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Acute hypobaric hypoxia and cardiac energetic response in prepubertal rats. Role of nitric oxide.

Running title: Hypobaric hypoxia in prepubertal heart.

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Abbreviations

DT: developed tension

eNOS: endothelial nitric oxide synthase

H/R: hypoxia/reoxygenation

HH: hypobaric hypoxia

HIF: hypoxic inducible factor

Ht: total heat production

iNOS: inducible nitric oxide synthase

ISO: isoproterenol hydrochloride

L-arg: L-arginine

L-NNA: N_{ω}-Nitro-L-arginine

LVP: left ventricular pressure

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N: normoxic

nNOS: neuronal nitric oxide synthase

NO: nitric oxide

NOS: nitric oxide synthase

Nx: Nitrites-nitrates

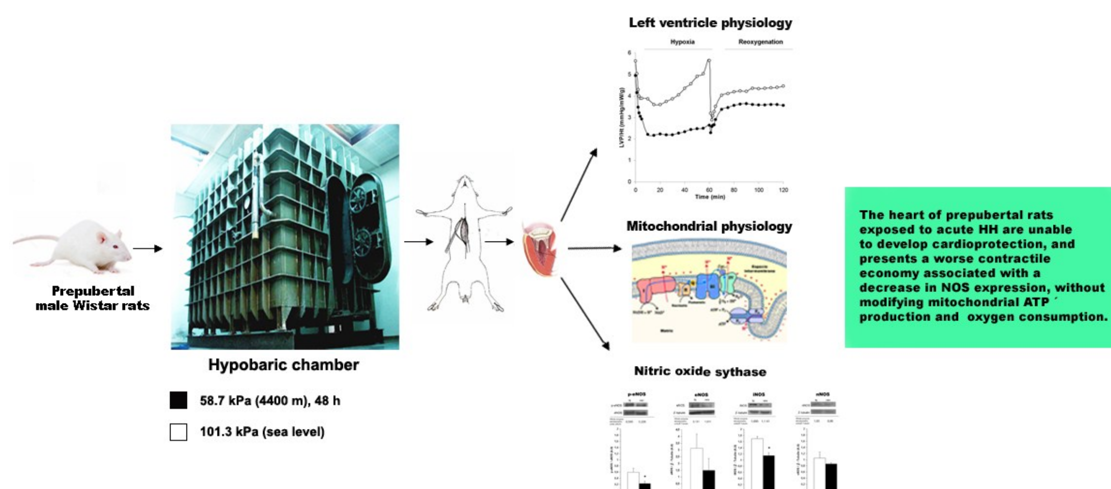
p-eNOS: phospho endothelial nitric oxide synthase-Ser1177

PM: papillary muscles

LVP/Ht: contractile economy

SD: standard deviation

Cover figure



What is the central question of this study?

Exposure to hypobaric hypoxia increased tolerance to hypoxia/reoxygenation, which is known as endogenous cardioprotection in heart of adult rats. This process involves the participation of the nitric oxide system and modulation of mitochondrial oxygen consumption. Taking in account the impact of the degree of somatic maturation on physiology, in the present work we evaluate the cardio energetic response in prepubertal rats exposed to hypobaric hypoxia.

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What is the main finding and its importance?

Prepubertal rats, as opposite to adult ones, were unable to increase tolerance to hypoxia/reoxygenation by acute exposure to hypobaric hypoxia, which impaired cardiac contractile economy. This finding could be related to the failure for increasing nitric oxide synthase expression, and thus modulation of mitochondrial oxygen consumption and ATP production.

Abstract

Studies in our laboratory showed that exposure of rats to hypobaric hypoxia (HH) increased the tolerance of heart to hypoxia/reoxygenation (H/R), involving mitochondrial and cytosolic NOS systems. The objective of the present study was to evaluate how the degree of somatic maturation could alter this healthy response.

Prepubertal male rats were exposed 48 h to 4400 m simulated altitude in a hypobaric chamber. The mechanic-energetic activity in perfused hearts and the contractile functional capacity of nitric oxide synthase (NOS) in isolated left ventricle papillary muscles (PM) were evaluated during H/R. Cytosolic nitric oxide (NO), nitrites/nitrates (Nx) production, NOS isoforms expression, mitochondrial O_2 consumption and ATP production were also evaluated.

Heart left ventricular pressure (LVP) during H/R was not improved by HH. However the energetic activity (Ht) was increased. Thus, the contractile economy (LVP/Ht) got worse in HH. Nitric oxide did not modify PM contractility after H/R. Cytosolic p-eNOS -Ser1177and iNOS expression were decreased by HH but no changes were observed in NO production. Interestingly, HH increased Nx levels but O_2 consumption and ATP production in mitochondria were not affected by HH.

Conclusions: prepubertal rats exposed to HH preserved cardiac contractile function, but with a high energetic cost, modifying contractile economy. Although this could be related to the decreased NOS

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expression detected, cytosolic NO production was preserved, may be through the Nx metabolic pathway, without modifying mitochondrial ATP production and O_2 consumption. In that scenario, the treatment was unable to increase tolerance to H/R as we observed in adult animals.

Keywords

Hypobaric hypoxia; Cardioprotection, Prepubertal heart, Cardiac economy, Nitric oxide Cardiac mitochondria.

1. Introduction

Several studies show that the acclimatized heart to chronic hypoxia develops cardioprotection through the involvement of the nitric oxide synthase (NOS) system, (Costa & La Padula, 2019). We previously reported that long-term exposure of rats to hypobaric hypoxia increased tolerance to one episode of hypoxia/reoxygenation (H/R) involving mitochondrial NOS and modulation of the respiratory chain (La Padula & Costa, 2005; Zaobornyj *et al.*, 2005). In a previous study, in which initially prepubertal rats (7-wk-old) were exposed to HH during lifetime, no changes in NO production or improvement of papillary muscle tolerance to H/R were observed after only seven days of HH (La Padula & Costa, 2005; Zaobornyj *et al.*, 2005). However, we found later an increase in cytosolic left ventricle nitric oxide (NO) production after 48 h exposure of 3-mo old rats to HH, associated to an improvement of the tolerance of papillary muscles to one episode of H/R (La Padula *et al.*, 2018). These results support the idea that cytosolic NO modulates left ventricle contractile machinery and has an impact on cardiac tolerance to H/R, besides the action on mitochondrial function. These HH studies (La Padula & Costa, 2005; Zaobornyj *et al.*, 2005; La Padula *et al.*, 2018), suggest that the apparent contradiction found between the observed cardioprotection after only 48

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h and the failure after 7 days of HH might be due to the difference in somatic maturity of the animals.

The mechanism triggered by acute hypoxia could have an important practical advantage in terms of clinical therapy over the chronic HH models. As in chronic hypoxia, resistance to myocardial ischemia developed by exposure to acute hypoxia has been consistently associated with modulation of NOS activity (Xi *et al.*, 2002; La Padula *et al.*, 2018). Nitric oxide system seems to play an essential role in the development of endogenous cardioprotection (Hare, 2003; Manukhina *et al.*, 2006). The three main isoforms of NO synthase, namely, neuronal NOS (nNOS), endothelial NOS (eNOS), and the inducible NOS (iNOS), have been implicated in cardioprotection in two different models of systemic hypoxia, at mitochondrial and cytosolic levels (Baker *et al.*, 1999; Xi *et al.*, 2002; Kolář & Ošťádal, 2004; Baker, 2004; La Padula *et al.*, 2008, 2018). Nitric oxide actions appeared to be site specific and concentration-dependent (Hare, 2003; La Padula *et al.*, 2008). At mitochondrial level, NO modulates oxygen consumption and free radicals production (Poderoso *et al.*, 1996; Cassina & Radi, 1996; Brown & Borutaite, 2007). Also, in cytosol, NO modulates specific myocytes calcium channels involved in mechanical activities (Xu *et al.*, 1998; Hare, 2003; Csordás *et al.*, 2006; Rastaldo *et al.*, 2007). In accordance, we previously reported an enhanced NOS activity and expression in mitochondrial fraction from left ventricles during acclimatization to hypobaric hypoxia (Zaobornyj *et al.*, 2005; La Padula *et al.*, 2008). On the other hand, during deacclimatization the normalization time of both NOS activity and expression decline at the same time as the loss of the cardioprotective effects, reaffirming the involvement of NO in cardioprotection after hypoxia exposure (La Padula *et al.*, 2008).

In addition, we recently found a positive interaction between NO and β-adrenergic systems after acute hypobaric hypoxia exposure but not specifically associated with tolerance to H/R (La Padula *et al.*, 2018). This observation together with many other evidences collected by both *in vivo* and *in vitro* experiments suggests a crosstalk between NO and β-adrenergic systems (Balligand, 1999; Conti *et*

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al., 2013; Vanhoutte & Gao, 2013). The importance of this interaction lies in the fact that both systems modulate contractility, being NO production directly linked to stimulation of β-adreno-receptors in many instances (Balligand, 1999; Queen & Ferro, 2006).

The objective of the present study was to evaluate the effect of acute HH in heart and the interaction of endogenous NO and cardiac β-adrenergic system before and after a single H/R episode, in somatically immature rats. For this purpose, mechanical and energetic activities were simultaneously determined in left ventricles of rats subjected to HH for 48 h, before and after cardiac H/R. Also, NO functional capacity was evaluated in response to β-adrenergic stimulation and during H/R. At biochemical level, left ventricle cytosolic NO production and the expression of NOS isoforms were characterized; mitochondrial oxygen consumption and ATP production as well as cytochrome oxidase activity were also evaluated.

2. Methods

2.1. Ethical Approval

Experimental protocol was conducted in compliance with the Principles and standards for reporting animal experiments in The Journal of Physiology and Experimental Physiology and in accordance with "Guide for the Care and Use of Laboratory Animals" prepared by the Institute of Laboratory Animal Research and published by the National Institutes of Health (NIH Publications No. 8023, revised 2011). - Also, the present study had the legal ethical accreditation from Ethics Committee for Laboratory Animal Handling of the School of Medicine from Universidad de Buenos Aires where the protocol was performed (RES (D) N° 981/2019).

2.2. Experimental design

Six and a half-week-old male Wistar rats of the CHbbTHOM albino strain (acquired from School of Medicine) were subjected during 48 h to a simulated 4,400 m altitude (58.7 kPa = 440 mmHg) in a

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hypopressure chamber as previously described (La Padula & Costa, 2005). A group of the same number of sibling rats remained as controls at sea level atmospheric pressure (101.3 kPa = 760 mmHg). Food and water were administered *ad-libitum*. 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 (22ºC) on a scheduled 12 h light-dark cycle. Rats received care in accordance with the 6344/96 regulation of the Argentinean National Drug, Food, and Medical Technology Administration (ANMAT) and the study was carried out in accordance with the "Guide for the Care and Use of Laboratory Animals" prepared by the Institute of Laboratory Animal Research and published by the National Institutes of Health (NIH Publications No. 8023, revised 2011). Immediately after removing hypoxic rats from the hypobaric chamber, as well as the control ones, the thoraxes were opened under anesthesia with heparinized (2000 U) pentobarbital overdose (60 mg/kg). The beating heart was excised and rinsed in warm Krebs solution (La Padula & Costa, 2005) before the isolation of papillary muscles (PM) or cannulation of the whole heart as described below.

2.3. *Functional study in the whole heart*

2.3.1. *Left ventricle mechanical and energetic measurements*

Hearts excised from the animals (n: 32) were quickly cannulated and arterially perfused at 6 ml/min/g through Langendorff technique with a Krebs-Ringer-$NaHCO_3$ solution that contained (in mM): 120 NaCl, 6 KCl, 25 $NaHCO_3$, 0.5 NaH_2PO_4, 1 $MgCl_2$, 1.35 $CaCl_2$, and 8 glucose (Krebs-C), continuously bubbled with a gas mixture (95% O_2-5% CO_2) at pH 7.4. Both atria were removed and a small cut in the interventricular septum close to the aorta was done to keep the ventricles at rest. A latex balloon of adjustable volume filled with water and connected to a pressure transducer (Gould Statham P23db, Hato Rey, Puerto Rico) was introduced into the left ventricle to measure the

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interventricular pressure (Bonazzola & Takara, 2010). The ventricles were mounted in the inner chamber of a flux calorimeter (Ponce-Hornos *et al.*, 1995). The calorimeter was submerged in a large water bath maintained at constant temperature (30°C), in which the perfusion solutions were also equilibrated. Ventricles were stabilized at optimal length in Krebs-C and were electrically stimulated at 2 Hz with 5 V, 5 ms duration pulses delivered from a Grass SD9 stimulator (Baintree, Quincy, Massachusets). Heat rate (Ht) and the left ventricular pressure (LVP) were continuously and simultaneously measured. Both signals were recorded at optimal volume in an 8 channels Grass polygraph (Grass Instruments, Quincy, Massachusetts) with A/D acquisition system (TL-1 DMA Axon Instruments Inc, Foster City, California). Ht was calculated at any time from the difference between the signal obtained with the heart into the inner chamber of the calorimeter and the baseline that comes from the perfused chamber without the organ. Calorimeter calibration was accomplished by applying a constant and known electrical power from a sinusoidal current generator (2 kHz, 1 V) through the heart that acts as impedance. Ht was expressed in mW/g wet weight. Total muscle economy was calculated as LVP/Ht ratio (in mm Hg. g/mW).

2.3.2 *Mechanical and energetic response to hypoxia/ reoxygenation*

After an equilibration period of about 90 min to reach steady LVP and Ht, the ventricles were challenged by 60 min of acute hypoxia perfusing with Krebs solution bubbled with 95% N_2-5% CO_2. Finally, ventricles were reoxygenated in Krebs-C during 60 min (Consolini & Bonazzola, 2008). During the whole protocol Ht and LVP were recorded. Eight experiments per group were performed.

2.3.3. *Mechanical sensibility to mitochondrial uncoupling protein 2 (UCP-2)*

After an equilibration period of about 90 min to reach steady LVP and Ht, the ventricles were perfused with 5 μM of genipin, an UCP-2 mitochondrial blocker. After the first 10 minutes of exposure to genipin, the ventricles were subjected to 60 min of acute hypoxia perfusion with Krebs solution added with genipin and bubbled with 95% N_2-5% CO_2. Finally, ventricles were reoxygenated

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in Krebs-C with genipin during 60 min. During the whole protocol Ht and LVP were recorded. Eight experiments per group were performed.

2.4. *Modulation of contractile papillary muscles activity by NO*

2.4.1. *Isolated papillary muscles mechanical preparation*

Twelve rats were kept in a hypobaric chamber for two days, and other twelve were maintained out of the chamber under normoxic conditions (controls). Once the heart was excised from the animal, it was rinsed and transferred to a Ringer solution of the following composition (mM): 128.3 NaCl, 4.7 KCl, 20.2 $NaHCO_3$, 0.35 NaH_2PO_4, 1.05 $MgSO_4$, 1.35 $CaCl_2$, and 5.5 glucose, pH 7.4, bubbled with 95% O_2-5% CO_2, at 30ºC. Left ventricle was opened, and both papillary muscles were removed while submerged in buffer. The chordae end of each muscle was tied with 10-0 nylon suture, which was attached to a Statham force transducer and 9853 coupler (Gould-Statham) mounted on a movable support controlled by a micrometer for accurate length adjustment. The bottom end of each papillary muscle was inserted into a stainless-steel spring clip, and the muscles were mounted vertically in two temperature-controlled chambers containing 30 ml of the Ringer solution each one. Solutions were equilibrated with a mixture of 95% O_2 and 5% CO_2, with pH and temperature kept constant at 7.4 and 30ºC, respectively. Heart, trimmed of atria and large vessels, was dissected into the left ventricle plus septum (LV) and right ventricle (RV), which were weighed separately (La Padula & Costa, 2005).

2.4.2. *Papillary muscle mechanical activity modulated by NO*

Papillary muscles were allowed to stabilize for 45 min after mounting. Rectangular pulses of 10 ms with amplitude 20% higher than the threshold of each preparation was digitally delivered by means of a stimulator controlled by a data acquisition and analysis software (FPE). Contraction frequency was kept constant at 12 beats min^{-1}. The muscles were then stretched until maximal developed tension occurred. Isometric mechanograms were recorded on a Beckman R511A connected to the

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force transducer, and simultaneously the computer utilizing FPE digitized and stored the force-pacing signal for later analysis. Mechanical activity was determined as developed tension (DT). Each data result was the mean of three successive twitches (La Padula & Costa, 2005). After recording basal contractility, papillary muscles (PM) were incubated 10 min with 2 mM L-arg, a NOS substrate, to obtain the maximal endogenous NO production or with 2 mM L-NNA, a NOS blocker, to obtain the minimum production of endogenous NO. After pretreatment with both drugs, an accumulative dose-response curves from 10^{-9} to 10^{-4} M isoproterenol hydrochloride (ISO) was performed. A 60-min period of hypoxia was then established by using a gas mixture of 95% N_2 and 5% CO_2, followed by a 30-min period of reoxygenation (95% O_2 and 5% CO_2), and mechanical events were recorded every 10 min (La Padula & Costa, 2005). At the end of each experiment, muscle length was measured with a caliper. The PM were then blotted dry and weighed, and cross-sectional area of each one was calculated, assuming the muscle to be a cylinder with a density of 1.0 g/cm^3. Mechanical parameters were normalized for muscle cross-sectional area (La Padula & Costa, 2005).

2.5. *Isolation of left ventricle cytosol and mitochondrial fraction*

Left ventricles deprived from the papillary muscles were weighed, chopped, and homogenized in an ice-cold homogenization medium (1:10) containing 0.23 M mannitol, 0.07 M sucrose, 10 mM Tris-HCl, and 1 mM EDTA, pH 7.4. Homogenates were centrifuged at 700 g for 10 min to discard nuclei and cell debris, and the supernatant was centrifuged at 8,000 g for 10 min. Supernatant, containing the cytosolic fraction, was separated from the mitochondrial pellet. Mitochondrial pellet was washed and resuspended in the homogenization medium. All these operations were carried out at 2–4°C (Boveris *et al.*, 2002). Protein concentration was determined with the Folin reagent and BSA as standard.

2.6. *Expression of cytosolic nitric oxide synthase isoforms*

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Protein expression of p-eNOS-Ser1177, eNOS, nNOS and iNOS was evaluated. Equal amounts of cytosolic protein (40 µg) were loaded into 7% SDS-PAGE separated and blotted onto PVDF 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 hour at room temperature. Blots were probed with 1:1000 dilution of primary antibody specific for p-eNOS (rabbit monoclonal, #9570, Cell Signaling Technology, Danvers, Ma, USA), eNOS (rabbit polyclonal, #sc-654, Santa Cruz Biotechnology, Santa Cruz, CA, USA), nNOS (rabbit, amino terminus, H-299, Santa Cruz Biotechnology, Santa Cruz, CA, USA), iNOS (mouse monoclonal, #sc-7271 Santa Cruz Biotechnology, Santa Cruz, CA, USA) or β-tubulin (mouse monoclonal #ab131205, Abcam). Primary antibodies were incubated in 1% BSA in PBS overnight at 4 ºC with rocking. The blots were rinsed three times for 15 min with PBST (PBS with 0.15% Tween 20). Biotin conjugated secondary anti-rabbit or anti mouse (Code: RPN1025V, GE healthcare, Buckingham, UK) and streptavidin-horseradish peroxidase complex (Code: RPN1051, GE healthcare, Buckingham, UK) were used at 1:10,000 dilution. Blots were rinsed three times for 10 min with PBS and then exposed to ECL reagent. Densitometric analysis of bands was performed using the NIH Image 1.54 software. All experiments were performed in triplicate.

2. 7. *Nitric oxide production*

NO production was measured in cytosolic fractions by following spectrophotometrically at 577–591 nm [molar extinction coefficient (e) = 11.2 mM^{-1} cm^{-1}] (Beckman DU 7400 diode array spectrophotometer) the oxidation of oxyhemoglobin to methemoglobin, at 37°C (Murphy & Noack, 1994; Boveris *et al.*, 2002). The reaction medium consisted of 50 mM phosphate buffer (pH 7.4), 50 µM L-arginine, 100 µM NADPH, 1 mM $CaCl_2$, 10 µM dithiothreitol, 4 µM Cu,Zn-SOD, 0.1 µM catalase, 20 µM oxyhemoglobin and cytosolic fractions (0.5– 0.8 mg protein/ml). NO production was expressed as nanomoles of NO/min. mg protein (Boveris *et al.*, 2002).

2.8. *Nitrites/nitrates content*

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The content of nitrites was evaluated spectrophotometrically in cardiac cytoplasmic fractions by the modified method of the Griess reaction. Total content of nitrites + nitrates was determined by the same method after reduction of the nitrates to nitrites, with vanadium (III) chloride (VCl_3). Initially, to deproteinize the samples, they were incubated with ethanol in a 1: 1 ratio (v:v), at -20 °C for 2 h. Subsequently, the mixture was centrifuged at 4,000 g for 10 minutes at 4 °C, and the pellet was discarded. The deproteinized samples were incubated with 8 mg/ml VCl_3 for 30 min at 37 °C to reduce the nitrates present to nitrites. Nitrite detection was performed by Griess reaction, by determining the absorbance at 540 nm. Calibration curves were performed using NO_2^- and NO_3^- as standard. The results were expressed as µM NO_x^- (Miranda *et al.*, 2001).

2.9. *Mitochondrial oxygen consumption*

A respirometer for high-resolution respirometry (Hansatech Oxygraph, Hansatech Instruments Ltd., Norfolk, England) was used. Mitochondrial respiratory rates were measured in a reaction medium containing 120 Mm KCl, 200 mM KH_2PO_4, 1mM EGTA, 3 mM HEPES, 1 mg/ml BSA, pH 7.2 and mitochondrial samples (0.5–1 mg protein/ml) at 30 °C. 2 mM malate and 5 Mm glutamate were used as substrates to measure state 4 respiration and 1 mM ADP was added to evaluate state 3 respiration. Oxygen consumption was evaluated at maximal levels of NO production by the addition of L-arg (0.1 mM). 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 whether isolation procedure or treatment affected mitochondrial physiology (Boveris *et al.*, 1999) (Estabrook, 1967).

2.10. *Determination of mitochondrial ATP production rates*

Mitochondrial ATP production was determined by luciferin-luciferase chemiluminescent method at maximal levels of NO production by the addition of L-arg 0.1 mM. Fresh isolated mitochondria (10-20 µg) were added to a medium containing 8 mM K_2HPO_4 / KH_2PO_4, 20 mM Tris–HCl, 120 mM KCl, 1.6 mM EDTA, 0.08% BSA, 0.08 mM $MgCl_2$, pH 7.4, 40 µM luciferine, 1 µg/ml luciferase at 28 °C. 6 mM

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malate, 6 mM glutamate, 1 mM ADP, and 0.15 mM di (adenosine) pentaphosphate were added to the reaction medium (Drew & Leeuwenburgh, 2003). Measurements were made for 3 minutes in a Varioskan Lux microplate reader (Thermo Scientific) with chemiluminescence detector. A calibration curve was performed using ATP as standard (0-20 nmoles), and the production of ATP in the presence of 2 µg/ml oligomycin was determined as a control. ATP production rate was expressed as nmol ATP/min. mg protein.

2.11. Cytochrome oxidase activity

Cytochrome oxidase activity (Complex IV) was assayed spectrophotometrically at 550 nm by following the rate of oxidation of reduced cytochrome c (Hatefi,.1985) Cytochrome c was reduced with dithionite that was removed afterwards by eluting through a Sephadex-G25 column with potassium phosphate buffer (10 mM), pH 7.4. The reaction was initiated by the addition of 50 µM reduced cytochrome c to submitochondrial membranes (0.5 mg/ml) and the rate of reduced cytochrome c oxidation was determined as a pseudo-first-order reaction constant (k') (expressed as k'/mg protein).

2.12. Statistics

Results are expressed as mean values ± SD. One-way ANOVA plus the post ANOVA Bonferroni *t* test for multiple comparisons were used for statistical analysis of the data as appropriate (Microcal Origin 6.0 statistical software and Graph Pad Prism version 6.0). For comparing only two samples, the unpaired Student t test was used. A value of $p < 0.05$ was considered statistically significant.

3. Results

3.1. *Biological parameters*

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After 48 h exposure to 4400 m simulated altitude, body weight decreased 2%, while in controls it increased 8% (t-test p = 0.0462). Heart weight was not affected: 0.66± 0.14 and 0.69 ±0.14 for N and HH respectively. Ventricles weight and papillary muscle areas were similar in both groups.

3.2. *Functional study in the whole heart*

3.2.1. *Mechanical and energetic responses*

In the N group, acute hypoxia for 60 min induced a fall in left ventricle pressure (LVP) to 19.4 ± 8,7 % from its initial value (61.8 ± 16.3 mm Hg). Simultaneously, the heat released (Ht) fell to 21.7 ± 11.6 % of its pre-hypoxic value (10.8±1.4 mW/g). Reoxygenation allowed a partial recovery of LVP (up to 62.1 ± 11.6%) and Ht (up to 82 ± 11 %). In the HH group, basal LVP was similar compared with N group, and the energetic cost (Ht) remained as in N hearts (12.1 ± 2.2 mW/g). The behavior of LVP during the whole period of H/R was similar as in N group. However, Ht was higher in HH during both periods of hypoxia (4.8 ± 1.7 vs. 2.4 ± 1.1 mW/g, for HH and N groups, respectively, p<0.05) and reoxygenation (10.7 ± 1.4 vs. 8.9 ± 1.1 mW/g for HH and N groups, respectively, p<0.05) (Fig. 1).

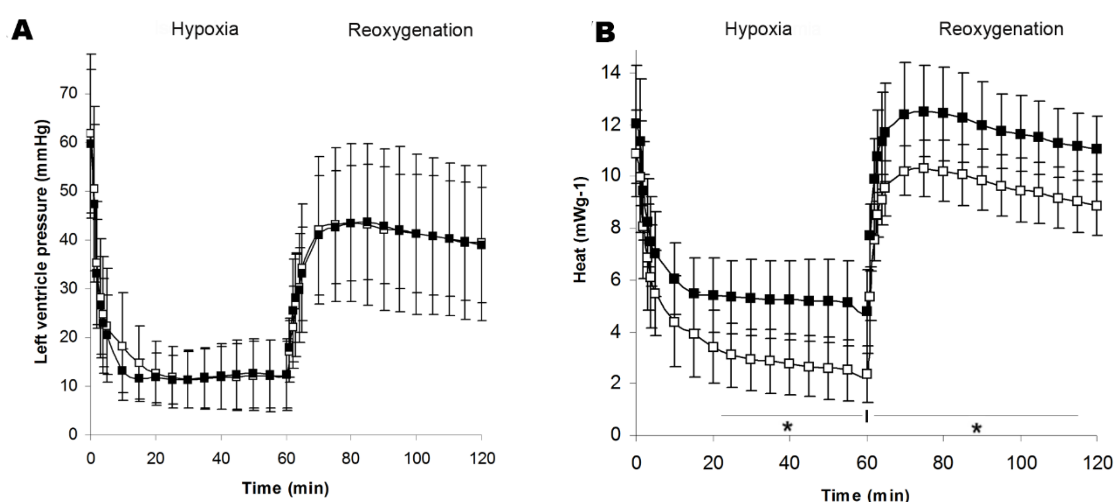

Fig 1. (A) Mechanical response to 60 min of hypoxia and 60 min of reoxygenation of left ventricle from normoxic rats (N) and rats submitted to hypobaric hypoxia (HH). (B) Energetic response to 60 min of hypoxia and 60 min of reoxygenation of left ventricle from normoxic rats (N) and rats submitted to hypobaric hypoxia (HH). Values are means ± SD. ANOVA. * *P* < 0.05, vs HH group. Open symbols, N; Closed symbols, HH.

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3.2.2. *Changes in contractile economy*

In normoxic ventricles, 60 min of acute hypoxia induced a transient reduction in contractile economy expressed as LVP/Ht and the following reoxygenation strongly reduced it. The behavior was different in HH group which presented a strongly reduced LVP/Ht, during H/R related to N group (Fig. 2).

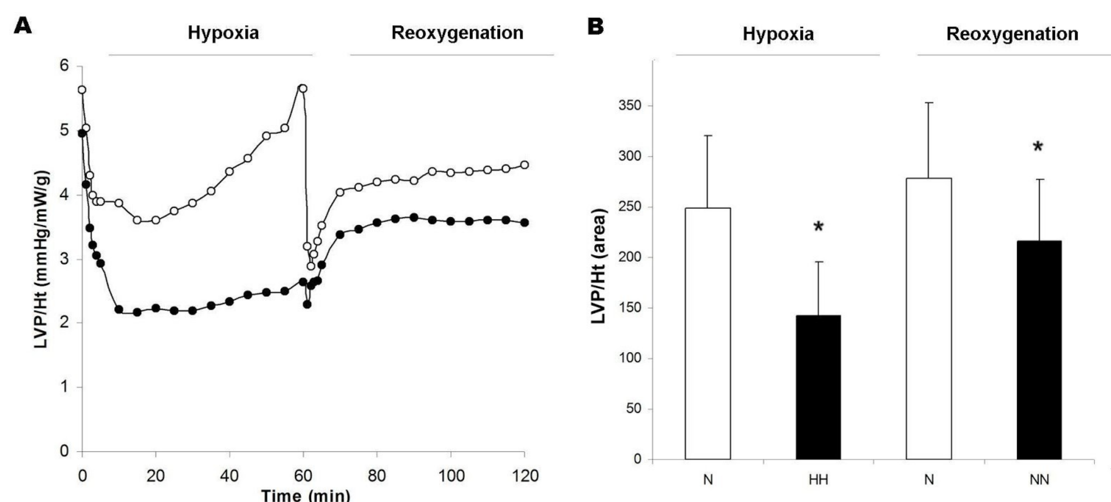

Fig. 2. (A) Contractile economy response to 60 min of hypoxia and 60 min of reoxygenation of left ventricle from normoxic (N) and submitted to acute hypobaric hypoxia (HH) rats. Only mean values are shown. (B) Graphical representation of the area under the curve in response to 60 min of hypoxia and 60 min of reoxygenation of left ventricle from normoxic (N) and rats submitted to hypobaric hypoxia (HH). Values are means ± SD (n:8). ANOVA. * *P* < 0.05, vs HH group. Open symbols or bars, N; Closed symbols or bars, HH.

3.2.3. *Contractility modulation by UCP-2*

The hypobaric treatment did not modify the response of the ventricles to genipin (G) indicating that UCP-2 does not participate in the effects observed during the exposure to acute hypobaric hypoxia. The blockage of UCP-2 modified the contractile response in the same way in both groups (N and HH), increasing the LVP recovery after H/R. The values expressed in % are: 62 ± 11 vs. 82 ± 17 for N and N+G, respectively, p: 0.0187 and 67 ± 17 vs. 88 ± 14 for HH and HH + G, respectively, p: 0.0326. The Ht released showed a tendency to decrease in both groups (N and HH). As a consequence, genipin improved the mechanical contractile economy evaluated as LVP/Ht ratio, in both, N and HH hearts. The values, expressed in % were: 78 ± 22 vs. 102 ± 14 for N and N+G, respectively, p: 0.0371, and 73 ± 22 vs. 104 ± 19 for HH and HH + G, respectively, p: 0.0214.

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3.3. *Modulation of contractile papillary muscles activity by NO.*

3.3.1. *Basal mechanical function*

Developed tension (DT) was similar in rats exposed to 48 h of simulated high altitude than in those from normoxic animals: 1.5 ± 1.0 ($g.mm^{-2}$) for both groups. Contractile activity was not affected by the addition of the NOS substrate L-arg or the NOS blocker L-NNA.

3.3. 2. *Mechanical ß–adrenergic response*

The normoxic papillary muscles response to isoproterenol (ISO) stimulation was modulated by NO. Under conditions of maximum and minimum NO production, the DT increase with ISO 10^{-4} M was 205 ± 42 % with substrate and 128 ± 17% with the NOS blocker (Fig. 3), respect to the pre-β-stimulation values (100%). Therefore, the mechanical functional capacity of NOS (LVP+L-arg - LVP+L-NNA) over the cardiac reserve was 77%. On the contrary, the β-adrenergic response of papillary muscles from HH was not modulated by NO: 173 ± 59 % vs 176 ± 62 %, for L-arg and L-NNA respectively (Fig. 3).

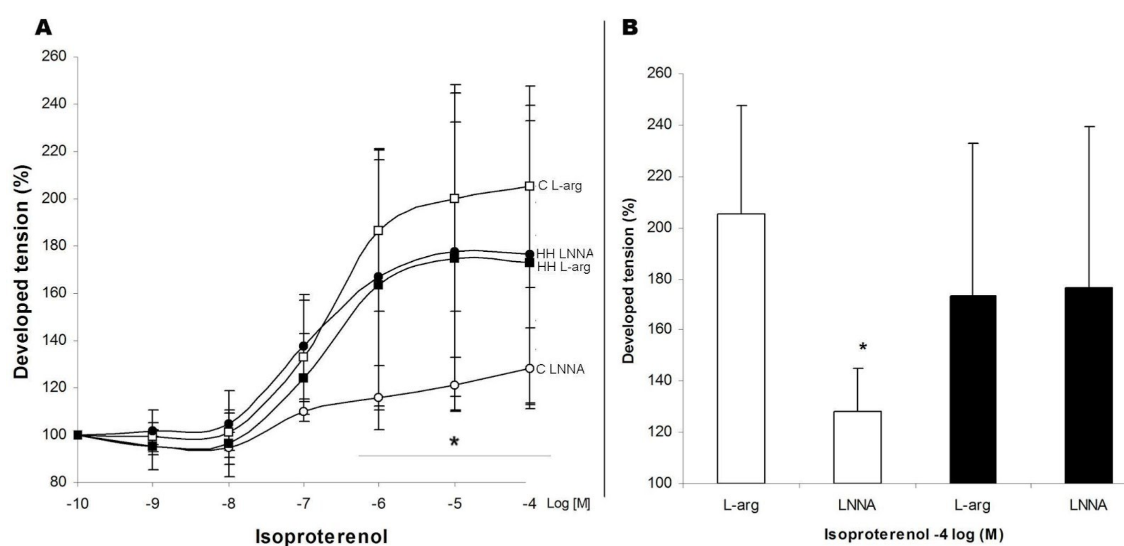

Fig. 3. Response to isoproterenol (ISO) of papillary muscles from N and HH rats expressed as the percentage increase respect to basal values taken as 100%. A: dose response curve to ISO, and B: percentage of change

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expressed in bars at maximum ISO dose. Values are means ± SD. ANOVA * $P < 0.05$ vs. all groups. Open symbols, N; Closed symbols, HH.

3. 3. 3. *Tolerance to hypoxia/reoxygenation*

At the end of 60 min of hypoxia, DT decreased similarly (93-88 %) in all the experimental groups. The recovery of contractile functions, expressed as % of pre hypoxic DT values is shown in Table 1. Similar results were observed for both normoxic and hypoxic groups.

Table 1. *Percentage of ISO- stimulated developed tension at 60 min of hypoxia and recovery during 30 min of reoxygenation in papillary muscles of rats submitted 48 h to 58.7 kPa (HH) and of the control group at 101.3 kPa (N) after L-arg or L-NNA addition*

Experimental group		60 min Hypoxia	10 min reoxygenation	20 min reoxygenation	30 min reoxygenation
N	L-arg	8 ± 5	35 ± 15	41 ± 15	49 ± 10
	L-NNA	12 ± 10	26 ± 12	34 ± 19	41 ± 12
HH	L-arg	7 ± 7	28 ± 17	41 ± 22	45 ± 19
	L-NNA	9 ± 5	25 ± 12	38 ± 19	40 ± 22

Values are means ± SD expressed as percentage.

3.4. *Cytosolic NOS expression and NO production*

Cytosolic fractions from N and HH left ventricles responded differently to anti-iNOS, anti-nNOS, anti-eNOS and anti-p-eNOS-Ser1177 antibodies (Fig. 4). As presented in Fig. 4 A and C, densitometric quantification of the western blot bands showed a significant decrease in the ratio p-eNOS-

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Ser1177/eNOS (62 %) and iNOS (32 %) protein expression and a non-significant tendency to decrease in nNOS (18 %) and eNOS (51%) expression in hypoxic hypobaric rats compared to normoxic ones (Fig. 4 B and D).

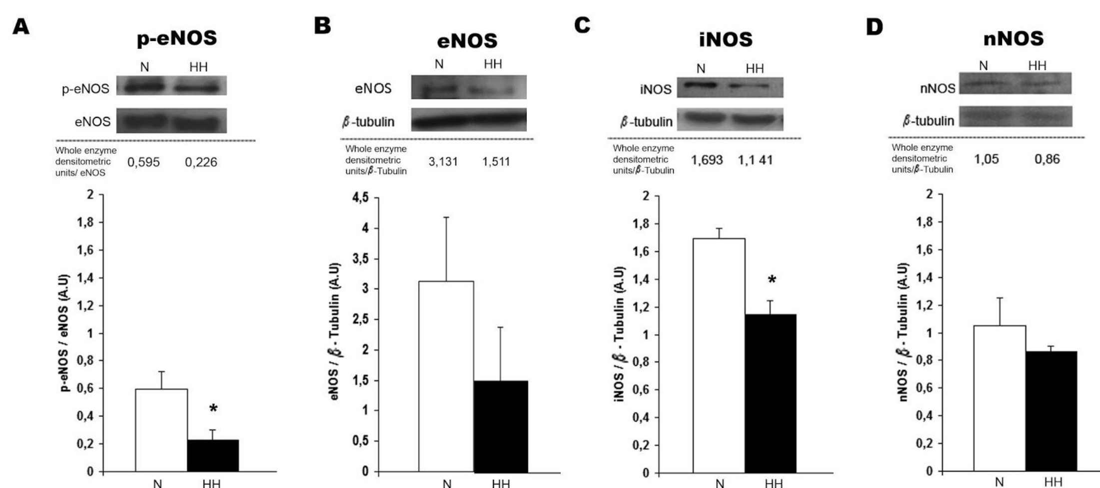

Fig. 4. (A) Left ventricle cytosolic p-eNOS-Ser1177 expression; (B) Left ventricle cytosolic eNOS expression; (C) Left ventricle cytosolic iNOS expression; (D) Left ventricle cytosolic nNOS expression. N: Normoxic group; HH: hypobaric hypoxia 48 h. Values are means ± SD. * $P < 0.05$ vs. N group.

Nitric oxide production rates were measured in cytosolic fractions of left ventricles from normoxic and hypoxic rats. Nitric oxide production was similar in both groups: 1.91 ± 1.72 nmol NO min^{-1} mg $protein^{-1}$ and 2.04 ± 0.54 nmol NO min^{-1} mg $protein^{-1}$, for normoxic and hypoxic rats, respectively (Fig. 5 A). The indirect evaluation of NO production by nitrites-nitrates detection was 1.83 ± 0.9 and 4.06 ± 3.2 nmol NO_2 / mg.protein, for normoxic and hypoxic groups, respectively (Fig. 5 B).

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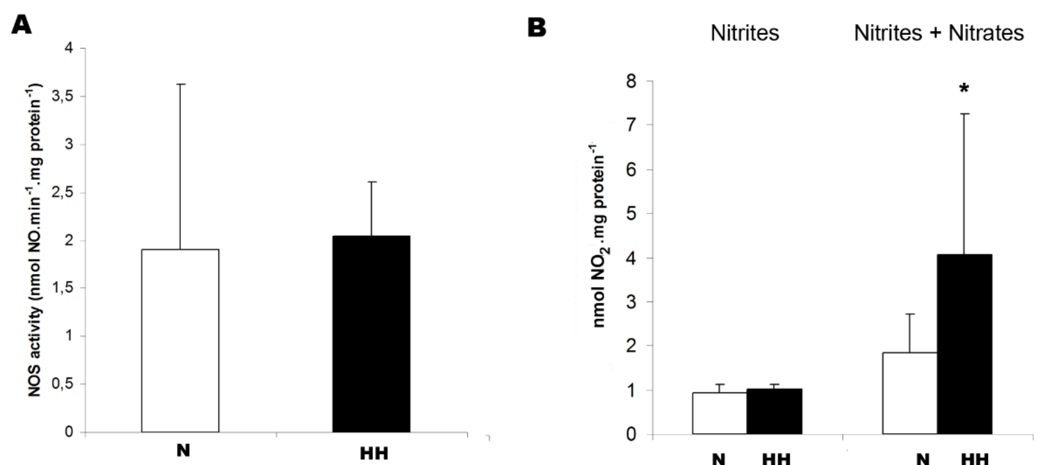

Fig. 5. (A) Left ventricle cytosolic NO production. (B) Left ventricle cytosolic nitrite-nitrate production. N: Normoxic group; HH: hypobaric hypoxia 48h. Values are means ± SD. * $P < 0.05$ vs. N group.

3. 5. *Cardiac mitochondrial function*

Mitochondrial function was assessed using two independent determinations: A) oxygen consumption and B) ATP production.

Table 2 shows oxygen consumption rates of left ventricle mitochondria isolated from both experimental groups. Mitochondria from animals exposed to hypobaric hypoxia showed similar respiratory rates to controls in, both, state 4 (resting or controlled respiration) and state 3 (active respiration, the maximal physiological rate of O_2 uptake and ATP synthesis) conditions. Accordingly, no significant changes were observed in respiratory control ratio after hypobaric hypoxia exposure, indicating the conservation of the mitochondrial inner membrane integrity and function. Furthermore, no significant changes were observed either in ATP production rates or in ATP/O ratio in hypoxic left ventricle mitochondria as compared to normoxic group. All together, these results indicated the preservation of mitochondrial function after exposure to hypobaric hypoxia. Oxygen consumption and ATP production were modulated in the same way in both groups after the addition of L-arginine. NOS substrate decreased oxygen consumption by 20% and 14% in N and HH groups, respectively. Similarly, ATP production was lowered by L-arg in the two groups: 63% and 56% for N

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and HH, respectively. As a consequence, the ATP/O ratio (calculated as ATP production rate/state 3 oxygen consumption) diminished in both groups: 52% and 44% for N and HH, respectively.

The cytochrome oxidase activity (cox) expressed as k´/min.mg.prot ± standard deviation in normoxic group was 146,3 ± 16,8 (N) and 135,3 ± 13,3 with the addition of L-arg (N-L-arg). The HH group presented 107,1 ± 20,8 (HH) and 102,4 ± 14,7 (HH-L-arg). The HH and HH-L-arg showed a decreased activity respect to normoxic tissue (* $P < 0.05$ vs N).

Table 2. *Oxygen consumption and ATP production in mitochondria from the left ventricle of rats submitted 48 h to 58.7 kPa (HH) and of their controls at 101.3 kPa (N).*

	Group	N	HH
Oxygen consumption	State 4	34 ± 14	31 ± 7
	State 3	176 ± 41	154 ± 27
	State 3+ L-arg	140 ± 38*	133 ± 25*
Respiratory control	State 3/ State 4	5.2 ± 3	5.0 ± 4
ATP production	State 3	426 ± 108	389 ± 92
	State 3 + L-arg	159 ± 56*	170 ± 78*
ATP/O	State 3	2.4 ± 0.8	2.5 ± 0.8
	State 3 + L-arg	1.1 ± 0.5*	1.4 ± 0.6

*Values are means ± SD. Oxygen consumption and ATP production were expressed in ng-at O/min.mg protein and nmol ATP/min.mg protein, respectively. * $P < 0.05$ vs. state 3 group.*

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4. Discussion

We have previously reported that acclimatization of rats to chronic hypobaric hypoxia improved cardiac tolerance to H/R, related to an increase in NO production and NOS protein expression (La Padula & Costa, 2005; Zaobornyj *et al.*, 2005; La Padula *et al.*, 2008). We showed later that young adult rats (3 mo old) exposed to acute hypobaric hypoxia (48 h at 4400 m simulated altitude) were able to develop cardioprotection involving similar mechanisms (La Padula *et al.*, 2018). The present study shows that prepubertal rats exposed to acute (48 h) HH decreased NOS expression without affecting mitochondrial oxygen consumption and ATP production, and worsening contractile economy, without improving tolerance to H/R (Fig. 6).

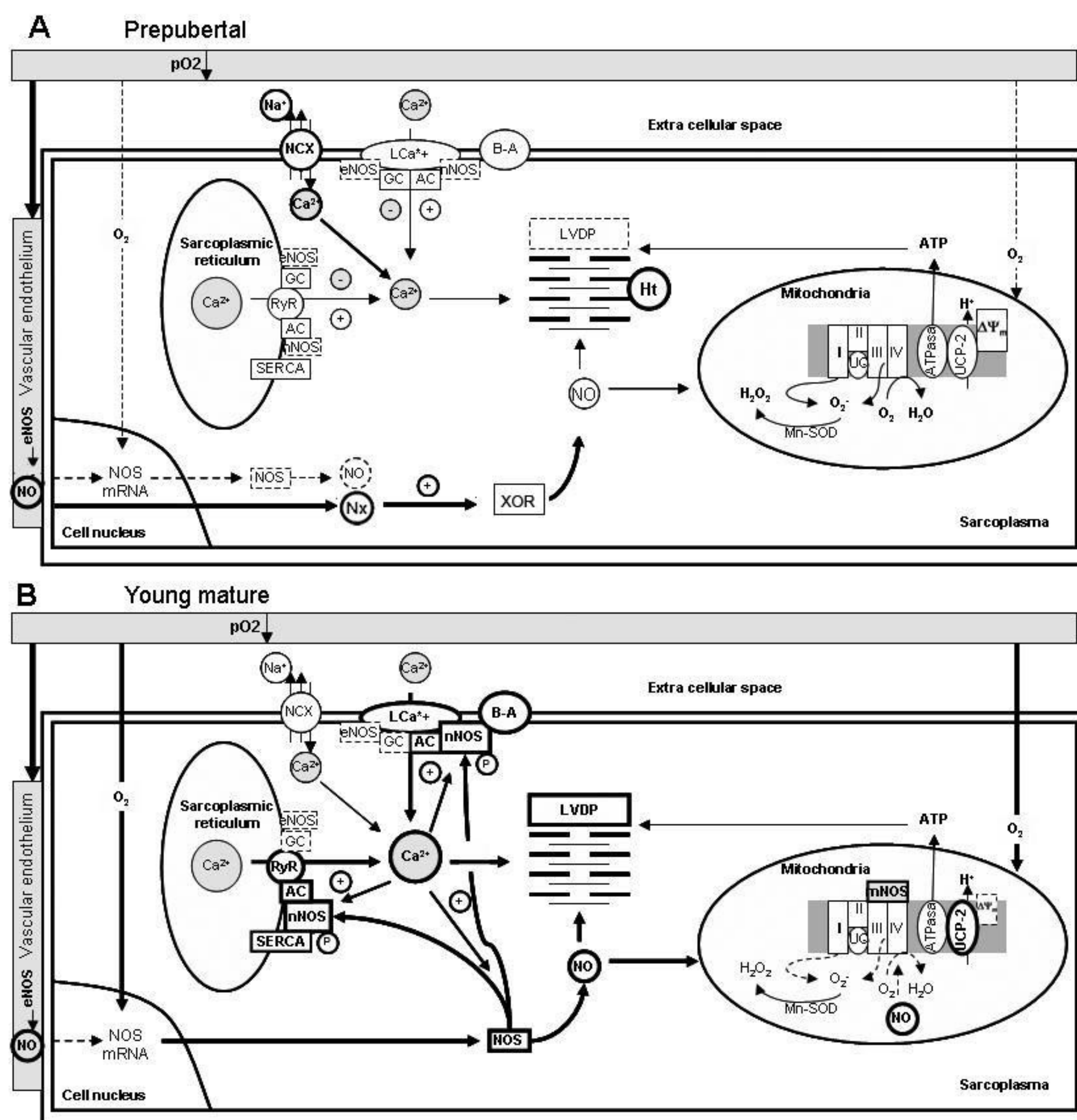

Fig.6. Hypothetical main myocyte differential molecular response activated during acute hypobaric hypoxia (HH) related to age. (A) Prepubertal. HH increased nitric oxide (NO) production by vascular endothelial cells through their own endothelial NOS (eNOS), triggering, by negative feedback over the nucleus of the

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surrounding myocytes a drop down of RNA messenger (mRNA) and nitric oxide synthase (NOS) synthesis (dash square). That drop in NO production by NOS may be compensated by the xanthine oxidoreductase (XOR), which would produce NO from the nitrites-nitrates (Nx) reservoir, coming largely from the diet (Moretti et al, 2019), and also possibly from the previous oxidized vascular NO cited above. This keeps normal intracellular NO levels, resulting in a maintenance of mitochondrial and contractile function (LVDP) as in normoxic conditions, preventing from developing endogenous cardioprotection (LVDP dash square). The increased heat (Ht) detected could be attributed to a major relative participation of active transport mechanisms like Na /Ca exchanger (NCX) and Na/K pump vs SR-Ca pump (SERCA). (B) Young mature. HH increased cytoplasmic and mitochondrial myocyte NOS synthesis (NOS and mNOS with solid square, respectively) and NO production. NO modulates mitochondrial function, as ATP availability and reduction of oxidative reactive species production (dashed lines). Also, it regulates contractile activity at lower oxygen pressure (pO_2), with cardioprotection (LVDP with solid square) related to NO nitrosylation of L-Ca Channel (L-Ca+) and ryanodine channel (RyR). Dashed lines indicate inhibition, solid lines stimulation and the width of the line the degree of stimulation: thick line: strong, thin line: steady state; B-A: Beta adrenergic receptor; AC: adenylate cyclase; GC: guanylate cyclase; UCP-2: uncoupling protein.

4.1. *Biological parameters*

Upon exposure of rats to simulated high altitude, the body weight decreased. Weight loss has been associated with hypoxia inducible factor (HIF) (Costa, 2007; Semenza, 2004*a*, 2004*b*) throughout a variation in leptin levels. This impacts in a decrease in food intake, with a marked reduction in fat content (Costa et al., 1979; Debevec *et al.*, 2016). In the present work, normoxic animals increased body weight 8% during 48 h growth, while hypoxic rats decreased body weight by 2%, resulting in the previously reported value of 10% of body weight loss by HH (La Padula *et al.*, 2018). On the other hand, the weight of the whole heart, left and right ventricles and papillary muscles areas were not affected by HH. These determinations guarantee the same physical conditions in normoxic and hypoxic rats tissues.

4.2. Mechanical and energetic responses in the whole heart

The tolerance to acute H/R evaluated by the LVP was low in prepubertal hearts from normoxic rats related to adult animals (La Padula *et al.*, 2018), and was unaffected by hypobaric hypoxia. The contractile economy evaluated as LVP/Ht was lower in hypoxic animals compared to the normoxic

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group during the whole experiment. This means that HH hearts spent more heat for the same contractile activity than control ones (Fig. 1). The increased heat could be related to active calcium ions transport systems involved in the contraction/relaxation cycle, like Na^{+}/Ca^{2+} exchanger via Na^{+}/K^{+} pump and SERCA (Chen *et al.*, 2006; Pei *et al.*, 2003; Guo *et al.*, 2011; Chen & Li, 2012; Ma *et al.*, 2014), and not to the mitochondrial respiratory chain, which appeared unaffected in these hypoxic conditions. The results obtained after addition of mitochondrial UCP-2 blockade with genipin reinforces that idea, because Ht was not decreased differentially in N and HH groups in presence of genipin. According to the "uncoupling to survive" hypothesis postulated by Brand (2000), UCP function could be involved in cardioprotective models, due to the fact that its activation leads to physiological mitochondrial depolarization and the consequent reduction of ROS production by the respiratory chain (Brand, 2000, Mookerjee et al, 2010). However, this mechanism does not seem to be involved in the present results, because HH heart does not respond to the UCP blocker genipin and it does not exhibit cardioprotection due to HH treatment. So, an increased cytosolic activation of sarcolemmal ionic transport systems by HH may be responsible for keeping cardiac contractile levels similar to N hearts working with lower oxygen levels and extra-energetic cost.

4.3. *Mechanical activity in papillary muscle. Modulation by NO*

Basal contractility in papillary muscles preparations, which are vascular tone independent, was not affected by NO, but cardiac reserve was modulated by 77% in the normoxic group (Fig. 4) probably by micro domains actions of the NO over the β-adrenergic receptor via AC-AMPc stimulation (Fig.6). Acute hypoxia treatment decreased NOS protein expression, impairing the cardiac relevance of NOS system in prepubertal rats. The percentage recovery of DT in normoxic rats after H/R (49 ± 4) was unaltered by HH treatment (45 ± 8), and it was similar to our previous studies in adult normoxic rats (51 ± 4), and opposite to the increased tolerance to H/R (78 ± 6) found after 48 h HH in adult rats (La Padula *et al.*, 2018). As mammalian fetus lives at an oxygen partial pressure corresponding to 8000

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m altitude, newborn mammals are normally adapted to hypoxia. Neonatal tolerance to oxygen deprivation seems to be primarily based on the ability to maintain tissue aerobiosis as long as possible. It seems that decreasing tolerance to ischemia during early postnatal life is counteracted by the development of endogenous protection (Oštádalová *et al.*, 1998; Oštádalová *et al.*, 2002). In our study, prepubertal rats have lost gestational acclimatization as expected and hypoxic cardioprotection cannot be evidenced yet, in contrast with results reported by other authors in younger animals (Vornanen, 1996*a*, 1996*b*; Oštádalová *et al.*, 1998; Richardson & Bocking, 1998; Oštádalová *et al.*, 2002).

4.4. NO production

At systemic level, NO main effect is to bring up peripheral vasodilation (Manukhina *et al.*, 2006; Ignarro, 2000) promoting a better supply of oxygen to tissues (Costa, 2007; Poderoso *et al.*, 1999). In the pulmonary system, NO functions to counteract the tendency to develop pulmonary hypertension (Eichstaedt *et al.*, 2015). On the other hand, increased NO production was involved in cardioprotection in adult rats after acute or chronic HH, preserving basal contractility and increasing the tolerance after one H/R episode (La Padula *et al.*, 2008, 2018). In the present work, we found that exposure of prepubertal animals to acute HH fails to induce an increase in cytosolic NO levels and to develop cardioprotection. The main NOS isoforms responsible for the establishment of cardioprotection during exposure to HH are the eNOS and nNOS throughout the modulation by cGMP and nitrosylation of ion channels like sarcolemmal calcium channels respectively, improving the mechanical heart activity. In the present work we found the three NOS isoforms decreased. The phosphorylated eNOS S1177, which stimulates the NO production (in the opposite way of the phosphorylation of residues T495 and Y657 which tends to attenuate nitric oxide generation) (Fleming, 2010), was also decreased (Fig.4 A).

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During acute hypoxia there is an early high vascular production of NO by eNOS (Chang *et al.*, 2004; Molina *et al.*, 2013) (Fig. 6). Inhibition of the myocyte cytosolic NOS expression by negative feedback, would preserve normal O_2 consumption and mitochondrial ATP production, to maintain the mechanical heart activity. On the other hand, the lack of modulation of respiratory chain by NO would impair oxygen diffusion to the deepest tissues, hindering the emergence of the hypoxic cardioprotection.

Increased nitrates found in this study, through the mammalian xanthine oxidoreductase (Jansson *et al.*, 2008; Huang *et al.*, 2010; Kapil *et al.*, 2020) may keep cytosolic NO levels invariable, compensating for the drop of NO generation by NOS isoforms (Zhang *et al.*, 1998; Dejam *et al.*, 2004; Gladwin, 2005; Gladwin *et al.*, 2005, 2006; Lundberg & Weitzberg, 2005; Jansson *et al.*, 2008; Molina *et al.*, 2013).The source of nitrates could be the vascular endothelial NO generated during the early exposure to HH, which could be oxidized and accumulated in the form of nitrates. This reservoir of nitrates could be then transformed by xanthine oxidoreductase (XOR) and proteins with nitrite reductase activity into NO to keep stable its physiological function on the mitochondrial respiration, which we found unaltered by HH.

4.5. Oxygen consumption and ATP production

Mitochondrial electron transport chain has long been suspected as a site of oxygen sensing (Guzy & Schumacker, 2006). In isolated papillary muscle, the increase in NO production was involved in the establishment of cardioprotective mechanisms developed during acclimatization to hypobaric hypoxia. This may be due to the effect of NO on mitochondrial function (La Padula & Costa, 2005; Zaobornyj *et al.*, 2005; La Padula *et al.*, 2008) in parallel to its cytosolic actions. In our previous work, cytochrome oxidase activity was decreased after 48 h of HH (La Padula *et al.*, 2018), probably modulated by NO. The present study shows that 48 h of HH did not increase NO production in prepubertal hearts, preserving O_2 consumption similar to normoxic group. This effect is in

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accordance to the unaffected ATP production, and the absence of a differential UCP-2 blocker action at the LVP mechanical activities and Ht delivered, which would keep unaltered the mechanical contractile function (Fig. 6). In this work, NO physiological effects on respiratory chain were confirmed by adding NOS substrate to the mitochondrial fraction, which decreased O_2 consumption and ATP production. Although non-statistically significant, we can see a tendency in the HH group to show a minor response to L-arg, so to present a minor modulation of the mitochondrial functions: inhibition of oxygen consumption 20 % for N and 14% for HH and 63% and 56% decrease in ATP production for N and HH respectively. So, the increased heat delivered during contraction/relaxation, must be sought outside the mitochondria, possibly at the active sarcolemma ions transport systems (Vornanen, 1996*b*; Guo *et al.*, 2011; Chen & Li, 2012; Ma *et al.*, 2014; Ponce-Hornos *et al.*, 1993), such as Na^+-Ca^{2+} exchanger and Na^+- K^+ pump, which are involved in the active control of calcium overload during exposure to HH (Pei *et al.*, 2003; Chen *et al.*, 2006). Finally, COX activity was significantly decreased in HH, may be following a tendency in the same way of O_2 consumption and ATP production.

Conclusion

The degree of somatic maturation modifies the response to HH. Immature animals exposed to acute hypoxia maintained normal NO production in spite of the decreased NOS expression, possibly through the accumulation of nitrates as a NO source, and then by the NO production by the nitrates-nitrites-nitric oxide pathway (Gladwin, 2005; Jansson *et al.*, 2008; Molina *et al.*, 2013). In this sense, HH prepubertal heart keeps O_2 consumption, ATP production and UCP-2 activity at physiological levels, preserving cardiac mechanical activity as normoxic animals with a high energetic cost, modifying contractile economy and without the ability to develop endogenous cardioprotection (Fig. 6). The lack of cardioprotection in the present model could be attributed to unaltered NO levels and lower expression of NOS, which would impact at cellular specific micro domains, close to active ions

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systems (Hare, 2003), probably involved in the high heat delivered during cardiac mechanical activity. Once more, this work presents evidence that cardiac tolerance to hypoxia/reoxygenation would be closely related to the NO system, but it depends on the degree of maturity of the organism. The present results are important for the translation to the experimental clinic, because the prepubertal organism does not respond as the canonical HH-NO-cardioprotective pathway in experimental models.

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Author Contributions

PHLP and LEC design of the experiments.

PHLP, AC, PB, VV and BP performed experiments and data analysis.

PHLP and AC wrote the manuscripts with input from all the authors mainly LEC and SLA.

All the authors discussed the results and contributed to the final version of the manuscript.

All authors have read and approved the final version of this manuscript and agree to

be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

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Data Availability Statement

The data that support the findings of this study are available from the corresponding author, upon reasonable request.

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