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Mitochondrial nitric oxide metabolism during rat heart adaptation to high altitude: effect of sildenafil, L-NAME, and L-arginine treatments

Tamara Zaobornyj,[1] Laura B. Valdez,[1] Darío E. Iglesias,[1] Manuel Gasco,[2] Gustavo F. Gonzales,[2] and Alberto Boveris[1]

[1]*Laboratory of Free Radical Biology, School of Pharmacy and Biochemistry, University of Buenos Aires, Buenos Aires, Argentina; and* [2]*Facultad de Ciencias y Filosofía, Departamento de Ciencias Biológicas y Fisiológicas, Instituto de Investigaciones de la Altura, Universidad Peruana Cayetano Heredia, Lima, Peru*

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Zaobornyj T, Valdez LB, Iglesias DE, Gasco M, Gonzales GF, Boveris A. Mitochondrial nitric oxide metabolism during rat heart adaptation to high altitude: effect of sildenafil, L-NAME, and L-arginine treatments. *Am J Physiol Heart Circ Physiol* 296: H1741–H1747, 2009. First published April 3, 2009; doi:10.1152/ajpheart.00422.2008.—Rats submitted to high altitude (Cerro de Pasco, Perú, 4,340 m, P_{O_2} = 12.2 kPa) for up to 84 days showed a physiological adaptive response with decreased body weight gain (15%), increased right ventricle weight (100%), and increased hematocrit (40%) compared with sea level animals. These classical parameters of adaptation to high altitude were accompanied by an increase in heart mitochondrial enzymes: complexes I-III activity by 34% and mitochondrial nitric oxide synthase (mtNOS) activity and expression by >75%. The hyperbolic increase for mtNOS activity during adaptation to high altitude was similar to the observed pattern for hematocrit. Hematocrit and mtNOS activity mean values correlated linearly (r^2 = 0.75, $P \leq 0.05$). Chronic treatment for 28 days with sildenafil (50 $mg \cdot kg^{-1} \cdot day^{-1}$) decreased the response of mtNOS to high altitude by 25%. Conversely, N^G-nitro-L-arginine methyl ester treatment (8.3 $mg \cdot kg^{-1} \cdot day^{-1}$) increased such response by 40%, whereas L-arginine treatment (106 $mg \cdot kg^{-1} \cdot day^{-1}$) had no effect. Nitric oxide (NO) production by mtNOS accounts for ~49% of total cellular NO production in sea level rats and for ~54% in rats exposed to high altitude for 84 days. It is concluded that mtNOS is a substantial source of cardiac NO, a factor in the adaptive response to sustained heart hypoxia that is susceptible to be modified by pharmacological treatments.

mitochondrial nitric oxide synthase activity; mitochondrial nitric oxide synthase expression; mitochondrial respiratory complexes; hematocrit; nitro-L-arginine methyl ester

HIGH ALTITUDE IS A MULTIFACTORIAL source of stress in which hypobaric hypoxia is the most important component. The O_2 gradient between atmospheric air and cells decreases from 105 mmHg at sea level to 49 mmHg at high altitude (20). There are a number of adaptive responses that are triggered by this situation, such as increased ventilation (28), pulmonary hypertension (40), erythropoiesis (39), and heart work load. The adaptation to high altitude constitutes a situation that has both advantageous and disadvantageous consequences for human health. The main long term effects are decreased physical activity and life span. Among the beneficial effects, there is a recognized cardioprotection with improvement of the myocardial tolerance to ischemic episodes. A number of studies involving exposure to natural (22) or simulated (29) hypobaric hypoxia reported that this adaptation is associated to lower incidence and smaller size of myocardial infarction (30).

Although the mechanism by which adaptation to hypoxia has cardioprotective effects remains not elucidated, nitric oxide (NO) has been extensively proposed as one of the molecular messengers involved in the process (19, 25, 50). Indeed, highlanders show augmented levels of NO catabolites in blood (41). In addition, L-arginine infusion increases arterial O_2 saturation at high altitude, and it decreases acute mountain sickness score in cases of acute mountain sickness (42). Therefore, the adaptive mechanisms of the cardiovascular system seem to trigger the activation of NO synthesis in the cells and tissues involved in the adaptation to high altitude.

NO is an intercellular messenger and an intracellular regulator of respiration and cellular processes. At submicromolar concentrations, it exhibits two main effects on the mitochondrial respiratory chain: the competitive inhibition of cytochrome oxidase (1, 8, 11) and the stimulation of superoxide anion ($O_2^{\cdot-}$) production by inhibition of electron transfer at complex III (38). This phenomenon is readily observed in isolated mitochondria where supplementation with L-arginine or with L-NMMA modulates O_2 consumption and hydrogen peroxide (H_2O_2) production. These effects have been named mitochondrial nitric oxide synthase (mtNOS) functional activity, as a description of mtNOS ability to regulate respiration (48).

Since mtNOS was first described (17, 18), its relevance in mitochondrial bioenergetics and physiology has been increasingly recognized. mtNOS activity has been found to be regulated by important physiological effectors; it is downregulated by ANG II (6) and thyroid hormones (15) and upregulated by insulin (16), the autonomic system (15), and during cold acclimation (37). Environmental O_2 pressure has been recognized as a physiological regulator of heart mtNOS activity. Rats kept in a hypobaric chamber at 53.8 kPa for 2–18 mo showed up to 60% increased mtNOS activity (47), associated with a retardation of the decline in the papillary muscle mechanical activity upon aging and with an improved recovery after anoxia-reoxygenation (50).

Previous work from our laboratory showed that exposure to high altitude (4,340 m, P_{O_2} = 12.2 kPa) for up to 21 days linearly increased heart mtNOS activity up to 58%, whereas heart cytosolic endothelial nitric oxide synthase (eNOS) and liver mtNOS activities were not affected (19). In this study, the observation is extended to 84 days with an analysis of the kinetics of the adaptive response considering classical physiological parameters such as body and organ weight and hematocrit, together with mtNOS activity and expression and the

Address for reprint requests and other correspondence: T. Zaobornyj, Fisicoquímica, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Junín 956, C1113AAD, Buenos Aires, Argentina (e-mail: tamaraz@ffyb.uba.ar).

activity of the mitochondrial electron transfer complexes. A growing number of studies in the last five years indicate that sildenafil, as a selective inhibitor of cGMP-specific phosphodiesterase 5 (PDE5), has a preconditioning-like cardioprotective effect in animal models of ischemia-reperfusion. Thus the goal of this work was to evaluate if the adaptive response involving heart mtNOS activity is affected by pharmacological treatment with drugs such as sildenafil, L-NAME, or L-arginine, which are involved in NO metabolism of mammalian organs.

MATERIALS AND METHODS

Chemicals. Chemicals were purchased from Sigma Chemical (St. Louis, MO). Antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Other reagents were of analytical grade.

Experimental design. Male Holtzman rats (240–310 g) from the animal facility of the Universidad Peruana Cayetano Heredia were housed under standardized conditions. Rats were divided at random into two groups, one was maintained at sea level (Lima, 150 m of altitude, Patm = 97.1 kPa, Po_2 = 20.6 kPa) and the second was kept at Cerro de Pasco [4,340 m of altitude, atmospheric pressure (Patm) = 58.2 kPa, Po_2 = 12.2 kPa]. Rats, both at sea level and at high altitude, were maintained in a temperature-controlled environment at 25°C under a 12:12-h light-darkness cycle and provided with Purina laboratory chow and water ad libitum.

Animals were killed by decapitation, and blood was collected from the cervical trunk. Hearts were removed immediately after death, weighed, frozen at 77 K (liquid nitrogen), and kept at that temperature and at 195 K (dry ice for air transport from Lima to Buenos Aires) until mitochondrial isolation.

The experimental protocol was approved by the Ethics Committee for Human and Animal Experimentation of the Universidad Peruana Cayetano Heredia (Lima, Peru) and is in accordance with the American Physiological Society's "Guiding Principles in the Care and Use of Animals."

Pharmacological treatments. Rats were randomly divided into the following eight groups: four groups of rats were maintained at sea level and four other groups at high altitude. At sea level and at high altitude, there was a control group, a group treated with sildenafil (50 $mg \cdot kg^{-1} \cdot day^{-1}$), a group treated with N^G-nitro-L-arginine methylester hydrochloride (L-NAME, 8.3 $mg \cdot kg^{-1} \cdot day^{-1}$), and a group treated with L-arginine (106 $mg \cdot kg^{-1} \cdot day^{-1}$). Rats were implanted with a gastric cannula daily to receive the corresponding drug dose.

Hematocrit. Hematocrit was determined using nonheparinized capillaries immediately after rats were killed, and blood samples were centrifuged at 1,000 *g* for 5 min.

Isolation of mitochondria and preparation of mitochondrial membranes from frozen heart. Hearts were thawed, and ventricles were excised and weighted. Organs were chopped and homogenized in an ice-cold homogenization medium (1/10) containing 0.23 M mannitol, 0.07 M sucrose, 1 mM EDTA, and 10 mM Tris·HCl, pH 7.4, for 30 s with a blade homogenizer (Kendro-Sorvall-Du Pont, Asheville, NC) and by five strokes in a glass-Teflon homogenizer. All of these operations were carried out at 2–4°C. The homogenates were centrifuged at 700 *g* for 10 min to discard nuclei and cell debris, and the supernatant was centrifuged at 7,000 *g* for 10 min (5). The supernatant of this centrifugation was used to determine nitric oxide synthase (NOS) activity in the postmitochondrial supernatant. The mitochondrial pellet was washed, resuspended in the homogenization medium, and frozen. The preparation to measure mitochondrial enzyme activities was obtained by twice freezing and thawing the mitochondrial preparation and homogenization by passage through a 25-gauge hypodermic needle (4). Protein concentration was determined with the Folin reagent and BSA as standard.

NO production. NO production was measured in mitochondrial membranes and in the postmitochondrial supernatant by following at 37°C the oxidation of oxyhemoglobin to methemoglobin, at 577–591 nm (ε = 11.2 $mM^{-1} \cdot cm^{-1}$) using a Beckman DU 7400 diode array spectrophotometer (4, 31). The reaction medium consisted of 50 mM phosphate buffer, pH 7.4, 1 mM L-arginine, 1 mM $CaCl_2$, 100 μM NADPH, 10 μM dithiothreitol, 4 μM Cu,Zn-superoxide dismutase, 0.1 μM catalase, 30 μM oxyhemoglobin heme, and 0.2–0.6 mg protein/ml. Control determinations adding 3 mM L-N^G-monomethyl-L-arginine (L-NMMA) and omitting L-arginine were performed to consider only L-NMMA-sensitive hemoglobin oxidation as due to NO formation. The absorbance changes that were inhibitable by L-NMMA (90–95%) were expressed as nanomoles NO per minute per milligram protein.

Mitochondrial electron transfer activities. The membrane-bound activities of NADH-cytochrome *c* reductase (complexes I-III), succinate-cytochrome *c* reductase (complexes II-III), and cytochrome oxidase (complex IV) were determined spectrophotometrically (550 nm, ε = 19 $mM^{-1} \cdot cm^{-1}$) at 30°C with mitochondrial membranes suspended in 100 mM phosphate buffer, pH 7.4 (33). For complexes I-III or II-III activities, mitochondrial membranes (0.25 mg protein/ml) were added with 0.2 mM NADH or 5 mM succinate, 25 μM cytochrome c^{3+} and 0.5 mM KCN. Cytochrome oxidase activity was determined in the same buffer added with 60 μM cytochrome c^{2+}. Reduced cytochrome *c* was prepared by reduction of cytochrome c^{3+} with sodium dithionite, followed by Sephadex G-25 chromatography. The rate of cytochrome c^{2+} oxidation was calculated as the pseudo-first order reaction constant, k'/mg protein.

Western blotting. The proteins were separated by SDS-PAGE (7.5%), blotted into nitrocellulose films, and probed with 1:500 diluted rabbit polyclonal anti-neuronal nitric oxide synthase (nNOS) (H-299, R-20, and K-20 antibodies) directed to different regions of NOS 1 protein or anti-inducible nitric oxide synthase (iNOS) (C-19) directed to the COOH-terminal region of NOS 2, and with a secondary anti-rabbit antibody (dilution 1:5,000) conjugated with horseradish peroxidase and revealed by chemiluminescence using enhanced chemiluminescence reagent (9, 19).

Statistics. Results are expressed as means ± SE. Dunnet's *t*-test was used to analyze the significance of differences between paired groups. The 5% probability level was used as a criterion for biological significance.

RESULTS

Body weight, heart weight, and right ventricle hypertrophy. At the end of exposure (84 days), sea level rats showed an overall weight increase of 35%, whereas rats exposed to high altitude showed an increase of only 20% (data not shown). Body weight gain was calculated for each animal as the difference between weight at a given exposure time and the initial weight (Fig. 1). Animals exposed to high altitude responded with a decrease of body weight during the first week. After 14 days, high altitude rats started to gain weight with a rate about similar to that showed by sea level rats.

The heart weight of rats exposed to high altitude for 84 days showed values 20% higher than those of sea level animals (Table 1). To note, heart and liver (data not shown) weight decreased by ~15% during the first week of exposure as a consequence of the general dehydration and anorexia suffered by the animals exposed to high altitude.

Right ventricle hypertrophy was noticeable and significant at 42 and 84 days of exposure, with weight values 56 and 100% higher in high altitude animals than in sea level rats, and with an approximate half-time ($t_{1/2}$) of 33 days (Table 1). Moreover, right ventricle hypertrophy was also evidenced by ratios of the right ventricle expressed as a percentage of heart weight. At *days 42* and *84* of exposure to high altitude, right ventricle

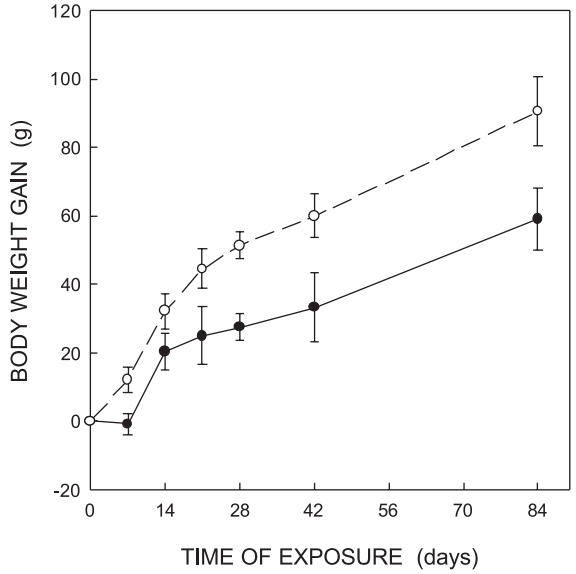

Fig. 1. Body weight gain in rats exposed to high altitude (●) and in control rats at sea level (○). Body weight gain was calculated as the difference between the rat weight at each time and at the beginning of the study. Values are expressed as means ± SE (n = 6 rats).

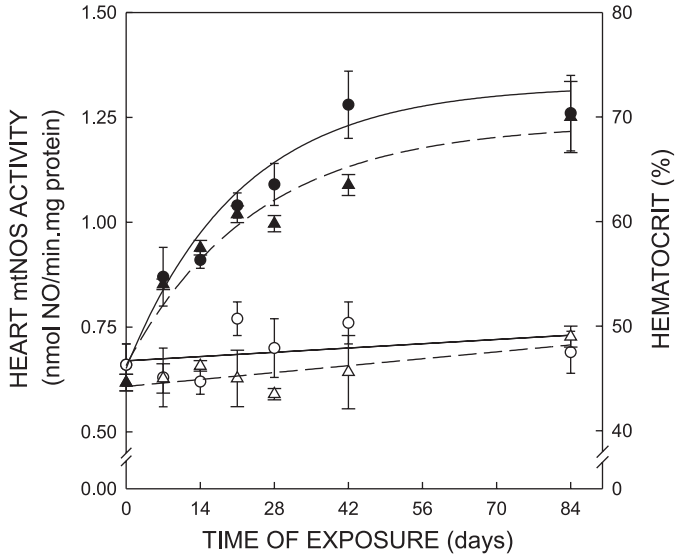

Fig. 2. Heart mitochondrial nitric oxide synthase (mtNOS) activity (○, ●) and hematocrit (△, ▲) of rats exposed to high altitude (●, ▲) and at sea level (○, △). Values are expressed as means ± SE (n = 6).

represented 33 and 34% of heart weight, values that were 50 and 70% higher than in sea level animals. Conversely, left ventricle weight did not exhibit significant differences between both groups of rats.

Heart mtNOS activity. Heart mtNOS enzymatic activity was markedly and significantly increased in mitochondrial membranes of rats exposed to high altitude for 7–84 days, showing a hyperbolic response with an approximate $t_{1/2}$ of 15 days (Fig. 2). After 42 days of exposure, this activity was 75% higher in high altitude than in sea level animals. A linear relationship between mtNOS activity and time of exposure during 21 days at high altitude was previously reported (19). The present results show a hyperbolic response of mtNOS activity in high altitude adaptation, by extending the exposure to 84 days. On the contrary, animals living at sea level showed no changes in heart mtNOS activity.

As shown in Fig. 2, the pattern of mtNOS activity was similar to the hyperbolic increase in hematocrit during adaptation to high altitude: hematocrit values of exposed rats showed a $t_{1/2}$ of 16 days and were up to 40% higher than sea level values after 84 days of exposure. Sea level animals showed no significant differences in hematocrit throughout the study.

Heart mtNOS expression. The expression levels of NOS in heart mitochondria were determined by Western blot using several anti-nNOS and anti-iNOS antibodies. It is worth noting that a band with a molecular mass between 116 and 205 kDa was recognized in heart mitochondria with the anti-nNOS (H-299) antibody, directed to an epitope corresponding to amino acids 2–300 mapping at the NH_2-terminus of nNOS (Fig. 3). Rats exposed to high altitude for 7–28 days showed an enhancement in heart mtNOS expression compared with sea level animals. The densitometric quantitation of the Western blot bands indicated an increase of between 30 and 100% in the expression of heart mtNOS in rats exposed to high altitude compared with those in sea level rats. This increase was in agreement with the observed increase in mtNOS biochemical activity. Neither an anti-nNOS raised against a peptide mapping near the COOH-terminus of NOS 1 [NOS 1 (R-20) antibody], nor an anti-nNOS raised against a peptide mapping near the NH_2-terminus of NOS 1 [NOS 1 (K-20) antibody] showed specific reactivity with heart mitochondrial fractions (data not shown).

In the conditions of the study, and as it was shown earlier by us (4, 19), heart mitochondrial membranes also reacted with anti-iNOS antibodies (C-19, raised against a peptide mapping near the COOH-terminus of iNOS). In this case, the expression

Table 1. *Heart and right ventricle weight of rats exposed to high altitude (Po_2 = 12.2 kPa) and sea level (Po_2 = 20.6 kPa)*

	Heart Weight, g		RV Weight, g		RV/H, %	
Time of Exposure, days	Po_2 20.6 kPa	Po_2 12.2 kPa	Po_2 20.6 kPa	Po_2 12.2 kPa	Po_2 20.6 kPa	Po_2 12.2 kPa
7	0.86±0.01	0.75±0.03*	0.19±0.05	0.22±0.03	22±3	29±1
14	0.99±0.01	0.89±0.01*	0.21±0.04	0.25±0.04	21±2	28±2
21	1.02±0.02	0.94±0.02	0.22±0.01	0.25±0.02	22±1	27±1
28	1.03±0.02	0.91±0.02	0.21±0.03	0.29±0.03	20±1	32±1
42	1.05±0.05	1.09±0.03	0.23±0.01	0.36±0.01*	22±1	33±1
84	1.03±0.05	1.24±0.05*	0.21±0.01	0.42±0.02*	20±1	34±1

Values are means ± SE. RV/H, right ventricle weight expressed as a percentage of heart weight. *$P < 0.05$ vs. sea level rats. At *day 0*, heart weight of rats at sea level and at high altitude was 0.82 ± 0.02 g. Ratios were calculated considering 4–6 independent determinations.

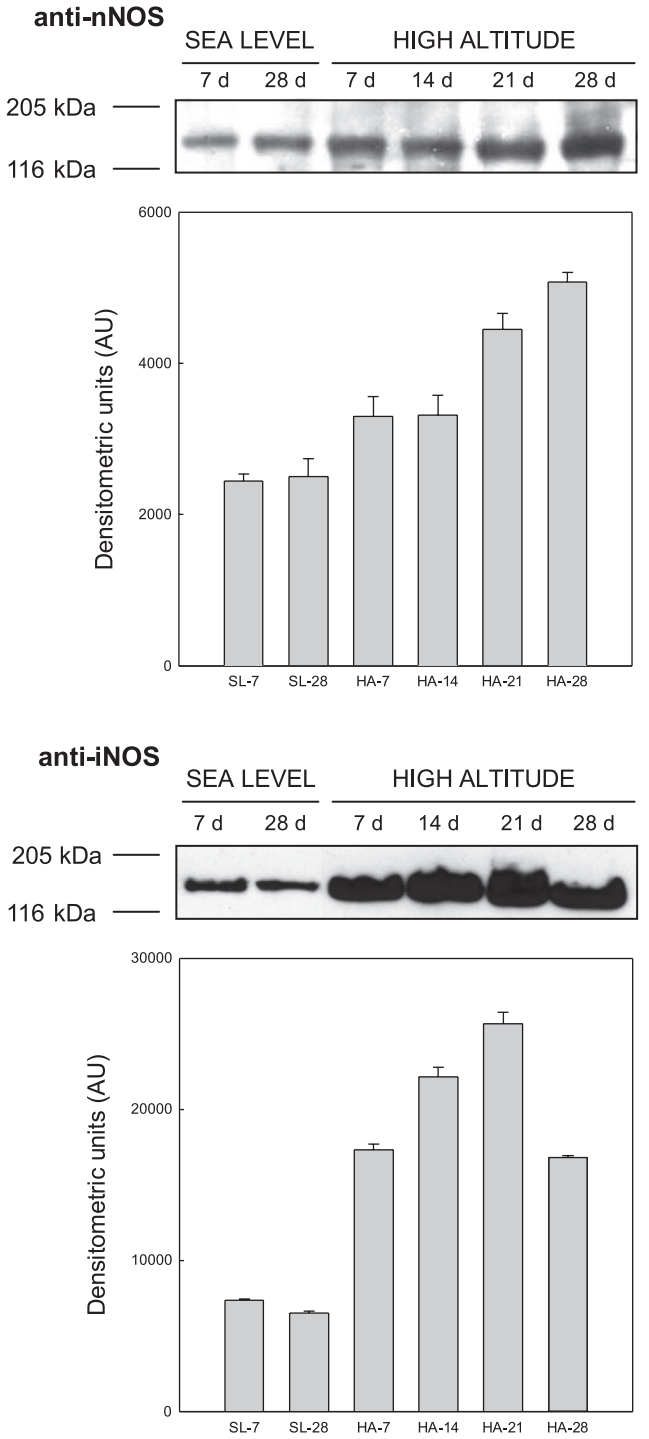

Fig. 3. Western blot analysis of heart mitochondrial membranes of rats exposed for 7–28 days to high altitude and rats maintained at sea level, using anti-neuronal nitric oxide synthase (nNOS) antibodies (H-299; Santa Cruz Biotechnology) and anti-inducible nitric oxide synthase (iNOS) antibodies (C-19; Santa Cruz Biotechnology).

levels of heart mtNOS of rats exposed to high altitude resulted between 140 and 270% higher than mtNOS of sea level rats (Fig. 3).

Activities of mitochondrial complexes I-III, II-III, and IV. The activities of the respiratory complexes of heart mitochondria were determined to assess overall mitochondrial function

Table 2. *Effect of high altitude (P_{O_2} = 12.2 kPa) on the enzymatic activity of rat heart mitochondrial respiratory complexes*

Time of Exposure, days	P_{O_2}, kPa	Complexes I-III, nmol·min^{-1}·mg protein^{-1}	Complexes II-III, nmol·min^{-1}·mg protein^{-1}	Complex IV, 1·min^{-1}·mg protein^{-1}
0	20.6	123±5	15.9±1.4	18.5±1.5
	12.2	124±4	14.2±1.5	20.6±1.7
21	20.6	127±4	16.6±1.6	19.0±1.3
	12.2	149±4*	14.8±1.3	21.2±1.9
42	20.6	128±6	15.5±1.1	16.2±1.1
	12.2	158±5*	15.8±1.4	17.5±1.9
84	20.6	124±5	16.3±1.9	18.2±1.6
	12.2	166±5*	18.7±2.3	21.5±1.9

Values are means ± SE; n = 6 rats. *$P < 0.05$ vs. sea level.

in rats exposed to high altitude. NADH-cytochrome *c* reductase activity, which accounts for the integrated activities of complexes I and III, of rats exposed to high altitude for up to 84 days was 34% higher than that of rats kept at sea level with an approximate $t_{1/2}$ of 17 days (Table 2). On the contrary, the activities of succinate-cytochrome *c* reductase (complexes II and III) and cytochrome oxidase (complex IV) were not affected by exposition to high altitude. In agreement, cytochrome aa_3 and *c* contents were similar in heart mitochondria of animals exposed to sea level and high altitude (data not shown).

Effect of sildenafil, L-NAME, and L-arginine on heart mtNOS activity and hematocrit. The duration of the treatment was selected taking into account that, at 28 days, the upregulation of mtNOS activity was present without significant right ventricle hypertrophy, an effect that is a consequence of the operation of heart remodeling processes.

Regarding the effect of the drugs on the mtNOS activity response of rats exposed to high altitude (Fig. 4), neither of the treatments assayed modified the hyperbolic adaptive response.

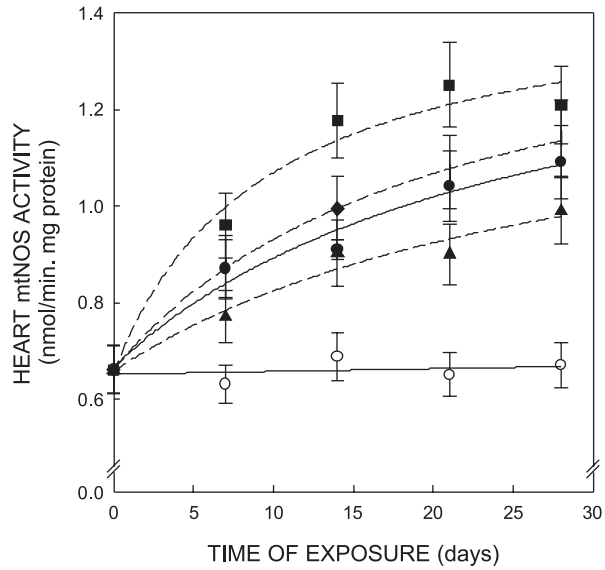

Fig. 4. Heart mtNOS activity of rats exposed to high altitude (●, ▲, ■, ◆) or maintained at sea level (○). Untreated (○, ●), treated with sildenafil (50 mg·kg^{-1}·day^{-1}; ▲), N^G-nitro-L-arginine methyl ester (8.3 mg·kg^{-1}·day^{-1}; ■) and L-arginine (106 mg·kg^{-1}·day^{-1}; ◆). Values are expressed as means ± SE (n = 6).

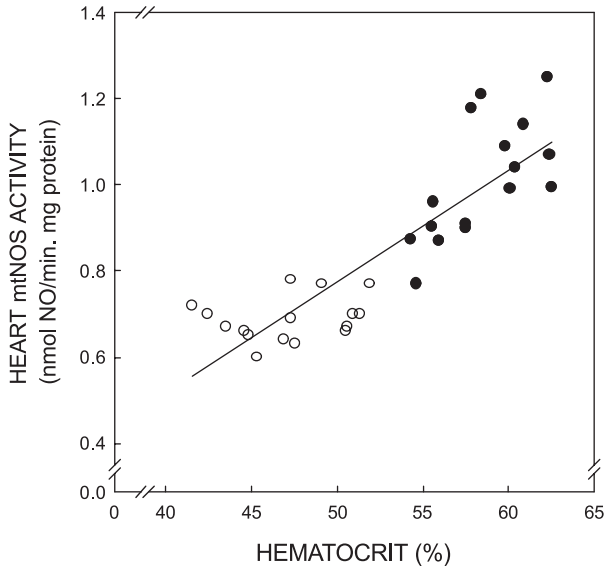

Fig. 5. Linear correlation between hematocrit and heart mtNOS activity of high altitude (●) and sea level (○) rats ($r^2 = 0.75$, $P \leq 0.05$).

Interestingly, the increase in mtNOS activity was different in the animals treated with sildenafil and L-NAME. Taking into account that heart mtNOS activity of rats exposed to high altitude for 28 days was 65% higher than the activity of animals at sea level, the combination of high altitude and L-NAME produced a faster enhancement in mitochondrial NO production, with values that were 90% higher than those of sea level animals after 28 days of exposure and treatment. L-Arginine produced no significant modification of mtNOS activity response to high altitude. Conversely, sildenafil treatment impaired this response with an overall enhancement that was only ~50%. Considering mtNOS activity of rats exposed to high altitude for 28 days, the adaptive response was increased by 40% in the animals treated with L-NAME, whereas it was decreased by 25% in the animals treated with sildenafil.

Furthermore, all of the groups maintained at high altitude showed the hyperbolic response of hematocrit as a function of time of exposure (data not shown). Figure 5 shows the correlation of hematocrit and heart mtNOS activity, considering all of the groups, with a positive linear relationship between those two adaptation indicators ($r^2 = 0.75$, $P < 0.05$).

DISCUSSION

When tissues are challenged by hypoxia, the expression of a number of physiologically important proteins such as erythropoietin, vascular endothelial growth factor, and glycolytic enzymes is increased (43). One remarkable effect observed during the exposure of rats to high altitude at Cerro de Pasco (4,340 m, P_{O_2} = 12.2 kPa, Patm = 58.2 kPa) was the parallel hyperbolic increase of mtNOS activity and hematocrit, with a similar $t_{1/2}$ of ~15 days. The similar time course observed for the increase in mtNOS activity and hematocrit and the linear correlation shown by both parameters suggest that a common signaling pathway is involved in the transcription of the genes encoding for erythropoietin (44) and mtNOS. The enhancement of mtNOS activity (75%) was accompanied by an increase in NOS protein expression as detected by anti-nNOS and anti-iNOS antibodies. Mitochondrial respiratory chain enzyme activities and cytochrome content were mostly unchanged, except for complex I activity that exhibited a 34% increase with a $t_{1/2}$ of 17 days. Complex I and mtNOS are mitochondrial enzymatic activities selectively decreased during different physiopathological processes such as senescence (33).

Ten years after the first reports about mtNOS activity (17, 18), the immunoreactivity of this enzyme is an issue that remains under debate (7, 26). There is positive and consistent evidence indicating that mtNOS is an integral protein of the inner mitochondrial membrane and the α splice variant of the nNOS isoform, with posttranslational modifications: acylation with myristic acid at the NH_2-terminal sequence and phosphorylation at the COOH-terminal region (13). Moreover, Kanai and coworkers (23) showed the presence of a NOS activity in mouse heart mitochondria by measuring the NO production in a single mitochondrion with a porphyrinic microsensor. The similarity of mtNOS to the neuronal isoform was deduced by the absence of NO production in the mitochondria of $nNOS^{-/-}$ mice. In this study, we detected an increased heart mtNOS expression after rat exposure to high altitude using anti-nNOS and anti-iNOS antibodies. It has been shown previously that heart mitochondria exhibit immunoreactivity with anti-iNOS antibodies (15, 19, 47). By means of a similar approach to the one of this study, La Padula et al. (27) recently reported a decrease in heart mtNOS expression during the regression of the cardioprotection conferred by hypoxia, using not only anti-nNOS but also anti-iNOS in Western blot experiments. The 51–57% homology reported for nNOS, iNOS, and eNOS, and the cross-reactivity of isoform-specific anti-NOS antibodies could contribute to explain the conflicting results (24, 26).

The relevance of mitochondrial NO production for heart function was assessed by estimating the relative contribution of heart mtNOS to total cellular NO production after 84 days of the study with rats exposed to high altitude or sea level (Table 3).

Table 3. *Mitochondrial contribution to heart NO production of rats exposed to high altitude (P_{O_2} = 12.2 kPa) and to sea level (P_{O_2} = 20.6 kPa) for 84 days*

P_{O_2}, kPa	Fraction	NOS Activity		Complex IV Activity, $1 \cdot min^{-1} \cdot mg\ protein^{-1}$
		nmol $NO \cdot min^{-1} \cdot mg\ protein^{-1}$	nmol $NO \cdot min^{-1} \cdot g\ tissue^{-1}$	
20.6	Mitochondrial membranes	0.74±0.08*	37.1±2.5	18.2±1.6†
	Postmitochondrial supernatant	0.48±0.03		3.7±0.2
	Extra mitochondrial cytoplasm	0.41	39.0	
12.2	Mitochondrial membranes	1.31±0.09*	65.5±1.8	21.5±1.9†
	Postmitochondrial supernatant	0.72±0.03		3.9±0.5
	Extra mitochondrial cytoplasm	0.58	55.6	

Values are means ± SE. NO, nitric oxide; NOS, nitric oxide synthase. *Data from Fig. 2. †Data from Table 2.

Complex IV (i.e., cytochrome oxidase) activity, specifically located in the mitochondrial inner membrane, was used to estimate the amount of mitochondrial protein present in the postmitochondrial supernatant as a consequence of the unavoidable mitochondrial rupture during the isolation of these organelles. The total NOS activity in heart is considered as the sum of the mtNOS activity (determined in mitochondrial membranes and in the postmitochondrial supernatant) and of other NOS activities located in the sarcoplasmic reticulum and in the caveolae (21) and also present in the postmitochondrial supernatant. Considering the determined protein contents of 50 mg mitochondrial protein/g heart (12, 19), and of 95 mg extra mitochondrial cytoplasmic protein/g heart (19), both mtNOS and extra mtNOS activities can be expressed as a function of heart mass. NO production by mtNOS accounts for 49% of total cellular NO production in sea level rats (37.1 of 76.1 nmol $NO \cdot min^{-1} \cdot g\ tissue^{-1}$) and for 54% in rats exposed to high altitude for 84 days (65.5 of 121.1 nmol $NO \cdot min^{-1} \cdot g\ tissue^{-1}$). The data indicate that mtNOS constitutes a substantial source of NO in cardiomyocytes and in heart adaptation to high altitude.

Considering the pivotal role of NO in the signaling processes that lead to heart adaptation to high altitude and the remarkable contribution of mtNOS to total heart NO production, we studied the effect of three drugs involved in NO metabolism on heart adaptation to high altitude. L-NAME and L-arginine were used as NOS inhibitor and substrate, respectively, whereas sildenafil was tested as a selective inhibitor of cGMP-specific PDE5. L-NAME had a moderate effect on the response of heart mtNOS activity to high altitude, enhancing it by ~40%, suggesting that sustained inhibition of mtNOS and the consequent decreased NO steady-state concentration in the cardiomyocytes result in an upregulation of mtNOS biosynthesis. Sildenafil treatment decreased mtNOS response to high altitude by 25%, suggesting that increased cGMP levels contribute to the development of the adaptive mechanisms triggered by heart hypoxia.

Of note, the extent of the treatment phase of the study that used L-NAME, L-arginine, and sidenafil was not enough to determine the drug effect on ventricle hypertrophy, since hypertrophy required a minimum of 42 days of exposure to be noticeable and significant (Table 1). Nevertheless, several groups have studied the involvement of heart NO metabolism in the hypertrophy and pathological remodeling that occur during adaptation to hypoxia (14, 32, 45, 49). In this regard, Nagendran et al. (32) reported that PDE5 is upregulated in right and left ventricle hypertrophy and that sildenafil ameliorates hypertrophied right ventricle heart contractility (32). Although further studies of left and right ventricle mtNOS activity need to be performed, it is possible that in our model the observed changes in mtNOS activity and expression are part of the early events involved in heart remodeling processes and right ventricle hypertrophy, and not only a direct consequence of myocardial hypoxia. Moreover, the effect of the drugs on heart mtNOS activity may be because of their action on heart NO steady-state levels or because of changes in pulmonary hemodynamics and vascular remodeling processes, which likely decrease right ventricle afterload caused by hypoxia-induced pulmonary hypertension (49).

The production of NO by mtNOS constitutes a regulatory mechanism that modulates mitochondrial and heart O_2 uptake, since NO inhibits cytochrome oxidase activity and mitochondrial respiration in a competitive and reversible manner (1, 11, 18). This phenomenon allows O_2 to reach longer distances along its gradient and extends the adequate tissue oxygenation away from the blood vessel, according to the Krogh model (3, 38, 46). Increased NO levels in highlanders could contribute to keep an adequate tissue perfusion and an effective O_2 supply, in spite of the decreased P_{O_2} in the inhaled air (41). In addition, an increase in NO production by mtNOS at high altitude enhances NO diffusion to cytosol, and this, in turn, may contribute to the observed mechanism of heart adaptation.

NO steady-state levels seem essential for the development of heart adaptation to hypoxia by its regulatory functions in respiration, signaling, and improved contractility (25, 50). At present, mitochondrial NO (10, 34, 35) and H_2O_2 (2, 10) are considered together as signals that are able to regulate genes involved in the control of cell metabolism and proliferation. In particular, NO was found to trigger mitochondrial biogenesis in diverse cell types (35, 36), an effect observed during chronic exposure to hypobaric hypoxia (12).

Through this study, we conclude that mtNOS is a substantial source of cardiac NO and constitutes a factor in the adaptive response to sustained heart hypoxia, susceptible to be modified by pharmacological treatments.

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