Inhaled Nitric Oxide Attenuates Hyperoxic Lung Injury in Lambs

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ABSTRACT: Cytochrome P450 (CYP) inhibition with cimetidine reduces hyperoxic lung injury in young lambs. Nitric oxide (NO), also a CYP inhibitor, has been shown to either aggravate or protect against oxidant stress depending on experimental context. The objective of this study was to determine whether NO, like cimetidine, would protect young lambs against hyperoxic lung injury, and whether its effect was associated with CYP inhibition. Three groups of lambs were studied: 1) room air exposure, 2) >95% O₂, and 3) >95% O₂ plus inhaled NO. After 72 h, hyperoxia alone resulted in a significant increase in arterial Pco₂ and number of polymorphonuclear leukocytes in bronchoalveolar lavage (BAL), and a significant decrease in arterial/alveolar O₂ tension (a/A). The addition of inhaled NO significantly decreased the hypercarbia and BAL polymorphonuclear cellular response associated with hyperoxia but had no beneficial effect on a/A ratio. There were no significant differences in F₂-isoprostanes or isofurans (markers of lipid peroxidation) measured in BAL or lung tissue among study groups. No intergroup differences were detected in BAL epoxyeicosatrienoic acid levels (index of CYP activity). The results of this study indicate that hypercarbia and inflammation accompanying hyperoxic lung injury in young lambs can be attenuated by inhaled NO. However, this study provides no direct evidence that NO is inhibiting CYP-mediated oxidant lung injury. (*Pediatr Res* 59: 142–146, 2006)

Previous work has shown that administration of a CYP inhibitor, cimetidine, would reduce hyperoxic lung injury in young lambs (1). In that work, lambs that breathed high oxygen concentrations exhibited impaired gas exchange (elevated Pco₂ and lower than expected Po₂) and evidence of an increase in pulmonary vascular permeability as indicated by a marked increase in lung lymph flow and protein clearance after 72–74 h of continuous hyperoxia. These effects of hyperoxia were attenuated by the administration of cimetidine before beginning high oxygen exposure. In further studies (2), oxygen breathing significantly increased lung CYP1A1 mRNA levels *in vivo*, and this increase preceded the increase in isozyme activity. Oxygen exposure also promptly increased CYP1A1 mRNA levels in cultured lamb lung microvascular

endothelial cells in these studies. As a monooxygenase, the catalytic activity of CYP is accompanied by the production and release of free radicals (3), and there is a variety of evidence linking CYP to oxidant lung injury (4). Also, the products of arachidonic acid metabolism catalyzed by the CYP system have biologic actions that could cause or aggravate lung injury (5). Taken together, these findings are consistent with the hypothesis that the CYP system in the lung provides a source of damaging oxygen free radicals and catalyzes the production of certain arachidonic acid metabolites which may cause or aggravate lung injury.

NO, an agent that mediates a wide variety of biologic actions, is used clinically as a vasodilator in the management of persistent pulmonary hypertension of the newborn (6,7). NO is also a potent CYP inhibitor that reacts with a heme moiety that is common to all CYP isoforms (8). The purpose of this study was to determine whether inhaled NO would have the same beneficial effects as cimetidine on gas exchange in young lambs exposed to hyperoxia. This study was designed to answer the following questions: 1) Will inhaled NO improve pulmonary gas exchange in young lambs exposed to high oxygen for 72 h; 2) Will inhaled NO decrease lung lipid peroxidation in young lambs exposed to high oxygen for 72 h; 3) Will inhaled NO decrease the production and release of CYP arachidonic acid metabolites into the airway of young lambs exposed to high oxygen for 72 h; 4) Will inhaled NO decrease lung inflammation in young lambs exposed to high oxygen for 72 h.

METHODS

Twenty-three 3- to 7-d-old full-term lambs delivered spontaneously were studied according to a protocol approved by the Institutional Animal Care and Use Committee of the Vanderbilt University Medical Center. Before study, catheters were placed into a lateral saphenous vein and a cranial tibial artery. The lambs were placed in a Plexiglas chamber (0.9 m high × 0.7 m wide × 1.2 m deep) designed to provide an environment of either room air or ≥95% O₂ when the chamber was flushed with a constant flow of either compressed air or 100% O₂ at 20 L/min. Additional CO₂ removal was accomplished with

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Abbreviations: BAL, bronchoalveolar lavage; bld, below limits of detection; CYP, cytochrome P450; EET, 14,15-epoxyeicosatrienoic acids; NICI-GCMS, negative ion-chemical ionization-gas chromatography-mass spectrometry; NO, nitric oxide; PMN, polymorphonuclear leukocytes

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a soda lime CO₂ scrubber placed in the chamber. Lambs were housed in the chamber for 72 h. Three to four times each day the lambs were removed from the chamber to nurse from their mothers for 5 min. A sample of arterial blood for blood gas analysis was taken in room air before exposure to the test gases and after exposure to the test gases for 72 h.

The 23 lambs were arbitrarily assigned to the following three groups: 1) RA: room air exposure ($n = 5$); 2) O₂: $\geq 95\%$ O₂ exposure ($n = 9$); 3) O₂-NO: $\geq 95\%$ O₂ exposure plus NO at 5–10 ppm ($n = 9$).

Sample sizes selected were similar to those in the previous study demonstrating the effects of cimetidine on oxidant lung injury in lambs (1). More lambs were included in the O₂ and O₂-NO groups than the RA group based on the assumption that any observed effect of NO on oxidant lung injury would be less than the effect of high O₂ exposure alone. These sample sizes were sufficient to detect a difference in BAL F₂-isoprostanes of 15 pg/mL between the RA and O₂ groups and 10 pg/mL between the O₂ and O₂-NO groups. They were also sufficient to detect between-group differences in BAL total EET levels of 1.2 ng/mL and 0.75 ng/mL, respectively. These differences were detectable with a power of 80% and a Type I error of 0.05.

Target NO concentrations in the chamber were achieved using a flow of 70–230 mL/min of a NO source containing 400 ppm NO (kindly provided by INO Therapeutics, Clinton, NJ). O₂, NO, NO₂, and CO₂ concentrations in the chamber were recorded just before removing the lamb from the chamber for feeding by the mother. Oxygen concentrations in the chamber were measured using an oxygen analyzer by Ventronics (Temecula, CA). NO and NO₂ concentrations were measured using a PulmoNOx II RT NO analyzer, Pulmonox Medical Corporation (Tofield, Alberta, Canada) kindly provided by INO Therapeutics. Carbon dioxide concentrations were measured using a LB-2 Medical Gas Analyzer by Beckman (Schiller Park, IL). Arterial pH, Po₂, and Pco₂ determinations were made using a model 248 pH/Blood Gas Analyzer (Chiron Diagnostics Ltd., Halstead, Essex, UK).

The lambs were killed by a rapid intravenous injection of thiamylal, 200 mg/kg, after 72 h exposure to the assigned gas mixture. After opening the chest, BAL was carried out on the left lung after clamping bronchi leading to the right lung. A portion of the right lower lobe was frozen in liquid nitrogen for studies detailed below.

BAL was carried out by infusing 10 mL/kg normal saline into the left lung followed immediately by aspiration of the lavage. One minute elapsed between the beginning of the infusion and the end of the aspiration, and $58 \pm 12\%$ (mean $\pm$ SD) of the infused saline was recovered in the aspirate. An uncentrifuged 10-mL aliquot of BAL was placed in a plastic tube containing 40–50 mg triphenylphosphine, a peroxide-reducing agent that prevents auto-oxidation of lipids during processing of the BAL fluid. This aliquot was thoroughly mixed, and stored at -70°C for later analysis of CYP metabolites. After removing a small sample for cell count, the remaining fluid was centrifuged at 2500 rpm for 10 min. The supernatant was stored at -70°C for later analysis of F₂-isoprostanes. A differential count of nucleated cells in the pellet was carried out using the cytospin technique (9).

F₂-isoprostanes, which serve as a highly sensitive marker of lipid peroxidation, were measured in BAL fluid and lung tissue with an assay employing stable isotope dilution techniques utilizing NICI-GCMS. The assay has been shown to be a highly accurate method to quantify oxidant injury *in vivo* (10). It has a precision within 6% and an accuracy of 96%. Isotransferrins, another highly sensitive marker of lipid peroxidation, were measured in lung tissue by NICI-GCMS as previously described (11).

CYP arachidonic acid metabolites (8,9-, 11,12-, and 14,15-EET) were assayed using methods previously described (5). The analysis of endogenous EET in BAL fluid involved preparation of a lipid extract of the fluid in the presence of an equimolar mixture of synthetic ¹⁴C-labeled 8,9-, 11,12-, and 14,15-EET as internal standards. Alkaline hydrolysis was followed by HPLC purification, pentafluorobenzyl esterification, repurification of the pentafluorobenzyl esters, and regioisomer quantification by NICI-GCMS (5).

Statistical methods. Data analysis was carried out using Systat Version 8 (Systat Software, Richmond, CA). To take into account skewed data, differences among study groups were assessed using the Kruskal-Wallis test which is the nonparametric analog of a one-way analysis of variance. Differences between groups at a p -value < 0.05 were considered statistically significant. Graphical data are presented as box plots which show the median, and the range within which the central 50% of the values fall, with the box edges at the first and third quartiles. More details are available in the Graphics Reference Manual for Systat 8.

All results are reported as the median and range for each group. F₂-isoprostanes in BAL that were bld were assigned a value of zero for analysis and designated bld in Table 2. Results of all measurements were available for all 23 lambs, except that values for F₂-isoprostanes and isotransferrins in lung tissue were available from only 9 lambs from the three groups because these tests were not undertaken until late in the study, when it became obvious that the anticipated differences in BAL F₂-isoprostanes were not being seen. Also,

measurements of CYP arachidonic acid metabolites were limited to 18 lambs in the three groups due to the intensity of labor and resources required coupled with the lack of any emerging trend toward intergroup differences.

RESULTS

The three groups of lambs were comparable with regard to arterial pH, Pco₂, Po₂, body weight, hematocrit, and respiratory rate before being placed into the chamber (data not shown). CO₂ concentrations in the chamber were 0.37% (0.17–0.68%) [median (range)]. There were no significant differences in inspired CO₂ concentrations across the study groups. Measured Fio₂ in the groups receiving high oxygen was always 1.0. NO concentrations in the chamber were 7 (5–14 ppm) [median (range)] for the O₂-NO group. NO₂ concentration in the chamber was 0 (0–0.2 ppm) [median (range)] for the O₂-NO group. Lambs in the two combined groups receiving high oxygen had less weight gain after 72 h than lambs in the RA group: 0.3 (–0.5–1.0 kg) *versus* 1.0 (0.4–1.4 kg) [median (range)], $p < 0.05$. Weight gain after 72 h was similar between the two groups receiving high oxygen. There were no differences in respiratory rate across the three groups after 72 h: RA, 76 (46–80); O₂, 60 (44–80); O₂-NO, 58 (40–120) [median (range)].

Arterial Pco₂ values after 72 h in the chamber are shown for the three groups in Figure 1. Lambs in the O₂ group had a significantly higher Pco₂ than lambs in the RA group ($p < 0.05$). This increase in Pco₂ was no longer significant in lambs that received NO in addition to high oxygen exposure ($p = 0.641$ for the O₂-NO group *versus* the RA group). Also, Pco₂ in the O₂-NO group was significantly lower than Pco₂ in the O₂ group ($p < 0.05$).

a/A oxygen ratios after 72 h in the chamber are shown for the three groups in Figure 2. As expected, high oxygen exposure had an adverse effect on oxygenation, resulting in a significant decrease in a/A oxygen ratio for the O₂ group when compared with the RA group ($p < 0.01$). No protective effect against the O₂-associated decrease in a/A ratio was seen with NO in that the a/A ratio for the O₂-NO group remained significantly decreased when compared with the RA group ($p < 0.01$). Complete arterial blood gas results are shown in Table 1.

Figure 1. Arterial Pco₂ 72 h after breathing study gas. RA: $n = 5$; O₂: $n = 9$; O₂NO: $n = 9$. * $p < 0.05$ vs RA group; † $p < 0.05$ vs O₂ group.

Figure 2. a/A oxygen tension 72 h after breathing study gas. RA: $n = 5$; O_2 : $n = 9$; O_2NO : $n = 9$. * $p < 0.01$ vs RA group.

Total, mononuclear, and PMN cell counts are shown in Figure 3 for the three study groups of lambs after 72 h exposure to study gases. In general, lambs exposed to high oxygen (groups O_2 and O_2 -NO) had higher cell counts (both total and fractionated) than the RA group. There were no significant differences in either total cells or mononuclear cells between the two groups exposed to high oxygen. However, the PMN cell count was significantly decreased in the lambs that received O_2 in combination with NO (Group O_2 -NO) compared with the lambs that received high O_2 alone (Group O_2).

There were no differences in F_2 -isoprostane levels in BAL fluid across the three study groups (Table 2). There were also no differences in lung tissue content of F_2 -isoprostanes or isofurans (data not shown). Finally, there were no differences in CYP arachidonic acid metabolites in BAL fluid across the three study groups (Table 3).

DISCUSSION

The results of this study indicate that hypercarbia and inflammation accompanying hyperoxic lung injury in young lambs can be attenuated by inhaled NO. Lambs in the O_2 group had significantly increased arterial P_{CO_2} tensions, significantly decreased a/A oxygen tension ratios, and significantly increased BAL PMN compared with the RA group. Treatment with inhaled NO significantly attenuated the effects of hyperoxia on arterial P_{CO_2} and BAL PMN, but not on a/A oxygen tension ratios.

These effects of NO on hyperoxic lung injury are similar to the effects of cimetidine that were observed in a previous study of hyperoxic lung injury in lambs (1). These findings are interesting in that both NO and cimetidine are inhibitors of

Figure 3. Nucleated cells in BAL according to study group. RA: $n = 5$; O_2 : $n = 9$; O_2NO : $n = 9$. * $p < 0.05$ vs RA group; † $p < 0.01$ vs RA group.

Table 2. F_2 -isoprostanes in BAL fluid

Group	F_2 -isoprostanes (pg/mL)
RA ($n = 5$)	15 (bld* - 17)
O_2 ($n = 9$)	bld (bld - 16)
O_2 -NO ($n = 9$)	10 (bld - 18)

Values shown as median (range).

* bld: below limit of detection (< 2 pg/mL).

CYP. However, this study provides no direct evidence that NO is inhibiting CYP-mediated oxidant injury in that 1) no increase in the markers of lipid peroxidation or in CYP-catalyzed arachidonic acid metabolites was seen in the lambs breathing high oxygen concentrations *versus* the control lambs breathing room air, and 2) no effect of NO on these markers was seen when the O_2 -NO group was compared with either the RA group or the O_2 group.

The two effects of inhaled NO on hyperoxic lung injury observed in this study were a reduction in the hypercarbia and a decrease in the number of PMN in BAL fluid compared with lambs exposed to high oxygen without NO. There are several explanations why inhaled NO might result in a decrease in arterial P_{CO_2} : 1) decreased pulmonary edema as a result of a protective effect of NO against endothelial and epithelial damage mediated by oxidant and inflammatory mechanisms (12); 2) reduced alveolar dead space consequent to normalizing the perfusion of ventilated alveoli (13); 3) improved minute ventilation secondary to counteracting the bronchoconstrictive effect of prolonged hyperoxia (14); and 4) protection of surfactant activity against free radical oxidants (15). It is possible that all of these mechanisms played a role in the beneficial effect of inhaled NO on hypercarbia in hyperoxic lung injury seen in this study.

The decrease in a/A ratio of oxygen tension in lambs breathing a high oxygen concentration was not affected by

Table 1. Arterial blood gases 72 h after breathing study gas

Group	pH	P_{CO_2} (kPa)	P_{O_2} (kPa)	Base excess (mEq/L)
RA	7.41 (7.36–7.43)	5.0 (4.5–5.9)	13.3 (12.8–14.3)	–0.6 (–6.6–3.3)
O_2	7.33 (7.20–7.40)*	7.0 (4.8–10.6)*	58.7 (52.7–62.0)†	–0.5 (–3.9–5.4)
O_2 -NO	7.43 (7.09–7.51)‡	5.3 (4.3–14.4)‡	54.3 (41.6–67.5)†	2.1 (–3.5–6.0)

Values shown as median (range).

* $p < 0.05$ vs RA group.

† $p < 0.01$ vs RA group.

‡ $p < 0.05$ vs O_2 group.

Table 3. P450 arachidonic acid metabolites in BAL fluid

Group	8,9-EET (ng/mL)	11,12-EET (ng/mL)	14,15-EET (ng/mL)	Total EET* (ng/mL)
RA (<i>n</i> = 4)	0.056 (0.028–0.068)	0.112 (0.020–0.126)	0.257 (0.073–0.317)	0.446 (0.122–0.476)
O ₂ (<i>n</i> = 7)	0.136 (0.039–0.476)	0.152 (0.064–0.896)	0.176 (0.075–0.548)	0.446 (0.228–1.912)
O ₂ -NO (<i>n</i> = 7)	0.059 (0.028–0.178)	0.117 (0.072–0.178)	0.195 (0.077–0.326)	0.435 (0.214–0.681)

Values shown as median (range).

* Sum of 8,9-, 11,12-, and 14,15-EET.

inhaled NO (Fig. 2). One explanation for the discordant effects of inhaled NO on P_{CO_2} and a/A ratio in lambs exposed to high oxygen concentrations is that inhaled NO may impair hypoxic regulation of the matching of perfusion to ventilation in poorly ventilated atelectatic areas of the lung. This effect has been demonstrated in humans with chronic obstructive pulmonary disease (16), in dogs with experimentally induced intrapulmonary shunt and ventilation-perfusion mismatch (17), and in horses during general anesthesia (18).

The finding that inhaled NO significantly reduced the PMN count in BAL compared with lambs breathing high oxygen without NO implies that NO may exert an anti-inflammatory effect in the context of this study. Increased pulmonary capillary permeability is a well-established component of hyperoxic lung injury and appears, at least in part, to be mediated by neutrophils (19). Inhaled NO has been shown to reduce lung neutrophil accumulation in severe experimental hyaline membrane disease (20), and to prevent neutrophil-mediated, oxygen radical-dependent leak in isolated rat lung (21) and in lung of intact rabbits (22). Other studies have shown that endogenous NO also has an inhibitory effect on neutrophil function (23).

Not all studies of the effects of inhaled NO on lung injury in prolonged hyperoxia have shown protective effects, and some have shown effects that are potentially damaging. For example, inhaled NO may prevent up-regulation of superoxide dismutase and catalase activity in piglets during prolonged hyperoxia (24). In another study by the same group, inhaled NO had no effect on pulmonary matrix degradation and the increased lung collagen content seen in piglets exposed to prolonged hyperoxia (25). Even though survival was improved, inhaled NO had no effect on lung neutrophil accumulation in rats exposed to hyperoxia for 60 h (26). These differences seen in NO effect from study to study may not necessarily override the clinical efficacy of inhaled NO to prevent or ameliorate lung injury. In a randomized, double-blind, placebo-controlled study of the effect of inhaled NO during the first week of life in premature infants who were undergoing mechanical ventilation for the respiratory distress syndrome, infants receiving NO therapy had a significant reduction in the incidence of death or chronic lung disease compared with the placebo group (27). This promising finding awaits confirmation by other trials presently underway.

We had expected the markers of lipid peroxidation to be elevated with exposure to high oxygen concentrations for several reasons. F₂-isoprostanes and isofurans have been shown to be highly sensitive and specific markers of lipid peroxidation in other contexts (10). Newborn infants with lung disease requiring treatment with high concentrations of in-

spired oxygen have elevated F₂-isoprostane levels in tracheal aspirate fluid when compared with infants not being treated with added oxygen (unpublished data). Measurements of F₂-isoprostanes in plasma have been used as evidence of oxidative stress in full-term healthy infants (28), and plasma 8-isoprostane has been shown to predict bronchopulmonary dysplasia and periventricular leukomalacia in premature infants (29). In addition, F₂-isoprostanes in BAL have been used as a biomarker of oxidative stress in patients with interstitial lung disease (30). It is possible, however, that a hyperoxia-induced increase in these compounds did occur in the present study, but that the increase was transient and their levels returned to baseline before the BAL and lung tissue samples were obtained. It has also been shown that in contrast to isofuran formation, F₂-isoprostane formation becomes limited in high oxygen tension (11), a phenomenon that may limit the value of the F₂-isoprostanes as a marker of oxidant lung injury.

We had also expected to find elevated levels of CYP arachidonic acid metabolites in BAL fluid from lambs in the O₂ group compared with lambs in the RA group. Newborn premature infants with lung disease requiring oxygen therapy have evidence of increased CYP activity in that their tracheal fluid aspirates have elevated levels of certain EET derivatives compared with infants not requiring oxygen therapy (unpublished data). It is not clear why the lambs in the O₂ group in this study did not also have elevated EET levels in BAL fluid compared with lambs in the RA group. Again, it is possible that the EET levels were only transiently elevated before obtaining the BAL samples. In regard to this possibility, it is interesting that in lamb lung, the oxygen-induced increase in CYP1A1 gene expression *in vitro* and *in vivo* was back to baseline levels by 48 h after peaking at 24 h of high oxygen exposure (2).

A limitation of this study is the small number of lambs from which lung tissue levels of F₂-isoprostanes and isofurans were measured. On the other hand, the analyses of BAL F₂-isoprostanes and EETs were sufficiently powered to detect differences in these markers that were seen previously in tracheal aspirates from premature infants exposed to either room air or added oxygen (unpublished data).

In conclusion, hypercarbia and inflammation accompanying hyperoxic lung injury in young lambs can be attenuated by inhaled NO. There was no direct evidence that these beneficial effects of NO resulted from the inhibition of CYP-mediated oxidant injury to the lung inasmuch as the biochemical markers of CYP activity and lipid peroxidation did not change with either oxygen exposure or inhaled NO.

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