Chronic intrauterine pulmonary hypertension compromises fetal pulmonary artery smooth muscle cell O₂ 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 smooth muscle cell O₂ 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) O₂ sensing, fluorescence microscopy was used to study the effect of an acute increase in PO₂ on the cytosolic Ca²⁺ concentration ([Ca²⁺]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 [Ca²⁺]i in control but not PHTN PA SMC. In control PA SMC, [Ca²⁺]i, increased after Ca²⁺-sensitive (KCa) and voltage-sensitive (Kv) K⁺ channel blockade and decreased after diazepam treatment. In PHTN PA SMC, KCa blockade had no effect, whereas Kv blockade and diazepam increased [Ca²⁺]i. Inhibition of sarcoplasmic reticulum Ca²⁺ ATPase activity caused a greater increase in [Ca²⁺]i in controls compared with PHTN PA SMC. Conversely, ryanodine caused a greater increase of [Ca²⁺]i, in PHTN compared with control PA SMC. KCa channel mRNA is decreased and Kv channel mRNA is unchanged in PHTN PA SMC compared with controls. We conclude that PHTN compromises PA SMC O₂ sensing, alters intracellular Ca²⁺ homeostasis, and changes the predominant ion channel that determines basal [Ca²⁺]i, from KCa to Kv.

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 Ca²⁺ channels, a decrease in cytosolic Ca²⁺ concentration ([Ca²⁺]i), and subsequent vasodilation (21).

Recent studies (9) from our laboratory have demonstrated that fetal PA SMC respond directly to changes in PO₂. Fetal PA SMC respond to acute hypoxia with an increase in [Ca²⁺]i. In response to an acute increase in PO₂ (acute normoxia), fetal PA SMC [Ca²⁺]i decreases (26). O₂ causes a decrease in PA SMC [Ca²⁺]i, via activation of a cGMP-sensitive kinase that mediates localized Ca²⁺ release from a ryanodine-sensitive intracellular Ca²⁺ store (24). Localized Ca²⁺ release results in activation of the Ca²⁺-sensitive K⁺ (KCa) channel and PA SMC membrane hyperpolarization, which leads to vasodilation (21).

Interestingly, the response of PA SMC to changes in PO₂ is developmentally regulated. Fetal, but not adult, PA SMC respond to an acute increase in PO₂ with a decrease in [Ca²⁺]i (23, 26). In contrast, adult PA SMC respond to acute hypoxia with a more rapid and greater increase in [Ca²⁺]i, than fetal PA SMC (8). The differential response to PO₂ between fetal and adult PA SMC may derive, in part, from changes in the predominant ion channel population that controls resting membrane potential (Eₘ). In the fetus, the KCa channel determines Eₘ, and, therefore, through subsequent effects on voltage-gated Ca²⁺ channels, basal levels of PA SMC [Ca²⁺]i. In contrast, the Eₘ and basal [Ca²⁺]i of PA SMC from the adult pulmonary circulation is mediated by voltage-sensitive K⁺ channels (Kv) (25). Thus fetal PA SMC are uniquely well adapted to respond to an acute increase in PO₂ and thereby enhance postnatal adaptation of the pulmonary circulation, whereas adult PA SMC are adapted to respond to an acute decrease in PO₂ 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 KCa 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 KCa 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 KCa channel activity determines resting membrane potential (25) and mediates the response of fetal PA SMC to an acute increase in Po$_2$ (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 Po$_2$ on [Ca$^{2+}$]i of PA SMC isolated from fetal lambs with chronic intrauterine pulmonary hypertension.

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 iberiotoxin, a selective KCa channel antagonist, and 4-aminopyridine (4-AP), a KCa antagonist on PA SMC [Ca$^{2+}$]. The effect of chronic intrauterine hypertension on intracellular calcium homeostasis was addressed by treating PHTN cells with thapsigargin, an inhibitor of sarcoplasmic reticulum (SR) ATPase, and ryanodine, a stimulant of Ca$^{2+}$ release from ryanodine-sensitive intracellular Ca$^{2+}$ stores (17). KCa channel mRNA levels were determined with the use of quantitative internally controlled RT-PCR.

**METHODS**

**Cell culture.** The techniques used for isolation and culture of ovine PA SMC have been previously described (9). Distal PAs were quickly excised from pentobarbital-anesthetized ovine fetuses ranging 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. Loose connective tissue and adventitia were removed and the vessels were liberally rinsed with minimal essential medium (MEM; 0.2 mM Ca$^{2+}$). Vessel segments were carefully cut into small pieces and placed into 50-ml conical flasks containing 5.0 ml of the enzymatic dissociation mixture, which consisted of 0.125 mg/ml elastase (Sigma; St. Louis, MO), 1 mg/ml collagenase (Worthington Biochemical; Freehold, NJ), 2.0 mg/ml bovine serum albumin (Sigma), 0.375 mg/ml soybean trypsin inhibitor (Sigma), and 4 ml of MEM. After incubation at 37°C for 60 min in a shaking bath, the tissue suspension was triturated 10 times every 15 min in a plastic pipette for a total incubation period of 90–120 min. The tissue suspension was then passed through a 100-μm nylon mesh (Nitex, Tetka; Elmsford, NJ) to separate dispersed cells from undigested vessel wall fragments and debris. The filtered suspension was centrifuged (200 g for 10 min) and the cell pellet was resuspended in 10 ml of MEM supplemented with 10% fetal bovine serum. The dispersed cell suspension was aliquoted onto 25-mm$^2$ glass coverslips and into 25-cm$^2$ tissue culture flasks (Falcon Plastics; Oxnard, CA) at a density of 5–10 x 10$^5$ cells/cm$^2$. The cells were incubated at 37°C in a humidified 10% O$_2$-5% CO$_2$-balance N$^2$ atmosphere (hypoxia) or humidified 95% air-5% CO$_2$. After 18–24 h, the cultures were washed once with Hanks’ balanced salt solution to remove nonadherent cells and debris and refed with fresh medium. Medium was routinely exchanged at 72-h intervals. Cells were studied between day 5 and day 14 of culture. Cell density stabilized as subconfluent monolayers after 3–5 days in culture. To verify the uniformity of the cell population, PA SMCs were routinely stained with a-actin-specific antibody after 5, 10, and 14 days in culture.

**Solutions.** Recording solutions consisted of (in mM) 10 HEPES, 10 glucose, 135 NaCl, 5.6 KCl, 1.8 CaCl$_2$, and 1.2 MgCl$_2$. All solutions were made with the use of nanopure distilled water. Osmolality was adjusted to ~300 mosM, and pH adjusted to 7.4.

**RT-PCR.** Arterial tissue was removed from the lung. Tissue was taken from pulmonary arteries that were greater than or equal to fourth generation. Pulmonary arterial tissue was suspended in liquid N$_2$ and ground to powder with a precooled mortar and pestle. Total RNA was extracted with the use of the guanidium thiocyanate-phenol-chloroform method (Tri reagent; 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 Kc, 2.1 cDNA were based on the human sequence (3) and were (forward) 5’ AAGAGGAACCCAAAGAGGAAGAC 3’ and (reverse) 5’ CACCTCTCATGAAGTTGACTTTA 3’. The use of these primers yielded a PCR product consistent with that expected for a fragment of the human Kc, 2.1 mRNA. The fragment size was 358 base pairs. The identity of the product was confirmed with sequence analysis. Oligonucleotide primers used to amplify KCa cDNA were based on the human sequence (34) and were (forward) 5’ CTACTGGGAT- GTTTCTACGTGTTG 3’ and (reverse) 5’ TGTCGTCACTCA-ACTGCA 3’. The use of these primers yielded a PCR product consistent with that expected for a fragment of the human KCa 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 224 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-
Experiments 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-deoxyoctonucleotide 5'-triphosphate mixture, 20 pM of 18S primer mixture (ratio of 1:9) of 18S primers/competimers, 1 U AmpliTaq 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). Densitometry 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 experiment. A control PCR experiment was run that contained no RNA template.

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 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 electroblotted 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 (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. Pura 2-AM was obtained from Molecular Probes (Eugene, OR) and the effect of an acute increase in P02 on [Ca²⁺]i was measured in PHTN and control fetal PA SMC. After stable baseline values in hypoxic recording solution were obtained, normoxic recording solution was superfused over the cells while [Ca²⁺] was measured in PHTN (n = 96) and control (n = 117) PA SMC.
Effect of potassium and voltage-operated Ca\textsuperscript{2+} channel antagonists on basal [Ca\textsuperscript{2+}], in hypoxic PHTN and control fetal PA SMC. In separate experiments, [Ca\textsuperscript{2+}], 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\textsuperscript{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\textsuperscript{2+} for 2 min. Cells were then treated with thapsigargin (10^{-6} M) while [Ca\textsuperscript{2+}], was measured in PHTN (n = 27) and control (n = 30) PA SMC. In a separate series of experiments, cells were treated with ryanodine (5 \times 10^{-5} M), whereas [Ca\textsuperscript{2+}], was measured in PHTN (n = 29) and control (n = 29) PA SMC.

Basal [Ca\textsuperscript{2+}], and the effect of an acute increase in PO\textsubscript{2} on [Ca\textsuperscript{2+}], 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\textsuperscript{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 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\textsuperscript{+} channel to 18S rRNA content from hypertensive animals was compared with the ratio of K\textsuperscript{+} 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\textsuperscript{2+}], and effect of acute increase in PO\textsubscript{2} on [Ca\textsuperscript{2+}], in chronically hypoxic PHTN and control fetal PA SMC. Basal [Ca\textsuperscript{2+}], did not differ between PHTN and control cells. In control fetal PA SMC, acute normoxia decreased [Ca\textsuperscript{2+}], by 25%, whereas in pulmonary hypertension (PHTN) cells, the median change was -5%.

PA SMC. Basal [Ca\textsuperscript{2+}], did not differ between PHTN and control cells. In control fetal PA SMC, acute normoxia decreased [Ca\textsuperscript{2+}], from 111 ± 10 to 83 ± 7 nM. Acute normoxia had no effect on PHTN PA SMC [Ca\textsuperscript{2+}], (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\textsuperscript{2+} channel antagonists on basal [Ca\textsuperscript{2+}], in PHTN and control fetal PA SMC. In control cells, iberiotoxin (Fig. 3) caused an increase of 224 ± 25 nM. In PHTN fetal PA

![Graph showing the effect of iberiotoxin on [Ca\textsuperscript{2+}] in PHTN and control fetal PA SMC.](image-url)
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).

Baseline [Ca^{2+}]_i and effect of acute increase in PO2 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 PO2.

αKCa subunit and Kv channel RT-PCR. KCa 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 αKCa 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). Kc, channel mRNA expression in PHTN PA SMC was not significantly different from control PA SMC.

KCa channel protein expression. Consistent with the observed effect on mRNA expression, Western blot analysis of KCa channel expression demonstrated decreased KCa 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 comprised...
stimuli that characterizes the clinical presentation of persistent pulmonary hypertension (16).

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

To further investigate the effect of chronic intrauterine hypertension on intracellular Ca$^{2+}$ 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$^{2+}$]. However, the effect of ryanodine was significantly greater in PHTN PA SMC. Previous work (20) suggests that oxygen causes a decrease in fetal PA SMC [Ca$^{2+}$], 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$^+$ channel. These small bursts of calcium have been termed calcium sparks. Ca$^{2+}$ sparks are localized events that do not contribute to the global [Ca$^{2+}$] and do not reach a high enough concentration to cause contraction. Ca$^{2+}$ spark activation of the K$\text{Ca}$ channel hyperpolarizes the cell, closing voltage-dependent Ca$^{2+}$ channels and causing a decrease in cytosolic Ca$^{2+}$ and, ultimately, vasodilation (11, 21). The relatively greater response to ryanodine in PHTN compared with normotensive PA SMC [Ca$^{2+}$], suggests chronic intrauterine hypertension affects the ryanodine-sensitive Ca$^{2+}$ stores such that the stores contain Ca$^{2+}$ in greater amounts than in controls. We speculate that Ca$^{2+}$ spark release may no longer be localized or in the region of the K$\text{Ca}$ channel, and Ca$^{2+}$ release from the store may lead to an increase in PA SMC [Ca$^{2+}$] 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$\text{Ca}$ channel activation results primarily from depletion of the ryanodine-sensitive store.

Consideration of these data in concert with the decrease in K$\text{Ca}$ channel activity noted in PA SMC from hypertensive animals leads to the conclusion that release of Ca$^{2+}$ from ryanodine-sensitive stores does not result in K$\text{Ca}$ channel activation. It is possible that chronic intrauterine pulmonary hypertension changes
the relationship of the ryanodine-sensitive intracellular stores and the K_{Ca} channel as has been described in cardiac myocytes derived from animals with congestive heart failure (13). The inability of Ca^{2+} release from the ryanodine-sensitive Ca^{2+} store to activate K_{Ca} channels, thereby causing membrane hyperpolarization and closure of voltage-operated Ca^{2+} 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 O_{2} (24) cause pulmonary vasodilation for this notion derives from the observation that both NO (29) and O_{2} (24) cause pulmonary vasodilation. Given the broadened decrease in responsiveness of hypertensive PA SMC, intracellular exposure to hypertension 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 [Ca^{2+}]_{i} between HTN and control PA SMC. Given clear evidence that chronic intrauterine pulmonary hypertension increases fetal pulmonary vascular tone, relatively higher levels of SMC [Ca^{2+}]_{i} might be anticipated in PHTN compared with control SMC. The similar levels of [Ca^{2+}]_{i} in these two cell populations suggests that the contractile state of the pulmonary vasculature is not determined solely by [Ca^{2+}]_{i}. Putative mechanisms include enhanced sensitivity of the contractile proteins to Ca^{2+}, upregulation of Rho kinase activity, or a decrease in phosphatase activity. 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 intrauterine pulmonary hypertension has direct effects on PA SMC. PA SMC from animals with chronic intrauterine pulmonary hypertension do not respond to an acute increase in P_{O_{2}} with a decrease in [Ca^{2+}]_{i}. Moreover, the K^{+} channel that determines basal [Ca^{2+}]_{i} changes from a K_{Ca} to a K_{v} channel. The physiological changes are consistent with changes in gene expression as K_{Ca} mRNA and protein levels are decreased and K_{v} mRNA levels are not significantly changed in PA SMC derived from animals with chronic intrauterine pulmonary hypertension. The observation that ryanodine causes a greater response in PA SMC [Ca^{2+}]_{i} in hypertensive compared with normotensive animals provides further insight into the mechanism whereby chronic intrauterine pulmonary hypertension alters perinatal pulmonary vascular reactivity. Further study is necessary to determine whether Ca^{2+} spark physiology is altered by chronic intrauterine pulmonary hypertension.

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DISCLOSURES

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