Long-term exposure to high-altitude chronic hypoxia during gestation induces neonatal pulmonary hypertension at sea level

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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. First published September 29, 2010; doi:10.1152/ajpregu.00123.2010.—We determined whether postnatal pulmonary hypertension induced by 70% of pregnancy at high altitude (HA) persists once the offspring return to sea level and investigated pulmonary vascular mechanisms operating under these circumstances. Pregnant ewes were divided into two groups: conception, pregnancy, and delivery at low altitude (580 m, LLL) and conception at low altitude, pregnancy at HA (3,600 m) from 30% of gestation until delivery, and return to lowland (LHL). Pulmonary arterial pressure (PAP) was measured in vivo. Vascular reactivity and morphometry were assessed in small pulmonary arteries (SPA). Protein expression of vascular mediators was determined. LHL lambs had higher basal PAP and a greater increment in PAP after Nω-nitro-L-arginine methyl ester (20.9 ± 1.1 vs. 13.7 ± 0.5 mmHg; 39.9 ± 5.0 vs. 18.3 ± 1.3 mmHg, respectively). SPA from LHL had a greater maximal contraction to K+ (1.34 ± 0.05 vs. 1.16 ± 0.05 N/m), higher sensitivity to endothelin-1 and nitropriasside, and persistence of dilatation following blockade of soluble guanylate cyclase. The heart ratio of the right ventricle-to-left ventricle plus septum was higher in the LHL relative to LLL. The muscle area of SPA (29.3 ± 2.9 vs. 21.1 ± 1.7%) and the protein expression of endothelial nitric oxide synthase (1.7 ± 0.1 vs. 1.1 ± 0.2), phosphodiesterase (1.4 ± 0.1 vs. 0.7 ± 0.1), and Ca2+-activated K+ channel (0.76 ± 0.16 vs. 0.30 ± 0.01) were greater in LHL compared with LLL lambs. In contrast, LHL had decreased heme oxygenase-1 expression (0.82 ± 0.26 vs. 2.22 ± 0.44) and carbon monoxide production (all P < 0.05). Postnatal pulmonary hypertension induced by 70% of pregnancy at HA promotes cardiopulmonary remodeling that persists at sea level.

pulmonary hypoxic vasoconstriction; pulmonary vascular reactivity; nitric oxide; pulmonary vasodilators; pulmonary vasoconstrictors

THE ETIOLOGY OF PULMONARY hypertension in the postnatal period is complex and not completely understood (2, 44, 48). Proposed mechanisms underlying the physiology mediating elevations in postnatal pulmonary arterial pressure include impaired endothelial function promoting an increase in pulmonary vascular resistance (1, 2, 44). Pulmonary hypertension in the postnatal period is associated with high mortality, and children who survive may have decreased postnatal growth and devastating neurological, respiratory, and cardiac complications that often persist into childhood (9, 44). One condition that may lead to elevations in pulmonary arterial pressure in the postnatal period is sustained fetal hypoxia (1, 44). In humans and animals, a common form of sustained fetal hypoxia is pregnancy at high altitude (16, 23). Pulmonary hypertension in the postnatal period due to this condition is an important problem, since currently nearly 140 million people reside at over 2,500 meters above sea level, being permanently exposed to chronic hypoxic conditions (34, 41). Sustained or partial exposure to high altitude of pregnant women, either permanently resident at high altitude or native to low altitude, is therefore a current problem.

Several mediators act upon the pulmonary vasculature, triggering alterations in vascular tone and structure. A potent vasoconstrictor is endothelin-1 (ET-1), which acts via the ETA receptor to stimulate both contraction and remodeling of the pulmonary vascular bed. ET-1 therefore plays an important role in the regulation of pulmonary vascular resistance (3, 4, 26). Interestingly, it has been reported that ET-1 function is increased in neonatal pulmonary hypertension (3). Nitric oxide (NO) is also another important modulator of the pulmonary circulation, and its vasodilator actions are mediated via several mechanisms, including the activation and opening of Ca2+-activated K+ channels (BKCa) and the balance between the synthesis of cGMP through the activation of soluble guanylate cyclase (sGC) and its degradation by the isoenzyme phosphodiesterase 5 (PDE5) (2, 42). The impairment of NO-dependent dilatation has also been closely related to pulmonary hypertension in the postnatal period (1, 44). In addition, the endogenous gas carbon monoxide (CO) is a dilator in the pulmonary vascular bed, and it protects against pulmonary vascular remodeling (31, 37, 48). In newborn llamas, augmented pulmonary CO, rather than pulmonary NO, helps to prevent pulmonary hypertension in the newborn period at high altitude (25).

Using ovine pregnancy at high altitude as an experimental model, we have previously reported that pregnancy and delivery at high altitude yields offspring with pulmonary hypertension, coupled with increased constrictor reactivity of isolated pulmonary vessels despite enhanced pulmonary NO function (23, 24, 25). In those studies, the in vivo and in vitro measurements were performed at high altitude. It remains unknown whether long-term exposure of the pregnancy to high altitude...
results in altered pulmonary vascular function and anatomy in offspring, even following return to sea level. Therefore, this study tested the hypothesis that long-term exposure of the pregnancy to high altitude results in postnatal pulmonary hypertension even following return to sea level and that this is associated with cardiopulmonary remodeling and alterations in the pulmonary vascular function. We used an integrative approach at the whole animal, isolated organ, and molecular level to determine the effects of 70% of gestation at high altitude on: 1) in vivo pulmonary arterial pressure under basal and acute hypoxic conditions, both before and after NO blockade; 2) the reactivity of isolated small pulmonary arteries to KCl, ET-1, and to sodium nitroprusside (SNP) before and after treatment with the sGC inhibitor 1H-[1,2,4]oxadiazolo[4,3-a]quinazolin-1-one (ODQ); 3) the mRNA and protein expression of endothelial NO synthase (eNOS), and protein expression of sGC, BKCa, PDE5, heme oxygenase-1 (HO-1), and CO production in the postnatal lung; and 4) the morphology of small pulmonary arteries and weight ratios of the heart and lungs. All studies were performed at sea level in lambs within the first two weeks of postnatal life.

MATERIALS AND METHODS

The Faculty of Medicine Ethics Committee of the University of Chile approved all experimental procedures (Protocol CBA No. 097, FMUCH). The studies on animals were performed according with the Guide for the Care and Use of Laboratory Animals published by the United States National Institutes of Health (NIH Publication No. 85-23, revised 1996) and adheres to the American Physiological Society’s Guiding Principles in the Care and Use of Animals.

Animals

Twenty-eight pregnant ewes (Ovis aries) were divided into the following two groups: conception, pregnancy, and delivery at lowland (Santiago, 580 m, LLL, n = 14) and conception at lowland, pregnancy at high altitude (Putre, 3,600 m) from 30% of gestation until delivery, and return to lowland (LHL, n = 14). Mothers and newborns were housed in an open yard with access to food and water ad libitum.

Surgical Preparation and In Vivo Experiments

A subgroup of the lambs was surgically prepared between 3 and 8 days of age for in vivo experimentation (LLL, n = 6; LHL, n = 5). In brief, the animals were anesthetized with ketamine (10 mg/kg im) and diazepam (0.1–0.5 mg/kg im) with additional local infiltration of 2% lidocaine. Polyvinyl catheters were placed in the descending aorta and return to lowland (LHL, n = 14). Mothers and newborns were removed and weighed. Lung weight-to-body weight ratio was calculated.

Histology.

We isolated and perfused the right lung with 4% formalin, fixed in 10% buffered formalin, dehydrated, cleared, and embedded in paraffin. Transversal sections of the left lung were stained with H.E. and analyzed by light microscopy.

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performed on 10-μm slides. At least four arteries (100–200 μm diameter) per lung were chosen, and an average of four measurements from each artery was recorded. Images of parenchymal arterioles were acquired using a workstation (Olympus trinocular microscope-BX51 plus digital camera QimagingGo3) linked to Image Pro software 6.3, and vascular areas were calculated using an image analysis program. The wall-to-vessel area ratio was calculated and expressed as a percentage, as previously described (24, 33). Briefly, the percent wall thickness was calculated as follows: wall thickness (%) = external area – internal area/external area × 100, where external area and internal area are the area bounded by external and internal elastic laminae, respectively. In addition, the area of vascular smooth muscle was calculated as follows: muscle area (%) = external muscle area – internal area/external muscle area × 100, where the external muscle area and the internal area are the external and internal boundaries of the tunica media, respectively.

Statistical Analysis

Data are expressed as means ± SE. Groups were compared by two-way ANOVA and the post hoc Newman-Keuls test, or the Student’s t-test for unpaired data, as appropriate. We used the Fisher Exact Test to compare survival between groups. For all comparisons, differences were considered statistically significant when \( P < 0.05 \) (18).

RESULTS

Survival and Weight

In marked contrast to sea level pregnancies (LLL) with 100% survival, pregnancies after 70% exposure to high altitude (LHL) had increased mortality, with 21% abortions and 14% stillbirths (\( P < 0.05 \)). No lambs died after birth in either group. Surviving LHL lambs were much lighter than LLL lambs (3.8 ± 0.3 kg, \( n = 9 \) vs. 7.0 ± 0.4 kg, \( n = 14 \), \( P < 0.001 \), weights at the time of experimentation between 6 and 11 days of age).

In Vivo Experiments

Because of differences in survival, eight LLL and four LHL lambs were studied in vivo. Values for basal \( P_H \), \( P_AO \), \( P_{CO_2} \), \( S_AO \), and [Hb] were similar in both groups of lambs (Table 1). During acute hypoxia on a background of saline infusion, a similar fall in \( P_AO \) and \( S_AO \) occurred in both groups of lambs, without any alteration to \( P_{CO_2} \) from baseline (Table 1). During recovery, all variables returned toward basal values in both groups. However, values for \( P_{CO_2} \) were significantly depressed from baseline in LLL lambs (Table 1). Treatment with L-NAME had no significant effect on arterial blood gas and acid base status either during basal or acute hypoxic conditions (Table 1).

Basal values for pulmonary arterial pressure, pulmonary vascular resistance, and cardiac output were significantly greater in LHL than LLL lambs (Fig. 1 and Table 1). Basal values for heart rate were similar between the groups (Table 1). Basal systemic arterial pressure was similar in LHL and LLL lambs (87 ± 3 vs. 82 ± 1 mmHg, respectively).

During acute hypoxia on a background of saline infusion, pulmonary arterial pressure, pulmonary vascular resistance, cardiac output, and heart rate increased significantly in both groups of lambs. However, in LHL relative to LLL lambs, values for pulmonary arterial pressure and cardiac output reached significantly greater values during the acute hypoxic challenge (Fig. 1 and Table 1). No changes in systemic arterial pressure were seen in either of the experimental groups during hypoxia. During recovery, pulmonary arterial pressure, and cardiac output remained significantly elevated from baseline, but heart rate and pulmonary vascular resistance returned toward basal values in LHL lambs. In contrast, all variables returned toward basal values in LLL lambs (Fig. 1 and Table 1).

Treatment of the lambs with L-NAME during the basal period led to an increase in pulmonary arterial pressure and pulmonary vascular resistance and a decrease in heart rate and cardiac output in both groups of lambs. Although the fall in heart rate and cardiac output was similar between the groups, the increment in pulmonary arterial pressure and in pulmonary vascular resistance was significantly greater in LHL than in LLL lambs (Fig. 1 and Table 1). Treatment with L-NAME did not affect the magnitude of the pulmonary hemodynamic and blood gas responses to acute hypoxia in either group of lambs, with the exception that values for pulmonary arterial pressure reached greater values in LHL than in LLL lambs (Fig. 1 and Table 1).

At the time of surgery up until the time of study, there was no evidence of a patent ductus arteriosus. At the time of dissection, after the last study, the ductus arteriosus was examined, and no lumen was visible in any of the studied animals. Additional evidence for the closure of the ductus arteriosus is provided by the similarities of oxygen saturation and \( P_{O_2} \) in samples obtained from the ascending and descending aorta (data not shown). This is further supported by the fact that systemic arterial pressure was always higher than the pulmonary arterial pressure in all animals. The presence of a left-to-right shunt is unlikely because there were no differences in pulmonary arterial pulse pressure, between LHL and LLL lambs, either during basal conditions or during hypoxia.

Ex Vivo Experiments

Isolated small pulmonary arteries from LHL relative to LLL lambs showed a greater maximal contraction to KCl (\( K_{max} \): 1.34 ± 0.05 vs. 1.16 ± 0.05 N/m, \( P < 0.05 \)) with similar sensitivity (\( EC_{50} \): 28.54 ± 2.48 vs. 31.53 ± 4.44; Fig. 2A). In contrast, the maximal contraction with ET-1 was similar in the two groups, although the sensitivity to the contraction elicited by ET-1 was significantly greater in LHL than in LLL lambs (PD\(_2\): 8.08 ± 0.13 vs. 7.22 ± 0.28, \( P < 0.05 \); Fig. 2B). The NO donor SNP evoked a similar maximal relaxation in pulmonary vessels from LLL and LHL lambs (\( %K_{max} \): 98.1 ± 3.0 vs. 100.0 ± 2.3%; Fig. 3A). However, the relaxant sensitivity of the pulmonary vessels to SNP was significantly greater in LHL than in LLL lambs (PD\(_2\): 7.31 ± 0.12 vs. 5.77 ± 0.07, \( P < 0.05 \); Fig. 3A). This SNP-induced vasorelaxation in the pulmonary vasculature was completely abolished by blocking sGC with ODQ in LLL lambs (Fig. 3B). In marked contrast, SNP-induced vasorelaxation in vessels isolated from LHL lambs persisted following treatment with ODQ, but the maximal relaxation and sensitivity were significantly diminished (before ODQ, \( %K_{max} \): 98.1 ± 3.0%; PD\(_2\): 7.31 ± 0.12; after ODQ, \( %K_{max} \): 37.7 ± 3.2%, PD\(_2\): 4.97 ± 0.13, \( P < 0.05 \); Fig. 3B).

Western Blot

The expression of eNOS mRNA and protein in lung tissue was significantly greater in LHL lambs than in LLL lambs (Figs. 4 and 5). This was associated with a significantly
greater protein expression of pulmonary BK$_{Ca}$ and PDE5 but not sGC in LHL than in LLL lambs (Fig. 5). Furthermore, protein expression of pulmonary HO-1 and the production of CO by the pulmonary circulation were both diminished in LHL compared with LLL lambs (Fig. 6).

**Heart and Lung Biometry**

The ratio of the weight of the right ventricle to the left ventricle plus septum was augmented in the LHL compared with the LLL group (0.370 vs. 0.328 ± 0.009, $P < 0.007$).
A striking difference between the groups of lambs in the present study was the much greater mortality and pronounced growth restriction in lambs born from pregnancies after prolonged exposure to high altitude. Pregnancy at high altitude induces maternal hypobaric hypoxia, and we have previously reported lower maternal and fetal arterial Po2 in a separate cohort of animals exposed to the same altitude during the whole pregnancy (12). A similar effect on fetal growth restriction and mortality during development at high altitude has been reported in highland human populations (16, 30, 34, 38) and in chick embryos following highland incubation (17, 43). Malnutrition during early gestation in high-altitude cattle also resulted in a higher incidence of elevated pulmonary arterial pressure and right ventricular hypertrophy compared with controls when measured in the offspring at 15 mo. This was associated with differential gene expression in the right ventricle, but the resulting interaction between undernutrition and high-altitude hypoxia is unclear (21). In our study, both groups of lambs received the same nutrition, so the changes observed in pulmonary arterial pressure and growth restriction appear to be independent of nutrition. Accordingly, the effects on fetal growth restriction and mortality of developmental hypoxia at high altitude have been shown to be independent of the maternal nutritional status and of highland hypobaria in other species, since fetal growth restriction persists in ewes undergoing pregnancy at high altitude with food intake values similar to those as sea level pregnancies (23, 39). These effects have also been shown in the chick embryo, where incubation at high altitude of sea level eggs with oxygen supplementation completely prevented the high altitude-induced fetal growth restriction and mortality (17). The present study extends these
findings and reports that 70% rather than 100% exposure to high altitude during fetal development can also have dramatic effects on the maintenance of pregnancy, on fetal growth, and on fetal mortality (abortion and stillbirth). In contrast, we did not have neonatal mortality.

Lambs born from pregnancies after 70% exposure to high altitude had a greater basal cardiac output, pulmonary vascular resistance, and pulmonary arterial pressure, even when their PaO₂ had recovered to normoxic levels, and also showed a greater pulmonary pressor response to L-NAME and to acute hypoxia. The greater basal cardiac output in the highland group is independent of differences in basal heart rate, suggesting a greater resting stroke volume in lambs from pregnancies after long exposure to high altitude. The differences in basal pulmonary arterial pressure and vascular resistance between the groups may be explained, in part, by the larger area of vascular smooth muscle, the greater pulmonary vessel maximal constrictr response to KCl, and the increased sensitivity to ET-1 in the LHL lambs. ET-1 is induced by chronic hypoxia and is a potent pulmonary vasoconstrictor and a mitogen, leading to smooth muscle cell proliferation (3). Previous studies have correlated an increased vascular response with greater smooth muscle cell remodeling (29, 45), conditions that were both
observed in LHL lambs in our study. Moreover, it has been suggested that the longer the exposure to high altitude, the greater the vascular smooth muscle remodeling (29, 40, 45). Dissociation between changes in vascular wall area and in wall thickness is a common finding with established explanations. Elegant studies by Baumbach and Heistad (5, 6) and by Mulvany (35, 36) have made it clear that an increase in the ratio of the vascular wall to lumen may be achieved by at least two very different situations. For instance, the ratio may be increased by a reduction in lumenal diameter without a change in medial volume. There is thus rearrangement of the same volume of vessel wall around a smaller-diameter lumen, what is now termed inward eutrophic vascular remodeling. Conversely, an increase in the vascular wall-to-lumen ratio may be achieved by an increase in wall material with or without a change in lumen diameter, what has been termed outward hypertrophic vascular growth. An increase in wall material with an increase in lumen diameter is what is occurring in the LHL vessels. Interestingly, the main driving forces that promote this type of vascular remodeling are increased flow and pressure (36), both of which are present in the pulmonary bed of LHL lambs.

The present study also reports an increase in right ventricular mass in LHL neonates. This is a common finding in humans and animals that have suffered arterial pulmonary hypertension (1, 15, 46, 47).

Other components contributing to basal pulmonary hypertension in lambs from pregnancies after prolonged exposure to high altitude may include alterations in the tonic balance between dilator and constrictor influences on the pulmonary vascular bed. For instance, we have previously reported in highland lambs reduced synthesis of dilators, such as CO (25). In this set of experiments, it was also found that LHL lambs had an important decrease in the production of CO by the pulmonary circulation concordant with the reduced HO-1 protein expression. Interestingly, a study in fetal lambs showed them to be unresponsive to CO (19). However, this was performed in ventilated (hypoxic, <10% FiO2) fetuses rather than normally oxygenated postnatal lambs. Lambs native to high altitude do not increase HO as do llamas, which suggests that they are insensitive to endogenous CO, although they may be responsive to induced CO production (19). CO is a dilator via activation of sGC (13, 27, 32) and via hyperpolarizing the vascular smooth muscle secondary to activation of BKCa channels (7, 10, 50). CO can also diminish the vasoconstrictor responses to phenylephrine and 20-hydroxyeicosatetraenoic acid while reducing the synthesis and release of ET (28, 51).

The diminished production of CO by the pulmonary circulation determined in this study may play a putative role in the maintenance of persistent pulmonary hypertension of the newborn at sea level. In addition, chronic developmental hypoxia is known to result in lung hypoplasia and immaturity, pulmonary edema, and altered endothelial function (2, 20, 39, 46). Alterations in the synthesis and function of vasoconstrictors such as ET-1, as reported in this paper, thromboxane, IGF,
serotonin, and leukotriene C₄/D₄ have also been implicated in the pulmonary hypertensive phenotype during chronic hypoxia (29, 45).

In the present study, the greater pulmonary hypertension under basal and stimulated conditions in lambs from pregnancies after 70% exposure to high altitude occurred despite evidence of enhanced NO-dependent dilator function in the pulmonary vascular bed. The greater pressor response to treatment with t-NAME, the increased expression of eNOS mRNA and protein, and the enhanced isolated vessel dilator response to SNP all strongly support enhanced NO function in the pulmonary vasculature of lambs from pregnancies after long-term exposure to high altitude. PDE is an enzyme that breaks down cGMP and thus halts the NO vasodilator cascade (42). In this study, LHL also showed greater pulmonary protein expression of PDE5, findings similar to those reported in hypertensive lambs and lambs native to high altitude (22, 24). Although a greater protein expression of pulmonary PDE5 may itself favor constriction in the pulmonary vascular bed, it is likely that the increased expression of PDE5 occurs to match all other components of the enhanced NO cascade, and it does not underlie a cause but is likely a consequence of the pulmonary hypertension in lambs from pregnancies exposed to high altitude. In the present study, blockade of sGC with ODQ completely prevented the pulmonary dilator response to the NO donor SNP in control lambs but not in lambs from pregnancies after prolonged exposure to high altitude. In the latter group, the dilator response to SNP persisted, albeit at a reduced level. This suggests that long-term exposure to high altitude during pregnancy may trigger an enhancement of NO dilatation pathways in addition to the activation of sGC in vascular smooth muscle. One possibility is the direct action of NO on the activation of K⁺ channels, as has already been described for the BKCa channel (8). Accordingly, in the present study, LHL lambs showed a significantly greater pulmonary BKCa protein expression. What is important to highlight is that, despite evidence of enhanced pulmonary NO function via at least two different signaling cascades, this adaptive response is insufficient to offset pulmonary hypertension and vascular remodeling in lambs even following return to sea level.

In conclusion, postnatal pulmonary hypertension induced by long-term exposure of the pregnancy to high altitude persists at sea level, despite enhanced pulmonary NO function. This condition is associated with a decrease in the production of pulmonary CO coupled with an increase in the vascular reactivity of constrictors associated with cardiopulmonary remodeling processes.

Perspectives and Significance

During acute episodes of hypoxia, the pulmonary vascular bed undergoes constriction to match the reduced oxygenation with reduced perfusion. During sustained hypoxia, this initial homeostatic response becomes maladaptive, triggering sustained increases in pulmonary vascular resistance, leading to the establishment of pulmonary hypertension. Our studies show that this maladaptive pulmonary constrictor response to hypoxia can be triggered in the newborn lamb following pregnancy at high altitude, when the measurements are performed at high altitude (23, 24, 25) and, even, following return to sea level. Sustained pulmonary hypertension and remodeling of the pulmonary vasculature suggest possible persistence of this maladaptive response until adulthood. The implications of these findings are not only relevant to women of reproductive age native to sea level countries, considering trips or work at high altitude, but also to the developmental programming of pulmonary hypertension in adulthood by prenatal hypoxia (14, 34).

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DISCLOSURES

No conflicts of interest are declared by the authors.

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