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Nitric oxide synthase activity in brain tissues from llama fetuses submitted to hypoxemia

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Abstract

The fetal llama (*Lama glama*; a species adapted to live in chronic hypoxia in the highlands of the Andes) did not increase cerebral blood flow and reduce the brain oxygen uptake during hypoxemia. Although nitric oxide (NO) is a normal mediator in the regulation of vascular tone and synaptic transmission, NO overproduction by hypoxemia could produce neuronal damage. We hypothesized that nitric oxide synthase (NOS) activity is either maintained or reduced in the central nervous system of the llama fetuses submitted to chronic hypoxemia. Approximately 85% of the Ca^{2+}-dependent NOS activity was soluble, at least 12% was associated with the mitochondrial fraction, and less than 5% remains associated with microsomes. To understand the role of NO in chronic hypoxemia, we determined the effect of 24-h hypoxemia on NOS activity in the central nervous system. No changes in activity or the subcellular distribution of NOS activity in brain tissues after hypoxemia were found. We proposed that the lack of changes in NOS activity in the llama under hypoxemia could be a cytoprotective mechanism inherent to the llama, against possible toxic effects of NO.
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1. Introduction

The umbilical vein Po_2 of fetuses of several species at sea level is 70% lower than maternal aortic Po_2, which is equivalent to Po_2 values obtained from the aorta in human climbers at very high altitudes (West et al., 1983). In their natural environment, the Andes mountains, llamas' Po_2 is lower than low land mammals. This peculiar hypoxemic condition has exerted upon the llama species (*Lama glama*) strong selective pressures allowing the fetal llama to develop mechanisms to cope with hypoxemia, as illustrated by the cardiovascular and metabolic responses to hypoxemia (Llanos et al., 1995; Giussani et al., 1996; Riquelme et al., 1997; Llanos et al., 2000). Compared to the fetal sheep, the llama fetus submitted to hypoxemia does not exhibit carotid and cerebral artery vasodilatation although it does present an intense peripheral vasoconstriction (Giussani et al., 1996). Therefore, cerebral blood flow does not increase and there is a marked reduction in femoral and carcass blood flow during hypoxemia (Llanos et al., 1995; Giussani et al., 1996). Contemporary with this response, cerebral oxygen delivery and consumption decrease from the very beginning of the hypoxemic insult (Llanos et al., 2000). To our knowledge, this is the only species whose fetuses do not protect their central nervous system by a substantial increase in the cerebral blood flow during hypoxemia (Llanos et al., 1995). Moreover, this is the only species whose fetuses do not maintain the cerebral oxygen consumption during an episode of moderate or severe hypoxemia (Riquelme et al., 1997; Llanos et al., 2000). Therefore, there is some basis to speculate that the llama fetus has defensive mechanisms in order to avoid hypoxemic neuronal damage. Among the mechanisms that could produce neuronal injury in hypoxemia is the overproduction of nitric oxide (NO) (Iadecola, 1997). Although NO is a normal mediator in the regulation of vascular tone and synaptic transmission, chronic hypoxemia induces an increase in either nitric oxide synthase (NOS) activity or mRNA expression in the cerebral tissue of sheep fetuses, a lowland species suggesting NO overproduction (Aguan et al., 1998). Furthermore, NOS blockade preserves neuronal integrity in fetuses and neonates of lowland species submitted to chronic hypoxemia (Malyshev et al., 1999). Gunn et al. (1992) examined the histologic and electrophysiologic changes associated with asphyxia due to uterine artery occlusion of varying duration in fetal sheep. They found that asphyxia for prolonged periods of up to 2 h might be accompanied by neuronal death. Their data support the suggestion that good cerebral perfusion is critical for surviving asphyxia without damage. Also neonatal mice lacking nNOS have been shown to be less vulnerable to acute hypoxic–ischemic injury (Ferriero et al., 1996). Both data suggest a role for NO in eliciting cerebral damage during hypoxemia.

Since there is no increase in cerebral blood flow and there is a marked reduction in cerebral oxygen consumption during hypoxemia in the fetal llama, we hypothesized that NOS activity is either maintained or reduced in the central nervous system of llama fetuses submitted to chronic hypoxemia.

2. Materials and methods

2.1. Use of animals

Time-dated pregnant llamas (10), were obtained from the University of Chile farm at the Rinconada de Maipú (580 m above sea level). Upon arrival to the laboratory in Santiago, also at 585 m above sea level, the llamas were housed in an open yard with access to food and water ad libitum and they were familiarized with the study metabolic cage and the laboratory conditions for 1–2 weeks prior to surgery.

2.2. Surgical preparation

Maternal surgery was carried out using well-established techniques previously described in detail (Benavides et al., 1989; Llanos et al., 1995). Briefly, maternal llamas were pre-medicated with atropine [1 mg intramuscular (i.m.) of atropine sulfate; Laboratorio Chile, Santiago, Chile]. Polyvinyl catheters (1.3 mm i.d.) were placed in the maternal descending aorta and inferior vena cava via a hindlimb artery and vein under light general anesthesia (ketamine, 10 mg kg^{-1} i.m.; Ketostop, Drug Pharma–Invetec, Santiago, Chile) with additional local infiltration of 2% lidocaine (dimecaine; Laboratorio Beta, Santiago, Chile). The catheters were then tunneled subcutaneously to exit the maternal flank. All animal care proce-

dures and experimentation were conducted in accordance with the *Guide for the Care and Use of Laboratory Animals* (1996, National Academy Press, Washington D.C., USA).

2.3. Experimental procedure

All experiments were based on a 25-h protocol: 1 h of normoxemia and 24 h of hypoxemia. Fetal hypoxemia was induced by placing a transparent polyethylene bag over the llama's head, into which known concentrations of O_2, N_2 and CO_2 (9% O_2 and 2–3% CO_2 in N_2) were flushed through at a rate of 50 $l \times min^{-1}$ to reduce the maternal Po_2 from ~96 mmHg to ~52 mmHg and hemoglobin saturation from ~97% to ~76%. This degree of maternal hypoxemia is known to reduce fetal descending aortic Po_2 and hemoglobin saturation to ~15mm Hg and to <25%, respectively, without altering fetal Pco_2 (Llanos et al., 1995; Giussani et al., 1996). Five fetuses were used in the chronic hypoxemic group, and five used as controls.

Arterial blood samples (0.5 ml) were taken from the mother after 30 and 60 min of normoxemia, at 30 min intervals during the hypoxemic period, in heparinized syringes. Arterial pH, Po_2 and Pco_2 were measured with a BMS 3Mk2 Blood Microsystem and PHM 73/Blood gas Monitor (Radiometer, Copenhagen, Denmark) and corrected for body temperature, i.e. 39°C. Hemoglobin saturation and hemoglobin concentration were measured with an OSM2 Hemoximeter (Radiometer, Copenhagen, Denmark). Maternal arterial and venous pressures, and heart rate were recorded continuously throughout the experiment (Statham pressure transducers, Hato Rey, Puerto Rico) and the signal displayed on a polygraph (Gilson ICM-5, Emeryville, CA). Perfusion pressure was calculated as mean systemic arterial blood pressure minus central venous pressure.

On completion of the experiments, the llama was anesthetized with intravenous thiopenthone sodium (1 g of sodium thiopental; Laboratorio Biosano SA, Santiago, Chile), and euthanized using saturated potassium chloride injected intravenously. Fetal cerebral hemispheres and cerebellum were quickly removed and frozen in liquid N_2 and stored at −80°C. The frozen tissues were pulverized in liquid N_2 and aliquots were taken to perform the subcellular fractionation.

2.4. Subcellular fractionation of NOS in cerebral tissues

Tissue aliquots between 1 and 2 g were homogenized in four volumes of cold Tris buffer containing 50 mM Tris–HCl, 0.1 mM EDTA, 0.1 mM EGTA, 12 mM 2-mercaptoethanol, 2 μM leupeptin, 1 μM pepstatin and 1 μM phenylmethylsulfonyl fluoride (Figueroa and Massmann, 1995). The homogenate was prepare using three 15-s bursts in an Ultra Turrax homogenizer set at 13 500 rev./min (Janke and Kunkel, IKA-Labortecnik, Staufen, Germany). After a low speed spin (800 × *g*) at 4°C for 15 min to eliminate tissue debris, the supernatant corresponding to crude fraction was subjected to differential centrifugation at 10 000 × *g* for 20 min to obtain a pellet corresponding to the mitochondrial fraction (P10K). The 10 K supernatant was centrifuged at 100 000 × *g* for 1 h to obtain the pellet corresponding to the microsomal fraction (P100K) and the supernatant corresponded to the cytosol (S100K). All the pellets were resuspended in buffer by sonication. Subcellular fractions of the cortex and cerebellum were frozen and stored for analysis at a later date.

Protein determination was measured according to the method of Bradford (1976).

2.5. NOS activity measurements.

NOS activity was determined by the conversion of arginine to citrulline using [^{3}H]-arginine (NEN, Boston, USA) in a medium containing: 1 mM NADPH, 1.25 mM $CaCl_2$, 20 μM FMN, 20 μM FAD and 10 μM BH_4 in 40 mM Tris–HCl pH 7.4 (Figueroa and Massmann, 1995). To establish the reaction specificity the following inhibitors were used: 0.8 mM L-NAME, 0.5-mM trifluoperazine, or 60 μM EGTA plus 340 μM EDTA. The reaction was carried at 30°C for 45 min and stopped with 1.8 ml of 30 mM HEPES (pH 5.5) with 3 mM EDTA. Separation of arginine from citrulline was performed by ion exchange chromatography with 1 ml of Dowex resin (50X8-400). The eluted fraction containing the [^{3}H]-citrulline was collected in 20-ml scintillation vials and 9 ml of scintillation fluid (Bioscint, Atlanta, Georgia, USA) were added. Optimization of the assay conditions for NOS activity was determined using pooled samples of crude fractions. To obtain full activity in the tissues tested it required the addi-

tion of NADPH, BH_4, FAD, FMN and Ca^{2+}. NOS activity decays after consecutive cycles of freezing and thawing, therefore we measured NOS activity in samples thawed only once. We used a high concentration of non-radioactive arginine of 25 μM. Increasing the NADPH concentration over 1 mM in the medium did not significantly increase the activity. Citrulline production was linear in the protein range used for the analysis.

2.6. Lactic and glutamic dehydrogenase activities measurements

LDH (cytosolic marker) and GDH (mitochondrial marker) activities were determined according to Leighton et al. (1968) and Schmidt and Schmidt (1987).

2.7. Immunowestern blot

Proteins from different cellular compartments were separated according to the method of Laemmli using 8.0% sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were transferred to PVDF membranes (Immobilon® Millipore, Marlborough, MA, USA) by electro-blotting. Samples containing between 10 and 50 μg of protein/lane and uniformity of loading confirm with Ponceau S staining of the membranes. The blot was incubated overnight at 4°C in blocking solution made of 6% dry non-fat milk in TBS-T buffer (20 mM Tris–HCl, 137 mM NaCl and 0.05% Tween 20, pH 7.6). After rinsing with TBS-T buffer, the membrane was incubated for 2 h either with anti-nNOS or anti-eNOS monoclonal antibody (Transduction Laboratories, Kentucky, USA; diluted 1:1000 in 6% dry non-fat milk in TBS-T buffer). After rinsing with TBS-T buffer, the blot was incubated for 1 h with horseradish peroxidase-conjugated secondary antibody (Transduction Laboratories, Kentucky, USA; diluted 1:2000 in 6% dry non-fat milk in TBS-T buffer). Positive reaction was identified with enhanced chemiluminescence (super signal west pico chemiluminescent substrate; Pierce, IL, USA), and relative intensity was normalized by loading equal protein amounts, and by the intensity of a standard preparation of llama cerebellum run in each gel. All bands were scanned to determine optical density, and quantified by using a computational program Uni-Scan-it.

Detection of von Willebrand factor as an endothelial marker (Abbott et al., 1992) was performed similarly, but using a polyclonal antibody (Sigma Chemical Co., St Louis, MO, USA; diluted 1:500) and an anti-rabbit IgG conjugated with peroxidase as a secondary antibody (Sigma Chemical Co., St. Louis, MO, USA; diluted 1:500). The presence of the protein was assessed by the appearance of a colored product using 4-chloro-1-naphtol dissolved in pure cold methanol 0.05% (mass/vol.) and H_2O_2 (0.05%) as substrates. Samples used contained 60 μg of crude, P10K, P100K and S100K of cerebellum and cerebral cortex, and a similar protein concentration of a standard preparation of llama pulmonary artery homogenate.

2.8. Statistical analysis

Values are expressed as means ± S.E.M. Changes in the measured variables between normoxemia and hypoxemia were tested for statistical significance using two-sample Student's *t*-test. A difference was considered significant when the *P* value was less than 0.05. Changes either in normoxemia or hypoxemia between the tissues studied were tested using one-way ANOVA, followed by a Newman–Keuls test (Zar, 1984).

3. Results

The ten fetal llamas had a mean weight of 2979 ± 1516 g, means ± S.E.M., which corresponds to 54–76% of gestation (gestational age approx. 350 days, where term is 7000–8000 g) (Fowler, 1989).

3.1. Maternal cardiorespiratory variables

Maternal blood gases and acid–base values during the experimental protocol are shown in Table 1. During the chronic hypoxemia, there was a reduction in maternal P_{O_2} and hemoglobin saturation in all mothers, without significant changes in pH, P_{CO_2} and hemoglobin concentration. There were no significant changes in the systemic perfusion pressure during the experimental protocol. A tachycardia was observed at the beginning of the hypoxemic period, but it came back to basal levels within 1 h.

Table 1
Maternal cardiorespiratory variables[a]

	Basal	1–12 h	13–24 h
pH	7.419 ± 0.017	7.412 ± 0.022	7.394 ± 0.019
Po_2 mmHg	98.43 ± 2.65	51.80 ± 0.40*	51.76 ± 0.09*
Pco_2 mmHg	32.16 ± 1.52	32.94 ± 1.43	34.12 ± 1.68
Hemoglobin g/dl	12.66 ± 0.66	12.56 ± 0.49	13.10 ± 0.59
Hemoglobin saturation (%)	97.42 ± 0.88	88.18 ± 0.35*	87.84 ± 0.60*

[a]Maternal pH, blood gases, hemoglobin and hemoglobin saturation measurements during a 24-h period. Data correspond to mean ± S.E.M.
*$P < 0.05$ vs. basal.

3.2. NOS activity in crude fraction of brain tissues under normoxemia and hypoxemia

NOS activity in the crude fraction of cerebral cortex was statistically significant higher than that of cerebellum, both in normoxemic and hypoxemic conditions (Fig. 1). Enzymatic activity was absolutely Ca^{2+}-dependent as illustrated by the marked inhibition when replacing $CaCl_2$ by 2.05 mM $MgCl_2$ and the addition of 0.15 mM EGTA and 0.9 mM EDTA. The calmodulin antagonist trifluoperazine was used to study the calmodulin dependence of the enzyme. At a concentration of 0.5 mM, the antagonist reduced completely the activity in both tissues studied, and its effect was reverted by the addition of 50 U/ml of calmodulin.

To confirm that the citrulline measured was produced by only the catalysis of nitric oxide synthase, we used the competitive NOS inhibitor L-NAME, an arginine analogue. The inhibition by this analogue was concentration-dependent, and we determined that a concentration of 1.0 mM completely suppressed the activity present in cerebral cortex and cerebellum.

Studies under hypoxemic conditions indicate that the reduction in the Po_2 did not affect the NOS activity in these fetal llama tissues (Fig. 1).

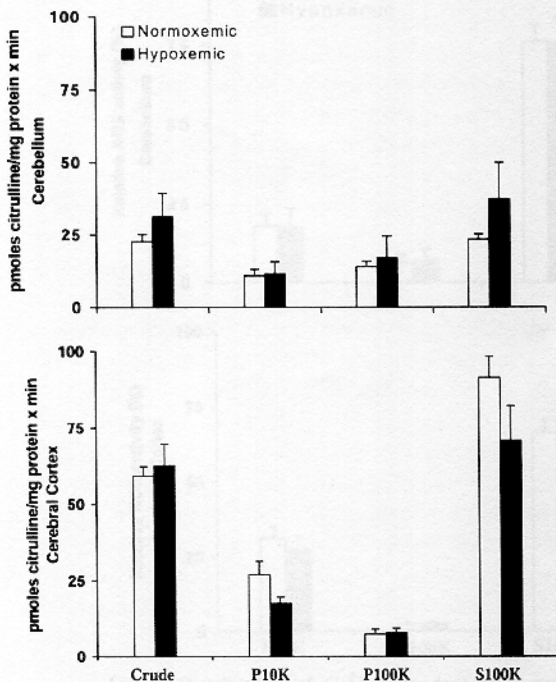

Fig. 1. Specific activity of Ca^{2+}-dependent NOS activity in the different subcellular fractions obtained from llama brain tissues under normoxemic ($n = 5$) and hypoxemic ($n = 5$) conditions. Values correspond to means ± S.E.M.

3.3. Subcellular distribution of NOS activity in fetal brain tissues under normoxemia and hypoxemia

Differential centrifugation of crude fraction from cerebral cortex and cerebellum of fetal llama under normoxemia and hypoxemia conditions revealed that the larger proportion of Ca^{2+}-dependent NOS activity was present in the soluble fraction (Fig. 2). Analysis of the NO synthase distribution among the various subcellular fractions showed that the specific activity of the S100K fraction from cerebellum was approximately two times higher compared with the mitochondrial and microsomal fractions. Whereas the soluble fraction isolated from the cerebral cortex was approximately 3.5–12-fold the specific activity determined in P10K and P100K fractions, respectively (Fig. 1). Hypoxemia did not produce any change either in the subcellular distribution or in the specific and total activity present in both.

Nearly 20% of the total NOS activity was found in the mitochondrial fraction of both brain tissues (Fig. 2). The larger proportion of GDH activity

found in the cerebellum would be attributed to the soluble forms of GDH report in bovine brain (Cho et al., 1995). We showed that these tissues were enriched in the mitochondrial marker GDH (Schmidt and Schmidt, 1987; Table 2). However, not all this activity is associated with the mitochondria because we also found between 7 and 10% of contamination with the cytosol deduced from the LDH activity (cytoplasmatic enzymatic marker) present in this subcellular fraction (Table 2). Thus the percentage of non-cytosolic NOS activity associated with both the mitochondrial fractions was at least 12% (Table 2).

Taking into consideration the observed contamination of the mitochondrial and microsomal fractions with LDH, close to 85% of the NOS activity corresponded to a soluble protein, and less than 5% was associated with the microsomal fraction (P100K).

3.4. Immunowestern blot analysis of NOS

We detected the presence of nNOS in all subcellular fractions of fetal cerebellum (Fig. 3). Since nNOS in the S100K represents almost 85% of fetal NOS activity, we analyzed only this subcellular compartment by western blot. The immunoreactive bands observed exhibit the same molecular weight of 160 kDa as the manufacturer positive control (rat pituitary, data not shown). To perform the quantification of the bands by densitometric analysis we used, as a control, a crude fraction of llama fetus cerebellum. The densitometric analysis of the 160-kDa positive band observed in normoxemic and hypoxemic conditions, expressed as a mean of arbitrary units ± S.E.M, gave the following values: 0.70 ± 0.24 and 0.77 ± 0.40, respectively, indicating no statistically significant differences between the two groups studied. In this subcellular compartment both eNOS and von Willebrand factor were not detected.

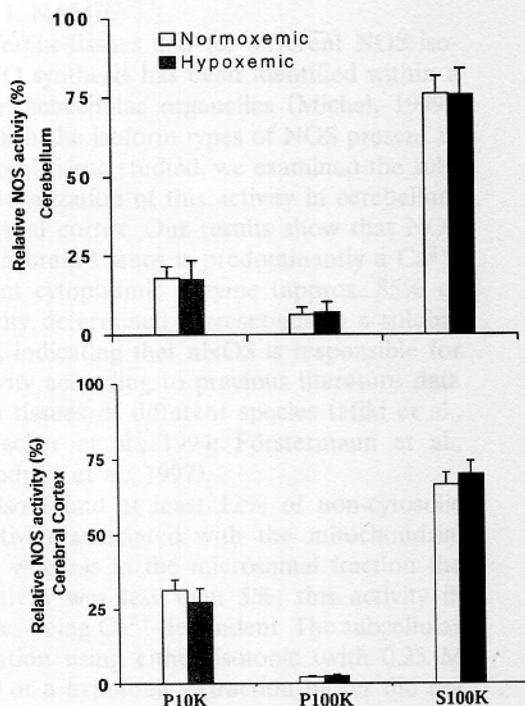

Fig. 2. Distribution of Ca^{2+}-dependent NOS activity in the different subcellular fractions from fetal llama brain tissues under normoxemic ($n = 5$) and hypoxemic ($n = 5$) conditions. A: cerebellum; B: cerebral cortex. Values correspond to means ± S.E.M.

Table 2
Relative activity of some subcellular fraction markers from cerebellar and brain fetal tissues[a]

	Subcellular fractions		
	P10K	P100K	S100K
	Enzymatic activity (%)		
Cerebellum			
GDH	56.4 ± 8.4	6.2 ± 0.9	37.4 ± 9.4
LDH	10.7 ± 4.1	5.5 ± 3.0	83.9 ± 6.8
NOS	18.6 ± 5.5	6.8 ± 3.4	76.4 ± 6.6
Non-cytosolic NOS	12.2 ± 3.5[b]	4.9 ± 2.0[b]	–
Cytosolic NOS	–	–	82.9 ± 2.4[c]
Cerebral cortex			
GDH	72.8 ± 1.5	4.6 ± 3.6	22.6 ± 2.1
LDH	7.0 ± 1.5	2.5 ± 0.7	88.5 ± 5.4
NOS	18.9 ± 4.5	5.6 ± 2.7	74.6 ± 9.3
Non-cytosolic NOS	11.9 ± 3.7[b]	3.3 ± 2.5[b]	–
Cytosolic NOS	–	–	84.8 ± 4.5[c]

[a] Data correspond to an average of three independent samples ± S.E.M. of cerebellum and cerebral cortex.
[b] Percentage of NOS determined in the fraction of each sample minus the percentage of LDH contamination in the same fraction.
[c] Percentage of total soluble NOS including the activity found contaminating both the mitochondrial and microsomal fractions.

4. Discussion

The results from this study were consistent with the hypothesis that NOS activity is either main-

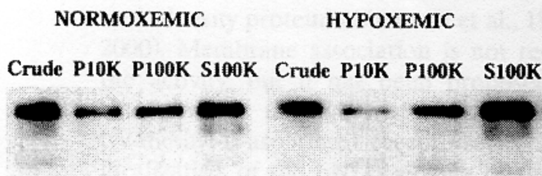

Fig. 3. Immunowestern blot analysis of nNOS in the different subcellular fractions of cerebellum of normoxemic (N) and hypoxemic (H) llama fetuses.

tained or reduced in the central nervous system of llama fetuses submitted to chronic hypoxemia, because NOS activity did not change with chronic hypoxemia (24 h) in the two tissues studied. We found differences in NOS activity in the cerebral cortex and in the cerebellum, being significantly higher in the cerebral cortex of the fetal llama.

This is the first report on NOS activity, measured as citrulline generation from arginine, in brain tissues obtained from fetal llama. The production of citrulline was the result of a constitutive NOS activity deduced from the NADPH, Ca^{2+}; calmodulin-dependence and the inhibitory effect of L-NAME.

In different tissues and for different NOS isoforms, NO synthesis has been identified within a variety of subcellular organelles (Michel, 1999). To establish the isoform types of NOS present in the cerebral tissue studied, we examined the subcellular localization of this activity in cerebellum and cerebral cortex. Our results show that NOS activity in brain tissues is predominantly a Ca^{2+}-dependent cytoplasmic enzyme (approx. 85% of the activity determined corresponds to a soluble enzyme), indicating that nNOS is responsible for this activity according to previous literature data on brain tissues of different species (Hiki et al., 1992; Hecker et al., 1994; Förstermann et al., 1998; Rodrigo et al., 1997).

We also found at least 12% of non-cytosolic NOS activity associated with the mitochondrial fraction, whereas in the microsomal fraction the NOS activity was less than 5%; this activity in both cases being Ca^{2+}-dependent. The subcellular fractionation using either isotonic (with 0.25 M sucrose) or a hypotonic extraction buffer did not significantly change the NOS activity associated with the mitochondrial fraction.

Recent reports have demonstrated the existence of a novel Ca^{2+}-dependent NOS subtype, mtNOS bound to the inner membrane of rat liver mitochondria with some similarities to the inducible isoform (iNOS), like tight association to calmodulin and antigenicity (Tatoyan and Giulivi, 1998). However, it is constitutively expressed, and it is Ca^{2+}-dependent. A recent report showed the existence of this mtNOS also in rat brains, considered as a variant of NOS I that could be the result of alternative splicing or post-translocational cleavage of the N-terminal (Riobó et al., 2000). This enzyme showed a different expression pattern during the rat development, only mtNOS was detected in the particulate fraction during postnatal days 0–4, while from day 6 up to the adult stage, the nNOS was co-expressed with the mtNOS (Riobó et al., 2000).

These antecedents made it necessary to investigate the type of NOS activity associated with the three fractions separated by differential centrifugation. Antibodies against nNOS protein (known to recognize enzymes from human, rat, mouse and dog origin) exhibited cross-reactivity with the four fractions studied (crude, mitochondrial, microsomal and cytosolic). Although our results suggest that NOS activity in the P10K has similarities to the already reported mtNOS found in rat liver and brain, i.e. to be Ca^{2+}-dependent, we found they immunoreact with monoclonal antibodies against nNOS.

The absence of Ca^{2+}-independent NOS activity makes us assume that the iNOS is not present at detectable amounts in both brain tissues studied.

In mammals, the nNOS and the eNOS were initially considered constitutively expressed and the activity was regulated by Ca^{2+}-calmodulin. However, recent evidence suggests that the expression of both of them can also be regulated under various circumstances (Förstermann et al., 1998). Neuronal NOS expression can be regulated by several physical, chemical and biological agents. Physical stimuli like hypoxia produce an up-regulation of this enzyme in the central nervous system of lowland rats (Prabhakar et al., 1996; Guo et al., 1997) and fetal sheep (Aguan et al., 1998), and also in lungs and culture cells (Shaul et al., 1995; North et al., 1996). The small proportion of nNOS associated with membranes compared with the soluble, can be explained by the already described large number of alternatively spliced forms of nNOS transcripts (Elliasson et al., 1997). Membrane association of nNOS in neurons appears to be mediated by the PDZ domain and interactions with proteins such as PSD-95 and 93 (postsyn-

aptic density protein) (Elliasson et al., 1997; Kone, 2000). Membrane association is not required for the activity, but it has been proposed that the activation of nNOS by stimulation of NMDA (*N*-methyl-D-aspartate) receptors depend on the localization of the nNOS vicinity of these receptors (Bolaños and Almeida, 1999).

Our results showed that hypoxemia did not produce an effect either on the NOS activity or an alteration of its intracellular localization in brain tissues of the llama fetus. This could be a unique characteristic of the llama fetus adapted to live over 4000 m above sea level. This proposal agrees with studies performed on piglets that showed that chronic exposure to hypoxia prevented changes in the eNOS activity, indicating an adaptation to this condition (Hislop et al., 1997). The adaptive response of fetal llama could be a neuroprotective mechanism to prevent the neurotoxic effect (oxidative damage) produced by the formation of peroxynitrite from NO and superoxide anions (Iadecola, 1997; Bolaños and Almeida, 1999; Andrew and Mayer, 1999). Peroxynitrite is a potent oxidant, which can decompose to the hydroxyl and nitrogen free radicals, being relevant in ischemia damage (Bolaños and Almeida, 1999). The neurotoxic effects of NO and peroxynitrite can be mediated by DNA damage, and decrease in brain energy metabolism by the inhibition of the mitochondrial respiration (Bolaños et al., 1998; Clementi et al., 1998; Giulivi, 1998; Sharpe and Cooper, 1998; López-Figueroa et al., 2000). Although NO may regulate cell respiration physiologically by its action on complex IV of the mitochondria, long-term exposure to NO leads to persistent inhibition of complex I due to its *S*-nitrosylation (Clementi et al., 1998), and could be potentially harmful for the brain function. The production of NO by mitochondria catalyzed by a mitochondrial enzyme has been proposed as a novel regulatory process designated to modulate cellular ATP production (Tatoyan and Giulivi, 1998). Thus it could be plausible that NOS targeted to mitochondria in a stress condition could play a role in the modulation of oxidative phosphorylation (Michel and Feron, 1997). However, our data showed that 24 h of hypoxemia did not produce any change in subcellular distribution of the NO synthases present in the brain of the llama fetus.

Previous work in our laboratory demonstrated that fetal cerebral blood flow was maintained and cerebral oxygen uptake decreased during hypoxemia, suggesting that these cardiovascular responses are an adaptive response in the llama fetus (Llanos et al., 1995, 1998, 2000). Whether the decreased brain oxygen uptake could be in part due to the NO production by the synthase activity present in the brain mitochondria remains to be investigated.

In summary, our data support the hypothesis that a period of 24 h of hypoxemia (with similar Po_2 values found at 4000 m of altitude) of the mother does not produce significant changes in NOS activity in cerebral cortex and cerebellum of llama fetus. This finding is consistent with the adaptation of this species to live at the high plateau of the Andes (4000–5000 m). The lack of susceptibility of NOS to hypoxemia could be a neuroprotective mechanism to prevent the possible negative neurological effects of hypoxemia.

Acknowledgements

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