causes the transcriptional activation of the IκBα gene and a rapid reaccumulation of cytoplasmatic IκBα (Baldwin, 1996; Ghosh et al., 1998). By

using cDNA microarray analysis in a murine macrophage cell line, Nemeth et al. (2003) recently reported that stimulation with LPS induced a sevenfold induction of IκBα gene and a threefold induction of NF-κB1 gene after 3 h. Therefore, at a given time point, NF-κB can simultaneously affect the transcription of pro-inflammatory and anti-inflammatory genes. To gain some insights into the inhibitory effects of anti-inflammatory drugs on TNF-α release, we investigated NF-κB activity by EMSA (as well as p50, p65/RelA, and phospho-IκBα assays) using nuclear (cytosolic for the phospho-IκBα assay) extracts from PMA- or LPS-stimulated monocytes. Cells were stimulated by 500 ng/ml LPS for 1 h or 10^{-6} M PMA for 4 h, which is the moment in which DNA binding activity of NF-κB is maximal (Fig. 2).

As expected, dexamethasone dose-dependently inhibited, with a maximum of about 50% at 10^{-5} M, NF-κB nuclear translocation in PMA- or LPS-challenged human monocytes. This observation is in good agreement with previous reports, indicating the steroid's ability to decrease by 40–50% NF-κB and AP-1 binding in PMA-stimulated monocytes and to suppress transcription of TNF-α in LPS-stimulated THP-1 cells (Adcock et al., 1995; Steer et al., 2000). Moreover, the suppression of NF-κB activity was observed at the concentration range required for dexamethasone inhibition of TNF-α production. The fact that anti-inflammatory and immunosuppressive actions of glucocorticoids mostly depend on inhibition of cytokine expression and/or cytokine release is widely accepted, as well as the appraisal that these effects are mainly achieved by antagonizing the activity of transcription factors, through direct and indirect molecular mechanisms (e.g., protein–protein interactions between the transcription factor and the activated glucocorticoid receptors or up-regulation of IκB) (see De Bosscher et al., 2003 for a review). The capacity of glucocorticoid receptor to associate with the transactivating domain of p65/RelA subunit of NF-κB (De Bosscher et al., 2000) and the fact that dexamethasone predominantly inhibited (at 50 μM, a concentration higher than the ones here used) p65 subunit expression in rat striatal neurones (Simpson and Morris, 1999) prompted us to evaluate if it could be the case in our system, too. Analysis of p65 content in nuclear extracts (as evaluated by ELISA kits) in PMA- or LPS-stimulated monocytes indicated that dexamethasone potently and dose-dependently inhibited the translocation of this NF-κB subunit, with maximal inhibition being achieved at 10 μM and reaching about 80%, with no significant variation between stimuli. However, p50 translocation was equally inhibited, so we can exclude a dexamethasone-selective effect on p65 in human monocytes. Dexamethasone also inhibited IKK kinase activity (as evaluated by measuring the amount of phosphorylated IκBα in cytosol) in both PMA- or LPS-stimulated monocytes. Therefore, we provided further experimental evidence that, in human monocytes, inhibition of p65/RelA and p50 binding to DNA, as well as inhibition of IKK, represent chief molecular mechanisms for the anti-inflammatory actions of dexamethasone.

Cyclooxygenase-independent actions of nonsteroidal anti-inflammatory drugs have gained a considerable attention and some of them (e.g., indomethacin, fenoprofen, and ibuprofen) have been claimed to activate PPARs (Jiang et al., 1998; Oates et al., 2002). In human monocytes and other cell types, activation of PPAR-γ receptors has been repetitively demonstrated to inhibit the production of inflammatory mediators (including TNF-α, COX-2, nitric oxide, interleukin-1, interleukin-2, and interleukin-6), with transrepression of the transcription factors NF-κB and AP-1 being involved (Oates et al., 2002).

In this study, indomethacin had no effect on LPS stimulation, but it dose-dependently inhibited TNF-α release in PMA-stimulated human monocytes, in keeping with observations by Jiang et al. (1998). Maximal inhibition was approximately 80% and the IC_{50} value (4.6 μM) measured in this condition could be of clinical relevance, as a high-dose therapy is expected to produce an indomethacin plasma concentration of about 10 μM (Jiang et al., 1998). In our hands, indomethacin failed to inhibit LPS-evoked NF-κB activation, thus confirming previous results in murine macrophages (Callejas et al., 2003), but exerted a modest inhibition on PMA-evoked one, in both EMSA and p50/p65 assays, but only at the high supratherapeutic (100 μM) concentration. Accordingly, at this high concentration, indomethacin also reduced the amount of phosphorylated IκBα induced by PMA in cytosolic extracts. Furthermore, indomethacin effects are significantly smaller than those afforded by refecoxib or dexamethasone. In other cellular models, indomethacin did not modify NF-κB activity (Tegeder et al., 2001; Yamamoto et al., 1999), but the experimental conditions (cell type, indomethacin concentrations, stimuli, etc.) varied largely from the ones we used.

In contrast to indomethacin, the selective COX-2 inhibitor, rofecoxib, inhibited PMA- or LPS-induced NF-κB activation in human monocytes, but had no effect on PMA- or LPS-induced release of TNF-α. Similar results have been recently published by others (Niederberger et al., 2003; Callejas et al., 2003). In the rat, rofecoxib administration at doses of 1, 10, and 50 mg/kg has been shown to inconsistently affect zymosan-induced TNF-α expression in lumbar spinal cord, with some reduction being observed at the intermediate 10 mg/kg dose only (Niederberger et al., 2003). In elicited peritoneal murine macrophage, the release of TNF-α by LPS-stimulated macrophages was inhibited by 25% after treatment with 10 μM rofecoxib (Callejas et al., 2003). However, the experimental conditions used (i.e., LPS concentration; 200 ng/ml, 20-fold higher than the 10 ng/ml we used), time of stimulation (8 h, instead of the 24 h we used), and the cell type (a mouse-activated inflammatory phagocyte) are quite different from our experimental conditions. Previous data indicate that LPS promotes the phosphorylation of ERK-1, ERK-2, p38 MAP kinase, and JNK, and activates the transcription factor NF-κB in human

monocytes (Meja et al., 2000); however, the extent to which any of these signalling molecules contribute to the generation of TNF-α is not fully elucidated.

Callejas et al. (2003) reported that the phosphorylation of p44 ERK, p38 MAP kinase, and p46 JNK, which are rapidly and transiently activated in response to LPS, was completely blocked in murine macrophages treated with 10 μM rofecoxib. At 2 μM, rofecoxib potently inhibited (about 80%) p46 JNK phosphorylation, but exerted a 30% reduction only vs. p44 ERK (Callejas et al., 2003).

The mechanisms by which rofecoxib affects NF-κB activation in human monocytes are currently under investigation. Results here reported indicate that at 10 μM, a concentration sufficient to completely block COX-2 (Chan et al., 1999), rofecoxib inhibited more than 60% NF-κB activity in PMA-stimulated monocytes, as evaluated by EMSA, and was just active at 0.1 μM. Interestingly, a peak plasma concentration around 10 μM has been reported in human volunteers given a high rofecoxib dose (375 mg), whereas a 3-μM plasma concentration has been achieved after the administration of 100 mg of rofecoxib (Depré et al., 2000). In the EMSA assays here reported, rofecoxib dose-dependently reduced, although to a lesser extent, LPS-induced NF-κB activity. To our knowledge, this is the first demonstration of such an effect for rofecoxib on human monocytes and represents a somewhat expected result, since COX-2 activity is largely regulated by the transcription factor NF-κB. Similar results have been obtained in mouse peritoneal macrophages and RAW 264.7 cells, a murine macrophage cell line (Callejas et al., 2003; Niederberger et al., 2003).

In RAW 264.7 macrophages, rofecoxib inhibited the DNA binding capacity of NF-κB at 10–100 μM, whereas the binding activity of the transcription factor AP-1 was significantly enhanced at the 100 μM in vitro concentration (Niederberger et al., 2003). In elicited peritoneal macrophages, 10 μM rofecoxib reduced the LPS-dependent COX-2 and iNOS expression, as well as prostaglandin E_2 and nitric oxide release, and also delayed the LPS-induced NF-κB activation (Callejas et al., 2003). The COX-2 inhibitor impaired IκBα phosphorylation and significantly inhibited IKK activity, thus suggesting IKK as a direct target of rofecoxib (Callejas et al., 2003). Our results are in keeping with and further extend these observations by indicating the PMA-induced NF-κB activity as another target for rofecoxib anti-inflammatory effects. By measuring the nuclear amounts of p50 and p65 subunits, which represent the main NF-κB complexes present in the nucleus of monocyte/macrophages in response to stimulation by LPS and inflammatory cytokines (Ghosh et al., 1998), we found that rofecoxib dose-dependently inhibited them. Our observation that, in cytosolic extracts, rofecoxib strongly inhibits the phosphorylation of IκBα evoked by LPS or PMA indirectly confirms the results by Callejas et al. (2003) and further indicates IKK inhibition as an important mechanism for rofecoxib-mediated anti-inflammatory effects. Experiments are ongoing to further elucidate this point, as well as the role, if any, of signal transduction inhibitors.

In conclusion, this study demonstrates that anti-inflammatory drugs differ largely in their ability to inhibit NF-κB activity and/or TNF-α release from monocytes. Inhibition of TNF-α release from monocytes could represent a relevant adjunctive feature for an anti-inflammatory drug to be clinically effective in rheumatoid arthritis, due to the role played by TNF-α in the "cytokine cascade" in inflammatory sites and in the activation of transcription factors, including NF-κB.

Acknowledgements

This study was supported by grants MURST 60% 2002 and 2003 from the Ministry of University and Research of Italy. We wish to thank Dr. L. Koch from Merck and Co. (USA) for the kind gift of rofecoxib.

References

Adcock, I.M., Brown, C.R., Gelder, C.M., Shirasaki, H., Peters, M.J., Barnes, P.J., 1995. Effects of glucocorticoids on transcription factor activation in human peripheral blood mononuclear cells. Am. J. Physiol. 268, C331–C338.

Almawi, W.Y., Melemedjian, O.K., 2002. Molecular mechanisms of glucocorticoid antiproliferative effects: antagonism of transcription factor activity by glucocorticoid receptor. J. Leukoc. Biol. 71, 9–15.

American College of Rheumatology Subcommittee on Rheumatoid Arthritis Guidelines, 2002. Guidelines for the management of rheumatoid arthritis. 2002 update. Arthritis Rheum. 46, 328–346.

Andreakos, E., Sacre, S.M., Smith, C., Lundberg, A., Kiriakidis, S., Stonehouse, T., Monaco, C., Feldmann, M., Foxwell, B.M., 2004. Distinct pathways of LPS-induced NF-κB activation and cytokine production in human myeloid and nonmyeloid cells defined by selective utilization of MyD88 and Mal/TIRAP. Blood 103, 2229–2237.

Baldwin, A.S., 1996. The NF-κB and IκB proteins: new discoveries and insights. Annu. Rev. Immunol. 14, 649–681.

Bondeson, J., Browne, K.A., Brennan, F.M., Foxwell, B.M., Feldmann, M., 1999. Selective regulation of cytokine induction by adenoviral gene transfer of IκBα into human macrophages: lipopolysaccharide-induced, but not zymosan-induced, proinflammatory cyokines are inhibited, but IL-10 is Nuclear Factor-κB independent. J. Immunol. 162, 2939–2945.

Brunelleschi, S., Penengo, L., Lavagno, L., Santoro, C., Col angelo, D., Viano, I., Gaudino, G., 2001. Macrophage Stimulating Protein (MSP) evokes superoxide anion production by human macrophages of different origin. Br. J. Pharmacol. 134, 1285–1295.

Callejas, N.A., Fernandez-Martinez, A., Castrillo, A., Bosca, L., Martin-Sanz, P., 2003. Selective inhibitors of cyclooxygenase-2 delay the activation of Nuclear Factor-κB and attenuate the expression of inflammatory genes in murine macrophages treated with lipopolysaccharide. Mol. Pharmacol. 63, 671–677.

Chan, C.C., Boyce, S., Brideau, C., Charleson, S., et al., 1999. Rofecoxib [Vioxx, MK-0966; 4-(4′ -methylsulphonylphenyl)-3-phenyl-2-(5*H*)-furanone]: a potent and orally active cycloxygenase-2 inhibitor. J. Pharmacol. Exp. Ther. 290, 551–560.

Choy, E.H.S., Panayi, G.S., 2001. Cytokine pathways and joint inflammation in rheumatoid arthritis. N. Engl. J. Med. 344, 907–916.

De Bosscher, K., van den Berghe, W., Vermeulen, L., Plaisance, S., Boone, E., Haegeman, G., 2000. Glucocorticoids repress NF-κB-driven genes

by disturbing the interaction of p65 with the basal transcription machinery, irrespective of coactivator levels in the cell. Proc. Natl. Acad. Sci. U. S. A. 97, 3919–3924.

De Bosscher, K., Vanden Berghe, W., Haegeman, G., 2003. The interplay between the glucocorticoid receptor and nuclear factor-kappaB or activator protein-1: molecular mechanisms for gene repression. Endocr. Rev. 24, 488–522.

Depré, M., Ehrilch, E., Van Hecken, A., De Lepeliere, I., Dallob, A., Wong, P., Porras, A., Gertz, B.J., De Schepper, P.J., 2000. Pharmacokinetics, COX-2 specificity, and tolerability of supratherapeutic doses of rofecoxib in humans. Eur. J. Pharmacol. 56, 167–174.

Feldmann, M., Brennan, F.M., Maini, R.N., 1996. Rheumatoid arthritis. Cell 85, 307–310.

Frankenberger, M., Pforte, A., Sternsdorf, T., Passlick, B., Bauerle, P.A., Ziegler-Heitbrock, H.W., 1994. Constitutive nuclear NF-κB in cells of the monocyte lineage. Biochem. J. 304, 87–94.

Ghosh, S., May, M.J., Kopp, E.B., 1998. NF-κB and Rel proteins: evolutionary conserved mediators of immune responses. Annu. Rev. Immunol. 16, 225–260.

Han, Z.N., Boyle, D.I., Manning, A.M., Firestein, G.S., 1998. AP-1 and NF-kappa B regulation in rheumatoid arthritis and murine collagen-induced arthritis. Autoimmunity 28, 197–208.

Jiang, C., Ting, A.T., Seed, B., 1998. PPAR-γ agonists inhibit production of monocyte inflammatory cytokines. Nature 391, 82–86.

Kaufman, P.A., Weinberg, J.B., Geene, W.C., 1992. Nuclear expression of the 50- and 65-kD Rel-related subunits of nuclear factor-κB is differentially regulated in human monocytic cells. J. Clin. Invest. 90, 121–129.

Lehmann, J.M., Lenhard, J.M., Oliver, B.B., Ringold, G.M., Kliewer, S.A., 1997. Peroxisome proliferator-activated receptors α and γ are activated by indomethacin and other non-steroidal anti-inflammatory drugs. J. Biol. Chem. 272, 3406–3410.

Makarov, S.S., Johnston, W.N., Olsen, J.C., Watson, J.M., Mondal, K., Rinehart, C., Haskill, J.S., 1997. NF-κB as a target for anti-inflammatory gene therapy: suppression of inflammatory responses in monocytic and stromal cells by stable gene transfer of IκBα cDNA. Gene Ther. 4, 846–852.

Meja, K.K., Seldon, P.M., Nasuhra, Y., Ito, K., Barnes, P.J., Lindsay, M.A., Giembycz, M.A., 2000. p38 MAP kinase and MKK-1 co-operate in the generation of GM-CSF from LPS-stimulated human monocytes by an NF-κB-independent mechanism. Br. J. Pharmacol. 131, 1143–1153.

Nemeth, Z.H., Leibovich, J., Deitch, E.A., Vizi, S., Szabo, C., Hasko, G., 2003. cDNA microarray analysis reveals a Nuclear Factor-κB-independent regulation of macrophage function by adenosine. J. Pharmacol. Exp. Ther. 306, 1042–1049.

Niederberger, E., Tegeder, I., Schaefer, C., Seegel, M., Groesch, S., Geisslinger, G., 2003. Opposite effects of rofecoxib on Nuclear Factor-κB and Activating Protein-1 activation. J. Pharmacol. Exp. Ther. 304, 1153–1160.

Oates, J.C., Reilly, C.M., Crosby, M.B., Gilkeson, G.S., 2002. Peroxisome proliferator-activated receptor γ agonists. Potential use for treating chronic inflammatory diseases. Arthritis Rheum. 46, 598–605.

Simpson, C.S., Morris, B.J., 1999. Activation of nuclear factor-κB by nitric oxide in rat striatal neurones: differential inhibition of the p50 and p65 subunits by dexamethasone. J. Neurochem. 73, 353–361.

Steer, J.H., Kroeger, K.M., Abraham, L.J., Joyce, D.A., 2000. Glucocorticoids suppress tumor necrosis factor-α expression by human monocytic THP-1 cells by suppressing transactivation through adjacent NF-κB and c-Jun-Activating Transcription Factor-2 binding sites in the promoter. J. Biol. Chem. 275, 18432–18440.

Tak, P.P., Firestein, G.S., 2001. NF-κB: a key role in inflammatory diseases. J. Clin. Invest. 107, 7–11.

Tegeder, I., Pfeilschifter, J., Geisslinger, G., 2001. Cyclooxygenase-independent actions of cyclooxygenase inhibitors. FASEB J. 15, 2057–2072.

Tran-Thi, T.A., Decker, K., Bauerle, P.A., 1995. Differential activation of transcription factors NF-κB and AP-1 in rat liver macrophages. Hepatology 22, 613–619.

Van der Goes, A., Hoekstra, K., van den Berg, T.K., Dijkstra, C.D., 2000. Dexamethasone promotes phagocytosis and bacterial killing by human monocyte/macrophages in vitro. J. Leukoc. Biol. 67, 801–807.

Yamamoto, Y., Yin, M.J., Lin, K.M., Gaynor, R.B., 1999. Sulindac inhibits activation of the NF-κB pathway. J. Biol. Chem. 274, 27307–27314.

Yin, M.J., Yamamoto, Y., Gaynor, R.B., 1998. The anti-inflammatory agents aspirin and salicylate inhibit the activity of IκB kinase-beta. Nature 396, 77–80.

British Journal of Pharmacology (2005) 145, 385–396
www.nature.com/bjp

Expression of functional NK_1 receptors in human alveolar macrophages: superoxide anion production, cytokine release and involvement of NF-κB pathway

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1 Substance P (SP) is deeply involved in lung pathophysiology and plays a key role in the modulation of inflammatory-immune processes. We previously demonstrated that SP activates guinea-pig alveolar macrophages (AMs) and human monocytes, but a careful examination of its effects on human AMs is still scarce.

2 This study was undertaken to establish the role of SP in human AM isolated from healthy smokers and non-smokers, by evaluating the presence of tachykinin NK_1 receptors (NK-1R) and SP's ability to induce superoxide anion (O_2^-) production and cytokine release, as well as activation of the nuclear factor-κB (NF-κB) pathway.

3 By Western blot analysis and immunofluorescence, we demonstrate that authentic NK-1R are present on human AMs, a three-fold enhanced expression being observed in healthy smokers. These NK-1R are functional, as SP and NK_1 agonists dose-dependently induce O_2^- production and cytokine release. In AMs from healthy smokers, SP evokes an enhanced respiratory burst and a significantly increased release of tumor necrosis factor-α as compared to healthy non-smokers, but has inconsistent effects on IL-10 release. The NK_1 selective antagonist CP 96,345 ((2*S*,3*S*)-*cis*-2-diphenylmethyl-*N*[(2-methoxyphenyl)-methyl]-1-azabicyclo-octan-3-amine)) competitively antagonized SP-induced effects.

4 SP activates the transcription factor NF-κB, a three-fold increased nuclear translocation being observed in AMs from healthy smokers. This effect is receptor-mediated, as it is reproduced by the NK_1 selective agonist [$Sar^9Met(O_2)^{11}$]SP and reverted by CP 96,345.

5 These results clearly indicate that human AMs possess functional NK-1R on their surface, which are upregulated in healthy smokers, providing new insights on the mechanisms involved in tobacco smoke toxicity.

British Journal of Pharmacology (2005) **145,** 385–396. doi:10.1038/sj.bjp.0706198
Published online 21 March 2005

Keywords: Substance P; human alveolar macrophages; NK_1 receptor; NF-κB activation; respiratory burst; cytokine release; TNF-α; IL-1β; IL-10

Abbreviations: AMs, alveolar macrophages; CP96,345, (2*S*,3*S*)-*cis*-2-diphenylmethyl-*N*[(2-methoxyphenyl)-methyl]-1-azabicyclo-octan-3-amine); GR82334, ([D-Pro^9(spiro-gamma-lactam)Leu^{10},Trp^{11}]physalaemin(1–11)); GR71251, ([Pro^9(s-piro-gamma-lactam) Leu^{10},Trp^{11}]SP); NF-κB, nuclear factor-κB; NK-1R, NK_1 receptor; O_2^-, superoxide anion; PMA, phorbol 12-myristate 13-acetate; SP, substance P

Introduction

The neuropeptide substance P (SP), a member of the tachykinin receptor family, is involved in many physiological processes, including nociception, vasodilation, exocrine and endocrine gland secretion, smooth muscle contraction, cell proliferation, and largely contributes to the local control of the immune responses (Severini *et al*., 2002). It induces lymphocyte proliferation (Payan *et al*., 1983), enhances immunoglobulin production by cloned B lymphoma cells (Pascual *et al*., 1991), degranulates rat mast cells (Mousli *et al*., 1989), modulates eosinophil and neutrophil activity (Brunelleschi *et al*., 1991; Iwamoto *et al*., 1993), stimulates human peripheral monocytes to produce inflammatory cytokines including IL-1, IL-6, IL-12 and tumor necrosis factor-α (TNF-α) (Lotz *et al*., 1988; Lavagno *et al*., 2001).

By using natural tachykinins and selective receptor agonists and antagonists, we previously demonstrated that guinea-pig alveolar macrophages (AMs) possess NK_1 receptors (NK-1R) and NK_2 receptors, their stimulation leading to superoxide anion (O_2^-) production and eicosanoid release, and that ovalbumin-sensitized AMs demonstrate an enhanced responsiveness to NK-2R stimulation (Brunelleschi *et al*., 1990; 1992). We also showed that SP, as well as neurokinin A (NKA)

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Published online 21 March 2005

and the selective NK_2 receptor agonist [β-Ala^8]-NKA(4–10), induces O_2^- production in AMs obtained from patients with active sarcoidosis (Brunelleschi *et al*., 1996).

The biological responses to SP are mediated by the G protein-coupled tachykinin NK-1R, although SP can also bind, with lower affinity, NK_2 and NK_3 tachykinin receptors (Severini *et al*., 2002; Pennefather *et al*., 2004). The presence of NK_1 receptors (NK-1R) on monocyte/macrophages has been demonstrated by evaluating the effects of selective receptor agonists and antagonists on functional parameters (for example, Brunelleschi *et al*., 1990; 1992; 1998) and/or by molecular biology and protein chemistry techniques. RT–PCR and *in situ* hybridization have been used to identify NK-1R mRNA expression in monocytes and macrophages (Ho *et al*., 1997; Germonpre *et al*., 1999), but little is known about NK_1 expression at the protein level. Marriott & Bost (2000) and Simeonidis *et al*. (2003) demonstrated the presence of NK-1R protein in murine peritoneal macrophages and THP-1 cells, respectively, but, to our knowledge, nobody has investigated this possibility in human AMs. Recent evidence indicates that NK-1R gene expression in THP-1 cells is increased after exposure to IL-1β and TNF-α: this effect is mediated by the transcription factor NF-κB, which binds to the promoter region of the NK-1R gene and so regulates its expression (Simeonidis *et al*., 2003).

In resting cells, nuclear factor-κB (NF-κB) is retained in the cytoplasm through an association with inhibitory proteins of the IκB family, which mask the nuclear localization signal (Baldwin, 1996). Upon stimulation, IκBα is phosphorylated, ubiquitinylated and degraded, thus allowing NF-κB to translocate to the nucleus. Once NF-κB enters the nucleus, it binds to the promoter region of various genes and induces their transcription (Baldwin, 1996). SP specifically activates NF-κB pathway in cells of the monocyte/macrophage lineage, for example, human astrocytoma cells, murine peritoneal macrophages and dendritic cells (Lieb *et al*., 1997; Marriott *et al*., 2000), but no information are available concerning human AMs.

The present study was undertaken to establish the role of tachykinin NK-1R in human AMs isolated from healthy smokers and non-smokers. We demonstrate the presence of authentic NK-1R, as determined by Western blot analysis and immunofluorescence, and indicate that the NK-1R expressed in human AM are functional, as demonstrated by the ability of SP to evoke O_2^- production and cytokine release. We also present direct evidence that SP activates the transcription factor NF-κB pathway, so providing new insights on the mechanisms involved in neuropeptidergic control of AM responsiveness.

Methods

Study population

This study and the research protocol were approved by the local Ethical Committee. A total of 25 individuals, 15 male and 10 female subjects, aged between 28 and 76 years, 13 smokers and 12 non-smokers, were studied. The characteristics and smoking history of the study population are presented in Table 1, Results section. None of the subjects received medical therapy at the time of the study. Broncho-alveolar lavage (BAL) was mainly performed for diagnostic purposes to have a further validation/confirmation of the suspected disease; healthy subjects were individuals who had no history of cardiopulmonary disease or other chronic disease, no diagnosed lung diseases and were not on medication. In a few cases, the attribution of a 'healthy' subject to the category was done after the BAL procedure.

Table 1 Study population

Subject	*Sex (F or M)*	*Age (years)*	*Smoker*	*Number of cigarette day^{-1}*	*Years on smoke*
1	F	51	Yes	20	25
2	M	54	Yes	12	20
3	F	43	No	—	—
4	M	63	Yes	15	40
5	M	50	No	—	—
6	F	28	Yes	10	10
7	M	43	Yes	30	18
8	M	50	No	—	—
9	M	46	No	—	—
10	F	29	No	—	—
11	M	37	Yes	20	15
12	F	35	No	—	—
13	M	69	No	—	—
14	F	56	Yes	25	32
15	M	48	No	—	—
16	M	62	Yes	16	34
17	F	70	No	—	—
18	F	45	Yes	20	15
19	M	39	No	—	—
20	M	45	Yes	35	20
21	M	55	No	—	—
22	F	76	No	—	—
23	F	33	Yes	12	8
24	M	67	Yes	10	30
25	M	58	Yes	12	33

Isolation of human AMs from BAL

AMs were isolated from BAL as described (Brunelleschi *et al*., 1996). After informed consent was obtained from each patient and pretreatment with parenteral atropine sulphate (0.5 mg), airways were anaesthetized with 2% lidocaine. 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 so 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 (35 mm diameter; Costar, U.K.). After 2 h at 37°C in humidified 5% CO_2 atmosphere, nonadherent cells (mainly lymphocytes) were gently removed and AMs 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.).

O_2^- production in AMs

Adherent AMs ($0.4–1 \times 10^6$ cells plate^{-1}) were washed twice with PBS, incubated in RPMI 1640 medium (without phenol red, no antibiotics and no FCS) and challenged with increasing concentrations of tachykinins for 30 min. SP is the major endogenous ligand for NK-1R, [Sar^{9}Met(O$_2$)^{11}]SP and Pro^{9}SP are selective NK$_1$ agonists. In the experiments with the NK-1R antagonists CP 96,345, GR82334 ([D-Pro^9(spiro-gamma-lactam)Leu^{10},Trp^{11}]physalaemin(1-11)) and GR71251 (([Pro^9(spiro-gamma-lactam) Leu^{10},Trp^{11}]SP)), AMs were pre-incubated for 15 min with these drugs and then challenged with tachykinins. The effects of tachykinins were compared with those evoked by phorbol 12-mirystate 13-acetate (PMA), a standard stimulus acting as a direct protein kinase C activator. The O_2^- production was evaluated by the superoxide dismutase (SOD)-inhibitable cytochrome *c* reduction, the absorbance changes being recorded at 550 nm in a Beckman DU 650 spectrophotometer. O_2^- production was expressed as nmol cytochrome *c* reduced/10^6 cells/30 min, using an extinction coefficient of 21.1 mM (Brunelleschi *et al*., 2001). To avoid interference with spectrophotometrical recordings of O_2^- production, AMs were incubated with RPMI 1640 without phenol red. Experiments were performed in duplicate or triplicate; control values (e.g., basal O_2^- production in the absence of stimuli) were subtracted from all determinations.

Release of TNF-α and other cytokines from AMs

Adherent AMs were challenged with the selected stimuli (SP, NK$_1$ selective agonists, PMA) for 24 h at 37°C to ensure maximal cytokine release. Supernatants were collected and stored at −20°C. TNF-α, IL-1β and 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 human 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 cells) were subtracted from all determinations. Results are expressed in pg ml^{-1}.

Immunofluorescence for NK-1R in AMs

Human AMs were cultured onto gelatin-coated glass slides. The cells were fixed in ice-cold 4% paraformaldahyde (20 min), washed twice with PBS, permeabilized with 0.5% Triton X-100 (15 min, 25°C), washed twice with PBS, and blocked with 10% FCS, 2% BSA, 1% glycine, 0.5% Triton X-100 in PBS (1 h, 25°C). The cells were then incubated in the presence of a rabbit antibody directed against the human NK-1R (Santa Cruz Biotechnology, U.S.A.) at a dilution of 1 : 120 in PBS overnight at 4°C. After washing, FITC-conjugated anti-rabbit immunoglobulins (1 : 30) (Dako Cytomation, Milan, Italy) were added (2 h, 25°C). After another washing with PBS, nuclear staining was performed using Hoechst 33258 (0.8 μg ml^{-1}, 1 h, 37°C) (Sigma-Aldrich, Milan, Italy). Fluorescence was visualized using 100-fold magnification.

Western blotting of the tachykinin NK-1R in AMs

Subconfluent AMs (10×10^6 cells) were washed twice with ice-cold PBS and lysed with 1 ml RIPA buffer (1% Triton X-100, 1% sodium deoxycolate, 0.1% SDS, 50 mM Hepes pH 7.4, 150 mM NaCl, 10% glycerol, 1.5 mM $MgCl_2$, 1 mM EGTA, 1 mM NaF) containing 1 mM Na_3VO_4 and protease inhibitors (10 μg ml^{-1} aprotinin, 10 μg ml^{-1} pepstatin, 50 μg ml^{-1} leupeptin, 1 mM phenylmethylsulphonyl fluoride-PMSF). Cells were placed on ice for 20 min and scraped. Cell lysates were sonicated on ice four times for 5 s each, cleared by centrifugation at $15{,}000 \times g$ for 10 min at 4°C and the supernatants (cell lysates) transferred into a new tube. If necessary, cell lysates were stored at −80°C. About 30 μg total extracts were separated on 8–10% SDS–PAGE and transferred to nitrocellulose filters (Protran, Perkin-Elmer Life Sciences, Boston, MA U.S.A.). Nonspecific binding sites on membrane were blocked at room temperature for 1 h in TBS-5% BSA nitrocellulose filters. Filters were probed with a commercial anti-human NK-1R antibody (NK-1R (H-83): sc-15323, Santa Cruz Biotechnology, U.S.A.; a rabbit polyclonal antibody mapping at the C-terminus of the human NK$_1$ receptor) (1 : 200 in TBS-5% BSA) for 2 h at room temperature. Proteins were visualized by using ECL Western blotting detection reagents (Perkin-Elmer Life Science, Boston, MA, U.S.A.). Quantification of Western blots was performed by densitometry using 'Quantity One, 1-D Analysis' software (Bio-Rad, U.S.A.) and expressed as intensity data units.

Evaluation of NF-κB activation

The activation of NF-κB induced by SP, NK$_1$ agonists or PMA 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 and Western blot). The methods we used are detailed below.

Preparation of nuclear and cytosolic cellular fractions

After challenge with stimuli, AMs (about 5×10^6 cells) were washed with ice-cold PBS, scraped and centrifuged at $1000 \times g$ for 5 min at 4°C. The cell pellet was resuspended in 300 μl of lysis buffer (10 mM Hepes, pH 7.6, 60 mM KCl, 1 mM EDTA, 1 mM PMSF, 1 mM dithiothreitol, 10 μl ml^{-1} protease cocktail inhibitors) and incubated on ice for 15 min. At the end of this incubation, 20 μl of 10% NP-40 was added and the tube vortexed for 10 s. After centrifugation at $13{,}000 \times g$ for 1 min at 4°C, supernatants (cytosolic fractions) were collected and stored at −80°C, whereas the pellets were further processed to obtain nuclear extracts. The pellets were resuspended in extraction buffer (20 mM Tris–HCl, pH 8, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.5 mM PMSF, 0.2 mM EDTA, 10 μl ml^{-1} protease cocktail inhibitors, glycerol 25% v v^{-1}) and incubated for 30 min at 4°C. Nuclear proteins were isolated by centrifugation at $13{,}000 \times g$ for 15 min. The supernatant was aliquoted and stored at −80°C until used for EMSA or p50/p60 ELISA assays. Protein concentrations were determined by using a protein assay (Bio-Rad, U.S.A.).

EMSA of NF-κB

Nuclear extracts (5 μg) were incubated with 2 μg poly (dI-dC) and the γ [^{32}P]ATP-labelled oligonucleotide probe (100,000–150,000 c.p.m.; Promega) in binding buffer (50% glycerol, 10 mM Tris–HCl, pH 7.6, 500 mM KCl, 10 mM EDTA, 1 mM dithiothreitol) in a final volume of 20 μl for 30 min at room temperature. The NF-κB consensus oligonucleotide (5′-AGTTGAGGGGACTTTCCCAGGC-3′) was obtained from Promega. The nucleotide–protein complex was separated on a 5% polyacrylamide gel in 0.5 × TBE buffer (100 mM Tris–HCl, 100 μM boric acid, 2 mM EDTA) at 150 V on ice. The gel was dried and radioactive bands were detected by autoradiography.

p50 and p65/RelA assays

Nuclear extracts were prepared as described above 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. Briefly, an equal amount (1 μg) of nuclear lysate was added to incubation wells precoated with an oligonucleotide containing the NF-κB consensus site (5′-GGGACTTTCC-3′) sequence, the active form of NF-κB contained in the cell extract specifically binding to this oligonucleotide. Sometimes, the cytosolic content of both subunits was also measured. 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). In some cases, the nuclear extracts (5 μg protein, for each sample) were also used to evaluate p50 and p65 subunits by Western blot. For these assays, commercial antibodies (anti-NF-κB p50: ab 7971 and anti-NF-κB p65: ab 7970) were obtained from Abcam (U.K.). Nuclear extracts were challenged for 2 h at room temperature with the antibody at a final concentration of 1 μg ml^{-1}.

Drugs and analytical reagents

Substance P, selective NK_1 agonists and NK_1 antagonists were obtained from Neo-System (Strasbourg, France). The anti-NK-1R-specific antibody (NK-1R (H-83): sc-15323) was from Santa Cruz Biotechnology (U.S.A.). The anti-NF-κB p50 and anti-NF-κB p65 antibodies were obtained from Abcam (U.K.). PBS, RPMI 1640 (with or without phenol red), BSA, glutamine, Hepes, streptomycin, penicillin, PMA, ethanol, SOD, cytochrome *c*, Na-deoxycholate, NaCl, EDTA, protease cocktail inhibitors (aprotinin 0.3 μM, bestatin 130 μM, leupeptin 1 μM), bromophenol blue, glycine, glycerol, methanol and Tween 20 were obtained from Sigma (Milwaukee, WI, U.S.A.). Poly(dI-dC) were obtained from Pharmacia (Uppsala, Sweden). Triton X-100 and β-mercaptoethanol were from Fluka (Buchs, Switzerland); PMSF was from Promega (Madison, WI, U.S.A.). SDS and DMSO were from Merck (Darmstadt, Germany). BCA Protein Assay Reagent kit was from Pierce (Rockford, IL, U.S.A.). Nitrocellulose filters (Hybond) and the enhanced chemiluminescence system were from Amersham (Buckinghamshire, U.K.). Tissue-culture plates were purchased from Costar Ltd (Buckinghamshire, U.K.). All cell culture reagents, with the exception of fetal bovine serum, were endotoxin-free according to details provided by the manufacturer. Fetal bovine serum (lot 40G3410 K, containing <10 EU ml^{-1}) was from Life Technologies Inc. (Rockville, U.S.A.). TNF-α, IL-1β and IL-10 immunoassay kit was obtained from CLB/Sanquin, Central Laboratory of the Netherlands Red Cross (Netherlands). Gel shift assay Core system and all the reagents for NF-κB EMSA were from Promega Corporation (St Louis, CA, U.S.A.).

Data and statistical analysis

Data are mean ± s.e.m. of duplicate determinations of '*n*' independent experiments. Concentration–response curves for SP and NK_1 agonists were constructed and EC_{50} values were interpolated from curves of best-fit. When required, statistical evaluation was performed by Student's *t* test.

Results

Study population, BAL and phenotype of AMs

In all, 25 individuals, 15 male and 10 female subjects (mean age = 50.2 ± 2.7 years; mean age of male and female subjects: 55 ± 3.7 and 44.5 ± 4.3 years, respectively, $P = 0.07$), were studied. A total of 13 (eight male and five female subjects) were smokers and 12 (seven male and five female subjects) were non-smokers; mean age of smokers (49.4 ± 3.3 years; $n = 13$) and non-smokers (50.8 ± 4 years, $n = 12$) being very similar. The characteristics and smoking history of the study population are listed in Table 1. None of the subjects received medical therapy at the time of the study. Total and differential cell counts in BAL and phenotype of AMs from smokers and non-smokers are presented in Table 2. As expected, a significant ($P < 0.05$) increase in the total cell number in BAL (with no significant differences in differential cell counts) was observed in smokers as compared to non-smokers. The great majority of AMs (96 ± 1%) in healthy smokers was CD68 + and a high percentage (86 ± 1 and 66 ± 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 receptor, whereas HLA-DR is related to antigen presentation. The expression CD14 and CD68 was significantly ($P < 0.05$) higher in AMs collected from healthy smokers as compared to healthy non-smokers (Table 2).

NK-1R expression in human AMs

We examined the expression pattern of the NK-1R gene products in human AMs. We collected AMs from healthy smokers and healthy non-smokers undergoing BAL for diagnostic procedures, after their informed consent. First, we performed immunofluorescence assays in AMs isolated from healthy non-smokers and observed the expression of the NK-1R protein localized primarily at the cell surface (Figure 1). A phase contrast photomicrograph of AM is shown in Figure 1a. Immunofluorescence with anti-NK-1R antibody and FITC-conjugated anti-rabbit antibody reveals a green colour (Figure 1b), nuclear staining with Hoechst 33258 (0.8 μg ml^{-1}, 30 min, 37°C) is depicted in blue (Figure 1c),

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

Subjects	*Total cell* ml^{-1} *BAL*	*AMS (%)*	*Lympho (%)*	*PMNs (%)*	*CD68+ (%)*	*HLA-DR+ (%)*	*CD14+ (%)*
Smokers ($n=13$)	390.000±6.000	90.8±1.9	8.2±2	1±0.5	96±1	86±1	66±3
Non-smokers ($n=12$)	139.000±5.100*	90.6±1	8.6±1	0.2±0.1	83±1*	84±2	50±2*

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

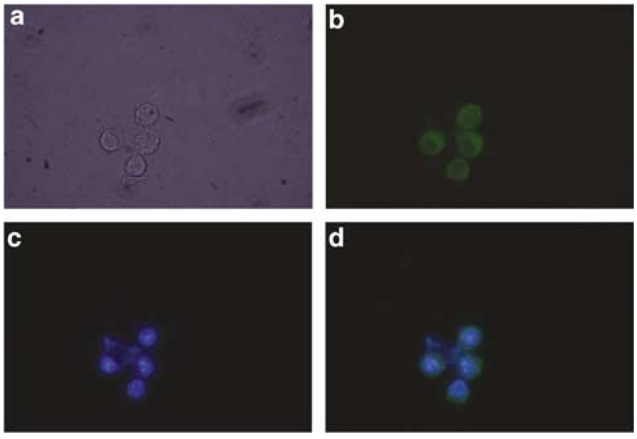

Figure 1 Immunocytochemical analysis of the expression and location of NK-1R in human AMs. (a) Phase contrast. (b) Immunofluorescence of anti-NK-1R polyclonal Ab followed by FITC-conjugated anti-rabbit immunoglubulins (green). (c) Nuclear staining with Hoechst 33258 (blue). (d) Merge of B and C. Fluorescence was visualized by a vertical fluorescence microscopy (100-fold magnification; Eclipse E600, Nikon, Tokyo, Japan).

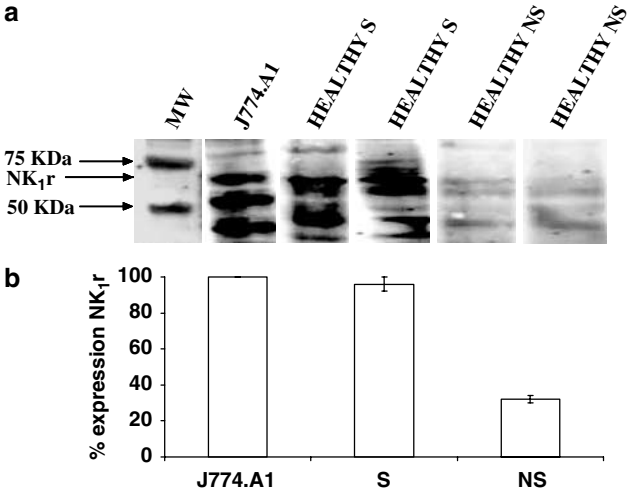

Figure 2 NK-1R expression in human AMs from healthy smokers (S) and non-smokers (NS). In (a), Western blot analysis of NK-1R in AM extracts of two smokers and two non-smokers. The blots are assembled from different single experiments in which AMs from S or NS have been evaluated. As a positive control for the presence of NK-1R, the macrophage cell line J774.A1 was used (for clarity, only one blot of J774.A1 cells, representative of seven others, is shown). The same protein amount (30 μg) was used in each experiment with AMs from smokers and non-smokers. An arrowhead indicates the 68-kDa band corresponding to the receptor. The migration of protein standards of known sizes is shown on the left. In (b), quantitative evaluation of NK-1R expression by densitometry. Intensity of the specific band of NK-1R in the macrophage J774.A1 cells amounted to 8710±250 intensity units (means±s.e.m. of eight experiments) and was taken as 100%. Results are expressed as % expression of the positive control; mean + s.e.m. of four experiments for S and NS.

and merge of both visualizes NK-1R on AM surface (Figure 1d).

We next performed Western blot in AMs isolated from healthy smokers and non-smokers to evaluate NK-1R expression at the protein level (Figure 2). AMs were obtained from four healthy smokers (subjects 1, 2, 14 and 20) and four healthy non-smokers (subjects 3, 5, 10 and 21); the experiments were performed separately (by using the same protein amount, 30 μg, for each individual assay) and were always compared with the NK-1R expression in the positive control, the cultured J774.A1 cells (a murine macrophage cell line). As shown in Figure 2a, Western blot analysis of J774.A1 cells, AMs from two healthy smokers (subjects 1 and 2) and two non-smokers (subjects 3 and 5), probed with the polyclonal anti-NK-1R antibody, reveals three prominent bands of 68, 53 and 42 kDa, respectively (Figure 2a). According to manufacturer's instructions, the commercial polyclonal antibody we used detected a protein of about 68 kDa. As known, NK-1R possess different sites for acetylation and phosphorylation and may be present as truncated forms (Li *et al*., 1997; Page & Bell, 2002; Caberlotto *et al*., 2003). In our experiments, we always observed a positive band at 68 kDa (as indicated by the manufacturer) and two bands of 53 and 42 kDa (Figure 2a). Such observations are in keeping with previous reports in which other authors, by using noncommercial monoclonal NK-1R antibodies, detected a 46 kDa protein in human antral tissue (Smith *et al*., 2000), a molecular mass band of 53 kDa in the monocyte/macrophage THP-1 cells (Simeonidis *et al*., 2003) or a 42 kDa protein in murine peritoneal macrophages and murine microglia (Marriott & Bost, 2000; Rasley *et al*., 2002).

Interestingly, densitometric evaluation of NK-1R expression revealed that AMs collected from healthy smokers demonstrated a >3-fold increase as compared to AMs isolated from healthy non-smokers (Figure 2b). The intensity of the specific band of 68 kDa in the positive control, the J774.A1 cell line, amounted to 8710±250 intensity units ($n=8$) and was taken as 100%. NK-1R expression in AMs from healthy non-smokers was 32±1.5% ($n=4$), whereas NK-1R expression in AMs from healthy smokers amounted to 96±4% ($n=4$).

NK-1R are functional in human AMs: O_2^- production and cytokine release

Basal values (O_2^- production from unstimulated AMs) in smokers and non-smokers were 13.5±2 ($n=6$) and 2.2±0.4

($n=5$; $P<0.01$) nmol cytochrome c reduced/10^6 AMs, respectively. These values were subtracted from those obtained after tachykinins or PMA challenge to obtain the net O_2^- production. PMA, used at 10^{-7} M (a near maximal concentration), produced 23.5 ± 2 ($n=6$) and 17 ± 0.6 ($n=5$; $P<0.05$) nmol cytochrome c reduced/10^6 AMs in smokers and non-smokers, respectively. In the concentration range 10^{-12}–10^{-6} M, SP dose-dependently evoked O_2^- production in AMs from both smokers and non-smokers, higher production being observed in smokers (Figure 3). As depicted in Figure 3, maximal activation by SP was observed at 10^{-6} M, EC_{50}s being 0.25 nM in smokers and 1 nM in non-smokers. In the presence of a cocktail of inhibitors (thiorphan, captopril and bestatin, all at 1 μM) of tachykinin degrading enzymes, SP effects were significantly enhanced (data not shown). The metabolically stable NK-1R agonist [Sar^{9}Met(O$_2$)^{11}]SP, although less potent than SP, evoked a significant respiratory burst in AMs collected from both smokers and non-smokers, EC_{50}s being about 3 nM in smokers and 10 nM in non-smokers (Figure 3). Pro^{9}SP, the other NK$_1$ selective agonist we used, acted dose-dependently, although less active and potent than SP or [Sar^{9}Met(O$_2$)^{11}]SP (Figure 3). EC_{50}s for Pro^{9}SP were about 10 nM in smokers and 30 nM in non-smokers (Figure 3). The nonpeptide NK$_1$ selective antagonist CP 96,345 at 1 nM competitively antagonized the effects of SP and NK$_1$ selective agonists: the dose–response curve for SP was shifted to the right about two orders of magnitude (Figure 4a) as were those for [Sar^{9}Met(O$_2$)^{11}]SP (about 1.5-fold; Figure 4b) and Pro^{9}SP (one order of magnitude; not shown). GR82334, a reversible NK$_1$ antagonist devoid of histamine-releasing properties in rat mast cells (Guo *et al*., 1995), and GR71251, a selective NK$_1$ antagonist that possesses GABA-releasing actions in rat spinal cord (Hagan *et al*., 1990), at 1 μM competitively antagonized the effects of SP and NK$_1$ selective agonists in AM (data not shown).

We also evaluated the release of proinflammatory cytokines, namely TNF-α (the most abundant cytokine in AMs) and IL-1β, as well as IL-10 release (the most relevant anti-inflammatory cytokine in AMs), after challenge with tachykinins or PMA. Basal values (that is the release from

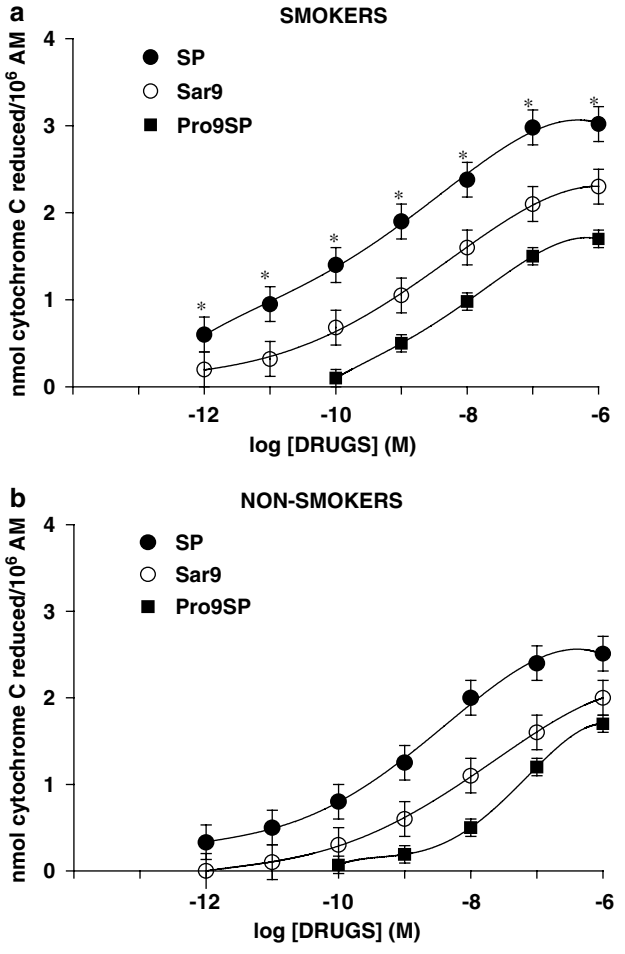

Figure 3 NK-1R agonists evoke O_2^- production in human AMs isolated from healthy smokers (a) and non-smokers (b). Cells were challenged with increasing concentrations of SP, [Sar^{9}Met(O$_2$)^{11}]SP and Pro^{9}SP for 30 min. Results are means$\pm$s.e.m. of five to six experiments in duplicate. *$P<0.05$ vs non-smokers.

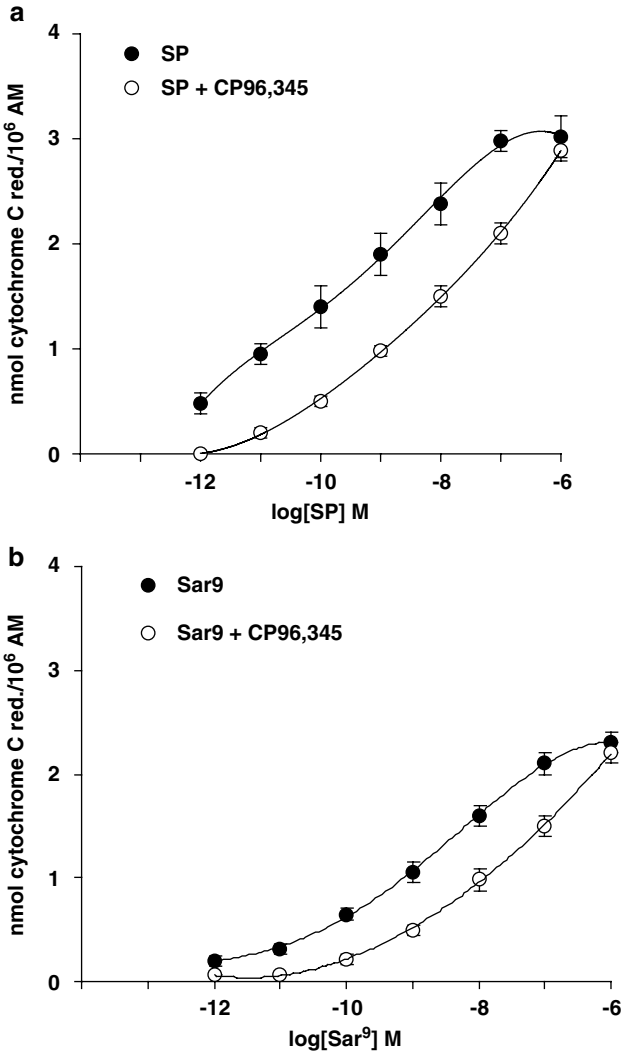

Figure 4 The NK$_1$ selective antagonist CP96,345 competitively antagonizes SP-induced O_2^- production (a) and [Sar^{9}Met(O$_2$)^{11}]SP-induced O_2^- production (b) in AMs from healthy smokers. Cells were preincubated with CP 96,345 at 1 nM for 15 min and then challenged with the NK$_1$ agonists for further 30 min. Results are means$\pm$s.e.m. of four experiments in duplicate.

Table 3 Basal release of cytokines in AMs collected from healthy smokers and non-smokers

Cytokine	*Smokers (n = 6)*	*Non-smokers (n = 6)*
TNF-α (pg ml^{-1})	42 ± 8*	11 ± 2
IL-1β (pg ml^{-1})	15 ± 4	10 ± 3
IL-10 (pg ml^{-1})	13 ± 5	15 ± 7

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

unstimulated AM) were subtracted from all determinations and are listed in Table 3. As shown in Figure 5, SP (Figure 5a) and the NK$_1$ agonist [Sar^{9}Met(O$_2$)^{11}]SP (Figure 5b) dose-dependently induced TNF-α release from AMs, a significantly higher effect being observed in AMs from healthy smokers as compared to non-smokers (Figure 5a and b; $P < 0.01$). CP 96,345, tested at 1 nM, competitively antagonized SP-induced TNF-α release in smokers' AMs (Figure 5c), so confirming that this effect is mediated by NK-1R activation. Also PMA, at 10^{-7} M, induced a higher TNF-α release in AMs from smokers (535 ± 60 pg ml^{-1}; $n = 8$) as compared to non-smokers (136 ± 20 pg ml^{-1}; $n = 6$; $P < 0.05$). By evaluating IL-1β production from human AMs, we observed that SP acted dose-dependently, maximal release (about 40 pg ml^{-1}) being detected at 0.1–1 μM, with no major difference between smokers and non-smokers (Figure 6a). PMA, at 10^{-7} M, also released similar amounts of IL-1β in both smokers and non-smokers (Figure 6a). SP, in a dose-independent way, released very small amounts of the anti-inflammatory cytokine IL-10 (Figure 6b) by human AMs, a higher but not significant release being observed in non-smokers. As depicted in Figure 6, PMA 10^{-7} M did not stimulate IL-10 secretion, while inducing IL-1β release from human AMs.

SP and NK-1R agonists induce NF-κB activation

Previous observations indicate that, in different cell models, SP can induce the activation of the transcription factor NF-κB. We checked this hypothesis in human AMs by first evaluating the nuclear translocation of NF-κB by EMSA and, to ensure a better quantitative evaluation, the amounts of translocated p50 and p65 subunits by an ELISA kit. As known, although different NF-κB forms have been described, the p50–p65 heterodimer is the predominant species in many cell types (Baldwin, 1996). As a positive control for the detection of NF-κB activation, human AMs were stimulated by PMA, as this agent has previously been demonstrated to induce NF-κB nuclear translocation in human monocytes (Lavagno *et al.*, 2004). To investigate the time- and dose-dependent effects of SP, AMs from healthy non-smokers (Figure 7a) and smokers (Figure 7b) were challenged with two different concentrations of SP (10^{-8} and 10^{-6} M) for different times (1–2 h). As reported in Figure 7, SP-induced NF-κB activation, just detectable after 1 h, was maximal at 2 h and had about the same intensity as PMA (Figure 7). Interestingly, AMs obtained from healthy smokers (Figure 7b) demonstrated a constitutively (control, unstimulated AMs, lane 7) enhanced nuclear translocation of the transcription factor NF-κB as compared to AMs isolated from non-smokers (Figure 7a, lane 7). Accordingly, PMA- and SP-induced nuclear translocation was higher in AMs from

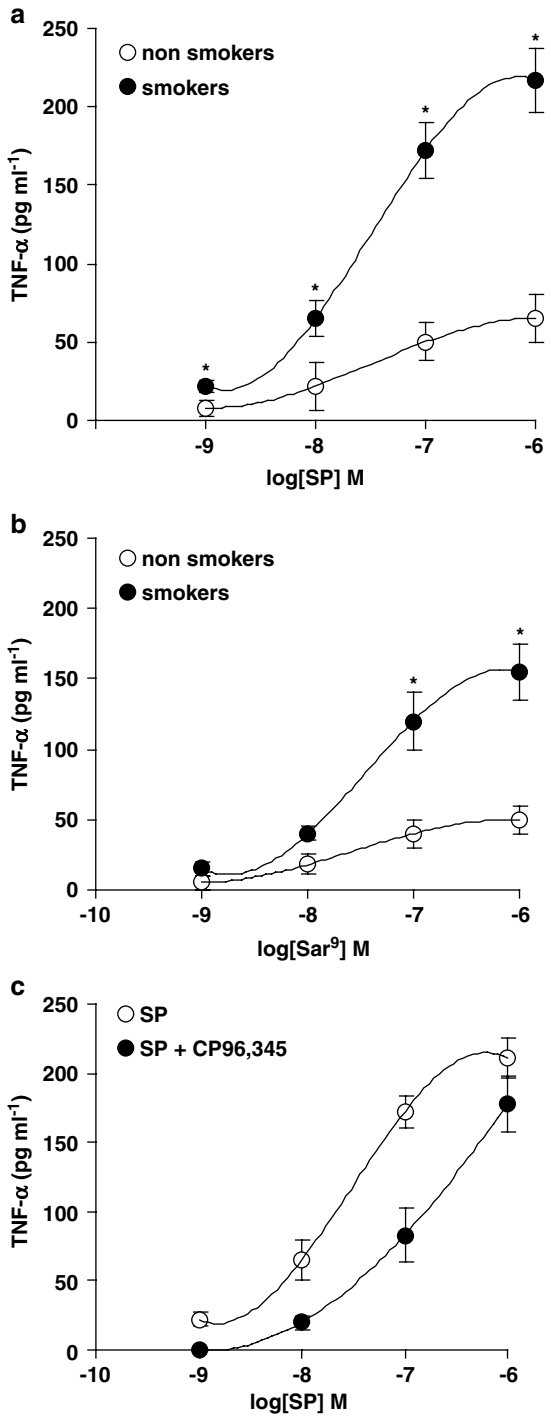

Figure 5 NK-1R stimulation induces TNF-α release in human AMs isolated from healthy smokers and healthy non-smokers. SP-induced TNF-α release in (a); [Sar^{9}Met (O$_2$)^{11}]SP-induced release in (b); reversal by CP 96,345 1 nM of SP-induced release in smokers in (c). Data are means ± s.e.m. of five to six experiments in duplicate. *$P < 0.01$ vs non-smokers. See text for further details.

smokers (Figure 7, lanes 1–2: PMA10^{-6} M, 2 and 1 h challenge, respectively; lanes 3–6: SP 10^{-6} and 10^{-8} M, 1 or 2 h challenge) as compared to non-smokers. The NK-1R antagonist CP96,345 ((2*S*,3*S*)-*cis*-2-diphenylmethyl-*N*[(2-methoxyphenyl)-methyl]-1-azabicyclo-octan-3-amine)), here evaluated at 1 μM, while not affecting *per se* PMA-stimulated translocation

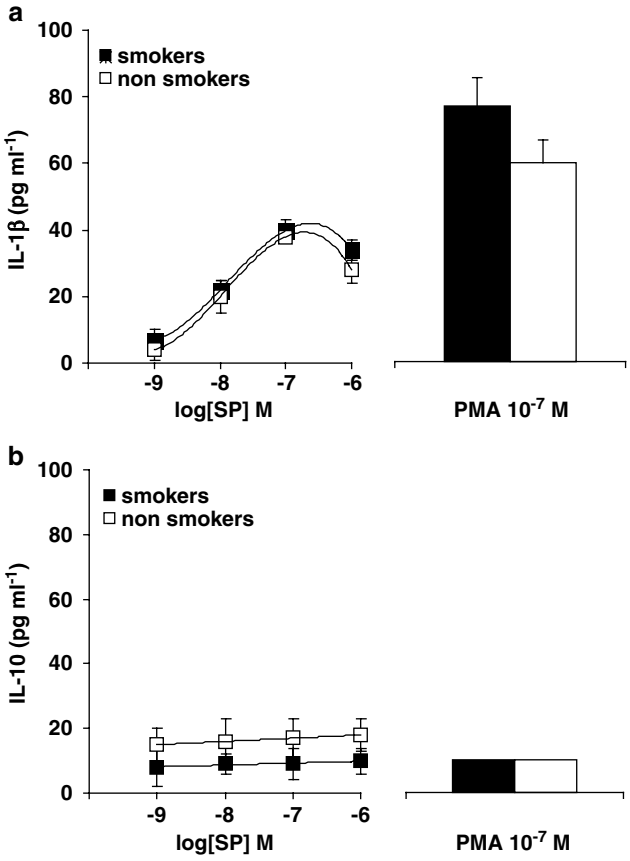

Figure 6 Effects of SP on IL-1β release (a) and IL-10 release (b) in human AMs isolated from healthy smokers and healthy non-smokers. PMA-induced release is shown for comparison. Data are means$\pm$s.e.m. of five to six experiments in duplicate.

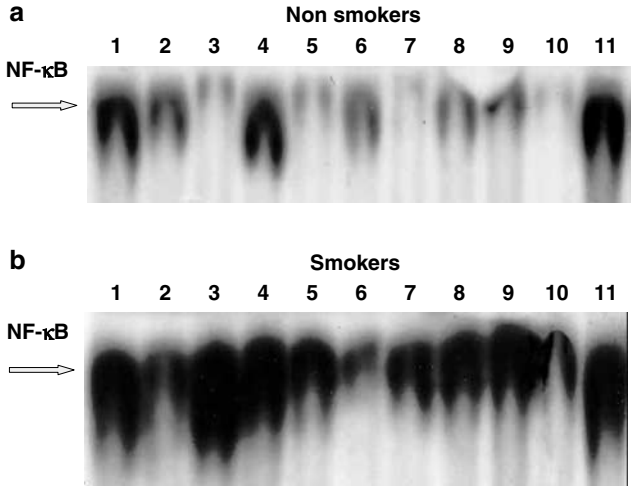

Figure 7 SP induces NF-κB activation in human AMs from healthy non-smokers (a) and healthy smokers (b) in a time- and dose-dependent manner. AMs were stimulated with SP (10^{-6} and 10^{-8} M) or PMA 10^{-6} M for 1 or 2 h, in the presence or absence of CP 96,345. The NK-1R antagonist was evaluated at 1 μM and preincubated for 15 min. Nuclear extracts (5 μg) were prepared and assayed for NF-κB activity by EMSA (see text for further details). In (a) (non-smoker): lane 1 = PMA 2 h; lane 2 = PMA 1 h; lane 3 = SP 10^{-6} M 1 h; lane 4 = SP 10^{-6} M 2 h; lane 5 = SP 10^{-8} M 1 h; lane 6 = SP 10^{-8} M 2 h; lane 7 = control, unstimulated AM; lane 8 = CP 96,345 + SP 10^{-8} M 2 h; lane 9 = CP 96.345 + SP 10^{-6} M 2 h; lane 10 = CP 96,345 alone; lane 11 = CP 96,345 + PMA 2 h. In (b) (smoker): lane 1 = PMA 2 h; lane 2 = PMA 1 h; lane 3 = SP 10^{-6} M 2 h; lane 4 = SP 10^{-6} M 1 h; lane 5 = SP 10^{-8} M 2 h; lane 6 = SP 10^{-8} M 1 h; lane 7 = control, unstimulated AM; lane 8 = CP 96,345 + SP 10^{-8} M 2 h; lane 9 = CP 96.345 + SP 10^{-6} M 2 h; lane 10 = CP 96,345 alone; lane 11 = CP 96,345 + PMA 2 h. This experiment was performed three times with similar results.

(Figure 7, lane 11) and basal constitutive activity (lane 10), potently reduced SP-induced effects (lanes 8 and 9), so confirming that SP-induced NF-κB nuclear translocation in human AMs is a receptor-mediated effect. Moreover, the NK_1 antagonist seemed to be less effective in reducing SP-induced nuclear translocation in smokers, as compared to smokers. The autoradiographs presented in Figure 7 (which are representative of two other additional experiments) are relative to AMs from smokers and non-smokers, which were obtained and contemporarily processed on the same day; the two gels presented as Figure 7a (non-smokers) and Figure 7b (smokers) were run concomitantly. Competition experiments performed with 100-fold excess unlabelled NF-κB sequence demonstrated the specificity of the induced NF-κB/DNA binding complex (not shown).

In AMs collected from healthy smokers, SP effects are reproduced, to about the same intensity, by the NK_1 agonist [$Sar^9Met(O_2)^{11}$]SP, evaluated at 10^{-8} and 10^{-6} M (Figure 8). The antagonist CP 96,345 potently reduced the effects of the NK_1 agonist, so confirming that NF-κB nuclear translocation is a receptor-mediated effect (Figure 8).

We also performed Western blot experiments to evaluate the nuclear translocation of p50 and p65 subunits of the NF-κB complex in AMs from both smokers and non-smokers. As depicted in Figure 9 (a: p50 subunit and b: p65 subunit), PMA, SP and the NK_1 agonist [$Sar^9Met(O_2)^{11}$]SP induced the translocation of both subunits, an increased p50 translocation being observed in smokers (Figure 9).

To ensure a better quantitative evaluation, we also assessed the translocation of p65 subunit and p50 subunit in AMs from both smokers and non-smokers, by using a commercially available ELISA kit. As depicted in Figure 10, SP dose-dependently induced p50 translocation (Figure 10a) and p65 translocation (Figure 10b) in AMs, a significantly ($P<0.05$) enhanced effect being observed in smokers especially for the p50 subunit. The NK_1 agonist [$Sar^9Met(O_2)^{11}$]SP, here evaluated at 1 μM, also increased p50 and p65 translocation, AMs from smokers depicting a significant enhanced p50 translocation. PMA-induced translocation is shown for comparison (Figure 10). CP 96,345 at 10^{-6} M significantly reduced SP-induced nuclear p50 and p65 translocation (not shown). Results are expressed as the nuclear/cytosolic ratio, which is the ratio between the amount of p50 (or p65) in nuclear extracts and cytosolic extracts.

Discussion

These results demonstrate that human AMs, isolated from both healthy smokers and non-smokers, possess functional NK-1R. In fact, we here report the presence of NK-1R protein (by Western blotting) as well as the ability of SP, the endogenous NK-1R ligand, to evoke O_2^- production and

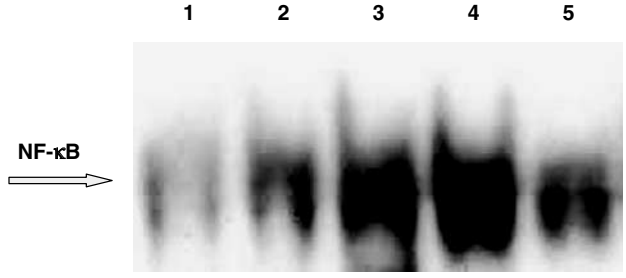

Figure 8 The NK_1 selective agonist [$Sar^9Met(O_2)^{11}$]SP induces NF-κB activation in human AMs from healthy smokers and its effects are reduced by the NK-1R antagonist CP 96,345. AMs were stimulated with [$Sar^9Met(O_2)^{11}$]SP 10^{-8} or 10^{-6} M for 2 h, in the presence or absence of CP 96,345 at 1 μM. The effects of SP 10^{-6} M are shown for comparison. Nuclear extracts (5 μg) were prepared and assayed for NF-κB activity by EMSA (see text for further details). Lane 1 = control, unstimulated AM; lane 2 = Sar^9 10^{-8} M; lane 3 = Sar^9 10^{-6} M; lane 4 = SP 10^{-6} M; lane 5 = CP 96,345 + Sar^9 10^{-6} M. This experiment was performed three times with similar results.

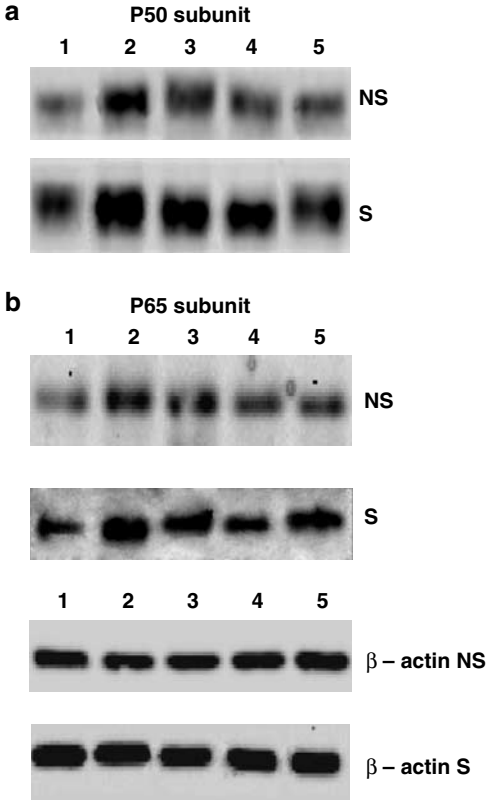

Figure 9 Western blots of p50 and p65 subunits in AMs from both non-smokers (NS) and smokers (S). The nuclear translocation of p50 is reported in (a); the translocation of p65 is reported in (b). Beta-actin is shown for comparison. Lane 1 = control; lane 2 = PMA 10^{-6} M; lane 3 = SP 10^{-6} M; lane 4 = SP 10^{-8} M; lane 5 = Sar^9 10^{-6} M.

cytokine release in AMs, as well as to induce activation of the transcription factor NF-κB. These effects are all receptor-mediated, as they are reproduced by NK_1 selective agonists and reverted by NK-1R antagonists.

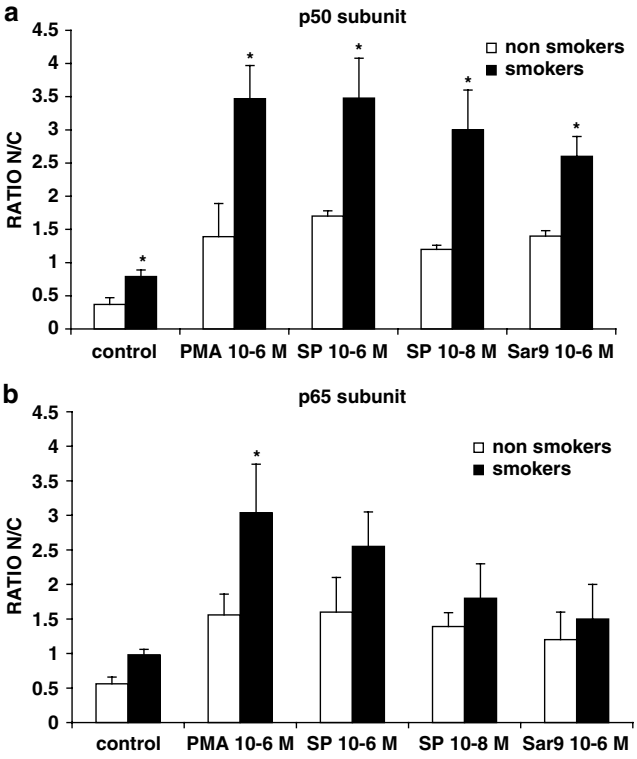

Figure 10 NK-1R stimulation induces the translocation of p50 (a) and p65 (b) subunits in human AMs from healthy smokers and healthy non-smokers. PMA-induced translocation is shown for comparison. AMs were challenged for 2 h with the stimuli; nuclear and cytosolic extracts were prepared and evaluated for their content in p50 and p65 subunits. Results are expressed as the nuclear/cytosolic ratio (ratio N/C) for both p50 and p65 subunits. Data are means ± s.e.m. of five experiments in duplicate. *$P<0.05$ vs non-smokers.

NK-1R expression has been evaluated by different authors in different cell types, by using RT–PCR technology, mainly. By this approach, human monocytes and monocyte-derived macrophages were shown to express SP and NK-1R, besides producing and releasing SP (Ho *et al*., 1997). Moreover, an NK-1R antagonist downregulated SP mRNA expression in monocyte-derived macrophages (Lai *et al*., 2002a). Lai *et al*. (2002b) quantified SP mRNA in different cells by real time RT–PCR and reported a large variability in the level of transcripts in monocyte-derived macrophages, the number of SP mRNA copies/μg total mRNA detected in preparations from four different donors ranging from 949 to 113,388 (Lai *et al*., 2002b). Therefore, given the demonstrated large variability in SP transcripts (Lai *et al*., 2002b) and depending on the number of collected AMs for the evaluation of all the other parameters (respiratory burst, cytokine release, NF-κB activation), in our AMs preparations, we evaluated NK-1R expression at the protein level, only. By using a noncommercial monoclonal anti-NK-1R antibody (raised in chicken against the final 15 amino acids at the C-terminus of the rat NK-1R), Smith *et al*. (2000) demonstrated the presence of NK-1R in human antrum and detected both a positive band at 46 kDa and a larger molecular mass band of 110 kDa, representing the glycosylated form of the NK-1R. Two NK-1R isoforms that differ in the length of the cytoplasmic carboxyl-terminus have

been reported (Fong *et al*., 1992; Mantyh *et al*., 1996; Li *et al*., 1997): in the rat, the full-length and the truncated receptor presented molecular weights of the receptor proteins of about 80 and 50 kDa, respectively, the deglycosylated receptors being 46-kDa and 37-kDa, respectively (Li *et al*., 1997). The truncated receptor, at variance from the full-length one, did not undergo rapid and long-lasting desensitization (Li *et al*., 1997); cells possessing the short NK-1R isoform would, therefore, be expected to have a prolonged responsiveness. By means of noncommercial NK-1R antibodies, a 42 kDa protein was detected in murine peritoneal macrophages and microglia (Marriott & Bost, 2000; Rasley *et al*., 2002), whereas a 53 kDa protein was demonstrated in the THP-1 cells (Simeonidis *et al*., 2003). Given the heterogeneity of NK-1R and the fact that we used a commercial polyclonal anti-NK-1R antibody, we detected three prominent bands of 68 (as indicated by the manufacturer), 53 and 42 kDa in human AMs, in accordance to what observed by others in cells of the monocyte/macrophage lineage. Interestingly, AMs from healthy smokers demonstrated a >3-fold increase in NK-1R expression as compared to healthy non-smokers: these results are in keeping with previous data indicating the key role for SP and NK-1R activation in tobacco-induced lung toxicity in both animals and humans (Dusser *et al*., 1989; Tomaki *et al*., 1995; Wu & Lee, 1999) and further extend observations from Bai *et al*. (1995), who detected an increased NK-1R expression in lung biopsies from smokers.

Furthermore, we have determined the functional nature of these NK-1R by demonstrating the ability of SP and selective NK_1 agonists to induce O_2^- production and cytokine release from human AMs. These observations extend our previous data in both human and guinea-pig AMs (Brunelleschi *et al*., 1990; 1992; 1996), demonstrating an enhanced responsiveness to tachykinins in AMs isolated from healthy smokers. By evaluating the respiratory burst, we measured a significantly increased O_2^- production when AMs from smokers were challenged with SP or selective NK_1 agonists, EC_{50}s being 0.25 nM in smokers and 1 nM in non-smokers for SP and 3 nM in smokers and 10 nM in non-smokers for $[Sar^9Met(O_2)^{11}]SP$. To further confirm the receptor nature of these effects, the non-peptide NK_1 antagonist CP 96,345 at 1 nM shifted to the right the concentration–response curve for both endogenous and synthetic NK_1 ligands.

TNF-α, IL-1β, IL-2 and IL-6 are frequently encountered proinflammatory cytokines, which are involved in a variety of immunological functions as well as interaction with different target cells. TNF-α is secreted by monocyte/macrophages mainly and, besides exerting cytotoxic activity to tumor cells, has a key role in chronic inflammation. TNF-α induces the expression of, and enhances cellular responsiveness to, other cytokines and growth factors, and affect signal transduction pathways leading to proliferation. SP and NK_1 selective agonists dose-dependently induce TNF-α release from human AMs, a more than doubled significant TNF-α release ($P<0.01$) being observed in AMs from healthy smokers. CP 96,345 at 1 nM competitively antagonize these effects, further confirming the receptor-mediated nature of SP-induced effects in human AMs. In the concentration range 1 nM–1 μM, SP also induces IL-1β release from AMs, no significant differences being observed between smokers and non-smokers. This result was somewhat unexpected also considering the fact (see below) that SP and NK-1R agonists activate the transcription factor NF-κB. As known, the regulation of TNF-α and IL-1β production is largely NF-κB-dependent, although evidence exists that TNF-α and other cytokines can also be induced through NF-κB-independent pathways (Bondeson *et al*., 1999; Andreakos *et al*., 2004). We have no definitive explanation for SP-induced IL-1β release being similar in smokers and non-smokers; however, PMA, too, although activating NF-κB and inducing cytokine release, did not release an enhanced amount of IL-1β in smokers. Moreover, AMs isolated from smokers and challenged with LPS released significantly decreased amounts of IL-1β as compared to non-smokers (Brown *et al*., 1989; Yamaguchi *et al*., 1989). Brown *et al*. (1989) concluded that 'there is a defect in release but not production of IL-1β from the AMs of chronic smokers'. In addition, SP exerts inconsistent effects on IL-10 release, in keeping with previous observations on peripheral blood mononuclear cells from healthy donors (Kim *et al*., 2003).

An important component controlling the synthesis of many cytokines and other proinflammatory gene products is the transcriptional activator NF-κB (reviewed in Baldwin, 1996). Five related mammalian gene products participate in NF-κB functions (p50/NF-κB1, p52/NF-κB2, p65/Rel A, c-Rel and RelB), the predominant species in many cell types being a p50-p65 heterodimer. As known, the transcription factor NF-κB regulates the expression of many proinflammatory genes, including those of TNF-α and IL-1β, and, in turn, these inflammatory cytokines are potent inducers of NF-κB activation (Baldwin, 1996).

SP specifically activates NF-κB in cells of the monocyte/macrophage lineage, for example, human astrocytoma cells, murine peritoneal macrophages and dendritic cells (Lieb *et al*., 1997; Marriott *et al*., 2000), but no information are available concerning human AM. We originally report here that activation of NK-1R by SP or $[Sar^9Met(O_2)^{11}]SP$ dose-dependently stimulates nuclear translocation of NF-κB, as evaluated by EMSA. This effect is reverted by the NK_1 antagonist CP 96,345. Interestingly, the entity of the effect is similar to the PMA-induced one, so indicating SP as a potent activator of this transcription factor in human AMs. Interestingly, SP induced a three-fold increase (as evaluated by densitometry) in NF-κB nuclear translocation in AMs isolated from healthy smokers as compared to non-smokers. Consistent with previous reports in which human AMs were used (Carter *et al*., 1998; Farver *et al*., 1998), a constitutive expression of NF-κB in the nucleus of unstimulated AMs was always observed in our experiments. By using specific NF-κB DNA-binding sequences for IL-6, IL-8 and TNF-α promoters, Carter *et al*. (1998) demonstrated that different NF-κB complexes are generated in AMs from healthy volunteers and that specific NF-κB complexes are used for the transcription of these cytokine genes. They found that both p50 and p65 proteins bound to the IL-6 sequence, whereas a p50 protein bound to the TNF sequence and a p65 protein bound to the IL-8 sequence (Carter *et al*., 1998).

By Western blot assays and ELISA kits, we have detected both p65 and p50 subunits in human AMs. In our experiments, the p50 subunit seems to be the most abundant one in AMs from both smokers and non-smokers, being more efficiently translocated in smokers. In fact, unstimulated AMs from healthy smokers presented a more than doubled nuclear translocation of p50 (but about the same for p65) as compared to AMs from non-smokers. When AMs were challenged by

PMA or NK-1R agonists, a further enhanced nuclear translocation of NF-κB subunits was observed: SP and [Sar^{9}Met(O$_2$)^{11}]SP were particularly effective on p50 translocation (about three-fold) and their effects were significantly enhanced in AMs from smokers. PMA, too, was very potent (more than three-fold) in inducing p50 translocation and also significantly stimulated (although to a lesser degree) p65 translocation in healthy smokers. On the contrary, SP and [Sar^{9}Met(O$_2$)^{11}]SP potentiated p65 nuclear translocation, but no significant variations were observed between smokers and non-smokers. This observation deserves further investigations but, in our opinion, is in keeping with the data demonstrating p50 as the major NF-κB subunit for the transcription of TNF-α gene (Carter *et al*., 1998). In fact, among the cytokines we evaluated, TNF-α is the one released to greater amounts by SP and NK$_1$ agonists, whereas inconsistent effect on IL-10 release are observed. As known, IL-10 exerts anti-inflammatory effects and inhibits NF-κB activation in LPS-stimulated human AMs (Raychaudhuri *et al*., 2000). Moreover, according to the fact that reactive oxygen intermediates (in particular hydrogen peroxide) have been proposed as second messenger molecules in the activation pathway of NF-κB and that antioxidants usually inhibit NF-κB activation (Lieb *et al*., 1997), the demonstrated SP ability to induce oxy-radical production could play a role in SP-induced translocation of the transcription factor. To our knowledge, this is the first paper that describes NK-1R expression and activation in human AMs as a whole, although other different reports have investigated, in monocyte/macrophages of different origin, some of the points we focused here. In conclusion, this paper indicates SP as a potent contributor for tobacco smoke toxicity.

We thank Pfizer (Italy) for the kind gift of the NK-1R antagonist CP 96,345. We also are indebted with Drs Donato Colangelo and Luigia Grazia Fresu (School of Medicine, Novara, Italy) for helpful discussion. Research described in this article was supported by Philip Morris U.S.A. Inc., by Philip Morris International and by MIUR ex-60% 2003 grants.

References

ANDREAKOS, E., SACRE, S.M., SMITH, C., LUNDBERG, A., KIRIAKIDIS, S., STONEHAUSE, T., MONACO, C., FELDMANN, M. & FOXWELL, B.M. (2004). Distinct pathways of LPS-induced NF-κB activation and cytokine production in human myeloid and nonmyeloid cells defined by selective utilization of MyD88 and Mal/TIRAP. *Blood,* **103,** 2229–2237.

BAI, T.R., ZHOU, D., WEIR, T., WALKER, B., HEGELE, R., HAYASHI, S., MCKAY, K., BONDY, G.P. & FONG, T. (1995). Substance P (NK-1)- and neurokinin A (NK-2)-receptor gene expression in inflammatory airway disease. *Am. J. Physiol.,* **269,** L309–L317.

BALDWIN, A.S. (1996). The NF-κB and IκB proteins: new discoveries and insights. *Annu. Rev. Immunol.,* **14,** 649–681.

BONDESON, J., BROWNE, K.A., BRENNAN, F.M., FOXWELL, B.M. & FELDMANN, M. (1999). Selective regulation of cytokine induction by adenoviral gene transfer of IκBα into human macrophages: lipopolysaccharide-induced, but not zymosan-induced, proinflammatory cytokines are inhibited, but IL-10 is nuclear factor-κB independent. *J. Immunol.,* **162,** 2939–2945.

BROWN, G.P., IWAMOTO, G.K., MONICK, M.M. & HUNNINGHAKE, G.W. (1989). Cigarette smoking decreases interleukin 1 release by human alveolar macrophages. *Am. J. Physiol.,* **256,** C260–C264.

BRUNELLESCHI, S., BORDIN, G., COLANGELO, D. & VIANO, I. (1998). Tachykinin receptors on human monocytes: their involvement in rheumatoid arthritis. *Neuropeptides,* **32,** 215–223.

BRUNELLESCHI, S., GUIDOTTO, S., VIANO, I., FANTOZZI, R., POZZI, E., GHIO, P. & ALBERA, C. (1996). Tachykinin activation of human alveolar macrophages in tobacco smoke and sarcoidosis: a phenotypical and functional study. *Neuropeptides,* **30,** 456–464.

BRUNELLESCHI, S., PARENTI, A., CENI, E., GIOTTI, A. & FANTOZZI, R. (1992). Enhanced responsiveness of ovalbumin-sensitized guinea-pig alveolar macrophages to tachykinins. *Br. J. Pharmacol.,* **107,** 964–969.

BRUNELLESCHI, S., PENENGO, L., LAVAGNO, L., SANTORO, C., COLANGELO, D., VIANO, I. & GAUDINO, G. (2001). Macrophage stimulating protein (MSP) evokes superoxide anion production by human macrophages of different origin. *Br. J. Pharmacol.,* **134,** 1285–1295.

BRUNELLESCHI, S., TARLI, S., GIOTTI, A. & FANTOZZI, R. (1991). Priming effects of mammalian tachykinins on human neutrophils. *Life Sci.,* **48,** PL1–PL5.

BRUNELLESCHI, S., VANNI, L., LEDDA, F., GIOTTI, A., MAGGI, C.A. & FANTOZZI, R. (1990). Tachykinins activate guinea-pig alveolar macrophages: involvement of NK$_1$ and NK$_2$ receptors. *Br. J. Pharmacol.,* **100,** 417–420.

CABERLOTTO, L., HURD, Y.L., MURDOCK, P., WAHLIN, J.P., MELOTTO, S., CORSI, M. & CARLETTI, R. (2003). Neurokinin 1 receptor and relative abundance of the short and long isoforms in the human brain. *Eur. J. Neurosci.,* **17,** 1736–1746.

CARTER, A.B., MONICK, M.M. & HUNNINGHAKE, G.W. (1998). Lipopolysaccharide-induced NF-κB activation and cytokine release in human alveolar macrophages is PKC-independent and TK- and PC-PLC-dependent. *Am. J. Respir. Cell. Mol. Biol.,* **18,** 384–391.

DUSSER, D.J., DJOKIC, T.D., BORSON, D.B. & NADEL, J.A. (1989). Cigarette smoke induces bronchoconstrictor hyperresponsiveness to substance P and inactivates airway neutral endopeptidase in the guinea pig. Possible role of free radicals. *J. Clin. Invest.,* **84,** 900–906.

FARVER, C.F., RAYCHAUDHURI, B., BUHROW, L.T., CONNORS, M.J. & THOMASSEN, M.J. (1998). Constitutive NF-κB levels in human alveolar macrophages from normal volunteers. *Cytokine,* **10,** 868–871.

FONG, T.M., ANDERSON, S.A., YU, H., HUANG, R.R. & STRADER, C.D. (1992). Differential activation of intracellular effector by two isoforms of human neurokinin-1 receptor. *Mol. Pharmacol.,* **41,** 24–30.

GERMONPRE, P.R., BULLOCK, G.R., LAMBRECHT, B.N., VAN DE VELDE, V., LUYTEN, W.H., JOOS, G. & PAUWELS, R.A. (1999). Presence of substance P and neurokinin 1 receptors in human sputum macrophages and U-937 cells. *Eur. Respir. J.,* **14,** 776–782.

GUO, J.Z., YOSHIOKA, K., ZHAO, F.Y., HOSOKI, R., MAEHARA, T., YANAGISAWA, M., HAGAN, R.M. & OTSUKA, M. (1995). Pharmacological characterization of GR82334, a tachykinin NK$_1$ receptor antagonist, in the isolated spinal cord of the neonatal rat. *Eur. J. Pharmacol.,* **281,** 49–54.

HAGAN, R.M., IRELAND, S.J., JORDAN, C.C., BERESFORD, I.J.M., STEPHENS-SMITH, M.L., EWAN, G. & WARD, P. (1990). GR71251, a novel, potent and highly selective antagonist at neurokinin-1 receptors. *Br. J. Pharmacol.,* **99,** 62P.

HO, W.Z., LAI, J.P., ZHU, X.H., UVAYDOVA, M. & DOUGLAS, S.D. (1997). Human monocytes and macrophages express substance P and neurokinin-1 receptor. *J. Immunol.,* **159,** 5654–5660.

IWAMOTO, I., NAKAGAWA, N., YAMAZAKI, H., KIMURA, A., TOMIOKA, H. & YOSHIDA, S. (1993). Mechanism for substance P-induced activation of human neutrophils and eosinophils. *Regul. Pept.,* **46,** 228–230.

KIM, K.H., PARK, K.C., CHUNG, J.H. & CHOI, H.R. (2003). The effect of substance P on peripheral blood mononuclear cells in patients with atopic dermatitis. *J. Dermat. Sci.,* **32,** 115–124.

LAI, J.P., DOUGLAS, S.D., SHAHEEN, F., PLEASURE, D.E. & HO, W.Z. (2002b). Quantification of Substance P mRNA in human immune cells by real-time reverse transcriptase PCR assay. *Clin. Diagn. Lab. Immunol.,* **9,** 138–143.

LAI, J.P., HO, W.Z., YANG, J.H., WANG, X., SONG, L. & DOUGLAS, S.D. (2002a). A non-peptide substance P antagonist down-regulates SP mRNA expression in human mononuclear phagocytes. *J. Neuroimmunol.,* **128,** 101–108.

LAVAGNO, L., BORDIN, G., COLANGELO, D., VIANO, I. & BRUNELLESCHI, S. (2001). Tachykinin activation of human monocytes from patients with rheumatoid arthritis: *in vitro* and *ex-vivo* effects of cyclosporin A. *Neuropeptides,* **35,** 92–99.

LAVAGNO, L., GUNELLA, G., BARDELLI, C., SPINA, S., FRESU, L.G., VIANO, I. & BRUNELLESCHI, S. (2004). Anti-inflammatory drugs and tumor necrosis factor-α production in monocytes: role of transcription factor NF-κB and implication in rheumatoid arthritis therapy. *Eur. J. Pharmacol.,* **501,** 199–208.

LI, H., LEEMAN, S.E., SLACK, B.E., HAUSER, G., SALTSMAN, W.S., KRAUSE, J.E., BLUSZTAJN, J.K. & BOYD, N.D. (1997). A substance P (neurokinin-1) receptor mutant carboxyl-terminally truncated to resemble a naturally occurring receptor isoform displays enhanced responsiveness and resistance to desensitization. *Proc. Natl. Acad. Sci. U.S.A.,* **94,** 9475–9480.

LIEB, K., FIEBICH, B.L., BERGER, M., BAUER, J. & SCHULZE-OSTHOFF, K. (1997). The neuropeptide substance P activates transcription factor NF-κB and κB-dependent gene expression in human astrocytoma cells. *J. Immunol.,* **159,** 4952–4958.

LOTZ, M., VAUGHAN, J.H. & CARSON, D.A. (1988). Effects of neuropeptides on production of inflammatory cytokines by human monocytes. *Science,* **241,** 1218–1221.

MANTYH, P.W., ROGERS, S.D., GHILARDI, J.R., MAGGIO, J.E., MANTYH, C.R. & VIGNA, S.R. (1996). Differential expression of two isoforms of the neurokinin-1 (substance P) receptor *in vivo*. *Brain Res.,* **719,** 8–13.

MARRIOTT, I. & BOST, K.L. (2000). IL-4 and IFN-γ up-regulate substance P receptor expression in murine peritoneal macrophages. *J. Immunol.,* **165,** 182–191.

MARRIOTT, I., MASON, M.J., ELHOFY, A. & BOST, K.L. (2000). Substance P activates NF-κB independent of elevations in intracellular calcium in murine macrophages and dendritic cells. *J. Neuroimmunol.,* **102,** 163–171.

MOUSLI, M., BRONNER, C., BUEB, J.L., TSCHIHART, E., GIES, J.P. & LANDRY, Y. (1989). Activation of rat peritoneal mast cells by substance P and mastoparan. *J. Pharmac. Exp. Ther.,* **250,** 329–335.

PAGE, N.M. & BELL, N.J. (2002). The human tachykinin NK_1 (short form) and tachykinin NK_4 receptor; a reappraisal. *Eur. J. Pharmacol.,* **437,** 27–30.

PASCUAL, D.W., XU-AMANO, J.C., KIYONO, H., MCGHEE, J.R. & BOST, K.L. (1991). Substance P acts directly upon cloned B lymphoma cells to enhance IgA and IgM production. *J. Immunol.,* **146,** 2130–2136.

PAYAN, D.G., BREWSTER, J.D. & GOETZL, E.J. (1983). Specific stimulation of human T lymphocytes by substance P. *J. Immunol.,* **131,** 1613–1615.

PENNEFATHER, J.N., LECCI, A., CANDENAS, M.L., PATAK, E., PINTO, F.M. & MAGGI, C.A. (2004). Tachykinins and tachykinin receptors: a growing family. *Life Sci.,* **74,** 1445–1463.

RASLEY, A., BOST, K.L., OLSON, J.K., MILLER, S.D. & MARRIOTT, I. (2002). Expression of functional NK-1 receptors in murine microglia. *Glia,* **37,** 258–267.

RAYCHAUDHURI, B., FISHER, C.J., FARVER, C.F., MALUR, A., DRAZBA, J., KAVURU, M.S. & THOMASSEN, M.J. (2000). Interleukin 10 (IL-10)-mediated inhibition of inflammatory cytokine production by human alveolar macrophages. *Cytokine,* **12,** 1348–1355.

SEVERINI, C., IMPROTA, G., FALCONIERI-ERSPAMER, G., SALVADORI, S. & ERSPAMER, V. (2002). The tachykinin peptide family. *Pharmacol. Rev.,* **54,** 285–322.

SIMEONIDIS, S., CASTAGLIUOLO, I., PAN, A., LIU, J.L., WANG, C.C., MYKONIATIS, A., PASHA, A., VALENICK, L., SOUGIOULTZIS, S., ZHAO, D. & POTHOULAKIS, C. (2003). Regulation of the NK-1 receptor gene expression in human macrophage cells *via* an NF-κB site on its promoter. *Proc. Natn. Acad. Sci. U.S.A.,* **100,** 2957–2962.

SMITH, V.C., SAGOT, M.A., WONG, H. & BUCHAN, A.M.J. (2000). Cellular expression of the neurokinin 1 receptor in the human antrum. *J. Autonom. Nerv. Syst.,* **79,** 165–172.

TOMAKI, M., ICHINOSE, M., MIURA, M., HIRAYAMA, Y., YAMAUCHI, H., NAKAJIMA, N. & SHIRATO, K. (1995). Elevated substance P content in induced sputum from patients with asthma and patients with chronic bronchitis. *Am. J. Respir. Crit. Care Med.,* **151,** 613–617.

WU, Z.X. & LEE, L.Y. (1999). Airway hyperresponsiveness induced by chronic exposure to cigarette smoke in guinea-pigs: role of tachykinins. *J. Appl. Physiol.,* **87,** 1621–1628.

YAMAGUCHI, E., OKAZAKI, N., ITOH, A., ABE, S., KAWAKAMI, Y. & OKUYAMA, H. (1989). Interleukin 1 production by alveolar macrophages is decreased in smokers. *Am. Rev. Respir. Dis.,* **140,** 397–402.

(Received January 21, 2005
Accepted February 9, 2005)

British Journal of Pharmacology (2006) 148, 478–489
www.nature.com/bjp

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.
British Journal of Pharmacology (2006) **148,** 478–489. doi:10.1038/sj.bjp.0706751;
published online 24 April 2006

Keywords: Macrophage-stimulating protein; human alveolar macrophages; tobacco smoke; NF-κB activation; respiratory burst; cytokine release; sarcoidosis; idiopathic pulmonary fibrosis; p50 subunit; IL-10; TNF-α

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

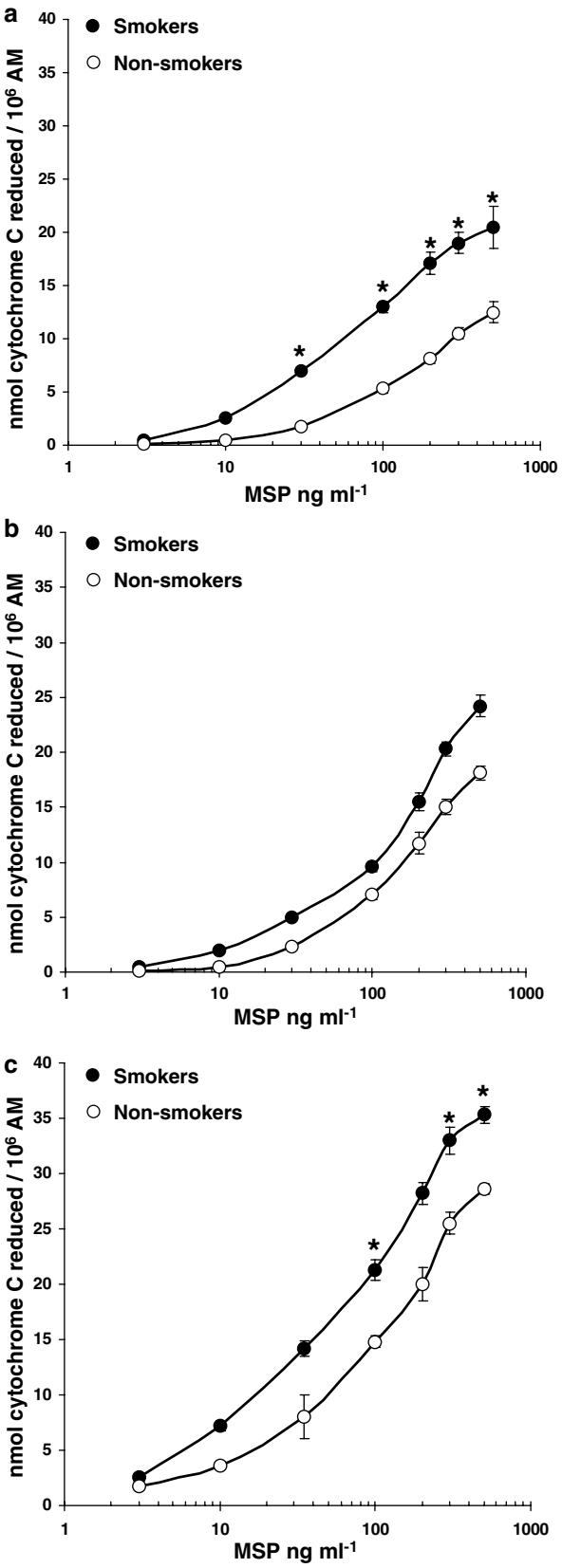

Figure 1 MSP evokes O_2^- production in AM. AM from healthy smokers and non-smokers (a), patients with sarcoidosis (b) and idiopathic pulmonary fibrosis (c) were challenged with increasing concentrations of MSP (3–500 ng ml^{-1}) for 30 min; ● = smokers, ○ = non-smokers. Results are means ± s.e.m. of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

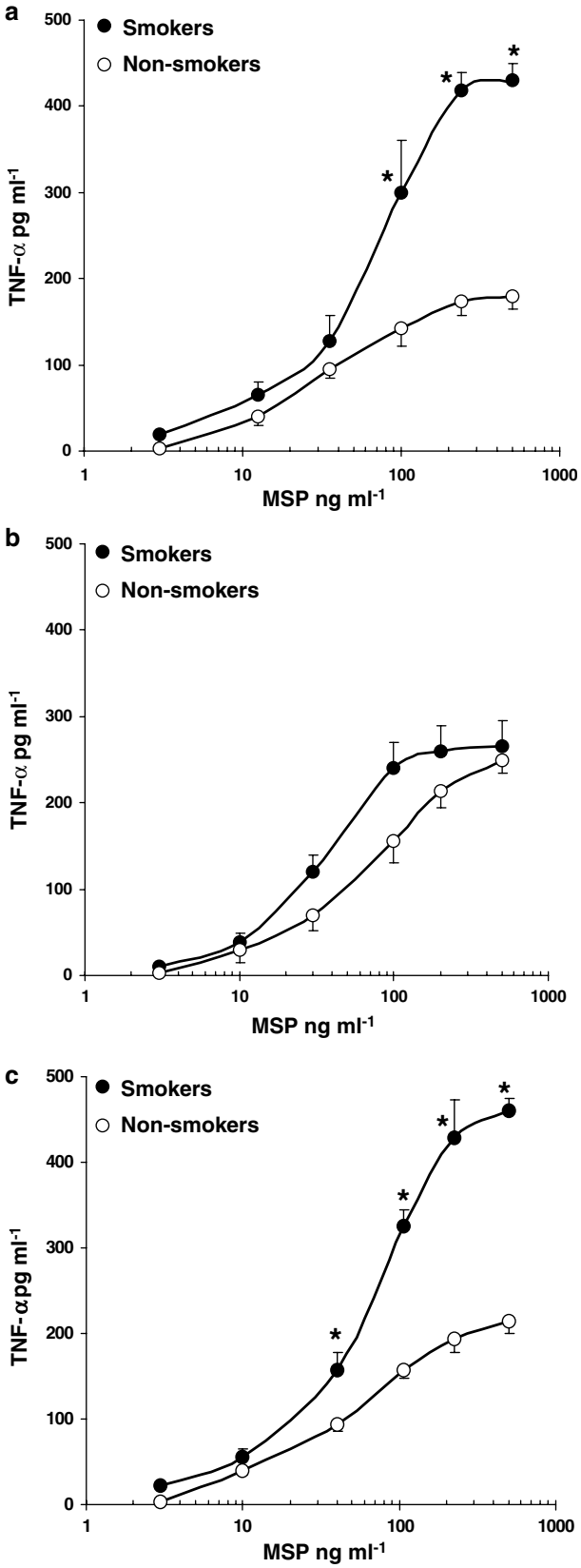

Figure 2 MSP evokes TNF-α release in AM. AM from healthy smokers and non-smokers (a), patients with sarcoidosis (b) and idiopathic pulmonary fibrosis (c) were challenged with increasing concentrations of MSP (3–500 ng/ml) for 24 h; ● = smokers, ○ = non-smokers. Results are means ± s.e.m. of five experiments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

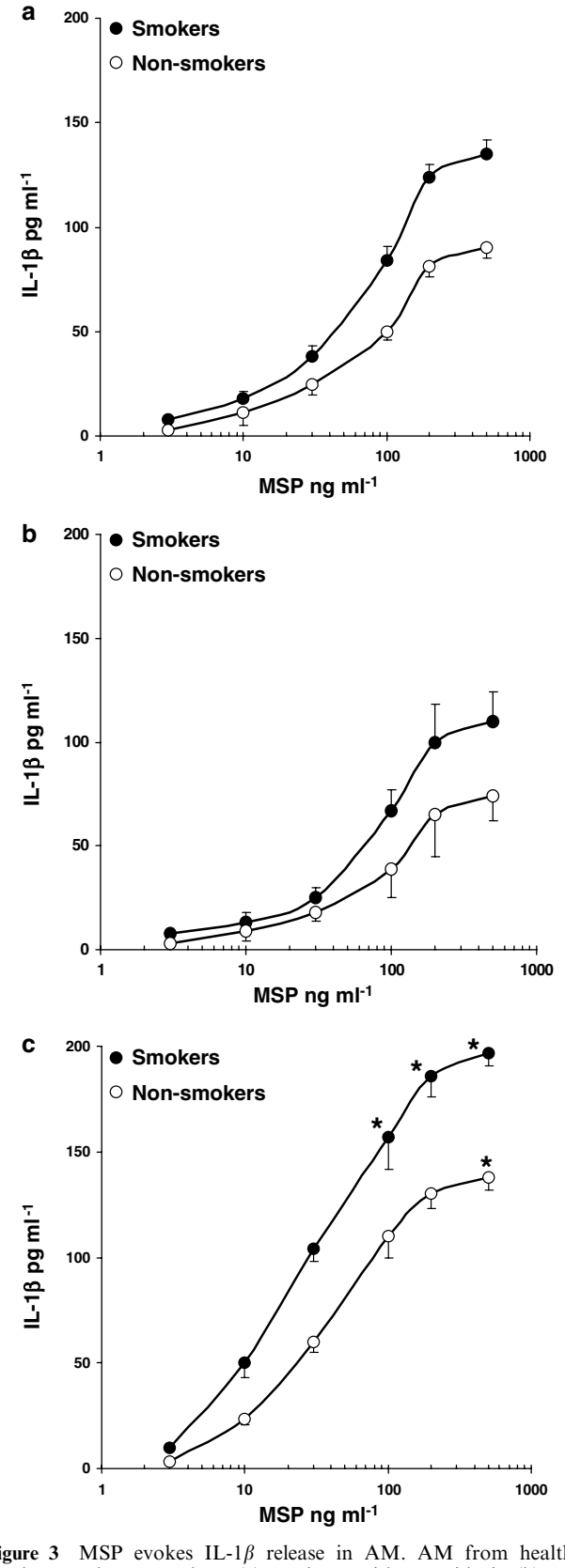

Figure 3 MSP evokes IL-1β release in AM. AM from healthy smokers and non-smokers (a), patients with sarcoidosis (b) and idiopathic pulmonary fibrosis (c) were challenged with increasing concentrations of MSP (3–500 ng/ml) for 24 h; ● = smokers, ○ = non-smokers. Results are means ± s.e.m. of five experiments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

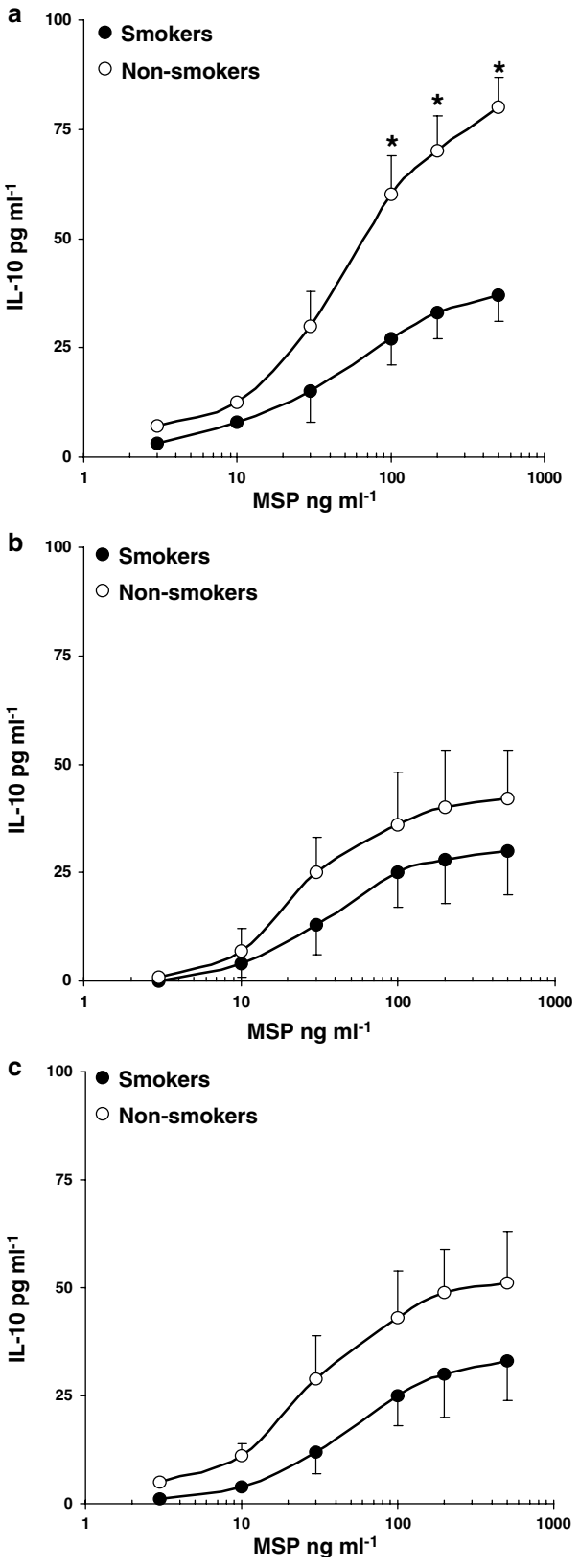

Figure 4 MSP evokes IL-10 release in AM from smokers and non-smokers. AM from healthy smokers and non-smokers (a), patients with sarcoidosis (b) and idiopathic pulmonary fibrosis (c) were challenged with increasing concentrations of MSP (3–500 ng/ml) for 24 h.; ● = smokers, ○ = non-smokers. Results are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

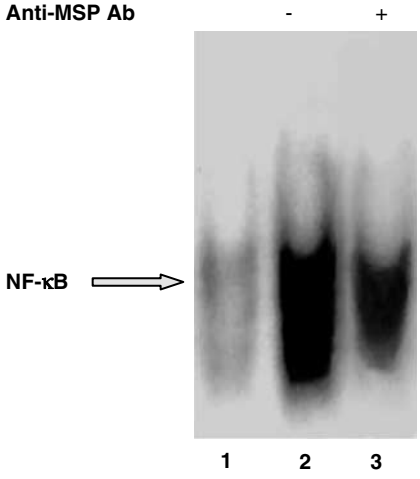

Figure 5 MSP evokes NF-κB activation in human MDM from healthy non-smokers. MDM from healthy non-smokers were challenged with MSP in the absence or presence of a monoclonal anti-MSP antibody ($2\,\mu g\,ml^{-1}$). Lane 1 = unstimulated, control MDM; lane 2 = MSP $100\,ng\,ml^{-1}$; lane 3: MSP + anti-MSP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

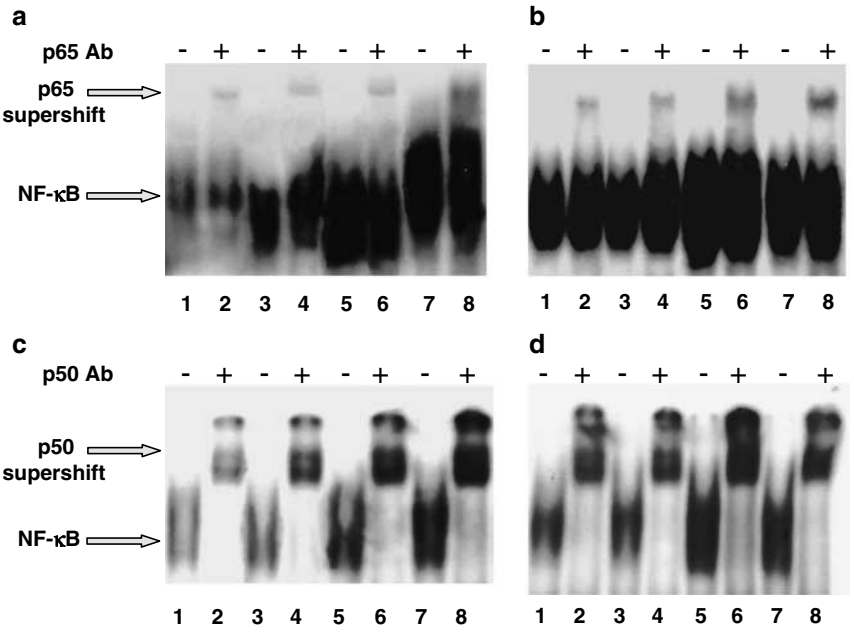

Figure 6 MSP evokes NF-κB activation in human MDM from healthy non-smokers and smokers: supershift assays. MDM from healthy non-smokers (a and c) and smokers (b and d) were challenged with MSP (50 and 100 ng ml^{-1}) or LPS 500 ng ml^{-1} for 2 h. Nuclear extracts (5 μg) were prepared and assayed for 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.

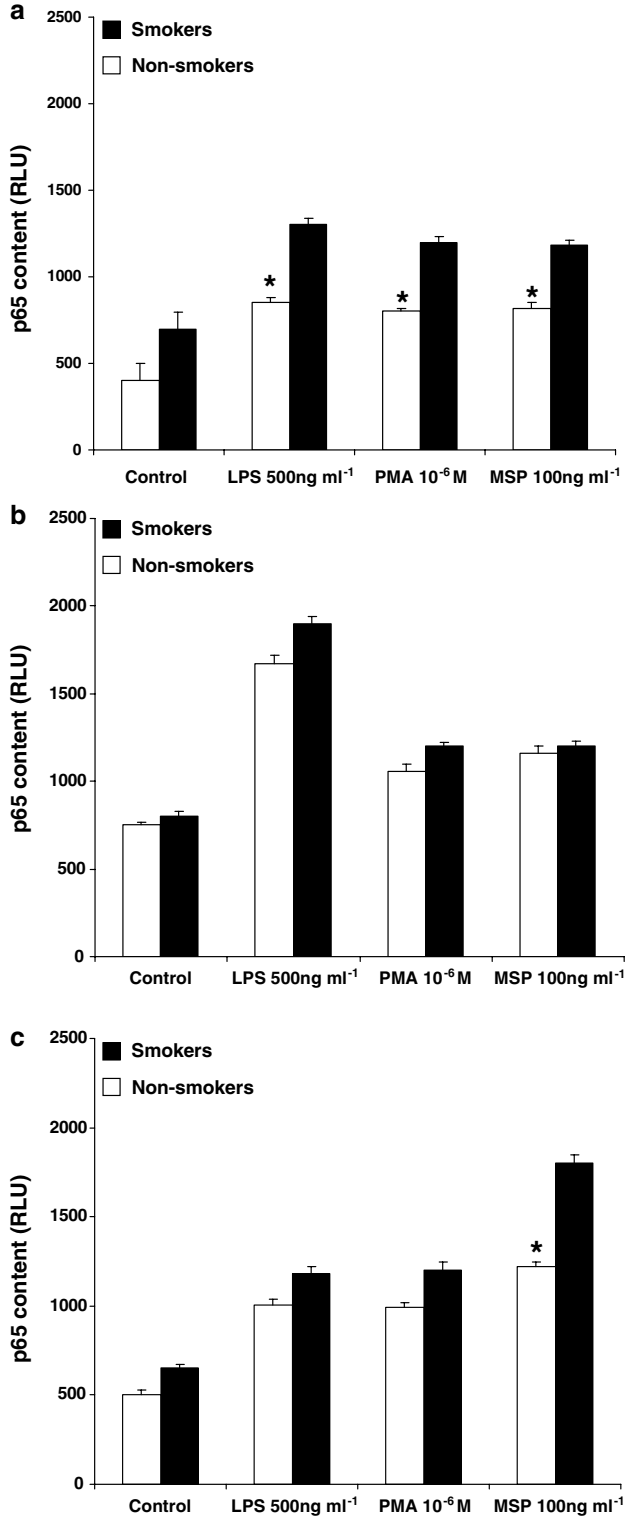

Figure 7 MSP induces the translocation of p65 subunit in AM. AM from healthy smokers and non-smokers (a), patients with sarcoidosis (b) and idiopathic pulmonary fibrosis (c) were challenged with MSP 100 ng ml^{-1}, PMA 10^{-6} M or LPS 500 ng ml^{-1} for 2 h. Nuclear extracts (1 μg) were prepared and 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.

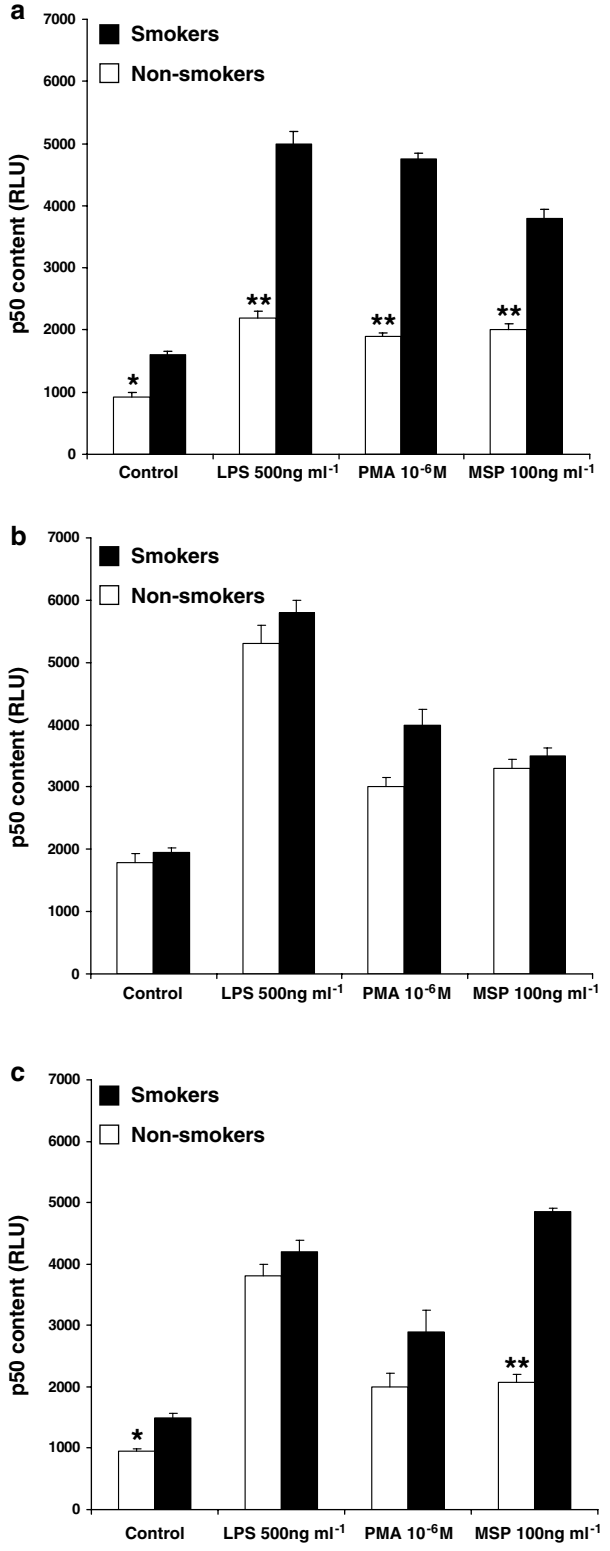

Figure 8 MSP induces the translocation of p50 subunit in AM. AM from healthy smokers and non-smokers (a), patients with sarcoidosis (b) and idiopathic pulmonary fibrosis (c) were challenged with MSP 100 ng ml^{-1}, PMA 10^{-6} M or LPS 500 ng ml^{-1} for 2 h. Nuclear extracts (1 μg) were prepared and 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.

Research described in this article was supported by Philip Morris U.S.A. Inc. and by Philip Morris International grants.

References

ALBINA, J.E. (1995). On the expression of nitric oxide synthase by human macrophages. Why no NO? *J. Leukoc. Biol.,* **58,** 643–649.

BALDWIN, A.S. (1996). The NF-κB and Iκ-B proteins: new discoveries and insights. *Annu. Rev. Immunol.,* **14,** 649–681.

BARDELLI, C., GUNELLA, G., VARSALDI, F., BALBO, P., DEL BOCA, E., SEREN BERNARDONE, I., AMORUSO, A. & BRUNELLESCHI, S. (2005). Expression of functional NK_1 receptors in human alveolar macrophages: superoxide anion production, cytokine release and involvement of NF-κB pathway. *Br. J. Pharmacol.,* **145,** 385–396.

BOWIE, A. & O'NEILL, L.A. (2000). Oxidative stress and nuclear factor-kappaB activation: a reassessment of the evidence in the light of recent discoveries. *Biochem. Pharmacol.,* **59,** 13–23.

BRUNELLESCHI, S., PENENGO, L., LAVAGNO, L., SANTORO, C., COLANGELO, D., VIANO, I. & GAUDINO, G. (2001). Macrophage stimulating protein (MSP) evokes superoxide anion production by human macrophages of different origin. *Br. J. Pharmacol.,* **134,** 1285–1295.

CARTER, A.B., MONICK, M.M. & HUNNINGHAKE, G.W. (1998). Lipopolysaccharide-induced NF-κB activation and cytokine release in human alveolar macrophages is PKC-independent and TK- and PC-PLC-dependent. *Am. J. Resp. Cell. Mol. Biol.,* **18,** 384–391.

CHEN, Y.Q., FISHER, J.H. & WANG, M.H. (1998). Activation of the RON receptor tyrosine kinase inhibits inducible nitric oxide synthase (iNOS) expression by murine peritoneal exudates macrophages: phosphatidylinositol-3 kinase is required for RON-mediated inhibition of iNOS expression. *J. Immunol.,* **161,** 4950–4959.

CONRON, M., ANDREAKOS, E., PANTELIDIS, P., SMITH, C., BEYNON, H.L.C., DUBOIS, R.M. & FOXWELL, B.M.J. (2002). Nuclear Factor-κB activation in alveolar macrophages requires IκB kinase-β, but not nuclear factor-κB inducing kinase. *Am. J. Resp. Crit. Care Med.,* **165,** 996–1004.

CONRON, M., BONDESON, J., PANTEDELIS, P., BEYNON, H.L.C., FELDMAN, M., DUBOIS, R.M. & FOXWELL, B.M.J. (2001). Alveolar macrophages and T cells from sarcoid, but not normal lung, are permissive to adenovirus infection and allow analysis of NF-κB-dependent signalling pathways. *Am. J. Resp. Cell Mol. Biol.,* **25,** 141–149.

CORRELL, P.H., IWAMA, S., TONDAT, G., MAYRHOFER, G., SUDA, T. & BERNSTEIN, A. (1997). Deregulated inflammatory response in mice lacking the STK/RON receptor tyrosine kinase. *Genes Funct,* **1,** 69–73.

CULVER, D.A., BARNA, B.P., RAYCHAUDHURI, B., BONFIELD, T.L., ABRAHAM, S., MALUR, A., FARVER, C.F., KAVURU, M.S. & THOMASSEN, M.J. (2004). Peroxisome proliferator-activated receptor γ activity is deficient in alveolar macrophages in pulmonary sarcoidosis. *Am. J. Resp. Cell. Mol. Biol.,* **30,** 1–5.

FARVER, C.F., RAYCHAUDHURI, B., BUHROW, L.T., CONNORS, M.J. & THOMASSEN, M.J. (1998). Constitutive NF-κB levels in human alveolar macrophages from normal volunteers. *Cytokine,* **10,** 868–871.

HOJO, S., FUJITA, J., YOSHINOU0CHI, T., YAMANOUCHI, H., KAMEI, T., YAMADORI, I., OTSUKI, Y., UEDA, N. & TAKAHARA, T. (1997). Hepatocyte growth factor and neutrophil elastase in idiopathic pulmonary fibrosis. *Resp. Med.,* **91,** 511–516.

IWAMA, A., WANG, M.H., YAMAGUCHI, N., OHNO, N., OKANO, K., SUDO, T, TAKEYA, M., GERVAIS, F., MORISETTE, C, LEONARD, E.J. & SUDA, T. (1995). Terminal differentiation of murine resident peritoneal macrophages is characterized by expression of the STK protein tyrosine kinase, a receptor for macrophage stimulating protein. *Blood,* **86,** 3394–3403.

KHALIL, N. & O'CONNOR, R. (2004). Idiopathic pulmonary fibrosis: current understanding of the pathogenesis and the status of treatment. *CMAJ,* **171,** 153–160.

KREIN, P.M. & WINSTON, B.W. (2002). Roles for insulin-like growth factor I and transforming growth factor-β in fibrotic lung disease. *Chest,* **122,** 289S–293S.

LEONARD, E.J. & SKEEL, A. (1976). A serum protein that stimulates macrophage movement, chemotaxis and spreading. *Exp. Cell Res.,* **102,** 434–438.

LIU, Q.P., FRUIT, K., WARD, J. & CORRELL, P.H. (1999). Negative regulation of macrophage activation in response to IFN-γ and lipopolysaccharide by the STK/RON receptor tyrosine kinase. *J. Immunol.,* **163,** 6606–6613.

MARCHAND-ADAM, S., MARCHAL, J., COHEN, M., SOLER, P., GERARD, B., CASTIER, Y., LESECHE, G., VALEYRE, D., MAL, H., AUBIER, M., DEHOUX, M. & CRESTANI, B. (2003). Defect of hepatocyte growth factor secretion by fibroblasts in idiopathic pulmonary fibrosis. *Am. J. Resp. Crit. Care Med.,* **168,** 1156–1161.

RAYCHAUDHURI, B., FISHER, C.J., FARVER, C.F., MALUR, A., DRAZBA, J., KAVURU, M.S. & THOMASSEN, M.J. (2000). Interleukin 10 (IL-10)-mediated inhibition of inflammatory cytokine production by human alveolar macrophages. *Cytokine,* **12,** 1348–1355.

SAKAI, T., SATOH, K., MATSUSHIMA, K., SHINDO, S., ABE, S., ABE, T., MOTOMIYA, M., KAWAMOTO, T., KAWABATA, Y., NAKAMURA, T. & NUKIWA, T. (1997). Hepatocyte growth factor in bronchoalveolar lavage fluids and cells in patients with inflammatory chest diseases of the lower respiratory tract: detection by RIA and *in situ* hybridization. *Am. J. Resp. Cell. Mol. Biol.,* **16,** 388–397.

SAKAMOTO, O., IWAMA, A., AMITANI, R., TAKEHARA, T., YAMAGUCHI, N., YAMAMOTO, T., MASUYAMA, K., YAMANAKA, T., ANDO, M. & SUDA, T. (1997). Role of macrophage-stimulating protein and its receptor, RON tyrosine kinase, in ciliary motility. *J. Clin. Invest.,* **99,** 701–709.

SCHWARTZ, M.D., MOORE, E.E., MOORE, F.A., SHENKAR, R., MOINE, P., HAENEL, J.B. & ABRAHAM, E. (1996). Nuclear factor-kappa B is activated in alveolar macrophages from patients with acute respiratory distress syndrome. *Crit. Care Med.,* **24,** 1285–1292.

TAKANO, Y., SAKAMOTO, O., SUGA, M., SUDA, T. & ANDO, M. (2000). Elevated levels of macrophage-stimulating protein in induced sputum of patients with bronchiectasis. *Resp. Med.,* **94,** 784–790.

WALTZ, S.E., TOMS, C.L., MC DOWELL, S.A., CLAY, L.A., MURAOKA, R.S., AIR, E.L., SUN, W.Y., THOMAS, M.B. & DEGEN, S.J. (1998). Characterization of the mouse Ron/Stk receptor tyrosine kinase gene. *Oncogene,* **16,** 27–42.

WANG, M.H., JULIAN, F.M., BREATHNAC, R., GODOWSKI, P.J., TAKEHARA, T., YOSHIKAWA, W., HAGIYA, M. & LEONARD, E.J. (1997). Macrophage stimulating protein (MSP) binds to its receptor via the MSP β chain. *J. Biol. Chem.,* **272,** 16999–17004.

WANG, M.H., ZHOU, Y.Q. & CHEN, Y.Q. (2002). Macrophage stimulating protein and RON receptor tyrosine kinase: potential regulators of macrophage inflammatory activities. *Scand. J. Immunol.,* **56,** 545–553.

WILLETT, C.G., SMITH, D.I., SHRIDHAR, V., WANG, M.H., EMANUEL, R.L., PATIDAR, K., GRAHAM, S.A., ZHANG, F., HATCH, V., SUGARBAKER, D.J. & SUNDAY, M.E. (1997). Differential screening of a human chromosome 3 library identifies hepatocyte growth factor-like/macrophage-stimulating protein and its receptors in injured lung. Possible implications for neuroendocrine cell survival. *J. Clin. Invest.,* **99,** 2979–2991.

WILLETT, C.G., WANG, M.H., EMANUEL, R.L., GRAHAM, S.A., SMITH, D.I., SHRIDHAR, V., SUGARBAKER, D.J. & SUNDAY, M.E. (1998). Macrophage-stimulating protein and its receptor in non-small-cell lung tumors: induction of receptor tyrosine phosphorylation and cell migration. *Am. J. Resp. Cell. Mol. Biol.,* **18,** 489–496.

YAMANOUCHI, H., FUJITA, J., YOSHINOUCHI, T., HOJO, S., KAMEI, T., YAMADORI, I., OHTSUKI, Y., UEDA, N. & TAKAHARA, J. (1998). Measurement of hepatocyte growth factor in serum and bronchoalveolar lavage fluid in patients with pulmonary fibrosis. *Respir. Med.,* **92,** 273–278.

ZHOU, Y.Q., CHEN, Y.Q., FISHER, J.H. & WANG, M.H. (2002). Activation of the RON receptor tyrosine kinase by macrophage stimulating protein inhibits inducible cyclooxygenase-2 expression in murine macrophages. *J. Biol. Chem.,* **277,** 38104–38110.

ZIEGENHAGEN, M.W. & MULLER-QUERNHEIM, J.M. (2003). The cytokine network in sarcoidosis and its clinical relevance. *J. Intern. Med.,* **253,** 18–30.

(Received November 21, 2005
Revised January 18, 2006
Accepted March 16, 2006
Published online 24 April 2006)