Expression of vascular endothelial growth factor and endothelial nitric oxide synthase is increased in the placenta of sheep at high altitude in the Andes

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Abstract

Fetal weight and the placenta of sheep at high altitude (HA) are affected by hypoxia. Placental changes (an increase in placental size and vascularization) are greater in ewes from populations that have lived for several generations at HA than in those exposed during just 1 gestation. This study investigated placental expression of vascular endothelial growth factor (VEGF) and endothelial nitric oxide synthase (eNOS), 2 molecules involved in placental angiogenesis that could be upregulated by hypoxia. Two groups of ewes were maintained at HA (3589 m) during pregnancy: HA-native ewes (group HH) and ewes native to lowlands but moved to HA immediately after the diagnosis of pregnancy (group LH). A control group (LL) was kept at sea level. Near term, placentomes were removed, weighed, and processed for immunohistochemical detection of VEGF and eNOS, as well as for vascular area measurement. Placental weight was significantly higher in the HH group than in the LH and LL groups; between the latter 2 groups there was no significant difference. The placental area occupied by vasculature was significantly greater in both the HA groups than in the LH group; the number of placentomes was greatest in the LL group. The density of VEGF and eNOS in the placentome tissue was significantly greater in both HA groups than in the LL group. Although the density of VEGF was significantly lower in the HH group than in the LH group, no differences were observed in eNOS density between the HH and LH animals. These results demonstrate that chronic hypoxia upregulates the expression of placental VEGF and eNOS, suggesting an important role of these molecules in the placental response to HA hypoxia. In addition, an attenuated response to hypoxia in VEGF synthesis may be part of the long-term process of adaptation to HA.

Résumé

Le poids fœtal et le placenta des moutons gardés en haute altitude (HA) sont affectés par l'hypoxie. Les changements placentaires (augmentation de la grosseur du placenta et de la vascularisation) sont plus marqués chez les brebis de populations ayant vécues pour plusieurs générations en HA comparativement à celles exposées uniquement durant une gestation. Dans la présente étude, nous avons étudié l'expression placentaire du facteur de croissance de l'endothélium vasculaire (VEGF) et de l'oxyde nitrique synthétase endothéliale (eNOS), 2 molécules impliquées dans l'angiogénèse du placenta qui pourraient être régulées à la hausse par une hypoxie. Deux groupes de brebis ont été maintenues en HA (3589 m) pendant la gestation : des brebis indigènes à HA (groupe HH) et des brebis indigènes aux basses altitudes mais déplacées en HA immédiatement après un diagnostic de gestation (LH). Un groupe témoin a été gardé au niveau de la mer (LL). Lorsque presque à terme, les placentomes ont été retirés, pesés, et traités pour détection immuno-histochimique de VEGF et eNOS, ainsi que pour une mesure de la région vascularisée. Le poids placentaire était significativement plus élevé dans le groupe HH que dans les groupes LH et LL; ces deux derniers ne présentant pas de différence significative. La superficie placentaire occupée par le réseau vasculaire était significativement plus grande dans les groupes HA comparativement au groupe LH : le nombre de placentomes était le plus grand dans le groupe LL. La densité de VEGF et de eNOS dans les tissus du placentomes était significativement plus grande dans les deux groupes HA comparativement au groupe LL. Bien que la densité de VEGF était significativement plus basse dans le groupe HH comparativement au groupe LH, aucune différence n'a été notée dans la densité de nNOS entre les animaux HH et LH. Ces résultats démontrent que l'hypoxie chronique régule à la hausse l'expression de VEGF placentaire et de eNOS, suggérant ainsi un rôle important pour ces molécules dans la réponse du placenta à une hypoxie HA. De plus, une réponse atténuée à l'hypoxie suite à la synthèse de VEGF pourrait être une composante du processus à long-terme d'adaptation à l'HA.

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Introduction

Knowledge of ovine reproduction at high altitude (HA) is important. Sheep were introduced to American highlands as a productive species by European settlers about 500 y ago. Despite this time under HA conditions, ovine reproductive efficiency is poor (1). However, the Latin-American Association of Small Ruminant Specialists has recently calculated that ovine breeding at altitudes above 2500 m involves more than 13 million animals (Latin-American Association of Small Ruminant Specialists). Sheep are of tremendous social and economic importance for native farmers at HA. Furthermore, about 25 million rural people in developing regions and transition countries live at such altitudes and can benefit from sheep breeding (2). Additionally, sheep are often used as a model for studies of human biology and medicine (3). Considering that approximately 140 million people live at altitudes above 2500 m and another 40 million visit HA regions yearly (4), an understanding of the effect of hypoxia on sheep gestation may have important implications for human reproductive health.

Ovine reproduction at HA is thought to be hampered by environmental hypobaric hypoxia. In addition to a low fertility rate (5), intrauterine growth restriction (IUGR) and low weight at birth (6) are among the detrimental effects of HA. The fetal changes are associated with structural modifications in the placenta: the number of cotyledons is decreased, and placental weight, cotyledon diameter, cotyledon–caruncle contact surface area, and cotyledon surface area occupied by vasculature are increased (7). The effects on birth weight are greater in ewes with short-term exposure to HA than in ewes from populations that have lived at HA for several generations. In contrast, the effects on placental traits are greater in ewes with long-term exposure to HA than in those with short-term exposure (7).

During pregnancy, the development and function of the placenta depend primarily on the growth of its vascular bed and a concomitant increase in uterine and umbilical cord blood flow. Placental vascular growth, or angiogenesis, is controlled by various peptides. Vascular endothelial growth factor (VEGF) and endothelial nitric oxide synthase (eNOS) play crucial roles in development of the ovine placenta (8), and in abnormal pregnancies the placental structure and function can be modified by altering the balance of such growth factors (9). The transcriptional activator hypoxia-inducible factor 1 (HIF-1) is the main molecule involved in the metabolic adaptation to oxygen deprivation (10). Hypoxia increases HIF-1 activity, which targets VEGF genes and allows an increase in VEGF expression (11). In human endothelial cells in vitro, the upregulation of eNOS by VEGF (12,13) is essential for VEGF angiogenic activity (14,15). Short-term hypoxia increases the expression of VEGF in the ovine fetal placenta (16), and long-term hypoxia upregulates eNOS expression in uterine artery endothelium in pregnant sheep (17). However, the effects of HA hypobaric hypoxia on the placental expression of these 2 factors have not been studied.

Considering that, in sheep, both placental size and vascularization are increased with HA hypoxia and that the placental changes are greater in HA-native ewes than in those native to sea level whose gestations proceeded at HA, we examined the placental expression of VEGF and eNOS in pregnant sheep with long-term or short-term exposure to HA and in those kept at sea level. Our main objective was to establish the role of these molecules in placental development in ewes exposed to HA and having different periods of adaptation.

Materials and methods

Animals

Fifteen time-mated pregnant Creole ewes were used. Five animals were native to HA and the other 10 were native to low altitude (LA). The pregnant LA ewes were separated into 2 groups. One group was used as the low-level (LL) control group: it was kept at 500 m above sea level (in the Lluta valley of northern Chile: 18°23′08″ S, 70°08′53″ W; mean barometric pressure 99.5 kPa; mean environmental temperature 18.8°C, range 15°C to 27°C) for the entire pregnancy. The other group (LH) was moved to HA immediately after the diagnosis of pregnancy by ultrasound examination (16 to 20 d after mating). This group was not allowed to become pregnant at HA because acute exposure abolishes fertility (5). The latter group, as well as the HA-native ewes (HH), were kept throughout gestation at our HA experimental station at the International Center for Andean Studies, University of Chile, which is 3589 m above sea level (18°11′48″ S, 69°33′11″ W; mean barometric pressure 66.7 kPa; mean environmental temperature 10.0°C, range 2°C to 21°C).

All the animals in this study were unshorn, had a singleton pregnancy, and had previously had 2 pregnancies with normal parturition. The average body weights at the beginning of pregnancy were similar in the 3 groups (HH, 39.3 ± 3.9 kg; LH, 40.7 ± 3.7 kg; LL, 40.6 ± 2.2 kg). During the entire pregnancy the animals were kept under similar management conditions and provided with alfalfa hay daily (~ 2.0 kg per animal: 85.6% dry matter, 14.1% crude protein, 10.3 MJ/kg mean energy intake) and fresh water ad libitum. The food supply was calculated to satisfy the daily requirement of sheep in late gestation (18).

Placental recovery and processing

Near term (at day 140 of pregnancy), when the fetal metabolic demands are highest, the ewes were anesthetized by an intravenous bolus injection of sodium thiopental (25 mg/kg of body weight). Immediately after anesthesia induction, carotid blood samples were collected for arterial blood gas measurements in an IL Synthesis 25 (Instrumentation Laboratory, Lexington, Massachusetts, USA) calibrated to local barometric pressure and ovine body temperature. After midventral laparotomy, hysterotomy, and fetal euthanasia by thiopental overdose, catheters were implanted in the umbilical and uterine arteries. The fetus was removed and weighed, and the ewe was euthanized by thiopental overdose. Once the umbilical and uterine veins had been cut, 0.2 M phosphate-buffered saline solution (PBS) was infused through the catheters to remove blood from the placental tissue. When the outflow had a clear appearance, the placenta was perfused with a fixative solution (4 L of 4% paraformaldehyde in 0.2 M PBS, pH 7.4). Placentomes were removed, counted, weighed, and submitted to an additional fixation period.

Ten type A placentomes (the predominant type) from each group of ewes (2 per animal, selected at random) were processed for paraffin-embedded immunohistochemical study and hematoxylin–eosin (H–E) staining.

Table I. Arterial blood variables in preterm pregnant ewes (at 140 d of gestation) at high and low altitudes

	Partial pressure (mm Hg)			Hemoglobin		
Group	Oxygen	Carbon dioxide	Packed cell volume (%)	Concentration (mg/dL)	Saturation (%)	pH
HH	63.4 ± 5.6[a]	25.9 ± 2.7[a]	30.4 ± 1.9[a]	12.3 ± 0.6[a]	83.9 ± 1.8[a]	7.503 ± 0.067
LH	56.9 ± 5.2[a]	29.3 ± 3.2[a]	35.1 ± 2.0[b]	14.3 ± 0.8[b]	82.2 ± 8.2[a]	7.504 ± 0.026
LL	97.9 ± 3.8[b]	39.9 ± 2.5[b]	29.6 ± 1.7[a]	10.7 ± 0.6[c]	98.2 ± 1.8[b]	7.460 ± 0.011

HH — ewes native to high altitude gestating at high altitude; LH — ewes native to low altitude gestating at high altitude; LL — ewes native to low altitude gestating at low altitude (control group).

[a,b,c] Distinct superscript letters indicate a significant difference ($P \leq 0.05$) among the groups.

Immunohistochemical detection of VEGF and eNOS

Serial transverse sections 7 μm thick were obtained from the middle part of each placentome. Four alternate sections were mounted on silanized slides for immunohistochemical procedures. After tissue rehydration, endogenous peroxidase activity was quenched with 3% hydrogen peroxide for 30 min, and the sections were gently washed in PBS. Nonspecific binding was blocked by incubation with 1% nonimmune bovine serum albumin (BSA) in PBS for 30 min.

The tissue sections were incubated for 20 h at 4°C with antibody against VEGF (sc-152; Santa Cruz Biotechnology, Santa Cruz, California, USA) or against eNOS (610296; BD Transduction Laboratories, BD Biosciences, San Jose, California, USA) diluted 1:200 in PBS and 1% BSA. After being washed with PBS, the sections were incubated for 1 h at room temperature with biotinylated goat antibody against rabbit (sc-2040; Santa Cruz Biotechnology) or goat secondary antibody against mouse (AB124; Chemicon, Millipore Corporation, Billerica, Massachusetts, USA) diluted 1:200 in PBS. Then a complex of streptavidin–peroxidase (SA202; Chemicon) was added.

The reaction was visualized by the addition of 3,3-diaminobenzidine (DAB) in a working solution of DAB Substrate Kit for Peroxidase (SK-4100; Vector Laboratories, Burlingame, California, USA) for 5 min. The sections were washed in PBS for 10 min between each step, dehydrated, and mounted by means of BIO-G medium (MCH30; Prolab, Santiago, Chile). Nonspecific background staining from the secondary antibody was tested through omission of the primary antibody from control sections and replacement with BSA. The staining for each specific protein was evaluated by light microscopy (5 fields per section, randomly selected) at a magnification of 400X. Digital images of each microscopic field were captured, stored in a computer, and subsequently analyzed for stain density with the use of free ImageJ software (US National Institutes of Health, Bethesda, Maryland, USA) and the methods described by Girish and Vijayalakshmi (19).

Staining for vascular area evaluation

Four additional alternate sections of each placentome were processed by conventional histologic methods and stained with H–E to evaluate the placental area occupied by vasculature. Five fields of each section, selected at random, were observed by light microscopy at a magnification of 400X. The images obtained were analyzed by means of ImageJ software and the same methods.

Statistical analysis

The immunostaining density of VEGF and eNOS in the HH and LH groups was expressed as a percentage of the LL average value and compared by Student's *t*-test after normalization of the data by means of angular transformation. Data for additional placental variables, fetal weight, and blood gas measurements in the various groups were compared by 1-way analysis of variance and then the Tukey–Kramer multiple comparisons test when needed. The statistical model was $y_i = \mu + G_{i=1-3} + \epsilon_i$, where y_i is the value for each trait, μ is the general average value for the trait, G_i is the effect of the group (HH, LH, or LL), and ϵ_i is the error. Analyses were carried out with the Statistical Analysis System, version 6.12 (SAS Institute, Cary, North Carolina, USA). A difference was considered significant when $P \leq 0.05$. The results for each characteristic were expressed as the average ± the standard deviation.

This protocol was in accordance with the guidelines of the Canadian Council on Animal Care on the care and use of experimental animals and the euthanasia guidelines of the American Veterinary Medical Association and was approved by the Bioethics Review Committee of the Faculty of Veterinary Sciences, University of Chile, and the Bioethics Committee of Chile's National Agency for Scientific Research (Comisión Nacional de Investigación Científica y Tecnológica, CONICYT).

Results

The sheep arterial blood gas measurements showed a hypoxic state in the HA animals (Table I): the partial pressures of oxygen and carbon dioxide and the oxygen saturation of hemoglobin were significantly lower in the HH and LH ewes than in the LL ewes. The packed cell volume was significantly increased in the LH ewes, whereas no difference was observed between the HH and LL groups. The hemoglobin concentration was highest in the LH group, intermediate in the HH group, and lowest in the LL group. No differences in pH were observed among the groups.

Placental weight was significantly higher in the HH group than in the other 2 groups (Table II); no difference was observed between the LH and LL groups. The highest number of placentomes was observed in the LL group and the lowest in the HH group; the intermediate number in the LH group was not significantly different from the numbers in the other groups. The placental area occupied by vasculature was significantly greater in the HH and LH groups than in the LL group. Fetal weight was lowest in the LH group and

Table II. Placental characteristics and fetal weight in the preterm pregnant ewes

Group	Placental weight (g)	Number of placentomes	Vascular area (%)	Fetal weight (g)
HH	523.7 ± 70.0[a]	57.2 ± 4.6[a]	14.0 ± 2.8[a]	3.1 ± 0.3[a,b]
LH	384.3 ± 29.9[b]	67.6 ± 5.3[a,b]	15.2 ± 3.1[a]	2.5 ± 0.3[a]
LL	317.3 ± 30.1[b]	74.5 ± 8.7[b]	9.2 ± 2.6[b]	3.8 ± 0.7[b]

[a,b] Distinct superscript letters indicate a significant difference ($P \leq 0.05$) among the groups.

highest in the LL group; the intermediate weight in the HH group was not significantly different from the means in the other groups.

Figure 1A illustrates the placental area occupied by vasculature, as demonstrated by H–E staining. Figure 1B shows that VEGF immunostaining was concentrated near the villous capillary vessels and was widely disseminated within the trophoblast villus. Figure 1C shows that eNOS immunostaining was localized closer to the villous capillaries, primarily around the peripheral trophoblast. No staining was observed in the control tissue sections.

The density of immunostaining for placental VEGF and eNOS, as assayed immunohistochemically, was significantly higher in both HA groups than in the LL group. Although for VEGF the density was significantly lower in the HH group (1.129 ± 0.013) than in the LH group (1.192 ± 0.023), there was no significant difference for eNOS between the HH (1.362 ± 0.035) and LH (1.363 ± 0.047) groups.

Discussion

The blood gas values and placental and fetal traits observed in this study agree with those from previous reports (7,20) and confirm the hypoxic state of pregnancies at HA and the effects on animals with long-term or short-term residence at HA. Although, in addition to hypoxia, various environmental factors can modify fetal and placental characteristics (for example, low food availability or high environmental temperature), these factors were absent from the experimental design, and their potential influence can also be disregarded because their effects are totally different from those of hypoxia, as discussed previously (6).

Angiogenesis and vasculogenesis are the main processes determining placental development and maturation, directly influencing fetoplacental blood flow and, hence, fetal development (21). Regulation of the dynamic equilibrium among several angiogenic factors and their receptors seems to be crucial for normal placental growth and development and for successful pregnancy. Furthermore, oxygen status at the uteroplacental level appears to regulate placental capillary organization, villous architecture, and vascular impedance (22). In addition, hemodynamic anomalies on either side of the placental circulation may affect these phenomena and thereby influence villous development (23). Pre-eclampsia (a hypertensive and hypoxemic state in pregnancy associated with IUGR), for instance, appears to depend on an imbalance between proangiogenic and antiangiogenic factors (24).

The expression patterns of VEGF, eNOS, and other molecules involved with the angiogenesis and vasculogenesis processes in the ovine placenta during normoxic pregnancies have previously been described (8). The present study was the first attempt to characterize the expression of both VEGF and eNOS in animals exposed to a natural hypoxic environment for different periods of time. The results show that the expression of VEGF is significantly greater at HA than it is nearer sea level. This was an expected result because it has previously been demonstrated that the VEGF gene is targeted by transcription factor HIF-1 (11), a heterodimeric protein consisting of the HIF-1α and HIF-1β subunits (25). Expression of the HIF-1α subunit is regulated by intracellular oxygen concentration: when the oxygen concentration declines, HIF-1α expression increases (10,26). As a result, HIF-1 target genes are upregulated in response to hypoxia, which leads to angiogenesis in order to increase oxygen availability to tissues (27).

The report that prolonged hypoxia increases VEGF mRNA expression in ovine placental tissue (16) supports our results. However, we found a significant difference in VEGF expression between the HH and LH groups, the LH ewes (the group without previous adaptation) showing the greater expression. A similar tendency, although not significant, was observed in VEGF mRNA expression of cultured umbilical endothelial cells after brief exposure to hypoxia (4 to 24 h) in cells from women adapted only briefly to HA (Han) compared with cells from women from families living at HA for several generations (Tibetans) (28). These results suggest that long-term adaptation to HA may decrease the expression of VEGF by means of decreased HIF-1 activity, as has been postulated by Tissot van Patot et al (29).

Placental eNOS immunoreactive expression was also higher at HA in our study. There is no previously published information about placental eNOS expression in a hypoxic environment. However, in a recent IUGR study in which sheep were exposed to hyperthermia for 80 d from day 35 of pregnancy, an upregulation of placental eNOS expression was observed at day 132 (30). The researchers attributed the eNOS increase to secondary hypoxia according to the IUGR model. These observations, although arising from a different experimental model, support our data on the effect of HA hypoxia on placental eNOS expression. Our results are also supported by previous reports of an upregulation of eNOS mRNA and protein levels in uterine artery endothelium of pregnant sheep at HA (17), as well as in human trophoblast cell cultures in a hypoxemic environment (31). In human pre-eclampsia (32) and in a murine model of pre-eclampsia-like syndrome (33), an upregulation of eNOS mRNA in placental tissue has also been demonstrated. In contrast, in umbilical endothelial cell cultures from Han and Tibetan women, exposure to hypoxia for 12 to 24 h induced a decrease in eNOS mRNA expression and a concomitant VEGF increase (28). The authors attributed the eNOS response to an absence of a hypoxia response element in the eNOS genes. In their study, among the women who had been

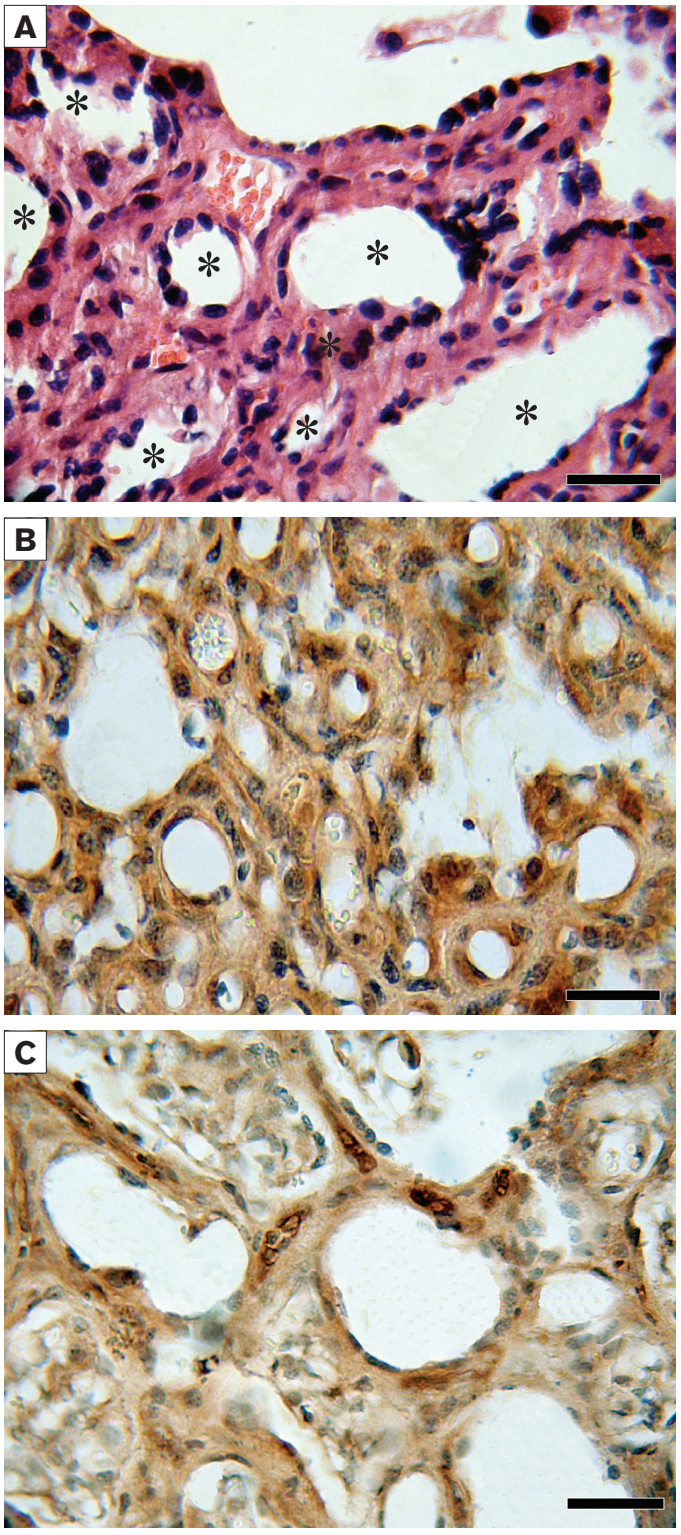

Figure 1. Ovine placental tissue obtained at 140 d of gestation at high altitude. A — Hematoxylin and eosin staining of blood vessels (asterisks) demonstrates placental surface area occupied by vasculature. B, C — Immunohistochemical staining of vascular endothelial growth factor and endothelial nitric oxide synthase (visualized in brown by use of 3,3-diaminobenzidine), respectively. Magnification ×400. Bar — 50 μm.

living for a long time under HA hypoxia the lack of eNOS response to VEGF stimulation may be part of the adaptation to HA. Another possible interpretation is that the different eNOS expression patterns observed in response to hypoxia are cell-type-dependent. Under the same hypoxic conditions eNOS expression differed among endothelial cells derived from uterine, femoral, and kidney arteries (17). Furthermore, despite the differences in VEGF expression, we observed no differences in eNOS protein expression between the HH and LH groups. This apparent inconsistency may reflect indirect effects of hypoxia on eNOS regulation.

Jensen et al (34) reported recently that low plasma levels of adrenal steroids in pregnant ewes (at ~ 130 d of gestation) induced by adrenalectomy and partial steroid replacement resulted in an increase in placental levels of VEGF protein without changes in eNOS levels. This effect may have been present in our LH group. We did not measure adrenal or ovarian steroids. However, in a previous study, using similar experimental groups, we found a trend to low plasma cortisol levels in LH pregnant ewes in comparison with HH ewes from the middle of gestation onwards, and the difference became significant during the last 5 d of pregnancy (35). This finding might be explained by the lower expression of key steroidogenic enzymes for cortisol synthesis and adrenocorticotropin receptors that has been observed in fetal adrenal glands after pregnant ewes have been exposed to HA hypoxia for a period similar to that of our LH ewes (36). Thus, it is probable that the effect on eNOS of high VEGF protein expression in placental tissue of LH ewes compared with HH ewes may be blunted by a mechanism dependent on low concentrations of corticosteroids.

Healthy pregnancy at HA depends on successful physiological adaptation to hypoxia. Understanding the mechanisms involved in adaptation to chronic hypoxia during pregnancy as well as identifying markers associated with a lack of adaptation will be critical for the development of therapeutic interventions for hypoxia-mediated pregnancy disorders. Our data show that long-term exposure of pregnant sheep to natural HA hypoxia upregulates the placental expression of VEGF and eNOS, which contributes to an increase in the placental mass and vascular bed as an attempt to improve blood flow and consequently oxygen delivery to the fetus. However, our data show that in ewes exposed to HA hypoxia for a short period, the observed placental changes are insufficient to counteract the effects of hypoxia on fetal growth. In contrast, in ewes from populations exposed to HA hypoxia for several generations, better fetal growth results from very large placental development even though overexpression of VEGF in the placental tissue is attenuated. This may be part of the process of adaptation to an HA hypobaric hypoxic environment.

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