# Inhaled Ethyl Nitrite Prevents Hyperoxia-impaired Postnatal Alveolar Development in Newborn Rats

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*Rationale*: **Inhaled nitric oxide (NO) has been used to prevent bronchopulmonary dysplasia, but with variable results. Ethyl nitrite (ENO) forms S-nitrosothiols more readily than does NO, and resists higher-order nitrogen oxide formation. Because S-nitrosylation is a key pathway mediating many NO biological effects, treatment with inhaled ENO may better protect postnatal lung development from oxidative stress than NO.**

*Objectives*: **To compare inhaled NO and ENO on hyperoxia-impaired postnatal lung development.**

*Methods*: **We treated newborn rats beginning at birth to air or 95% $O_2$ ± 0.2–20.0 ppm ENO for 8 days, or to 10 ppm NO for 8 days. Pups treated with the optimum ENO dose, 10 ppm, and pups treated with 10 ppm NO were recovered in room air for 6 more days.**

*Measurements and Main Results*: **ENO and NO partly prevented 95% $O_2$–induced airway neutrophil influx in lavage, but ENO had a greater effect than did NO in prevention of lung myeloperoxidase accumulation, and in expression of cytokine-induced neutrophil chemoattractant-1. Treatment with 10 ppm ENO, but not NO, for 8 days followed by recovery in air for 6 days prevented 95% $O_2$–induced impairments of body weight, lung compliance, and alveolar development.**

*Conclusions*: **Inhaled ENO conferred protection superior to inhaled NO against hyperoxia-induced inflammation. ENO prevented hyperoxia impairments of lung compliance and postnatal alveolar development in newborn rats.**

**Keywords:** bronchopulmonary dysplasia; *O*-nitrosoethanol; S-nitrosylation

Inhaled nitric oxide (NO) has been used to prevent and treat bronchopulmonary dysplasia (BPD) in premature newborns, with mixed success (1–4). The rationale underlying clinical trials has included improvement of ventilation–perfusion matching through classical effects on guanylyl cylase–mediated pulmonary vascular tone and remodeling (5), alveolar capillary development (6), and reducing inflammation (7). However, many, if not most NO biological effects are mediated through reversible S-nitrosylation of critical thiols that govern multiple biological pathways (8–10).

Inhaled agents preferentially forming S-nitrosothiols may "deliver" NO biological effects more efficiently, which may be of particular benefit in treating patients subjected to high levels of inhaled oxygen, as NO tends to rapidly form peroxynitrite in the presence of superoxide. Inhaled ethyl nitrite (ENO) has been used to treat newborns with pulmonary hypertension (11), and efficiently achieves S-nitrosation of glutathione compared with NO, without releasing significant amounts of reactive •NO species (12), so it should be less susceptible to forming higher-order nitrogen oxides.

We therefore compared the effects of inhaled NO and ENO in hyperoxia-exposed newborn rats, which we have previously shown to develop an acute inflammatory response followed by alveolar hypoplasia, both features of clinical BPD (13). To determine if inhaled ENO could prevent pulmonary inflammation and protect against hyperoxia-impaired alveolar development *in vivo*, we treated newborn rat pups with 95% $O_2$ with or without inhaled ENO at 0.2–20 ppm, and measured effects on S-nitroso (SNO) accumulation in bronchoalveolar lavage (BAL), and on pulmonary leukocyte influx, inflammatory chemokine expression, nuclear factor (NF)-κB activation, antioxidant enzyme activity, 3-nitrotyrosine, static lung compliance, and alveolar development, and compared effects with groups treated with inhaled NO at 10 ppm, a dose previously used in clinical trials to prevent BPD (14). We have previously reported effects of ENO on *in vitro* NF-κB activation in the form of an abstract (15).

### AT A GLANCE COMMENTARY

#### Scientific Knowledge on the Subject

S-nitrosylation may affect a broad number of regulatory cell responses, such as inflammation. Inhaled agents that promote S-nitrosylation may provide targeted protection from inflammation.

#### What This Study Adds to the Field

Inhaled ethyl nitrite dose-dependently prevents hyperoxia-induced lung inflammation in newborn rats, and, at an equivalent dose, afforded better protection of postnatal lung function and development than nitric oxide.

## METHODS

### Materials

Time-mated pregnant Sprague-Dawley rats were obtained from Charles River Laboratories (Raleigh, NC). Reagents were from Sigma-Aldrich (St. Louis, MO), unless otherwise specified. Gas sources and descriptions are in the online supplement.

### Animal Exposures

Procedures were approved by the institutional animal care and use committee. Exposure conditions and details are in the online supplement. Animals were exposed, beginning on the day of birth, to air,

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95% $O_2$+ 5% $N_2$, 95% $O_2$ + 0.2, 1.0, 10, and 20 ppm ENO, or 10 ppm NO (balance gas: $N_2$). Exposures were continued for 8 days, body weights were recorded, and pups were killed with sodium pentobarbital. Additional litters were exposed to air or 95% $O_2$ ± ENO or NO, both at 10 ppm, for 8 days, recovered in air for 6 more days (total = 14 d), and then killed.

### Methemoglobin

Methemoglobin was measured in blood samples obtained by cardiac puncture at Day 8 in six pups per treatment group (Model 482 co-oximeter; Instrumentation Laboratories, Lexington, MA).

### Pulmonary Leukocyte Influx

BAL was performed at 8 days, as previously described (13). After lavage and pulmonary artery perfusion with 0.9% NaCl, lungs from Day-8 pups were snap frozen in liquid nitrogen. Lung extracts were reacted with *o*-dianisidine to detect myeloperoxidase (MPO) activity (13).

### SNO Accumulation

BAL fluid (BALF) samples were snap frozen, and NO was measured in thawed samples by chemiluminescense after UV-photolysis before and after reduction of SNO by $HgCl_2$, as previously described, using an S-nitrosoglutathione standard curve (16). Equal volumes of BALF were analyzed from each pup.

### Cytokine-induced Neutrophil Chemoattractant-1 Expression

Lung extracts from Day-8 pups in each group were analyzed in duplicate by ELISA for cytokine-induced neutrophil chemoattractant-1 (CINC-1), as previously described (13). Random sections from four animals per treatment group were immunostained with anti–CINC-1 (*see* details in the online supplement). Real-time polymerase chain reaction measurements of CINC-1 and L32 cyclin (loading control) mRNA were performed, as previously described, (17) (*see* details in the online supplement). Mean CINC-1/L32 crossing-point ratios from hyperoxia-exposed groups were expressed as a proportion of the mean CINC-1/L32 ratio in the air-exposed group.

### 3-Nitrotyrosine

Lung homogenates, 0.4 mg total protein/lung from pups in each treatment group (n = 6 or 7/group) at Day 8 were analyzed in duplicate for 3-nitrotyrosine by ELISA (Northwest Life Science Specialties, Vancouver, WA). Results below the limit of detection for the assay were assigned a value of one half of the detection limit for the purposes of statistical comparisons.

### NF-κB Activation

Nuclear proteins were extracted from Day-8 pups, as described in the online supplement. Nuclear protein from tumor necrosis factor-α–activated Jurkat cells was supplied with the kit as a positive control, along with nuclear protein extracted from lungs from LPS (*Escherichia coli* serotype 055, 6 mg/kg intraperitoneally)-treated adult rats (18), with vehicle-treated adult rats as additional negative controls. Activated NF-κB was detected using a commercial kit (TransAM NFκB; Active Motif, Carlsbad CA), binding nuclear protein extracts to NF-κB–binding DNA sequence covalently bound to an ELISA plate (19). Samples (10 μg/pup) were assayed in duplicate from three pups per group.

Activated NF-κB was detected in histologic sections from each treatment group by immunohistochemistry using an antibody directed against the nuclear localization epitope of the p65 subunit, which is exposed during activation. Details are in the online supplement.

### Antioxidant Enzyme Expression

Whole-lung homogenates from pups at 8 days (n = 4/group) were analyzed in triplicate for total superoxide dismutase (SOD) (WST-1; Dojindo, Molecular Technologies, Gaithersburg, MD) and catalase (AmplexRed; Invitrogen, Carlsbad, CA) activities, according to the manufacturers' directions, using bovine SOD and catalase as standards. SOD1, -2, and -3 isoforms and catalase expression were detected by immunoblot and quantified by image analysis, normalizing to β-actin signal, as previously described (17). Details are in the online supplement.

### Lung Mechanics

Pups exposed to air or 95% $O_2$ ± ENO or NO, 10 ppm, for 8 days, then recovered in air for 6 days (total, 14 postnatal days) were anesthetized and connected to a small animal ventilator. Pressures and flows at specified tidal volumes were measured using forced oscillatory maneuvers with a small animal ventilator (FlexiVent; SCIREQ, Montreal, PQ, Canada) to generate pressure–volume loops, and to calculate compliance, as previously described in detail (20).

### Lung Morphometry

Random sections of lungs from pups exposed to air or 95% $O_2$ ± ENO or NO, 10 ppm for 8 days, then recovered in air, as described above (n = 6/group), were stained with malachite green according to the manufacturer's directions (Sigma), and with Hart's elastin, as previously described (21). Random images were obtained from each section for measurements of alveolar volume density (estimates alveolar number), alveolar surface density (estimates surface area), and secondary crest density normalized to alveolar volume density to account for inflation differences. Details are provided in the online supplement.

### Data Analysis

Data are expressed as the mean ± SEM, except where noted. Significant differences between treatment groups were determined by analysis of variance. *Post hoc* differences were identified by Tukey-Kramer tests. Significance was accepted for comparisons at p less than 0.05, and calculations were made using statistical analysis software (SPSS version 14; SPSS, Inc., Chicago, IL).

## RESULTS

### Survival, Weight Gain, Methemoglobin

Survival was slightly less in hyperoxia-exposed groups (95%) compared with air-exposed groups (97%), but was not statistically different between hyperoxia and hyperoxia + ENO– or NO-exposed groups at Days 8 and 14. No gross abnormalities in behavior or appearance were seen by inspection in any treatment group. As expected, hyperoxia exposure impaired postnatal weight gain, measured as body weight at Days 8 and 14 (Figure 1), but this effect was prevented in pups treated with hyperoxia + 20 ppm ENO at 8 days, and in pups treated with 10 ppm ENO at 8 or 14 days. Inhaled NO + hyperoxia–treated pups were smaller than hyperoxia-treated pups at 8 days, but not at 14 days. Whole-blood methemoglobin measured at Day 8 was not significantly affected by exposure to hyperoxia or to hyperoxia + ENO or NO (Figure 1). No gross abnormalities were observed by visual inspection in thoracic or abdominal organs in any treatment group.

### SNO Accumulation

We measured SNO in lavage from 4 or 5 pups per group treated with air, 95% $O_2$, or 95% $O_2$ + 10 or 20 ppm ENO for 8 days, or 10 ppm NO for 8 days. ENO treatment showed a trend toward increased SNO concentrations, but the differences were not statistically significant, as shown in Figure 1.

### Effects on Inflammatory Responses

As shown in Figure 2, treatment with ENO for 8 days dose-dependently decreased 95% $O_2$–induced BALF leukocyte and neutrophil accumulation and tissue MPO activity (n = 8/group). NO at 10 ppm also prevented BALF leukocyte and neutrophil influx, but was less effective at preventing MPO accumulation. There were no significant differences between ENO and NO effects on BALF neutrophils. ENO, but not NO, at 10 ppm, prevented hyperoxia-induced total lung CINC-1 mRNA accumulation at Day 8, and both ENO and NO partly prevented CINC-1 protein accumulation in whole lung, as measured by ELISA (Figure 3). ENO treatment partly prevented hyperoxia-induced

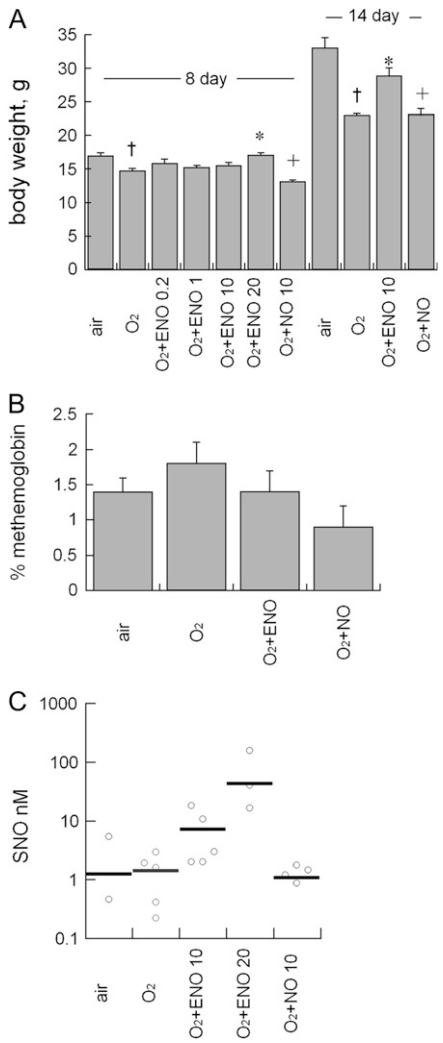


***Figure 1.*** (*A*) Effect of air or 95% oxygen ($O_2$) ± ethyl nitrite (ENO; 0.2–20 ppm) × 8 days, or 10 ppm nitric oxide (NO) × 8 days on body weight (*left-hand bars*: n = 16/group; †$p < 0.05$ vs. 8-day air; *$p < 0.01$ vs. 8-day $O_2$ control group; +$p < 0.05$ vs. 95% $O_2$ + 10 ppm ENO). Effect of air × 14 days or 95% $O_2$ ± 10 ppm ENO or NO × 8 days, followed by recovery in air for 6 days, on body weight (*right-hand bars*: †$p < 0.05$ vs. air control group; *$p < 0.005$ vs. 14 days $O_2$ control; +$p < 0.05$ vs. 95% $O_2$ + 10 ppm ENO; mean + SEM). (*B*) Effect of air or 95% $O_2$ ± 10 ppm ENO or NO × 8 days on whole-blood methemoglobin; mean + SEM; n = 6/group. (*C*) Effect of air or 95% $O_2$ ± 10 or 20 ppm ENO or 10 ppm NO on S-nitroso concentration in lavage. *Bars* represent means of average values from four or five pups per treatment group.

alveolar epithelial and bronchiolar epithelial CINC-1 immunostaining, whereas NO treatment only reduced immunostaining in bronchiolar epithelium.

### 3-Nitrotyrosine

Hyperoxia induced a statistically significant increase in whole-lung 3-nitrotyrosine, compared with air-exposed animals at Day 8 (Figure 4). In contrast, hyperoxia did not significantly increase 3-nitrotyrosine in animals treated with ENO or NO at 10 ppm.

### NF-κB Activation

Hyperoxia activated NF-κB in a subpopulation of cells, mainly in bronchiolar epithelial cells (Figure 5), but also in alveolar epithelium, as detected by immunohistochemistry. This was in marked contrast to the widespread activation observed in lungs from LPS-treated adult rat. Treatment with ENO, but not NO, decreased bronchiolar epithelial NF-κB activation. Effects on alveolar epithelium were modest and heterogeneous in all hyperoxia-exposed groups at 8 days. Hyperoxia did not significantly increase NF-κB activation in whole lung compared with air-exposed newborn pups, and this was unaffected by ENO or NO. The overall activation in lungs from each of the neonatal exposure groups was small compared with the activation in LPS-treated adults (or in stimulated Jurkat cells), in accord with the immunohistochemical studies (Figure 5).

### Lung Antioxidant Enzyme Expression

Whole-lung SOD activity was unaffected by hyperoxia or hyperoxia + 10 ppm ENO (Figure 6), but was modestly increased in NO-treated pups. Catalase activity was decreased in hyperoxia-exposed pups, but this was not statistically significant ($p = 0.08$). There was no significant effect of hyperoxia + ENO or NO on catalase activity. Whole-lung SOD1 and SOD2 abundance in immunoblots normalized to β-actin were unaffected by 95% $O_2$ ± ENO or NO at 8 days. SOD3 was increased in all hyperoxia-exposed groups compared with air-exposed pups, but with no differences among hyperoxia-exposed treatment groups. Parallel to the effects on activity, catalase abundance was decreased in 95% $O_2$–exposed pups, but not in 95% $O_2$ + ENO– or NO-exposed pups compared with air-exposed pups.

### Lung Mechanics and Alveolar Development after Recovery in Air

To determine longer-term effects of ENO and NO on hyperoxia-impaired postnatal lung function and development, pups were exposed to air, 95% $O_2$, 95% $O_2$ + 10 ppm ENO, or 95% $O_2$ + 10 ppm NO for 8 days, followed by recovery in air for 6 days. Hyperoxia significantly decreased lung compliance measured at Day 14, and this was prevented in ENO-treated pups, but not in NO-treated pups, which showed worse compliance than $O_2$-exposed control animals (Figure 7).

Effects on alveolar development were parallel to effects on static lung compliance. Using lung morphometry in random sections from each treatment group, we found parallel effects of hyperoxia, ENO, and NO treatment on alveolar surface density, alveolar volume density, and secondary alveolar septal crest density (elastin-stained) normalized to septal tissue. Hyperoxia exposure impaired alveolar volume density, alveolar surface density, and secondary septal crest densities, as expected. Treatment with 10 ppm ENO for 8 days significantly ameliorated these effects. NO treatment did not prevent hyperoxia-impaired alveolar development (Figure 8).

## DISCUSSION

Increasing evidence points to S-nitrosylation as a key pathway that mediates many of the physiologic responses to endogenous and exogenous NO (8). Our rationale for treatment with inhaled ENO was based on its superior ability to achieve *in vitro* S-nitrosylation of glutathione (12), abundant in alveolar lining fluid (22), compared with NO treatment. A trend toward higher concentrations of SNO were detected in BALF from ENO-treated pups in the small number of samples analyzed, consistent with earlier *in vitro* comparisons of ENO and NO gases (12). In preliminary studies, we attempted to measure SNO in lung homogenates, but were unable to consistently detect SNO groups, possibly due to rapid intracellular SNO group metabolism via transnitrosation. Alternatively, SNOs are avidly taken

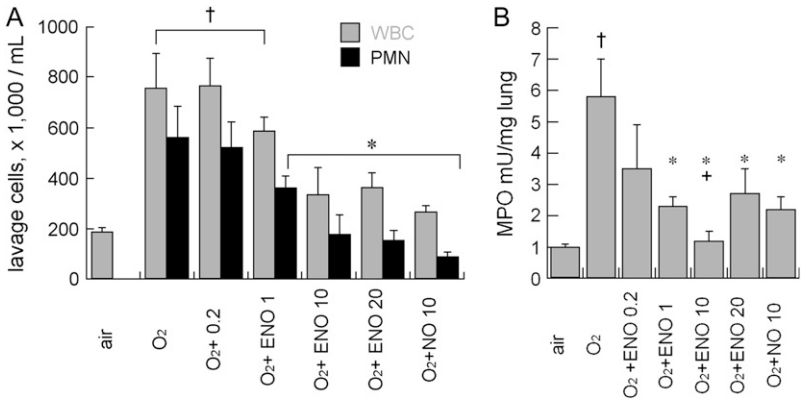


*Figure 2.* (*A*) Effect of air or 95% $O_2$ ± ENO at 0.2, 1, 10, or 20 ppm, or NO at 10 ppm, × 8 days, on bronchoalveolar lavage leukocytes (WBC) and neutrophils (PMN); n = 8/group; mean ± SEM; †p < 0.05 versus air; *p < 0.05 versus $O_2$ control group. (*B*) Effect of air or 95% $O_2$ ± ENO at 0.2, 1, 10, or 20 ppm, or NO at 10 ppm, × 8 days, on myeloperoxidase (MPO) activity in perfused, lavaged whole lung; n = 8/group; mean ± SEM; †p < 0.05 versus air; *p < 0.05 versus $O_2$ control group; +p < 0.05 versus NO.

up by hemoglobin, and it may be that residual blood in the perfused lungs interfered with detection in lung homogenates (23), but this was not directly addressed.

We found that both inhaled ENO and NO partly prevented hyperoxia-induced leukocyte influx in BALF in newborn rats. In contrast, 10 ppm ENO treatment was superior to 10 ppm NO at preventing hyperoxia-induced accumulation of MPO. Because the lungs were lavaged and perfused, the tissue MPO should reflect both adherent intravascular as well as interstitial leukocytes. We did not perform detailed analysis of interstitial inflammation. ENO treatment prevented hyperoxia induction of CINC-1 mRNA, a neutrophil chemokine, and both ENO and NO partly prevented hyperoxia-induced CINC-1 protein in whole lung. CINC-1 immunostaining was more widespread, including alveolar septal tissue in NO-treated pups compared with ENO-treated pups. These differences in local chemokine accumulation may have contributed to the differing effects on leukocyte adhesion reflected in the MPO activities. Effects of inhaled ENO and NO on inflammation may differ because of disparate effects on adhesion molecules that govern leukocyte trafficking. NO has been shown to inhibit leukocyte adhesion (24) and viability (25). S-nitrosoglutathione inhibits monocyte adhesion (26), and S-nitrosoacetylpenicillamine, another NO donor, downregulates expression of CXCR2, a neutrophil chemokine receptor (27). No comparable studies have been done with ENO, as far as we know. Differences between ENO and NO effects may depend on the dosing strategies, which can be further defined in future studies.

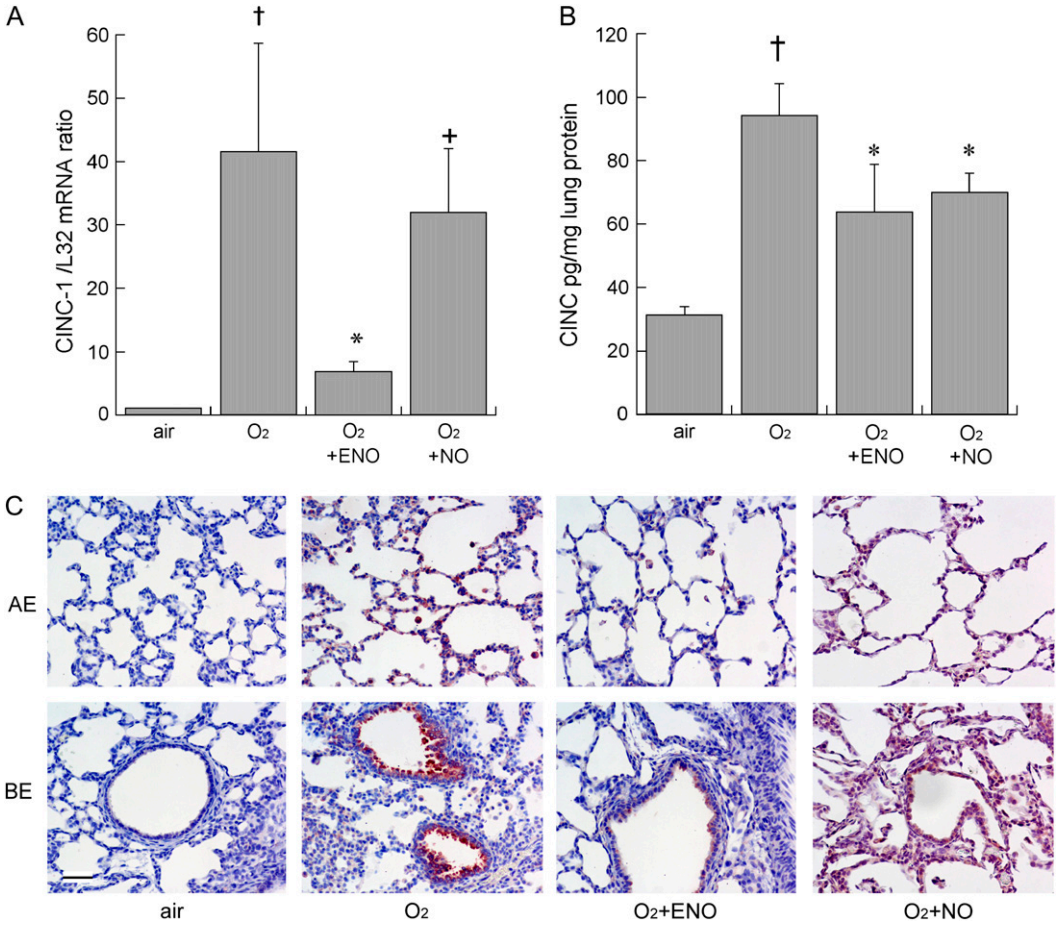


*Figure 3.* (*A*) Effect of air or 95% $O_2$ ± ENO or NO, 10 ppm × 8 days on whole lung cytokine-induced neutrophil chemoattractant-1 (CINC-1) mRNA (normalized to L32 cyclin) expressed as fold difference versus air control group (n = 4/group) and (*B*) CINC-1 protein measured by ELISA (n = 8/group); mean + SEM; †p < 0.05 versus air; *p < 0.05 versus $O_2$ control group; and +p < 0.05 versus $O_2$ ENO group. (*C*) CINC-1 immunolabeling in alveolar and bronchiolar epithelium (AE and BE, respectively); *scale bar* = 50 μm.

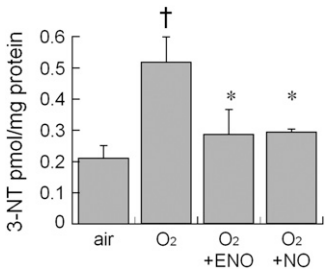


***Figure 4.*** Effect of air or 95% $O_2$ ± 10 ppm ENO or NO, × 8 days on 3-nitrotyrosine detected by ELISA; mean + SEM; n = 6 or 7/group; †p < 0.05 versus air; *p < 0.05 versus $O_2$ control group.

We focused our assessments of inflammatory cytokines on CINC-1 because of the importance of the CINC-1/CXCR2 pathway to hyperoxia-impaired postnatal lung development (13, 21, 28). Although other neutrophil chemokines that we did not measure could have contributed to inflammation, our previous studies using this model system showed that treatment with neutralizing anti–CINC-1 (13), or with an antagonist to its receptor, CXCR2 (21), were sufficient to substantially protect against hyperoxia-impaired postnatal lung development.

Localized differences in cellular uptake of inhaled NO or ENO could also account for some of the observed differences in inflammation and local CINC-1 expression. Because the ease of formation of S-nitrosothiol is a key distinction between ENO and NO (12), differences in pulmonary S-nitrosothiol uptake from airway-formed SNOs could be important as an explanation for differing biological effects. The role of metabolism of airway-formed SNOs and its contribution to lung biology is incompletely understood, as are differences in diffusion from the airway to the interior of lung epithelial cells, and downstream metabolism and trafficking. Extracellularly formed S-nitrosoglutathione appears to require importation via amino acid transporters to achieve intracellular S-nitrosylation in some cell types (29). If true in lung epithelium, this may partly explain the more robust effect of ENO, as it forms S-nitrosothiol more avidly than NO.

We therefore hypothesized that ENO might be superior to NO in its ability to inactivate NF-κB, an important regulator of lung inflammation via induction of inflammatory cytokine transcription factors (30). SNO modifications inactivate I-κ kinase-kinase β *in vitro*, which derepresses NF-κB activation (31). S-nitrosylation inactivates the p50 subunit of NF-κB, preventing transcriptional activation (32). Preliminary studies show that ENO can block superoxide-induced NF-κB activation *in vitro* (15). To determine if hyperoxia induced NF-κB activation in newborn rat lung, or if inhaled ENO or NO blocked hyperoxia-induced pulmonary NF-κB activation *in vivo*, we measured NF-κB activation in nuclear protein extracts from whole lung in each treatment group at Day 8. We found that the overall magnitude of NF-κB activation in whole-lung nuclear extracts was small compared with the more robust stimulus of LPS injection in adult rat lung. We found that hyperoxia exposure increased activation in a subset of cells identified by immunohistochemistry—most prominently, the bronchiolar epithelium—which was prevented in ENO-treated pus, but not in NO-treated pups. In contrast with the LPS-treated adult rats, alveolar epithelial NF-κB activation was qualitatively decreased and heterogeneously expressed.

NF-κB abundance is reportedly induced by hyperoxia in neonatal rats, but activation and nuclear localization were not directly examined in the previous report (33). Hyperoxia-exposed transgenic newborn mice expressing NF-κB–driven luciferase demonstrate NF-κB activation, which persisted until 72 hours of hyperoxia exposure (later time points were not examined), but not in all cells (34), in agreement with our findings in newborn rats. NF-κB activation may protect some cells against oxidative

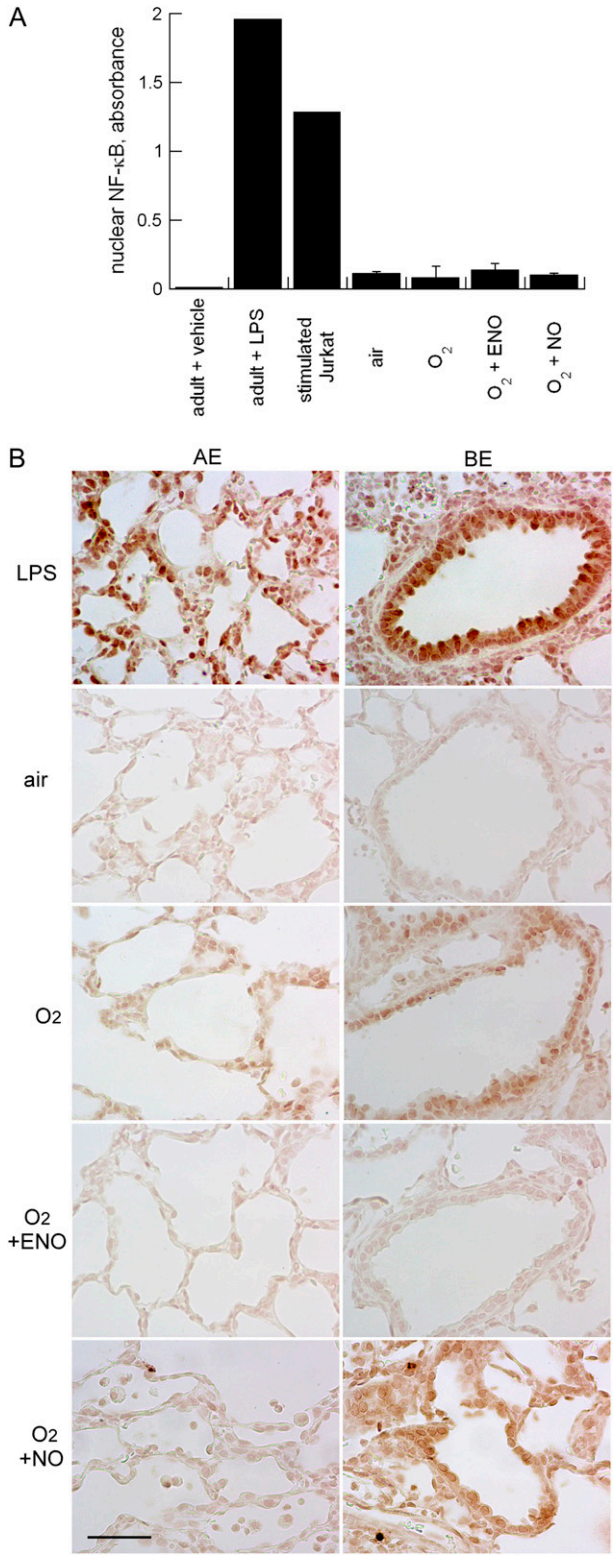


***Figure 5.*** (*A*) Nuclear factor (NF)-κB activation in air (negative control group)-exposed adult rat lung, LPS-treated adult rat lung, or tissue plasminogen activator–stimulated Jurkat cells (positive control group), 8-day-old rat lung from air, 95% $O_2$–exposed, 95% $O_2$ + ENO, and 95% $O_2$ + NO treatment groups; mean + SD; n = 3/group. (*B*) Immunohistochemical localization of activated (nuclear localization signal) NF-κB in representative sections from each treatment group. AE = alveolar epithelium; BE = bronchiolar epithelium. *Scale bar* = 50 μm.

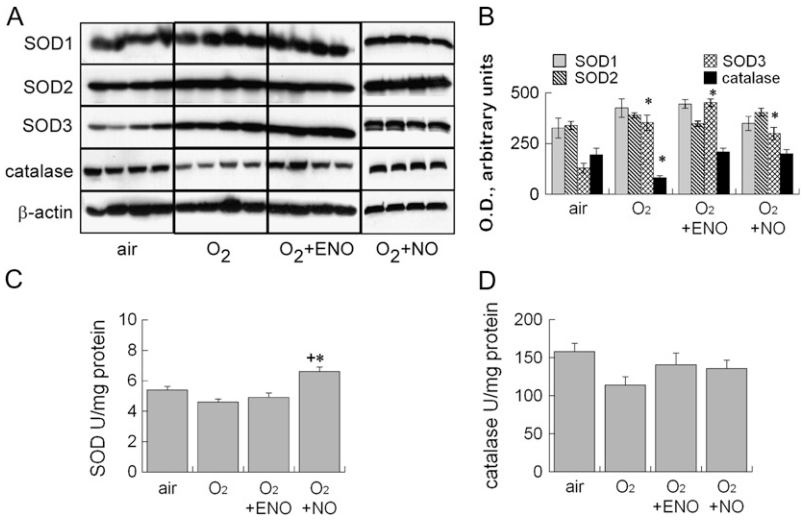


*Figure 6.* (*A*) Effect of air or 95% $O_2$, or 95% $O_2$ + 10 ppm inhaled ENO or NO, × 8 days on superoxide dismutase (SOD)-1, -2, and -3, catalase, and β-actin immunoblot expression in whole-lung homogenates, 20 μg/lane; n = 4/group. (*B*) Blots were quantified by image analysis, mean optical density ± SEM. (*C*) Effects on total lung SOD activity (*$p < 0.05$ vs. air control group; $^{+}p < 0.05$ vs. ENO) and (*D*) catalase activity; n = 4/group; mean + SEM.

stress–induced apoptosis (35), and may serve as an adaptive response under certain circumstances. Conducting similar studies using the NF-κB luciferase transgenic mouse could help to clarify timing and location of inhaled ENO or NO effects on pulmonary NF-κB activation.

Our main finding was that ENO significantly prevented hyperoxia-impaired lung compliance and alveolar development in animals recovered in air at Postnatal Day 14. This was not observed in NO-treated pups. The physiologic impact of this benefit is reflected by the superior protection against hyperoxia-impaired body growth in ENO- versus NO-treated pups at Days 8 and 14. The effects on static compliance could have been due, in part, to differences in inflammation, with most, but not all, parameters superior in ENO- versus NO-treated animals. Other possible factors include effects on surfactant that could have been a significant contributor to observed compliance differences. The effect of NO on the surfactant system is variable, with inhibition of surfactant function at high NO doses, (36) but apparent augmentation at low doses (1). Improvement or impairment of surfactant function *in vitro* does not necessarily correlate with *in vivo* effects on lung compliance (1). Interstitial edema could have contributed to the disparate effects on lung compliance, but this was not examined directly.

The beneficial effects of ENO on hyperoxia-impaired alveolar development were significant. Alveolar volume density, which estimates alveolar number, was significantly protected in ENO-treated pups, but not in NO-treated pups. ENO treatment also preserved alveolar surface area, estimated by surface density. Because interpretation of volume or surface density could have been confounded by differences in lung inflation, we measured secondary crest abundance normalized to septal tissue, which demonstrated even more striking differences between ENO and NO effects on alveolar development in hyperoxia-exposed rat pups. We did not observe obvious differences in elastin deposition in alveolar walls or in septal tips between the air-, $O_2$-, $O_2$ + ENO–, and $O_2$ + NO–exposed animals at 14 days (data not shown). This contrasts with the finding of preserved elastin morphology in the NO-treated baboon model of BPD (37). This may be attributable to species differences, or the other important differences between the models, including the degree of immaturity, and the lack of mechanical ventilation as a contributor in our studies.

Because NO and ENO effects are likely to be mediated by a number of pathways, we measured effects of ENO and NO on antioxidants, as differences in ENO and NO effects on antioxidant defenses might have contributed to the disparity in effects

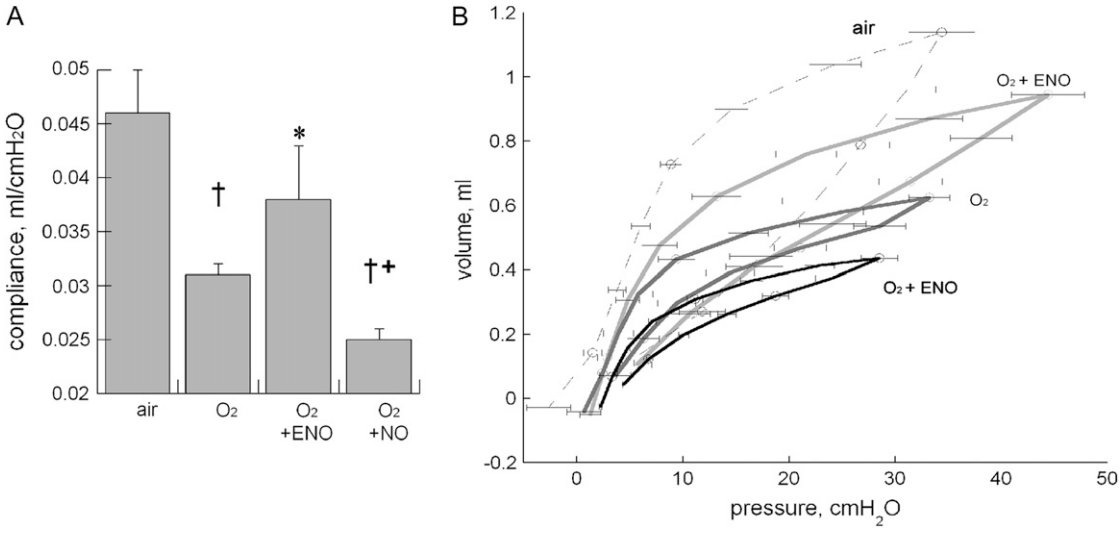


*Figure 7.* Effect of air or 95% $O_2$ ± 10 ppm ENO or NO for 8 days followed by recovery in air for 6 days on (*A*) static compliance ($^{\dagger}p < 0.05$ vs. air; *$p < 0.05$ vs. $O_2$; $^{+}p < 0.05$ vs. $O_2$ + ENO and vs. $O_2$) and (*B*) pressure–volume loops; n = 5 or 6/group; mean ± SEM.

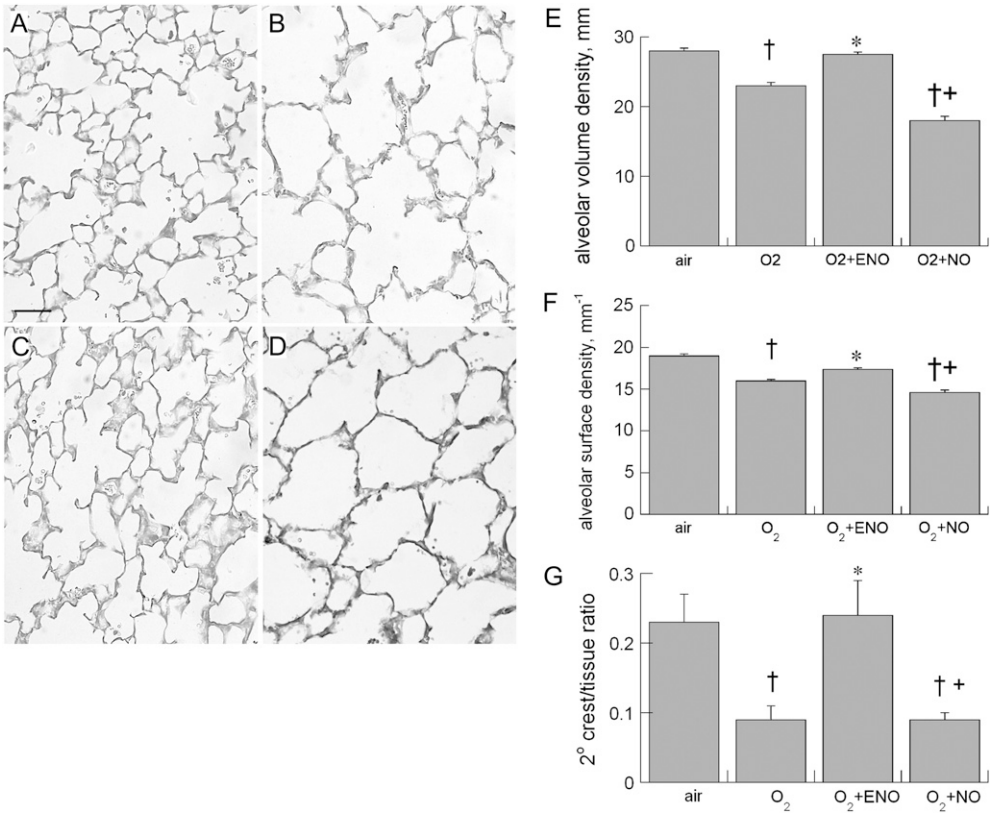


***Figure 8.*** Representative lung photomicrographs of the effects of (*A*) air, (*B*) 95% $O_2$, (*C*) 95% $O_2$ + ENO, or (*D*) 95% $O_2$ + NO × 8 days followed by recovery for 6 days; *scale bar* = 50 μm. Effects of air, $O_2$→air recovery, $O_2$+ENO→air recovery, and $O_2$+NO→air recovery on (*E*) alveolar volume density, (*F*) alveolar surface density, and (*G*) 2° septal crest:tissue ratios; n = 6 or 7 pups/group. $^{\dagger}p < 0.05$ versus air; $^{*}p < 0.05$ versus $O_2$; $^{+}p < 0.05$ versus $O_2$ + ENO.

on lung compliance and development. Low levels of NO induce catalase *in vitro* (38), and S-nitrosylation has been implicated as potentially regulating catalase expression (39). Sp1 transcription factor, which regulates extracellular SOD3 (40), appears to be affected by S-nitrosylation (41), although the specific thiol targets have not been identified. We found no large effects of either ENO or NO treatment on SOD or catalase activity. Total lung SOD activity was statistically greater in the NO-treated group, but the increment was modest. Increases in SOD activity unaccompanied by changes in catalase could potentially increase hydrogen peroxide accumulation, depending on the local conditions. Whole-lung expression of SOD1, -2, or -3, or catalase, were unaffected by either ENO or NO treatment in hyperoxia-exposed pups. Hyperoxia increased SOD3 expression, as expected (42). We cannot exclude disparities in local effects on antioxidant enzyme regulation, as these enzymes (43), particularly SOD3 (17, 42), are not expressed ubiquitously in the lung.

We examined the effects of NO and ENO inhalation on hyperoxia-induced 3-nitrotyrosine formation, as ENO should, theoretically, be less able to form peroxynitrite, the precursor of 3-nitrotyrosine, than is NO, assuming conditions of excess superoxide, as the reaction proceeds at essentially diffusion-limited rates (44). Peroxynitrite formation is associated with disrupted protein function and cellular metabolism (*see* Reference 45 for review). Both ENO- and NO-exposed animals had reduced 3-nitrotyrosine in whole lung compared with hyperoxia-exposed control animals. ENO and NO suppression of neutrophil accumulation and, therefore, neutrophil-generated superoxide may have been sufficient to prevent peroxynitrite formation and 3-nitrotyrosine accumulation.

There are a large number of pathways important to postnatal lung development that could be differentially affected by ENO and NO. Cui and colleagues demonstrated many cell-cycle, inflammation, and apoptosis-related genes that were induced *in vitro* by NO, independent of its effects on guanylyl cyclase, and, therefore, most likely affected via S-nitrosylation (9). We speculate that the improvements in lung development that we observed are partly attributable to preservation of lung cell proliferation and differentiation, which likely depend on a number of these S-nitrosylation–modified pathways. Disordered epithelial proliferation has been linked to the development of BPD in a number of animal models (46, 47).

The lack of beneficial effect of inhaled NO in our model system contrasts with beneficial effects in other systems designed to model BPD. This likely depends on a number of interacting factors, including, but not limited to, the overall burden of oxidative stress, the dose and timing of NO therapy, and the magnitude of endogenous NO production. For example, inhaled NO after hyperoxia exposure in newborn rats rescued impaired alveolar development (6), but treatment with an NO donor during hyperoxia exposure did not (48). In model systems with less oxidative stress (e.g., prematurely delivered lambs [49] and baboons [37]), inhaled NO, generally at doses lower than 10 ppm, improved lung development. It would be important to determine whether or not similar protective effects are conferred with less stringent oxidative stress (e.g., lower, more chronic oxidative stress [21], and in models that include mechanical ventilation, to more closely mimic clinical BPD).

In summary, we found that inhaled ENO and NO both inhibited hyperoxia-induced lung inflammation, ENO more so than NO, and prevented protein nitration in postnatal rats, but that ENO alone prevented hyperoxia-impaired weight gain, lung compliance, and alveolar development. The magnitude of differences in effects of ENO and NO on hyperoxia-induced inflammation can only partly account for the differences in lung development. Because the dosing regimens of ENO and NO were the same, we speculate that S-nitrosylation–dependent regulatory pathways account for the beneficial effects of ENO on lung

development. Under conditions of oxidative stress, inhaled ENO may restore or augment airway lining fluid S-nitrosothiols that may be important to processes, yet undetermined, that govern postnatal alveolar development.

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