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Endothelin-1 and O_2-Mediated Pulmonary Hypertension in Neonatal Rats: A Role for Products of Lipid Peroxidation

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ABSTRACT

We hypothesized that reactive O_2 species, or their intermediary products, generated during exposure to elevated O_2 lead to pathologic endothelin-1 expression in the newborn lung. Endothelin-1 expression and 8-isoprostane content (an *in vivo* marker of lipid peroxidation) were examined and found to be elevated ($p < 0.05$) in the lungs of newborn rats with abnormal lung morphology and pulmonary hypertension, as assessed by right ventricular hypertrophy, after a 14-d exposure to 60% O_2. The antioxidant and lipid hydroperoxide scavenger, U74389G (10 mg/kg), given by daily i.p. injection prevented O_2-dependent right ventricular hypertrophy ($p < 0.05$ compared with vehicle-treated controls), but had no effect on abnormal lung morphology. Additionally, we observed that 8-isoprostane caused marked endothelin-1 mRNA up-regulation *in vitro* in primary rat fetal lung cell cultures. We conclude that reactive O_2 species, or their bioactive intermediaries, are causative in O_2-mediated pulmonary hypertension and endothelin-1 up-regulation. It is likely that the bioactive lipid peroxidation product, 8-isoprostane, plays a key role in pathologic endothelin-1 expression and pulmonary hypertension during oxidant stress. **(*Pediatr Res* 48: 289–298, 2000)**

Abbreviations

BPD, bronchopulmonary dysplasia
ET-1, endothelin-1
GAPDH, glyceraldehyde 3-phosphate dehydrogenase
RVH, right ventricular hypertrophy

The use of sustained supplemental O_2 and mechanical ventilation in premature infants with respiratory failure is associated with the development of bronchopulmonary dysplasia (BPD), a chronic neonatal lung injury (1). Characteristic features of BPD include a dysregulation of lung growth, including fibroproliferative changes (2). This condition remains an important cause of mortality and morbidity during early infancy and in later childhood (3). There are currently no rational or effective therapies available for the prevention or treatment of BPD and its accompanying complications. The putative central role for O_2 toxicity in the pathogenesis of BPD has led to considerable interest in the use of antioxidant therapies as preventive and therapeutic agents (4).

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Pulmonary hypertension is a common complication in infants with established BPD (5) and early respiratory failure (6), and is an important adverse factor in their outcome (7). An increased expression of ET-1 is observed in adult patients with pulmonary hypertension (8), which may play a causative role. The endothelins are a family of 21-amino acid peptides that are the most potent vasoconstrictor substances yet described (9). ET-1 is the dominant form in the lung and is expressed by multiple cell types, principally vascular endothelium and bronchial epithelium (10, 11). Both ET_A and ET_B receptors mediate the effects of ET-1. These receptors are both expressed in vascular and bronchial smooth muscle cells (11, 12), whereas vascular endothelial cells express only ET_B receptors (13).

Factors known to up-regulate ET-1 include hypoxia (14, 15), proinflammatory cytokines (16), and shear stress (17). We have recently described a newborn rat model with BPD-like histopathology (18) and pulmonary hypertension (19) induced by exposure to 60% O_2 for 14 d. Current evidence implicates reactive O_2 species, generated in excess of antioxidant defenses, either directly or through the generation of bioactive

intermediary products, in the pathogenesis of pulmonary O_2 toxicity (20–22). Whether reactive O_2 species and reactive O_2 species-induced products directly stimulate ET-1 synthesis in the lung is not known.

We hypothesized that hyperoxia leads to lung injury, pulmonary vascular abnormalities, and ET-1 up-regulation in this model through the increased production of reactive O_2 species (21), which may exert their effect through the peroxidation of membrane lipids and the consequent release of reactive products such as lipid hydroperoxides and 8-isoprostane (22). To test this hypothesis, we examined the effect of 60% O_2 on ET-1 expression in neonatal rat lung tissue and the modifying effect of the synthetic 21-aminosteroid (lazaroid) compound, U74389G. The 21-aminosteroids are derivatives of methylprednisolone that are devoid of glucocorticoid activity (23). This class of compounds is lipophilic and has been reported to scavenge lipid hydroperoxide and superoxide radicals, and to effectively inhibit iron-dependent hydroxyl radical formation and lipid peroxidation (24–27). U74389F, another 21-aminosteroid with a different spectrum of antioxidant properties, has been reported to protect against the alveolar structural changes and pulmonary hypertension observed in neonatal rats exposed to 95% O_2 (28).

METHODS

Materials. U74389G and CS-4 vehicle (20 mM citric acid monohydrate, 3.2 mM sodium citrate dihydrate, 77 mM NaCl, pH 3.0) were kindly provided by Dr. D. Zimmerman (Pharmacia & Upjohn, Kalamazoo, MI, U.S.A.). Radioisotopes and nylon membranes were purchased from Amersham Canada (Oakville, Ontario), and restriction enzymes were from Amersham Pharmacia Biotech (Baie D'Urfé, Québec). BSA type V, Ficoll 400, polyvinylpyrrolidone, and guanidine thiocyanate were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Herring sperm DNA was from Roche Molecular Biochemicals (Laval, Québec). Organic solvents were of HPLC grade. A partial prepro-ET-1 cDNA probe (insert size, 543 bp) was synthesized and amplified by reverse transcriptase PCR based on the rat pulmonary endothelial cell cDNA sequence, as described by Terada *et al.* (29). A plasmid containing a full-length cDNA (insert size, 1.27 kb) of the rat GAPDH (EC 1.2.1.12) was a generous gift from Dr. M. Rabinovitch (University of Toronto, Ontario). Rabbit antiserum to human ET-1 was from Biochem Immunosystems (Montreal, Québec), and goat anti-rabbit IgG-biotin antibody was from GIBCO (Burlington, Ontario). Mouse MAb to α-smooth muscle actin was obtained from Lab Vision (Fremont, CA, U.S.A.), and sheep anti-mouse IgG-biotin antibody was from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Avidin-biotin-peroxidase and alkaline phosphatase complex immunohistochemistry kits were purchased from Vector Laboratories (Burlingame, CA, U.S.A.). 8-Isoprostane and an 8-isoprostane enzyme immunoassay kit were from Cayman (Ann Arbor, MI, U.S.A.), total protein assay kit was from Bio-Rad (Hercules, CA, U.S.A.), and Sep Pak C18 cartridges were from Waters (Mississauga, Ontario).

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 Samuel Lunenfeld Research Institute, Mount Sinai Hospital.

Exposure system. This has been described in detail previously (18). Briefly, pathogen-free timed pregnant Sprague Dawley rats (250–275 g) were obtained from Charles River (St. Constant, Quebec). Experiments were conducted as paired exposures with one chamber receiving 60% O_2 and the other receiving air. On the anticipated day of delivery, each dam was placed in a 60 × 48 × 25-cm plastic chamber with 12 h/12 h light-dark cycles, a temperature maintained at 25 ± 1°C, minimal humidity, and a CO_2 concentration of < 0.5%. Food and water were available *ad libitum*. Equal litter sizes (10–12 pups) were maintained between paired chambers. Dams were exchanged daily between chambers to prevent maternal O_2 toxicity. At the termination of each exposure period, animals were killed by ether inhalation.

Interventions. Pups were maintained in four paired chambers (air and O_2) for a 14-d exposure period, each pair receiving either CS-4 vehicle (vehicle control) or U74389G in CS-4 vehicle (2 mg/mL and 10 mg/kg/d). Daily injections (5 μL/g), commenced within 6 h of birth, were via a 30-gauge needle into the right iliac fossa.

Examination of RVH. RVH is a well-established index of pulmonary hypertension (30) and has been shown to have a direct correlation with vascular smooth muscle hyperplasia in this model (19). At death, the thoracic contents were removed *en bloc*. The heart was then separated from the lungs, and the right ventricle was dissected free from the left ventricle and septum. Each component was freeze-dried and weighed separately.

Isolation of lung RNA. The lungs were dissected away from large vessels and airways to be flash frozen in liquid N_2 immediately after weighing. Total (nuclear and cytoplasmic) RNA was isolated by lysis of tissue in 4 M guanidine thiocyanate, followed by phenol/chloroform extraction according to the method of Chomsczynski and Sacchi (31).

RNA analyses. For Northern blot analyses, 20 μg of total lung RNA was fractionated on 1.3% (wt/vol) agarose gels containing 0.66 M formaldehyde and transferred to a nylon membrane. Prepro-ET-1 and GAPDH cDNAs were labeled

Table 1. *Effect of U74389G on survival rate, body weights, and wet lung weights after a 14-d exposure to 60% O_2*

Treatment group	Survival rate (%)	Body weight (g)	Lung wet weight (mg)
Air/Control	97.92 ± 2.08	28.22 ± 0.73	456.62 ± 14.01
Air/U74389G	97.92 ± 2.27	25.99 ± 0.49†	394.60 ± 10.65†
60% O_2/Control	91.47 ± 3.41	27.48 ± 0.61	422.05 ± 8.82
60% O_2/U74389G	89.39 ± 2.03*	24.86 ± 0.39†	396.49 ± 7.70

Newborn rats were exposed to room air or 60% O_2 for 14 d, and were treated with daily i.p. injections of either 10 mg/kg of U74389G in CS-4 vehicle, or CS-4 vehicle alone. Survival data are presented as the mean ± SEM for four litters (46 pups in total) and other data as mean ± SEM for 12 pups from a single litter. * $p < 0.05$, by one-way ANOVA, compared with air-exposed animals given the same treatment. † $p < 0.05$, by one-way ANOVA, compared with vehicle controls given the same gas.

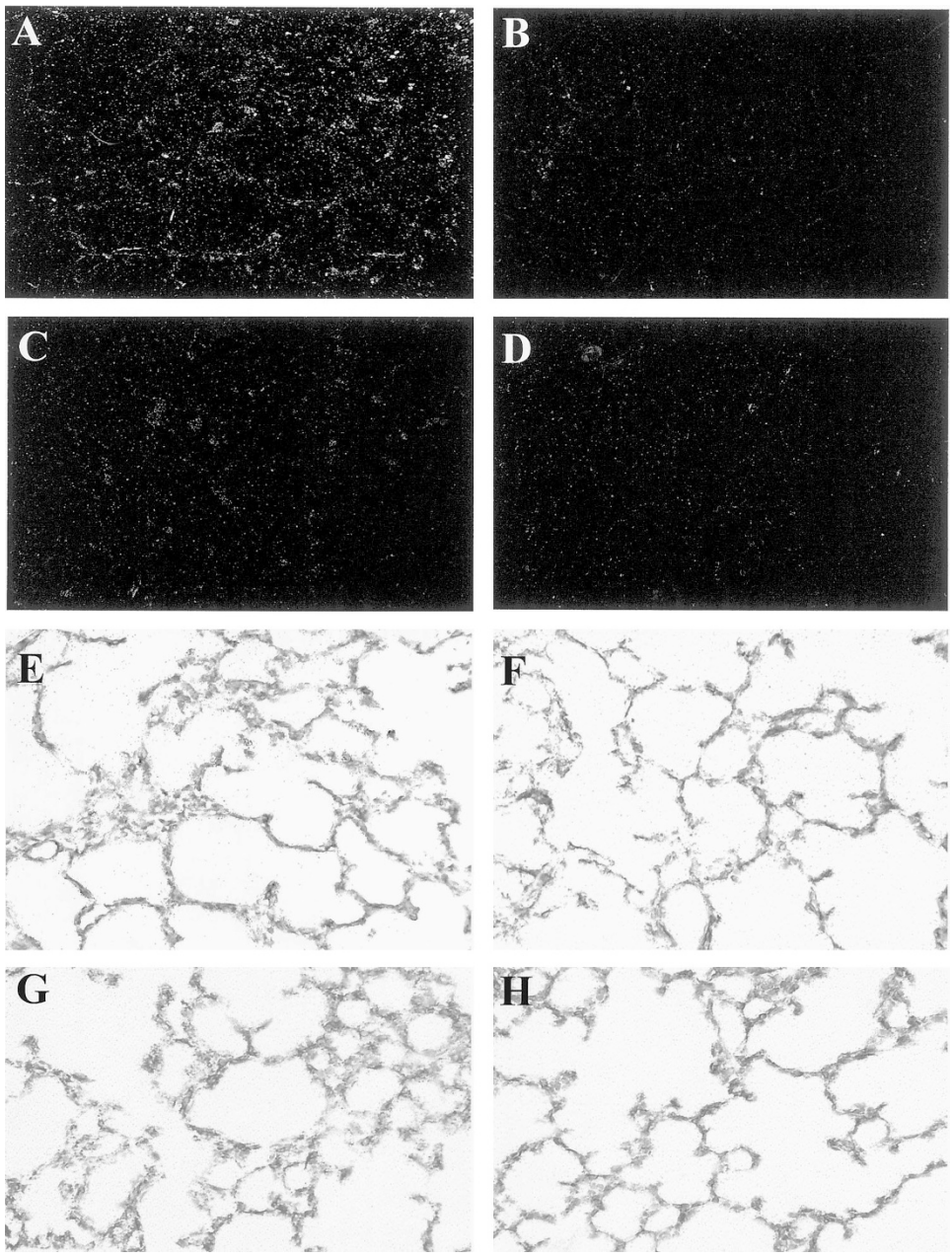

Figure 1. [^{3}H]Thymidine autoradiography of lung tissue after exposure to air or 60% O_2 for 14 d. Newborn rats received daily i.p. injections of 10 mg/kg U74389G in CS-4 vehicle or CS-4 vehicle alone. Dark- (*A, B, C,* and *D*) and bright- (*E, F, G,* and *H*) field microscopy is shown. Original magnification, ×200. *A, E,* air-exposed animals that received vehicle. *B, F,* 60% O_2-exposed animals that received vehicle. Reduced DNA synthesis is evident compared with air-exposed animals. *C, G,* air-exposed animals that received U74389G. Markedly reduced DNA synthesis is evident compared with vehicle-treated animals. *D, H,* 60% O_2-exposed animals that received U74389G. No recovery of DNA synthesis was observed in U74389G-treated animals.

with deoxycytidine 5′-[α-^{32}P]triphosphate by a random priming labeling system (Amersham, Arlington Heights, IL, U.S.A.), with specific activities of 0.5–2.9 × 10^9 counts·min^{-1}·μg DNA^{-1}. For GAPDH mRNA, prehybridization (> 4 h) and hybridization (overnight) were performed in 50% (vol/vol) formamide, 5× SSPE, [750 mM NaCl, 50 mM sodium phosphate, 5 mM EDTA, pH 7.4], 5× Denhardt's solution [0.1% (wt/vol) each of BSA, Ficoll, and polyvinylpyrrolidone], 0.5% (wt/vol) SDS, and 100 μg/mL denatured herring sperm DNA at 42°C. For prepro-ET-1 mRNA, prehybridization and hybridization were performed in 5× SSPE, 5× Denhardt's solution, 0.5% (wt/vol) SDS, and 100 μg/mL denatured herring sperm DNA at 65°C. After hybridization, GAPDH blots were washed in 2× SSC [300 mM NaCl, 30 mM sodium citrate, pH 70]; 0.1% SDS for 10 min at room temperature. Prepro-ET-1 blots were washed in 2× SSC; 0.1% SDS for 10 min at room temperature followed by 1× SSC; 0.1% SDS for 10 min at 65°C. All blots were exposed for 24–72 h at −70°C to Kodak Biomax MS film and intensifying screen (Eastman Kodak, Rochester, NY, U.S.A.). The films were electronically scanned and the band densities were quantified using Scion Image software (Version 1.6, National Institutes of Health, Bethesda, MD, U.S.A.). To correct for variations in RNA

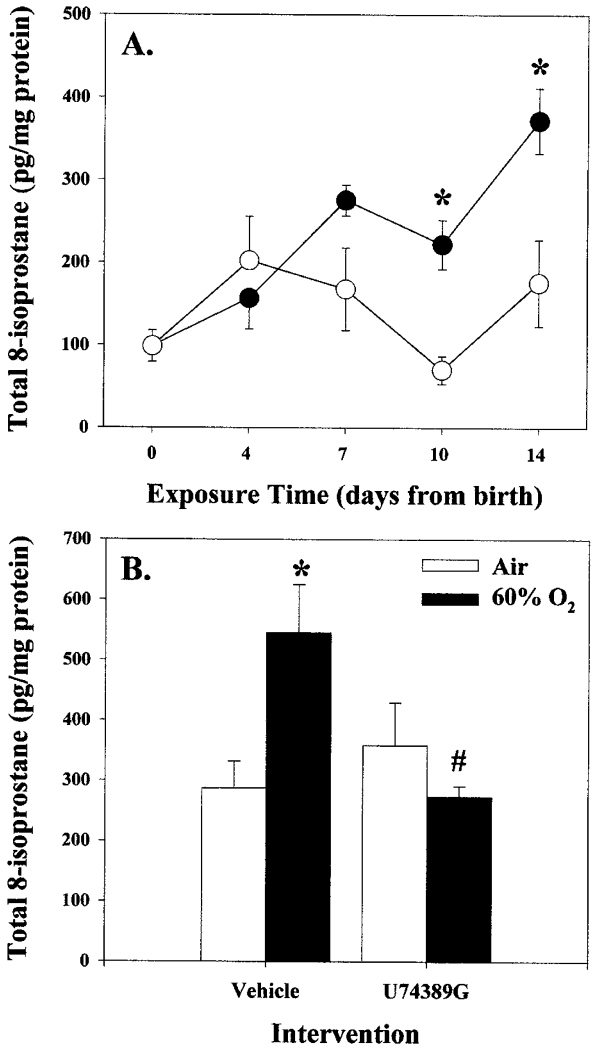

Figure 2. Total (free and esterified) 8-isoprostane in lung tissue. *A,* the effect of air (○) or 60% O_2 (●) on total 8-isoprostane content in lungs from newborn rats. *B,* total 8-isoprostane content in lungs after exposure to air (*open bars*) or 60% O_2 (*filled bars*) for 14 d. Newborn rats received daily i.p. injections of 10 mg/kg U74389G in CS-4 vehicle or CS-4 vehicle alone. Plot points and bars represent mean ± SEM for four litters. $*p < 0.05$, by one-way ANOVA, for animals exposed to 60% O_2 compared with those in air in the same treatment group at d 14. $\#p < 0.05$, by one-way ANOVA, for animals exposed to 60% O_2 for 14 d and treated with U74389G in CS-4 vehicle, compared with those in 60% O_2 and treated with CS-4 vehicle alone.

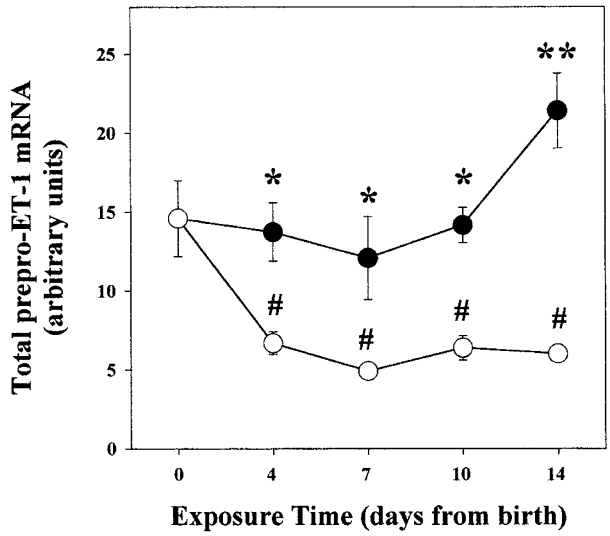

Figure 3. The effect of air (○) or 60% O_2 (●) on whole lung mRNA for prepro-ET-1 in newborn rats, as assessed by Northern analysis. All data points represent the mean ± SEM for four litters. Where error bars are not evident, they fall within the plot point. $*p < 0.05$, by one-way ANOVA, for 60% O_2-exposed animals compared with air controls at the same time. $\#p < 0.05$, by one-way ANOVA, for air-exposed animals compared with all other groups. $**p < 0.05$, by one-way ANOVA, for 60% O_2-exposed animals at d 14 compared to d 7 and d 10.

loading of gels and transfer to membranes, all results were normalized to GAPDH mRNA, which we determined to be unaffected by O_2 exposure.

Immunohistochemistry and autoradiography. Animals were anesthetized with i.p. ketamine (80 mg/kg) and xylazine (20 mg/kg). The pulmonary circulation was flushed with PBS containing 1 U/mL heparin to clear the lungs of blood and fixed with 4% (wt/vol) paraformaldehyde by perfusion under a constant airway pressure of 10 cm H_2O via a tracheal catheter. For autoradiography, animals received 1 μCi/g i.p. [^{3}H]thymidine 2 h before lung fixation. Before immunohistochemical staining, lung sections were examined to confirm a normal lung structure from air-exposed control pups and the presence of patchy areas of interstitial thickening and emphysema in 60% O_2-exposed pups (18). When lung sections were examined for the presence of ET-1, dilutions of the primary and secondary antisera were 1:650 and 1:520, respectively. When lung sections were examined for the presence of α-smooth muscle actin, dilutions of the primary and secondary antisera were 1:1000 and 1:300, respectively. Antibody specificity was verified by omitting the primary antiserum. After completion of immunohistochemical studies, using an avidin-biotin-peroxidase complex method (32), slides were lightly counterstained with Carazzi hematoxylin, dehydrated, cleared in xylene, and mounted. Slides were coated with Kodak NBT-3 emulsion for autoradiography and developed after 4 wk at 4°C for examination by light microscopy to identify dense areas of silver granule concentration over cell nuclei undergoing active DNA synthesis.

Lung volume-pressure loops. Animals were anesthetized with sodium pentobarbitone (5–10 mg/kg) and paralyzed with pancuronium bromide (0.3 mg/kg). After tracheotomy, the lungs were degassed and volume-pressure loops in the closed chest recorded as previously described (18).

Total (free and esterified) lung 8-isoprostane measurement. To prevent auto-oxidation, lung tissue was immediately flash frozen in liquid N_2 at harvest and stored at −80°C until analysis. Upon thawing, the tissue sample was homogenized in 1× PBS with 0.005% (vol/vol) butylated hydroxytoluene, and 5000 cpm of [^{3}H]$PGF_{2\alpha}$ was added to quantify recovery during purification. Proteins were precipitated with ethanol and removed by centrifugation. The supernatant was incubated with an equal volume of 15% (wt/vol) potassium hydroxide at 40°C for 1 h for alkaline hydrolysis before solid-phase extraction using Sep Pak C18 cartridges. After purification, samples were analyzed in duplicate for 8-isoprostane content using a commercially available ELISA kit as previously described (33). Recovery from the purification step was analyzed by liquid

scintillation counting of the extract and factored into the calculation along with correction for tissue protein content.

Effect of 8-isoprostane on prepro-ET-1 mRNA expression. Primary mixed lung cell cultures were prepared from d 22 rat fetuses as previously described (34). Briefly, after removal of the heart, major vessels, and airways, the combined lungs were minced with scissors. The tissue was then subjected to sequential enzymatic dissociation, initially using 1 mg/mL trypsin and 0.01 mg/mL DNase. Cells were grown to confluence in Eagles' minimum essential medium with 10% (vol/vol) fetal bovine serum at a gas phase of 21% O_2, 5% CO_2, and 74% N_2. 8-Isoprostane (10, 100, and 1000 nM) was added, and cultures were incubated in 21% O_2 at 37°C in minimum essential medium without serum or antibiotics for 48 h. Cells were gently scraped from the culture flasks for RNA isolation and analysis as previously described.

Data presentation. Unless otherwise stated, all values are for mean ± SEM of four litters. Statistical significance ($p < 0.05$) was determined by one-way ANOVA followed by assessment of differences using Duncan's multiple range test (35). Where error bars are not evident in figures, they fall within the plot point.

RESULTS

The effects of U74389G on survival and wet lung and body weights are shown in Table 1. Animals exposed to 60% O_2 for 14 d had an approximately 10% mortality, which was greater, although not significantly so ($p > 0.05$), than that for air-exposed pups. Treatment with U74389G slightly reduced survival in 60% O_2-exposed animals, such that the increase in mortality in 60% O_2 became statistically significant ($p < 0.05$) when compared with U74389G-treated air controls. Animals in both air and O_2 that had been treated with U74389G had significantly lower body weights and wet lung weights than vehicle-treated animals ($p < 0.05$). U74389G caused a marked reduction in lung cell DNA synthesis of air-exposed animals after 14 d, compared with vehicle-treated animals, when assessed by [^{3}H]thymidine autoradiography (Fig. 1, *A* and *C*). This finding is in keeping with previous observations using proliferating cell nuclear antigen immunohistochemistry (33) as an index of DNA synthesis. U74389G did not lead to an increase in lung cell DNA synthesis of 60% O_2-exposed animals after 14 d, when assessed by [^{3}H]thymidine autoradiography (Fig. 1*D*).

As previously described (18), the lungs of rat pups exposed to 60% O_2 for 14 d have areas of emphysema interspersed with patchy areas of parenchymal thickening and, consistent with these structural changes, abnormal lung mechanics. When compared with these previously reported changes (18), we could not detect any obvious effect of treatment with U74389G on either macroscopic structural changes or on lung mechanics (data not shown).

Lipid hydroperoxide formation in lung tissue, as assessed by measurement of lung 8-isoprostane content, was significantly increased ($p < 0.05$) after exposure to 60% O_2 for 10 and 14 d (Fig. 2*A*). In contrast to gross structural changes, the increase in lipid peroxidation evident after a 14-d exposure to 60% O_2 was completely attenuated ($p < 0.05$) by treatment with U74389G (Fig. 2*B*).

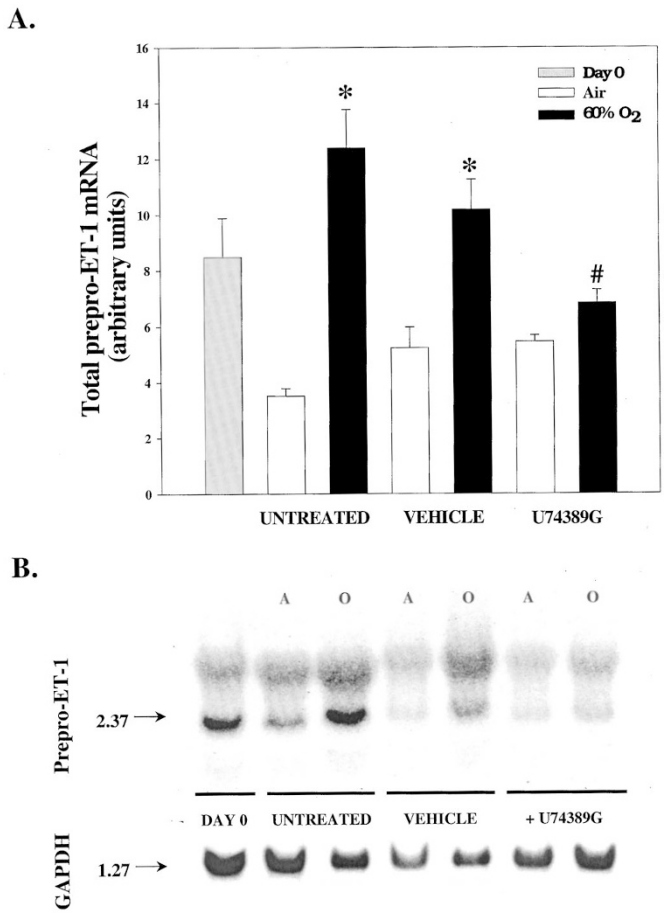

Figure 4. Effect of U74389G on total lung prepro-ET-1 mRNA. *A,* whole lung prepro-ET-1 mRNA after exposure to air (*open bars*) or 60% O_2 (*filled bars*) for 14 d. Newborn rats received daily i.p. injections of 10 mg/kg U74389G in CS-4 vehicle (U74389G), CS-4 vehicle alone (VEHICLE), or no intervention (UNTREATED). Bars represent mean ± SEM for four litters. *$p < 0.05$, by one-way ANOVA, for animals exposed to 60% O_2 compared with those in air in the same treatment group. #$p < 0.05$, by one-way ANOVA, for U74389G-treated animals compared with untreated or vehicle-treated animals exposed to 60% O_2. *B,* Northern blot analysis of whole lung prepro-ET-1 mRNA at birth (DAY 0) and after exposure to air (*A*) or 60% O_2 (*O*) for 14 d. Newborn rats received either no injections (UNTREATED) or daily i.p. injections of either 10 mg/kg U74389G (U74389G) or vehicle alone (VEHICLE). To correct for differences in loading of RNA and transfer to membranes, all results were normalized to GAPDH mRNA. Transcript sizes are in kilobases.

Results for whole lung prepro-ET-1 mRNA expression are shown in Figures 3 and 4, *A* and *B*. Prepro-ET-1 mRNA expression in air-exposed animals declined significantly after birth ($p < 0.05$) and remained low, as would be expected with the normal reduction in pulmonary vascular resistance that occurs after delivery. In contrast, animals exposed to 60% O_2 had a significantly ($p < 0.05$) increased expression of prepro-ET-1 mRNA (similar to levels at birth), relative to air-exposed pups, on d 4, 7, and 10. By d 14 of exposure to 60% O_2, expression of prepro-ET-1 mRNA had increased to values significantly greater than those present at birth (Fig. 3). Rat pups treated with U74389G for 14 d had no evidence of a 60% O_2-mediated increase in prepro-ET-1 mRNA expression (Fig.

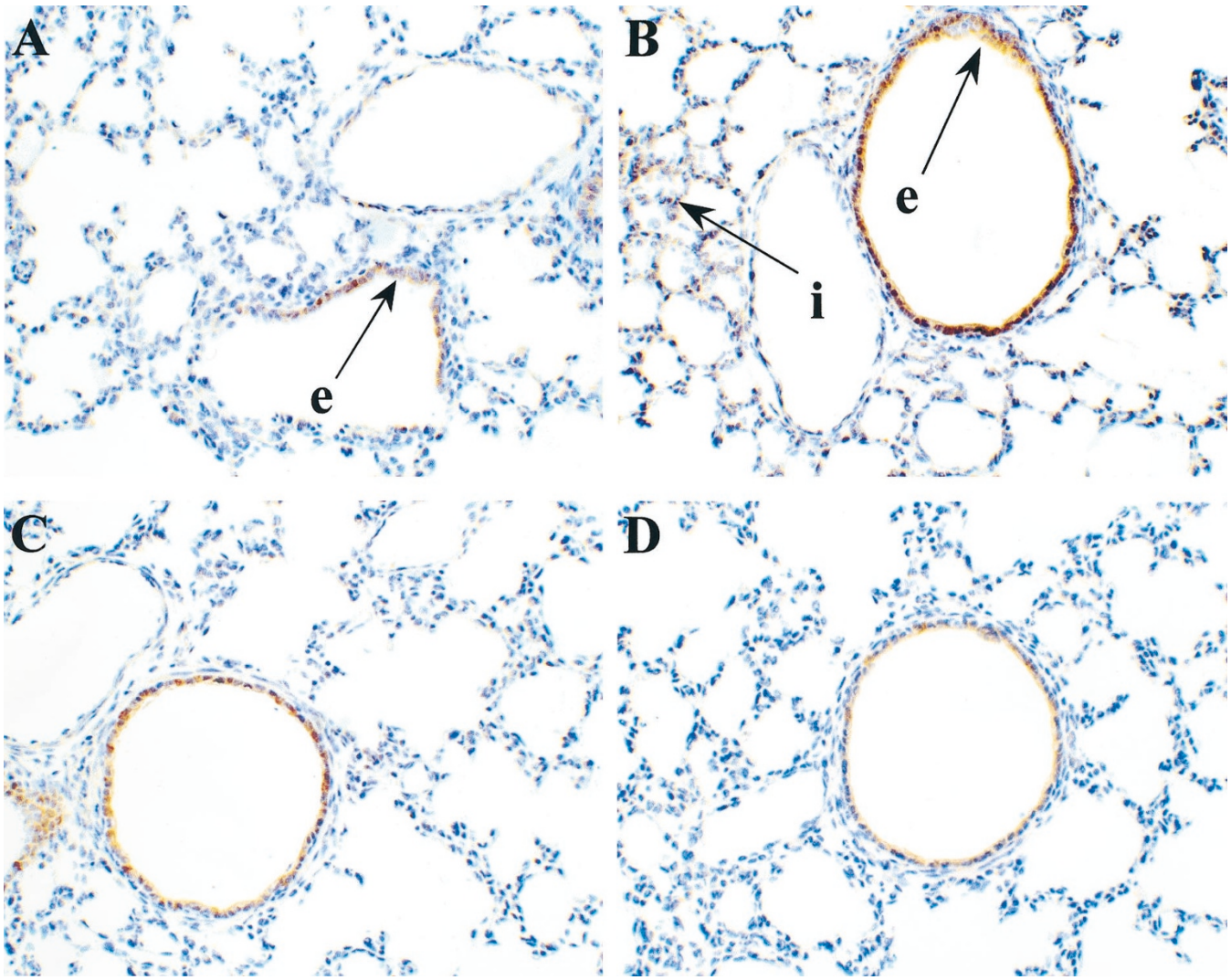

Figure 5. Immunohistochemistry for ET-1 in lung tissue after exposure to air or 60% O_2 for 14 d. Newborn rats received daily i.p. injections of 10 mg/kg U74389G in CS-4 vehicle or CS-4 vehicle alone. Original magnification ×200. *A,* air-exposed pups that received vehicle. The *arrow* indicates immunoreactive staining of bronchial epithelium (*e*). *B,* 60% O_2-exposed pups that received vehicle. Markedly increased immunoreactive staining is evident in bronchial epithelial (*e*) and parenchymal interstitial (*i*) cells. *C,* air-exposed pups that received U74389G. *D,* 60% O_2-exposed pups that received U74389G. Increased immunoreactive staining is not evident in O_2-exposed U74389G-treated animals.

4, *A* and *B*) compared with vehicle-treated and untreated animals. ET-1 protein content in lung tissue was assessed by immunohistochemistry (Fig. 5). When compared with air-exposed controls (Fig. 5*A*), vehicle-treated animals exposed to 60% O_2 for 14 d (Fig. 5*B*) had markedly increased ET-1 immunoreactivity localized to bronchial epithelium and interstitial cells in the lung parenchyma. Animals treated with U74389G did not have this 60% O_2-mediated increase in ET-1 immunoreactivity (Fig. 5, *C* and *D*), in keeping with the effect of U74389G on expression of prepro-ET-1 mRNA.

Vascular smooth muscle in lung tissue was assessed by α-smooth muscle actin immunohistochemistry (Fig. 6). Vehicle-treated 60% O_2-exposed animals (Fig. 6, *B* and *D*) showed marked arterial smooth muscle hypertrophy, and endothelial cell loss, compared with air-exposed controls (Fig. 6, *A* and *C*). Animals treated with U74389G did not have evidence of O_2-induced smooth muscle hypertrophy (Fig. 6*F*). Consistent with these smooth muscle changes, there was significant RVH (Fig. 7), as assessed by the ratio of the right ventricle to the left ventricle and septum dry weights, in 60% O_2-exposed vehicle-treated animals at d 14 compared with the similarly treated animals in air. This 60% O_2-induced RVH, an index of pulmonary hypertension, was completely attenuated ($p < 0.05$) in animals treated with U74389G.

To determine whether there could be a direct link between the products of 60% O_2-induced lipid peroxidation and the expression of ET-1 after birth, we examined the effect of 8-isoprostane on fetal lung cell prepro-ET-1 mRNA expression. Compared with controls, 8-isoprostane (10, 100, and 1000 nM) caused a significant ($p < 0.05$) increase in prepro-ET-1 mRNA expression in d 22 rat fetal lung cells (Fig. 8).

DISCUSSION

The ontogeny of ET-1 during fetal and early postnatal life is consistent with it playing an important role in maintaining the

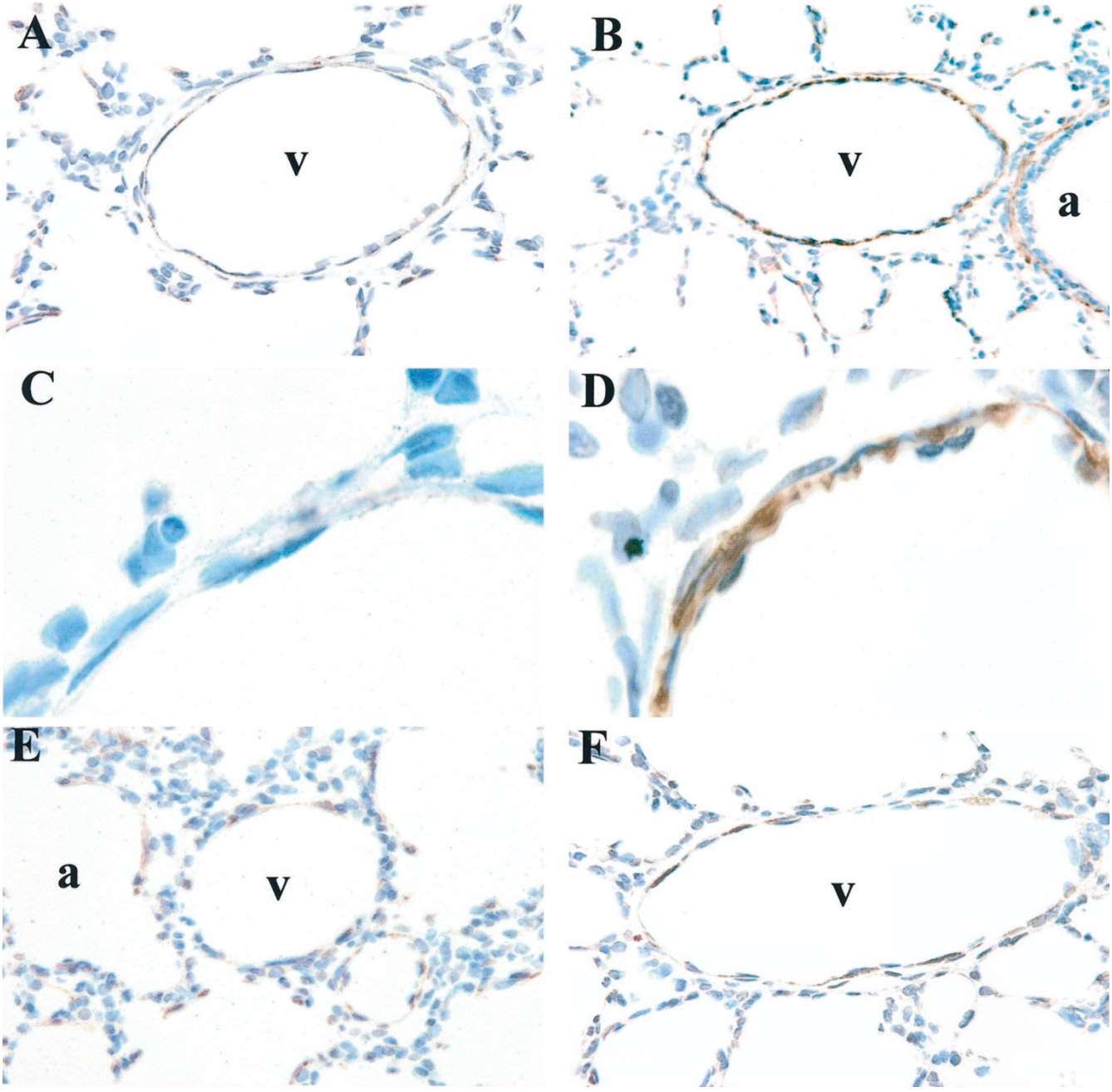

Figure 6. Effect of U74389G on 60% O_2-mediated pulmonary vascular smooth muscle hypertrophy. Immunohistochemistry for α-smooth muscle actin in vessels (*v*) and airways (*a*) after exposure to air or 60% O_2 for 14 d. Newborn rats received daily i.p. injections of 10 mg/kg U74389G in CS-4 vehicle or CS-4 vehicle alone. Original magnification ×200 (*A, B, E,* and *F*) or ×800 (*C* and *D*). *A, C,* air-exposed pups that received vehicle. *B,* 60% O_2-exposed pups that received vehicle. *D,* 60% O_2-exposed animals that received vehicle. Marked vascular smooth muscle hypertrophy and endothelial cell loss is seen. *E,* air-exposed pups that received U74389G. *F,* 60% O_2-exposed pups that received U74389G. O_2-mediated vascular smooth muscle hypertrophy is not evident in U74389G-treated animals.

normally high fetal pulmonary vascular resistance, which decreases after adaptation of the pulmonary circulation to extrauterine life (36–38). In accordance with our observations in the newborn rat, abnormally elevated levels of ET-1 in plasma and tracheobronchial aspirates of the human newborn have been correlated with persistent pulmonary hypertension and with pulmonary hypertension associated with respiratory distress and BPD (39–43).

ET-1 elicits a number of biologic effects in the lung, including pulmonary vascular and bronchial smooth muscle contraction and proliferation (39, 44), in addition to fibroblast proliferation and chemotaxis (45). Endothelin receptor antagonists have been effective in the prevention, or reversal, of pulmonary hypertension in laboratory animals induced by hypoxia (46, 47) and monocrotaline injection (48). These data are consistent with ET-1 being a critical mediator of the pulmonary vascular pathology of BPD, which includes vasoconstriction, vascular remodeling, and RVH. ET-1 may also contribute to the pathologic changes observed in lung injury through inhibition of apoptosis (49) or enhancement of pulmonary fibrosis (50).

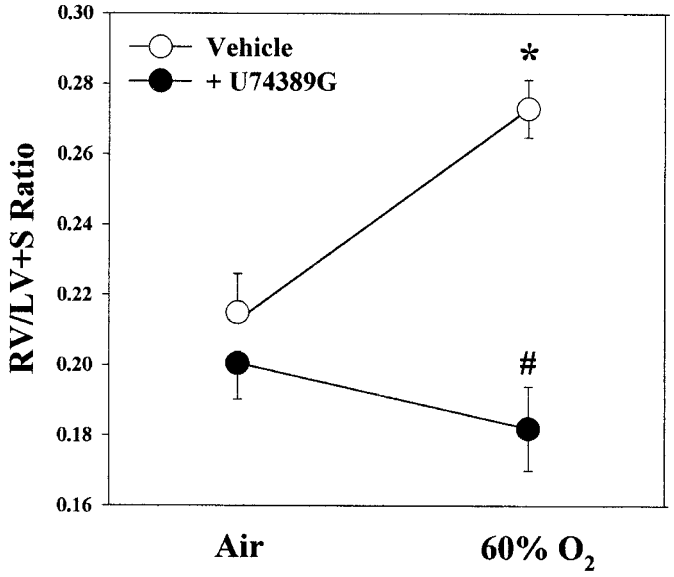

Figure 7. Right ventricular (RV) dry weight compared with that of left ventricle and septum (LV+S), as an index of RVH, after exposure to air or 60% O_2 for 14 d. Newborn rats received daily i.p. injections of 10 mg/kg U74389G in CS-4 vehicle (U74389G) or CS-4 vehicle alone (Vehicle). Plot points represent mean ± SEM for 12 pups from a single litter. *$p < 0.05$, by one-way ANOVA, for control animals exposed to 60% O_2 compared with those in air. #$p < 0.05$, by one-way ANOVA, for U74389G-treated animals compared with control animals exposed to 60% O_2.

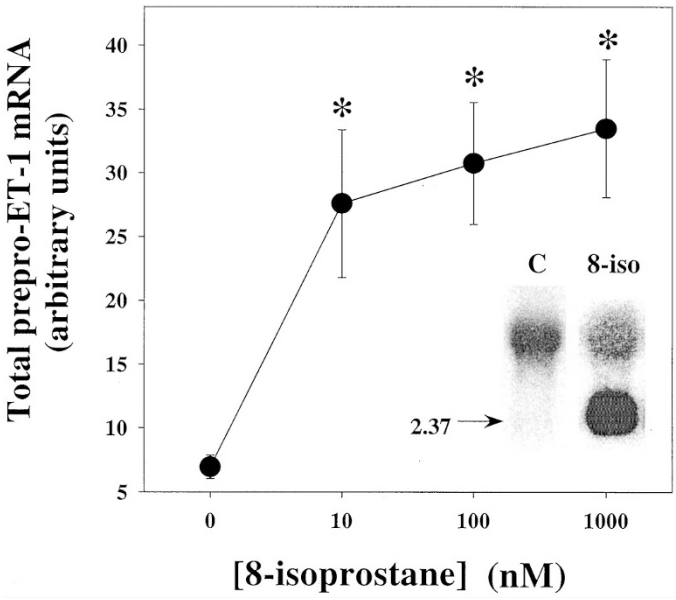

Figure 8. Effect of 8-isoprostane on prepro-ET-1 mRNA expression in primary cultures of d 22 mixed rat fetal lung cells. Plot points represent mean ± SEM for three separate experiments. *$p < 0.05$, by one-way ANOVA, compared with controls. *Inset,* Northern blot analysis of prepro-ET-1 mRNA expression in control (*C*) cells and cells exposed to 10 nM 8-isoprostane (*8-iso*). Transcript size is in kilobases.

As we have also recently reported elsewhere (33), antioxidants can cause inhibition of lung cell DNA synthesis in air-exposed neonatal rats. Our findings with 60% O_2-exposed animals differed somewhat from previous observations with animals exposed to 95% O_2 for 7 d, assessed using proliferating cell nuclear antigen immunohistochemistry (33), as well as [^{3}H]thymidine autoradiography (unpublished observations), in which some protection against O_2-mediated inhibition of DNA synthesis was observed. This may reflect the differing exposure times, or the very different lung injuries, observed with these exposure protocols. The inhibition of lung cell DNA synthesis observed in air-exposed animals treated with U74389G presumably reflects the critical physiologic role played by reactive O_2 species in cell signaling and growth (51). Our current finding that U74389G-treated animals also had attenuated somatic and lung growth is of concern, although a nutritional effect cannot be ruled out. If antioxidant therapies inhibit lung growth, already an adverse consequence of BPD (52), targeted approaches to specific aspects of O_2-induced lung injury may be preferable to an antioxidant approach for the newborn.

Our findings of a marked effect on ET-1 expression by hyperoxia, and the efficacy of U74389G in its prevention, imply a novel mechanism for ET-1 regulation. We have previously reported that U74389G does not scavenge hydrogen peroxide, and is ineffective at preventing O_2-induced cytotoxicity mediated by hydrogen peroxide in distal fetal rat lung epithelial cells (24). These findings indicate that pulmonary hypertension and ET-1 up-regulation are caused, directly or indirectly, by reactive O_2 species other than hydrogen peroxide during prolonged exposure of neonatal rats to 60% O_2. The extensive increase in immunoreactive ET-1, and its immunolocalization, in the lungs of pups exposed to 60% O_2 is consistent with the findings reported for adult rats subjected to another reactive O_2 species-mediated lung injury, bleomycin-induced pulmonary fibrosis (53).

Peroxidation of membrane phospholipids by reactive O_2 species leads to the production of lipid hydroperoxides (54) and stable, diffusible degradation products such as aldehydes (55) and isoprostanes (22). These products are themselves biologically active, and appear to act as second messengers for a wide range of effects induced by reactive O_2 species. Among these, the F_2-isoprostanes are a recently described group of prostaglandin $F_{2\alpha}$ isomers formed by reactive O_2 species-mediated peroxidation of arachidonic acid, independent of the action of cyclooxygenase. The best-characterized isoprostane produced *in vivo* is 8-isoprostane, a stable compound found in all biologic fluids that is increased by oxidative stress and antioxidant deficiency (22). 8-Isoprostane is highly vasoactive, causing vasoconstriction in the kidney (56) and retina (57) and increased microvascular permeability in the lung (58).

The bioactive properties of 8-isoprostane, and its marked increase in neonatal rat lung tissue after exposure to 60% O_2, suggested to us that it may also contribute directly to the O_2-mediated pulmonary hypertension observed in this model. We further postulated that the preventive effect of U74389G on 60% O_2-induced ET-1 up-regulation was through inhibition of 8-isoprostane formation. In support of this postulate was the preventive effect of U74389G on lung 8-isoprostane content *in vivo* and the striking *in vitro* effect of 8-isoprostane on prepro-ET-1 mRNA production. Recent *in vitro* evidence, in bovine aortic endothelial cells and the kidney, has also suggested that the biologic activity of 8-isoprostane may be attributable in part to up-regulation of ET-1 (56, 59, 60). The above findings are also consistent with data from Moore *et al.* (61), who noted that plasma ET-1 levels are markedly increased in the hepato-

renal syndrome, a condition in which 8-isoprostane is most likely the causative mediator (62). Our observations are the first, to our knowledge, to link an O_2-mediated induction of 8-isoprostane with up-regulation of ET-1 and pulmonary hypertension.

The biologic effects of 8-isoprostane are probably mediated by its interaction with an uncharacterized unique receptor similar to, but distinct from, the thromboxane A_2 receptor (56). Work by Lahaie and colleagues (57) has shown that the thromboxane receptor antagonist L670596 attenuated the vasoconstrictor action of 8-isoprostane on retinal vessels, possibly owing to blockade of 8-isoprostane receptors. The dominant role of the thromboxane receptor on 8-isoprostane-induced retinal vasoconstriction reported in this study may also be explained by the observation that ET-1, which is up-regulated by 8-isoprostane, causes phospholipase A_2 activation and subsequent thromboxane release through stimulation of the ET_B receptor (63, 64). If the same pathways are active in the O_2-injured lung, thromboxane receptor antagonists may also be effective in the prevention of O_2-induced pulmonary hypertension.

The observations reported above have implications for the development of interventions designed to limit O_2-mediated lung injury. First, the observation that a single antioxidant therapy can prevent the pulmonary vascular effects of O_2 toxicity in newborn rats, without correcting dysregulated growth of lung interstitial cells, suggests that these outcomes are regulated by different reactive O_2 species. Any antioxidant, or combination of antioxidants, intended to completely prevent O_2-mediated lung injury will need to have a broad spectrum of activities. Second, the recognition of a reversible up-regulation of ET-1 in O_2-mediated pulmonary hypertension suggests that alternative therapeutic approaches, such as endothelin or thromboxane receptor antagonists, may be effective.

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