Time course of regression of the protection conferred by simulated high altitude to rat myocardium: correlation with mtNOS

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Submitted 13 March 2008; accepted in final form 13 June 2008

During acclimatization to sustained hypobaric hypoxia, up-regulation of mitochondrial nitric oxide synthase (mtNOS). To evaluate the time course of regression of these effects on deacclimatization, rats exposed to 53.8 kPa in a hypopressure chamber for 5 mo were returned to 101.3 kPa, whereas controls remained at 101.3 kPa throughout the study. At three time points, contractile function in response to calcium and to hypoxia-reoxygenation (H/R) were determined in papillary muscle, and NO S activity and expression were determined in mitochondria isolated from left ventricle. Developed tension was, before H/R, 65, 58, and 40%, and, after H/R, 129, 107, and 71% higher than in controls at 0.4, 2, and 5 mo of normoxia, respectively. Maximal rates of contraction and relaxation followed a similar pattern. All three parameters showed a linear decline during deacclimatization, with mean half-time ($t_{1/2}$) of 5.9 mo for basal mechanical activity and 5.3 mo for posthypoxic recovery. Left ventricle mtNOS activity was 42, 27, and 20% higher than in controls at 0.4, 2, and 5 mo of normoxia respectively. The expression of mtNOS showed similar behavior. The correlation of mtNOS activity with muscle contractility sustained a biphasic modulation, suggesting an optimal mtNOS activity. This experimental model would provide the most persistent effect known at present on regulation of left ventricle mtNOS was associated to the age-associated decline in left ventricle mechanical activity and improved posthypoxic recovery.

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phological activity and recovery after in vitro hypoxia declined linearly during deacclimatization; half-time decays of more than 5 mo, well above those of other changes occurring at high altitude, were found, thus resulting the most persistent effects of hypoxia reported in the literature. Heart mtNOS activity and expression accompanied the changes in basal contractility and posthypoxic recovery.

METHODS

Animals. Seven-week-old male Wistar rats of the CHbbTHOM albino strain were submitted to a simulated altitude of 5,000 m (53.8 kPa) at sea level atmospheric pressure (101.3 kPa). Chamber pressure was interrupted 20–30 min three times per 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 12:12-h light-dark schedule. After 5 mo of acclimatization, hypoxic rats were removed from the hypobaric chamber and kept at normoxic conditions similar to those of the control group. After 0.2–0.5 (mean = 0.4), 2, and 5 mo, five rats of each group (one at a time, alternating pre-hypoxic and controls) were used for the study of papillary muscle mechanical activity. For globular value determination, three heparinized microhematocrits were filled with blood obtained by cutting the tip of the tail under ether anesthesia, immediately before heart removal. Rats received care in accordance with the 6344/96 regulation of the Argentinean National Drug, Food, and Medical Technology Administration (ANMAT) and the “Guiding Principles for Research Involving Animals and Human Beings” of the American Physiological Society. All experimental procedures and manipulations were reviewed and approved by the ANMAT.

Heart muscle preparations. The thorax was opened under ether anesthesia, and the heart was excised, rinsed, and transferred to Ringer solution of the following composition (in mM): 128.3 NaCl, 4.7 KCl, 20.2 NaHCO$_3$, 0.35 NaH$_2$PO$_4$, 1.05 MgSO$_4$, 0.6 CaCl$_2$, and 5.5 glucose, pH 7.4, flushed with 95% O$_2$-5% CO$_2$ at 30°C. The left ventricle was opened, and both papillary muscles were removed while submerged in buffer. The chordae 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, each containing 30 ml of the Ringer solution. The solutions were equilibrated with a mixture of 95% O$_2$-5% CO$_2$, with pH and temperature kept constant at 7.4 and 30°C, respectively. The heart, trimmed of atria and large vessels, was dissected into the left ventricle plus septum (LV) and right ventricle (RV), which were weighed separately.

Papillary muscle mechanical activity. Papillary muscles were allowed to stabilize for 45 min after mounting. Rectangular pulses of 10 ms with an amplitude 20% higher than the threshold of each preparation were 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. The muscles were then stretched until maximal developed tension occurred. The isometric mecanograms were recorded on a Beckman R511A connected to the force transducer, and simultaneously the computer utilizing FPE digitized and stored the force-pacing signal for later analysis. Maximal developed tension (DT), maximal rate of rise in DT (+T), and maximal velocity of relaxation (−T) were determined. Each data result was the mean of three successive twitches. Calcium concentration was increased every 10 min, and mechanical activity was recorded at 0.60, 0.84, 1.35, 1.81, 2.30, and 2.75 mM Ca$^{2+}$. A 60-min period of hypoxia was then established by using a gas mixture of 95% N$_2$-5% CO$_2$, followed by a 30-min period of reoxygenation (95% O$_2$-5% CO$_2$), and mechanical events were recorded every 10 min. At the end of each experiment, muscle length was measured with a caliper. Both muscles 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. Mechanical parameters were normalized for muscle cross-sectional area.

Isolation of left ventricle mitochondria. Left ventricles deprived of 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, for 30 s with a blade homogenizer (Kendro-Sorvall-Du Pont Institute, Asheville, NC) and by five strokes in a glass Teflon homogenizer. All these operations were carried out at 2–4°C (7, 18). The 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. The mitochondrial pellet was washed and resuspended in the homogenization medium.

Submitochondrial membranes. Mitochondria were frozen and thawed three times and homogenized by passage through a 29-gauge hypodermic needle (7). Protein concentration was determined with the Folin reagent and BSA as standard.

Heart mtNOS activity. NO production was measured in submitochondrial membranes (SMM) by following spectrophotometrically at 577–591 nm [molar extinction coefficient (ε) = 11.2 M$^{-1}$·cm$^{-1}$] (Beckman DU 7400 diode array spectrophotometer) the oxidation of oxyhemoglobin to methemoglobin, at 37°C (7, 18). 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-SOD, 0.1 μM catalase, 20 μM oxyhemoglobin, and SMM 0.5–0.8 mg protein/ml. Control experiments adding 2 mM N$_6$-monomethyl-l-arginine (l-NMMMA) were performed, and l-NMMA-sensitive hemoglobin oxidation was considered due to NO formation that was expressed as nanomoles of NO per minute per milligram of protein.

Western blot analysis. The proteins of mitochondrial membranes (0.1 mg protein) were separated by SDS-PAGE (7.5%) and blotted into nitrocellulose films. Membranes were probed with 1:500 diluted rabbit polyclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) against eNOS, iNOS, and nNOS. The nitrocellulose membrane was subsequently incubated with a secondary goat anti-rabbit antibody conjugated with horseradish peroxidase (dilution 1:1,000) and revealed by chemiluminescence with ECL reagent (10). Densitometric analysis of the bands were performed using SCION Image software.

Statistics. Results are expressed as means ± SE. One-way ANOVA, plus the post-ANOVA Bonferroni t-test for multiple comparisons, were used for statistical analysis of the data as appropriate. Single linear and nonlinear regression analyses were performed to determine relationships between variables (Microcal Origin 6.0 statistical software). A value of $P < 0.05$ was considered statistically significant.

RESULTS

Biological parameters. Body weight gain was delayed during the whole period of acclimatization; when rats were returned to sea-level atmospheric pressure, body weight was normalized within 2 mo (Fig. 1). Hematocrit value, which was highly significantly increased in rats submitted to hypoxia (34) and is usually a very sensitive index of adaptation, was plotted for individual rats during deacclimatization (Fig. 2), showing a
decline with a half-time of 27 days and normalization at ~2 mo. RV weight was more than 100% increased by hypoxia and declined under normoxia with a half-time of 3.7 mo (Fig. 3). After 5 mo, RV was still significantly hypertrophied, whereas LV weight remained normal throughout this study (Fig. 3) as it was during acclimatization (34). Ventricle weights were not normalized per body weight or area because hypoxic rats experience a marked decrease in body fat content (16).

**Papillary muscle contractile parameters: response to Ca\(^{2+}\).** Papillary muscle mechanical activity, measured as DT, +T, and −T, at 1.35 mM Ca\(^{2+}\), was age dependent and was markedly increased in rats submitted to similar conditions of hypoxia as in the present study (34). Confirming that during the period in which this investigation was carried out (from 7 to 12 mo old) mechanical activity was not influenced by the age of the animals (34), data obtained in the control groups were not significantly different from each other and were pooled for further analysis and presentation. Figure 4 shows Ca\(^{2+}\) response of DT in deacclimatized and in control rats. Over the whole range of Ca\(^{2+}\) concentration assayed, DT was significantly higher in all groups of hypoxic than in control animals. At maximal Ca\(^{2+}\), DT was 65, 58, and 40% higher than in controls at 0.4, 2, and 5 mo, respectively. Although at this point differences among deacclimatized groups did not result in statistical significance, DT showed a clear tendency to decrease with time under normoxia; +T and −T followed a similar pattern (Table 1). The mean percent increase of the three parameters (see Fig. 7) showed a linear decline during deacclimatization with a half-time of 5.9 mo. At lower Ca\(^{2+}\) concentration (up to 1.81 mM), DT was significantly higher in the group of rats most recently hypoxic than in the other groups, suggesting a higher Ca\(^{2+}\) sensitivity of cardiac myofilaments during the early phase of deacclimatization.
Hypoxia and reoxygenation. Recovery of papillary muscle DT, +T, and −T after 60 min hypoxia and 30 min reoxygenation was highly improved in adult hypoxic rats compared with their normoxic controls (34). This advantage was still significant 5 mo after returning to sea-level atmospheric pressure (Fig. 5 and Table 1). DT was 129, 107, and 71% higher in rats deacclimatized for 0.4, 2, and 5 mo, respectively, than in controls; +T and −T showed similar increases. All three parameters showed a linear decline during deacclimatization with a mean half-time of 5.3 mo (see Fig. 7).

Heart mitochondria NO production. LV mtNOS activity was significantly higher in rats submitted to hypobaric hypoxia than in their sibling controls kept at sea-level atmospheric pressure (51). Production of NO by SMM was still 42, 27, and 20% increased after 0.4, 2, and 5 mo of deacclimatization, whereas DT age-associated decline was retarded (34). Because DT during deacclimatization was measured only at 1.35 mM Ca\(^{2+}\) (34), DT values corresponding to this particular Ca\(^{2+}\) concentration (see Fig. 4) were used to plot deacclimatization data. Basal DT showed a Gaussian relationship with mtNOS activity \((R^2 = 0.94)\) and defined the optimal NO production as 0.74 ± 0.01 nmoL NO·min\(^{-1}\)·mg protein\(^{-1}\), whereas +T and −T (not shown) behaved similarly, with \(R^2 = 0.95\) and 0.80 and maximum at 0.73 ± 0.01 and 0.70 ± 0.02, respectively.

Heart mitochondria NO expression. In the conditions of the assay, NO from heart mitochondrial membranes (mtNOS) reacted with anti-iNOS and anti-nNOS antibodies (Fig. 9). There was no reaction with anti-eNOS antibody. The densitometric quantitation of the Western blot spots indicated a higher level of expression of mtNOS in prehypoxic than in control rats, with a similar increase and behavior during deacclimatization to mtNOS activity measured by the biochemical assay. Half-time decline was 5.3 and 4.7 mo for iNOS and nNOS, respectively, consistent with 1, 10, 26, 45, and 74 wk in our previous work (34) and during deacclimatization in the present study were plotted vs. LV mtNOS activity (51 and Fig. 6). During aging under simulated high altitude, mtNOS activity increased (51), whereas DT age-associated decline was retarded (34). Because DT during deacclimatization was measured only at 1.35 mM Ca\(^{2+}\) (34), DT values corresponding to this particular Ca\(^{2+}\) concentration (see Fig. 4) were used to plot deacclimatization data. Basal DT showed a Gaussian relationship with mtNOS activity \((R^2 = 0.94)\) and defined the optimal NO production as 0.74 ± 0.01 nmoL NO·min\(^{-1}\)·mg protein\(^{-1}\), whereas +T and −T (not shown) behaved similarly, with \(R^2 = 0.95\) and 0.80 and maximum at 0.73 ± 0.01 and 0.70 ± 0.02, respectively.

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with the values determined for NO production, basal mechanical activity, and tolerance to hypoxia-reoxygenation.

DISCUSSION

We have previously reported that papillary muscle improved DT, \( \frac{d}{dt} \), and \( \frac{V}{dt} \), and recovery of these parameters after hypoxia-reoxygenation developed during acclimatization to sustained hypobaric hypoxia. The present study shows that they gradually decline when rats are returned to sea-level atmospheric pressure but remain significantly higher than in controls at least for 5 mo. These effects persist longer than classical changes occurring during acclimatization as impaired body weight gain and increased hematocrit, which are normalized after 2 mo. In contrast, complete regression of LV adaptive changes would require more than 10 mo. This is the first report to our knowledge where improved basal contractility and recovery of posthypoxic LV function have been documented months after the termination of exposure to chronic hypoxia.

Right ventricle resistance against acute hypoxic injury conferred by intermittent chronic hypobaric hypoxia in adult rats persisted for 4 mo after removal from the hypoxic environment (39). Right ventricle hypertrophy develops during acclimatization as a consequence of pulmonary vasoconstriction in response to hypoxia, which increases pulmonary arterial pressure. This effect is usually considered an adverse influence of high-altitude hypoxia on the cardiopulmonary system (29, 38), and many well-adapted people live at high altitude without pulmonary hypertension or cardiac hypertrophy. The reversibility of these changes following removal from a chronically hypoxic environment has been described (23). In our model of sustained hypobaric hypoxia, RV weight declined with a half-time of 3.7 mo when hypopressure was interrupted and was still significantly increased after 5 mo of normoxia. On the other hand, the fact that LV was not hypertrophied during acclimatization (34) implies that the observed effects on contractile function and recovery from hypoxia-reoxygenation would only be ascribed to adaptation to hypoxia.

Similarly to papillary muscle contractile parameters before and after recovery from in vitro hypoxia, LV mtNOS activity, which significantly increased during acclimatization (51), showed a tendency to decline when rats were returned to sea-level atmospheric pressure. All three effects of LV acclimatization, namely, preservation of age-associated decline in mechanical activity, improvement of posthypoxic recovery, and upregulation of mtNOS, showed similar half-time decay during deacclimatization; this fact sustains our hypothesis previously advanced (34, 51) toward an involvement of NO generated by mitochondria in the mechanisms underlying cardioprotection by chronic hypoxia.

A biphasic relationship between LV mtNOS activity and papillary muscle DT at 1.35 mM \( \text{Ca}^{2+} \) during acclimatization (\( \blacklozenge \)) and deacclimatization (\( \blacktriangle \)) was reported in our previous study (51); an optimal mtNOS activity of 0.69 nmol NO/min/mg protein was associated with the highest contractile parameters in the LV of young rats (2 mo old). During acclimatization mtNOS activity increased (51), and age-associated mechanical activity decline was attenuated (34). The Gaussian relationship resulting when jointly plotting the data values obtained during acclimatization in the previous study and deacclimatization in the present one sets the optimal
mtNOS activity for basal contractility within a range of 0.70–0.74 nmol NO·min⁻¹·mg protein⁻¹.

Excitation-contraction coupling is driven by an ion channel-mediated calcium cycle that produces myofilament contraction and relaxation, and NO synthesis by constitutive NOS (including mtNOS) is calcium dependent. NO concentrations fluctuate with the cardiac cycle, in the submicromolar range, strongly supporting a physiological role for NO in contractility (28, 42, 45). Many of the controversies in the literature have arisen regarding the directionality of NO effects (35). Indeed, the effect of NO on contractility is bimodal in a concentration-dependent manner, and there are NOS isoform-specific responses within the heart (28, 45).

NO generated by eNOS stimulates guanylate cyclase to produce cGMP in adjacent smooth muscle cells, leading to vasodilation and increased blood flow and O₂ delivery to the tissue (14, 44). Besides this classical concept, NO is known to act as a physiological regulator of mitochondrial respiration through cGMP-independent pathways. NO inhibits mitochondrial respiration by a rapid, selective, potent, and reversible inhibition of cytochrome oxidase (complex IV) (8, 9, 11, 12, 43). The inhibition occurs in competition with O₂, so that NO dramatically increases the O₂ concentration that yields half-maximal rate of O₂ uptake (6, 9). The recognition of NO as the first known physiological regulator to act directly on the mitochondrial respiratory chain revealed the importance that NO could have in mitochondrial adaptation to hypoxia (5, 6, 16). The NO-inhibited respiration lowers the steepness of intracellular O₂ gradients and allows O₂ to diffuse further along its gradient, extending the space of adequate tissue oxygenation away from the blood vessel (16, 43, 48). Moreover, NO was found to trigger mitochondrial biogenesis in cardiomyocytes and other cell types (37) and acclimatization to hypobaric hypoxia increased the number of mitochondria per volume unit in rat LV (17). On the other hand, too high NO production may be associated with excessive cytochrome oxidase inhibition and increased peroxynitrite (ONOO⁻) formation, which would have detrimental effects (3, 9, 35, 40).

In addition, NO modulates the activity of several key calcium channels involved in excitation-contraction coupling (28, 45). The mechanisms by which NO influences myocardial Ca²⁺ cycling remain controversial. NOS isoforms have specific spatial localization in cardiac myocytes; nNOS, found in cardiac sarcoplasmic reticulum (SR) (28, 45) and mitochondria (32, this study), would preferentially regulate Ca²⁺ release and reuptake, resulting in potentiation of the cardiac force response. There is evidence that cytosolic Ca²⁺ signals are efficiently transmitted to the mitochondria, providing a means for coupling cardiac muscle excitation to oxidative energy production (21, 24). Nitric oxide is recognized as a mediator of calcium homeostasis in a highly complex and cell-specific manner (13), which can affect mitochondrial calcium homeostasis as well (22).

Although many potential factors have been proposed to play a role in the long-term cardioprotective effect of chronic hypoxia (1, 33), the detailed molecular mechanism remains unknown (31). There is no consensus about the NOS isoform involved in myocardial protection by chronic hypoxia. Either eNOS or iNOS have been previously implicated in different models (1, 33), and nNOS is now added. In our previous study (51), mtNOS was estimated to account for 55% of total heart cytosolic NO. Indeed, there are reports indicating even higher contribution of mtNOS (27). The activity of cytosolic NOS assayed in the postmitochondrial supernatant did not change after exposure of rats to 4,340 m for up to 21 days (27). However, under chronic hypoxia, both mtNOS and eNOS of caveolae and plasma membrane (2, 46) appear to significantly contribute to an increased NO level in the cytosol as part of the mechanism of heart adaptation to hypoxia.

Considering the extended memory of cardioprotection conferred by adaptation to chronic sustained hypoxia, the unraveling of the molecular basis of this phenomenon would be of utmost importance for the development of therapeutic strategies (4, 47). NO generated by mtNOS appears to have a key role, possibly through regulation of the rate of O₂ consumption and reactive oxygen species production by the respiratory chain, modulation of intramitochondrial calcium concentration, activation of mitochondrial ATP-sensitive potassium channels, and cellular signaling through mitochondrial NO release to the cytosol (16).

In summary, increased heart functional capacity and resistance to O₂ deprivation developed during long-term exposure to simulated high altitude lasted for more than 5 mo after return to sea-level atmospheric pressure. Decline in mtNOS activity and expression during deacclimatization closely accompanied the loss of myocardial function and protection, supporting a role for this enzyme in the mechanism involved.

GRANTS

This work was supported by Grant PIP 6320 from Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Argentina. L. E. Costa is Career Investigator from CONICET.

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