Therapeutic effects of hypercapnia on chronic lung injury and vascular remodeling in neonatal rats

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Submitted 24 April 2009; accepted in final form 9 September 2009

Management strategies, utilized in an attempt to reduce the likelihood of infants developing BPD, have included minimizing the duration of mechanical ventilation, avoiding the use of high inspired O2 concentrations while maintaining adequate oxygenation, fluid restriction, bronchodilators, diuretic therapy, and improved nutritional support, none of which has had any major effect on the ultimate outcome (4). A very promising approach, developed from adult intensive care practices, has been that of permissive hypercapnia, which has improved survival in patients suffering from acute respiratory distress syndrome (1, 6, 33) and acute lung injury (30). The initial rationale underlying the use of permissive hypercapnia had been that ventilation with reduced tidal volumes should protect against stretch-mediated lung injury (36). An alternative, or complementary, explanation for the beneficial effects of ventilation with reduced tidal volumes is a direct protective effect of hypercapnia (25, 31). Permissive hypercapnia is now a widely used strategy in ventilation of the newborn, with a target arterial partial pressure of CO2 (PaCO2) of 45–55 mmHg (50), which appears to have no identifiable short-term adverse consequences (35).

Multicenter trials of permissive hypercapnia conducted on premature human infants, using a target PaCO2 of ~52 or 55–65 mmHg to reduce minute ventilation below that of control infants, have failed to demonstrate an altered incidence of death or BPD (9, 48). Logical explanations for this lack of benefit include: inadequate sample sizes, in that both of these trials had to be terminated before full patient recruitment; the neonatal lung is resistant to the benefits of hypercapnia; and the target PaCO2 in these clinical trials was less than that required to obtain benefit.

So-called therapeutic hypercapnia, in which the CO2 concentration in the inspired gas has been increased, has been used to demonstrate a protective effect of an increased PaCO2, in both acute studies of ventilator-induced (44, 45) and ischemia-reperfusion-induced (32) lung injuries in animal models. We applied this same approach to an established rat model of chronic neonatal lung injury induced by exposure to 60% O2 for 14 days (15) to determine whether either the neonatal lung is unresponsive to hypercapnia or whether a greater degree of hypercapnia than that applied clinically to date. Neonatal rats inhaled air or 60% O2 for 14 days with or without 5.5% CO2. Lung parenchymal neutrophil and macrophage numbers were significantly increased by hyperoxia alone, which was associated with interstitial thickening and reduced secondary crest formation. The phagocyte influx, interstitial thickening, and impaired alveolar formation were significantly attenuated by concurrent hypercapnia. Hyperoxic pups that received 5.5% CO2 had a significant increase in alveolar number relative to air-exposed pups. Increased tyrosine nitration, a footprint for peroxynitrite-mediated reactions, arteriolar medial wall thickening, and both reduced small peripheral pulmonary vessel number and VEGF and angiopoietin-1 (Ang-1) expression, which were observed with hyperoxia, was attenuated by concurrent hypercapnia. We conclude that evolving chronic neonatal lung injury in a rat model is responsive to the beneficial effects of hypercapnia. Inhaled 5.5% CO2 provided a significant degree of protection against parenchymal and vascular injury in an animal model of chronic neonatal lung injury likely due, at least in part, to its inhibition of a phagocyte influx.

carbon dioxide; lung growth; inflammation

BRONCHOPULMONARY DYSPLASIA (BPD) is a chronic neonatal lung injury that affects extremely premature infants of <32-wk gestation who have required mechanical ventilation and O2 therapy following birth (3, 26). The incidence of BPD is inversely proportional to the gestational age at which these infants are born, approaching 60% in the smallest survivors. The cardinal features of BPD, as observed in the current era, are parenchymal thickening with an inhibition/arrest of alveolar growth (10). Pulmonary hypertension is a major adverse outcome in those infants most seriously affected (46, 47).

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to 60% O2 for 14 days have a lung histology similar to that observed in human infants with BPD (15) in that they have an heterogeneous injury with impaired alveolar development and thickening of the lung parenchyma (53). The parenchymal lung injury is heterogeneous, with areas of interstitial thickening and active DNA synthesis mixed with areas of arrested alveolarization and growth (15). This parenchymal thickening appears to be due to an increase in cell number in that there is no effect of 60% O2 on lung wet-to-dry weight ratios (15) and that 60% O2 induces no qualitative difference in collagen deposition, as assessed by Sirius red staining (data not shown) as previously described (22). The cells represented in the thickened parenchyma appear to be largely of epithelial origin (41).

Neonatal rats exposed to 60% O2 for 14 days also develop a macrophage influx-dependent pulmonary hypertension (20). Based on the protective effects observed with adult human patients with acute respiratory distress when subjected to a target Paco2 of 67 mmHg (16), we studied the effect of adding 5.5% carbon dioxide to the inspired gas of neonatal rat pups, which we estimated, from the alveolar gas equation, should achieve a Paco2 of ~70 mmHg.

**MATERIALS AND METHODS**

**In vivo interventions.** Animal experiments were conducted according to Canadian Council on Animal Care guidelines. Approvals were obtained from the Animal Care Review Committees of the Sunnybrook and Hospital for Sick Children Research Institutes. Rat pups (10–12 per litter) were exposed to air or 60% O2 in paired chambers for up to 14 days as previously described (8, 15, 20, 41, 53). Half the litters in air or 60% O2 had 5.5% CO2 included in their inspired gas. Pups delivered inside the chambers. Dams were exchanged daily between chambers to avoid maternal O2 toxicity.

**Blood gas analyses and assessment of respiratory and heart rates.** An initial study was conducted to confirm that the Paco2 predicted from the alveolar gas equation had been achieved. Pups that had been exposed to air or concurrent air and 5.5% CO2 were anesthetized in their sealed exposure chambers on day 14 of life using intraperitoneal ketamine-xylazine (80:20 mg/kg) for carotid artery puncture. Analyses were performed using CG4+ cartridges with an i-STAT portable clinical analyzer (Abbott Diagnostics, Abbott Park, IL). Effects of anesthesia on heart and respiratory rates were assessed using a tail sensor pulse oximeter (MouseOx; STARR Life Sciences, Oakland, PA).

**Immunohistochemistry.** Lungs were initially flushed with PBS containing 1 U/ml heparin, while undergoing manual inflations, to clear the pulmonary circulation of blood and then perfused with 4% (wt/vol) freshly dissolved paraformaldehyde before fixing over 12 h suspended in 4% (wt/vol) freshly dissolved paraformaldehyde. Throughout the fixation process, a constant airway pressure of 20 cmH2O was maintained with air, via a tracheal catheter, to prevent lung recoil. For vessel counts, and measurement of vessel medial wall thickness, a vascular perfusion pressure of 100 cmH2O was maintained after ligation of the pulmonary veins. The lungs were briefly washed three times in PBS, followed by a graded ethanol series, and then embedded in paraffin at 60°C. Randomly oriented lung blocks were cut into 5-µm sections for staining with hematoxylin and eosin or for immunohistochemistry. Immunostaining was performed using an avidin-biotin-peroxidase complex method (18). Slides were incubated overnight at 4°C with the primary antibody. After a 1-h incubation with biotin-conjugated secondary antibody, the labeled Vectorstain ABC system (Vector Laboratories, Burlingame, CA) was used with a substrate of 3,3-diaminobenzidine (DAB peroxidase substrate kit; Vector Laboratories). Slides were mounted in Permount mounting medium. Immunostaining for myeloperoxidase was performed with 1:2,000 primary rabbit polyclonal antibody to von Willebrand factor (Thermo Fisher Scientific, Fremont, CA) to identify endothelial cells (38), and with 1:100 rabbit polyclonal antibody to von Willebrand factor (Upstate Biotechnology, Lake Placid, NY) to identify nitrotyrosine residues (20). Secondary species-specific antibody concentrations were 1:200. A fluorescent secondary antibody was used for von Willebrand factor immunolocalization (Alexa Fluor 488; A-11008; Invitrogen, Carlsbad, CA). Counterstaining was performed with Nuclear Red for myeloperoxidase and with Carazzi hematoxylin for nitrotyrosine, CD68, and α-smooth muscle actin (Neomarkers, Fremont, CA), with 1:50 rabbit polyclonal antibody (Upstate Biotechnology, Lake Placid, NY) to identify nitrotyrosine residues (20). Secondary species-specific antibody concentrations were 1:200. A fluorescent secondary antibody was used for von Willebrand factor immunolocalization (Alexa Fluor 488; A-11008; Invitrogen, Carlsbad, CA). Counterstaining was performed with Nuclear Red for myeloperoxidase and with Carazzi hematoxylin for nitrotyrosine, CD68, and α-smooth muscle actin. Peptides against which antibodies to myeloperoxidase, CD68, and α-smooth muscle actin were raised were not commercially available. Specificity of antibodies for their target proteins was therefore assessed by Western blot. Appropriately sized single protein bands only, at 42 and 98 kDa, were observed for α-smooth muscle actin and rat CD68, respectively. For myeloperoxidase, only the expected four bands at 10, 15, 55, and 60 kDa were observed.

**Western analyses and ELISA.** Western blots of lysates and immunoprecipitated lysates from perfused lung tissue were performed as previously described (54). Protein content was measured as described by Bradford (7). Membranes were incubated overnight at 4°C with 1:2,500 rabbit polyclonal antibody to nitrotyrosine (Upstate Biotechnology) or 1:500 rabbit polyclonal antibody to GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA) for normalization in blocking solution. After a thorough wash, the membranes were incubated with secondary horseradish peroxidase-conjugated anti-rabbit antibody (Cell Signaling Technology, Beverly, MA), diluted 1:3,000, for 90 min. Protein bands on blots were quantified by enhanced chemiluminescence detection, with integrated band densities being calculated after subtraction of background values, as previously described (20). For studies of α-smooth muscle actin nitration, α-smooth muscle actin was immunoprecipitated and then separated with protein A sepharose beads (Sigma, St. Louis, MO) and centrifugation. Because GAPDH could not be used to normalize immunoprecipitated bands, Ponceau S protein stain was used for normalization. Angiopoietin-1 (Ang-1), VEGF-A, and its receptor, Flt-1, were quantified by ELISA using commercially available kits (R&D Systems, Minneapolis, MN).

**Table 1. Blood gases from day 14 rat pups exposed to air or air + 5.5% CO2**

<table>
<thead>
<tr>
<th>Group</th>
<th>PaO2, mmHg</th>
<th>Paco2, mmHg</th>
<th>pH</th>
<th>HCO3−, meq/l</th>
</tr>
</thead>
<tbody>
<tr>
<td>Air</td>
<td>84 ± 3</td>
<td>46 ± 3</td>
<td>7.35 ± 0.02</td>
<td>25 ± 1</td>
</tr>
<tr>
<td>Air + 5.5% CO2</td>
<td>106 ± 1*</td>
<td>70 ± 3*</td>
<td>7.25 ± 0.03*</td>
<td>31 ± 2*</td>
</tr>
</tbody>
</table>

Data are presented as means ± SE (n = 4 or 5 animals per group), *P < 0.05 vs. air control group by 1-way ANOVA. PaO2, arterial PO2; Paco2, arterial PCO2; meq, milliequivalents.

**Table 2. Respiratory and heart rates of day 14 rat pups before and after anesthesia following exposure to air or air + 5.5% CO2**

<table>
<thead>
<tr>
<th>Group</th>
<th>RR Before</th>
<th>RR After</th>
<th>HR Before</th>
<th>HR After</th>
</tr>
</thead>
<tbody>
<tr>
<td>Air</td>
<td>147 ± 4</td>
<td>146 ± 6</td>
<td>475 ± 17</td>
<td>400 ± 25</td>
</tr>
<tr>
<td>Air + 5.5% CO2</td>
<td>143 ± 1</td>
<td>142 ± 2</td>
<td>407 ± 6*</td>
<td>352 ± 16</td>
</tr>
</tbody>
</table>

Data are presented as means ± SE (n = 4 or 6 animals per group). When assessed by 2-way ANOVA, no overall statistically significant effect of anesthesia on respiratory (RR) or heart rate (HR) was observed. There was an overall significant effect of CO2 on HR. *Significantly different (P < 0.05) on pairwise testing from HR before in air.
Table 3. Lung body weights and postfixation displacement lung volumes in 14-day-old rat pups exposed to air or 60% O2 ± 5.5% CO2

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Air</th>
<th>Air + 5.5% CO2</th>
<th>60% O2</th>
<th>60% O2 + 5.5% CO2</th>
</tr>
</thead>
<tbody>
<tr>
<td>LW, mg</td>
<td>467±4</td>
<td>477±7</td>
<td>468±1</td>
<td>488±9</td>
</tr>
<tr>
<td>BW, g</td>
<td>30.1±0.5</td>
<td>30.4±0.1</td>
<td>31.4±0.2</td>
<td>31.4±0.3</td>
</tr>
<tr>
<td>LW/BW</td>
<td>1.55±0.03</td>
<td>1.57±0.02</td>
<td>1.49±0.01</td>
<td>1.56±0.02</td>
</tr>
<tr>
<td>LV, μl</td>
<td>1,069±16</td>
<td>1,087±22</td>
<td>1,015±10</td>
<td>1,098±36</td>
</tr>
</tbody>
</table>

Data are presented as means ± SE (n = 5 pups per group). Both O2 [body weight (BW)] and CO2 [lung weight (LW)] had significant effects (P < 0.05) by 2-way ANOVA. No differences were detected for pairwise comparisons between individual groups. LV, lung volume.

**Morphometric analyses.** Morphometric analyses of whole left lungs were performed as previously described (53). Observers were masked from group allocation for all counting procedures. Ten random images were captured from nonoverlapping fields from each section, with four randomly oriented sections per animal and six animals from different litters per group. Mean linear intercept was calculated as described by Dunnill (11). Estimated alveolar surface area per unit lung volume was calculated as described by Kawakami et al. (27). The tissue fractions per image were calculated using a 130-point contiguous counting grid superimposed on each (×200) image and counting the proportion of grid points that fell on tissue. This same grid was used to determine secondary crest volume density. Secondary crests were identified by immunoreactive α-smooth muscle actin in their tips, and their density per unit area was derived from the total number of attached secondary crests in an image of known area, irrespective of their length. Ratios of secondary crests to tissue were derived using the superimposed grid and counting the number of points overlying secondary crests or other tissue. The number of total crests per field was counted manually without the use of the overlaying grid. Estimated total alveolar numbers were calculated as described by Weibel and Gomez (52). Postfixation lung volumes were measured by water displacement. These morphometric approaches are relatively crude compared with the elegant, precise, and technologically advanced approaches applied by some investigators (43), and we readily acknowledge that they provide only estimates of alveolar surface area and number. However, they have the advantage of simplicity and are well-suited to identifying large differences between groups. Some of our derived numbers could be subject to fixation artifact, if lung inflation was inconsistent. This would not appear to be the case in our studies based on the standard errors of 0.99–3.3% for postfixation lung volumes.

**Vessel numbers and medial wall thickness.** Tissue sections that had been paraffin-embedded were stained using Weigert’s resorcin-fuchsin solution (Elastin Products, Owensville, MO) diluted in acidic 70% (vol/vol) ethanol. Dewaxed and rehydrated sections were rinsed in distilled water and placed in Weigert’s solution overnight. After a 10-min wash in lukewarm tap water, slides were counterstained with 0.5% (wt/vol) tartrazine in 0.25% (vol/vol) acetic acid and then rinsed in distilled water. Sections were then dehydrated, cleared in xylene, and mounted using a Permount-xylene (1:1 vol/vol) mixture. Concentric rings with diameters of 20 and 65 μm were superimposed on images, and only those vessels with both inner and outer elastic lamina to identify arterioles and outer elastic band diameters within this range were counted. The choice of 20- and 65-μm diameters was somewhat arbitrary, with the intent of excluding capillaries yet including the most distal arterioles. The decision to only include vessels with both inner and outer elastic lamina was to identify only fully muscle-laced vessels, which may be contributing to any observed pulmonary hypertension. The number of peripheral vessels of 20–65 μm in diameter was counted in 10 random, nonoverlapping fields per lung with all fields being within 435 μm of the edge of the lung on tissue cross-section. Fields containing no identifiable vessels within the selected range were included in the analysis. Medial wall thickness of vessels of 20- to 65-μm outer diameter was calculated from the formula “percent wall thickness = (2 × wall thickness/external diameter) × 100” as previously described (28), with the following modification. Sixty to eighty vessels per pup were photographed under an oil-immersion lens at a 1,000-fold magnification. Vessels with an external diameter within 20–65 μm and having external diameters measured at 2 perpendicular planes within 33% of each other were included in the counts. Applying this exclusion eliminated ~20% of the total vessels that were cut too obliquely and therefore had the potential to introduce bias into the medial wall thickness measurement.

**Data presentation.** All values are presented as means ± SE. Data for multiple groups were subjected to 1- or 2-way ANOVA, followed...
by the Tukey test, using the SigmaStat (SPSS, Chicago, IL) analysis program. A $P$ value $<0.05$ was regarded as statistically significant.

**RESULTS**

The initial assessment of terminal blood gases confirmed that the predicted increase in $P_{aCO2}$ following exposure to 5.5% CO$_2$ had been achieved (Table 1). The assessment of respiratory and heart rates before and after anesthesia (Table 2) did not indicate any obvious effect of anesthesia on either parameter. Visual assessment of respiratory rates correlated with the values determined by oximeter.

Exposure to neither 60% O$_2$ nor 5.5% CO$_2$ had significant effects on lung or body weights, the lung-to-body weight ratios, or postfixation lung volumes (Table 3). As shown in Fig. 1A, exposure to 60% O$_2$ caused marked interstitial thickening of the lung that appeared, microscopically, to be attenuated by exposure to concomitant hypercapnia. Neonatal rats exposed to both air and 5.5% CO$_2$ had occasional areas that appeared to have an increase in interstitial thickening compared with those exposed to air alone. Calculation of the tissue fraction (proportion of field occupied by tissue) confirmed a significant increase following exposure to 60% O$_2$, which was attenuated in pups exposed to the combination of hyperoxia and hypercapnia (Fig. 1B). The mild patchy increase in interstitial thickening induced in air-exposed pups by hypercapnia was not statistically significant.

That exposure to 60% O$_2$ resulted in heterogeneous changes in alveolar diameter is well-demonstrated in Fig. 1, yet there was no apparent effect on mean linear intercept in Fig. 2A. As discussed elsewhere (53), the process of averaging values for all fields in a section masks heterogeneity occurring between individual fields. To assess this, we analyzed the variance of values for mean linear intercepts between fields within each section as a measure of heterogeneity (53), as shown in Fig. 2B. Exposure to 60% oxygen induced significant heterogeneity. Concurrent exposure to hypercapnia both reduced mean linear intercept values...
Secondary crests growing in from the walls of precursor saccules initiate the process of alveolar formation. A statistically significant increase in the number of secondary crests per unit area was observed in the pups exposed to 60% O₂ and 5.5% CO₂ for 14 days, relative to all other exposure groups (Fig. 3A). To exclude the possibility that this increase was due to an inflation artifact, the number of secondary crests was also expressed as a fraction of total tissue. Following this correction, it was evident that the combination of hyperoxia and hypercapnia had indeed stimulated secondary crest formation beyond that seen in the other exposure groups (Fig. 3B). There was a significant reduction in the secondary crest-to-tissue ratio following exposure to 60% O₂. This could reflect the previously noted increase in tissue fraction and also the fact that many of the secondary crests in 60% O₂-exposed pups are stunted in length, as previously described (53). The enhanced secondary crest formation, following exposure to a combination of hyperoxia and hypercapnia, resulted in a significant increase in estimated gas exchange surface areas (Fig. 3C) and estimated total alveolar numbers (Fig. 3D).

We have previously reported enhanced secondary crest and alveolar formation in 60% O₂-exposed pups when neutrophil influx into their lungs was prevented (53). One possible explanation of the increased secondary crest formation observed in animals exposed to both hyperoxia and hypercapnia was that the hypercapnia was suppressing an inflammatory cell influx. Myeloperoxidase staining for neutrophils at day 14 revealed an anticipated (53) increase in number with 60% O₂. The neutrophil influx appeared to be reduced by concomitant hypercapnia (Fig. 3A). Direct cell counts confirmed a significant but incomplete hypercapnia-dependent reduction in the neutrophil influx induced by hyperoxia (Fig. 4B). CD68 immunostaining for...
macrophages confirmed the anticipated (20) influx of macrophages induced by exposure to 60% O2, which also appeared to be attenuated by concomitant hypercapnia (Fig. 4C). A complete inhibition of the 60% O2-mediated macrophage influx by concomitant hypercapnia was confirmed by direct cell counts (Fig. 4D). Because lung tissue was fixed during inflation with air, macrophages retained their position on alveolar walls rather than floating into the lumen of the alveolus, as would have occurred during inflation with fluid.

Based on previous observations (20), we hypothesized that the observed inhibition of a macrophage influx into the 60% O2-exposed lungs by concomitant hypercapnia would also be associated with a reduction both in pulmonary hypertension and of nitrotyrosine formation. When assessed by immunohistochemistry for α-smooth muscle actin (Fig. 5A), the normal spiral pattern of perivascular smooth muscle observed around vessels of 30- to 50-μm diameter in air-exposed pups appeared thickened and formed a complete ring following exposure to hyperoxia, consistent with the development of pulmonary hypertension-induced vascular remodeling. This change appeared to be attenuated by concomitant hypercapnia. To assess pulmonary hypertension-induced vascular remodeling, we measured vessel medial wall thickness. As shown in Fig. 5B, exposure to 60% O2 significantly increased medial wall thickness, which was prevented by concomitant hypercapnia. Hypercapnia alone had no effect on medial wall thickness.

As shown in Fig. 6, the marked increase in nitrotyrosine immunoreactivity observed following exposure to 60% O2 was apparently attenuated by concomitant hypercapnia. That hypercapnia completely attenuated the marked increase in nitrotyrosine immunoreactivity observed following exposure to 60% O2 was confirmed by measurement of nitrotyrosine content by Western blot (Fig. 7A). Peroxynitrite is both a potent vasoconstrictor and an inhibitor of relaxation of small pulmonary vessels (5). One mechanism by which relaxation could be impaired is through nitration of smooth muscle proteins. We therefore specifically immunoprecipitated α-smooth muscle actin to assess its degree of tyrosine nitrination by Western blot. After densitometry, it became apparent that exposure to 60% O2 significantly increased tyrosine nitrination of α-smooth muscle actin compared with controls, which was attenuated by concomitant exposure to hypercapnia (Fig. 7B).

When examining slides for changes in α-smooth muscle actin immunoreactivity, as described above, we noted an apparent reduction in small vessels in the lung periphery induced by hyperoxia and restored by concomitant hypercapnia. When endothelial cells were identified, using an antibody to von Willebrand factor, there was a qualitatively obvious reduction of vessels of all sizes, including capillaries (Fig. 8). We did not attempt to perform capillary counts, which we find challenging due to the uncertainties involved in labeling immunoreactive cell clusters with no apparent lumen. However, vessel depletions included an apparent 60% O2-dependent reduction in the number of arterioles, which was restored by concomitant hypercapnia. Hart’s elastin stain was used to better demonstrate this finding (Fig. 9) and to allow direct counts of small vessels with both inner and outer elastic lamina and an outer elastic lamina diameter of 20–65 μm in the lung periphery. As shown in Fig. 10A, hyperoxia caused a significant reduction in the number of small vessels in the lung periphery, which was completely attenuated by concomitant hypercapnia. The pattern of a reduction in vessel number in hyperoxia, which was restored by concomitant hypercapnia, was matched by parallel reductions in VEGF (Fig. 10B) and Ang-1 (Fig. 10C) contents, which were also restored by concomitant hypercapnia. Flt-1 content was unaffected by either hyperoxia or hypercapnia (Fig. 10D).

**DISCUSSION**

Outside the neonatal period, hypercapnia is well-recognized to have anti-inflammatory effects on the injured lung (29). Intuitively, this would be a likely significant contributor to any protective effect on lung injury. However, a recent study of...
experimental pneumonia-induced lung injury suggested that the protective effect of hypercapnia was neutrophil-independent (39). The only laboratory study, of which we are aware, that has addressed the question of whether hypercapnia is protective against injury in the developing lung was a short-term (6-h) study conducted in ventilated preterm lambs (45). The authors of that study found that a mean PaCO₂ of 95 mmHg was protective against acute lung injury. Hypercapnia was also associated with a reduction in indicators of inflammation, although only a reduction in airway inflammatory cells achieved statistical significance. Most likely because our model is a chronic preparation, we were able to detect a significantly reduced influx of neutrophils in the lung tissue compared to the control group.

Fig. 6. Nitrotyrosine formation, a marker for peroxynitrite-mediated reactions, in the lung tissue of neonatal rats exposed to air or 60% O₂ for 14 days with or without 5.5% CO₂. As a negative control, sections were immunostained after immunoadsorption of the primary antibody. As a positive control, sections were immunostained after exposure to peroxynitrite. Bar = 100 μm.

Fig. 7. Lung homogenates from neonatal rats exposed to air (a) or 60% O₂ (o) for 14 days with or without 5.5% CO₂ (a+c; o+c) were studied by Western blot and densitometric analysis for nitrotyrosine content (A) and after immunoprecipitation for the content of nitrated α-smooth muscle actin (B). Both O₂ (A) and CO₂ (A and B) had significant effects (P < 0.05) by 2-way ANOVA. *Significantly different (P < 0.05) from the air group. §Significantly different (P < 0.05) from the 60% O₂ group. Values are means ± SE for 4 average-sized pups from 4 different litters.
of inflammatory cells into the lung, including the lung parenchyma, in response to hypercapnia. One other study examined gene expression in air-exposed neonatal mouse lungs and found hypercapnia to downregulate the expression of a number of immune response genes, but no corresponding protein measurements were made (34). We are unaware of other studies showing hypercapnia-mediated inhibition of pulmonary inflammation or protection against lung injury in the neonate.

Our initial assessment of blood gases suggested that we had achieved the target PaCO₂. Subsequent assessment of the impact of anesthesia on respiratory and heart rates did not suggest any obvious impact. However, we acknowledge that we cannot exclude some degree of anesthesia effect with absolute certainty. We observed hypercapnia to completely protect against 60% O₂-mediated perivascular smooth muscle hyperplasia and vascular pruning. This we attribute, at least in part, to the

Fig. 8. Immunofluorescent staining for von Willebrand factor (white), a marker for endothelial cells, in the lungs of neonatal rats exposed to air or 60% O₂ for 14 days with or without 5.5% CO₂. Bar = 100 μm.

Fig. 9. Hart’s elastin staining of lung tissue from neonatal rats exposed to air or 60% O₂ for 14 days with or without 5.5% CO₂ was used to identify vessels with elastic lamina. Bar = 200 μm.
previously described critical role of a macrophage influx in both protein nitration and pulmonary hypertension in this model (20, 23). We speculate that the two processes may be linked through the potent vasoconstrictive properties of peroxynitrite (5), which could result in sustained pulmonary hypertension with secondary smooth muscle cell hyperplasia and an eventual reduction in vessel number. Alternatively, or additionally, the reduction in vessel number could be due to a failure of arteriolar formation induced by hyperoxia. Consistent with this latter explanation was the reduced concentrations of VEGF and Ang-1 induced by hyperoxia, which was restored by concomitant hypercapnia. Some caution needs to be exerted with respect to this interpretation of the VEGF/Ang-1 data, since only a single time point was studied, and expression could have been quite different at earlier time points.

We (5) have previously documented that peroxynitrite impairs relaxation of perivascular smooth muscle. We speculate, based on our observations herein, that this could result, at least in part, from protein modification of α-smooth muscle actin by nitration. Hypercapnia reduced the protein nitration associated with exposure to 60% O₂, which is consistent with our previous findings in a different lung injury model (32) but conflicts with expectations based on ex vivo analyses (14).

The effect of therapeutic hypercapnia on preventing the neutrophil influx during lung injury, although statistically significant, was incomplete. The partial limitation of a neutrophil influx was, however, sufficient to unmask the stimulation of secondary crest formation by 60% O₂, as previously described (53), which resulted in increased alveolar formation. Inflammation has long been recognized as an aggravating or exacerbating factor both in human BPD (14) and in hyperoxic rat models of chronic neonatal lung injury (2, 20, 51, 53). Based on one published study that used neonatal rat pups exposed to 95% O₂ (51), we might have expected the observed CO₂-mediated inhibition of a macrophage influx to have been accompanied by a similarly effective inhibition of neutrophil influx. However, this was not the case, which is probably due to the very different inflammatory cell responses observed in rat pups exposed to 60% O₂ rather than 95% O₂. In rat pups exposed to 60% O₂, the neutrophil influx precedes the macrophage influx (20), suggesting that in the 60% O₂-mediated neonatal lung injury it is not the macrophage that initiates the neutrophil influx, as seen with pups in 95% O₂.

Our study, although clearly demonstrating the protective effects of therapeutic hypercapnia in this model, leaves unanswered a number of important mechanistic questions. These include defining the mediators of pulmonary hypertension that are downregulated by hypercapnia. In previous studies, we have demonstrated O₂-mediated upregulation of the vasoconstrictors endothelin-1, 8-isoprostanone (19, 21), and peroxynitrite (20). In this study, we did not examine the effect of hypercapnia on the expression of endothelin-1 or 8-isoprostanone but did observe reduced nitrotyrosine immunoreactivity, a marker for peroxynitrite-mediated reactions. Determining whether peroxynitrite is a critical mediator of pulmonary hypertension will require intervention studies using, for example, a peroxynitrite decomposition catalyst (40). Additionally, we did not determine whether hypercapnia affects apoptosis in vascular smooth muscle cells or in lung parenchymal cells. We have previously demonstrated that endothelin-1 inhibits apoptosis of neonatal rat pulmonary artery smooth muscle cells (24).

Our primary objective in these studies was to determine whether the chronically injured neonatal lung was capable of a beneficial response to therapeutic hypercapnia. Our observations that hypercapnia, in the presence of moderate hyperoxia, limited pulmonary parenchymal injury, promoted alveolar

Fig. 10. Neonatal rats exposed to air (a) or 60% O₂ (o) for 14 days with or without 5.5% CO₂ (a+c, o+c). A: counts of lung peripheral vessels with an outer elastic lamina diameter (o.d.) of 20–65 μm expressed as vessel density per unit area. B: VEGF-A concentrations in lung homogenates. C: angiopeptin-1 (Ang-1) concentrations in lung homogenates. D: VEGF-A receptor Flt-1 concentrations in lung homogenates. Both O₂ (A and B) and CO₂ (A) had significant effects (P < 0.05) by 2-way ANOVA. *Significantly different (P < 0.05) from the air group. §Significantly different (P < 0.05) from the 60% O₂ group. Values are means ± SE for 6 average-sized pups from different litters.
growth, and prevented vascular remodeling during neonatal lung injury clearly demonstrated that the neonatal lung does have such a capacity. However, whether such a beneficial effect can be extrapolated to the human clinical arena remains unclear. Our calculated target PaCO$_2$ was greater than that accepted in normal clinical practice within neonatal intensive care units. Although adult humans may tolerate extreme levels of hypercapnia without adverse effects (37, 49), the population of human infants most prone to develop BPD are at particular risk of developing intracranial hemorrhage, retinopathy of prematurity and neurodevelopmental impairment. Their risk of developing the latter morbidities may be increased by hypercapnia (12, 17, 48), and any future studies designed to test the potential benefits of a less conservative PaCO$_2$ than previously used (9) would need to be undertaken with an appropriate degree of caution.

GRANTS

This work was supported by group [J. Belik, B. P. Kavanagh, and A. K. Tanswell (MGC-250291) and operating [J. Belik (MOP-49473), R. P. Jankov (MOP-74506 and -84290), B. P. Kavanagh (MOP-69006), and A. K. Tanswell (MOP-152761)] grants from the Canadian Institutes of Health Research (CIHR) and an infrastructure grant from the Canada Foundation for Innovation New Opportunities Fund (R. P. Jankov). R. P. Jankov holds a CIHR Independent Investigator award.

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