Chronic intrauterine pulmonary hypertension compromises fetal pulmonary artery smooth muscle cell O$_2$ sensing

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Linden, Bradley C., Ernesto R. Resnik, Kristine J. Hendrickson, Jean M. Herron, Timothy J. O’Connor, and David N. Cornfield. Chronic intrauterine pulmonary hypertension compromises fetal pulmonary artery (PA) smooth muscle cell (SMC) O$_2$ sensing. Am J Physiol Lung Cell Mol Physiol 285: L1354–L1361, 2003. First published July 25, 2003; 10.1152/ajplung.00091.2003.—To test the hypothesis that chronic intrauterine pulmonary hypertension (PHTN) compromises pulmonary artery (PA) smooth muscle cell (SMC) O$_2$ sensing, fluorescence microscopy was used to study the effect of an acute increase in PO$_2$ on the cytosolic Ca$^{2+}$ concentration ([Ca$^{2+}$]$_i$) of chronically hypoxic subconfluent monolayers of PA SMC in primary culture. PA SMCs were derived from fetal lambs with PHTN due to intrauterine ligation of the ductus arteriosus. Acute normoxia decreased [Ca$^{2+}$]$_i$ in control but not PHTN PA SMC. In control PA SMC, [Ca$^{2+}$]$_i$ increased after Ca$^{2+}$-sensitive (K$_{Ca}$) and voltage-sensitive (K$_V$) K$^+$ channel blockade and decreased after diltiazem treatment. In PHTN PA SMC, K$_{Ca}$ blockade had no effect, whereas K$_V$ blockade and diltiazem increased [Ca$^{2+}$]$_i$. Inhibition of sarcoplasmic reticulum Ca$^{2+}$ ATPase activity caused a greater increase in [Ca$^{2+}$]$_i$, in controls compared with PHTN PA SMC. Conversely, ryanodine caused a greater increase of [Ca$^{2+}$]$_i$ in PHTN compared with control PA SMC. K$_{Ca}$ channel mRNA is decreased and K$_V$ channel mRNA is unchanged in PHTN PA SMC compared with controls. We conclude that PHTN compromises PA SMC O$_2$ sensing, alters intracellular Ca$^{2+}$ homeostasis, and changes the predominant ion channel that determines basal [Ca$^{2+}$]$_i$ from K$_{Ca}$ to K$_V$.

THE MECHANISMS RESPONSIBLE for the maintenance of the high tone, low-flow fetal pulmonary vasculature and the rapid increase in pulmonary blood flow that occurs at birth are not completely understood. At birth, pulmonary blood flow increases 8- to 10-fold and pulmonary artery (PA) pressure declines steadily over the first several hours of life (27). While physical factors and vasoactive products elaborated by the pulmonary vascular endothelium are involved in the regulation of perinatal pulmonary vascular tone (1, 4, 10), sustained and progressive perinatal pulmonary vasodilation requires PA smooth muscle cell (PA SMC) K$^+$ channel activation (33). PA SMC K$^+$ channel activation causes membrane hyperpolarization, closure of voltage-operated Ca$^{2+}$ channels, a decrease in cytosolic Ca$^{2+}$ concentration ([Ca$^{2+}$]$_i$), and subsequent vasodilation (21).

Recent studies (9) from our laboratory have demonstrated that fetal PA SMC respond directly to changes in PO$_2$. Fetal PA SMC respond to acute hypoxia with an increase in [Ca$^{2+}$]$_i$. In response to an acute increase in PO$_2$ (acute normoxia), fetal PA SMC [Ca$^{2+}$]$_i$ decreases (26). O$_2$ causes a decrease in PA SMC [Ca$^{2+}$]$_i$, via activation of a cGMP-sensitive kinase that mediates localized Ca$^{2+}$ release from a ryanodine-sensitive intracellular Ca$^{2+}$ store (24). Localized Ca$^{2+}$ release results in activation of the Ca$^{2+}$-sensitive K$^+$ (K$_{Ca}$) channel and PA SMC membrane hyperpolarization, which leads to vasodilation (21).

Interestingly, the response of PA SMC to changes in PO$_2$ is developmentally regulated. Fetal, but not adult, PA SMC respond to an acute increase in PO$_2$ with a decrease in [Ca$^{2+}$]$_i$ (23, 26). In contrast, adult PA SMC respond to acute hypoxia with a more rapid and greater increase in [Ca$^{2+}$]$_i$ than fetal PA SMC (8). The differential response to PO$_2$ between fetal and adult PA SMC may derive, in part, from changes in the predominant ion channel population that controls resting membrane potential ($E_m$). In the fetus, the K$_{Ca}$ channel determines $E_m$, and, therefore, through subsequent effects on voltage-gated Ca$^{2+}$ channels, basal levels of PA SMC [Ca$^{2+}$]$_i$. In contrast, the $E_m$ and basal [Ca$^{2+}$]$_i$ of PA SMC from the adult pulmonary circulation is mediated by voltage-sensitive K$^+$ channels (K$_V$) (25). Thus fetal PA SMC are uniquely well adapted to respond to an acute increase in PO$_2$ and thereby enhance postnatal adaptation of the pulmonary circulation, whereas adult PA SMC are adapted to respond to an acute decrease in PO$_2$ and thereby match ventilation and perfusion to prevent intrapulmonary shunting and subsequent hypoxemia (36).

In some newborn infants, pulmonary vascular resistance remains elevated after birth, resulting in a clinical syndrome termed persistent pulmonary hypertension of the newborn (PPHN). PPHN is characterized by extrapulmonary right-to-left shunting of blood across the ductus arteriosus or patent foramen ovale causing...
severe hypoxemia (16). Infants with PPHN respond incompletely to perinatal pulmonary vasodilator stimuli. Evidence suggests that adverse intrauterine stimuli, such as chronic hypoxia or hypertension (19), can decrease endothelial nitric oxide (NO) synthase (eNOS) gene and protein expression, NOS activity, and limit perinatal NO production, thereby contributing to the pathophysiology of PPHN (2, 30, 35). A recent study (7) from an animal model of PPHN demonstrated a decrease in Kc_{a} channel gene expression in whole lung tissue of fetal lambs with chronic intrauterine pulmonary hypertension. It remains unknown whether 1) the decrease in fetal lung Kc_{a} channel gene expression is sufficient to compromise fetal pulmonary vascular oxygen sensing; and 2) chronic intrauterine pulmonary hypertension directly affects PA SMC oxygen sensing.

Because PA SMC Kc_{a} channel activity determines resting membrane potential (25) and mediates the response of fetal PA SMC to an acute increase in P_{O2} (24), as well as NO (29), we hypothesized that chronic intrauterine pulmonary hypertension has direct effects on PA SMC O_{2} sensing. To test this hypothesis, we used fluorescence microscopy to study the effect of an acute increase in P_{O2} on [Ca^{2+}]_{i} of PA SMC isolated from fetuses from normotensive and hypertensive ovine PA. PA SMC isolated from normotensive and hypertensive ovine PAs were quickly excised from pentobarbital-anesthetized fetuses in gestational age from 135 to 140 days (term = 147 days) and placed in physiological saline solution composed of (in mM) 120 NaCl, 5.9 KCl, 11.5 dextrose, 25 NaHCO_{3}, 1.2 NaH_{2}PO_{4}, 1.2 MgCl_{2}, and 1.5 CaCl_{2}. PA SMC were isolated from fourth- or fifth-generation resistance PAs. To determine any changes in K^{+} channel activity due to chronic intrauterine pulmonary hypertension, the effect of K^{+} channel antagonists (5) was studied. PA SMC isolated from normotensive and hypertensive fetal ovine PAs were treated with iiberotoxin, a selective Kc_{a} channel antagonist, and 4-aminopyridine (4-AP), a K_{a} antagonist on PA SMC [Ca^{2+}]_{i}. The effect of chronic intrauterine hypertension on intracellular calcium homeostasis was addressed by treating PHTN with the use of the guanidium thiocyanate-phenol-chloroform method (Trireagent; Sigma). After homogenization, the samples were processed according to the reagent instructions and the RNA was dissolved in diethyl pyrocarbonate-treated water and stored at −70°C. Optical density was measured to determine the RNA concentration. One microgram of RNA was added to 11 μl of First Strand cDNA Synthesis reagent (Pharmacia) with random hexamers as primers in a final volume of 33 μl. Two microliters of this RT reaction were added to each PCR reaction. Oligonucleotide primers used to amplify K_{a}, 2.1 cDNA were based on the human sequence (3) and (reverse) 5’ ACAGAGCAACCAAAAGGAAAGAC 3’ and (reverse) 5’ CACCGCTCAAGTTGAGGTCTTTA 3’. The use of these primers yielded a PCR product consistent with that expected for a fragment of the human Kc_{a} mRNA. The fragment size was 385 base pairs. The identity of the product was confirmed with sequence analysis. Oligonucleotide primers used to amplify Kc_{a} cDNA were based on the human sequence (34) and were (forward) 5’ GTAATGGGATGTCCRATGTTGTGTTGTTTCTGCCAGTTGTTCTGCTTTTCTG 3’ and (reverse) 5’ TCACCTGCTAGTGAATCAGCTGTAC 3’. The use of these primers yielded a PCR product consistent with that expected for a fragment of the human Kc_{a} mRNA. The fragment size was 446 base pairs. Identity of the product was confirmed with sequence analysis.

18S rRNA was analyzed concurrently in RT-PCR as an internal control. 18S cDNA was amplified with a QuantumRNA primer/competimer set (Ambion) to act as an internal control for the quantitation of relative expression of the ethidium bromide-stained bands. This control band appears as 324 base pairs. Because 18S rRNA is far more abundant than the mRNA under study, the 18S amplification reaction was modulated by the addition of “competimers.” These competimer primers are modified to block extension by DNA polymerase. When combined with the functional primers for 18S cDNA the amplification efficiency is reduced. Pilot ex-
periments determined the correct ratio of primers/competimers, cycle number, and RT input to yield multiplex PCR products that are all in the linear range of amplification. The PCR cocktail consisted of 1× PCR buffer (Perkin Elmer) with 1.5 mM Mg²⁺, 10 pM each K, 2.1 primer, 10 nM 2-deoxynucleotide 5'-triphosphate mixture, 20 pM of 18S primer mixture (ratio of 1:9) of 18S primers/competimers, 1 U AmpliTag polymerase and water to make 50 μL. PCR was performed in an MJ Research thermocycler with a heated lid and 0.2-ml thin-walled tubes. The PCR reaction was 2 min at 90°C, followed by 28–32 cycles of 1 min at 94°C, 1 min at 54°C, 2 min at 72°C, then an extension of 2 min at 65°C. Samples without RT were evaluated in PCR; the products were absent. The identity of the band was confirmed by sequencing the product (>91% homology with known sequences). Densityometry was used to quantify the RT-PCR product (NIH Image software; Scion, Frederick, MD) and the internal control 18S rRNA to correct for variability in lane loading. Each gel contained PCR product from both hypertensive and control animals. The relative density of the 18S ribosomal and potassium channel PCR products were compared in each gel. If the gel was run that day, the control and test lungs were run that day to ensure consistency.

Western blot analysis for KCa protein. Cultured smooth muscle cell monolayers were rinsed with cold PBS and scraped from their 25-mm coverslips in RIPA buffer with protease inhibitor cocktail and 0.1% Triton X-100. The homogenate was sonicated briefly (2 s) on ice and then centrifuged at 1,000 g for 5 min. The supernatant protein concentration was determined with the bicinchoninic acid protein assay (Pierce). Protein (75 μg) was combined with SDS-PAGE reducing sample buffer and electrophoresed in a 4–20% gradient gel. The proteins were electrobotted onto polyvinylidene difluoride membrane (Bio-Rad). Skim milk (6%) in 20 mM Tris-buffered saline (TBS) was used for blocking and washing the membranes. Antibody against KCa channel (Alomone) was diluted 1:200 in milk-TBS. In blocking experiments the antibody was incubated 1 h as recommended with specific antigen provided with the antibody and then diluted 1:200. The membranes were rotated in the solutions overnight at 4°C and washed. Second antibody was anti-rabbit IgG horseradish peroxidase conjugate (Jackson), 1:3,000 in milk-TBS with 0.01% Tween 20, rocked for 2 h at room temperature. After being washed, the membranes were incubated 10 min at room temperature with Super Signal West Pico chemiluminescent reagent (Pierce) and the membrane was exposed to Kodak X-omat fs-1 film. The KCa channel band was seen at 125 kDa, and antibody binding to these bands was blocked by antigen preincubation.

Animals. The procedures used in these studies were previously reviewed and approved by the Animal Care and Use Committee at the University of Minnesota Medical School. Fetal sheep were used in this study. At the time of tissue procurement, fetal ages ranged from 135 to 140 days gestation (term is 147 days). Ewes with time-dated pregnancies were fasted for 24 h and were sedated with pentobarbital sodium (10 g total dose). Fetal lambs were rapidly delivered through a hysterotomy and an injection of pentobarbital sodium was given in the umbilical artery to prevent spontaneous breathing. After thoracotomy, the lungs were isolated, and samples were preserved for RNA analysis by freeze clamping and storage at −70°C. 

Experiment 1: chronic intrauterine pulmonary hypertension. Surgical ligation of the ductus arteriosus was performed as previously described (20, 28). Mixed-breed (Columbia-Rambouillet) pregnant ewes between 126 and 128 days gestation (term is 147 days) were fasted for 24 h before surgery. Ewes were sedated with intravenous pentobarbital sodium (total dose: 2–4 g) and anesthetized with 1% tetracaine hydrochloride (3 mg) by lumbar puncture. The ewes were kept sedated but breathed spontaneously throughout the operation. Under sterile conditions, the gravid uterus was delivered through a midline laparotomy. The fetal lamb’s left forelimb was withdrawn through a small hysterotomy. A skin incision was made under the left forelimb after local infiltration with lidocaine (2–3 ml, 1% solution). A left thoracotomy exposed the heart and great vessels. The ductus arteriosus was visualized. A 2-0 silk suture was placed around the ductus arteriosus and tied. The ribs and skin were reapproximated. The hysterotomy was closed, and the uterus was returned into the maternal abdominal cavity. The ewes recovered rapidly from surgery and were generally standing in their pens within 6 h. Food and water were provided ad libitum. After 7–12 days, animals were euthanized rapidly after high-dose maternal and fetal infusions of pentobarbital sodium, and the lung tissue was harvested as described above. 

Drugs used. Thapsigargin and 4-AP were obtained from Sigma. Fura 2-AM was obtained from Molecular Probes (Eugene, OR), and the KCl solution was obtained from Alomone Laboratories (Jerusalem, Israel). Ionomycin and diltiazem were obtained from Calbiochem (La Jolla, CA). All drug solutions were adjusted to pH 7.4 before use and solubilized in normal saline.

Ca²⁺ imaging. To assess dynamic changes in [Ca²⁺], in individual PA SMC, we used the Ca²⁺-sensitive fluorophore fura 2-AM (Molecular Probes). Subconfluent fetal PA SMC on 25-mm² glass coverslips were placed on the stage of an inverted microscope (Nikon Diaphot). Cells were loaded with 100 nM fura 2-AM plus 2.5 mg/ml Pluronic acid (Molecular Probes) for 20 min in Ca²⁺-free solution, followed by a 20-min wash in calcium-containing solution before the start of the experiment. Ratiometric imaging was performed with the use of excitation wavelengths of 340 and 380 nm and an emission wavelength 560 nm. Imaging was performed with an ICCD camera (Photonic Science; Robertsbridge, UK) using Axon Instruments (Foster City, CA) image capture and analysis software. Ca²⁺ calibration was achieved by measuring a maximum (with ionomycin 1 mM) and a minimum (with EGTA 10 mM) for each cell. PO₂ was controlled by aerating the recording solution reservoir with either 21% O₂ with balance N₂ or 100% N₂. PO₂ was monitored throughout the use of an O₂ electrode (Microelectrodes; Bedford, NH). Further control of PO₂ was obtained by aerating the stage microincubator with 21% O₂ with balanced N₂ or 100% N₂. pH was 7.40 ± 0.05 and did not change during the experiments. Intracellular free Ca²⁺ was calculated assuming a dissociation constant of 220 (31). For each experiment, 5–12 cells were visualized and cytosolic Ca²⁺ measurements were made from individual cells.

Measurement of [Ca²⁺], [Ca²⁺]i and the effect of an acute increase in PO₂ on [Ca²⁺]i. Measurement of [Ca²⁺], [Ca²⁺]i, and the effect of an acute increase in PO₂ on [Ca²⁺]i, in chronically hypoxic PHTN and control fetal PA SMC. After stable baseline values in hypoxic recording solution (25 mmHg) or normoxic (21% O₂; PO₂ = 117) PA SMC.
Effect of potassium and voltage-operated Ca\(^{2+}\) channel antagonists on basal [Ca\(^{2+}\)]\(_i\), in hypoxic PHTN and control fetal PA SMC. In separate experiments, [Ca\(^{2+}\)]\(_i\), was measured in control and PHTN PA SMC during the application of the following: 1) iberiotoxin (10^{-9} M) in PHTN (n = 17) and control (n = 51) PA SMC; 2) 4-AP (10^{-3} M) in PHTN (n = 47) and control (n = 38) PA SMC; and 3) diltiazem (10^{-5} M) in PHTN (n = 19) and control (n = 29) PA SMC.

Intracellular Ca\(^{2+}\) stores in hypoxic PHTN and control fetal PA SMC. After obtaining stable baseline values in hypoxic recording solution, the recording solution was changed to hypoxic solution without added Ca\(^{2+}\) for 2 min. Cells were then treated with thapsigargin (10^{-6} M) while [Ca\(^{2+}\)]\(_i\) was measured in PHTN (n = 27) and control (n = 36) PA SMC. In a separate series of experiments, cells were treated with ryanodine (5 \times 10^{-5} M), whereas [Ca\(^{2+}\)]\(_i\) was measured in PHTN (n = 29) and control (n = 29) PA SMC.

Basal [Ca\(^{2+}\)]\(_i\), and the effect of an acute increase in P\(_O_2\) on [Ca\(^{2+}\)]\(_i\), in serum-starved chronically hypoxic PHTN PA SMC. SMC from PHTN lambs were isolated and maintained in primary culture under hypoxic conditions as described above. In separate sets of experiments, the medium over the cells was changed from 10% to 0.1% fetal bovine serum for 1 or 7 days. Ca\(^{2+}\) imaging was performed while the conditions were changed to acute normoxia (n = 30 cells).

Statistical analysis. 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 each experimental protocol. Values are expressed as means ± SE. P values <0.05 were considered significant.

In each individual RNA experiment, the ratio of K\(^+\) channel to 18S rRNA content from hypertensive animals was compared with the ratio of K\(^+\) channel to 18S rRNA content in control animals. Total RNA was isolated from four control animals and six PHTN animals. The Student’s t-test was used to assess differences between experimental groups. P values <0.05 were considered significant.

RESULTS

Basal [Ca\(^{2+}\)]\(_i\), and effect of acute increase in P\(_O_2\) on [Ca\(^{2+}\)]\(_i\) in chronically hypoxic PHTN and control fetal PA SMC. Basal [Ca\(^{2+}\)]\(_i\), did not differ between PHTN and control cells. In control fetal PA SMC, acute normoxia decreased [Ca\(^{2+}\)]\(_i\) from 111 ± 10 to 83 ± 7 nM. Acute normoxia had no effect on PHTN PA SMC [Ca\(^{2+}\)]\(_i\), (Fig. 1) (24). The response to acute normoxia was not uniform. The heterogeneity of the response is illustrated in Fig. 2.

Effect of potassium and voltage-operated Ca\(^{2+}\) channel antagonists on basal [Ca\(^{2+}\)]\(_i\), in PHTN and control fetal PA SMC. In control cells, iberiotoxin (Fig. 3) caused an increase of 224 ± 25 nM. In PHTN fetal PA
SMC, iberiotoxin had no effect. In control fetal PA SMC, 4-AP caused an increase of 15 ± 3 nM, whereas in PHTN cells, 4-AP caused an increase of 32 ± 5 nM. In control PA SMC, diltiazem caused [Ca^{2+}]_{i} to decrease by 34 ± 4 nM from baseline, whereas in PTHN PA SMC [Ca^{2+}]_{i} increased by 21 ± 5 nM.

Intracellular Ca^{2+} stores in hypoxic PHTN and control fetal PA SMC. Ryanodine caused an increase in [Ca^{2+}]_{i} in both control and PHTN fetal PA SMC. In control PA SMC, ryanodine increased [Ca^{2+}]_{i} by 147 ± 28 nM, while in PHTN PA SMC [Ca^{2+}]_{i} increased by 347 ± 46 nM (Fig. 4A). In control fetal PA SMC, thapsigargin increased [Ca^{2+}]_{i} by 45 ± 12 nM. In PHTN fetal PA SMC, thapsigargin had no effect on [Ca^{2+}]_{i} (Fig. 4B).

Basal [Ca^{2+}]_{i} and effect of acute increase in P O_{2} on [Ca^{2+}]_{i} in serum-starved chronically hypoxic PHTN PA SMC. Serum deprivation had no effect on basal [Ca^{2+}]_{i} or on the response to an acute increase in P O_{2}.

αKCa subunit and K_v channel RT-PCR. K_Ca mRNA expression was decreased in PHTN, compared with control fetal PA SMC as determined by quantitative, internally controlled RT-PCR (Fig. 5, A and B). The ratio of αK_Ca RNA to 18S RNA levels was 1.562 ± 0.136 in controls (n = 4) and 1.41 ± 0.103 in animals with PHTN (n = 6 animals). K_c channel mRNA expression in PHTN PA SMC was not significantly different from control PA SMC.

K_Ca channel protein expression. Consistent with the observed effect on mRNA expression, Western blot analysis of K_Ca channel expression demonstrated decreased K_Ca channel protein (125-kDa band) in the PHTN PA SMC compared with controls (Fig. 6).

DISCUSSION

The present series of experiments demonstrates that chronic intrauterine pulmonary hypertension compro-
within the intracellular K \textsuperscript{v} channel in PA SMC (6). Furthermore, the voltage-operated Ca\textsuperscript{2+} channel blocker diltiazem increased [Ca\textsuperscript{2+}]\textsubscript{i} in hypertensive fetal PA SMC, while causing a significant decrease in [Ca\textsuperscript{2+}]\textsubscript{i} in normotensive fetal PA SMC. Molecular data provides further support for these observations as K\textsubscript{Ca} channel expression is decreased and K\textsubscript{v} channel gene expression is unchanged in hypertensive, compared with normotensive, fetal PA SMC. Whereas previous investigators (2, 20) have clearly demonstrated that chronic intrauterine pulmonary hypertension attenuates perinatal pulmonary vasodilation, the present study provides the first evidence that alterations in PA SMC O\textsubscript{2} sensing and intracellular Ca\textsuperscript{2+} homeostasis may contribute to abnormal perinatal pulmonary vasoreactivity.

Previous studies have demonstrated that perinatal pulmonary vasodilator stimuli act, at least in part, through activation of the pulmonary vascular K\textsubscript{Ca} channel. In specific ventilation (33) and shear stress (32), an acute increase in P\textsubscript{O\textsubscript{2}} (6) and NO (29) causes perinatal pulmonary vasodilation through activation of the K\textsubscript{Ca} channel. From a teleologic perspective, the normal perinatal pulmonary circulation is uniquely well adapted to respond to an acute increase in P\textsubscript{O\textsubscript{2}} as fetal, but not adult, PA SMC respond to an acute increase in P\textsubscript{O\textsubscript{2}} with a decrease in [Ca\textsuperscript{2+}]\textsubscript{i} (26). The inability of PA SMC derived from fetal lambs with chronic intrauterine pulmonary hypertension to respond to an acute increase in P\textsubscript{O\textsubscript{2}} is consistent with previous reports of a decrease in pulmonary K\textsubscript{Ca} channel expression in this model (7). In addition, recently published electrophysiology data indicate that the predominant potassium channel contributing to E\textsubscript{m} shifts from the K\textsubscript{Ca} channel to the K\textsubscript{v} channel in PA SMC from lambs with chronic intrauterine pulmonary hypertension (22). Moreover, the present observation is consistent with the incomplete response to vasodilator stimuli that characterizes the clinical presentation of persistent pulmonary hypertension (16).

To address the possibility that the attenuated Ca\textsuperscript{2+} response in hypertensive PA SMC results from increased Ca\textsuperscript{2+}-ATPase activity in the SR, cells were treated with thapsigargin. In normotensive fetal PA SMC maintained under hypoxic conditions, thapsigargin caused a substantial increase in [Ca\textsuperscript{2+}]\textsubscript{i}, implying that Ca\textsuperscript{2+}-ATPase activity plays a role in the maintenance of basal Ca\textsuperscript{2+} stores. In contrast, thapsigargin had no effect on the basal [Ca\textsuperscript{2+}]\textsubscript{i} of hypertensive PA SMC maintained under hypoxic conditions. Thus, in PHTN SMC, the SR Ca\textsuperscript{2+}-ATPase activity is diminished. Consequently, the absence of the normoxia-induced decrease in [Ca\textsuperscript{2+}]\textsubscript{i} in PHTN SMC is unlikely to result from augmented Ca\textsuperscript{2+}-ATPase activity. Further studies are necessary to determine the significance of the relatively diminished role of Ca\textsuperscript{2+}-ATPase activity in the maintenance of basal Ca\textsuperscript{2+} in PA SMC from animals with chronic intrauterine pulmonary hypertension.

To further investigate the effect of chronic intrauterine hypertension on intracellular Ca\textsuperscript{2+} handling, PA SMC from hypoxic normotensive and hypertensive animals were treated with ryanodine, which causes release of calcium from ryanodine stores in the SR (14, 17). Ryanodine caused an increase in both normotensive and PHTN PA SMC [Ca\textsuperscript{2+}]\textsubscript{i}. However, the effect of ryanodine was significantly greater in PHTN PA SMC. Previous work (29) suggests that oxygen causes a decrease in fetal PA SMC [Ca\textsuperscript{2+}]\textsubscript{i}, through the quantal, localized release of intracellular calcium from a ryanodine-sensitive store close to the cell membrane in the region of the calcium-sensitive K\textsuperscript{v} channel. These small bursts of calcium have been termed calcium sparks. Ca\textsuperscript{2+} sparks are localized events that do not contribute to the global [Ca\textsuperscript{2+}]\textsubscript{i} and do not reach a high enough concentration to cause contraction. Ca\textsuperscript{2+} spark activation of the K\textsubscript{Ca} channel hyperpolarizes the cell, closing voltage-dependent Ca\textsuperscript{2+} channels and causing a decrease in cytosolic Ca\textsuperscript{2+} and, ultimately, vasodilation (11, 21). The relatively greater response to ryanodine in PHTN compared with normotensive PA SMC [Ca\textsuperscript{2+}]\textsubscript{i} suggests chronic intrauterine hypertension affects the ryanodine-sensitive Ca\textsuperscript{2+} stores such that the stores contain Ca\textsuperscript{2+} in greater amounts than in controls. We speculate that Ca\textsuperscript{2+} spark release may no longer be localized or in the region of the K\textsubscript{Ca} channel, and Ca\textsuperscript{2+} release from the store may lead to an increase in PA SMC [Ca\textsuperscript{2+}]\textsubscript{i} from PHTN animals. While the underlying reason for the relatively replete ryanodine-sensitive stores is unknown, this observation seems to preclude the possibility that diminished K\textsubscript{Ca} channel activation results primarily from depletion of the ryanodine-sensitive store.

Consideration of these data in concert with the decrease in K\textsubscript{Ca} channel activity noted in PA SMC from hypertensive animals leads to the conclusion that release of Ca\textsuperscript{2+} from ryanodine-sensitive stores does not result in K\textsubscript{Ca} channel activation. It is possible that chronic intrauterine pulmonary hypertension changes

**Fig. 6.** A representative Western blot for K\textsubscript{Ca} channel protein expression in PA SMC from culture. The 125-kDa K\textsubscript{Ca} channel band is shown above the \(\alpha\)-smooth muscle actin-loading control band in SMC from control and PHTN animals. The bands were quantified by densitometry with the use of NIH Image software. The K\textsubscript{Ca} protein band was normalized to the \(\alpha\)-smooth muscle actin loading control by expressing the result as a ratio against the loading control signal (K\textsubscript{Ca}/\(\alpha\)-smooth muscle actin).
the relationship of the ryanodine-sensitive intracellular stores and the KCa channel as has been described in cardiac myocytes derived from animals with congestive heart failure (13). The inability of Ca²⁺ release from the ryanodine-sensitive Ca²⁺ store to activate KCa channels, thereby causing membrane hyperpolarization and closure of voltage-operated Ca²⁺ channels, may underlie the incomplete response to pulmonary vasodilator stimuli that characterizes persistent pulmonary hypertension of the newborn. Further support for this notion derives from the observation that both NO (29) and O₂ (24) cause pulmonary vasodilation through ryanodine-dependent activation of KCa channels. Thus, if calcium release from ryanodine-sensitive stores cannot activate KCa channels, then both oxygen and NO-induced vasodilation may be compromised.

Given the generalized decrease in responsiveness of hypertensive PA SMC, intracellular exposure to hypoxia may prompt PA SMC to change from a contractile to a synthetic phenotype (12). To address such a possibility, PA SMC from hypertensive animals were serum deprived for periods of time ranging between 24 h and 7 days before the study. Serum deprivation had no effect on the responsiveness of these cells to any of the agents included in the present manuscript. On the basis of these results, we conclude that diminished responsiveness of these cell types cannot be explained on the basis of a change in phenotype.

There was no difference in basal [Ca²⁺]i between HTN and control PA SMC. Given clear evidence that chronic intracellular pulmonary hypertension increases fetal pulmonary vascular tone, relatively higher levels of SMC [Ca²⁺]i might be anticipated in PHTN compared with control SMC. The similar levels of [Ca²⁺]i in these two cell populations suggests that the contractile state of the pulmonary vasculature is not determined solely by [Ca²⁺]i. Putative mechanisms include enhanced sensitivity of the contractile proteins to Ca²⁺, upregulation of Rho kinase activity, or a decrease in phosphatase activity (15). Alternatively, changes in intracellular calcium buffering, such as augmented cADP-ribose activity, may modulate SMC contractility (18).

In summary, the present study provides data that chronic intracellular pulmonary hypertension has direct effects on PA SMC. PA SMC from animals with chronic intracellular pulmonary hypertension do not respond to an acute increase in PO₂ with a decrease in [Ca²⁺]i. Moreover, the K⁺ channel that determines basal [Ca²⁺]i changes from a KCa to a Kₐ channel. The physiological changes are consistent with changes in gene expression as KCa mRNA and protein levels are decreased and Kₐ mRNA levels are not significantly changed in PA SMC derived from animals with chronic intracellular pulmonary hypertension. The observation that ryanodine causes a greater response in PA SMC [Ca²⁺]i in hypertensive compared with normotensive animals provides further insight into the mechanism whereby chronic intracellular pulmonary hypertension alters perinatal pulmonary vascular reactivity. Further study is necessary to determine whether Ca²⁺ spark physiology is altered by chronic intruterine pulmonary hypertension.

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**DISCLOSURES**

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Pulmonary Hypertension Limits Vascular O2 Sensing


