Susceptibility to ozone-induced acute lung injury in iNOS-deficient mice

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Kenyon, Nicholas J., Albert van der Vliet, Bettina C. Schock, Tatsuya Okamoto, Gabrielle M. McGrew, and Jerold A. Last. Susceptibility to ozone-induced acute lung injury in iNOS-deficient mice. Am J Physiol Lung Cell Mol Physiol 282: L540–L545, 2002. First published October 19, 2001; 10.1152/ajplung.00297.2001. — Mice deficient in inducible nitric oxide synthase (iNOS; C57Bl/6Ai-KO; NOS2 N5) or wild-type C57Bl/6 mice were exposed to 1 part/million of ozone 8 h/night or to filtered air for three consecutive nights. Endpoints measured included lavagable total protein, macrophage inflammatory protein (MIP)-2, matrix metalloproteinase (MMP)-9, cell content, and tyrosine nitration of whole lung proteins. Ozone exposure caused acute edema and an inflammatory response in the lungs of wild-type mice, as indicated by significant increases in lavage protein content, MIP-2 and MMP-9 content, and polymorphonuclear leukocytes. The iNOS knockout mice showed significantly greater levels of lung injury by all of these criteria than did the wild-type mice. We conclude that iNOS knockout mice are more susceptible to acute lung damage induced by exposure to ozone than are wild-type C57Bl/6 mice and that protein nitration is associated with the degree of inflammation and not dependent on iNOS-derived nitric oxide.

nitrotyrosine; nitric oxide; inflammation; matrix metalloproteinase-9; macrophage inflammatory protein-2

THE MOLECULAR AND CELLULAR mechanisms responsible for lung injury in animals exposed to ozone are not yet well understood, although components of this response have been studied in detail for many years. It is well recognized that injury to epithelial cells, changes in lung permeability that give rise to pulmonary edema, and airway inflammation are all components of the acute response of the lung to inhalation of ozone (14). However, many questions about the details of these processes remain unanswered.

One broad category of such unanswered questions is the detailed pathway of chemical reactions occurring upon inhalation of ozone. The combination of high chemical reactivity of ozone and its proclivity to initiate free radical reactions leading to peroxidation of (poly)unsaturated fatty acids in lipids, and there is direct evidence in vivo that this process occurs in the lungs of rats exposed to ozone (3). Products arising from interactions of ozone and free radicals arising from ozone with biological molecules may also be important in its mechanism of action.

One potential biological target for ozone or its secondary oxidation products is nitric oxide (NO), which can be converted to various reactive nitrogen oxides that might contribute to development of lung damage or inflammation (22). There is an obvious linkage to the concept that ozone-induced lung damage is related to endogenous or induced NO levels in the lung, and this relationship has been suggested by several recent studies (8, 10). Exposure to ozone is associated with increased levels of mRNA for the inducible form of nitric oxide synthase (iNOS) in alveolar macrophages and alveolar type II cells (16). Although induction of NO production may contribute to (oxidative) lung injury, NO may also exhibit anti-inflammatory actions and exert a beneficial role in lung injury models (13). Hence, the precise role of NO in the pathogenesis of acute lung injury, such as in response to ozone inhalation, remains to be defined.

Additional unanswered questions with regard to the effects of ozone on the lung relate to the elicitation of cell signaling pathways that directly and indirectly regulate the inflammatory response in this complex organ. Exposure of laboratory rodents to ozone is associated with very rapid recruitment of neutrophils to the lung (5, 9, 25). This observation has been directly linked to increased production of neutrophil chemoattractant cytokines such as macrophage inflammatory protein-2 (MIP-2, the murine homolog of interleukin [IL]-8) by pulmonary alveolar macrophages (5, 9) as well as by lung epithelial cells (25). The ubiquitous NO may also play a role in this process. In a recent paper, Inoue et al. (8) reported that treatment of guinea pigs with inhibitors of iNOS attenuated expression of IL-8 and decreased the accumulation of neutrophils in lung lavage fluid from animals exposed to 3 parts/million ozone.
(ppm) of ozone for 2 h. Exposure of rats and mice to ozone is also known to cause lung fibrosis (14). Because acute increases in matrix metalloproteinase (MMP)-9 (also known as gelatinase B) are known to be associated with lung fibrosis, we also measured the activity of MMP-9 in lavage fluid from the mice as a putative biomarker of early events that might lead to fibrosis (23).

These considerations led us to design experiments to specifically attempt to examine the role of iNOS in the response to ozone exposure of mice. To accomplish this, we used a line of mice in which the gene for iNOS had been knocked out by deletion of the first four exons and part of the promoter region of the NOS2 gene (15). We proposed to test the specific hypothesis that iNOS knockout mice would be relatively resistant to acute injury by ozone because of their potentially decreased ability to produce reactive nitrogen species in the lung.

**METHODS**

**Mice.** Wild-type mice were C57Bl/6 obtained from Bantam and Kingman Laboratories (Gilroy, CA). The iNOS knockout mice were initially purchased from Taconic Farms (Germantown, NY) and were rederived by embryo transfer to establish a breeding colony in the Targeted Genomics Laboratory of the Mouse Biology Program barrier facility at University of California, Davis. They are on a C57Bl/6 background and are designated C57Bl/6Ai[KO]NOS2 N5.

**Exposure to ozone.** Mice were exposed for 8 h/night (midnight to 8:00 AM) to 1 ppm of ozone for three consecutive nights or 2 h. Actual exposure concentration to the corresponding aminotyrosine derivative by sodium acetylation followed by 10.220.32.246 on April 30, 2017 http://ajplung.physiology.org/ Downloaded from L541

AJP-Lung Cell Mol Physiol • VOL 282 • MARCH 2002 • www.ajplung.org

serum albumin were run with each assay and were linear ($r^2 = 0.98-0.99$) between 0 and 50 µg of protein. Results are presented as pooled data from the two independent experiments, which gave very similar outcomes.

**MIP-2 determination.** MIP-2 was determined by ELISA, using a commercially available microplate assay kit (Quantikine M Mouse MIP-2 assay, lot no. 0018173; RD Systems, Minneapolis, MN), which gave a linear response between 0 and 250 pg/ml ($r^2 = 0.99$).

**Determination of gelatinase B.** Gelatinase B (MMP-9) levels in lung lavage fluid were determined by gelatin zymography, as described in detail by Suga et al. (21). Precast 10% Tris-glycine polyacrylamide gels from Invitrogen (Novex Zymogram gels; Carlsbad, CA) were used for this assay with 0.1% gelatin incorporated as a substrate. Gelatinolytic bands were analyzed by densitometry using NIH Image software.

**Determination of nitrite and nitrate.** Nitrite and nitrate were measured as an indicator of NO production by chemical reduction to NO in acidified vanadium(III) and subsequent NO analysis by ozone-induced chemiluminescence (1).

**Determination of protein nitrotyrosine.** Lung tissue sections from wild-type and iNOS-deficient mice were homogenized in two volumes of 100 mM sodium acetate (pH 7.2), and proteins were precipitated by the addition of an equal volume of acetonitrile. Precipitated proteins were centrifuged for 5 min at 3,000 g and washed four times with 2 ml of sodium acetate buffer (pH 7.2)-acetonitrile (50:50 vol/vol) to remove any soluble contaminants that could cause artifactual nitration during protein hydrolysis.

The washed proteins were hydrolyzed under reduced pressure in 6 M HCl for 18 h at 110°C and subsequently dried under a stream of N2 gas. Amino acids were reconstituted in 100 mM sodium acetate buffer (pH 7.2) and derivatized by acetylation followed by O-deacetylation to generate N-acetyl derivatives. The resulting N-acetyltyrosine was reduced to the corresponding aminotyrosine derivative by sodium dithionite. The resulting N-acetylaminoacrylate from 3-nitrotyrosine and N-acetylaminoacrylate from unmodified tyrosine residues were analyzed by reverse-phase HPLC with tandem electrospray ionization and ultraviolet detection (20).

**Statistical analysis of data.** Data were analyzed for significance using one-way analysis of variance, with the Tukey-Kramer multiple comparisons test used as a post hoc analysis to correct for multiple comparisons with the control group.

**RESULTS**

Mice were exposed to either 1 ppm of ozone for 8 h/night for three consecutive nights or filtered air. Exposed wild-type animals and controls appeared to be normal in all respects, whereas the iNOS knockout mice exhibited rapid shallow breathing immediately after the cessation of exposure. Lungs from all groups of animals appeared to be grossly normal visually.

Total protein content of lung lavage fluid was used as a primary indicator of lung injury in response to exposure of mice to ozone (7). As shown in Fig. 1, protein content of the lavage fluid from mice exposed to filtered air was 0.39 ± 0.04 µg/ml (n = 10). The wild-type mice exposed to ozone showed a significant threefold increase in protein content ($P < 0.001$) to 1.24 ± 0.11 µg/ml (n = 10). An even larger increase in protein content ($P < 0.001$) to 1.72 ± 0.17 µg/ml (n = 9) was observed in ozone-exposed iNOS-deficient mice. The difference between the wild-type and knockout mice...
was statistically significant (P < 0.05). No significant differences were observed in the protein content of lung lavage fluid from wild-type and iNOS knockout mice exposed to filtered air, nor was there any significant difference between male and female mice, either in their baseline content of protein in lung lavage fluid or in their response to exposure to ozone.

Differential cell counts in the lung lavage fluid were also examined. In the mice exposed to filtered air, essentially all of the cells recovered by lavage were pulmonary alveolar macrophages, as might have been expected. There were no significant differences between the different exposure groups in numbers of pulmonary alveolar macrophages counted per field, as illustrated in Fig. 2A. There were, however, significant differences in the numbers of neutrophils in the lavage fluid from the different groups of mice, as shown in Fig. 2B. Lavage fluids from control mice exposed to filtered air contained essentially no neutrophils, but similar fluids from wild-type mice exposed to ozone had 21.5 ± 4.9 (n = 10) neutrophils per high power field, illustrating an inflammatory response. Neutrophil numbers in lavage fluids from iNOS knockout mice exposed to ozone were significantly higher, at 31.5 ± 69 (n = 9) neutrophils/field. This finding illustrates a dramatically amplified inflammatory response in iNOS-deficient mice upon ozone exposure.

Levels of MIP-2, the murine homolog of IL-8, were measured in lavage fluid from the three groups of mice tested (Fig. 3) and were 1.9 ± 0.8 pg/ml (n = 4) and 8.8 ± 1.7 pg/ml (n = 4) for wild-type mice exposed to air or ozone, respectively. MIP-2 levels were markedly higher in lavage fluids from iNOS knockout mice exposed to ozone, 33.9 ± 9.9 pg/ml (n = 3), consistent with the markedly increased neutrophil influx in these mice.

MMP-9 levels in lung lavage fluid, as determined by gelatin zymography, were minimal in unexposed wild-type and iNOS-deficient mice (Fig. 4), but there was a significant increase of approximately threefold in

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Fig. 1. Protein content of lung lavage fluid from mice exposed to ozone or to filtered air. Pooled data are presented from 2 independent experiments. Mice were lavaged with a total volume of 2 ml of PBS, and aliquots of 25, 50, and 100 μl of the supernatant prepared from each sample (see Fig. 2 legend) were analyzed using the bicinchoninic acid assay for total protein content. Standard curves were constructed between 0 and 50 μg of protein using bovine serum albumin as the standard. Straight lines were fit to the data points by linear regression analysis, forcing the line through the origin. Correlation coefficients (r² values) were routinely 0.98-0.99. *Significantly different from air-exposed group; †significantly different from wild-type mice exposed to ozone. Bars indicate 1 SE.

Fig. 2. Cell content of lung lavage fluid from mice exposed to ozone or to filtered air. Pooled data are presented from 2 independent experiments. Mice were lavaged with a total volume of 2 ml of PBS, and the resulting suspension was centrifuged to prepare a cell pellet. The pellet was suspended in 200 μl of PBS; 50 or 100 μl of this cell suspension was processed in a cytocentrifuge to prepare slides. Slides were stained with Diff Quik to allow differential counts to be performed. All of the cells in a total of 10 randomly chosen high power fields were counted for each slide, and the sum of each cell type in 10 fields was calculated. A: macrophages; B: neutrophils.

*Significantly different from air-exposed group; †significantly different from wild-type mice exposed to ozone. Bars indicate 1 SE.
MMP-9 activity in the lungs of the wild-type mice exposed to ozone. A more dramatic, ~12-fold increase was observed in iNOS knockout mice. Presumably, infiltrated neutrophils are the major source of the increased MMP-9 activity, and the increased MMP-9 levels observed were consistent with the degree of neutrophil influx.

To determine the formation of reactive nitrogen species, protein tyrosine nitration was measured in lung tissue by protein hydrolysis, amino acid derivatization, and HPLC analysis, as shown in Fig. 5. There appeared to be higher levels of 3-nitrotyrosine in the lung proteins of mice exposed to ozone; however, these apparent increases were not statistically significant.

Despite the deficiency in iNOS, we observed no significant difference between concentrations of nitrite plus nitrate in lavage fluid from control and exposed mice of either genotype. Lavage fluids from mice exposed to filtered air contained 1.83 ± 0.20 μM NO$_3$ / NO$_2$ (means ± SE), compared with 1.30 ± 0.26 μM and 1.41 ± 0.29 μM for ozone-exposed wild-type and iNOS knockout mice, respectively.

**DISCUSSION**

Exposure of laboratory animals in vivo to ozone causes a loss of the epithelial cell barrier to permeability, usually most easily measured as an increase in the protein content of whole lung lavage fluid (7). Thus in this study, we chose as our primary indicator of ozone-induced lung damage, quantification of the total protein content of lavage fluid from the exposed mice. Wild-type mice exposed to ozone showed a significant increase of more than threefold in the protein content of their lung lavage fluid, a clear indication that the exposure protocol used in the current study was efficacious. The iNOS knockout mice showed a significantly greater increase in protein content of their lavage fluid, ~40% higher than the value in the wild-type animals. Thus the iNOS knockout mice were more susceptible to...
lung damage, estimated by protein content of their lung lavage fluid, than were otherwise isogenic control mice. Thus we can reject our initial hypothesis that a decreased ability of the iNOS knockout mice to produce reactive nitrogen species such as peroxynitrite in the lung would make them resistant to ozone-induced inflammation. This unexpected finding raises several questions. First and foremost is whether there are plausible mechanisms that might account for the increased susceptibility of the iNOS knockout mice to ozone. Our results provide several clues to the answer to this question. We found 1) increased neutrophils and neutrophil marker enzymes in the lavage fluid from the iNOS knockout mice (Fig. 2; 2) increased MIP-2 in the lavage fluid from these mice (Fig. 3); and 3) increased protein content in the lavage fluid from these mice (Fig. 1). These results are consistent with an enhanced inflammatory reaction in the iNOS knockout mice and focus attention on the neutrophil as a possible effector cell contributing to the increased response to ozone that we observed in the iNOS knockout animals. The increased MIP-2 levels in the lung lavage fluid might have been produced from pulmonary alveolar macrophages or epithelial cells and are likely to account, at least in part, for the increased number of neutrophils in the airways of the knockout mice exposed to ozone. The greatly increased MMP-9 levels in the lavage fluid from these mice demonstrate that these infiltrated neutrophils were most likely activated. Neutrophils are thought to use MMP-9 to facilitate their migration across the basement membrane (4), which may have also contributed to their accumulation in the airways of these animals. In addition, MMP-9 may also contribute directly to lung injury by virtue of its proteolytic activity (23).

There are clear differences in the relative sensitivity of different strains of mice to acute lung injury caused by exposure to ozone (17). In the Prows et al. (17) study, mice of the C57Bl/6 strain were defined as resistant to ozone, relative to A/J mice, which are more sensitive. On the other hand, C57Bl/6 mice are more susceptible to ozone than are mice of the C3H/HeJ strain (11). The relative susceptibility to ozone was traced to three specific gene loci, one of which might be tnf, the gene encoding tumor necrosis factor-α (2, 11). Given the genetic background of our iNOS knockout mice, we were constrained to work in mice of the C57Bl/6 strain in the current study. However, this would appear to be an appropriate model based on previous studies of others on ozone-induced lung injury in mice (2, 5, 11, 25).

Our results would appear to be inconsistent with those of Inoue et al. (8), who found decreased ozone-induced airway inflammation in guinea pigs treated with inhibitors of NOS activity, including less neutrophil accumulation and less IL-8 production by epithelial cells. However, it should be noted that different exposure protocols (concentration and duration of exposure) and different species were used in their study and ours, and that a combination of pharmacological NOS inhibitors was used in their study, which would have also affected other NOS isozymes. Our results also appear to be inconsistent with those of Kleeberger et al. (10), who found decreases in ozone-induced epithelial permeability in mice treated with the NOS inhibitor Nω-monomethyl-l-arginine (l-NMMA) and in a strain of iNOS knockout mice. It should be pointed out that l-NMMA inhibits all three isoforms of NOS, not just iNOS. Moreover, their exposure conditions (0.3 ppm of ozone continuously for 72 h) were different from ours. We would expect that the higher concentration of ozone (1 ppm) used in our experiments would result in a much greater level of pulmonary edema than that elicited by 0.3 ppm of ozone, and, therefore, that the underlying pathogenic basis of injury might have been different in the two studies. Furthermore, Kleeberger et al. (10) did not observe differences in ozone-induced airway inflammation in wild-type or iNOS-deficient mice, contrary to our results. There might also be minor differences between the strains of mice used in our study and by Kleeberger et al. because different suppliers were used (Taconic Farms vs. Jackson Laboratories). According to the Taconic Farms website, the company has been breeding its animals from founders in a closed colony since 1995.

Our findings are more consistent with results reported recently by Kobayashi et al. (12), who exposed wild-type and iNOS-deficient knockout mice to hyperoxia. Lung injury, as measured by increased protein content of lavage fluid and by other parameters, was greater in the iNOS knockout mice than in the wild-type animals. In addition, similar to our results, substantial amounts of nitrotyrosine were found in the lungs of iNOS knockout mice exposed to hyperoxia, demonstrating that protein nitration proceeds by an iNOS-independent pathway. Finally, they also observed increased accumulation of neutrophils in lung lavage fluid from the iNOS-deficient mice exposed to hyperoxia. In this regard, our results are consistent with those of Kobayashi et al. (12) in that nitrotyrosine formation seems to be associated with the degree of inflammation and does not require iNOS expression or induction. The requisite NO for such nitration reactions presumably arises from the action of endothelial and/or neuronal NOS (1 and 3).

We can summarize our findings from this study as follows. iNOS knockout mice are more susceptible to acute lung damage induced by exposure to ozone than are wild-type C57Bl/6 mice. This increased susceptibility is associated with a very large increase in the number of neutrophils recovered by lung lavage, which in turn appears to be a response to elevated levels of MIP-2 in the lungs of the iNOS knockout mice exposed to ozone. Lack of iNOS may contribute to neutrophil accumulation in the lung by inhibiting apoptosis of neutrophils or by affecting the expression of adhesion molecules (19). NO is known to participate in one of the major pathways to apoptosis, which may be initiated by IL-1β and results from decreased expression of the antiapoptotic protein bax (24). Despite the lack of one of the three known enzymes responsible for generation of NO in the iNOS knockout mice, 3-nitrotyrosine is
found in the lung proteins of these mice exposed to ozone. Overall, our results suggest that iNOS-derived NO displays anti-inflammatory properties in the lung, possibly by inhibition of inflammatory cytokine expression and neutrophil adhesion or by modulation of neutrophil apoptosis.

This research was supported, in part, by a pilot grant from The Center for Environmental Health Sciences at University of California, Davis; National Institutes of Health Grants ES-05707, HL-47628, and HL-60812; Tobacco-Related Research Program 7RT-0167; and by a University of California Health Systems award.

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