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Differentiation of the Roles of NO from Airway Epithelium and Inflammatory Cells in Ozone-Induced Lung Inflammation

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Abstract

Mice lacking inducible nitric oxide synthase (NOS2^{-/-}) are more susceptible to ozone-induced lung inflammation and injury than their isogenic wild-type (NOS2^{+/+}) counterparts, demonstrating an apparent protective effect for NOS2 in murine lungs. We hypothesized that nitric oxide (NO) generated from either NOS2 in the airway epithelial cells or the bone marrow-derived inflammatory cells was responsible for the protective effect of NOS2. To test this hypothesis, we prepared chimeric mice by killing their endogenous bone marrow cells by whole body irradiation, followed by bone marrow transplantation from a heterologous donor mouse. We exposed C57BL/6 (NOS2^{+/+}), NOS2^{-/-}, and chimeric NOS2 mice (NOS2^{-/+}, NOS2^{+/-}) to 1 ppm of ozone for 3 consecutive nights. NOS2^{-/-} mice were more severely injured after exposure to ozone than C57BL/6 mice, including a more robust inflammatory cell influx ($4.14 \times 10^5 \pm 2.19 \times 10^5$ vs. $2.78 \times 10^5 \pm 1.36 \times 10^5$ cells respectively; $p=0.036$) and greater oxidation of total protein sulfhydryls (R-SH) in their blood plasma. Chimeric NOS2^{-/+} mice, which had bone marrow from NOS2^{-/-} mice transplanted into C57BL/6 recipients, had a significantly greater response to ozone (increased numbers of neutrophils in lung lavage and decreased concentrations of exhaled NO) as compared to the reciprocal chimeric strain (NOS2^{+/-}). We conclude that NOS2 has a protective effect against acute lung injury caused by ozone inhalation, which may be mediated, in part, by NO generated by NOS2 from inflammatory cells, predominantly neutrophils, recruited into the lung.

Keywords

Nitric oxide synthase; Exhaled nitric oxide; Peroxynitrite; Neutrophil; Lung compliance; Reactive oxygen species

INTRODUCTION

Nitric oxide (NO) chemistry is fundamental to understanding ozone-associated acute lung injury. Endogenous NO is known to modulate ozone-induced lung injury (Inoue et al., 2000; Kleeberger et al., 1997). For example, increased levels of mRNA for the inducible form of nitric oxide synthase (NOS2) have been found in alveolar macrophages and alveolar type II cells after exposure of animals to ozone (Pendino et al., 1996). We, and others, have found that

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knockout of the NOS2 gene increases injury to the lungs of animals exposed to ozone (Kenyon et al., 2002; Fakhrzadeh et al., 2002). Kristof and colleagues (1998) showed, for example, that NO produced by NOS2 could exert anti-inflammatory properties and protect against oxidative insults in the lung. The precise role of NO in the pathogenesis of lung injury associated with ozone inhalation remains poorly understood.

Acute exposure of animals to high levels of ozone causes characteristic pathogenic responses in the airway and lung parenchyma (Last, 1988). Ozone can 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 (Cueto et al., 1992). Acute lung injury after ozone exposure is probably modulated, in part, by NO and by reactive nitrogen species derived from NO, including peroxynitrite (ONOO-) and nitrogen dioxide (Laskin et al., 2001; van der Vliet et al., 1999).

NOS2 and, presumably, NOS2-derived NO confer a protective effect against neutrophil recruitment into the lung after exposure to ozone (Kenyon et al., 2002). This protective effect may be mediated in part through macrophage inhibitory protein-1 (MIP-1); NO is known to modulate MIP-1 expression (Kenyon et al., 2002; Bhattacharyya et al., 2002; Martinez-Mier et al., 2002; Wishah et al., 2002). Despite previous investigations focusing on NO in ozone-induced injury, neutrophil recruitment, and ozone-associated lung injury, key questions remain unanswered. Three questions we sought to answer were: 1) Is there evidence of up-regulation of NOS1 and/or NOS3 in the lungs of NOS2^{-/-} mice exposed to ozone that can account for NOS2-independent generation of protective NO? 2) Does nitric oxide in the lungs of mice exposed to ozone arise mainly from the inflammatory cell influx (mainly neutrophils under the exposure conditions used in this study) or from the airway epithelial cells? 3) Does the clinically relevant marker FeNO (NO concentration in the expired air) accurately reflect the amount of NO in the lung after ozone exposure?

To answer these questions, we developed two strains of chimeric mice that have airway inflammatory cells that contain NOS2 (NOS2^{+/+}) and airway epithelial cells that lack NOS2 (NOS2^{-/-}), or the reciprocal situation. This approach is well established (Scott et al., 2002; Wang et al., 2001); for example, it has been used to study a murine model of sepsis. This approach allows us to ask whether changing the genotype of selected cell populations in an animal (in our case the presence or absence of NOS2 in the inflammatory cells) affects the response of a target organ, e.g. the lung, in the chimeric animal. In these experiments, we exposed NOS2^{-/-}, C57 BL/6 (NOS2^{+/+}), and chimeric (NOS2^{-/+} and NOS2^{+/-}) mice to 1 ppm of ozone for 8 hours per night for three consecutive nights. In addition to measuring lung inflammation, we also quantified total protein sulfhydryl groups in blood plasma as a measure of systemic injury and oxidant stress, and lung NO generation by measuring exhaled nitric oxide and nitrate and nitrite concentrations produced by cells obtained by lung lavage and cultured in vitro with stimulation by LPS and IFN- α .

MATERIALS AND METHODS

Animals

All procedures were performed under an IACUC approved protocol. Specific pathogen-free C57BL/6 (NOS2^{+/+}) mice aged about 6 weeks (16–20g) were purchased from Charles River Breeding Laboratories (Wilmington, MA). Animals were housed until used in Bio-Clean facilities in the Animal Resources Center at our facility on a 12-hour light, 12-hour dark cycle and fed standard rodent feed ad libitum. The NOS2 knockout mice (NOS2^{-/-}) were initially purchased from Taconic Laboratories (Germantown, N.Y.), and were re-derived by embryo transfer to establish a breeding colony in the Targeted Genomics Laboratory of the Mouse

Biology Program barrier facility at U.C. Davis. These mice are on a C57BL/6 genetic background and are designated C57BL/6Ai-[KO] NOS2 N5.

Bone marrow transplantation and chimera development

Chimeric NOS2 strains (NOS2 +/-, NOS2 -/+) of mice were developed using techniques described previously (Scott et al., 2002; Wang et al., 2001). In brief, we began with C57BL/6 (NOS2+/+) and NOS2 knockout (NOS2-/-) mice. Recipient mice were treated with total body irradiation (⁶⁰Co source, 400cGy × 2 sessions 4 hours apart) to kill endogenous bone marrow cells prior to transplantation. Two strains of chimeric mice were prepared by bone marrow transplantation: NOS2+/- (NOS2+/+donor bone marrow transplanted into NOS2-/- recipient mice) and the reciprocal strain (NOS2-/- donor bone marrow transplanted into NOS2+/+ hosts: NOS-/+). Donor and recipient mice were of approximately equal age (6–8 weeks old). Bone marrow was harvested by flushing the femurs and tibias with RPMI-1640 medium (containing 3% fetal calf serum (FCS), 1% penicillin and 1% streptomycin, GIBCO BRL, Grand Island, NY). The resulting suspension was passed through a sterile mesh filter to obtain a single cell suspension. Cells were washed twice in Hanks' balanced salt solution (HBSS with 3% FCS, 1% penicillin and streptomycin; GIBCO BRL) and suspended in sterile 0.9% saline to a final concentration of 5×10^7 cells per ml. Four hours after irradiation, the mice received 1.5×10^7 donor bone marrow cells in 0.3 ml normal saline via tail vein injection. After transplantation, mice were allowed free access to food and water and housed in individual sterile cages fitted with cover filters and sterile bedding. For the first 2 weeks after transplantation, mice were given sterile drinking water containing 100 mg of neomycin and 10 mg of polymixin B (Sigma, St. Louis, MO) per liter. Animals were allowed to recover from transplantation for at least 3–4 weeks prior to exposure to ozone.

Exposure to ozone

Mice were exposed for 8 hours per night (midnight to 8:00 AM) to 1 ppm of ozone or to filtered air for three consecutive nights, using methods described in detail previously (Kenyon et al., 2002). Briefly, ozone was produced from vaporized liquid medical grade oxygen with a silent arc discharge ozonizer (Erwin Sander Corp., Giessen, Germany). Both the ozone and the oxygen were conveyed through Teflon lines to the mixing inlet of the exposure chamber. Ozone concentrations in the chambers were monitored by ultraviolet photometry with calibrated Dasibi ultraviolet ozone monitors (Model 1003-AH), with data recorded every 2 minutes directly onto an IBM-AT computer for analysis. Calibration was checked against an absolute ozone monitor (Dasibi Model 1008-PC). The three-day interval was chosen to maximize the inflammatory response (Guth et al., 1986). This protocol gives the same (or greater) response as exposure to the same total dose of ozone for 24 hours (Gelzleichter et al., 1992). Two independent experiments with separate exposures and analyses were performed. Actual exposure concentration in the first experiment was 1.00 ± 0.01 (mean $\pm$ 1 SD) ppm (n = 357 determinations), and in the second was 1.00 ± 0.005 ppm (n = 357). Matched control animals were treated identically as ozone exposed, except the exposure chambers contained only filtered air.

Whole lung lavage

Mice were killed by an overdose of phenobarbital and dilantin administered intraperitoneally. Animals were placed on a restraining board and the lungs were lavaged with two 1-ml aliquots of phosphate-buffered saline (pH=7.4); the lavage with each 1-ml aliquot was repeated twice. The resulting 2 ml of lavage fluid was combined and centrifuged for 10 minutes at 2500 rpm in a desktop centrifuge. The supernatant was aliquoted into plastic test tubes, quick frozen on dry ice, and stored at -20° C until analyzed (usually within 1-2 days). The pellets were resuspended in 0.5 ml of phosphate-buffered saline, pH 7.4. Aliquots (100 µl) of this cell

suspension were processed onto glass slides in a cytocentrifuge at 1650 rpm for 15 minutes and then dried in air. Slides were stained with hematoxylin and eosin and protected with coverslips. Cells were counted in at least 10 fields under a 40X objective, and classified as pulmonary alveolar macrophages, polymorphonuclear leukocytes (neutrophils), lymphocytes, or “other” based upon staining color and characteristic morphology. Results are presented as pooled data from the two independent experiments, which gave similar independent results.

Protein determination

Samples were analyzed by a colorimetric assay based upon the reaction of protein with bis-cinchonic acid to form a colored product with a peak absorbance at 562 nm (Pierce [Rockford, IL] micro BCA protein assay kit). Standard curves with bovine serum albumin were run with each assay, and were linear ($r^2 = 0.98\text{--}0.99$) between 0 and 50 μg of protein.

Plasma R-SH group content

We determined the total protein sulfhydryl R-SH content of blood plasma by a colorimetric reaction using Ellman's reagent (Sedlak and Lindsay, 1968) and modified the assay to use a 96-well plate format. Blood was collected by cardiac puncture, using EDTA as an anticoagulant, and plasma was separated by centrifugation for 10 minutes at 2,000 rpm. We routinely analyzed 10 μl aliquots of plasma.

Lung Compliance measurements

We measured dynamic lung compliance and airway resistance on anesthetized, tracheotomized and ventilated mice. Mice were deeply anesthetized and sedated with medetomidine, 0.5 mg/kg (Domitor, Orion Pharma, Finland), and tiletamine/zolpidem (Telazol, Fort Dodge Laboratories, Fort Dodge, IA), 50 mg/kg. Mice were ventilated at 7–8 cc/kg with a mouse ventilator (MiniVent, Harvard Apparatus, Cambridge, MA) for the duration of the procedure. Measurements were made with the mouse in the supine position using a plethysmograph for restrained animals (Buxco Inc, Troy, NY). Lung compliance and resistance measurements were made at baseline and immediately following serial 3-minute nebulizations of saline and methacholine (0.1–2.0 mg/ml).

Measurement of exhaled NO

Exhaled gas was collected during the first 5 minutes the mouse was on the ventilator with a collection bag connected to the exhalation port. Placement of the bag did not affect pressure measurements. Exhaled NO concentrations were measured from this sample with a Sievers NO Analyzer (Sievers Inst., Boulder, CO).

Nitrate/nitrite measurements

Bone marrow cells and lavaged cells were collected from each animal in these experiments and stimulated with 100 $\mu\text{g/ml}$ LPS and 200 $\mu\text{g/ml}$ IFN- γ in F-12 medium (low NO_x) for 24 hours at 37°C. Supernatants were collected and nitrate and nitrite were measured as an indicator of NO production through reduction with acidified vanadium III using the Sievers NO Analyzer (Sievers, Boulder, CO). We also collected blood from the mice, and stored their plasma at -80°C prior to analysis.

Western Blot analysis of NOS isoforms

Fifteen microgram samples of microdissected airway homogenates were mixed with equal volumes of 2x SDS electrophoresis sample buffer (0.25M Tris [pH=6.8], 2% SDS, 10% glycerol, 0.1% bromphenol blue), boiled for 3 minutes, and separated by SDS gel electrophoresis under reducing conditions (Bio-Rad Laboratories, Hercules, CA). Proteins

were transferred to a PVDF Western blotting membrane using an electroblotting apparatus according to the manufacturer's protocol (Invitrogen Corp., Grand Island, NY). Membranes were blocked with 5% Blotto [5% non-fat powdered milk in Tris-buffered saline with 0.1% Tween 20 (TBST)] for 60 minutes at room temperature. The membranes were then incubated with either NOS1, NO2, or NOS3 goat anti-rabbit primary antibodies (Santa Cruz Biotechnologies, Santa Cruz, CA) diluted (1:200 for all 3 antibodies) in 5% Blotto for 1 hour at room temperature. The membranes were washed 6 times for 5 min. each with TBST and then incubated with the horseradish peroxidase (HRP)-conjugated secondary antibody (1:10,000) for 1 h at room temperature. After 6 washes with TBST, the membranes were incubated with ECL reagent for 1 minute and band quantification was performed with a scanner using Kodak 1D software (Eastman Kodak, Rochester, NY). Band density measurements for each blot were normalized to the average band density of the least exposed or lightest blot.

Statistical analysis of data

Instat 2.0 or Prism 3.0 (GraphPad Software, San Diego, CA) and the 'R' package (R Foundation for Statistical Computing, Vienna, Austria, URL: <http://www.R-project.org>), a common open source statistical computing package, were used for data analysis. Data are routinely expressed in figures and text as mean values $\pm$ 1 SE. For parametric analysis of data, a t-test with appropriate correction for multiple comparisons was used. For non-parametric analysis, a Wilcoxin or a Pearson test was used.

RESULTS

Animal exposures

C57BL/6 (NOS2^{+/+}), NOS2^{-/-} and the two chimeric mouse strains (NOS2^{+/-} and NOS2^{-/+}) were exposed to 1 ppm of ozone for 8 hours per night for three consecutive nights. All animals tolerated the exposure without incident and appeared normal. One chimeric mouse (of a total of 34) died on day 2 after irradiation and transplantation, several weeks prior to ozone exposure, apparently from acute radiation toxicity.

Ozone-induced toxicity: oxidant stress

To test the hypothesis that exposure to ozone could cause systemic oxidative stress, we analyzed the total sulfhydryl (R-SH) content of blood plasma collected from C57BL/6, chimeric, and NOS2^{-/-} mice exposed to ozone, and from their matched controls that breathed only filtered air. Most of the SH content of plasma is protein associated, so we are measuring mainly total protein sulfhydryl groups with this assay. As shown in Figure 1, there was a trend ($P=0.124$) towards less R-SH in the blood plasma from the C57BL/6 filtered air controls (130.0 ± 19.0 uM, $n=9$) as compared to the NOS2^{-/-} filtered air controls (162.2 ± 10.8 uM, $n=16$), suggesting lower quantities of total R-SH in the blood of the C57BL/6 animals. Air controls from the chimeric mice gave results consistent with the plasma-SH concentrations in the donor strains. In the mice exposed to ozone, we found evidence for decreased total R-SH in blood plasma (Figure 1). There was an apparent trend ($P=0.286$) towards less total R-SH in the C57BL/6 mice, and a significant ($P=0.02$) decrease in total R-SH in the plasma from the NOS2^{-/-} mice. In the same set of experiments, the NOS2^{+/-} mice (C57BL/6 donors, NOS2^{-/-} recipients) behaved comparably to the C57BL/6 mice exposed to ozone, with a significant ($P=0.016$) decrease to 54.6 ± 19.3 uM R-SH groups in their plasma, while the NOS2^{-/+} chimeras (NOS^{-/-} donors, C57BL/6 recipients) behaved similarly to the NOS2^{-/-} animals, with a significant ($P=0.006$) decrease in plasma R-SH groups to 116.8 ± 11.1 uM.

However, we have previously reported (Last et al., 2005) that mice exposed to 1 ppm of ozone for 3 days, using an identical protocol to that used in this study, lose body weight and demonstrate a systemic cachexic response. Thus, any apparent reduction in total plasma R-SH

could have been either due to oxidation of the R-SH groups on plasma proteins or to a decreased total content of protein, predominantly albumin, in the plasma. Therefore, we also determined total protein concentration in the same plasma samples we analyzed for their total R-SH content. In the case of the C57BL/6 animals, which showed an apparent (not significant) decrease of about 20% in their plasma R-SH concentration, we also found a corresponding decrease of about 20% in their plasma protein content. Thus, there was no net change in total plasma R-SH content expressed per mg of protein in the C57BL/6 mice. However, with the NOS2^{-/-} mice we did find a statistically significant decrease in total plasma R-SH content. The decrease in plasma protein content in the NOS2^{-/-} mice exposed to ozone was about 11%, while the decrease in total plasma R-SH concentration was about 33%.

Airway inflammation: lung lavage cell counts

Air control animals for all four strains evaluated showed only alveolar macrophages to be present. As shown in Figure 2, there were equal numbers of cells found in the NOS2^{+/+} and NOS2^{-/-} strains when air controls were examined. Greater numbers of macrophages were lavaged from the lungs of the two chimeric strains exposed only to filtered air (suggesting the possibility of residual effects from irradiation or bone marrow transplantation, which is currently under study); there was no significant difference between the number of cells lavaged from the two chimeric strains in the air controls. As rapidly as possible after the last exposure of the mice to ozone, lung lavage cells were collected and counted. There were significantly more cells collected from NOS2^{-/-} mice compared with C57BL/6 mice ($4.14 \times 10^5 \pm 2.19 \times 10^5$ vs. $2.78 \times 10^5 \pm 1.36 \times 10^5$; $p=0.036$) (Figure 2). The differential count on these cells showed the vast majority (>95%) to be macrophages and neutrophils in the ozone-exposed animals. There was a significant increase in the percentage of neutrophils in the NOS2^{-/-} mice compared to the NOS2^{+/+} mice (NOS2^{-/-} mice transplanted with bone marrow cells from C57BL/6 mice) (20.3 ± 12.9 vs. $11.5 \pm 8.2\%$ respectively, $p=0.037$) (Figure 3). This result is consistent with our earlier study (Kenyon et al., 2002) and suggests that the NOS2^{-/-} animals are more susceptible to ozone-induced neutrophil infiltration into the lung. There were fewer total cells and fewer neutrophils in the NOS2^{+/-} chimeric mice after ozone exposure than in the NOS2^{-/-} strain ($2.46 \pm 2.4 \times 10^5$ vs. $4.14 \pm 2.19 \times 10^5$ cells; $p=0.037$, Figures 2 and 3). On the other hand, the NOS2^{+/+} chimeras (NOS^{-/-} donors) behaved similarly to the NOS2^{-/-} animals. Hematoxylin and eosin-stained lung sections showed neutrophil and macrophage infiltration in the airway mucosa and submucosa in all animals exposed to ozone; however, there were no distinguishable differences in the number of inflammatory cells in the parenchyma after lung lavage between the four mouse strains.

Decreased total cell counts and decreased neutrophils in the lung lavage fluid from chimeric mice exposed to ozone could conceivably have reflected deficiencies in the bone marrow-derived pool of circulating inflammatory cells after irradiation. Thus, in order to determine whether poor bone marrow engraftment had occurred, we flushed and counted the total marrow cells from the tibias and femurs of mice. Marrow engraftment appeared adequate; total marrow cell counts were $1.76\text{--}2.03 \times 10^7$ cells/ml and there were no significant differences among the C57BL/6, NOS2^{-/-} and chimeric mice. We did not attempt to do differential counts of circulating blood cells in the pellets after preparation of plasma.

Lung compliance

Dynamic lung compliance (C_{dyn}) was measured in anesthetized, tracheotomized mice 1 to 3 hours after their last ozone exposure, immediately after the collection of exhaled gas. There was not a significant difference between the C_{dyn} of the C57BL/6 mice and the NOS2^{-/-} animals after exposure to ozone (0.0240 ± 0.004 (n=7) vs. 0.0276 ± 0.003 (n=10) ml/cm H₂O, $p=0.069$) (Figure 4). There was a significant difference in the C_{dyn} measurements between the chimeric groups, however. NOS2^{+/-} chimeric mice had significantly higher mean C_{dyn}

than the reciprocal chimeric strain (0.028 ± 0.003 (n=14) vs. 0.023 ± 0.003 (n=4) ml/cm H₂O respectively, $p=0.005$). All of the mice exposed to ozone had significantly lower lung compliance measurements than either the C57BL/6 mice (0.031 ± 0.005 µl/cm H₂O, n=3) or the NOS2^{-/-} mice (0.034 ± 0.003 µl/cm H₂O, n=4) exposed to filtered air for 4 days. However, the compliance values in all four strains of mice exposed to ozone were similar to the values observed in both chimeric strains exposed only to filtered air.

Exhaled NO concentration

In mice breathing clean filtered air, exhaled NO levels in NOS2^{-/-} mice were comparable with those in C57BL/6 mice and in the two chimeric strains (Figure 5). This result suggests that the exhaled NO in NOS2^{+/+} mice breathing only filtered air arises from the action of NOS isoforms other than NOS2. Exhaled NO concentrations increased after ozone exposure, but it was clear that the actual values of FeNO were critically affected by the elapsed time after exposure cessation. Overall, NO levels were similar in NOS2^{-/-} mice after exposure to ozone compared to animals breathing filtered air [4.62 ± 2.48 ppb (n=15) vs 4.16 ± 0.74 ppb (n=7)]. The increase in FeNO in C57BL/6 mice after exposure to ozone compared to animals breathing only filtered air was also not significant. There was less FeNO in the NOS2^{-/-} mice compared to the C57BL/6 mice after ozone exposure (4.62 ± 2.48 ppb (n=15) vs. 6.49 ± 3.09 ppb (n=13); $p=0.0437$, 1-tailed test). The NOS2^{+/-} chimeric mice generated significantly more exhaled NO than the NOS2^{-/-} mice after 3 days of exposure to ozone (7.55 ± 2.51 ppb (n=17) vs. 4.62 ± 2.48 ppb (n=15); $p=0.002$, 2-tailed test). This finding suggests that inflammatory cells contribute significantly to the measurable levels of exhaled NO in mice exposed to ozone.

To further analyze these data, we pooled the four subgroups of mice into two large groups based on their bone marrow type (NOS2^{+/+} and NOS2^{+/-}, NOS2^{-/-} and NOS2^{-/+}). The cell count and exhaled NO data were normalized by subtraction of the mean measured values for all groups and division by the standard deviation. Figures 2 and 5 illustrate that the response to exposure was in the opposite direction for the two assays in the NOS2^{-/-} mice as compared to the NOS2^{+/+} (wild-type) mice, so the normalized values of the exhaled NO levels were multiplied by negative one. These normalized values could then be meaningfully compared statistically. For all of those mice that were used for both assays, we examined the correlation between the lavage cell counts and exhaled NO within each of the four groups, and found that there was no significant correlation. Therefore, we treated each measurement as an independent entity.

Table 1 contains the results of these tests. The first three tests were conducted to confirm that the measurements we treated as coming from the same group were indistinguishable. These were examined using two-tailed t-tests because we had no a priori reason to predict a response in a given direction. We conducted one-tailed t-tests for the final group comparisons because our previous results (Figures 2 and 5) indicated the direction of the predicted change in the specific assay performed. The difference between the groups with the NOS2⁺ and the NOS2⁻ bone marrow (NOS2^{+/+} and NOS2^{+/-} vs. NOS2^{-/-} and NOS2^{-/+}) is highly significant. Thus, we would conclude that the source of the bone marrow cells was the critical determinant of the lung response of the mice to ozone in each of these two assays. We also used this model to ask whether the difference in response of the two strains of chimeric mice (NOS2^{+/-} vs NOS2^{-/+}) to exposure to ozone was significantly different, and it was ($P=0.039$, Table 1, last row).

Nitrate/nitrite measurements in supernatants from lung lavage and in bone marrow cells

Measurements of nitrate/nitrite (NO_x) from cells recovered by lung lavage and cultured with LPS and IFN-α confirm and reinforce the differences between groups seen with the FeNO data. After ozone exposure, the concentration of NO_x was significantly higher in the C57BL/6 mice

than the NOS2^{-/-} mice ($6.06 \pm 1.45 \mu\text{M}$ (n=15) vs $3.96 \pm 1.43 \mu\text{M}$ (n=18); $p=0.002$) (Figure 6). There were significant differences in the NO_x concentrations in both chimeras compared to the host strains after ozone (C57BL/6: $6.06 \pm 1.45 \mu\text{M}$ (n=15) vs. NOS2^{-/+}: $4.94 \pm 0.93 \mu\text{M}$ (n=11); $p=0.04$, NOS2^{-/-}: $3.96 \pm 1.43 \mu\text{M}$ (n=18) vs. NOS2^{+/-}: $6.12 \pm 2.82 \mu\text{M}$; $p=0.02$). The chimeric mice produced NO_x at levels most closely resembling those of the donor strain and different from the host animals. The source of the inflammatory cells thus seemed to be the major contributor to the detectable NO_x and to the mouse lung phenotype as determined by this assay.

NO_x levels were also measured in bone marrow of chimeric mice after exposure to ozone. There were large differences in the concentrations of NO_x produced by (LPS + IFN- γ)-stimulated bone marrow cells between the C57BL/6 mice and the NOS2^{-/-} mice sampled (29.5 ± 12.4 (n=4) vs. 0.89 ± 0.72 (n=3) μM , respectively, $p=0.016$) (Figure 7). Transplantation of bone marrow cells from C57BL/6 mice into NOS2^{-/-} hosts led to a significant increase in the concentration of NO_x produced by these cells as compared to the NOS2^{-/-} mice (7.92 ± 4.86 (n=8) vs. 0.89 ± 0.72 , $p=0.028$). This finding confirms that engraftment of the bone marrow is occurring in the chimeric mice, and that the phenotype of these mice is altered. The inflammatory cells that are derived from the bone marrow in chimeric mice are fundamentally different in their capacity to generate NO than the native alveolar macrophages and neutrophils of the host strain.

Plasma NO_x concentrations were approximately 6 μM in either NOS2^{-/-} or NOS2^{+/+} mice exposed to filtered air. Upon exposure of either strain of mice to ozone, we observed a significant increase in plasma NO_x concentrations to about 9–11 μM . Both of the chimeric mouse strains showed a trend towards higher concentrations of NO_x in their plasma (about 8 μM) after exposure to ozone, but these apparent increased concentrations were not statistically significant (data not shown).

NOS protein determinations

Western blot analysis of homogenates prepared from microdissected airways from mice exposed to ozone showed a significant increase in NOS3 mean band density in the chimeric strains compared to the NOS2^{+/+} mice (NOS2^{+/-}: 72.1 ± 1.6 (n=7) vs. NOS2^{+/+}: 63.7 ± 1.0 (n=8), $p=0.0004$; NOS2^{-/+}: 71.4 ± 3.5 (n=5) vs. NOS2^{+/+}: 63.7 ± 1.0 , $p=0.02$) and a trend to increased NOS3 in the NOS2^{-/-} mice (67.3 ± 1.5 (n=7) vs. 63.7 ± 1.0 , $p=0.06$) (Figure 8). In contrast, there was a notable decrease in NOS1 protein band intensity in the NOS2^{+/-} chimeric mice compared to the NOS2^{+/+} wild-type strain (69 ± 2.7 (n=7) vs. 79.4 ± 2.4 (n=11), $p=0.01$) (Figure 9). It appears that there is up-regulation of NOS3 enzyme in the mouse strains containing either the epithelial or inflammatory cell compartment from the NOS2^{-/-} mice after exposure to ozone (Figure 8). As expected, NOS2 protein was not detectable in the NOS2^{-/-} animals. In complementary work, we have not found any NOS2 gene expression in gene array experiments in the NOS2^{-/-} animals (data not shown).

DISCUSSION

Our overall experimental goal was to compare the response to acute ozone exposure of wild type C57BL/6 mice to that of a NOS2^{-/-} strain derived from the C57BL/6 strain. We then asked whether chimeric mice with a reconstituted bone marrow-derived immune system from either C57BL/6 or NOS2^{-/-} donors responded more like the donor or recipient strain, thereby allowing us to distinguish the relative importance of inflammatory cells versus constitutive lung cells in the responses we measured.

In our previous studies we have reported increased protein content of lung lavage fluid as a quantitative index of lung damage in mice exposed to ozone (Kenyon et al., 2002). In the

experiments reported here, there was not a significant increase in this parameter in the pooled data from several experiments, even though we found a non-significant trend toward increased lung lavage protein in the NOS2^{-/-} mice compared to the C57BL/6 mice (data not shown). Thus, we measured oxidation of R-SH groups in plasma from these same animals as a primary index of oxidant-induced toxicological response. Unlike C57BL/6 mice, NOS2^{-/-} mice show a significant decrease in the amount of total protein sulfhydryl groups (R-SH) in their blood plasma after ozone exposure. This finding presumably reflects systemic oxidation of protein-bound -SH groups by oxidants arising directly or indirectly from the interaction of ozone with the airway lining fluid of the lung. Our data suggest that there is a higher concentration of total R-SH groups in plasma from NOS2^{-/-} mice, as compared to C57BL/6 mice, breathing only filtered air (Figure 1). We know that normal NOS2^{-/-} mice produce less NO than the NOS2^{+/+} strain (Figure 5), and can speculate that they produce less endogenous peroxynitrite and other NO-derived reactive species. Therefore, there is less endogenous oxidation of R-SH groups in their blood under normal conditions, and presumably a greater capacity of the blood proteins to respond to exogenous oxidative stress. We also observed that NOS2^{-/-} mice produce less NO in their lungs than C57BL/6 mice after exposure to 1 ppm of ozone, but significantly more than they produce with no ozone exposure (Figure 5). Thus, there is the potential for reactive nitrogen species to be a significant cause of systemic oxidant stress in the NOS2^{-/-} strain after exposure to ozone, consistent with our findings in Figure 1.

An alternative explanation for our observations is that NO, because of its “antioxidant” properties, can itself protect against decrements in protein R-SH groups caused by oxidants arising from ozone exposure, and that the availability of this “antioxidant” NO would be decreased in the NOS2^{-/-} mice, as we have demonstrated. Especially worthy of note is the significantly lower exhaled NO concentration in the NOS2^{-/+} than in the NOS2^{+/+} chimeric mice (Figure 5); we compared these two strains by a one-tailed test based upon the a priori hypothesis that FeNO would be lower in the chimeric strain with inflammatory cells reconstituted from the NOS2^{-/-} mice, and found $P = 0.053$. NOS2^{-/+} mice were also the chimeric mouse strain that showed a significant decrease in plasma total R-SH concentration in the animals exposed to ozone. Collectively, our data suggest that circulating cells derived from bone marrow utilize NOS2-derived NO to protect against systemic oxidative stress associated with ozone inhalation, a protective mechanism that is compromised in NOS2^{-/-} mice and in the NOS2^{-/+} chimeric animals.

Previously, we demonstrated that NOS2^{-/-} mice were more susceptible to lung injury and neutrophil infiltration into the lung than were C57BL/6 mice (Kenyon et al., 2002). This appeared to result from a NOS2-independent mechanism, as we found approximately equal concentrations of 3-nitrotyrosine in the NOS2^{-/-} mice as compared to those of NOS2^{+/+} control mice, which did not lack this enzyme. These previous observations demonstrate two important points that are consistent with our current results. First, decreased production of NO in NOS2^{-/-} mice exposed to ozone would theoretically provide a ratio of oxidants to NO that would create conditions for optimal catalysis of tyrosine nitration and other bimolecular oxidative reactions. Second, the relative decrease in NO production in NOS2^{-/-} mice presumably increases the bioavailability of superoxide anion and increased production of hydrogen peroxide after ozone exposure, which would enhance neutrophil-mediated oxidative damage by both myeloperoxidase-dependent (Eiserich et al., 1998; Andreadis et al., 2003) and peroxynitrite-dependent mechanisms (Beckman and Koppenol, 1996). The increase in myeloperoxidase-dependent production of reactive oxygen species and peroxynitrite-dependent reactions could facilitate increased systemic oxidation of plasma sulfhydryl groups in NOS2^{-/-} mice after ozone exposure, as observed in this study (Figure 1).

Beyond these points, two further questions arose from our previous observations. 1) Is there evidence of up-regulation of NOS1 and/or NOS3 in the lungs of NOS2^{-/-} mice exposed to

ozone that can account for the NOS2-independent tyrosine nitration? 2) Does nitric oxide in the lungs of mice exposed to ozone arise mainly from the inflammatory cell influx (mainly neutrophils at the duration of exposure in this study) or from the airway epithelial cells?

NOS3, or eNOS, was increased in the airways of our NOS2^{-/-} mice after exposure to ozone. NOS3 expressed in the airway epithelium and in endothelial cells of the lung may be responsible for the NO detectable in the exhaled breath and the NO_x in the lavage fluid of NOS2^{-/-} mice. Our data in the NOS2^{-/-} mice illustrate that NOS2, while it is a major contributor to total NO production in wild-type mice, may not be the only enzyme responsible for NO production in response to a lung irritant such as ozone. Thus, we would answer our first question that there is indeed evidence of NOS3 up-regulation in the lungs of NOS2^{-/-} mice exposed to ozone that could also account for the NOS2-independent tyrosine nitration known to occur in these animals (Kenyon et al., 2002). Alternatively, these findings could also result from an alteration of the balance of oxidants produced to NO produced, as discussed above.

The primary cellular source(s) of exhaled NO from mouse lung is unclear from the existing literature. Evidence supports both the airway epithelial cells and inflammatory cells as key contributors of NO in models of lung inflammation and lung injury (Wang et al., 2001; Hickey et al., 2002; Razavi et al., 2004). Using chimeric NOS2 mice, Wang and colleagues showed that the majority of NO exhaled after LPS injection into the tail vein, as measured by serum levels of NO_x, was produced primarily by lung parenchymal cells (Wang et al., 2001). In contrast, a recent paper by this same group also using the chimeric NOS2 model, but this time after cecal perforation in mice, showed that NO generated by NOS2 in the neutrophils was primarily responsible for modulating neutrophilic migration into the lung (Razavi et al., 2004). Hickey and colleagues (2002) reported that leukocyte-derived NOS2, rather than lung epithelial NOS2, was the principal source of NO in mice after an infusion of endotoxin for 4 hours. The lung was unique in this regard as compared to other solid organs after the endotoxin infusion (Hickey et al., 2002).

Our exhaled NO and lavage NO_x data show that both the inflammatory cells and the airway epithelium generate NO. Transplantation of NOS2^{-/-} bone marrow cells into NOS2^{+/+} mice led to the infiltration of more inflammatory cells into the lung after ozone exposure than were found in the NOS2^{+/+} mice. NO production in (LPS + INF- γ)stimulated bone marrow cells was strikingly different between the groups. Our assay showed a 10-fold difference in the amount of nitrate and nitrite produced by cultured bone marrow cells when the C57BL/6 and the NOS2^{-/-} mice were compared. Transplantation of the bone marrow cells from the C57BL/6 mice into the NOS2 knockout strain gave chimeric mice with an intermediate level of NO_x generation. This result demonstrates that the stem cells that we infused into the tail veins engraft in the bone marrow and propagate. Our findings suggest that the transplanted bone marrow cells generate additional NO in the lungs of their previously naïve NOS2^{-/-} hosts and affect the response of the host strain to ozone. The answer to our second question, which was whether nitric oxide in the lungs of mice exposed to ozone arises mainly from the inflammatory cell influx (mainly neutrophils at the duration of exposure in this study) or from the airway epithelial cells, is that bone marrow-derived inflammatory cells are an important source of NO in the lungs of NOS2^{+/+} (and NOS2^{+/-}) mice exposed to ozone, but the airway epithelium also plays a significant role in NO production.

The chimeric mouse model is well established. The major benefit of this approach is that it mimics the conditional knockout animal. In the chimera model, Wang and colleagues found that the airway epithelial cells, rather than inflammatory cells, were the primary contributors of NO that contributed to sepsis-induced lung injury and microvascular leakage (Wang et al., 2001). Our results in the ozone model point to the opposite conclusion. This apparent difference

probably reflects the different routes of administration and sources of injury used in the two systems. Acute lung injury in Wang and colleagues' model was caused by an indirect systemic sepsis response triggered by ligation and perforation of the intestine (Wang et al., 2001). Inhalation of ozone causes direct toxic damage to the lung, and the mechanisms of activation and influx of inflammatory cells are much different. The relative contributions of NO from the airway epithelial cells and from the inflammatory cells may depend on the mechanism and route of lung injury.

Our results demonstrate increased susceptibility to ozone in strains of mice lacking NOS2 in their neutrophils and pulmonary macrophages. We can speculate that this is due to their decreased ability to make NO in a critical compartment of the lung—the airway lumen. Compartmentalization of the NOS2 gene may alter the functions of NOS2-generated NO, but this is difficult to study. Speyer and colleagues found that NOS^{-/-} mice suffered more lung injury and increased lung neutrophilia after exposure to intratracheal lipopolysaccharide (LPS) than did NOS2^{+/+} mice (Speyer et al., 2003). They suggested that this might be due to the decreased ability of NOS2^{-/-} mice to modulate the production of MCP-1, a CC chemokine and neutrophil chemoattractant, in lung macrophages. Evidence supporting their suggestion came from in vitro experiments; for example, endothelial cells and peritoneal macrophages from NOS2^{-/-} mice after exposure to LPS produced more MCP-1 than did cells from LPS-treated NOS2^{+/+} mice.

We performed measurements of dynamic lung compliance (C_{dyn}) on anesthetized, tracheotomized mice immediately prior to euthanasia to determine whether ozone exposure affected lung function in the experimental animals (Figure 5). We found that all mice exposed to ozone had decreased C_{dyn} as compared with the groups exposed to air, a finding that is consistent with the development of alveolar edema in these animals. There was no measurable difference in lung compliance between the NOS2^{-/-} and C57BL/6 strains after exposure, despite the differences in their inflammatory cell response as demonstrated in Figures 2 and 3. Higher numbers of neutrophils in the lung lavage do not directly correlate, therefore, with a measurable decline in lung compliance. The two strains of chimeric mice we studied showed lung lavage data fully consistent with the bone marrow donor strain (Figure 6), and lung compliance that also decreased in response to ozone exposure with no significant differences between the mouse strains.

In summary, experiments with chimeric NOS2 mice have shown that NO is generated primarily from the bone marrow-derived inflammatory cells after exposure to ozone. NOS2 is not the sole enzyme responsible for the production of NO during such an exposure, and in fact we find up-regulation of NOS3 in the NOS2^{-/-} mice exposed to ozone, perhaps as a compensatory mechanism. Mice that produce significant amounts of NO from both the inflammatory cells and host airway epithelial cells are partially protected from the lung injury that results from exposure to ozone. Acute exposure to ozone also causes systemic oxidant stress, manifested here as increased oxidation of R-SH groups in blood plasma proteins. The oxidant species involved include nitrogenous compounds or radicals, based on the increased susceptibility of NOS2^{-/-} mice and of chimeric animals with their leukocytes derived from NOS2^{-/-} mice. Therefore, NO produced from NOS2 appears to play a protective role in the lung inflammatory responses associated with ozone exposure in mice, as revealed by the responses of the chimeric mouse strains constructed for the present study.

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Figure 1.

Total R-SH content of 10 ul aliquots of blood plasma determined by a colorimetric reaction using Ellman's reagent. The responses of C57BL (NOS2+/+) and NOS2-/- mice, as well as of the reciprocal chimeric strains prepared from these mice, to exposure to ozone are compared.

* Symbol denotes $p < 0.02$ compared to the corresponding group exposed only to filtered air.

Figure 2.

Total cell counts from lung lavage fluid. The number in parentheses is number of experimental animals in each group. * Symbol denotes $p < 0.05$ compared to C57BL/6 (NOS2^{+/+}) mice exposed to ozone; + symbol denotes $p < 0.05$ compared NOS2^{-/-} group exposed to ozone. There are significantly more total cells in each group exposed to ozone than in their corresponding filtered air controls (not indicated on figure).

Figure 3.

Percentage of neutrophils in lung lavage fluid. Air controls for each of the four mouse strains studied contained 0% neutrophils (the only cell type present in the air controls was pulmonary alveolar macrophages), so the air control results are not shown on the figure. * Symbol denotes $p < 0.05$ compared to the NOS2^{-/-} group.

Figure 4.

Dynamic lung compliance measured in anesthetized, tracheotomized, and ventilated mice after exposure to air or ozone. Mice were deeply anesthetized and sedated with medetomidine, 0.5 mg/kg, and tiletamine/zolpidem, 50 mg/kg and ventilated at 7–8 cc/kg with a mouse ventilator for the duration of the procedure. Measurements were made with the mouse in the supine position using a plethysmograph. * Symbol denotes $P=0.005$ as compared to the corresponding value in the reciprocal chimeric strain.

Figure 5.

Exhaled nitric oxide concentration in ventilated mice after exposure to air and ozone. Exhaled gas was collected during the first 5 minutes the mouse was on the ventilator with a collection bag connected to the exhalation port. Exhaled NO concentrations were measured from this sample with a Sievers NO Analyzer. + Symbol denotes a p value < 0.05 compared to NOS2+/+ mice exposed to ozone. # Symbol denotes p value < 0.05 compared to NOS2-/- mice after exposure to ozone.

Figure 6.

Nitrate and nitrite (NOx) concentrations found from lung lavage cells stimulated with LPS + IFN- α from mice exposed to ozone. * Symbol denotes p value <0.05 compared to NOS2+/+ mice. + Symbol denotes p value <0.05 compared to NOS2-/- mice. # Symbol denotes p value < 0.05 compared to NOS2+/+ mice.

Figure 7.

Nitrate and nitrite concentration found from bone marrow cells stimulated with LPS + IFN- α from mice exposed to ozone. * Symbol denotes p value < 0.03 compared to both of the other groups.

Figure 8.

NOS3 Western blot band density from the airways of each of the four groups of mice studied after exposure to ozone (n=5–7 each). *, + Symbols denote p value < 0.02 compared to C57BL (NOS2+/+) and NOS2-/- mice, respectively.

Figure 9.

NOS1 Western blot band density from the airways of each of the four groups of mice studied after exposure to ozone (n=5–7 each). Data were log-transformed to better fit the figure. * Symbol denotes p value <0.05 compared to C57BL/6 (NOS2+/+) mice.

Table 1

Analysis of groups of mice based on results in Figures 2 and 5

Group 1	Group 2	P value
NOS2+ (lavage cells)	NOS2+ (exhaled NO)	0.8
NOS2+	NOS2+/-	0.28
NOS2-	NOS2-/+	0.77
NOS2+ and NOS2+/-	NOS2- and NOS2-/+	0.0002
NOS2+/-	NOS2-/+	0.039

Each pair of groups was analyzed by Student's t-test, as explained in the text. Exact P values for each comparison are given; a value of $P < 0.05$ is taken to indicate significance.

NOS2+ and NOS2- groups indicate pooled data from both normal and chimeric mice with the same bone marrow source, as explained in the text.