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# Carbon monoxide: a novel pulmonary artery vasodilator in neonatal llamas of the Andean *altiplano*

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**Aims** To study the nitric oxide (NO) and carbon monoxide roles in the regulation of the pulmonary circulation in lowland and highland newborn sheep and llamas.
**Methods and results** We used neonatal sheep (*Ovis aries*) and llamas (*Lama glama*) whose gestation and delivery took place at low (580 m) or high (3600 m) altitude. *In vivo*, we measured the cardiopulmonary function basally and with a NO synthase (NOS) blockade and calculated the production of carbon monoxide by the lung. *In vitro*, we determined NOS and soluble guanylate cyclase (sGC) expression, NOS activity, and haemoxygenase (HO) expression in the lung. Pulmonary arterial pressure was elevated at high altitude in sheep but not in llamas. Sheep at high altitude relative to sea level had significantly greater total lung NOS activity and eNOS protein, but reduced sGC and HO expression and carbon monoxide production. In contrast, llamas showed no difference in NO function between altitudes, but a pronounced increase in pulmonary carbon monoxide production and HO expression at high altitude.
**Conclusions** In the llama, enhanced pulmonary carbon monoxide, rather than NO, protects against pulmonary hypertension in the newborn period at high altitude. This shift in pulmonary dilator strategy from NO to carbon monoxide has not been previously described, and it may give insight into new treatments for excessive pulmonary vasoconstriction.

## 1. Introduction

Pulmonary hypertension is seen in mammals at high altitude.[1–3] Appropriate increases in pulmonary arterial vascular resistance are adaptive, matching pulmonary perfusion to the reduced oxygenation. However, excessive increases in pulmonary vascular resistance lead to pathology, expressed as high-altitude pulmonary hypertension and oedema in adult humans, persistent pulmonary hypertension in newborn infants, and brisket disease in cattle.[4,5] This is the case of newborn sheep gestated and born at high altitude, as they show marked pulmonary hypertension when compared with their lowland counterparts.[6] In contrast, species adapted to high altitude may have developed protection against the effects of chronic hypoxia on pulmonary vascular resistance by enhancing pulmonary vasodilator function. For instance, Tibetans and Bolivian *Aymaras* show an increase in pulmonary nitric oxide (NO) synthesis relative to sea level dwellers.[7] Another potent pulmonary vasodilator is carbon monoxide, an endogenous gas synthesized by the haemoxygenase (HO) pathway.[8] Like NO, carbon monoxide operates by activating soluble guanylate cyclase (sGC) and potassium channels in the pulmonary vasculature.[9,10] Carbon monoxide also contributes to a decrease in cardiovascular remodelling and smooth muscle cell proliferation induced by hypoxia.[8,11] The llama (*Lama glama*) is a camelid that has lived at high altitude for at

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least 2-million years.[12] There are studies that indicate that this species has selected mechanisms to adapt to the chronic hypobaric hypoxia of high altitude.[13–16] In this study, we have tested the hypothesis that the HO–carbon monoxide system is enhanced during the newborn period in high-altitude species, like the llama, protecting its pulmonary vasculature against the deleterious effects of chronic hypoxia.

## 2. Methods

All animal-care procedures and experimentation were conducted in accordance with 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 were approved by the Ethics Committee of the Faculty of Medicine, University of Chile.

### 2.1 Animals

Lowland and highland newborn sheep (*Ovis aries*, $n = 5$ and $n = 6$, respectively) and llama (*Lama glama*, $n = 7$ and $n = 5$, respectively) were investigated near sea level (Santiago, 580 m, 710 mmHg barometric pressure) and at high altitude (Putre, 3600 m, 480 mmHg barometric pressure).

### 2.2 *In vivo* studies

We compared pulmonary NO and carbon monoxide functions in highland and lowland newborn sheep and llama, investigated near sea level (Santiago, 580 m, 710 mmHg barometric pressure) and at high altitude (Putre, 3600 m, 480 mmHg barometric pressure). The newborns were submitted to a surgical procedure at 4–5 days of age and studied at 7–10 days of age. Under general anaesthesia ketamine–diazepam association (10 mg $kg^{-1}$ i.m. Ketostop, Drag Pharma-Invectec, Santiago, Chile: 0.1–0.5 mg $kg^{-1}$ i.m. Diazepam, Laboratorio Biosano, Santiago, Chile) and additional local infiltration of 2% lidocaine (Dimecaína, Laboratorio Beta, Santiago, Chile), polyvinyl catheters (1.2 mm i.d.) were placed in the descending aorta and inferior vena cava via a hindlimb artery and vein, exteriorized subcutaneously through the animal flank and kept in a pouch sewn onto the skin. In addition, a Swan-Ganz catheter (Edwards Swan-Ganz 5 French, Baxter Healthcare Corporation, Irvine, CA, USA) was inserted into the pulmonary artery via an external jugular vein, exteriorized and placed in a pouch around the neck of the animal. All vascular catheters were filled with a heparinized saline solution (500 IU heparin $mL^{-1}$ in 0.9% NaCl) and plugged with a copper pin. Ampicillin 10 mg $kg^{-1}$ i.v. (Ampicilina, Laboratorio Best-Pharma, Santiago, Chile) was administered every 12 h while the animals were catheterized. The experiments commenced 3 days after surgery. We measured the blood pH and gases, pulmonary arterial pressure (PAP), and cardiac output of the chronically instrumented conscious newborn sheep and llamas at near sea level and at high altitude.[6]

We measured pulmonary arterial pressure of the newborn sheep and llamas by a Swan-Ganz catheter (5 or 7 French) installed via the jugular vein connected to a pressure transducer. The role of NO in maintaining PAP was investigated *in vivo* by an infusion of a NO synthase (NOS) blocker (L-NAME, Sigma, bolus 20 mg $kg^{-1}$ +0.5 mg $kg^{-1}$ $min^{-1}$ infusion for 15 min, via inferior *vena cava*). The controls received a NaCl 0.9% infusion in the same period. *In vivo* pulmonary circulation carbon monoxide production was determined by the difference in the concentration of carbon monoxide between the aorta and pulmonary artery (percent of carboxyhaemoglobin; OSM3 Hemoximeter, Radiometer), multiplied by the cardiac output (determined by thermodilution with the Swan-Ganz catheter).

### 2.3 Biochemistry and molecular biology studies

A comparable set of not instrumented lowland and highland animals (sheep, $n = 5$ and $n = 5$, respectively; llama, $n = 5$ and $n = 5$, respectively) were euthanized with an anaesthetic overdose of thiopentone, and their lungs were obtained by dissection. We assessed total lung NOS activity by measuring the conversion of L-[$^3$H]-arginine to L-[$^3$H]-citrulline.[17] In total lung lysates, the expression of eNOS, sGC and β-actin proteins were measured by immunoblot with specific anti-eNOS monoclonal antibody (Transduction Laboratories), anti-sGC polyclonal antibody (Cayman Laboratories), and anti-β-actin monoclonal antibody (Sigma), respectively. Signals were developed by incubation with horseradish peroxidase (HRP)-coupled anti-mouse IgG or anti-rabbit IgG secondary antibodies (Jackson Immunoresearch) and evidenced by chemiluminescence (ECL, SuperSignal, Pierce).

In addition, the haemoxygenase-1 (HO-1) protein expression in total lung extract was determined by immunoblot with monoclonal anti-HO-1 antibody (Transduction Laboratories) and HRP-coupled anti-mouse IgG secondary antibody (Jackson Immunoresearch) followed by chemiluminescence (ECL, SuperSignal, Pierce).

### 2.4 Statistical analysis

Data were expressed as mean ± SEM. Statistical analysis was performed using a two-way analysis of variance (ANOVA). Differences between means were assessed using the Newman–Keuls test. Statistical significance was accepted when $P < 0.05$.

## 3. Results

At high altitude, basal $PaO_2$, $SaO_2$, and $O_2$ content were decreased compared with sea level controls in both sheep and llamas (*Table 1*). Although there was an increase in haemoglobin (Hb) concentration in newborn sheep at high altitude, Hb concentration in highland and lowland llamas was similar. Conversely, a fall in $PCO_2$ occurred in llamas but not in sheep at altitude. Values for $SaO_2$ at any altitude were higher in llamas than in sheep, suggesting a leftward shift in their Hb–oxygen dissociation curve (*Table 1*). Basal PAP was significantly elevated at high altitude only in newborn sheep but not in newborn llamas (*Figure 1A*). *In vivo* treatment of newborn animals with L-NAME revealed a greater increment in PAP in sheep than in llamas at both altitudes (*Figure 1B*). Newborn sheep at high altitude relative to sheep at sea level had significantly greater total lung NOS activity and eNOS protein, but reduced lung sGC expression (*Figure 2*) and reduced carbon monoxide production and HO-1 expression (*Figure 3*). In marked contrast, newborn llamas showed no difference in NO function

**Table 1** Arterial blood acid–base status and gases in sea level (SL) and high altitude (HA) newborn sheep and llama infused with L-NAME

| | | | Basal | Basal + L-NAME |
|---|---|---|---|---|
| pHa | Sheep | SL | 7.46 ± 0.01 | 7.44 ± 0.01 |
| | | HA | 7.47 ± 0.01 | 7.46 ± 0.01 |
| | Llama | SL | 7.45 ± 0.01 | 7.44 ± 0.01 |
| | | HA | 7.45 ± 0.02 | 7.43 ± 0.02 |
| $PaO_2$ (mmHg) | Sheep | SL | 78 ± 3 | 83 ± 5 |
| | | HA | 41 ± 4[a] | 37 ± 4[a] |
| | Llama | SL | 94 ± 3[b] | 88 ± 3[b] |
| | | HA | 52 ± 4[a,b] | 46 ± 5[a,b] |
| $PaCO_2$ (mmHg) | Sheep | SL | 33 ± 1 | 33 ± 1 |
| | | HA | 32 ± 2 | 32 ± 2 |
| | Llama | SL | 37 ± 1[b] | 36 ± 2[b] |
| | | HA | 32 ± 2[a] | 31 ± 3[a] |
| $SaO_2$ (%) | Sheep | SL | 95 ± 1 | 94 ± 2 |
| | | HA | 66 ± 4[a] | 62 ± 6[a] |
| | Llama | SL | 97 ± 1 | 96 ± 2 |
| | | HA | 92 ± 2[a,b] | 86 ± 6[a,b] |
| Hb (g $dL^{-1}$) | Sheep | SL | 9.7 ± 0.7 | 9.5 ± 0.8 |
| | | HA | 11.6 ± 0.7[a] | 12.0 ± 0.7[a] |
| | Llama | SL | 9.9 ± 0.4 | 10.3 ± 0.4 |
| | | HA | 8.7 ± 0.7[b] | 8.8 ± 1.6[b] |
| $O_2$ cont (mL $dL^{-1}$) | Sheep | SL | 12.5 ± 0.9 | 12.0 ± 1.0 |
| | | HA | 10.5 ± 0.9[a] | 10.3 ± 1.1[a] |
| | Llama | SL | 12.8 ± 0.5 | 13.1 ± 0.5 |
| | | HA | 10.7 ± 0.9[a] | 10.2 ± 1.3[a] |

Arterial blood acid–base status and gases in newborn sheep and llama during basal conditions and following i.v. treatment with the nitric oxide synthase (NOS) blocker L-NAME. Values represent the mean ± SEM of measurements performed in five sheep and seven llamas at sea level (SL) and in six sheep and five llamas at high altitude (HA). Significant differences are $P < 0.05$.

[a]High altitude vs. sea level in the same species (two-way ANOVA and Newman–Keuls test).

[b]Llama vs. sheep at the same altitude (two-way ANOVA and Newman–Keuls test).

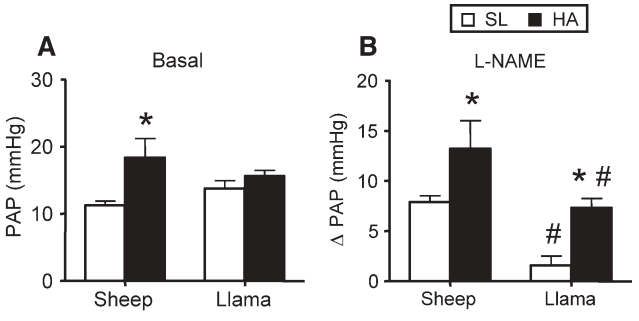


**Figure 1** Pulmonary arterial pressure in the newborn sheep and llama. Pulmonary arterial pressure (PAP) in newborn sheep and llama during basal conditions (*A*) and the increment in PAP following i.v. treatment with the nitric oxide synthase (NOS) blocker L-NAME (*B*). Values represent the mean ± SEM of measurements performed in five sheep and seven llamas at sea level (white bars, SL) and in six sheep and five llamas at high altitude (black bars, HA). Significant differences are $P < 0.05$: *, high altitude vs. sea level in the same species; #, llama vs. sheep at the same altitude (two-way ANOVA and Newman–Keuls test).

between altitudes, but a pronounced increase in pulmonary carbon monoxide production and HO-1 expression at high altitude compared with sea level (*Figures 2* and *3*). The opposing effects of high altitude on pulmonary carbon

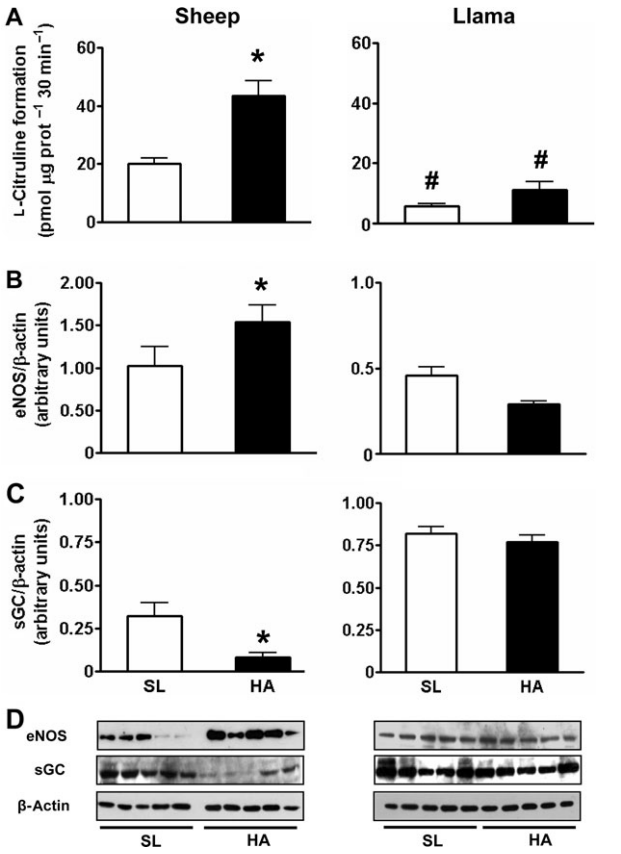


**Figure 2** Pulmonary nitric oxide function in the newborn sheep and llama. Total lung nitric oxide synthase (NOS) activity (*A*), western blot of endothelial nitric oxide synthase (eNOS, *B*), and western blot of soluble guanylate cyclase (sGC, *C*) of pulmonary tissue of newborn sheep and llamas. Values represent the mean ± SEM of measurements performed in five sheep and five llamas at sea level (white bars, SL) and in five sheep and five llamas at high altitude (black bars, HA). Pictures of the different western blots are shown (*D*). Significant differences are $P < 0.05$: *, high altitude vs. sea level in the same species; #, llama vs. sheep at the same altitude (two-way ANOVA and Newman–Keuls test).

monoxide production in sheep and llamas could not be explained by different ventilatory responses to altitude between the species, as the partial pressure of $CO_2$ ($PCO_2$) in the aortic blood, a function of alveolar ventilation, was similar in sheep and llamas at high altitude (*Table 1*).

## 4. Discussion

The data in this study show that in the llama, enhanced pulmonary carbon monoxide, rather than pulmonary NO, protects against pulmonary hypertension in the newborn period at high altitude. In contrast, in newborn sheep at high altitude, pulmonary carbon monoxide production and HO-1 expression are not increased, but markedly reduced. Further, the relative enhancement of the pulmonary NO system in sheep appears insufficient to restrain increases in PAP at high altitude. The data strongly support the hypothesis tested that an alternative vasodilator, such as the HO–carbon monoxide system, is enhanced during the newborn period in high-altitude species like the llama, protecting against the deleterious effects of chronic hypoxia on the pulmonary vasculature.

A limited number of studies have reported that the HO–carbon monoxide system is altered in the lung during

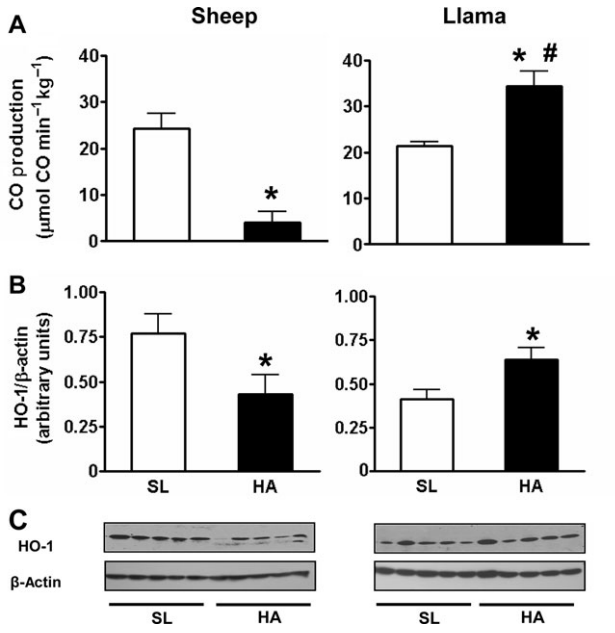


**Figure 3** Pulmonary carbon monoxide function in the newborn sheep and llama. Carbon monoxide lung production (CO production, *A*) and western blot of hemoxygenase-1 (HO-1, *B*) of pulmonary tissue from newborn sheep and llamas. Values represent the mean ± SEM of measurements performed in five sheep and five llamas at sea level (white bars, SL) and in six sheep and five llamas at high altitude (black bars, HA) for carbon monoxide production; and in five sheep and five llamas at sea level (white bars, SL) and altitude (black bars, HA) for HO-1 expression. Pictures of the western blots are shown (*C*). Significant differences are $P < 0.05$: *, high altitude vs. sea level in the same species; #, llama vs. sheep at the same altitude (two-way ANOVA and Newman–Keuls test).

hypoxia. In lowland species, carbon monoxide has been reported to be up-regulated in acute hypoxia, followed by a down-regulation in response to chronic hypoxia.[8,18] Recent studies have reported no significant differences in lung HO activity between normoxic and chronic hypoxic adult and foetal sheep.[19] In contrast, we show in newborn sheep that the pulmonary HO–carbon monoxide system is depressed at high altitude. However, in the llama, the pulmonary HO–carbon monoxide system in the newborn period is not only resilient but also enhanced. The pulmonary HO–carbon monoxide system may protect against the development of hypoxic pulmonary vasoconstriction and vascular remodelling. The pulmonary vasodilatation via carbon monoxide is mediated by cGMP and $K_{Ca}$ channels, providing an effective mechanism in the regulation of this vascular territory.[11,20,21] Further advantages of this system result from the products of the enzymatic reactions in the haem degradation pathway, such as biliverdin and bilirubin, which have important biological effects including antioxidant, anti-inflammatory, and cytoprotective functions.[8,11,22,23] Moreover, the HO–carbon monoxide system has been proposed as an oxygen sensor that controls channel activity during oxygen deprivation.[10] Mice null for HO-1 gene have cardiac complications, such as right ventricular infarction and dilatation, when submitted to hypoxia,[8] whereas targeted lung over-expression of HO-1 diminishes pulmonary hypertension and inflammation triggered by hypoxia.[21] Indeed, the use of carbon monoxide to treat pulmonary hypertension either as inhalatory carbon monoxide[24] or by haemin administration (a HO-1 inducer) has been recently proposed.[20] Accordingly, we have previously reported that inhalatory carbon monoxide reduced pulmonary vascular resistance in hypoxic adult sheep.[25]

Several studies in foetal and adult llama support the idea that this animal is genetically adapted to the hypobaric hypoxia of high altitude.[13–16,26] Among these adaptations, high-altitude neonatal llamas show higher levels of $SaO_2$ than highland sheep suggesting an enhanced Hb oxygen affinity, as is the case in adult llamas.[27] Furthermore, in contrast to newborn sheep, the neonatal llama did not have an increase in Hb concentration at high altitude. This may indicate a different set point in the hypoxic stimulation of erythropoietin secretion in highland than in lowland species. We propose that the llama has selected over generations preferential up-regulation of the pulmonary carbon monoxide over the NO system to offset the biological *sequelae* of living under the influence of the chronic hypoxia of the Andean *altiplano*, preventing the development of neonatal pulmonary hypertension and lung injury at high altitude. This shift in pulmonary dilator strategy from NO to carbon monoxide has not been previously described in highland dwellers, and it may give insight into new treatments for excessive pulmonary vasoconstriction in humans.

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**Conflict of interest**: none declared.

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