Regulation of cytokine production in human alveolar macrophages and airway epithelial cells in response to ambient air pollution particles: Further mechanistic studies

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Abstract

In order to better understand how ambient air particulate matter (PM) affect lung health, the two main airway cell types likely to interact with inhaled particles, alveolar macrophages (AM) and airway epithelial cells have been exposed to particles in vitro and followed for endpoints of inflammation, and oxidant stress. Separation of Chapel Hill PM 10 into fine and coarse size particles revealed that the main proinflammatory response (TNF, IL-6, COX-2) in AM was driven by material present in the coarse PM, containing 90–95% of the stimulatory material in PM10. The particles did not affect expression of hemoxygenase-1 (HO-1), a sensitive marker of oxidant stress. Primary cultures of normal human bronchial epithelial cells (NHBE) also responded to the coarse fraction with higher levels of IL-8 and COX-2, than induced by fine or ultrafine PM. All size PM induced oxidant stress in NHBE, while fine PM induced the highest levels of HO-1 expression. The production of cytokines in AM by both coarse and fine particles was blocked by the toll like receptor 4 (TLR4) antagonist E5531 involved in the recognition of LPS and Gram negative bacteria. The NHBE were found to recognize coarse and fine PM through TLR2, a receptor with preference for recognition of Gram positive bacteria.

Compared to ambient PM, diesel PM induced only a minimal cytokine response in both AM and NHBE. Instead, diesel suppressed LPS-induced TNF and IL-8 release in AM. Both coarse and fine ambient PM were also found to inhibit LPS-induced TNF release while silica, volcanic ash or carbon black had no inhibitory effect. Diesel particles did not affect cytokine mRNA induction nor protein accumulation but interfered with the release of cytokine from the cells. Ambient coarse and fine PM, on the other hand, inhibited both mRNA induction and protein production. Exposure to coarse and fine PM decreased the expression of TLR4 in the macrophages. Particle-induced decrease in TLR4 and hyporesponsiveness to LPS may be related to LPS tolerance induced by low levels of LPS.

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Introduction

Epidemiological studies have associated exposure to ambient particulate matter (PM) with adverse cardiorespiratory effects (Dockery et al., 1993; Pope and Dockery, 1998; Pope et al., 1995; Schwartz et al., 1996). Proposed mechanisms behind PM-induced health effects are oxidant stress (Nel et al., 2001; Tao et al., 2003) and inflammation-associated injury (Dick et al., 2003; Ghio and Devlin, 2001). Recent in vitro studies with airway epithelial cells have demonstrated that exposure to pollution particles induces IL-8 production and various responses associated with oxidant stress, (Hetland et al., 2004; Li et al., 2002). Lung macrophages respond to PM by releasing a variety of cytokines, while showing decreased phagocytic function (Becker et al., 2003; Soukup and Becker, 2001).

Previous studies (Becker et al., 2002) have shown that ambient air pollution particles contain components which stimulate Toll like receptors 2 and 4 (TLR2 and TLR4). It was therefore proposed that components of whole Gram positive and Gram negative bacteria were associated with the particles, including the ubiquitously present lipopolysaccharide (LPS). In AM, the cytokine response to particles was inhibited by antibody to CD14 and an antagonist of TLR4, emphasizing the role of bacterial products in the response (Becker et al., 2002; Soukup and Becker, 2001). Also, airway epithelial cells have been shown to respond to various bacterial pathogens by the release of cytokines (Becker et al., 2004; Larsson et al., 1999). These cells have been shown to express several members of the TLR family of receptors (Becker et al., 2000), which are known to be involved in the recognition components of Gram-positive and -negative bacteria, viruses as well as fungal elements (Janssens and Beyaert, 2003). Therefore, epithelial cells may also respond to microbial products, other than LPS, contained in ambient air PM.

Diesel particles, which constitute the main mass of fine PM in some locations, induce negligible amounts of cytokine in AM compared to amounts induced following stimulation with ambient PM (Becker et al., 1996). Instead, the suppressive effect of diesel on cytokine production by LPS or other microbial products have been emphasized as a possible mechanism whereby diesel exerts its health effects (Amakawa et al., 2003; Yang et al., 2001). Fine pollution particles may have a similar effect to diesel since the major component of this size fraction in the area of collection (Chapel Hill, NC) is diesel dust. Experiments were conducted to compare suppressive effect of diesel, ambient fine and coarse PM, on LPS-induced cytokine production. Both PM size fractions were found to be strongly suppressive, and the effect of at least coarse PM unlikely to be related to organic compounds of diesel. Since cytokine induction by coarse PM, and fine PM, involved stimulation of TLR4, it is possible that these particles induce an state similar to well-documented LPS tolerance (Dobrovolskaia and Vogel, 2002; Nomura et al., 2000; Schwartz, 2002).

Materials and methods

Particle collection. Ambient air particles: Particles were collected in Chapel Hill, North Carolina, using a ChemVol high Volume Cascade Impactor (Rupprecht and Patashnick Co. Albany, NY). Coarse PM and fine PM particles were collected for 72 h onto polyurethane foam ( McMaster-Carr, Atlanta, GA), which were previously cleaned with methanol and water and dried under sterile conditions. Ultrafine particles were collected onto G5300 filters (Monadnock Non-Wovens LLC, Mt. Pocono, PA). The foams/filters cut into strips were placed in a 50-ml culture tube, then wetted with a small amount of 70% ethanol, before addition of endotoxin-free water to a total of 40 ml. The particles were sonicated for 30 min in a sonicating water bath (FS220, Fisher Scientific). The supports were removed and particles were then lyophilized.

Diesel exhaust particles used were Standard Reference Materials 2975 (SRM 2975 obtained from the National Institute of Standards and Technology (NIST; Gaithersburg MD). Silica, volcanic ash and carbon black were from stocks used in other studies (18).

Isolation and culture of normal human bronchial epithelial cells (NHBE) and alveolar macrophages. Volunteers, 20–35 years old, were recruited to undergo airway biopsy and bronchoalveolar lavage (Ghio et al., 1998), under a research protocol approved by the Internal Review Board at University of North Carolina Medical School. None of the subjects had a history of asthma, allergic rhinitis, chronic respiratory disease or cardiac disease. Subjects were excluded from the study if they had a recent acute respiratory illness and were asked to avoid exposure to air pollutants such as tobacco smoke and paint fumes. Biopsies of the epithelial cell lining of the distal trachea or the proximal bronchus were taken using cytology brushes (BARD International Products, Tewksbury MA). The removal of cells from the brushes, their disaggregation and culture has been previously described in detail (Wu et al., 2001). At confluence, NHBE were seeded into 12-well cluster plates ( Costar 3512) at a density of 50,000–100,000 cells/well in 2 ml BEGM. Medium was replaced every 48 h until cells were >90% confluent. At that point, NHBE were exposed to equal concentrations of coarse, fine or ultrafine PM in BEGM for
18 h, unless otherwise noted. Supernatants were collected and stored at −80 °C until analyzed. RNA was extracted from the cells for RT-PCR analysis.

BAL cells containing AM at 2–3 × 10^5 cells in 1 ml of RPMI-1640 supplemented with 2% fetal bovine serum (FBS) (Life Tech., Rockville, MD) were exposed to particles for 18 h in 5 ml polypropylene tubes. Supernatants were collected for cytokine assessment and cells for mRNA assessment. In some experiments, AM cultures were further challenged with 10 ng/ml LPS and supernatants collected and cells lysed after 6 h of exposure with 0.1% Triton X containing protease inhibitors.

**ELISA assays.** Cytokine ELISA kits for IL-6, TNF and IL-8 were purchased from R&D Systems and used according to the manufacturer’s directions.

**Inhibition of cytokine release.** Endotoxin activity present on particles was inhibited by pre-incubation with Polymixin B Sulfate (Sigma, St. Louis, MO) at a concentration of 10 µg/ml. TLR4 activity was blocked using the lipid A agonist E5531 (Kawata et al., 1999), at 3 µg/ml in NHBE and AM cultures. This reagent was kindly shared by Dr. M. Fenton (Boston University) in whose laboratory the preliminary experiments were conducted, and was gift of Eisai Corporation, Boston MA. TLR2 activity was blocked using a TLR2 antibody (2239), a kind gift of Genentech, at a concentration of 50 and 25 µg/ml in NHBE cultures. An IgG1 isotype mouse monoclonal antibody was used as control. The cells were incubated with the inhibitors E5531 and anti TLR2 for 30 min before addition of particles or bacteria.

**Isolation of RNA.** Total RNA from NHBE and AM was isolated on cesium chloride gradients according to previously described procedure (Brenner et al., 1989). The RNA was reverse transcribed to generate cDNA for PCR analysis using M-MLV reverse transcriptase (Promega).

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**Fig. 1.** Effects of ambient PM size on NHBE and alveolar macrophage mediators of inflammation and antioxidant defense. mRNA levels of IL-8, IL-6, COX-2 and HO-1 were assessed in NHBE (panel A) and in AM (panel B) after 18 h of exposure to coarse, fine and ultrafine particles. The data points represent the means ± SEM of 3–4 separate experiments.
Real-time quantitative PCR. Relative gene expression in NHBE and AM was quantified using Real-Time Quantitative PCR. Oligonucleotide primer pairs for GAPDH, COX-2, TNF, IL-8, TLR2 and TLR4 and fluorescent probes were designed using a primer design program (Primer Express, Applied Biosystems, Foster City, CA) and obtained from Integrated DNA Technologies (Coralville, IA). Quantitative fluorogenic amplification of cDNA was performed using the ABI Prism 7700 Sequence Detection System (Applied Biosystems), TaqMan Universal PCR Master Mix (Applied Biosystems), and the primers and probes. The relative abundance of mRNA levels was determined from standard curves generated from a serially diluted standard pool of cDNA prepared from stimulated NHBE cells or LPS-stimulated AM. The relative abundance of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was used to normalize levels of the mRNAs of interest. Primer sequences have been previously cited (Becker et al., 1991, 2005; Samet et al., 1996).

Statistical analysis. Analysis was performed by one-way ANOVA and Tukey’s multiple comparison post-test. \( P < 0.05 \) was considered significant.

Results

Oxidant stress

Although epidemiological studies have suggested that fine particles may be more detrimental to human health than coarse mode particles (Sarnat et al., 2001; Schwartz et al., 1996), in vitro studies (Becker et al., 2003; Hetland et al., 2004), and animal in vivo data (Dick et al., 2003; Dybing et al., 2004; Schins et al., 2004; Tao et al., 2003) have provided equivocal support for the preferential toxicity of fine particles. It was shown that coarse particles in AM and airway epithelial cells are responsible for most of the proinflammatory effects induced, as well as inflammation and cytokine production in vivo. Here, mRNA expression for proinflammatory cytokines and COX-2 was compared in AM and epithelial cells exposed to the three PM size fractions and these inflammatory mediators were preferentially induced by the coarse fraction (Figs. 1A and B) in both NHBE and AM. On the other hand, a sensitive measure of oxidant stress (Choi and Alam, 1996; Li et al., 2002), i.e., the expression of heme oxygenase-1 was unaffected in AM, or possibly reduced while strongly induced in NHBE preferentially by the fine fraction. Oxidant stress has been suggested to be one of the stresses on the cardiopulmonary system responsible for health effects (Gurgueira et al., 2002; Nel et al., 2001; Rhoden et al., 2004; Tao et al., 2003). In direct measures of oxidant generation in the cells using fluorescent substrates dichlorofluorescin and dihydrodernmine-123, we found no significant difference in generation between the three size fractions, neither in NHBE nor AM (data not shown). While NHBE cells generated both H2O2 and superoxide, AM only generated H2O2. This is in agreement with studies using diesel particles on epithelial cells and a macrophage like cell line THP-1 (Li et al., 2002). The epithelial cells responded by O2•− production and HO-1 induction while the RAW cells did not. Part of the stress response may be caused by organics or metals in the fine fraction (Donaldson et al., 1997; Li et al., 2002). Thus, it appears that the proinflammatory and oxidant stress responses are driven by different components in PM.

Fig. 2. Involvement of TLR2 in airway epithelial cell IL-8 response to ambient pollution particles. NHBE cells were exposed to 250 \( \mu \)g/ml particles or bacteria (10 bacteria/cell) in the presence of anti-TLR2 antibody or an irrelevant control antibody. Supernatants were collected after 18 h and assessed for IL-8. The bars represent the means ± SEM of three separate experiments.

Fig. 3. Involvement of TLR4 in alveolar macrophage and airway epithelial cell cytokine response to ambient pollution particles. NHBE (panel A) and AM (panel B) were exposed to coarse, fine and ultrafine particles in the presence or absence of polymixin B or E5531. Supernatants were collected after 18 h and assessed for IL-8 (NHBE) or IL-6 (AM). The bars represent means ± SEM of three separate experiments.
Role of TLR in epithelial cell cytokine production

Since PM10 contain material which is recognized by Toll like receptors 2 and 4 (Becker et al., 2002), the possibility that NHBE cells use these receptors in their cytokine response to PM was explored. NHBE express mRNA for several known TL receptors (Becker et al., 2000). Quantitative comparison of TLR2 and TLR4 expression in NHBE and AM showed that AM express at least 10 times the amount of TLR4 than expressed in NHBE, while both cell types express similar amounts of TLR2 mRNA (not shown). To demonstrate the involvement of TLR2 in the response of NHBE to PM as well as Gram+ and Gram− bacteria, activation was blocked by antibody to TLR2 as shown in Fig. 2. Neither LPS nor TLR4 was involved in the NHBE response, since neither polymixin B nor E5531 had any effect of NHBE cytokine production (Fig. 3A). This was contrasted the response of AM (Fig. 3B). With these cells, the response to both coarse and fine particles was blocked by polymixin B and E5531. The results imply that microbial material can affect both epithelial cells and AM. When discussing urban air pollution, it is important to take into consideration that human activity and waste increase microbial material in the air. It is proposed that degradation products of bacteria are preferentially attached to coarse pollution particles, and that bacteria themselves, dead or alive are collected as components of coarse more than fine PM. This may vary with particle collection method. Fine particles collected in the high volume sampler contain more cytokine stimulatory activity than particles collected by a dichotomous sampler reported in previous studies (Becker et al., 2003; Soukup and Becker, 2001).

The results, obtained with both AM and NHBE emphasize the importance of biological materials, ubiquitously present in or carried on pollution particles. However, others have shown that also oxidant stress induced cytokine induction may involve TLR2 (Kirschning and Schumann, 2002). While it is less likely that the ultrafine particle fraction contain microbes, the oxidant stress induced by this fraction may be responsible for cytokine induction.

Inhibition of LPS response by pollution particles

Pollution particles may also inhibit cytokine production. The suppressive effects of diesel particles on LPS-induced cytokine production has been investigated by several

![Fig. 4. Effect of coarse and fine particles on TLR4 expression in alveolar macrophages. AM were exposed to 50 μg/ml coarse or fine particles for 18 h. Expression of TLR4 mRNA was determined by quantitative RT/PCR, and relative expression of mRNA determined. The bars represent the means ± SEM of three separate experiments.](image)

![Fig. 5. Effect of diesel and ambient pollution particles on alveolar macrophage TNF production induced by LPS. AM were exposed to particles overnight, the cells were washed and then stimulated for 6 h with 10 μg/ml LPS for TNF protein determination in supernatants and cell lysates (panels A and B), and for 4 h for TNF mRNA determination (panels C and D). The bars represent the means ± SEM of 2–4 separate experiments.](image)
laboratories (Amakawa et al., 2003; Yang et al., 2001). Also, exposure of macrophages to low levels of LPS has been shown to inhibit cytokine production by a subsequent exposure to LPS. Dysregulation of TLR4, both the expression and its signaling pathway, has been associated with this tolerance (Medvedev et al., 2000). The possibility that TLR4 in AM was affected by the low levels of LPS/TLR4 stimulatory components on pollution particles was investigated, as was the response to LPS after exposure to pollution particles. Fig. 4 shows that AM exposed to coarse PM express reduced levels of TLR4 mRNA. A similar downregulation of TLR4 expression has been observed in mouse macrophages (Dobrovolskaia and Vogel, 2002). Diesel particles known to inhibit TNF and IL-8 release by LPS did not show this response. Diesel was found to inhibit release of the cytokine rather than the induction of cytokine mRNA and protein production (Fig. 5A). The cytokines, both IL-6 and TNF, were retained in the diesel exposed cells while total levels of cytokine found in the LPS-stimulated previously unexposed and diesel exposed cells were the same. In contrast, AM exposed to ambient air pollution particles showed decreased levels of total TNF protein, but not IL-8 (Fig. 5B). This appeared to be a consequence of a decrease in TNF mRNA induction following exposure to LPS. It is likely that the inhibition of LPS-induced TNF production by air ambient pollution particles, but not diesel, is related to LPS and possibly other microbial material in the ambient particles.

Conclusion

Taken together, the selected experiments shown here demonstrate possible mechanisms whereby pollution particles affect lung health. If inflammation is involved as in asthma and inflammatory lung diseases, particles may exacerbate symptoms or obstruct a normal response. Prolinflammatory measures appear to be the dominant response to coarse PM, which affect cytokine production and proaglandin synthesis. This response appears to be driven by TLR2 and TLR4 stimulating compounds in both NHBE and AM. Involvement of TLR4 in the AM response to ambient pollution particles may lead to tolerance to LPS induced cytokine production. This could result in an inability to respond appropriately to infection.

References

positive bacteria induce IL-6 and IL-8 production in human alveolar macrophages and epithelial cells. Inflammation 23, 217 – 230.


