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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 h) 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-κB binding activity and expression of TNFα 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.

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 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 (TNFα) 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, 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-κB) and upregulation of proinflammatory mediators including TNFα 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

Abbreviations: 4-HNE, 4-hydroxynonenal; BAL, bronchoalveolar lavage; EMSA, electrophoretic mobility shift assay; iNOS, inducible nitric oxide synthase; NF-κB, nuclear factor-kappa B; SP, surfactant protein; TLR4, toll-like receptor 4; TNFα, tumor necrosis factor-alpha.

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Academy of Sciences. Mice were placed in a Plexiglass chamber and exposed for 3 h 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) Ca^{2+}/Mg^{2+}-free Hank's balanced salt solution (HBSS) containing 25 mM HEPES, 0.5 mM EGTA and 4.4 mM $NaHCO_3$ 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 (BSA) as the standard. BAL fluid was centrifuged (300×*g* for 8 min), supernatants collected, aliquoted, and stored at −80 °C until analysis. Cell pellets were washed 4 times with HBSS containing 2% fetal bovine serum (FBS) and then enumerated using a hemocytometer. Viability was 98% as determined by trypan blue dye exclusion, and cell purity > 98% macrophages as assessed morphologically after Giemsa staining.

Western blotting

BAL fluid (500 μl) was concentrated by centrifugation at 14,000×*g* for 33 min using a 10 K 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 h 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 h 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 μm) were deparaffinized, then incubated for 30 min with 3% hydrogen peroxide to quench endogenous peroxidase. This was followed by incubation for 1 h 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, Bellerica, 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. Louise, MO) following the manufacturer's instructions. Binding reactions were carried out at room temperature for 30 min in a total volume of 15 μl containing 5 μg of nucleic extract protein, 5 μl of 5X gel shift binding buffer (37.5% glycerol, 5 mM $MgCl_2$, 0.25 mM dithithreitol (DTT), 175 mM NaCl, 37.5 mM HEPES, pH 8.0), 0.1% BSA, 1 μg poly dI-dC and 2 μl of γ[^{32}P]ATP (3000 Ci/mmol at 10 mCi/ml)-labeled NF-κB (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 run 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-κB oligonucleotide was added to the reaction mixture 2 h 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 μl of water was denatured at 65 °C for 4 min, rapidly cooled on ice and then resuspended in a 20 μl final volume containing 250 mM Tris–HCl pH 8.3, 375 mM KCl, 15 mM $MgCl_2$, 100 mM DTT, 10 mM dNTP, 200 μM random hexamers and 200 units Superscript II Reverse Transcriptase (Invitrogen, Carlsbad, CA). After 1 h 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 μl) contained 1 μl cDNA template, 0.8 mM mouse TNFα primer pair and a 1:9 ratio of 18S rRNA competimer/primer (Ambion, Austin, TX), 2 μl of 10X PCR buffer, 10 mM dNTP, 0.2 μl α[^{32}P] 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 s, 58 °C for 25 s and 72 °C for 90 s. 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 h 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 h 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 (Fig. 3).

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. 1). In addition, ozone-induced increases in BAL SP-D, 24p3 and 4-HNE modified