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Mitochondrial function in rat cerebral cortex and hippocampus after short- and long-term hypobaric hypoxia

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ABSTRACT

Taking into account the importance of aerobic metabolism in brain, the aim of the present work was to evaluate mitochondrial function in cerebral cortex and hippocampus in a model of sustained hypobaric hypoxia (5000 m simulated altitude) during a short (1 mo) and a long (7 mo) term period, in order to precise the mechanisms involved in hypoxia acclimatization.

Hippocampal mitochondria from rats exposed to short-term hypobaric hypoxia showed lower respiratory rates than controls in both states 4 (45%) and 3 (41%), and increased NO production (1.3 fold) as well as eNOS and nNOS expression associated to mitochondrial membranes, whereas mitochondrial membrane potential decreased (7%). No significant changes were observed in cortical mitochondria after 1 mo hypobaric hypoxia in any of the mitochondrial functionality parameters evaluated.

After 7 mo hypobaric hypoxia, oxygen consumption was unchanged as compared with control animals both in hippocampal and cortical mitochondria, but mitochondrial membrane potential decreased by 16% and 8% in hippocampus and cortex respectively. Also, long-term hypobaric hypoxia induced an increase in hippocampal NO production (0.7 fold) and in eNOS expression. A clear tendency to decrease in H_2O_2 production was observed in both tissues.

Results suggest that after exposure to hypobaric hypoxia, hippocampal mitochondria display different responses than cortical mitochondria. Also, the mechanisms responsible

Abbreviations: $DiOC_6$, 3, 3''-dihexyloxacarbocyanine iodide; EDTA, ethylenediaminetetraacetic acid; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; HRP, horseradish peroxidase; H_2O_2, hydrogen peroxide; $^{\bullet}NO$, nitric oxide; ROS, reactive oxygen species; SOD, superoxide dismutase

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for acclimatization to hypoxia would be time-dependent, according to the physiological functions of the brain studied areas. Nitric oxide metabolism and membrane potential changes would be involved as self-protective mechanisms in high altitude environment.

1. Introduction

The mechanisms of adaptation to low O_2 pressures have been a puzzle for many years. The well-known systemic responses are not energetically economic mechanisms, can only buffer but not prevent the fall in mixed venous blood PO_2 below normal limits, and are mostly absent or attenuated in organisms genotypically adapted to high altitude, suggesting that complete adaptation would take place at cellular level, mainly through changes in O_2 utilization by mitochondria. However, although *in vivo* oxidative functions are O_2 dependent at relatively high arterial concentrations, affinity of isolated mitochondria for O_2 was reported to be too high to be involved in the control of O_2 consumption, and measurements of mitochondrial respiration and oxidative phosphorylation, as well as the activity of respiratory complexes, were mostly unchanged in animals acclimatized to hypoxia (Costa, 2007). The finding that nitric oxide ($^{\bullet}$NO) has an essential role as a physiological regulator of the respiratory chain provides a hypothesis to fill the gap between the observations previously mentioned. The $^{\bullet}$NO-inhibited respiration lowers the steepness of intracellular O_2 gradients and allows O_2 to diffuse further along its gradient, extending the space of adequate tissue oxygenation away from the blood vessel. Endogenous$^{\bullet}$NO production has been shown to inhibits tissue O_2 consumption in hippocampal slices at physiological O_2 concentration, strongly supporting the current paradigm for O_2 and $^{\bullet}$NO interplay in the regulation of cellular respiration (Ledo et al., 2010).

Previous studies of our research group showed that nitric oxide synthase (NOS) was modulated in heart mitochondria during acclimatization to hypobaric hypoxia in association with cardioprotection (La Padula et al., 2008). Brain is particularly sensitive to oxygen deficiency. Morphological changes in the hippocampus have been observed by Hale et al. in hypobaric hypoxia (Shukitt-Hale et al., 1996). Hypoxia adaptative responses such as metabolic reprogramming and reactive oxygen species (ROS) neutralization depends on the different brain regions and cell types (Van Elzen et al., 2010). Cerebral cortex exposed to acute hypobaric hypoxia (3, 6, 12 and 24 h) is less vulnerable to hypoxia than hippocampus due to its high content of antioxidant enzymes (Sharma et al., 2013). Maiti and colleagues (2006) reported that hippocampus and striatum are more susceptible to hypoxia than cerebral cortex after animal exposure for three or seven days to a simulated altitude of 6100 m, showing increased free radicals production, $^{\bullet}$NO levels, lipid peroxidation and decreased amount of antioxidant defenses (Maiti et al., 2006). Furthermore, the level of oxidative stress is time-dependent. Exposure to a simulated altitude of 6100 m during 14 days was found to reduce the oxidative stress in rat hippocampus when compared to seven days of exposure (Hota et al., 2007).

Mitochondrial oxygen consumption is required for ATP generation, and cell survival is threatened when cells are deprived of oxygen. Due to the fact that mitochondria represent the final step of the interaction with molecular oxygen, they are able to sense the concentration gradient of oxygen arriving from the environment (Guzy and Schumacker, 2006; Lukyanova, 2013). Then, the mitochondrial respiratory chain acts as a signal-transforming metabolic system which activates the functional response to hypoxia.

Previous data from hypobaric hypoxia models have shown enhanced $^{\bullet}$NO production with the implication of nNOS in the CNS. Acute hypobaric hypoxia has been demonstrated to increase nNOS mRNA expression in Purkinje cells (Prabhakar et al., 1996) and $^{\bullet}$NO levels production in extracts from whole brain (Malyshev et al., 1999), in the supraoptic and paraventricular nuclei (Luo et al., 2000) and in cerebellum (Serrano et al., 2003).

Studies in chronic hypoxic conditions compatible with acclimatization are scarce, even though clinically relevant, because adaptation to hypoxia renders protection to nerve cells of brain (Goryacheva et al., 2010; Manukhina et al., 2008; Mashina et al., 2006). Taking into account the importance of mitochondria in oxygen homeostasis, the aim of the present work was to evaluate mitochondrial function in cerebral cortex and hippocampus in a model of sustained hypobaric hypoxia (1 and 7 months) that simulates high-altitude (5000 m), in order to precise the mechanisms involved in hypoxia acclimatization.

2. Results

2.1. *Oxygen consumption*

Malate-glutamate dependent oxygen consumption was measured in state 4 (resting or controlled respiration) and in state 3 (active respiration, the maximal physiological rate of O_2 uptake and ATP synthesis) (Boveris et al., 1999). The respiratory control ratio (the most sensitive indicator of mitochondrial oxidative phosphorylation coupling) was calculated as the relationship between state 3/state 4 respiration rates.

Table 1 shows oxygen consumption rates of cortical and hippocampal intact mitochondria isolated from different experimental groups. Hippocampal mitochondria from animals exposed to short-term hypobaric hypoxia showed lower respiratory rates than controls, 45% in state 4 ($p<0.05$) and 41% in state 3 ($p<0.05$). No significant changes were observed in state 4 and state 3 respiratory rates in cortical mitochondria. Respiratory control of cortical and hippocampal mitochondria from 1 mo hypoxic rats were similar to control

Table 1 – Mitochondrial oxygen consumption in rats submitted to 53.8 kPa and their controls at 101.3 kPa.

	Short-term hypobaric hypoxia				Long-term hypobaric hypoxia			
	Cortex		Hippocampus		Cortex		Hippocampus	
Oxygen consumption (natgO/min.mg protein)	Control	Hypoxia	Control	Hypoxia	Control	Hypoxia	Control	Hypoxia
State 4	14±2	14±1	10.3±0.1	5.5±0.5*	16±1	18±5	8±3	9±3
State 3	47±2	49±3	27±3	16±1*	55±1	45±5	31±3	29±4
Respiratory Control	3.4±0.1	3.5±0.1	2.6±0.1	2.9±0.1	3.5±0.3	2.9±0.6	3.9±1.1	3.2±0.5

Values are mean±SEM;
* $p<0.05$ vs. control.

values, indicating the preservation of the mitochondrial function.

State 4 and state 3 respiratory rates, as well as respiratory control values were not affected by long-term hypoxia (7 mo) in either of the brain studied areas.

2.2. *Hydrogen peroxide production*

Hydrogen peroxide production from cerebral cortex and hippocampus was measured in intact mitochondria from 1 mo and 7 mo hypoxic animals and their respective controls. Cortical mitochondria from 1 mo hypoxic animals showed hydrogen peroxide production rates of 0.91±0.08 nmol/min.mg protein. No significant changes were observed in H_2O_2 production in hypoxic animals as compared with controls (0.88±0.05 nmol/min.mg protein). Hydrogen peroxide production of hippocampal mitochondria showed a tendency to decrease (14%) in hypoxic rats (0.97±0.08 vs. 1.13±0.08 nmol/min.mg protein in controls). A tendency to decrease by 20% in hippocampal and by 12% in cortical mitochondria of rats exposed to high altitude for 7 mo was also observed.

2.3. *Mitochondrial membrane potential*

Mitochondrial potential is an important parameter of mitochondrial function. Intact mitochondria isolated from different brain areas of normoxic and hypoxic animals were used after loading with the potentiometric probe $DiOC_6$ to determine membrane potential by flow cytometry.

The gated mitochondrial population (R1) and the autofluorescence level of unloaded samples are shown for cortex and hippocampus from 1 mo hypoxic animals in Fig. 1A. As described in the histograms, high altitude conditions induced a decrease in FL-1-$DiOC_6$ fluorescence, indicating mitochondrial depolarization in hippocampal mitochondria. Quantification of $DiOC_6$ fluorescence in the different conditions is presented in Fig. 1C. High altitude did not disturb cortical mitochondrial polarization but induced hippocampal mitochondrial depolarization by 7% ($p<0.05$), as compared with control mitochondria. As expected, high depolarization was detected in mitochondria from both brain studied areas after 0.5 μM FCCP addition (Fig. 1C).

The effect of 7 months of hypoxia on cortical and hippocampal mitochondrial membrane potential is shown in Fig. 1B. Autofluorescence from unloaded mitochondria is presented at the insets. After 5000 m simulated altitude, a significant decrease in FL-1-$DiOC_6$ fluorescence was observed in both brain areas as observed in the histograms, indicating mitochondrial depolarization. Statistical values of the relative fluorescence intensity of cortical and hippocampal mitochondria from control and treated animals are presented in Fig. 1D. Animals exposed for seven months to hypobaric conditions showed decreased cortical and hippocampal mitochondrial polarization by 8% ($p<0.01$) and 16% ($p<0.001$), respectively, as compared with mitochondria from control animals. Mitochondrial pre-treatment with FCCP was able to induce a strong depolarization in both brain areas.

2.4. *Nitric oxide metabolism*

2.4.1. *eNOS and nNOS expression*

Endothelial and neuronal nitric oxide synthase expression in cortical and hippocampal mitochondria from control and 1 mo hypoxic animals is presented in Fig. 2A. Increased eNOS and nNOS expression was clearly observed in hippocampal mitochondria from 1 mo hypoxic animals as compared with control mitochondria. Ratios of eNOS or nNOS/VDAC were calculated by band quantification. One month of hypoxic acclimatization significantly increased eNOS/VDAC and nNOS/VDAC ratios by 1.5 and 0.5 fold respectively in hippocampal mitochondria ($p<0.01$). However, no significant changes in eNOS/VDAC and nNOS/VDAC ratios were observed in cortical mitochondria.

Fig. 2B shows eNOS and nNOS expression in cortical and hippocampal mitochondria from control and 7 mo hypoxic animals. Hippocampal eNOS/VDAC ratio was increased by 1.4 fold in hypoxia. No significant changes were observed in nNOS/VDAC ratio after 7 mo of hypoxic acclimatization, as compared with control values. Furthermore, long-term hypoxic conditions did not modify eNOS/VDAC and nNOS/VDAC ratios in cortical mitochondria.

2.4.2. *Nitric oxide production*

Nitric oxide production was measured in submitochondrial membranes from cerebral cortex and hippocampus after short- and long-term hypobaric hypoxia. As described in Fig. 3, no significant changes were observed in cortical $^{\bullet}NO$ levels for both exposure times to hypobaric conditions. However, 1 mo (Fig. 3A) and 7 mo (Fig. 3B) hypobaric hypoxia significantly increase hippocampal $^{\bullet}NO$ production

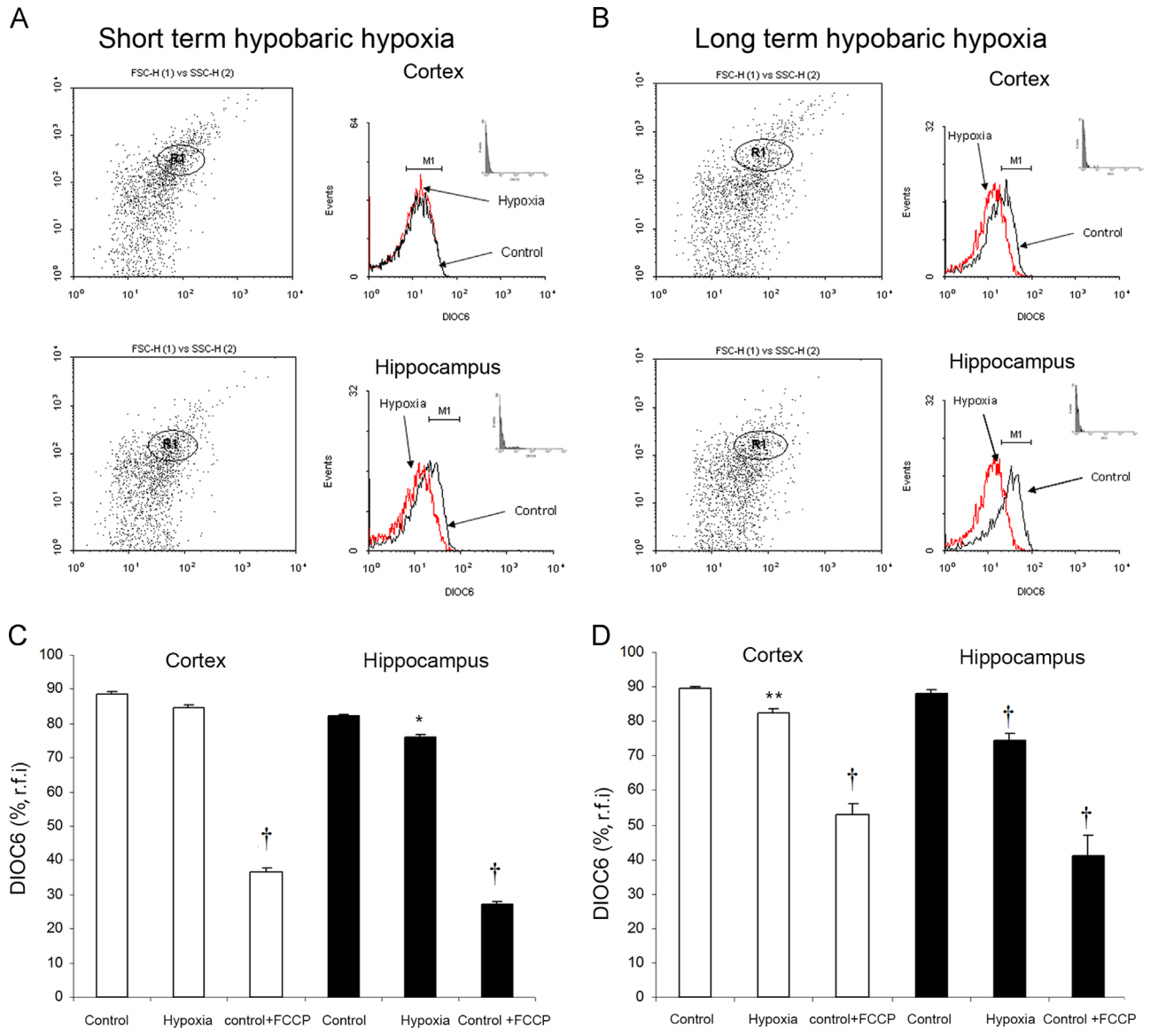

Fig. 1 – Evaluation of $\Delta\Psi_m$ through the changes in $DiOC_6$ fluorescence intensity.
Histograms of cortical and hippocampal gated mitochondrial events (R1) versus relative fluorescence intensity (FL-1) from 1 month (A) and 7 months (B) hypoxic animals and its respective controls. Assessment of $\Delta\Psi_m$ was performed as described in *Materials and Methods*. Each histogram represents a typical experiment out of three. Autofluorescence was evaluated without probe (insets). Quantification of $DiOC_6$ fluorescence corresponding to mitochondrial $DiOC_6$ relative fluorescence intensity (%) of cortical and hippocampal mitochondria from 1 month (C) and 7 months (D) hypoxic and control animals. Bars represent the mean ± SEM. (*$p < 0.05$; **$p < 0.01$, †$p < 0.001$ as compared with control value).

by approximately 1.3-fold and 0.7-fold, respectively, as compared with control values.

3. Discussion

Adaptation to hypoxia was early recognized to imply an increased resistance of the cells themselves, leading to an enhanced resistance to hypoxia and other stressors. It is now appreciated that all cells in the organism sense O_2 concentration and respond to hypoxia, activating adaptive processes that will improve the likelihood of survival.

In isolated papillary muscle, an increase in •NO production would be involved in the mechanisms of protection to an acute hypoxia/reoxygenation episode developed during the acclimatization of rats to hypobaric hypoxia, probably through the effect of •NO on mitochondrial function (La Padula and Costa, 2005; La Padula et al., 2008; Zaobornyj et al., 2005). Taking into account that the brain is particularly sensitive to oxygen deficiency and that similar mechanisms to those described for heart could be involved in brain acclimatization; in the present study we analyzed the effects of chronic sustained hypobaric hypoxia on mitochondrial parameters in cerebral cortex and hippocampus, at short- and long-term acclimatization.

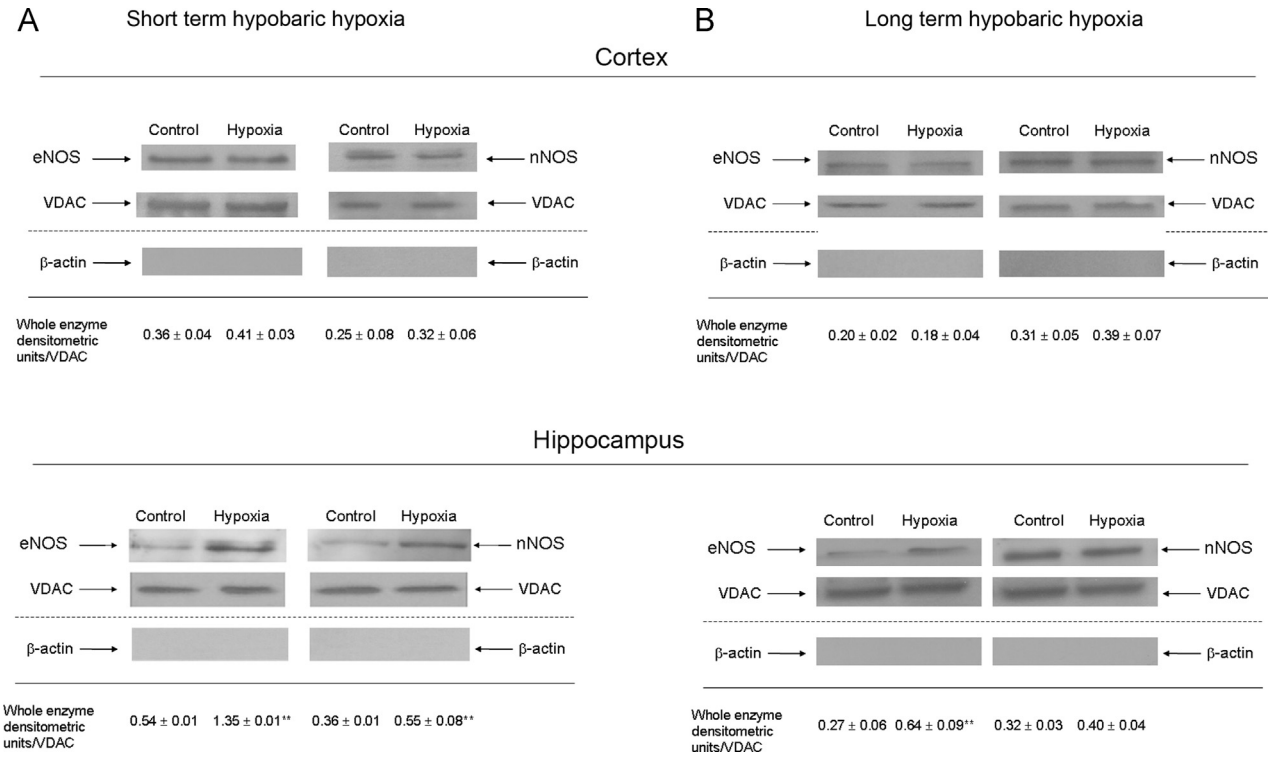

Fig. 2 – Endothelial and neuronal nitric oxide synthases expression.
Typical examples of Western blots of hippocampal submitochondrial membranes from 1 month (A) and 7 months (B) hypobaric animals. Voltage-dependent anion channel (VDAC) was used as loading control. β-actin was used in order to evaluate contamination from the cytoplasm. The results are representative of three independent studies. Values [means ± SEM] below blots represent mean of densitometric units after background subtraction ($p < 0.01$ as compared with control value).**

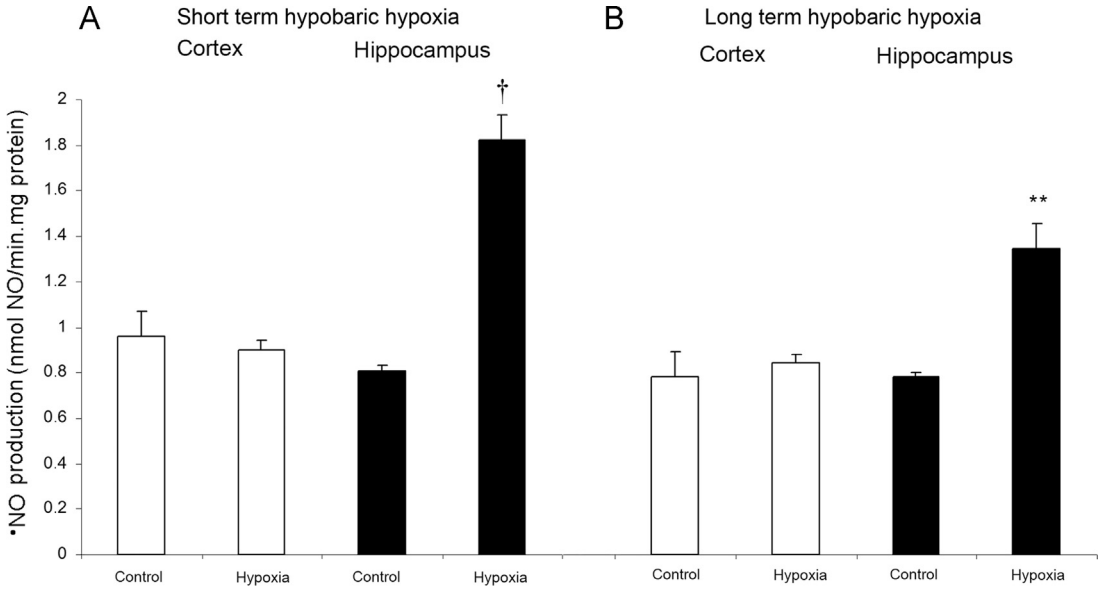

Fig. 3 – NO production after short and long hypobaric hypoxia.
NO production in cortical and hippocampal submitochondrial membranes from 1 month (A) and 7 months (B) hypobaric animals was measured. Bars represent the mean ± SEM. ($p < 0.01$, †$p < 0.001$ as compared with control value).**

Mitochondrial electron transport chain has long been suspected a likely site of oxygen sensing (Guzy and Schumacker, 2006). The present study shows that 1 mo of hypobaric hypoxia increased eNOS and nNOS expression as well as •NO production associated to mitochondrial membranes and decreased hippocampal state 4 and state 3

respiratory rates. The inhibition of active respiration in hippocampal mitochondria would be a consequence of a direct effect of increased •NO on cytochrome oxidase activity. Our results are in accordance with previous studies showing hypobaric hypoxia increases calcium-dependent NOS activity in different brain areas (Castro-Blanco et al., 2003; Encinas et al., 2004; Mathiesen et al., 2013; Serrano et al., 2003). Also, the inhibition of mitochondrial cytochrome oxidase is one of the earliest events occurring under hypoxia (Anderson et al., 1997; Horvat et al., 2006; LaManna et al., 1996; Prabu et al., 2006; Taylor and Moncada, 2010). Respiratory control of hippocampal mitochondria from 1 mo hypoxic rats was similar to control values, indicating the preservation of the mitochondrial function. A moderate inhibition of mitochondrial respiration by •NO would extend the space of adequate tissue oxygenation and ATP production. Ledo et al. (2010) showed, in a tissue model, that •NO derived from increased neuronal activity can in fact modulate tissue O_2 consumption rate toward decreased O_2 usage, thus increasing its diffusion (Ledo et al., 2010). Taking into account that different •NO diffusional and inactivation mechanisms of (e.g. peroxinitrite formation and hemoglobin scavenging) coexist *in vivo*, the effect of •NO in cellular respiration in isolated mitochondria is difficult to extrapolate to *in vivo* conditions.

Previous studies indicate that complex III is the primary site of ROS production during hypoxia through an effect of O_2 within the mitochondrial inner membrane on the lifetime of the ubisemiquinone radical, the relative release of mitochondrial ROS toward the matrix compartment versus the intermembrane space or the ability of O_2 to access the ubisemiquinone radical in complex III (Guzy and Schumacker, 2006). Also, •NO is capable of inhibiting the mitochondrial electron transfer at the ubiquinone-cytochrome *b* region of the respiratory chain leading to an increase in superoxide anion and H_2O_2 (Poderoso et al., 1996). In our study, H_2O_2 production of hippocampal mitochondria not only did not increase after 1 mo hypobaric hypoxia but tended to decrease; indicating that increase in •NO would not be high enough to inhibit electron transfer through Complex III. Also, a lower H_2O_2 production could be related to the decrease in hippocampal membrane potential observed after 1 mo hypobaric hypoxia. This idea is in accordance with the fact that pathophysiologic levels of ROS are produced in the presence of hyperpolarized mitochondria (Kadenbach, 2003).

Summing up, we propose that hippocampal mitochondria displayed adaptive changes in order to increase the efficiency of O_2 utilization after 1 mo of hypoxic conditions. Primarily, the increment in •NO levels as a consequence of a higher expression eNOS and nNOS would decrease mitochondrial oxygen consumption, leaving oxygen available to diffuse further and reach more mitochondria and to be used as well in other physiological processes. Moreover, a mild mitochondrial membrane depolarization would contribute to reduce ROS production after 1 mo of hypobaric hypoxia probably through UCPs-dependent mechanisms which have been described in hypoxia (Andrews et al., 2005; Kim-Han and Dugan, 2005; Sullivan et al., 2004) (Fig. 4).

It is important to mention that no significant changes were observed in cortical mitochondria after 1 mo hypobaric hypoxia in any of the parameters evaluated, probably because an increased irrigation, which we could observe in this tissue, would compensate the decrease in blood pO_2.

As described previously, the mechanisms involved in hypoxia response/acclimatization are time-dependent (Hota et al., 2007). For this reason, we also evaluated cortical and hippocampal mitochondrial changes after 7 mo of hypobaric hypoxia. It has been hypothesized that partial dissipation of the proton-motive force by mild uncoupling of oxidative phosphorylation is a mechanism used by mitochondria to attenuate ROS production (Brand, 2000; Toime and Brand, 2010). In fact, proton leak enhancement and membrane potential decrease has been correlated with the increased activity of UCPs in different experimental models of hypoxia (Xu et al., 2013; Zhou et al., 2000). It has been described that non-esterified fatty acids can regulate UCPs mechanism after acute high altitude hypoxia (Xu et al., 2013). In this work, we found that long-term hypobaric hypoxia induced hippocampal mitochondrial depolarization and this effect was enhanced as compared with 1 mo hypoxic animals. The observed decrease in H_2O_2 production would be consistent with the mild uncoupling acting as a defense against ROS generation by the electron transport chain.

It is important to note that despite 7 mo of hypobaric hypoxia induced eNOS expression and •NO production, no significant changes were observed in oxygen consumption. Recent evidence suggests the existence of a threshold of •NO in the hundred nanomolar range, below which •NO can inhibit cytochrome oxidase without affecting the net O_2 consumption (Ledo et al., 2010). We hypothesize that the increase in eNOS will maintain •NO concentration under this threshold or rather that the inhibition of mitochondrial respiration by •NO will be masked by an increment in oxygen consumption as a result of mitochondrial membrane depolarization.

Taking together, hippocampal mitochondria of hypoxic animals showed a two-step adaptive mechanism. An increase in •NO metabolism was already shown at 1 mo. Also, mitochondrial depolarization was enhanced at 7 mo and appeared to be established as a protective mechanism. The dissipation of proton gradient would reduce ROS damage (Fig. 4).

In cerebral cortex, respiratory rates and •NO production as well as eNOS and nNOS expression associated to mitochondrial membranes were unchanged after long-term exposure to hypobaric hypoxia, similarly to the results obtained at 1 mo. The increased mitochondrial depolarization in cerebral cortex after 7 mo of hypoxia could be the reason for the observed tendency to decrease in H_2O_2 production.

4. Conclusions

At the same stimulus, biological systems would present different kind of strategies related to energy cell economy. Mitochondria play an important role in this response. The present work suggests that the •NO effects on the electron transport chain and the mild uncoupling are involved as self-protective mechanisms in hippocampal mitochondria of rats exposed to a high altitude environment. On the other hand, cerebral cortex appears to be protected by changes in vascular system in response to hypoxia. In addition, our study

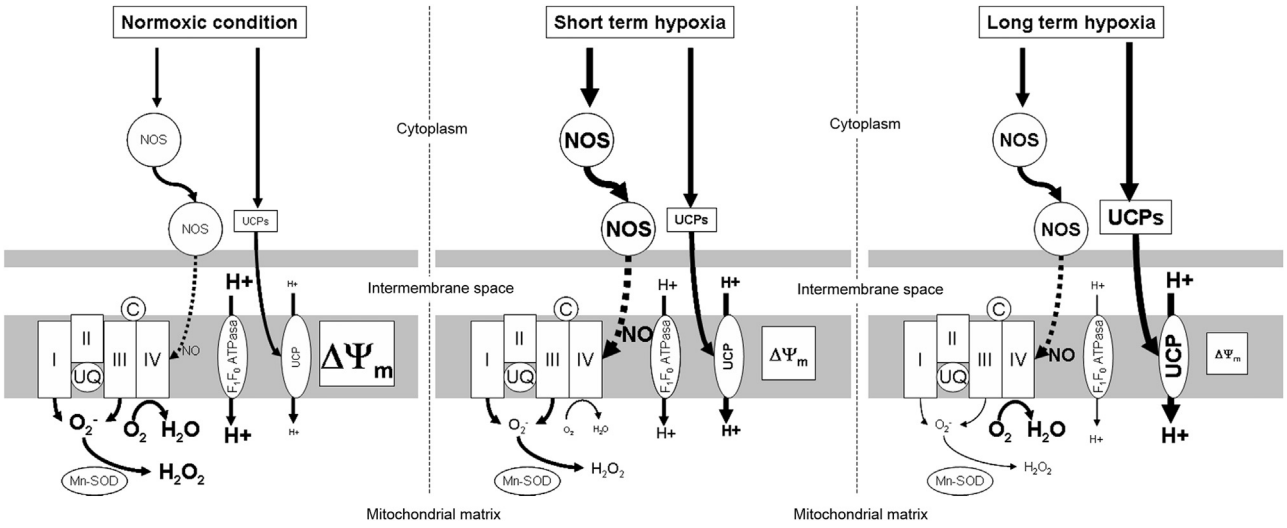

Fig. 4 – Hippocampal mitochondrial function during acclimatization.
After *short-term hypoxia*, the increased NOS expression inhibits mitochondrial respiration through NO levels as compared with *normoxic conditions*, most likely through increased cytochrome oxidase inhibition. Dissipation of membrane potential probably mediated by UCPs activity reduces H_2O_2 production. After *long-term hypoxia*, mitochondrial H_2O_2 formation tends to decrease further possibly by the increased mitochondrial membrane depolarization. The persisted inhibition of mitochondrial respiration by NO would mask the expected increment in oxygen consumption in response to mitochondrial depolarization. The thickness of the lines and letters indicates the level of activation. Dotted lines indicate inhibition.

points out a substantive difference in the temporal response according to the brain studied area.

5. Experimental procedure

5.1. *Animals and experimental design*

Seven-week-old male Wistar rats of the CHbbTHOM albino strain ($N=16$) were submitted to a simulated altitude of 5,000 m (53.8 kPa=404 mmHg) in a hypopressure chamber as previously described (La Padula and Costa, 2005), whereas the same number of sibling rats remained as controls at sea level atmospheric pressure (101.3 kPa=760 mmHg). Chamber pressure was interrupted 20–30 min three times a week for cleaning, replacement of food and water, which were administered ad-libitum, and periodic body weight control. Pressure changes were achieved slowly, and the renewal of air in the chamber was sufficient to ensure the composition of atmospheric air. The partial pressure of O_2 in the inspired air was, therefore, 11.3 kPa=85 mmHg and 21.2 kPa=159 mmHg, for hypoxic and control rats, respectively. Both groups were maintained at the same temperature (22 °C) on a schedule of 12 h of light and 12 h of dark. After 1 and 7 mo, control and hypoxic animals ($N=8$ in each group) were sacrificed and brains were quickly removed. Dissection of cerebral cortex and hippocampus was performed as described by Madison and Edison (Madison and Edson, 2001).

Rats received care in accordance with the 6344/96 regulation of the Argentinean National Drug, Food, and Medical Technology Administration (ANMAT). All experimental procedures and manipulations were reviewed and approved by the ANMAT.

5.2. *Isolation of mitochondria*

Immediately after dissection, cerebral cortex and hippocampus were weighed and homogenized (1:5 w/v) in an ice-cold medium consisting of 0.23 M mannitol, 0.07 M sucrose, 5 mM Hepes and 1 mM EDTA, pH 7.4 (MSHE buffer). Homogenates were centrifuged at 700 *g* for 10 min to discard nuclei and cell debris and the supernatant obtained was centrifuged at 8000 *g* for 10 min. The resulting pellet containing mitochondria was washed and resuspended in 0.23 M mannitol, 0.07 M sucrose, 5 mM Hepes, pH 7.4 (MSH buffer) at a protein concentration of 20–25 mg/ml (Czerniczyniec et al., 2011). All the procedure was carried out at 0–2 °C. Mitochondrial samples were less than 2–4% contaminated with cytosolic components according to the amount of lactate dehydrogenase present in the samples.

Submitochondrial membranes were obtained by twice freezing and thawing the mitochondrial preparation and were homogenized by passage through a tuberculin syringe with a needle. The preparation obtained in this way consists in a fraction of outer and inner membranes which do not present restriction to substrate access (Boveris et al., 2002).

Protein content was assayed by using Folin phenol reagent and bovine serum albumin as standard (Lowry et al., 1951).

5.3. *Oxygen consumption*

A two-channel respirometer for high-resolution respirometry (Oroboros Oxygraph, Paar KG, Graz, Austria) was used. Mitochondrial respiratory rates were measured in a reaction medium containing 0.23 M mannitol, 0.07 M sucrose, 20 mM Tris–HCl (pH 7.4), 1 mM EDTA, 4 mM $MgCl_2$, 5 mM phosphate, 0.2% bovine serum albumin and mitochondrial samples

(0.5–1 mg protein/ml) at 30 °C. Malate 6 mM and glutamate 6 mM were used as substrates to measure state 4 respiration and 1 mM ADP was added to measure state 3 respiration (Boveris et al., 1999). Oxygen uptake was expressed in ng-at O/min. mg protein. The respiratory control ratio (state 3 respiration/state 4 respiration) was determined in order to evaluate if isolation procedure or treatment affected mitochondrial physiology (Estabrook, 1967).

5.4. *Hydrogen peroxide production*

Hydrogen peroxide generation was determined in intact brain mitochondria (0.1-0.3 mg protein/ml) by the scopoletin-HRP method, following the decrease in fluorescence intensity at 365–450 nm (λexc-λem) at 37 °C (Boveris, 1984). The reaction medium consisted of 0.23 M mannitol, 0.07 M sucrose, 20 mM Tris–HCl (pH 7.4), 0.8 μM HRP, 1 μM scopoletin, 6 mM malate, 6 mM glutamate, 0.3 μM SOD and mitochondrial samples (0.05-0.1 mg protein/ml). A calibration curve was made using H_2O_2 (0.05-0.35 μM) as standard to express the fluorescence changes as nmol H_2O_2/min.mg protein.

5.5. *Mitochondrial membrane potential*

Flow cytometry assay was performed in a FACScalibur (Becton-Dickinson) equipped with a 488 nm argon laser and a 615 nm red diode laser.

Population with high FSC and SSC was chosen according to the typical mitochondrial size (3-5 μm) calibrated using Beads Calibrite (6 μm) (Becton Dickinson) for all the cytometric studies.

Equal protein concentration of intact isolated mitochondria were loaded with 30 nM of the potentiometric cationic probe $DiOC_6$ during 20 min at 37 °C in MSH buffer (0.23 M mannitol, 0.07 M sucrose, 5 mM Hepes) supplemented with 5 mM malate, 5 mM glutamate and 1 mM phosphate. The procedure was carried out in a dark room (Bustamante et al., 2004). Mitochondrial fluorescence with no probe and after 0.5 μM FCCP (carbonyl cyanide p-trifluoromethoxyphenylhydrazone) treatment was measured as negative and positive controls, respectively. In order to quantify the resulting changes in membrane potential, a common marker indicating the relative fluorescence intensity of the mitochondrial population analyzed was used.

5.6. *eNOS and nNOS expression*

Cortical and hippocampal submitochondrial membranes were prepared in Laemmli buffer with 2-mercaptoethanol (1:2 v/v) and boiled for 1 min. Equal amounts of protein (80 μg) were loaded onto SDS-PAGE (7.5% or 15%), separated and blotted onto nitrocellulose membranes in Tris-glycine-MeOH buffer. Non-specific binding was blocked by incubation of the membranes with 5% non-fat dry milk in PBS for 1 h at room temperature. The blots were probed with a dilution 1:500 of polyclonal primary antibodies specific for nNOS (rabbit, amino terminus, H-299, Santa Cruz Biotechnology, Santa Cruz, CA, USA), eNOS (rabbit, amino terminus, N-20, Santa Cruz Biotechnology, Santa Cruz, CA, USA), VDAC (goat, N-18, Santa Cruz Biotechnology, Santa Cruz, CA, USA) or β-Actin (mouse, (C4): sc-47778, Santa Cruz Biotechnology, Santa Cruz, CA, USA). Primary antibodies were incubated in 1% BSA in PBS overnight at 4 °C with rocking. The blots were rinsed three times for 15 min with PBST (PBS with 0.15% Tween 20) and then incubated with horseradish peroxidase-conjugated secondary antibodies (anti-rabbit or anti-goat) at 1:5000 in 2.5% non-fat dry milk in PBS for 1 h at room temperature with rocking. The blots were rinsed three times for 10 min with PBS and then exposed with ECL reagent (Boveris et al., 2002; Bustamante et al., 2002). Densitometric analysis of bands was performed using the NIH Image 1.54 software. All experiments were performed in triplicate.

5.7. *Nitric oxide production*

Nitric oxide production was measured in submitochondrial membranes, by following the oxidation of oxyhemoglobin (HbO_2) to methemoglobin (metHb) at 37 °C. The $^{\bullet}NO$ assay was performed using a Beckman-Coulter Serie DU 7400 diode array spectrophotometer in which the active wavelength is set at 577 nm and the reference wavelength at the isosbestic point at 591 nm ($\varepsilon = 11.2\ mM^{-1}\ cm^{-1}$) (Boveris et al., 2002). The method is based on the original assay developed by Murphy and Noack (Murphy and Noack, 1994) for perfused organs in which the HbO_2 γ band is used to follow $^{\bullet}NO$ production. The α band is more suitable for high light scattering conditions of cellular and mitochondrial suspensions due to the close vicinity of the active (577 nm) and the reference (591 nm) wavelengths (Boveris et al., 2002). The measurements were carried out in a reaction medium containing 50 mM phosphate buffer pH 5.8, 1 mM $CaCl_2$, 50 μM L-arginine, 100 μM NADPH, 10 μM DTT, 4 μM Cu-Zn superoxide dismutase (SOD), 0.1 μM catalase, submitochondrial membranes (0.5-1.0 mg protein/ml) and 25 μM oxyhemoglobin (expressed per heme group). Oxyhemoglobin reacts efficiently with $^{\bullet}NO$ if the reaction between $^{\bullet}NO$ and superoxide anion ($O_2^{\bullet-}$) is prevented. Superoxide dismutase is added to abrogate any other reaction with $O_2^{\bullet-}$, including a direct oxidation of HbO_2 to metHb or reduction of metHb to Hb. In the presence of SOD, H_2O_2 could be produced. H_2O_2 can oxidize both HbO_2 and metHb to higher oxidation states. For this reason, catalase is added to the assay (Murphy and Noack, 1994). Controls adding 0.5 mM N_ω-nitro-L-arginine (L-NNA) and N_ω-nitro-L-arginine-methyl ester (L-NAME) as NOS inhibitors were performed in all cases to give specificity to the assay.

5.8. *Drugs and chemicals*

ADP, L-arginine, Catalase, Dithiothreitol, EDTA, Glutamic Acid, Malic Acid, Mannitol, NADPH, N_ω-nitro-L-arginine, Haemoglobin, Scopoletin, Horseradish Peroxidase, Succinate, Sucrose, Superoxide Dismutase, Cytochrome c, and Trizma Base were purchased from Sigma Chemical Co. (St. Louis, Missouri). Other reagents were of analytical grade.

5.9. *Statistics*

Results were compared by unpaired independent Student's *t*-test in order to analyze the significance of differences between two groups and presented as mean ± SEM. A

difference was considered to be statistically significant when $p<0.05$.

Conflict of interest

The authors declare that there are no conflicts of interest

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