Nitric oxide plays a role in the regulation of adrenal blood flow and adrenocorticomedullary functions in the llama fetus

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The hypothesis that nitric oxide plays a key role in the regulation of adrenal blood flow and plasma concentrations of cortisol and catecholamines under basal and hypoxaemic conditions in the llama fetus was tested. At 0.6–0.8 of gestation, 11 llama fetuses were surgically prepared for long-term recording under anaesthesia with vascular and amniotic catheters. Following recovery all fetuses underwent an experimental protocol based on 1 h of normoxaemia, 1 h of hypoxaemia and 1 h of recovery. In nine fetuses, the protocol occurred during fetal I.V. infusion with saline and in five fetuses during fetal I.V. treatment with the nitric oxide synthase inhibitor L-NAME. Adrenal blood flow was determined by the radiolabelled microsphere method during each of the experimental periods during saline infusion and treatment with L-NAME. Treatment with L-NAME during normoxaemia led to a marked fall in adrenal blood flow and a pronounced increase in plasma catecholamine concentrations, but it did not affect plasma ACTH or cortisol levels. In saline-infused fetuses, acute hypoxaemia elicited an increase in adrenal blood flow and in plasma ACTH, cortisol, adrenaline and noradrenaline concentrations. Treatment with L-NAME did not affect the increase in fetal plasma ACTH, but prevented the increments in adrenal blood flow and in plasma cortisol and adrenaline concentrations during hypoxaemia in the llama fetus. In contrast, L-NAME further enhanced the increase in fetal plasma noradrenaline. These data support the hypothesis that nitric oxide has important roles in the regulation of adrenal blood flow and adrenal corticomedullary functions during normoxaemia and hypoxaemia functions in the late gestation llama fetus.

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In lowland species, such as cattle, sheep, pig and dog, a component of the control of adrenal function is regulated by the innervation to the adrenal gland (Ehrhart-Bornstein *et al.* 1998). For example, Edwards and colleagues have shown that stimulation of the splanchnic innervation of the gland, doubles the output of cortisol (Edwards & Jones, 1987*a*), while section of the splanchnic nerves halves the cortisol response to an exogenous infusion of ACTH in conscious, hypophysectomized calves (Edwards & Jones, 1987*b*). More recently, it has become clear that in the adult rat, pig, cow and human, a component of splanchnic control of adrenal function may be regulated by the release of peptides, such as vasoactive intestinal peptide (VIP; Ehrhart-Bornstein *et al.* 1998). Intra-aortic infusions of VIP in functionally hypophysectomized calves given exogenous ACTH mimics the increase in adrenal cortisol output produced by splanchnic nerve stimulation (Bloom *et al.* 1987). That VIP may contribute to adrenal glucocorticoid release has also been demonstrated in the pig (Ehrhart-Bornstein *et al.* 1991) and the rat (Hinson *et al.* 1994). In turn, peptidergic regulation of adrenal function in the adult animal may involve nitric oxide (NO). It has been reported that VIPergic neurotransmission in both the enteric and autonomic nervous systems depends on the presence, or *de novo* synthesis of NO. This has been shown both presynaptically where NO mediates the release of the peptide, and postsynaptically where it modulates certain responses to it (Edwards & Garrett, 1993; Grider & Jin, 1993; Buckle *et al.* 1995; Edwards *et al.* 1996).

Neural control of adrenal function also occurs during fetal life in late gestation sheep as section of either the carotid sinus nerves or the splanchnic nerves affects the

steroidogenic response without affecting the increase in ACTH during acute hypoxaemia or acute hypotension (Myers *et al.* 1990; Giussani *et al.* 1994). Previously, we have shown that neural control of adrenal function during fetal life is greater in highland than in lowland species, since section of the carotid sinus nerves completely prevented the increase in plasma cortisol in acute hypoxaemia in the llama fetus (Riquelme *et al.* 1998), but only delayed this increase in the sheep fetus (Giussani *et al.* 1994), without affecting the plasma ACTH response in either species. Enhanced neural control of adrenal function in the llama fetus may be an adaptation to the chronic hypoxia of life at altitude. It is known that most of such adaptations to high altitude in the llama are genetic since they persist in llamas born and living at sea level (Banchero *et al.* 1971; Moraga *et al.* 1996; Giussani *et al.* 1999*b*).

To date, the role of NO in the regulation of adrenocortical or adrenomedullary function in the fetus of either highland or lowland species is unknown. Therefore, this study tested the hypothesis that NO plays a role in the regulation of adrenal function in the llama fetus, since this species demonstrates potent neural control of adrenal activity, by investigating the effects of NO blockade on adrenal blood flow, and plasma concentrations of cortisol and catecholamines under normoxaemic and hypoxaemic conditions.

METHODS

Animals

Fourteen time-dated pregnant llamas carrying fetuses at 0.6–0.8 of gestation (3.498 ± 0.304 kg, means ± S.E.M., where term weight is 10.7 ± 0.7 kg, Fowler, 1989) were obtained from the University of Chile farm at Rinconada de Maipú, at 580 m above sea level. Upon arrival in Santiago, 585 m above sea level, the llamas were studied in the Laboratory for Developmental Physiology and Pathophysiology at the Faculty of Medicine, University of Chile. The animals were housed in an open yard with access to food and water *ad libitum* and they were familiarized with the study cage and the laboratory conditions for 1–2 weeks prior to surgery.

Surgical preparation

Maternal and fetal surgeries were carried out on consecutive days using well-established techniques previously described in detail (Llanos *et al.* 1998). Briefly, following food and water deprivation for 24 h, the pregnant llamas were pre-medicated with atropine (1 mg, I.M., Atropina Sulfato, Laboratorio Chile, Santiago, Chile). Polyvinyl catheters (i.d. 1.3 mm) were placed in the maternal descending aorta and inferior vena cava via a hindlimb artery and vein under light general anaesthesia (ketamine, 10 mg kg^{-1} I.M., Ketostop, Biosano Laboratorio, Santiago, Chile) with additional local infiltration of lidocaine (lignocaine; 2% lidocaine hydrochloride, Dimecaína, Laboratorio Sanderson SA, Santiago, Chile). The catheters were then tunnelled subcutaneously to exit through a small incision in the maternal flank.

The following day the fetuses were surgically prepared under maternal general anaesthesia (5–7 mg kg^{-1} sodium thiopentone, Tiopental Sódico, Laboratorio Biosano SA, Santiago, Chile for induction and 0.5–2% halothane in 50:50 O_2 and N_2O for maintenance). Following a midline laparotomy, a fetal hindlimb was withdrawn through a small hysterotomy. Polyvinyl catheters (i.d. 0.8 mm) were inserted into the fetal aorta via a hindlimb artery and into the inferior vena cava via a hindlimb vein. A fetal forelimb was exposed through a second hysterotomy and a catheter (i.d. 0.8 mm) was placed into the brachial artery. A catheter was placed in the amniotic cavity for pressure reference, and the uterine and abdominal incisions were closed. All catheters were filled with heparinized saline (1000 i.u. heparin ml^{-1}), plugged with copper pins, exteriorized through the incision in the maternal flank and kept in a pouch sewn onto the maternal skin.

During surgery, all llamas were constantly hydrated with a warm I.V. solution of 0.9% NaCl at a rate of 15–20 ml kg^{-1} h^{-1}, to compensate for fluid loss during the procedures. At the end of the surgery, ampicillin (500 mg; Ampicilina, Laboratorio Best Pharma, Santiago, Chile) and gentamicin (80 mg; Gentamicina Sulfato, Laboratorio Sanderson SA, Santiago, Chile) were given intra-amniotically. The animals were returned to the yard and at least 4 days of postoperative recovery were allowed before the experiments were started. Vascular catheters were maintained patent by daily flushing with heparinized saline (200 i.u. heparin ml^{-1}).

All experimental protocols were reviewed and approved by the Faculty of Medicine Ethics Committee of the University of Chile. All animal procedures, maintenance and experimentation were conducted in accordance with the recommendations in the Guiding Principles for Research Involving Animals and Human Beings of the American Physiological Society and the UK Animals (Scientific Procedures) Act 1986.

Experimental protocol

All experiments were based on a 3 h protocol divided into three periods of 60 min: 1 h of normoxaemia, 1 h of hypoxaemia and 1 h of recovery. A transparent respiratory hood was placed over the llama's head into which known concentrations of O_2, N_2 and CO_2 were passed at a rate of *ca* 50 l min^{-1}. Following 1 h of breathing air (normoxaemia), fetal hypoxaemia was induced by maternal inhalation of a hypoxic gas (9% O_2 in N_2; 2–3% CO_2), which reduced maternal arterial P_{O_2} (partial pressure of O_2) from ~96 to ~35 mmHg and haemoglobin saturation from ~97 to ~76%. This, in turn, reduced fetal descending aortic P_{O_2} and haemoglobin saturation to ~12 mmHg and to ~21%, respectively, without alteration in fetal arterial P_{CO_2} from baseline. After the hour of hypoxaemia the maternal llama was returned to breathing air for a further 60 min (recovery).

In nine fetuses, acute hypoxaemia was induced during fetal I.V. infusion with saline (0.33 ml min^{-1}) and in five fetuses acute hypoxaemia was induced during I.V. treatment with the NO synthase inhibitor, N^{ω}-nitro-L-arginine, dissolved in saline (L-NAME; 20 mg kg^{-1} bolus + 0.5 mg kg^{-1} min^{-1} infusion at 0.5 ml min^{-1}. Fetal infusion with saline or treatment with L-NAME started 15 min prior to hypoxaemia and continued until the end of the hypoxaemic challenge. Assignation of fetuses to saline infusion or L-NAME groups was randomized.

Maternal and fetal arterial blood samples (0.5 ml) were taken into chilled, heparinized syringes after 15, 45 and 60 min of normoxaemia, at 15 min intervals during hypoxaemia and after 15 and 45 min of recovery for measurement of arterial pH, P_{O_2}, P_{CO_2} (BMS 3MK2 Blood Microsystem and PHM 73/Blood Gas Monitor, Radiometer, Copenhagen, Denmark; measurements corrected to 39 °C) and percentage saturation of haemoglobin and haemoglobin concentration (OSM2 Haemoximeter, Radiometer). In addition, maternal and fetal arterial blood samples were taken

into chilled, heparinized syringes (3.5 ml) at 15, 45 and 60 min of normoxaemia, 15 and 45 min of hypoxaemia and at 45 min of recovery for measurement of plasma hormone concentrations. The overall volume of blood samples during the experimental protocol in any one control or L-NAME-treated fetus was *ca* 10 %. Fetal arterial, venous and amniotic pressures (Statham, Hato Rey, Puerto Rico) and fetal heart rate were recorded continuously throughout the experiment on a polygraph (Gilson ICM-5, Emeryville, CA, USA).

Fetal combined ventricular output and organ blood flows were determined at 45 and 60 min of normoxaemia and at 45 min of hypoxaemia and at 45 min of recovery. Radionuclide-labelled microspheres of 15 μm diameter (^{57}Co, ^{113}Sn, ^{46}Sc and ^{103}Ru; New England Nuclear, Boston, MA, USA; 1.0–1.2 million) were injected into the inferior vena cava while reference samples were obtained from the ascending and descending aorta (Heymann *et al.* 1977). The rate at which the reference samples were drawn was 3.2 ml min^{-1} for 1.5 min. This method allows blood flow determination to all organs except the lung (Heymann *et al.* 1977). In the present paper, we report only flow to the adrenal glands. Blood flow distribution to other organs will be published elsewhere.

On completion of the experiments, the llama was anaesthetized with I.V. sodium thiopentone (1 g; Tiopental Sódico, Laboratorio Sanderson SA, Santiago, Chile) and killed with I.V. saturated potassium chloride.

Adrenal blood flow, vascular resistance and oxygen and ACTH deliveries

Post mortem, the uterus and individual fetal organs were dissected and weighed. All dissected material was carbonized, ground to coarse powder, placed in vials and counted with a multichannel gamma pulse analyser (Minaxi 5000, Packard, Canberra, Australia). Microsphere mixing was considered appropriate when the percentage difference in calculated blood flow to either cerebral hemisphere and to either kidney was < 10 %. To minimize error in the calculation of organ blood flow, sufficient microspheres were injected to ensure a distribution of > 400 microspheres per organ (Heymann *et al.*1977).

In each fetus, blood flow to the adrenal gland was calculated by comparing the organ radioactivity with the activity and flow rate of the descending aorta reference sample. Adrenal blood flow was calculated as follows:

$$\mathrm{ABF} = C_{\mathrm{Adr}} \times \dot{Q}_{\mathrm{reference}}/C_{\mathrm{reference}},$$

where ABF is adrenal blood flow (ml min^{-1}) and C_{Adr} is the radioactivity in the adrenal gland in counts min^{-1} (c.p.m.), $\dot{Q}_{\mathrm{reference}}$ is the reference flow of the descending aorta (ml min^{-1}) and $C_{\mathrm{reference}}$ is the radioactivity in the reference sample (c.p.m.).

Adrenal vascular resistance was calculated by dividing perfusion pressure (arterial–venous pressure) at the time of microsphere injection by adrenal blood flow. In addition, blood oxygen content (O_2 content, ml O_2 (ml blood)$^{-1}$) and deliveries of oxygen and ACTH to the adrenal gland (Adr O_2 delivery, ml O_2 (100 g)$^{-1}$ min^{-1}; Adr ACTH delivery, ng ACTH (100 g)$^{-1}$ min^{-1}) were calculated as follows:

$$O_2\ \text{content} = \text{Hb concentration} \times \text{Hb saturation} \times 100^{-1} \times 1.34,$$

$$\text{Adr}\ O_2\ \text{delivery} = O_2\ \text{content} \times \text{ABF},$$

$$\text{Adr ACTH delivery} = \text{ACTH concentration} \times \text{ABF}.$$

1.34 is a constant and its units are ml O_2 (g Hb)$^{-1}$. Units of measurement were as follows: Hb concentration (g ml^{-1}), Hb saturation (%), ABF (ml (100 g)$^{-1}$ min^{-1}), and ACTH concentration (ng ml^{-1}).

Hormone analyses

Blood samples taken for hormone analyses were immediately centrifuged at 800 *g* for 5 min. The plasma was removed and divided into several aliquots. A 1 ml aliquot of plasma was acidified with 0.1 ml of 35 % perchloric acid, shaken and centrifuged at 800 *g* at 4 °C for 5 min. Two aliquots (0.3 ml each) of untreated plasma were stored at −80 °C for determination of cortisol and ACTH concentrations. All plasma samples were assayed within 2 months of collection.

Catecholamines. Plasma adrenaline and noradrenaline concentrations were determined using solid phase extraction and HPLC. In brief, catecholamines were removed from plasma using the formation of a complex-ionic pair, in alkaline medium, between diphenylborate and the diol group of catecholamines (Smedes *et al.* 1982). Dihydroxybenzylamine (DHBA) was used as an internal standard. The catecholamine complex was adsorbed in a Bakerbond SPE C18 column. Catecholamines were eluted with 0.4 ml of 1 M acetic acid and 100 μl aliquots were measured by HPLC (Merck Hitachi L-6200 A) with amperometric detection (Metrohm, model 656), in a C18 reverse-phase column (5 μm, 30 × 6 mm i.d.). The mobile phase consisted of a mixture of 0.008 M citric acid, 0.008 M Na_2HPO_4, 0.01 % EDTA, 0.05 % octansulphonic acid (pH 4.5) and methanol (90:10, v/v) (Williams *et al.* 1985). Detection was performed at a potential of +0.60 V, in the range of 0.5–1.0 nA, registered at full scale. Peak height ratios were then determined by dividing the peak height of the catecholamines by the peak height of the internal standard (Monari *et al.* 1992). These ratios were interpolated in a standard curve of adrenaline and noradrenaline and processed as described for the samples. The lower limit of detection was 10 pg ml^{-1} for both adrenaline and noradrenaline. The coefficient of variation was 13 % for noradrenaline and 8.5 % for adrenaline.

ACTH. Plama ACTH concentrations were measured by a commercial radioimmunoassay kit (Incstar, Stillwater, MN, USA). The intra- and inter-assay coefficients of variation were 9.6 and 11.9 %, respectively. The lower limit of detection was 8 pg ml^{-1}. This ACTH antibody cross reacts at a level of < 0.01 % with α-melanocyte-stimulating hormone (MSH), β-MSH, β-endorphin and β-lipotropin, and does not recognize pro-ACTH or propio-melanocortin (Jeffray *et al.* 1998). The antibody also showed > 95 % immunoreactivity with ACTH$^{1–39}$ when samples of fetal sheep taken in normoxaemia or hypoxaemia were assayed after HPLC separation of ACTH-related peptides (Challis *et al.* 1989).

Cortisol. Cortisol was measured by radioimmunoassay for human plasma (Sufi *et al.* 1998). Parallelism between standard curves and llama plasma (30 to 120 μl) was obtained (data not shown). Samples were analysed in duplicate either in 60 or in 120 μl aliquots of plasma using an antiserum raised against cortisol-21-hemisuccinate-BSA antiserum, cortisol standard and 1,2,6,7-[^{3}H]cortisol (65.0 Ci mmol^{-1}). Reagents were obtained from the Programme for the Provision of Matched Assays Reagents for the Radioimmunoassay of Hormones in Reproductive Physiology, WHO Special Programme of Research, Development and Training in Human Reproduction. The assay was performed directly in aliquots of plasma diluted with 0.1 M phosphate-buffered saline, pH 7.4, and heated at 60 °C to destroy transcortin. The assay range was 0.06–2.00 ng of cortisol per tube.

Table 1. L-NAME effect upon cardiorespiratory variables in the fetal llama

	0.9 % NaCl treated				L-NAME treated			
	Normox.	Normox. + NaCl	Hypox. + NaCl	Recovery	Normox.	Normox. + L-NAME	Hypox. + L-NAME	Recovery
pH (units)	7.37 ± 0.02	7.37 ± 0.02	7.31 ± 0.03 †	7.23 ± 0.02 †	7.37 ± 0.02	7.37 ± 0.03	7.30 ± 0.03	7.16 ± 0.06 †
P_{O_2} (mmHg)	23 ± 1	23 ± 1	12 ± 1 †	20 ± 1 †	20 ± 1	19 ± 1	12 ± 1 †	19 ± 2
P_{CO_2} (mmHg)	43 ± 1	43 ± 1	40 ± 2	40 ± 1	43 ± 1	45 ± 2	43 ± 1	46 ± 2
Hb Sat (%)	53 ± 3	53 ± 3	19 ± 2 †	42 ± 3 *	46 ± 3	43 ± 3	21 ± 3 †	35 ± 3 *
Hb (g dl^{-1})	11.6 ± 0.3	11.6 ± 0.3	12.7 ± 0.3 †	11.8 ± 0.3	11.6 ± 0.6	11.9 ± 0.6	13.5 ± 0.5 *‡	13.2 ± 0.7 *‡
MAP (mmHg)	51 ± 2	50 ± 2	53 ± 2 †	50 ± 2	49 ± 2 †	57 ± 3	62 ± 5 ‡	61 ± 4 ‡
HR (min^{-1})	118 ± 4	118 ± 5	116 ± 6	131 ± 2	119 ± 8	91 ± 7 *	95 ± 9 *	118 ± 11

Values are means ± S.E.M. for arterial pH, P_{O_2}, P_{CO_2}, haemoglobin saturation and haemoglobin concentration. Normoxaemia represents the mean value of samples taken at 15 and 45 min prior to starting the infusions. Normoxaemia + 0.9 % NaCl or L-NAME represents the value for samples taken at 60 min of normoxaemia. Hypoxaemia + 0.9 % NaCl or L-NAME represents the mean value of samples taken at 15, 30, 45 and 60 min after the start of hypoxaemia during infusion. Recovery represents the mean value of samples taken at 15 and 45 min after the end of hypoxaemia and infusions. Measurements for mean arterial pressure (MAP) and heart rate (HR) were made every minute during each treatment period. Significant differences are: $P < 0.05$: * *vs.* normoxaemia; † *vs.* all periods of the same treatment and ‡ *vs.* 0.9 % NaCl group.

The antiserum used cross-reacts with cortisone (< 0.1 %), corticosterone (9.2 %), 11-deoxycortisol (27.1 %), 17α-OH-progesterone (0.8 %), and progesterone (0.2 %), measured at 50 % displacement. Inter- and intra-assay coefficients of variation were 11.9 and 4.8 %, respectively.

Data and statistical analyses

Values for arterial blood gas status, plasma hormone concentrations and cardiovascular variables during normoxaemia, hypoxaemia and recovery were averaged over each experimental period. All values are expressed as means ± S.E.M. Statistical analyses of the different variables over time were performed using a one-way ANOVA with repeated measures and the *post hoc* Newman-Keuls test. Comparisons between the saline infused and L-NAME-treated fetuses were made by two way ANOVA with repeated measures and the Tukey test. For all statistical comparisons, significance was accepted when $P < 0.05$ (Zar, 1984).

RESULTS

Effect of L-NAME on fetal arterial blood gas status, fetal arterial blood pressure and fetal heart rate

Arterial blood gas status and cardiovascular variables were similar in saline-infused and L-NAME-treated fetuses during normoxaemia (Table 1). While infusion of fetuses with saline had no effect on fetal arterial blood gas status or cardiovascular variables during normoxaemia, fetal treatment with L-NAME led to an increase in arterial blood pressure and a fall in heart rate (Table 1). During acute hypoxaemia, the falls in fetal arterial P_{O_2} and haemoglobin saturation and the increase in fetal haemoglobin concentration were of similar magnitude in both groups of treated fetuses. In contrast, the increase in fetal haemoglobin concentration was higher in the L-NAME-treated group (Table 1). While heart rate remained unchanged from normoxaemic baseline and arterial blood pressure increased during acute hypoxaemia in saline-infused fetuses, arterial blood pressure remained elevated and heart rate depressed in L-NAME-treated fetuses during acute hypoxaemia (Table 1). Furthermore, arterial blood pressure values were higher during hypoxaemia and recovery in the L-NAME-treated group compared to saline infused fetuses (Table 1). Since the results in heart rate shown in Table 1 represent the average of 1 h, the initial bradycardia registered under hypoxaemia in the saline-infused fetuses (Giussani *et al.* 1996) is not reflected. During recovery, significant acidaemia developed and fetal arterial P_{O_2} and haemoglobin saturation returned towards basal levels in both groups of fetuses, although saturation remained decreased relative to baseline values in both groups of fetuses (Table 1).

Effect of L-NAME on fetal adrenal blood flow, fetal adrenal vascular resistance and oxygen delivery to the fetal adrenal gland

Adrenal blood flow, adrenal vascular resistance and oxygen delivery to the adrenal glands were similar in both groups of fetuses during normoxaemia (Fig. 1). While saline infusion had no effect on any of these variables during normoxaemia, a fall in adrenal blood flow and oxygen delivery, and an increase in adrenal vascular resistance, occurred in L-NAME-treated fetuses during normoxaemia (Fig. 1). During acute hypoxaemia in saline-infused fetuses adrenal blood flow increased, adrenal vascular resistance decreased and adrenal oxygen delivery remained unchanged (Fig. 1). In contrast, adrenal blood flow and oxygen delivery remained low and adrenal vascular resistance remained elevated, in fetuses treated with L-NAME during hypoxaemia (Fig. 1). Whilst adrenal blood flow and vascular resistance returned to normoxaemic basal values in saline-infused fetuses, adrenal blood flow and vascular resistance remained different from normoxaemic baseline in L-NAME-treated fetuses during recovery.

Effect of L-NAME on adrenal corticomedullary functions and on ACTH delivery to the adrenal gland

Plasma concentrations of ACTH, cortisol and catecholamines, and ACTH delivery to the adrenal gland were similar in both groups of fetuses during normoxaemia (Figs 2 and 3). While saline infusion during normoxaemia had no effect on plasma hormone concentrations or ACTH delivery to the adrenal gland, treatment with L-NAME led to a fall in ACTH delivery to the adrenal gland and produced a marked increase in plasma noradrenaline and adrenaline concentrations, but it did not affect fetal plasma concentrations of ACTH or cortisol (Figs 2 and 3). During acute hypoxaemia, an increase in fetal plasma concentrations of ACTH, cortisol, noradrenaline and adrenaline, and an increase in ACTH delivery to the adrenal gland, occurred in saline-infused fetuses. In fetuses treated with L-NAME during acute hypoxaemia, the increase in fetal plasma ACTH was unaffected and a significant increase in ACTH delivery to the adrenal gland still occurred, albeit at a markedly attenuated level, relative to saline-infused fetuses. Despite this, the increase in fetal plasma cortisol in L-NAME treated fetuses was completely prevented during acute hypoxaemia (Fig. 2). In marked contrast, whilst fetal plasma concentrations of noradrenaline became further enhanced, plasma concentrations of adrenaline reverted towards basal values in L-NAME-treated fetuses during acute hypoxaemia (Fig. 3). During recovery, all variables

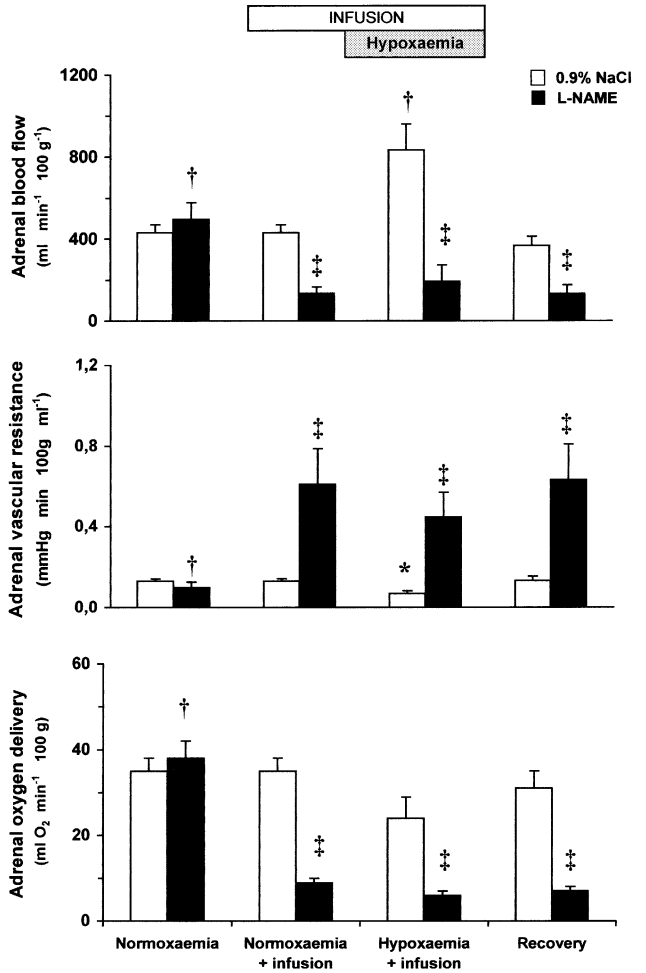

Figure 1. Effects of treatment with L-NAME on adrenal blood flow, adrenal vascular resistance and adrenal oxygen delivery in the llama fetus

Values are means ± S.E.M. Significant differences are $P < 0.05$: * *vs.* normoxaemia, † *vs.* all periods of the same treatment and ‡ *vs.* 0.9 % NaCl group. Normoxaemia represents the value of measurements performed at 45 min of normoxaemia. Normoxaemia + infusion represents the measurements performed 15 min after the start of the infusions. Hypoxaemia + infusion represents the measurements performed at 45 min after the start of hypoxaemia during infusion. Recovery represents the measurements performed at 45 min after the end of hypoxaemia and infusions.

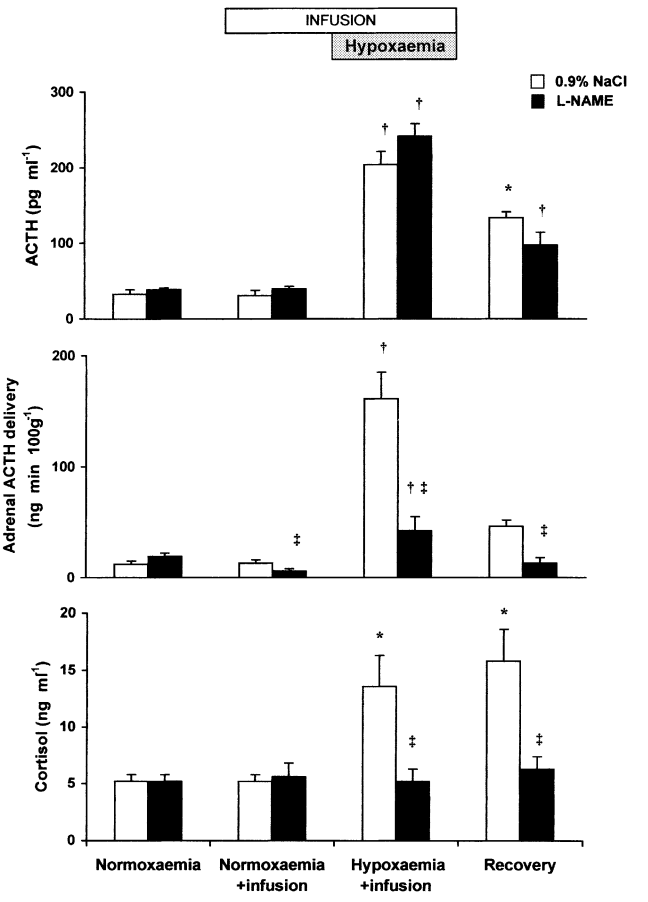

Figure 2. Effects of treatment with L-NAME on plasma ACTH and cortisol concentrations and on ACTH delivery to the adrenal gland in the llama fetus

Values are means ± S.E.M. Significant differences are $P < 0.05$: * *vs.* normoxaemia, † *vs.* all periods of the same treatment and ‡ *vs.* 0.9 %NaCl group. Normoxaemia represents the mean value of samples taken at 45 and 15 min prior to starting the infusions. Normoxaemia + infusion represents the value for samples taken at 15 min after the start of the infusions. Hypoxaemia + infusion represents the mean value of samples taken at 15 and 45 min after the start of hypoxaemia during infusion. Recovery represents the value for samples taken at 45 min after the end of hypoxaemia and infusions.

returned towards baseline, but plasma concentrations of ACTH and cortisol remained significantly elevated from basal values in saline-infused fetuses (Figs 2 and 3). In L-NAME-treated fetuses, fetal plasma ACTH and ACTH delivery to the adrenal gland also returned to basal values, fetal plasma cortisol and adrenaline concentrations still remained unaltered from basal values, and plasma concentrations of noradrenaline remained significantly elevated from baseline during the recovery period (Figs 2 and 3).

DISCUSSION

This study tested the hypothesis that NO plays a key role in the regulation of adrenal corticomedullary functions in the fetus of the llama, a species which demonstrates potent neural control of adrenal activity during fetal life. The studies were performed in conscious animals, which had been surgically prepared under general anaesthesia for long-term recording. The hypothesis of the study was tested by investigating the effects of NO blockade on fetal adrenal blood flow, and fetal plasma concentrations of cortisol and catecholamines during normoxaemic and hypoxaemic conditions. The results show that during fetal life in this high altitude species: (1) NO plays a key role in the control of adrenal blood flow during basal and hypoxaemic conditions; (2) NO is an important regulator of fetal plasma cortisol concentrations during acute hypoxaemia; and (3) NO is an important regulator of fetal plasma catecholamine concentrations during acute hypoxaemia.

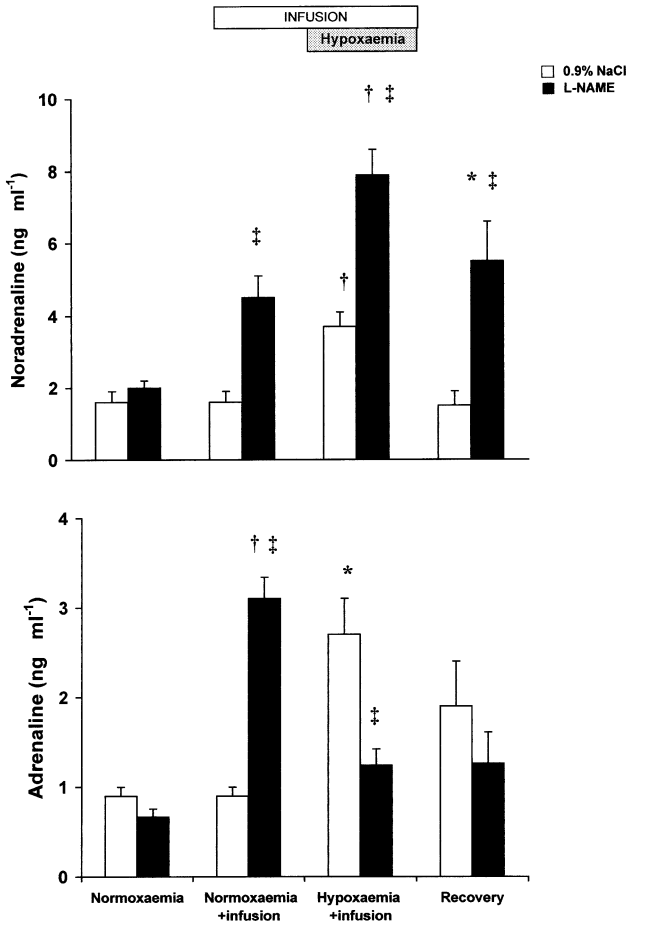

Figure 3. Effects of treatment with L-NAME on plasma cathecholamine concentrations in the llama fetus

Values are means ± S.E.M. Significant differences are $P < 0.05$: * *vs.* normoxaemia, † *vs.* all periods of the same treatment and ‡ *vs.* 0.9 %NaCl group. Normoxaemia represents the mean value of samples taken at 45 and 15 min prior to starting the infusions. Normoxaemia + infusion represents the value for samples taken at 15 min after the start of the infusions. Hypoxaemia + infusion represents the mean value of samples taken at 15 and 45 min after the start of hypoxaemia during infusion. Recovery represents the value for samples taken at 45 min after the end of hypoxaemia and infusions.

NO and adrenal blood flow in the llama fetus

Treatment of the llama fetus with the NO synthase inhibitor, L-NAME, markedly diminished the well-documented increase in adrenal blood flow during acute hypoxaemia in this species (Giussani *et al.* 1996; Llanos *et al.* 1998), in spite of a pronounced decrease in fetal arterial P_{O_2} and a marked increase in fetal plasma ACTH concentrations. In addition, the present study showed that NO blockade produced a marked increase in adrenal vascular resistance in normoxaemia, indicating that NO also plays an important role in the maintenance of adrenal blood flow under basal conditions in the llama fetus. It is possible that the lack of a significant increase in adrenal blood flow during acute hypoxaemia following treatment with L-NAME is the result of an overriding influence on normal vasodilator factors released during hypoxaemia of the marked increase in adrenal vascular tone caused by NO blockade during basal conditions. The source of NO regulating adrenal blood flow in the fetus either during normoxaemic or during hypoxaemic conditions may be vascular, through the activity of endothelial NO synthase (eNOS) and/or neural, as nNOS, has also been detected in adrenal medullary neurons (Bredt *et al.* 1990; Schwarz *et al.* 1998). These results in the llama fetus are consistent with findings in fetal and adult animals from other species. For example, Harris *et al.* (2001) have recently shown that NO has an important role in the control of adrenal blood flow under basal and hypoxaemic conditions in term fetal sheep. Furthermore, NO has been shown to have a role in the control of the adrenal blood flow in the adult anaesthetized dog since treatment with L-NAME reduced both medullary and cortical blood flows by 42 and 60 %, respectively (Breslow *et al.* 1993). Furthermore, Giussani *et al.* (1999*a*) have shown that treatment of the conscious, hypophysectomized calf with L-NAME suppressed the increase in adrenal blood flow in response to stimulation of the peripheral end of the splanchnic nerves, indicating that adrenal vasodilatation during sympathetic activation in the calf is also dependent on the production of NO. During fetal life, NO contributes to the regulation of blood

flow in many other vascular beds, including the umbilical (Chang *et al.* 1992; Chlorakos *et al.* 1998; Gardner *et al.* 2001), systemic (Smolich, 1998), cerebral (van Bel *et al.* 1995; McCrabb & Harding, 1996), myocardial (Reller *et al.* 1995; Harris *et al.* 2001), gastrointestinal (Fan *et al.* 1998; Harris *et al.* 2001) and in the femoral and carotid arteries (Green *et al.* 1996).

NO and adrenocortical function in the llama fetus

The influence of NO in the control of plasma steroid concentrations has not been previously determined during fetal life in any species. In the llama fetus, acute hypoxaemia elicits a pronounced increase in fetal plasma ACTH and cortisol concentrations (Riquelme *et al.* 1998). In addition, section of the carotid sinus nerves in the llama fetus completely prevented the increase in fetal plasma cortisol, without affecting the increase in fetal plasma ACTH or its increased delivery to the adrenal gland in response to acute hypoxaemia (Riquelme *et al.* 1998). That study provided good evidence of an important neural contribution to the control of plasma glucocorticoid concentration in the llama fetus. The data reported in the present study further extend these findings to show that treatment of the llama fetus with L-NAME also completely prevented the rise in fetal plasma cortisol, without affecting the increase in plasma ACTH and preserving a significant increase in ACTH delivery to the adrenal gland, albeit at a much reduced level relative to that calculated in control fetuses, in response to acute hypoxaemia. Clearly, the attenuation of the increase in delivery of ACTH to the adrenal gland in L-NAME-treated fetuses relative to saline-infused controls in response to acute hypoxaemia is primarily due to an effect of the synthase blocker on adrenal blood flow rather than on plasma ACTH concentration. Combined, past and present data therefore suggest that NO is not an important regulator of plasma ACTH concentration during hypoxaemic conditions in this species during fetal life. Furthermore, an important component of the control of plasma cortisol concentration during hypoxaemia in the llama fetus is determined by increased plasma ACTH and its delivery to the gland. However, a remaining, minor component of the control of plasma cortisol concentration in the llama fetus is dependent on increased NO activity during acute hypoxaemia, independent of increased plasma ACTH or its delivery to the adrenal cortex. We hypothesize that a carotid chemoreflex sensitizes the adrenal cortex to ACTH delivery during acute hypoxaemia, that this reflex is mediated via splanchnic nerve efferents involving increased activity of NO, that this reflex is operative in fetal life during late gestation, and that this neural contribution to adrenocortical function is exacerbated in high altitude species. It is possible that, as in the adult, a component of neural control of adrenal function in the fetus may be regulated by the release of VIP (Bloom *et al.* 1987; Ehrhart-Bornstein *et al.* 1991, 1998; Hinson *et al.* 1994) and that sympathetic peptidergic sensitization of adrenocortical function may involve NO, as it does in the enteric and autonomic nervous systems (Edwards & Garrett, 1993; Grider & Jin, 1993; Buckle *et al.* 1995; Edwards *et al.* 1996).

One cannot exclude the possibility that the decrease in P_{O_2} in the fetal llama arterial circulation during acute hypoxaemia may have *per se* affected adrenocortical cortisol secretion, since treatment of the llama fetus with L-NAME led to a marked fall in oxygen delivery to the adrenal gland during both basal and hypoxaemic conditions. However, we do not favour this possibility as cortisol secretion was not inhibited by a fall in P_{O_2} in bovine adrenocortical cells *in vitro* (Raff *et al.* 1989). In contrast, low oxygen selectively inhibited aldosterone secretion under the same conditions (Raff *et al.* 1989). Finally, it is also possible that inhibition of NO synthesis by L-NAME may have affected mechanisms mediating the clearance and/or inactivation of plasma glucocorticoid from the fetal llama circulation. For example, another possible explanation for the lack of an increase in plasma cortisol during hypoxaemia in the llama fetus following treatment with L-NAME is that under these circumstances adrenocortical glucocorticoid secretion may be limited by the lower venous outflow to carry cortisol away from the gland, resulting in local concentrations of the steroid high enough to limit production via negative feedback.

NO and adrenomedullary function in the llama fetus

The role of NO in the synthesis, secretion or clearance of catecholamine under basal or hypoxaemic conditions has also not been previously addressed during fetal life. Results of the present study show that treatment of the llama fetus with L-NAME led to an increase in plasma noradrenaline and adrenaline concentrations during basal conditions. In addition, treatment of the llama fetus with L-NAME under hypoxaemic conditions further enhanced the increment in plasma noradrenaline, but diminished plasma adrenaline towards concentrations measured during basal conditions.

The increase in plasma catecholamine concentration during normoxaemia under conditions of NO blockade may be due to a marked reduction in oxygen delivery to the adrenal gland, secondary to the pronounced adrenal vasoconstriction induced by the treatment. Oxygen-sensing mechanisms are present in the chromaffin cells in the adrenal medulla of fetal animals of other species (Rychkov *et al.* 1998), eliciting secretion of catecholamines into the systemic circulation.

The dissociation in the plasma noradrenaline and adrenaline levels of the fetal llama to acute hypoxaemia under conditions of NO blockade in the present study is interesting. While plasma adrenaline is mainly derived from the adrenal medulla (Jones *et al.* 1987), sources of plasma noradrenaline include both the adrenal medulla

and overspill from the sympathetic nerve terminals. The further increment in plasma noradrenaline concentrations during acute hypoxaemia under conditions of NO blockade in the llama fetus may be explained, in part, by an increased rate of neurotransmission at sympathetic nerve terminals, coupled with a further reduction in oxygen delivery to the adrenal gland augmenting the stimulation of adrenal medullary chemoreceptors (Rychkov *et al.* 1998). In addition, several reports suggest an inhibitory influence of NO on sympathetic neurotransmission and catecholamine synthesis, which may explain elevations in plasma noradrenaline during both basal and hypoxaemic conditions in llama fetuses following treatment with L-NAME. For example, in adult pigs, inhibition of NO during hypoxaemia stimulates sympathetic neuronal activity in the ventrolateral region of the medulla (Zazinger *et al.* 1998) and in anaesthetized adult dogs NO plays an inhibitory role in adrenal catecholamine production in response to cholinergic stimulation (Nagayama *et al.* 1998).

The inability to maintain high plasma concentrations of adrenaline during acute hypoxaemia under conditions of NO blockade may be due to an effect of L-NAME on the adrenomedullary synthesis of adrenaline. The production of adrenaline is limited by the activity of phenylethanolamine-*N*-methyltransferase (PNMT), the enzyme that catalyses the conversion of noradrenaline to adrenaline. It is known that a component of PNMT activity is regulated by increased glucocorticoids during fetal and adult life. For example, glucocorticoid administration induces an increase in PNMT activity in the adrenal medulla of the fetal rat (Parker & Noble, 1967) and it stimulates an increase in the levels of PNMT mRNA in bovine medullary cells in culture (Wan & Livett, 1989). In fetal sheep, PNMT mRNA, the content of adrenaline within the adrenal medulla and plasma adrenaline concentrations all increase with advancing gestational age, in parallel with the prepartum increase in fetal plasma cortisol (Llanos *et al.* 1979; Adams *et al.* 1998). Therefore, in the present study, one possibility is that treatment of the llama fetus with L-NAME may have affected the activity of PNMT directly, or indirectly secondary to a reduction in glucocorticoid synthesis, thereby favouring an increase in the ratio of noradrenaline:adrenaline concentrations in plasma during hypoxaemia. However, it is possible that this effect of cortisol may require a longer time course since its action is likely to be at the genomic level. Finally, one cannot exclude the possibility that treatment of the fetal llama with L-NAME may have an effect on the metabolic disposition of catecholamines. Therefore, modifications in plasma catecholamine concentrations could also reflect changes in catecholamines clearance (Esler *et al.* 1990).

In summary, the data reported in the present study support the hypothesis that NO has an important role in the regulation of adrenal blood flow and adrenal corticomedullary functions in the llama fetus during normoxaemia and hypoxaemia in the last third of gestation.

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