Chronic O₂ exposure in the newborn rat results in decreased pulmonary arterial nitric oxide release and altered smooth muscle response to isoprostane


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Chronic O₂ exposure induces pulmonary hypertension and increased lipid peroxidation in the newborn rat (14). The mechanisms responsible for these changes are not fully understood; however, recent evidence from our laboratory (10, 11) indicates that endothelin-1, and 8-iso-prostaglandin F₂α (8-iso-PGF₂α) may be involved in this process. Isoprostanes are produced as a result of peroxidation of arachidonic acid by reactive O₂ species (12). The most widely studied isoprostane molecule is 8-iso-PGF₂α (12). In the adult rat pulmonary artery, this isoprostane isomer has been shown to have dual effects, inducing vasoconstriction in relaxed vessels and relaxation at lower concentrations in precontracted vessels (13). 8-iso-PGF₂α-induced relaxation of vascular smooth muscle is dependent on nitric oxide (NO) release because it can be suppressed by a NO synthase (NOS) inhibitor (13).

Pulmonary hypertension in humans is associated with increased lipoperoxidation (5), yet little is known about effects of the isoprostanes on pulmonary vascular smooth muscle in this condition. Induction of oxidative stress in adult rats causes severe systemic hypertension and depressed NO availability (23), suggesting that isoprostanes may modulate the regulation of vascular resistance. To the best of our knowledge, an effect of 8-iso-PGF₂α on pulmonary arteries in neonatal pulmonary hypertension has not been studied. Thus the purpose of this study was to evaluate the pulmonary arterial NO pressor and relaxant effect of 8-iso-PGF₂α in a newborn hyperoxia-induced chronic lung injury model of pulmonary hypertension. We have previously reported that increased pulmonary arterial muscle contraction is present in O₂-exposed newborn rats. We therefore hypothesized that 8-iso-PGF₂α-induced force would also be increased.

We also evaluated vascular NO production in this model. Hyperoxia has been shown to increase lung endothelial NOS (eNOS) expression (24) but not NO release (6) in adult rats. In the chronically O₂-exposed newborn rat, we have recently documented sequestration of NO, due to peroxynitrite formation (9). Thus we hypothesized that the 8-iso-PGF₂α-induced relaxation of pulmonary arterial muscle is diminished in the newborn rat exposed to chronic hyperoxia.

METHODS

Institutional review. All procedures involving animals were conducted according to criteria established by the Canadian Council for Animal Care. Approval for the study was obtained from the Animal Care Review Committee of the University Health Network, Toronto Western Hospital, and Hospital for Sick Children Research Institute. Exposure system. Pathogen-free, timed-pregnant or nonpregnant female adult Sprague-Dawley rats (250–275 g) were obtained from Charles River (St. Constant, Quebec, Canada). Experiments were conducted as paired exposures with one chamber receiving 60% O₂ and the other receiving air. The animals were randomized to one or the other chamber. O₂ and CO₂ concentrations, temperature, and humidity were continuously monitored, recorded, and regulated by a computer with customized software (AnaWin2 Run-Time, version 2.2.18, Watlow-Anafaze, St. Louis, MO). Gas delivery was regulated by customized hardware (Oxycycler model A84XOV, Biospherix, Redfield, NY) and software (AnaWin2 Run-Time, Watlow-Anafaze), to maintain an O₂ concentration within 0.1% of the set point. O₂ sensors were calibrated weekly. On the anticipated day of delivery, each dam was placed in a 80 × 60 × 50-cm plastic chamber maintained on either air or 60% O₂ with 12:12-h light-dark cycles and the temperature maintained at 25 ± 1°C, a humidity of 50%, and a CO₂ concentration of <0.5%. Equal litter sizes (10–12 pups) were maintained in the paired chambers. Food and water were available ad libitum. Dams were exchanged daily between chambers to prevent maternal O₂ toxicity. Pups were maintained in the chambers (air and O₂) for a 14-day exposure period. Air-treated animals served as controls. At the termination of each exposure period, animals were killed by an overdose of pentobarbital sodium (40 mg/kg ip).

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Organ bath studies. Fourth-generation left lung intralobar pulmonary artery ring segments (average diameter = 100 μm; length = 2 mm) were dissected free and mounted in a wire myograph (Danish Myo Technology). Isometric changes were digitized and recorded online (Myodaq, Danish Myo Technology). Tissues were bathed in Krebs-Henseleit buffer ([in mM] 115 NaCl, 25 NaHCO₃, 1.38 NaHPO₄, 2.51 KCl, 2.46 MgSO₄, 1.91 CaCl₂, and 5.6 dextrose) bubbled with air-6% CO₂ and maintained at 37°C.

After 1 h of equilibration, the optimal resting tension of the tissue was determined by repeated stimulation with 128 mM KCl until maximum active tension was reached. All subsequent force measurements were obtained at optimal resting tension.

Pulmonary vascular muscle contractile responses were normalized to the tissue cross-sectional area as (width × diameter) × 2 (expressed in mN/mm²). The relaxant response to 8-iso-PGF₂α was determined by precontracting with 128 mM KCl. The thromboxane A₂ (TXA₂)/prostaglandin H₂ (TP) receptor blocker L-705956 (6 × 10⁻⁶ M) and the NOS inhibitor N⁵-nitro-L-arginine methyl ester (L-NAME; 10⁻⁵ M) were used.

Ex vivo vascular NO accumulation. NO accumulation from pulmonary vessels and aorta was measured polarographically at 37°C by using a sealed water-jacketed cell fitted with a NO electrode (ISO-NOP) and monitor (NOMKD, World Precision Instruments, Sarasota, FL). Before each experiment, the electrode was calibrated at the same temperature by using a standard nitrite solution (World Precision FL). Before each experiment, the electrode was calibrated at the same temperature by using a standard nitrite solution (World Precision Instruments). Isometric changes were digitized and recorded online (Myodaq, Danish Myo Technology). Tissues were bathed in Krebs-Ringer phosphate buffer [(in mM) 130 NaCl, 5.9 KCl, 1.4 CaCl₂, 1.3 MgSO₄ and 150 NaHCO₃, pH 7.4] equilibrated in 21% O₂ and maintained at 37°C. After a 5-min equilibration period, NO accumulation was quantitated from the slope of the polarograph output over a 5-min period and expressed as nanomoles per minute. Given that the tissue could not be accurately weighed (<1 mg), we measured the length and diameter of the vessel rings and calculated their area in millimeters squared (length × diameter).

Western blot analysis. Intralobar third- to fourth-generation pulmonary arterial tissue extract protein (30 μg) was applied to 4–20% SDS-polyacrylamide gradient gels and electroblotted to nitrocellulose paper. The efficiency of transfer was verified by staining the gel with Coomassie blue after the transfer. The blot was blocked overnight with 5% nonfat dry milk in Tween-20 Tris-base buffer (10 mM Tris buffer, pH 7.6, 150 mM NaCl, and 0.05% Tween 20). The blots were incubated at 4°C overnight with 1:1,000 eNOS or inducible NOS (iNOS) (Transduction Laboratories, Lexington, KY) monoclonal antibodies and with 1:5,000 secondary antibody for 1 h at room temperature. For eNOS, the secondary antibody was goat anti-mouse IgG (heavy + light chains)-alkaline phosphatase ([H+L]-AP) conjugate (Bio-Rad Laboratories, Hercules, CA), and for the iNOS the antiamouse IgG horseradish peroxidase (Amersham, Arlington Heights, IL) was utilized. The cross-reactivity of the iNOS antibody was detected by chemiluminescence by using a mixture of equal volumes of enhanced luminol reagent and oxidizing reagent (enhanced chemiluminescence Western blotting analysis system, Amersham). For eNOS, after washing with Tween-20 Tris-base sodium, the blots were developed with the nitro blue tetrazolium-5-bromo-4-chloro-3-indolyl phosphate p-toluidine salt substrate (Roche Diagnostics, Laval, Quebec, Canada). Quantification was obtained by two-dimensional scanning of the autoradiograms of the blots with a high-resolution scanner (ImageMaster Analyser, Pharmacia, Peapack, NJ). The results are expressed as arbitrary units (pixel intensity per cross-sectional area of the band), as read by the scanner.

Immunohistochemistry. Lungs were removed immediately after death, and the pulmonary vascular bed was perfused with phosphate-buffered saline, and inflated (10 cmH₂O pressure) and immersed in 4% paraformaldehyde in a 0.2 M sodium phosphate buffer (pH 7.4).

The tissue was postfixed and embedded in paraffin and further processed for immunohistochemistry by using standard techniques.

Primary antibodies eNOS and iNOS (Transduction Laboratories) were used at a dilution of 1:100 and incubated overnight at 4°C. The secondary antibody goat anti-mouse IgG (H+L) biotinylated (Oncogene Research Products, Boston, MA) was utilized at 1:200 dilution and incubated for 30 min at room temperature. Sections were further incubated in avidin-biotin-peroxidase complex (ABC kit PK-4000, Vectorstain, Vector Laboratories, Burlingame, CA) for 45 min. Color was developed by a 3,3-diaminobenzidine tetrahydrochloride substrate kit (SK-4100 Vector Labs), and sections were counterstained with Carazzi’s hematoxylin before mounting. Negative controls were provided by primary antibody omission. Lung slices from archival material of a newborn rat that was injected intraperitoneally with Escherichia coli serotype 0111:B4 LPS (Sigma Chemical, St. Louis, MO), served as a positive control for the iNOS antibody.

Drugs. L-705956 was obtained from Merck Frost (Kirkland). 8-iso-PGF₂α, was obtained from Cayman Chemical (Ann Arbor, MI). All other chemicals were obtained from Sigma Chemical (Oakville, Ontario, Canada) and dissolved in Krebs-Henseleit buffer.

Sample size and data analysis. Newborn animals utilized in the study were obtained from different litters. Four air-treated and four 60% O₂-exposed litters were studied. The specific number or animals utilized for each experiment is described in the figure legends.

Data were evaluated by Student’s t-test or two-way ANOVA, with multiple comparisons obtained by the Tukey-Kramer test when appropriate. Statistical significance was accepted if P < 0.05. All statistical analysis was performed with the Number Cruncher Statistical System (NCSS, Kaysville, UT). Data are presented as means ± SE.

RESULTS

8-iso-PGF₂α-induced dose-dependent increase in pulmonary artery muscle force development (Fig. 1). A significant increase in 8-iso-PGF₂α-induced force was found in vessels from experimental animals (P < 0.01), relative to controls exposed to air (Fig. 1). In the control group, pretreatment of vessels with the NOS inhibitor L-NAME increased the 8-iso-
PGF<sub>2α</sub>-induced force to levels comparable to the experimental group (Fig. 1).

To test for the 8-iso-PGF<sub>2α</sub>-induced relaxation response, pulmonary arteries were prestimulated with KCl (128 mM). In control arteries, 8-iso-PGF<sub>2α</sub>-induced relaxation was observed only at the highest concentration tested (10<sup>-5</sup> M). However, in the presence of L-670596, a TxA<sub>2</sub> receptor blocker, a significant (P < 0.01) dose-dependent relaxation was seen at all concentrations, which was suppressed by the addition of L-NAME (Fig. 2). When 8-iso-PGF<sub>2α</sub>-induced relaxation of air-exposed control and 60% O<sub>2</sub>-exposed experimental arteries were compared in the presence of TP-receptor blockade, the arteries from experimental animals showed a significantly decreased (P < 0.01) relaxant response (Fig. 3).

Pulmonary artery and aorta from control and experimental animals were examined for basal NO accumulation (Fig. 4). Compared with air controls, NO accumulation was significantly decreased in the pulmonary arteries (P < 0.05) and aortas (P < 0.01) of the experimental animals (Fig. 4). The group differences were not related to the amount of tissue present in the polarographic chamber, because the pulmonary arterial and aorta tissue area for the control (5.5 ± 0.4 and 7.1 ± 0.4 mm<sup>2</sup>, respectively) and experimental (5.8 ± 1.2 and 10.2 ± 1.8 mm<sup>2</sup>, respectively) groups were not statistically different.

Finally, we evaluated pulmonary arterial NOS protein content (Figs. 5 and 6). As shown in Fig. 5 by Western blot and Fig. 6 by immunohistochemistry, we found no differences in eNOS content between control and experimental groups. The tissue iNOS protein content was significantly reduced (P < 0.01) in the 60% O<sub>2</sub>-exposed animals (Fig. 5) but was below detection limits by immunohistochemistry in the lungs of both groups (Fig. 6).

**DISCUSSION**

Exposure of newborn rats to 60% O<sub>2</sub> for 14 days results in pulmonary vascular remodeling and changes in pulmonary arterial smooth muscle contractile properties (2, 8). Our group has recently reported (9) that enhanced reactive O<sub>2</sub> species-mediated inactivation and sequestration of NO, attributed to peroxynitrite formation, is present in the lungs of hyperoxia-exposed newborn rats on the basis of increased lung nitrotyrosine formation. In this study, together with a reduced pulmonary vascular iNOS protein content, we have confirmed that NO release is significantly decreased in experimental animals. This finding, which indicates decreased functional release of NO, may well account for alterations in pulmonary arterial smooth muscle response to 8-iso-PGF<sub>2α</sub> in hyperoxia-exposed animals.

8-iso-PGF<sub>2α</sub> has a dual effect on the pulmonary arterial muscle of the adult rats: it may cause either contraction of relaxed vessels or relaxation of precontracted vessels in a

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**Fig. 2.** 8-iso-PGF<sub>2α</sub> dose-response curves for the control animals’ pulmonary arterial muscles precontracted with 128 mM KCl in the absence (n = 7) and presence of thromboxane A<sub>2</sub>prostaglandin H<sub>2</sub> (TP) receptor (L-670596) blockade [TP(-); n = 4] as well TP receptor and NO synthase (NOS) blockade [TP(-) + L-NAME; n = 4]. **P < 0.01 by 2-way ANOVA compared with other group values. The KCl-induced stress was 2.3 ± 0.1, 1.9 ± 0.1, and 2.2 ± 0.1 mN/mm<sup>2</sup> in the control, TP(-), and TP(-) + L-NAME groups, respectively. Note that the 8-iso-PGF<sub>2α</sub>-induced relaxation is unmasked after TP-receptor blockade.

**Fig. 3.** 8-iso-PGF<sub>2α</sub>-induced relaxation for the control (n = 7) and 60% O<sub>2</sub>-treated (n = 4) animals’ pulmonary arterial muscles precontracted with 128 mM KCl in the presence of TP-receptor blocker (L-670596). The KCl-induced stress was 2.3 ± 0.1 and 4.1 ± 0.2 mN/mm<sup>2</sup> in the control and 60% O<sub>2</sub>-treated groups, respectively. Note that the 8-iso-PGF<sub>2α</sub>-induced relaxation is significantly decreased (**P < 0.01) in the O<sub>2</sub>-treated animals.

**Fig. 4.** NO release from control and 60% O<sub>2</sub>-treated pulmonary arterial (n = 4 and n = 6, respectively) and aortic (n = 4 and n = 9, respectively) tissue. **P < 0.01, *P < 0.05 compared with control values. Note that pulmonary arterial and aorta tissue NO releases are significantly reduced in the experimental animals.
concentration-dependent manner. The relaxant response to 8-iso-PGF\(_2\alpha\) in the presence of TP-receptor blockade is consistent with there being two receptors for this isoprostane (7). The TP receptor is localized on the smooth muscle membrane and is involved in the contraction response. The other receptor has not been characterized, but it is believed to be located on the endothelial cell membrane and responsible for the 8-iso-PGF\(_2\alpha\)-induced relaxation. Our laboratory has previously demonstrated...

Fig. 5. Western blot analysis of pulmonary arterial tissue from control (n = 4) and 60% \(\text{O}_2\)-treated animals (n = 4) for endothelial NOS (eNOS) and inducible NOS (iNOS). Although no change in eNOS protein content was observed, 60% \(\text{O}_2\) treatment resulted in a significant decrease (**P < 0.01) in iNOS protein content.

Fig. 6. Representative pulmonary arterial immunohistochemistry for eNOS and iNOS. No obvious group staining difference was noted for eNOS. iNOS immunostaining was absent in the control (C) and 60% \(\text{O}_2\)-treated groups but present in the positive-control (+control) sample of a pulmonary arterial vessel of an Escherichia coli LPS-treated newborn rat. Magnification = ×100, ×200, and ×400, as illustrated.
shown (3) that the newborn pulmonary vascular muscle response to 8-iso-PGF_2α is clearly distinct from the adult. In the newborn, 8-iso-PGF_2α induces less muscle contraction than the adult, and its relaxant effect is only evident after TP-receptor blockade.

In this study, we have shown that blockade of NOS with l-NAME resulted in increased 8-iso-PGF_2α-induced force and decreased relaxation of precontracted control pulmonary arteries. These changes mirror the pattern observed in pulmonary arterial tissue of chronic hyperoxia-exposed animals. Such evidence, in conjunction with the documented reduced NO release from pulmonary arteries of experimental animals, suggests that the altered response to 8-iso-PGF_2α was caused by an effect of chronic hyperoxia on the pulmonary vascular NO metabolism.

Conflicting data exist concerning the effect of chronic hyperoxia on NO production in the adult rat. A <72-h exposure to 85% O_2 in adult rats increased lung iNOS but not eNOS content without an effect on exhaled NO content (6). Exposure to 85% O_2 for 28 days resulted in increased lung vascular tissue eNOS content and activity (21), whereas 95% O_2 exposure for up to 5 days caused a decrease in eNOS activity (1) in adult rats. Isolated and perfused adult lungs exposed to 90% O_2 for 48 h showed loss of the hypoxic pulmonary vasoconstrictor response, which was interpreted as being related to upregulation of NO production by the lung (22).

In 3-day-old rats exposed to 95% O_2 for up to 14 days, eNOS activity was decreased, but the lung tissue protein content increased, whereas iNOS activity increased and its protein content decreased, compared with age-matched controls (18). Ours is only the second study to address the effect on chronic hyperoxia on the pulmonary vascular tissue NOS content of newborn rats. It contrasts with the former study in subjecting the newborn rats to 60% O_2 for 14 days starting from birth. As mentioned previously, no changes in eNOS protein content or lung immunohistochemical staining were noted, but iNOS protein content was significantly reduced in experimental animals’ pulmonary vascular tissue in the present study, in keeping with the findings of Radomsky et al. (18). The mechanism accounting for the decrease in the pulmonary artery iNOS content is presently unclear. The apparent discrepancy between the absent immunostaining but positive Western results for iNOS likely reflects the low tissue iNOS protein content, which makes detection by immunohistochemistry technically difficult. It is clear that the antibody utilized in this study can recognize iNOS in paraffin-prepared tissue, because positive immunostain was present in the LPS-stimulated positive control newborn lung. Although not measured in this study, the pulmonary arterial eNOS activity and phosphorylation in the 60% O_2-exposed newborns is likely normal given our previously reported data (2) in this model relative to unaltered acetylcholine-induced relaxation in these vessels.

Last, the aorta NO release in the experimental animals was significantly lower than the control tissue, which suggests that chronic hyperoxia has a similar effect on systemic vascular NO metabolism, as observed in the pulmonary vessels. This finding is not surprising given that endothelial-derived NO is reduced in vascular diseases such as systemic hypertension in humans (17) and animal models of atherosclerosis (20). Furthermore, induction of chronic oxidative stress by inhibition of glutathione synthase induces systemic hypertension in adult rats via a mechanism involving NO inactivation by reactive O_2 species (24).

The novel data reported in this study may be relevant to our understanding of the pathogenesis of pulmonary hypertension in the human neonate. Pulmonary disease-induced lipid peroxidation (4, 15, 16) and the therapeutic use of supplemental O_2 (19) result in isoprostane production in the lung. At low concentrations, isoprostanes may have a physiological role in the lung by promoting pulmonary vasodilation via NO release (12). Yet, in pulmonary hypertension, where an altered vascular NO metabolism is present, increased 8-iso-PGF_2α and possibly other isoprostane production will promote pulmonary vascular muscle contraction, contributing to the maintenance of a high pulmonary vascular resistance in this disease.

In conclusion, we have shown that chronic exposure to 60% O_2 in newborn rats results in decreased pulmonary vascular tissue NO release. This finding likely accounts for the observed enhanced pulmonary arterial muscle contraction and reduced relaxation in response to 8-iso-PGF_2α. Considering that the lung levels of isoprostane are markedly increased in experimental animals exposed to 60% O_2, these changes are likely to play a major role in the pathogenesis of pulmonary hypertension in this animal model.

REFERENCES


