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Ozone-induced Lung Injury and Sterile Inflammation. Role of Toll-Like Receptor 4

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Abstract

Inhalation of toxic doses of ozone is associated with a sterile inflammatory response characterized by an accumulation of macrophages in the lower lung which are activated to release cytotoxic/proinflammatory mediators that contribute to tissue injury. Toll-like receptor 4 (TLR4) is a pattern recognition receptor present on macrophages that has been implicated in sterile inflammatory responses. In the present studies we used TLR4 mutant C3H/HeJ mice to analyze the role of TLR4 in ozone-induced lung injury, oxidative stress and inflammation. Acute exposure of control C3H/HeOuJ mice to ozone (0.8 ppm for 3 hr) resulted in increases in bronchoalveolar lavage (BAL) lipocalin 24p3 and 4-hydroxynonenal modified protein, markers of oxidative stress and lipid peroxidation. This was correlated with increases in BAL protein, as well as numbers of alveolar macrophages. Levels of surfactant protein-D, a pulmonary collectin known to regulate macrophage inflammatory responses also increased in BAL following ozone inhalation. Ozone inhalation was associated with classical macrophage activation, as measured by increased NF- κ B binding activity and expression of TNF α mRNA. The observation that these responses to ozone were not evident in TLR4 mutant C3H/HeJ mice demonstrates that functional TLR4 contributes to ozone-induced sterile inflammation and macrophage activation.

Keywords

ozone; sterile inflammation; TLR4; macrophages; NF- κ B; TNF α

Introduction

Ozone is a highly reactive gas present in photochemical smog. Inhalation of toxic levels of ozone results in constriction of the airways, increased bronchial reactivity and decreased lung functioning (Mudway and Kelly, 2004; Savov et al., 2004). Ozone also targets alveolar epithelial cells in the lower lung disrupting barrier functioning and allowing proteins to enter the alveolar space (Fakhrzadeh et al., 2004; Kleeberger et al., 2000). This leads to an

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Conflict of interest statement

The authors declare no conflict of interest.

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accumulation of inflammatory macrophages in the lung and proliferation and transformation of type II pneumocytes, a key step in repair of the alveolar epithelium. In response to oxidative stress and products released from injured tissues, lung macrophages are classically activated to release cytotoxic/proinflammatory mediators such as tumor necrosis factor alpha (TNF α) and highly reactive oxygen and nitrogen species which contribute to the pathogenic response (Laskin et al., 2011). The observation that pulmonary damage induced by ozone is prevented or ameliorated by blocking classical macrophage activation or the production of proinflammatory mediators provides support for this idea (Cho et al., 2001; Fakhrzadeh et al., 2004; Fakhrzadeh et al., 2008; Giri et al., 1975; Haddad et al., 1995; Pendino et al., 1995).

Toll-like receptor 4 (TLR4) belongs to a family of pattern recognition receptors, which are rapidly upregulated on macrophages in response to pathogens, proinflammatory cytokines and environmental stress (Kono and Rock, 2008; Lin et al., 2011). Engagement of TLR4 leads to the recruitment of adaptor proteins and triggering of downstream signaling molecules culminating in activation of nuclear factor-kappa B (NF- κ B) and upregulation of proinflammatory mediators including TNF α , and enzymes that generate cytotoxic mediators such as inducible nitric oxide synthase (iNOS) (Akira and Takeda, 2004). TLR4 has previously been reported to play a role in ozone-induced hyperpermeability and inflammation (Bauer et al., 2011; Kleeberger et al., 2001). The present studies demonstrate that ozone-induced oxidative stress, lipid peroxidation, and macrophage accumulation and activation in the lung are also dependent on functional TLR4. These findings are important as they suggest a general role of TLR4 signaling in sterile inflammatory responses to tissue injury.

Materials and methods

Animals and treatments

Male TLR4 mutant C3H/HeJ and control C3H/HeOuJ mice (11–12 weeks) were purchased from The Jackson Laboratory (Bar Harbor, ME). Mice were housed in sterile microisolation cages and provided autoclaved food and water *ad libitum*. Animal care was in compliance with Rutgers University guidelines as outlined in the *Guide for the Care and Use of Laboratory Animals* prepared by the National Academy of Sciences. Mice were placed in a Plexiglass chamber and exposed for 3 hr to ozone (0.8 ppm) or air control. Ozone was generated from oxygen gas via ultraviolet light ozone generator (Orec Corp., Phoenix, AZ) and mixed with the inlet air of the exposure chamber. Ozone concentrations in the chamber were stabilized by adjusting both the intensity of the ultraviolet light and the flow rate of ozone into the chamber and were continuously monitored using an ozone monitor (Model 1008 AH, Dasibi Environmental Corp., Glendale, CA).

Sample Collection

Mice were anesthetized by i.p. injection of Nembutal (200 mg/kg). The lung was perfused (10 ml/min) with 50 ml of warm (37°C) Ca²⁺/Mg²⁺-free Hank's balanced salt solution (HBSS) containing 25 mM HEPES, 0.5 mM EGTA and 4.4 mM NaHCO₃ at pH 7.3. The trachea was cannulated and the lung removed from the chest cavity. Bronchoalveolar lavage (BAL) was collected by slowly instilling and withdrawing 1 ml of HBSS 7–10 times through the cannula. Total protein was quantified in the first ml of BAL fluid using the BCA Protein Assay kit (Pierce Biotechnologies Inc., Rockford, IL) with bovine serum albumin as the standard. BAL fluid was centrifuged (300 $\times$ g for 8 min), supernatants collected, aliquoted, and stored at –80°C until analysis. Cell pellets were washed 4 times with HBSS containing 2% FBS and then enumerated using a hemocytometer. Viability was 98% as

determined by trypan blue dye exclusion, and cell purity >97% macrophages as assessed morphologically after Giemsa staining.

Western blotting

BAL fluid (500 μ l) was concentrated by centrifugation at $14,000 \times g$ for 33 min using a 10K centrifugal filter (Millipore, Billerica, MA) and then fractionated on SDS 10.5–14% Tris-HCl polyacrylamide Criterion™ Precast gels (Bio-Rad, Hercules, CA). After transferring to Trans-Blot pure nitrocellulose membranes (Bio-Rad, Hercules, CA), non-specific binding was blocked by incubation of the membrane with 5% FBS for 1 hr at room temperature. Blots were incubated overnight with a 1:500 dilution of rabbit anti-lipocalin 24p3 or mouse monoclonal anti-4-hydroxynonenal (4-HNE, Abcam, Cambridge, MA), or a 1:2000 dilution of rabbit anti-SP-D (Millipore, Billerica, MA) antibodies in 5% FBS. This was followed by incubation with a 1:20,000 dilution of horseradish peroxidase-conjugated secondary antibody (Cell Signaling, Danvers, MA) in 5% FBS for 1 hr at room temperature. Bands were visualized using a SuperSignal® West Pico Chemiluminescent Substrate kit (Thermo Scientific, Rockford, IL). Densitometry was performed using Image Processing and Analysis in Java (ImageJ) gel analyzer software.

Immunohistochemistry

Perfused lung was inflation-fixed in 10% formalin buffer overnight at room temperature, followed by 50% ethanol. Lung sections (6 μ m) were deparaffinized, then incubated for 30 min with 3% hydrogen peroxide to quench endogenous peroxidase. This was followed by incubation for 1 hr at room temperature with normal goat serum to block non-specific binding. Sections were then incubated overnight at 4 °C with rabbit antibody to pro-SP-C (1:2000, Millipore, Billerica, MA), or normal rabbit IgG. Antibody binding was visualized using a Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA).

Electrophoretic mobility shift assays (EMSA)

Nuclear extracts were prepared from BAL cells using Nuclear Extraction Reagent (NER) (Pierce, Rockford, IL) supplemented with a 1:50 dilution of Protease Inhibitor Cocktail (Sigma, St. Louis, MO) following the manufacturer's instructions. Binding reactions were carried out at room temperature for 30 min in a total volume of 15 μ l containing 5 μ g of nuclear extract protein, 5 μ l of 5X gel shift binding buffer (37.5% glycerol, 5 mM $MgCl_2$, 0.25 mM DTT, 175 mM NaCl, 37.5 mM HEPES, pH 8.0), 0.1% BSA, 1 μ g poly dI-dC and 2 μ l of γ [^{32}P]ATP (3000 Ci/mmol at 10 mCi/ml)-labeled NF- κ B (5'-AGT TGA GGG GAC TTT CCC AGG C-3') consensus oligonucleotide (Santa Cruz Biotechnologies, Santa Cruz, CA). Protein-DNA complexes were separated on 7% non-denaturing polyacrylamide gels ran at 150 V in 0.5X TBE (45 mM Tris-borate, 1 mM EDTA, pH 8.0). The gels were dried and autoradiographed. For competitor reactions, a 50-fold excess unlabeled NF- κ B oligonucleotide was added to the reaction mixture 2 hr prior to the addition of labeled probe.

Relative reverse transcription-polymerase chain reaction (RT-PCR)

DNase I treated total RNA was extracted from BAL cells using RNeasy Mini kit (QIAGEN Inc, Valencia, CA) according to the manufacturer's protocol. RNA concentrations were determined by absorbance at 260 nm. For cDNA synthesis, RNA (200 ng) in 9 μ l of water was denatured at 65 °C for 4 min, rapidly cooled on ice and then resuspended in a 20 μ l final volume containing 250 mM Tris-HCl pH 8.3, 375 mM KCl, 15 mM $MgCl_2$, 100 mM DTT, 10 mM dNTP, 200 μ M random hexamers and 200 units Superscript II Reverse Transcriptase (Invitrogen, Carlsbad, CA). After 1 hr incubation at 37 °C, 2 units RNase H was added and samples incubated for additional 20 min. The samples were denatured at 95 °C for 5 min and chilled on ice. Each sample reaction (20 μ l) contained 1 μ l cDNA

template, 0.8 mM mouse TNF α primer pair and a 1:9 ratio of 18S rRNA competitor/primer (Ambion, Austin, TX), 2 μ l of 10X PCR buffer, 10 mM dNTP, 0.2 μ l α [³²P] dCTP (10 mCi/ml; >3000 Ci/mmol), and 0.5 units Taq DNA polymerase (Invitrogen). Using GeneAmp PCR System 9600 (Perkin, Elmer), amplification was initiated at 94 °C for 1 min, followed by 23 cycles at 94 °C for 15 sec, 58 °C for 25 sec and 72 °C for 90 sec. The amplified PCR products were then run on a 5% denaturing polyacrylamide gel. The gel was dried and radioactive bands from the PCR products excised and counted in a scintillation counter. Amplifications for all samples were performed at the same time and run on the same gel to minimize variability.

Statistics

All experiments were repeated at least three times. Data were analyzed by one-way ANOVA; a p-value < 0.05 was considered significant.

Results

Initially we analyzed the role of TLR4 in lung injury and oxidative stress induced by acute exposure to ozone. Treatment of control C3H/HeOuJ mice with ozone resulted in a significant increase in BAL protein which peaked 12–24 hr post exposure, demonstrating alveolar epithelial injury (Fig. 1, upper panel). Subsequently protein levels began to decline. This was correlated with significant increases in BAL levels of lipocalin 24p3, a marker of oxidative stress (Roudkenar et al., 2007; Sunil et al., 2007), and the lipid peroxidation product, 4-HNE, as indicated by the appearance of a Mr = 50,000 modified protein (Fig. 2). As observed with total BAL protein, these were most prominent 12–24 hr post exposure. Ozone-induced lung injury and oxidative stress were followed by an accumulation of inflammatory cells in the lung, as measured by increased BAL cell content (Fig. 1). Differential analysis revealed that the majority of these cells (>98%) were macrophages.

SP-D is a pulmonary collectin known to play a role in regulating macrophage inflammatory responses (McCormack and Whitsett, 2002). Following ozone exposure, increased levels of SP-D were detected in BAL of C3H/HeOuJ control mice. C3H/HeJ TLR4 mutant mice were found to be significantly less sensitive to ozone than C3H/HeOuJ control mice; thus, no changes in BAL protein content or inflammatory cell accumulation were evident in these animals following ozone inhalation (Fig. 3). In addition, ozone-induced increases in BAL SP-D, 24p3 and 4-HNE modified protein were diminished in these animals relative to control C3H/HeOuJ mice.

NF- κ B is transcription factor known to regulate inflammatory gene expression in macrophages and is considered a marker of classically activated proinflammatory macrophages (Lawrence and Natoli, 2011). We next analyzed the role of TLR4 in ozone-induced activation of macrophage NF- κ B nuclear binding activity. In alveolar macrophages from C3H/HeOuJ control mice, a time-related increase in NF- κ B nuclear binding activity was observed following ozone inhalation; this was evident within 30 min and remained elevated for at least 24 hr after exposure (Fig. 4). Competition experiments using 50-fold excess unlabeled NF- κ B probe blocked NF- κ B binding demonstrating the specificity of NF- κ B DNA binding activity. Although ozone inhalation also induced NF- κ B nuclear binding activity in macrophages from C3H/HeJ TLR4 mutant mice, this activity was reduced when compared to C3H/HeOuJ mice; maximal activity was also delayed until 12 hr post exposure.

TNF α is a proinflammatory cytokine under the transcriptional control of NF- κ B (Lawrence et al., 2005). It is released by classically activated macrophages and has been shown to play an important role in the pulmonary toxicity of ozone (Cho et al., 2007; Fakhrzadeh et al., 2008). In C3H/HeOuJ mice ozone inhalation resulted in a significant increase in TNF α .

mRNA expression in alveolar macrophages, which was evident after 12 hr (Fig. 5). In contrast, only small increases in TNF α mRNA expression were observed in macrophages from TLR4 mutant mice 24 hr following ozone exposure.

In response to ozone-induced tissue injury, type II cells begin to proliferate in order to replace damaged epithelium (Mudway and Kelly, 2000). ProSP-C is a marker of proliferating type II epithelial cells (Weaver and Conkright, 2001). In C3H/HeOuJ mice, but not C3H/HeJ mice, ozone inhalation resulted in a time-dependent increase in pro-SP-C expression in type II epithelial cells beginning 24 hr after exposure and increasing for at least 72 hr (Fig. 6).

Discussion

Recent studies have shown that pattern recognition receptors including TLR4, are upregulated in macrophages, not only in response to microbial products, but also following exposure to cell-derived danger-associated molecular patterns and other products released by injured cells and tissues (Lorne et al., 2010; Matzinger, 2002). Like inflammatory responses to microbial products, the resulting “sterile” inflammatory response, is characterized by an accumulation of neutrophils and macrophages in the tissue, and the generation of chemokines and pro-inflammatory cytokines, such as TNF α , as well as reactive oxygen and nitrogen species (Chen and Nunez, 2010). The present studies demonstrate that functional TLR4 plays a role in lung inflammation, injury and oxidative stress induced following acute exposure to toxic doses of ozone. These data provide additional support for the essential contribution of TLR signaling to sterile inflammatory diseases (Lin et al., 2011).

Ozone is a strong oxidizing agent that reacts with cell membranes damaging epithelial cells in the lower lung leading to increased vascular permeability (Bhalla, 1999). Consistent with this observation, we found that exposure of control C3H/HeOuJ mice to ozone resulted in a significant increase in BAL protein. Findings that this response was blunted in TLR4 mutant C3H/HeJ mice support the idea that TLR4 signaling is important in ozone-induced lung hyperpermeability (Kleeberger et al., 2000; Kleeberger et al., 2001). In contrast, studies with TLR4 knockout mice have suggested that TLR4 is required for airway hyperresponsiveness after ozone inhalation, but not alveolar epithelial barrier dysfunction (Hollingsworth et al., 2004; Williams et al., 2007). These differences may be due to differential responses of TLR4 mutant versus TLR knockout mice to pulmonary irritants (Ewart et al., 2000; Savov et al., 2004; Takeda et al., 2001). We found that ozone-induced increases in BAL protein were correlated with elevated levels of lipocalin 24p3, an acute phase protein and marker of oxidative stress (Roudkenar et al., 2007; Sunil et al., 2007). Lipocalin 24p3 has been detected in the lung after exposure of rodents to endotoxin or particulate matter, as well as in sputum from patients with chronic obstructive pulmonary disease, and in blood from Cynomolgus monkeys exposed to ozone (Andre et al., 2006; Hicks et al., 2010; Keatings et al., 1997; Sunil et al., 2007; Sunil et al., 2009). Our findings that lipocalin 24p3 is increased in the lung after ozone are novel, and suggest that it may be a sensitive marker of acute oxidant-induced lung injury. In TLR4 mutant mice, ozone exposure had minimal effects on BAL lipocalin 24p3 content indicating that TLR4 signaling is also important in oxidative stress induced by this pulmonary irritant.

Ozone-induced oxidative stress is associated with lipid peroxidation resulting in the generation of toxic aldehydes such as 4-HNE, which form protein adducts. This leads to altered protein function, cytotoxicity and apoptosis (Grimsrud et al., 2008; Hamilton et al., 1998; Kirichenko et al., 1996; Li et al., 1996; Pryor and Church, 1991). Following exposure of C3H/HeOuJ mice to ozone, a 4-HNE-protein-adduct was identified in BAL. These

findings are in accord with previous observations in humans and rodents exposed to ozone (Hamilton et al., 1996; Hamilton et al., 1998; Kirichenko et al., 1996). The fact that levels of BAL 4-HNE-protein adducts and total protein levels peaked at the same time suggests that lipid peroxidation may contribute to alveolar epithelial injury following ozone exposure. Only low levels of protein and 4-HNE protein-adducts were present in BAL from C3H/HeJ TLR4 mutant mice after ozone exposure, which indicates a role of TLR4 in ozone-induced lipid peroxidation resulting from oxidative stress. A similar contribution of TLR4 to oxidative stress and lipid peroxidation has previously been described in murine liver during the development of steatosis or ischemia, in the heart after ischemia-reperfusion injury, or following doxorubicin intoxication, and in the brain after stroke (Caso et al., 2007; Oyama et al., 2004; Spruss et al., 2009; Tsung et al., 2007).

Inhalation of ozone by C3H/HeOuJ mice also resulted in increased numbers of alveolar macrophages in BAL, which was evident 48 hr after exposure. This was not observed in C3H/HeJ mice, providing additional support for TLR4 signaling in sterile lung inflammation induced by ozone. Previous studies have shown no effect of either acute or subchronic ozone on macrophage accumulation in BAL 24 hr after exposure in mice with defective TLR4 signaling (Williams et al., 2007). Our findings are consistent with these reports, and indicate that the macrophage response to ozone is delayed in C3H/HeOuJ mice.

Accumulating evidence suggests that macrophage functioning in the lung is regulated by the pulmonary collectin SP-D (Guo et al., 2008; King and Kingma, 2011; Sano and Kuroki, 2005). SP-D has been reported to bind to a soluble form of TLR4 and its adaptor proteins MD-2 (Nie et al., 2008; Ohya et al., 2006). This results in suppression of macrophage activation (Liu et al., 2010; Yamazoe et al., 2008). Following ozone exposure, we noted a significant increase in BAL SP-D levels. This may reflect an attempt by the host immune system to limit ozone-induced inflammation and tissue injury. This is supported by findings that loss of SP-D results in exacerbated inflammation in response to ozone (Kierstein et al., 2006). Loss of functional TLR4 was associated with decreased SP-D protein in the lungs after ozone exposure. This is most likely a consequence of reduced lung injury and inflammation in C3H/HeJ mice.

Engagement of TLR4 results in sequential activation of cytoplasmic Toll/IL-1 receptor domain, adaptor molecules, mitogen activated protein kinases and I κ B kinase (Akira and Takeda, 2004). This culminates in activation of the transcription factor NF- κ B which regulates expression of proinflammatory genes such as TNF α (Lin et al., 2011). In control C3H/HeOuJ mice, but not C3H/HeJ TLR4 mutant mice, ozone inhalation resulted in a time-related increase in NF- κ B nuclear binding activity which peaked after 24 hr. This was correlated with increased TNF α gene expression. Similar coordinate increases in NF- κ B and TNF α have been described in inflammation sensitive C57BL/6J mice following ozone exposure (Fakhrzadeh et al., 2004). Findings that mice lacking NF- κ B p50 are protected from ozone toxicity suggest that TLR4 dependent activation of NF- κ B is an important mechanism leading to oxidant-induced inflammatory mediator production and toxicity. This is supported by reports that loss of functional TLR4 results in a blunted NF- κ B response, reduced production of proinflammatory mediators, and decreased sensitivity of mice to hyperoxia (Ogawa et al., 2007).

Previous studies have shown that excessive production of TNF α by alveolar macrophages after ozone inhalation contributes to tissue injury (Cho et al., 2007; Cho et al., 2001; Fakhrzadeh et al., 2004; Fakhrzadeh et al., 2008). Moreover, TNF α polymorphisms in humans are associated with exacerbation of ozone-induced alterations in lung functioning (Yang et al., 2005). The present studies demonstrate that ozone-induced increases in TNF α mRNA expression are significantly reduced in C3H/HeJ mice relative to C3H/HeOuJ mice.

These findings are in line with reduced NF- κ B activity in the TLR4 mutant mice. Similar decreases in TNF α have been described in the lungs of TLR4 mutant mice exposed to hyperoxia (Ogawa et al., 2007). These data indicate that TLR4 signaling is critical for production of TNF α by alveolar macrophages following irritant exposure.

In response to ozone-induced tissue injury, Type II cells begin to proliferate to repair damaged epithelium (Fehrenbach, 2001). In accord with this response, we noted increased staining of Type II cells with proSP-C, a specific marker of their proliferation, in lungs of control C3H/HeOuJ mice following ozone exposure. Our findings that proSP-C staining remained elevated for 72 hr after ozone indicates that processes of injury and consequent repair are persistent. In contrast to C3H/HeOuJ mice, proSP-C staining was not evident in C3H/HeJ mice. These data are consistent with reduced injury and oxidative stress in these mice. The role of TLR4 signaling in alveolar epithelial barrier dysfunction is unclear (Bauer et al., 2011; Hollingsworth et al., 2004; Kleeberger et al., 2000; Williams et al., 2007). The present studies support the idea that TLR4 contributes to this pathologic response. The reason for the disparate findings between the present studies and previous reports may be due to different mouse strains utilized and/or different ozone exposure protocols. It is also likely that multiple genetic polymorphisms contribute to oxidant induced lung injury, and this remains to be investigated. Data are also presented that TLR4 plays a role in ozone-induced macrophage accumulation and classical activation in the lung. Evidence suggests that macrophages possessing a classically activated phenotype contribute to cytotoxicity induced by pulmonary irritants (Laskin et al., 2011). These cells exhibit a Th1-like phenotype, promoting inflammation, extracellular matrix destruction and apoptosis. During sterile inflammatory responses, a number of products are generated that can function as endogenous ligands for TLR4. These include extracellular matrix degradation products such as hyaluronan and products released from dying cells like HMGB1 (Lafferty et al., 2010; Lennon and Singleton, 2011). Recent studies have demonstrated that hyaluronan is released following ozone intoxication (Li et al., 2011). Hyaluronan has been reported to upregulate macrophage production of TNF α and expression of iNOS, in a process this is dependent on NF- κ B (Jiang et al., 2010; Li et al., 2011; McKee et al., 1996). These data suggest that classical macrophage activation in the lung following ozone inhalation may involve stimulation of TLR4 via endogenous ligands such as hyaluronan. It remains to be determined if these ligands are also important in initiating tissue remodeling and repair of acute lung injury.

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Abbreviations

4-HNE	4-hydroxynonenal
BAL	bronchoalveolar lavage
EMSA	electrophoretic mobility shift assays
iNOS	nitric oxide synthase
NF-κB	nuclear factor-kappa B
SP	surfactant protein
TLR4	toll-like receptor 4

TNF α tumor necrosis factor alpha

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Highlights

- Ozone induces a sterile inflammatory response in the lung characterized by an accumulation of classically activated macrophages.
- The effects of ozone are significantly blunted in mice with a mutated nonfunctional TLR4.
- TLR4 is key to oxidant-induced lung injury, sterile inflammation and classical macrophage activation.

Fig. 1. Effects of loss of functional TLR4 on ozone-induced markers of lung injury and inflammation. BAL was collected 0.5–48 hr following exposure of C3H/HeOuJ or C3H/HeJ mice to air or ozone. *Upper panel:* Protein content was analyzed by the BCA assay with bovine serum albumin as the standard. Each bar represents the mean $\pm$ SE (n=14–18). *Lower panel:* Viable cells were enumerated using trypan blue exclusion. Each bar represents the mean $\pm$ SE (n=7–11). *Significantly different (p < 0.05) from air control.

Fig. 2.

Effects of ozone on markers of oxidative stress. BAL was collected 12–48 hr after exposure of C3H/HeOuJ or C3H/HeJ mice to air or ozone. *Upper panel:* BAL was analyzed for the presence of lipocalin 24p3 and 4-HNE-modified proteins by western blotting. Note that only a single 50 kDa molecular weight protein was detected in BAL following ozone inhalation. *Lower panel:* Gels were scanned and analyzed by densitometry. Each bar represents the mean $\pm$ SE in (n=3–5). *Significantly different (p < 0.05) from air control.

Fig. 3. Effects of ozone on SP-D levels. BAL was collected 12–48 hr after exposure of C3H/HeOuJ or C3H/HeJ mice to air or ozone. *Upper panel:* BAL was analyzed for the presence of SP-D by western blotting. *Lower panel:* Gels were scanned and analyzed by densitometry using ImageJ. Each bar represents the mean $\pm$ SE (n=3–5). *Significantly different (p < 0.05) from air control.

Fig. 4.

Effects of ozone on NF-κB nuclear binding activity. Alveolar macrophages were isolated 0.5–24 hr following exposure of C3H/HeOuJ or C3H/HeJ mice to air or ozone. NF-κB nuclear binding activity was quantified by EMSA. One representative of three independent experiments is shown. Cold competitor (CC) oligo was incubated with nucleic extracts 2 hr before addition of ^{32}P -labeled NF-κB consensus oligonucleotide.

Fig. 5. Effects of ozone on TNF α mRNA expression. Alveolar macrophages were isolated 0.5–48 hr following exposure of C3H/HeOuJ or C3H/HeJ mice to air or ozone. TNF α mRNA was quantified by relative quantitative RT-PCR. *Upper panel:* Representative PCR gel. *Lower panel:* TNF α was quantified by scintillation counting of excised bands. Each bar represents the mean $\pm$ SE (n=3). *Significantly different (p < 0.05) from air control.

Fig. 6. Effects of ozone on proSP-C expression. Lung sections, collected 12–48 hr following exposure of C3H/HeOuJ or C3H/HeJ mice to air or ozone, were stained with anti-proSP-C antibody. Binding was visualized using a Vector DAB peroxidase substrate kit. Slides were counterstained with hematoxylin. Original magnification, 1000x.