Sustained therapeutic hypercapnia attenuates pulmonary arterial Rho-kinase activity and ameliorates chronic hypoxic pulmonary hypertension in juvenile rats

Gary Peng,1 Julijana Ivanovska,1 Crystal Kantores,1 Todd Van Vliet,1,3 Doreen Engelberts,1 Brian P. Kavanagh,1,4,5 Masahiro Enomoto,1 Jaques Belik,1,2,3,5 Amish Jain,1,3 Patrick J. McNamara,1,3,5 and Robert P. Jankov1,2,3,5

1Physiology & Experimental Medicine Program, Hospital for Sick Children Research Institute, Toronto, Ontario, Canada; 2Heart and Stroke Richard Lewar Centre of Excellence, University of Toronto, Toronto, Ontario, Canada; 3Division of Neonatology, Department of Paediatrics, University of Toronto, Toronto, Ontario, Canada; and the 4Department of Anaesthesia, University of Toronto, Toronto, Ontario, Canada; and 5Department of Physiology, University of Toronto, Toronto, Ontario, Canada

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Peng G, Ivanovska J, Kantores C, Van Vliet T, Engelberts D, Kavanagh BP, Enomoto M, Belik J, Jain A, McNamara PJ, Jankov RP. Sustained therapeutic hypercapnia attenuates pulmonary arterial Rho-kinase activity and ameliorates chronic hypoxic pulmonary hypertension in juvenile rats. Am J Physiol Heart Circ Physiol 302: H2599–H2611, 2012. First published April 13, 2012; doi:10.1152/ajpheart.01180.2011.—Sustained therapeutic hypercapnia prevents pulmonary hypertension in experimental animals, but its rescue effects on established disease have not been studied. Therapies that inhibit Rho-kinase (ROCK) and/or augment nitric oxide (NO)-cyclic guanosine monophosphate (cGMP) signaling can reverse or prevent progression of chronic pulmonary hypertension. Our objective in the present study was to determine whether sustained rescue treatment with inhaled CO2 (therapeutic hypercapnia) would improve structural and functional changes of chronic hypoxic pulmonary hypertension. Synchronously breathing pups were exposed to normoxia (21% O2) or hypoxia (13% O2) from postnatal days 1–21 with or without 7% CO2 (Paco2, elevated by ~25 mmHg) or 10% CO2 (Paco2, elevated by ~40 mmHg) from days 14 to 21. Compared with hypoxia alone, animals exposed to hypoxia and 10% CO2 had significantly (P < 0.05) decreased pulmonary vascular resistance, right-ventricular systolic pressure, right-ventricular hypertrophy, and medial wall thickness of pulmonary resistance arteries as well as decreased lung phosphodiesterase (PDE) V, RhoA, and ROCK activity. Rescue treatment with 10% CO2, or treatment with a ROCK inhibitor (15 mg/kg ip Y-27632 twice daily from days 14 to 21), also increased pulmonary arterial endothelial nitric oxide synthase and lung NO content. In contrast, cGMP content and cGMP-dependent protein kinase (PKG) activity were increased by exposure to 10% CO2, but not by ROCK inhibition with Y-27632. In vitro exposure of pulmonary artery smooth muscle cells to hypercapnia suppressed serum-induced ROCK activity, which was prevented by inhibition of PKG with Rp-8-Br-PET-cGMPs. We conclude that sustained hypercapnia dose-dependently inhibited ROCK activity, augmented NO-cGMP-PKG signaling, and led to partial improvements in the hemodynamic and structural abnormalities of chronic hypoxic PHT in juvenile rats. Increased PKG content and activity appears to play a major upstream role in CO2-induced suppression of ROCK activity in pulmonary arterial smooth muscle.

carbon dioxide; hypoxia; nitric oxide; RhoA; protein kinase G

CHRONIC PULMONARY HYPERTENSION (PHT) is a debilitating disease characterized by a progressive increase in pulmonary arterial pressure and resistance, secondary to sustained pulmonary vasoconstriction and arterial wall remodeling. In early life, chronic PHT is most frequently observed in the context of severe bronchopulmonary dysplasia (51, 64), vascular hypoplasia syndromes, including congenital diaphragmatic hernia (43), and in a subset of cases of idiopathic persistent PHT of the newborn (46). Efforts to treat chronic PHT in this population are frequently hampered by nonresponsiveness to pulmonary vasodilators, such as inhaled nitric oxide (NO), and a relatively rapid progression toward end-stage pathology (9, 21, 66). In experimental animals, sustained vasoconstriction and vascular remodeling in chronic PHT involve activation of RhoA/Rho-kinase (ROCK) (41, 74) and decreased function of the NO-cyclic guanosine monophosphate (cGMP) (52) signaling pathways.

There is substantial evidence to suggest that hypercapnia, induced by sustained inhalation of carbon dioxide (CO2), may ameliorate injury to the lung (39) and pulmonary vasculature (30, 33, 39). Sustained exposure to CO2 has been reported to prevent chronic hypoxic PHT in both adult (50) and neonatal (33) rats and to reverse acute hypoxic (3, 10, 12, 15) or vasoconstrictor-induced (10) PHT, particularly when acute changes in pH are buffered. Hypercapnia is thought to prevent pulmonary vascular injury via multiple pathways, among which include attenuated oxidative and nitrative stress (10, 33, 39, 49) and downregulated expression of critical G protein-coupled receptor (GPCR) ligands, such as endothelin-1 (33), all of which have potential to activate ROCK in pulmonary arterial smooth muscle. Additionally, vasodilatory effects of CO2 on the ex vivo pulmonary vasculature have been shown to be most evident when vascular tone is high (11), to occur independent of changes in pH (11, 67), and to persist in the absence of endothelium-derived NO (11), suggesting a direct effect of CO2 on smooth muscle.

Activation of the small GTPase, RhoA, and its effector protein ROCK (40, 60) is strongly implicated as a key pathway regulating changes in pulmonary vascular tone and smooth muscle phenotype, which contribute to increased pulmonary arterial pressure and resistance. Inhibition of ROCK blunts vasoconstrictor responses to a variety of stimuli, including GPCR activation, inhibition of NO signaling, and smooth muscle calcium entry in both the pulmonary and systemic circulations (8). As opposed to intermittent (or phasic) contraction, which is regulated by changes in cytosolic calcium, ROCK activation leads to sustained contraction of smooth muscle at a given level of calcium (known as calcium sensitiv-
zation), which results from persistent phosphorylation of myosin regulatory light chain (MLC2) (14, 17, 59). The substrate of ROCK upstream of MLC2 is myosin light chain phosphatase (MLCP) (17). Phosphorylation of threonine-696 and/or -853 (850 in the rat) of the myosin phosphatase target (MYPT)-1 regulatory subunit of MLCP by ROCK leads to repression of its activity (22). Known effects of ROCK inhibition in chronic PHT include reversal of sustained vasoconstriction through smooth muscle calcium desensitization (17, 41, 59), attenuated remodeling through decreased proliferation and enhanced apoptosis of arterial wall smooth muscle (74, 75), and augmented vascular NO bioavailability and signaling (1, 16, 19, 42, 53, 65).

Given that rescue treatment of chronic PHT is of greater relevance, in the clinical context, than prevention, our objective in the present study was to examine the rescue effects of sustained hypercapnia on established chronic hypoxic PHT in young rats. We hypothesized that sustained exposure to CO2 would attenuate RhoA/ROCK activity, decrease pulmonary arterial pressure and resistance, and attenuate vascular remodeling.

MATERIALS AND METHODS

Materials. Y-27632 (a ROCK inhibitor) and Rp-8-Br-PET-cGMPS [a cGMP-dependent protein kinase (PKG) type I inhibitor] were from Axxora (San Diego, CA). Phos-tag acrylamide was from NARD Institute (Amagasaki City, Hyogo, Japan). A nitrate/nitrite (NOx) assay kit was from Cayman Chemical (Ann Arbor, MI). Dulbecco’s modified Eagle’s medium (DMEM), trypsin, and heat-inactivated fetal bovine serum (FBS) were from GIBCO-BRL (Burlington, Ontario, Canada). Avidin-biotin-peroxidase complex immunohistochemistry kits, 3,3’-diaminobenzidine staining kits, 4-

<table>
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<th>Parameter/Group</th>
<th>Air + Normocapnia</th>
<th>Hypoxia + Normocapnia</th>
<th>Air + 7% CO2</th>
<th>Hypoxia + 7% CO2</th>
<th>Air + 10% CO2</th>
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<td>46.5 ± 0.6</td>
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<td>pH</td>
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<td>7.27 ± 0.03</td>
<td>7.24 ± 0.02</td>
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<td>80.4 ± 1.6</td>
<td>73.4 ± 1.0†</td>
<td>94.2 ± 2.8**</td>
<td>94.4 ± 2.2***</td>
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<td>PaO2, mmHg</td>
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<td>99.0 ± 3.7</td>
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<td>106.7 ± 7.6*</td>
<td>49.2 ± 3.6*</td>
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<td>28.6 ± 0.4*</td>
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<td>Mean SBP, mmHg</td>
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<td>101 ± 6</td>
<td>102 ± 4</td>
<td>112 ± 7</td>
<td>114 ± 9</td>
<td>124 ± 7</td>
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Values represent means ± SE. n = 6–8 animals/group. SBP, systemic blood pressure. *P < 0.01, by ANOVA, compared with respective air group exposed to the same level of CO2. †P < 0.05, by ANOVA, compared with respective normocapnia group exposed to the same level of O2. ‡P < 0.01, by ANOVA, compared with all other groups. Δ P < 0.01, by ANOVA, compared with all normocapnia-exposed groups. **P < 0.01, by ANOVA, compared with all 7% CO2-exposed groups.
was estimated from the parasternal short-axis view according to the formula: [(LV end-diastolic diameter - LV systolic diameter)/LV end-diastolic diameter] × 100. RV-to-left ventricle + septum dry weight ratios (n = 10–12 animals representing 4 litters/group) and histological assessment of percentage medial wall thickness (%MWT) of pulmonary arteries (left lungs from n = 4 animals representing 4 litters/group) were measured as previously reported (29, 33, 41, 74). All measurements were carried out by an observer blinded to group identity. For assessment of %MWT, pulmonary arteries (20–100 μm external diameter) were identified by the presence of both inner and outer elastic lamina using Hart’s stain of paraffin-embedded lungs (33, 74).

Frozen sections. Lungs from four animals (representing 2 litters) per group were inflated and snap-frozen in optimum-cutting temperature compound, as previously described (26), then cut by cryostat into 10-μm sections, which were mounted, fixed in ice-cold acetone, and incubated with anti-vWF (diluted 1:60, 1 h at room temperature) as a marker of endothelial cells (39) followed by Alexa Fluor 546-conjugated secondary antibody (diluted 1:300, at room temperature in the dark for 30 min), before aqueous mounting with DAPI. Images were digitally captured using an epifluorescent microscope with appropriate filter sets and merged using Image-Pro Plus software (version 7.0; Media Cybernetics, Bethesda, MD).

Capillary counts. To quantify vWF-immunoreactive capillaries (taken as vessels <10 μm diameter, including cell clusters with no apparent lumen), two noncontiguous paraffin-embedded left lung sections per animal (6 animals representing 2 litters/group) were immunostained for vWF and aqueous mounted with DAPI. High-power images from 10 random nonoverlapping fields per section were acquired, merged, and counted by an observer blinded to group identity. Counts were normalized to tissue fraction (75).

Arterial contraction and relaxation studies. Isometric contraction of ex vivo intralobar pulmonary arteries, or mesenteric arteries (4 arteries from 4 pups representing 2 litters/group), in response to a thromboxane analog, U-46619, or relaxation responses to an eNOS stimulator (acetylcholine; ACh) were measured (8 arteries from 8 pups representing 4 litters/group) as previously described (5, 28).

In vitro studies. Primary pulmonary artery smooth muscle cell (PASMC)-enriched cultures were obtained from explants of pooled intrapulmonary arteries from a litter (10 pups) of normal (normoxia-exposed) day 14 Sprague-Dawley rats, as previously described in detail (26). All experiments were duplicated in explants derived from a second litter of pups to ensure reproducibility. We have previously shown that treatment with 10% FBS induces a marked upregulation of ROCK activity (74). Cells were passaged by trypsinization using 0.05% (wt/vol) trypsin/EDTA and centrifugation at 300 g for 5 min, followed by reseeding in six-well plates. At passage 2–3, cells were treated with DMEM + 0.1% (vol/vol) FBS (negative control) or 10% (vol/vol) FBS at two different concentrations of ambient CO2: 5% (control); 21% O2-balance N2 or 10% (hyperoxia – PCO2 in medium elevated by ~40 mmHg; 21% O2-balance N2) for 4 h, before collection of cells for lysis (2 wells/sample). Some cells were pretreated with Rp-8-Br-PET-cGMPS (10 μM) 1 h before addition of serum.

Western blot analyses. Third- or fourth-generation intrapulmonary arteries were dissected from four litters per group (the pooled vessels of 2 animals from each litter representing 1 sample). Lung tissue, pulmonary artery tissue, or PASMCs were lysed in RIPA buffer with protease [Protease Inhibitor Cocktail Set I (catalog no. 539131); Calbiochem, San Diego, CA] and phosphatase [Phosphatase Inhibitor Cocktail Set 2 (catalog no. P5726); Sigma] inhibitors and sonicated on ice at 40 watts for 30 s. Protein samples (50 μg/lane) were boiled in Laemmli buffer, fractionated by SDS-PAGE on 4–20% gradient Tris-glycine gels, transferred to polyvinylidene difluoride membranes, and blotted, as previously described (27). Dilutions of primary antibodies were 1:200 for VASP (50 kDa), 1:1,000 for eNOS and phospho-serine-1177 eNOS (135 kDa), MLC2 (20 kDa), phospho-serine-695/Thr-783 eNOS (135 kDa), MLC2, and phospho-serine-1177 eNOS and MLC2 (135 kDa), respectively.

Fig. 1. Rescue therapy with 10% CO2 improves pulmonary hemodynamics. Inverse pulmonary arterial acceleration time (PAAT)-to-right ventricular ejection time (RVET) ratio, as a measure of pulmonary vascular resistance (PVR; n = 6–8 animals/group) (A), right ventricular systolic pressure (RVSP; n = 4–6 animals/group) (B), and left ventricular (LV) systolic function (n = 7–8 animals/group) (C). Pups were exposed from postnatal days 1 to 21% O2 to 21% O2 (normoxia-exposed groups) or 13% O2 (hypoxia; filled bars) while receiving concurrent exposure to normoxia (<0.5%), 7 or 10% CO2 from days 14 to 21. Values represent means ± SE. *P < 0.01, by ANOVA, compared with normoxia-exposed groups. #P < 0.05, by ANOVA, compared with other hypoxia/normoxia groups.

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RESULTS

Body weights, arterial blood gas, and systemic blood pressure measurements. Values are shown in Table 1. Animals exposed to hypoxia for 21 days had significantly decreased body weight, relative to normoxia-exposed controls. Treatment of hypoxia-exposed animals with either 7 or 10% CO2, from days 14 to 21, led to increased somatic growth, relative to animals in normocapnia, with 10% CO2 having a greater effect. Exposure to 7% CO2 increased PaCO2 by ~25 mmHg and exposure to 10% CO2 by ~40 mmHg above levels measured in normocapnia-exposed controls. Exposure to either level of hypercapnia caused significant acidosis with partial metabolic correction, as reflected in lower pH and higher HCO3 levels than normocapnic controls. Values for PaCO2 obtained after a 30-min exposure to 7 or 10% CO2 (data not shown) were similar to those obtained after 7 days (Table 1), suggesting that PaCO2 remained stable throughout the exposure protocol. Chronic exposure to hypoxia and/or either level of CO2 did not significantly alter systemic blood pressure by day 21 (Table 1).

Effects on pulmonary hemodynamics and LV systolic function. To distinguish acute from chronic effects of hypercapnia on PVR index, preliminary measurements were first obtained while animals continued to breathe CO2 and were repeated following a 15-min period of recovery in room air. PVR index was found to be unchanged (P > 0.5; data not shown) by removal from CO2; therefore, all reported data were obtained while animals were breathing room air. As previously reported (74), exposure to hypoxia for 21 days led to a significantly increased PVR index (Fig. 1A) and RVSP (Fig. 1B), relative to normoxia-exposed controls. Hypoxia-induced increases in PVR index (Fig. 1A) and RVSP (Fig. 1B) were partially decreased by exposure to 10% CO2 from days 14 to 21 (P < 0.05), but were not significantly reduced by 7% CO2 (P > 0.05). LV fractional shortening was significantly (P < 0.01) increased by exposure to 10% CO2 in normoxia-exposed animals (Fig. 1C). Exposure to hypoxia, with or without 10% CO2, caused no significant changes in LV fractional shortening (Fig. 1C).

Effects on structural changes of PHT and lung development. As previously shown (74), exposure to hypoxia for 21 days led to significant right ventricular hypertrophy (RVH) (Fig. 2A) and increased %MWT (Fig. 2B) in pulmonary resistance arteries. Exposure to 10% CO2 from days 14 to 21 significantly attenuated RVH (Fig. 2A) and normalized %MWT in hypoxia-exposed animals (Fig. 2B), whereas exposure to 7% CO2 did
not lead to significant changes in either parameter (Fig. 2, A and B). Relative differences in medial wall thickness between groups are illustrated by high-power images of elastin-stained pulmonary arteries (Fig. 2C). We have previously reported that chronic exposure to hypoxia from birth for 14 days leads to altered distal airway structure characterized by enlarged distal airspaces, septal thinning, and greatly reduced numbers of secondary crests and peripheral (20–65 μm) pulmonary arteries (75). As shown in Fig. 2, D and E, these changes are also evident in rat pups exposed to 21 days of hypoxia. In addition, staining for vWF (Fig. 2E) suggested an increase in capillary numbers secondary to hypoxia, in keeping with a stimulatory effect of hypoxia on angiogenesis, as previously reported in adult rats (25). When vWF-immunoreactive capillary numbers were quantified, an upward trend in hypoxia-exposed animals approached statistical significance (P = 0.06 vs. normoxia; Fig. 2F). Capillary numbers were unchanged in normoxia- or hypoxia-exposed animals by exposure to 10% CO₂ (Fig. 2F).
Changes in RhoA/ROCK pathway. Increased activation of RhoA (Fig. 3A) and ROCK (Fig. 3, B and C) was observed in the lungs and intrapulmonary arteries, respectively, of animals exposed to hypoxia. Increased RhoA/ROCK activity (Fig. 3, A–C) secondary to hypoxia was normalized by exposure to 10% CO₂ (P < 0.05), but not by 7% CO₂ (P > 0.05). Changes in ROCK activity induced by 10% CO₂ were not accounted for by any change in ROCK-I or ROCK-II content (P > 0.05), which were unchanged by exposure to hypoxia, as previously reported (74), or by exposure to 10% CO₂ (Fig. 3D).

Changes in NO-cGMP signaling. Chronic exposure to hypoxia for 21 days caused no change (P > 0.05) in pulmonary arterial eNOS (Fig. 4A) or lung NOx (Fig. 4C) and a trend toward decreased phospho-serine-1177 eNOS content, a known contributor to endothelial dysfunction (45), which was close to statistical significance (P = 0.06 vs. normoxic control;
Exposure to 10% CO2 significantly increased pulmonary arterial eNOS (Fig. 4A) and lung NOx content in hypoxia-exposed animals (Fig. 4C) while there was a nonsignificant trend toward increased phospho-serine-1177 eNOS content (Fig. 4B). Chronic exposure to hypoxia led to a small, but significant, increase in lung PDE V activity (Fig. 5A). Exposure to 10% CO2 led to a significant (P < 0.05) attenuation in PDE V activity in both normoxia- and hypoxia-exposed animals (Fig. 5A); exposure to 7% CO2 had no effect (P > 0.05). Changes in PDE V were inversely correlated with altered cGMP content in pulmonary arteries (quantified by VASP phosphorylation), which was decreased in animals exposed to hypoxia alone and increased in animals exposed to hypoxia and 10% CO2 (Fig. 5B). Exposure to 10% CO2 (P < 0.05), but not 7% CO2 (P > 0.05), led to increased content (Fig. 5C) and activity (Fig. 5D) of PKG in pulmonary arteries from hypoxia-exposed animals. PKG activity, but not content, was also increased in normoxia-exposed control animals exposed to 10% CO2 (Fig. 5D).

ROCK inhibitor-induced changes in NO-cGMP signaling. Similar to findings with 10% CO2 exposure (Fig. 4), twice daily treatment with Y-27632 (a ROCK inhibitor), from days 14 to 21, significantly (P < 0.05) increased pulmonary arterial eNOS and lung NOx contents (Fig. 6A) in hypoxia-exposed animals. However, unlike 10% CO2 (Fig. 5, C and D), treatment with Y-27632 had no significant effects (P > 0.05) on

Fig. 4. Rescue therapy with 10% CO2 increases endothelial nitric oxide synthase (eNOS) and nitric oxide (NO) (NOx) contents. Western blot analyses of pulmonary arterial eNOS (n = 4 samples/group) (A) and serine-1177 phosphorylated eNOS (n = 6 samples/group) (B). C: lung tissue NOx (n = 6 samples/group). Values represent means ± SE. Pups were exposed from postnatal days 1 to 21 to 21% O2 (normoxia; open bars) or 13% O2 (hypoxia; filled bars) while receiving concurrent exposure to normocapnia (<0.5%), 7 or 10% CO2 from days 14 to 21. *P < 0.05, by ANOVA, compared with other hypoxia-exposed groups. #:P < 0.01, by ANOVA, compared with all other groups. Representative immunoblots are shown below each graph with noncontiguous gel lanes demarcated by black lines.

n = 6 samples/group). Exposure to 10% CO2 significantly (P < 0.05) increased pulmonary arterial eNOS (Fig. 4A) and lung NOx content in hypoxia-exposed animals (Fig. 4C) while there was a nonsignificant (P = 0.24) trend toward increased phospho-serine-1177 eNOS content (Fig. 4B). Chronic exposure to hypoxia led to a small, but significant (P < 0.05), increase in lung PDE V activity (Fig. 5A). Exposure to 10% CO2 led to a significant (P < 0.05) attenuation in PDE V activity in both normoxia- and hypoxia-exposed animals (Fig. 5A); exposure to 7% CO2 had no effect (P > 0.05). Changes in PDE V were inversely correlated with altered cGMP content in pulmonary arteries (quantified by VASP phosphorylation), which was decreased in animals exposed to hypoxia alone and
pulmonary arterial PKG content/activity or on cGMP content (Fig. 6B).

Changes in arterial contraction and relaxation function. As shown in Fig. 7, chronic exposure to hypoxia significantly increased pulmonary arterial force contraction in response to U-46619 and led to attenuated eNOS-dependent arterial relaxation. Force contraction was normalized in animals exposed to 10% CO2 while the relaxation response to eNOS stimulation with ACh was significantly (P<0.05, by ANOVA, compared with normocapnia group exposed to the same level of O2, #P<0.05, by ANOVA, compared with normocapnia group exposed to the same level of O2, †P<0.01, by ANOVA, compared with other normoxia-exposed groups. Representative immunoblots are shown adjacent to each graph with noncontiguous gel lanes demarcated by black lines.

In vitro effects of hypercapnia and PKG inhibition on ROCK activity. Compared with serum-starved PASMCs exposed to normocapnia (5% CO2), ROCK activity was increased in serum-stimulated PASMCs, which was significantly (P<0.05) attenuated by exposure to hypercapnia (10% CO2; Fig. 8A). Pretreatment with Rp-8-Br-PET-cGMPS, a PKG type I inhibitor, prevented suppression of serum-induced ROCK activity by hypercapnia (Fig. 8A), suggesting a major upstream role for PKG in this phenomenon. Exposure to hypercapnia had no significant effects on either ROCK-I or ROCK-II content in serum-treated PASMCs (Fig. 8B).

A schematic summarizing the effects of sustained inhaled CO2 on RhoA/ROCK and NO-cGMP signaling is shown in Fig. 9.

DISCUSSION

This is the first in vivo study, to our knowledge, to examine the rescue effects of sustained hypercapnia on established chronic PHT. Our group has previously shown that sustained inhalation of CO2, when commenced at the onset of injury, prevented chronic hypoxic or hyperoxic PHT (33, 39) in newborn rats. Such preventive effects appear to involve diverse, but interrelated, pathways, including attenuation of ox-
idative/nitrative stress (28, 30, 33, 39) and GPCR signaling (33), as well as increased NO bioavailability and signaling (5). Given recent evidence of a critical role for ROCK signaling downstream and upstream of these pathways (41, 74), we were interested to determine the effects of hypercapnia on ROCK signaling in the setting of rescue therapy. The concentrations of CO₂ examined were intended to reproduce what are generally considered, in the clinical setting, to represent moderate (7% CO₂; mean elevation of PaCO₂ 25 mmHg) and severe (10% CO₂; mean elevation of PaCO₂ 40 mmHg) levels of hypercapnia. Our findings were that sustained rescue treatment with 10% CO₂ decreased PVR and RVSP, attenuated arterial wall remodeling, and reduced RVH in chronic hypoxia-exposed juvenile rats. Effects of CO₂ on hemodynamic and structural changes of chronic hypoxic PHT were found to be concentration (or dose)-dependent in that lesser, nonstatistically significant, differences in many of these parameters were observed when animals were exposed to 7% CO₂. Exposure to 10% CO₂ (vs. normocapnia) also led to functional changes in pulmonary arteries from hypoxia-exposed animals, in which contractile potential was normalized and endothelium-dependent arterial relaxation (endothelial dysfunction) was improved.

Examination of arterial acid-base status revealed that a 7-day exposure to either level of CO₂ caused a similar degree of acidosis, due to differing degrees of metabolic correction. A possible implication of these observations is that benefits of CO₂ on PHT were related to the dose of CO₂ itself, rather than the degree of acidosis induced, in keeping with several ex vivo studies (11, 67). Alternatively, recent evidence suggests that arterial ROCK activity is highly pH-sensitive, being decreased at both low and high intracellular pH (6). It is not possible to separate the effects of acidosis from hypercapnia by buffering in this chronic model. In short-term lung injury models, however, normalizing pH by buffering has been shown to worsen injury (35) where inhaled CO₂ has otherwise been protective (36, 57, 63). The finding of acidosis in pups exposed to hypoxia for 21 days differs from previous data obtained after 14 days (33). Given that the acidosis was metabolic, rather than

Fig. 6. Rescue therapy with a ROCK inhibitor, Y-27632, increases eNOS and NO (NOx) contents but has no effects on PKG or cGMP. Y-27632-induced changes in contents of pulmonary arterial eNOS (n = 4 samples/group), lung tissue NOx (n = 6 samples/group) (A), or pulmonary arterial contents of PKG type I (bars on left), phosphorylated serine-695 MYPT-1 (as a marker of PKG activity; bars in middle), and VASP phosphorylation ratio (as a marker of cGMP content; bars on right; n = 4 samples/group) (B). Pups were exposed from postnatal days 1 to 21 to 13% O₂ while receiving saline vehicle (open bars) or 15 mg/kg ip Y-27632 twice daily (filled bars) from days 14 to 21. Values represent means ± SE relative to vehicle-treated hypoxia-exposed animals, which were assigned a mean value of 1. *P < 0.05, by ANOVA, compared with vehicle-treated group.

Fig. 7. Rescue therapy with 10% CO₂ normalizes thromboxane-induced arterial contractile force and improves endothelium-dependent relaxation. Thromboxane analog-induced isometric force tension [mN/mm²; n = 4 animals (4 arteries/group) (A) and percentage (%) endothelium-dependent relaxation (following U-46619-mediated preconstriction) in isolated 3rd- to 4th-generation intrapulmonary arteries [n = 8 animals (8 arteries/group)] (B). Values represent means ± SE. Where error bars are not visible, they fall within the plot point. Pups were exposed from postnatal days 1 to 21 to 21% O₂ (Air; open circles), 13% O₂ (Hypoxia; closed circles), or 13% O₂ with concurrent exposure to 10% CO₂ from days 14 to 21 (Hypoxia + 10% CO₂; gray triangles). *P < 0.05, by ANOVA, compared with air group at the same dose. #P < 0.05, by ANOVA, compared with other groups at the same dose.
Fig. 8. Hypercapnia limits ROCK activation in pulmonary arterial smooth muscle cells (PASMCs) through increased PKG activity. A: effects on serum stimulation with or without hypercapnia and/or Rp-8-Br-PET-cGMPS, a PKG type I inhibitor, on ROCK activity. B: effects on serum stimulation with or without hypercapnia on ROCK-I or ROCK-II content in PASMCs. Values represent means ± SE for n = 3–4 samples/group. Cells were treated with DMEM + 0.1% (vol/vol) FBS in 5% ambient CO2 (control; open bars), DMEM + 10% (vol/vol) FBS in 5% ambient CO2 (normocapnia; filled bars), or DMEM + 10% (vol/vol) FBS in 10% ambient CO2 (hypercapnia; dark gray bars). *P < 0.01, by ANOVA, compared with all other groups.

Fig. 9. Schematic illustrating putative effects of sustained inhaled CO2 on RhoA/ROCK and NO-cGMP signaling in the pulmonary circulation.

respiratory, we believe it was unlikely to have represented an anesthetic effect. While hypoxia would be expected to acutely cause hyperventilation and hypocapnia, this is not sustained (44), perhaps in part due to blunted carotid chemoreceptor sensitivity (61). A limitation of measurement in anesthetized animals is the possibility of respiratory suppression, which may have confounded data on PaCO2, although we anticipate that all groups were affected equally. The main purpose of providing such data was to estimate the degree of change in PaCO2 and acid-base status to provide some clinical context. Unfortunately, the method used was the only feasible approach due to our inability to reliably maintain an arterial catheter in nonanesthetized animals of this size.

We observed that exposure to 10% CO2 significantly increased LV systolic function, as measured by increased fractional shortening, in normoxia-exposed animals, while causing no change in this parameter in hypoxia-exposed animals. Therapeutic hypercapnia has potential to decrease systolic function, secondary to acidosis (69), but this effect may be counterbalanced by increased oxygenation, preload, and coronary blood flow secondary to hypercapnia (71), potentially leading to a net increase in cardiac output (7, 24, 37, 62, 68). A limitation of the present study is that we were unable to quantify LV diastolic function, which may also be adversely affected by hypercapnic acidosis (70), due to an inability to reliably acquire the required four- and five-chamber views to perform transmural Doppler, described in mature murine models (56, 72). Although our finding that PVR was significantly improved and that LV systolic function was not adversely affected (or was perhaps increased) by chronic hypercapnia suggests a decreased likelihood of diastolic dysfunction (38), this issue warrants future study employing catheter-based measurement.

Understanding the relative contributions and temporal relationships between the NO-cGMP and RhoA/ROCK pathways is complicated by the two pathways having well-described reciprocal interactions both in vitro and in vivo. For example, therapies that enhance cGMP-mediated signaling, such as PDE V inhibitors, have also been shown to inhibit RhoA/ROCK activity in multiple experimental models (13, 20, 23). In addition, ROCK inhibition has been reported to enhance expression of eNOS (1, 16, 42, 58, 65), as in the present study, and to increase the vasodilatory effects of cGMP (19). Effects of exposure to 10% CO2 on hemodynamic and structural markers of chronic PHT were paralleled by attenuated activity of RhoA/ROCK and by upregulated content and activity of
eNOS and of its downstream effector cGMP (perhaps in part through attenuated PDE V activity). Given previous observations in the same model on rescue effects of a ROCK inhibitor, Y-27632 (74), it is reasonable to suggest that improvements in PVR and arterial remodeling secondary to inhaled CO\(_2\) may have resulted from attenuated ROCK activity, although this remains unproven. Our observation that a dose of CO\(_2\) that led to decreased remodeling (10\%, but not 7\%) also led to attenuated RhoA/ROCK activity lends further support to this hypothesis.

Attenuated activity of ROCK by 10\% CO\(_2\) was unaccompanied by changes in content of either of its known isoforms, ROCK-I and ROCK-II, indicating that inhibitory effects were at a functional level. Attenuated NO signaling is also known to play a major role in the pathogenesis of sustained vasoconstriction and vascular remodeling (2, 48, 65), since endogenous vascular NO production (in major part, by eNOS) leads to relaxation through PKG-induced calcium desensitization and has anti-proliferative and pro-apoptotic effects on smooth muscle (2, 48, 65). Because cGMP-induced vasorelaxation is known to be mediated, in part, through attenuating effects of PKG (a downstream effector of cGMP) on RhoA activation (47, 54, 55) or through serine-695/852 phosphorylation of MYPT-1, which directly inhibits ROCK activity (18, 31, 73), we also explored the effects of 10\% CO\(_2\) on pulmonary arterial PKG content and activity, which were found to be increased. In vitro exposure of PASMCs to hypercapnia attenuated serum-induced ROCK activation, which was prevented by a PKG type I inhibitor. Taken together, these findings suggest that effects of CO\(_2\) on upregulated cGMP/PGK and downregulated ROCK activity were mechanistically related and that PKG was acting upstream of RhoA/ROCK and not vice versa. This premise is further supported by our observation that treatment with a ROCK inhibitor, Y-27632, had no effects on pulmonary arterial PKG activity or cGMP content, whereas eNOS and NOX were increased. The latter finding implicates decreased ROCK activity as the mechanism leading to improved endothelial function secondary to 10\% CO\(_2\). We did not examine the effects of increased CO\(_2\) on eNOS content, NO production, or function of NO-dependent pathways in primary cultured pulmonary arterial endothelial cells, changes in which could contribute to the regulation of ROCK activity in smooth muscle. Therefore, a more precise understanding of the contribution of enhanced endogenous NO production toward pulmonary vasorelaxation, augmented PKG signaling, and attenuated smooth muscle ROCK activity secondary to CO\(_2\) will require further study. However, work by others has suggested that pulmonary vasodilatory effects of CO\(_2\) may be independent of NO (11). Other likely upstream mechanisms by which CO\(_2\) may have limited RhoA/ROCK activity, not explored in this study, include attenuated oxidative and nitrative stress in the lung (33, 39, 49) and downregulated expression of endothelium-derived GPCR ligands, such as endothelin-1 (33, 74). Potential use of ROCK inhibitors in humans is complicated by the recognition that ROCK is also a critical regulator of systemic vascular tone and is expressed in multiple nonvascular tissues and cell types. Indeed, we and others have reported significant adverse effects with systemic ROCK inhibition, including severe hypotension (8, 74) and growth restriction (75), although not attenuation of lung growth or development (75), highlighting the potential for efficacy of more pulmonary-selective strategies. Importantly, we observed that CO\(_2\)-exposed animals had better weight gain than their normocapnia-exposed counterparts. Coupled with observations indicating a lack of effect of CO\(_2\) on systemic blood pressure or on mesenteric artery contraction ex vivo, our findings suggest that inhibitory effects of inhaled CO\(_2\) on ROCK-mediated vasoconstriction were greatest in the pulmonary vasculature.

In conclusion, our findings indicate that rescue therapeutic hypercapnia may represent an easily applied means by which attenuation of ROCK activity can be achieved in the lung, leading to attenuated vasoconstriction and vascular remodeling. Given that chronic PHT in early life is caused by other factors in addition to hypoxia, these observations require confirmation in neonatal models where pulmonary inflammation, increased pulmonary blood flow, and/or severe vascular hypoplasia are contributory. It also remains to be determined whether inhaled CO\(_2\)-mediated effects result from direct exposure of the pulmonary vasculature to CO\(_2\) secondary to diffusion through distal airways or whether they are related to systemic venous CO\(_2\) to which the pulmonary arteries are exposed. Given the potential for harmful effects of hypercapnia, or hypercapnic acidosis, on the immature brain (30, 32), making this distinction will be important before consideration of clinical translation in this population. If via direct diffusion from distal airways, it is conceivable that a strategy could be devised to minimize the potential for adverse effects of systemic hypercapnia on other organ systems.

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DISCLOSURES
No conflicts of interest are declared by the authors.

AUTHOR CONTRIBUTIONS

REFERENCES


