Ultrafine carbon particles induce interleukin-8 gene transcription and p38 MAPK activation in normal human bronchial epithelial cells

Yu-Mee Kim,1,2 William Reed,2 Anke G. Lenz,3 Ilona Jaspers,2 Robert Silbajoris,5 Harry S. Nick,4 and James M. Samet5

1Department of Environmental Sciences and Engineering, School of Public Health and 2Center for Environmental Medicine, Asthma, and Lung Biology, University of North Carolina, Chapel Hill; and 3Human Studies Division, National Health and Environmental Effects Research Laboratory, United States Environmental Protection Agency, Chapel Hill, North Carolina; 4Department of Neurosciences, College of Medicine, University of Florida, Gainesville, Florida; and 5Institute for Inhalation Biology, National Research Center for Environment and Health, Munich, Germany

Submitted 26 July 2004; accepted in final form 11 October 2004

Ultrafine carbon particles induce interleukin-8 gene transcription and p38 MAPK activation in normal human bronchial epithelial cells. Am J Physiol Lung Cell Mol Physiol 288: L432–L441, 2005; doi:10.1152/ajplung.00285.2004.—Epidemiological studies suggest that ultrafine particles contribute to particulate matter-induced adverse health effects. Interleukin (IL)-8 is an important proinflammatory cytokine in the human lung that is induced in respiratory cells exposed to a variety of environmental insults, including ambient air ultrafine particles. In this study, we examined the effect of a model ultrafine particle on IL-8 expression and the cellular mechanisms responsible for this event. Here, we report that carbonaceous ultrafine particles consisting of synthetic elemental carbon particles (UfCP) markedly increase the expression of IL-8 mRNA and protein in normal human bronchial epithelial (NHBE) cells. IL-8 promoter activity was increased by UfCP exposure in NHBE cells, indicating UfCP-induced IL-8 expression is transcriptionally regulated. IL-8 expression in NHBE is known to be regulated by nuclear factor (NF)-κB activation. However, UfCP did not induce inhibitory factor κB (IκB) degradation, NF-κB-DNA binding, or NF-κB-dependent promoter activity in NHBE cells, indicating that UfCP induces IL-8 expression through a mechanism that is independent of NF-κB activation. Additionally, we observed that UfCP exposure induces the phosphorylation and activation of p38 mitogen-activated protein kinase (MAPK) in a biphasic manner and that the inhibition of p38 MAPK activity can block IL-8 mRNA expression induced by UfCP in NHBE cells. These results demonstrate that UfCP-induced expression of IL-8 involves a transcriptional mechanism and activation of p38 MAPK in NHBE cells.

human bronchial epithelial cell; p38 mitogen-activated protein kinase; particulate matter; ultrafine particles; nuclear factor-κB

Epidemiological studies have demonstrated a positive association between ambient particulate matter (PM) levels and mortality and morbidity (7, 41, 43, 50, 52). Human health effects resulting from PM exposure include decreased pulmonary function, exacerbation of respiratory disease, elevated risk of cancer, and increased acute mortality from cardiovascular/ pulmonary disease (44, 53). Multiple physicochemical properties of PM, such as chemical composition, number, mass, and size, have been proposed as factors responsible for the health effects of PM inhalation. Ultrafine PM are particles <0.1 μm in diameter that are emitted in very large numbers by combustion processes, including automobile and industry. The composition of ambient ultrafine particles includes organic and elemental carbon, nitrate, sulfate, and metals. Typically, elemental carbon serves as the core for organic material adsorption of these constituents (30).

Despite many studies conducted over the last decade, the mechanisms underlying these health effects induced by PM are still not understood but may involve inflammatory responses. A number of epidemiological studies suggest that increased ultrafine particles associated with PM are potent contributors to decreased pulmonary function, increased frequency of symptoms, and medication use in asthmatics (15, 42, 60). A number of epidemiological, animal, and in vitro studies indicate that exposure to ultrafine particles causes proinflammatory events characterized by oxidative stress, lipid mediator synthesis, transcription factor activation, and cytokine release (2, 4, 32, 55). These particles are reported to be more inflammatory than both fine and coarse particles (3, 31). Furthermore, they may be able to translocate to other organs, such as the blood, brain, and liver (25, 38–40, 54).

One of the proinflammatory cytokines induced by ultrafine particle exposure is interleukin (IL)-8, a potent neutrophil chemoattractant that plays an important role in pathogenic responses in the human lung. Elevated levels of IL-8 in the lung is a feature of a number of respiratory diseases, including cystic fibrosis, asthma, and acute respiratory distress syndrome (48). IL-8 is synthesized by airway epithelial cells in response to a variety of extracellular stimuli, including the cytokines tumor necrosis factor (TNF)-α and IL-1β, viral and bacterial infection, and air pollutants (17, 19, 24, 35). IL-8 expression is regulated at both transcriptional and posttranscriptional levels under the control of multiple signaling pathways. In addition to a CCAAT box, multiple regulatory elements have been identified in the 5′-flanking region of the IL-8 gene, including glucocorticoid, hepatocyte nuclear factor-1, interferon regulatory factor-1, activator protein-1 (AP-1), CCAAT/enhancer binding protein β (C/EBPβ), and nuclear factor-κB (NF-κB) response elements (22, 37). The transcription factor NF-κB is the best-understood regulator of IL-8 transcription in human airway epithelial cells (24, 26, 58). Other pathways, including mitogen-activated protein kinase (MAPK) pathways, have also

http://www.ajplung.org

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
been identified as regulators controlling IL-8 expression (11, 14). The four major MAPK families are the extracellular signal-related kinase 1/2 (ERK1/2), the c-Jun NH2-terminal kinase (JNK), the p38 kinases, and big MAPK1 (also called ERK5) (13). The p38 pathway has been associated with transcriptional and posttranscriptional regulation of IL-8 expression (14, 46).

Although IL-8 release induced by ultrafine particle exposure has been demonstrated in in vitro and animal studies, the cellular mechanisms responsible for IL-8 expression induced by ultrafine particle exposure have not been characterized. In this study, we examined the effects of exposure to a model carbonaceous ultrafine particle consisting of synthetic elemental carbon particles generated by spark discharge across graphite rods of high purity in an inert atmosphere on IL-8 expression in normal human bronchial epithelial (NHBE) cells. We report here that ultrafine elemental carbon particles (UfCP) markedly increase IL-8 expression in NHBE cells through a transcriptional mechanism that involves p38 MAPK, but not NF-κB activation.

MATERIALS AND METHODS

Cell culture. NHBE cells were obtained by brush scraping during bronchoscopy of human volunteers following a protocol approved by the Committee on the Protection of the Rights of Human Subjects of the University of North Carolina at Chapel Hill. NHBE cells were initially plated in bronchial epithelial cell basal medium (BEBM) supplemented with 0.5 ng/ml human epidermal growth factor (EGF), 0.5 μg/ml hydrocortisone, 5 μg/ml insulin, 10 μg/ml transferrin, 0.5 μg/ml epinephrine, 6.5 ng/ml gentamicin, 50 ng/ml amphotericin-B, 52 μg/ml bovine pituitary extract, and 0.1 ng/ml retinoic acid [bronchial epithelial growth medium (BEGM)] on tissue culture plates coated with human collagen (Sigma, St. Louis, MO); grown to confluence; and then passaged two or three times in BEGM on ordinary tissue culture plates. Confluent cultures were deprived of EGF for 15 h to decrease the basal level of activation before treatment with stimuli such as UfCP, TNF-α, IL-β, or vanadyl sulfate. To assess cell viability after introduction of inhibitors, 50 μl supernatant from the cell culture were taken, and lactate dehydrogenase (LDH) level was measured using CytoTox 96 (Promega, Madison, WI) in accordance with the manufacturer’s instructions.

Reagents. Tissue culture media, supplements, and supplies were obtained from Clonetics (San Diego, CA). SDS-PAGE supplies were obtained from Bio-Rad (Richmond, CA). BSA, β-mercaptoethanol, α-amanitin, and other common laboratory chemicals were purchased from Sigma Chemical. Guanidine isothiocyanate and cesium chloride were purchased from Boehringer Mannheim (Indianapolis, IN). MAPK inhibitors, such as SB-202190 [4-(4-fluorophenyl)-2-(4-hydroxyphenyl)-5-(4-pyridyl)-1H-imidazole] and SP-600125 [anthra(1,9-cd)-amine-3-phosphate dehydrogenase in first-strand cDNAs was measured by TaqMan methodology using oligonucleotide primers and probes previously described (18, 19).

Measurement of IL-8 protein levels. Release of IL-8 protein by NHBE cells into the supernatant on the cell culture plate was assayed with a human IL-8 ELISA kit (R & D Systems, Minneapolis, MN) according to the instructions given by the manufacturer.

Preparation of cytoplasmic and nuclear fractions and electrophoretic mobility shift assay. Preparation of nuclear extracts from primary human airway epithelial cells and electrophoretic mobility shift assay (EMSA) were performed as described (19). Cytoplasmatic extraction buffer (CEB) consisted of 10 mM Tris-HCl (pH 7.9), 60 mM KCl, 1 mM EDTA, 1 mM DTT, and protease inhibitors (PI; 1 mM Pefabloc, 50 μg/ml antipain, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 40 μg/ml bestatin, 3 μg/ml E-64, and 100 μg/ml chymostatin; all purchased from Boehringer Mannheim). Cells were scraped into CEB, transferred into a microcentrifuge tube, and placed on ice for 15 min. Nonidet P-40 (NP-40; Sigma) was added to a final concentration of 0.1% and vortexed for 10 s. Nuclei were pelleted by centrifugation at 15,000 g for 30 s, and this was repeated after washing the nuclei with CEB. The supernatant was removed, and the nuclei were incubated for 10 min on ice in a nuclear extraction buffer (consisting of 20 mM Tris-HCl, pH 8.0, 400 mM NaCl, 1.5 mM MgCl2, 1.5 mM EDTA, and 1 mM DTT, 25%) with PI to extract nuclear proteins. After brief centrifugation, the supernatants, containing the nuclear proteins, were stored at −80°C until ready for use.

An oligonucleotide containing NF-κB consensus sequence was labeled by incubation with 15 units of T4 polynucleotide kinase (New England Biolabs, Beverly, MA), 100 ng double-stranded probe, and 100 μCi [γ-32P]ATP (ICN, Irvine, CA) at 37°C for 30 min, as described previously (18). Nuclear protein extract (4 μg) was mixed with 1 μl labeled probe, 10 μl running buffer [10 mM Tris-HCl (pH 7.5), 50 mM NaCl, 2 mM EDTA, 1 mM DTT, and 5% glycerol], and 2 μg poly(dI/dC) (Boehringer Mannheim) for DNA-protein binding
reaction at room temperature for 10 min. Samples were run on 4.5% nondenaturing polyacrylamide gels in 25 mM Tris base-190 mM glycine-1 mM EDTA. Gels were electrophoresed for 4 h at 250 volts, dried, and analyzed by PhosphorImaging (Molecular Dynamics, Sunnyvale, CA).

**Western blotting.** Gel electrophoreses were performed by Western blotting were performed essentially as described by Laemmli (27) and Towbin et al. (59). Cells were lysed with RIPA lysis buffer containing 1% NP-40, 0.5% deoxycholate, and 0.1% SDS in PBS at pH 7.4, phosphatase inhibitor cocktail set I and II, and PI cocktail set III purchased from Calbiochem. Each sample was normalized for a protein content of 50–100 μg before loading on a SDS-PAGE gel. Lysates were mixed with one volume of reducing SDS-PAGE loading buffer containing 0.125 M Tris (pH 6.8), 4% SDS, 20% glycerol, 10% β-mercaptoethanol, and 0.05% bromphenol blue. The samples were heated for 1 min at 90°C, run on 11% SDS-PAGE gels with precasted molecular weight markers (Bio-Rad) in Tris-glycine-SDS buffer (Bio-Rad), and electroblotted on nitrocellulose membranes. The blots were blocked with 5% nonfat milk and incubated with primary antibodies. HRP-conjugated goat anti-rabbit was used as a secondary antibody. Protein bands on the membrane were detected using the enhanced chemiluminescence detection solution (Amersham Pharmacia Bio-Tech) and exposed on high-performance chemiluminescence film (Amersham Pharmacia Biotech). The bands were quantified by a Kodak electrophoresis documentation and analysis system (Rochester, NY).

**MAPK activity assays.** Cells were lysed in a low-salt buffer containing 150 mM NaCl, 20 mM Tris-HCl, pH 7.5, 5 mM EDTA, 1% Triton X-100, 50 mM NaF, 10% glycerol, 1 mM sodium metavanadate, 4 μg/ml aprotinin, 20 μg/ml phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 1 μg/ml microcystin. p38 MAPK protein was immunoprecipitated from 50–100 μg cell lysate using agarose-conjugated anti-P38 antibodies for 2 h at 4°C. The immunoprecipitates were then washed first with lysis buffer and then with a kinase buffer consisting of 20 mM HEPES, pH 7.4, 10 mM MgCl2, 50 mM NaCl, 1 mM DTT, 5 mM β-glycerophosphate, 1 μg/ml microcystin, and 1.5 mM EGTA. Kinase activity of p38 MAPK immunoprecipitate was assayed in kinase buffer containing 50 μM ATP, 10 μM [γ-32P]ATP, and 1 μg AUF-2. This reaction was incubated for 20 min at 30°C with vigorous agitation in an Eppendorf Thermomixer (Brinkman Instruments, Westbury, NY) and was subsequently terminated by adding 20 μl 100 mM EDTA or 20% (final) TCA, and a fraction was adsorbed onto P81 cellulose phosphate paper (Whatman) that was washed extensively with 1% phosphoric acid. Radioactivity on the P81 paper was quantified by liquid scintillation counting (LKB Wallace, Gaithersburg, MD).

**NF-κB-luciferase promoter-reporter constructs, transduction, and promoter-reporter assay.** A Moloney murine leukemia virus-based self-inactivating (SIN) retroviral promoter-luciferase plasmid, pHermes-luc, was constructed that included a retroviral 5’-long terminal repeat (LTR) and ψ-packaging signal followed by the transcription silencing sequence, multicloning site, and firefly luciferase coding sequence of pGL3basic (Promega) followed by a bovine growth hormone polyadenylation sequence and a SIN 3’-LTR. The NF-κB-luciferase promoter-reporter plasmid, pHermes-κBluc, was constructed by inserting the 5’ NF-κB response element and TATA box (bases 2660–2805) of pNF-κB[Δluc] (Stratagene, San Diego, CA) in the multicloning site of pHermes-luc. The SV40-REnilla luciferase promoter-reporter plasmid, pHERMES-SV40Bluc, was constructed by replacing the transcription silencing sequence, multicloning site, and firefly luciferase coding sequence of pHermes-luc with the SV40 promoter, chimeric intron, and Renilla luciferase coding sequence (bases 7–1,643) of pRl-SV40 (Promega). Retrovirus was packaged using human embryonic kidney 293T cells. Cells were plated at 2 x 107/10-cm tissue culture dish and transiently transfected the following day by calcium phosphate DNA coprecipitation as follows. Retroviral promoter-reporter plasmid (10 μg), 14 μg pcI-GPZ, 6 μg pcI VSV-G (62), 438 μl sterile water, and 61 μl 2 M CaCl2 were mixed with 0.125 M Tris (pH 7.4), 10 mM MgCl2, 50 mM NaCl, 10 mM KCl, 1.5 mM Na2HPO4, and 12 mM glucose, pH 7.05) were added dropwise. After addition of the DNA-CaPO4 precipitate to the culture, it was returned to the incubator for 24 h. Transfections were stopped by replacement of culture medium. Retroviral-laden culture supernatants were harvested 48 h later and sedimented at 400 g to remove nonadherent cells. The cleared supernatants were stored frozen at −80°C until used.

**NHBE cells grown to 70% confluence were cotransduced with pHermes-κBluc and pHermes-SV40Bluc retroviruses for 6 h. A 24-h recovery period was followed by challenge with UICP or TNF-α (10 ng/ml) and IL-β (5 ng/ml). Firefly and Renilla luciferase activities were measured using a dual luciferase assay system (Promega) and an autoinjector luminometer (Autolumat LB953, Berthold Technologies). Firefly luciferase activity was normalized to Renilla luciferase activity.**

**Adenoviruses, transduction, and IL-8-enhanced green fluorescent protein promoter-reporter assay.** Ad5SIEBo (16), a dominant negative adenoviral construct encoding hemagglutinin-tagged inhibitory factor κBo (IκBo; S32A, S36A), was a generous gift of Dr. D. A. Brenner (College of Physicians and Surgeons, Columbia University, New York, NY). Ad5CMV3, a nonrecombinant adenoviral expression vector, was obtained from the University of North Carolina Gene Therapy Vector Core. Ad5IL-8pro-enhanced green fluorescent protein (EGFP), an adenoviral IL-8 promoter-reporter vector, was prepared as follows.

An IL-8 promoter-reporter cassette was generated that was composed of the transcription-silencing sequence (bases 2,193–2,689) from pNF-κBluc (Stratagene) upstream of a 1.46-kb fragment of the IL-8 gene (−1,410 to +54) followed by the coding sequence of EGFP and an SV40 polyadenylation sequence. This cassette was inserted in the multicloning site of pShuttle (Clontech, Palo Alto, CA). A recombinant plasmid, psh-IL8pro-EGFP, bearing the desired sequence was used by the University of North Carolina Vector Core Facility to generate a recombinant adenovirus.

**NHBE cells grown to 80% confluence in 24-well plates were transduced with AdIL-8pro-EGFP at a multiplicity of infection of 500 focus-forming units/cell for 8 h. Adenoviral dominant-negative IκBo (Ad5SIEBo), an adenoviral expression vector encoding hemagglutinin-tagged IκBo (S32A, S36A), was cotransduced with adenoviral IL-8 EGFP promoter-reporter at a multiplicity of infection of 100 focus-forming units/cell. The recovery period of 24 h was followed by the challenge with UICP or TNF-α (10 ng/ml). IL-8 promoter activity was measured as follows: Cells were put in suspension by brief treatment with trypsin-EDTA at 37°C and assessed for mean EGFP fluorescence intensity using FACTsort flow cytometry (Becton-Dickenson, San Jose, CA).

**Statistical analysis.** Data are presented as means ± SE of at least three separate experiments. Data comparisons were carried out using repeated-measures ANOVAs followed by Dunnett’s posttest, and paired or unpaired one- or two-tailed Student’s t-test with or without Bonferroni correction.

**RESULTS**

UICP exposure induces IL-8 expression in NHBE cells. To study inflammatory responses to ultrafine PM exposure by the airway epithelium, we examined expression of IL-8 after noncytotoxic exposure of NHBE cells to UICP. NHBE cells were incubated with 66 μg/ml (17.4 μg/cm2) or 33 μg/ml (8.7 μg/cm2) UICP up to 30 h. Levels of IL-8 mRNA were measured using real-time RT-PCR. IL-8 mRNA levels were expressed as the degree of IL-8 mRNA in challenged cells over unstimulated control cells. As shown in Fig. 1A, UICP exposure resulted in statistically significant increases in levels of
IL-8 mRNA as early as 3 h, with a peak increase of ~6.5-fold over control seen at 16 h, followed by a gradual decrease. Smaller increases in levels of IL-6 and cyclooxygenase-2 mRNA were also observed in response to NHBE exposure to UfCP (data not shown).

Compared with the marked increase in IL-8 mRNA, levels of IL-8 protein released by NHBE cells treated with UfCP appeared to be disproportionately small. Preliminary experiments showed this to be because of strong nonspecific binding of IL-8 to UfCP. Therefore, the data on IL-8 protein release may underestimate the actual IL-8 protein release in response to UfCP stimulation. In spite of this limitation, UfCP induced a statistically significant 4.6- and 4.2-fold increase in IL-8 protein in the culture media at 33 and 66 g/ml, respectively (Fig. 1).

IL-8 mRNA as early as 3 h, with a peak increase of ~6.5-fold over control seen at 16 h, followed by a gradual decrease. Smaller increases in levels of IL-6 and cyclooxygenase-2 mRNA were also observed in response to NHBE exposure to UfCP (data not shown).

Compared with the marked increase in IL-8 mRNA, levels of IL-8 protein released by NHBE cells treated with UfCP appeared to be disproportionately small. Preliminary experiments showed this to be because of strong nonspecific binding of IL-8 to UfCP. Therefore, the data on IL-8 protein release may underestimate the actual IL-8 protein release in response to UfCP stimulation. In spite of this limitation, UfCP induced a statistically significant 4.6 ± 0.6- and 4.2 ± 0.9-fold increase in IL-8 protein in the culture media at 33 and 66 μg/ml, respectively (Fig. 1).

UfCP-induced expression of IL-8 is transcriptionally regulated in NHBE cells. Because IL-8 expression is known to be regulated at the transcriptional level, we next tested the effect of pretreatment of NHBE cells with α-amanitin (3 μg/ml), an inhibitor of eukaryotic RNA-polymerase II, on IL-8 mRNA levels induced by UfCP. α-Amanitin pretreatment for 30 min blocked UfCP-induced IL-8 mRNA expression in a statistically significant manner, demonstrating that transcription is essential for this response to UfCP in NHBE cells (Fig. 2A).

Fig. 1. Ultrafine particles consisting of synthetic elemental carbon particles (UfCP) induce interleukin (IL)-8 mRNA and protein expression in normal human bronchial epithelial (NHBE) cells. A: NHBE cells were treated with UfCP (66 μg/ml) for 1, 3, 7, 11, 16, 24, or 27 h. RNA was extracted, and levels of IL-8 mRNA were analyzed by real-time RT-PCR. IL-8 mRNA induction was normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) levels and expressed as the degree of increase over control. Data represent means ± SE of 4 independent experiments. *P < 0.05 compared with control. B: NHBE cells were incubated with UfCP (33 or 66 μg/ml) for 24 h. The concentration of IL-8 protein in the culture supernatant was determined by ELISA and expressed as the degree of increase over control. Shown are means ± SE of 3 independent experiments. *P < 0.05 compared with control.

Fig. 2. UfCP-induced IL-8 expression is transcriptionally regulated in NHBE cells. A: NHBE cells were treated with medium with 66 μg/ml UfCP or medium (bronchial epithelial growth medium) alone, in the presence or absence of α-amanitin (3 μg/ml) for 1, 3, 7, or 11 h. IL-8 mRNA level was quantified by real-time PCR and normalized to GAPDH mRNA levels. IL-8 mRNA induction was expressed as the degree of increase over control. Shown are means ± SE of 4 independent experiments. *P < 0.05 compared with control. B: NHBE cells were infected with an adenoviral IL-8-enhanced green fluorescent protein (EGFP) promoter reporter vector at a multiplicity of infection of 500 focus-forming units/cell for 8 h. After a 24-h recovery period, the cells were challenged with UfCP (66 μg/ml) or tumor necrosis factor (TNF)-α (10 ng/ml). IL-8 promoter activity (mean EGFP fluorescence intensity/cell) was determined by flow cytometry 18 and 34 h after challenge. Shown are means ± SE of 3 independent experiments. *P < 0.05 compared with control. C: IL-8 mRNA levels at each time point in B. Shown are means ± SE of 3 independent experiments. Dotted lines represent the levels in the respective media control.
To determine whether specific IL-8 transcription was induced by UfCP exposure, we transduced NHBE cells with an adenovirus carrying an IL-8-EGFP promoter-reporter construct. After the incubation of NHBE cells with UfCP or TNF-α (as positive control), EGFP fluorescence intensity was measured by flow cytometry to assess IL-8 promoter activity. Exposure of NHBE cells to UfCP for 18–34 h resulted in a statistically significant (~2-fold) increase in IL-8 promoter activity (Fig. 2B). As expected, treatment with TNF-α induced a pronounced increase in IL-8 promoter activity. The accompanying levels of IL-8 mRNA induced by UfCP or TNF-α are shown in Fig. 2C.

UfCP-induced IL-8 expression is independent of NF-κB activation in NHBE cells. IL-8 gene transcription can be induced by NF-κB (26, 36), a transcription factor that is activated by a variety of extracellular stimuli. In many instances, NF-κB activation involves the breakdown of inhibitor proteins (IκBs) that sequester NF-κB in the cytoplasm followed by translocation of NF-κB in the nucleus (9). To examine whether the UfCP-induced IL-8 gene expression was NF-κB dependent, we determined whether UfCP exposure causes IκBα breakdown and increased nuclear NF-κB DNA binding activity.

NHBE cells left untreated or treated with 66 μg/ml UfCP for 30 min or 2 or 4 h or with 10 ng/ml TNF-α for 30 min were subjected to immunoblotting using a specific anti-IκBα antibody. Although TNF-α caused a clear degradation of IκBα, there was no discernible IκBα breakdown in cells treated with UfCP (Fig. 3A).

Next, EMSAs were performed to determine whether UfCP treatment increased nuclear NF-κB DNA binding activity. Nuclear extracts were prepared from NHBE cells left untreated or incubated with 66 μg/ml UfCP for 1, 2, and 6 h or with 100 μM vanadyl sulfate (which is known to cause NF-κB DNA binding) for 1 h. Although treatment with vanadyl sulfate induced a clear shift in the electromobility of the NF-κB radioprobe, UfCP had no such effect, suggesting no increase in NF-κB DNA binding (Fig. 3B).

In addition to the translocation of cytosolic p50-p65 and other dimers, the ability of NF-κB to promote transcription is...
regulated by phosphorylation of the transactivation domains of certain NF-κB subunits (33, 34, 61, 65). Because transactivation of NF-κB can occur in the absence of nuclear translocation (8, 19, 56), we determined whether UfCP treatment transactivates constitutive nuclear NF-κB. To examine this possibility, NHBE cells were transduced with a κB-dependent promoter-reporter retrovirus before treatment with UfCP (66 μg/ml) or TNF-α (10 ng/ml) and IL-1β (5 ng/ml) for 18 h. As shown in Fig. 3C, UfCP exposure did not increase κB-dependent transcription in NHBE cells. In contrast, stimulation of NHBE cells with the cytokines resulted in a marked increase in NF-κB-dependent promoter activity (Fig. 3C).

To independently probe the role of NF-κB in UfCP-induced IL-8 expression, we next determined whether the overexpression of a Ad5IkBα could block IL-8 promoter activity induced by UfCP. As a result of the substitution of alanines for Ser32 and Ser36 of IkBα, the phosphorylation sites that normally target IkBα for degradation, overexpression of this mutant IkBα can sequester NF-κB into IkBα (S32A, S36A)-NF-κB complexes, thus preventing NF-κB-dependent signaling and transcription (19). Ad5IkBα was cotransduced with the adenoviral IL-8-EGFP promoter-reporter vector into NHBE cells. As the results in Fig. 3D show, dominant-negative IkBα expression had no effect on IL-8 promoter activity induced by UfCP. In contrast, TNF-α-induced IL-8 promoter activity was almost completely blocked (Fig. 3D). Taken together, these results strongly suggested that UfCP-induced IL-8 expression does not induce NF-κB activation and that UfCP-induced IL-8 expression occurs through a separate signaling mechanism from that induced by TNF-α.

**UfCP activate p38 MAPK in NHBE cells.** We next examined the activation of MAPK as an alternative signaling mechanism that potentially mediates UfCP-induced IL-8 expression. The effect of UfCP on p38 phosphorylation in NHBE cells was first assessed by immunoblotting using a phosphospecific p38 antibody. Analysis of cell lysates from NHBE cells incubated with 66 μg/ml UfCP for 0.5, 1, 2, 4, 8, 12, or 20 h showed that p38 MAPK was phosphorylated in a biphasic manner in response to UfCP exposure, with early phosphorylation seen before 30 min followed by a second phase after 2 h of exposure (Fig. 4A).

To corroborate the immunoblotting findings, we next examined the effect of UfCP on the functional activation of p38 MAPK. As shown in Fig. 4B, UfCP induced p38 MAPK activity in NHBE cells, with the strongest activation seen at 8 h of exposure. To determine whether p38 MAPK activation is necessary for UfCP-induced IL-8 expression, we used a specific p38 MAPK inhibitor, SB-202190. As shown in Fig. 4C, preincubation with SB-202190 (15 μg/ml) for 30 min completely inhibited UfCP-induced IL-8 expression. These results strongly suggest that p38 MAPK activation is necessary for UfCP-induced IL-8 expression.

**Fig. 4. p38 mitogen-activated protein kinase (MAPK) activation is involved in IL-8 mRNA expression induced by UfCP exposure.** A: UfCP induce p38 MAPK phosphorylation in NHBE cells. NHBE cells were treated with UfCP (66 μg/ml) for 30 min and 1, 2, 4, 8, 12, or 20 h or vanadyl sulfate (100 μM) for 30 min. Cell lysates were separated by SDS-PAGE and immunoblotted with p38 and anti-phospho-p38 MAPK antibodies. Densitometric analyses were carried out on 3 independent experiments and are shown as the degree of increase over control. B: UfCP induce p38 MAPK activity in NHBE cells. NHBE cells were treated with UfCP (66 μg/ml) for 30 min and 1, 2, 4, 8, 12, and 20 h or vanadyl sulfate (100 μM) for 1 h. p38 MAPK was immunoprecipitated from cell lysates using anti-p38 MAPK antibodies, and p38 kinase activity was determined against ATF-2 using radiolabeled [32P]ATP. Data represent means ± SE of 3 independent experiments. *P < 0.05 compared with control. C: inhibition of p38 activation blocks UfCP-induced IL-8 expression. Cells pretreated with SB-202190 (15 μg/ml) for 30 min were incubated with UfCP (66 μg/ml) for 18 h. IL-8 mRNA normalized by GAPDH mRNA are shown in arbitrary units. Fold increases are shown over bars. Shown are means ± SE of 3 independent experiments. Significance from untreated controls (*) and significance from UfCP control condition (#), P < 0.05 compared with control.
MAPK. p38 MAPK activity was quantified by measuring the ability of p38 MAPK to transfer a phosphate group from \(^{32}\text{P}\)ATP to the p38 substrate ATF-2 by anti-p38 MAPK immunoprecipitates prepared from cells treated with UfCP or media alone.

In concordance with the immunoblotting data, p38 MAPK activity data also exhibited a biphasic activation of p38 MAPK induced by UfCP exposure with an early response at 30 min followed by a second phase after 2 h of exposure. The increased level of p38 activity was ~5.5-fold over control at 30 min and 4-fold over control at the later time point (Fig. 4B). However, the data were not statistically significant when analyzed by ANOVA.

Inhibition of p38 activation partially blocks UfCP-induced IL-8 expression. We next assessed the role of p38 MAPK activation in UfCP-induced IL-8 mRNA expression. NHBE cells were pretreated with the pyridinyl imidazole SB-202190, a p38 kinase activity inhibitor, before being stimulated with 66 \(\mu\text{g/ml UfCP for 12 h. As shown in Fig. 4C, SB-202190 partially blocked IL-8 mRNA expression, suggesting that p38 is at least partially responsible for UfCP-induced IL-8 expression.}

To rule out the possibility of cytotoxicity resulting from SB-202190, we measured cell viability by analyzing LDH release in the culture media. NHBE cell viability in the presence of SB-202190 did not differ from that of untreated cells under the experimental conditions (data not shown).

DISCUSSION

Although ultrafine particles contribute negligibly to the overall mass of PM in ambient air, they grossly outnumber particles of larger size and have been shown to be an important contributor to PM-induced toxicity. In addition, ultrafine particles are of interest because of their high deposition efficiency in the respiratory tract and their ability to reach into the deep lung (6, 7, 50, 52). Also intriguing is the fact that the inflammation of ultrafine particles is associated with the very high surface areas of these particles (4, 31). Although it is likely that the dosages used in this in vitro mechanistic study are probably considerably greater than those attainable in the environment, the concentrations of UfCP used in this study were intended for an initial examination of the specific effects of these particles on IL-8 expression and its underlying mechanism. The fact that the noncytotoxic concentrations of UfCP were used increases the likelihood that the events described in this study are representative of those that occur under “real-world” conditions.

Stimulation with ambient air pollutants, such as ozone, diesel exhaust particles, and metals, including sodium arsenite, vanadyl sulfate, and oil fly ash, has previously been shown to induce IL-8 expression in airway epithelial cells in vitro (11, 17–19, 24, 57, 58). This study demonstrates that UfCP exposure induces elevated expression of IL-8 mRNA and protein in NHBE cells. IL-8 expression is known to be upregulated by both mRNA transcription and stabilization (21, 47). In this study, we show that IL-8 expression induced by UfCP in NHBE cells involves increased IL-8 promoter activity. The magnitude of the UfCP-induced IL-8 mRNA increase exceeded that of the IL-8 promoter activity, suggesting that UfCP might activate transcriptional elements outside of those present in the IL-8 promoter construct used in this study, or that IL-8 mRNA stabilization may also contribute to UfCP-induced IL-8 expression. Additional studies will be required to examine this possibility.

The IL-8 promoter contains functional binding sites for the transcription factors NF-\(\kappa\)B, AP-1, and C/EBP\(\beta\) (1, 46). Activation of all three of these transcription factors is required for maximal transcriptional activity, although there are tissue- and stimulus-specific differences. Previous studies have shown that a variety of xenobiotic stimuli, including PM\(_{2.5}\), copper, diesel exhaust particles, vanadium, and ultrafine carbon black, activate NF-\(\kappa\)B-dependent transcription characterized by NF-\(\kappa\)B-DNA binding and NF-\(\kappa\)B-dependent promoter reporter activity (18, 19, 24, 45, 55, 58). In contrast, our study shows that NF-\(\kappa\)B activation does not appear to contribute to UfCP-induced IL-8 expression in NHBE cells. This conclusion is based on four independent lines of evidence. First, immunoblotting analyses using specific anti-I\(\kappa\)B\(\alpha\) antibody showed that I\(\kappa\)B\(\alpha\) degradation is not induced by UfCP exposure of NHBE cells. Second, NF-\(\kappa\)B DNA binding was not observed in NHBE cells exposed to UfCP, as assessed by EMSA analysis. Third, UfCP did not induce I\(\kappa\)B-dependent transcriptional activity. Fourth, dominant-negative I\(\kappa\)B\(\alpha\) overexpression did not alter IL-8 promoter-reporter activity induced by UfCP exposure. The activation of transcription factors shows cell type- and stimuli-specific patterns. For example, H\(_2\)O\(_2\) activates AP-1 but not NF-\(\kappa\)B, whereas TNF-\(\alpha\) activates AP-1 and NF-\(\kappa\)B in A549 epithelial cells. In HMEC-1 endothelial cells, however, TNF-\(\alpha\) activated NF-\(\kappa\)B but not AP-1, whereas H\(_2\)O\(_2\) did not activate either transcription factor (28). Thus, although the activation of NF-\(\kappa\)B is known to be a major mechanism regulating IL-8 gene expression induced by a variety of stimuli, our data do not support a role for NF-\(\kappa\)B activation in UfCP-induced IL-8 expression by NHBE cells. The implication of this finding is that another transcriptional regulatory pathway, such as AP-1 or C/EBP\(\beta\), is responsible for IL-8 gene transcription in response to UfCP exposure of NHBE cells. Additional studies will be needed to examine the role of these transcription factors in UfCP-induced IL-8 expression in NHBE cells.

The MAPK pathways play a very important role in signal transduction, leading to transcription in response to a variety of extracellular growth factors, cytokines, and cellular stresses, including ultraviolet and ionizing radiation, hyperosmolarity, and heat shock (5). Acute exposure to diesel exhaust particles and metallic components of ambient PM, including As, Cr, Cu, V, or Zn, results in the activation of ERK, JNK, and p38 MAPK pathways and induces the phosphorylation of the transcription factors c-Jun and ATF-2 and IL-8 expression in human bronchial epithelial cell lines (11, 18, 51, 63, 64). Our study demonstrates that p38 MAPK is phosphorylated and functionally activated in NHBE cells exposed to UfCP. Both immunoblotting and p38 MAPK activity assay showed that the activation of p38 MAPK induced by UfCP exposure is biphasic, with an early activation occurring before 30 min followed by a late phase after 2 h. However, there was no accompanying biphasic pattern in the IL-8 expression time course in response to UfCP exposure. The significance of this discrepancy is not known but could reflect a bimodal mechanism of activation that involves a protein intermediate(s) that participates in the expression of IL-8, such as transactivation of transcription...
factors or induction of IL-8 mRNA stabilization. A similar pattern of p38 MAPK activation was reported for cyclooxygenase-2 expression induced in response to stimulation with IL-1α (29).

Previous studies have revealed that the p38 MAPK pathway is involved in cytokine production in response to certain stimuli, including TNF-α, IL-1β, and IL-17, and extracellular stressors, such as hyperosmolality and diesel exhaust particles (11, 12, 20, 22). In this study, we examined the involvement of p38 MAPK activation in IL-8 expression induced by UfCP exposure. For this purpose, we used a drug (SB-202190) that competitively inhibits ATP binding to p38 MAPK. Inhibition of p38 MAPK activation reduced IL-8 mRNA expression induced by UfCP significantly. There are several possible reasons that might account for the fact that we did not observe a complete inhibition of UfCP-induced IL-8 expression upon pretreatment of NHBE with SB-202190. For instance, the prolonged time course may have resulted in degradation of the inhibitor or the activation of a compensatory cellular mechanism. Alternatively, it may not be possible to use a sufficiently high dose of SB-202190 to produce a complete inhibition of p38 activity for the duration required to induce IL-8 expression without inducing cytotoxicity. Additionally, the p38 γ-isoform, which is not inhibited by SB-202190, may mediate UfCP-induced IL-8 expression (10). Finally, it is possible that other signaling pathways are involved in UfCP-induced expression of IL-8 in NHBE cells. In preliminary studies, we have observed phosphorylation of JNK and ERK in UfCP-exposed NHBE cells, and that inhibition of JNK, but not the MAP kinase kinase MEK, blocks IL-8 mRNA expression induced by UfCP exposure (Kim, unpublished observations).

p38 MAPK regulates the activity of multiple transcription factors in a phosphorylation-dependent manner. The AP-1 family of transcription factors consists of homodimers and heterodimers of Jun, Fos, and members of the ATF family (23). For instance, ATF-2 is one of the transcription factors that is phosphorylated in the p38 pathway, and it is also necessary for forming AP-1, which is a transcription factor involved in IL-8 expression (23). Thus, although NF-κB-dependent transcription is not the major determinant of the marked increase in IL-8 mRNA expression induced by UfCP in our study, the involvement of p38 MAPK suggests the involvement of other transcription factors, possibly AP-1.

In summary, carbonaceous ultrafine particles appear to be a potent inducer of proinflammatory responses in NHBE cells. UfCP induce a marked increase in IL-8 mRNA and protein expression in NHBE cells. This study shows that UfCP-induced IL-8 expression is transcriptionally dependent, does not appear to involve NF-κB, and is controlled by the p38 MAPK signaling pathway. We conclude that the p38 MAPK signaling contributes to the activation of IL-8 expression induced by UfCP in NHBE cells through a mechanism that likely involves other signaling intermediates.

ACKNOWLEDGMENTS

We thank Drs. D. A. Brenner for the Ad5IeBα virus, R. Jude Samulski [Gene Therapy Vector Core, University of North Carolina (UNC) School of Medicine, Chapel Hill, NC] and the UNC Vector Core for preparation of the Ad5IeBα and Ad5SCMV3 viruses, and Dr. Philip A. Bromberg for critical review of the manuscript. The technical assistance of Lisa A. Dailey is also gratefully acknowledged.

DISCLOSURES

The research described herein has been reviewed by the National Health and Environmental Effects Research Laboratory and has been approved for publication. Approval does not signify that the contents necessarily reflect the views and policies of the U.S. EPA, nor does mention of trade names constitute endorsement of recommendation for use.

REFERENCES

ULTRAFINE PARTICLES INDUCE IL-8 EXPRESSION


