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Cardiovascular function in term fetal sheep conceived, gestated and studied in the hypobaric hypoxia of the Andean *altiplano*

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Key points

- High altitude developmental hypoxia causes intrauterine growth restriction and cardiovascular programming. However, some mammals exposed chronically to high-altitude hypoxia have less growth restriction suggesting certain protection.
- Cardiovascular defence mechanisms during acute fetal hypoxia divert blood flow from the periphery towards the brain, heart and adrenals. In contrast, little is known about the cardiovascular defence mechanisms during chronic fetal hypoxia.
- Here, we established the cardiovascular responses in fetal sheep that were conceived, gestated, born and studied at 3600 m. The data suggest that chronically hypoxic pregnant ewes and their fetuses have evolved different mechanisms from sea level pregnancies to withstand chronic hypoxia.
- The cardiovascular responses to acute hypoxia are blunted in the chronically hypoxic fetus. These findings points towards compensatory mechanisms in the highland fetus at the level of the cells and molecules rather than mounting major cardiovascular responses, saving oxygen not easily available in the *Alto Andino*.

Abstract High-altitude hypoxia causes intrauterine growth restriction and cardiovascular programming. However, adult humans and animals that have evolved at altitude show certain protection against the effects of chronic hypoxia. Whether the highland fetus shows similar protection against high altitude gestation is unclear. We tested the hypothesis that high-altitude fetal sheep have evolved cardiovascular compensatory mechanisms to withstand chronic hypoxia that are different from lowland sheep. We studied seven high-altitude (HA; 3600 m) and eight low-altitude (LA; 520 m) pregnant sheep at ∼90% gestation. Pregnant ewes and fetuses were instrumented for cardiovascular investigation. A three-period experimental protocol was performed *in vivo*: 30 min of basal, 1 h of acute superimposed hypoxia (∼10% O_2) and 30 min of recovery. Further, we determined *ex vivo* fetal cerebral and femoral arterial function. HA pregnancy led to chronic fetal hypoxia, growth restriction and altered cardiovascular function. During acute superimposed hypoxia, LA fetuses redistributed blood flow favouring the brain, heart and adrenals, whereas HA fetuses showed a blunted cardiovascular response. Importantly, HA fetuses have a marked reduction in umbilical blood flow *versus* LA. Isolated cerebral

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arteries from HA fetuses showed a higher contractile capacity but a diminished response to catecholamines. In contrast, femoral arteries from HA fetuses showed decreased contractile capacity and increased adrenergic contractility. The blunting of the cardiovascular responses to hypoxia in fetuses raised in the *Alto Andino* may indicate a change in control strategy triggered by chronic hypoxia, switching towards compensatory mechanisms that are more cost-effective in terms of oxygen uptake.

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Abbreviations ABF, adrenal blood flow; BBF, brain blood flow; CBF, carotid blood flow; CVR, carotid vascular resistance; FBF, femoral blood flow; FVR, femoral vascular resistance; HA, high-altitude; HBF, heart blood flow; HR, heart rate; IUGR, intrauterine growth restriction; LA, low-altitude; MCA, middle cerebral artery; MSAP, mean systemic arterial pressure; NA, noradrenaline; NO, nitric oxide; NOS, nitric oxide synthases; pD_2, $-\log EC_{50}$; Phe, phenylephrine; UBF, umbilical blood flow.

Introduction

Pregnant women and their unborn babies from several regions of the world are chronically exposed to the low oxygen milieu of high altitude mountains or plateaus. Such women come from different ethnicities, and some have resided in highlands for hundreds of generations (Beall, 2007; Simonson *et al.* 2010; Wang *et al.* 2011; Browne *et al.* 2015). Despite acclimatization, pregnancy at high altitude is clearly a potential burden for both mother and fetus. In fact, the incidence of pregnancy complications and neonatal morbidity, such as intrauterine growth restriction (IUGR), fetal hypoxia, stillbirth and respiratory distress in neonates is significantly increased in high altitude populations (Giussani *et al.* 2001; Keyes *et al.* 2003; Gonzales *et al.* 2008; Soria *et al.* 2013). Thus, in Andean high-altitude locations (> 3200 m) there is an increased infant mortality rate (Keyes *et al.* 2003; Gonzales *et al.* 2008) relative to lowland populations. Interestingly, human populations that have resided for the longest time period at high altitude in Tibet and Bolivia have the greatest reduction in the incidence of growth restriction, suggesting graded adaptation to this milieu (Moore, 2001; Giussani *et al.* 2001; Soria *et al.* 2013). Similarly, sheep brought by the Spanish *Conquistadores* 500 years ago and now permanently resident for many generations in the Andean *altiplano* (or *Alto Andino*, the high-altitude plateau) have substantially smaller fetuses and newborn lambs compared to those residing at low altitude (Parraguez *et al.* 2005; Herrera *et al.* 2007). However, these animals show a reduced neonatal morbidity and mortality when compared with sheep newly arrived in the highlands (Herrera *et al.* 2010, 2011).

In normal pregnancy, acute hypoxia is a common challenge to the fetus, for instance during transient compression of the umbilical cord. In the last decades, the fetal cardiovascular responses to acute hypoxia have been extensively studied and described (Cohn *et al.* 1974; Parer, 1980; Alonso *et al.* 1989; Pérez *et al.* 1989; Giussani *et al.* 1993; Fletcher *et al.* 2006; Bennet & Gunn, 2009; Thakor *et al.* 2010; Kane *et al.* 2012; Herrera *et al.* 2012). These responses are coordinated by the autonomic nervous system, unleashing an initial peripheral vasoconstriction triggered by a carotid chemoreflex (Giussani *et al.* 1993) and mediated by increased sympathetic activity (Giussani *et al.* 1993; Bennet & Gunn, 2009). Fetal hormones liberated into the blood stream maintain the peripheral constriction (Pérez *et al.* 1989; Fletcher *et al.* 2006) and there is now evidence of a local oxidant tone acting directly at the level of the fetal vasculature (Thakor *et al.* 2010, 2015; Kane *et al.* 2012; Herrera *et al.* 2012). However, the effect of chronic hypoxia on the fetal cardiovascular responses to superimposed acute hypoxia is less well understood and very much understudied. Until now, the few attempts have involved mostly sea level animal models made chronically hypoxic during pregnancy via several techniques (see Giussani & Davidge, 2013) and an elegant series of studies investigating the effects on fetal physiology of sea level sheep exposed to high altitude (Kamitomo *et al.* 1993; Longo *et al.* 1993; Gilbert *et al.* 2003; Pereyra *et al.* 2007). To the best of our knowledge, the basal cardiovascular effects and the responses to acute hypoxia on fetuses conceived, gestated and studied at actual high altitude have never been investigated. The present study used an established cohort of sheep exposed to the high altitude of the *Alto Andino* for several generations (Llanos *et al.* 2003; Herrera *et al.* 2007) to test the hypothesis that highland fetal sheep have evolved cardiovascular compensatory mechanisms to withstand basal and superimposed acute hypoxia that are different from lowland fetal sheep.

Methods

The Faculty of Medicine Bioethics Committee of the University of Chile approved all experimental procedures

(Protocol CBA (Animal Bioethics Committee) No. 097, Faculty of Medicine, University of Chile (FMUCH)). The studies on animals were performed according to the Animal Research: Reporting In Vivo Experiments (ARRIVE) guidelines and the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and adhered to the American Physiological Society's *Guiding Principles in the Care and Use of Animals.*

Animals

Seven time-mated pregnant sheep (*Ovis aries*) from several generations at high altitude (HA; Putre Research Station, University of Chile, 3600 m above sea level) and eight pregnant sheep from low altitude (LA; Santiago, Faculty of Medicine, University of Chile, 520 m above sea level) were bred and maintained under standard housing conditions. The HA fetuses were conceived, gestated, instrumented and studied at the Andean *altiplano* (Putre Research Station), while the low altitude animals were bred and studied at near sea-level. Both groups of fetuses were studied at the gestational age of 133 ± 3 days for the *in vivo* protocols (term ∼148 days). Similar in gestational age but uninstrumented fetuses (HA: $n = 5$; LA: $n = 5$; 134 ± 2 days) were used for the collection of small resistance arteries for the *ex vivo* myography studies.

Surgical preparation and *in vivo* experiments

All surgical procedures were made under aseptic conditions following food and water deprivation for 24 h. Between 125 and 130 days of gestation, pregnant sheep were premedicated with atropine (0.04 mg kg^{-1} I.M., Atropina Sulfato, Laboratorio Chile, Santiago, Chile), induced for intubation (5–7 mg kg^{-1} I.V. sodium thiopentone, Tiopental Sódico, Laboratorio Biosano SA, Santiago, Chile), and anaesthetized with 0.5–2% isofluorane in 50:50 oxygen and nitrous oxide. A midline laparotomy and hysterotomy was performed, and polyvinyl catheters (0.8 mm ID) were inserted into the fetal carotid artery and in the abdominal aorta and cava vein via hind limb fetal vessels. Ultrasonic flow probes (2R, Transonic Systems, Ithaca, NY, USA) were implanted around fetal femoral and carotid arteries. Additionally, polyvinyl catheters were placed into the femoral artery and amniotic cavity of the ewes (Giussani *et al.* 1993, 1994). After surgery, animals were returned to the yard and received a prophylactic treatment of ampicillin I.M. (500 mg; Ampicilina, Laboratorio Best Pharma, Santiago, Chile), and gentamicin (80 mg; Gentamicina Sulfato, Laboratorio Biosano SA, Santiago, Chile) into the amniotic cavity. Then catheters were filled with heparinized saline (250 IU heparin per ml 0.9% NaCl) and maintained patent by daily flushing.

Experimental procedure. All the *in vivo* experiments started at least 5 days after surgery and were based on a three-period protocol: 30 min of basal, 1 h of hypoxia and 30 min of recovery. Hypoxia was induced by a transparent respiratory hood placed over the maternal head supplied with a controlled mixture of gases (50 l min^{-1}, ∼12–18% O_2 and 2–3% CO_2 in N_2) designed to reduce the fetal P_{O_2} to 12–14 Torr in the ascending aorta. During basal and recovery periods the animals breathed atmospheric air.

Measurements and calculations. Maternal and fetal arterial blood samples (0.5 ml) were taken in heparinized syringes at 0, 15 and 30 min of basal, at 15 min intervals during hypoxia, and at 15 and 30 min of recovery. These samples were analysed for pH, P_{O_2}, P_{CO_2} (BMS 3Mk2 Blood Microsystem and PHM 73 pH/Blood Gas Monitor, Radiometer, Copenhagen, Denmark), percentage of oxygen saturation of haemoglobin (S_{aO_2}) and haemoglobin concentration [Hb] (OSM2 Haemoximeter, Radiometer, Copenhagen, Denmark).

Systemic arterial pressure was obtained continuously using a pressure transducer. In addition, fetal carotid (CBF) and femoral (FBF) blood flows were measured with the Transonic flow probes. All cardiovascular variables were recorded with a data acquisition system (Powerlab/8SP System and Chart v5.0 Software; ADInstruments, Bella Vista, NSW, Australia) connected to a personal computer. From these data, mean systemic arterial pressure (MSAP), heart rate (HR), and carotid and femoral vascular resistance (CVR and FVR respectively) were calculated.

In addition, the fetal organ blood flows to brain, heart, adrenals and umbilico-placental vascular bed were determined by the fluorescent microspheres method. Different coloured microspheres (10^6) were injected into inferior vena cava for each experimental period (Fluospheres, Molecular Probes, USA). During the injection, reference blood samples were drawn at 3.2 ml min^{-1} for 90 s from the ascending aorta for brain and heart blood flow determinations and from descending aorta for adrenal and umbilico-placental determinations (Heymann *et al.* 1977; Sanhueza *et al.* 2005). A crimson colour was used as an internal standard for all the experiments (Prinzen & Bassingthwaighte, 2000). On completion of experiments, ewes and fetuses were killed with a sodium thiopentone overdose (200 mg kg^{-1} slow I.V.; Tiopental Sódico, Laboratorio Biosano SA, Santiago, Chile). Fetuses and their organs were weighed and dissected. The organs in which we measured blood flow were digested in 3 M ethanolic KOH with 0.5% Tween 80,

filtered with 10 μm pore (Gaiser, Kappel-Grafenhausen, Germany) and rinsed with phosphate-buffered saline (PBS) and demineralised water. Dye fluorescence retained in the filtered microspheres was extracted dissolving the microspheres for 2 h with 10 ml of diethylene glycol monoethyl ether (Aldrich) and measured with a luminescence spectrophotometer (Perkin-Elmer L55, Waltham, MA, USA), using the specific excitation/emission wavelength and slit widths for each coloured sphere as follows:

$$\dot{Q}_{\text{organ}}\ (\text{ml}\ min^{-1}) = F_{\text{organ}} \times \dot{Q}_{\text{reference}}\ (\text{ml}\ min^{-1})/F_{\text{reference}}$$

where, $\dot{Q}$ is blood flow in millilitres per minute and F the measured fluorescence intensity (Tan *et al.* 1997). The organ vascular resistance was calculated by dividing perfusion pressure by the blood flow. To assess relative weight reduction we calculated the fetal organs to body weight ratio, and uteroplacental efficiency was calculated as the ratio of fetal body weight to uterus plus placental weight. Further, to determine growth symmetry, we calculated the brain to liver weight ratio.

Ex vivo studies

Uninstrumented pregnant sheep (gestational age 134 ± 2 days) were killed with a sodium thiopentone overdose (200 mg kg^{-1} slow I.V. bolus; Tiopental Sódico, Laboratorio Biosano SA, Santiago, Chile), and the fetal brain and hind limb were removed and immediately immersed in cold saline. Middle cerebral arteries (MCAs) and third generation femoral arteries were dissected and mounted in an isometric force transducer (model DSC 6; Kistler Morce, Seattle, WA, USA) of a wire myograph (dual-wire myograph; Danish Myo Technologies, Aarhus, Denmark). During experimentation the myograph bath was filled with Krebs buffer maintained at 39°C and aerated with 95% O_2 and 5% CO_2. Each artery was stretched to its individual optimal lumen diameter, considered as the diameter at which it developed the strongest contractile response to 125 mM K^+, as previously described (Herrera *et al.* 2007). Contractile agonists were evaluated under basal tone. A concentration–response curve was constructed for potassium chloride (KCl, 4.75–125 mM) with Krebs buffer washes between the different concentrations.

Cumulative concentration–response curve for noradrenaline (NA; 10^{-10} to 10^{-3} M) and phenylephrine (Phe; 10^{-10} to 10^{-3} M) were constructed for femoral and middle cerebral arteries. Each concentration–response was determined 2 min after adding each dose.

Solutions and drugs. Krebs buffer contained (mM): 118.5 NaCl, 25 $NaHCO_3$, 4.7 KCl, 1.2 KH_2PO_4, 1.2 $MgSO_4$, 2.5 $CaCl_2$, and 5.5 glucose with a pH of 7.4. In 125 mM K^+ buffer, all of the NaCl was replaced by an equimolar amount of KCl. All reagents and drugs were obtained from Sigma-Aldrich (St Louis, MO, USA).

Statistical analysis

Data are expressed as means ± SEM and significance was accepted when $P < 0.05$. For some *in vivo* experiments (MSAP, HR, CBF, CVR, FBF and FVR), we calculated the average of 15 min intervals, basal period (0–15; 16–30 min); early hypoxia (31–45; 46–60 min); late hypoxia (61–75; 76–90 min); and recovery (91–105; 106–120 min). Next, we utilized a two-way analysis of variance (ANOVA) followed by Newman–Keuls *post hoc* test to determine statistical differences. These two tests were also used to determine statistical comparisons between experimental time and groups in organ blood flows. For *ex vivo* experiments, the dose–response curves were analysed in terms of sensitivity (EC_{50} or pD_2) and maximal response (K_{max}) by fitting the experimental data to a sigmoidal equation (Prism 5.0; GraphPad Software, La Jolla, CA, USA). Contractile responses were expressed in absolute tension (mN mm^{-1}) or as a percentage of maximal response to KCl (%K_{max}). Sensitivity was calculated as pD_2, where $pD_2 = -\log [EC_{50}]$, EC_{50} being the concentration at which 50% of the maximal response was obtained. Differences between mean values were compared by Student's t test for unpaired data (Glantz & Slinker, 2001). For all comparisons, statistical significance was accepted when $P < 0.05$.

Results

Fetal and organ weights

The HA fetal lambs showed a significant reduction of 26% in fetal weight relative to LA at the same gestational age (133 ± 3 days). In addition, absolute brain and liver weights were lower and the brain to liver weight ratio was increased in HA relative to LA fetuses. Further, the adrenals relative to body weight, and the placental absolute and relative weight were increased in the HA group (Table 1). Accordingly, the calculated uteroplacental efficiency was markedly diminished in HA pregnancies (Table 1).

Maternal arterial blood gases and cardiovascular variables

The LA pregnant ewes showed blood gas and cardiovascular values within the normal range for this gestational age during basal conditions (Pérez *et al.* 1989). HA ewes had lower P_{O_2}, P_{aCO_2} and S_{aO_2}, higher pH and [Hb] compared to LA ewes. However, HA ewes had similar

Table 1. Fetal body and organ weights

		LA	HA	% change
Body	Absolute	3613 ± 176	2680 ± 168[a]	74.2[a]
Brain	Absolute	55.0 ± 1.9	44.5 ± 1.0[a]	80.9[a]
	Relative	1.549 ± 0.058	1.683 ± 0.114	108.7
Heart	Absolute	27.12 ± 1.69	22.75 ± 2.28	83.9
	Relative	0.736 ± 0.026	0.853 ± 0.082	115.9
Liver	Absolute	125.7 ± 6.5	81.8 ± 8.6[a]	65.1[a]
	Relative	3.839 ± 0.329	3.030 ± 0.168	78.9
Adrenals	Absolute	0.377 ± 0.018	0.410 ± 0.038	108.8
	Relative	0.0105 ± 0.0008	0.0156 ± 0.0020[a]	148.6[a]
Placenta+uterus	Absolute	1812 ± 211	2840 ± 83[a]	156.7[a]
	Relative	52 ± 6	107 ± 8[a]	205.8[a]
Uteroplacental efficiency	Relative	205 ± 16.4	96 ± 6.1[a]	46.8[a]
Brain/liver weight	Relative	0.445 ± 0.024	0.592 ± 0.060[a]	133.3[a]

Fetal body weight (g) and organ absolute weight (g) and relative to body weight ratio. Uteroplacental efficiency was calculated and fetal body weight to uteroplacental weight. Growth symmetry was calculated as brain/liver weight. Values are means ± SEM. Significant difference ($P \leq 0.05$): *a vs.* LA; Student's *t* test for all variables.

Table 2. Arterial blood gases and cardiovascular variables in pregnant ewe

		Basal	Hypoxia	Recovery
pH	LA	7.484 ± 0.018	7.485 ± 0.019	7.504 ± 0.015
	HA	7.520 ± 0.01[a]	7.511 ± 0.014[a]	7.501 ± 0.016
P_{aO_2} (Torr)	LA	100 ± 3	52 ± 5[c]	105 ± 5
	HA	48 ± 2[a]	44 ± 3[a]	49 ± 1[a]
P_{aCO_2} (Torr)	LA	32.2 ± 1.0	31.1 ± 1.2	30.7 ± 1.5
	HA	25.5 ± 1.4[a]	23.9 ± 1.1[a]	23.8 ± 0.5[a]
[Hb] (g dl^{-1})	LA	9.1 ± 0.5	8.9 ± 0.5	9.1 ± 0.6
	HA	11.7 ± 0.4[a]	11.3 ± 0.3[a]	11.4 ± 0.3[a]
S_{aO_2} (%)	LA	100 ± 1	77 ± 3[c]	100 ± 1
	HA	80 ± 3[a]	75 ± 3	82 ± 3[a]
O_2 cont (ml O_2 dl^{-1})	LA	12.2 ± 0.7	10.1 ± 0.9[c]	12.3 ± 0.9
	HA	12.3 ± 0.4	11.1 ± 0.5	12.0 ± 0.6
MSAP (mmHg)	LA	89 ± 6	94 ± 7	91 ± 7
	HA	87 ± 8	90 ± 9	89 ± 9
HR (min^{-1})	LA	119 ± 9	121 ± 7	121 ± 7
	HA	117 ± 8	122 ± 10	116 ± 9

Arterial pH, blood gases, haemoglobin concentration ([Hb]), percentage of oxyhaemoglobin (S_{aO_2}), arterial oxygen content (O_2 cont), mean systemic arterial pressure (MSAP) and heart rate (HR), in low altitude (LA) and high altitude (HA) pregnant sheep during basal, hypoxia and recovery. Values are means ± SEM. Significant differences ($P \leq 0.05$): *a vs.* LA; *b vs.* basal and *c vs.* all in the same group; ANOVA + Newman–Keuls test for all variables.

O_2 content relative to LA ewes (Table 2). During the acute hypoxic episode, the P_{aO_2}, S_{aO_2} and O_2 content decreased only in the LA ewes relative to their basal period. In addition, P_{aCO_2} (isocapnic hypoxia), MSAP and HR were maintained unchanged from baseline in both groups during the experimental protocol (Table 2).

Fetal arterial blood gases

During basal conditions, LA fetal sheep showed blood gas values within the normal range for this gestational age (Pérez *et al.* 1989). HA fetal sheep showed lower P_{aO_2}, P_{aCO_2} and S_{aO_2}, and higher [Hb] relative to LA fetal sheep. In contrast, HA and LA fetuses had similar values for pH and O_2 content (Table 3). During the acute hypoxic episode the P_{aO_2}, S_{aO_2} and O_2 content were reduced relative to basal values in both groups. In addition, pH was reduced only in the LA fetal sheep. During recovery, all variables returned towards basal values in both groups, with the exception of pH, which was maintained at a reduced level during recovery in the LA fetuses (Table 3).

Table 3. Ascending aorta blood gases in fetal sheep

		Basal	Hypoxia	Recovery
pH	LA	7.416 ± 0.007	7.354 ± 0.022^{b}	7.346 ± 0.019^{b}
	HA	7.436 ± 0.007	7.413 ± 0.008^{a}	7.414 ± 0.007^{a}
P_{aO_2} (Torr)	LA	23.4 ± 1.0	14.6 ± 0.6^{c}	23.9 ± 1.1
	HA	14.8 ± 1.0^{a}	11.7 ± 0.3^{c}	14.9 ± 0.9^{a}
P_{aCO_2} (Torr)	LA	46.5 ± 1.3	44.2 ± 1.5	43.8 ± 1.6
	HA	34.3 ± 1.4^{a}	32.4 ± 1.4^{a}	32.4 ± 1.6^{a}
[Hb] (g dl^{-1})	LA	9.3 ± 0.3	9.4 ± 0.2	9.0 ± 0.3
	HA	11.1 ± 0.4^{a}	10.6 ± 0.3^{a}	10.7 ± 0.3^{a}
S_{aO_2} (%)	LA	59.2 ± 2.0	25.5 ± 2.6^{c}	53.0 ± 2.8
	HA	42.3 ± 4.5^{a}	28.4 ± 1.3^{c}	42.2 ± 4.3^{a}
O_2 cont (ml O_2 dl^{-1})	LA	7.3 ± 0.1	3.2 ± 0.3^{c}	6.4 ± 0.4
	HA	6.2 ± 0.7	4.1 ± 0.3^{c}	6.0 ± 0.6

Arterial pH, blood gases, haemoglobin concentration ([Hb]), percentage of oxyhaemoglobin (S_{aO_2}) and arterial oxygen content (O_2 cont) in low altitude (LA) and high altitude (HA) fetal sheep during basal, hypoxia and recovery. Values are means ± SEM. Significant differences ($P \leq 0.05$): *a vs.* LA; *b vs.* basal and *c vs.* all in the same group; ANOVA + Newman–Keuls test for all variables.

Fetal systemic circulation

During basal conditions, HA fetal sheep showed a slightly but significantly lower mean systemic arterial pressure (MSAP) and an elevated fetal heart rate relative to LA fetal sheep. While MSAP increased in LA fetal sheep during acute hypoxia and recovery, HA fetal sheep did not exhibit the classic increase in MSAP during an episode of superimposed acute hypoxia and showed no changes in the MSAP throughout the experimental protocol (Fig. 1). Similarly, the classic fall in fetal heart rate at the onset of acute hypoxia seen in LA animals was not present in HA fetuses (Fig. 1). During recovery, both groups returned to basal HR values.

Fetal carotid circulation

During basal conditions, the CBF was higher in the HA fetal sheep compared to LA fetal sheep. Consequently, the CVR was decreased in HA compared to LA fetuses (Fig. 2). During the superimposed hypoxic episode, the CBF and CVR did not change in the HA fetal lambs, whereas in the LA group the CBF increased and CVR decreased, significantly compared to baseline. During the recovery period, both variables returned to basal levels in the LA group (Fig. 2).

Fetal femoral circulation

During basal conditions, HA fetal sheep showed a higher FBF compared to the LA group. However, FVR was similar between the two groups (Fig. 3). While FBF decreased and FVR increased significantly during acute hypoxia in LA fetuses, these changes were markedly diminished or absent in HA fetuses. These variables returned to basal levels in the recovery period in both groups (Fig. 3).

Fetal organ blood flow, vascular resistance and oxygen delivery

During basal conditions, the fetal sheep showed no differences in the blood flow (Fig. 4), vascular resistance (Table 4) and oxygen delivery (Table 5) to the brain between the two groups. During the acute hypoxic episode, both groups of fetal sheep increased the cerebral blood flow (Fig. 4), decreased the cerebral vascular resistance (Table 4) and maintained the O_2 delivery to the brain (Table 5), relative to baseline. During recovery, values for brain blood flow and vascular resistance returned to baseline in both groups (Fig. 4; Table 4).

Similarly, myocardial blood flow (Fig. 4), vascular resistance (Table 4) and O_2 delivery (Table 5) followed a comparable basal and response pattern throughout the experiment, without differences between groups. In contrast, adrenal vascular resistance (Table 4) was diminished in HA relative to LA during baseline. However, fetal adrenal blood flow (Fig. 4), vascular resistance (Table 4) and O_2 delivery (Table 5) showed the same response pattern to superimposed hypoxia observed for the brain and heart, in both groups of fetuses. All of the adrenal variables returned towards basal values during recovery.

Values for the umbilical blood flow (Fig. 4), vascular resistance (Table 4) and O_2 delivery (Table 5) were similar between the two groups during basal conditions. However, during acute hypoxia and recovery, the umbilical blood flow increased (Fig. 4) in LA relative to HA fetuses. In marked contrast, the umbilical blood flow and resistance were maintained during the entire protocol in the HA

group. Consequently, the umbilical O_2 delivery during hypoxia was significantly decreased in HA fetuses (Fig. 4; Table 4, 5).

Contractile function of middle cerebral arteries

The contractile responses of MCAs to potassium chloride showed a higher maximal response in HA fetuses relative to the LA fetal sheep (2.06 ± 0.15 *vs.* 4.16 ± 0.46 mM mm^{-1}, $P < 0.05$, respectively), while no differences in sensitivities were observed between groups (EC_{50}, 28.41 ± 1.71 *vs.* 25.64 ± 4.09, respectively; Fig. 5*A*). The adrenergic contractile response to noradrenaline and phenylephrine was assessed in MCAs, where the HA fetal group showed a marked decrease in the maximal response to both drugs relative to LA animals (Fig. 5*B* and *C*). The sensitivity to phenylephrine was enhanced in HA relative to LA fetuses (Fig. 5*C*).

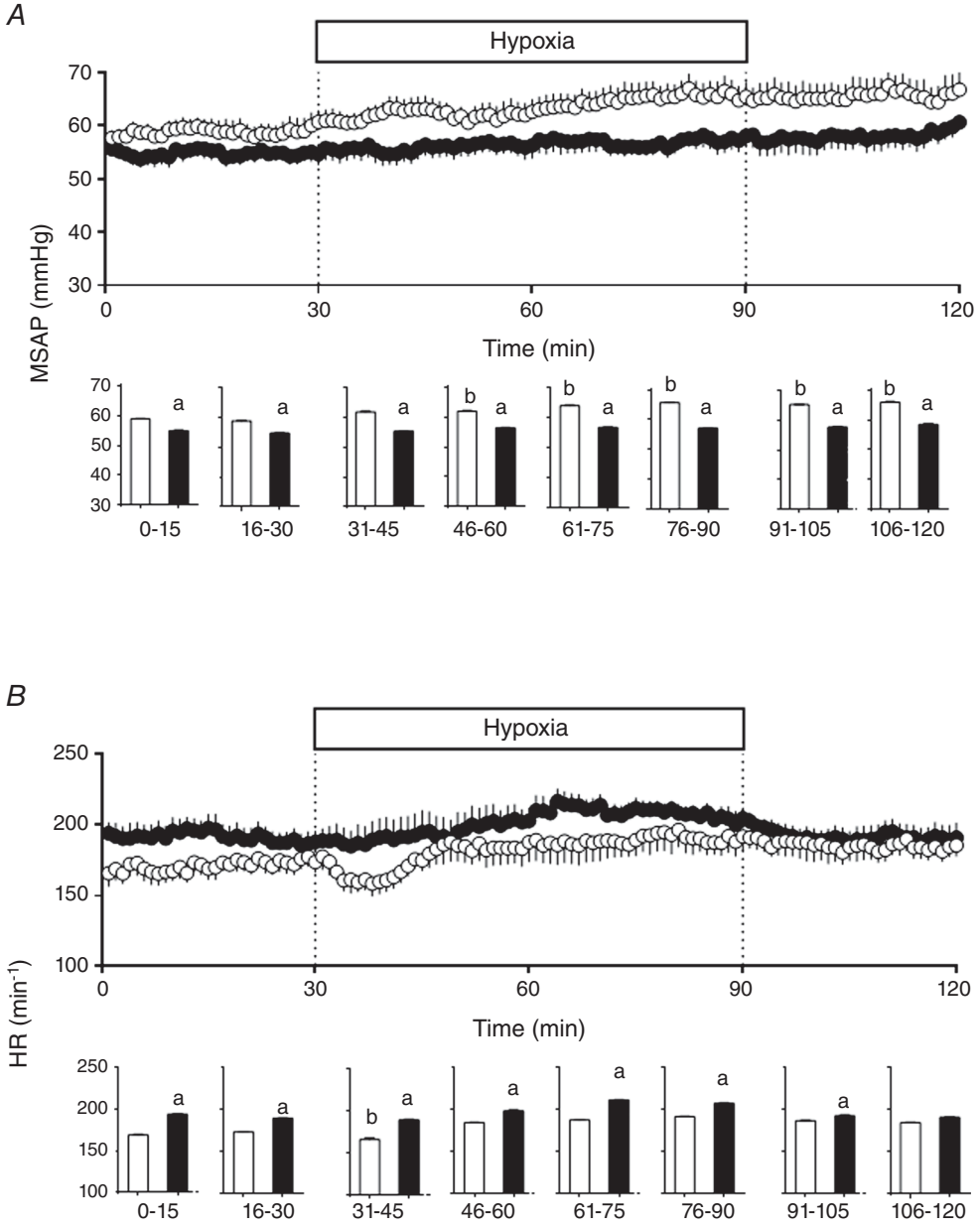

Figure 1. Cardiovascular function in fetal sheep
Mean systemic arterial pressure (MSAP, *A*) and heart rate (HR, *B*) and in low altitude (LA, open circles/bars) and high altitude (HA, filled circles/bars) fetal lambs. Histograms represent 15 min average of the experimental periods (basal, early hypoxia, late hypoxia and recovery). Values are means ± SEM. Significant differences ($P \leq 0.05$): *a vs.* LA and *b vs.* basal.

Contractile function of femoral arteries

Small resistance femoral arteries from HA fetuses showed a diminished contractile response and sensitivity to K^+ compared to the LA fetuses (Fig. 6*A*). However, in contrast to cerebral arteries, femoral arteries from HA fetuses showed a marked increase in the maximal response (214.1 ± 15.9 *vs.* 47.8 ± 3.3 %, $P < 0.05$, respectively) and sensitivity (pD_2: 5.11 ± 0.14 *vs.* 4.58 ± 0.10, $P < 0.05$, respectively) to noradrenaline relative to the LA fetuses (Fig. 6*B*). Further, the maximal response to phenylephrine was enhanced while the sensitivity was decreased in femoral arteries isolated from HA compared to LA fetuses (Fig. 6*C*).

Discussion

To the best of our knowledge, this is the first study to investigate fetal cardiovascular function *in vivo* and *ex vivo* in high altitude adapted sheep at the *Alto Andino*. The data support the hypothesis tested that high-altitude fetal sheep have evolved cardiovascular compensatory mechanisms to withstand basal and superimposed acute hypoxia that are different from lowland sheep, demonstrating markedly blunted cardiovascular responses to superimposed acute hypoxia.

The HA fetal sheep showed a reduced body weight for the gestational age relative to lowlanders. This reduction signifies intrauterine growth restriction (IUGR) and it has been described previously as an effect of altitude independent of nutrition (Lichty *et al.* 1957; Giussani *et al.* 2001; Moore, 2003; Herrera *et al.* 2007, 2010; Soria *et al.* 2013). The reduction in the HA fetal weight may be attributed to a reduction in the O_2 supply (Soria *et al.* 2013), which involves adaptations like reductions in metabolic processes such as ATP production and demand (Wheaton & Chandel, 2011), reducing the Na^+–K^+-ATPase activity and protein synthesis, consistent with a total decrease of fetal oxygen uptake (Richardson & Bocking, 1998; Wheaton & Chandel, 2011). The

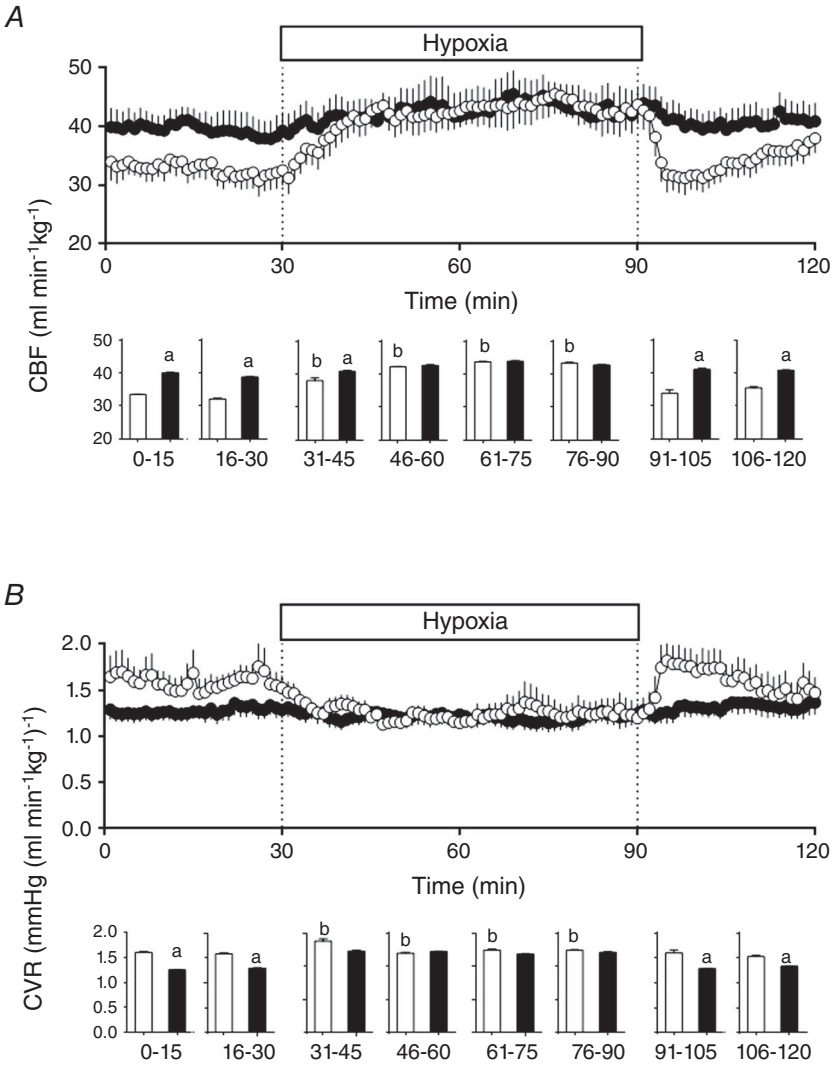

Figure 2. Carotid function in fetal sheep
Carotid blood flow (CBF, *A*) and carotid vascular resistance (CVR, *B*) in low altitude (LA, open circles/bars) and high altitude (HA, filled circles/bars) fetal lambs. Histograms represent 15 min average of the experimental periods (basal, early hypoxia, late hypoxia and recovery). Values are means ± SEM. Significant differences ($P \leq 0.05$): *a vs.* LA and *b vs.* basal.

brain-to-liver weight ratio, an established index of asymmetric growth restriction, is significantly greater in HA compared to LA fetuses, as previously established in chronic fetal hypoxia at sea level (Llanos *et al.* 1980). Additionally, the absolute and relative placenta plus uterine weight was increased in HA pregnancies. Nevertheless, this increase in uteroplacental weight was insufficient to maintain fetal growth, yielding fetal growth restriction and thereby a fall in uteroplacental efficiency (see Fowden *et al.* 2009 for review; Londero *et al.* 2013).

There is disagreement about the effects of chronic hypoxia on placental phenotype, which may be due to varying duration, and/or magnitude and/or onset of hypoxia exposure as well as due to species differences. Some studies suggest decreased placental weight (Huang *et al.* 2004) while others reported increased weight and decreased efficiency under the influence of chronic hypoxia (Parraguez *et al.* 2006; Richter *et al.* 2012; Herrera *et al.* 2014). However, there is a common agreement that the failure of the placenta to meet the increasing demand for oxygen and substrate of the developing fetus ultimately ends in fetal growth restriction (Herrera *et al.* 2014). Under chronic hypoxia there is an increased oxidative stress that may be inducing damage to the developing placenta and fetus (Tissot van Patot *et al.* 2012; Richter *et al.* 2012; Giussani *et al.* 2012; Herrera *et al.* 2014). However, placentas from women of high altitude populations developed under hypoxic conditions may have less oxidative damage, providing a greater cushion to protect adverse effects on the fetus (Tissot van Patot *et al.* 2012; Herrera *et al.* 2014).

The hypoxic environment of high altitude triggers cardiorespiratory compensatory responses that tend to adapt the organism to harness the oxygen bioavailability. For instance, an increased haemoglobin concentration in the fetal and maternal blood increases the oxygen transport capacity (Kitanaka *et al.* 1989) and leads to maintained arterial O_2 content at similar levels to those calculated for LA fetal sheep and mothers. Additionally, the ventilatory response in the HA pregnant we leads to a better P_{aO_2}, with a mild alkalaemia and a decreased arterial P_{aCO_2}, which may help the haemoglobin loading by O_2

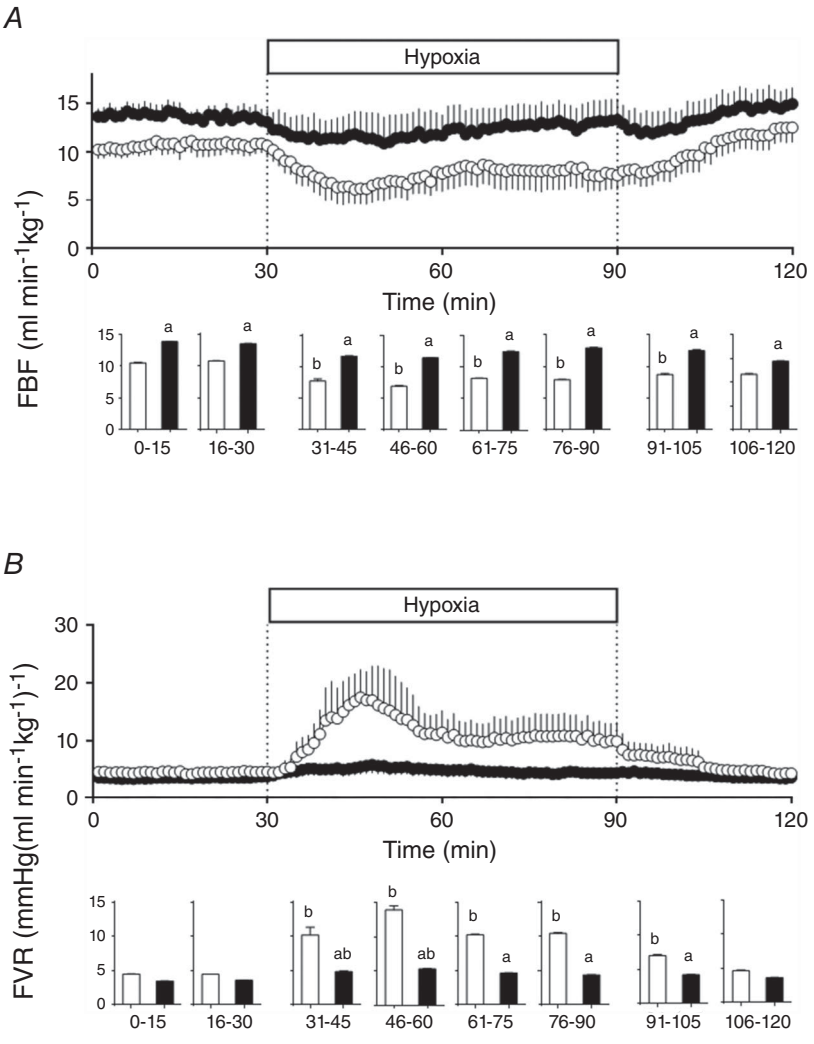

Figure 3. Femoral function in fetal sheep
Femoral blood flow (FBF, *A*) and femoral vascular resistance (FVR, *B*) in low altitude (LA, open circles/bars) and high altitude (HA, filled circles/bars) fetal lambs. Histograms represent 15 min average of the experimental periods (basal, early hypoxia, late hypoxia and recovery). Values are means ± SEM. Significant differences ($P \leq 0.05$): *a vs.* LA and *b vs.* basal.

in the lung, by shifting to the left the O_2 dissociation curve. All of the above are well-known compensatory responses to high altitude hypoxia. Regarding the systemic blood pressure, HA pregnant sheep showed no differences relative to LA pregnant ewes in both diastolic and systolic pressures in agreement with no blood pressure changes in acclimatized highlanders compared to lowlanders (León-Velarde *et al.* 2010). In contrast to their mothers, the HA fetal sheep showed lower MSAP than LA fetuses during basal conditions. Furthermore, during acute hypoxia LA fetal sheep showed an increase in MSAP, a response attributed to increases in the sympathetic tone and in circulating catecholamine levels, among others factors (Cohn *et al.* 1974; Pérez *et al.* 1989; Giussani *et al.* 1993, 1994). This slight and transient hypertension in the LA fetal sheep during acute hypoxia (Cohn *et al.* 1974, Pérez *et al.* 1989) involves peripheral vasoconstriction with anaerobic metabolism, particularly in circulations that undergo constriction, such as the femoral vascular bed (Boyle *et al.* 1990). The latter induces lactate production in peripheral tissues, explaining the fall in the blood pH observed only in this group (Giussani *et al.* 1993). In contrast, the HA fetal lambs showed similar basal MSAP, but a blunted pressor response during superimposed acute hypoxia relative to LA sheep. This suggests either a lower neutrally triggered chemoreflex drive and sympathetic tone, and/or reduced vasoconstrictor hormones and/or a greater vasodilator influence masking the constrictor responses induced by fetal gestation under chronic hypoxia (Tissot van Patot *et al.* 2012). In lowland fetal sheep, in response to acute hypoxia the bradycardia and the initial increase in femoral vascular resistance are established carotid chemoreflexes, as both are abolished by selective carotid body denervation (Giussani *et al.* 1993). Therefore, the lesser bradycardic response in HA fetuses in the present study is likely to be due to a blunted carotid chemoreflex in fetal life triggered by the chronic hypoxia of pregnancy at high altitude. This effect is similar to the blunted chemoreflex hyperventilatory response to hypoxia in highland natives (Monge & León-Velarde, 1991). Alternatively, additional results in the present manuscript show that femoral arteries from uninstrumented HA fetuses have lower contractile capacity and sensitivity to K^+, but an increased response and sensitivity to NA relative to LA fetuses. Thus, the *in vivo* blunted contractile response could be associated with the decreased contractile capacity of the femoral vascular bed but not to alterations related to vascular adrenergic pathways.

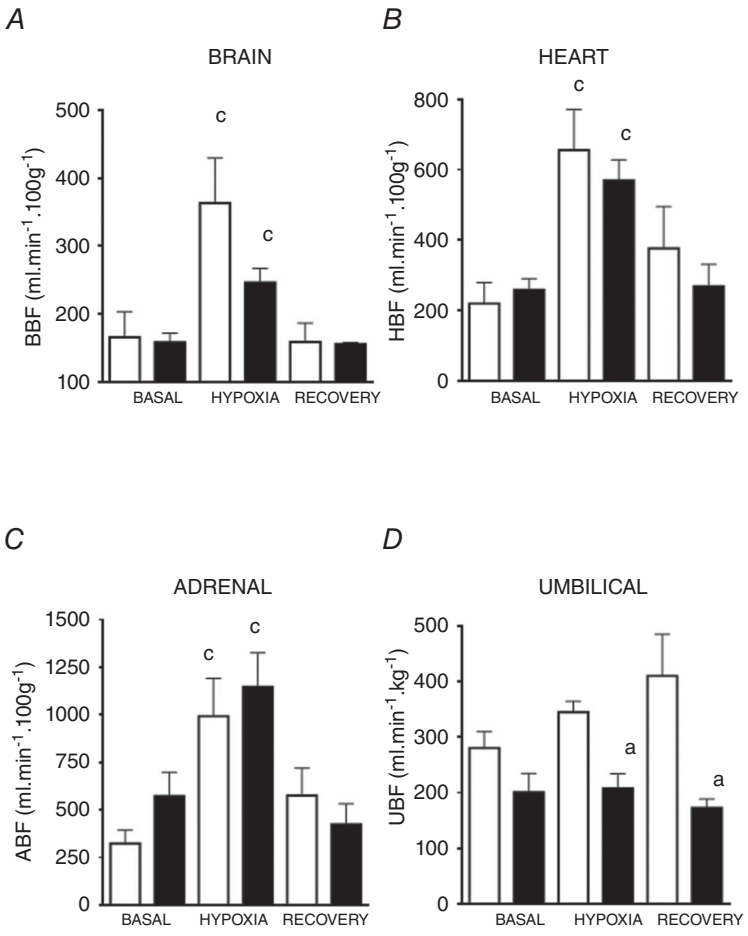

Figure 4. Organ blood flow in fetal sheep
Blood flow to brain (BBF, *A*), heart (HBF, *B*), adrenal (ABF, *C*) and umbilical (UBF, *D*) vascular beds in low altitude (LA, open circles/bars) and high altitude (HA, filled circles/bars) fetal lambs. Values are means ± SEM. Significant differences ($P \leq 0.05$): *a vs.* LA and *c vs.* all periods in the group.

Table 4. Organ vascular resistance in fetal sheep ((ml min^{-1})$^{-1}$)

		Basal	Hypoxia	Recovery
Brain	LA	0.419 ± 0.078	0.191 ± 0.021^{c}	0.445 ± 0.070
	HA	0.359 ± 0.038	0.222 ± 0.017^{c}	0.365 ± 0.031
Heart	LA	0.293 ± 0.049	0.156 ± 0.060^{b}	0.209 ± 0.078
	HA	0.222 ± 0.025	0.102 ± 0.003^{c}	0.225 ± 0.040
Adrenals	LA	0.220 ± 0.034	0.070 ± 0.010^{c}	0.178 ± 0.040
	HA	0.116 ± 0.022^{a}	0.053 ± 0.008^{c}	0.165 ± 0.022
Umbilical	LA	0.226 ± 0.032	0.195 ± 0.007	0.166 ± 0.040
	HA	0.286 ± 0.046	0.278 ± 0.026^{a}	0.351 ± 0.035^{a}

Fetal organs vascular resistance in low altitude (LA) and high altitude (HA) fetal sheep during basal, hypoxia and recovery. Values are means ± SEM. Significant differences ($P \leq 0.05$): *a vs*. LA; *b vs*. basal and *c vs*. all in the same group; ANOVA + Newman–Keuls test for all variables.

Table 5. Organ oxygen delivery in fetal sheep (ml O_2 min^{-1})

		Basal	Hypoxia	Recovery
Brain	LA	14.97 ± 3.87	9.37 ± 1.55	9.80 ± 3.57
	HA	11.78 ± 0.78	9.83 ± 1.23	10.13 ± 1.35
Heart	LA	17.05 ± 5.67	21.40 ± 4.51	21.48 ± 7.60
	HA	19.18 ± 2.08	22.55 ± 2.22	16.20 ± 2.34
Adrenals	LA	24.95 ± 6.84	30.47 ± 8.80	28.63 ± 8.89
	HA	36.48 ± 7.14	34.68 ± 2.95	25.53 ± 4.63^{c}
Umbilical	LA	16.10 ± 1.43	8.97 ± 0.69^{c}	16.18 ± 3.65
	HA	12.75 ± 0.85	6.38 ± 0.37^{b}	9.80 ± 1.30^{a}

Fetal organs O_2del in low altitude (LA) and high altitude (HA) fetal sheep during basal, hypoxia and recovery. Values are means ± SEM. Significant differences ($P \leq 0.05$): *a vs*. LA; *b vs*. basal and *c vs*. all in the same group; ANOVA + Newman–Keuls test for all variables.

The fetal lambs chronically exposed to HA showed relative higher basal CBF, an effect that should maintain an adequate delivery of oxygen and nutrients to the developing brain in hypoxia (Tissot van Patot *et al.* 2012). However, carotid blood flow did not have a significant increase in this group during the superimposed acute hypoxia (Pereyra *et al.* 2007; Tissot van Patot *et al.* 2012). The increase in CBF during acute hypoxia in LA fetal sheep is known to be mediated by vasodilators, including nitric oxide (NO) (Green *et al.* 1996; Hunter *et al.* 2003; Sanhueza *et al.* 2005). Therefore, the enhanced basal CBF in HA fetuses may indicate a greater NO tone induced by exposure to high altitude. This appears to be the case, since preliminary data from our laboratory show that CBF decreases more in HA than LA fetuses after the infusion of N^{G}-nitro-L-arginine methyl ester, a nitric oxide synthases (NOS) inhibitor (unpublished data). In our study, basal cerebral blood flow in HA fetuses was not different from the LA fetuses, in agreement with the data of Kamitomo *et al.* (1993), both measurements performed by the microspheres method. Furthermore, there was an increase in cerebral blood flow in HA and LA fetuses, with no difference between them, as also described previously (Kamitomo *et al.* 1993). In addition, the brain oxygen delivery was not different among HA and LA fetal lambs, suggesting that HA fetuses are able to maintain the brain oxygenation in spite of the low P_{O_2} (Kamitomo *et al.* 1993; Pereyra *et al.* 2007). Moreover, the *ex vivo* studies of MCA reactivity in the present paper showed a higher contractile capacity and reduced response to noradrenaline in HA fetal lambs relative to controls. Thus, the increase in the brain blood flow and the fall in the vascular resistance observed in the HA fetus during superimposed hypoxia could be attributed to a blunted sensitivity to catecholamines and/or an augmented vasodilator NO effect, which is known to be an important modulator in the fetal brain (Green *et al.* 1996). The cerebral and carotid blood flow responses to acute hypoxia in the HA fetus are different from a species adapted to the life in the Andean *altiplano*, such as the fetal llama. Llama fetuses respond to acute hypoxia decreasing the cerebral metabolism, mediated by reductions in Na^{+}–K^{+}-ATPase activity and expression of voltage-gated Na^{+} channels (NaV) (Ebensperger *et al.* 2005) and a marked peripheral vasoconstrictor response (Giussani *et al.* 1996, 1999; Llanos *et al.* 2003; Sanhueza *et al.* 2005). Clearly, we are in the presence of dissimilar acquired adaptations to high altitude between sheep and llamas, probably due

to differential evolutionary exposure to chronic hypoxia in the Andean highlands (Stanley *et al.* 1994).

The increased potassium contractile response in the MCAs of HA fetal sheep may reflect increases in both the wall thickness and L-type calcium channel function (Gilbert *et al.* 2003), raising the calcium flux into the fetal smooth muscle cells, triggering contraction. In contrast, in the femoral circulation, decreases in femoral wall thickness and/or L-type calcium channel function may have taken place. Moreover, NA and Phe contractile responses in the MCAs from HA fetuses were completely blunted compared to LA fetal sheep. Arteries from chronically hypoxic fetuses are exposed to enhanced plasma concentrations of catecholamine (Kitanaka *et al.* 1989). Further, chronic hypoxia reduced the capacity of NA to cause contraction, mainly due to hypoxic downregulation of adrenergic and lesser IP_3 receptor densities (Gilbert *et al.* 2003), resulting in lower intracellular Ca^{2+} concentration and smooth muscle contraction in HA MCAs. In marked contrast, in HA fetal sheep the catecholamine response is clearly enhanced in the femoral circulation *ex vivo*. This finding may be partially explained by upregulation of the α_1-adrenergic receptor in these arteries *ex vivo*, as described for smooth muscle cells isolated from fresh or cultured segment of mesenteric arteries *in vitro* (Cao *et al.* 2006).

The reduced levels of umbilical blood flow measured in the HA fetus in the present study could suggest an impaired placental vasodilator function at altitude, possibly due to an umbilical or chorionic endothelial dysfunction (Krause *et al.* 2012; Herrera *et al.* 2014). In fact intrauterine growth restricted fetuses show impaired

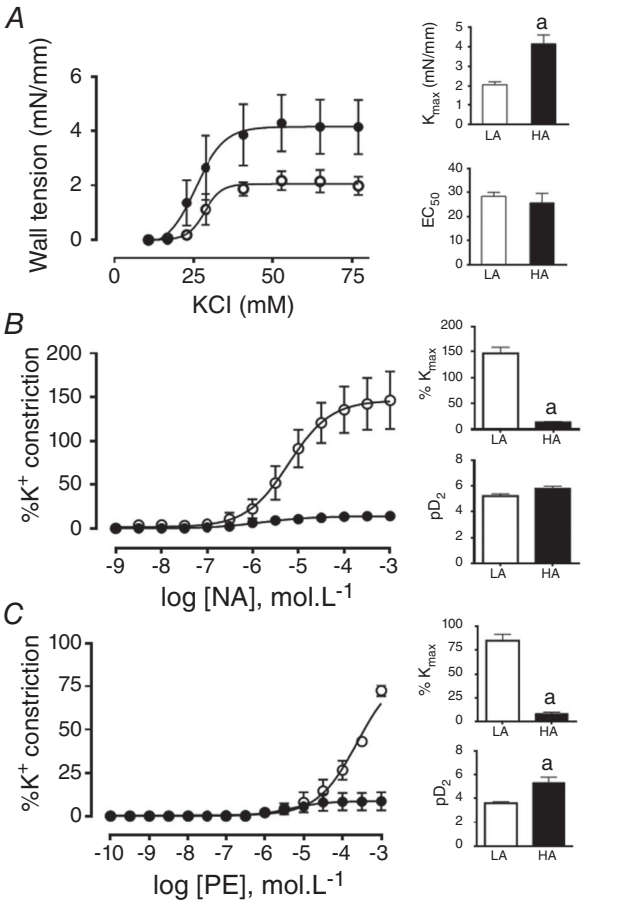

Figure 5. Contractile function in middle cerebral arteries from fetal sheep
Vasoconstrictor response of middle cerebral arteries to potassium chloride (KCl, *A*), noradrenaline (NA, *B*), and phenylephrine (Phe, *C*) in low altitude (LA, open circles/bars) and high altitude (HA, filled circles/bars) fetal lambs. Values are means ± SEM. Significant differences ($P \leq 0.05$): *a vs*. LA.

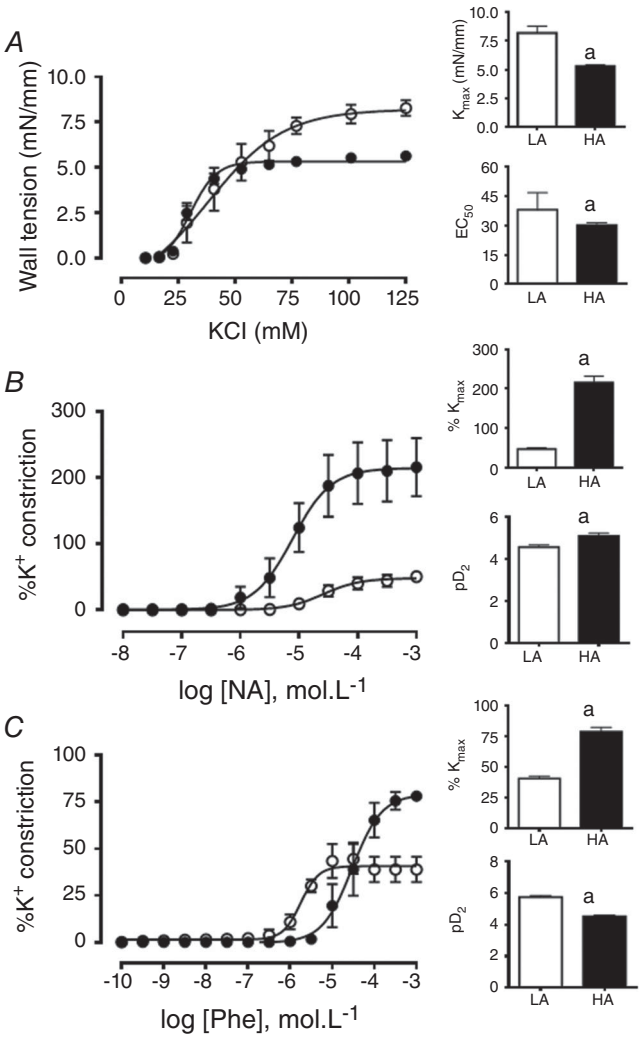

Figure 6. Contractile function in femoral arteries from fetal sheep
Vasoconstrictor response of femoral arteries to potassium chloride (KCl, *A*), noradrenaline (NA, *B*), and phenylephrine (Phe, *C*) in low altitude (LA, open circles/bars) and high altitude (HA, filled circles/bars) fetal lambs. Values are means ± SEM. Significant differences ($P \leq 0.05$): *a vs* LA.

eNOS function in umbilical vessels (Krause *et al.* 2012). Further, in the presence of oxidative stress, reactive oxygen species will decrease the NO bioavailability with increased peroxynitrites resulting in endothelial cell damage or placental apoptosis and reduced umbilical perfusion (Miller *et al.* 1996; Giussani *et al.* 2012). Accordingly, Thakor *et al.* (2010) reported that treatment of sheep with antioxidants such as melatonin and vitamin C could increase umbilical conductance via increasing NO bioavailability. The decrease in umbilical blood flow promotes a lower O_2 and nutrients delivery to the fetus, and impaired removal of waste products towards the mother in the chronic hypoxic pregnancy, an important cause of IUGR, as determined in this study.

We believe that our findings highlight the need to diagnose, in human fetuses, the increase in carotid and decrease in umbilical blood flow observed in our studies. These indices are already in use in some health centres in the *Alto Andino* and widely used in hospitals and clinics at lowlands. Additionally, the lack of further increase in carotid blood flow during the fall of fetal P_{O_2}, as may occur during the uterine contractions of labour at the end of highland pregnancy, may signal exhausted mechanisms to augment the cerebral blood flow. The latter could eventually be added to the clinical repertoire used to diagnose the fragile chronically hypoxic fetus.

In conclusion, this study determined for the first time the fetal cardiovascular responses to chronic and acute hypoxia in sheep at high altitude. We reported markedly blunted cardiovascular responses to acute superimposed hypoxia in highland relative to lowland fetal sheep. Whether these blunted cardiovascular responses are beneficial or detrimental during development under chronic hypoxia is still unclear. More studies are required to determine if these effects are short or long lasting after delivery and if the changes are genetically fixed by selection pressure, epigenetically determined or simply a long term physiological acclimatization to a chronic hypoxic milieu.

We speculate that blunting the cardiovascular responses in chronically hypoxic fetuses reflects a change in the focus of the compensatory mechanisms towards cells, tissues and molecules, instead of mounting major cardiovascular modifications, which require a high oxygen consumption, oxygen not easily available in the Andean *altiplano*.

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Additional information

Competing interests

No conflicts of interest are declared by the authors.

Author contributions

The experiments in this study were performed in Putre Research Station of the International Center for Andean Studies (INCAS) and in the Program of Pathophysiology, ICBM, Faculty of Medicine, University of Chile. EAH, RTR, BJK, GE, RVR, DAG, JTP and AJLL conceived and designed the experiments. EAH, RTR, BJK, GE, RVR, DAG, JTP and AJLL collected, analysed and interpreted the experimental data. EAH, RTR, DAG, JTP and AJLL drafted the article. All authors revised and approved the final version of the article.

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