Therapeutic hypercapnia prevents chronic hypoxia-induced pulmonary hypertension in the newborn rat

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PULMONARY HYPERTENSION, characterized by increased pulmonary artery resistance and pressure, is a common complication of sick newborn infants that carries a high mortality and is associated with considerable long-term morbidity in survivors (43, 59, 60). Studies suggest that the newborn is uniquely susceptible to remodeling of pulmonary resistance arteries (49). Vascular remodeling contributes to increased resistance and pulmonary arterial remodeling induced by chronic hypoxia, which we speculate derives from antioxidant properties of CO2 on the lung and consequent modulating effects on the endothelial pathway.

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The development of more severe pulmonary hypertension in later life (10, 17). Thus therapies aimed at preventing or ameliorating vascular remodeling in the newborn have great potential to improve mortality and long-term outcome.

A major pathological role for reactive oxygen species (ROS) in pulmonary hypertension is likely, as evidenced by observational studies showing increased oxidant stress in the lungs of adult humans with idiopathic pulmonary arterial hypertension (6, 13) and by the reported efficacy of various antioxidant interventions in the prevention of experimental pulmonary hypertension in both adult and newborn rats (11, 21, 26, 39). A putative mechanism for the beneficial effects of antioxidants is the decreased generation of oxidant-induced pulmonary vasoconstrictors, such as 8-isoprostane (5), peroxynitrite (4), and endothelin (ET)-1 (23, 25, 26, 63). ET-1 in particular is known to be a critical mediator of hypoxic pulmonary hypertension in fetal lambs (12) and of both hypoxia- and hyperoxia-induced pulmonary hypertension in newborn rodents (1, 25).

Studies on adult animals indicate that carbon dioxide (CO2) may have beneficial effects on the pulmonary circulation. In common with the well-described dilatory effects of CO2 on the systemic circulation, short-term inhalation of CO2 can also lead to pulmonary vasodilation, but only where acute changes in pH are normalized (3, 8, 58). Furthermore, chronic exposure to 10% CO2 for up to 3 wk was protective against hypoxia-induced pulmonary hypertension in adult rats (47), although the precise mechanisms for these effects were not elucidated. Hypoxia is a potent stimulus to sustained vasoconstriction but also causes oxidant stress (18, 21, 44), which is critical to the pathogenesis of chronic hypoxic pulmonary hypertension (39). Hypercapnia has been demonstrated to possess potent antioxidant properties, both in vitro and in vivo (38, 51, 55). Therefore, a plausible mechanism by which hypercapnia may protect against hypoxic pulmonary hypertension is through attenuation of oxidant stress in the lung.

The therapeutic use of hypercapnia has received much attention in adults, both experimentally and clinically (37). However, to our knowledge, the effects of chronic hypercapnia have not been examined on lung injury in newborns. Given that inhaled CO2 has great potential as an inexpensive and easily applied therapy, but also because it has the potential to cause harm (40, 41, 65), we sought to test the hypothesis that “therapeutic” hypercapnia would limit oxidant stress, prevent lung ET-1 upregulation, and thus protect against the develop-
ment of pulmonary hypertension and vascular remodeling in newborn rats chronically exposed from birth to moderate normobaric hypoxia (13% O₂).

MATERIALS AND METHODS

Materials. Plexiglass animal exposure chambers and automated O₂ and CO₂ controllers (OxyCycler model AX480CVC) were purchased from BioSpherix (Redfield, NY). SB217242, a combined ET receptor antagonist, was a generous gift from Dr. Douglas Hay (Glaxo Smith Kline, King of Prussia, PA). Tris-glycine gels were from Bio-Rad (Mississauga, ON, Canada). Polyvinylidene difluoride (PVDF) membranes were from VWR (Mississauga, ON, Canada). Acids, alcohols, organic solvents, paraformaldehyde, Permount, and Superfrost/Plus microscope slides were from Fisher Scientific (Whitby, ON, Canada). Protease inhibitors were from Roche (Laval, QC, Canada). A mouse monoclonal antibody against α-smooth muscle actin was from NeoMarkers (Fremont, CA). A rabbit polyclonal antibody against glycer-aldehyde-3-phosphate dehydrogenase (GAPDH; EC 1.2.1.12) and goat anti-mouse and rabbit IgG-biotin secondary antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). A rabbit polyclonal antibody against prepro-ET-1 was from Peninsula Laboratories (San Carlos, CA). Goat anti-mouse and anti-rabbit IgG-peroxidase antibodies were from Cell Signaling Technology (Beverly, MA). Avidin-biotin-peroxidase complex immunohistochemistry kits, 3,3′-diaminobenzidine staining kits, and normal goat serum were from Vector Laboratories (Burlingame, CA). 8-Isoprostane affinity purification columns, Sep Pak C18 cartridges, 8-isoprostane, and ET-1 enzyme immunoassay kits were purchased from Cayman Chemical (Ann Arbor, MI). All other chemicals and reagents were purchased from Bioshop (Burlington, ON, Canada).

Animal exposures. All procedures involving animals were in accordance with the standards of the Canadian Council on Animal Care and were approved by the Animal Care Committees of the Sunnybrook and Hospital for Sick Children Research Institutes. On their anticipated day of delivery, pathogen-free pregnant Sprague-Dawley dams (Taconic, Germantown, NY) were placed in sealed 80 × 60 × 50 cm exposure chambers with 12:12-h light-dark cycles, with the temperature maintained at 25 ± 1°C and humidity at ~50%. Each litter, maintained at n = 10–12 pups to control for nutritional effects, was nursed in one of the following gas concentrations from birth until 14 days: 1) normoxia (21% O₂) and normocapnia (<0.5% CO₂), 2) normoxia and hypercapnia (5.5 or 10% CO₂), 3) 13% O₂ and normocapnia, 4) 13% O₂ and hypercapnia (5.5 or 10% CO₂), or 5) 10.5% O₂ and 10% CO₂.

O₂ and CO₂ concentrations, temperature, and humidity were continuously monitored, recorded, and regulated by computer using customized software (AnaWin2 Run-Time, v2.2.18; Watlow-Anafaze, St. Louis, MO). O₂ and CO₂ sensors were calibrated weekly. Dams were exchanged daily between paired normoxia and hypoxia chambers to prevent any maternal toxicity and consequent nutritional effects on the pups. In preliminary experiments, we determined that maternal exchange between normocapnia and hypercapnia chambers was not necessary, as this had no impact on the average pup weights at 14 days compared with litters where the mother remained in CO₂ (either 5.5 or 10%) for the duration of the experiment (data not shown). The degree of hypoxia (13% O₂) used to induce chronic pulmonary hypertension was found to be the lowest that did not lead to excess mortality by 14 days. In contrast, exposure to 10.5% O₂: a level of hypoxia commonly used to induce pulmonary hypertension in adult rodents, led to 100% mortality by 14 days (n = 4 litters). For some experiments, pups received daily ip injections of SB217242 or vehicle alone as previously described (24, 25). At the end of each exposure period, pups were either killed by pentobarbital sodium overdose or exsanguinated after anesthesia.

Arterial blood gas measurement. Pups were anesthetized with a low dose ip of ketamine (20 mg/kg), and the neck was dissected to expose the external carotid artery. After 15–30 min of recovery in the appropriate concentrations of O₂ and CO₂, the artery was transected, and blood was rapidly collected using a heparinized capillary tube and analyzed (ABL500; Radiometer, Copenhagen, Denmark).

Hematocrit measurement. Anesthetized pups had their thoracic cavity opened and left atrium transected. Blood was collected using a heparinized capillary tube and spun for 5 min in a hematocrit centrifuge (ZIPOcrit; LW Scientific, Lawrenceville, GA).

Two-dimensional echocardiography and Doppler ultrasound. Echocardiographic evaluation of pulmonary hemodynamics and ventricular dimensions was performed as previously described (14) using the Vivid 7 Advantage cardiovascular ultrasound system (GE Medical Systems, Milwaukee, WI) with a small (0.8 × 2.5 cm) high-frequency linear probe (i13L) that allowed image acquisition at a rate of 400 frames/s. The ultra-high frame rate provided high image quality, which was found to be particularly suitable for small animal imaging, where the heart rate is very high. After induction of anesthesia with ketamine (40 mg/kg ip) and xylazine (10 mg/kg ip), the animal was laid supine, and, while spontaneously breathing the appropriate concentration of O₂ and CO₂ through a modified face mask, the probe was gently applied to the chest. For measurements of right heart loading, short-axis views of the right and left ventricle at a level just distal to the mitral valve were obtained. Right ventricular end-diastolic area (RVEDA) and left ventricular end-diastolic area (LVEDA) were measured by manually tracing the endocardial border. An average of three readings were made, and the ratio of mean RVEDA to LVEDA (RVEDA/LVEDA) was calculated as a measure of right heart loading. For measurements of pulmonary artery hemodynamics, a short-axis view at the level of the aortic valve was obtained, and the pulmonary artery was identified using color flow Doppler. Filters were set for maximum ranges for volumes and velocity settings with a medium degree of motion discrimination. The diameter of the pulmonary artery was measured at the hinge-point of the pulmonary valve leaflets. The pulsed Doppler gate was placed proximal to the pulmonary valve leaflets and aligned, with an angle of insonation < 20°, to maximize laminar flow. The pulmonary artery acceleration time (PAAT) and right ventricular ejection time (RVET) of the pulmonary Doppler profile were measured. PAAT was measured as the time from the onset of systolic flow to peak pulmonary outflow velocity, and RVET was measured as the time from onset to completion of systolic pulmonary flow. Because RVET was found to be unaffected by chronic exposure to hypoxia (data not shown), we used decreased PAAT alone as a measure of increased pulmonary artery pressure. PAAT was not found to be significantly influenced by variations in heart rate, as determined by the pulmonary artery Doppler peak-peak distance (data not shown). From the same Doppler interrogation of the pulmonary artery, right ventricular stroke volume (RVSV) was also calculated as a measure of right ventricular performance according to the following formula: (pulmonary artery diameter/2)² × 3.14 × pulmonary artery velocity time integral. Left ventricular systolic performance was estimated from the parasternal short-axis view by calculating the shortening fraction according to the following formula: left ventricular end diastolic dimension (LVEDD) – left ventricular end systolic dimension (LVESD) × 100.

Right ventricular hypertrophy. Right ventricular hypertrophy (RVH) was quantified by measuring the right ventricle (RV)-to-left ventricle and septum (LV+S) dry weight ratios from the pooled hearts of each litter, as previously described (23, 25, 26).

Histological and morphometric studies. Four animals from each group (2 from each of 2 separate litters) were anesthetized with ketamine (80 mg/kg ip) and xylazine (20 mg/kg ip). After opening of the thoracic cavity and cannulation of the trachea, the pulmonary veins were divided. The pulmonary circulation was flushed with 1× PBS containing 1 U/ml heparin to clear the lungs of blood via a needle inserted in the right ventricle. The lungs were then perfusion-fixed with ice-cold 4% (wt/vol) paraformaldehyde in 1× PBS via a needle.
in the right ventricle, while the lungs were inflated at a constant pressure of 20 cmH2O. For morphometric studies, while the lungs were constantly inflated at 20 cmH2O pressure, the pulmonary circulation was flushed with 1× PBS containing 1 U/ml heparin followed by perfusion of a hot (60°C) saturated barium-10% (wt/vol) gelatin mixture for 5 min at 100 cmH2O pressure through the right ventricle. Paraffin-embedded lung sections (4/animal) were immunostained for α-smooth muscle actin, as previously described (24, 26), or stained for elastin (for measurement of medial wall thickness; MWT), as described below. For measurements of MWT, intra-acinar pulmonary arteries (associated with respiratory bronchioles, alveolar ducts and alveolar walls), where the complete circumference of the vessel was visible (>100/animal), were digitally photographed using a Penguin 600CL camera and Viewfinder capture software (Pixera, Los Gatos, CA) by an observer blinded to group identity. Percent MWT was calculated using the following formula: distance between internal and elastic laminae × 2 mean external diameter × 100 (48). Mean external diameter was calculated from measurements in two perpendicular planes to account for any irregularities in vessel shape. For measurement of arterial muscularization, distal pulmonary arteries of ≤80-µm external diameter were identified by eyepiece reticle and counted (>50/animal) on α-actin-immunostained sections by an observer blinded to group identity. Vessels were characterized as either completely muscular (complete muscle coat around the circumference of the vessel), partially muscular (incomplete muscle coat), or nonmuscular.

Hart’s elastin staining. Staining of paraffin-embedded tissue sections for elastin fibers was performed using a commercially available Weigert’s (resorcin-fuchsin) solution (Elastin Products, Owensville, MO) diluted in acidic 70% (vol/vol) ethanol, according to the supplier’s instructions. Sections were dewaxed and rehydrated, rinsed in distilled water (dH2O), and left overnight in Weigert’s solution. After further washing in dH2O, sections were counterstained using 0.5% (wt/vol) tartrazine in 0.25% (vol/vol) acetic acid, then dehydrated and cleared in xylene. Slides were mounted using a 50% (vol/vol) Permount-50% (vol/vol) xylene mixture.

Prepro-ET-1 immunostaining. Paraffin-embedded tissue sections were stained for immunoreactive prepro-ET-1 by an avidin-biotin-peroxidase method, as previously described (23). Negative controls were generated by omitting the primary antisem.

Total lung α-actin. Lung actin concentration was quantified by Western blot analysis on total protein lysates from four litters per group (the pooled lungs of 2 animals were used from each litter), which were prepared as previously described (24). Protein (50 µg/lane) was fractionated on Tris-glycine 4–20% gradient gels under reducing conditions by SDS-PAGE for 2 h at 120 V and subsequently transferred to PVDF membranes. Membranes were blocked with 5% (wt/vol) nonfat milk in TBST [20 mM Tris base, 137 mM NaCl, pH 7.6, with 0.1% (vol/vol) Tween 20] overnight at 4°C and then incubated with anti-α-smooth muscle actin (43 kDa; 1/5,000 = 0.04 µg/ml) for >2 h at room temperature. To control for differences in

<table>
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<tr>
<th>Parameter/Group</th>
<th>21% O2 + Normocapnia</th>
<th>13% O2 + Normocapnia</th>
<th>21% O2 + 5.5% CO2</th>
<th>13% O2 + 5.5% CO2</th>
<th>21% O2 + 10% CO2</th>
<th>13% O2 + 10% CO2</th>
<th>10.5% O2 + 10% CO2</th>
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<tr>
<td>Body wt, g</td>
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<td>22.8 ± 1.2</td>
<td>28.4 ± 1.4</td>
<td>23.9 ± 0.05</td>
<td>27.5 ± 1.4</td>
<td>25.7 ± 1.1</td>
<td>19.8 ± 1.4</td>
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<tr>
<td>pH</td>
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<td>7.33 ± 0.01</td>
<td>7.34 ± 0.04</td>
<td>7.27 ± 0.05</td>
<td>7.20 ± 0.02</td>
<td>7.17 ± 0.02</td>
<td>7.10 ± 0.05</td>
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<td>pCO2, mmHg</td>
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<td>47.2 ± 2.2</td>
<td>59.5 ± 5.9</td>
<td>63.3 ± 8.6</td>
<td>100.9 ± 4.2</td>
<td>103.6 ± 8.3</td>
<td>113.8 ± 10.0</td>
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<tr>
<td>NO2, mmHg</td>
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<td>77.1 ± 14.6</td>
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<td>30.1 ± 2.1</td>
<td>27.7 ± 0.9</td>
<td>37.6 ± 0.8</td>
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<td>Hematocrit, l/l</td>
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<td>0.32 ± 0.01</td>
<td>0.33 ± 0.02</td>
<td>0.28 ± 0.01</td>
<td>0.32 ± 0.02</td>
<td>0.40 ± 0.04</td>
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Values (n = 6–8 animals/group) are means ± SD. 1P < 0.01 by ANOVA compared with air + normocapnia group. 2P < 0.01 by ANOVA compared with normocapnia and 5.5% CO2 groups. 3P < 0.01 by ANOVA compared with normocapnia groups. 4P < 0.01 by ANOVA compared with 5.5% CO2 groups. 5P < 0.01 by ANOVA compared with all other groups. 6P < 0.01 by ANOVA compared with all other groups except 10.5% O2 + 10% CO2. 7P < 0.01 by ANOVA compared with all other groups except 13% O2 + normocapnia.

Fig. 1. Right ventricle (RV)/left ventricle + septum (LV+S) dry weight ratios (A) and percent (%) arterial medial wall thickness (MWT; B) as markers of pulmonary vascular remodeling. Animals were exposed to 21% O2 (Air; open bars), 13% O2 (black bars), or 10.5% O2 (gray bar) and either normocapnia (NC), 5.5% CO2, or 10% CO2 from birth until 14 days (n = 4 litters or animals/group). Values are expressed as means ± SE. *P < 0.01 by ANOVA, compared with normoxia-exposed animals receiving the same concentration of CO2. #P < 0.01, by ANOVA, compared with 13% O2 and NC or 5.5% CO2 groups. †P < 0.01 by ANOVA, compared with 13% O2 and NC group.
protein loading, each blot was co-incubated with anti-GAPDH (30 kDa; 1/1,000/H11005/0.2 μg/ml). Blots were washed and placed in the appropriate secondary antibody for >1 h at room temperature. After further washing, the protein bands were imaged by enhanced chemiluminescence (Pierce Biotechnology, Rockford, IL) and exposed to blue film (CL-XPosure, Pierce). Films were then electronically scanned, and the densities of nonsaturated bands were quantified using ImageJ software (v1.30; NIH, Bethesda, MD).

**Total lung ET-1.** Tissue samples from four litters per group (the pooled lungs of 2 animals were used from each litter) were homogenized in 1× PBS with protease inhibitors and sonicated on ice for 30 s. A small aliquot was taken for total protein measurement, and the remaining sample was centrifuged at 3,000 rpm for 5 min. Supernates were diluted 1:3 in 4% (vol/vol) acetic acid, passed through a Sep Pak C18 column, eluted with 4% (vol/vol) acetic acid in 86% (vol/vol) ethanol, and evaporated to dryness under a stream of N2. ET-1 in purified samples and standards was quantified using a commercially available enzyme immunoassay kit, according to the manufacturer’s instructions. Values are expressed as picograms of ET-1 per milligram total lung protein.

**Lung 8-isoprostane.** Total (free and esterified) 8-isoprostane was extracted from the homogenized lung tissue of four litters per group (the pooled lungs of 2 animals were used from each litter) using immunosorbent affinity columns (Cayman), according to the manufacturer’s instructions. 8-Isoprostane was quantified in affinity-purified samples and standards using a commercially available enzyme immunoassay kit, as previously described (23, 25, 26). Values are expressed as picograms of 8-isoprostane per milligram total lung protein.

**Data presentation and analysis.** Unless otherwise stated, all values are expressed as means ± SE. Statistical significance (P < 0.05) was determined by two-way ANOVA followed by pairwise multiple comparisons using Tukey’s test if significant differences were found.

**RESULTS**

**Body weight, arterial blood gas, and hematocrit values.** Values after 14 days of exposure to the different concentrations of O2 and CO2 are shown in Table 1. Pups exposed to hypoxia (with and without hypercapnia) all had significantly (P < 0.05) lower body weights than normoxia controls, although there was a trend toward increasing body weight with increasing levels of hypercapnia in 13% O2-exposed groups. Increased PaCO2 levels were observed in animals exposed to 5.5% CO2 (by ∼10–15 mmHg relative to animals in normocapnia; P < 0.05) and to a much greater extent in animals exposed to 10% CO2 (∼50 mmHg relative to animals in normocapnia; P < 0.05). Animals chronically exposed to 10% CO2 developed significant hypercapnic acidosis with partial metabolic correction, as reflected in lower pH and higher HCO3 levels (P < 0.05 compared with normocapnia groups). Exposure to 13% O2 led to significantly decreased PaO2 levels (P < 0.05 relative to normoxia-exposed animals), which were normalized by exposure to CO2 (both 5.5 and 10%; P > 0.05 compared with normocapnia groups). Animals exposed to normoxia and 10% CO2, but not normoxia and 5.5% CO2, had significantly increased PaO2 levels (P < 0.05 compared with normoxia and normocapnia or 5.5% CO2 groups). Hematocrit was significantly increased by a 14-day exposure to 13% O2 (P < 0.05 relative to normoxia-exposed animals), which was
normalized by exposure to either 5.5 or 10% CO₂ (P > 0.05 compared with normoxia and normocapnia group). Exposure to 10.5% O₂ and 10% CO₂ was associated with 96% survival by 14 days (compared with no survivors in pups exposed to 10.5% O₂ alone, most of which died after day 7), and pups had comparable PaO₂ and hematocrit values to pups exposed to 13% O₂ alone (P > 0.05).

Hypoxia-induced RVH was attenuated by exposure to 10% CO₂. As shown in Fig. 1A, significant RVH, as demonstrated by a twofold increase in the RV/LV+S dry weight ratio after 14 days, was observed in animals exposed to 13% O₂ (P < 0.05 compared with normoxia and normocapnia group). A nonsignificant trend toward a decrease in 13% O₂-induced RVH was observed in animals that were exposed to 5.5% CO₂ (P > 0.05 compared with hypoxia and normocapnia group), whereas a greater, and statistically significant, reduction in hypoxia-induced RVH was observed in animals exposed to 13% O₂ (or to 10.5% O₂) as well as 10% CO₂ (P < 0.05 compared with hypoxia and normocapnia group).

Hypoxia-induced arterial medial wall thickening was attenuated by exposure to 10% CO₂. Percent MWT in intra-acinar pulmonary arteries is shown in Fig. 1B. Animals exposed to 13% O₂ from birth developed a marked increase in percent MWT (P < 0.05) after 14 days compared with animals exposed to normoxia. A nonsignificant trend toward a decrease in 13% O₂-induced MWT was observed in animals that were exposed to 5.5% CO₂ (P > 0.05 compared with hypoxia and normocapnia group), whereas a greater, and statistically significant, reduction in hypoxia-induced RVH was observed in animals exposed to 13% O₂ (or to 10.5% O₂) as well as 10% CO₂ (P < 0.05 compared with hypoxia and normocapnia group).

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Hypoxia-induced distal arterial muscularization and increased lung α-smooth muscle actin concentration were attenuated by exposure to 10% CO₂. Percentage of completely muscularized distal arteries, as an index of distal arterial extension of smooth muscle, is shown in Fig. 3A. Animals exposed to 13% O₂ from birth showed a marked increase in numbers of completely muscularized distal vessels (P < 0.05) after 14 days compared with animals exposed to normoxia. A nonsignificant trend toward a decrease in 13% O₂-induced distal muscularization was observed in animals that were exposed to 5.5% CO₂ (P > 0.05 compared with hypoxia and normocapnia group), whereas a greater, and statistically significant, reduction in hypoxia-induced MWT was observed in 13% O₂-exposed animals that were also exposed to 10% CO₂ (P < 0.05 compared with hypoxia and normocapnia group). As shown by Western blot analyses (Fig. 3B), animals exposed to 13% O₂ had a significant (P < 0.05) increase in total lung actin concentration, which includes both vascular and airway smooth muscle, relative to animals exposed to normoxia. This 13% O₂-induced increase was completely attenuated by exposure to 10% CO₂ (P < 0.05 compared with hypoxia and normocapnia). There was a trend toward a persistent increase in total lung actin content in hypoxia and 5.5% CO₂-exposed animals, relative to the normoxia-exposed groups; however, these differences were not statistically significant (P > 0.05) because of considerable variations in actin expression among litters. Representative immunohistochemistry for α-smooth muscle actin is shown in Fig. 4. Compared with normoxia-exposed animals, there was a marked overall increase in vascular wall smooth muscle and in the proportion of distal arteries that were completely muscularized in animals exposed to 13% O₂. Similar to our findings on Western blot analyses of smooth muscle actin (Fig. 3B), this 13% O₂-induced change was largely attenuated by exposure to 10% CO₂.
Abnormal pulmonary hemodynamics induced by hypoxia was attenuated by exposure to CO₂. High-resolution two-dimensional echocardiography and Doppler ultrasound measurements were made while animals were receiving the same O₂ and CO₂ concentrations to which they were chronically exposed from birth (Fig. 5). Compared with normoxia, pups exposed to 13% O₂ had significantly increased (P < 0.05) RVEDA/LVEDA ratios (Fig. 5A), indicating right ventricular dilation, and significantly decreased (P < 0.05) RVSV (Fig. 5B), indicating impaired right ventricular performance. Increased pulmonary arterial resistance, as reflected by a decrease in PAAT (Fig. 5C), was also seen in animals exposed to 13% O₂ (P < 0.05 compared with normoxia and normocapnia group). Consistent with our pathological findings, there was a nonsignificant trend (P > 0.05 compared with hypoxia and normocapnia group) toward increased PAAT (decreased pulmonary arterial pressure) in animals exposed to 13% O₂ and 5.5% CO₂, whereas exposure to 10% CO₂ led to a greater, and statistically significant, increase (P < 0.05 compared with hypoxia and normocapnia group). There was also a nonsignificant trend toward increased RVSV in animals exposed to normoxia and hypercapnia. We observed no significant (P > 0.05) differences between groups in heart rate and left ventricular systolic performance (data not shown).

Hypoxia-induced oxidant stress in the lung was attenuated by exposure to 10% CO₂. 8-Isoprostane, a robust in vivo marker of oxidant stress (50), was quantified in lung tissue (Fig. 6). The lungs of animals exposed to 13% O₂ for 14 days from birth had increased oxidant stress (P < 0.05 compared with normoxia and normocapnia group) as evidenced by a twofold increase in 8-isoprostane content. A nonsignificant trend toward a decrease in 13% O₂-induced oxidant stress was observed in animals that were exposed to 5.5% CO₂ (P > 0.05 compared with hypoxia and normocapnia group), whereas a complete, and statistically significant, reduction was observed in 13% O₂-exposed animals that were also exposed to 10% CO₂ (P < 0.05 compared with hypoxia and normocapnia group).

Hypoxia-induced upregulation of arterial wall prepro-ET-1 and total lung ET-1 expression was attenuated by exposure to 10% CO₂. Representative immunohistochemistry for prepro-ET-1, an intracellular precursor of ET-1, is shown in Fig. 7. Compared with normoxia-exposed animals, where immunoreactivity was mostly localized to bronchial epithelium, there was a marked increase in immunoreactivity on the intimal and medial walls of small pulmonary arteries in animals exposed to 13% O₂. This hypoxia-induced increase in vascular wall expression of ET-1 was only partially attenuated by 5.5% CO₂.
and completely attenuated by exposure to 10% CO₂. As shown in Fig. 8A, the lungs of animals exposed to 13% O₂ for 14 days from birth had a more than twofold increase in total ET-1 (P < 0.05 compared with normoxia and normocapnia group). A nonsignificant trend toward a decrease in 13% O₂-induced ET-1 upregulation was observed in animals that were exposed to 5.5% CO₂ (P > 0.05 compared with hypoxia and normocapnia group), whereas an almost constant, and statistically significant, reduction was observed in 13% O₂-exposed animals that were also exposed to 10% CO₂ (P < 0.05 compared with hypoxia and normocapnia group).

Endothelin receptor blockade prevented increased arterial medial wall thickening induced by exposure to 13% O₂. As shown in Fig. 8B, increased medial wall thickening of intraacinar pulmonary arteries induced by exposure to 13% O₂ (P < 0.05 compared with normoxia-exposed, vehicle-treated group) was completely attenuated by daily treatment with the endothelin receptor blocker SB217242 (P < 0.05 compared with 13% O₂-exposed, vehicle-treated group).

DISCUSSION

We report that chronic hypercapnic acidosis, induced by spontaneous breathing of 10% CO₂, attenuated pathological and Doppler-derived indexes of hypoxic pulmonary hypertension in neonatal rats. These effects were associated with attenuation of oxidant stress and upregulation of ET-1 in the lung. We also confirmed, using a combined ET receptor antagonist intervention, that ET-1 mediates vascular remodeling in hypoxia-exposed newborn rats. Estimation of arterial acid-base status indicated that a 14-day exposure to 10% CO₂, but not 5.5% CO₂, caused a persistent respiratory acidosis, which may have at least partly been the result of an immaturity-related insufficiency or delay in metabolic correction (30). A possible implication of these observations is that exposure to a level of CO₂ sufficient to induce persistent hypercapnic acidosis may be required to lead to significant benefit, as has been suggested in studies on the injured ex vivo rabbit lung, where buffering of acidosis led to worsening of injury (34).
alternative interpretation is that benefits of CO$_2$ on pulmonary hypertension are simply dose dependent, regardless of the degree of acidosis induced. We did not examine for incremental improvements in pulmonary hypertension and vascular remodeling at concentrations $>$ 10% CO$_2$, since we anticipated that higher levels would have increased mortality, as our group has reported in adult rats (35). It was not possible in our noninstrumented newborn rat model to separate the effects of acidosis from hypercapnia by chronically buffering pH, which would be required to ascertain the underlying basis for the beneficial effects of chronic hypercapnia.

The concentrations of CO$_2$ that we targeted in this study were based on previously reported acute observations in cats (2) suggesting that exposure to 5.5 and 10% CO$_2$ would be expected to elevate PaCO$_2$ from normal levels by 20 mmHg (PaCO$_2$ $\sim$ 55–65 mmHg or “mild-moderate” hypercapnia) and 40 mmHg (PaCO$_2$ $\sim$ 75–85 mmHg or “severe” hypercapnia), respectively. That our blood gas measurements did not closely reflect these anticipated PaCO$_2$ values was probably related to depressant effects of anesthesia on respiratory drive, a likelihood substantiated by our findings of lower PaO$_2$ and pH values and higher PaCO$_2$ values than would be anticipated in control (normoxic and normocapnic) animals. Variations from antici-

Fig. 6. Total lung 8-isoprostane as a marker of oxidant stress. Animals were exposed to 21% O$_2$ (Air; open bars) or 13% O$_2$ (black bars) and either normocapnia (NC) or hypercapnia (5.5 or 10% CO$_2$) from birth for 14 days ($n$ = animals from 4 litters/group). Values are expressed as means $\pm$ SE. *$P < 0.01$, by ANOVA, compared with respective normoxia groups. #$P < 0.05$, by ANOVA, compared with 13% O$_2$ and NC or 5.5% CO$_2$ groups.

Fig. 7. Representative immunohistochemistry for prepro-endothelin (ET)-1. Animals were exposed to 21% O$_2$ (Air; left) or 13% O$_2$ (Hypoxia; middle) and either normocapnia or hypercapnia (+5.5 or +10% CO$_2$) for 14 days. Bar = 25 $\mu$m. Markedly increased immunoreactivity was observed on distal airway epithelium and the walls of small pulmonary resistance arteries in a pup exposed to hypoxia and normocapnia (Hypoxia) compared with an animal exposed to normoxia and normocapnia (Air), where expression was predominantly evident on bronchial epithelium. Increased hypoxia-induced pulmonary arterial wall immunoreactivity was partially attenuated by exposure to 5.5% CO$_2$ (Hypoxia + 5.5% CO$_2$). Increased hypoxia-induced immunoreactivity on both distal airway epithelium and pulmonary arterial walls was completely attenuated by 10% CO$_2$ (Hypoxia + 10% CO$_2$). Right: enlarged views of pulmonary arteries enclosed by boxes. Inset (at top left): complete lack of staining in a section from a hypoxia-exposed control animal where the primary antibody was omitted.
Interestingly, both levels of hypercapnia examined in this study increased arterial $P_{O_2}$ and decreased hematocrit values in 13% $O_2$-exposed animals to levels similar to normoxia controls. Our findings on $P_{A_{O_2}}$ are in keeping with previously reported observations in both rats (19, 35) and humans (22, 42) indicating that mild-moderate hypercapnia improves systemic oxygenation. Possible mechanisms for an effect of hypercapnia on systemic oxygenation include improved right ventricular performance, as shown in this study, and reduced metabolic activity and $O_2$ consumption, as reported by others (22, 42). To separate the contributions of improved oxygenation from any direct beneficial effects of hypercapnia on chronic hypoxic pulmonary hypertension, we also examined the effect of 10% $CO_2$ on pulmonary vascular remodeling in animals exposed to an otherwise universally fatal degree of hypoxia (10.5% $O_2$). In animals exposed to 10.5% $O_2$ and 10% $CO_2$, $P_{A_{O_2}}$, hematocrit, and mortality were comparable with that of animals exposed to 13% $O_2$ alone, but markers of pulmonary vascular remodeling were significantly improved. We interpret this as being consistent with an effect of hypercapnia that is largely independent of any associated improvements in $P_{A_{O_2}}$.

The present study adds to a growing body of experimental evidence (35, 36, 38, 47) indicating that hypercapnia can have beneficial effects on the pulmonary circulation. Whereas there is a relatively large body of literature examining the acute pulmonary vascular effects of hypercapnia (54), we are aware of only one other study (47) where the chronic effects of hypercapnia were examined. Our observations are in agreement with Ooi et al. (47), who reported that 10% $CO_2$ prevented chronic hypoxia-induced pulmonary vascular remodeling in adult rats. In the present study, we explored potential mechanisms for the benefits of $CO_2$ by examining effects on two factors that are known to be critical to the pathogenesis of hypoxia-induced pulmonary hypertension: oxidant stress and upregulation of the potent vasoconstrictor peptide ET-1. Our observation on increased lung 8-isoprostane, which was completely attenuated by exposure to 10% $CO_2$, is in keeping with an important antioxidant effect of hypercapnia on the lung. We speculate that this antioxidant effect was likely to have accounted for the attenuation of vascular ET-1 upregulation induced by hypoxia. This is based on reports of attenuating effects of antioxidants on ET-1 upregulation in hypoxia-exposed adult rats (64) and by our own previous work using an antioxidant intervention in hyperoxia-exposed newborn rats (26). Antioxidant properties of hypercapnia may occur through attenuation of a number of pathways with putative roles in hypoxic pulmonary hypertension, including superoxide-generating xanthine oxidase (21, 51) and pulmonary inflammation (55), or by direct scavenging of peroxynitrite (38, 52).

An important caveat to the present findings is that there is parallel literature to suggest that hypercapnia can be harmful to the lung. Such adverse effects have included inhibition of surfactant function through increased protein nitrification (41, 65) or, when hypercapnia was induced by low rate and tidal volume ventilation, an enhancement of inflammatory (lipopolysaccharide induced) lung injury (40). These findings, coupled with evidence that hypercapnia can also have adverse effects on brain and retinal development in newborn animals (16, 20), indicates that a great deal of further study is required before hypercapnia can be considered as a potential therapy in the human newborn. Extrapolation of our current findings to

Fig. 8. A: total lung ET-1. Animals were exposed to 21% $O_2$ (Air; open bars) or 13% $O_2$ (Hypoxia; black bars) and either normocapnia (NC) or hypercapnia (5.5 or 10% $CO_2$) from birth for 14 days ($n$ = animals from 4 litters/group). Values are expressed as means ± SE. *$P < 0.01$, by ANOVA, compared with respective normoxia groups. #$P < 0.01$, by ANOVA, compared with 13% $O_2$ and NC or 5.5% $CO_2$ groups. B: inhibitory effect of the nonselective ET receptor antagonist SB217242 on hypoxic pulmonary vascular remodeling, as measured by increased %MWT. Animals were exposed to 21% $O_2$ (Air; open bars) or 13% $O_2$ (black bars) and received daily ip injections of vehicle or SB217242 (5 mg/kg) from birth until 14 days ($n = 4$ animals/group). Values are expressed as means ± SE. *$P < 0.01$, by ANOVA, compared with all other groups.
human newborns with pulmonary hypertension is also limited by the fact that chronic hypoxia is only an initiating or perpetuating factor in some infants. Nonetheless, because hypercapnia may be an easily applicable protective strategy, and because permissive hypercapnia is commonplace in newborn intensive care, as a consequence of the low tidal volume ventilatory strategies that are now widely applied (9, 56), we believe it is imperative to understand the effects of CO₂ on both the normal and injured newborn lung.

The present study is the first, to our knowledge, to adapt the technique of high-resolution two-dimensional echocardiography to the neonatal rat and was among the first (33) to report pulse-wave Doppler measurements in animals of this size. This not only complemented our pathological data but also provided, for the first time, a reliable and reproducible in vivo measure of myocardial performance. Although pulmonary arterial Doppler-derived indexes of resistance provide only an indirect measure of myocardial performance. Although pulmonary arterial Doppler-derived indexes of resistance, they have been reported by others to correlate with direct measurement of pulmonary arterial pressure (27, 28) and, as we have demonstrated in the present study, were found to closely correlate with the degree of pulmonary vascular remodeling. Using these techniques, we also made the intriguing observation that mild hypercapnia (5.5% CO₂) normalized right ventricular performance in hypoxia-exposed animals while having a much smaller and nonsignificant effect on increased pulmonary arterial resistance. Furthermore, we observed a trend toward increased RVSV in control animals exposed to hypercapnia. A possible underlying basis for these observations may be a hypercapnia-induced increase in myocardial blood flow (62).

In conclusion, our data provide evidence of a protective role for therapeutic hypercapnia in the prevention of chronic hypoxia-induced neonatal pulmonary hypertension. This could have important implications for the clinical management of human infants with this condition. We speculate that these protective effects of CO₂ were related to antioxidant properties, the precise mechanisms of which remain to be elucidated, as well as inhibition of ET-1 upregulation in the lung.

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