**Chronic intrauterine pulmonary hypertension selectively modifies pulmonary artery smooth muscle cell gene expression**

**Ernesto Resnik,** Jean Herron, Maggie Keck, David Sukovich, Bradley Linden, and David N. Cornfield

Departments of Pediatrics and Surgery, University of Minnesota Medical School, Minneapolis, Minnesota

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Chronic intrauterine pulmonary hypertension selectively modifies pulmonary artery smooth muscle cell gene expression. Am J Physiol Lung Cell Mol Physiol 290: L426–L432, 2006; doi:10.1152/ajplung.00281.2005.—Pulmonary artery smooth muscle cell (PASMC) relaxation at birth results from an increase in cytosolic cGMP, cGMP-dependent and kinase-mediated activation of the Ca2+-sensitive K+ channel (KCa), and closure of voltage-operated Ca2+ channels (VOCC). How chronic intrauterine pulmonary hypertension compromises perinatal pulmonary vasodilation remains unknown. We tested the hypothesis that chronic intrauterine pulmonary hypertension selectively modifies gene expression to mitigate perinatal pulmonary vasodilation mediated by the cGMP kinase-KCa-VOCC pathway. PASMC were isolated from late-gestation fetal lambs that had undergone either ligation of the ductus arteriosus (hypertensive) or sham operation (control) at 127 days of gestation and were maintained under either hypoxic (70 Torr) or normoxic (100 Torr) conditions in primary culture. We studied mRNA levels for cGMP kinase-1α (PKG-1α), the α-chain of VOCC (Ca,1.2), and the α-subunit of the KCa channel. Compared with control PASMC, hypertensive PASMC had decreased VOCC, KCa, and PKG-1α expression. In response to sustained normoxia, expression of VOCC and KCa channel decreased and expression of PKG-1α increased. In contrast, sustained normoxia had no effect on PKG-1α levels and an attenuated effect on VOCC and KCa channel expression in hypertensive PASMC. Protein expression of PKG-1α was consistent with the mRNA data. We conclude that chronic intrauterine pulmonary hypertension decreases PKG expression and mitigates the genetic effects of sustained normoxia on pulmonary vasodilation, because gene expression remains compromised even after sustained exposure to normoxia.

fetal; oxygen sensing; nitric oxide

**IN UTERO,** oxygen tension is low and pulmonary vascular resistance is greater than systemic vascular resistance (36). At birth, the pulmonary circulation undergoes an unprecedented and unparalleled transition, given that pulmonary blood flow increases 8- to 10-fold and arterial pressure decreases by 50% within 24 h, concomitant with an increase in oxygen tension, establishment of an air-liquid interface, and rhythmic distention of the lung (9, 15, 44).

Recent data suggest that activation of the large-conductance Ca2+-sensitive K+ channel (KCa, also known as BKCa or MaxiK) plays a critically important role in mediating the response to perinatal pulmonary vasodilator stimuli such as oxygen (11), nitric oxide (NO) (38), shear stress (41), and ventilation (45). Further work has provided insight into the cellular mechanisms whereby NO (5, 8) and, in particular, oxygen cause relaxation of pulmonary arterial smooth muscle cells (34). Both these molecules activate guanylate cyclase to increase cGMP concentration and activate protein kinase G (PKG) (34). PKG both directly and indirectly activates the KCa channel (7). The indirect effect includes receptor phosphorylation of the intracellular ryanodine-sensitive Ca2+ store, causing a localized release of a so-called Ca2+ spark and activation of the KCa channel (30). An additional indirect effect includes the relatively recent observation that PKG decreases Ca2+ transit via voltage-operated Ca2+ channels (VOCC), thereby further limiting PASMC cytosolic Ca2+ concentration ([Ca2+]i) and promoting vasodilation (20). PKG may also directly activate the KCa channel by phosphorylation of its α-chain (42). Activation of the channel results in membrane hyperpolarization, closure of voltage-gated Ca2+ channels, a decrease in cytosolic Ca2+, and vasodilation.

Recent data indicate the existence of several different isoforms of cGMP-dependent protein kinase. In vascular smooth muscle cells, relaxation is contingent on activation of the type I α-isoform (PKG-1α) (33). Nitrate-mediated relaxation is mediated by the type I α-isoform, because an important phosphorylation target of the enzyme is the KCa channel (5, 35). PKG-1α may be sufficient to activate KCa channels via the NO/cGMP signaling pathway (42). Nitrate tolerance, a condition wherein sensitivity to nitrovasodilators is diminished, is likely related to reduced PKG-1α expression (27, 40, 48).

In some newborn infants, pulmonary vascular resistance remains elevated after birth, resulting in shunting of blood away from the lungs and severe central hypoxemia (21). Infants with this condition, termed persistent pulmonary hypertension of the newborn (PPHN), often respond only incompletely to administration of high concentrations of supplemental oxygen or inhaled NO (22). Given that an incomplete response to pulmonary vasodilator stimuli characterizes PPHN (17), we sought to determine whether chronic intrauterine pulmonary hypertension, an animal model of PPHN, affects the mechanisms of the relaxation signaling cascade and the response to sustained increase in oxygen tension, and we investigated the expression of the molecular components involved in such mechanisms.

**METHODS**

**Animals.** The procedures followed in these studies were previously reviewed and approved by the Animal Care and Use Committee at the University of Minnesota.

**Cell cultures.** Techniques used for cell isolation and culture have been previously described (13, 14). Late-gestation fetal sheep (term =
147 days) from ewes with time-dated pregnancies were used in this study. Ewes were fasted for 24 h and sedated with pentobarbital sodium. Fetal lambs were partially delivered through a hysterotomy incision, with the head remaining inside the womb to prevent spontaneous breathing, and intracardiac pentobarbital sodium was administered. After thoracotomy, the lung and heart block was isolated.

Methods for dissection of distal (≥4th generation) pulmonary arteries (PA) and isolation and culturing of smooth muscle cells (SMC) were described previously (11). Subconfluent monolayers of cells were studied between days 5 and 14 of primary culture. PASMC were maintained in a low-oxygen tension environment (25 mmHg). In experiments examining the effects of sustained normoxia, oxygen tension was increased to 120 mmHg after 72 h in culture under hypoxic conditions.

Chronic intrauterine pulmonary hypertension model. Surgical ligation of the ductus arteriosus (DA) was performed as previously described (28). Pregnant ewes between 126 and 128 days of gestation were killed rapidly after high-dose maternal and fetal infusions of paraformaldehyde in PBS and permeabilized with 0.1% Triton X-100. Primary antibodies for PKG-1α (Stressgen, Victoria, BC, Canada) or negative control IgG were diluted in blocking agent and incubated with the cells overnight at 4°C, followed by a FITC-labeled secondary antibody (Jackson Immunoresearch, West

Reverse transcriptase-polymerase chain reaction. Total RNA was extracted from PASMC in primary culture using TRI reagent (Sigma). cDNA synthesis reaction (Invitrogen, Carlsbad, CA). Oligonucleotide primers used to amplify PKG-1α were designed using the human sequence and were (forward) 5′-GAGGGCAAGCGGCTGACAGAAG-3′ and (reverse) 5′-TGGTCGACTTCTGTCGAACCGCA-3′ (generating an 850-bp fragment). Primers for the voltage-gated Ca2+-channel α1c-subunit (Ca1.2) were (forward) 5′-GCCCTCTTTCTCCAGGGACTGTT-3′ and (reverse) 5′-TGAGGGCTATACCTTGGCAGGA-3′ (516-bp product). Primers directed against the α-chain of the KCa channel were designed on the basis of the consensus among human, bovine, and dog (slo) sequences and were (forward) 5′-CTACCTGGGATGTTCTTACTGTGTT-3′ and (reverse) 5′-TGCTGTCTAGAAGACTGCTCAATACTGATAA-3′ (446-bp product). The identity of each product was confirmed with sequence analysis. Gel densitometry was performed to quantify the RT-PCR product (NIH Image software; Scion, Frederick, MD). 18S rRNA cDNA was amplified concurrently in RT-PCR with an internal loading control. The relative density of the 18S and the α-actin loading control. *P 0.01 vs. normotensive.

Ca2+ imaging. Dynamic changes in [Ca2+]i in individual SMC were assessed with the Ca2+-sensitive fluorophore fura-2 AM (Molecular Probes). Subconfluent fetal PASMC on 25-mm2 glass coverslips were placed on the stage of an inverted microscope (Nikon Diaphot). Cells were loaded with 10 nM fura-2 AM and 2.5 μM Fluo-4 (Molecular Probes) for 20 min, followed by 20 min in Ca2+-containing solution to allow for deesterification before the experiment. Ratiometric imaging was performed with excitation wavelengths of 340 and 380 nm and an emission wavelength of 510 nm. Imaging was performed with an intensified charge-coupled device camera (Photonic Science, Robertsbridge, UK) using Axon Instruments (Foster City, CA) or Metaffluor (Fryer, Bloomingon, MN) image capture and analysis software. Ca2+ calibration was achieved by measuring a maximum (with 1 mM ionomycin) and a minimum (with 10 mM EGTA). Intracellular free Ca2+ was calculated by assuming a dissociation constant of 220 nM (18). For each experiment, 8–10 cells were visualized and ratiometric data were acquired from individual cells.

Immunohistochemistry. Cells grown on glass coverslips were fixed in 4% paraformaldehyde in PBS and permeabilized with 0.1% Triton X-100 in PBS. Primary antibodies for PKG-1α (Stressgen, Victoria, BC, Canada) or negative control IgG were diluted in blocking agent and incubated with the cells overnight at 4°C, followed by a FITC-labeled secondary antibody (Jackson Immunoresearch, West

Fig. 1. cGMP-kinase Iα (PKG-1α) expression in chronic intrauterine hypertension under normoxic and low oxygen tension. Protein and mRNA levels were determined in pulmonary artery smooth muscle cells (PASMC) isolated from normal and hypertensive fetal lambs. A: aggregate PKG-1α mRNA expression data obtained from RT-PCR using PKG-1α-specific primers. Band density was normalized to the 18S rRNA internal control. *P < 0.05; **P < 0.01 vs. hypoxia. ***P < 0.01 vs. normotensive. B: representative RT-PCR gel and Western blot (No, normoxic; Hy, hypoxic). *P < 0.01 vs. normotensive. B: representative RT-PCR gel and Western blot (No, normoxic; Hy, hypoxic). C: PKG-1α protein levels (aggregate data from Western blot). The 70-kDa PKG band was quantified and normalized to the 42-kDa α-actin loading control. *P < 0.01 vs. normotensive.
Grove, PA). Digital images were obtained with a Spot camera (Diagnostic Instruments, Sterling Heights, MI) mounted on a Zeiss Atto Arc fluorescence microscope (Carl Zeiss MicroImaging, Thornwood, NY).

**Western blotting.** Cultured cells were rinsed twice in cold PBS and then lysed in RIPA buffer. Protein (75 μg) was electrophoresed in a 4–20% gradient gel (Bio-Rad) and electroblotted onto polyvinylidene difluoride membrane (Bio-Rad). Antibodies against PKG-1α and anti-rabbit IgG horseradish peroxidase conjugate were from Stressgen.

**Statistical analysis.** Throughout, results are presented as means ± SE. Statistical significance was tested with Student’s t-test (paired or unpaired as appropriate). \( P < 0.05 \) was taken as the threshold level for statistical significance. Experiments were designed to have a statistical power of at least 90% at a probability level of \( P < 0.05 \). A two-way ANOVA with repeated measures and a Student-Newman-Keuls post hoc test were used to assess the differences between and among groups in the manganese quenching experimental protocol.

**RESULTS**

**Effect of sustained normoxia on PKG-1α expression in control and hypertensive PASMC.** Under hypoxic conditions, PKG-1α mRNA expression was 0.82 ± 0.15 in control (\( n = 6 \) animals; 13 PCR) and 0.88 ± 0.16 (\( n = 4 \) animals; 7 PCR) in hypertensive PASMC. In sustained normoxia, PKG-1α mRNA expression increased to 0.96 ± 0.11 in control (\( n = 6 \) animals; 10 PCR; \( P < 0.05 \) vs. hypoxia) but decreased to 0.52 ± 0.10 in hypertensive (\( n = 4 \); 6 PCR; \( P < 0.01 \) vs. normoxia) PASMC (Fig. 1A). Under both hypoxic and normoxic conditions, PKG-1α expression was greater in control (\( n = 4 \) animals), compared with hypertensive (\( n = 4 \) animals) PASMC (Fig. 1C). Immunohistochemistry was consistent with the protein data, because staining intensity was diminished in hypertensive compared with normotensive PASMC (Fig. 2).

**Effect of sustained normoxia on \( K_{Ca} \) channel and voltage-operated \( Ca^{2+} \) channel mRNA expression in control and hypertensive PASMC.** Under hypoxic conditions, \( K_{Ca} \) channel α-subunit mRNA expression was 1.56 ± 0.14 in control (\( n = 4 \) animals; 7 PCR) and 1.14 ± 0.10 (\( n = 4 \) animals; 8 PCR) in hypertensive (\( P = 0.03 \), control vs. hypertensive) PASMC. In sustained normoxia, \( K_{Ca} \) α-subunit mRNA expression decreased to 1.09 ± 0.18 in control (\( n = 4 \) animals; 8 PCR; \( P < 0.05 \) vs. hypoxia) but increased to 1.42 ± 0.12 in hypertensive (\( n = 4 \); 5 PCR; \( P < 0.02 \) vs. hypoxia, control normoxia) PASMC (Fig. 3A). Sustained normoxia decreased \( K_{Ca} \) channel α-subunit mRNA expression by 29 ± 10% in control compared with an increase of 24 ± 7% in hypertensive PASMC (Fig. 3B, \( P < 0.01 \), control vs. hypertensive).

Under hypoxic conditions, \( Ca_{1.2} \) mRNA expression was 1.54 ± 0.14 in control (\( n = 4 \) animals; 7 PCR) and 1.26 ± 0.11 (\( n = 5 \) animals; 9 PCR) in hypertensive PASMC. In sustained normoxia, \( Ca_{1.2} \) expression decreased to 1.09 ± 0.09 in control (\( n = 4 \) animals; 9 PCR; \( P < 0.001 \) vs. hypoxia) and decreased to 1.076 ± 0.12 in hypertensive (\( n = 4 \); 9 PCR; \( P < 0.02 \) vs. hypoxia) PASMC (Fig. 4A). Sustained normoxia decreased \( Ca_{1.2} \) mRNA expression by 29 ± 6% in control compared with a decrease of 13 ± 6% in hypertensive PASMC (Fig. 4B, \( P < 0.01 \), control vs. hypertensive).

**Effect of 8-bromo-cGMP on control and hypertensive PASMC.** Under low-oxygen tension conditions, cells were treated with the cell-permeant analog of cGMP, 8-bromo-cGMP. In control PASMC (\( n = 79 \)), 8-bromo-cGMP (10⁻² M) decreased the fluorescence ratio by 4.7 ± 0.2%, whereas in hypertensive PASMC (\( n = 83 \)), 8-bromo-cGMP had no effect on fura-2 fluorescence (Fig. 5, \( P < 0.001 \) vs. hypertensive).

**DISCUSSION**

In this report, we present evidence that the molecular response of PASMC to sustained increase in oxygen tension, similar to the normal transition to air-breathing life, is affected in a model of chronic intrauterine hypertension. In response to sustained exposure to normoxia, the expression of molecules that favor diminished tone in PASMC normally increases. Gene and protein expression of PKG-1α, a molecule that
affects SMC tone at different but complementary levels (27, 40, 42), increases in response to a sustained increase in oxygen tension. Emerging evidence indicates that PKG has three distinct effects that promote diminished SMC tone. First, receptor phosphorylation by PKG enables ryanodine-sensitive Ca\(^{2+}\)/H\(_{11001}\) stores to produce a local and quantal release of Ca\(^{2+}\), resulting in KCa channel opening, membrane hyperpolarization, and subsequent closure of Ca\(^{2+}\) channels to decrease cytosolic Ca\(^{2+}\) (19, 35). Second, PKG directly activates the KCa channel through phosphorylation at serine 1072 of the \(\alpha\)-chain (4, 16). Third, PKG may inhibit the voltage-operated Ca\(^{2+}\) channel by direct phosphorylation of the channel or by PKG-induced activation of a phosphatase (20). In addition to the effects on PKG-1\(\alpha\) expression, sustained normoxia decreases expression of PASMC KCa and VOCC.

Whereas chronic intrauterine pulmonary hypertension compromises postnatal adaptation of the pulmonary circulation (1, 29, 37), the mechanisms that account for sustained elevation of pulmonary vascular resistance remain incompletely understood. As previously reported (12, 39, 47), chronic intrauterine pulmonary hypertension alters the expression of molecules that modulate perinatal pulmonary vascular tone. The present data add to the current knowledge by demonstrating that chronic intrauterine pulmonary hypertension decreases protein expression of cGMP kinase (PKG-1\(\alpha\)). More important, perhaps, we

**Fig. 3. Effect of oxygen tension on Ca\(^{2+}\)-sensitive K\(^+\) channel (KCa) mRNA expression in chronic intrauterine hypertension.** Aggregate RT-PCR data were obtained for KCa mRNA levels in PASMC isolated from normal and hypertensive fetal lambs. A: relative KCa mRNA levels in PASMC from normotensive and hypertensive fetal lambs were obtained under either normoxic or hypoxic conditions by using KCa\(\alpha\)-specific primers. Band intensity was normalized to the 18S rRNA internal control. *P = 0.03 vs. normotensive. **P < 0.05 vs. hypoxia. ***P < 0.02 vs. hypoxia. B: relative %change in KCa mRNA when switching normal and hypertensive PASMC from hypoxia to normoxia. *P < 0.01 vs. normotensive. C: representative RT-PCR gel and Western blot.

**Fig. 4. Effect of oxygen tension on voltage-gated Ca\(^{2+}\) channel \(\alpha_{1C}\)-subunit (Cav1.2) mRNA expression in chronic intrauterine hypertension.** Aggregate RT-PCR data were obtained for Cav1.2 mRNA levels in PA SMC isolated from normal and hypertensive fetal lambs. A: relative Cav1.2 mRNA levels in PA SMC were obtained under normoxic or hypoxic conditions by using Cav1.2-specific primers. Band intensity was normalized to the 18S rRNA internal control. *P < 0.001 vs. hypoxia. **P < 0.02 vs. hypoxia. B: relative %change in Cav1.2 mRNA (Cav) when switching normal and hypertensive PA SMC from hypoxia to normoxia. *P < 0.01 vs. normotensive. C: representative RT-PCR gel.
have presented data indicating that the intrauterine experience of the pulmonary vasculature informs the more long-term genetic response of PASMC to sustained increases in oxygen tension. In response to sustained normoxia, PKG-1α mRNA and protein expression remains substantially elevated in control compared with hypertensive PASMC. Our immunohistochemistry studies are consistent with these observations.

In the pulmonary circulation, an acute increase in oxygen tension (3), shear stress (10), and NO (2) production each results in perinatal pulmonary vasodilation. Each of these essential vasodilator stimuli acts through cGMP-mediated activation of the KCa channel (11, 38, 41), thereby causing membrane hyperpolarization (31) and a decrease in pulmonary artery smooth muscle cytosolic Ca2+, a key determinant of the SMC contractile state (46). Diminished cGMP kinase and the decrease in PASMC KCa channel expression that has been previously reported in an ovine model of PPHN (12) is entirely consistent with the persistent elevation of pulmonary vascular resistance after birth. Several clinical studies have demonstrated that in a subset of infants with PPHN, neither high concentrations of inspired oxygen nor inhaled NO causes resistance decreases to 20% of systemic vascular resistance (24). Postnatal alveolarization of lung occurs concomitantly with pulmonary vasculogenesis. Exposure to atmospheric levels of oxygen tension may be among the key signals for the long-term adaptation of the pulmonary circulation, because perinatal hypoxia (25, 26) results in remodeling of the pulmonary circulation, diminished radial alveolar counts, and increased pulmonary vascular reactivity (43). The present data suggest that the intrauterine experience of the pulmonary vasculature influences not only the histology and physiology of the neonatal pulmonary vascular SMC but also the molecular response to sustained levels of increased oxygen tension. The present observations have implications for postnatal alveolarization, because elevated vascular tone likely diminishes lung growth (6). Because PKG-1α has both direct and indirect effects on the contractile state of vascular smooth muscle cells, it may be centrally involved in mediating both the immediate and long-term responses of the pulmonary vasculature to sustained normoxia.

The present findings demonstrate that pulmonary artery smooth muscle cells exposed to chronic intrauterine pulmonary hypertension show diminished expression of molecules that mediate the response of the pulmonary circulation to vasodilator stimuli and promote pulmonary vasodilation. Whereas control PASMC respond to prolonged exposure to normoxia with changes in the expression of genes critical for the maintenance of low pulmonary vascular tone, the effect of sustained normoxia on these molecules is attenuated in PASMC derived from animals with chronic intrauterine pulmonary hypertension. The present study is the first to report that sustained exposure to normoxia selectively modulates gene expression to limit pulmonary artery smooth muscle cell tone. Moreover, the present data provide evidence that chronic intrauterine pulmonary hypertension affects gene expression even after cells have
been removed from the hypertensive environment. The long-lived effects of chronic intrauterine pulmonary hypertension include an inability of cells to respond to an acute increase in oxygen tension with an increase in the expression of PKG and a decrease in the expression of ion channels centrally important in maintaining the low pulmonary vascular tone that characterizes air-breathing life and promotes postnatal alveolarization.

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Present address for D. N. Cornfield: Dept. of Pediatrics, Stanford University School of Medicine, 300 Pasteur Dr., Stanford, CA 94304.

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REFERENCES


