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Effect of smoking on MAP kinase-induced modulation of IL-8 in human alveolar macrophages

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ABSTRACT: Inflammatory cytokine production by alveolar macrophages (AMs) is regulated by transcriptional activation and may be increased by cigarette smoking.

The smoking-induced regulation of interleukin (IL)-8 by extracellular signal-regulated kinase (ERK)-1 and -2, p38 mitogen-activated protein kinase (MAPK) and the transcription factor nuclear factor-κB (NF-κB) in lipopolysaccharide-stimulated AMs was assessed in nine smokers compared with nine healthy nonsmokers.

IL-8 production was dependent on phosphorylation of ERK-1 and -2 and p38 MAPK, as examined by PD 098059 (10 μM), an inhibitor of the upstream activator of MAPK kinase (MKK)-1, and SB 203580 (10 μM), an inhibitor of p38 MAPK. IL-8 release and the inhibitory effect of PD 098059 were increased in AMs from smokers. Moreover, ERK-2 messenger ribonucleic acid expression, as examined by reverse transcriptase polymerase chain reaction and phosphorylation of ERK-2 using Western blots, were increased in AMs from smokers, indicating a smoking-induced modulatory role of ERK-1 and -2. Lipopolysaccharide-induced IL-8 production was dependent on activation of NF-κB, as examined by SN 50 (100 μM), an inhibitor of NF-κB translocation, and the specific NF-κB inhibitor kinase-2 inhibitor, AS 602868 (10 μM), with no differences in AMs from smokers and nonsmokers. SN 50 but not PD 098059 and SB 203580 blocked NF-κB deoxyribonucleic acid-binding, and this occurred to the same extent in AMs from smokers and nonsmokers, as examined by electromobility shift assay.

It is concluded that cigarette smoking enhances mitogen-activated protein kinase activation more than nuclear factor-κB activation to increase lipopolysaccharide-induced interleukin-8 production in alveolar macrophages.

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Alveolar macrophages (AMs) play a pivotal role in the regulation of immune and inflammatory events [1, 2] and airway remodelling [3, 4] in chronic respiratory diseases such as asthma [5] and smoking-induced chronic obstructive pulmonary disease (COPD) [6, 7]. This includes the capacity of phagocytosis, production of inflammatory mediators such as arachidonate metabolites (*e.g.* leukotriene B_4 and prostaglandin E_2), nitric oxide and reactive oxygen metabolites and enzymes (elastase and metalloproteinases), and the expression of a variety of inflammatory cytokines such as interleukin (IL)-1, -2, -4, -6 and -8, tumour necrosis factor-α (TNF-α), interferon gamma and granulocyte-macrophage colony-stimulating factor (GM-CSF) [8]. Furthermore, AMs collaborate with lymphocytes in generating specific immune responses, including antigen presentation [9]. A variety of morphological, physiological, biochemical and enzymatic changes can be observed in AMs from cigarette smokers [10–14]. Increased numbers of AMs and neutrophils have been demonstrated in bronchoalveolar lavage (BAL) fluid (BALF) from cigarette smokers with normal lung function and smokers with COPD [6]. It has been proposed that the influx of these inflammatory cells occurs because of chemotactic factors generated in the lung in response to cigarette smoke [15].

IL-8, a member of the CXC chemokine family, activates adhesion molecule expression on endothelial cells [16] and is an important activator and chemoattractant for neutrophils [17, 18], T-cells [19] and monocytes [20]. In the dog, intratracheal instillation of IL-8 *in vivo* causes the migration of neutrophils to bronchial tissue and into the luminal space [18]. Increased levels of IL-8 have been found in induced sputum [21] and BALF from patients with smoking-related COPD associated with increased numbers of activated neutrophils and eosinophils [6] and increased colocalisation with T-lymphocytes predominantly of type-1 T-helper cell/type-1 cytotoxic T-cell phenotype [22, 23]. Therefore, IL-8 has been implicated in the initiation and maintenance of chronic airway inflammation, possibly *via* activation of the transcription factor nuclear factor-κB (NF-κB) [24]. There is evidence that cigarette smoke may induce infiltration of neutrophils into the airways through NF-κB and IL-8 gene expression in AMs [25]. These mechanisms include smoking-induced

differences in surface expression of a subset of cell adhesion molecules (intercellular adhesion molecule-1(ICAM-1), vascular cell adhesion molecule-1(VCAM-1) and endothelial leukocyte adhesion molecule-1(ELAM-1)), associated with an increase in the binding activity of NF-κB to the consensus motif common to cell adhesion molecule genes in monocytes [25] and heightened binding of NF-κB to specific DNA sequences by dysregulated activation of protein kinase C in endothelial cells [25].

Transcriptional regulation of inflammatory genes, such as that encoding IL-8, in response to extracellular stimuli is achieved by a variety of different signal transduction mechanisms. One of the most extensively studied signalling pathways into the nucleus involves the mitogen-activated protein kinase (MAPK) cascade [26]. At present, there are at least three MAPK pathways, defined according to the MAPK activated: 1) the extracellular signal-regulated kinase (ERK)-1 and -2 pathway; 2) the c-Jun N-terminal kinase pathway; and 3) the p38 MAPK pathway. These are activated by different stimuli, including growth factors, cytokines, stress, pharmacological agonists and endotoxins such as lipopolysaccharide (LPS) [27]. In the present study, the potential role of these protein kinases in modulating NF-κB-dependent LPS-induced expression of IL-8 was examined. In addition, since cigarette smoking may modify these signalling pathways, it was also examined whether this regulatory role is different in AMs from cigarette smokers.

Methods

Subjects

Nine normal nonatopic nonsmoking volunteers (six males/three females) and nine normal nonatopic smoking subjects (seven males/two females; cigarette consumption $\geqslant$10 pack-yrs) who had no history of asthma or any respiratory disease, with normal lung function and airway responsiveness to methacholine (provocative concentration of methacholine causing a 20% fall in forced expiratory volume in one second of >16 mg·mL^{-1}), were included in the present study (table 1). All volunteers underwent fibreoptic bronchoscopy and BAL after giving written informed consent. The study was approved by the ethics committees of the Royal Brompton Hospital (London, UK) and the University of Cologne (Cologne, Germany).

Table 1. – Characteristics of nonsmoking and smoking subjects and BALF cells

	Nonsmokers	Smokers
Females/males	3/6	2/7
Age yrs	27.2±2.4	28.8±2.8
FEV1 % pred	102.6±4.2	92.9±4.6
Smoking pack-yrs	0	11.2±0.3
BALF		
Volume recovered mL	54.5±4.7	56.8±3.8
Inflammatory cells 10^3 cells·mL BALF^{-1}	31.8±3.6	79.9±9.4
Macrophages %	90.0±2.3	95.9±0.5
Neutrophils %	3.2±0.8	1.7±0.2
Eosinophils %	0.9±0.1	0.9±0.2
Lymphocytes %	3.9±1.7	1.4±0.5

Data are presented as mean±SEM. FEV1: forced expiratory volume in one second.

Fibreoptic bronchoscopy

After an overnight fast, subjects were sedated with intravenous midazolam (5–10 mg). Oxygen (3 L·min^{-1}) was administered *via* nasal prongs, and oxygen saturation was monitored by digital pulse oximetry. Using local anaesthesia with lidocaine (2% weight (w)/volume (v)) to the upper airways and larynx, a fibreoptic bronchoscope (Olympus BF10; Olympus, Hamburg, Germany) was passed through the nasal passages into the trachea. The bronchoscope was wedged in the right middle lobe and four 60-mL aliquots of prewarmed sterile 0.9% NaCl were instilled. This solution was aspirated through the bronchoscope, collected in prechilled bottles and stored on ice. BALF recovery was 120–200 mL.

Separation of alveolar macrophages from BALF

The BALF was filtered through sterile gauze to exclude mucus plugs and then centrifuged for 10 min at 1,000x*g* at 4°C to obtain a cell pellet. The cell pellet was washed once in 50 mL Ca^{2+}/Mg^{2+}-free Hank's balanced salt solution (HBSS; Sigma, Munich, Germany, and Welwyn Garden City, UK). The cells were counted using a haemocytometer (Neubauer chamber) slide and a Kimura counterstain and viability assessed by trypan blue exclusion. Cytospins were performed using 2.5x10^4 cells per slide, and stained with May-Grünwald Giemsa in order to obtain differential cell counts. The remaining cells were resuspended in Roswell Park Memorial Institute (RPMI; Sigma) 1640 medium supplemented with 10% (v/v) heat-inactivated foetal calf serum (Sigma), 2 mM L-glutamine (Sigma), 100 U·mL^{-1} penicillin (Sigma) and 100 µg·mL^{-1} streptomycin (Sigma). AMs were plated at 3x10^6 cells·well^{-1} on to six-well plates (for electrophoretic mobility shift assay (EMSA) and Western blotting) and at 1x10^6 cells·well^{-1} on to 12-well plates (for enzyme-linked immunosorbent assay (ELISA)) and allowed to adhere for a minimum of 3 h in a humidified incubator in 95% air/5% carbon dioxide (v/v) at 37°C. Nonadherent cells were removed by washing with RPMI 1640, leaving the adherent AMs, which were >99% pure, as assessed by staining and morphological analysis. The AMs were either harvested with a cell scraper or stimulated, cultured and then recovered. Cells were >85% viable, as assessed by trypan blue exclusion.

Cell culture

AMs were cultured in RPMI 1640 supplemented with 10% (v/v) heat-inactivated foetal calf serum, 2 mM L-glutamine, 100 U·mL^{-1} penicillin and 100 µg·mL^{-1} streptomycin in a humidified atmosphere containing 5% CO_2 at 37°C. After 12 h, cells were stimulated in fresh RPMI 1640 supplemented as above at 1x10^6 cells·mL^{-1} for ELISA for 24 h and with RPMI 1640 in the absence of foetal calf serum for EMSA and Western blotting for 1 h. The ERK-1/-2 inhibitor PD 098059 (Calbiochem-NovaBiochem UK Ltd, Nottingham, UK; 10 µM), p38 MAPK inhibitor SB 203580 (Calbiochem-NovaBiochem UK Ltd; 10 µM), NF-κB inhibitor SN 50 (BioMol, Hamburg, Germany; 100 µM) and NF-κB inhibitor (IκB) kinase (IKK)-2 inhibitor AS 602868 (Serono, Basel, Switzerland; 1 and 10 µM) [28] were added 30 min before stimulation with LPS in different concentrations and in a time-dependent manner. Drugs were dissolved in dimethyl sulphoxide (DMSO) (PD 098059 and SB 203580) or distilled water diluted to final concentrations of <0.1% (v/v). DMSO

had no effect on activation of NF-κB or cytokine transcription or expression (data not shown).

Preparation of cytoplasmic and nuclear extracts

Extracts were prepared from AMs according to the method of OSBORN *et al.* [29]. Cells were washed twice with ice-cold HBSS before resuspension in 10 mM hydroxyethylpiperazine ethanesulphonic acid (HEPES; pH 7.9) containing 10 mM KCl, 1.5 mM $MgCl_2$, 0.1% v/v Nonidet P40, 0.5 mM phenylmethylsulphonyl fluoride (PMSF) and 1 mM dithiothreitol (DTT) (buffer A). After a 2-min incubation on ice, nuclei were separated by centrifugation for 10 min at 1000x*g*. Supernatants (cytoplasmic extracts) were retained. For EMSA, nuclei were resuspended in 20 mM HEPES (pH 7.9) containing 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM ethylenediamine tetra-acetic acid (EDTA), 25% v/v glycerol, 0.5 mM PMSF and 1 mM DTT (buffer B) and incubated for 60 min on ice with vigorous mixing. Nuclear debris was removed by centrifugation, and supernatants (nuclear extracts) were diluted four-fold in 20 mM HEPES (pH 7.9) containing 50 mM KCl, 0.2 mM EDTA, 0.5 mM PMSF and 1 mM DTT (buffer C).

Electrophoretic mobility shift assay

Nuclear proteins (5 μg) were used in binding reactions as originally described by OSBORN *et al.* [29]. Double-stranded oligonucleotides encoding the consensus target sequence of NF-κB (5′-AGTTGAGGGGACTTTCCCAGG-3′) were end-labelled using [γ-^{32}P]ATP and T4 polynucleotide kinase. Nuclear proteins from each sample were incubated with 5 μL binding buffer (20% v/v glycerol, 250 mM NaCl, 2.5 mM EDTA, 2.5 mM DTT, 5 mM $MgCl_2$ and 0.4 mg·mL^{-1} sonicated salmon sperm DNA in 50 mM tris(hydroxymethyl)aminomethane (Tris)-HCl (pH 7.5)) for 20 min on ice. Radiolabelled oligonucleotide (0.0175 pmol) was then added and the volume made up to 25 μL with buffer C. This was incubated for 45 min on ice. Specificity was determined by the prior addition of a 100-fold excess of unlabelled competitor consensus oligonucleotide. In order to confirm the identity of the components of the retarded complexes, supershift experiments were conducted, using antibodies directed against the human p50 subunit of NF-κB (Promega, Southampton, UK) at 0.4 μg·mL^{-1} 30 min before the addition of radiolabelled oligonucleotide. Reaction products were separated on 6% nondenaturing polyacrylamide gels in 0.25x1 M Tris-buffered ethyleneglycol tetra-acetic acid (EGTA; 0.5 M) (pH 8.0). Gels were vacuum-dried, and protein/DNA complexes were visualised by autoradiography at -70°C using Kodak X-OMAT film (Protein Databases Inc., New York, NY, USA). The retarded bands were quantified by laser densitometry. Band density measurements were expressed as the amount of NF-κB per microgram of protein loaded.

Enzyme-linked immunosorbent assay for IL-8

IL-8 was assayed using a quantitative sandwich enzyme immunoassay technique. A commercially available kit was used (DuoSet; R&D Systems, Abingdon, UK). Briefly, 100 μL mouse monoclonal antihuman IL-8 (4 μg·mL^{-1}) was coated on to each well of a 96-well microtitre plate (Nunc, Inc., Wiesbaden, Germany) and incubated overnight at ~25°C. After blocking for 1 h with phosphate-buffered saline (pH 7.4) containing 0.05% Tween 20® and three washes, standards and samples were added and the plate incubated for 2 h. Biotinylated goat antihuman IL-8 (20 ng·mL^{-1}) was added to the wells to sandwich immobilised IL-8. Addition of horse radish peroxidase-conjugated streptavidin (1:200 dilution of 1.25 mg·mL^{-1} solution) and a stabilised chromogen and hydrogen peroxide (R&D Systems) permitted colour development in proportion to the amount of IL-8 present, assayed by measurement of optical density using a spectrophotometer set to 450 nm with wavelength correction at 540 nm and quantified by interpolation from a standard curve. The lower limit of detection was 15.6 pg·mL^{-1}.

Sodium dodecyl sulphate-gel electrophoresis and Western blotting

The activation status of ERK-1 and -2 was assessed by Western immunoblot analysis using antibodies that recognise the dually phosphorylated (activated) form of the enzymes, as previously described [30]. Denatured proteins (40 μg) were subsequently separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis on 10% vertical slab gels and transferred to Hybond electrochemoluminescence (ECL) membranes (Amersham Biosciences, Freiburg, Germany). The nitrocellulose was incubated for 1 h at ~25°C in 25 mM Tris-buffered saline (TBS; pH 7.6; 150 mM NaCl) containing 0.1% Tween 20 (TBS-Tween 20) and 10% w/v nonfat milk and then for 2 h in TBS-Tween 20 containing 3% bovine serum albumin and primary antibodies directed against either phospho-ERK-1 and -2 (New England Biolabs, UK Ltd, Hitchin, UK) or β-actin (Santa Cruz Biotechnology/Autgen Bioclear, Calne, UK) (diluted 1:1,000). Following three 10-min washes in TBS-Tween 20, the membranes were incubated for 60 at ~25°C min with goat antirabbit peroxidase-conjugated immunoglobulin (Ig) G (Dako, Glostrup, Denmark) diluted 1:5,000 in TBS-Tween 20 supplemented with 1% nonfat milk for ERK-1 and -2, or for 60 min with rabbit antimouse peroxidase-conjugated IgG (Dako) diluted 1:4,000 in TBS-Tween 20 supplemented with 5% nonfat milk for β-actin, and subsequently visualised by ECL. The relevant bands were quantified by laser-scanning densitometry and normalised to the housekeeping protein β-actin.

Reverse transcriptase polymerase chain reaction for total ERK-2 and reduced glyceraldehyde-3-phosphate dehydrogenase mRNA expression

RNA was extracted using the Qiagen kit (RNeasy Mini kit; Qiagen, Hilden, Germany) according to the manufacturer's instructions, and purity assessed spectrophotometrically. RNA was denatured for 10 min at 65°C. Reverse transcription of 1 μg total RNA was performed using avian myeloblastosis virus (AMV) reverse transcriptase (RT; 15 U); deoxyadenosine triphosphate (dATP), deoxycytidine triphosphate (dCTP), deoxyguanosine triphosphate (dGTP) and deoxythymidine triphosphate (dTTP) (each 10 mM); oligo-dT15 primer (0.2 μg); ribonuclease inhibitor (30 U) and 5xAMV buffer in a total volume of 10 μL (Promega). The remaining ingredients were then added as a master mix and the samples incubated for 60 min at 42°C followed by 4 min at 90°C. The complementary DNA (cDNA) was subsequently diluted to a final volume of 60 μL in nuclease-free water. For PCR, 5 μL cDNA solutions were used. PCR was performed using forward and reverse primers (each 0.5 μg·μL^{-1}); dATP, dGTP, dTTP and dCTP (each 10 mM); *Taq* polymerase (0.5 U) and buffer A in a final volume of 20 μL (Promega) for

23 cycles for total ERK-2 mRNA and 24 cycles for reduced glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA, for 30 s at a denaturing temperature of 94°C, 30 s at a specific annealing temperature of 62°C for total ERK-2 mRNA and 60°C for GAPDH mRNA, and 30 s at an extension temperature of 72°C. The primers for total ERK-2 mRNA were 5′-GAGCACCAGACCTACTGCCAG-3′ (forward) and 5′-AATTTCTGGAGCCCTGTACCA-3′ (reverse), giving a product of 416 base pairs. The primers for GAPDH mRNA were 5′-TCTAGACGGCAGGCTAGGTCCACC-3′ and 5′-ACGGTACCTTAAACGGTACCCACC-3′, giving a product of 598 base pairs. PCR was carried out in a Techne multiwell thermocycler (Techne, Cambridge, UK). The number of cycles was chosen after determination of the linear phase of the product amplification curve from serial sampling with increasing numbers of cycles of amplification. Products were distinguished by electrophoresis on a 2% agarose ethidium bromide-stained gel and then visualised using ultraviolet luminescence.

Lipopolysaccharide

LPS from *Salmonella enteriditis* (phenol extracted, gel filtered, chromatographically purified and gamma irradiated) was obtained from Sigma, Munich, Germany. The phenol extract contains up to 60% RNA and <1% protein. Purification by gel filtration chromatography removes much of the protein present in the phenol-extracted LPS, but leaves a product that still contains 10–20% nucleic acids. Only one batch of LPS was used for all experiments in the present study.

Data analysis

Data are presented as mean±SEM, and were analysed by paired t-test or one-way analysis of variance. Bonferroni correction for multiple comparisons was applied. The null hypothesis was rejected when $p<0.05$.

Results

Lipopolysaccharide-induced expression and release of IL-8

There was constitutive release of IL-8 from unstimulated human AMs from nonsmokers and smokers (fig. 1a). IL-8 release was significantly increased 24 h after stimulation with LPS (10 ng·mL^{-1}). Although there was no significant difference in IL-8 release when stimulated with LPS at a concentration of 10 ng·mL^{-1}, more IL-8 was obtained at the higher concentration of 1 μg·mL^{-1} from the AMs of smokers (fig. 1a). IL-8 mRNA expression, as measured by RT-PCR, was maximal 12 h after LPS stimulation at 10 ng·mL^{-1} without further increase at 1 μg·mL^{-1}, but there were no differences between smokers and nonsmokers (fig. 1b).

Lipopolysaccharide-induced phosphorylation of ERK-1 and -2

Under basal (control) conditions, phosphorylation of ERK-1 (ERK-1/β-actin ratio 0.46±0.30; n=5) and ERK-2 (ERK-2/β-actin ratio 0.47±0.15; n=5) was detectable by Western blotting (fig. 2a and b). There was significantly greater basal phosphorylation of ERK-2 (ERK-2/β-actin ratio 1.06±0.17; $p<0.05$; n=5) in AMs from smokers compared to nonsmokers. In nonsmokers, LPS (10 ng·mL^{-1}) did not cause a significant increase in phosphorylation of ERK-1 and -2 compared to control (ERK-1/β-actin ratio 0.73±0.38; ERK-2/β-actin ratio 0.82±0.29; NS; n=5). In contrast, in smokers, there was a significant increase in LPS-induced ERK-2 phosphorylation (ERK-2/β-actin ratio 3.58±0.61 in smokers; $p<0.01$ *versus* nonsmokers; n=5) (fig. 2a and b).

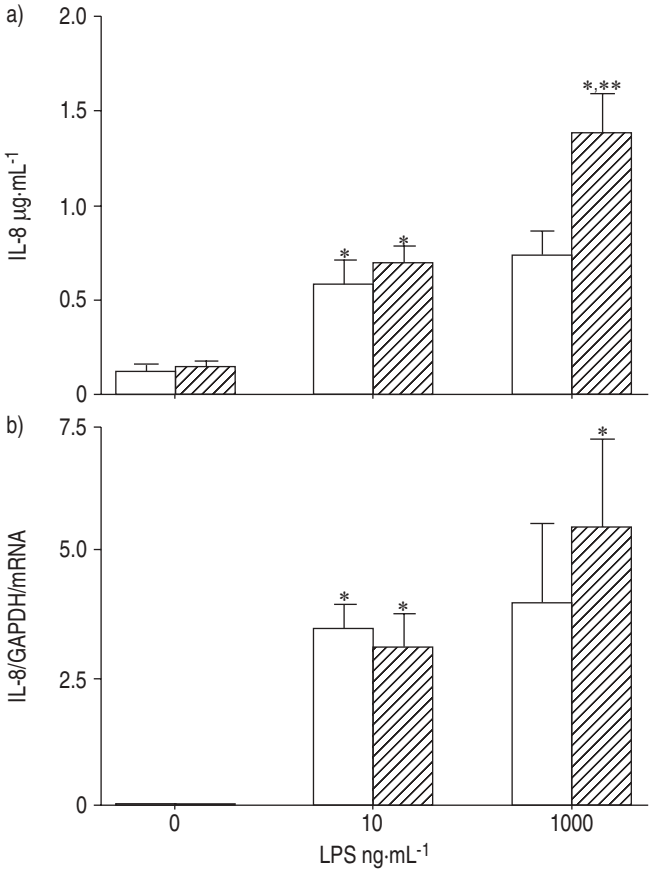

Fig. 1. – Concentration-dependence of lipopolysaccharide (LPS)-induced interleukin (IL)-8 generation by adherent human alveolar macrophages in culture: a) IL-8 generation (n=8 for both groups); and b) IL-8 mRNA expression as a ratio of reduced glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA expression (n=5 for smokers; n=4 for nonsmokers) in smoking (▨) and nonsmoking (□) subjects. Data are presented as mean±SEM. *: $p<0.05$ *versus* controls (no LPS); **: $p<0.01$ *versus* nonsmokers.

Lipopolysaccharide-induced total ERK-2 mRNA

There was a significant increase in basal (control) total ERK-2 mRNA expression in AMs from smokers (ERK-2/GAPDH: nonsmokers 0.37±0.02, n=9; smokers 2.52±0.81, $p<0.05$, n=9) using RT-PCR (fig. 2c and e). Stimulation with LPS (10 ng·mL^{-1}) did not induce further total ERK-2 mRNA expression in smokers and nonsmokers compared with controls (ERK-2/GAPDH: nonsmokers 0.34±0.03, n=4; smokers 1.97±0.71, n=9) (fig. 2g).

Role of ERK-1 and -2 and p38 MAPK in lipopolysaccharide-induced IL-8 release

PD 098059, an inhibitor of the upstream activator of ERK-1 and -2, MAPK kinase (MKK)-1, inhibited LPS

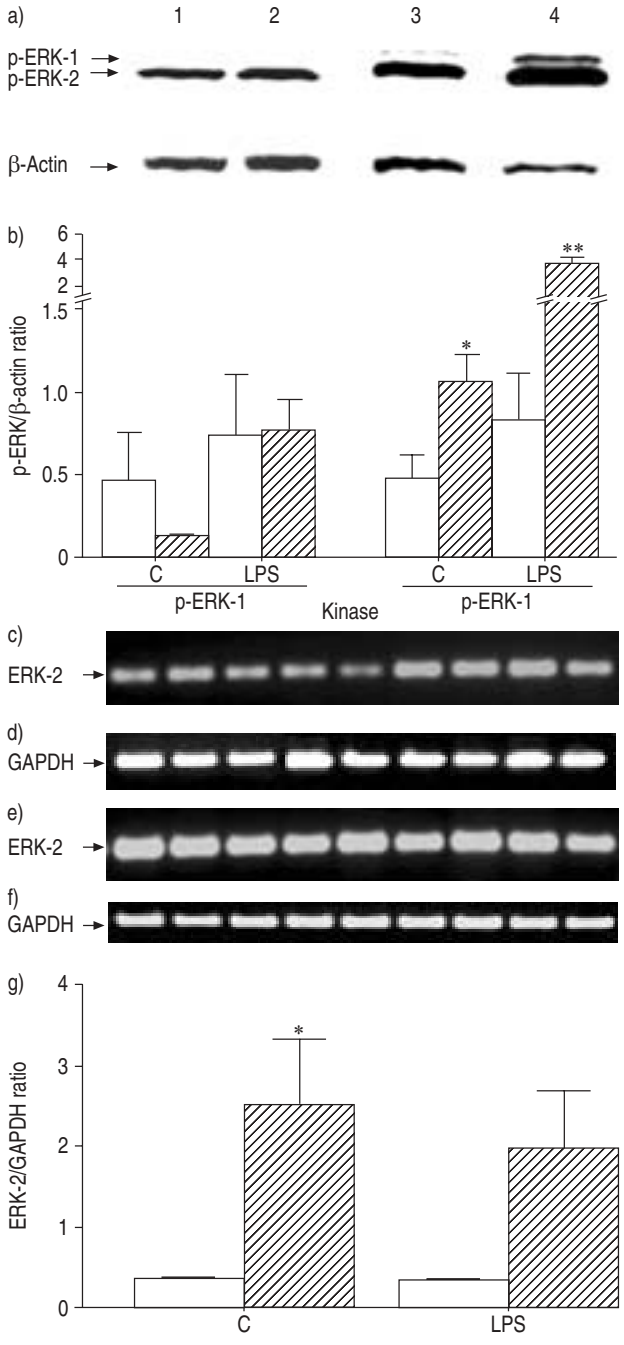

Fig. 2. – a, b) Basal (control (C)) and lipopolysaccharide (LPS; 10 ng·mL^{-1})-induced phosphorylation of extracellular signal-regulated kinase (ERK)-1 and -2 (p-ERK-1 and p-ERK-2) and β-actin expression in alveolar macrophages from smokers and nonsmokers: a) representative Western blots from nonsmokers (lanes 1 and 2) and smokers (lanes 3 and 4) in the presence (lanes 2 and 4) and absence (lanes 1 and 3) of LPS (n=1 for both groups); and b) densitometric normalisation to expression of the housekeeping protein β-actin (□: nonsmokers; ▨: smokers). Data are presented as mean±SEM (n=5 for both groups). *: p<0.05 *versus* nonsmokers; **: p<0.01 *versus* nonsmokers for LPS-induced increase in phosphorylation. c–g) Control and LPS (10 ng·mL^{-1})-stimulated total ERK-2 mRNA expression: c–f) representative reverse transcriptase PCR gels from nonsmokers (c, d) and different smokers (e, f) showing ERK-2 (c, e) and glyceraldehyde-3-phosphate dehydrogenase (d, f) (GAPDH) mRNA expression in unstimulated cultured alveolar macrophages; and g) densitometric normalisation to expression of the housekeeping protein GAPDH mRNA (□: nonsmokers; ▨: smokers). Data are presented as mean±SEM (n=9 for both groups). *: p<0.05 *versus* nonsmokers.

(10 ng·mL^{-1})-induced IL-8 release by 20.3±4.4% (n=6) (fig. 3). However, in AMs from smokers, the inhibition of IL-8 release by PD 098059 was greater (32.0±2.8; p<0.05; n=7) compared to nonsmokers (fig. 3). In contrast, inhibition of IL-8 release by SB 203580, an inhibitor of the α- and β-isoform of p38 MAPK, was not different in smokers (18.3±4.0%; n=6) compared to nonsmokers (12.6±6.8%; n=5; p=NS) (fig. 3).

Role of nuclear factor-κB in lipopolysaccharide-induced expression and generation of IL-8

SN 50, a cell-permeable peptide that contains the nuclear localisation sequence of the p50 subunit of NF-κB and inhibits subcellular traffic of NF-κB/Rel complexes from the cytoplasm to the nucleus, reduced LPS (10 ng·mL^{-1})-induced IL-8 release by 71.7±23.1% in nonsmokers and 65.0±14.6% in smokers, with no significant difference between groups (n=4) (fig. 3). This result was confirmed by the finding that the IKK-2-inhibitor, AS 602868 (10 μM), reduced LPS-induced IL-8 release by 61.3±8.1% in nonsmokers (n=3) and 77.4±2.4% in smokers (n=3), whereas, at 1 μM, AS 602868 gave only a small reduction in IL-8 release, of 25.0±4.6% in smokers and 10.6±2.5% in nonsmokers (n=3). PD 098059 and SB 203580 did not modulate the AS 602868-induced inhibition of IL-8 release in smokers and nonsmokers (n=3) (data not shown).

Effects of SN 50, PD 098059 and SB 203580 on lipopolysaccharide-induced nuclear factor-κB DNA-binding

Induction of the NF-κB/DNA complex was specific to the NF-κB consensus oligonucleotide, since an excess of unlabelled oligonucleotide prevented binding of the labelled probe. Supershift analysis was performed to identify the Rel proteins involved in these responses. In resting and LPS (10 ng·mL^{-1})-stimulated AMs, NF-κB/DNA complexes contained p50 subunits, as demonstrated by the retardation and reduction in the intensity of DNA-binding complexes caused by anti-p50 antisera, as shown recently [30]. LPS induced an

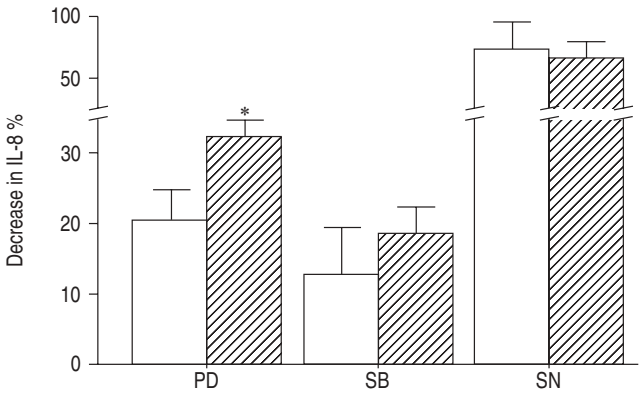

Fig. 3. – Inhibitory effect of PD 098059 (PD; 10 μM), SB 203580 (SB; 10 μM) and SN 50 (SN; 100 μM) pretreatment on maximal lipopolysaccharide (10 ng·mL^{-1})-induced interleukin (IL)-8 generation in adherent alveolar macrophages from smoking (▨; n=7) and nonsmoking (□; n=6) subjects. The amount of IL-8 released into the culture supernatant after 24 h was measured by sandwich enzyme-linked immunosorbent assay. Data are presented as mean±SEM from separate experiments. *: p<0.05 *versus* nonsmokers.

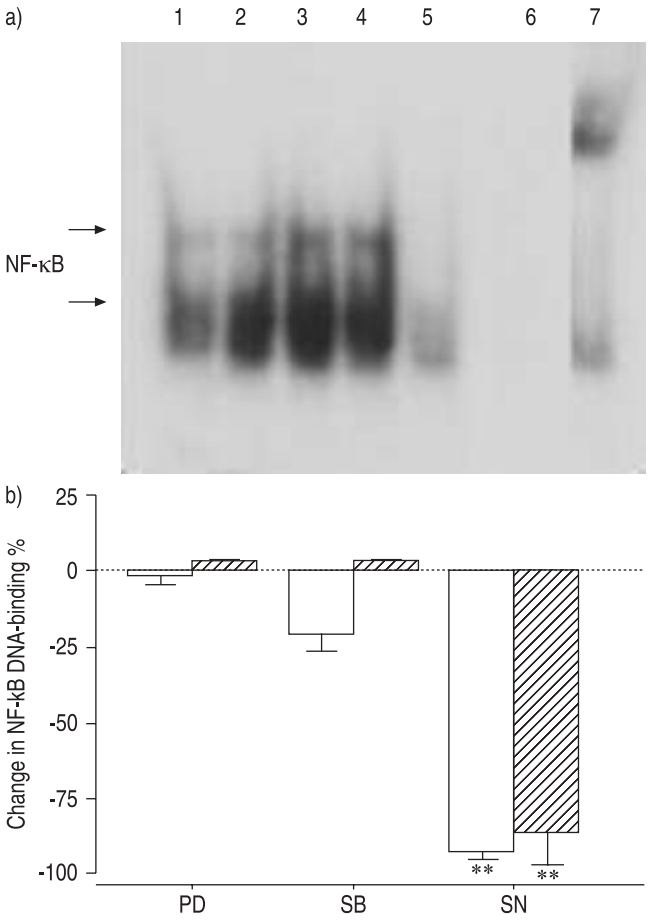

Fig. 4. – Electrophoretic mobility shift assay showing changes in maximal binding of nuclear factor-κB (NF-κB; two bands) to the DNA of nuclear protein extracts from alveolar macrophages stimulated by lipopolysaccharide (LPS; 10 ng·mL^{-1}). a) Representative autoradiogram from a nonsmoking individual demonstrating the lack of effect of PD 098059 (10 μM; lane 3) and SB 203580 (10 μM; lane 4) on NF-κB DNA-binding (lane 1: unstimulated cells; lane 2: LPS stimulation alone). In contrast, SN 50 (100 μM; lane 5) completely inhibited the binding. Supershift analysis was performed using antisera raised against the amino-terminus of the nuclear translocation sequence of p50 subunit of NF-κB (lane 7; lane 6: competition probe). b) Densitometry showing the lack of effect of PD 098059 (PD) and SB 203580 (SB) and equal inhibition of binding by SN 50 (SN) in macrophages from nonsmokers (□; n=5) and smokers (▨; n=5). Data are presented as mean±SEM from separate experiments. **: $p<0.01$ *versus* LPS alone.

increase in NF-κB: DNA-binding in nonsmokers and smokers (p=NS; n=5), without differences in the degree of inhibition of NF-κB: DNA-binding in the presence of SN 50 (91.9±3.0% in nonsmokers; 85.7±11.0% in smokers; n=5). There was no inhibitory effect of NF-κB: DNA-binding in the presence of PD 098059 and SB 203580 compared with LPS alone in smokers or nonsmokers (fig. 4).

Discussion

IL-8 was constitutively expressed in nonstimulated AMs, and there were no differences between smokers and nonsmokers. The generation of IL-8 was promoted by LPS in a concentration-dependent manner, with significantly increased release in AMs from smokers compared to nonsmokers. This finding confirms previous work showing that IL-8 release from bronchial epithelial cells from cigarette smokers is higher than that from nonsmokers [31]. Evidence is provided that activation of NF-κB and of the ERK-1/-2 and p38 MAPK pathways can induce IL-8 expression in human AMs. It is also shown that AMs from cigarette smokers produce more LPS-induced IL-8 by modulating MAPK pathways than through NF-κB signalling pathways.

In many inflammatory cells, including monocytes [32], cytokine generation induced by LPS appears to be regulated by the transcription factor NF-κB [33]. It has been shown previously that, in human AMs, LPS promotes time-dependent activation of NF-κB; the specificity of the NF-κB activation was confirmed by the finding that NF-κB/DNA binding complexes were supershifted by antibodies directed against the p50 subunit of NF-κB [30]. Furthermore, the synthetic cell-permeable peptide SN 50, which has been shown to specifically antagonise the nuclear translocation of NF-κB in a diverse variety of other cell types, including mouse LE II lung endothelial cells, fibroblasts and cells of the THP-1 human monocytic cell line [34, 35], blocked the binding of NF-κB to DNA in LPS-stimulated AMs [30]. In addition, SN 50 decreased LPS-induced IL-8 release by ~70% in AMs from nonsmokers and smokers, indicating that LPS-induced IL-8 release in AMs is mainly controlled by the transcription factor NF-κB without smoking-induced differences. This was confirmed by inhibition of IL-8 release using the IKK-2 inhibitor, AS 602868, in both groups. Interestingly MOCHIDA-NISHIMURA *et al.* [36] stimulated total BALF cells with 1 μg·mL^{-1} LPS and found that cells from smokers are more sensitive to LPS-induced degradation of IκB-α and activation of NF-κB compared to BALF cells from nonsmokers, indicating that LPS-induced NF-κB activation differs depending on cell type and LPS concentration used.

A role for the MAPKs ERK-1 and -2 and p38 MAPK in LPS-induced IL-8 generation was indicated by the ability of their specific inhibitors, PD 098059 and SB 203580 [37], to modulate the release of IL-8 [38]. The high degree of specificity of PD 098059 *in vitro* and *in vivo* is indicated by its failure to inhibit 18 protein serine/threonine kinases (including two other MAPK kinase (MAPKK) homologues) *in vitro* and by its failure to inhibit the *in vivo* activation of MAPKK and MAPK homologues that participate in stress and IL-1-stimulated kinase cascades in KB and PC12 cells, and by its lack of inhibition of the activation of p70 S6 kinase by insulin or epidermal growth factor in Swiss 3T3 cells [39]. PD 098059 inhibited the activation of MAPKK2 by Raf with a much higher median inhibitory concentration (IC_{50}) (50 μM) and did not inhibit the phosphorylation of other Raf or MAPK kinase (MEK) kinase substrates, indicating that it exerts its effect by binding to the inactive form of MAPKK1 [39]. PD 098059, which is 10–20-fold selective for MKK-1 over MKK-2, was found to suppress LPS-induced TNF-α generation from human monocytes only at a concentration (50 μM) that approaches the IC_{50} for the inhibition of other kinases such as MKK-2, and, in addition, significant inhibition of other kinases, including MKK-3 and MKK-6, can be expected [40]. Thus, at concentrations of PD 098059 of >10 μM, the greater inhibitory action might be attributable to the inhibition of an ERK-independent MKK-2 signalling pathway.

The pyridinylimidazole compound SB 203580 is known to inhibit the p38 α and β isoforms but not the γ and δ isoforms [41]. This specificity is due largely to the presence of threonine at position 106 in p38α and -β but also involves histidine 107 and leucine 108 and exhibited significant activity against the tyrosine kinases p56*lck* and *c-src* (IC_{50} 5 μM) [42]. In human monocytes, SB 203580 inhibited GM-CSF generation in a concentration-dependent manner (IC_{50} 7.31±0.13) [32]. At a concentration of 10 μM, SB 203580 reduced LPS-induced GM-CSF release by ~80%.

There was a greater, 32%, decrease in PD 098059-induced IL-8 release in smokers compared to only 20% in nonsmokers, and a nonsignificant, ~18%, decrease in SB 203580-induced IL-8 release in AMs from smokers compared with ~13% in AMs from nonsmokers, indicating that cigarette smoke increased MAPK-modulating effects in LPS-induced IL-8 expression.

One explanation for the differences in smoking-induced MAPK-modulated IL-8 release could be that cigarette smoke may lead to an increase in IL-8 production through a higher level of ERK-2 phosphorylation and activation. This was supported by the finding that smoking induced an increase in basal (2.2 times) and LPS-induced (3.4 times) phosphorylation of ERK-2 in smokers compared to nonsmoking individuals.

Another explanation for post-transcriptional MAPK-related IL-8 generation could be the fact that cigarette smoke reduces histone deacetylase (HDAC) 2 expression in AMs, as shown previously by Ito *et al.* [43]. The reduced HDAC2 activity correlated inversely with the induction of IL-8 production in AMs from smokers [43]. Repression of HDAC2 activity may lead to enhanced histone acetylation and increased post-transcriptional IL-8 expression by modulation of DNA polymerase II [44]. Increased histone acetylation causes local unwinding of DNA, allowing increased inflammatory gene expression [43].

The fact that smoking neither changed the inhibition of p50/NF-κB translocation by SN 50 nor influenced the reduction in LPS-induced IL-8 release by SN 50 or the IKK-2 inhibitor AS 602868, may indicate that smoking modulates mainly the MAPK rather than the NF-κB pathway. The observation that NF-κB DNA-binding was unaffected by the inhibitor of ERK-1 and -2, PD 098059, and the inhibitor of p38 MAPK, SB 203580, in LPS-stimulated AMs and without modulating the AS 602868-induced inhibition of IL-8 release underscores the thesis that ERK-1 and -2 and p38 MAPK do not inhibit NF-κB-driven transcription by blocking the translocation of NF-κB to the nucleus.

In conclusion, the present study demonstrates that lipopolysaccharide-induced interleukin-8 generation in human alveolar macrophages is mainly nuclear factor-κB-dependent, and is also modulated by the mitogen-activated protein kinase pathways, p38 mitogen-activated protein kinase and extracellular signal-regulated kinase-1 and -2. Furthermore, cigarette-smoke induced modulation of lipopolysaccharide-induced interleukin-8 expression predominantly occurs at the level of mitogen-activated protein kinase activation rather than through the nuclear factor-κB pathway.

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