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Store-operated channels in the pulmonary circulation of high- and low-altitude neonatal lambs

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Parrau D, Ebensperger G, Herrera EA, Moraga F, Riquelme RA, Ulloa CE, Rojas RT, Silva P, Hernandez I, Ferrada J, Diaz M, Parer JT, Cabello G, Llanos AJ, Reyes RV. Store-operated channels in the pulmonary circulation of high- and low-altitude neonatal lambs. *Am J Physiol Lung Cell Mol Physiol* 304: L540–L548, 2013. First published February 15, 2013; doi:10.1152/ajplung.00024.2012.—We determined whether store-operated channels (SOC) are involved in neonatal pulmonary artery function under conditions of acute and chronic hypoxia, using newborn sheep gestated and born either at high altitude (HA, 3,600 m) or low altitude (LA, 520 m). Cardiopulmonary variables were recorded in vivo, with and without SOC blockade by 2-aminoethyldiphenylborinate (2-APB), during basal or acute hypoxic conditions. 2-APB did not have effects on basal mean pulmonary arterial pressure (mPAP), cardiac output, systemic arterial blood pressure, or systemic vascular resistance in both groups of neonates. During acute hypoxia 2-APB reduced mPAP and pulmonary vascular resistance in LA and HA, but this reduction was greater in HA. In addition, isolated pulmonary arteries mounted in a wire myograph were assessed for vascular reactivity. HA arteries showed a greater relaxation and sensitivity to SOC blockers than LA arteries. The pulmonary expression of two SOC-forming subunits, TRPC4 and STIM1, was upregulated in HA. Taken together, our results show that SOC contribute to hypoxic pulmonary vasoconstriction in newborn sheep and that SOC are upregulated by chronic hypoxia. Therefore, SOC may contribute to the development of neonatal pulmonary hypertension. We propose SOC channels could be potential targets to treat neonatal pulmonary hypertension.

hypoxia; pulmonary vasoconstriction; pulmonary hypertension; 2-aminoethydiphenylborinate; pulmonary vascular reactivity

PULMONARY ARTERIES HAVE AN intrinsic vasoconstrictor response to low oxygen levels when exposed to acute hypoxia. This is a reversible, rapid and physiological response, known as hypoxic pulmonary vasoconstriction (HPV), that redirects blood flow from poorly oxygenated to better oxygenated alveoli. Thus, when total lung is exposed to hypoxia, HPV results in an increase in pulmonary artery pressure (PAP) that reverses when normoxia is reestablished (31). However, exposure to chronic hypoxia produces an imbalance between vasodilator and vasoconstrictor mechanisms, and there is pulmonary vascular remodeling that includes proliferation of pulmonary artery myocytes among other cellular processes (46). The result is a pathological and persistent increase in pulmonary artery contractile tone and pulmonary arterial hypertension, which in many cases leads to right ventricular hypertrophy, right heart failure, and eventually death (13).

In pulmonary artery smooth muscle cells, an increase in intracellular calcium concentration ($[Ca^{2+}]_i$) is essential for HPV, proliferation, and remodeling (13, 19, 20, 42). This increase in $[Ca^{2+}]_i$ greatly depends on an influx of extracellular calcium (13, 59), which may enter the smooth muscle cell through store-operated channels (SOC) among other pathways (5, 8, 22, 40). These are channels physiologically activated by internal calcium store depletion induced by agonists of inositol-(1,4,5)-triphosphate receptor and/or the ryanodine receptor such as endothelin-1 (ET-1), among other effects of the latter. They can also be experimentally stimulated by agents that block the sarcoplasmic reticulum calcium pump like cyclopiazonic acid or thapsigargin (7, 40) and blocked by 2-aminodiphenylborinate (2-APB) or 1-[2-(4-methoxyphenyl)−2-[3-(4-methoxyphenyl) propoxy]ethyl]imidazole (SKF-96365) (1, 22, 57).

SOC-forming molecules of the TRPC, ORAI, and STIM families are expressed in lung, isolated pulmonary arteries, and cultured smooth muscle from pulmonary arteries (9, 25, 27, 30, 35, 36, 50, 62, 63). Pharmacological inhibition and gene suppression experiments have shown that SOC are involved in the pulmonary vascular $[Ca^{2+}]_i$ increase and contractile response to acute hypoxia (27, 28, 48, 55, 57, 58).

On the other hand, chronic hypoxia upregulates SOC in pulmonary artery myocytes from rat, mouse, and human (25, 52, 54). SOC upregulation is also observed in myocytes stimulated to proliferate as happens in pulmonary artery remodeling (12, 22, 53, 61, 62, 63).

Despite the substantial advances in the SOC-related mechanisms regulating pulmonary vascular function, most of these data have been obtained from ex vivo and in vitro approaches

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using isolated cells or organs from adult individuals. Nevertheless, studies covering the in vivo role of SOC on the neonatal pulmonary circulation are lacking.

Neonatal pulmonary hypertension in humans is associated with developmental chronic hypoxia and results in high mortality, decreased postnatal growth and long lasting neurological, respiratory, and cardiac complications (4, 10, 14, 21, 45). We have developed a model of neonatal pulmonary hypertension in sheep, gestated and born at high altitude in the Andean Altiplano, characterized by an increased PAP, impaired vascular reactivity, and altered arterial structure in the pulmonary circulation (14, 15, 16, 18).

In the present work, we tested the hypotheses that SOC contribute to the control of PAP in low-altitude and high-altitude newborn sheep and that SOC function is enhanced at high altitude. We used an integrative approach at the whole animal, isolated organ, and molecular levels in comparing the two groups of animals. Our studies include *1*) in vivo cardiopulmonary function under normoxia and acute hypoxia, with or without SOC blockade with 2-APB; *2*) ex vivo relaxation induced by SOC blockade with 2-APB, in isolated small pulmonary arteries; and *3*) the pulmonary expression of putative SOC-forming molecules TRPC1, TRPC3, TRPC4, TRPC5, TRPC6, ORAI1, and STIM1.

MATERIALS AND METHODS

The Faculty of Medicine Ethics Committee of the University of Chile approved all experimental procedures (protocols CBA N° 040 and CBA 0232 FMUCH). The studies on animals were performed according with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication no. 85-23, revised 1996) and adhere to the American Physiological Society's Guiding Principles in the Care and Use of Animals.

Animals. The animals used in the present study consisted of 10 newborn sheep gestated, born, and raised at the University of Chile farm, Rinconada de Maipú, at 580 m altitude (LA, 13.9 ± 0.5 days old; 7.4 ± 0.3 kg) and 10 newborn sheep gestated, born, and raised at Putre Research Station, International Center for Andean Studies (INCAS), at 3,600 m altitude (HA, 13.7 ± 0.7 days old; 5.1 ± 0.3 kg; $P < 0.05$, HA vs. LA for body weight).

Surgical preparation and in vivo experiments. Five lambs per group were surgically prepared between 6 and 8 days of age for in vivo experimentation as described previously (16). In brief, the animals were anesthetized with a ketamine (10 mg/kg im) and diazepam (0.1–0.5 mg/kg im) association with additional local infiltration of 2% lidocaine. Polyvinyl catheters were placed into the descending aorta and inferior vena cava and a Swan-Ganz catheter was placed into the pulmonary artery. Following 3–4 days of postsurgical recovery, the animals were subjected to a 3-h experimental protocol, consisting of 1 h of basal recording (breathing room air), 1 h of acute isocapnic hypoxia, and 1 h of recovery during which they were returned to room air breathing. This protocol was performed twice on separate days on each animal in random order either with vehicle infusion (DMSO-0.9% NaCl, 1:10) or under SOC blockade with 2-APB (8 mg/kg bolus in vehicle). Infusion of vehicle or 2-APB started 15 min prior to hypoxia, ran continuously for 15 min, and finished before the beginning of the hypoxic challenge. Acute isocapnic hypoxia was induced via a transparent, loosely tied polyethylene bag placed over the animal's head into which a known mixture of air, N_2 and CO_2 (~14% O_2 in Santiago and 17.5% O_2 in Putre; 2–3% CO_2 in N_2) was passed at a rate of 20 l/min to reach an arterial P_{O_2} of ~30 mmHg. Arterial blood samples were taken during the experimental protocol to determine arterial pH, P_{CO_2}, P_{O_2}, hemoglobin concentration and percentage saturation of hemoglobin (SO_2) (IL-Synthesis 25, Instrumentation Laboratories measurements corrected to 39°C). PAP and systemic arterial pressure (SAP) were recorded continuously via a data-acquisition system (PowerLab/8SP System and LabChart v7.0 Software; ADInstruments) connected to a computer. Cardiac output (CO) was determined at set intervals with the thermodilution method by the injection of 3 ml of chilled (0°C) 0.9% NaCl into the pulmonary artery through the Swan-Ganz catheter connected to a cardiac output computer (COM-2 model, Baxter). Heart rate (HR), mean PAP (mPAP), mean SAP, pulmonary vascular resistance (PVR), and systemic vascular resistance (SVR) were calculated as described previously (14).

Ex vivo and in vitro experiments. Five uninstrumented lambs per group underwent euthanasia with an overdose of sodium thiopentone (100 mg/kg iv) for collection of their lungs.

WIRE MYOGRAPHY. The lungs were removed by dissection and immediately immersed in cold saline. Parenchymal pulmonary arteries (200–300 μm) were dissected, isolated, and mounted on a wire myograph and maintained at 37°C aerated with 95% O_2-5% CO_2 in Krebs buffer. The resting tension, defined as transmural pressure exerted in the vessels (at the pulmonary arterial circulation this pressure is ~17–25 mmHg, as seen on our in vivo experiments), was calculated by stretching the vessel in a stepwise manner to a standardized tension equivalent to the physiological transmural pressure (32). This was done to simulate conditions in vivo for two main reasons: first, because the stimulated vascular response is dependent on the degree of stretch; and secondly, because this degree of stretch gives the maximal vascular response (32, 44, 56).

Concentration-response curves (CRCs) were constructed for contraction induced by potassium chloride (KCl, 6.25 to 125 mM) and ET-1 (10^{-12} to 10^{-7} M) to assess contractile function. The ex vivo relaxant effect of SOC blockade with 2-APB and SKF-96365 was determined as follows: tension of pulmonary arteries was previously recorded in a calcium-free Krebs buffer (calcium was omitted and traces were removed with 2×10^{-4} M EGTA) under voltage-dependent calcium channel blockade with nifedipine (10^{-5} M). Next, ET-1 (10^{-7} M) was added to promote depletion of internal calcium stores and to evaluate contraction under these conditions, followed by external calcium restitution (2×10^{-3} M) to promote additional contraction. When maximal contraction was reached under these conditions, the relaxation promoted by increasing cumulative concentrations of 2-APB (10^{-7} to 10^{-4} M) or SKF-96365 (10^{-7} to 10^{-4} M) was recorded to have a CRC for SOC blockade. The response to every CRC dose was recorded 3 min after each condition. Contractile responses were expressed in terms of tension (N/m) or percentage relative to a submaximal dose of KCl (62.5 mM). Relaxation responses to 2-APB and SKF-96365 were expressed as a percentage of decrease of the tension reached after external calcium restitution. CRCs were fitted to a nonlinear equation (Prism 5.0, GraphPad Software, La Jolla, CA), and differences between groups were compared by calculating the area under the curve and the sensitivity as pD2, where $pD2 = -\log[EC_{50}]$, EC_{50} being the concentration at which 50% of the maximal response was achieved (14, 17).

RT-PCR. Total RNA purification from lung tissue, cDNA synthesis, and PCR amplification were performed as described previously (6, 17). Primers for amplification of partial DNA sequences from TRPC1 (forward 5′-ATGGGACAGATGTTACAAGATTTTGGG-3′ and reverse 5′-AGCAAACTTCCATTCTTTATCCTCATG-3′, accession number NM_053558), TRPC3 (forward 5′-TGACCTCTGTTGTGCTCAAATATG-3′ and reverse 5′-CCACTCTACATCACTGTAATCC-3′, accession number NM_021771), TRPC4 (forward 5′-TCTGCAGATATCTCTGGGAAGGATGC-3′ and reverse 5′-AAGCTTTGTTCGAGCAAATTTCCATTC-3′, accession number NM_080396), TRPC5 (forward 5′-AACTCCCTCTACCTGGCAACTA-3′ and reverse 5′-GGATATGAGACGCCACGAACTT-3′, accession number NM_080898), TRPC6 (forward 5′-AAAGATATCTTCAAATTCATGGTCATA-3′ and reverse 5′-ATCCGCATCATCCTCAATTTC-3′, accession number

NM_053559), ORAI1 (forward 5′-AGGTGATGAGCCTCAACGAG-3′ and reverse 5′-CTGATCATGAGCGCAAACAG-3′, accession number NM_001013982), and STIM1 (forward 5′-GGCCAGAGTCTCAGCCATAG-3′ and reverse 5′-CATAGGTCCTCCACGCTGAT-3′, accession number NM_001108496) were derived from the corresponding rat genes after alignment and identification of conserved sequences from rat, mouse, bovine, and human orthologs, whereas the 18S-rRNA (forward 5′-GTAACCCGTTGAACCCCATT-3′ and reverse 5′-CCATCCAATCGGTAGTAGCG-3′, the housekeeping gene, accession number DQ013885) was derived from the corresponding ovine sequence. All the PCR products were sequenced to verify their identity. The PCR products were visualized under UV light and the signals obtained on RT-PCR determinations were quantified by densitometric analysis using the Scion Image Software (Scion Image Beta 4.02 Win; Scion, Frederick, MD).

Statistical analysis. Data are expressed as means ± SE. Groups were compared by two-way ANOVA and the post hoc Newman-Keuls test or by Student's *t*-test for unpaired data, as appropriate. For all comparisons, differences were considered statistically significant when $P < 0.05$ (11).

RESULTS

In vivo cardiopulmonary function with and without 2-APB. Arterial blood gases and acid-base status. Basal P_{O_2}, SO_2, and P_{CO_2} were lower, whereas pH was higher in HA than LA lambs (Table 1). During acute hypoxia with either vehicle or 2-APB infusion, a fall to similar values of P_{O_2} and SO_2 occurred in both groups of lambs, without any changes of P_{CO_2} relative to basal period (Table 1). During recovery, all variables returned toward basal values in both groups (Table 1). Treatment with 2-APB had no significant effect on arterial blood gas and acid-base state either during basal and acute hypoxic conditions relative to controls (Table 1).

Cardiovascular and pulmonary functions. HA lambs showed higher mPAP than LA lambs throughout the experimental protocol. Furthermore, during acute superimposed hypoxia, mPAP increased in both LA and HA animals infused with vehicle (Fig. 1, *A* and *B*). SOC blockade with 2-APB did not have any effects during basal, but significantly attenuated the increase of mPAP induced by acute hypoxia in LA lambs (Fig. 1*A*). SOC blockade with 2-APB reduced mPAP under hypoxia and recovery in HA lambs (Fig. 1*B*). The net decrease of mPAP elicited by SOC blockade, expressed as the difference between mPAP under vehicle infusion and 2-APB infusion during acute hypoxia was greater in HA than in LA lambs (Fig. 1*C*). Consequently, PVR was higher in HA relative to LA newborns during all of the experimental

Table 1. *Arterial pH and blood gases in LA and HA lambs*

	Basal	Basal + Infusion	Hypoxemia	Recovery
pH				
LA				
Vehicle	7.432 ± 0.014	7.432 ± 0.009	7.419 ± 0.014	7.419 ± 0.013
2-APB	7.400 ± 0.009	7.411 ± 0.013	7.393 ± 0.028	7.412 ± 0.012
HA				
Vehicle	7.496 ± 0.016*	7.489 ± 0.020*	7.469 ± 0.014*	7.464 ± 0.017*
2-APB	7.484 ± 0.014*	7.489 ± 0.015*	7.477 ± 0.021*	7.475 ± 0.017*
P_{CO_2}, mmHg				
LA				
Vehicle	40.1 ± 1.1	40.6 ± 1.3	39.3 ± 1.5	39.3 ± 1.3
2-APB	41.1 ± 1.2	39.4 ± 1.5	38.8 ± 1.5	37.5 ± 1.1
HA				
Vehicle	29.2 ± 0.8*	28.8 ± 1.1*	29.1 ± 0.9*	28.6 ± 1.6*
2-APB	27.8 ± 0.8*	26.8 ± 0.8*	27.6 ± 0.7*	26.7 ± 0.9*
P_{O_2}, mmHg				
LA				
Vehicle	79.6 ± 1.8	81.9 ± 2.6	31.3 ± 0.3‡	81.9 ± 1.5
2-APB	79.5 ± 1.8	82.0 ± 2.1	30.4 ± 0.5‡	84.8 ± 3.8
HA				
Vehicle	42.7 ± 1.9*	43.0 ± 2.2*	29.3 ± 0.6‡	44.3 ± 1.6*
2-APB	44.0 ± 1.9*	47.2 ± 1.7*	29.6 ± 0.4‡	46.8 ± 1.3*
SO_2, %				
LA				
Vehicle	100.2 ± 1.0	100.9 ± 1.0	56.7 ± 2.0‡	100.4 ± 0.9
2-APB	94.3 ± 0.9	100.1 ± 0.7	52.9 ± 2.2‡	100.8 ± 1.0
HA				
Vehicle	74.4 ± 2.0*	75.2 ± 2.8*	49.2 ± 2.3‡	75.1 ± 2.3*
2-APB	74.6 ± 1.9*	78.4 ± 1.2*	49.6 ± 3.9‡	77.6 ± 2.5*
Hb, g/dl				
LA				
Vehicle	9.4 ± 0.3	8.9 ± 0.3	9.6 ± 0.2	9.2 ± 0.2
2-APB	9.9 ± 0.4	9.8 ± 0.5	10.2 ± 0.4	9.7 ± 0.5
HA				
Vehicle	11.0 ± 0.7	10.6 ± 0.6	12.5 ± 1.5* ‡	10.5 ± 0.5
2-APB	10.6 ± 0.5	10.5 ± 0.4	11.3 ± 0.5‡	10.7 ± 0.6

Values are the means ± SE for arterial pH (pH), partial pressure of carbon dioxide (P_{CO_2}), partial pressure of oxygen (P_{O_2}), saturation of hemoglobin with oxygen (SO_2) and hemoglobin concentration ([Hb]). LA, conception pregnancy and delivery at low altitude (580 m, $n = 5$); HA, conception pregnancy and delivery at high altitude (3,600 m, $n = 5$). Vehicle, dimethylsulfoxide: NaCl 0.9% (1:10); 2-APB, 2-aminoethyldiphenylborinate. Blood samples were taken and measured during preinfusion (Basal), during infusion with vehicle or 2-APB (Basal+Infusion), during acute hypoxia, and during recovery. Significant differences ($P < 0.05$): *LA vs. HA; ‡vs. all in the same group.

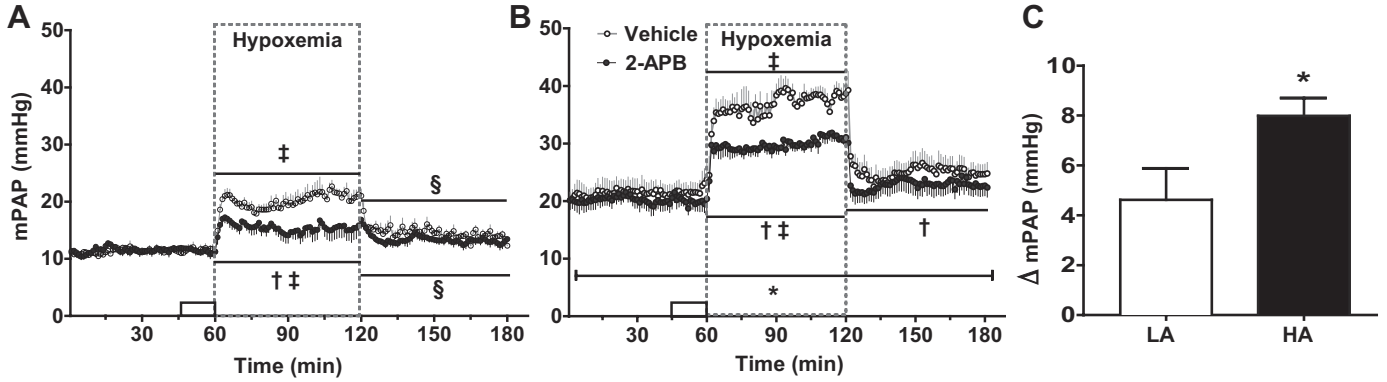

Fig. 1. Mean pulmonary arterial pressure (mPAP) during in vivo acute hypoxia protocol in LA (*A*) or HA (*B*) lambs; LA, conception pregnancy and delivery at low altitude (580 m, $n = 5$); HA, conception pregnancy and delivery at high altitude (3,600 m, $n = 5$). Acute hypoxia was induced following a vehicle infusion (○) or with the store-operated channel (SOC) blocker 2-aminoethyldiphenylborinate (2-APB; ●). The horizontal gray bar indicates the infusion period. Values are means ± SE. calculated every minute during the experimental protocol. The sensitivity of the acute hypoxic response to 2-APB, was calculated according to the following formula: $\Delta mPAP = mPAP_{vehicle}\text{-}mPAP_{2\text{-}APB}$, where $mPAP_{vehicle}$ and $mPAP_{2\text{-}APB}$ represents the mean pulmonary artery pressure (mPAP) during 1 h of hypoxia, in the presence of 2-APB or its vehicle, respectively (*C*). Significant differences ($P < 0.05$): *LA vs. HA; †vs. vehicle; ‡vs. all in the same group; §vs. Basal, Basal+Infusion.

protocol. LA and HA lambs showed an increased PVR during acute hypoxia and exhibited a partial attenuation of this response by 2-APB (Fig. 2, *C* and *D*).

CO was similar in LA and HA lambs under basal conditions. During acute hypoxia, CO increased and returned to basal values during recovery in both LA and HA lambs. The infusion of 2-APB did not have any effect on CO throughout the experimental protocol (Fig. 2, *A* and *B*).

Basal HR was similar in both vehicle-infused LA and HA groups; it increased during acute superimposed hypoxia but returned to basal values during recovery. Infusion of 2-APB did not modify HR in basal or acute hypoxic conditions, but it maintained an elevated HR during recovery in both groups (Table 2).

SAP remained stable during all of the experimental protocols, either with vehicle or 2-APB in LA and HA lambs (Table 2). In contrast, SVR was similar during baseline but diminished during acute hypoxia and returned to basal values during recovery in LA animals. Infusion of 2-APB did not induce any changes of SVR relative to the vehicle in this group (Table 2).

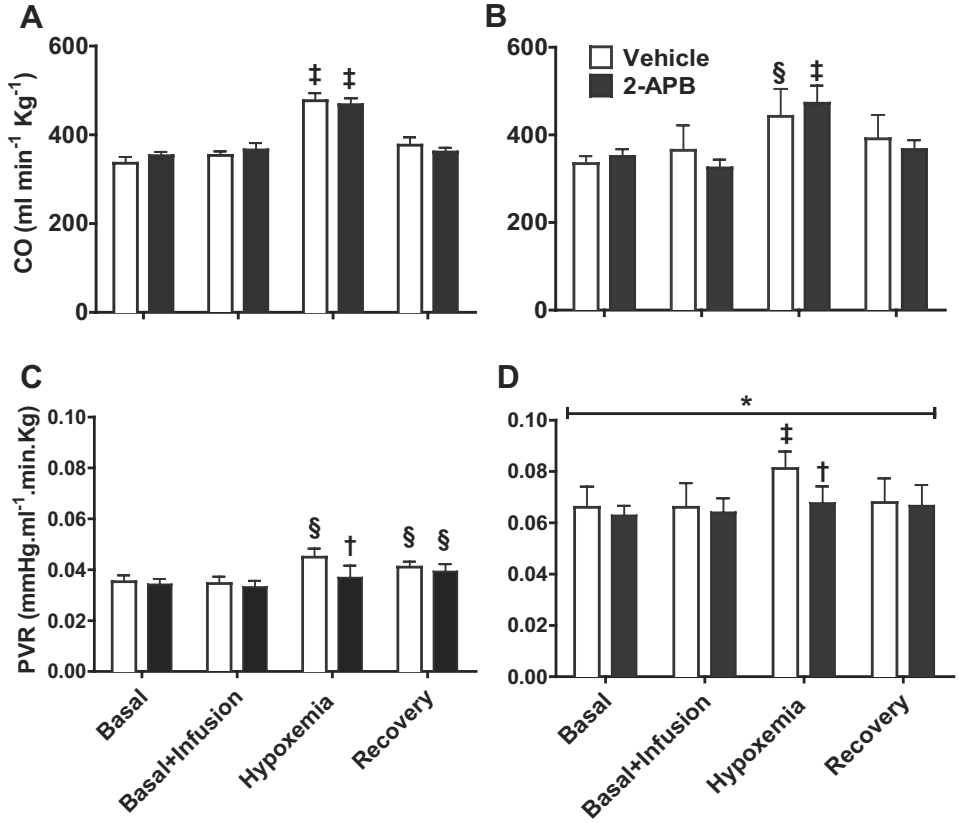

Fig. 2. Cardiac output (CO) and pulmonary vascular resistance (PVR) in LA (*A* and *C*) and HA (*B* and *D*) newborn sheep. Values are expressed as means ± SE. for the different experimental periods under vehicle (open bars) or 2-APB administration (solid bars). Significant differences ($P < 0.05$): *LA vs. HA; †vs. vehicle; ‡vs. all in the same group; §vs. Basal, Basal+Infusion.

Table 2. *Cardiovascular variables in LA and HA lambs*

	Basal	Basal + Infusion	Hypoxemia	Recovery
HR, bpm				
LA				
Vehicle	189.8 ± 12.0	190.8 ± 15.2	254.4 ± 42.3‡	218.7 ± 14.8
2-APB	180.0 ± 10.8	194.6 ± 18.5	293.3 ± 32.3‡	251.0 ± 46.2§
HA				
Vehicle	174.6 ± 15.4	167.8 ± 14.8	240.5 ± 35.8‡	202.3 ± 14.7
2-APB	186.5 ± 23.5	201.0 ± 24.4	271.0 ± 44.9‡	230.2 ± 23.8§
SAP, mmHg				
LA				
Vehical	79.1 ± 6.1	78.3 ± 5.6	79.6 ± 5.5	82.0 ± 7.3
2-APB	79.6 ± 6.0	78.6 ± 6.2	82.6 ± 7.8	78.8 ± 4.9
HA				
Vehicle	82.9 ± 6.7	84.1 ± 6.4	90.3 ± 7.1	86.0 ± 7.0
2-APB	85.6 ± 7.9	90.9 ± 14.5	96.1 ± 14.3	88.7 ± 9.6
SVR, mmHg. min/l				
LA				
Vehicle	0.24 ± 0.02	0.22 ± 0.02	0.17 ± 0.02‡	0.22 ± 0.03
2-APB	0.23 ± 0.02	0.22 ± 0.03	0.18 ± 0.02‡	0.22 ± 0.02
HA				
Vehicle	0.27 ± 0.03	0.27 ± 0.07	0.23 ± 0.05	0.25 ± 0.06
2-APB	0.28 ± 0.05	0.28 ± 0.05	0.21 ± 0.04§	0.25 ± 0.01

Values are the means ± SE for heart rate (HR), systemic arterial pressure (SAP), and systemic vascular resistance (SVR) The cardiovascular variables were recorded and calculated during preinfusion (Basal), during infusion with vehicle or 2-APB (Basal+Infusion), during acute hypoxia and during recovery. Significant differences ($P < 0.05$): ‡vs. all in the same group; §vs. Basal, Basal+Infusion.

Vehicle-infused HA lambs did not decrease their SVR under acute hypoxia, but 2-APB-infused HA animals did (Table 2).

Ex vivo function of pulmonary arteries. Isolated small pulmonary arteries reached maximal contraction to ET-1 at 10^{-7} M, reaching similar tensions in both groups of animals (HA: 5.5 ± 0.7 vs. LA: 5.1 ± 1.5 N/m). In the absence of external calcium and under voltage-dependent calcium channel blockade with nifedipine, ET-1 promoted contraction in both groups, but this contraction was greater in HA than LA newborn sheep (Fig. 3). Restitution of external calcium elicited additional contraction in both groups, and the tension reached was also greater in HA than LA animals (Fig. 3). When increasing concentrations of 2-APB were added after external calcium restitution, small pulmonary arteries from HA showed a greater relaxation and sensitivity than those from LA lambs (Fig. 4). To verify SOC specific blockade by 2-APB, we used SKF-96365 ($n = 5$ LA, $n = 3$ HA), another SOC blocker, that also relaxed pulmonary arteries with a greater sensitivity in HA lambs (pD2, LA 4.35 ± 0.35 vs. HA 5.26 ± 0.18, $P < 0.05$; Fig. 4 *inset*).

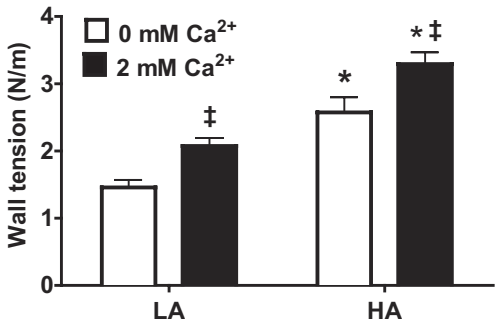

Fig. 3. Contractile response to external calcium restoration in isolated small pulmonary arteries. Pulmonary arteries were preincubated with nifedipine in the absence of Ca^{2+} and tension developed after the addition of 10^{-7} M endothelin-1 (ET-1) was recorded. Thereafter, external Ca^{2+} was restored and the additional tension developed was recorded. The contraction in the absence (0 mM Ca^{2+}) and the presence of external calcium (2 mM Ca^{2+}) is compared in LA (open bars) and HA (solid bars) newborn sheep. Values are expressed as means ± SE. Significant differences ($P < 0.05$): *LA vs. HA; ‡vs. zero external calcium in the same group.

Pulmonary expression of SOC-forming subunits. HA lamb showed a greater expression of TRPC4 and STIM1 transcripts (Fig. 5). In contrast the expression of TRPC1, TRPC3, TRPC5, TRPC6, and ORAI1 was similar in LA and HA newborns (Fig. 5).

DISCUSSION

This study supports the idea that SOC contribute to hypoxic pulmonary vasoconstriction in LA and HA newborn sheep. We showed that SOC blockade with 2-APB limits the increase of PAP and PVR during acute hypoxia in both groups of animals. We also showed an enhanced function of these channels in the neonatal pulmonary circulation at high altitude, since the reduction of the pulmonary pressure response to acute hypoxia evoked by SOC blockade was greater in HA lambs with pulmonary hypertension than LA control lambs. These effects occurred without basal systemic cardiovascular modification. This enhanced SOC function is consistent with an increased response to 2-APB and SKF-96365 in isolated pulmonary arteries and an augmented expression of the TRPC4 and STIM1 subunits of SOC channels in high altitude.

As expected, HA lambs had a lower basal P_{O_2} than LA lamb, and consequently, their basal PAP and PVR were higher. HA sheep hyperventilate relative to LA animals, as evidenced by their lower arterial P_{CO_2} and higher pH. This condition may partially compensate the marked pulmonary vasoconstriction observed in these lambs. All these observations are in agreement with our previously published work (14, 16).

Support for our in vivo results concerning the 2-APB inhibition of hypoxic pulmonary vasoconstriction comes from studies showing the abolition by 2-APB of calcium influx in cultured pulmonary artery myocytes stimulated with hypoxia

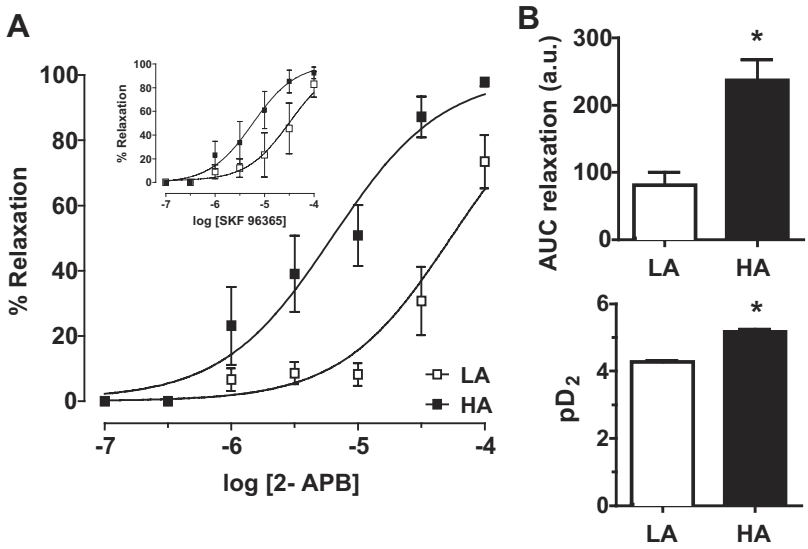

Fig. 4. Vasodilator response in isolated pulmonary arteries. Relaxation induced by increasing concentrations of 2-APB and SKF-96365 (*inset*) in small pulmonary arteries precontracted with ET-1 in LA (□) and HA (■) (*A*). The area under the curve (AUC), in arbitrary units (a.u.), and sensitivity (pD_2) were calculated (*B*). LA group (2-APB $n = 5$; SKF-96365 $n = 5$), HA group (2-APB $n = 5$; SKF-96365 $n = 3$). The arteries were preincubated with nifedipine and ET-1 in a calcium-free medium, and contraction was induced by restoration of external calcium. Then, 2-APB or SKF-96365 was added in cumulative doses. Values are expressed as means ± SE. Significant differences ($P < 0.05$): *LA vs. HA.

(48). Furthermore, SOC stimulated by internal calcium store depletion with thapsigargin, cyclopiazonic acid, or 5-hydroxytryptamine are also inhibited by 2-APB, in freshly dissociated pulmonary artery myocytes (30, 34).

The ex vivo results obtained in pulmonary artery myocytes are consistent and supportive of our observations given that the 2-APB concentration used to block SOC in the previously mentioned experiments is in the 10–100 μM range; this is also the case in experiments performed in HEK293 cell line transfected with genes coding for SOC-forming subunits like ORAI1/STIM1 (3, 37) or TRPC3, 6, and 7 (24). We estimate that this concentration is on the same order of magnitude reached in our in vivo experiments assuming a volume distribution of the drug equivalent of 40% of animal weight. In addition this is in the range of concentration of 2-APB used in our ex vivo experiments with isolated pulmonary arteries. Other potential actions of 2-APB different than SOC inhibition must be considered, such as inhibition of inositol-(1,4,5)-triphosphate-dependent calcium release (29), of TRPM7 channels [a type of Mg-inhibitable cation channel (2)], of gap junction intercellular communications (23), and of mitochondrial calcium efflux, as well as potentiation and inhibition of store-operated currents, depending on the concentration of the drug (39), are described in other experimental models. However, we had similar results with SKF-96365, another SOC blocker, in the relaxation of the isolated small pulmonary arteries, suggesting that the main 2-APB effect is to block these channels. The in vivo effect of 2-APB is selective for the pulmonary vascular bed, since it did not significantly modify SAP or SVR. These findings are supported by previous observations in isolated rat pulmonary and femoral arteries, where store depletion with thapsigargin induced a store-operated calcium entry in both vascular beds, but it induced contraction only in the pulmonary circulation (43).

In further agreement with our results, SKF-96365 was able to block calcium entry induced by acute hypoxia in rat pulmonary artery myocytes (51) and HPV and ligand-gated vasoconstriction in isolated lung (57).

The finding that 2-APB evokes a greater attenuation of HPV in HA than in LA sheep is consistent with an enhanced function of SOC as the result of perinatal chronic hypoxia. This increased function of SOC is also observed as an increased ability of SKF-96365 to reduce $[Ca^{2+}]_i$ and tension in pulmonary artery myocytes and vessel rings from chronically hypoxic rats (52).

An enhanced role of SOC is also seen in pulmonary artery myocytes from fetal sheep with increased shear stress and pulmonary hypertension induced by ductus arteriosus ligation (41), as well as in cultured pulmonary arteries from adult rats with pulmonary hypertension (26).

Our experiments in isolated pulmonary arteries show that in the absence of external calcium, ET-1 promotes a contraction in HA and LA vessels. The contraction increased in both groups after the restoration of external calcium, and the tension reached was greater in HA than in LA pulmonary arteries. The experiments were conducted in the presence of nifedipine to preclude any contribution of voltage-dependent calcium channels. Therefore, the observed effects must be mediated by a voltage-independent channel such as SOC (60). Thus the relaxations promoted by 2-APB and SKF-96365 support this point.

The greater contraction reached after external calcium restoration, as well as the greater relaxation and sensitivity observed for 2-APB and SKF-96365 in HA than LA newborn arteries, also suggests that SOC are upregulated by chronic hypoxia. These results are in agreement with our in vivo experiments as well as with results observed in adult rat pulmonary artery smooth muscle and rings (52).

Both in vivo and ex vivo data suggest an enhanced function of SOC in pulmonary vasculature from newborn sheep that have been submitted to in utero chronic hypoxia. This assumption could be explained by an increase of the expression of SOC-forming subunits such as TRPC1, TRPC3, TRPC4, TRPC5, TRPC6, and ORAI1 that contribute to the pore constitution, or by an increase of the expression of STIM1, which senses calcium depletion in internal stores and activates the pore-forming subunits (36, 47, 60). We found an upregulation of transcripts coding for STIM1 and TRPC4 in HA newborn sheep relative to LA lambs. These results are consistent with previous studies reporting that TRPC4 is a SOC component in

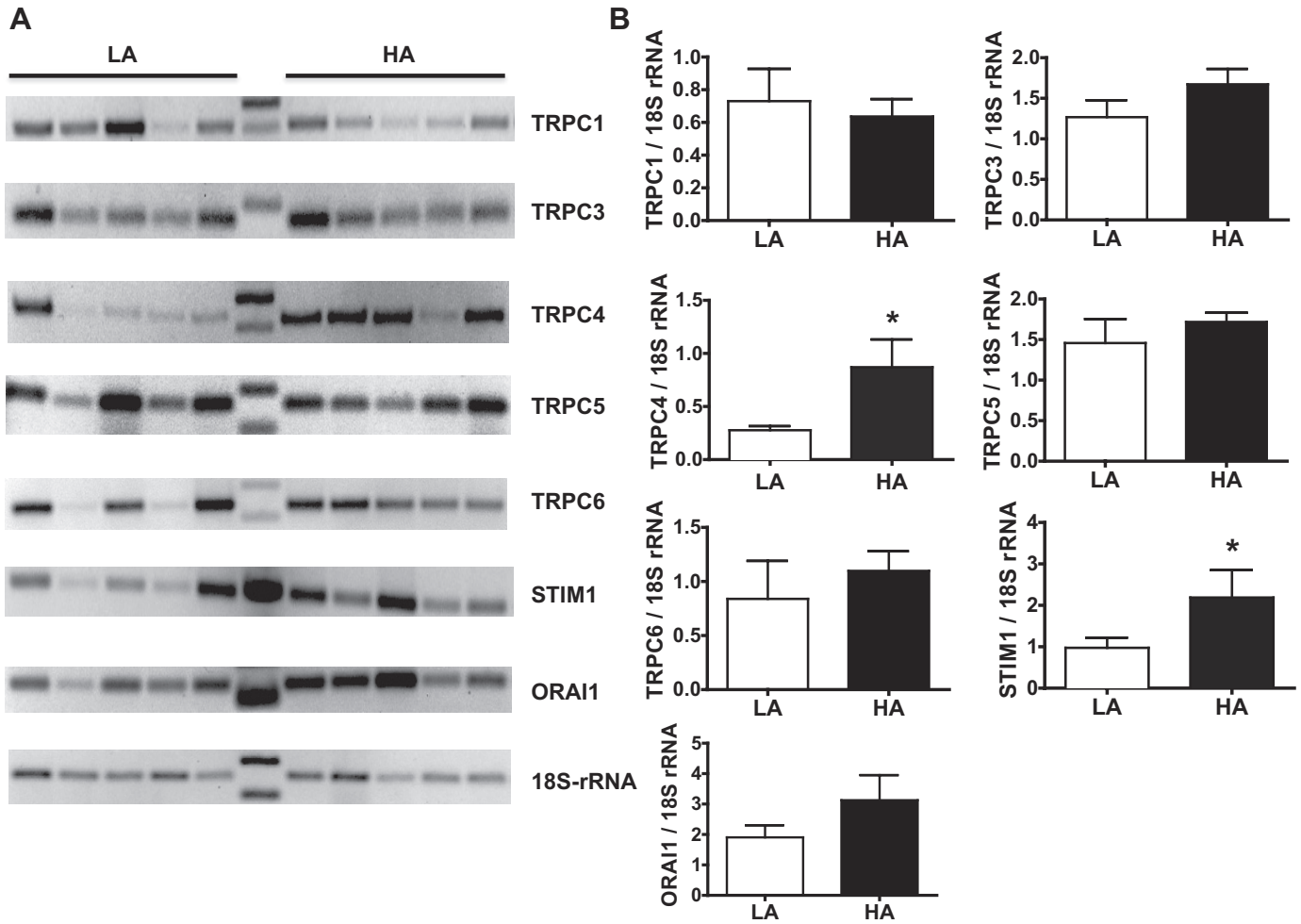

Fig. 5. Expression of SOC-forming subunits in lung from LA (open bars) and HA (closed bars) newborn sheep. Values are expressed as means ± SE. Significant differences ($P < 0.05$): *LA vs. HA.

pulmonary artery smooth muscle cells (60) and could be responsible for the robust acute hypoxic vasoconstrictor response of distal pulmonary arteries (27). Furthermore, TRPC4 upregulation is also observed in rats with monocrotaline-induced pulmonary hypertension (26). Other authors have observed that hypoxia evokes an increase in TRPC1 and TRPC6 expression in cultured pulmonary artery smooth muscle cells from adult rat and mouse (25, 52). These differences with our results could arise from species specific response (rodent vs. sheep), from the developmental stage (adult vs. newborn) of the model used and/or from the preparation (cultured smooth muscles cells vs. lung) used for expression studies.

Another feature that is present in pulmonary hypertension is pulmonary vascular remodeling, characterized by thickening of the pulmonary artery wall that in turn results from pulmonary artery smooth muscle cell proliferation among other processes (46). According to previous studies (16, 18), we observed the thickening of pulmonary artery medial layer in our hypertensive HA lambs. Nevertheless, further experiments are needed to establish whether there is a cause-effect relationship between enhanced SOC function and pulmonary arterial remodeling in the hypoxic newborn sheep.

Taken together, our results suggest that upregulation of Stim1 and TRPC4 may contribute to pulmonary hypertension by enhancing vasoconstrictor response to acute hypoxia. However, SOC contribution to increased vascular remodeling reported in these experimental models cannot be discarded and merits further studies.

In summary, we have demonstrated by in vivo, ex vivo, and in vitro studies that SOC are involved in the pulmonary vascular response to acute and chronic hypoxia of the newborn sheep.

Perspectives and significance. Our findings contribute to the knowledge of mechanisms involved in the neonatal pulmonary vascular function. Furthermore, these data are relevant not only for newborns from mothers who spend their pregnancy and subsequently deliver at high altitude, but also for lowland women whose babies are exposed to in utero hypoxia. This study adds knowledge to the currently known NO-dependent and independent treatments, such as PDE inhibitors, Ca^{2+} channel blockers, ET-1 blockers, and PGI_2 analogs (33, 38). So far, no treatment has shown 100% efficiency, and a number of these babies have persistent pulmonary hypertension and respiratory distress refractory to treatments with vasodilators and inhalatory nitric oxide (38, 49). Administration of SOC block-

ers may be a complementary treatment, with specific effects on the pulmonary vascular bed and absence of systemic vasodilation that merits to be investigated. As with any innovative treatment, this should be done after careful evaluation of risks, benefits, and alternatives, and under strictly controlled conditions.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

D.P., G.E., E.A.H., A.J.L., and R.V.R. conception and design of research; D.P., G.E., E.A.H., F.A.M., R.A.R., C.E.U., R.T.R., P.S., I.H., J.F., M.D., J.T.P., G.C., A.J.L., and R.V.R. performed experiments; D.P., G.E., E.A.H., F.A.M., R.A.R., C.E.U., R.T.R., P.S., I.H., J.F., M.D., J.T.P., A.J.L., and R.V.R. analyzed data; D.P., G.E., E.A.H., F.A.M., R.A.R., P.S., I.H., J.F., M.D., J.T.P., G.C., A.J.L., and R.V.R. interpreted results of experiments; D.P., G.E., E.A.H., C.E.U., R.T.R., I.H., M.D., and A.J.L. prepared figures; D.P., G.E., E.A.H., R.A.R., C.E.U., J.T.P., A.J.L., and R.V.R. drafted manuscript; D.P., G.E., E.A.H., F.A.M., R.A.R., J.T.P., G.C., A.J.L., and R.V.R. edited and revised manuscript; D.P., G.E., E.A.H., F.A.M., C.E.U., R.T.R., P.S., I.H., J.F., M.D., J.T.P., G.C., A.J.L., and R.V.R. approved final version of manuscript.

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