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The role of nitric oxide signaling in pulmonary circulation of high- and low-altitude newborn sheep under basal and acute hypoxic conditions

Emilio A. Herrera[a,b], Germán Ebensperger[a,b], Ismael Hernández[a], Emilia M. Sanhueza[a], Aníbal J. Llanos[a,b], Roberto V. Reyes[a,b,*]

[a] Programa de Fisiopatología, Instituto de Ciencias Biomédicas (ICBM), Facultad de Medicina, Universidad de Chile, Santiago, RM, Chile
[b] International Center for Andean Studies (INCAS), Universidad de Chile, Santiago, RM, Chile

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ABSTRACT

Nitric oxide (NO) is the main vasodilator agent that drives the rapid decrease of pulmonary vascular resistance for the respiratory onset during the fetal to neonatal transition. Nevertheless, the enhanced NO generation by the neonatal pulmonary arterial endothelium does not prevent development of hypoxic pulmonary hypertension in species without an evolutionary story at high altitude. Therefore, this study aims to describe the limits of the NO function at high-altitude during neonatal life in the sheep as an animal model without tolerance to perinatal hypoxia. We studied the effect of blockade of NO synthesis with L-NAME in the cardiopulmonary response of lowland (580 m) and highland (3600 m) newborn lambs basally and under an episode of acute hypoxia. We also determined the pulmonary expression of proteins that mediate the actions of the NO vasodilator pathway in the pulmonary vasoactive tone and remodeling. We observed an enhanced nitrergic function in highland lambs under basal conditions, evidenced as a markedly greater increase in basal mean pulmonary arterial pressure (mPAP) and resistance (PVR) under blockade of NO synthesis. Further, acute hypoxic challenge in lowland lambs infused with L-NAME markedly increased their mPAP and PVR to values greater than baseline, whilst in highland animals under NO synthesis blockade, these variables did not show additional increase in response to low PO_2. Highland animals showed increased pulmonary RhoA expression, decreased PSer188-RhoA fraction, increased PSer311-p65-NFκβ fraction and up-regulated smooth muscle α-actin, relative to lowland controls. Taken together our data suggest that NO-mediated vasodilation is important to keep a low pulmonary vascular resistance under basal conditions and acute hypoxia at low-altitude. At high-altitude, the enhanced nitrergic signaling partially prevents excessive pulmonary hypertension but does not protect against acute hypoxia. The decreased vasodilator efficacy of nitrergic tone in high altitude lambs could be in part due to increased RhoA signaling that opposes to NO action in the hypoxic pulmonary circulation.

1. Introduction

Nitric oxide (NO) is the main vasodilator allowing the rapid decrease of pulmonary vascular resistance during the fetal to neonatal transition at birth [1]. NO is produced by endothelial nitric oxide synthase (eNOS) and diffuses to pulmonary artery smooth muscle cells to stimulate a cGMP-protein kinase G (PKG-1) signaling pathway that finally modulates membrane potential, Ca^{2+} influx, Ca^{2+} sensitivity of contractile machinery, cell migration and proliferation [2,3,4,5]. Most of the studies regarding the role of NO signaling in the adaptation of pulmonary circulation to extrauterine life have been conducted at near sea level. Under these environmental conditions, NO signaling is important to keep a low postnatal pulmonary arterial resistance and pressure, in normoxia and acute or chronic hypoxia [6]. Although there is a significant increase of NO production and function at birth, this does not avoid the increase of pulmonary arterial resistance and pressure in humans and most mammals at high altitude during neonatal life [3,7,8]. Moreover, hypobaric hypoxia during gestation and early neonatal life in lambs increases the magnitude of the vasoconstrictor response to a new hypoxic challenge [7,8]. Nevertheless, the role of the NO-mediated pulmonary vasodilation as a defense mechanism against neonatal pulmonary hypertension and particularly during the response to acute superimposed hypoxia, has been poorly studied in neonatal mammals with short ancestry at high-altitude. We tested the hypothesis that chronically hypoxic neonatal lambs have an enhanced NO function, which is insufficient to prevent pulmonary arterial hypertension

* Corresponding author. Programa de Fisiopatología, Instituto de Ciencias Biomédicas (ICBM), Facultad de Medicina, Universidad de Chile, Santiago, RM, Chile.
E-mail address: virreyc@gmail.com (R.V. Reyes).

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either in basal and acute hypoxic conditions. Therefore we aimed to evaluate the role of NO signaling in the control of cardiopulmonary function either in basal conditions or acute hypoxic challenge, in both highland and lowland newborn lambs, and to assess if perinatal chronic hypoxia modifies the expression of signaling proteins that regulate the vasodilator and anti-remodeling efficacy of NO To accomplish these aims, we did the following: 1) *in vivo* we recorded pulmonary and systemic arterial pressures, heart rate and cardiac output, and we calculated the pulmonary and systemic vascular resistances, under basal or acute hypoxic conditions. These measurements were done in low- and high-altitude newborn lambs, in presence or absence of L-NAME, an eNOS blocker; 2) *in vitro,* we measured in lung tissue from lowland and highland sheep, the pulmonary expression of proteins involved in vasodilator/vasoconstrictor and Ca^{2+} sensitization/desensitization balances downstream to NO, such as phosphodiesterase-5 (PDE5), its phosphorylated and more active form (PSer92-PDE5), protein kinase G (PKG1), big conductance calcium-activated potassium channels (BKCa), Rho-associated kinase 1 (ROCK1), RhoA and its phosphorylated and less active fraction (PSer188-RhoA), protein kinase *C*-potentiated inhibitory protein of phosphatase-1 (CPI17), its more active phosphorylated form (PThr38-CPI17), myosin phosphatase target 1 (MYPT1), its more activated phosphorylated form (PSer695-MYPT1), and total and phosphorylated myosin light chain (MLC20 and PSer19-MLC20); 3) we finally also measured the pulmonary expression of markers of remodeling and cell cycle progression, such as smooth muscle α-actin, cyclinD1, p50-NFKβ, p65-NFKβ and its phosphorylated active fraction PSer311-p65-NFKβ in low and high altitude newborn lambs.

2. Methods

All animal care, procedures and experimentation were approved by the Ethics Committee of the Faculty of Medicine, University of Chile (CBA # 097 FMUCH) and they were carried out according to international standards following 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).

2.1. Animals

Thirteen lowland lambs (LA, weight = 7.0 ± 0.4 kg), gestated, born and raised at the Estación Experimental Germán Greve Silva, Universidad de Chile (Santiago, 580 m altitude), and seven highland lambs (HA, weight = 5.6 ± 0.4 kg), gestated, born and raised at Estación Experimental Putre, International Center for Andean Studies (INCAS, Putre, 3600 m altitude), Universidad de Chile were used for *in vivo* experiments. Both LA and HA lambs were 10–12 days-old at the moment of the experiments. Tissues for immunoblot experiments were obtained from a separate group of non-instrumented LA (n = 5) and HA (n = 5) newborn at equivalent ages. The newborn sheep and their mothers were housed in an open yard with access to water and food *at libitum*.

2.2. Surgical instrumentation

The newborn lambs were instrumented according to surgical protocols previously established at our laboratory [7,9,10,11,12,13,14]. Briefly, the animals were pre-medicated with atropine (0.04 mg kg^{-1} i.m., atropine sulphate, Laboratorio Chile, Santiago, Chile) and submitted to general anesthesia with ketamine (10 mg kg^{-1} i.m., ketostop, Drag Pharma-Invetec, Santiago, Chile) and diazepam (0.1–0.5 mg kg^{-1} i.m., Laboratorio Biosano, Santiago, Chile) with additional local infiltration of lidocaine (2% lidocaine hydrochloride, Dimecaína, Laboratorio Beta, Santiago, Chile) in the incision area. Polyvinyl catheters (0.8 mm i.d.) were installed in the descending aorta and inferior vena cava via femoral artery and vein, respectively. The polyvinyl catheters were exteriorized subcutaneously through the flank and kept in a pouch attached to the skin. A Swan-Ganz catheter (Edwards Swan-Ganz 5 French, Baxter Healthcare, Irvine, CA, USA) was inserted into the pulmonary artery via external jugular vein, exteriorized and located in a scarf around the neck of the animal. All vascular catheters were filled with heparinized solution (500 IU heparin ml^{-1} in 0.9% NaCl) and plugged with a copper pin. Oxytetracycline (10 mg kg^{-1} i.m., Liquamicina LA, Pfizer, Santiago, Chile) and sodium metamizole (0.1 mg kg^{-1} i.m., Dipirona, Laboratorio Chile), were administered during the surgical preparation. After the instrumentation, and once awake, the lambs were returned to the flock with their mothers. The experiments began 3 days after surgery, and the lambs were previously allowed to familiarize with the metabolic cage. All the cardiovascular recording was performed with the lambs awake and without any sedation or physical restrain.

2.3. Recording of cardiopulmonary variables

LA and HA lambs were subjected to experiments based on a 3 h protocol divided into three periods: 1 h of baseline, 1 h of hypoxia and 1 h of recovery. After baseline recording (breathing room air), a loosely tied transparent polyethylene bag was placed over the newborn's head into which a controlled mixture of air, N_2 and CO_2 was passed at *ca.* 15 L min^{-1}. Hypoxia (~9%O_2 and 2–3% CO_2 in N_2) was induced to reduce the PO_2 to *ca.* 30 mmHg, without altering PCO_2 [7,8,10,11,13,14]. After the hour of isocapnic hypoxia the animal was returned to breathing atmospheric air for a further 60 min (recovery). During the first experiment, the lambs received an infusion of a solution of 0.9% NaCl (Control vehicle treatment). The following day, the same protocol was performed but with the administration of N^G-nitro-L-arginine methyl ester (L-NAME, Sigma Chemical Co; 20 mg kg^{-1} bolus plus infusion at 0.5 mg kg^{-1} min^{-1} dissolved in 0.9% NaCl) to inhibit *in vivo* NOS activity [15,16]. Infusions were given via the inferior vena cava, starting 15 min before hypoxia, until the end of the hypoxic challenge. Arterial blood samples (0.3 ml) were taken in heparinized syringes at 15 and 45 min of baseline, at intervals of 15 min during the hypoxic hour, and at 15 and 45 min during the recovery period. Arterial pH, PCO_2, PO_2 (ABL 555, Blood gas Monitor, Radiometer, Denmark; measurements corrected at 38.5 °C), hemoglobin concentration (Hb), percentage saturation of hemoglobin (Hb sat) and oxygen content (O_2 cont) were measured (OSM3 Hemoximeter, Radiometer, Denmark). Systemic and pulmonary arterial pressure (PAP) were measured and recorded continuously (Statham P23 transducers BB-db, Hato Rey, Puerto Rico) using a data acquisition system connected to a PC (PowerLab, ADInstruments, Australia). Heart rate (HR), mean pulmonary (mPAP) and systemic (mSAP) arterial pressures were obtained from this recording.

Cardiac output (CO) was determined by thermodilution as the average of three measurements after injection of 3 ml 0.9% NaCl chilled to 0 °C (Baxter COM-2, Irvine, CA, USA) [7]. Pulmonary (PVR) and systemic vascular resistance (SVR) were calculated using the following equations:

$$PVR = \frac{mPAP - \text{wedge pressure (mm Hg)}}{CO\ (ml\ min^{-1}.\ kg^{-1})}$$

$$SVR = \frac{mSAP - \text{Right atrial pressure (mmHg)}}{CO(ml\ min^{-1} kg^{-1})}$$

The day after experiments terminated, lambs were submitted to an extra surgical protocol for catheters extraction and were maintained under veterinary care for 1 week to assure healthy recovery. Another set of un-instrumented lambs (n = 10) underwent euthanasia with an overdose of sodium thiopentone (100 mg kg^{-1}, slow I.V. infusion) for tissue extraction and *in vitro* analyses. Considering clinical features, lung histology and alveolar arterial PO_2 difference (data not shown), we did not find any suggestion of pulmonary edema in the newborn sheep utilized in this study.

2.4. Immunoblot experiments

Lung lysates were prepared in RIPA buffer and subjected to immunoblot analysis as described elsewhere [9,17,18]. The intensities of the bands corresponding to the immunoblot experiments were quantified by densitometric analysis, using Scion Image Software (Scion Image Beta 4.02 Win; Scion Image Corporation, MD, USA). The specific primary antibodies used were *anti*-BKCa (monoclonal, Neuromab, clone L6/60), anti-PKG-1 (polyclonal, Enzo LifeSciences), *anti*-PSer92-PDE5 (polyclonal, FabGennix), *anti*-PDE5 (monoclonal, BD Transduction Labs), anti-ROCK1 and *anti*-MYPT (polyclonal, Thermofisher Scientific), *anti*-MLC_{20} and *anti*-PSer19 MLC_{20} (polyclonal, Cell Signaling), *anti*-RhoA, *anti*-PSer965MYPT1, *anti*-p50-NFKβ, *anti*-p65-NFKβ, *anti*-PSer311-p65-NFKβ and anti-CPI17 (monoclonal, Santa Cruz Biotechnology), *anti*-PThr38-CPI17 (polyclonal, Abcam), *anti*-PSer188-RhoA (polyclonal, Merck Millipore), *anti*-p21 and *anti*-cyclinD1 (polyclonal, Santa Cruz Biotechnology), *anti*-α-actin and *anti*-β-actin antibodies (monoclonal, Sigma, San Louis, MO, USA).

2.5. Statistics

All values are expressed as means ± S.E.M. *In vivo* experiments were analyzed using a two-way analysis of variance (ANOVA) followed by a Newman-Keuls *post hoc* test. For *in vitro* experiments, differences between mean values were assessed by unpaired Student's *t* or Mann-Whitney tests as appropriate. For all comparisons, statistical significance was accepted when $p < 0.05$ [19].

3. Results

3.1. Acid-base and arterial gases

3.1.1. NaCl (control) experiments

At baseline, HA lambs showed higher arterial pH than LA lambs, whilst no pH differences between both groups were observed during hypoxemia and recovery (Table 1). PCO_2 was lower in HA lambs than LA lambs during basal period, but no changes were observed during hypoxemia, whereas during recovery period, it decreased in LA lambs relative to their baseline period (Table 1). As expected, PO_2, Hb saturation and O_2 content were lower in HA relative to LA lambs during basal and recovery periods (Table 1). However, these variables decreased to similar values in both LA and HA lambs during hypoxic challenge while all the other variables did not change (Table 1).

3.1.2. L-NAME experiments

LA lambs showed lower pH during acute hypoxia and recovery periods whilst in HA lambs, pH remained unchanged during all the experimental periods (Table 2). PCO_2 was stable during all the experimental periods in LA lambs except for recovery that showed a significant decrease relative to basal period. In contrast, pCO_2 was stable during the experimental protocol in HA lambs (Table 2). PO_2, Hb saturation, and O_2 content were lower in HA than LA lambs during basal and recovery periods and decreased to similar values during acute hypoxic challenge in both groups of animals (Table 2).

3.2. Cardiopulmonary variables

3.2.1. NaCl 0.9% (control) experiments

HA lambs had greater mPAP than LA lambs during basal and recovery periods whilst during acute hypoxia mPAP increased to similar values in both HA and LA lambs (Fig. 1A). Basal CO was higher in HA than LA lambs whereas it did not differ between LA and HA lambs in the other experimental periods. CO increased during acute hypoxia in LA lambs and decreased but did not reach basal values during recovery. In contrast, CO was stable along all the experimental protocol in HA lambs (Fig. 2A). Consequently, PVR was similar in LA and HA lambs during basal and recovery periods, and it increased in both groups under acute hypoxia, but the rise was higher in HA group (Fig. 2B).

3.2.2. L-NAME experiments

In LA lambs, L-NAME infusion evoked a significant increase of mPAP under basal conditions. This variable further increased under acute hypoxia and decreased during recovery but remained higher than basal period before infusion in LA lambs. The net mPAP value of L-NAME-infused lambs was greater than their NaCl-infused counterparts under basal conditions and acute hypoxia but did not differ under recovery in LA group (Fig. 1 A, B, C and D). In the HA group, the L-NAME infusion evoked an intense rise of basal mPAP, of markedly greater magnitude than in LA counterparts (Fig. 1 A, B, C and D). Despite the acute superimposed hypoxic challenge, mPAP did not increased further than in basal conditions under NO blockade in HA lambs. Moreover, during recovery mPAP remained elevated in L-NAME infused HA lambs (Fig. 1C and D). L-NAME infusion decreased CO during basal, hypoxia and recovery periods in both LA and HA lambs (Fig. 2C). However, the blockade did not prevent the increase of CO during acute hypoxia in LA group, but it abolished this response in HA group (Fig. 2A and C). CO was lower in LA lambs infused with L-NAME relative to their NaCl-infused counterparts during all the experimental periods, whilst in HA lambs, L-NAME infusion resulted in lower CO than NaCl infusion only during acute hypoxia (Fig. 2A and C). Moreover, L-NAME infusion evoked a rise of basal PVR, a further increase during acute hypoxia and a partial decrease during recovery in LA lambs, since it was still elevated compared to basal values before infusion. PVR increased during L-NAME infusion, under basal, acute hypoxia and recovery periods in HA lambs. The PVR value was higher in HA compared to LA group under basal condition during L-NAME infusion (Fig. 2D). HA lambs infused with L-NAME had greater PVR than their counterparts infused with NaCl during basal and acute hypoxia periods, whilst L-NAME infused LA

Table 1
pH and arterial gases in Lowland (LA) and Highland (HA) newborn sheep under saline infusion (NaCl 0.9%). Statistical differences ($p \leq 0.05$): † vs LA, * vs Basal, § vs all periods in the same group.

		BASAL/BASAL + i			HYPOXEMIA + i			RECOVERY		
pH	LA	7.412	±	0.014	7.391	±	0.019	7.423	±	0.018
	HA	7.455	+	0.006†	7.427	+	0.012	7.415	+	0.013
PCO_2	LA	36.8	±	1.1	35.8	±	1.2	32.4	±	1.4*
mmHg	HA	32.1	±	1.6†	31.8	±	1.4	30.6	±	1.6
PO_2	LA	79.5	±	1.9	30.9	±	0.6§	82.4	±	3.8
mmHg	HA	41.5	±	2.4†	31.2	±	0.5§	43.4	±	3.4†
Hb	LA	10.9	±	0.5	11.3	±	0.4	10.3	±	0.4
g/dL	HA	11.8	±	0.7	12.3	±	0.7	11.7	±	0.7
Hb sat	LA	94.7	±	0.7	52.7	±	3.1§	96.0	±	0.7
%	HA	66.1	±	2.6†	48.6	±	3.1§	65.5	±	3.2†
O_2 cont	LA	13.9	±	0.6	8.0	±	0.5§	13.3	±	0.6
$mlO_2.dL^{-1}$	HA	10.8	±	0.8†	8.4	±	0.8§	10.6	±	0.9†

Table 2
pH **and arterial gases in Lowland (LA) and Highland (HA) newborn sheep under LNAME infusion.** Statistical differences ($p \leq 0.05$): † vs LA, * vs Basal, § vs all periods in the same group.

		BASAL/BASAL + i			HYPOXEMIA + i			RECOVERY		
pH	LA	7.459	±	0.007	7.393	±	0.027*	7.409	±	0.033*
	HA	7.467	±	0.015	7.437	±	0.016	7.444	±	0.017
PCO_2	LA	33.3	±	0.8	32.5	±	0.8	28.9	±	1.2*
mmHg	HA	36.3	±	3.0	33.7	±	2.4	33.5	±	3.2
PO_2	LA	78.3	±	2.8	32.0	±	0.7§	77.8	±	6.1
mmHg	HA	40.6	±	3.6†	31.5	±	1.0§	39.3	±	4.3
Hb	LA	9.7	±	0.7	10.8	±	0.5	10.1	±	0.4
g/dL	HA	11.6	±	0.7	12.4	±	0.7	11.9	±	0.6
Hb sat	LA	94.9	±	1.1	55.9	±	5.6§	90.9	±	4.6
%	HA	66.0	±	4.4†	51.7	±	3.1§	63.5	±	6.3
O_2 cont	LA	12.5	±	0.9	8.3	±	1.2§	12.5	±	0.8
$mlO_2.dL^{-1}$	HA	10.5	±	0.9†	9.0	±	0.9§	10.5	±	1.2

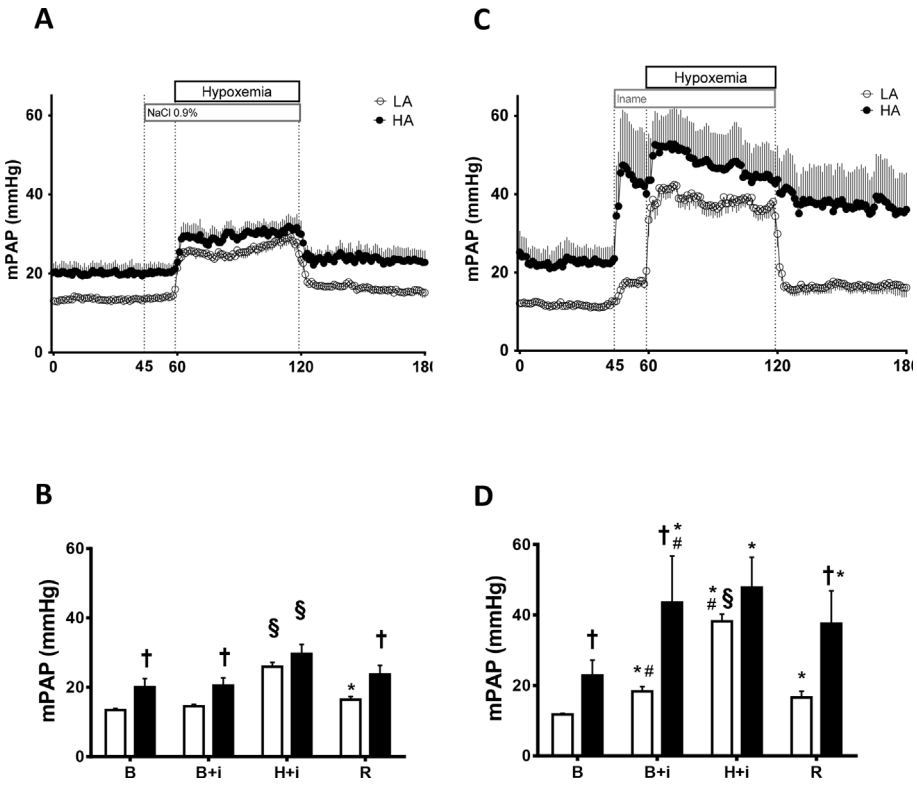

Fig. 1. Mean pulmonary arterial pressure. Groups are low-altitude (LA, open symbols) and high-altitude (HA, filled symbols) newborn sheep infused with NaCl 0.9% (left panel, A and B) or L-NAME (right panel, C and D), basally and during a period of superimposed acute hypoxia. A and C represent continuous recording of mPAP, whilst B and D represent bar graphs with the mean ± s.e.m of mPAP for basal (B), basal + infusion of NaCl or L-NAME (B + i), acute hypoxia + infusion (H + i) and recovery (R) periods. Significant differences, $p < 0.05$ (ANOVA + Student – Newman Keuls test): *, vs B; §, vs all in the same group; ǂ, LA vs HA; #, L-NAME vs NaCl, ǂ, vs B + i.

lambs had higher PVR in relation to their NaCl counterparts during all the experimental protocol (Fig. 2B and D).

3.3. Systemic variables

3.3.1. NaCl (control) experiments

Basal HR was similar in LA and HA lambs, it increased to similar values during acute hypoxia, and returned to resting values during recovery (Fig. 3A). SAP was similar in LA and HA lambs and remained stable during all the experiment (Fig. 3B). In contrast, SVR was lower in HA than LA lambs during basal and recovery periods, but similar in both groups during acute hypoxia. In fact, SVR decreased during acute hypoxia in LA lambs and recovered partially without reaching basal values while remained unchanged during all the experiment in HA lambs (Fig. 3C).

3.3.2. L-NAME experiments

L-NAME infusion decreased HR during basal and recovery periods and there was an increase related to basal plus L-NAME and recovery. Nevertheless, the HR was similar to the value observed under basal condition previous L-NAME infusion. The net HR was lower in L-NAME- than NaCl-infused LA lambs during all the experimental periods (Fig. 3A and D). In HA lambs, L-NAME infusion decreased HR which was maintained low during the entire experimental protocol (Fig. 3D). SAP increased with L-NAME infusion in both LA and HA lambs, remaining increased during all the experimental protocol, relative to their NaCl-infused counterparts (Fig. 3B and E). Further, L-NAME infusion enhanced SVR in both LA and HA lambs, but net SVR values also remained lower in HA than LA lambs during basal and recovery periods. A partial decrease of SVR was observed during acute hypoxia in LA lambs infused with L-NAME, not seen in HA lambs. Noteworthy, SVR during acute hypoxic challenge with L-NAME was similar in LA and HA lambs (Fig. 3C and F).

3.4. Pulmonary expression of proteins involved in the NO downstream signaling

The pulmonary expression of total phosphodiesterase-5 (PDE5), its phosphorylated and more active form (PSer92-PDE5), protein kinase G (PKG1), big conductance calcium-activated potassium channels (BKCa)

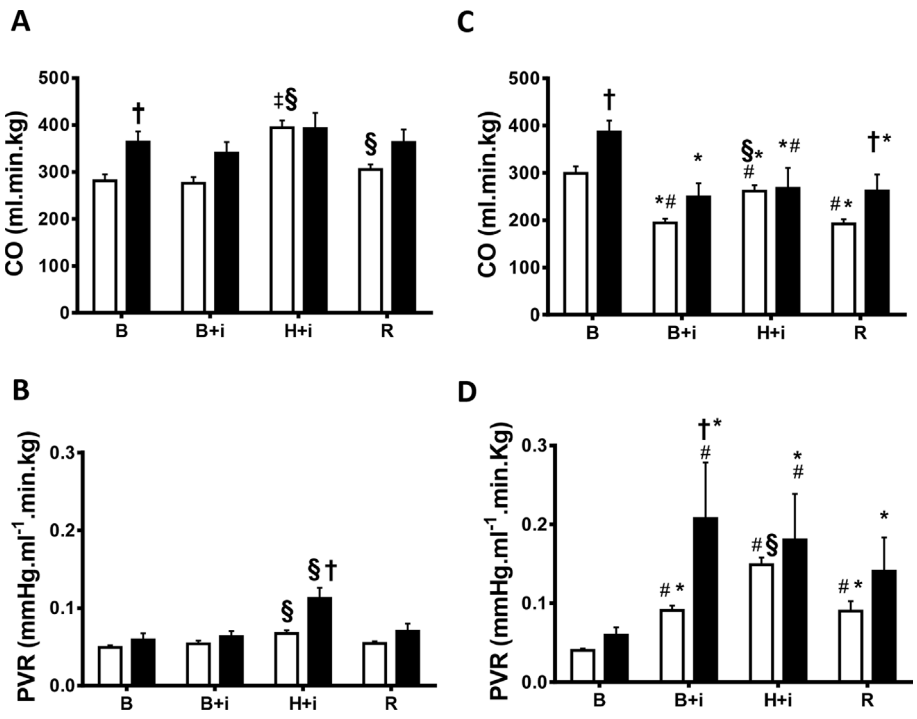

Fig. 2. Cardiac output and pulmonary vascular resistance. Groups are low-altitude (LA, open symbols) and high-altitude (HA, filled symbols) newborn sheep infused with NaCl 0.9% (left panel, A and B) or L-NAME (right panel, C and D), basally and during a period of superimposed acute hypoxia. Bar graphs with the mean ± s.e.m of basal (B), basal + infusion of NaCl or L-NAME (B + i), acute hypoxia + infusion (H + i) and recovery (R) periods are shown for cardiac output (A and C) and pulmonary vascular resistance (B and D). Significant differences, $p < 0.05$ (ANOVA + Student – Newman Keuls test): *, vs B; §, vs all in the same group; ǂ, LA vs HA; #, L-NAME vs NaCl, ǂ, vs B + i.

and Rho-associated kinase 1 (ROCK1) were similar in LA and HA lambs (Fig. 4). Nevertheless, total RhoA expression was higher, whilst its phosphorylated and less active fraction (PSer188-RhoA) was lower in HA than LA lambs (Fig. 4). The total kinase *C*-potentiated protein inhibitor of phosphatase-1 (CPI17) and myosin phosphatase target 1 (MYPT1) expression, and their corresponding phosphorylated and

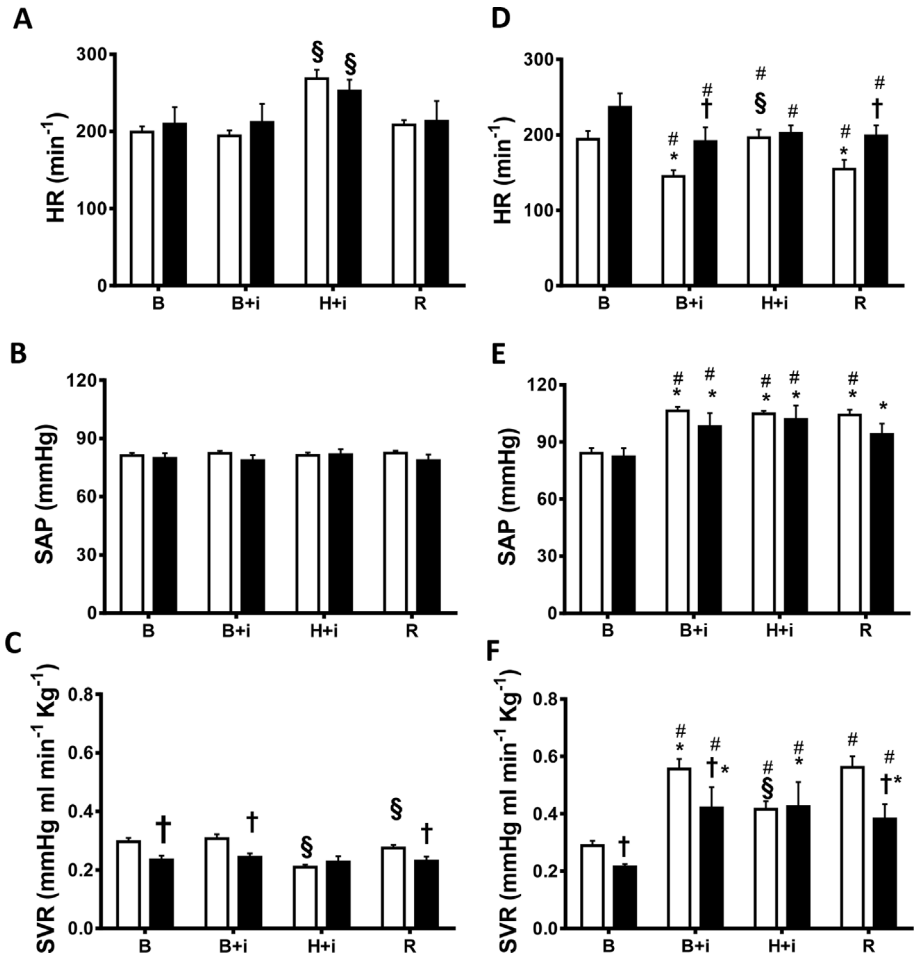

Fig. 3. Heart rate, mean systemic arterial pressure and resistance. Groups are low-altitude (LA, open symbols) and high-altitude (HA, filled symbols) newborn sheep infused with NaCl 0.9% (left panel, A, B and C) or L-NAME (right panel, D, E and F), basally and during a period of superimposed acute hypoxia. Bar graphs show the mean ± s.e.m of basal (B), basal + infusion of NaCl or L-NAME (B + i), acute hypoxia + infusion (H + i) and recovery (R) periods for heart rate (A and D), mean systemic arterial pressure (B and E) and systemic vascular resistance (C and F). Significant differences, $p < 0.05$ (ANOVA + Student – Newman Keuls test): *, vs B; §, vs all in the same group; ǂ, LA vs HA; #, L-NAME vs NaCl.

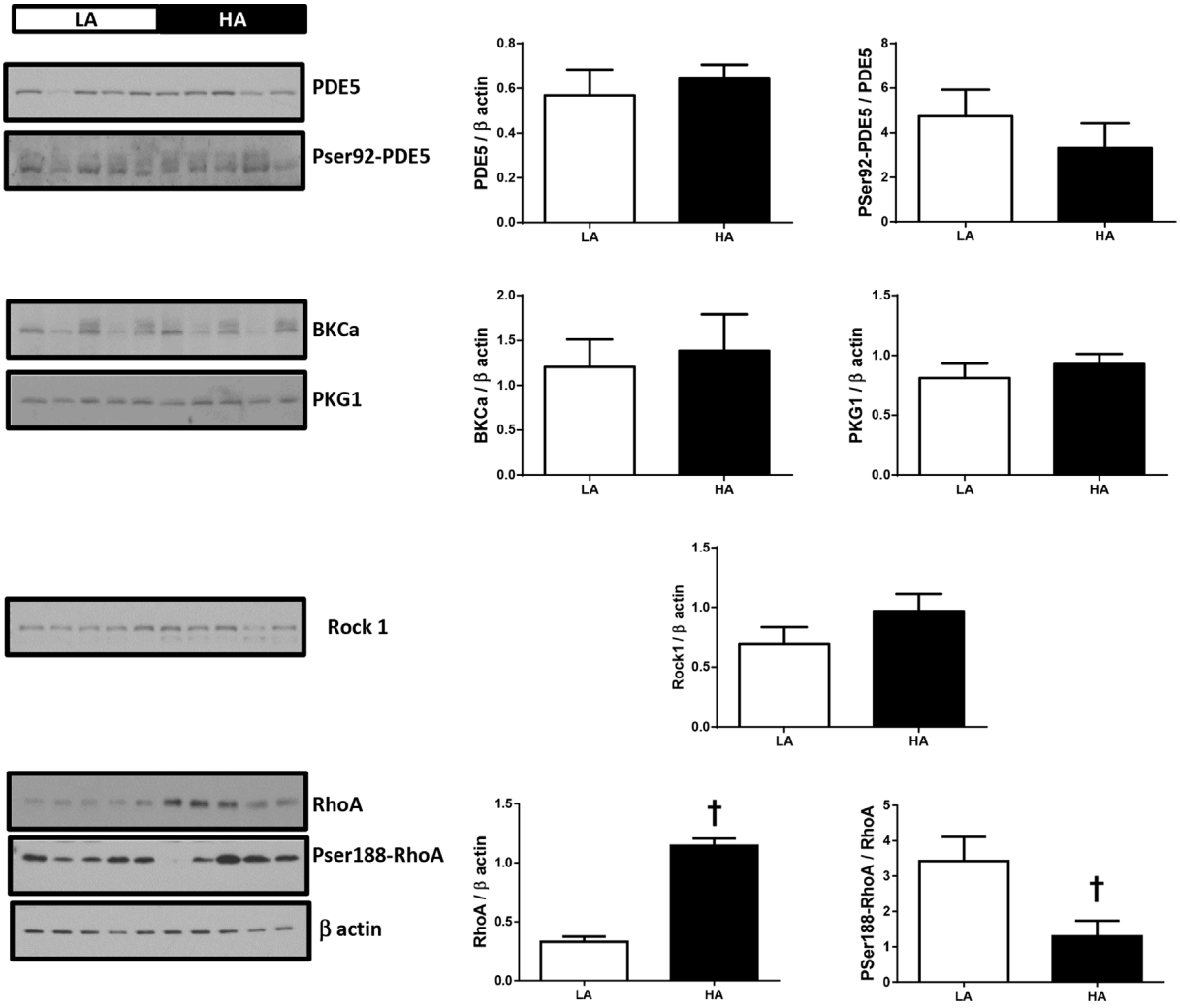

Fig. 4. Pulmonary expression and phosphorylation of proteins of the PDE5 - PKG - RhoA signaling axis. PSer92-PDE5, BKCa, PKG1, ROCK1, RhoA and Pser188-RhoA were evaluated in lowland (LA, open bars) and highland (HA, filled bars) newborn sheep. Values are mean ± standard error of the mean. Significant differences, $P < 0.05$, student's unpaired *t*-test or Mann-Whitney test, **Ɨ**, LA vs HA.

activated fractions (PThr38-CPI17 and PSer695-MYPT1 respectively) had similar expression in both groups (Fig. 5). Additionally, total and phosphorylated myosin light chain (MLC20 and PSer19-MLC20) were also similar in LA and HA groups (Fig. 5). Finally, CyclinD1, p50-NFKβ and total p65-NFKβ did not change, whilst the phosphorylated active fraction of the latter, PSer311-p65-NFKβ, was higher in lungs from HA than LA lambs (Fig. 6). Pulmonary smooth alpha-actin expression was also higher in HA than LA lambs (Fig. 6).

4. Discussion

This study was performed under field conditions with newborn lambs conceived, gestated, born and raised under natural environments, either near sea-level (580 m) or hypobaric hypoxia at 3600 m altitude at the Andean *Altiplano*. The sheep was introduced to South America during the colonization and despite we cannot guarantee the exact amount of generations, we know that sheep have been raised for more than 100 years in the community of Putre, where this study was carried out. This experimental design allowed us to compare the role of NO signaling in the cardiopulmonary function of species like the sheep, considered as a "newcomer" to high altitude in terms of evolution. Our findings add knowledge to the cardiovascular and pulmonary responses to high altitude hypobaric hypoxia, highlighting some the NO-dependent mechanisms opposing the pulmonary hypertension of the neonate.

4.1. Significance of increased NO-signaling in neonatal pulmonary circulation at high altitude

Various studies in mammals have suggested that up-regulation of NO- signaling is related to partial or total adaptation/acclimatization of pulmonary circulation to chronic hypoxia. Studies carried out in animal models from species with either low- or high-ancestry at high-altitude like the sheep, and the yak or the llama, respectively, also suggest that NO-dependent mechanisms contribute to chronic hypoxia adaptation to different degrees. Our group has previously reported that eNOS activity and expression are up-regulated in lungs from high-altitude newborn sheep compared to lowland controls. However, this defense mechanism does not prevent the development of pulmonary hypertension [9]. Consistent with these observations, our new data show that eNOS blockade *in vivo* induces an increase of basal mPAP in both low- and high-altitude lambs. In addition, the magnitude of this response at high-altitude [9]. Taken together, these data provide a robust evidence of increased synthesis of NO in the pulmonary circulation of high-altitude lambs. A similar observation has been reported in species tolerant to hypoxia that do not develop pulmonary hypertension like the yak and the llama, where eNOS blockade evoked a greater increase of mPAP and PVR in high-than low-altitude animals [9,17,20]. In humans, exhaled NO is higher in adult Tibetans and Aymaras living at altitudes between 3900 m and 4200 m, than in lowland dwellers [21]. Collectively, these

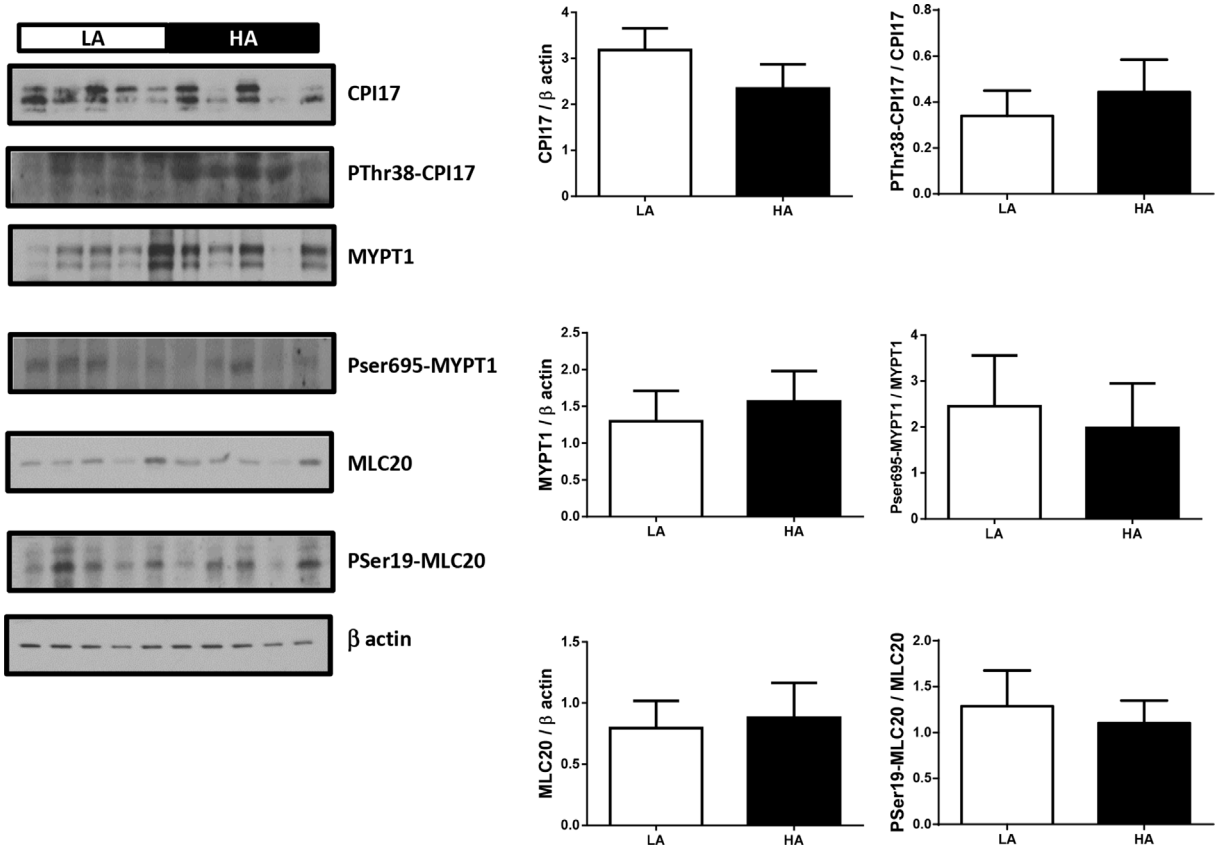

Fig. 5. Pulmonary expression and phosphorylation state of proteins of regulating calcium sensitivity and contraction of smooth muscle. CPI17, PThr38-CPI17, MYPT1, PSer695-MYPT1, MLC20 and Pser-19MLC20 were evaluated in lowland (LA, open bars) and highland (HA, black bars) newborn sheep. Values are mean ± standard error of the mean.

observations suggest that up-regulation of NO-signaling is necessary, but not enough by itself to offset the hypoxic increase of basal mPAP in some species like the sheep, while NO action is probably potentiated by other mechanisms in species like the yak and the llama. In the present work, we have confirmed our previous observations: in the regulation of basal mPAP and PVR, the nitrergic tone in more important in high- than low-altitude lambs. Nevertheless, we have also observed that eNOS blockade results in additional elevation of mPAP and PVR during acute hypoxic challenge in low-altitude but not in high-altitude lambs. Moreover, these variables reverse pre-hypoxic values after the end of the hypoxic challenge in low- but not in high-altitude sheep. Furthermore, in high-altitude newborn lambs, the NO function seems to be maximally enhanced, since under L-NAME administration, mPAP is the same basally and during acute hypoxic challenge. Apparently, there is no further room for mPAP increment. This observation may have clinical relevance. Human neonates with persistent pulmonary hypertension may develop acute hypoxia crisis, reaching PO2 values similar and even lower than those observed in our experimental acute hypoxic challenge. Therefore, the cardiorespiratory response of the newborn lambs suggests that endogenous NO does not provide any additional vasodilator tone to cope with this intense hypoxia. This is consistent with failure of inhaled NO therapy in close to 30% of cases of persistent pulmonary hypertension of the newborn [22,23].

Collectively, these findings suggest that nitrergic tone is preventing a deleterious augmentation in pulmonary arterial pressure and resistance, under acute hypoxia in lowland sheep, but is unable to do so in highland sheep, probably because in the latter, other mechanisms limit nitrergic vasodilation.

4.2. Hypoxic up regulation of pathways that antagonize increased NO-signaling in neonatal pulmonary circulation in the newborn sheep

Normally, NO activates sGC to catalyze the conversion of GTP into cGMP, which in turn activates PKG-1. The cGMP content of the vascular smooth cell depends on the ratio of its synthesis by sGC and its degradation by PDE5, and the latter may be phosphorylated to yield the more active PSer92-PDE5 [4,24,25]. PKG-1 may directly phosphorylate and activate BKCa channels on Ser1072 at plasma membrane, to promote pulmonary artery smooth muscle hyperpolarization, resulting in decrease of intracellular Ca^{2+} [25,26]. In addition, PKG-1 may phosphorylate MYPT1 on Ser695, the regulatory subunit of myosin light chain phosphatase, to increase myosin light chain dephosphorylation and decrease the sensitivity to Ca^{2+} at the contractile machinery (Ca^{2+} desensitization) [4,25]. Also, myosin light chain phosphatase may be negatively regulated through alternative MYPT1 phosphorylation on Thr696 by ROCK1 or 2, and through binding of CPI-17 to its catalytic subunit [4,27]. ROCK1/2 activity is stimulated through RhoA binding, a small G protein that is in turn negatively regulated by PKG- and PKA-mediated phosphorylation on Ser188 [4,28,29]. On the other hand, CPI-17 may be potentiated through phosphorylation on Thr38 by PKC [30]. Thus, RhoA and CPI17 phosphorylation results in either Ca^{2+} -desensitization or -sensitization respectively, modulating in this way the vasodilator efficacy of NO signaling. In the present work, we did not find differences between low- and high-altitude lambs in the pulmonary expression of total PDE5, PSer92-PDE5 fraction, PKG1, BKCa, ROCK1, CPI17, or PThr38-CPI17 fraction. Nevertheless, we found a significant increase of total RhoA in high-altitude lambs, as previously reported for this model [13], and we also observed, for the first time, a decrease of the PSer188-RhoA less active fraction under chronically hypoxic conditions. Collectively our data suggest that in newborn from species

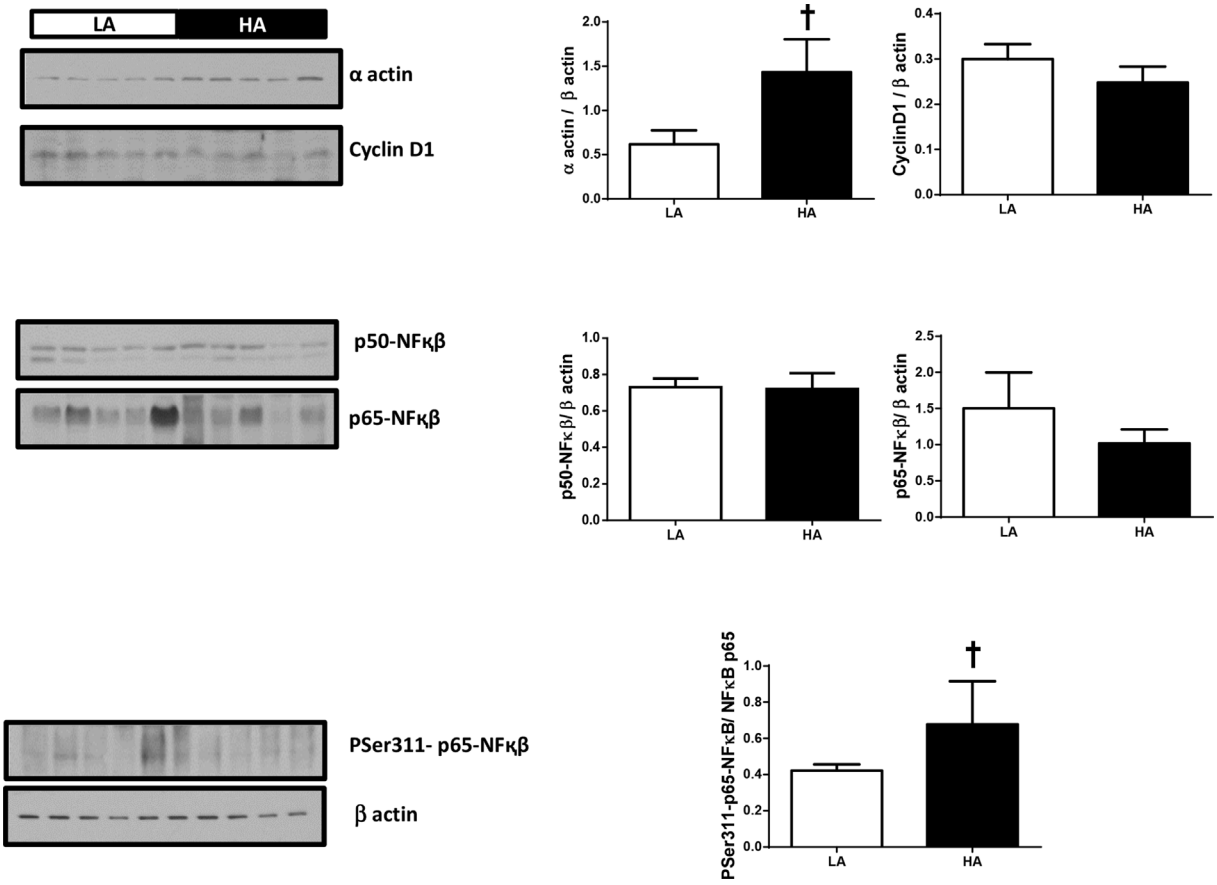

Fig. 6. Pulmonary expression and phosphorylation state of RhoA-dependent signaling proteins of cell cycle and smooth muscle remodeling. Smooth muscle α-actin, cyclin D1, p50-NFκβ, p65-NFκβ and PSer311-p65-NFκβ were evaluated in lowland (LA, open bars) and highland (HA, black bars) newborn sheep. Values are mean ± standard error of the mean. Significant differences, $P < 0.05$, student's unpaired *t*-test or Mann-Whitney test, ǂ, LA vs HA.

without ancestry at high altitude like the sheep, the enhancement of NO signaling is an important defense mechanism against hypoxia that favors pulmonary vasodilation, Ca^{2+} de-sensitization and decrease of pathological remodeling of pulmonary arteries. However, the efficacy is counteracted by parallel up-regulation of RhoA signaling, among other mechanisms, that favors pulmonary vasoconstriction, Ca^{2+}-sensitization and remodeling. In agreement with our interpretation, we did not observe increase in the pulmonary expression of MYPT1, PSer695-MYPT1 fraction, MLC_{20} or PSer19-MLC_{20} fraction in highland lambs.

Further support for this interpretation comes from the comparison of our data with those observed in species that have evolved at high altitude and have developed more efficient mechanisms to cope with sustained hypoxia. Under L-NAME infusion, the increase of basal mPAP is lower in newborn llama than sheep at equivalent altitudes [9]. Despite that, the nitrergic tone of the llama provides significant protection against increase of mPAP under basal conditions, but particularly against an acute hypoxic challenge to high-altitude newborn llamas. This increased efficacy of nitrergic tone is associated with the ability to modulate other signaling molecules downstream of NO signaling [17]. For instance, high-altitude llamas have greater expression of MYPT1, Pser695-MYPT1 fraction and decreased ROCK2 expression in lungs, compared to their lowland counterparts, suggesting that Ca^{2+}-desensitization is a mechanism that improves the vasodilation mediated by NO in these species, as reflected by a lower Pser19MLC_{20}/MLC_{20} ratio under chronic hypoxia [17]. Ca^{2+} de-sensitization through blunted ROCK function as protective strategy against hypoxic pulmonary hypertension has been also documented in adult yak [31]. Other mechanisms that explain the difference of NO-mediated vasodilation between species tolerant and non-tolerant to hypoxia are related to the expression and function of the soluble guanylate cyclase, the hemoxygenase-carbon monoxide (HO–CO) vasodilator signaling and the regulation of substrates and activity of eNOS by arginase activity and asymmetric dimethylarginine (ADMA), respectively. Both soluble guanylate cyclase (sGC) and hemoxygenase-1 (HO1) expression, as well as pulmonary carbon monoxide are decreased in the highland newborn sheep, whilst sGC expression is preserved and HO1 and pulmonary carbon monoxide are increased in the highland llama [9]. ADMA is a competitive endogenous inhibitor of eNOS, while arginase hydrolyzes arginine, the main substrate for NO production. Newborn sheep have higher plasma ADMA concentration and pulmonary arginase activity than newborn llamas, and when they are exposed to hypoxia, the sheep increase their ADMA concentration and arginase activity [32]. The up-regulation of arginase is also reported in pulmonary endothelial and smooth muscle cells from humans submitted to chronic hypoxia [33,34]. This could lead to a decrease in the cellular arginine content under low oxygen conditions in species prone to hypoxic pulmonary hypertension like the human and the sheep. In contrast, in response to hypoxia, the llama does not change either arginase or ADMA [31], while the yak decreases ADMA [35].

Finally, another aspect to take in consideration is that despite NO production is increased during chronic hypoxia, its bioavailability maybe reduced through rapid inactivation by oxidative stress in species that are less adapted, compared to those better adapted to high altitude, like the sheep and llama respectively [12,36,37]. Species that are tolerant to hypoxia like the mole rat, show increased expression of antioxidant enzymes like catalase, superoxide dismutase, glutathione peroxidase and hemoxygenase-1 [38,39]. Moreover, antioxidant treatment with melatonin increases NO bioavailability and reduces pulmonary arterial pressure in high altitude newborn sheep [12]. This evidence supports the concept that NO bioavailability is decreased at high

altitude due to excessive reactive oxygen species. Therefore, despite a compensatory increased synthesis of NO, the vasodilator effectiveness is markedly depressed.

In addition to over-constriction, pulmonary circulation also responds to chronic hypoxia exposure with pathological remodeling mainly characterized by thickening of the medial layer of the wall from distal pulmonary arteries. This is often the result of proliferation of pulmonary artery smooth muscle cells and *trans*-differentiation of pulmonary artery fibroblast to myofibroblasts, a hybrid cell phenotype that express smooth muscle cell markers like α-actin but is poorly responsive to vasodilator stimuli [40]. Inhalation of NO reduces this pathological remodeling of pulmonary arteries in rats with pulmonary hypertension [41]. Nevertheless, this pulmonary arterial remodeling with thickening of medial layer of small pulmonary is present in high altitude lambs despite up-regulation of eNOS [10]. Pulmonary arterial remodeling is a complex process that involves multiple differentiation and proliferative pathways of pulmonary smooth muscle cells and fibroblasts. We observed an increase of RhoA expression and a decrease of its less active fraction PSer188-RhoA in lungs of high-altitude lambs. RhoA activation is at the center of several pathways that stimulate transcription factors such as activator protein-1 (AP-1), myocardin-related transcription factor-A (MRTF-A) and nuclear factor kappa-light-chain-enhancer of activated B Cells (NFκβ), which in turn can stimulate cyclin D1, smooth muscle α-actin or VEGF receptor expression among others, to finally regulate cell cycle progression, proliferation, differentiation and angiogenesis [42]. We did not find changes in the pulmonary expression of cyclin D1 or total p50-NFKβ and p65-NFKβ, but its activated PSer311-p65-NFKβ fraction increased. We also observed an increased smooth muscle α-actin expression, suggesting that pulmonary arterial remodeling mediated through RhoA is opposing to the potential vasodilator and anti-remodeling action of NO.

5. Conclusions

Our results support the following conclusions: (i) NO synthesis partially contributes to set low pulmonary arterial pressure and resistance under basal conditions and under acute hypoxia in low altitude lambs; (ii) NO synthesis is greatly up-regulated in newborn lambs at 3600 m altitude, where it contributes to partially offset the hypoxic increase of pulmonary arterial pressure and resistance under basal conditions but it does not provide additional vasodilator defense under an acute hypoxic challenge of greater magnitude; (iii) NO function is not up-regulated in systemic circulation of chronically hypoxic lambs, but it contributes to control systemic arterial pressure and resistance under basal conditions and under acute hypoxia in both low and high altitude lambs; (iv) Nitrergic tone is also important to positively regulate heart rate and cardiac output under basal conditions and acute hypoxia in low- and high-altitude newborn lambs; (v) the efficacy of increased pulmonary vascular nitrergic tone in high-altitude lambs, to counteract hypoxic pulmonary hypertension, is limited because RhoA signaling is also up-regulated and is opposing to the vasodilator action of NO, probably through increase of Ca^{2+}-sensitization and remodeling.

In summary, the results of this study indicate that NO is a major determinant of the pulmonary vascular tone during the neonatal life in the chronically hypoxic and pulmonary hypertensive neonatal lambs. Further, the high levels of NO production in the neonatal sheep appears to partially offset a dangerous increase in pulmonary pressure in highlands, preventing clinical pictures such as pulmonary edema or right heart failure. Moreover, NOS inhibition by L-NAME, uncovered other vasoactive agent, like RhoA, that seems to play an important role in the control of the neonatal pulmonary circulation at highlands.

Conflicts of interest

No conflicts of interest, financial or otherwise, are declared by the authors.

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