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 \( P_O_2 \) 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 \( Ca^{2+} \) channel blockade and decreased after dil-tiazem treatment. In PHTN PA SMC, \( K_{Ca} \) blockade had no effect, whereas \( K_v \) blockade and dil-tiazem 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 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\( v \)). Thus fetal PA SMC are uniquely well adapted to respond to an acute increase in \( P_O_2 \) and thereby enhance postnatal adaptation of the pulmonary circulation, whereas adult PA SMC are adapted to respond to an acute decrease in \( P_O_2 \) and thereby match ventilation and perfusion to prevent intrapulmonary shunting and subsequent hypoxemia.

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 intruterine 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 K_{ca} channel gene expression in whole lung tissue of fetal lambs with chronic intruterine pulmonary hypertension. It remains unknown whether 1) the decrease in fetal lung K_{ca} channel gene expression is sufficient to compromise fetal pulmonary vascular oxygen sensing; and 2) chronic intruterine pulmonary hypertension directly affects PA SMC oxygen sensing. Because PA SMC K_{ca} 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 intruterine 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 intruterine pulmonary hypertension.

To determine any changes in K^{+} channel activity due to chronic intruterine 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 K_{ca} channel antagonist, and 4-aminopyridine (4-AP), a K^{+} antagonist on PA SMC [Ca^{2+}]_{i}. The effect of chronic intruterine hypertension on intracellular calcium homeostasis was addressed by treating PHTN fth-generation resistance PAs. To determine any changes in K^{+} channel activity due to chronic intruterine 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 K_{ca} channel antagonist, and 4-aminopyridine (4-AP), a K^{+} antagonist on PA SMC [Ca^{2+}]_{i}. The effect of chronic intruterine hypertension on intracellular calcium homeostasis was addressed by treating PHTN fth-generation resistance PAs.

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 tissue was cut into small pieces and placed into 50-ml conical flasks (Falcon Plastics; Oxnard, CA) at a density of 5–10 × 10^{6} 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 prechilled mortar and pestle. Total RNA was extracted 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_{ca} cDNA were based on the human sequence (3) and were (forward) 5’ ACAGAGCAAAACAAGGGAAGAAC 3’ and (reverse) 5’ GCCCTTCAATGAGTGTGAATTT 3’. The use of these primers yielded a PCR product consistent with that expected for a fragment of the human K_{ca} 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’ CTACTGGGATGTTTCTACGTGTT 3’ and (reverse) 5’ TGCTGTTCATCAACTGCTA 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 Quantum RNA 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 a decrease in KCa channel gene expression in whole lung tissue of fetal lambs with chronic intruterine 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 intruterine pulmonary hypertension directly affects PA SMC oxygen sensing.
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 MgCl₂, 10 pM each K₇, 2.1 primer, 10 nM 2-deoxyuridine 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, and then an extension of 2 min at 72°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 animal and the densitometric values were run through a calibration curve for serum Na⁺.

Western blot analysis for KCa protein. Cultured smooth muscle cell monolayers were rinsed with cold PBS and trypsinization 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 collection, fetal ages ranged from 135 to 140 days gestation (term is 147 days). Ewes were 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 lung tissue was harvested as described above.

Ca²⁺ imaging. To assess dynamic changes in [Ca²⁺]i 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 μM) 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 with the use of an O₂ electrode (Microelectrodes; Bedford, NH). Further control of PO₂ was obtained by aerating the stage microincubator with 21% O₂ with balance 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⁺]i, [Ca²⁺]c, measurements were made after stable baseline Ca²⁺ values were obtained in either hypoxic (PO₂ = 25 mmHg) or normoxic (21% O₂; PO₂ = 120 mmHg) recording solution. The recording solution was superfused over the cells at a rate of ~2 ml/min in all experiments. Measurements of [Ca²⁺]i, were made for at least 10 min after a change in conditions or addition of a drug. KCl (60 mM) was superfused over the cells at 2 ml/min at the conclusion of the experiment to ensure cellular viability.
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 = 30) 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 PO$_2$ 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 PO$_2$ 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 (10$^{-9}$ M) increased [Ca$^{2+}$]$_i$ to increase 22% ± 5% in PHTN PA SMC (n = 19) [Ca$^{2+}$]$_i$, to increase 32 ± 9%. Voltage-operated Ca$^{2+}$ channel blockade with 10$^{-5}$ M Diltiazem led to a decrease in [Ca$^{2+}$]$_i$, in control but not in PHTN PA SMC [Ca$^{2+}$]$_i$. All experiments were performed under hypoxic conditions. *P < 0.01 vs. baseline, †P < 0.01 vs. control, ‡P < 0.05 vs. baseline.
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 ± 7 nM.

In control PA SMC, diltiazem caused [Ca\(^{2+}\)]\(_i\) to decrease by 34 ± 4 nM from baseline, whereas in PHTN 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 Po\(_2\) 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 Po\(_2\).

αK\(_{Ca}\) 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\(_{Ca}\) 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 compr...
pertensive fetal PA SMC is determined by 4-aminopyr-
spond to an acute increase in PO2 is consistent with
chronic intrauterine pulmonary hypertension to re-
inability of PA SMC derived from fetal lambs with
consistent with the incomplete response to vasodilator
pertension (22). Moreover, the present observation is
from lambs with chronic intrauterine pulmonary hy-
monary vasoreactivity.

Previous investigators (2, 20) have clearly demon-
channel gene expression is unchanged in hypertensive,
channel expression is decreased and Kv
lecular data provides further support for these obser-
ations in PA SMC O2 sensing and intracellular Ca2+
homeostasis may contribute to abnormal perinatal pul-
monary vasoreactivity.

Previous studies have demonstrated that perinatal
channel stimuli act, at least in part, through activation of the pulmonary vascular Kca
channel. In specific ventilation (33) and shear stress
(32), an acute increase in Po2 (6) and NO (29) causes
perinatal pulmonary vasodilation through activation of the
channel. From a teleologic perspective, the
normal perinatal pulmonary circulation is uniquely
well adapted to respond to an acute increase in Po2 as
fetal, but not adult, PA SMC respond to an acute
increase in Po2 with a decrease in [Ca2+]i (26). The
inability of PA SMC derived from fetal lambs with
chronic intrauterine pulmonary hypertension to re-
spend to an acute increase in Po2 is consistent with
previous reports of a decrease in pulmonary Kca
channel expression in this model (7). In addition, recently
published electrophysiology data indicate that the
predominant potassium channel contributing to Em shifts from the Kca channel to the Kv channel in PA SMC
from lambs with chronic intrauterine pulmonary hy-
pertension (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 Ca2+
response in hypertensive PA SMC results from in-
creased Ca2+-ATPase activity in the SR, cells were
reated with thapsigargin. In normotensive fetal PA
SMC maintained under hypoxic conditions, thapsigar-
gin caused a substantial increase in [Ca2+]i, implying
that Ca2+-ATPase activity plays a role in the mainte-
nance of basal Ca2+ stores. In contrast, thapsigargin
had no effect on the basal [Ca2+]i of hypertensive PA
SMC maintained under hypoxic conditions. Thus, in
PHTN SMC, the SR Ca2+-ATPase activity is dimin-
ished. Consequently, the absence of the normoxia-in-
duced decrease in [Ca2+]i in PHTN SMC is unlikely to
result from augmented Ca2+-ATPase activity. Further
studies are necessary to determine the significance of
the relatively diminished role of Ca2+-ATPase activity in
the maintenance of basal [Ca2+]i in PA SMC from
animals with chronic intrauterine pulmonary hyper-

To further investigate the effect of chronic intrauter-
ine hypertension on intracellular Ca2+ handling, PA
SMC from hypoxic normotensive and hypertensive an-
imals were treated with ryanodine, which causes re-
lease of calcium from ryanodine stores in the SR (14, 17). Ryanodine caused an increase in both normoten-
sive and PHTN PA SMC [Ca2+]i. However, the effect of
ryanodine was significantly greater in PHTN PA SMC.
Previous work (20) suggests that oxygen causes a de-
crease in fetal PA SMC [Ca2+]i through the quantal,
localized release of intracellular calcium from a ryan-
odine-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. Ca2+ sparks are localized events that do not
contribute to the global [Ca2+]i and do not reach a high
enough concentration to cause contraction. Ca2+ spark
activation of the Kca channel hyperpolarizes the cell,
closing voltage-dependent Ca2+ channels and causing
a decrease in cytosolic Ca2+ and, ultimately, vasodila-
tion (11, 21). The relatively greater response to ryan-
odine in PHTN compared with normotensive PA SMC
[Ca2+]i, suggests chronic intrauterine hypertension af-
facts the ryanodine-sensitive Ca2+ stores such that the
stores contain Ca2+ in greater amounts than in con-
trols. We speculate that Ca2+ spark release may no
longer be localized or in the region of the Kca channel,
and Ca2+ release from the store may lead to an in-
crease in PA SMC [Ca2+]i from PHTN animals. While
the underlying reason for the relatively replete ryan-
odine-sensitive stores is unknown, this observation
seems to preclude the possibility that diminished Kca
channel activation results primarily from depletion of the
ryanodine-sensitive store.

Consideration of these data in concert with the de-
crease in Kca channel activity noted in PA SMC from
hypertensive animals leads to the conclusion that re-
lease of Ca2+ from ryanodine-sensitive stores does not
result in Kca 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 and closure of voltage-operated \( Ca^{2+} \) channels. Thus, if calcium release from ryanodine-sensitive stores cannot activate \( K_{Ca} \) channels, then both oxygen and NO-induced vasodilation may be compromised.

Given the generalized decrease in responsiveness of hypertensive PA SMC, intraterine 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 (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 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 \( O_2 \) with a decrease in \([Ca^{2+}]_i\). Moreover, the \( K^+ \) channel that determines basal \([Ca^{2+}]_i\) changes from a \( K_{Ca} \) to a \( K_\alpha \) channel. The physiological changes are consistent with changes in gene expression as \( K_{Ca} \) mRNA and protein levels are decreased and \( K_\alpha \) 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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