



Activation of arginase II by asymmetric dimethylarginine and homocysteine in hypertensive rats induced by hypoxia: a new model of nitric oxide synthesis regulation in hypertensive processes?

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Abstract

In recent years, the increase in blood pressure at high altitudes has become an interesting topic among high-altitude researchers. In our animal studies using Wistar rats, we observed the existence of two rat populations that exhibit differential physiological responses during hypoxic exposure. These rats were classified as hypoxia-induced hypertensive rats and nonhypertensive rats. A decrease in nitric oxide levels was reported in different hypertension models associated with increased concentrations of asymmetric dimethylarginine (ADMA) and homocysteine, and we recently described an increase in arginase type II expression under hypoxia. ADMA and homocysteine decrease nitric oxide (NO) bioavailability; however, whether ADMA and homocysteine have a regulatory effect on arginase activity and therefore regulate another NO synthesis pathway is unknown. Therefore, the aim of this study was to measure basal ADMA and homocysteine levels in hypoxia-induced hypertensive rats and evaluate their effect on arginase II activity. Our results indicate that hypoxia-induced hypertensive rats presented lower nitric oxide concentrations than nonhypertensive rats, associated with higher concentrations of homocysteine and ADMA. Hypoxia-induced hypertensive rats also presented lower dimethylarginine dimethylaminohydrolase-2 and cystathionine β -synthase levels, which could explain the high ADMA and homocysteine levels. In addition, we observed that both homocysteine and ADMA had a significant effect on arginase II activation in the hypertensive rats. Therefore, we suggest that ADMA and homocysteine have dual regulatory effects on NO synthesis. The former has an inhibitory effect on eNOS, and the latter has a secondary activating effect on arginase II. We propose that arginase II is activated by ADMA and homocysteine in hypoxia-induced hypertensive rats.

Keywords Arginine · Dimethylarginine · Hypoxia · Hypertension · Homocysteine

Introduction

Systemic arterial hypertension at high altitudes has become an interesting topic among high-altitude researchers. There have been conflicting reports on this issue, with some researchers reporting a slight increase in blood pressure soon after arrival at a high altitude [1, 2] and others reporting no

such change [3, 4] or a decrease in blood pressure followed by an increase in blood pressure [5, 6]. In our animal studies using Wistar rats, we observed the existence of two groups of rats that exhibit differential physiological responses during hypoxic exposure [7]. These groups were classified as tolerant and intolerant to hypobaric hypoxia. Intolerant rats typically developed arterial hypertension with average pressure values of 183 ± 3.7 mmHg and hematocrit levels of 74.0 ± 4.0 , leading to death when hypoxic exposure persisted. Therefore, rats that are intolerant to our chronic intermittent hypobaric hypoxia model constitute a hypertensive group. In contrast, tolerant rats did not develop arterial hypertension, and their physiological parameters were similar to those of control rats (normoxia), therefore, these rats constitute a nonhypertensive group.

Hypertensive responses can arise as a result of changes in endothelial function [8]. In this context, one of the common

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causes of deregulated endothelial vasodilator function is the alteration of nitric oxide (NO) synthesis pathways [9, 10]. Our previous results showed that hypoxia-induced hypertensive rats exhibited lower NO production, indicating that the regulation of NO synthesis is crucial to the development of hypertensive hypoxia [7]. In fact, various studies in hypertension models have described that downregulation of the NO synthetic pathway is accompanied by an increase in asymmetric dimethylarginine (ADMA) and homocysteine plasma levels [11–15].

ADMA and homocysteine are inhibitors of nitric oxide synthase (NOS), and ADMA has also been identified as a regulator of NO synthesis in vivo [16]. Increased levels of both of these metabolites are cardiovascular risk factors [14–16].

An increase in ADMA was described in rats and humans exposed to hypoxia [17] and was recently shown to be a predictor for the development of hypoxia-induced pulmonary hypertension [18]. On the other hand, we recently demonstrated that low baseline levels of ADMA and homocysteine in llamas (*Lama glama*), an animal genetically adapted to life at a high altitude, is an adaptive mechanism against the development of hypoxia-induced pulmonary hypertension [19].

Almost 80% of ADMA is degraded by a group of hydrolases called dimethylarginine dimethylaminohydrolases (DDAHs) [20]. There are two types of DDAHs known for their ability to hydrolyze ADMA: DDAH-1 and dimethylarginine dimethylaminohydrolase-2 (DDAH-2). Recently, we reported that DDAH-2 participates in ADMA regulation under hypoxic conditions [19]. In addition, cystathionine β -synthase (CBS) participates in the regulation of homocysteine degradation. Currently, both DDAH-2 and CBS are considered molecular risk targets for the development of cardiovascular diseases due to the existence of polymorphisms in their genes that dysregulate their metabolism [21, 22]. However, whether NO production can also be regulated by L-arginine plasma levels as a result of the competition between NOS and arginase has been less well studied [23–25].

Arginase plays an important role in the pathogenesis of several hypertension models. For instance, an increase in arginase activity was associated with several pulmonary and systemic hypertension models [26–28]. Similarly, we reported an increase in arginase II expression that led to the development of hypertension associated with decreased plasma NO in intolerant rats [7]. However, no scientific evidence regarding the effect of ADMA and homocysteine on the arginase pathway and the participation of the arginase pathway in the regulation of hypertensive processes is available. Therefore, the main objective of this study was to determine the baseline levels of ADMA and homocysteine in hypertensive and nonhypertensive rats induced by

hypobaric hypoxia and to study the effect of ADMA and homocysteine on arginase type II activity. The study of new regulatory NO synthesis mechanisms will help to identify novel therapeutic targets for the pharmacological treatment of hypertension.

Materials and methods

Animal study groups

A total of 40 young and healthy Wistar rats (age: 3.0 ± 0.4 months; weight: 250 ± 25 g) were randomly divided into two groups: the hypobaric hypoxia (2 days of hypoxia, 2 days of normoxia, $n = 30$) and normoxia ($n = 10$) groups. Hypobaric hypoxia was simulated in a hypobaric chamber at 428 torr, which is equivalent to an altitude of 4600 m, maintained with an average internal flow of room air of 13.14 L/min (0.438 L/min of air per rat) at 22 ± 2 °C on a 12 h light–dark cycle. Rats were intermittently kept in the hypobaric chamber, spending 2 days inside the chamber (hypoxic conditions) followed by 2 days outside the chamber, for a period of 1 month (30 days). Rats in the control group were placed in the same room under the same conditions to which the hypoxic group was exposed (with the exception of hypoxia). Four animals were housed per cage, and the animals were provided food (10 g/day of pellets per rat) and water using bottles dispensers. The variables weight (W, g) and systolic blood pressure (SBP, mmHg) were measured at sea level under normoxic conditions immediately after the rats had been removed from the hypobaric chamber, starting with basal measurements and every 4 days thereafter for 1 month. Weight was measured using an Acculab V-1200 electronic balance 3 h after rats descended from the chamber to allow for hydration. Blood pressure and heart rate were measured using an inflatable tail-cuff and a pressure sensor (RTBP1003-220, Kent Scientific, Torrington, CT, USA) as previously described [29]. The signal was sent through a preamplifier to a workbench data acquisition system, RTBP-001DS worksheet (Kent Scientific), and the average value of six consecutive measurements was calculated. Measurements were performed by placing the animals inside a movement-limiting Plexiglas chamber for 10–20 min.

Classification of hypoxia-induced hypertensive and nonhypertensive rats

Hypertension in rats in Wistar rats with adverse outcomes due to hypoxia was determined according to the predictive factors previously described in our laboratory [7]. Briefly,

hypoxia-induced hypertensive rats were identified as those that developed arterial hypertension. The criteria used to determine whether rats were hypertensive were blood pressure increased by $\geq 20\%$ compared to baseline, body weight loss $\geq 30\%$ compared to baseline, and increased hematocrit $\geq 20\%$ compared to baseline. Non-hypertensive rats were identified as those that did not develop arterial hypertension and exhibited an increase in body weight and increase in Hct of $< 20\%$ compared to baseline. Rats were sacrificed when they met these criteria (average days of life: 30 days, $n = 10$). The rats were handled in accordance with guidelines for the humanitarian treatment of laboratory animals, and all experiments were conducted according to the NIH Guide for the Care and Use of Laboratory Animals (NIH publication No. 8023, revised 1978)

The animals were sacrificed using anesthesia, and the Ethics Committee at the Universidad Católica del Norte, Coquimbo-Chile, approved the design of the protocol.

Enzyme preparation

The hearts were excised, frozen using liquid nitrogen and stored at -80°C until further use. Heart tissue was homogenized in a solution of 10 mM Tris-HCl (pH 7.5), 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM DTT, and 5 mM EDTA and centrifuged at $5000 \times g$ for 10 min at 4°C . Subsequently, the enzyme solution (supernatant) was separated by chromatography on a DEAE-cellulose column equilibrated with 5 mM Tris-HCl (pH 7.5). The active eluted fractions obtained by washing the column corresponded to arginase I, and the fractions obtained with a linear gradient of Tris/KCl at 0.10–0.15 M corresponded to arginase II [30]. The presence of arginase I and II in the wash fraction and eluted fraction, respectively, was confirmed by Western blotting. Finally, the active fractions were pooled and stored at -20°C .

Western blotting

All Western blotting was performed according to standard methods. Briefly, total aortic artery tissues were frozen in liquid nitrogen and then homogenized in homogenization buffer containing 50 mM Tris-HCl, 150 mM NaCl, 100 mM NaF, 1% Triton X-100, 1 mM dithiothreitol (DTT), 0.1 mM PMSF, 1 mM leupeptin, 0.02 M HEPES, 10^{-3} M EDTA, 10^{-3} M EGTA, and 20% glycerol, followed by centrifugation to $10000 \times g$ for 10 min. The supernatant was removed, and protein quantification was carried out by Bradford assay. Equal amounts of protein (25 μg) were resolved by 7.5–12% SDS-PAGE, and the proteins were transferred to PVDF membranes. Non-specific binding to the membranes was blocked using 5%

nonfat dry milk in TBS-T buffer (10 mM Tris-HCl, 150 mM NaCl, 0.05% Tween 20 (pH 7.4) for 1 h. Membranes were incubated with the primary polyclonal antibodies rabbit anti-eNOS, anti-iNOS, (1:500; Santa Cruz Biotechnology, Dallas, Texas, USA), anti-DDAH-2, anti-CBS (1:500; Abcam, Biosonda, Chile), anti-arginase type II (1:5000; Invitrogen, Thermo Scientific, Chile), anti- β actin, and anti-GDPH (1:5000; Sigma Chile Ltd., Chile); washed in TBS with 0.1% Tween; and incubated (1 h, 22°C) in TBS/0.1% Tween containing horseradish peroxidase-conjugated goat antirabbit or anti-mouse secondary antibodies. Proteins were detected by enhanced chemiluminescence and quantified by densitometry using ImageJ (NIH). Expression levels of eNOS were normalized to GAPDH expression.

Measurement of arginase activity

Specific arginase activity in heart tissues was determined based on urea production using a colorimetric method with α -isonitrosopropiophenone [29]. Briefly, 50 μL of the fractions obtained from DEAE-cellulose chromatography were incubated in 30 mM Tris-HCl (pH 7.5) containing 10 mM L-arginine at 37°C for 10 min. The reaction was stopped by the addition of 1 ml of an acidic mixture containing 9% (v/v) H_3PO_4 and 23% (v/v) H_2PO_4 . To evaluate the effect of homocysteine and ADMA on arginase activity, a specific arginase activity assay was performed in the presence of ADMA and homocysteine at physiological concentrations (0.5, 1.0, 3.0, 6.0, and 10.0 μM) in a solution of 50 mM Tris-HCl (pH 7.5).

Protein assays

Total protein was determined by using the Bio-Rad protein assay (Bio-Rad CA, USA) with bovine serum albumin as a standard [31].

Collection of blood and plasma

A 2 ml blood sample was obtained by cardiac puncture under anesthesia (0.3 mg of ketamine). The plasma was then separated from the blood by centrifugation ($4500 \times g$, 12 min, 4°C) and stored at -80°C until further analyses to measure ADMA, homocysteine and NO. After this procedure, the rat was comfortably placed in a separate cage under veterinary care. Once awake and able to mobilize, drink water, and eat food, the rat was moved to the chamber.

At the end of the protocol, the rats were euthanized with an overdose of ketamine (7 mg/k), and the hearts and aortic arteries were surgically removed, dissected, immediately frozen in liquid nitrogen and stored at -80°C .

Detection of NO metabolites

Total plasma nitrate and nitrite (NO_x) concentrations were determined using a kit based on the Griess reaction (Cayman Chemical). The kit enabled the detection of NO_x (sum of nitrate and nitrite) by providing an index of the NO content. The results are reported as μmol/L NO_x.

Measurement of ADMA and homocysteine plasma concentrations

Plasma levels of ADMA (Enzo Life Sciences, Farmingdale, New York, USA) and homocysteine (Alpco Diagnostics, Salem, New Hampshire, USA) were measured by enzyme-linked immunosorbent assay according to the manufacturer's instructions.

Measurement of endothelial nitric oxide synthase activity

NOS activity was measured by assessing the conversion of L-[³H]-arginine to L-[³H]-citrulline [32]. Briefly, one volume of tissue extract from the aortic artery containing 300–400 mg of total protein in 20 mmol/L CHAPS was added to 200 μL of reaction mixture (50 mmol/L Tris buffer (pH 7.4) containing 10 μmol/L DTT, 10 μmol/L THB4, 10 μg/mL calmodulin, 1 mmol/L NADPH, 4 μmol/L flavin adenine dinucleotide, 4 mol/L flavin mononucleotide, 2 μmol/L L-arginine, and 10⁻³ mCi/mL L-[³H]-arginine). The assay was carried out at 37 °C and terminated with 2 mL of ice-cold stop buffer (20 mmol/L CH₃COONa (pH 5.5) containing 2 mmol/L EDTA, 0.2 mmol/L EGTA, and 1 μmol/L L-citrulline). As L-[³H]-citrulline is neutral at pH 5.5, it was separated from the incubation mixture with cation exchange resin (Dowex AG 50W-X8; Bio-Rad) to which L-[³H]-arginine adhered. After elution with 2 mL of water, L-[³H]-citrulline was quantitated by 16 mL liquid scintillation counting (SL 3000; Intertechnique, Plaisir, France). Background radioactivity (as a percentage of total radioactivity) was measured to assess the quality of the L-[³H]-arginine stock (background radioactivity below 3%). Arginase activity was determined by measuring the formation of L-[³H]-citrulline from the homogenized aortic artery of nonhypertensive and hypoxia-induced hypertensive rats.

Extraction and quantification of oxide endothelial nitric synthase (eNOS), dimethyl-aminohydrolase (DDAH-2), cystathionine β-Synthase (CBS), and arginase Type II (ARG II) mRNA Levels by qRT-PCR

For qRT-PCR analysis, total RNA was prepared from the aortic artery tissue by homogenization of the sample in the presence of TRIzol reagent (Invitrogen Corp., Carlsbad, CA,

USA). The RNA concentration and purity were assessed by ultraviolet (UV) spectrophotometry ($1.8 < A_{260}/A_{280} < 2.0$). RNA integrity was evaluated using electrophoresis. Reverse transcription was carried out using 4 μg of total RNA and the First Strand Complementary DNA (cDNA) Synthesis Kit (Thermo Scientific, Boston, MA, USA).

To make standard curves, 1 μL of first-strand cDNA was amplified with an AffinityScript cDNA Synthesis Kit, and quantified polymerase chain reaction (PCR) products were used to plot standard curves. Quantitative real-time PCR was carried out using an HT-7500 thermocycler (Applied Biosystems) and Brilliant III Ultra-Fast SYBR Green QPCR Master Mix. The PCR cycling conditions consisted of 95 °C for 3 min, followed by 40 cycles of 95 °C for 30 s and 60 °C for 30 s. GAPDH was used as an internal control. DDAH-2, CBS, eNOS, and ARG II mRNA levels were normalized to GAPDH (Glyceraldehyde 3-phosphate dehydrogenase) mRNA levels to compensate for variations in initial amounts of RNA. Normalization was carried out by dividing the logarithmic of DDAH-2, CBS, and eNOS expression levels by the logarithmic of GAPDH expression levels.

Data analyses and statistics

All statistical analyses were performed using SPSS 12.0.1 software (SPSS Inc., Chicago, Illinois, USA). The mean values and standard errors were calculated for each parameter. The normality of quantitative variables was established using the Kolmogorov–Smirnov test. The statistical validity of differences across all testing conditions was established using one-factor analysis of variance (ANOVA), and repeated measures ANOVA was performed to evaluate differences within the group. Pearson's correlation was also performed. Linear regression analysis was carried out to establish the association between arginase activity (dependent variable) and the other variables. Significance was established by a *p* value < 0.05.

Results

Characteristics of hypoxia-induced hypertensive and nonhypertensive rats

After 1 month of hypoxia exposure, ~30% of the rats presented an increase in SBP (140 ± 2.4 vs. 199 ± 1.3 without and with hypoxia exposure, respectively) associated with an increase in hematocrit (42 ± 1.5 vs. $73 \pm 2.5\%$) without and with hypoxia exposure, respectively). These rats were classified as hypoxia-induced hypertension rats. Meanwhile, the remaining rats exposed to hypoxia (~70%) did not show an increase in blood pressure and were therefore classified as nonhypertensive rats (Fig. 1). These characteristics were

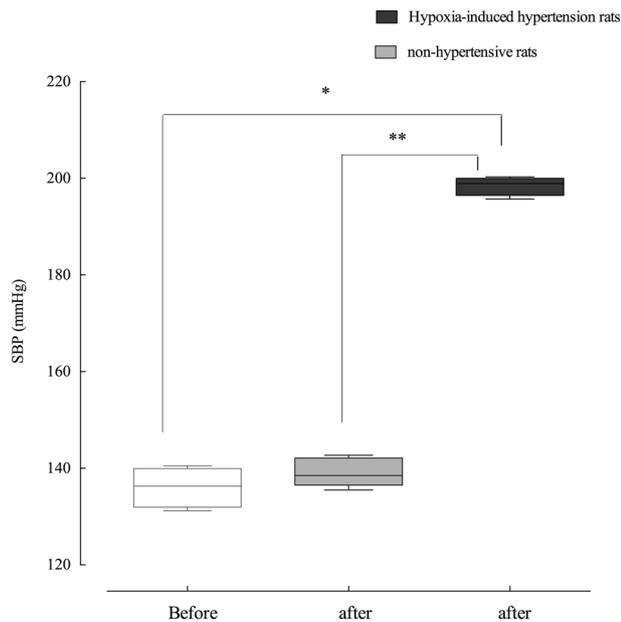


Fig. 1 Effect of exposure to hypobaric hypoxia on SBP in rats. The mean shows the SBP in rats ($n = 30$) before exposure to hypoxia and after an exposure time of 30 days. SBP values were significantly higher in the group of hypoxia-induced hypertension rats ($n = 10$, $*p < 0.001$). Differences in SBP between hypertensive and non-hypertensive rats after exposure to hypoxia ($n = 10$, $**p < 0.001$)

some of the criteria used to determine tolerance to hypoxia in our model. In addition, control rats (rats under normoxia, data not shown) did not present variations in SBP during the experiment (1 month). The average SBP of nonhypertensive rats after hypoxia exposure was not significantly different than that of control rats after hypoxia exposure (Fig. 1).

Hypoxia-induced hypertensive rats exhibited a lower NO plasma concentration

As shown in Fig. 2A, hypoxia-induced hypertensive rats exhibited significantly lower NO levels after 1 month of exposure than nonhypertensive rats. In fact, the NO plasma levels in the hypoxia-induced hypertensive rats and non-hypertensive rats were $7.7 \pm 0.4 \mu\text{mol/L}$ and $12.5 \pm 0.59 \mu\text{mol/L}$, respectively ($n = 10$, $*p < 0.001$), nonhypertensive and control rats (normoxia) presented the same NO plasma levels (12 ± 0.59 and $13.5 \pm 0.6 \mu\text{mol/L}$, respectively; $n = 10$, $*p > 0.05$).

Hypoxia-induced hypertensive rats exhibited higher plasma ADMA and homocysteine levels

To evaluate whether the decreased NO levels in hypertensive rats were also due to increased levels of NO synthesis inhibitors, plasma levels of homocysteine and ADMA were measured. As seen in Fig. 2B, ADMA plasma concentrations were increased after 1 month of exposure to hypoxia. In the

hypertensive rats, a significant increase in ADMA concentration compared to the baseline concentration prior to hypoxia exposure was observed (0.80 ± 0.05 vs. 2.08 ± 0.08 , respectively; $n = 10$, $*p < 0.001$). In addition, non-hypertensive rats showed a discrete increase in ADMA concentration compared to the baseline concentration prior to exposure (0.80 ± 0.05 vs. 1.53 ± 0.16 ; $n = 10$, $**p < 0.01$). Hypoxia-induced hypertensive rats exhibited a significant increase in homocysteine plasma levels after 1 month of exposure to hypoxia (15.83 ± 0.59 vs. $29.36 \pm 1.27 \mu\text{mol/L}$ before and after hypoxia, respectively; $n = 10$, $*p < 0.001$), and homocysteine plasma levels after 1 month of exposure to hypoxia were higher in hypertensive rats than in non-hypertensive rats ($29.36 \pm 1.27 \mu\text{mol/L}$ vs. $16.02 \pm 0.98 \mu\text{mol/L}$, respectively; $n = 10$, $*p < 0.001$) (Fig. 2C). The average homocysteine levels in nonhypertensive rats after hypoxia exposure did not significantly differ from those in control rats (under normoxia). In fact, homocysteine plasma levels were $15.83 \pm 0.59 \mu\text{mol/L}$ and $16.02 \pm 0.98 \mu\text{mol/L}$ in the control and nonhypertensive groups, respectively ($n = 10$, $*p > 0.05$).

Hypoxia-induced hypertensive rats exhibited decreased eNOS activity

To explain the low levels of NO found in hypoxia-induced hypertensive rats, eNOS activity and mRNA levels were measured. Exposure to hypoxia decreased eNOS activity in hypoxia-induced hypertensive rats ($n = 10$, $*p < 0.001$), which showed significantly lower levels of eNOS activity than those found in nonhypertensive rats after 1 month of exposure to hypoxia (Fig. 3A) ($n = 10$, $**p < 0.001$). The eNOS activity levels in nonhypertensive rats were not significantly lower after exposure to hypoxia. In contrast, when evaluating eNOS mRNA levels, we observed that exposure to hypoxia induced a slight increase in eNOS expression in nonhypertensive rats (Fig. 3B); however, we did not detect the expression of other NOS isoforms, such as iNOS (data not shown). In addition, the results regarding eNOS mRNA levels correlate with those regarding eNOS protein levels (Western blotting analysis).

Hypoxia-induced hypertensive rats exhibited decreased DDAH-2 and CBS levels and increased arginase type II levels

To explain the high ADMA and homocysteine levels found in the hypoxia-induced hypertensive rats, DDAH-2 and CBS expression levels were evaluated. Hypoxia induced a decrease in DDAH-2 protein and mRNA levels (Fig. 4), and this decrease was more substantial in hypoxia-induced hypertensive rats. To evaluate the potential L-arginine levels available for NO synthesis, arginase type II levels were measured. Hypoxia-induced hypertensive rats exhibited a significant increase in arginase type II expression (Fig. 4C).

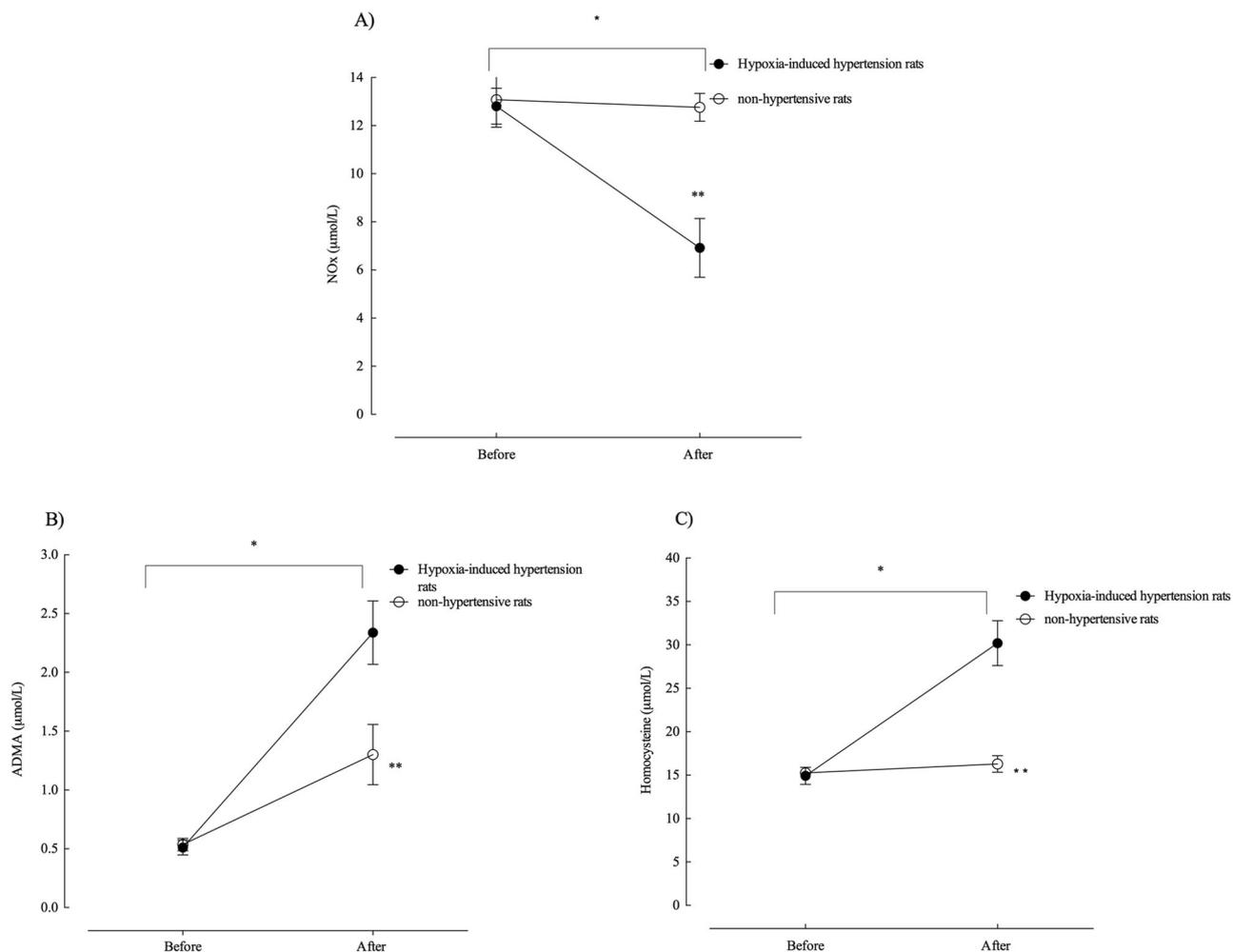


Fig. 2 Effect of exposure to hypobaric hypoxia on plasma levels of NO, homocysteine, and ADMA in rats. **A** Plasma NO levels before and after exposure for 30 days. Total NO plasma levels in the hypoxia-induced hypertensive rats were significantly lower than those after exposure to hypoxia ($n = 10$, $*p < 0.001$) and significantly lower than those in nonhypertensive rats ($n = 10$ nonhypertensive rats vs. hypoxia-induced hypertension rats, $**p < 0.001$). **B** Plasma ADMA levels before and after exposure for 30 days. Total ADMA plasma levels in hypoxia-induced hypertensive rats were significantly higher

than those after exposure to hypoxia ($n = 10$, $*p < 0.001$) and significantly higher than those in nonhypertensive rats ($n = 10$ nonhypertensive rats vs. hypoxia-induced hypertensive rats, $**p < 0.001$). **C** Plasma homocysteine levels before and after exposure for 30 days. The total homocysteine plasma levels in the hypoxia-induced hypertensive rats were significantly higher than those in nonhypertensive rats ($n = 10$ nonhypertensive rats vs. hypoxia-induced hypertensive rats, $*p < 0.001$)

Arginase activity was increased by homocysteine and ADMA in hypoxia-induced hypertensive rats

Arginase I and II activity was poorly inhibited by ADMA in both control rats and rats exposed to hypoxia (Fig. 5A). Contrary to our expectations, arginase II activity was significantly increased by ADMA ($p < 0.01$) in hypertensive rats (Fig. 5B).

Homocysteine was a potent activator of arginase I in the control rats and rats exposed to hypoxia (Fig. 6A). However, homocysteine had no effect on arginase II activity in the control group (Fig. 6B, triangles, normoxia). Therefore, exposure to hypoxia generated a particular form of arginase

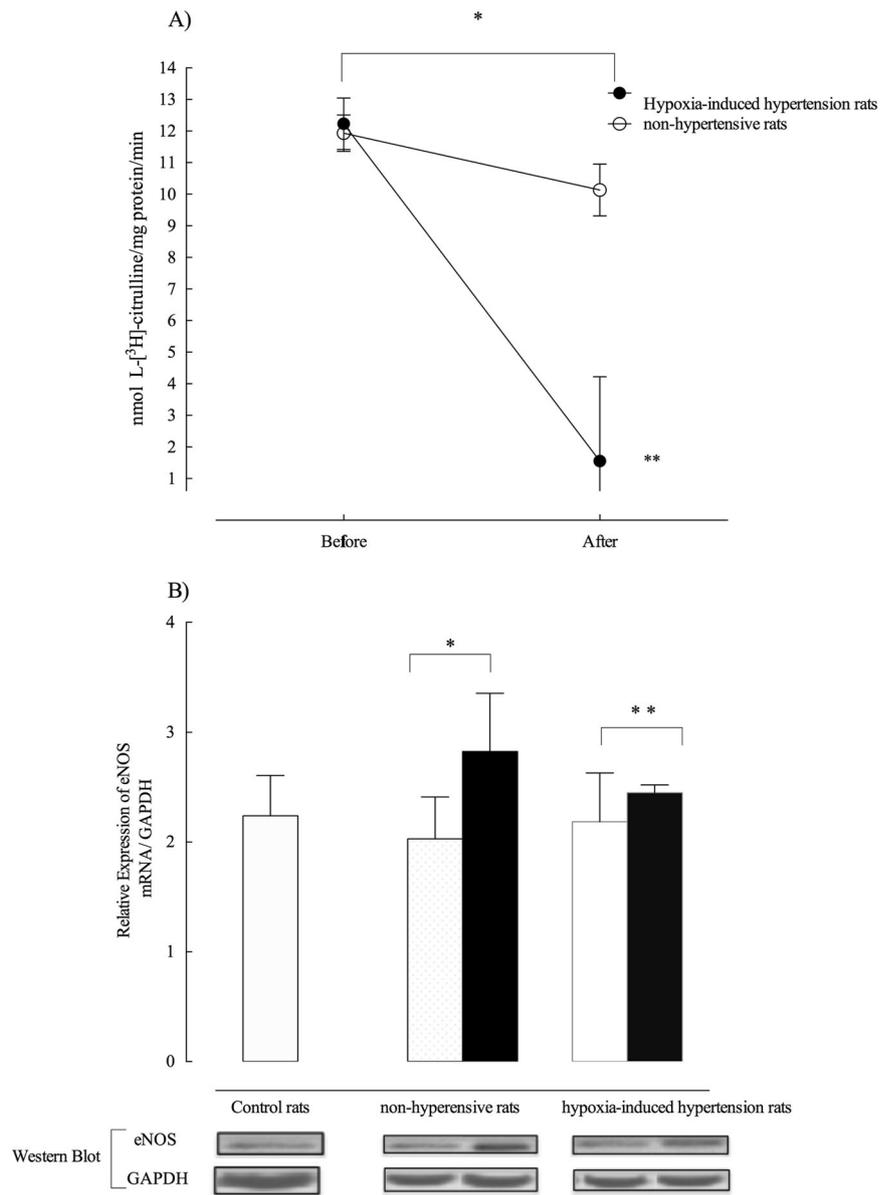
II that was sensitive to homocysteine-mediated activation (Fig. 6B).

Correlations between homocysteine levels, ADMA levels, arginase activity, and SBP

To determine the relationships between homocysteine and ADMA with relevant molecular and physiological parameters induced by hypobaric hypoxia in rats, we evaluated the correlation between homocysteine levels, ADMA levels, arginase activity, and SBP variables.

ADMA plasma levels exhibited a positive correlation with increases in arginase activity ($r = 0.594$, $r^2 = 0.352$,

Fig. 3 Endothelial NOS synthase activity and expression. **A** Measurement of eNOS activity was performed in a homogenate of the aortic arteries of rats exposed to hypoxia by measuring L-[³H]-citrulline. NOS synthase activity in hypoxia-induced hypertensive rats was significantly lower after exposure to hypoxia ($n = 10$ * $p < 0.001$) and significantly higher than that in nonhypertensive rats ($n = 10$, ** $p < 0.001$ nonhypertensive rats vs. hypoxia-induced hypertension rats). **B** Effect of hypoxia exposure on eNOS mRNA expression in hypoxia-induced hypertensive and nonhypertensive rats; levels before exposure to hypoxia (white bar) and after 30 days of exposure (black bar) ($n = 6$ per group, * $p < 0.001$ and ** $p < 0.01$) in homogenized aortic arteries are shown. The boxes correspond to the Western blot analysis from aortic artery homogenates



$p < 0.01$) and SBP ($r = 0.927$, $r^2 = 0.850$, $p < 0.0001$) (Fig. 7A, B). Likewise, we found that increased homocysteine plasma levels exhibited a positive correlation with arginase activity ($r = 0.872$, $r^2 = 0.761$, $p < 0.001$) and SBP ($r = 0.950$, $r^2 = 0.904$, $p < 0.001$; Fig. 8A, B).

Discussion

Hypoxia-induced hypertensive rats exhibited lower NO concentrations

To assess whether hypoxia-induced hypertension is caused by endothelial dysfunction, we measured NO plasma concentrations. Hypertensive rats exhibited lower NO plasma

concentrations (Fig. 2A), which suggests decreased NO production due to the inhibition of NOS as a consequence of increased ADMA and homocysteine levels (Fig. 2B, C). This would explain why although eNOS expression was increased (Fig. 3B), NO concentrations were decreased. Specifically, we suggest that hypertensive rats presented an increase in eNOS expression and a decrease in NO synthesis due to inhibition of the enzyme eNOS as a consequence of the increase in ADMA concentrations. The inhibitory mechanisms of these metabolites on eNOS activity are currently unclear. To resolve this, we are currently carrying out kinetic characterization of the inhibitory effect of ADMA and homocysteine on eNOS in hypertensive and nonhypertensive rats.

On the other hand, a decrease in NO production could be explained by a reduction in the L-arginine concentration

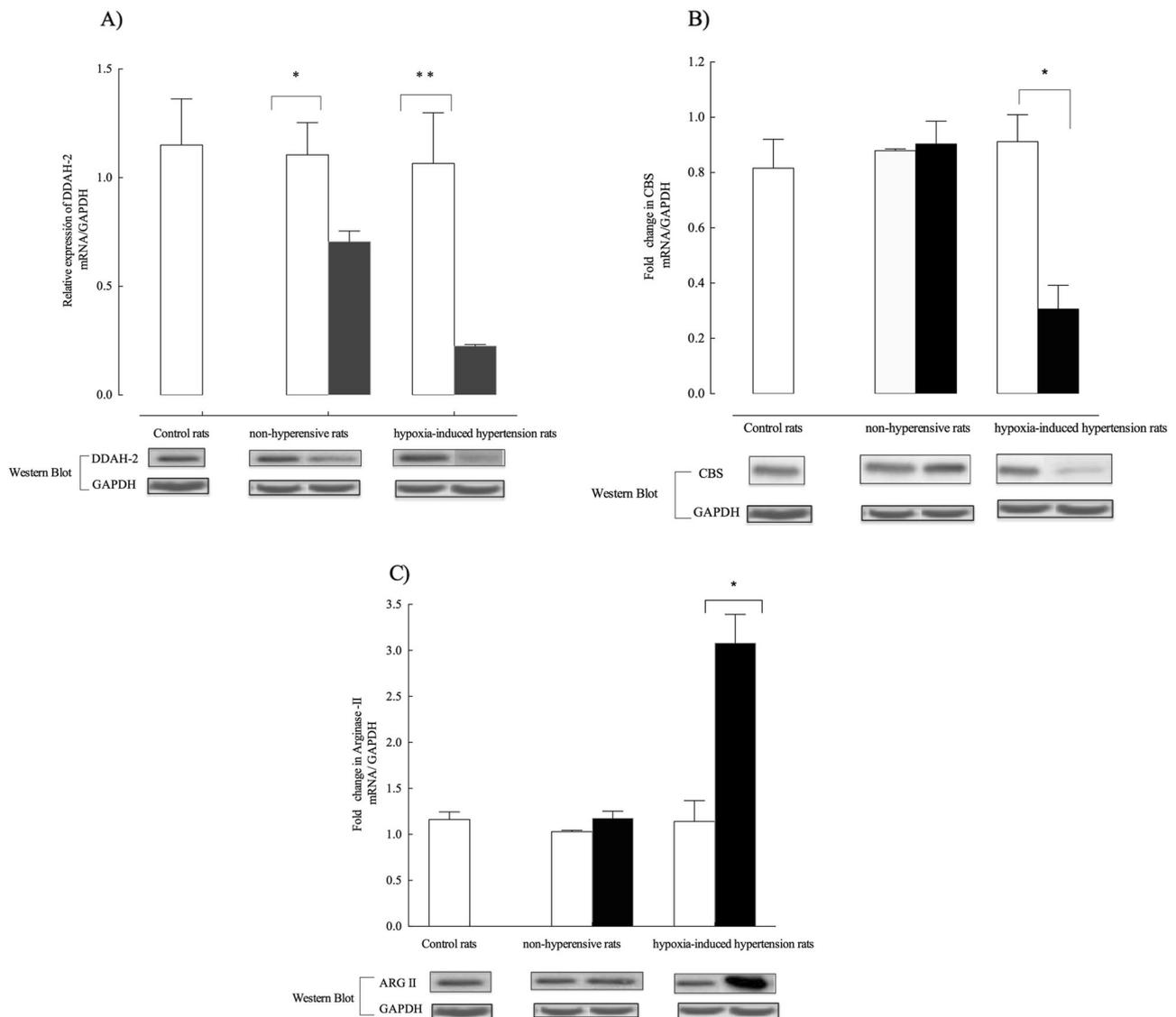


Fig. 4 Effect of hypobaric hypoxia exposure on mRNA expression and the protein levels of DDAH-2, CBS, and arginase type II. **A** Quantitative PCR of DDAH-2 in hypoxia-induced hypertensive and non-hypertensive rats before exposure to hypoxia (white bar) and 30 days after exposure (black bar) ($n = 6$ per group, $*p < 0.001$ and $**p < 0.001$). **B** Quantitative PCR of CBS mRNA expression in hypoxia-induced hypertensive and nonhypertensive rats before exposure to hypoxia (white bar) and after 30 days of hypoxia exposure (black bar)

($n = 6$ per group, $*p < 0.001$). **C** Quantitative PCR of arginase type II mRNA expression in hypoxia-induced hypertensive and non-hypertensive rats before exposure to hypoxia (white bar) and after 30 days of exposure to hypoxia (black bar) ($n = 6$ per group, $*p < 0.001$). Measurements were made in aortic artery tissue homogenates. The mRNA levels were normalized to GAPDH mRNA levels. The boxes correspond to Western blots analysis of aortic artery homogenates

available for NO synthesis due to increased arginase II expression (Fig. 4C). We recently showed that *Lama glama* (*L. glama*) an animal genetically resistant to the development of hypoxia-induced pulmonary hypertension [33], present 10 times less arginase type II than other species, allowing increased NO synthesis. Therefore, low expression of type II arginase in these animals could constitute an adaptive mechanism to the development of hypertension [19]. Our results suggest that increased type II arginase levels are involved in the development of hypoxia-induced

hypertension. Although L-arginine levels were not measured in this study, we suggest that the bioavailability of L-arginine for NO synthesis would be lower [25].

Hypoxia-induced hypertensive rats have higher ADMA concentrations and increased arginase type II activity

ADMA is a competitive inhibitor of endogenous eNOS [25] and can inhibit NO synthesis. In this context, ADMA was

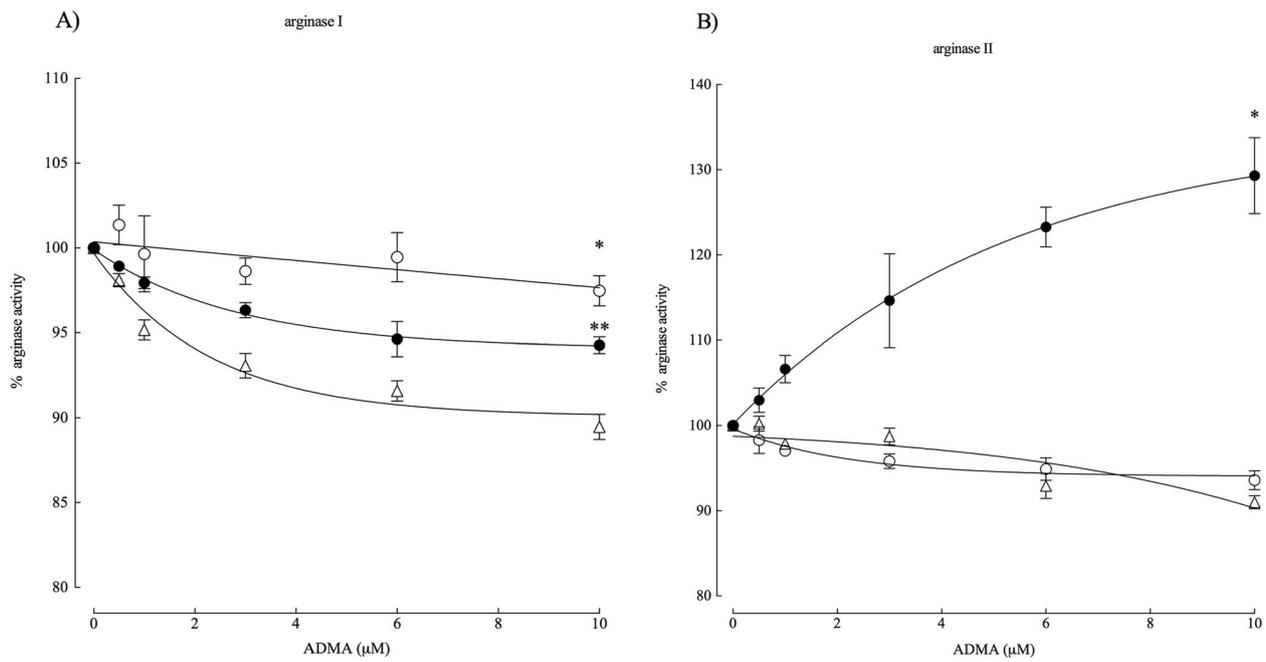


Fig. 5 Effect of ADMA concentrations on arginase type I and II activity in homogenized heart tissue from controls (normoxia) and rats exposed to hypoxia. Control (open triangles), nonhypertensive (open circles), and hypertensive (black circles) rats. **A** Effect of ADMA concentrations on arginase type I activity. An asterisk indicates a significant difference ($p < 0.01$) for the control group vs. the hypertensive and nonhypertensive groups. Double asterisk indicates a

significant difference ($p < 0.01$) for the hypertensive group vs. the nonhypertensive group. **B** Effect of ADMA concentrations on arginase type II activity. An asterisk indicates a significant difference ($p < 0.01$) for the hypertensive group vs. the control and nonhypertensive groups. Arginase activity is expressed as the percent specific activity in $\mu\text{mol}/\text{urea}/\text{mg}/\text{min}$. Data are expressed as the mean \pm SE ($n = 10$ per group)

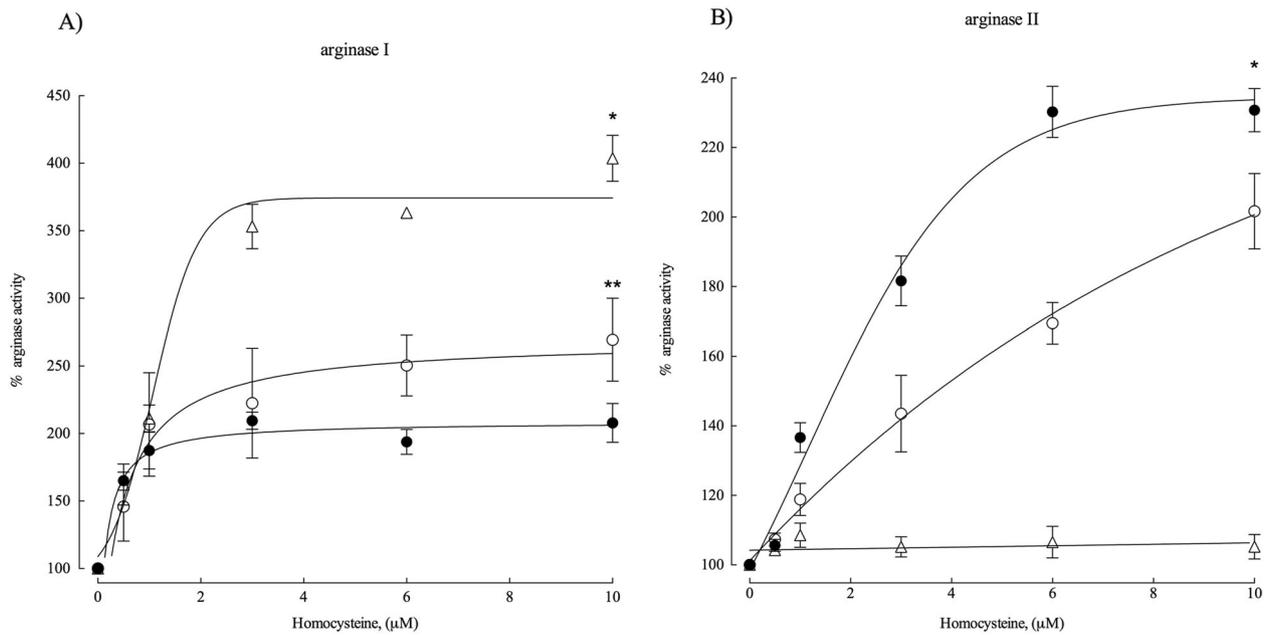


Fig. 6 Effect of homocysteine concentrations on arginase type I and II activity in homogenized heart tissue from control rats (normoxia) and rats exposed to hypobaric hypoxia. Control (open triangles), nonhypertensive (open circles), and hypertensive (black circles) rats. **A** Effect of homocysteine concentrations on arginase type I activity. An asterisk indicates a significant difference ($p < 0.01$) for the control group vs. the hypertensive and nonhypertensive groups; Double

asterisk indicates a significant difference ($p < 0.01$) for the nonhypertensive group vs. the hypertensive group. **B** Effect of homocysteine concentrations on arginase type II activity. An asterisk indicates a significant difference ($p < 0.01$) for the hypertensive group vs. The nonhypertensive group. Arginase activity is expressed as a percent specific activity in $\mu\text{mol}/\text{urea}/\text{mg}/\text{min}$. Data are expressed as the mean \pm SE ($n = 10$ per group)

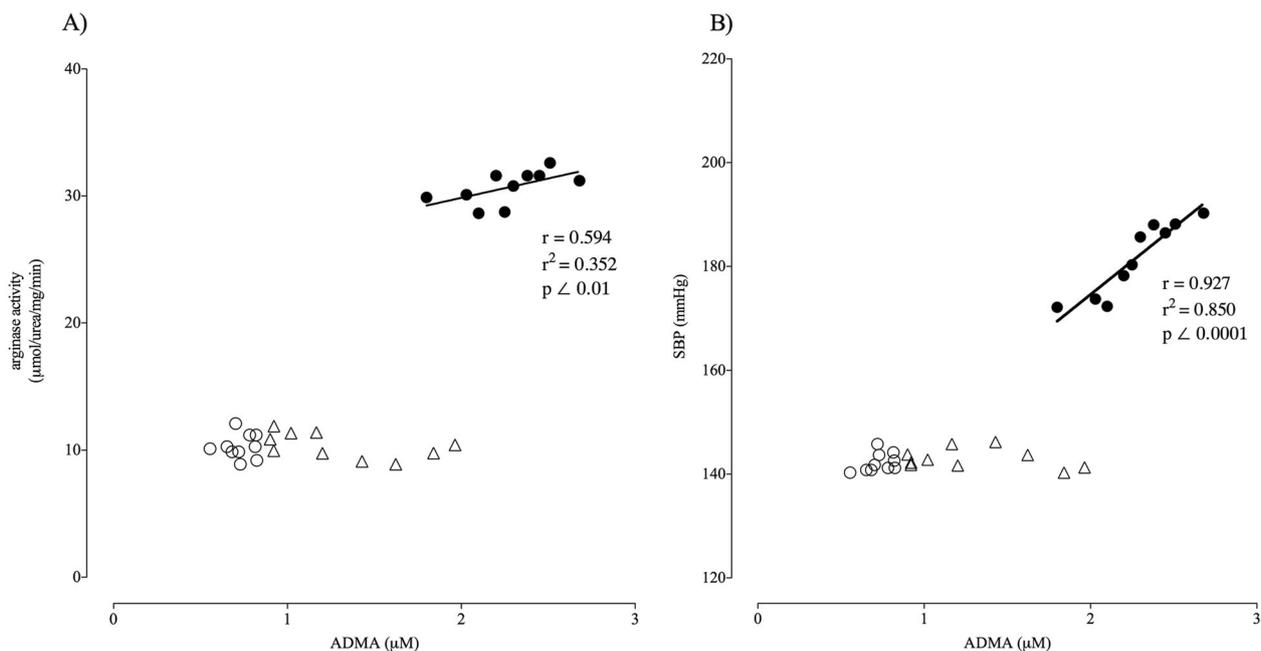


Fig. 7 Correlations between plasma ADMA concentration with arginase II activity and systolic blood pressure (SBP). **A** Correlation between ADMA and arginase II activity. **B** Correlation between ADMA and SBP. Control (open triangles), nonhypertensive (open

circles), and hypertensive (black circles) rats. Pearson's r values, r^2 , and p values correspond to the hypertensive rat group. No significant difference was found between the nonhypertensive rat group and the control group ($n = 10$ per group)

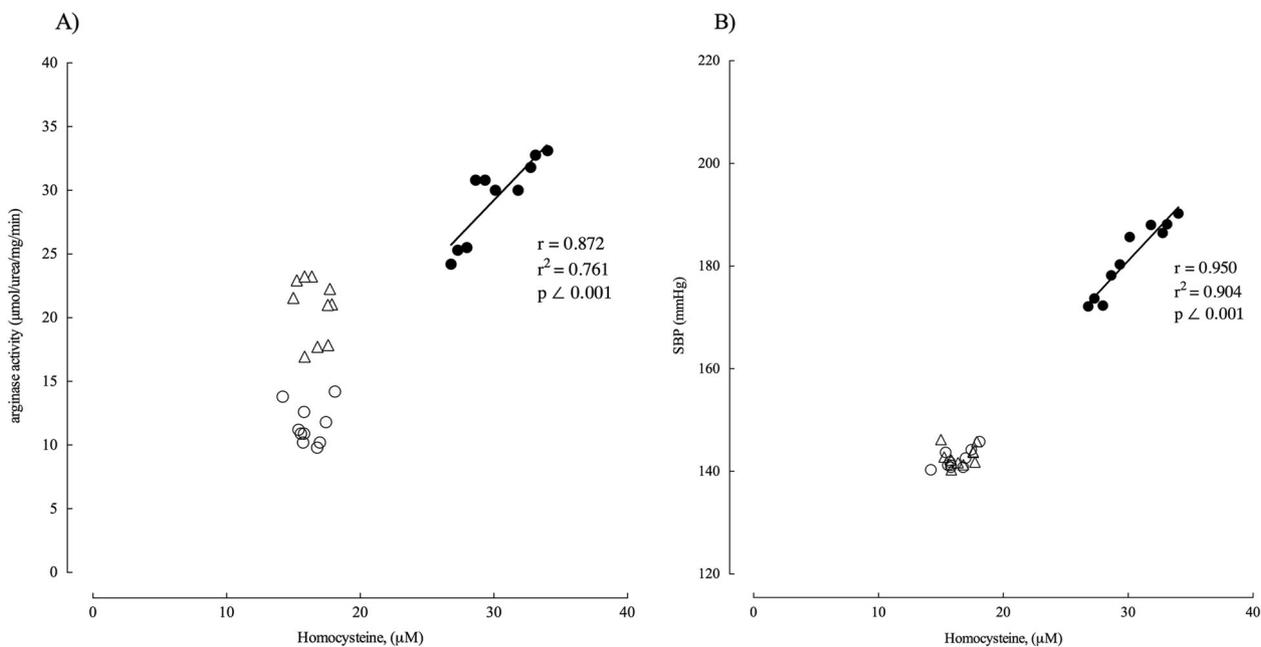


Fig. 8 Correlations between homocysteine concentration with arginase type II activity and systolic blood pressure (SBP). **A** Correlation between homocysteine and arginase II activity. **B** Correlation between homocysteine and SBP. Control (open triangles), nonhypertensive

(open circles), and hypertensive (black circles) rats. (Pearson's r values, r^2 , and p values correspond to the hypertensive rat group. No significant difference was found between the nonhypertensive rat group and the control group ($n = 10$ per group)

proposed as a risk marker for cardiovascular disease [34–37] and other pathologies, such as atherosclerosis, diabetes mellitus, rheumatoid arthritis, and preeclampsia [38–41]. In

this study, we observed a significant increase in ADMA levels in hypertensive rats. According to our results, one possible explanation for the high ADMA levels is a decrease

in DDAH-2 expression [20]. According to the literature, dimethylarginine dimethylaminohydrolase-2 (DDAH-2) has been proposed as a “target molecule” due to the presence of a polymorphism that induces the imbalance of ADMA metabolism and therefore contributes to the development of cardiovascular diseases [21].

Contrary to expectations, ADMA proved to be a poor inhibitor of arginase I and II (Fig. 5A, B). However, ADMA was a potent activator of arginase II in hypertensive rats (Fig. 5B). One putative mechanism by which ADMA production was mediated by the activation of arginase II in hypertensive rats may be the existence of polymorphisms in the arginase II gene, which may generate a specific regulatory site for ADMA. In this context, ADMA-induced arginase II activation may result in the decreased bioavailability of L-arginine for NO synthesis, explaining the increased arterial blood pressure observed in hypertensive rats. Nevertheless, the discovery of specific polymorphisms in the arginase II gene opens new avenues into the study of new mechanisms of hypoxia tolerance and predisposition to the development of hypertension triggered by environmental factors.

On the other hand, elevated ADMA plasma concentrations correlated with increases in arginase II activity and systolic blood pressure in hypertensive rats (Figs. 7A and 8B, respectively). We have described for the first time differential responses among rats exposed to hypoxia. Specifically, our results indicate that hypertensive rats have high ADMA concentrations and that arginase type II activity is susceptible to the effect of this metabolite, probably due to the existence of a regulatory site for ADMA.

Hypoxia-induced hypertensive rats exhibited higher homocysteine concentrations and increased arginase activity

A wide range of biological and pathological conditions associated with high levels of plasma homocysteine, such as diabetes, hypertriglyceridemia, endothelial dysfunction, apoptosis, oxidative stress, and renal and hepatic impairment, has been described [14, 42–44]. Increased homocysteine was recently described as a risk factor for neurodegenerative and neuropsychiatric diseases (e.g., vascular dementia) [45–47]. Nevertheless, most research on homocysteine conducted over the last two decades has focused on its role as a risk factor for cardiovascular [48–51] and other associated diseases, such as thrombosis, aortic aneurysm, and atherosclerosis [50, 52, 53]. In this study, we describe for the first time an increase in plasma homocysteine levels in hypoxia-induced hypertension (Fig. 2C).

The differences in plasma homocysteine between non-hypertensive and hypertensive rats may be related to differences in the regulation of key enzymes involved in

homocysteine metabolism. The presence of genetic polymorphisms in the CBS enzyme gene has been described [52, 54, 55], and these polymorphisms may be associated with increases in plasma homocysteine levels and the risk of cardiovascular diseases. In 2014 [52], Sarov et al. observed an association between mutations in the CBS enzyme and the prevalence of hyperhomocysteinemia and homocystinuria in patients with acute deep vein thrombosis (DVT). Furthermore, patients with DVT completely recovered after treatment with heparin, a methionine-restricted diet, and vitamin B12 supplements.

In the present study, the differences in plasma homocysteine between hypertensive and nonhypertensive rats may be explained by reduced CBS expression during exposure to hypoxic conditions, suggesting that hypoxia may decrease CBS expression in hypertensive rats, probably due to the presence of polymorphisms associated with this gene. Based on these results, we suggest that the increased homocysteine concentrations observed in hypertensive rats may be due to the existence of polymorphisms in the CBS gene, producing changes in the activity and expression of these enzymes with a subsequent increase in plasma homocysteine levels.

In addition, in this study, we describe for the first time the role of homocysteine as an important trigger of arginase isoforms (Fig. 6A, B). However, as mentioned above, homocysteine was not observed to affect arginase II under normoxic conditions (Fig. 6B). These results correlate with those reported by López et al. in 2009, who showed that arginase II activity and arginase I activity displayed different kinetics. Based on these observations, we suggest the existence of a regulatory site for homocysteine in arginase II that leads to a significant increase in its activity.

Previously, we reported that hypoxia-induced hypertensive rats exhibit higher arginase II expression [7], which leads to lower arginine bioavailability for NO synthesis, and that a lower NO concentration leads to the development of arterial hypertension. Based on these observations, we suggest that the insensitivity of homocysteine to arginase II could represent a protective mechanism against endothelial dysfunction under normoxic conditions that avoids alterations to the vasoconstrictor systems dependent on NO. Nevertheless, exposure to hypoxia may generate an arginase II conformation that is sensitive to homocysteine-mediated activation (Fig. 6B). This suggests that under hypoxic conditions, arginase II may adopt a structural conformation with an allosteric site for homocysteine. We are currently undergoing studies in our laboratory to confirm his hypothesis.

The results from this study reveal that the development of hypertension induced by hypoxia in hypertensive rats may be the result of increased ADMA and homocysteine levels and activation of the arginase II pathway, which in

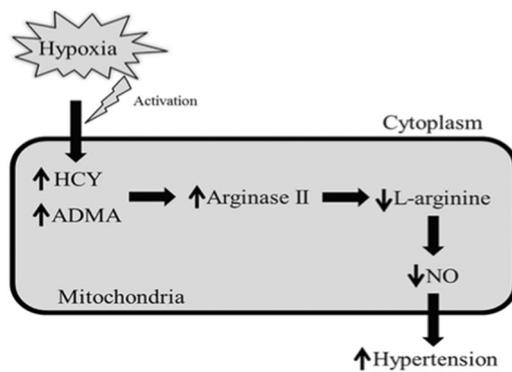


Fig. 9 Proposed mechanism for the development of hypoxia-induced hypertension in hypertensive rats

turn could inhibit NO synthesis and reduce L-arginine bioavailability (Fig. 9).

Finally, based on our findings, we propose a model for the development of hypertension induced by hypobaric hypoxia. Hypoxia-induced hypertension is caused by arginase II activation mediated by an increase in homocysteine and ADMA concentrations, suggesting decreased L-arginase bioavailability for the synthesis of NO (Fig. 9). ADMA and homocysteine have dual inhibitory effects on NO synthesis. The first involves the inhibition of eNOS, and the second, described here for the first time, involves the effect of ADMA and homocysteine in activating type II arginase. This is likely due to the existence of regulatory sites for these metabolites, such as those generated by polymorphisms in the arginase type II gene. However, this hypothesis needs to be evaluated.

Finally, our study proposes regulation of the arginase II pathway by homocysteine and ADMA as a new mechanism for NO synthesis regulation and that this regulation may play an important role as a molecular target for clinical applications.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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