Pulmonary and Hepatic Effects of Inhaled Ozone in Rats

Debra L. Laskin, Kimberly J. Pendino, Chitra J. Punjabi, Marina Rodriguez del Valle, and Jeffrey D. Laskin

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

Nitric oxide is a highly reactive molecule that has been implicated in host defense and in tissue injury. In the present studies we analyzed the effects of brief exposure of rats to inhaled ozone on production of this mediator by lung macrophages and type II epithelial cells. We found that ozone exposure (1–2 ppm, 3 hr) induced a marked increase in spontaneous nitric oxide production by alveolar (AM) and interstitial macrophages, as well as type II cells. These effects were apparently due to increased expression of inducible nitric oxide synthase protein and mRNA, which was evident in vitro in isolated cells and in situ in histologic sections. Macrophages and epithelial cells from ozone-treated rats were also sensitized to produce increased amounts of nitric oxide in response to inflammatory cytokines such as interferon-γ, a response that was also mediated by inducible nitric oxide synthase. Unexpectedly, we also discovered that brief inhalation of ozone caused dramatic effects on the liver, including increased production of nitric oxide by hepatocytes and enhanced protein synthesis. These data suggest that this inhaled irritant induces an acute phase response. Additional studies indicated that AM from ozone-treated rats produced significantly more tumor necrosis factor-α and interleukin-1 than did cells from control animals. Elevated levels of tumor necrosis factor-α were also noted immunohistochemically in both lung and liver tissue. These results indicate that the extrapulmonary effects of ozone may be mediated by inflammatory cytokines released by activated lung macrophages. Taken together our data demonstrate that acute exposure of rats to ozone activates lung macrophages and type II epithelial cells to release cytokotic and proinflammatory mediators that we speculate contribute to the pathophysiologic effects of this oxidant on the lung and the liver. — Environ Health Perspect 102(Suppl 1):61–64 (1994)

Key words: ozone, nitric oxide, macrophages, type II cells, acute phase response

Introduction

Ozone is a highly reactive oxidant that is present in ambient air at low concentrations. Acute inhalation of this pulmonary irritant causes damage to epithelial cells in the terminal bronchioles and in the proximal alveolar regions (1). This is accompanied by a marked inflammatory response, characterized by macrophage and neutrophil accumulation at these sites and the release of highly reactive and potentially cytotoxic mediators, as well as cytokines (1–6). Although cytokines are well recognized for their local proinflammatory and immunoregulatory actions, recent studies suggest that they can also act at distal sites to induce biologic responses. For example, in response to cytokines such as interleukin-1 (IL-1), interleukin-6 (IL-6), and tumor necrosis factor-α (TNF-α) released systemically during bacterial infections, a reorganization in the pattern of gene expression of several plasma proteins by hepatocytes is observed (7). This reaction is part of a complex series of local and systemic responses, collectively termed the acute phase response, that is thought to function primarily to maintain homeostasis. Our laboratory has been investigating the pulmonary effects of brief inhalation of ozone. We found that there are increased numbers of alveolar macrophages (AM) and interstitial macrophages (IM) in the lungs of exposed rats and that these cells are activated to release nitric oxide (NO) and hydrogen peroxide (H₂O₂), two mediators that we hypothesize are involved in tissue injury (5,6). Surprisingly, during the course of our studies, we also discovered that hepatic protein synthesis was markedly elevated in animals exposed briefly to ozone. These data, together with the observation that there is increased production of the inflammatory cytokines, IL-1, IL-6, and TNF-α, known to induce acute phase protein production, in the lung following ozone inhalation (3,4), suggest that this pulmonary irritant induces an acute phase response. Thus, the response of rats to acute inflammatory injury in the lung induced by ozone is similar to that observed during respiratory infections or trauma associated with surgery.

Materials and Methods

Ozone Exposures

Female specific pathogen-free Sprague-Dawley rats (200–225 g, Taconic Farms, Germantown, NY) were exposed to ozone (0.5–2 ppm) or ultra pure air in 5.5 ft³ air-tight Plexiglass chambers for 3 hr. Ozone was generated from oxygen gas via a 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 48 hr after treatment of rats with air or ozone. AM were isolated from perfused rat lung by lavage (8). IM and type II cells were obtained from lavaged lung by differential centrifugation and digestion of the tissue with collagenase or elastase, respectively (8,9). Hepatocytes
were recovered from perfused liver by collagenase digestion (10).

**Measurement of Nitric Oxide Production**

Nitric oxide, quantified by the accumulation of nitrite in the culture medium, was measured by a microplate assay based on the Greiss reaction with sodium nitrite as the standard (11).

**Measurement of Protein Synthesis**

Hepatocytes were cultured in 96 well dishes (2 × 10⁶ cells/well) for 44 hr. The cells were then pulse-labeled for 4 hr with ³H-leucine (1 μCi/well, specific activity 3.7 MBq/μCi) (ICN Biomedicals, Irvine, CA) in medium containing 160 μM leucine. The cells were then lysed with 1 N NaOH in 0.025% Triton X-100, proteins precipitated with 40% trichloroacetic acid, collected on filter paper and counted for radioactivity.

**Results**

**Activation of Alveolar and Interstitial Macrophage and Type II Alveolar Epithelial Cells for Nitric Oxide Production by Ozone**

In initial studies we quantified production of NO by AM and IM after ozone exposure. This reactive nitrogen intermediate is released by activated macrophages in response to inflammatory stimuli (12–14). Freshly isolated AM and IM from control rats were found to produce NO in response to in vitro treatment with the inflammatory mediators, interferon-γ (IFN-γ), lipopolysaccharide (LPS), and TNF-α, as measured by the accumulation of nitrite in the culture medium (Table 1) (4,14). Production of NO by these cells was time dependent, reaching a maximum after 24 to 48 hr in culture, and blocked by the NO synthase inhibitor, N°C-monomethyl-L-arginine (4,12,14). Northern and Western blot analysis revealed that this was due to increased expression of mRNA and protein, respectively, for the inducible form of nitric oxide synthase (4; data not shown). After brief exposure of rats to ozone (1–2 ppm, 3 hr), AM as well as IM spontaneously produced significantly more NO than cells from control rats (Table 1) (4,6), a response that was due, at least in part, to increased expression of mRNA and protein for inducible NO synthase (4; data not shown). Macrophages isolated from ozone-exposed rats were also sensitized to produce more NO in response to inflammatory stimuli (Table 1) (4,6), and to express greater quantities of protein and mRNA for NO synthase (4; data not shown). In addition, mRNA for inducible nitric oxide synthase was observed in these cells within 2 hr, compared to 12 hr for cells from control rats. Taken together, these data demonstrate that lung macrophages from ozone treated rats are “activated” to produce reactive nitrogen intermediates and that this response is due, in part, to increased inducible NO synthase.

In further studies we determined if type II alveolar epithelial cells also have the capacity to be activated to release NO. Type II cells stimulated with IFN-γ and/or TNF-α, but not LPS, were found to produce significant quantities of NO in a time- and concentration-dependent manner (Table 1) (15). In addition, after exposure of rats to ozone, type II cells, like AM, produced significantly more NO than cells from control animals, both spontaneously and in response to IFN-γ (Table 1). This was due, at least in part, to increased expression of mRNA for inducible NO synthase, as determined by Northern blot analysis and in situ hybridization studies (15).

To determine if ozone exposure has the potential of modifying NO production in vivo, sections of lung tissue from control and ozone-exposed rats were stained with an antibody to inducible NO synthase. Microscopic examination of these sections revealed specific staining of lung tissue from ozone-treated rats with this antibody (4). The enzyme appeared to be localized throughout the alveolar epithelium, in particular the cytoplasm of lung macrophages, as well as in epithelial cells, interstitial cells, and fibroblasts lining the alveolar sacs. No specific staining was evident in sections from lungs of control animals or in sections stained with an antibody to the constitutive form of the enzyme (4). These data suggest that enhanced expression of inducible NO synthase may underlie increased spontaneous NO production by isolated lung phagocytes and type II cells following ozone treatment of rats (Table 1) (4,6).

**Effects of Ozone Inhalation on the Liver**

In additional studies, we analyzed the effects of acute ozone inhalation on liver function. Unexpectedly, we found that hepatocytes isolated from rats 48 hr after ozone exposure produced significantly more NO spontaneously and in response to the inflammatory stimuli, IFN-γ and LPS, than did cells from control animals (Table 2). This was apparently due to increased inducible NO synthase which was determined by Western and Northern blot analysis, respectively (16). These data demonstrate that ozone “primes” hepatocytes for NO production. Remarkably, ozone exposure was also found to cause a dramatic increase in hepatocyte protein synthesis (Table 2). These effects were dose dependent in the range of 0.5 to 2 ppm ozone.

In further studies, we began to analyze potential mechanisms underlying the extra-pulmonary effects of inhaled ozone. We speculated that increased hepatocyte NO production and protein synthesis were mediated by cytokines released from activated macrophages in the lung. Two

| Ozone, ppm | Nitric oxide²(nmol/10⁶ cells) | Protein synthesis,³ cpm/well |
|-----------|-------------------------------|-----------------------------|
| 0.0       | 140 ± 10                      | 420 ± 100                   |
| 0.5       | 145 ± 10                      | 614 ± 112                   |
| 1.0       | 230 ± 28*                     | 1637 ± 426*                 |
| 2.0       | 389 ± 20*                     | 3040 ± 322*                 |

*Hepatocytes were isolated 48 hr after exposure of rats to 0 to 2 ppm ozone (3 hr). Cells were cultured in the presence of LPS (5 μg/ml) and IFN-γ (50 U/ml). Nitric accumulation in the culture medium was quantified 48 hr later. Each point is the average ± SE of three samples from one representative experiment.

*Cells were cultured for 44 hr and then pulse-labeled for 4 hr with ³H-leucine. Each value is the average ± SE of three samples from one representative experiment. *Significantly different (p < 0.05) from control (0 ppm ozone).
cytokines of particular relevance are TNF-α and IL-1, known to stimulate both of these biologic processes in hepatocytes (17,18). After exposure of rats to ozone, AM were found to produce increased amounts of TNF-α (3). This was evident in vitro in isolated cells and in vivo in histologic sections. Furthermore, examination of immunohistochimically stained sections of livers from ozone-treated rats revealed extensive staining of hepatocytes with TNF-α. These data provide support for our hypothesis that inflammatory cytokines such as TNF-α mediate at least some of the effects of inhaled ozone on the liver.

Discussion

The present studies demonstrate that acute inhalation of ozone has dramatic effects on NO production by macrophages and epithelial cells in the lung, as well as by hepatocytes in the liver. Furthermore, following ozone exposure, hepatocytes are stimulated to produce increased amounts of protein. These results were surprising and demonstrate that there are extrapulmonary effects of this inhaled oxidant. Hepatocytes are known to produce a wide array of proteins that function in normal and pathophysiological processes. One group of these proteins, termed acute phase proteins, are produced in response to injury or trauma (7). Our finding that inhalation of ozone stimulates hepatocyte protein synthesis suggests that this pulmonary irritant may induce an acute phase response similar to that observed during a respiratory infection. Interestingly, the effects of ozone on hepatocyte protein production were observed at concentrations below those required to induce a significant inflammatory response in the lungs of rats (4). These data are intriguing and suggest that the extrapulmonary effects of ozone may be a more sensitive indicator of toxicity, at least in the rat model. The relationship between NO production and induction of hepatocyte protein synthesis is unknown. Induction of acute phase protein production following endotoxemia has also been reported to be associated with enhanced NO production (19). Whether nitric oxide produced in the liver is involved in xenobiotic-induced toxicity or acts to protect the tissue from oxidant-induced damage remains to be determined.

After brief inhalation of ozone, both AM and IM in the lung produced increased amounts of NO spontaneously and in response to relatively low concentrations of inflammatory mediators. These effects were dose dependent in the range of 1 to 2 ppm ozone and were maintained for at least 4 days after exposure (4). These data suggest that lung macrophages are "activated" to produce NO following ozone inhalation. Our observations of increased protein and mRNA for inducible NO synthase suggest that ozone may act to augment NO production by elevating levels of this enzyme. Additional studies revealed that type II cells are also activated after ozone exposure to produce increased amounts of NO. The results of these studies were surprising because alveolar epithelial cells have not generally been considered to participate in tissue inflammatory responses to inhaled irritants. Our results, together with recent reports of production of eicosanoids (20), chemotactic factors (27,22), and H2O2 (15,23) by type II cells, suggest that they may contribute to this process.

The contribution of nitric oxide to ozone-induced inflammation and injury is unknown. NO is a highly reactive molecule that can complex with iron in home-containing proteins leading to inhibition of mitochondrial respiration and DNA synthesis and to cytotoxicity (24–26). NO has been reported to mediate glutamate neurotoxicity in rat primary cortical cultures (27) and carrageenin-induced increases in epidermal vascular permeability and edema (28). Mulligan et al. (29,30) have also suggested that immune complex-induced injury to rat lung and to skin is mediated by nitric oxide. Similarly, NO produced by lung macrophages and epithelial cells after ozone exposure may damage surrounding tissue and/or modify type II cell growth, thus influencing alveolar epithelial regeneration. Alternatively, NO may combine with superoxide anion, effectively reducing levels of both of these oxidants and protecting against toxicity (31). Further studies are in progress to analyze the precise role of reactive nitrogen intermediates in the pathogenesis of ozone-induced tissue injury.

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