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Evolving in thin air—Lessons from the llama fetus in the *altiplano*

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Abstract

Compared with lowland species, fetal life for mammalian species whose mothers live in high altitude is demanding. For instance, fetal llamas have to cope with the low fetal arterial P_{O_2} of all species, but also the likely superimposition of hypoxia as a result of the decreased oxygen environment in which the mother lives in the Andean *altiplano*. When subjected to acute hypoxia the llama fetus responds with an intense peripheral vasoconstriction mediated by alpha-adrenergic mechanisms plus high plasma concentrations of catecholamines and neuropeptide Y (NPY). Endothelial factors such as NO and endothelin-1 also play a role in the regulation of local blood flows. Unlike fetuses of lowland species such as the sheep, the llama fetus shows a profound cerebral hypometabolic response to hypoxia, decreasing cerebral oxygen consumption, Na–K-ATPase activity and temperature, and resulting in an absence of seizures and apoptosis in neural cells. These strategies may have evolved to prevent hypoxic injury to the brain or other organs in the face of the persistent hypobaric hypoxia of life in the Andean *altiplano*.

Keywords: Hypoxia; High altitude; Hypometabolism; Vasoconstriction; Adrenergic; Cardiovascular

1. Introduction

Hypoxia during fetal development or shortly after birth induces abnormal growth and development. It can cause short (death, pulmonary hypertension) and long-term consequences such as abnormal neurodevelopment and an increase in risk for diseases later in life (Fowden et al., 2006). Causes for hypoxia are found in placental pathology, umbilical cord abnormalities, asphyxia during delivery or neonatal pathologies such as metabolic diseases, lung pathology (immaturity, pulmonary hypertension, aspiration of meconium or infection (Alonso-Spilsbury et al., 2005). Its treatment is often very invasive where the neonate is artificially ventilated, a practice that can produce inflammation, lung injury and infection. Moreover, powerful pharmaceutical adrenergic drugs are administered to increase the function of the heart and thus move more oxygen to the organs and tissues. The use of such drugs may damage the heart. A more informed approach to prevent hypoxia in seriously ill newborns is required. A great deal can be learnt from

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nature in this respect by studying appropriate animal models. In this review we concentrate on ideas derived from the South American camelids and particularly the domestic species, the llama (*Lama glama*), which develops, resides and reproduces in chronic environmental hypoxia.

Most mammalian species live and develop in an environment close to sea level, with an atmospheric pressure near to 760 mmHg and a partial pressure of 150 mmHg of O_2 in the air. Although most mammals are very susceptible to even modest oxygen shortages, some species have developed a particular tolerance to hypoxia, since their evolution has taken place in an environment of low oxygen availability. Among these species are seals and other diving mammals, animals that live in burrows, and those which live and develop in the hypobaric hypoxia of high altitudes. The latter comprises the South American *Camelidae*, which includes the domestic species, llama (*L. glama*) and alpaca (*Lama pacos*), and, the wild species, vicuña (*Vicugna vicugna*) and guanaco (*Lama guanicoe*).

2. The adult llama

To thrive at the high altitudes of the Andean *altiplano*, the llama shows characteristics, which appear to have evolved in several physiological functions. These are now genetically determined, since they continue to be present in llamas born and living at low altitudes. These physiological changes include high hemoglobin oxygen affinity (low P_{50}), small elliptical red cells with high hemoglobin concentration, a small increase in blood hemoglobin concentration, high muscle myoglobin concentration, more efficient O_2 extraction at the tissue and high lactate dehydrogenase activity (Llanos et al., 2003). Lowland species such as the sheep, or indeed humans, develop pulmonary hypertension when they reside at high altitude. This produces structural changes in the pulmonary blood vessels (Harris et al., 1982). However, the llama prevents the occurrence of pulmonary arterial hypertension by having less highly muscularised pulmonary arterioles (Harris et al., 1982). To examine further such species differences, in adult llamas raised at sea level, we measured pulmonary vascular responses to acute hypoxia and compared them relative to those of lowland sheep. While pronounced increases in pulmonary arterial pressure and pulmonary vascular resistance occurred in sheep, these changes were markedly attenuated in llamas (Fig. 1). In both species cardiac output increased, systemic vascular resistance decreased and mean systemic arterial pressure was maintained during acute hypoxia. These data show that the llama responds to acute hypoxia with diminished cardiovascular responses, showing only a mild pulmonary hypertension relative to the sheep. Blunted cardiopulmonary responses to acute hypoxia may be a beneficial adaptation in the Andean camelid to the chronic hypobaric hypoxia of life at high altitude.

In contrast to pulmonary arterial pressure, systemic arterial blood pressure changes with the head position, since the llama is a long necked animal. Arterial blood pressure was higher when the head was above the heart level (Fig. 2). These results indicate a significant variation in the systemic arterial pressure with vertical variations in the head position as in the giraffe (Goetz et al., 1960; Mitchell et al., 2006). Nevertheless, in spite of a lower systemic arterial pressure when the llama's head was down, there was no tachycardia, suggesting a lower baroreflex threshold for eliciting an increase in heart rate (Fig. 2).

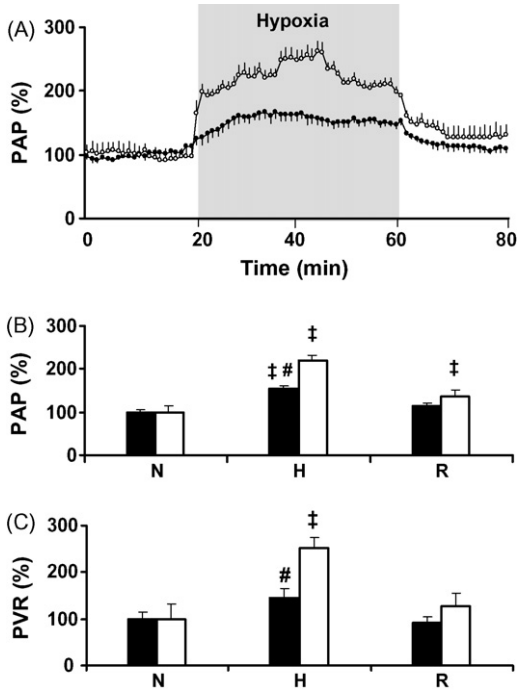

Fig. 1. Pulmonary arterial pressure (PAP) and vascular resistance (PVR) in adult llama ($n = 4$, solid circles-bars) and sheep ($n = 5$, open circles-bars) during acute hypoxia. (A) PAP was recorded continuously during 20 min of normoxia, 40 min of hypoxia and 20 min of recovery. (B) Average PAP during 20 min of normoxia, 40 min of hypoxia and 20 min of recovery. (C) Average PVR during 20 min of normoxia, 40 min of hypoxia and 20 min of recovery. Data are expressed as percentage of change for normoxic period. Means ± S.E.M., $p < 0.05$, ‡vs. normoxia, #vs. sheep (ANOVA and Newman-Keuls).

3. The fetal llama

Compared with lowland species, fetal life for mammalian species whose mothers live in an environment of oxygen shortage is demanding. For instance, fetal llamas have to cope with the low fetal arterial P_{O_2} of all species, plus the likely superimposition of low P_{O_2}, as a result of the decreased oxygen environment in which the mother lives in the Andean *altiplano* (Llanos et al., 2003). The fetal llama has a higher hemoglobin oxygen affinity compared with the adult llama, however the difference in hemoglobin oxygen affinity between mother and fetus is less that that between maternal and fetal sheep (Moraga et al., 1996). Basal cardiovascular function is characterized by lower cardiac output and organ blood flows and higher total peripheral vascular resistance than fetuses of lowland species such as the sheep, showing more efficient total O_2 extraction (Benavides et al., 1989; Llanos et al., 1995; Pérez et al., 1989).

Since the maternal llama can modify systemic arterial pressure and utero-placental blood flow by changes in the position of the head, we studied the influence of pregnancy on the variations of this cardiovascular variable. We also measured fetal oxygenation during such changes. As in the non-pregnant

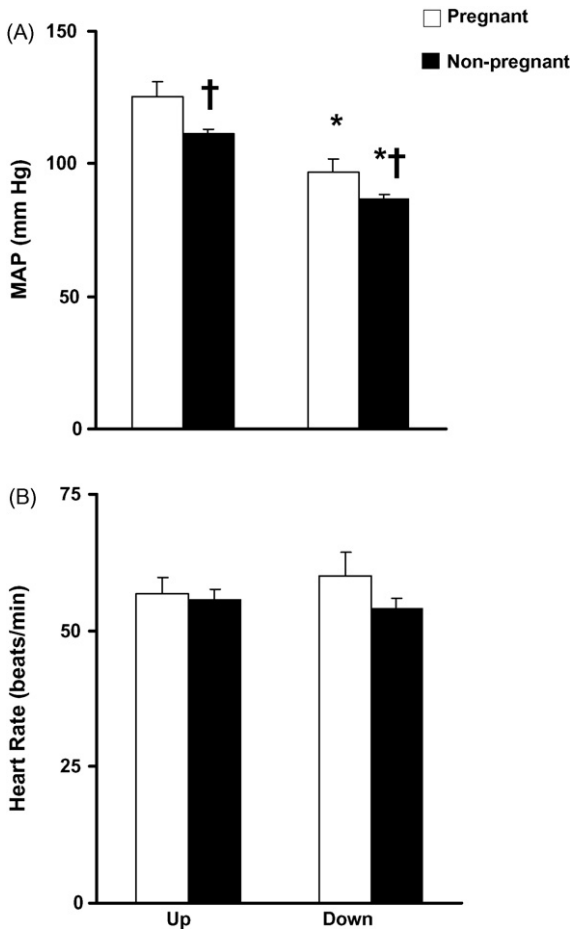

Fig. 2. Effects of adult llama's head position on mean arterial pressure and heart rate in pregnant ($n = 5$, open bars) and non-pregnant animals ($n = 6$; closed bars). (A) Mean arterial pressure with the llama's head above (up) or under (down) the heart level. (B) Heart rate with the llama's head above (up) or under (down) the heart level. Data are expressed as means ± S.E.M., $p < 0.05$, *vs. up, †vs. pregnant (ANOVA and Newman-Keuls).

llamas, arterial blood pressure varied with modifications of head position during pregnancy, the pressure being higher when the head was above the heart level (Fig. 2). In contrast with humans, pregnant llamas had a higher arterial blood pressure than non-pregnant animals (Fig. 2). Fetal pH and blood gases did not however change with variations in the maternal head position, indicating that no major changes in utero-placental blood flow occurred in spite of marked changes in maternal systemic arterial pressure. The latter suggest autoregulation of the utero-placental blood flow in contrast to what is observed in humans.

4. The fetal llama in acute and chronic hypoxia

The llama fetus responds to acute hypoxia with a marked peripheral vasoconstriction and a small increase in cerebral blood flow (Llanos et al., 1995, 1998, 2002). In contrast, fetuses of lowland species show a substantial increase in cerebral blood flow, with vasoconstrictor responses being less than that of the fetal llama (Llanos et al., 2003). In addition, the hypoxic fetal llama shows a greater increase in cardiac blood flow, a smaller increase in adrenal blood flow and a greater decrease in kidney and carcass blood flow than the fetal sheep (Llanos et al., 2003). Since the carotid chemoreflex is partially responsible of the increase in peripheral vascular resistance in the sheep fetus, we wondered whether this intense peripheral vasoconstriction in response to acute hypoxia in the fetal llama was due to an accentuated carotid chemoreflex. Therefore, we denervated the carotid sinuses by sectioning the sinus nerves bilaterally. We found no modification of the severe peripheral vasoconstriction during acute hypoxia in the fetal llama (Giussani et al., 1996), indicating that the carotid chemoreceptors are not involved in the mechanism by which the marked vasoconstriction occurred and suggesting a major role of alternative mechanisms such as endocrine and local mechanisms. However, in the chemodenervated llama fetuses the plasma cortisol concentration did not rise during an episode of acute hypoxia, as was observed in controls, notwithstanding the comparable values for plasma ACTH levels and adrenal blood flows (Riquelme et al., 1998). On the contrary, chemodenervated fetal sheep did enhance cortisol plasma concentration only during late hypoxia, 45 min after the onset of the insult (Giussani et al., 1994). Taken together all these results show a significant neural contribution in the regulation of adrenal cortex function, but not in producing the intense peripheral vasoconstriction in the llama fetus submitted to acute hypoxia.

One of the candidates for mediating the striking peripheral vasoconstriction during hypoxia in the llama fetus is the sympathetic nervous system. Consequently, when phentolamine, an α_1 and α_2-adrenergic blocker, was administered intravenously at the beginning of an acute hypoxia insult, there was a pronounced systemic arterial hypotension with decreases in carotid and femoral blood flow, leading rapidly to fetal death (Giussani et al., 1999). In preliminary experiments in 3 fetal llamas, when the more specific α_1-adrenergic blocker prazosin was administered during hypoxia, a similar cardiovascular collapse and death was observed as with phentolamine. In contrast, with administration of the α_2-adrenergic blocker yohimbine, no cardiovascular effects were observed either in normoxia or in hypoxia. Therefore, the major α-adrenergic effect in the fetal circulation in the hypoxic llama fetus appears to involve α_1-adrenergic receptors. The role of the α-adrenergic system in cardiovascular regulation is also observed in the chronically hypoxic fetal sheep, while α-adrenergic blockade also produced severe systemic hypotension and fetal death (Block et al., 1984). These results suggest that chronic hypoxia in lowland species or adaptation to chronic hypoxia over an evolutionary timescale in highland species increases the contribution of the α-adrenergic system in the responses to episodes of acute hypoxia.

We have found higher plasma catecholamine concentrations in the fetal llama compared to fetal sheep of the same gestational age, in both normoxia and hypoxia, indicating the high functionality of the α-adrenergic system in both normoxia and hypoxia in the former (Riquelme et al., 2002). In agreement with this, exposure of fetal sheep to chronic hypoxia produces an increase in basal fetal plasma norepinephrine but not necessarily in epinephrine concentrations (Gardner et al., 2001).

Because vascular responses to acute hypoxia in the llama are dominated by α-adrenergic mechanisms, we studied the

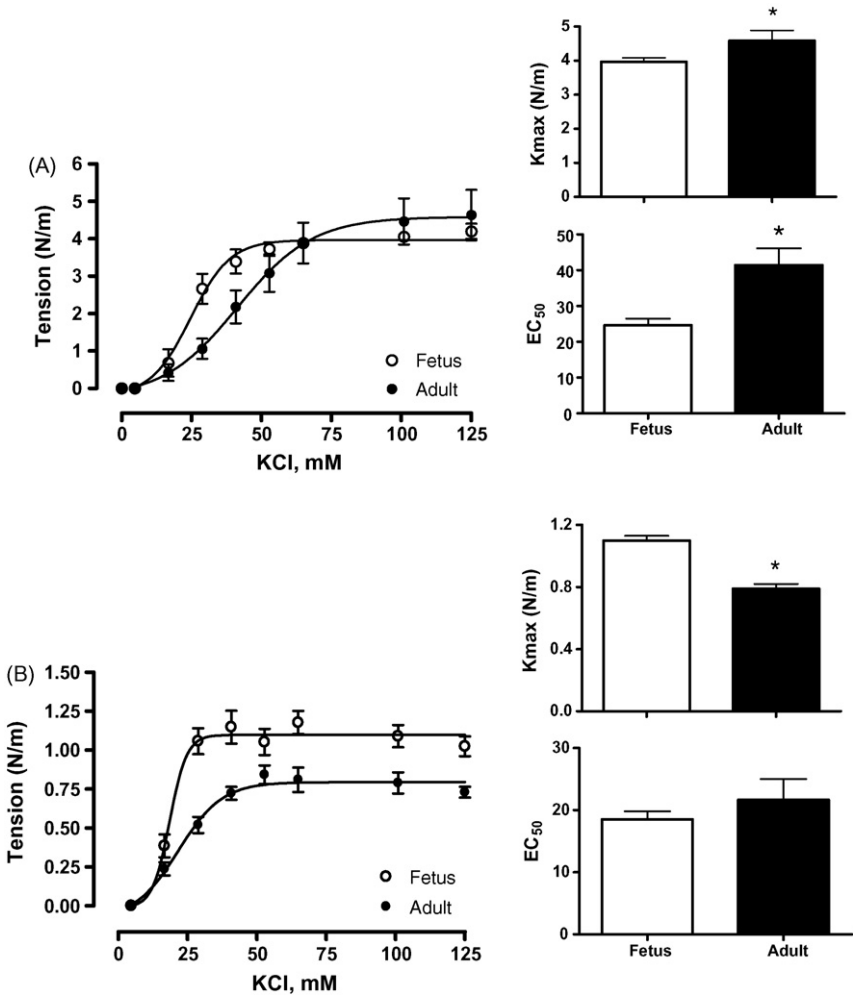

Fig. 3. Concentration-dependent contractile effects of potassium chloride (KCl) in mesenteric (A) and pulmonary (B) small arteries from fetal (◯) and adult (●) llamas. Each point represents the means ± S.E.M. of 10 fetal and 6 adult animals. Maximal responses and sensitivity are presented as histograms for fetal (open) and adult (closed) llama. Significant differences $p < 0.05$ (t-test): *adult vs. fetal.

contractile responses and sensitivity of systemic (mesenteric) and pulmonary small arteries of fetal and adult llamas utilizing wire myography (Mulvany and Halpern, 1977). Both the fetuses and adults studied were from high altitude, over 4000 m above sea level. The sensitivity of the contractile responses to K^+ and to norepinephrine was more marked in both vascular beds in the fetus than in the adult, with a marked contractile response to K^+ and norepinephrine in the fetal small pulmonary artery (Figs. 3 and 4). After precontraction with KCl, endothelium-independent (sodium nitroprusside, SNP) relaxation was higher in both fetal vascular beds than in adult arteries (Fig. 5). The strong adrenergic responses of fetal llama mesenteric and pulmonary arteries support the concept of an adaptive general mechanism, which has evolved to withstand the hypoxia at high altitude. As observed in terms of blood pressure and organ blood flows in the whole fetus in utero, the effects of NO to relax small arteries were also enhanced in the llama fetus, perhaps as a mechanism to counteract the pronounced α-adrenergic mechanisms.

The increased systemic vascular resistance of the fetal llama compared to the fetal sheep persists into the neonatal period. The femoral vascular resistance of the llama neonate is higher compared to the sheep, in both normoxia and hypoxia. Furthermore, we found that the α_1-adrenergic vasoconstrictor responses of isolated small femoral vessels from the newborn llama were greater than those of the newborn sheep. This difference could be explained by the greater expression of a high affinity α_{1B} receptor and the lower expression of a low affinity α_{1A} receptor in the newborn llama than in newborn sheep femoral arteries (Moraga, 2006).

Therefore, one of the mechanisms that may explain the marked peripheral vasoconstriction observed in the llama fetus and neonate during normoxia and acute hypoxia is a stronger endocrine vasoconstrictor tone. This is the result of greater elevations in plasma catecholamines and enhanced α-adrenergic mediated mechanisms compared to fetus and neonates of lowland species.

The pronounced peripheral vasoconstriction of the llama fetus also involves other vasoconstrictor hormones, such as arginine vassopresin (AVP) and neuropeptide Y (NPY). Their plasma concentrations increase more than in the fetal sheep (Giussani et al., 1996; Sanhueza et al., 2003). In contrast,

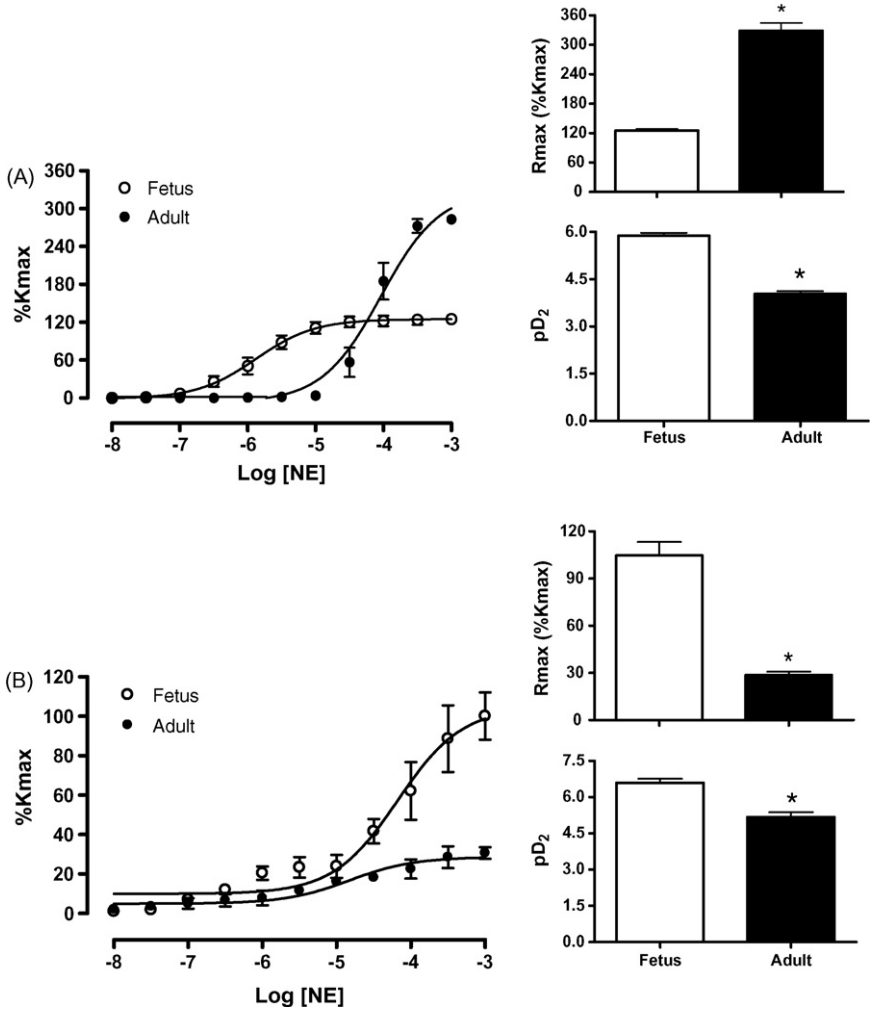

Fig. 4. Response to norepinephrine (NE) in mesenteric (A) and pulmonary (B) small arteries from fetal (◯) and adult (●) llamas. Each point represents the means ± S.E.M. of 10 fetal and 6 adult animals. Maximal responses and sensitivity are presented as histograms for fetal (open) and adult (closed) llama. Significant differences $p < 0.05$ (t-test): *adult vs. fetal.

angiotensin II plasma concentration did not rise during hypoxia in the llama fetus (Giussani et al., 1996). Nevertheless, a V_1 AVP receptor antagonist did not modify either the blood flow to any organ (Herrera et al., 2000), or the carotid or femoral blood flow during hypoxia (Giussani et al., 1999).

Vascular endothelial cells synthesize and release various factors, which regulate vascular tone, angiogenesis, inflammatory responses and permeability. Among these factors, nitric oxide (NO), carbon monoxide (CO), prostanoids including prostacyclin, thromboxane A2 and/or isoprostanes, endothelin-1, etc., actively contribute to the regulation of vascular tone. Such mediators can modify local and regional vascular resistances and organ blood flows (Félétou and Vanhoutte, 2006).

As noted above, NO is central to the regulation of the circulation in the fetal llama, counteracting the strong vasoconstrictor action of α-adrenergic agonists. With the administration of L-NAME, a NO synthase blocker, cerebral, carotid and femoral vascular resistance increased and blood flow decreased to carotid and femoral beds, while cerebral blood flow did not change significantly in normoxia. However, during hypoxia cerebral and carotid vascular resistance fell by 44% from its value in normoxia after L-NAME, while femoral vascular resistance gradually increased and remained high during recovery (Sanhueza et al., 2005). In addition, adrenal blood flow decreased and adrenal vascular resistance increased while oxygen delivery fell, in llama fetuses treated with L-NAME during normoxia and hypoxia (Riquelme et al., 2002). Furthermore, NO plays a vital role in modifying heart blood flow. For example, the heart blood flow increased in 3.7 times during an episode of acute hypoxia in the fetal llama, but when L-NAME was given this increase was only 2.8 times (Fig. 6).

Fetal plasma norepinephrine and epinephrine concentrations increased markedly with L-NAME treatment in normoxia, but those of ACTH or cortisol were not affected. As expected, fetal plasma concentrations of ACTH, cortisol, norepinephrine and epinephrine increased during acute hypoxia in saline-infused fetuses. The increased plasma cortisol level observed in acute hypoxia was completely prevented in L-NAME treated fetuses. In L-NAME treated fetuses during acute hypoxia, fetal plasma concentrations of norepinephrine became further elevated, whereas concentrations of epinephrine returned back to near basal values (Riquelme et al., 2002). Putting together all

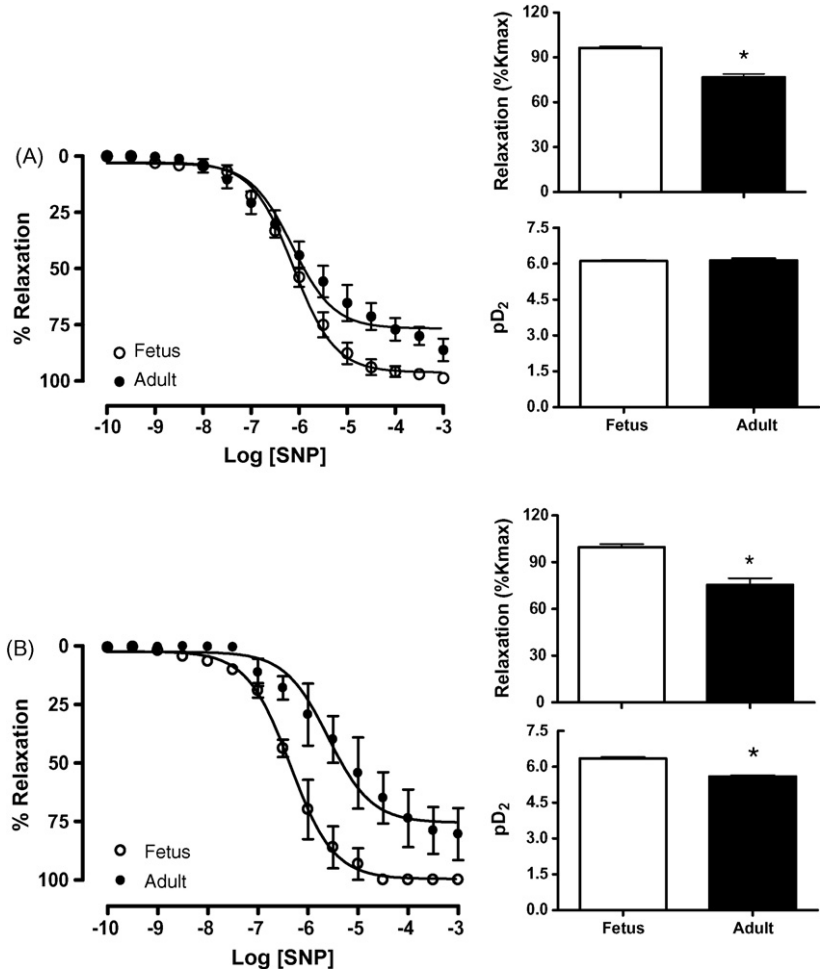

Fig. 5. Response to sodium nitroprusside (SNP) in mesenteric (A) and pulmonary (B) small arteries from fetal (◯) and adult (●) llamas. Each point represents the means ± S.E.M. of 10 fetal and 6 adult animals. Maximal responses and sensitivity are presented as histograms for fetal (open) and adult (closed) llama. Significant differences $p < 0.05$ (t-test): * adult vs. fetal.

these data, it appears that NO plays a crucial function in the control of adrenal blood flow during basal and hypoxic conditions, totally precluding the well established increase in adrenal blood flow during hypoxia in the llama fetus (Llanos et al., 2003). In addition, these data strongly suggest that NO plays a critical role in the regulation of adrenal secretion of cortisol and catecholamines during acute hypoxia in the llama fetus.

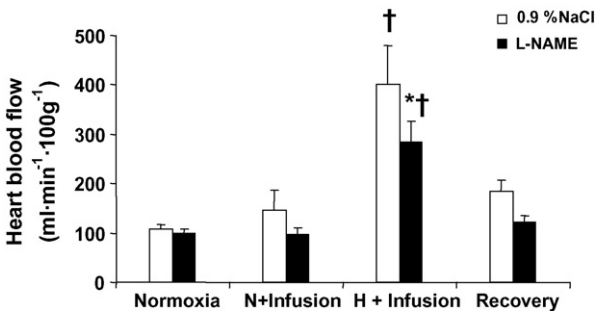

Fig. 6. Heart blood flow in fetal llama during infusion of L-NAME (20 mg kg^{-1} I.V. bolus followed by I.V. infusion of 0.5 mg kg^{-1}, $n = 3$, solid bars) or saline infusion (control, 0.9% NaCl, $n = 4$, open bars). The infusion started 15 min prior to hypoxia (H) and ran continuously during 60 min of hypoxia. Data expressed are as means ± S.E.M., $p < 0.05$, * vs. 0.9% NaCl and † vs. normoxia (ANOVA and Newman-Keuls).

NO production has been involved in eliciting neuronal damage during hypoxia, even though NO is also a normal mediator in synaptic transmission (Galleguillos et al., 2001). We found no changes in NOS activity or in its subcellular distribution in brain tissues of fetal llama submitted to 24 h of hypoxia. These responses could be a cytoprotective mechanism inherent to the fetal llama against possible toxic effects of NO (Galleguillos et al., 2001).

Another endothelial product, endothelin-1, has been established as one of the most powerful vasoconstrictors in the pulmonary and systemic circulation. The endothelial cells secrete endothelin-1 (ET-1), which acts as the natural counterpart of the vasodilator NO. ET-1 contributes to vascular tone and regulates cell proliferation due to activation of ET_A and ET_B receptors. Shear stress, thrombin, epinephrine, angiotensin II, growth factors, cytokines and free radicals augment secretion of ET-1. By contrast, mediators such as NO, cyclic GMP, atrial natriuretic peptide, and prostacyclin diminish the secretion of endogenous ET-1. Thus, the effects of ET-1 are tightly regulated by inhibition or stimulation of its release from the endothelium

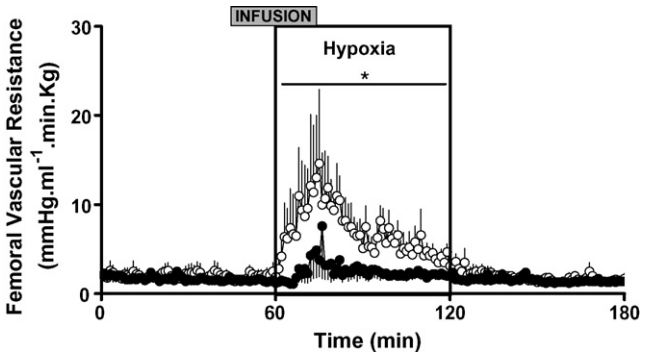

Fig. 7. Femoral vascular resistance (FVR) in fetal llama during infusion of BQ 123 (30 μg kg^{-1} min^{-1}, $n = 3$, solid circles) or saline infusion (control, 0.9% NaCl, $n = 4$, open circles). The infusion was started 15 min prior to hypoxia and ran continuously during 15 min during hypoxemia. Data expressed as means ± S.E.M., $p < 0.05$, *vs. 0.9% NaCl (ANOVA and Newman-Keuls).

(Marasciulo et al., 2006). Since the llama fetus had a potent peripheral vasoconstriction during acute hypoxia, we examined whether endothelin-1 participated in the pronounced femoral vascular tone during acute hypoxia, by using the endothelin A receptor antagonist, BQ123. Administration of BQ123 had no effect on cardiovascular variables during normoxia, but completely prevented the increase in femoral vascular resistance, indicating a central role for endothelin-1 in the regulation of the femoral vascular tone during acute hypoxia in the llama fetus (Fig. 7) (Riquelme et al., 2003).

But the critical question remains of the function(s) of the marked peripheral vasoconstriction to cope with hypoxia in the fetal llama. First, by reducing blood flow to peripheral organs it permits increased flow to others, although, this only applies to the heart and adrenals since the brain blood flow does not increase with hypoxia in the fetal llama. Secondly, the intense vasoconstriction could induce preconditioning in the tributary organ of the constricted artery or induce remote ischemic preconditioning in some other organ(s) (Pérez-Pinzon, 2007). This can occur when a short ischemic episode is followed by a period of reperfusion, enhancing the resistance of the organ to successive ischemic events. However, at least in late gestation fetal sheep, a series of five 1-min total umbilical cord occlusions did not result in robust cardio- and neuroprotection, in contrast to ischemic preconditioning reported in adults (Lotgering et al., 2004). Thirdly, the striking peripheral vasoconstriction could trigger the hypometabolic adaptation of several organs in the fetal llama. Such hypometabolism (Milton and Prentice, 2007; Pérez-Pinzon, 2007; Storey, 2007; Bickler and Buck, 2007; Ramirez et al., 2007) will be discussed further below.

5. The brain during hypoxia in the llama fetus

We measured cerebral blood flow and calculated cerebral oxygen consumption in the llama fetus, and the data obtained were surprisingly different from those of the fetal sheep (Field et al., 1990; Pearce, 2006; Pena et al., 2007). The fetal llama did not increase cerebral blood flow during hypoxia, so there was a progressive fall in cerebral oxygen consumption when the hypoxic condition became more severe (Llanos et al., 2002). Carotid blood flow was less in the high voltage–low frequency state, condition of lower cerebral oxygen consumption than in the low voltage high frequency state (Blanco et al., 1997). The same pattern is observed in the fetal sheep (Richardson et al., 1985). These results can be interpreted as evidence of another strategy used by the llama fetus to conserve energy (Blanco et al., 1997).

But a fundamental question is how the cerebral oxygen consumption can decrease in the fetal llama during hypoxia without producing neural damage. We have shown that the electrocorticogram flattens under these conditions of hypoxia, but in contrast to the fetal sheep, seizure activity does not occur (Llanos et al., 2000). This implies absence of asphyxial damage (although we do not yet have histological data in the llama fetus to confirm the lack of injury) and suggests adaptive brain hypometabolism. To seek further evidence that fetal llama responds to hypoxia with adaptive brain hypometabolism, we measured brain temperature, Na^+ channel density and Na–K-ATPase activity in llama fetuses submitted to an episode of prolonged hypoxia of 24 h. In addition, we determined whether there was evidence of cell death in the brain cortex by looking at poly ADP-ribose polymerase (PARP) protein degradation. We found a reduction of 0.56 °C in brain cortex temperature that went together with 51% decrease in brain cortex Na–K-ATPase activity and a 44% decrease in protein level of NaV1.1, a voltage-gated sodium channel. These effects occurred in the absence of changes in PARP protein degradation, suggesting that brain cell death was not increased in the fetal llama during hypoxia. Taken together these results support the hypothesis that the fetal llama responds to prolonged hypoxia with adaptive brain hypometabolism, partly mediated by decreases in Na–K-ATPase activity and expression of NaV channels (Ebenspeger et al., 2005). Preliminary data from our laboratory suggests that little production of lactate by the fetal llama brain occurs during hypoxia, further supporting the idea of an active hypometabolism during this condition.

Hypometabolic responses and hypoxia tolerance have been described in a wide range of species including fish, amphibians, and reptiles (Bickler and Buck, 2007), in mammals and birds (Ramirez et al., 2007), invertebrates (Haddad, 2006), turtles (Pek-Scott and Lutz, 1998; Buck and Bickler, 1998), carp (Nilsson, 2001), fetal sheep (Blood et al., 2003; Hunter et al., 2003a,b) and the issue is extensively reviewed elsewhere (see Milton and Prentice, 2007; Pérez-Pinzon, 2007; Storey, 2007). Remarkably, cerebral hypometabolism is also described in Quechua Indians, indigenous people of South America who have about 10,000–15,000 years of residence at high altitude (Hochachka et al., 1994). The strategy of hypoxic hypometabolism, which appears to be shared by the llama, may have developed to prevent hypoxic injury in cerebral or other tissues, and be a potent adaptation selected to resist the prolonged risk of hypoxia resulting from the persistent hypobaric hypoxia of life in the Andean *altiplano*.

The challenge is now to investigate the physiological, cellular and molecular mechanisms of the adaptive hypometabolic response triggered by hypoxia. Nature gives us some clues from animals living in diverse hypoxic environments. This will help

us to understand further how the fetal, newborn and maternal llamas have evolved to cope with hypoxia, a pathological condition of great scientific, and medical and social impact.

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