Published in final edited form as:

Toxicol Appl Pharmacol. 2008 March 15; 227(3): 380–389. doi:10.1016/j.taap.2007.11.012.

Regulation of Caveolin-1 Expression, Nitric Oxide Production and Tissue Injury by Tumor Necrosis Factor- α following Ozone Inhalation

Ladan Fakhrzadeh^a, Jeffrey D. Laskin^b, and Debra L. Laskin^a

^aDepartment of Pharmacology and Toxicology, Rutgers University, Piscataway, New Jersey

^bDepartment of Environmental and Occupational Medicine, University of Medicine and Dentistry of New Jersey-Robert Wood Johnson Medical School, Piscataway, New Jersey

Abstract

Alveolar macrophages (AM) and inflammatory mediators including nitric oxide and peroxynitrite contribute to ozone-induced lung injury. The generation of these mediators is regulated, in part, by the transcription factor NF- κ B. We previously demonstrated a critical role for NF- κ B p50 in the ozone-induced injury. In the present studies mechanisms regulating NF- κ B activation in the lung after ozone inhalation were analyzed. Treatment of wild type (WT) mice with ozone (0.8 ppm, 3 h) resulted in a rapid increase in NF- κ B binding activity in AM, which persisted for at least 12 h. This was not evident in mice lacking TNF α which are protected from ozone-induced injury; there was also no evidence of nitric oxide or peroxynitrite production in lungs from these animals. These data demonstrate that TNF α plays a role in NF- κ B activation and toxicity. TNF α signaling involves PI-3-kinase (PI3K)/protein kinase B (PKB), and p44/42 MAP kinase (MAPK) which are important in NF- κ B activation. Ozone Inhalation resulted in rapid and transient increases in p44/42 MAPK and PI3K/PKB in AM from WT mice, which was evident immediately after exposure. Caveolin-1, a transmembrane protein that negatively regulates PI3K/PKB and p44/42 MAPK signaling, was downregulated in AM from WT mice after ozone exposure. In contrast, ozone had no effect on caveolin-1, PI3K/PKB or p44/42 MAPK expression in AM from TNF α knockout mice. These data, together with our findings that TNF α suppressed caveolin-1 in cultured AM, suggest that TNF α and downstream signaling mediate activation of NF- κ B and the regulation of inflammatory genes important in ozone toxicity, and that this process is linked to caveolin-1.

Keywords

Ozone; TNF- α ; caveolin-1; lung; alveolar macrophages

© 2007 Elsevier Inc. All rights reserved.

Address correspondence to: Dr. Debra L. Laskin, Rutgers University, Department of Pharmacology and Toxicology, 160 Frelinghuysen Road, Piscataway, New Jersey 08854, Tel: 732-445-5862. Fax: 732-445-2534. laskin@eohsi.rutgers.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Introduction

Tumor necrosis factor alpha (TNF α) is a macrophage-derived proinflammatory cytokine implicated in the pathogenesis of oxidant-induced tissue injury (Mukhopadhyay *et al.*, 2006). The biological actions of TNF α are mediated by two structurally related, but functionally distinct receptors: TNFR1 (p55) and TNFR2 (p75) (Locksley *et al.*, 2001). TNF α signaling through the p55 receptor is the primary pathway leading to inflammatory responses (Vandevorde *et al.*, 1997; Peschon *et al.*, 1998). Binding of TNF α to TNFR1 causes recruitment of cytoplasmic adaptor proteins important in initiating signal transduction. This leads to activation of various signaling molecules, including phosphatidylinositol 3'-kinase (PI3K)/protein kinase B (PKB), and p44/42 mitogen activated protein kinase (MAPK), and downstream transcription factors such as nuclear factor- κ B (NF- κ B), which is known to regulate genes controlling proinflammatory mediator and antioxidant production, as well as apoptosis and cellular proliferation (Sebban and Courtois, 2006; Perkins, 2007). In the lung, TNF α is thought to contribute to a variety of inflammatory conditions including acute respiratory distress syndrome, idiopathic pneumonia, chronic obstructive pulmonary disease and asthma (reviewed in Mukhopadhyay *et al.*, 2006).

Ozone is a highly reactive oxidant present in photochemical smog. Inhalation of toxic levels of ozone leads to airway inflammation and damage to the alveolar epithelium in the lower lung (Laskin *et al.*, 2003; Uysal and Schapira, 2003; Hollingsworth *et al.*, 2007). A number of studies have suggested that at least one mechanism underlying the toxicity of ozone involves excessive production of cytotoxic mediators including reactive oxygen and nitrogen intermediates by activated lung macrophages (reviewed in (Laskin *et al.*, 2003)). In this regard, we have demonstrated that acute exposure of rodents to ozone results in increased production of nitric oxide and peroxynitrite by lung macrophages (Pendino *et al.*, 1993b; Fakhrzadeh *et al.*, 2002; Fakhrzadeh *et al.*, 2004a; Fakhrzadeh *et al.*, 2004b). Moreover, transgenic mice lacking nitric oxide synthase II (NOSII) or over expressing superoxide dismutase are protected from ozone toxicity (Fakhrzadeh *et al.*, 2002; Fakhrzadeh *et al.*, 2004a). TNF α expression has also been reported to be upregulated in mouse lung following ozone inhalation (Cho *et al.*, 2001; Fakhrzadeh *et al.*, 2004b; Lu *et al.*, 2006). The present studies were aimed at analyzing the role of TNF α in ozone-induced nitric oxide production and toxicity. Mechanisms regulating TNF α signaling in ozone-exposed animals were also investigated. We found that macrophages from transgenic mice with a targeted disruption of the gene for TNF α were unable to generate reactive nitrogen intermediates and that these mice were protected from ozone toxicity. This was associated with a marked attenuation of ozone-induced activation of PI3K/PKB and p44/42 MAPK and suppression of caveolin-1 (Cav-1), a negative regulator of TNF α signaling (Williams and Lisanti, 2004). These findings are novel and may lead to the identification of new targets for therapeutic intervention aimed at regulating lung inflammation induced by pulmonary irritants.

Materials and methods

Animals and treatments

TNF α knockout mice (C57BL/6X129) were kindly provided by Dr. Michael Marino (Memorial Sloan Kettering Cancer Center, NY). Wild type B6J129SV F2 mice were purchased from Jackson Laboratories (Bar Harbor, ME). Animals were housed in microisolator cages and received food and sterile pathogen-free water *ad libitum*. Female mice, 8–12 weeks of age, were placed in whole-body Plexiglas chambers and exposed to ultra-pure air (control) or 0.8 ppm ozone for 3 h. Ozone was generated from oxygen gas via an ultraviolet light ozone generator (Orec Corp., Phoenix, AZ). Ozone concentrations in the

chamber were maintained by adjusting both the intensity of the ultraviolet light (mJ/cm^2) and the flow rate (ml/min). Concentrations of ozone in ppm were continuously monitored using an ozone analyzer (Model 1008 AH; Dasibi Environmental Corp., Glendale, CA). All animals received humane care in compliance with the institution's guidelines, as outlined in the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health.

Reagents

Mouse recombinant interferon-gamma ($\text{IFN-}\gamma$) was purchased from GIBCO (Grand Island, NY). Salmonella *enteritidis* lipopolysaccharide (LPS) and DNase I were obtained from Sigma Chemical Co. (St. Louis, MO). Rabbit polyclonal antibodies against NOSII and Cav-1, goat polyclonal antibodies against TNF α PI3K-p85, and horseradish peroxidase (HRP) conjugated anti-rabbit and anti-goat IgG were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Rabbit polyclonal antibody against nitrotyrosine was from Upstate Biotechnology (Lake Placid, NY) and rabbit polyclonal antibodies against NF- κ B p50 and p65 from Stressgen (Victoria BC, Canada). Rabbit antibodies against PKB/AKT, phospho-PKB/AKT, p44/42 MAPK, and phospho-p44/42 MAPK were from Cell Signaling Technology (Danvers, MA). FITC-conjugated anti-rabbit and anti-goat IgG were from Molecular Probes (Eugene, OR).

Cell isolation and preparation of extracts

Alveolar macrophages were isolated from the lung as previously described (Fakhrzadeh *et al.*, 2002). Briefly, mice were anesthetized, the lung excised, and the trachea and major bronchi removed. The lung was then cut into uniform 500 μm slices (MacIlwain Tissue Chopper, Brinkmann Instruments, Westbury, NY) and incubated in ice cold $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free Hank's balanced salt solution (HBSS) containing 0.005% DNase I (HBSS-DNase) for 30 min. This was followed by mixing, using a Vortex Genie 2 (Fisher Scientific, Pittsburgh, PA) at speed 3. The cells released during these steps were filtered through a 220 μm filter, washed and subjected to Metrizamide gradient centrifugation using a Beckman TJ-6 centrifuge for elimination of red blood cells, dead cells and debris. Cell viability was greater than 98%, as assessed by trypan blue dye exclusion. Purity was greater than 97% macrophages based on differential staining with Giemsa (Fisher Scientific, Springfield, NJ). To prepare extracts, cells were lysed in buffer (10 mM HEPES, pH 7.4, 10 mM KCl, 2 mM MgCl_2 , 2 mM EDTA) on ice for 10 min with intermittent mixing for 2 sec using a Vortex Genie at a setting of 3. NP-40 was added (final concentration 0.1%) and the cells incubated for an additional 5 min on ice. Cells were then centrifuged at 4 $^{\circ}\text{C}$ ($16,000 \times g$) using an Eppendorf microcentrifuge for 5 min and supernatants containing cytoplasmic extracts collected and aliquots stored at -70°C . The pellet was resuspended in buffer (50 mM HEPES pH 7.4, 50 mM KCl, 300 mM NaCl, 0.1 mM EDTA and 10% glycerol). After 20 min on ice, the sample was centrifuged at 4 $^{\circ}\text{C}$ ($16,000 \times g$) for 5 min, supernatants containing nuclear extracts collected, and aliquots stored at -70°C . Protein determinations were performed using a BCA protein assay kit (Pierce, Rockford, IL) with bovine serum albumin (BSA) as the standard.

Quantitation of bronchoalveolar lavage protein

Animals were sacrificed and the trachea cannulated with polyethylene tubing (PE-90; Clay Adams, Parsippany NJ) attached to a 3 cc Bectin-Dickinson (Franklin Lakes, NJ) single use syringe. The lung was then instilled with 1 ml of $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free phosphate-buffered saline (PBS) at 37 $^{\circ}\text{C}$ and the fluids slowly withdrawn and instilled three times. The lavage fluid was centrifuged ($350 \times g$ for 10 min, 4 $^{\circ}\text{C}$) and protein content in supernatants quantified using the Bradford protein assay (Bio-Rad Laboratories, Hercules, CA) with BSA as the standard.

Measurement of nitric oxide production

Cells were cultured in 96-well dishes (2×10^5 cells/well) in phenol red-free Dulbecco's modified Eagle's medium in the presence or absence of LPS (100 ng/ml) and IFN- γ (100 U/ml) or medium control. Nitric oxide was quantified after 48 h by the accumulation of nitrite in the culture medium using the Griess reaction with sodium nitrite as the standard as previously described (Fakhrzadeh *et al.*, 2002). For nitrate determinations, samples were treated with nitrate reductase and NADPH for 30 min prior to analysis. We found that the ratio of nitrate to nitrite produced by alveolar macrophages was 1:1 and that this ratio did not change in cells from ozone treated mice.

Western blot analysis—Alveolar macrophages, lysed in buffer containing 10 mM Tris-HCl (pH 7.4) and 1% sodium dodecylsulfate (SDS), were run on 10% or 15% SDS-polyacrylamide gels (5 μ g protein/lane) as described previously (Fakhrzadeh *et al.*, 2002; Fakhrzadeh *et al.*, 2004b). Proteins were then transferred to nitrocellulose and blocked overnight at 4°C with 5% non-fat powdered milk. The nitrocellulose membrane was then incubated overnight with anti-Cav-1 (1:1000), anti-PKB (1:500), or anti-PI3K (1:200) antibodies, followed by HRP-conjugated anti-rabbit IgG (1:5000) for 1 h. The blots were developed using an Enhanced Chemiluminescence detection kit (Amersham Life Science, Arlington Heights, IL). Blots were stained with Ponceau S (Sigma) to confirm equal loading of proteins on the gels.

Electrophoretic mobility shift assays

Binding reactions were carried out at room temperature for 30 min in a total volume of 15 μ l and contained 2–5 μ g of nuclear extract protein, 5 μ l of the 5X gel shift binding buffer (20% glycerol, 5 mM MgCl₂, 2.5 mM EDTA, 2.5 mM DTT, 250 mM NaCl, 50 mM Tris-HCl pH 7.5, 2 μ g poly (dI-dC) and 3×10^4 cpm/ml of a γ [³²]P-labeled NF- κ B (AGTTGAGGGTTTCCCAGGC) (Promega Gel Shift Assay Systems) consensus oligonucleotide. Probes were labeled using γ [³²]P-ATP (3000 Ci/mmol, NEN, Boston, MA). Protein-DNA complexes were separated on 5% non-denaturing polyacrylamide gels run at 250 V at 400 mAmps for 1.5 h in 0.5X TBE running buffer and visualized after the gels were dried and autoradiographed. For supershift reactions, the samples were incubated with antibodies (1 μ g) to NF- κ B p50 or p65 on ice for 20 min prior to the labeled oligonucleotide. For competitor reactions, samples were preincubated with a 100-fold excess of the respective unlabeled oligonucleotides.

Immunostaining

Tissue sections (6 μ m) were prepared for immunohistochemistry from paraffin-embedded perfused lungs that were inflation-fixed with 3% paraformaldehyde for 4 h at 4°C (Pendino *et al.*, 1993b; Pendino *et al.*, 1994; Pendino *et al.*, 1995). Sections were deparaffinized prior to analysis. For immunostaining, slides containing tissue sections or cells cytocentrifuged onto slides, were preincubated for 30 min in 3% hydrogen peroxide to quench endogenous peroxidase. This was followed by incubation for 20 min in PBS containing 1% BSA and 0.05% sodium azide and overnight incubation with antibody to TNF α (1:1000), Cav-1, PKB/AKT, phospho-PKB/AKT, nitrotyrosine, or with the appropriate nonimmune IgG. A Vectastain ABC kit (Vector Laboratories, Burlingame, CA) was utilized to visualize antibody binding.

Flow cytometry

Macrophages were washed in PBS, permeabilized and fixed with a Leucoperm kit (Serotec, NC) according to the manufacturers protocol. Cells (1×10^6 cells/ml) were labeled with 1:50 dilution of antibody to TNF α , Cav-1, total-p44/p42, phospho-p44/p42 or IgG control for 1 h

at room temperature. Cells were then washed with PBS and incubated with FITC-conjugated secondary antibody. After 45 min, the cells were washed with PBS and analyzed by flow cytometry on a Coulter Cytomics FC500 flow cytometer (Beckman Coulter). For each analysis, at least 10,000 events were collected and analyzed using CXP software. The percentage positive cells were calculated using Overton's cumulative subtraction routine (Coulter software).

Statistics

All experiments were repeated 3–5 times using 3–6 animals per experiment. Data were analyzed using a non-paired, two-tailed student's *t* test. A *P* value of 0.05 was considered statistically significant.

Results

We have previously demonstrated that macrophages play an important role in ozone toxicity (Pendino *et al.*, 1995). Macrophages are known to release a number of mediators that have been implicated in tissue injury (Duffield, 2003; Laskin *et al.*, 2003). Of particular interest is TNF α which promotes inflammation and cytotoxicity (Mukhopadhyay *et al.*, 2006). In the present studies we analyzed the role of TNF α in ozone-induced lung injury.

Immunohistochemical evaluation of lung sections revealed low levels of TNF α in alveolar macrophages from air-exposed mice (Fig. 1, upper panels). Ozone inhalation resulted in a rapid induction of TNF α in alveolar macrophages, which was most pronounced immediately after exposure. Subsequently, expression of TNF α began to decline. These findings were confirmed by flow cytometric analysis of isolated alveolar macrophages stained with anti-TNF α antibody. Whereas low basal levels of TNF α were detectable in cells from air-exposed mice, expression increased 3-fold after ozone inhalation (Fig. 1, lower panels). These experiments also revealed a single homogeneous population of alveolar macrophages that expressed TNF α from both control and ozone-treated mice. To analyze the role of TNF α in ozone-induced tissue injury, we used transgenic mice with a targeted disruption of the gene for this cytokine. TNF α was not detectable in lung sections, or in isolated alveolar macrophages from TNF α knockout mice, even after ozone inhalation (Fig. 1). Whereas lung injury, as measured by significant increases in bronchoalveolar lavage fluid protein, was observed in wild type mice treated with ozone, this was not evident in TNF α knockout mice (Fig. 2). These findings demonstrate that TNF α knockout mice are protected from ozone-induced toxicity.

Following ozone inhalation, alveolar macrophages generate increased quantities of nitric oxide and peroxynitrite which contribute to lung toxicity (Pendino *et al.*, 1993b; Fakhrzadeh *et al.*, 2002; Fakhrzadeh *et al.*, 2004a; Fakhrzadeh *et al.*, 2004b). We next assessed the effects of loss of TNF α on this response. Consistent with our previous studies (Fakhrzadeh *et al.*, 2002; Fakhrzadeh *et al.*, 2004a; Fakhrzadeh *et al.*, 2004b), we found that macrophages isolated from wild type mice readily generated nitric oxide in response to the inflammatory stimuli, LPS and IFN γ (Fig. 3, upper panel). This activity increased two-fold in macrophages from wild type mice following ozone inhalation. In contrast, macrophages from TNF α knockout mice were unable to generate significant quantities of nitric oxide, and ozone had no major effect on this activity. Nitric oxide can rapidly react with superoxide anion to form peroxynitrite, a potent nitrating species (Koppenol *et al.*, 1992). We found that ozone inhalation resulted in the appearance of nitrotyrosine residues in proteins in the lung, an *in vivo* marker of peroxynitrite formation (Fig. 3, lower panel). This was prominent in alveolar macrophages. Nitrotyrosine staining was not detectable in TNF α knockout mice.

TNF α receptor binding activates several downstream signaling molecules including the transcription factor NF- κ B, which regulates the activity of inflammatory genes implicated in

ozone toxicity including nitric oxide synthase-2 and cyclooxygenase-2 (Hazucha *et al.*, 1996; Fakhrzadeh *et al.*, 2002). We have previously shown that alveolar macrophages from mice with a targeted disruption of NF- κ B p50 do not generate reactive nitrogen intermediates and that these mice are protected from ozone toxicity (Fakhrzadeh *et al.*, 2004b). In further studies we analyzed the role of TNF α in ozone-induced NF- κ B activation. NF- κ B nuclear binding activity was not detectable in alveolar macrophages from air exposed wild type mice (Fig. 4). Following ozone inhalation, a time-related increase in NF- κ B binding activity was observed in alveolar macrophages. This was evident immediately after ozone exposure and persisted for at least 12 h. In contrast, no major changes in NF- κ B nuclear binding activity were observed in cells from TNF α knockout mice following ozone exposure. In activated macrophages from wild type mice, NF- κ B binding activity was blocked by incubating the samples with a 100-fold excess of unlabeled probe, demonstrating the specificity of the probe. Moreover, supershift assays using antibodies to p50 or p65 slowed the migration of the NF- κ B complex in the gels indicating that both of these proteins were involved in the responses to ozone.

A number of biochemical signaling molecules have been implicated in TNF α -induced activation of NF- κ B including PI3K and its downstream target, PKB (Hazucha *et al.*, 1996; Wallach *et al.*, 1999; Grivennikov *et al.*, 2006), and expression of these proteins was analyzed next. Alveolar macrophages from air-exposed wild type mice were found to express low levels of PI3K and PKB as determined by western blotting and immunocytochemistry (Fig. 5). Ozone inhalation caused a marked increase in expression of both total and phospho-PI3K and PKB. Whereas expression of PI3K reached a maximum after 3 h, peak PKB expression was observed after 6 h. Subsequently, expression of both of these proteins declined toward control levels. To determine if PI3K is involved in ozone-induced increases in nitric oxide production by alveolar macrophages, we used wortmanin and LY294002, two structurally distinct inhibitors of this enzyme (Walker *et al.*, 2000). As described above, following ozone inhalation, alveolar macrophages produced two-fold more nitric oxide in response to LPS and IFN γ than cells from air-exposed mice (Fig. 6). Pretreatment of cells from ozone exposed mice with wortmanin and LY294002 prevented this increase. These inhibitors also blocked ozone-induced increases in NF- κ B activation (Fig. 4). In contrast, wortmanin and LY294002 had no effect on nitric oxide production or NF- κ B binding activity in macrophages from air-exposed animals (Fig. 6 and not shown).

NF- κ B activation is also induced via mitogen activated protein kinases such as p44/42 (Wallach *et al.*, 1999; Chen *et al.*, 2001; Grivennikov *et al.*, 2006). Flow cytometric analysis revealed the presence of one homogeneous population of alveolar macrophages from air-exposed wild type mice that expressed low levels of phospho- and total p44/42 MAPK. Ozone inhalation resulted in a rapid increase in phospho-p42/44 MAPK in alveolar macrophages, which was evident immediately after exposure and peaking after 6 h (Fig. 7 upper panel). In contrast, in TNF α -/- mice, constitutive expression of MAPK was not detected, and ozone had no effect on expression of this protein.

We next analyzed the effects of ozone inhalation on expression of Cav-1, a membrane scaffolding protein known to negatively regulate PI3K and p44/42 MAPK (Williams and Lisanti, 2004; Schwencke *et al.*, 2006). Western blot analysis revealed that alveolar macrophages from air exposed wild type mice constitutively expressed Cav-1 (Fig. 8, upper panel). Ozone inhalation caused a dramatic reduction in Cav-1 protein expression, which was observed immediately following exposure and persisted for at least 6 h. Subsequently levels of Cav-1 began to return towards control. These findings were confirmed in intact cells by flow cytometry (Fig. 8, middle panel). This analysis showed that the macrophages from air and ozone-exposed mice consisted of one homogeneous population of cells that expressed Cav-1. Following ozone inhalation, expression of Cav-1 was not detectable. The

rapid and transient inhibitory effects of ozone exposure on constitutive Cav-1 expression in alveolar macrophages were also evident in histologic sections stained with anti-Cav-1 antibody (Fig. 8, lower panel). In contrast to the effects observed in wild type mice, ozone had minimal or no effects on Cav-1 expression in TNF α knockout mice. These data suggest that TNF α is required for ozone-induced suppression of Cav-1 (Fig. 8). To analyze this further, the effects of TNF α on Cav-1 expression was assessed in alveolar macrophages isolated from wild type mice. Figure 9 shows that alveolar macrophages from control mice expressed significant quantities of Cav-1. Moreover, treatment of the cells with TNF α for 20 min suppressed Cav-1 expression.

Discussion

TNF α is a multifunctional cytokine implicated in the pathogenesis of a number of pulmonary disorders including acute respiratory distress syndrome and idiopathic pneumonia, as well as tissue injury induced by endotoxin, bleomycin, silica and asbestos (Liu *et al.*, 2001; Pryhuber *et al.*, 2003; Shimabukuro *et al.*, 2003; Bhatia and Mochhala, 2004; Zhao *et al.*, 2004; Shukla *et al.*, 2005; Ke *et al.*, 2006; Mukhopadhyay *et al.*, 2006). We found that ozone inhalation was associated with a rapid and transient induction of TNF α in the lung. These findings are in accord with previous reports showing increased TNF α , as well as TNF receptor-1 (TNFR1, p55) and TNFR2 (p75), in the lung and/or in bronchoalveolar lavage fluid of humans and experimental animals exposed to ozone (Pendino *et al.*, 1994; Kleeberger *et al.*, 1997; Peschon *et al.*, 1998; Cho *et al.*, 2001; Shore *et al.*, 2001; Cho *et al.*, 2007). The fact that TNF α and its receptors are detectable in the lung prior to ozone-induced increases in epithelial cell permeability and lavageable inflammatory cells (Ishii *et al.*, 1997), suggests a role of this cytokine in the progression of the pathogenic response. Our findings that mice lacking TNF α are protected from ozone-induced injury are consistent with this idea. A similar protective effect against ozone-induced injury has been reported in TNFR1 and/or TNFR2 knockout mice (Cho *et al.*, 2001; Shore *et al.*, 2001; Cho *et al.*, 2007), and in mice pretreated with anti-TNF α antibodies (Piguet and Vesin, 1994; Young and Bhalla, 1995; Kleeberger *et al.*, 1997; Cho *et al.*, 2001; Bhalla *et al.*, 2002). TNF α plays an important role in inflammatory cell trafficking into sites of injury and activation, and this may contribute to the toxicity of ozone. This is supported by findings that administration of antibodies to TNF α attenuates ozone-induced increases in macrophage adherence, neutrophil accumulation and IL-1 and IL-6 expression in the lung, and that ozone-induced inflammation and macrophage activation are reduced in TNFR1 knockout mice (Young and Bhalla, 1995; Kleeberger *et al.*, 1997; Pearson and Bhalla, 1997; Cho *et al.*, 2001; Bhalla *et al.*, 2002).

Reactive nitrogen intermediates including nitric oxide and peroxynitrite are cytotoxic mediators produced in excessive quantities after ozone inhalation (Pendino *et al.*, 1993b; Laskin *et al.*, 1996; Fakhrzadeh *et al.*, 2002; Fakhrzadeh *et al.*, 2004b). Reactive nitrogen intermediates generated via NOSII have been shown to be important in ozone-induced injury and inflammation (Inoue *et al.*, 2000; Kleeberger *et al.*, 2001; Fakhrzadeh *et al.*, 2002). TNF α stimulates nitric oxide production by macrophages (Lavnikova *et al.*, 1993; Frankova and Zidek, 1998), and this may also be important in the pathogenesis of injury induced by ozone. In this regard, we found that macrophages from TNF α -/- mice were unable to generate nitric oxide, even after ozone inhalation. These findings demonstrate a direct role for TNF α in activating NOSII in the lung *in vivo* after ozone exposure. Our results are in accord with reports that TNF α is involved in regulating nitric oxide production in idiopathic pneumonia syndrome (Shukla *et al.*, 2005). Consistent with our previous studies (Pendino *et al.*, 1993a; Fakhrzadeh *et al.*, 2002; Fakhrzadeh *et al.*, 2004a; Fakhrzadeh *et al.*, 2004b), we also found that alveolar macrophages from ozone treated mice produced increased quantities of nitric oxide suggesting that these cells are primed to respond to

inflammatory mediators. This was correlated with peroxynitrite-induced tissue injury as assessed by nitrotyrosine staining of the lung. The fact that nitrotyrosine staining was not apparent in TNF α –/– mice provides further support for the concept that these mice are protected from ozone-induced injury and that reactive nitrogen intermediates are critical mediators of toxicity (Inoue *et al.*, 2000; Kleeberger *et al.*, 2001; Fakhrzadeh *et al.*, 2002; Zeidler and Castranova, 2004).

TNF α receptor binding leads to the recruitment of adaptor proteins and activation of a cascade of signaling molecules including PI 3-kinase/PKB, p44/42 MAPK, and NF- κ B, a transcription factor important in the regulation of many inflammatory genes and proteins involved in ozone toxicity including NOSII (Aggarwal *et al.*, 2002; Fakhrzadeh *et al.*, 2002; Shishodia and Aggarwal, 2002). The present studies show that increased TNF α expression in the lung following ozone inhalation is correlated with activation of NF- κ B which we have previously demonstrated is important in the toxicity of this oxidant (Fakhrzadeh *et al.*, 2004b). Interestingly, this activity was absent in mice lacking TNF α . These findings are consistent with recent reports on the effects of continuous exposure of TNFR1–/– mice to ozone for 48 hr (Cho *et al.*, 2007) and provide additional support for the idea that activation of NF- κ B in this model of lung injury is dependent on TNF α signaling.

TNF α expression in the lung following ozone inhalation was also associated with upregulation of PI3K and PKB in alveolar macrophages. These findings, together with the observation that ozone-induced increases in nitric oxide production by alveolar macrophages were markedly reduced by PI3K inhibitors suggest that this signaling pathway is likely to be involved in controlling TNF α -induced NOSII activity in the lung following ozone inhalation. Similar decrements in nitric oxide production by PI3K inhibitors have previously been described in macrophages, as well as astrocytes and microglia (Jung *et al.*, 1999; Kim *et al.*, 2005; Bani-Hani *et al.*, 2006; Keshewani and Sodhi, 2007). We also found that culturing macrophages from both air and ozone-exposed animals with PI3K inhibitors blocked NF- κ B nuclear binding activity. This indicates that PI3K/PKB signaling plays a role in ozone-induced NF- κ B activation. Our findings are in accord with previous reports demonstrating that PI3K inhibitors decrease fMLP-induced nitric oxide production and NF- κ B nuclear binding activity in murine peritoneal macrophages (Sodhi and Biswas, 2002). Ozone inhalation also caused upregulation of p44/42 MAPK in alveolar macrophages which is in accord with recent findings in whole lung (Cho *et al.*, 2007). This signaling molecule has been reported to be a key transducer of NF- κ B activation and nitric oxide production (Barton and Medzhitov, 2003; Takeda *et al.*, 2003), and it may also be important in ozone-induced toxicity. The fact that p44/42 activation was not evident in TNF α –/– mice which are resistant to ozone-induced injury is consistent with this idea. A similar dependency of ozone-induced MAPK activation on TNF α has recently been described (Cho *et al.*, 2007). Taken together, these data support the idea that the NF- κ B and MAPK pathways are important in TNF α mediated pulmonary injury induced by ozone.

Both PI3K and p44/42 MAPK are negatively regulated by the membrane scaffolding protein, Cav-1 (Galbiati *et al.*, 1998; Williams and Lisanti, 2004). By maintaining these molecules in an inactive form, downstream biological responses mediated by PI3K and p44/42 MAPK are inhibited (Engelman *et al.*, 1998; Okamoto *et al.*, 1998). Ozone inhalation caused a rapid and transient decrease in Cav-1 expression in alveolar macrophages. This was correlated with increased expression of PI3K, PKB and p44/42 MAPK activity suggesting a regulatory role for Cav-1 in ozone-induced activation of these signaling pathways. Our findings are in accord with the observation that Cav-1 knockout mice exhibit hyperactivation of PI3K/PKB and p44/42 MAPK (Park *et al.*, 2002; Cohen *et al.*, 2003). Results from the present studies also indicate that TNF α plays a role in down-regulating Cav-1 following ozone inhalation. This is supported by the fact that decreases in Cav-1 are

in general not observed in ozone-treated TNF α -/- mice, and that TNF α reduces Cav-1 expression in cultured alveolar macrophages. The mechanism mediating TNF α -induced down-regulation of Cav-1 is unknown. Studies have suggested that phosphorylation of Cav-1 by TNF α via p44/42 MAPK results in a conformational change in Cav-1 leading to dissociation of the scaffolding domain from the inhibitory binding motifs (Engelman *et al.*, 1998; Galbiati *et al.*, 1998; Volonte *et al.*, 2005). Alternatively, TNF α may cause activation of putative kinases resulting in phosphorylation of residues within the scaffolding domain. This would lead to dissociation and activation of p44/42 MAPK. The observation that p44/42 MAPK is rapidly upregulated in the lung following ozone inhalation supports the idea that p44/42 MAPK is a key enzyme involved in this process. Interestingly, a small and transient decrease in Cav-1 expression was observed in alveolar macrophages isolated immediately after exposure of TNF α -/- mice to ozone. These findings suggest that there are TNF α -independent mechanisms mediating Cav-1 down regulation in the lung at early times following ozone inhalation.

The present studies demonstrate that TNF α is a critical mediator of ozone-induced lung injury, NF- κ B activation, and macrophage production of nitric oxide. Moreover, TNF α signaling in lung macrophages appears to be negatively regulated by the membrane scaffolding protein, Cav-1. These findings are novel and suggest new potential therapeutic targets that may be useful for abrogating oxidant-induced lung injury.

Acknowledgments

This work was supported by National Institutes of Health Grants ES004738, GM034310, CA100994, AR055073, and ES005022.

References

- Aggarwal BB, Shishodia S, Ashikawa K, Bharti AC. The role of TNF and its family members in inflammation and cancer: lessons from gene deletion. *Current drug targets*. 2002; 1:327–341. [PubMed: 14561180]
- Bani-Hani MG, Greenstein D, Mann BE, Green CJ, Motterlini R. A carbon monoxide-releasing molecule (CORM-3) attenuates lipopolysaccharide- and interferon-gamma-induced inflammation in microglia. *Pharmacol Rep*. 2006; 58(Suppl):132–144. [PubMed: 17332683]
- Barton GM, Medzhitov R. Toll-like receptor signaling pathways. *Science (New York, N.Y.)*. 2003; 300:1524–1525.
- Bhalla DK, Reinhart PG, Bai C, Gupta SK. Amelioration of ozone-induced lung injury by anti-tumor necrosis factor-alpha. *Toxicol Sci*. 2002; 69:400–408. [PubMed: 12377989]
- Bhatia M, Moolchhala S. Role of inflammatory mediators in the pathophysiology of acute respiratory distress syndrome. *J Pathol*. 2004; 202:145–156. [PubMed: 14743496]
- Chen C, Chou C, Sun Y, Huang W. Tumor necrosis factor alpha-induced activation of downstream NF-kappaB site of the promoter mediates epithelial ICAM-1 expression and monocyte adhesion. Involvement of PKCalpha, tyrosine kinase, and IKK2, but not MAPKs, pathway. *Cell Signal*. 2001; 13:543–553. [PubMed: 11483407]
- Cho HY, Morgan DL, Bauer AK, Kleeberger SR. Signal transduction pathways of tumor necrosis factor--mediated lung injury induced by ozone in mice. *Am J Respir Crit Care Med*. 2007; 175:829–839. [PubMed: 17255564]
- Cho HY, Zhang LY, Kleeberger SR. Ozone-induced lung inflammation and hyperreactivity are mediated via tumor necrosis factor-alpha receptors. *Am J Physiol Lung Cell Mol Physiol*. 2001; 280:L537–L546. [PubMed: 11159038]
- Cohen AW, Razani B, Wang XB, Combs TP, Williams TM, Scherer PE, Lisanti MP. Caveolin-1-deficient mice show insulin resistance and defective insulin receptor protein expression in adipose tissue. *Am J Physiol Cell Physiol*. 2003; 285:C222–C235. [PubMed: 12660144]

- Duffield JS. The inflammatory macrophage: a story of Jekyll and Hyde. *Clin Sci (Lond)*. 2003; 104:27–38. [PubMed: 12519085]
- Engelman JA, Chu C, Lin A, Jo H, Ikezu T, Okamoto T, Kohtz DS, Lisanti MP. Caveolin-mediated regulation of signaling along the p42/44 MAP kinase cascade in vivo. A role for the caveolin-scaffolding domain. *FEBS Lett*. 1998; 428:205–211. [PubMed: 9654135]
- Fakhrzadeh L, Laskin JD, Gardner CR, Laskin DL. Superoxide dismutase-overexpressing mice are resistant to ozone-induced tissue injury and increases in nitric oxide and tumor necrosis factor- α . *Am J Respir Cell Mol Biol*. 2004a; 30:280–287. [PubMed: 12855403]
- Fakhrzadeh L, Laskin JD, Laskin DL. Deficiency in inducible nitric oxide synthase protects mice from ozone-induced lung inflammation and tissue injury. *Am J Respir Cell Mol Biol*. 2002; 26:413–419. [PubMed: 11919077]
- Fakhrzadeh L, Laskin JD, Laskin DL. Ozone-induced production of nitric oxide and TNF- α and tissue injury are dependent on NF- κ B p50. *Am J Physiol Lung Cell Mol Physiol*. 2004b; 287:L279–L285. [PubMed: 15064226]
- Frankova D, Zidek Z. IFN- γ -induced TNF- α is a prerequisite for in vitro production of nitric oxide generated in murine peritoneal macrophages by IFN- γ . *Eur J Immunol*. 1998; 28:838–843. [PubMed: 9541578]
- Galbiati F, Volonte D, Engelman JA, Watanabe G, Burk R, Pestell RG, Lisanti MP. Targeted downregulation of caveolin-1 is sufficient to drive cell transformation and hyperactivate the p42/44 MAP kinase cascade. *Embo J*. 1998; 17:6633–6648. [PubMed: 9822607]
- Grivennikov SI, Kuprash DV, Liu ZG, Nedospasov SA. Intracellular signals and events activated by cytokines of the tumor necrosis factor superfamily: From simple paradigms to complex mechanisms. *Int Rev Cytol*. 2006; 252:129–161. [PubMed: 16984817]
- Hazucha MJ, Madden M, Pape G, Becker S, Devlin R, Koren HS, Kehrl H, Bromberg PA. Effects of cyclo-oxygenase inhibition on ozone-induced respiratory inflammation and lung function changes. *Eur J Appl Physiol Occup Physiol*. 1996; 73:17–27. [PubMed: 8861665]
- Hollingsworth JW, Kleeberger SR, Foster WM. Ozone and pulmonary innate immunity. *Proceedings of the American Thoracic Society*. 2007; 4:240–246. [PubMed: 17607006]
- Inoue H, Aizawa H, Nakano H, Matsumoto K, Kuwano K, Nadel JA, Hara N. Nitric oxide synthase inhibitors attenuate ozone-induced airway inflammation in guinea pigs. Possible role of interleukin-8. *Am J Respir Crit Care Med*. 2000; 161:249–256. [PubMed: 10619828]
- Ishii Y, Yang H, Sakamoto T, Nomura A, Hasegawa S, Hirata F, Bassett DJ. Rat alveolar macrophage cytokine production and regulation of neutrophil recruitment following acute ozone exposure. *Toxicol Appl Pharmacol*. 1997; 147:214–223. [PubMed: 9439717]
- Jung YD, Kim MS, Lee KS, Kang IC, Nah AS, Song DU, Yang SY, Kim JK, Ahn BW. 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002) inhibits nitric oxide production in cultured murine astrocytes. *Pharmacol Res*. 1999; 40:423–427. [PubMed: 10527657]
- Ke Q, Li J, Ding J, Ding M, Wang L, Liu B, Costa M, Huang C. Essential role of ROS-mediated NFAT activation in TNF- α induction by crystalline silica exposure. *Am J Physiol Lung Cell Mol Physiol*. 2006; 291:L257–L264. [PubMed: 16489119]
- Keshewani V, Sodhi A. Differential activation of macrophages in vitro by lectin Concanavalin A, Phytohemagglutinin and Wheat germ agglutinin: production and regulation of nitric oxide. *Nitric Oxide*. 2007; 16:294–305. [PubMed: 17208474]
- Kim YH, Choi KH, Park JW, Kwon TK. LY294002 inhibits LPS-induced NO production through a inhibition of NF- κ B activation: independent mechanism of phosphatidylinositol 3-kinase. *Immunol Lett*. 2005; 99:45–50. [PubMed: 15894110]
- Kleeberger SR, Levitt RC, Zhang LY, Longphre M, Harkema J, Jedlicka A, Eleff SM, DiSilvestre D, Holroyd KJ. Linkage analysis of susceptibility to ozone-induced lung inflammation in inbred mice. *Nature genetics*. 1997; 17:475–478. [PubMed: 9398854]
- Kleeberger SR, Reddy SP, Zhang LY, Cho HY, Jedlicka AE. Toll-like receptor 4 mediates ozone-induced murine lung hyperpermeability via inducible nitric oxide synthase. *Am J Physiol Lung Cell Mol Physiol*. 2001; 280:L326–L333. [PubMed: 11159012]
- Koppenol WH, Moreno JJ, Pryor WA, Ischiropoulos H, Beckman JS. Peroxynitrite, a cloaked oxidant formed by nitric oxide and superoxide. *Chem Res Toxicol*. 1992; 5:834–842. [PubMed: 1336991]

- Laskin, DL.; Gardner, CR.; Gerecke, DR.; Laskin, JD. Ozone-induced lung injury: role of macrophages and inflammatory mediators. In: Vallyathan, V.; Shi, X.; Castranova, V., editors. *Reactive Oxygen/Nitrogen Species: Lung injury and Disease*. New York: Marcel Dekker; 2003. p. 289-316.
- Laskin JD, Heck DE, Laskin DL. Nitric oxide production in the lung and liver following inhalation of the pulmonary irritant ozone. *Adv Exp Med Biol*. 1996; 387:141–146. [PubMed: 8794206]
- Lavnikova N, Drapier JC, Laskin DL. A single exogenous stimulus activates resident rat macrophages for nitric oxide production and tumor cytotoxicity. *Journal of leukocyte biology*. 1993; 54:322–328. [PubMed: 8409754]
- Liu JY, Sime PJ, Wu T, Warshamana GS, Pociask D, Tsai SY, Brody AR. Transforming growth factor-beta(1) overexpression in tumor necrosis factor-alpha receptor knockout mice induces fibroproliferative lung disease. *Am J Respir Cell Mol Biol*. 2001; 25:3–7. [PubMed: 11472967]
- Locksley RM, Killeen N, Lenardo MJ. The TNF and TNF receptor superfamilies: integrating mammalian biology. *Cell*. 2001; 104:487–501. [PubMed: 11239407]
- Lu FL, Johnston RA, Flynt L, Theman TA, Terry RD, Schwartzman IN, Lee A, Shore SA. Increased pulmonary responses to acute ozone exposure in obese db/db mice. *Am J Physiol Lung Cell Mol Physiol*. 2006; 290:L856–L865. [PubMed: 16373670]
- Mukhopadhyay S, Hoidal JR, Mukherjee TK. Role of TNFalpha in pulmonary pathophysiology. *Respir Res*. 2006; 7:125. [PubMed: 17034639]
- Okamoto T, Schlegel A, Scherer PE, Lisanti MP. Caveolins, a family of scaffolding proteins for organizing "preassembled signaling complexes" at the plasma membrane. *J Biol Chem*. 1998; 273:5419–5422. [PubMed: 9488658]
- Park DS, Lee H, Frank PG, Razani B, Nguyen AV, Parlow AF, Russell RG, Hulit J, Pestell RG, Lisanti MP. Caveolin-1-deficient mice show accelerated mammary gland development during pregnancy, premature lactation, and hyperactivation of the Jak-2/STAT5a signaling cascade. *Mol Biol Cell*. 2002; 13:3416–3430. [PubMed: 12388746]
- Pearson AC, Bhalla DK. Effects of ozone on macrophage adhesion in vitro and epithelial and inflammatory responses in vivo: the role of cytokines. *J Toxicol Environ Health*. 1997; 50:143–157. [PubMed: 9048958]
- Pendino KJ, Gardner CR, Laskin JD, Laskin DL. Induction of functionally active platelet-activating factor receptors in rat alveolar macrophages. *J Biol Chem*. 1993a; 268:19165–19168. [PubMed: 8396125]
- Pendino KJ, Laskin JD, Shuler RL, Punjabi CJ, Laskin DL. Enhanced production of nitric oxide by rat alveolar macrophages after inhalation of a pulmonary irritant is associated with increased expression of nitric oxide synthase. *J Immunol*. 1993b; 151:7196–7205. [PubMed: 7505023]
- Pendino KJ, Meidhof TM, Heck DE, Laskin JD, Laskin DL. Inhibition of macrophages with gadolinium chloride abrogates ozone-induced pulmonary injury and inflammatory mediator production. *Am J Respir Cell Mol Biol*. 1995; 13:125–132. [PubMed: 7542894]
- Pendino KJ, Shuler RL, Laskin JD, Laskin DL. Enhanced production of interleukin-1, tumor necrosis factor-alpha, and fibronectin by rat lung phagocytes following inhalation of a pulmonary irritant. *Am J Respir Cell Mol Biol*. 1994; 11:279–286. [PubMed: 8086166]
- Perkins ND. Integrating cell-signalling pathways with NF-kappaB and IKK function. *Nat Rev Mol Cell Biol*. 2007; 8:49–62. [PubMed: 17183360]
- Peschon JJ, Torrance DS, Stocking KL, Glaccum MB, Otten C, Willis CR, Charrier K, Morrissey PJ, Ware CB, Mohler KM. TNF receptor-deficient mice reveal divergent roles for p55 and p75 in several models of inflammation. *J Immunol*. 1998; 160:943–952. [PubMed: 9551933]
- Piguet PF, Vesin C. Treatment by human recombinant soluble TNF receptor of pulmonary fibrosis induced by bleomycin or silica in mice. *Eur Respir J*. 1994; 7:515–518. [PubMed: 7516893]
- Pryhuber GS, Huyck HL, Baggs R, Oberdorster G, Finkelstein JN. Induction of chemokines by low-dose intratracheal silica is reduced in TNFR I (p55) null mice. *Toxicol Sci*. 2003; 72:150–157. [PubMed: 12604844]
- Schwencke C, Braun-Dullaeus RC, Wunderlich C, Strasser RH. Caveolae and caveolin in transmembrane signaling: Implications for human disease. *Cardiovasc Res*. 2006; 70:42–49. [PubMed: 16412403]

- Sebban H, Courtois G. NF-kappaB and inflammation in genetic disease. *Biochem Pharmacol*. 2006; 72:1153–1160. [PubMed: 16965764]
- Shimabukuro DW, Sawa T, Gropper MA. Injury and repair in lung and airways. *Crit Care Med*. 2003; 31:S524–S531. [PubMed: 12907882]
- Shishodia S, Aggarwal BB. Nuclear factor-kappaB activation: a question of life or death. *Journal of biochemistry and molecular biology*. 2002; 35:28–40. [PubMed: 16248967]
- Shore SA, Schwartzman IN, Le Blanc B, Murthy GG, Doerschuk CM. Tumor necrosis factor receptor 2 contributes to ozone-induced airway hyperresponsiveness in mice. *Am J Respir Crit Care Med*. 2001; 164:602–607. [PubMed: 11520723]
- Shukla M, Yang S, Milla C, Panoskaltsis-Mortari A, Blazar BR, Haddad IY. Absence of host tumor necrosis factor receptor 1 attenuates manifestations of idiopathic pneumonia syndrome. *Am J Physiol Lung Cell Mol Physiol*. 2005; 288:L942–L949. [PubMed: 15608149]
- Sodhi A, Biswas SK. fMLP-induced in vitro nitric oxide production and its regulation in murine peritoneal macrophages. *Journal of leukocyte biology*. 2002; 71:262–270. [PubMed: 11818447]
- Takeda K, Kaisho T, Akira S. Toll-like receptors. *Annu Rev Immunol*. 2003; 21:335–376. [PubMed: 12524386]
- Uysal N, Schapira RM. Effects of ozone on lung function and lung diseases. *Curr Opin Pulm Med*. 2003; 9:144–150. [PubMed: 12574695]
- Vandevoorde V, Haegeman G, Fiers W. Induced expression of trimerized intracellular domains of the human tumor necrosis factor (TNF) p55 receptor elicits TNF effects. *J Cell Biol*. 1997; 137:1627–1638. [PubMed: 9199176]
- Volonte D, Liu Y, Galbiati F. The modulation of caveolin-1 expression controls satellite cell activation during muscle repair. *Faseb J*. 2005; 19:237–239. [PubMed: 15545301]
- Walker EH, Pacold ME, Perisic O, Stephens L, Hawkins PT, Wymann MP, Williams RL. Structural determinants of phosphoinositide 3-kinase inhibition by wortmannin, LY294002, quercetin, myricetin, and staurosporine. *Molecular cell*. 2000; 6:909–919. [PubMed: 11090628]
- Wallach D, Varfolomeev EE, Malinin NL, Goltsev YV, Kovalenko AV, Boldin MP. Tumor necrosis factor receptor and Fas signaling mechanisms. *Annu Rev Immunol*. 1999; 17:331–367. [PubMed: 10358762]
- Williams TM, Lisanti MP. The Caveolin genes: from cell biology to medicine. *Ann Med*. 2004; 36:584–595. [PubMed: 15768830]
- Young C, Bhalla DK. Effects of ozone on the epithelial and inflammatory responses in the airways: role of tumor necrosis factor. *J Toxicol Environ Health*. 1995; 46:329–342. [PubMed: 7473861]
- Zeidler PC, Castranova V. Role of nitric oxide in pathological responses of the lung to exposure to environmental/occupational agents. *Redox Rep*. 2004; 9:7–18. [PubMed: 15035823]
- Zhao HW, Hu SY, Barger MW, Ma JK, Castranova V, Ma JY. Time-dependent apoptosis of alveolar macrophages from rats exposed to bleomycin: involvement of tnfr receptor 2. *J Toxicol Environ Health A*. 2004; 67:1391–1406. [PubMed: 15371238]

Fig. 1.

Effects of ozone inhalation on TNF α expression. *Upper panels:* Histological sections were prepared immediately (0 h) or 3 h after exposure of wild type (A, B, C, D) or TNF α ^{-/-} (E, F, G, H) mice to air (A, E) or ozone (B, C, D, F, G, H). Sections were stained with anti-TNF α antibody (A, B, C, E, F, G) or IgG control (D, H). Original magnifications 1000X. Arrows indicate macrophages. *Lower panels:* Alveolar macrophages isolated immediately after exposure of wild type (WT) or TNF α ^{-/-} mice to air or ozone were stained with anti-TNF α antibody or IgG control followed by FITC-labeled secondary antibody, and then analyzed by flow cytometry as described in Materials and Methods.

Fig. 2. Mice lacking TNF α cytokine are protected from ozone-induced lung injury. Wild type (WT) or TNF α ^{-/-} mice were exposed to air or ozone. Lung injury was assessed 48 h later by quantifying bronchoalveolar lavage fluid protein levels. Each bar represents mean $\pm$ SE of 3–6 animals. *Significantly different ($P < 0.05$) from air control.

Fig. 3. Effects of ozone inhalation on production of reactive nitrogen intermediates. *Upper Panel:* Alveolar macrophages were isolated 48 h after exposure of wild type (WT) or TNF α ^{-/-} mice to air or ozone. Cells were cultured with IFN- γ (100 u/ml) and LPS (100 ng/ml) for 48 h. Supernatants were collected and analyzed for nitrite content. Each bar is the mean $\pm$ SE of 3–6 experiments. *Significantly different ($p < 0.05$) from air control. *Lower panels:* Histological sections were prepared 48 h following exposure of wild type (A, B, C, F) or TNF α ^{-/-} (D, E) mice to air (A, D) or ozone (B, C, E, F). Sections were stained with anti-nitrotyrosine antibody (A, B, D, E), IgG control (C), or anti-nitrotyrosine preincubated with nitrotyrosine (F). Original magnification 1000X. Arrows indicate macrophages.

Fig. 4.

Ozone induced activation of NF-κB in alveolar macrophages following ozone inhalation.

Upper panel: Cells were isolated 0–12 h after exposure of wild type or TNFα-/- mice to air or ozone. Nuclear extracts from wild type mice prepared 6 h after ozone inhalation were incubated with anti-p50 or anti-p65 antibody, or 100X excess unlabeled probe (CC) prior to the labeled probe. *Lower panel:* Cells isolated 6 h after exposure of wild type mice to ozone were treated with IFN-γ (100 U/ml) and LPS (100 ng/ml) with and without 100 nM wortmanin (W) and 10 μM LY294002 (Ly) for 1 h as indicated. NF-κB nuclear binding activity was analyzed by EMSA.

Fig. 5.

Effects of ozone inhalation on PI3K and PKB expression. *Upper panel:* Alveolar macrophages were isolated 0–48 h following exposure of wild type (WT) or TNF α -/- mice to air or ozone. PI3K and PKB expression was analyzed by western blotting. *Lower panel:* Alveolar macrophages isolated 6 h after exposure of WT or TNF α -/- mice to air or ozone were analyzed for PKB or p-PKB by immunocytochemistry.

Fig. 6. Effects of PI3K inhibitors on ozone-induced production of reactive nitrogen intermediates. Alveolar macrophages were isolated 3 h after exposure of wild type (WT) mice to air or ozone. Cells were cultured with IFN- γ (100 u/ml) and LPS (100 ng/ml) for 48 h together with 100 nM wortmanin and 10 μ M LY294002 (Inhibitors) or medium. Supernatants were collected and analyzed for nitrite content. Each bar is the mean $\pm$ SE of 3–4 experiments. *Significantly different (p < 0.05) from air control. **Significantly different (p < 0.05) from medium (med).

Fig. 7.

Effects of ozone inhalation on p42/p44 MAPK expression. *Upper panels:* Alveolar macrophages, isolated from WT or TNF- α -/- mice immediately following exposure to air or ozone were stained with antibodies to total or phospho- p42/p44 or IgG control, followed by FITC-labeled secondary antibody and then analyzed by flow cytometry as described in the Materials and Methods. *Lower panels:* Alveolar macrophages, were isolated from wild type (WT) or TNF α -/- mice 0–12 h following air or ozone exposure and analyzed for phospho-p42/p44 expression by western blotting.

Fig. 8.

Effects of ozone inhalation on Cav-1 expression. *Upper panel:* Alveolar macrophages isolated from wild type (WT) or TNF α -/- mice 0–12 h following air or ozone exposure were analyzed for Cav-1 expression by western blotting. +, positive control for anti-Cav-1 antibody binding. *Middle panel:* Alveolar macrophages isolated from wild type (WT) or TNF α -/- mice immediately following air or ozone exposure were stained with anti-Cav-1 antibody or IgG control, followed by FITC-labeled secondary antibody and then analyzed by flow cytometry. *Lower panel:* Cav-1 staining of lung. Histological sections were prepared 0 (B, F, D, H) and 3 h (C, G) after exposure of wild type (A, B, C, D) or TNF α -/- (E, F, G, H) mice to air (A, E) or ozone (B, C, D, F, G, H). Sections were stained with anti-TNF α .

antibody (A, B, C, E, F, G) or IgG control (D, H). Arrows indicate macrophages. Original magnifications 1000X.

\$watermark-text

\$watermark-text

\$watermark-text

Fig. 9.

Effects of TNF α on Cav-1 expression. Alveolar macrophages isolated from wild type mice were cultured with medium control (Med) or 1000 U/ml TNF α for 20 min and then analyzed for Cav-1 expression by western blotting. Data were quantified by densitometry (OneD-Scan) and presented in arbitrary units. One representative blot from 3 separate experiments is shown.