Am J Physiol Lung Cell Mol Physiol 311: L788–L799, 2016.
First published August 19, 2016; doi:10.1152/ajplung.00230.2016.

CALL FOR PAPERS | *Ion Channels and Transporters in Lung Function and Disease*

2-Aminoethyldiphenylborinate modifies the pulmonary circulation in pulmonary hypertensive newborn lambs partially gestated at high altitude

S. Castillo-Galán,[1,2]* S. Quezada,[1,2]* F. Moraga,[3] G. Ebensperger,[2,4] E. A. Herrera,[2,4] F. Beñaldo,[2] I. Hernández,[1,2] R. Ebensperger,[1] S. Ramírez,[2] A. J. Llanos,[2,4] and R. V. Reyes[1,2]

[1]*Laboratorio de Bioquímica y Biología Molecular de la Hipoxia, Universidad de Chile, Santiago, Chile;* [2]*Programa de Fisiopatología, Instituto de Ciencias Biomédicas (ICBM), Facultad de Medicina, Universidad de Chile, Santiago, Chile;* [3]*Facultad de Medicina, Universidad Católica del Norte, Coquimbo, Chile; and* [4]*International Center for Andean Studies (INCAS), Universidad de Chile, Santiago, Chile*

Submitted 6 June 2016; accepted in final form 14 August 2016

Castillo-Galán S, Quezada S, Moraga F, Ebensperger G, Herrera EA, Beñaldo F, Hernández I, Ebensperger R, Ramírez S, Llanos AJ, Reyes RV. 2-Aminoethyldiphenylborinate modifies the pulmonary circulation in pulmonary hypertensive newborn lambs partially gestated at high altitude. *Am J Physiol Lung Cell Mol Physiol* 311: L788–L799, 2016. First published August 19, 2016; doi:10.1152/ajplung.00230.2016.—Calcium signaling through store-operated channels (SOC) is involved in hypoxic pulmonary hypertension. We determined whether a treatment with 2-aminoethyldiphenylborinate (2-APB), a compound with SOC blocker activity, reduces pulmonary hypertension and vascular remodeling. Twelve newborn lambs exposed to perinatal chronic hypoxia were studied, six of them received a 2-APB treatment and the other six received vehicle treatment for 10 days in both cases. Throughout this period, we recorded cardiopulmonary variables and on *day 11* we evaluated the response to an acute hypoxic challenge. Additionally, we assessed the vasoconstrictor and vasodilator function in isolated pulmonary arteries as well as their remodeling in lung slices. 2-APB reduced pulmonary arterial pressure between the 3rd and 10th days, cardiac output between the 4th and 8th days, and pulmonary vascular resistance at the 10th day of treatment. The pulmonary vasoconstrictor response to acute hypoxia was reduced by the end of treatment. 2-APB also decreased maximal vasoconstrictor response to the thromboxane mimetic U46619 and endothelin-1 and increased maximal relaxation to 8-bromoguanosine 3′,5′-cyclic monophosphate (8-BrcGMP). The maximal relaxation and potency to phosphodiesterase-5 and Rho-kinase inhibition with sildenafil and fasudil, respectively, were also increased. Finally, 2-APB reduced the medial and adventitial layers' thickness, the expression of α-actin, and the percentage of Ki67-positive nuclei of small pulmonary arteries. Taken together, our results indicate that 2-APB reduces pulmonary hypertension, vasoconstrictor responses, and pathological remodeling in pulmonary hypertensive lambs. We conclude that SOC targeting may be a useful strategy for the treatment of neonatal pulmonary hypertension; however, further testing of specific blockers is needed.

hypoxia; neonatal pulmonary hypertension; 2-aminoethyldiphenylborinate; pulmonary artery remodeling

NEONATAL PULMONARY HYPERTENSION is a syndrome related to the failure to reduce the pulmonary artery pressure (PAP) and resistance (PVR) at birth. This condition hinders the establishment of a normal cardiopulmonary function of the newborn and is often complicated by right ventricular hypertrophy, cardiac failure, and death (1). One of main causes of neonatal pulmonary hypertension is perinatal hypoxia, as seen in high altitude pregnancies and births (18, 19, 21, 23, 26, 42, 49). In pulmonary circulation, hypoxia triggers changes in gene expression and posttranslational modifications of several proteins involved in the generation of vasoconstrictor and vasodilator responses. In addition, chronic hypoxia induces differentiation and proliferation of pulmonary vascular smooth muscle cells and fibroblasts (56, 67). These changes result in both increased vasoconstrictor and decreased vasodilator functions and in pathological remodeling characterized by wall thickening with increased medial and adventitial layer (19, 50). Inhaled nitric oxide (iNO) and extracorporal membrane oxygenation (ECMO) are the only known treatments universally accepted for the neonatal pulmonary hypertension. Nevertheless, iNO fails to lower mPAP in 40% of the cases, and there is no concluding evidence that it contributes to reverse the pathological remodeling (18). When iNO fails, the last treatment is ECMO, but this treatment has high morbidity and mortality rates (2). Moreover, in some cases there is a reappearance of the disease once the drug is removed. Currently, there are other treatments available to treat pulmonary hypertension in adults and children, such as prostacyclin analogs, endothelin-1 (ET-1) receptor blockers, phosphodiesterase 5 (PDE5) inhibitors, and guanylate cyclase stimulators, but their use is still not approved for treating neonatal pulmonary hypertension. (2, 35, 60). Therefore, the development of treatments efficient in reducing overconstriction as well as pathological remodeling is critically needed.

Calcium signaling is key for contraction, differentiation, and proliferation of pulmonary vascular cells. In the past 10 years, a significant body of evidence regarding the store-operated channels (SOC), a calcium-permeable class of plasma membrane cationic channels, led to the proposal that these channels were important players in both the intrinsic vasoconstrictor response and in the pulmonary artery remodeling induced by chronic hypoxia (15, 16, 34). Most of this evidence comes from pharmacological or genetic suppression experiments performed in cultured cells, isolated organs or vessels, or transgenic mice in adult models of pulmonary hypertension. How-

* S. Castillo-Galán and S. Quezada contributed equally to this work.
Address for reprint requests and other correspondence: R. Reyes, Programa de Fisiopatología, Instituto de Ciencias Biomédicas, Facultad de Medicina, Campus Oriente, Universidad de Chile. Avenida Salvador 486, Providencia, Código Postal 7500922, Santiago, Chile (e-mail: vicreyes@med.uchile.cl).

ever, in vivo studies using inhibitors of SOC are scarce, particularly using neonatal models of the disease. Despite some recent progress in the identification of SOC inhibitors, like pyrazole-, imidazole-, and diphenylborinate-derived compounds, the few studies describing their selectivity, potency, and toxicity are not sufficient to initiate clinical trials. Furthermore, many inhibitors are still not easily available for studies in large animal models (9, 61). Until that point is reached, in vivo assays with other SOC inhibitors, regardless of their specificity, could be helpful to characterize the effect of long term SOC targeting. We have previously observed that a single dose of 2-APB and SKF-96365, two drugs with inhibitory action on SOC, reduce hypoxic- and agonist-induced pulmonary vasoconstriction in newborn lambs that had been gestated, born, and studied under chronic hypoxia at high altitude (47). In addition, we have also reported that newborn lambs with partial gestation under chronic hypoxia have elevated mean pulmonary arterial pressure (mPAP), increased pulmonary artery reactivity, and pathological pulmonary artery remodeling that persist at sea level under normoxic conditions (26). In the present study, we hypothesized that a SOC blockade treatment would be able to revert the pulmonary hypertension and vascular remodeling observed in a model using lambs partially gestated under chronic hypoxia and studied at sea level. To test this hypothesis, we used pulmonary hypertensive lambs to evaluate the efficacy of a 10-day treatment with 2-APB to revert the elevated pulmonary arterial pressure and vascular remodeling. We studied and compared newborn lambs treated with 2-APB or its vehicle with an integrative approach: *1*) in vivo cardiopulmonary response under basal and acute hypoxic conditions; *2*) ex vivo reactivity of small pulmonary arteries; and *3*) pulmonary artery remodeling through morphometric and immunohistochemical analyses.

METHODS

All experimental protocols were reviewed and approved by the Faculty of Medicine Bioethics Committee of the University of Chile (CBA No. 0476 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 National Institutes of Health (NIH Publication No. 85-23, Revised 1996) and adhered to American Physiological Society's "Guiding Principles in the Care and Use of Animals."

Animals. Twelve pregnant ewes (*Ovis aries*) spent the first 30% of their gestation (50 days) at lowlands (Lluta Research Station, 60-m altitude, Faculty of Medicine, University of Chile), and the last 70% of their gestation under chronic hypoxia at high altitude (Putre Research Station, INCAS, University of Chile, 3.600-m altitude) until delivery. Two days after birth, the newborns and their mothers returned to low altitude (Lluta Research Station, 60-m altitude) where they were housed in an open yard with access to food and water ad libitum.

Surgical preparation and in vivo experiments. At 3 days of age, lambs were chronically instrumented for in vivo studies as described previously (23, 24, 26, 47). Briefly, animals were anesthetized with an association of ketamine (10 mg/kg im) and xylacine (0.05 mg/kg im) and also an additional local infiltration of 2% lidocaine. Oxytetracyclin (20 mg/kg sc) and sodium metamizole (0.1 mg/kg im) were given for 3 days after surgery. Polyvinyl catheters were placed into the descending aorta and inferior vena cava and a Swan-Ganz catheter was placed into the pulmonary artery to record cardiopulmonary variables.

At day four after birth, the lambs were randomly divided in two groups and submitted to a ten-day treatment with a single daily dose of infusion vehicle [dimethyl sulfoxide (DMSO): NaCl 1:10 iv, $n = 6$] or 2-APB infusion (10 mg/kg bolus in vehicle iv, $n = 6$) every morning. Pulmonary arterial pressure (PAP), systemic arterial pressure (SAP), and heart rate (HR) were recorded via a data acquisition system (PowerLab/8SP System and LabChart v7.0 Software; ADInstruments) connected to a computer. Cardiac output (CO) was determined 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 CO computer (COM-2 model, Baxter). Mean PAP (mPAP), mean SAP (mSAP), pulmonary (PVR), and systemic (SVR) vascular resistances were calculated as described previously (23, 26, 47). Arterial blood samples were taken daily to determine arterial pH, P_{O_2}, P_{CO_2}, hemoglobin saturation percentage (Sa_{O_2}), total hemoglobin (THb), and oxygen content (O_{2ct}) (IL-Synthesis 25, Instrumentation Laboratories; measurements corrected to 39°C). Cardiopulmonary variables and arterial blood gases were measured every day, 5 min before the infusion, so that the first day corresponds to the basal condition before the start of the treatment, and the following days are values recorded 24 h after the infusion of the previous day.

Twenty-four hours after the last infusion, animals were subjected to a 3-h experimental protocol as previously described (23, 26, 47), consisting in 1 h of basal recording (breathing room air), 1 h of acute isocapnic hypoxia, and 1 h of recovery during which they returned to breath room air. 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 was passed at a rate of 20 l/min to reach an arterial P_{O_2} of ~30 mmHg with a constant P_{CO_2}. Cardiopulmonary variables and arterial blood gases were also recorded during this protocol. The contractile response of the pulmonary circulation to acute hypoxia was expressed as the ratio between the increase of mPAP and the decrease of arterial P_{O_2} according to the formula:

$$\Delta mPAP/\Delta P_{O_2} = \left(mPAP_{Hypoxia} - mPAP_{Basal}\right)/\left(P_{O_{2Basal}} - P_{O_{2Hypoxia}}\right)$$

where $mPAP_{Hypoxia}$ and $P_{O_{2Hypoxia}}$ are the average values of mPAP and arterial P_{O_2} during acute hypoxia and $mPAP_{Basal}$ and $P_{O_{2Basal}}$ are the average values of mPAP and arterial P_{O_2} during the basal period.

Ex vivo and in vitro experiments. Twenty-four hours after the acute hypoxic challenge test, the lambs underwent euthanasia with an overdose of sodium thiopental (100 mg/kg iv) and lung samples were taken for ex vivo and in vitro analyses.

Wire myography. The lungs were removed and immediately immersed in cold Krebs solution. Parenchymal small pulmonary arteries (100–300 μm) were dissected and isolated from the right lung, mounted on a wire myograph, and maintained at 37°C and aerated with 95% O_2-5% CO_2 in Krebs buffer. The resting tension, defined as transmural pressure exerted in the vessels (17–25 mmHg, as seen in the in vivo studies), was calculated by stretching the vessel in a stepwise manner to a standardized tension equivalent to the physiological transmural pressure (43). 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 second, because this degree of stretch gives the maximal vascular response (43, 55, 64).

Concentration-response curves (CRCs) were constructed for contraction induced by potassium chloride (KCl; 6.25 to 125 mM), endothelin-1 (ET-1; 10^{-13} to 10^{-7} M), U46619 (10^{-13} to 10^{-5} M), serotonin (5-HT; 10^{-10} to 10^{-4} M), and for relaxation induced by sodium nitroprusside (SNP; 10^{-10} to 10^{-3} M), 8-bromoguanosine 3′,5′-cyclic monophosphate (8-BrcGMP; 10^{-10} to 10^{-4} M), sildenafil (10^{-10} to 10^{-5} M), and fasudil (10^{-10} to 10^{-4} M). Relaxation curves were performed after the addition of 10^{-7} M ET-1 as a preconstrictor agent. The response to every CRC dose was recorded 2 min after each

addition. Contractile responses were expressed in terms of tension (N/m) for KCl or percentage relative to the tension evoked by a submaximal dose of potassium (62.5 mM) for ET-1, U46619, or 5-HT, to normalize for differences arising from variability between different vessels analyzed. Dilator responses were expressed as percentage relative to the maximal contraction given by the preconstrictor agent. CRCs were analyzed in terms of sensitivity and maximal responses by fitting experimental data to the Boltzmann equation or an Agonist-response function as appropriate (Prism 5.0; GraphPad Software, La Jolla, CA) (26).

Histological staining and immunohistochemistry. The left lung was perfused with saline and then fixed in 4% paraformaldehyde in PBS for 24 h. The fixed tissue was embedded in paraffin, cut in 5- to 10-μm serial slices, and submitted to a van Gieson staining. The percentage of wall thickness and adventitia for the small arteries (150–300 μm) was calculated as described previously (24, 26, 41).

Immunohistochemical detection for α-actin and Ki-67 was performed with commercial antibodies (Sigma, dilution: 1:200 and Clone MIB-1, Dako, Glostrup, Denmark, dilution 1:100, respectively). For all procedures the antigen retrieval was performed at 100°C with citrate buffer pH: 6, 1X. (Dako). Primary antibody incubations were performed for 4 h for α-actin and overnight for Ki-67 at 4°C and labeling was developed using a HRP-diaminobenzidine kit (Envision TM+; Dako). The α-actin immunoreactivity of small arteries muscle layer was calculated as described previously (27) and the percentage of Ki67-positive nuclei in the medial layer of pulmonary arteries smooth muscle cells was calculated as a ratio of positive Ki-67 vs. total nuclei (72).

Statistical analysis. Data are expressed as means ± SE. Groups were compared by two-way ANOVA for repeated measures 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 $P < 0.05$ (20).

RESULTS

Neonatal weights and crown-rump length. Animals from both experimental groups had similar initial weights (3.92 ± 0.39 vs. 4.30 ± 0.19 kg for vehicle group and 2-APB group respectively). Also, there was no difference in the weight by the end of the treatment (5.62 ± 0.43 vs. 6.07 ± 0.39 kg for vehicle group and 2-APB group, respectively). The crown-rump length was also similar at the beginning (41.38 ± 1.48 vs. 40.28 ± 1.22 cm for vehicle and 2-APB lambs) and after the end of the treatment (49.73 ± 1.45 vs. 48.46 ± 1.66 cm).

Basal cardiopulmonary variables during the treatment. Before starting the treatment, both 2-APB and vehicle-infused lambs showed similar cardiopulmonary variables (Fig. 1). The 2-APB treatment decreased significantly the mPAP from the 3rd day of administration, until the end of the treatment (14.28 ± 0.63 vs. 19.90 ± 1.28 mmHg at *day 1*0 for 2-APB and vehicle, respectively; Fig. 1*A*). The daily recording of HR showed similar values between 2-APB- and vehicle-treated animals (Fig. 1*B*). Furthermore, 2-APB transiently decreased CO between the 4th and the 8th day of treatment, but by the end of the treatment there were no differences (Fig. 1*C*). The PVR was similar in both experimental groups between the 1st and the 9th day of infusion, but it was lower in the 2-APB-treated lambs at the 10th day of treatment (0.040 ± 0.002 vs. 0.051 ± 0.002 mmHg·ml·min^{-1}·kg^{-1}at *day 10* for 2-APB and vehicle, respectively; Fig. 1*D*). In contrast, mSAP was similar in both experimental groups along the treatment (Fig. 2*A*), while SVR was higher in the 2-APB lambs relative to control lambs between the 6th and 8th days of treatment, recovering to similar levels as control lambs by the end of the experimental period (Fig. 2*D*).

Arterial ph and blood gases during acute hypoxia. Values for basal pH, P_{CO_2}, P_{O_2}, THb, Sa_{O_2}, and O_2 content (O_{2ct}) were similar in both groups (Table 1). During the acute hypoxic challenge, a similar fall in P_{O_2}, Sa_{O_2}, and O_{2ct} occurred in both groups of lambs, with no changes in P_{CO_2} (Table 1). pH decreased slightly in vehicle-infused animals during acute hypoxia compared with the basal value; however, in the 2-APB-treated lambs' pH showed only a tendency to decline ($P = 0.059$) in the same period (Table 1). During recovery, all these variables returned to similar values in both groups (Table 1).

Cardiovascular response during acute hypoxia. Basal mPAP was lower in the 2-APB-treated group than control lambs (15.88 ± 1.38 vs. 20.69 ± 0.96 mmHg, respectively, $P = 0.03$). During acute hypoxia, both groups significantly increased their mPAP. Furthermore, both groups reduced mPAP during recovery but did not reach baseline value during the time of recording (Fig. 3*A*). Nevertheless, the mPAP was also lower during acute hypoxia and recovery in the 2-APB-treated lambs than in the control lambs (Fig. 3*A*). Moreover, when normalized by the decrease of P_{O_2}, the acute increase of mPAP observed in the treated lambs was nearly half of the value observed in the control lambs (Fig. 3*B*). Both groups showed similar basal CO and a similar increase during acute hypoxia. However, during recovery only 2-APB group returned to basal levels while in control animals CO remained elevated (Fig. 3*C*). In addition, there were no differences in PVR between both groups during basal and recovery periods, but under acute hypoxia, the 2-APB-treated lambs showed a lower PVR than the vehicle-infused lambs (Fig. 3*D*). Furthermore, PVR significantly increased during recovery in the 2-APB lambs (Fig. 3*D*). Finally, mSAP remained unchanged during all the hypoxemic challenge, while SVR decreased during acute hypoxia and was restored during recovery in both groups (Fig. 4, *A* and *B*).

Isolated pulmonary arteries reactivity. Newborn lambs treated with 2-APB and vehicle showed similar concentration-response curves to potassium and to 5-HT without significant modification of their corresponding maximal contraction, EC_{50} or pD_2 (Fig. 5, *A* and *B*). In contrast, 2-APB-treated lambs exhibited a significantly lower maximal contraction in response to the thromboxane mimetic U46619 compared with vehicle-treated control lambs, without a difference in their pD_2 (Fig. 5*C*). The concentration-response curve to ET-1 was attenuated in the 2-APB-treated lambs relative to their vehicle-treated counterparts, with decreased maximal contraction but similar pD_2 (Fig. 5*D*).

Regarding the vasodilator stimuli, 2-APB-treated animals showed no difference in SNP-driven relaxation compared with the vehicle-infused animals (Fig. 6*A*). However, 2-APB-treated lambs improved their relaxation to increasing concentrations of 8-BrcGMP and showed a significantly higher maximal relaxation and reduced pD_2 than control lambs (Fig. 6*B*). In addition, the treatment with 2-APB also enhanced the relaxation to increasing concentrations of sildenafil and fasudil, with increased maximal relaxation and pD_2 relative to the vehicle group (Fig. 6, *C* and *D*).

Morphometry and remodeling markers. Morphometric analysis of small pulmonary arteries revealed a significant decrease in both vascular medial and adventitial layer thickness in

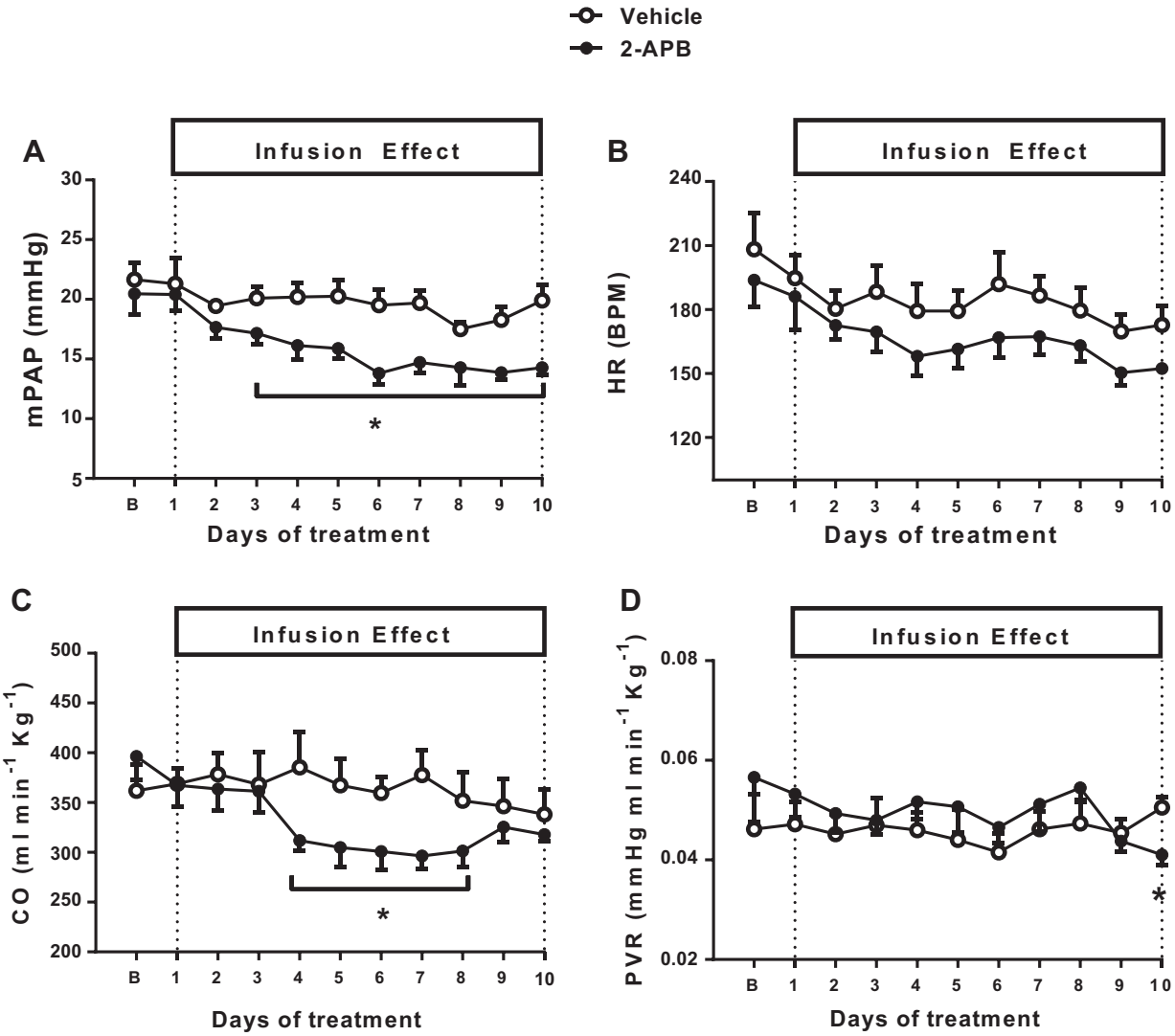

Fig. 1. Cardiopulmonary variables during 2-aminoethyldiphenylborinate (2-APB) treatment. Basal values for mean pulmonary artery pressure (mPAP; *A*), heart rate (HR; *B*), cardiac output (CO; *C*) and pulmonary vascular resistance (PVR; *D*) were measured 5 min before the daily infusion of vehicle (white circles) or 2-APB (black circles). The B day corresponds to basal values before the start of the treatment and the following days to values recorded 24 h after the infusion of the previous day. Values are the average ± SE. $*P < 0.05$, significant differences for vehicle vs. 2-APB, two-way ANOVA for repeated-measures with Newman-Keuls post hoc test.

2-APB-treated compared with vehicle-treated lambs (Fig. 7*A*). Furthermore, 2-APB animals showed a decreased immunoreactivity to α-actin smooth muscle (Fig. 7*B*). This was associated with a diminished percentage of Ki67-positive nuclei in the medial layer of small pulmonary arteries from 2-APB lambs relative to vehicle-treated lambs (3.72 ± 0.55 vs. 7.50 ± 1.50, respectively, $P = 0.039$).

DISCUSSION

In this study we tested the hypothesis that therapy with 2-APB is able to reduce the elevated pulmonary arterial pressure and the pathological pulmonary vascular remodeling in newborn lambs with partial gestation under chronic hypoxia. To the best of our knowledge, this is the first study assessing the daily cardiopulmonary changes with a long-term 2-APB treatment in neonates. Our results showed a decrease of the pulmonary arterial pressure and vasoconstrictor response to acute hypoxia in vivo, a decrease in the response to vasoconstrictor, and an increase in the response to vasodilator stimuli of the small pulmonary arteries ex vivo, as well as a marked diminution in the pathological pulmonary artery remodeling.

The 2-APB-infusion reduced the mPAP in 5 - 6 mmHg at the end of treatment compared with the initial values before starting the treatment and also to the control vehicle-infused group, reaching about 14 mmHg, a value that is close to the 10–12 mmHg observed in lowland newborn lambs (23, 24, 25, 26, 47). The initial decrease in mPAP may be in part caused by a transient drop in CO since there is a decrease of ~20 to 25% in this variable between the 4th and the 8th day of treatment. This transient decrease of CO may be due to a reduction in intracellular cardiomyocyte calcium concentration as an indirect effect of 2-APB on ryanodine receptor (RyR)-mediated calcium release through its potential effects on cardiac SOC or ultimately on inositol triphosphate receptors (IP_3R) (12, 14, 71). Furthermore, there is also an effect of 2-APB on the contractility of the pulmonary arteries, considering the obser-

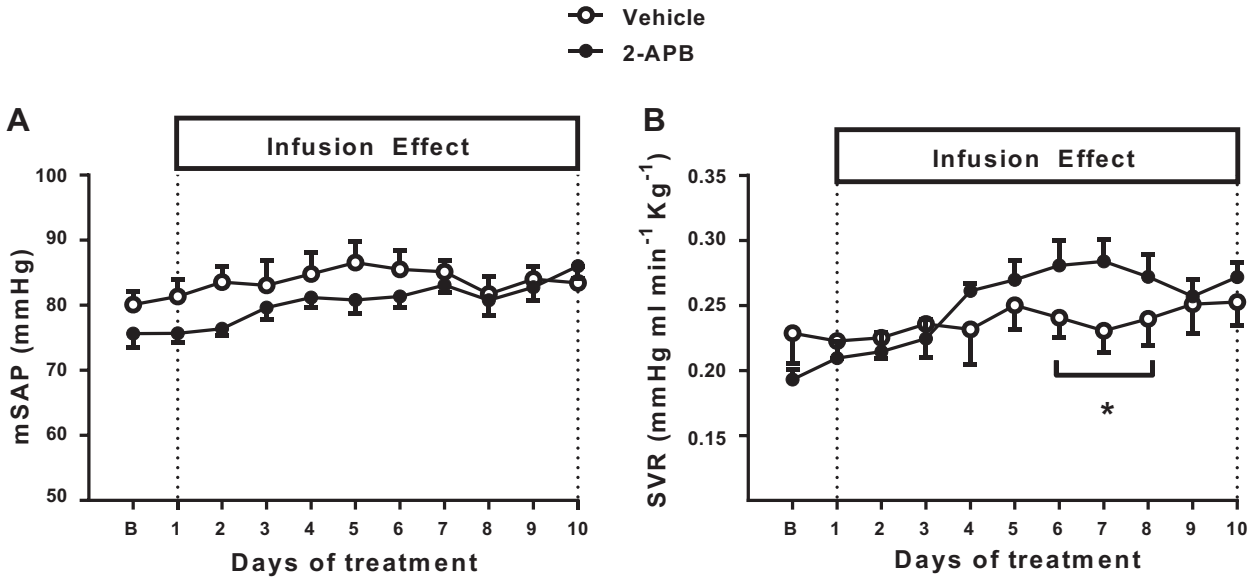

Fig. 2. Cardiovascular variables during 2-APB treatment. Basal values for mean systemic artery pressure (mSAP; *A*) and systemic vascular resistance (SVR; *B*) were recorded 5 min before the daily infusion of vehicle (white circles) or 2-APB (black circles). The B day corresponds to basal values before treatment, and the following days to values recorded 24 h after the infusion of the previous day. Values are the average ± SE. $*P < 0.05$, significant differences for vehicle vs. 2-APB, two-way ANOVA for repeated measures with Newman-Keuls post hoc test.

vation that CO recovered to values similar to those of control lambs on the ninth and tenth day and PVR decreased on the last day of the treatment, reaching a value close to the PVR value observed in lowland newborn lambs of an equivalent age (37). Concerning the systemic variables, mSAP remained unchanged during the treatment, consistent with the lack of effect of 2-APB on mSAP in highland lambs treated with single doses of 2-APB (47) or on the independence of rat femoral arteries contractility on store-operated calcium entry (SOCE) (53). The SVR was higher in the 2-APB-infused lambs between the 6th and the 8th day of treatment. This transient increase of SVR could be the result of the decrease of CO triggering the baroreflex response to maintain constant the mSAP (22). An alternative explanation could be a local peripheral vascular response to the transient decrease of CO. Lower CO may impact by diminished local peripheral blood flow and decreased shear stress and therefore less vasodilator synthesis such as NO and prostacyclin (54). We speculate that the SVR increase may represent a peripheral vasodilator decrease in response to either lesser stimuli by shear stress or a direct effect of 2-APB on the endothelial cells, decreasing Ca^{2+} release.

Table 1. *Arterial pH and blood gases during an acute hypoxic challenge*

	Basal	Hypoxia	Recovery
pH			
Vehicle	7.459 ± 0.008	7.385 ± 0.029*	7.420 ± 0.014
2-APB	7.448 ± 0.010	7.410 ± 0.014	7.433 ± 0.013
P_{CO_2}, mmHg			
Vehicle	40.3 ± 0.6	40.1 ± 1.3	37.1 ± 1.5
2-APB	41.4 ± 1.4	41.0 ± 0.8	39.0 ± 1.2
P_{O_2}, mmHg			
Vehicle	96.3 ± 1.3	30.4 ± 0.5*	101.4 ± 2.6
2-APB	92.5 ± 3.8	29.5 ± 0.4*	95.9 ± 1.2
S_{O_2}, %			
Vehicle	99.4 ± 0.5	43.1 ± 1.6*	99.9 ± 0.3
2-APB	98.0 ± 1.0	42.4 ± 0.6*	97.2 ± 1.5
O_{2ct}, ml O_2/dl			
Vehicle	13.6 ± 0.8	6.3 ± 0.4*	13.1 ± 0.7
2-APB	14.3 ± 0.8	6.5 ± 0.4*	14.3 ± 0.8
THb, g/dl			
Vehicle	10.5 ± 0.6	10.9 ± 0.5	10.1 ± 0.6
2-APB	11.2 ± 0.7	11.2 ± 0.8	11.2 ± 0.7

Values are the average ± SE for arterial pH, P_{O_2}, P_{CO_2}, S_{O_2}, total hemoglobin (THb), and oxygen content (O_{2ct}). 2-APB, 2-aminoethyldiphenylborinate. $*P < 0.05$, significant differences vs. all in the same group, two-way ANOVA for repeated measures with Newman-Keuls post hoc test.

Ca^{+2} signaling is ubiquitous and pivotal for function and structure in many cell types, including vascular cells, but it may be also involved in maturation and development in a neonatal model. This concern raises the question about the potential effect of 2-APB on normal developmental processes in newborn lambs. Nevertheless, the postnatal cardiopulmonary variables recently published for untreated lowland lambs showed that they remain stable between *days 4* and *14* of age (37). Cardiopulmonary variables of vehicle-treated lambs with partial gestation under hypoxia also remained constant during the same time window. In addition, the neonatal weight and the crown-rump length are not by modified by 2-APB. The contractile response of isolated pulmonary arteries to potassium is proposed as developmentally regulated in both lowland and highland sheep (45), and this response is not modified by 2-APB in our experiments. All these observations suggests that the treatment does not interfere with normal postnatal development. Nevertheless, a group of lowland lambs treated with 2-APB is necessary to answer this question and this is beyond the scope of this work.

SOCs play an important role during the hypoxic pulmonary vasoconstriction (57, 58, 66). The blockade with 2-APB or SKF-96365 reduces the calcium entry and hypoxic contraction in rodent and ovine pulmonary arteries (47, 65). In fact, in high-altitude lambs that had been gestated, born, and studied under chronic hypoxia at the 3,600-m altitude, the attenuation of the in vivo hypoxic increase of mPAP, as well as the ex vivo

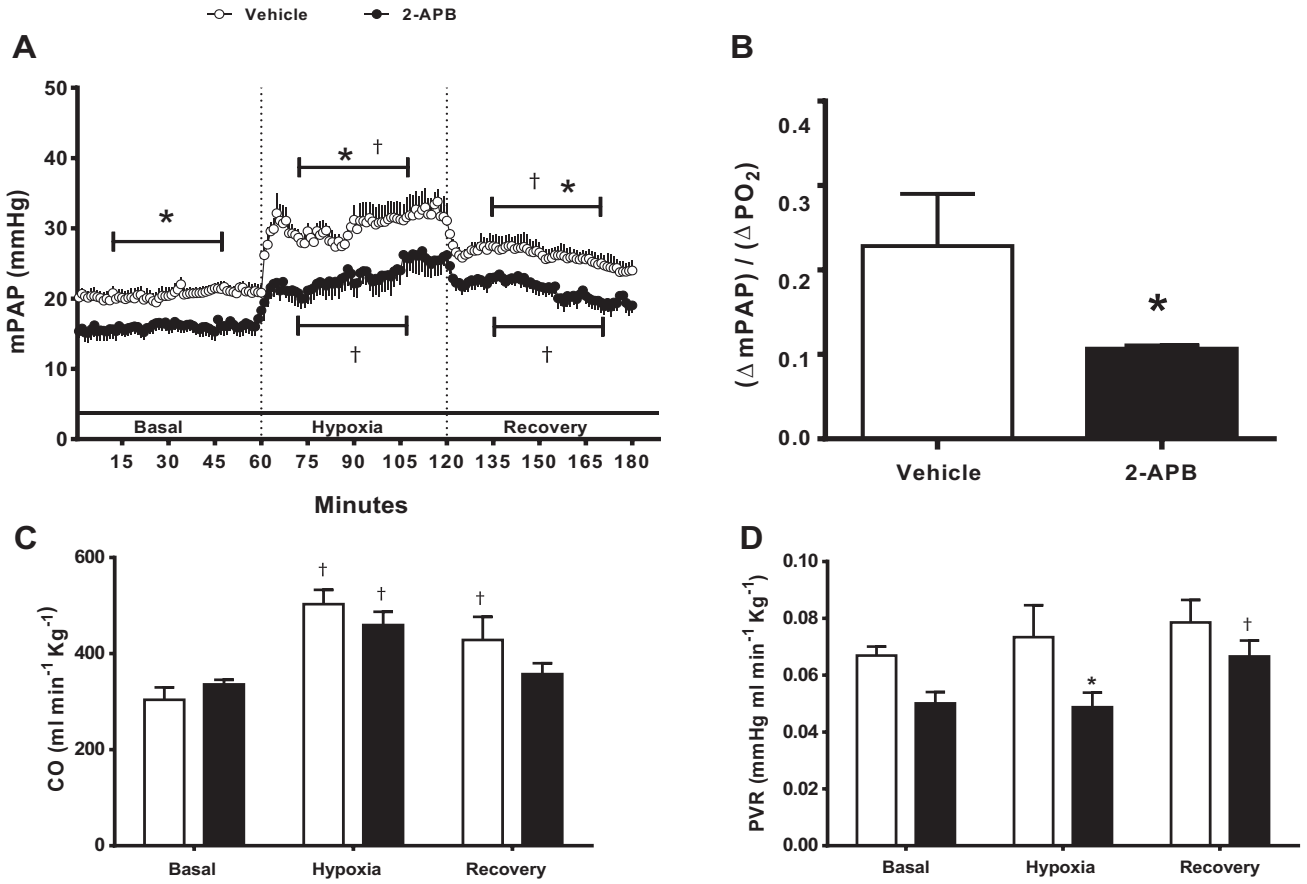

Fig. 3. Cardiopulmonary response to an acute hypoxic challenge. Vehicle-treated (white) and 2-APB-treated (black) lambs were submitted to an acute hypoxic challenge 1 day after the end of the treatment and mPAP (*A*), CO (*C*), and PVR (*D*) were determined as indicated in METHODS. The in vivo sensitivity to hypoxia (*B*) was calculated as $\Delta mPAP/\Delta Po_2 = (mPAP_{Hypoxia} - mPAP_{Basal})/(Po_{2Basal} - Po_{2Hypoxia})$. The values for CO (*C*) and PVR (*D*) are expressed as the average ± SE. for each experimental period. $^*P < 0.05$, significant differences for vehicle vs. 2-APB; †$P < 0.05$, significant differences vs. all in the same group, two-way ANOVA for repeated measures with Newman-Keuls post hoc test.

relaxation, promoted by 2-APB or SKF-96365, is greater than what is seen in control lowland lambs. These observations correlate well with the greater pulmonary canonical transient receptor potential 4 (TRPC4) and Stim1 expression in the former (47). Moreover, preliminary data from our laboratory show that the newborn lambs with partial gestation under chronic hypoxia but studied under normoxia, as used in this study, partially match with these observations, showing a greater pulmonary expression of TRPC4 and Orai1 than control lowland lambs, while IP_3R expression is unaffected (Reyes

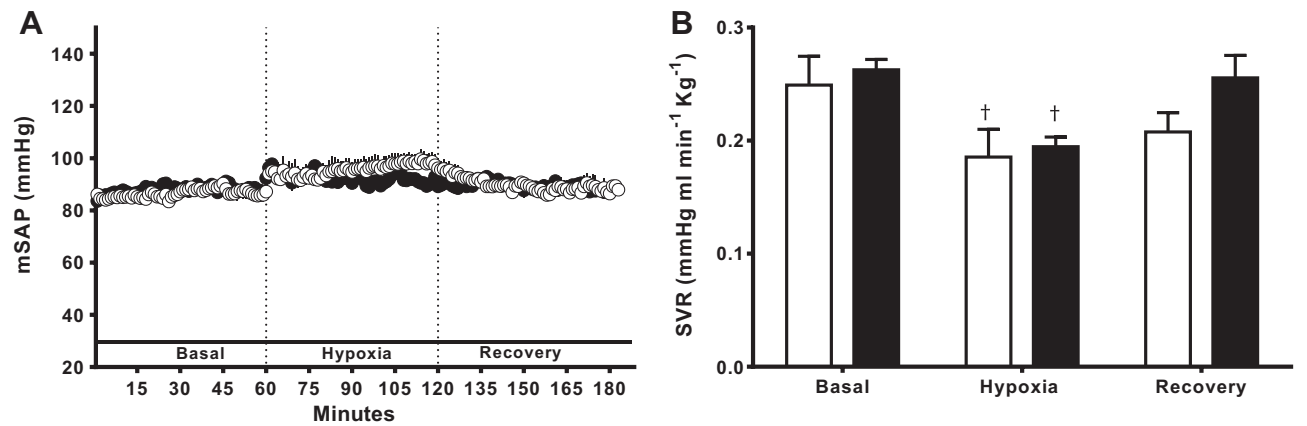

Fig. 4. Cardiovascular response to an acute hypoxic challenge. Vehicle-treated (white) and 2-APB-treated (black) animals were submitted to an acute hypoxic challenge 1 day after the end of the treatment and mSAP and SVR were determined as indicated in METHODS. *A*: continuous recording of mSAP. *B*: SVR expressed as the average ± SE. for each experimental period. †$P < 0.05$, significant differences vs. all in the same group, two-way ANOVA for repeated measures with Newman-Keuls post hoc test.

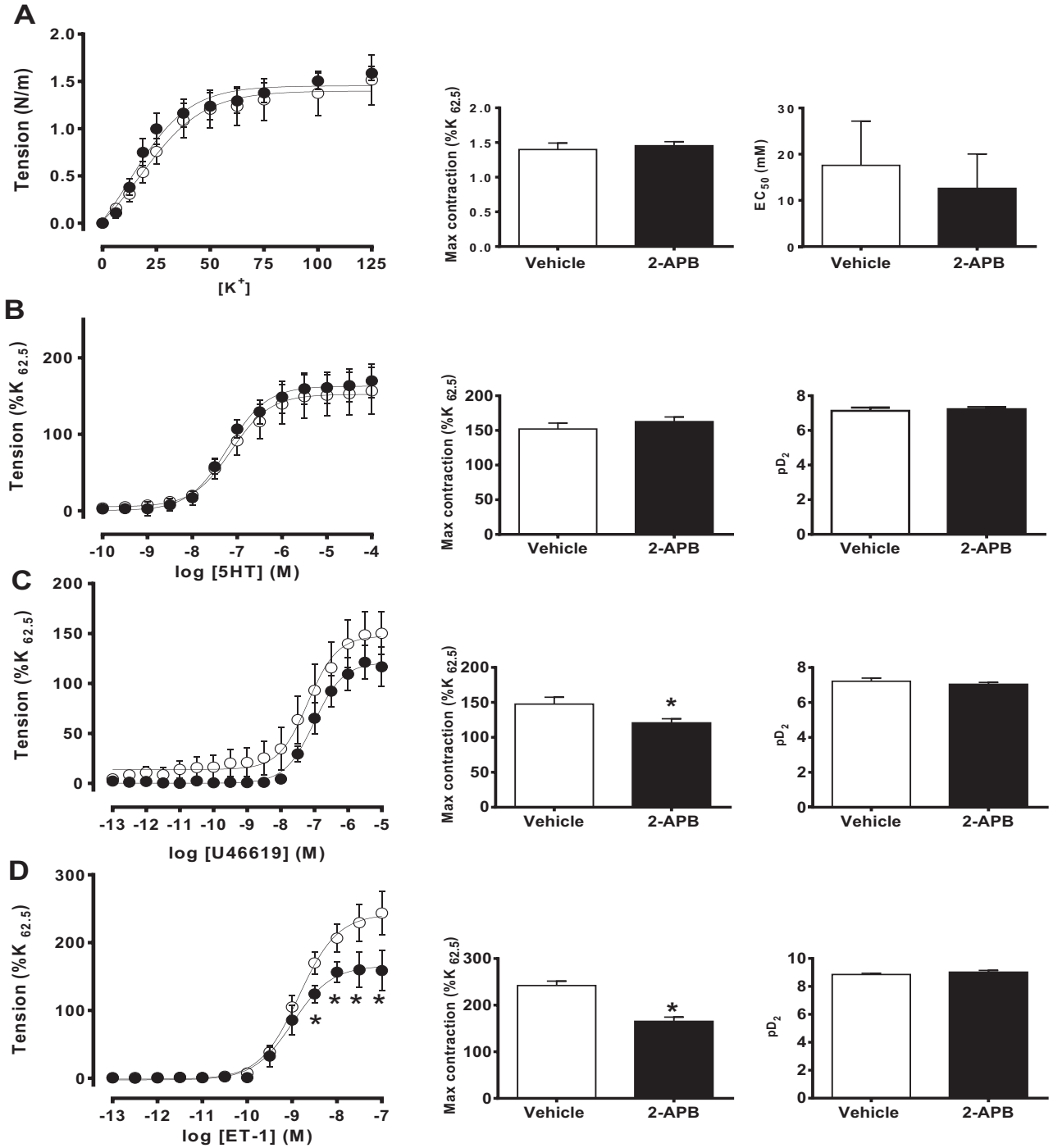

Fig. 5. Vasoconstrictor function in isolated pulmonary arteries. Small pulmonary arteries isolated 48 h after the end of the treatment were induced to contract by increasing concentrations of potassium (*A*, *left*), serotonin (5-HT; *B*, *left*), U46619 (*C*, *left*), and endothelin-1 (ET-1; *D*, *left*) in vehicle-treated (white circles) and 2-APB-treated (black circles) lambs, and the corresponding maximal contraction and potency (pD_2) were calculated as indicated in METHODS (*A–D*, *middle* and *right*). Values are the average ± SE. *$P < 0.05$, significant differences for vehicle vs. 2-APB, two-way ANOVA for repeated measures with Newman-Keuls post hoc test for concentration-response curves and unpaired *t*-test for maximal response and pD_2.

RV, unpublished observations). In 2-APB-treated lambs, we observed a smaller increase in mPAP in response to a similar decrease of PO_2, than in vehicle-treated lambs, suggesting a decrease in the sensitivity of the vasoconstrictor response to acute hypoxia that persists 24 h after the end of the treatment. Moreover, the lower PVR during acute hypoxia in the 2-APB-treated lambs compared with the vehicle-treated lambs, without variations in the CO, mSAP, or SVR, is consistent with this assumption.

Today, it is accepted that 2-APB markedly inhibits calcium influx through SOC (9, 10, 13, 36, 48, 61), despite its potential effects on other molecular targets able to directly or indirectly modify intracellular calcium, smooth muscle tension, or remodeling such as IP_3R (38), TRPM7 channels (11) or GAP

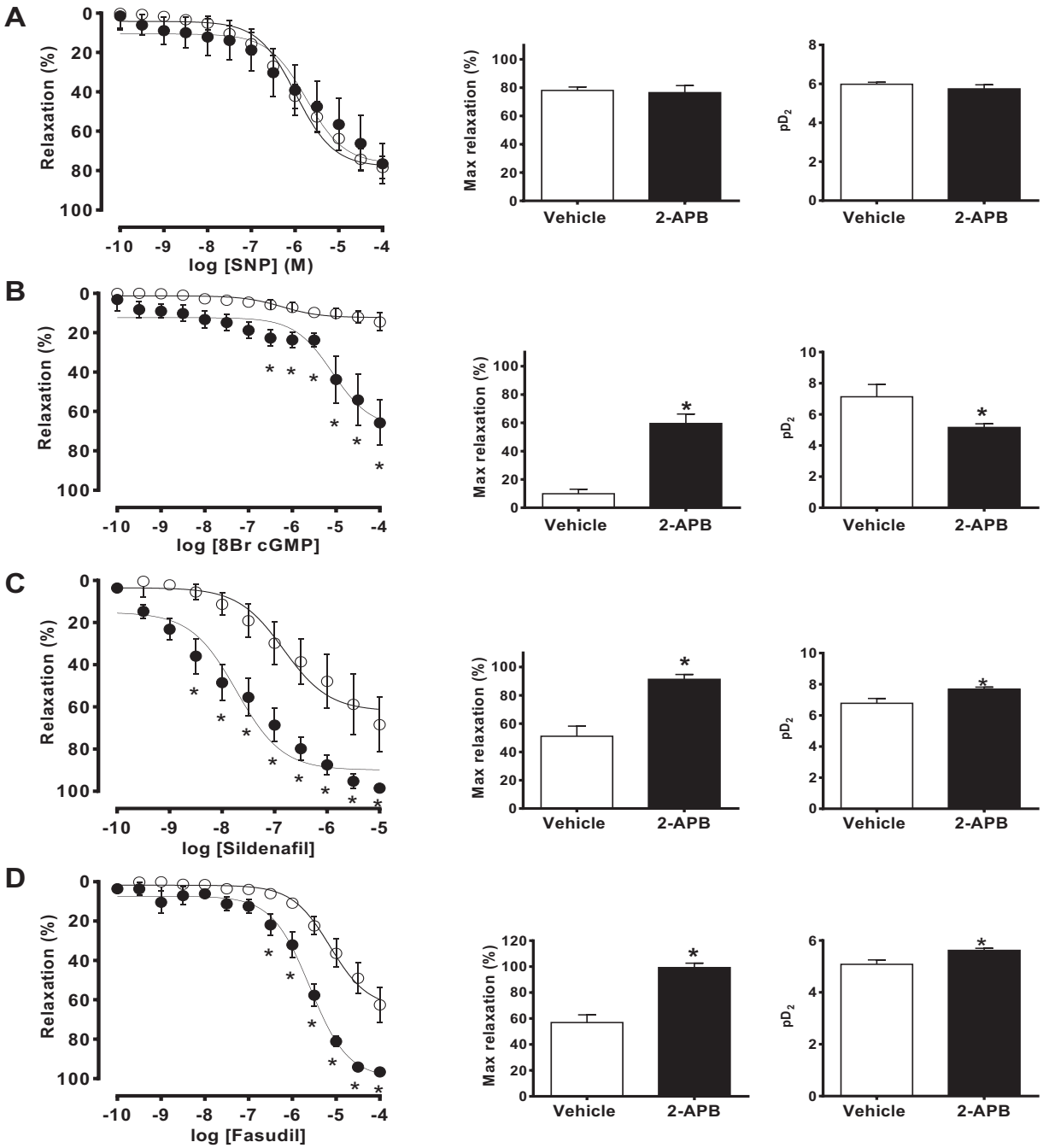

Fig. 6. Vasodilator function in isolated pulmonary arteries. Small pulmonary arteries isolated 48 h after the end of the treatment were precontracted with 10^{-7} ET-1 and then induced to relax with increasing concentrations of SNP (*A*, *left*), 8-bromoguanosine 3′,5′-cyclic monophosphate (8-BrcGMP; *B*, *left*), fasudil (*C*, *left*), and sildenafil (*D*, *left*) in vehicle-treated (white circles) and 2-APB-treated (black circles) lambs, and the corresponding maximal relaxation and sensitivity (pD_2) were calculated (*A-D*, *middle* and *right*). Values are the average ± SE. *$P < 0.05$, significant differences for vehicle vs. 2-APB, two-way ANOVA for repeated measures with Newman-Keuls post hoc test for concentration-response curves and unpaired *t*-test for maximal response and pD_2.

junction proteins (4). We do not have pharmacokinetic studies regarding 2-APB distribution and clearance; however, using hypoxic lambs at high altitude we have estimated that it should reach a concentration of the order of 10–100 μM in the extracellular space (47). A similar concentration of 2-APB inhibits SOC in HEK293 cell lines transfected with Orai/Stim or TRPC subunits (13, 36, 48) and IP_3R (38). Initial IP_3-mediated calcium release is necessary for hypoxic sustained Ca^{2+} entry in rat distal pulmonary artery myocytes (63) while it is not needed for hypoxic Ca^{2+} influx and contraction in canine pulmonary myocytes and rat main pulmonary arteries respectively (32, 44). Also, in pulmonary arteries from hypoxic newborn lambs, SKF-96365, a SOC blocker without known effect on IP_3R, induces a relaxation similar to 2-APB (47). Whether 2-APB is acting on IP_3R, SOC or both, SOC finally account for the sustained calcium increase in response to hypoxia in pulmonary artery myocytes (57). A concentration range of 20–100 μM 2-APB also blocks connexin (Cx) pro-

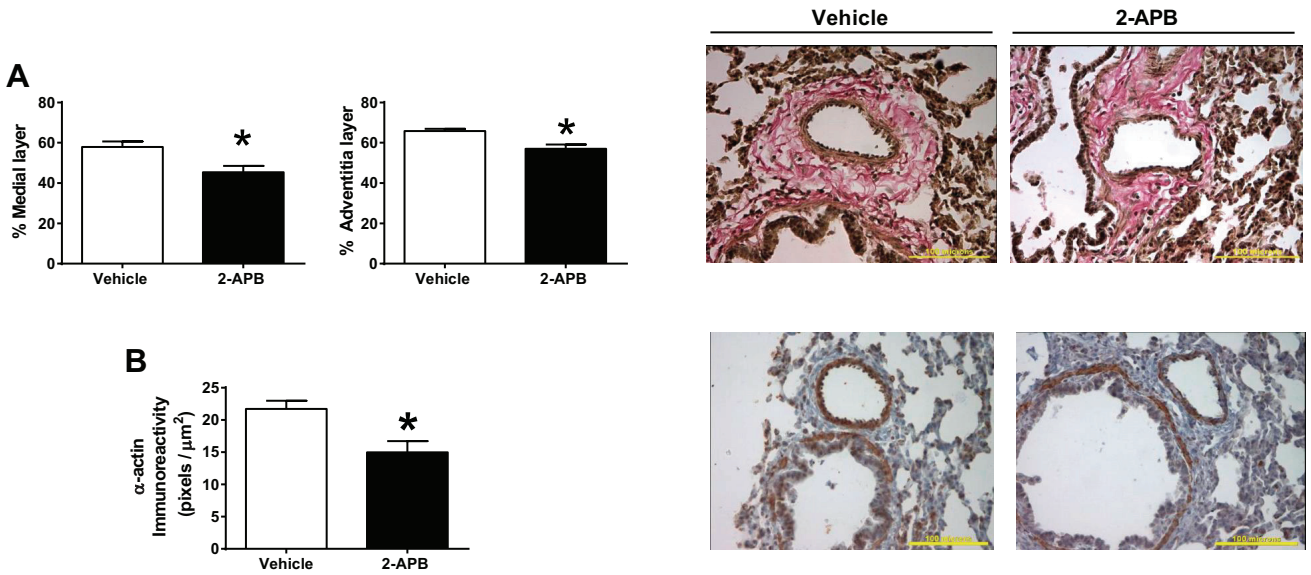

Fig. 7. Small pulmonary arteries remodeling. Medial and adventitia layer area (*A*) and α-actin (*B*) immunostaining of pulmonary arteries, performed in lung slices 48 h after the end of the treatment. Bar = 100 μm; magnification: ×40. Values are the average ± SE. *$P < 0.05$, significant differences for vehicle vs. 2-APB, unpaired *t*-test.

teins Cx32, Cx36, Cx40, and Cx50 of the gap junctions (4, 59). Nevertheless, the rat pulmonary vasculature expresses Cx37, Cx40, and Cx43 and the specific blockade of Cx40 does not modify the vasoconstrictor response to ET-1 (6), while in our experiments, 2-APB treatment reduced ET-1 vasoconstriction, suggesting than in the neonatal lambs, 2-APB is not targeting gap junctions.

Concerning the potential inhibition of TRPM7, a inhibitory effect is reached at high concentrations (100–300 μM) of 2-APB and depends on acidification of the intracellular milieu rather than on a direct action on the channel (11). Taken together, these observations suggest that in our sheep neonatal model, 2-APB is preferentially acting on SOC, but we cannot rule out an effect of long term administration of the drug on the other targets previously mentioned, and further experiments are needed to address this question.

The ex vivo responses of isolated pulmonary arteries to various vasoconstrictor and vasodilator stimuli were also assessed. Contractile responses to potassium or 5-HT did not change but the maximal contraction to ET-1 and to the TxA2 receptor agonist U46619 was reduced. It has been previously reported that there is an increased maximal contraction to potassium and increased sensitivity to ET-1 in pulmonary arteries of this model of lambs exposed to perinatal hypoxia, compared with lowland lambs (26). Increased vasoconstrictor responses to potassium and endothelin-1, with unchanged contraction to serotonin when the latter is normalized to potassium contraction, have been also reported in chronically hypoxic piglets and reviewed in sheep (7, 46). Hyperreactivity to thromboxane receptor stimulation is also observed in pulmonary artery myocytes from chronically hypoxic piglets (28). The decrease in hyperreactivity to both endothelin and thromboxane receptor stimulation observed ex vivo by cumulative doses of 2-APB may indicate mechanisms that are contributing to the beneficial in vivo effects of this treatment in the pulmonary circulation and are consistent with the contribution of SOC to the contraction elicited by both signaling systems (3, 29, 39, 47). The absence of change for the contraction in response to potassium in our experiments is consistent with the dependence on voltage-dependent calcium entry rather than SOCE for this vasoconstrictor stimulus observed in pulmonary arteries from both fetal and adult sheep (45). Nevertheless, the contraction curve to 5-HT is also unaffected by 2-APB treatment in our lambs and this is not consistent with the strong attenuation of the contraction to 5-HT observed by SKF-96365 blockade in pulmonary arteries from hypoxic lambs (7). In our experiments, lambs were exposed to hypoxia during the last 100 days of gestation, and the first 2 days after birth, however, they continued their development in the lowlands under normoxic conditions during all the study until the collection of the samples at 15 days of age. In the study of Blood et al. (7), the lambs were exposed to hypoxia during the last 120 days of gestation and continued to be exposed to hypoxia throughout postnatal life, until the moment of the sample collection between 10 and 20 days of age. We speculate that the different window of exposure to hypoxia may be related to this difference in the dependence of 5-HT contraction on SOC entry.

Concerning the response to vasodilator stimuli, the 10 days of 2-APB treatment did not modify the relaxation to SNP, a NO donor, but increased the maximal relaxation to 8-BrcGMP, suggesting a significant recovery of protein kinase G (PKG) function or downstream signaling. In lambs with perinatal hypoxia of different duration, pulmonary hypertension is observed despite increased sensitivity to SNP, increased pulmonary NO synthase activity and endothelial NO synthase expression (25, 26). This is probably the combined result of a blunted NO signaling resulting from decreased soluble guanylyl cyclase (sGC), increased PDE5 expression and activity and increased NO degradation by superoxide anion in the lungs of these animals (24, 25, 26, 62).

On the other hand, the function of PKG-1 may be also blunted under chronic hypoxia. A reduced vasodilation in response to cGMP analogs is observed with pulmonary arteries from hypoxic adult rats despite an increased expression of

PKG-1 (31), while in cultured fetal ovine pulmonary artery smooth muscle cells, hypoxia downregulates PKG-1 and hinders its interaction with MYPT1, the regulatory subunit of myosin light chain phosphatase (MLCP). This may favor the action of Rho kinases and sensitize the contractile machinery to calcium (51). The recovery of maximal capacity of PKG to promote relaxation after 2-APB treatment and despite the reduction of sensitivity could indicate a mechanism that counteracts the blunted NO signaling and promotes calcium desensitization. The improvement of PKG function may also contribute to the reduction of the contractile response to thromboxane receptor stimulation observed in our model and is consistent with the inhibition of thromboxane receptor signaling by PKG-1 (17, 33). An inhibitory effect of 2-APB on calcium influx leading to a decrease of the activity of myosin light chain kinase (MLCK), a calcium calmodulin-dependent enzyme, cannot be ruled out as a mechanism contributing to the increased PKG-dependent relaxation (30).

The 2-APB treatment also increased the maximal relaxation and the sensitivity to sildenafil and fasudil, suggesting an increase of PDE5 and Rho-kinase functions, respectively. The increased vasodilator response of pulmonary arteries to PDE5 inhibition may be also related to the gain of PKG-1 function already discussed. It is described that phosphorylation of PDE5 by PKG activates the enzyme to increase cGMP degradation rate as a negative feedback mechanism to avoid the overactivation of cGMP pathway. Nevertheless, this phosphorylation also increases the enzyme affinity to inhibitors like sildenafil and derivatives, which, in turn, results in an amelioration of potency and efficacy of these inhibitors (5, 8, 17). Whether the increased vasodilation to sildenafil observed after 2-APB treatment is the result of increased PDE5 function, increased affinity for sildenafil, or both remains to be established. The enhanced function of Rho kinases after 2-APB treatment, as evidenced as an increased relaxation to fasudil, is an unexpected result, since upregulation of the RhoA/Rho-kinase pathway is associated with increase of PAP in hypoxic rodents and lambs (37, 40, 70) rather than a reduced mPAP as we observed in our lambs. We do not know if this is a rebound effect resulting from the suspension of the treatment or a direct effect of 2-APB on RhoA/Rho-kinase signaling. The increase in PKG vasodilation function, along with the decrease in ET-1 and thromboxane contraction could be partially counteracted by enhanced Rho-kinase and PDE5 functions observed in the 2-APB-treated neonatal lambs and could explain the decrease in mPAP and PVR without normalizing these variables to similar values observed in lowland lambs. Experiments with combined therapies with 2-APB, or any other SOC blocker, and fasudil or sildenafil are needed to answer this question.

A characteristic finding of pulmonary hypertension is the pathologic remodeling of pulmonary arteries. This is a maladaptive response of pulmonary arteries to hypoxia, characterized by the thickening of medial layer, muscle extension to the normally nonmuscularized distal arterioles and sometimes a thickening of the adventitia with increased extracellular matrix deposition. The whole process is the result of an imbalance of the ratio between proliferation and apoptosis in pulmonary artery myocytes, as well as changes in the differentiation state of pulmonary artery smooth muscle cells and fibroblasts, and secretion of vasoactive compounds from pulmonary artery endothelium (50). Moreover, hypoxic pulmonary hypertension is associated with a thicker pulmonary artery medial layer and increased immunoreactivity smooth muscle markers like α-actin and myosin heavy chain (24, 26, 52, 69). There is a significant amount of information linking intracellular calcium, particularly SOCE, to changes in the differentiation and proliferative state of the different type of pulmonary vascular cells, to activity of signaling pathways regulating their cell cycle, and to the secretion of mitogens and smooth muscle markers (34). This is consistent with the decrease of the area of both medial and adventitial layer observed in the pulmonary arteries of 2-APB-treated lambs, as well with the decrease in the percentage of Ki67 positive cells and the expression α-actin. These observations suggest that the treatment effectively decreased both pulmonary artery remodeling through a proliferative arrest of both pulmonary artery smooth muscle and fibroblasts.

Taken together our results suggest that 2-APB treatment reduces pulmonary arterial hypertension and acute hypoxic vasoconstriction, decreasing ET-1 and TxA_2 receptor-mediated effects and significantly increasing cGMP dependent vasodilation. There is a transient decrease of CO during the intermediate phase of the treatment, suggesting that 2-APB also affects calcium in cardiomyocytes. Nevertheless, this effect on cardiac function disappears at the end of the treatment, where the effect on pulmonary arterial pressure could be related initially to the decrease in CO and in the last of treatment to a drop in PVR.

SOC are ubiquitous channels formed by combinations of different isoforms of TRPC and Orai pore-forming subunits together, with Stim proteins acting as endoplasmic reticulum calcium sensors. They are involved in the regulation of a myriad of functions in different cell types, which include smooth muscle and endothelial vascular cells, platelets, T and B lymphocytes, mast cells, fibroblasts, neurons and cardiac and skeletal muscle cells, among others (9, 61). Therefore, the beneficial cardiovascular effects observed after administration of any SOC blocker, like 2-APB or other small molecules on a whole animal model, need to be interpreted with caution. SOC blockade treatment could be a useful complementary strategy to heal neonatal pulmonary hypertension, and the development of new drugs with selective inhibitory action on pulmonary artery SOC isoforms, with reduced risk of cardiac and other systemic side effects, and in vivo testing are needed.

Perspectives and Significance

This study provides evidence about the cardiopulmonary effects of 2-APB, a drug that inhibits SOC, to reverse neonatal pulmonary arterial hypertension, extending our previous report about this blockade with a single dose of 2-APB in high altitude hypoxic lambs (47). These data are relevant for newborns that spent their gestation at high altitude and for lowland babies exposed to in utero chronic hypoxia. Our approach including the whole animal, isolated organ, and tissue is, to the best of our knowledge, the first characterization of the cardiopulmonary effects of a treatment with cumulative doses of 2-APB. The SOC blockade may be a potential pharmacological strategy to revert both pulmonary artery excessive vasoconstriction and remodeling in neonates. New SOC blockers, with improved specificity and potency, need to be assayed in whole animal models of neonatal pulmonary hypertension, their car-

diopulmonary and systemic effects and their long-term toxicity must be carefully evaluated.

ACKNOWLEDGMENTS

We are grateful to Carlos López, José López, Jaime Figueroa, Patricio Alvarez, and José Beretta from Liceo Agrícola Francisco Napolitano, Lluta, Chile for collaboration in the studies. We thank Carlos Brito and Gabino Llusco for excellent technical assistance. We also thank Dr. Catherine Connelly for careful editing for grammar and spelling.

GRANTS

This work was supported by National Fund of Scientific and Technological Development (FONDECYT) Grants 1120605, 1130424, 1140647, and 1151119, and Vicerrectoría de Investigación y Desarrollo, Universidad de Chile VID-Enlace.

DISCLOSURES

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

AUTHOR CONTRIBUTIONS

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

REFERENCES

1. **Abman SH.** Abnormal vasoreactivity in the pathophysiology of persistent pulmonary hypertension of the newborn. *Pediatr Rev* 20: e103–e109, 1999.
2. **Abman SH.** Recent advances in the pathogenesis and treatment of persistent pulmonary hypertension of the newborn. *Neonatology* 91: 283–290, 2007.
3. **Alapati VR, McKenzie C, Blair A, Kenny D, MacDonald A, Shaw AM.** Mechanisms of U46619-and 5-HT-induced contraction of bovine pulmonary arteries: role of chloride ions. *Br J Pharmacol* 151: 1224–1234, 2007.
4. **Bai D, Del Corsso C, Srinivas M, Sray DC.** Block of specific gap junction channel subtypes by 2-aminoethoxydiphenyl borate (2-APB). *J Pharmacol Exp Ther* 319: 1452–1458, 2006.
5. **Bessay EP, Blount MA, Zoraghi R, Beasley A, Grimes KA, Francis SH, Corbin JD.** Phosphorylation increases affinity of the phosphodiesterase-5 catalytic site for tadalafil. *J Pharmacol Exp Ther* 325: 62–68, 2008.
6. **Billaud M, Dahan D, Marthan R, Savineau JP, Guibert C.** Role of the gap junctions in the contractile response to agonists in pulmonary artery from two rat models of pulmonary hypertension. *Respir Res* 12: 30, 2011.
7. **Blood AB, Terry MH, Merritt TA, Papamatheakis DG, Blood Q, Ross JM, Wilson SM.** Effect of chronic perinatal hypoxia on the role of Rho-kinase in pulmonary artery contraction in newborn lambs. *Am J Physiol Regul Integr Comp Physiol* 304: R136–R146, 2013.
8. **Blount MA, Beasley A, Zoraghi R, Sekhar KR, Bessay EP, Francis SH, Corbin JD.** Binding of tritiated sildenafil, tadalafil, or vardenafil to the phosphodiesterase-5 catalytic site displays potency, specificity, heterogeneity, and cGMP stimulation. *Mol Pharmacol* 66: 144–152, 2004.
9. **Bon RS, Beech DJ.** In pursuit of small molecule chemistry for calcium-permeable non-selective TRPC channels–mirage or pot of gold? *Br J Pharmacol* 170: 459–474, 2013.
10. **Bootman MD, Collins TJ, MacKenzie L, Roderick HL, Berridge MJ, Peppiatt CM.** 2-Aminoethoxydiphenyl borate (2-APB) is a reliable blocker of store-operated Ca^{2+} entry but an inconsistent inhibitor of InsP3-induced Ca^{2+} release. *FASEB J* 16: 1145–1150, 2002.
11. **Chokshi R, Fruasaha P, Kozak JA.** 2-Aminoethyl diphenyl borinate (2-APB) inhibits TRPM7 channels through an intracellular acidification mechanism. *Channels* 6: 362–369, 2012.
12. **Collins HE, Zhu-Mauldin X, Marchase RB, Chatham JC.** STIM1/Orai1-mediated SOCE: current perspectives and potential roles in cardiac function and pathology. *Am J Physiol Heart Circ Physiol* 305: H446–H458, 2013.
13. **DeHaven WI, Smyth JT, Boyles RR, Bird GS, Putney JW.** Complex actions of 2-aminoethyldiphenyl borate on store-operated calcium entry. *J Biol Chem* 283: 19265–19273, 2008.
14. **Domeier TL, Zima AV, Maxwell JT, Huke S, Mignery GA, Blatter LA.** IP_3 receptor-dependent Ca^{2+} release modulates excitation-contraction coupling in rabbit ventricular myocytes. *Am J Physiol Heart Circ Physiol* 294: H596–H604, 2008.
15. **Earley S, Brayden JE.** Transient receptor potential channels in the vasculature. *Physiol Rev* 95: 645–690, 2015.
16. **Fernandez RA, Sundivakkam P, Smith KA, Zeifman AS, Drennan AR, Yuan JX.** Pathogenic role of store-operated and receptor-operated channels in pulmonary arterial hypertension. *J Signal Transduct* 2012: 951497, 2012.
17. **Francis SH, Busch JL, Corbin JD.** cGMP-dependent protein kinases and cGMP phosphodiesterases in nitric oxide and cGMP action. *Pharmacol Rev* 62: 525–563, 2010.
18. **Galiè N, Palazzini M, Leci E, Manes A.** Current therapeutic approaches to pulmonary arterial hypertension. *Rev Esp Cardiol* 63: 708–724, 2010.
19. **Gao Y, Raj JU.** Hypoxic pulmonary hypertension of the newborn. *Comp Physiol* 1: 61–79, 2011.
20. **Glantz SA, Slinker BK.** *Primer of Applied Regression & Analysis of Variance* (2nd ed.). New York: McGraw-Hill, 2001, p. 418–507.
21. **Guibert C, Marthan R, Savineau JP.** Modulation of ion channels in pulmonary arterial hypertension. *Curr Pharm Des* 13: 2443–2455, 2007.
22. **Heesch CM.** Reflexes that control cardiovascular function. *Am J Physiol (Adv Phsyiol Educ 22)* 277: S234–S243, 1999.
23. **Herrera EA, Pulgar VM, Riquelme RA, Sanhueza EM, Reyes RV, Ebensperger G, Llanos AJ.** High-altitude chronic hypoxia during gestation and after birth modifies cardiovascular responses in newborn sheep. *Am J Physiol Regul Integr Comp Physiol* 292: R2234–R2240, 2007.
24. **Herrera EA, Ebensperger G, Krause BJ, Riquelme RA, Reyes RV, Capetillo M, Llanos AJ.** Sildenafil reverses hypoxic pulmonary hypertension in highland and lowland newborn sheep. *Pediatr Res* 63: 169–175, 2008a.
25. **Herrera EA, Reyes RV, Giussani DA, Riquelme RA, Sanhueza EM, Ebensperger G, Llanos AJ.** Carbon monoxide: a novel pulmonary artery vasodilator in neonatal llamas of the Andean altiplano. *Cardiovasc Res* 77: 197–201, 2008b.
26. **Herrera EA, Riquelme RA, Ebensperger G, Reyes RV, Ulloa CE, Cabelo G, Llanos AJ.** Long-term exposure to high-altitude chronic hypoxia during gestation induces neonatal pulmonary hypertension at sea level. *Am J Physiol Regul Integr Comp Physiol* 299: R1676–R1684, 2010.
27. **Herrera EA, Macchiavello R, Montt C, Ebensperger G, Díaz M, Ramírez S, Llanos AJ.** Melatonin improves cerebrovascular function and decreases oxidative stress in chronically hypoxic lambs. *J Pineal Res* 57: 33–42, 2014.
28. **Hinton M, Gutsol A, Dakshinamurti S.** Thromboxane hypersensitivity in hypoxic pulmonary artery myocytes: altered TP receptor localization and kinetics. *Am J Physiol Lung Cell Mol Physiol* 292: L654–L663, 2007.
29. **Horinouchi T, Terada K, Higashi T, Miwa S.** Endothelin receptor signaling: new insight into its regulatory mechanisms. *J Pharm Sci* 123: 85–101, 2013.
30. **Jernigan NL, Resta FC.** Calcium homeostasis and sensitization in pulmonary arterial smooth muscle. *Microcirculation* 21: 259–271, 2014.
31. **Jernigan NL, Walker BR, Resta TC.** Pulmonary PKG-1 is upregulated following chronic hypoxia. *Am J Physiol Lung Cell Mol Physiol* 285: L634–L642, 2003.
32. **Jin N, Packer CS, English D, Rhoades RA.** Inositol trisphosphate is involved in norepinephrine- but not in hypoxia-induced pulmonary arterial contraction. *Am J Physiol Lung Cell Mol Physiol* 264: L160–L164, 1993.
33. **Kelley-Hickie LP, O'Keeffe MB, Reid HM, Kinsella BT.** Homologous desensitization of signalling by the alpha (α) isoform of the human thromboxane A 2 receptor: a specific role for nitric oxide signalling. *Biochim Biophys Acta* 1773: 970–989, 2007.
34. **Kuhr FK, Smith KA, Song MY, Levitan I, Yuan JX.** New mechanisms of pulmonary arterial hypertension: role of Ca^{2+} signaling. *Am J Physiol Heart Circ Physiol* 302: H1546–H1562, 2012.
35. **Lakshminrusimha S, Mathew B, Leach CL.** Pharmacologic strategies in neonatal pulmonary hypertension other than nitric oxide. *Semin Perinatol* 640: 160–173, 2016.
36. **Lievremont JP, Bird GS, Putney JW.** Mechanism of inhibition of TRPC cation channels by 2-aminoethoxydiphenylborane. *Mol Pharmacol* 68: 758–762, 2005.

37. **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.
38. **Maruyama T, Kanaji T, Nakade S, Kanno T, Mikoshiba K.** 2APB, 2-aminoethoxydiphenyl borate, a membrane-penetrable modulator of Ins (1, 4, 5) P3-induced Ca^{2+} release. *J Biochem* 122: 498–505, 1997.
39. **McKenzie C, MacDonald A, Shaw AM.** Mechanisms of U46619-induced contraction of rat pulmonary arteries in the presence and absence of the endothelium. *Br J Pharmacol* 157: 581–596, 2009.
40. **McNamara PJ, Murthy P, Kantores C, Teixeira L, Engelberts D, Van Vliet T, Jankov RP.** Acute vasodilator effects of Rho-kinase inhibitors in neonatal rats with pulmonary hypertension unresponsive to nitric oxide. *Am J Physiol Lung Cell Mol Physiol* 294: L205–L213, 2008.
41. **Minamino T, Christou H, Hsieh CM, Liu Y, Dhawan V, Abraham NG, Perella MA, Mitsialis SA, Kourembanas S.** Targeted expression of heme oxygenase-1 prevents the pulmonary inflammatory and vascular responses to hypoxia. *Proc Natl Acad Sci USA* 98: 8798–8803, 2001.
42. **Moore LG.** Human genetic adaptation to high altitude. *High Alt Med Biol* 2: 257–279, 2001.
43. **Mulvany MJ, Halpern W.** Contractile properties of small arterial resistance vessels in spontaneously hypertensive and normotensive rats. *Circ Res* 41: 19–26, 1997.
44. **Ng LC, Wilson SM, McAllister Hume JR.** Role of InsP3 and ryanodine receptors in the activation of capacitive Ca^{2+} entry by store depletion or hypoxia in canine pulmonary arterial smooth muscle cells. *Br J Pharmacol* 152: 101–111, 2007.
45. **Papamatheakis DG, Patel JJ, Blood Q, Merritt TT, Longo LD, Wilson SM.** Depolarization-dependent contraction increase after birth and preservation following long term hypoxia in sheep pulmonary arteries. *Pulm Circ* 2: 41–53, 2012.
46. **Papamatheakis DG, Chundu M, Blood AB, Wilson SM.** Prenatal programming of pulmonary hypertension induced by chronic hypoxia or ductal ligation in sheep. *Pulm Circ* 3: 757–780, 2013.
47. **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.
48. **Peinelt C, Lis A, Beck A, Fleig A, Penner R.** 2-Aminoethoxydiphenyl borate directly facilitates and indirectly inhibits STIM1-dependent gating of CRAC channels. *J Physiol* 586: 3061–3073, 2008.
49. **Peñaloza D, Arias-Stella J.** The heart and pulmonary circulation at high altitudes healthy highlanders and chronic mountain sickness. *Circulation* 115: 1132–1146, 2007.
50. **Shimoda LA, Laurie SS.** Vascular remodeling in pulmonary hypertension. *J Mol Med* 91: 297–309, 2013.
51. **Singh DK, Sarkar J, Raghavan A, Reddy SP, Raj JU.** Hypoxia modulates the expression of leucine zipper-positive MYPT1 and its interaction with protein kinase G and Rho kinases in pulmonary arterial smooth muscle cells. *Pulm Circ* 1: 487–498, 2011.
52. **Sluiter I, van Heijst A, Haasdijk R, Kempen MB, Boerema-de Munck A, Reiss I, Rottier RJ.** Reversal of pulmonary vascular remodeling in pulmonary hypertensive rats. *Exp Mol Pathol* 93: 66–73, 2012.
53. **Snetkov VA, Aaronson PI, Ward J, Knock GA, Robertson TP.** Capacitative calcium entry as a pulmonary specific vasoconstrictor mechanism in small muscular arteries of the rat. *Br J Pharmacol* 140: 97–106, 2003.
54. **Spieker LE, Flammer AJ, Lüscher TF.** The vascular endothelium in hypertension. *Hand Exp Pharmacol* 176: 249–283, 2006.
55. **Spiers A, Padmanabhan N.** A guide to wire myography. *Methods Mol Med* 108: 91–104, 2005.
56. **Stenmark KR, Tuder RM, El Kasmi KC.** Metabolic reprogramming and inflammation act in concert to control vascular remodeling in hypoxic pulmonary hypertension. *J Appl Physiol* 119: 1164–1172, 2015.
57. **Sylvester JT, Shimoda LA, Aaronson PI, Ward JP.** Hypoxic pulmonary vasoconstriction. *Physiol Rev* 92: 367–520, 2012.
58. **Tang C, To WK, Meng F, Wang Y, Gu Y.** A role for receptor-operated Ca^{2+} entry in human pulmonary artery smooth muscle cells in response to hypoxia. *Physiol Res* 59: 909–918, 2010.
59. **Tao L, Harris AL.** 2-Aminoethoxydiphenyl borate directly inhibits channels composed of connexin 26 and/or connexin 32. *Mol Pharmacol* 71: 570–579, 2007.
60. **Thomas M.** Pharmacological targets for pulmonary vascular disease: vasodilation versus anti-remodeling. In: *Membrane Receptors, Channels and Transporters in Pulmonary Circulation*. edited by Yuan JX, Ward JP. New York: Humana, 2010, p. 475–490.
61. **Tian C, Du L, Zhou Y, Li M.** Store-operated CRAC channel inhibitors: opportunities and challenges. *Future Med Chem* 8: 817–832, 2016.
62. **Torres F, González-Candia A, Montt C, Ebensperger G, Chubretovic M, Serón-Ferré M, Herrera EA.** Melatonin reduces oxidative stress and improves vascular function in pulmonary hypertensive newborn sheep. *J Pineal Res* 58: 362–373, 2015.
63. **Wang J, Shimoda L, Sylvester JT.** Ca^{2+} responses of pulmonary arterial myocytes to acute hypoxia require release from ryanodine and inositol trisphosphate receptors in sarcoplasmic reticulum. *Am J Physiol Lung Cell Mol Physiol* 303: L161–L168, 2012.
64. **Ward J, Snetkov VA.** Determination of signaling pathways responsible for hypoxic pulmonary vasoconstriction: use of the small vessel myograph. *Methods Enzymol* 381: 71–87, 2004.
65. **Weigand L, Foxson J, Wang J, Shimoda LA, Sylvester JT.** Inhibition of hypoxic pulmonary vasoconstriction by antagonists of store-operated Ca2+ and nonselective cation channels. *Am J Physiol Lung Cell Mol Physiol* 289: L5–L13, 2005.
66. **Weissmann N, Dietrich A, Fuchs B, Kalwa H, Ay M, Dumitrascu R, Gudermann T.** Classical transient receptor potential channel 6 (TRPC6) is essential for hypoxic pulmonary vasoconstriction and alveolar gas exchange. *Proc Natl Acad Sci USA* 103: 19093–19098, 2006.
67. **Welsh DJ, Peacock AJ.** Cellular responses to hypoxia in the pulmonary circulation. *High Alt Med Biol* 14: 111–116, 2013.
68. **Yang XR, Lin MJ, Sham JS.** Physiological functions of transient receptor potential channels in pulmonary arterial smooth muscle cells. *Adv Exp Med Biol* 661: 109–122.
69. **Yeager ME, Frid MG, Stenmark KR.** Progenitor cells in pulmonary vascular remodeling. *Pulm Circ* 1: 3–16, 2011.
70. **Ziino AJ, Ivanovska J, Belcastro R, Kantores C, Xu EZ, Lau M, Jankov RP.** Effects of rho-kinase inhibition on pulmonary hypertension, lung growth, and structure in neonatal rats chronically exposed to hypoxia. *Pediatr Res* 67: 177–182, 2010.
71. **Zima AV, Bare DJ, Mignery GA, Blatter LA.** IP3-dependent nuclear Ca^{2+} signalling in the mammalian heart. *J Physiol* 584: 601–611, 2007.
72. **Zuckerbraun BS, Chin BY, Wegiel B, Billiar TR, Czsimadia E, Rao J, Otterbein LE.** Carbon monoxide reverses established pulmonary hypertension. *J Exp Med* 203: 2109–2119, 2006.