Increased Nitric Oxide Synthase in the Lung after Ozone Inhalation Is Associated with Activation of NF-κB

Debra L. Laskin, Vasanthi Sunil, Yan Guo, Diane E. Heck, and Jeffrey D. Laskin

Environmental and Occupational Health Sciences Institute, Rutgers University and University of Medicine and Dentistry of New Jersey-Robert Wood Johnson Medical School, Piscataway, New Jersey

Acute inhalation of ozone is associated with an inflammatory response characterized by the accumulation of macrophages at sites of tissue injury. These cells, along with resident alveolar epithelial cells, become activated and release cytoketic and proinflammatory mediators, such as nitric oxide (NO), that we speculate contribute to toxicity. In these studies we analyzed mechanisms regulating increased NO synthase activity in lung macrophages and type II cells after ozone inhalation. Brief exposure of rats to ozone (2 ppm for 3 hr) resulted in an increase in NO production by alveolar macrophages as well as type II cells in response to the inflammatory mediators lipopolysaccharide and interferon γ. These effects were apparently due to increased expression of inducible NO synthase (iNOS) protein and mRNA, which were evident in vitro and in situ in histologic sections. NO production and iNOS protein expression by both macrophages and epithelial cells were blocked by pyrrolidine dithiocarbamate (PDTC), an agent that inhibits activity of nuclear transcription factor kappa B (NF-κB). Cells from ozone-treated animals were less sensitive to the effects of PDTC than cells from control animals. Using electrophoretic mobility shift assays, we measured NF-κB binding activity in nuclear extracts of cells from control and ozone-exposed animals. Treatment of rats with ozone resulted in a time-dependent increase in NF-κB binding activity in both cell types, reaching a maximum in cells isolated 12 to 24 hr after ozone inhalation. Taken together, these data suggest that changes in the activity of NF-κB signaling are important in the response of lung macrophages and type II epithelial cells to cytokines after ozone inhalation. — Environ Health Perspect 106(Supp 5):1175–1178 (1998); http://ehpnet1.niehs.nih.gov/docs/1998/Suppl-5/1175-1178/laskin/abstract.html

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Introduction

Lung inflammation and injury are common consequences of inhaling irritants. Of particular concern is ozone, a ubiquitous urban air pollutant known to damage the alveolar epithelium. In humans, inhalation of ozone in amounts as low as 0.1 to 0.2 ppm is sufficient to cause toxicity characterized by edema, airway hyperresponsiveness, alveolar epithelial damage, and impaired host defense (1). Our laboratory has been interested in examining mechanisms by which ozone damages the lung. Using a rat model, we found that ozone readily induces an inflammatory reaction characterized by the rapid accumulation of neutrophils and macrophages into alveolar regions of the lung (2,3). This is followed by macrophage and type II epithelial cell activation. Thus, these cells display enhanced functional activity that includes increased production of reactive oxygen intermediates and nitric oxide (NO) (3,4). Following ozone inhalation, alveolar macrophages also release increased amounts of proinflammatory cytokines such as interleukin (IL)-1β and tumor necrosis factor alpha (TNF-α) (5). It is believed that mediators released by these cells contribute to lung injury (6,7). In this regard we found that rats pretreated with agents that block macrophages or reduce oxidant generation in the lung are protected from pulmonary toxicity induced by inhaled ozone (8,9). Many inflammatory cytokines mediate their action by activating the nuclear factor kappa B (NF-κB) family of transcription factors. Members of the NF-κB family constitute an important network of transcription factors and regulatory proteins that control the expression of genes crucial for immune and inflammatory responses (10). These include inducible nitric oxide synthase (iNOS), IL-1β, and TNF-α. Our studies focused on analyzing mechanisms by which alveolar macrophages and type II cells become activated after exposure to ozone. We hypothesized that lung cell activation and increased mediator production involve alterations in NF-κB binding activity.

Materials and Methods

Ozone Exposures

Female specific pathogen-free Sprague-Dawley rats (200–225 g; Taconic Farms, Germantown, NY) were exposed to ozone (2 ppm) or ultrapure air in 5.5-ft3 air-tight plexiglass chambers for 3 hr. Ozone was generated from oxygen gas via an ultraviolet (UV) light ozone generator (Orec Corp., Phoenix, AZ) and mixed with the inlet air of the exposure chamber. Ozone concentrations in the chamber were maintained by adjusting both the intensity of the UV light and the flow rate of ozone into the chamber. Concentrations were continuously monitored using an ozone monitor (Model 1008 AH, Dasibi, Glendale, CA).

Cell Isolation

Cells were isolated 3 to 48 hr after treatment of rats with air or ozone. Alveolar macrophages were isolated from perfused rat lung by lavage (11). Type II cells were obtained from lavaged lung by differential centrifugation and digestion of the tissue with elastase (12).
Measurement of 'NO Production and iNOS Protein Expression

Nitric oxide was quantified by the accumulation of nitrite and nitrate in the culture medium and was measured by a microplate assay based on the Greiss reaction, with sodium nitrite as the standard (13). For nitrate determinations, samples were treated with nitrate reductase and NADPH for 30 min prior to analysis. We found that the ratio of nitrate:nitrite produced by macrophages was 1:8:1 and that this ratio was unaffected by ozone inhalation. iNOS protein expression was measured in cell lysates by Western blot analysis using an iNOS-specific antibody (3). Proteins were visualized using an alkaline phosphatase detection system.

Nuclear Extraction and Electrophoretic Mobility Shift Assay for NF-κB

Nuclear protein was extracted from 2 to 3×10⁶ cells/treatment group as previously described (14). For electrophoretic mobility shift assay (EMSA), nuclear proteins (10–30 μg) were incubated for 30 min at room temperature with [32P]-dCTP-labeled iNOS NF-κB oligonucleotide probe (5’GAAGCTTGAGGAGTTCCCTTG-3’). For supershift assays, 10 μg of anti-p65 or anti-p50 antibody (Santa Cruz Biotech, Santa Cruz, CA) was added to the incubation mixture 20 min prior to the addition of the labeled probe. Protein–DNA complexes were separated on 5% non-denaturating polyacrylamide gels. Gels were run at 250 V, dried, and autoradiographed.

Results

In initial studies we quantified the effects of ozone inhalation on 'NO production by alveolar macrophages and type II alveolar epithelial cells. Freshly isolated macrophages and type II cells from control rats produced 'NO in response to in vitro treatment with the combination of lipopolysaccharide (LPS) and interferon γ (IFN-γ), as measured by the accumulation of nitrite and nitrate in the culture medium (Table 1; data not shown). Alveolar macrophages produced significantly more 'NO in response to these inflammatory mediators than did type II cells. Production of 'NO by both macrophages and type II cells was time dependent, reaching a maximum after 24 to 48 hr in culture and blocked by the iNOS inhibitor aminoxyguanidine (not shown). Northern and Western blot analyses revealed that this was due to increased expression of mRNA and protein, respectively, for the iNOS (Figure 1) (4, 9). Treatment of rats with ozone resulted in a time-dependent increase in 'NO production by both cell types (15). This was correlated with increased expression of iNOS mRNA and protein that was observed in vitro in isolated cells and in situ in histologic sections by immunohistochemistry and in situ hybridization (Figure 1) (9).

NF-κB is a nuclear transcription factor involved in regulating the activity of several genes involved in the inflammatory response including iNOS (10). In initial studies to determine if NF-κB mediated ozone-induced increases in 'NO production by lung macrophages and epithelial cells, we used the inhibitor pyrrolidine dithiocarbamate (PDTC), an antioxidant known to block NF-κB activity (16). We found that PDTC caused a dose-dependent inhibition of 'NO production by both cell types (Table 1; data not shown). Cells from ozone-treated rats were less sensitive to PDTC than were cells from control animals. PDTC treatment was also found to reduce iNOS expression by the cells (Figure 1). These effects were more pronounced in cells isolated from control animals and are consistent with our results on 'NO production. In further studies we determined if ozone inhalation modified expression of NF-κB binding activity in macrophages and type II cells. We found low levels of NF-κB activity in alveolar macrophages from control animals (Figure 2). Treatment of rats with ozone resulted in a time-dependent increase in NF-κB binding activity in the nucleus of both cell types (Figure 2; data not shown). Maximum

**Figure 1.** Effects of ozone inhalation on iNOS protein expression in alveolar macrophages (AM) and type II cells. Cells isolated 24 hr after exposure of rats to air (lanes 1–3) or ozone (lanes 4–6) were cultured with medium control (lanes 1, 4), 1 μg/ml LPS + 100 U/ml IFN-γ (lanes 2, 5) or LPS + IFN-γ + 1 μM PDTC (lanes 3, 6). Cell lysates were prepared 24 hr later and analyzed by Western blot analysis. The arrows show the position of iNOS. One of two similar experiments is shown.

**Figure 2.** Effects of ozone inhalation on expression of NF-κB in alveolar macrophages (AM) and type II cells. Cells isolated 24 hr after exposure of rats to air were analyzed for expression of NF-κB by EMSA as described in "Materials and Methods." One of two similar experiments is shown.

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**Table 1.** Effects of acute inhalation of ozone on nitric oxide production by lung macrophages and alveolar type II cells.

|                  | Air     | Ozone   |
|------------------|---------|---------|
| **Alveolar macrophages** |         |         |
| Control          | 0.5 ± 0.2 | 1.8 ± 0.2 |
| LPS + IFN-γ      | 11.4 ± 0.8 | 24.2 ± 1.6 |
| LPS + IFN-γ + PDTC, 0.5 μM | 7.1 ± 0.7 | 19.9 ± 1.4 |
| LPS + IFN-γ + PDTC, 1 μM | 3.2 ± 0.9 | 15.3 ± 1.1 |
| **Type II cells** |         |         |
| Control          | 0.3 ± 0.1 | 4.3 ± 0.4 |
| LPS + IFN-γ      | 6.9 ± 1.4 | 18.8 ± 1.0 |
| LPS + IFN-γ + PDTC, 0.5 μM | 3.2 ± 0.7 | 11.3 ± 1.3 |
| LPS + IFN-γ + PDTC, 1 μM | 1.1 ± 0.4 | 8.6 ± 1.6 |

*Cells isolated 24 hr after exposure of rats to air or ozone (2 ppm for 3 hr) were cultured with medium control, LPS (1 μg/ml) + IFN-γ (100 U/ml), or LPS + IFN-γ + PDTC. Nitrite accumulation in the culture medium was quantified 48 hr later. Each value is the average nmol nitrite/2×10⁶ cells ± SE (n=3–6). Results are significantly different from air control (p<0.05).
activity was observed in cells isolated 12 to 24 hr after ozone inhalation (15). This binding activity was specific, as it was blocked by excess unlabeled oligonucleotide probe. We also performed supershift assays to identify which NF-κB are involved in the response of macrophages and epithelial cells to ozone. These experiments demonstrated the presence of the p50 subunit in the nucleus of lung macrophages and epithelial cells (not shown).

Discussion

Macrophages have been implicated in the pathophysiology of tissue injury induced by a variety of pulmonary toxicants including ozone, endotoxin, silica, and asbestos (reviewed by Laskin and Pendino (6) and Laskin and Laskin (7)). In response to inflammatory mediators generated at sites of tissue injury, macrophages undergo a process of cellular activation. This is characterized by biochemical and functional alterations in the cells leading to the increased production of proinflammatory and cytotoxic mediators. Recent studies have suggested that lung epithelial cells also have the capacity to become activated and release inflammatory mediators (4,15,17–19). Thus these cells may also contribute to tissue injury associated with inflammation. The mechanisms regulating macrophage and epithelial cell activation in the lung after toxicant exposure are unknown and represent the focus of the present studies.

'NO is a highly reactive cytotoxic oxidant produced by both macrophages and type II cells via an iNOS (3,4). 'NO reacts with various molecular targets, in particular, proteins with iron- and iron–sulfur-containing centers. This can result in inhibition of critical enzymes in intermediary metabolism, mitochondrial respiration, and/or DNA synthesis and can lead to decreased cellular proliferation and nucleic acid biosynthesis, cytotoxicity, and apoptosis (20,21). 'NO also reacts with superoxide anion radicals, forming peroxynitrite, a relatively long-lived cytotoxic oxidant (22). Peroxynitrite can initiate lipid peroxidation and also reacts directly with sulfhydril groups in cell membranes (23). Thus, increased production of 'NO or its oxidation products in the lung after ozone exposure may contribute to tissue injury. We found that brief inhalation of ozone results in a marked increase in iNOS expression and 'NO production by both macrophages and type II cells in response to inflammatory mediators. 'NO and its oxidation products (nitrate, nitrite, peroxynitrite) have been implicated in the pathophysiology of lung injury induced by a number of pulmonary irritants (20,21), and similar mechanisms may contribute to alveolar epithelial damage induced by ozone.

A question arises as to the mechanism underlying increased expression of iNOS in alveolar macrophages and type II cells after exposure to ozone. We hypothesize that increased iNOS activity is due to alterations in the activity of transcription factors that regulate expression of this gene. The promoter region of the iNOS gene contains binding sites for several different transcription factors including NF-κB (24). It is generally believed that binding of cytokines such as TNF-α and IL-1β or bacterially derived LPS to cell surface receptors on responsive cell types initiates an intracellular signaling pathway leading to phosphorylation and proteolysis of IκB (inhibitory protein-κB), an inhibitory protein that functions to retain NF-κB in an inactive form in the cytoplasm. When IκB is inactivated, active NF-κB is released, translocates to the nucleus, and binds to regulatory motifs of responsive genes such as iNOS. The present studies demonstrate that PDTC, an inhibitor of NF-κB (16), blocked 'NO production by both macrophages and epithelial cells. This was correlated with decreased iNOS protein expression. Moreover, cells from ozone-treated rats were less sensitive to the inhibitory effects of PDTC than cells from control animals. EMSA assays revealed that NF-κB nuclear binding activity was increased in cells from ozone-treated rats. Taken together, these data suggest that changes in the activity of NF-κB signaling may be important in the response of lung cells to cytokines after ozone inhalation. However, these results have to be interpreted with caution since PDTC is an antioxidant and nonspecific inhibitor of NF-κB. Further studies using more specific inhibitors are required to analyze more precisely the role of this transcription factor in ozone-induced increases in 'NO production and pulmonary toxicity.

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