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Diesel exhaust activates redox-sensitive transcription factors and kinases in human airways

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Pourazar, Jamshid, Ian S. Mudway, James M. Samet, Ragnberth Helleday, Anders Blomberg, Susan J. Wilson, Anthony J. Frew, Frank J. Kelly, and Thomas Sandström. Diesel exhaust activates redox-sensitive transcription factors and kinases in human airways. *Am J Physiol Lung Cell Mol Physiol* 289: L724–L730, 2005. First published March 4, 2005; doi:10.1152/ajplung.00055.2005.— Diesel exhaust (DE) is a major component of airborne particulate matter. In previous studies we have described the acute inflammatory response of the human airway to inhaled DE. This was characterized by neutrophil, mast cell, and lymphocyte infiltration into the bronchial mucosa with enhanced epithelial expression of IL-8, Gro- α , and IL-13. In the present study, we investigated whether redox-sensitive transcription factors were activated as a consequence of DE exposure, consistent with oxidative stress triggering airway inflammation. In archived biopsies from 15 healthy subjects exposed to DE [particulates with a mass median diameter of $<10 \mu\text{m}$, $300 \mu\text{g}/\text{m}^3$] and air, immunohistochemical staining was used to quantify the expression of the transcription factors NF- κB (p65) and AP-1 (c-jun and c-fos), as well their upstream MAPKs, p38 and JNK, in the bronchial epithelium. In addition, phosphorylation of tyrosine residues was examined. DE induced a significant increase in the nuclear translocation of NF- κB ($P = 0.02$), AP-1 ($P = 0.02$), phosphorylated JNK ($P = 0.04$), and phosphorylated p38 ($P = 0.01$), as well as an increase in total (cytoplasmic + nuclear) immunostaining of phosphorylated p38 ($P = 0.03$). A significant increase in nuclear phosphorylated tyrosine was also observed ($P < 0.05$). These observations demonstrate that DE activates redox-sensitive transcription factors in vivo consistent with oxidative stress triggering the increased synthesis of proinflammatory cytokines.

particulate matter

EPIDEMIOLOGICAL STUDIES HAVE DEMONSTRATED a consistent association between the exacerbations of respiratory disease and concentrations of airborne particulate matter, especially with fine particles with an aerodynamic diameter of $<2.5 \mu\text{m}$ (PM_{2.5}) (22, 23). Particles derived from diesel engines are considered to comprise a significant proportion of PM_{2.5} in urban areas, and both in vitro and in vivo studies have demonstrated that they have potent effects on the lung (20, 36). Exposure of human subjects to whole diesel exhaust (DE)

(particulates and the associated gas phase) results in an acute inflammatory response characterized by neutrophil, lymphocyte, and mast cell influx into the airways. These cellular changes are associated with upregulation of vascular endothelial adhesion molecules as well as enhanced expression of IL-8, IL-6, Gro- α , and IL-13 in the bronchial epithelium (24, 30, 31). Instillation of DE particle (DEP) extracts into the nose has also been shown to increase the production of mucosal T helper type 2 (Th2)-related cytokines in atopic volunteers (10). Similarly, bronchial epithelial and macrophage cell lines, as well as primary cultures of bronchial epithelial cells, have been shown to release a variety of chemokines and cytokines when treated with DEP or their organic extracts (2, 4, 6, 7, 14, 15, 18, 26, 37). The cellular and molecular mechanisms underlying these inflammatory responses remain unresolved.

DE-induced cytokine release requires upregulation of signaling pathways modulating cytokine gene expression. Much attention has focused on the capacity of DEP to elicit oxidative stress in the lung as the trigger for upregulating these pathways, specifically the redox-sensitive transcription factors NF- κB and activator protein-1 (AP-1). Consistent with this, addition of DEP to bronchial epithelial cell cultures has been shown to induce cytokine production associated with activation of NF- κB and AP-1 and their associated upstream MAPKs (2, 4, 6, 7, 14, 15, 18, 26, 37). Furthermore, treatment of epithelial cells and macrophages with antioxidants has been shown to reduce DEP-induced cytokine production by downregulating the activation of these signaling pathways (6, 14, 15, 18, 37).

The capacity of DEP to cause oxidation reactions in vivo has been attributed to their content of metals (20), polyaromatic hydrocarbons (PAH) (4), and quinones (33). Redox-active metals, such as iron, copper, and chromium, undergo redox cycling, generating reactive oxygen species (ROS), whereas redox-inactive metals, such as lead, cadmium, mercury, and others can deplete cellular antioxidants, particularly thiols (34), resulting in upregulation of oxidative stress-sensitive signaling pathways. The removal of metals from PM samples has been shown to reduce their proinflammatory capacity (19). The toxicity of PAH is related to their bioactivation in vivo by cytochrome P-450 1A1, leading to production of electrophilic

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and reactive metabolites, including ROS (4). Quinones are known to generate an oxidative stress through redox cycling (33) and are suspected to be responsible for the production of $O_2\cdot^-$ and $\cdot OH$ radicals detected by electron paramagnetic resonance in DEP methanol extracts (29). Furthermore, ROS have been detected in bronchial and nasal epithelial cells exposed to DEP or to organic extracts obtained from DEP (4).

In light of these observations, we hypothesized that the increased production and release of proinflammatory cytokines from the human airway after DE exposure could be attributed to the activation of redox-sensitive transcription factors (NF- κ B and AP-1) and their associated upstream stress-related MAPKs (p38 and JNK). To investigate these signaling cascades, we utilized immunohistochemical techniques to quantify the epithelial expression of these signaling molecules in human bronchial biopsies obtained 6 h after exposure to DE or filtered air.

METHODS

Subjects and exposure. The study included 15 nonatopic nonsmokers (11 males and 4 females) with a mean age of 24 years (range 21–28 years). They had normal lung function and negative skin prick tests to common airborne allergens and were free from respiratory tract infections for at least 6 wk before or during the study period. Each subject was exposed to air or DE for 1 h in a specially designed diesel exposure chamber according to earlier described standard protocols (28, 30) on two separate occasions in a randomized sequence, at least 3 wk apart. The DE was generated by using an idling Volvo diesel engine, and exposure was standardized by keeping the concentration of particulates with a mass median diameter of $<10 \mu\text{m}$ (PM_{10}) at $300 \mu\text{g}/\text{m}^3$, which was associated concentration of NO_2 of 1.6 ppm, nitric oxide (NO) of 4.5 ppm, CO of 7.5 ppm, total hydrocarbons of 4.3 ppm, formaldehyde of $0.26 \text{ mg}/\text{m}^3$, and 4.3×10^6 suspended particulates/ cm^3 (28). The subjects were unaware of the actual exposure, and during each exposure they performed moderate exercise (minute ventilation = $20 \text{ l} \cdot \text{min}^{-1} \cdot \text{m}^2$) on a bicycle ergometer alternated with rest at 15-min intervals. The study was performed according to the Declaration of Helsinki and approved by the local ethics committee. All subjects gave their written informed consent.

Bronchoscopies and processing of biopsies. Six hours after the end of exposure, bronchoscopy with endobronchial biopsy sampling was performed in alternating lungs on two occasions. Biopsies were fixed in chilled acetone containing protease inhibitors (20 mM iodoacetamide and 2 mM phenylmethylsulfonyl fluoride) at -20°C overnight (16–20 h). After fixation, biopsies were processed into glycolmethacrylate (GMA) resin as previously described (8). The GMA-embedded biopsies were stored in air-tight containers at -20°C until used for cutting and immunostaining. Biopsies from each subject obtained during the two separate bronchoscopies were processed with all the different antibodies (Table 1) in the same batch of immunostaining. Two sections from one biopsy with proper morphological structure from each subject and exposure were cut at $2\text{-}\mu\text{m}$ thickness and

placed onto poly-L-lysine-treated slides. Tris-buffered saline (TBS) with 0.3% Triton X-100 was applied for 30 min, and after being rinsed in TBS with 0.1% Triton X-100 (TBST), the endogenous peroxidases were inhibited using 0.1% sodium azide and 0.3% hydrogen peroxide in distilled water. After $3 \times 5\text{-min}$ washes in TBST, nonspecific antibody binding was blocked with undiluted culture medium (DMEM, Sigma) containing 10% fetal calf serum and 1% bovine serum albumin (BSA) for 30 min, followed by incubation with rabbit normal serum. After 30 min, the primary antibodies (Table 1) diluted in 0.05% TBST with 1% BSA were applied and incubated overnight. After being rinsed with TBST for $3 \times 5 \text{ min}$, the biotinylated rabbit anti-mouse [IgG F(ab')₂; Dako, Glostrup, Denmark] was applied and incubated for 2 h. After further rinsing with TBS $3 \times 5 \text{ min}$, streptavidin-biotin-horseradish peroxidase complex (Dako) diluted in Tris-HCl was added and incubated for another 2 h, followed by $3 \times 5\text{-min}$ washes with TBS. The sections were then visualized with diaminobenzidine to yield a brown color and counterstained with Mayer's hematoxylin. IgG and TBST with 1% BSA were used as negative controls.

Quantification of immunostaining. MAPKs and transcription factor immunoreactivity was quantified using a color video camera (Sony DXC-950P 3-charge-coupled device 3-chip power HAD) containing 380,000 effective picture elements (pixels) (Sony, Tokyo, Japan). The camera was connected to a Leica imaging workstation, with highly specific PC software (Leica Q500IW; Leica, Cambridge, UK). The image setting included the option to carefully adjust the individual color components being displayed by the system to ensure a close match between what was being viewed directly and the image as displayed. Detection of an appropriate color was quantified using binary definition of color images as displayed on the screen (Fig. 1). The binary image required the user to define which pixel in the image was to be considered for measurement. In short, the positively stained area was managed as a grid of pixels containing the binary value 1 (the pixel is "set"), and the remaining area contained the binary value 0 (the pixel is not set). All intact epithelia from both sections on a slide were used for quantification of epithelial area. The appropriate area was selected using image software that provides automatic area calculation. The analyzed epithelial area was median $67,600 \mu\text{m}^2$, with an interquartile range of $32,000\text{--}109,000 \mu\text{m}^2$. The immunoreactivity was determined as positive cytoplasmic plus nuclear (total) staining, given as a percentage of the epithelial area selected with the image system, as previously reported (27). Nuclear translocation was determined as the number of positively stained nuclei/ mm^2 of the selected epithelial area.

Statistical analysis. Subjects acted as their own controls, and comparison of postair and DE stainings were performed using Wilcoxon's paired rank test using Windows version 11 (SPSS, Chicago, IL). A P value <0.05 was considered significant. Data are presented as medians and interquartile range (Tables 2 and 3). Correlation analyses were carried out using Spearman's rank order correlation with a P value <0.05 considered significant. Comparisons were performed using either the change in a given parameter, value post-diesel minus that of postair, or using the absolute values after the air or diesel exposure.

Table 1. Monoclonal antibodies used for immunohistochemical staining of the bronchial epithelium

Antibody	Clone	Dilution	Specificity Against	Source
p-p38	D-8	1:20	Tyr-182 p-p38	Santa Cruz Biotechnology (Santa Cruz, CA)
p-JNK	G-7	1:20	Tyr-185 and Thr-182	Santa Cruz Biotechnology
p-Tyr	PY99	1:10	Phosphotyrosine	Santa Cruz Biotechnology
p65 (NF- κ B)	G96-347	1:25	p65 subunit	BD Pharmingen
p-c-jun	KM-1	1:20	p-c-jun on Ser-63	Santa Cruz Biotechnology
c-Fos	D-1	1:10	c-Fos	Santa Cruz Biotechnology

p, Phosphorylated; Tyr, tyrosine.

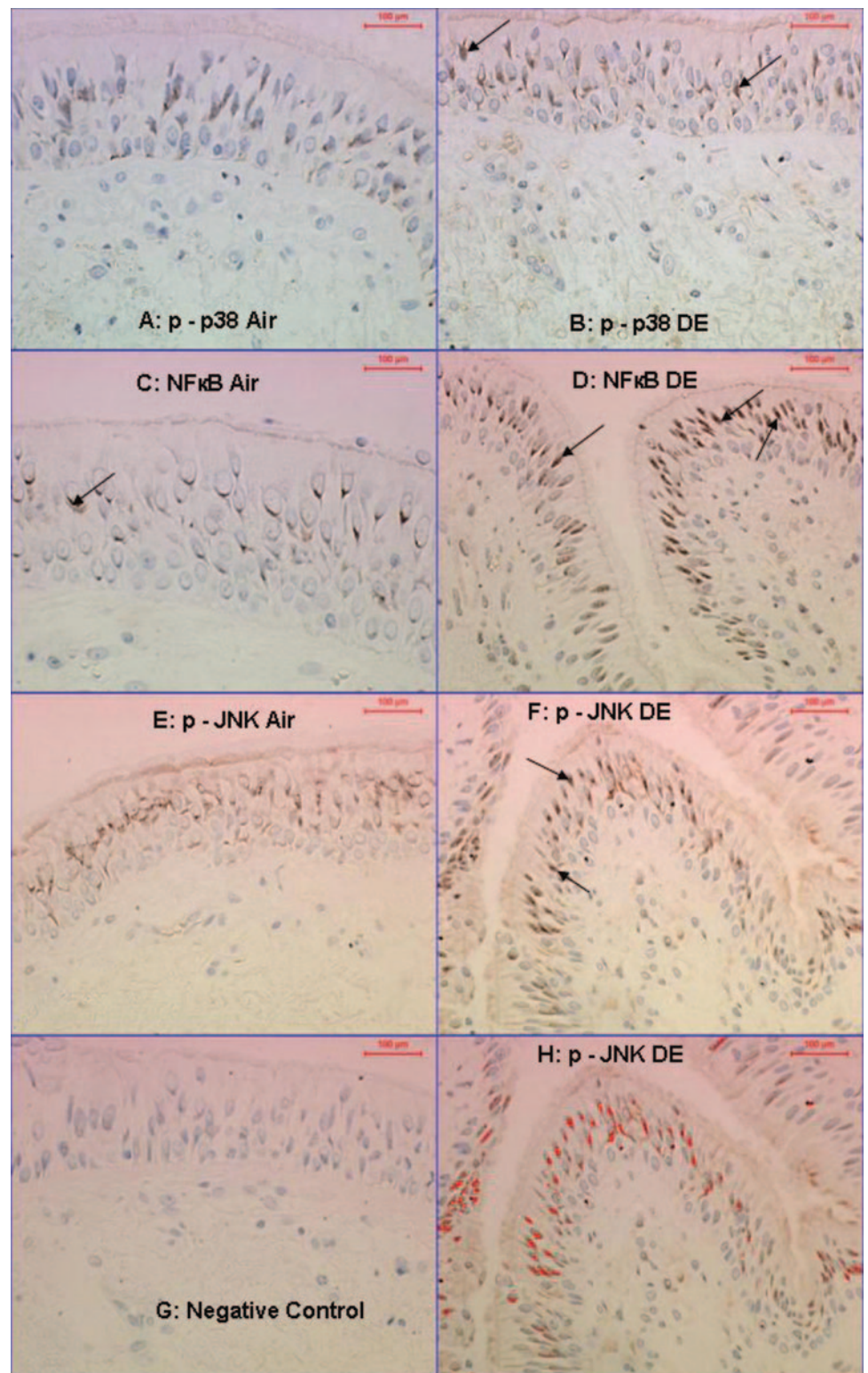


Fig. 1. Immunostaining of bronchial epithelium with monoclonal antibodies, phosphorylated p38 (p-p38), NF- κ B (p65), and phosphorylated JNK (p-JNK) is shown. Arrows indicate nuclear stainings. A, C, and E: stainings after air exposure. B, D, and F: corresponding stainings after diesel exhaust (DE) exposure. G: a negative control. H: demonstrates (on the same section as F) how the computer-aided system has selected the brown diaminobenzidine staining and visualizes this with red color.

RESULTS

Short-term exposure to DE ($300 \mu\text{g}/\text{m}^3$) increased total (cytoplasmic + nuclear) immunoreactivity of phosphorylated p38 MAPK in the bronchial epithelium ($P = 0.03$). We also observed increased nuclear translocation of phosphorylated p38 and JNK MAPK in bronchial epithelium after DE ($P =$

0.01 and $P = 0.04$, respectively). This increased nuclear translocation of phosphorylated MAPK was accompanied by elevated nuclear phosphorylated tyrosine immunoreactivity [$P < 0.05$ (Table 2)]. The translocation of phosphorylated MAPKs to the nucleus was associated with a significant increase in nuclear translocation of the p65 subunit of NF- κ B

Table 2. Bronchial epithelium expression of MAP kinase and phosphotyrosine in biopsies obtained 6 h after exposure of healthy subjects to DE vs. filtered air

Exposure	Total Staining* p-p38	Nuclear p-p38	Total Staining* p-JNK	Nuclear p-JNK	Total Staining* p-Tyr	Nuclear p-Tyr
Air	0.49 (0.03–1.18)	483 (210–641)	1.75 (1.12–2.56)	908 (782–1,145)	4.72 (1.56–5.83)	626 (499–952)
DE	1.31 (0.33–2.02)	681 (518–870)	1.66 (1.09–2.36)	1286 (1,115–1,638)	5.73 (3.51–7.37)	906 (793–1,026)
Sig.	$P = 0.03$	$P = 0.01$	NS	$P = 0.04$	NS	$P = 0.05$

Values are given as medians (1st–3rd quartiles). Total staining is given as percentage (%) of positively stained area of total epithelial area. Nuclear staining is given as the number of positively stained cells/mm² epithelium. All values are quoted as medians with interquartile ranges. Statistical comparisons are shown by Wilcoxon's paired rank test. *Cytoplasmic + nuclear immunostaining. DE, diesel exhaust; Sig., significance. NS, not significant.

($P = 0.02$) as well as the phosphorylated c-jun subunit of AP-1 ($P = 0.02$; Table 3). Neither total nor nuclear c-fos immunoreactivity was altered following DE exposure (Table 3). The increased nuclear translocation of phosphorylated JNK following DE exposure was significantly associated with the phosphorylation of its target c-jun in the nucleus ($r_s = 0.70$, $P = 0.004$) as well as with the increase in nuclear p65 ($r_s = 0.71$, $P = 0.004$). The latter association was primarily driven by an underlying positive correlation between the changes in nuclear staining for p65 (NF- κ B) and for nuclear phosphorylated c-jun (AP-1; $r_s = 0.72$, $P = 0.004$). Typical bronchial stainings for phosphorylated JNK and phosphorylated c-Jun as well as p65 postair and DE exposures are illustrated in Fig. 1. Comparison of these results with those previously obtained for epithelial IL-8, Gro- α (31), and IL-13 (24) immunoreactivity after air and DE revealed that the expression of IL-8 was positively associated with nuclear phosphorylated p38 ($r_s = 0.74$, $P = 0.002$) and c-jun ($r_s = 0.77$, $P = 0.001$) and to a lesser degree nuclear p65 ($r_s = 0.61$, $P = 0.02$) after DE, but not after filtered air (Fig. 2).

DISCUSSION

In this study, we sought to test the hypothesis that the upregulation of proinflammatory cytokines observed following DE challenge could be related to the activation of redox-sensitive transcription factors. The rationale for this was based on literature demonstrating that DEP are capable of inducing oxidative stress (17, 20) as well as the fact that many cytokines and chemokines have NF- κ B and AP-1 binding sites in their upstream regulatory domains (1, 21). We assessed total cytoplasmic and nuclear expression of the redox-sensitive transcription factors (NF- κ B and AP-1) as well as their upstream stress-related MAPKs (JNK and p38 MAPK) in the epithelium of bronchial biopsies obtained from healthy subjects 6 h after a high-dose DE challenge (300 μ g/m³ for 1 h), a concentration that occasionally occurs in certain occupational and diesel-dominated traffic situations (12, 35).

Using archived biopsies, we were able to demonstrate that exposure to DE induced activation/phosphorylation and nuclear translocation of stress-related MAPKs (p38, JNK) into the bronchial epithelium, associated with increased nuclear translocation of the p65 subunit of NF- κ B and the phosphorylated c-jun, but not the c-fos subunit, of AP-1. The activation of the MAPKs after DE was evidenced by a significant increase in nuclear phosphorylated tyrosine immunoreactivity.

DE comprise both particulate components and gases, mainly oxides of nitrogen, with the level of NO₂ in this study being 1.6 ppm. We do not suggest NO₂ to have influenced the epithelial and submucosal inflammatory events by DE (24, 30, 31), since we were unable to identify any such events in an earlier set of studies that included NO₂ at a higher industrial concentration of 2 ppm for 4 h (5).

These findings were therefore consistent with the assumption that DE-induced oxidative stress acts as the trigger for the subsequent inflammatory response. Notably, while the activation of these pathways were quantitatively related to the upregulation of IL-8, no associations were seen with Gro- α or IL-13, suggesting that these mediators are under the regulation of separate pathways.

It is well established that DE particles can elicit the increased synthesis and release of proinflammatory cytokines from airway epithelial cells in vitro associated with the activation of NF- κ B (6, 15, 37). In contrast, the majority of studies examining AP-1 activation by DEP have yielded negative results (37), despite evidence for activation of the JNK cascade in bronchial epithelial cells treated with organic extracts from DEP (18). The upregulation of these redox-sensitive signaling pathways after diesel challenge has been proposed to be related to the imposition of oxidative stress at the air-lung interface. The capacity of DE particles to generate free radicals in the lung has been attributed to their content of metals (20), PAH (4), and quinones (33). Consistent with this, ROS have been detected in bronchial and nasal epithelial cells exposed to DEP or to their organic extracts (4). Further evidence for a key role

Table 3. Bronchial epithelium expression of transcription factors in biopsies obtained 6 h after exposure of healthy subjects to DE vs. filtered air

Exposure	Total* p-c-jun	Nuclear p-c-jun	Total* c-Fos	Nuclear c-Fos	Total* p65	Nuclear p65
Air	1.41 (1.15–2.65)	626 (459–1,102)	0.92 (0.82–1.96)	620 (262–739)	1.39 (0.87–1.99)	527 (311–637)
DE	2.08 (1.42–2.63)	1,138 (865–1,489)	1.04 (0.60–2.70)	643 (367–1,017)	1.48 (1.38–3.95)	810 (597–1,039)
Sig.	NS	$P = 0.02$	NS	NS	NS	$P = 0.02$

Total staining is given as percentage (%) of positively stained area of total epithelial area. Nuclear staining is given as the number of positively stained cells/mm² epithelium. All values are quoted as medians with interquartile ranges. Statistical comparisons are shown by Wilcoxon's paired rank test. *Cytoplasmic + nuclear immunostaining.

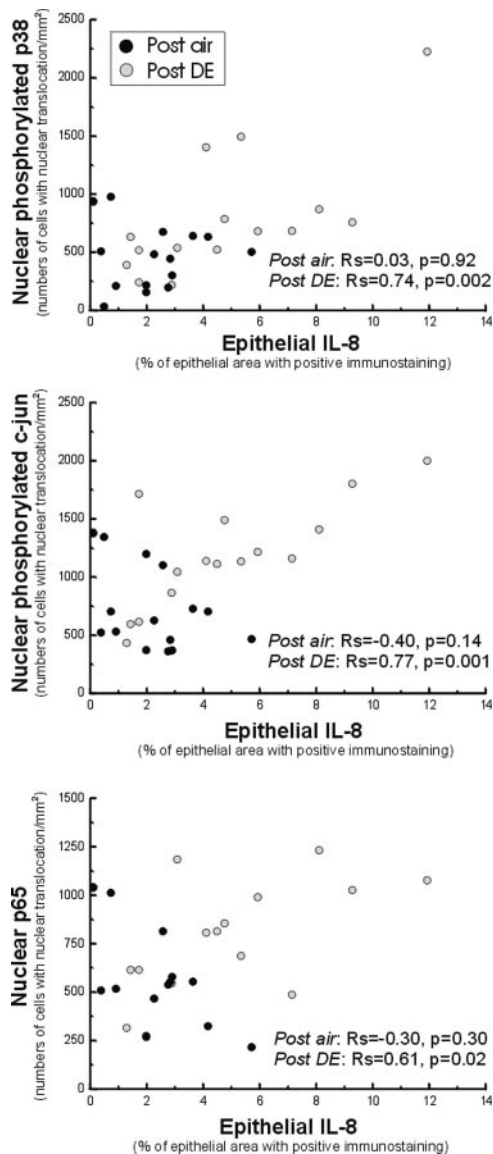


Fig. 2. Association between IL-8 immunoreactivity in human bronchial epithelium and the translocation and phosphorylation of key subunits and activators of the redox-sensitive transcription factors NF- κ B and AP-1.

of oxidative stress in the upregulation of proinflammatory cytokines has been demonstrated by the capacity of antioxidants to reduce both NF- κ B activation and cytokine release from cells challenged with DEP (6, 14, 15, 18, 37).

NF- κ B is a general term used to describe a number of dimeric combinations of members of the Rel family of gene regulatory proteins. The most common form of NF- κ B consists of a heterodimer of p50 (NF- κ B1) and p65 (RelA) proteins. This complex has the ability to bind with promoter sequences in DNA and to inaugurate transcription for many proinflammatory mediators, both cytokines (3) and vascular adhesion molecules (9). In quiescent cells, these heterodimers are kept in the cytoplasm by an inhibitor protein, I κ B. NF- κ B translocation and DNA binding is dependent on I κ B kinase, which phosphorylates I κ B on serines within the NH₂-terminal domain, resulting in I κ B degradation, thereby allowing NF- κ B translocation to the nucleus. The upstream regulation of NF- κ B

has been reviewed elsewhere (11). We examined both nuclear translocation of p65 and p38 activation (phosphorylation and nuclear translocation) to demonstrate increased NF- κ B activity after DE. NF- κ B binding sites have been identified in the promoter sequences of IL-8, IL-13, and Gro- α (1, 21), cytokines previously demonstrated to be upregulated in bronchial epithelium after DE challenge in healthy humans (24, 31). In the current study, we demonstrated increased nuclear p65 immunoreactivity as well as increased phosphorylation and nuclear translocation of p38 after DE. This was associated with increased nuclear phosphorylated tyrosine immunoreactivity, suggesting functional activation of this pathway. This upregulation of p65 and phosphorylation of p38 was associated with the degree of IL-8 immunoreactivity seen after DE. No association was seen with either IL-13 or Gro- α , despite both genes containing putative NF- κ B binding sites in their promoter regions (1), suggesting that these inflammatory mediators may be regulated through different signaling pathways.

The proinflammatory transcriptional element AP-1 has been shown to be an important regulator of the expression of Th2 cytokines IL-4, IL-5, and IL-13. AP-1 is composed of a mixture of heterodimeric protein complexes derived from the Fos and Jun families, including c-Fos, FosB, Fra-1, Fra-2, c-Jun, JunB, and JunD. Like NF- κ B, AP-1 expression is induced by multiple stimuli (16), including oxidative stress (32, 39). The activation of AP-1 requires both the nuclear translocation and phosphorylation of c-Jun, which is dependent on the JNK (25). Activation of JNK by hydrogen peroxide has been observed in several cell types and transformed cell lines (13). Furthermore, ambient PM has been reported to increase JNK activity and the downstream expression of phosphorylated c-jun in airway epithelial cells (38). In the present study, we confirmed an increased expression of nuclear phosphorylated JNK and nuclear phosphorylation of its target c-jun after DE exposure, which were furthermore strongly correlated to each other. Although both IL-8 and IL-13 have AP-1 binding sites in their promoters (21), no associations were seen between either phosphorylated JNK or c-jun and the epithelial expression of IL-13. However, as with NF- κ B, a strong association was observed between c-jun and IL-8, suggesting that AP-1 was also involved in the regulation of this cytokine. Moreover, a strong correlation was observed between the nuclear staining for p65 (NF- κ B) and phosphorylated c-jun (AP-1), suggesting possible coactivation following DE exposure.

In this study, we examined two of the three major groups of MAPKs that have been implemented in the activation of NF- κ B and AP-1, but we did not examine the extracellular signal-regulated kinase (ERK) pathway. ERKs are typically involved in the response to growth factors but are also upregulated in response to ROS. Given the lack of an association between either p38 or JNK activation and epithelial IL-13 or Gro- α expression, examination of the ERK pathway and further redox-sensitive transcription factors (such as HIF-1 and STAT) is warranted in future studies (40).

In conclusion, we demonstrated that exposure of healthy subjects to DE results in upregulation of redox-sensitive transcription factors and cell-signaling pathways in the bronchial epithelium. We believe that upregulation of these pathways may be the molecular mechanism linking DEP-induced extracellular oxidative stress to the induction of proinflammatory

cytokines. Moreover, the induction of IL-8, Gro- α , and IL-13 after DE exposure may be regulated by different pathways.

GRANTS

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