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Role of the RhoA/ROCK pathway in high-altitude associated neonatal pulmonary hypertension in lambs

Nandy C. Lopez,[1]* German Ebensperger,[1,2]* Emilio A. Herrera,[1,2] Roberto V. Reyes,[1,2] Gloria Calaf,[3] Gertrudis Cabello,[4] Fernando A. Moraga,[5] Felipe A. Beñaldo,[1] Marcela Diaz,[1,6] Julian T. Parer,[7] and Anibal J. Llanos[1,2]

[1]*Laboratorio de Fisiología y Fisiopatología del Desarrollo, Programa de Fisiopatología, ICBM, Facultad de Medicina, Universidad de Chile, Santiago, Chile;* [2]*International Center for Andean Studies (INCAS), Universidad de Chile, Santiago, Chile;* [3]*Instituto de Alta Investigación, Universidad de Tarapacá, Arica, Chile;* [4]*Departamento de Biología, Facultad de Ciencias, Universidad de Tarapacá, Arica, Chile;* [5]*Departamento de Ciencias Biomédicas, Facultad de Medicina, Universidad Católica del Norte, Coquimbo, Chile;* [6]*Departamento de Promoción de la Salud de la Mujer y el Recién Nacido, Facultad de Medicina, Universidad de Chile, Santiago, Chile; and* [7]*Department of Obstetrics, Gynecology and Reproductive Sciences, University of California San Francisco, San Francisco, California*

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Lopez NC, Ebensperger G, Herrera EA, Reyes RV, Calaf G, Cabello G, Moraga FA, Beñaldo FA, Diaz M, Parer JT, Llanos AJ. Role of the RhoA/ROCK pathway in high-altitude associated neonatal pulmonary hypertension in lambs. *Am J Physiol Regul Integr Comp Physiol* 310: R1053–R1063, 2016. First published February 24, 2016; doi:10.1152/ajpregu.00177.2015.—Exposure to high-altitude chronic hypoxia during pregnancy may cause pulmonary hypertension in neonates, as a result of vasoconstriction and vascular remodeling. We hypothesized that susceptibility to pulmonary hypertension, due to an augmented expression and activity of the RhoA/Rho-kinase (ROCK) pathway in these neonates, can be reduced by daily administration of fasudil, a ROCK inhibitor. We studied 10 highland newborn lambs with conception, gestation, and birth at 3,600 m in Putre, Chile. Five highland controls (HLC) were compared with 5 highland lambs treated with fasudil (HL-FAS; 3 mg·kg^{-1}·day^{-1} iv for 10 days). Ten lowland controls were studied in Lluta (50 m; LLC). During the 10 days of fasudil daily administration, the drug decreased pulmonary arterial pressure (PAP) and resistance (PVR), basally and during a superimposed episode of acute hypoxia. HL-FAS small pulmonary arteries showed diminished muscular area and a reduced contractile response to the thromboxane analog U46619 compared with HLC. Hypoxia, but not fasudil, changed the protein expression pattern of the RhoA/ROCKII pathway. Moreover, HL-FAS lungs expressed less pMYPT1^{T850} and pMYPT1T^{696} than HLC, with a potential increase of the myosin light chain phosphatase activity. Finally, hypoxia induced RhoA, ROCKII, and PKG mRNA expression in PASMCs of HLC, but fasudil reduced them (HL-FAS) similarly to LLC. We conclude that fasudil decreases the function of the RhoA/ROCK pathway, reducing the PAP and PVR in chronically hypoxic highland neonatal lambs. The inhibition of ROCKs by fasudil may offer a possible therapeutic tool for the pulmonary hypertension of the neonates.

Rho kinase; hypoxia; pulmonary hypertension; newborn; high altitude

EXPOSURE to chronic hypoxia causes pulmonary arterial hypertension (PAH), characterized by a progressive elevation of pulmonary artery pressure and resistance, associated with vasoconstriction and vascular remodeling (14, 43). Although PAH can develop at any stage during an individual's lifetime, neonates are especially vulnerable to this syndrome due to the marked changes that take place in the pulmonary circulation at this period of life. At birth, there is a rapid transition from a high resistance-low flow pulmonary circulation in the fetus to a very low resistance condition in the newborn capable of accommodating the total cardiac output (1, 2). Exposure to chronic hypoxia in utero, as seen in some complicated pregnancies at low altitude and in high altitude populations, may lead to persistent pulmonary hypertension of the newborn (37). This is a life-threatening syndrome with a prevalence from 0.43 to 6.82 per 1,000 live births in lowlands (52), and some authors suggested that at high altitude this prevalence is higher than in lowlands (37, 40). Despite the available therapeutic strategies, a high percentage of patients are refractory to these treatments and the mortality rate ranges from 4 to 33%, accompanied by significant morbidity (45, 52).

The mechanisms of chronic hypoxia inducing PAH are complex and not completely understood, but the involvement of the RhoA/Rho-kinase (ROCK) pathway has been demonstrated in the rat and mouse, contributing to vascular remodeling and vasoconstriction through Ca^{2+} sensitization (38, 54). Thus, ROCK-specific pharmacological inhibitors, fasudil and Y-27632, have been shown to inhibit adult and fetal hypoxic pulmonary artery myogenic responses (9, 51), reversing hypoxic pulmonary vasoconstriction (12). Furthermore, treatment with ROCK inhibitors suppresses the development of hypoxic PAH in adult rodents (12, 35).

ROCKs are cytoplasmic serine/threonine kinases translocated to the membrane after activation of the small GTPase RhoA (26). The binding of active RhoA to ROCK RBD (RhoA binding domain) stimulates the phosphotransferase activity of ROCKs (4, 30). Once activated in the smooth muscle, ROCK acts on the regulatory subunit of myosin light chain phosphatase MYPT1 and phosphorylates MYPT1's threonine 850 and 696. This results in a decreased myosin light chain phosphatase (MLCP) activity and increased levels of phosphorylated myosin light chain (MLC) at a constant cytosolic Ca^{2+} concentration, a mechanism known as Ca^{2+} sensitization (48, 50). There are two ROCK isoforms, ROCKI and ROCKII, and both are expressed in vascular smooth muscle cells (VSMC) and phosphorylate MLCP (36). Nevertheless, these proteins seem to

* N. C. Lopez and G. Ebensperger contributed equally to this work.

Address for reprint requests and other correspondence: A. J. Llanos, Laboratorio de Fisiología y Fisiopatología del Desarrollo, Programa de Fisiopatología, ICBM, Facultad de Medicina, Universidad de Chile, Avda. Salvador 486, Providencia, CP 7500922, Santiago, Chile (e-mail: allanos@med.uchile.cl).

have isoform-specific roles, since ROCKII but not ROCKI binds directly to MYPT1 and predominantly regulates VSMC contractility. In addition, both proteins have opposing effects on VSMC morphology through the control of actin cytoskeletal organization (53). However, their role in the pathogenesis of PAH induced by chronic hypoxia remains largely unknown.

While vasoconstriction is an important factor in vascular lesion formation in pulmonary arterial hypertension (PAH), vascular remodeling is the main cause of the persistent high vascular resistance in this disease. It has been demonstrated in chronically hypoxic mice (12) and rats (24, 39) that sustained ROCK inhibition prevents and even reverses this process (57). More recently, the role of ROCKII was demonstrated in the pathogenesis of PAH through enhanced VSMC migration and proliferation in mouse (47).

Despite the large amount of accumulated data concerning the important role of ROCK in the development and maintenance of hypoxia-induced PAH in adult animal models (12, 48), there are few studies that focus on the role of the RHOA/ROCK pathway during the neonatal stage (61). Studies in neonatal rats with PHT secondary to chronic hypoxia have shown that the RhoA/ROCK pathway is activated in pulmonary arteries, and that a single bolus of either Y-27632 or fasudil completely normalizes pulmonary vascular resistance (31). Further, a daily intraperitoneal injection of ROCK inhibitors in these neonatal animals decreases ROCK activity, pulmonary vascular resistance, right ventricular hypertrophy, and arterial medial wall thickening (61).

Recent reports show that ROCK modulates tone in the perinatal pulmonary circulation through effects on both pulmonary artery endothelial cells (PAEC) and PASMC. In late-gestation ovine fetuses, ROCK inhibition increases NO production in PAECs, enhances barrier function, and produces pulmonary vasodilatation, affecting smooth muscle cells in a NO-independent manner (3). Further, the expression of ROCK II and activity of ROCKs were increased in chronically hypoxic arteries (18) and veins from near-term sheep fetuses (17). In contrast, Blood et al. (7) reported that an acute single intravenous bolus of fasudil had no effect on PAP or PVR responses to acute hypoxia in highland newborn lambs studied at sea level. Thus there are few and contradictory studies about the role of ROCKs in the perinatal pulmonary circulation.

In the present study, we hypothesized that daily administration of fasudil, the first and only clinically available ROCK inhibitor that exerts its action via targeting the ROCK ATP-dependent kinase domain, will reduce vasoconstriction and vascular remodeling in the neonatal pulmonary arterial hypertension in the *Alto Andino*.

To test this hypothesis, we used an integrative approach at the whole animal, isolated organ, and molecular level to determine the effects of the ROCK inhibitor, fasudil, in high-altitude pulmonary hypertensive newborn lambs, studied at 3,600 m. We determined *1*) in vivo pulmonary and systemic arterial blood pressure, basally and under a superimposed episode of acute hypoxia; *2*) ex vivo vasoconstrictor function of isolated small pulmonary arteries; *3*) morphology of small pulmonary arteries; and *4*) in vitro expression of RhoA/ROCK related proteins in the neonatal lungs and smooth muscle cell cultures in basal and hypoxic conditions.

MATERIALS AND METHODS

Animals. All experimental protocols were reviewed and approved by the Faculty of Medicine Bioethics Committee of the University of Chile (CBA no. 0315 and CBA no. 0561 FMUCH). Animal care, maintenance, procedures, and experimentation were performed in accordance 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 adheres to American Physiological Society's "Guiding Principles in the Care and Use of Animals."

Ten highland newborn lambs, whose ancestors have lived for many generations at high altitude, were conceived, gestated, and born at the Putre Research Station (3,600 m altitude), International Center for Andean Studies (INCAS), University of Chile. Lambs were randomly divided in two groups: 5 control lambs treated with vehicle (0.9% NaCl; HLC, weight 4.94 ± 0.55 kg), and 5 lambs treated with fasudil (Fasudil, LC Laboratories) for 10 days (HL-FAS, 3 $mg \cdot kg^{-1} \cdot day^{-1}$ iv during 15 min, every morning; weight 7.20 ± 1.13 kg). Additionally, two groups of 5 lowland newborn sheep (LLC, weight of 8.54 ± 0.40 kg: $P < 0.05$ vs. HLC) conceived, gestated, and born at Lluta (50 m altitude) were used as lowland control groups for myography, histology, and molecular biology studies in the first group of 5 and cardiovascular lowland data in the second group of 5. The neonatal body weights correspond to 15 days of age at euthanasia.

In vivo experiments. All in vivo experimental procedures were performed at Putre (HLC and HL-FAS groups) or Lluta (LLC) Research Stations. Lambs were instrumented under general anesthesia with ketamine, 10 mg/kg im (Ketostop; Drag Pharma-Invectec, Santiago, Chile), diazepam 0.1–0.5 mg/kg im (Laboratorio Biosano, Santiago, Chile) and atropine (0.04 mg/kg im; Atropina Sulfato; Laboratorio Chile, Santiago, Chile), with additional local infiltration of 2% lidocaine (Dimecaina; Laboratorio Beta, Santiago, Chile). Polyvinyl catheters (1.2 mm internal diameter) were placed into the descending aorta and inferior vena cava, and a Swan-Ganz catheter (Edwards Swan-Ganz 5 French, Baxter Healthcare) was placed in the pulmonary artery. All catheters were exteriorized and kept in a pouch sewn onto the skin. Antibiotic (Oxitetraciclin 20 mg/kg sc; Liquamicina LA, Pfizer, Chile) and analgesic (Sodium metamizole; Metamizol sodico, Laboratorio Chile, Chile) agents were given immediately postoperatively for 3 days.

The treatment (vehicle or fasudil) commenced the day after surgery, every morning and daily for 10 days. Before treatment injection, we measured pulmonary and systemic arterial pressures and heart rate via a data acquisition system (Powerlab/8SP System and Chart v4.1.2 Software; ADInstruments, New South Wales, Australia) connected to a computer. Mean pulmonary (mPAP), and systemic (mSAP) arterial blood pressures and heart rate (HR) were obtained from this record. Additionally, we determined the cardiac output (CO) by the thermodilution method by the injection of 3 ml of chilled (0°C) 0.9% NaCl into the pulmonary artery via the Swan-Ganz catheter connected to a cardiac output computer (COM-2 model; Baxter, Irvine, CA). We also calculated pulmonary and systemic vascular resistances as described previously (23). Arterial blood samples were taken daily to determine arterial pH, P_{O_2}, P_{CO_2}, hemoglobin concentration ([Hb]), and percentage of hemoglobin oxygen saturation ($\%SaO_2$) (IL-Synthesis 25, Instrumentation Laboratories, Lexington, MA); measurements were corrected to 39°C (22).

After 10 days of treatment and daily measurements, the newborn lambs were subjected to a superimposed episode of hypoxia. Experiments were based on a 3 h protocol: 60 min of basal (breathing room air), 60 min of hypoxia (P_{O_2}: 32 ± 1 mmHg), and 60 min of recovery. Hypoxia was induced by a transparent loosely tied polyethylene bag, placed over the animal's head; we introduced a controlled mixture of air, N_2, and CO_2 (~10% O_2 and 2–3% CO_2 in N_2) passed at ~20 l/min. Arterial blood samples were taken during the experimental protocol to determine arterial pH, P_{CO_2}, P_{O_2}, [Hb], and $\%SaO_2$.

Pulmonary (PAP) and systemic (SAP) arterial pressure, cardiac output (CO) and heart rate (HR) were determined in vivo during the protocol. Further, pulmonary (PVR) and systemic (SVR) vascular resistances were calculated. Measurements and calculations were performed as described previously (22).

At 15 days of age, the HLC, HL-FAS, and LLC lambs were euthanized with an overdose of sodium thiopentone 100 mg/kg slow iv (Tiopental; Laboratorio Biosano, Santiago, Chile). Lungs were removed by dissection and immediately immersed in cold saline, for further myography, cell culture, molecular biology and histology studies.

Wire myography. Myographic procedures were performed at Putre (HLC and HL-FAS lambs) and Lluta (LLC lambs) Research Stations. Fourth- to sixth-generation pulmonary arteries (counting from the pulmonary artery trunk) were dissected from the caudal lobule of the right lung. Isolated arteries were mounted in a wire myograph, maintained at 37°C, and aerated with 95% O_2-5% CO_2 (22). Optimal diameter was obtained stretching the vessel in a stepwise manner to a standardized tension equivalent to a physiological transmural pressure of 25 mmHg (23). Concentration-response curves (CRCs) were constructed for endothelin-1 and U46619 (thromboxane mimetic). Concentration-response curves (CRCs) were analyzed in terms of sensitivity and maximal responses by fitting experimental data to a sigmoidal equation (Prism 5.0, GraphPad Software, La Jolla, CA). Contractile responses were determined in terms of tension (N/m) and expressed as percentage of maximal response to KCl (%K_{max}, 125 mM). Sensitivity was calculated as pD_2, where $pD_2 = -\log[EC_{50}]$, EC_{50} being the concentration at which 50% of the maximal response was obtained (21, 32).

Histology. Isolated left lungs were perfused (~25 mmHg) with 4% paraformaldehyde for 24 h at 4°C and embedded in paraffin. Thereafter, van Gieson staining was performed in 10-μm slides. At least 8 arteries (100–300 μm diameter) per lung sample were chosen, and an average of 4 measurements from each artery was recorded. Images of parenchymal arterioles were acquired utilizing a workstation (Olympus trinocular microscope-BX51 plus digital camera QimaginGO3) linked to ImagePro software 6.3 and the vascular areas were calculated using the same software. The percentage of wall thickness was calculated as: wall thickness (%) = [(external area − internal area)/external area] × 100, where external and internal area are the areas bounded by external and internal elastic laminae, respectively. The area of vascular smooth muscle was calculated as: muscle area (%) = [(external muscle area − internal area)/external muscle area] × 100, where the external muscle area and the internal area are the external and internal boundaries of the tunica media, respectively (21).

Western blot. Pulmonary tissue lysates were resolved by electrophoresis in SDS-polyacrylamide gels and electrotransferred to a nitrocellulose membrane, performed at sea level (Universidad de Tarapacá, Arica). Nonspecific binding of antibody was blocked by washing with Tris-buffered saline containing 5% skim-milk for 1 h, and then membranes were incubated with one of the following primary antibodies: anti-β-actin (cat. no. A 5316, monoclonal, dilution 1:20,000; Sigma); anti-ROCK-II (cat. no. 610624, monoclonal, dilution 1:500; BD Biosciences); anti-RhoA (cat. no. SC179, polyclonal, dilution 1:2,000; Santa Cruz); anti-phospho-MYPT1 at Thr850 (cat. no. 36-003, polyclonal; dilution 1:500; Upstate Biotechnology) and anti-phospho-MYPT1 at Thr696 (cat. no. 07–251, polyclonal, dilution 1:500; Upstate Biotechnology). Signals were developed by incubation with horseradish peroxidase (HRP)-coupled anti-mouse IgG (cat. no. SC2031; Santa Cruz), anti-goat IgG (cat. no. SC2020; Santa Cruz) or anti-rabbit IgG (cat. no. SC2301; Santa Cruz) secondary antibodies and detected by chemiluminescence (SuperSignal West Pico Luminol/Enhancer Solution, Pierce). The relative intensities of immunoreactive bands were quantified by densitometry, and beta actin was used as normalizing protein (Scion Image Beta 4.02 Win; Scion, MD).

PASMC culture. Cultured smooth muscle cells from pulmonary arteries of HL-FAS, HLC, and LLC newborn lambs were prepared using an explant method at sea level (Universidad de Tarapacá, Arica). A section of the pulmonary artery trunk was excised and cleaned and the endothelium was removed by a scalpel under sterile conditions. Vessels were cut into small pieces and placed in growth medium (M199 medium, Hyclone) with 10% FBS (Hyclone), 2 mM glutamine, and 100 units/ml penicillin-streptomycin (Invitrogen) in gelatin-coated culture plates. Cells were cultured in a humidified atmosphere with 5% CO_2-95% air at 37°C. After 7–10 days, cells migrated from the explants, and explant fragments were removed after ~10 days of culture. A monoclonal antibody against smooth muscle α-actin was used to assess the purity of the smooth muscle cells culture (cat. no. A 5228, Sigma).

Hypoxic treatment. Cells (passage 2 or 3) were exposed (0, 15, 30, 60, 90, and 120 min, 37°C) to a gas mixture (5% CO_2-balanced N_2) to obtain 1% O_2 (medium $Po_2 \approx 7.13$ mmHg) in an automated PROOX 110-sealed hypoxia chamber (BioSpherix). Following hypoxia treatment, cells were homogenized in TRIzol (Invitrogen Life Technologies) and stored at −80°C for total RNA extraction.

Real time PCR. Cell RNA quality and integrity was assured by gel visualization and spectrophotometric analysis (OD260/280) and quantitated at 260 nm. Aliquots (2 μg) of total RNA were reversed transcribed into cDNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, CA). Procedures were carried out following the manufacturers' instructions.

Real time PCR experiments for ROCKI, ROCKII, RhoA, PKG and 18S-rRNA were performed using a C1000 thermal cycler and a CFX96 Real-Time PCR Detection System (Bio-Rad). Reactions in 20 μl volume included 0.2 μmol/l primers, and dNTPs, Taq DNA polymerase and reaction buffer provided in the Brilliant II SYBR Green QPCR Master Mix (Agilent Technologies). HotStart Taq DNA polymerase was activated (10 min, 95°C), and a 2-step protocol was performed, with denaturation (15 s at 95°C), and annealing/extension steps (45 s at 61 or 63.6°C), after which, fluorescent data were collected. Relative mRNA levels were calculated based on the Ct values and normalized to 18S-rRNA. Product specificity was confirmed by melting curve analysis and agarose gel electrophoresis (2% vol/vol) followed by DNA sequencing. The product Tm values were 78.5°C for ROCKI, 78°C for ROCKII, 82.5°C for RhoA, 87.5°C for PKG and 81.5°C for 18S-rRNA.

Specific primers used for sequence detection were ROCKI (forward 5′-CCACCAGGAAGGTGTACGCTATGA-3′, reverse 5′-AATCGGGCCCATTTTTCA GGCACG-3′), ROCKII (forward 5′-GCCCGGTTAAGGAAAACACAGGCA-3′, reverse 5′- TCCATGGGTTCGGTCCCTCCT-3′), RhoA (forward 5′-ACGACGAGCACACAAGGCGG-3′, reverse 5′- ACGTCTGGCTTGCAGAGCAGC-3′) all designed using Primer3 software. PKG and 18S primers have been previously described (16, 33). Expected size products were ROCKI 241 bp, ROCKII 177 bp, RhoA 182 bp, PKG 253 bp, and 18S-rRNA 152 bp.

Statistical analysis. Data were expressed as means ± SE. For in vivo studies, groups were compared by two-way ANOVA and the post hoc Newman-Keuls test. For ex vivo studies and Western blot analysis we performed one-way ANOVA followed by Tukey's Multiple Comparison test. Dunnett's test was used for real-time PCR analysis. For all comparisons, differences were considered statistically significant when $P < 0.05$.

RESULTS

In vivo experiments. Mean PAP (mPAP) and PVR were significantly higher in the HLC than in the LLC group, as has been previously described (21, 22). In contrast, high-altitude newborn lambs treated with fasudil had a significantly lower mPAP during the whole extension of the treatment compared with highland control (HLC) neonates (Fig. 1*A*), although the

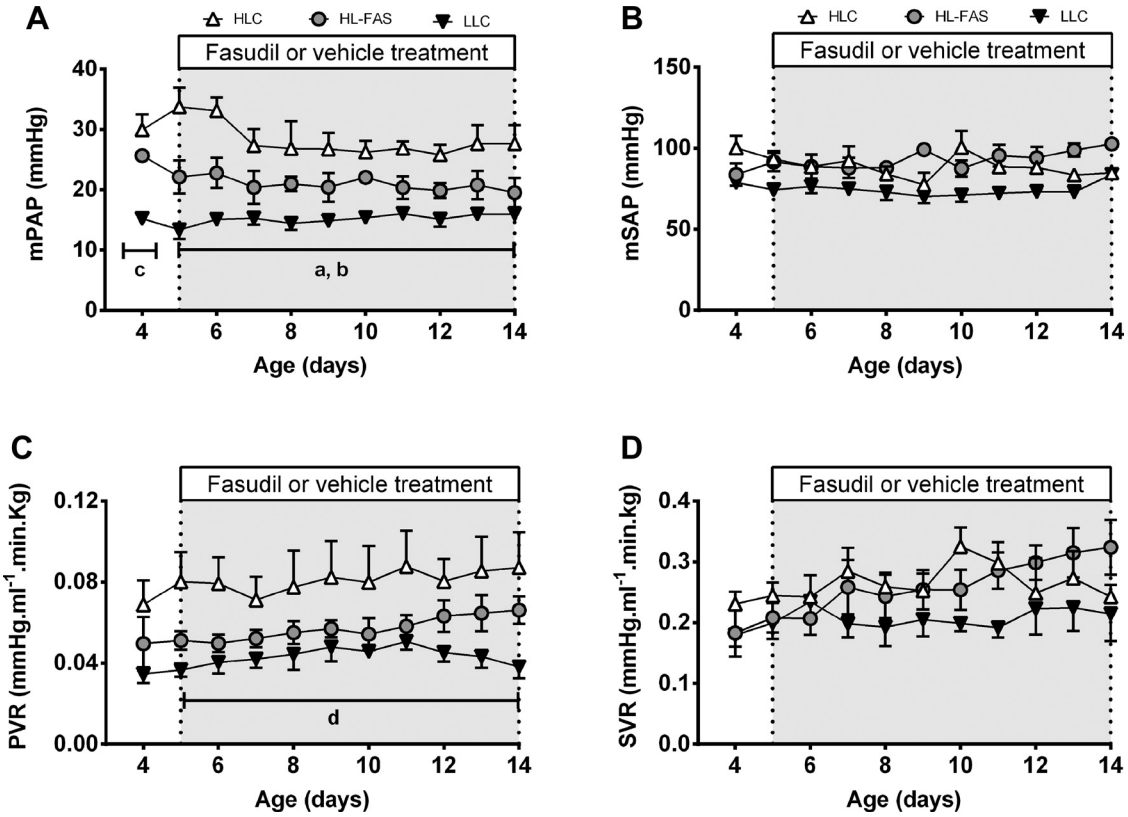

Fig. 1. Hypoxia and fasudil effects on the cardiopulmonary and systemic circulation. Mean pulmonary arterial pressure (mPAP; *A*), mean systemic arterial pressure (mSAP; *B*), pulmonary vascular resistance (PVR; *C*), and systemic vascular resistance (SVR; *D*) in highland control newborn lambs (HLC, white triangles, $n = 5$), highland newborn lambs treated with fasudil (HL-FAS, gray circles, $n = 5$), and lowland control newborn lambs (LLC, black triangles, $n = 5$). Values are means ± SE. Significant differences ($P < 0.05$): [a]HL-FAS vs. all; [b]HLC vs. LLC; [c]LLC vs. all; [d]HLC vs. all.

difference from the HLC group was more prominent in the first 2 days. The decrease in mPAP in HL-FAS animals is consistent with the reduction in PVR in the same period, reaching similar values observed in LLC group (Fig. 1*C*). In contrast, the 3 experimental groups showed similar cardiac output (CO) (Fig. 2*A*). Vehicle administration did not elicit any systemic cardiovascular responses during the entire protocol. Importantly, mSAP, SVR and heart rate (HR) were similar between groups (Figs. 1, *B* and *D*, and 2*B*).

During acute superimposed hypoxia, fasudil-treated animals at high altitudes had a decreased mPAP and PVR compared with the HLC group (Fig. 3, *A* and *C*), this response was similar to the LLC group. Furthermore, there was no difference in the PVR between basal, hypoxia, and recovery periods in fasudil-treated lambs. In contrast, HLC and LLC groups responded with an increased PVR during hypoxia. Additionally, cardiac output increased in all three groups during hypoxia (Fig. 3*B*). No changes in mSAP were seen during basal, hypoxia and recovery periods in the 3 experimental groups (data not shown).

Arterial blood gases were similar to those reported in previous studies. The Pa_{O_2}, Pa_{CO_2}, %Sat Hb and O_2 content were lower in HLC animals, relative to LLC, during the basal period (22, 23). Further, fasudil had no effects on arterial blood gases and acid base status with the exception of Pa_{CO_2} which was higher than in HLC group. Hypoxia decreased Pa_{O_2}, %Sat Hb and O_2 content in all highland and lowland animal groups to similar levels. During the recovery all the affected parameters returned to basal levels (Table 1).

Ex vivo experiments. We evaluated the effect of fasudil treatment on contractile responses of small pulmonary arteries of highland lambs, compared with control highland and lowland neonatal lambs. We did not find any differences between HL-FAS and HLC groups in maximal contraction to ET-1 or sensitivity (Fig. 4*A*). However, both highland groups had a greater maximal contraction to ET-1, relative to LLC, with similar sensitivity between the 3 groups of lambs (pD_2: 8.264 + 0.197 HLC, 8.136 + 0.136 HL-FAS and 7.976 + 0.167 LLC; Fig. 4*A*). In contrast, the highland lambs treated with fasudil showed a diminished maximal contraction to the thromboxane mimetic U46619 relative to highland control lambs (Fig. 4*B*). Additionally, lowland control lambs (LLC) showed also a smaller maximal contraction to U46619 relative to HLC newborns. The sensitivity to U46619 was similar in the 3 groups (pD_2: 6.978 + 0.106 HLC, 6.602 + 0.169 HL-FAS and 7.218 + 0.287 LLC; Fig. 4*B*).

Histology. As reported previously, HLC had a thicker wall and muscle layer relative to LLC (Fig. 5) (20, 23). Ten days of treatment with fasudil reduced the marked pulmonary vascular remodeling found in the chronically hypoxic and pulmonary hypertensive neonatal lamb. Thus, fasudil induced a substantial reduction in the small pulmonary artery wall thickness (Fig. 5*A*), and its vascular smooth muscle (Fig. 5*B*).

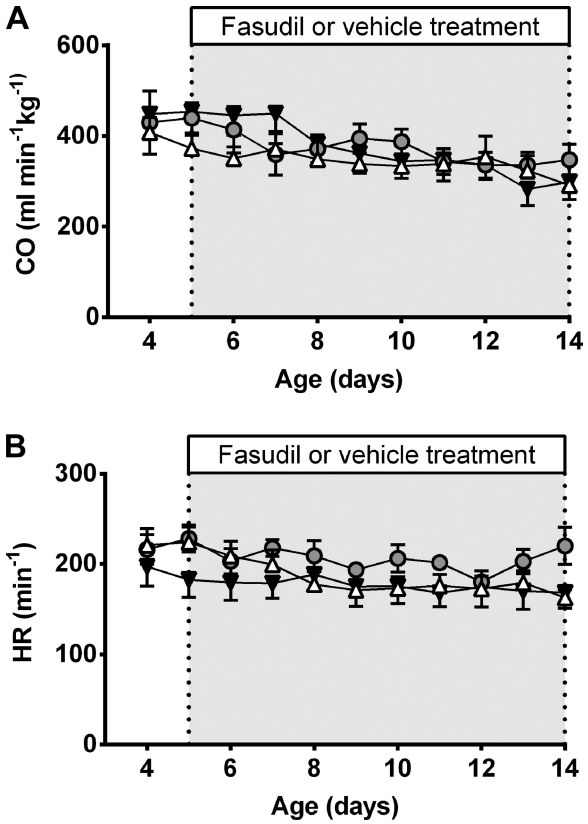

Fig. 2. Hypoxia and fasudil effects on cardiac output and heart rate. Cardiac output (CO; *A*) and heart rate (HR; *B*) in highland control newborn lambs (HLC, white triangles, $n = 5$), highland newborn lambs treated with fasudil (HL-FAS, gray circles, $n = 5$), and lowland control newborn lambs (LLC, black triangles, $n = 5$). Values are means ± SE.

Western blot. The pulmonary protein expression of RhoA from neonates exposed to chronic hypoxia in utero increased compared with LLC group (Fig. 6*A*). In contrast, high-altitude gestation inhibits ROCKII protein expression relative to LLC group (Fig. 6*B*). Treatment with fasudil did not affect RhoA or ROCKII protein levels in both groups of chronically hypoxic newborn lambs (Fig. 6, *A* and *B*).

The protein levels of MYPT1 phosphorylated at Thr850 and Thr696 (the regulatory subunits of MLCP) were not affected by chronic hypoxia during gestation. In contrast, fasudil treatment markedly decreased pMYPT1^{T850} and pMYPT1^{T696} protein levels (Fig. 6, *C* and *D*).

Real time PCR. Two hours of hypoxia induced RhoA, ROCKII, and PKG mRNA in HLC pulmonary artery smooth muscle cells (PASMCs) compared with LLC PASMCs (Fig. 7). Fasudil-treated cells prevented these responses, showed by a similar mRNA expression of RhoA, ROCKII, and PKG in HL-FAS and LLC PASMCs. In the case of RhoA and PKG (Fig. 7, *A* and *C*), we observed a peak at 60 min of hypoxia, after which mRNA expression decreased to basal levels. In contrast, ROCKII mRNA showed higher expression from 60 min of hypoxia until the end of the experiment (Fig. 7*B*).

DISCUSSION

In this study, we showed that 10 days of fasudil treatment in pulmonary hypertensive newborn sheep conceived, gestated, and born at high altitude, inhibited ROCK, decreased mPAP and PVR, and reduced vascular remodeling and contractile response, diminishing pMYPT1T^{850} and pMYPT1T^{696} expression in the lung.

Our results support the hypothesis that the RhoA/ROCK pathway may play an important role in causing pulmonary vasoconstriction and vascular remodeling in chronically hypoxic, pulmonary hypertensive neonatal lambs. Although there are studies that demonstrate the involvement of the RhoA/ROCK pathway in the development of pulmonary arterial hypertension induced by hypoxia in different species, there is no information about its role in chronically hypoxic neonates studied in high altitude to the best of our knowledge. The results of the present study support the potential use of pharmacological inhibitors of the RhoA/ROCK pathway as possible alternative treatments in newborns undergoing chronic hypoxia during gestation, both in highlands and potentially in lowlands, although further studies are needed at lowlands to clarify this issue.

The beneficial clinical effects of acute and more prolonged fasudil treatment on adult PH patients were demonstrated over the past decade (13, 15, 25, 56), improving the mPAP and cardiac index, and acting selectively on the pulmonary circulation. These effects have been also observed in pediatric patients (27).

The PAP attenuation induced by fasudil is the result of a lower PVR, since no changes in CO were observed. Fasudil prevented the marked elevation of PAP as observed in the highland controls (HLC) and the rise in PAP during hypoxemia was similar to that of lowland controls (LLC). Fasudil completely blunted the increase in PVR of these animals when submitted to a superimposed episode of acute hypoxia. These findings correlate with the decreased contractile response of the pulmonary arteries to the thromboxane mimetic U46619. They are also consistent with the histologic results, which demonstrated that HL-FAS neonates have lower pulmonary vascular remodeling, measured by wall thickness or smooth muscle area. We observed the decrease in mPAP and PVR in HL-FAS neonatal lambs beginning with the very first days of fasudil treatment, indicating that ROCKs inhibition initially produced vasodilatation followed by a vascular remodeling prevention. Lung RhoA/ROCK protein expression pattern was changed by hypoxia, and fasudil treatment inhibited the protein levels of MYPT1 phosphorylated at Thr850 and Thr696, the targets of ROCK to inactivate myosin light chain phosphatase (MLCP) activity, consistent with the reduced pulmonary arterial resistance and pressure, and the diminished small pulmonary artery remodeling.

Previously it has been reported that fasudil reduces PAP in pulmonary hypertensive neonatal rats. McNamara et al. (31) reported that ROCK inhibitors, delivered as a single intraperitoneal bolus, normalized PVR in a neonatal rat model of hypoxia-induced PHT. In this animal model, Ziino et al. (61) demonstrated that chronic treatment with ROCK inhibitors significantly attenuate PVR and arterial medial wall thickening in hypoxia-exposed pups. In the present study, 3 $mg \cdot kg^{-1} \cdot day^{-1}$ of fasudil was given intravenously to lamb neonates, a dose very similar to that used in the work reported by Blood et al. (7). Similar to their study, the pulmonary arterial blood pressure and resistance increased in our highland control lambs, and consistent with these findings, pulmonary

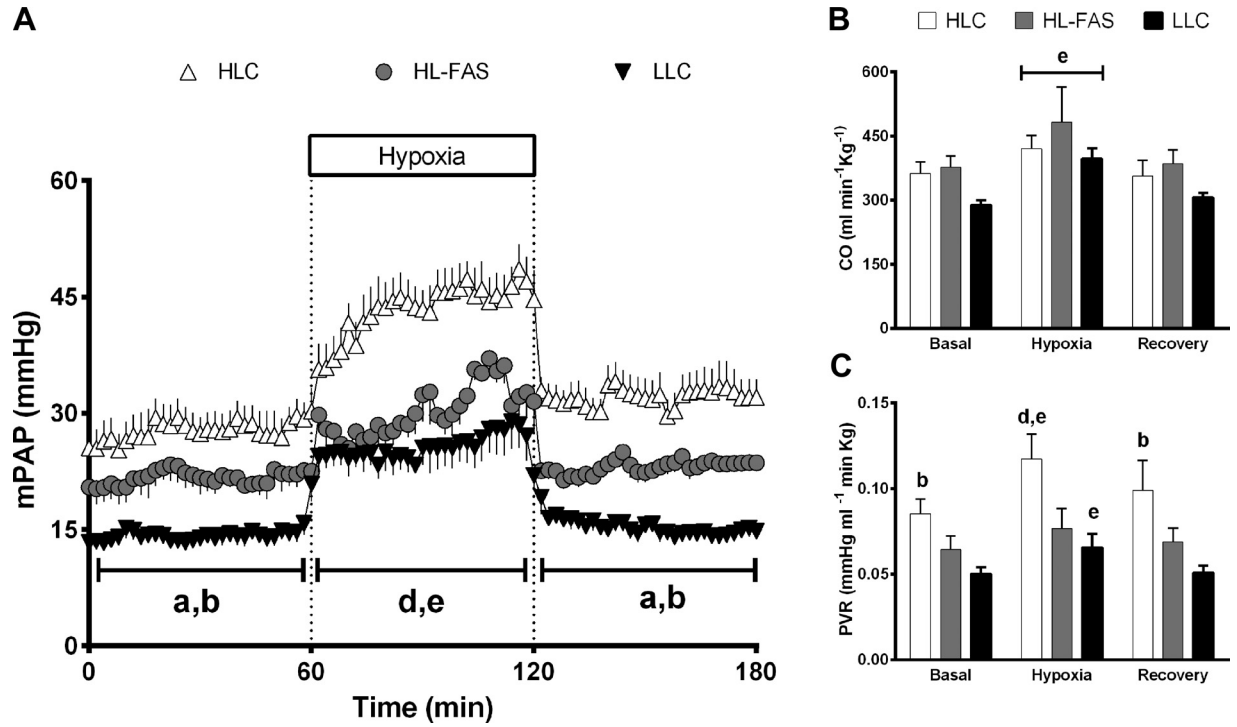

Fig. 3. Hypoxia and fasudil effects on the pulmonary circulation during superimposed hypoxia. mPAP (*A*), CO (*B*), and PVR (*C*) in highland control newborn lambs (HLC, white triangles/bars, $n = 5$), highland newborn lambs treated with fasudil (HL-FAS, gray circles/bars, $n = 5$), and lowland control newborn lambs (LLC, black triangles/bars, $n = 5$). Values are means ± SE. Significant differences ($P < 0.05$): [a]HL-FAS vs. all; [b]HLC vs. LLC; [d]HLC vs. all; [e]Hypoxia vs. Basal and Recovery.

arteries from highlands controls had enhanced contractile responses to vasoconstrictor agents. However, in contrast to our results, Blood et al. reported that fasudil administration had no effect on PAP or PVR in highland newborn lambs (7). These dissimilar results could be explained, among other things, by certain important differences between both experimental protocols. First, our pregnant ewes and newborn lambs were always at high altitude, from conception up to the in vivo and ex vivo studies in the neonates. In contrast, Blood et al. used ewes that were bred at near sea level and then transported to high altitude where delivery took place and the neonates brought to low altitude where they were studied in a hypoxic chamber (7). These authors studied the acute effect of a bolus of fasudil on the pulmonary circulation under acute hypoxia whereas our lambs received fasudil for 10 days, while we recorded the cardiopulmonary data daily. Further, after the treatment, the lambs were submitted to a superimposed episode of acute hypoxia.

An interesting finding was that fasudil-treated animals in our work are hypoventilating relative to the high-altitude controls, suggesting some involvement of the RhoA/ROCK pathway in the control of pulmonary ventilation.

An important issue relative to treatment of pulmonary hypertension with vasodilators is the lack of pulmonary selectivity of the therapies. Small decreases in systemic arterial blood pressure have been reported following systemic administration of ROCK inhibitors in adult animals (34) and in pilot studies on adult humans with idiopathic PHT (25). Interestingly, fasudil treatment in our neonates induced a slight decrease in mSAP (less than 10%) immediately after the infusion, but a complete recovery took place 15–20 min after the end of the infusion.

In the present study we have found that contractile function of small pulmonary arteries of newborn lambs, gestated and

Table 1. *Arterial blood gases in newborn sheep*

		Basal	Hypoxia	Recovery
pHa	HLC	7.474 ± 0.013	7.444 ± 0.013	7.429 ± 0.009
	HL-FAS	7.489 ± 0.008	7.479 ± 0.013	7.495 ± 0.009
	LLC	7.404 ± 0.015[c]	7.364 ± 0.022[c]	7.396 ± 0.024
Pa_{O_2}, mmHg	HLC	44.0 ± 1.1	30.4 ± 0.6[e]	48.0 ± 1.3
	HL-FAS	38.9 ± 1.2	30.5 ± 0.3[e]	40.5 ± 3.4
	LLC	82.6 ± 2.6[c]	30.1 ± 0.3[e]	89.0 ± 3.2[c]
Pa_{CO_2}, mmHg	HLC	28.3 ± 0.5[d]	27.9 ± 0.6[d]	26.2 ± 0.9[d]
	HL-FAS	33.5 ± 1.8[f]	32.9 ± 1.6[f]	31.2 ± 1.7
	LLC	38.7 ± 1.2	38.2 ± 1.4	34.1 ± 1.3
%Sat Hb, %	HLC	71.3 ± 1.3	45.9 ± 1.7[e]	74.5 ± 1.8
	HL-FAS	67.9 ± 2.4	50.8 ± 3.3[e]	70.7 ± 3.4
	LLC	95.5 ± 0.5[c]	50.9 ± 4.0[e]	96.4 ± 0.7[c]
Hb, g/dl	HLC	11.7 ± 0.8	12.0 ± 1.1	11.5 ± 0.8
	HL-FAS	12.2 ± 0.8	11.8 ± 1.1	12.0 ± 0.8
	LLC	10.8 ± 0.5	11.1 ± 0.4	10.3 ± 0.4
O_2 content, ml O_2/dl	HLC	11.2 ± 0.6	7.5 ± 0.5[e]	11.4 ± 0.6
	HL-FAS	10.9 ± 0.6	8.4 ± 0.7[e]	11.1 ± 0.8
	LLC	14.2 ± 0.6[c]	7.7 ± 0.8[e]	13.6 ± 0.6[c]

Values are means ± SE taken as basal, hypoxia, and recovery. Highland newborn sheep were treated for 10 days either with fasudil (HL-FAS, $n=5$) or NaCl 0.9%, highland control (HLC, $n=5$) and lowland control (LLC, $n=5$), during an episode of hypoxia. Significant differences at $P < 0.05$ (ANOVA + Newman-Keuls test) are shown as [c]LLC vs. all; [d]HLC vs. all; [e]Hypoxia vs. Basal and Recovery; [f]HL-FAS vs. LLC. pHa, arterial pH; Pa_{O_2}, arterial O_2 partial pressure; Pa_{CO_2}, arterial CO_2 partial pressure; %Sat Hb, percent saturation of hemoglobin with oxygen; Hb, hemoglobin concentration.

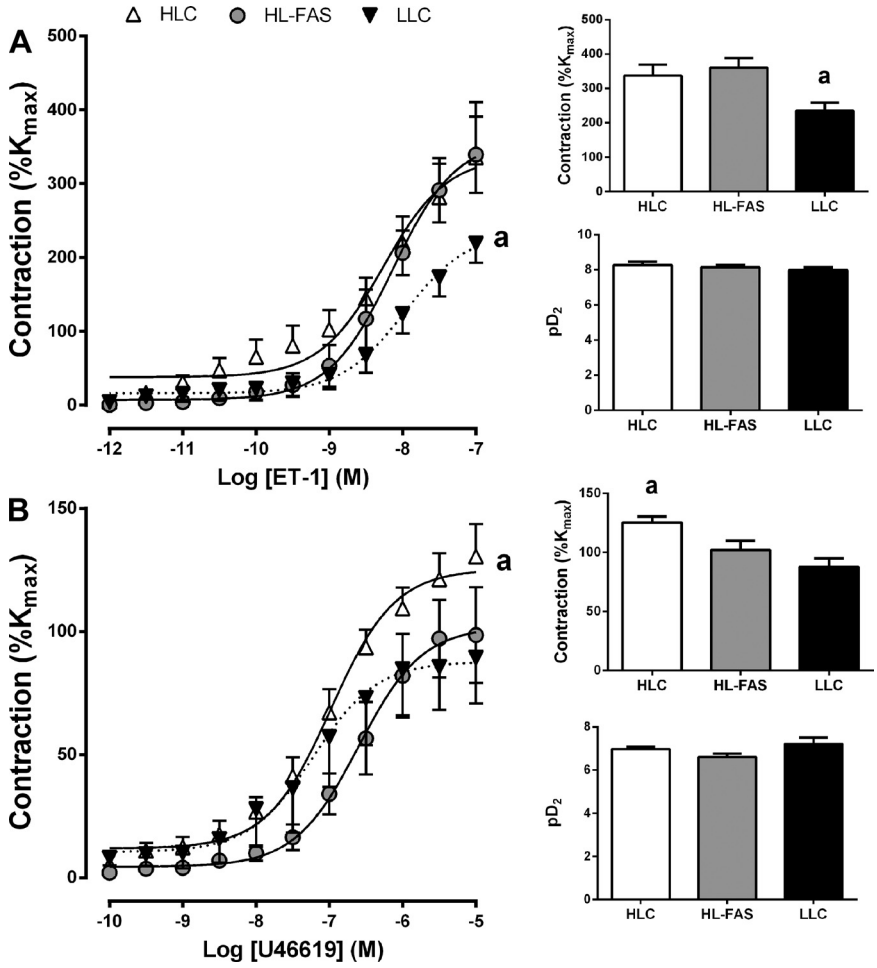

Fig. 4. Hypoxia and fasudil effects on the vasoconstrictor function in small pulmonary arteries. Responses to ET1 (*A*) and thromboxane analog U46619 (*B*) in small pulmonary arteries from highland control newborn lambs (HLC, white triangles/bars, $n = 5$), highland newborn lambs treated with fasudil (HL-FAS, gray circles/bars, $n = 5$), and lowland control newborn lambs (LLC, black triangles/bars, $n = 5$). Histograms show maximal contraction ($\%K_{max}$) and sensitivity (pD2). Values are means ± SE. Significant differences ($P < 0.05$): [a]vs. all.

born in the chronic hypoxia of the Andean altiplano, is enhanced, as has been previously reported (22). The response to U46619 is dependent on ROCKs, in agreement with a report that demonstrated that in PASMCs, the RhoA/ROCK pathway is a mediator of the response induced by the stimulation of the thromboxane A_2/prostaglandin H_2 (TP) receptor (58). Therefore, fasudil restored thromboxane maximal response as seen in the pulmonary circulation of lowlanders. In contrast, fasudil had no effects on the responses to ET-1 in the pulmonary vessels at high altitude. It has been shown that RhoA can be activated by ET-1 (35) and it was demonstrated in the Fawn-Hooded hypertensive rat model, that the RhoA/ROCK pathway mediates the increased pulmonary vascular response to ET-1 (6). Nevertheless, ET-1 causes vasoconstriction through numerous mechanisms in VSMC, RhoA/ROCK being only one of them. ET-1 exerts its biological actions mainly through the activation its receptor ET-A, inducing the generation of second messengers, such as 1,2-diacylglycerol (DAG) and inositol-1′,4′,5′-trisphosphate (IP3), activation of calcium channels in the sarcoplasmic reticulum which leads to an increase in Ca^{2+} levels, and activation of protein kinase C (PKC), which is a regulatory enzyme involved in the mobilization of intracellular Ca^{2+} stores and sensitivity to Ca^{2+} (48, 49). Also, at least another two signaling pathways are activated by ET-1 in VSMC; the mitogen-activated protein kinase (MAPK) cascade and phosphatidylinositol-3 kinase/protein kinase B (PI3K) (8). Therefore, it is possible that the molecular mechanisms involved in different types of PAH differ, and the RhoA/ROCK pathway may not be a relevant mediator of the ET-1 responses in our experimental model.

The U46619 myographic results are consistent with our histologic findings that showed a marked pulmonary vascular remodeling in the chronically hypoxic and pulmonary hypertensive neonatal lambs, compared with lowland control (LLC) and fasudil-treated neonates (HL-FAS). These results clearly demonstrate a role for the RhoA/ROCK pathway in the newborn pulmonary vascular remodeling induced by perinatal hypoxia. The mechanisms could include the participation of RhoA/ROCK signaling in the hypoxia-induced secretion of matrix metalloproteinase 2 of PASMC (28), degradation of p27, a cyclin inhibitor (60), and inhibition of apoptosis (29). Recently it was reported in a sheep model of persistent pulmonary hypertension of the newborn, that the exposure to chronic hemodynamic stress during late gestation alters extracellular matrix remodeling of proximal pulmonary arteries (PA) (11). This proximal PA stiffness may be involved in the increased right ventricular afterload in the chronically hypoxic fetal and neonatal lamb, contributing to pulmonary hypertension and right ventricle hypertrophy. This phenomenon may be true as well for the distal arteries seen in our study, contributing to the pulmonary hypertension.

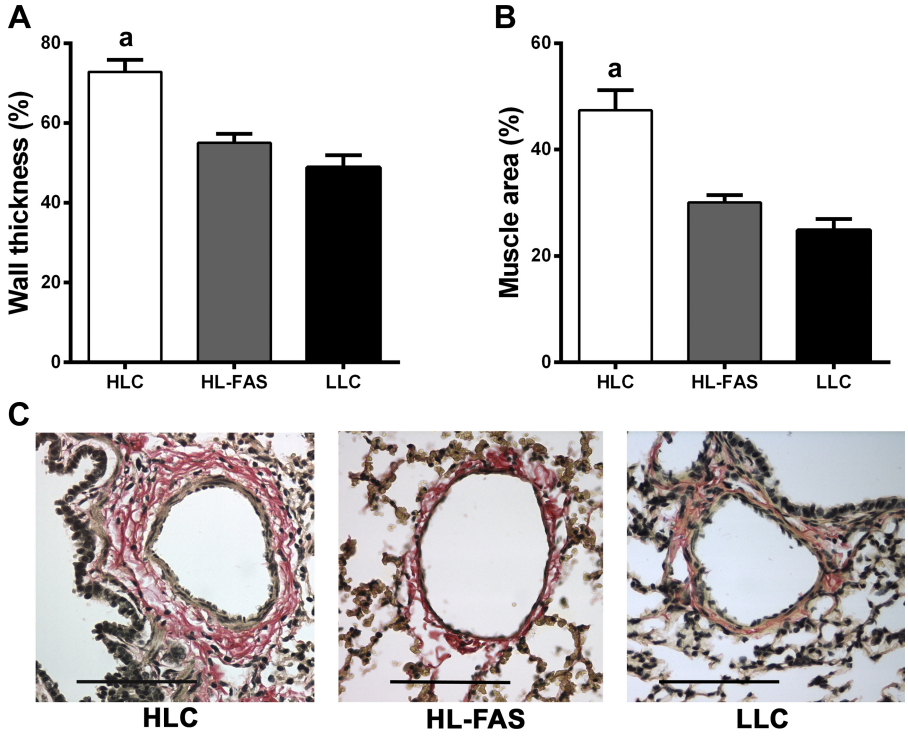

Fig. 5. Hypoxia and fasudil effects on pulmonary vascular remodeling. Percentage of wall thickness (*A*) and muscle area (*B*) of small pulmonary arteries from highland control newborn lambs (HLC, white bars, $n = 5$), highland newborn lambs treated with fasudil (HL-FAS, gray bars, $n = 5$), and lowland control newborn lambs (LLC, black bars, $n = 5$). Representative micrograph of small pulmonary arteries (*C*) from HLC, HL-FAS and LLC lambs. van Gieson staining. Bar: 100 μm. Magnification: 40×. Values are means ± SE. Significant differences ($P < 0.05$): [a]HLC vs. all.

The two isoforms of ROCKs, ROCKI and ROCKII, are expressed in vascular smooth muscle. Still, it is not clear which isoform is involved in pulmonary hypertension induced by hypoxia. In adult animal models, the RhoA/ROCKII pathway plays a central role in VSMC-mediated vasoconstriction. The mechanisms involved by the RhoA/ROCKII pathway generating pulmonary vasoconstriction requires binding to and phosphorylating the myosin-binding subunit of myosin phosphatase (53), ROCKII being the main isoform involved in the pathogenesis of PAH through enhanced VSMC migration and proliferation (47). McNamara et al. (31) reported that activity of the RhoA/ROCK pathway and ROCKI and II expression were increased in hypoxia-induced pulmonary hypertension in their neonate rat model. In contrast, Gao et al. (18) found higher ROCK activity but only ROCKII protein expression increased in near-term fetuses delivered from ewes exposed to chronic high-altitude hypoxia. In the present study, we showed that chronic hypoxia in utero decreased ROCKII expression by

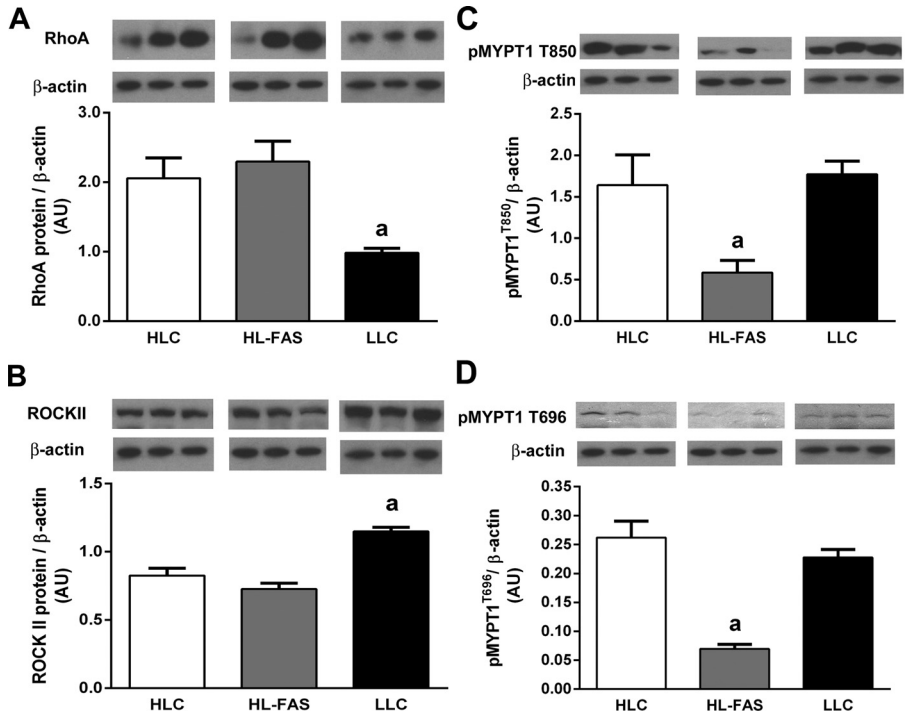

Fig. 6. Hypoxia and fasudil effects on RhoA and ROCKII expression and pMYPT1 phosphorylation in lung. Representative Western blots and densitometric scanning of RhoA (*A*) and ROCKII (*B*), pMYPT1T850 (*C*) and pMYPT1T696 (*D*) in pulmonary tissue from highland control newborn lambs (HLC, white bars, $n = 5$), highland newborn lambs treated with fasudil (HL-FAS, gray bars, $n = 5$), and lowland control newborn lambs (LLC, black bars, $n = 4$). AU, arbitrary units. Values are means ± SE. Significant differences ($P < 0.05$): [a]vs. all.

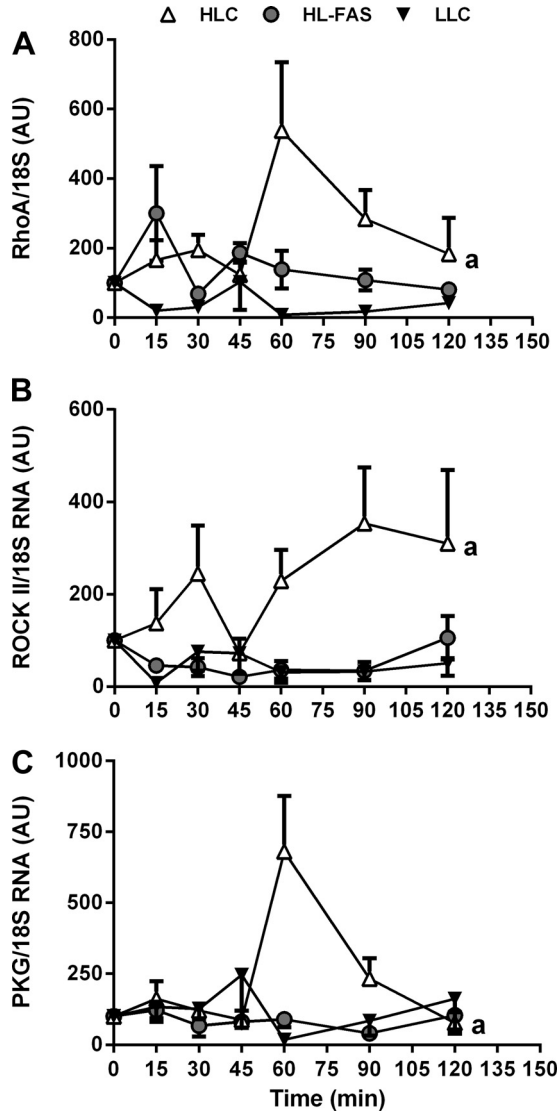

Fig. 7. Hypoxia and fasudil effects on RhoA/ROCK/PKG in PASMCs. Real time RT-PCR of RhoA (*A*), ROCKII (*B*), and PKG (*C*) in PASMCs undergoing different times of hypoxia, from highland control newborn lambs (HLC, white triangles, $n = 5$), highland newborn lambs treated with fasudil (HL-FAS, gray circles, $n = 4$), and lowland control newborn lambs (LLC, black triangles, $n = 4$). AU, arbitrary units. Values are means ± SE. Significant differences ($P < 0.05$): [a]HLC vs. all.

28%, relative to lowlands animals. In contrast, RhoA protein expression increases more than twofold in pulmonary tissue. Although we did not measure activated RhoA, we speculate that the change in the protein expression pattern of the RhoA/ROCKII pathway could result in a larger activation of ROCKs under stimulation, and together with our histologic findings, could explain the in vivo results.

Previous studies suggest that hypoxia may regulate ROCK in pulmonary hypertension through the generation of reactive oxygen species (ROS). Specifically, in pulmonary hypertension induced by chronic hypoxia, the NADPH oxidase systems subunits are upregulated in pulmonary arteries, and xanthine oxidase lung activity and vascular production are increased by hypoxia in newborn rats. Also, ROS are able to stimulate RhoA activity in pulmonary arteries and induce the translocation of ROCKII from nucleus to the cytosol, evoking a vasoconstriction sensitive to the ROCK inhibitor Y27632, in rat pulmonary arteries (41). We further assessed phosphorylation of MYPT1, the regulatory subunit of MLCP, at Thr850 and Thr696, as these processes are associated with inhibition of the holoenzyme binding to myosin (48). The results demonstrated that fasudil inhibited the expression of MYPT1 phosphorylated at these two amino acids, suggesting a decrease in the ROCK activity, thus correlating with the in vivo and ex vivo studies. The change in the level of MYPT1 phosphorylation is even more interesting, since we performed the experiments in a basal state and these differences between HL-FAS and HLC groups could be more marked under stimulation, as it has been described (18). We cannot exclude a change in the expression of total MYPT1 as we were unable to detect it. Nevertheless, this possibility seems very unlikely, according to previous studies reporting fasudil inhibition of MYPT1 phosphorylation but not of its expression (5, 18, 19).

Finally, we investigated if a superimposed hypoxic event could induce the expression of the RhoA/ROCK pathway in PASMCs of HLC, LLC and HL-FAS lamb neonates. We also were interested in the expression of PKG since it seems that vasoconstrictor tone of pulmonary arteries is tightly regulated by the action and interaction between RhoA and PKG (18). PKG may stimulate MLCP activity through interaction between its leucine zipper motifs and MYPT1 (48) and this may also antagonize phosphorylation of MYPT1 induced by ROCKs by phosphorylating amino acids that are immediately adjacent to ROCKs targets (55). Moreover, Gao et al. (18) demonstrated that chronic hypoxia in utero attenuates PKG-mediated relaxation in pulmonary arteries, partly due to inhibition of PKG activity and partly due to enhanced ROCK activity. In addition, PKG1 phosphorylates RhoA at Ser188 and thus negatively regulates the RhoA/ROCK pathway (46). Recently, it was reported that a selective mutation in the NH_2 terminus leucine zipper domain of PKG1 alpha results in a progressive increase in pulmonary artery pressure, under normoxic conditions in mice. This result is in part due to lack of inhibition of the RhoA/ROCK pathway by a defective PKG1 in PASMC and the resultant vasoconstrictor effect of ROCK (44). We found that in HLC PASMCs, RhoA, ROCKII, and PKG mRNAs were induced under hypoxic exposure. RhoA and PKG showed a peak at 60 min of hypoxia, after which mRNA expression decreased to basal levels, but the expression of ROCKII remained elevated until the end of the experiment. In contrast to our results, it has been reported that exposure of adult human PASMCs to 2% hypoxia for 2 h or longer has no effect on ROCKI and ROCKII mRNA, although there was an increase in protein expression (39, 60). It is not clear how hypoxia could affect ROCK expression, but it has been reported that the Na^+/H^+ exchanger (NHE) expression is induced in PASMCs, mediated by HIF-1 (42), and it has been proposed that NHE1 may promote pulmonary vascular remodeling by regulating ROCK expression and activity in mouse (59). Importantly, in PASMCs of fasudil-treated neonatal lambs in Putre, the mRNA increases of the three proteins were inhibited, indicating a role for the RhoA/ROCK pathway in the control of its own gene expression. It has been reported that ROCKI activates transcriptional factors in the smooth muscle cells through phosphorylation. We speculate that this mecha-

nism could also be involved in the transcriptional inhibition of ROCKII, RhoA, and PKG by fasudil treatment (10, 20).

In conclusion, using an integrative approach at the whole animal, isolated organ, and molecular level we provide evidence for the involvement of the RhoA/ROCK pathway in the development of the pulmonary hypertension induced by chronic hypoxia during gestation and birth of lambs at high altitude. The inhibition of ROCKs by fasudil may offer a possible therapeutic tool in the pulmonary hypertension of the neonate.

Perspectives and Significance

This study provides evidence for the involvement of the RhoA/ROCK pathway in pulmonary hypertension during gestation and birth of lambs undergoing chronic hypoxia at high altitude. Our approach includes studies at the whole animal, isolated organ, molecular and structural levels. The present studies extend reports on the use of the RhoA/ROCK blocker, fasudil, in adult and pediatric pulmonary hypertensive patients. The inhibition of this pathway by fasudil may offer a therapeutic tool for pulmonary hypertension in the neonate, after appropriate clinical studies.

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DISCLOSURES

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

AUTHOR CONTRIBUTIONS

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

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