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Hypobaric hypoxia induced arginase expression limits nitric oxide availability and signaling in rodent heart

Manjulata Singh, Gayatri Padhy, Praveen Vats, Kalpana Bhargava *, Niroj Kumar Sethy **

Defence Institute of Physiology and Allied Sciences, Defence Research and Development Organisation, Lucknow Road, Timarpur, Delhi 110054, India

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ABSTRACT

Background: This study was aimed to evaluate regulation of cardiac arginase expression during hypobaric hypoxia and subsequent effect on nitric oxide availability and signaling.
Methods: Rats were exposed to hypobaric hypoxia (282 mm Hg for 3 h) and ARG1 expression was monitored. The expression levels of eNOS and eNOS^{Ser1177} were determined by Western blotting, cGMP levels were measured by ELISA and amino acid concentrations were measured by HPLC analysis. Transcription regulation of arginase was monitored by chromatin immunoprecipitation (ChIP) assay with anti-c-Jun antibody for AP-1 consensus binding site on ARG1 promoter. Arginase activity was inhibited by intra-venous dose of N-(ω)-hydroxy-nor-L-arginine (nor-NOHA) prior to hypoxia exposure and subsequent effect on NO availability and oxidative stress were evaluated.
Results: Hypobaric hypoxia induced cardiac arginase expression by recruiting c-Jun to AP-1 binding site on ARG1 promoter. This increased expression redirected L-arginine towards arginase and resulted in limited endothelial nitric oxide synthase (eNOS) activity, nitric oxide (NO) availability and cGMP mediated signaling. Inhibition of arginase restored the eNOS activity, promoted cardiac NO availability and ameliorated peroxynitrite formation during hypoxia.
Conclusions: Hypoxic induced arginase under transcription control of AP-1 reciprocally regulates eNOS activity and NO availability in the heart. This also results in cardiac oxidative stress.
General significance: This study provides understanding of hypoxia-mediated transcriptional regulation of arginase expression in the heart and its subsequent effect on eNOS activity, NO availability and signaling as well as cardiac oxidative stress. This information will support the use of arginase inhibitors as therapeutics for pathological hypoxia.

1. Introduction

Inadequate supply of oxygen from the blood stream to the cells of tissue results in hypoxia. Several pathological conditions such as pulmonary disease, cardiac failure, orthologic hypotension, stroke, obstructive sleep apnea as well as physiological conditions including the exercising muscle and environmental conditions at high altitude can result in acute or chronic hypoxia. High altitude associated hypobaric hypoxia is an extreme environmental stress which can result in pulmonary hypertension, vascular resistance, high altitude pulmonary edema (HAPE) and high altitude cerebral edema (HACE) with potentially fatal consequences [1]. Exposure to high altitude induces pulmonary arterial vasoconstriction and increases pulmonary arterial systemic pressure (PASP). In contrast, a net systemic vasodilatation and a variable clinical blood pressure have been observed for hypoxia [2].

Compelling evidences suggest that hypobaric hypoxia impairs systemic endothelial function in individuals prone to high altitude diseases [3,4]. This limits the bioavailability of endothelium-produced nitric oxide (NO) which plays an integral part of human physiological response to hypoxia [4–7]. This was further substantiated by lower levels of exhaled NO in mountaineers both susceptible to HAPE and suffering from HAPE [8,9]. In contrast, high altitude native Tibetans exhibit more than double and in some cases higher orders of magnitude lung, plasma and red blood cell NO levels than other populations regardless of altitude. These higher levels of NO and biologically active NO metabolites enable higher blood flow, normal oxygen delivery and offset physiological hypoxia [9]. These studies suggest that hypoxia differentially regulates hypoxic NO formation and bioavailability.

NO is produced by endothelial NO synthase (eNOS) which converts L-arginine to NO and L-citrulline in an oxygen dependent manner which also requires NADPH and cofactors FAD, FMN and BH_4. Once produced in the endothelium, NO freely diffuses into smooth muscle cells to

* Correspondence to: K. Bhargava, Ph.D., Peptide and Proteomics Division, Defence Institute of Physiology and Allied Sciences, Lucknow Road, Timarpur, Delhi-110054, India. Tel.: +91 11 23883010; fax: +91 11 23914790.
** Correspondence to: N.K. Sethy, Ph.D., Peptide and Proteomics Division, Defence Institute of Physiology and Allied Sciences, Lucknow Road, Timarpur, Delhi-110054, India. Tel.: +91 11 23883003; fax: +91 11 23932869.
E-mail addresses: kalpanab2006@gmail.com (K. Bhargava), nksethy@gmail.com (N.K. Sethy).

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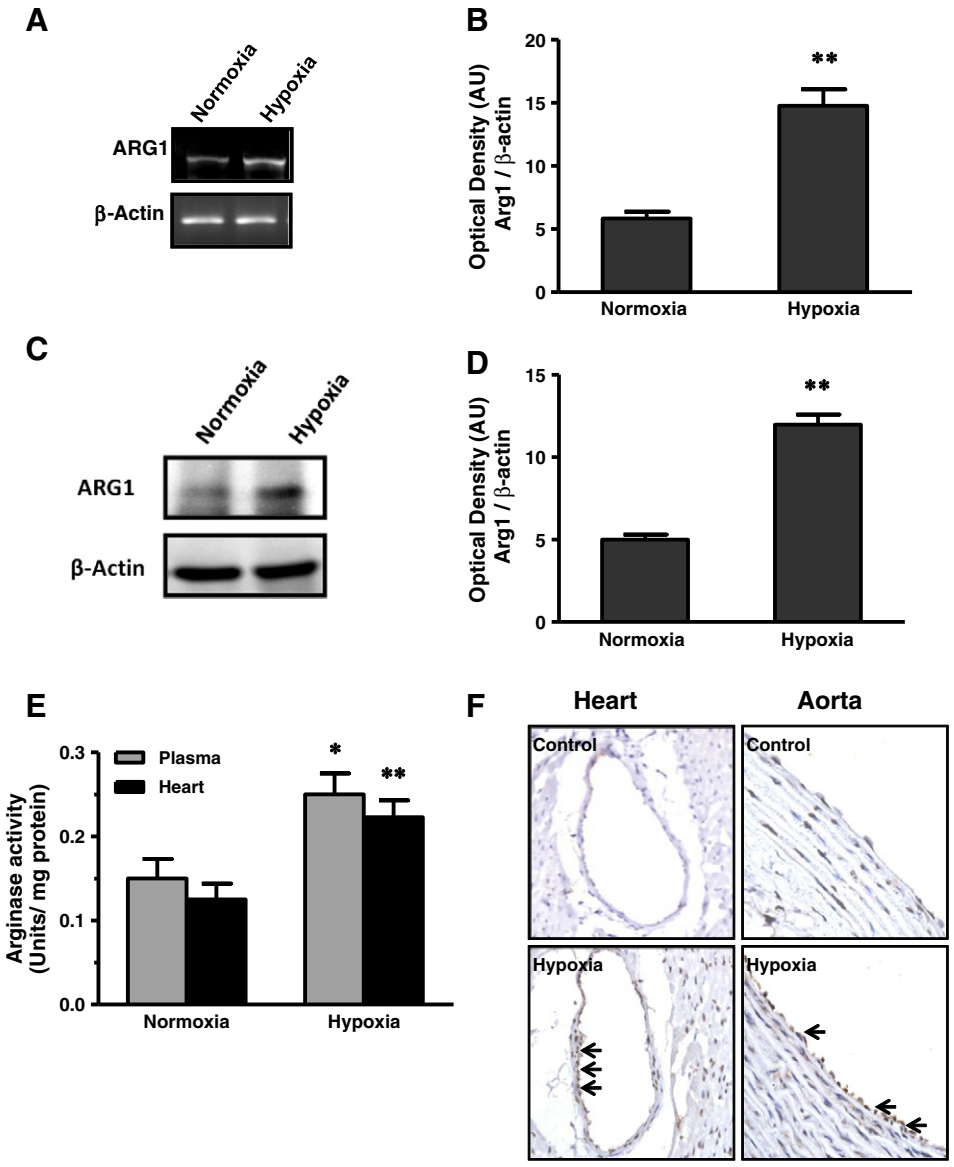

Fig. 1. Hypobaric hypoxia induces cardiac arginase expression. The mRNA and protein expression levels of ARG1 were measured in normoxia and hypoxia rat hearts. Both mRNA expression (A, B) and protein expression (C, D) of ARG1 were increased in hypoxic hearts as compared to normoxic hearts. Total arginase enzymatic activity was estimated in plasma and heart samples of both normoxia and hypoxia exposed animals (E). Hypobaric exposure has increased the total arginase activity both in plasma and in cardiac tissue. In situ ARG1 expression was estimated by immunohistochemical staining of heart and aortic tissues of normoxic and hypoxic animals (F). The endothelial linings of the aorta and small capillaries of the heart showed more DAB stained cells indicating higher arginase expression. Data were represented as mean ± SEM (n = 6 rats per group; * $p < 0.05$, ** $p < 0.01$, with respect to normoxia).

activate soluble guanylate cyclase to produce cyclic guanosine monophosphate (cGMP) which leads to vasodilatation. Interestingly, arginase competes with eNOS for the common substrate L-arginine and increased arginase activity results in decreased NO production and activity [10,11]. Arginase reportedly inhibits NO production via several potential mechanisms, including competition with NOS for the substrate L-arginine, uncoupling of NOS, repression of the translation and stability of inducible NOS protein, inhibition of inducible NOS activity via the generation of urea, and sensitization of NOS to its endogenous inhibitor, asymmetric dimethyl-L-arginine [11–14]. Recent studies have provided evidences for increased arginase activity in causing eNOS uncoupling which results in vascular oxidative stress and inflammatory response [12,13]. Similarly, higher levels of arginase have been demonstrated in heart and lung tissues of hypobaric hypoxia-induced hypertensive rats [15,16]. However, the molecular regulation of hypobaric hypoxia-induced arginase expression and its subsequent effect on NO bioavailability and oxidative stress remains to be evaluated.

We hypothesized that hypobaric hypoxia exposure activates cardiac arginase expression which reciprocally regulates NO availability and signaling. After exposing rats to hypobaric hypoxia, we evaluated cardiac arginase transcript and protein levels, eNOS expression and phosphorylation along with cardiac cGMP content. We studied the transcriptional regulation of arginase by hypoxia using chromatin immunoprecipitation (ChIP) analysis. Inhibiting arginase activity by nor-NOHA during hypoxia, we also studied eNOS activity, NO availability and oxidative stress.

2. Materials and methods

2.1. Chemicals and reagents

All the chemicals of purest available grade were procured from Sigma-Aldrich (St. Louis, MO, USA). All the antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, California, USA), except

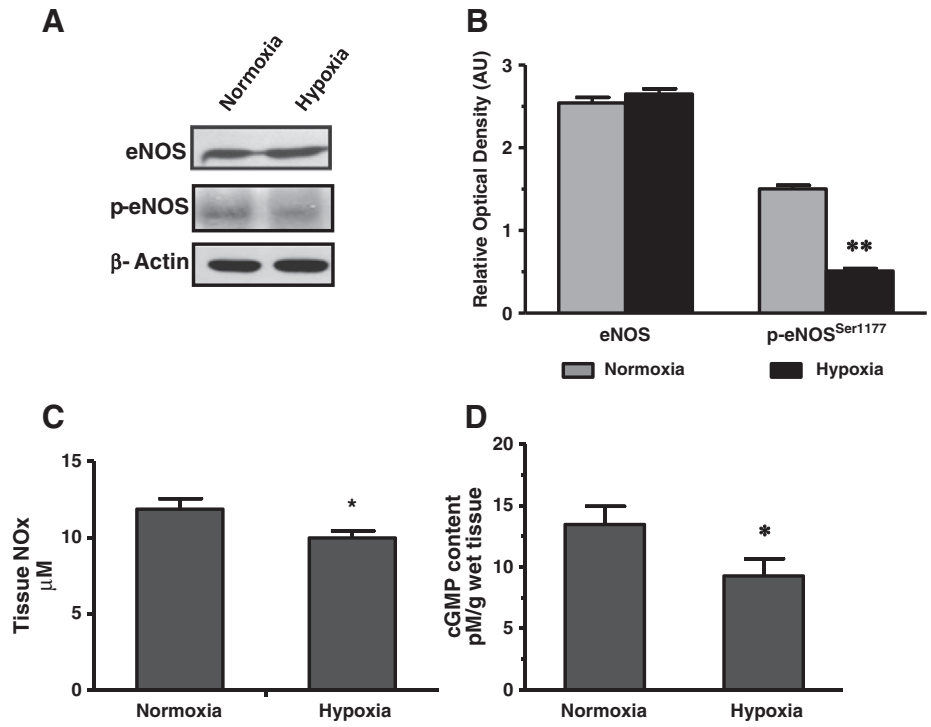

Fig. 2. Hypobaric hypoxia limits NO availability and signaling in cardiac tissue. Expression levels of both eNOS and p-eNOS^{Ser1177} were estimated in heart tissue of normoxic and hypoxic animals (A, B). Hypobaric hypoxia significantly decreased p-eNOS^{Ser1177} expression in hypoxic hearts. Similarly lower levels of cardiac total nitrate + nitrite (NOx) were also observed for hypoxia exposed animals (C) suggesting lower NO availability. Estimation of cardiac cGMP levels also revealed significant lower cGMP levels limiting NO-cGMP mediated signaling (D). Data were represented as mean ± SEM (n = 6 rats per group; * $p < 0.05$, ** $p < 0.01$ with respect to normoxia).

anti-c-Jun antibody-ChIP Grade (Abcam, MA, USA) and phospho-eNOS^{Ser1177} antibody (Cell Signaling Technologies, Danvers, MA, USA). Nitrate/nitrite colorimetric assay kit, cGMP EIA kit, and protein carbonyl assay kit were procured from Cayman Chemicals (MI, USA). OxiSelect™ HNE-His Adduct ELISA Kit and OxiSelect™ Nitrotyrosine ELISA kits were obtained from Cell Biolabs (CA, USA). RNeasy spin columns, universal PCR master mix and EpiTectChIP qPCR primer for rat ARG1 were procured from Qiagen (Valencia, USA). The high capacity cDNA reverse transcription kit and TaqMan probes were obtained from Applied Biosystems (Appelara, USA).

2.2. Animals and exposure to hypobaric hypoxia

Male Sprague–Dawley rats (200 ± 10 g) were maintained at 25 °C with a fixed 12 h light–dark cycle and provided free access to standard commercial diet and reverse osmosis water. The animals were randomly divided (n = 8) into (1) normoxia (C), (2) hypoxia (H), (3) nor-NOHA + normoxia (NoN), and (4) nor-NOHA + hypoxia (NoH) groups. For arginase inhibition, single intravenous dose of N-(ω)-hydroxy-nor-arginine (nor-NOHA, 100 mg/kg body weight) was injected one hour prior to hypoxia exposure [17]. Animals of H and NoH groups were exposed to 282 mm Hg (25,000 ft) of hypobaric hypoxia for 3 h while animals of C and NoN groups were maintained at normoxia (738 mm Hg) [18]. Post-hypoxia blood samples along with control animals were collected from tail vein into heparinized tubes for biochemical measurements. Hearts from both control and hypoxia exposed animals were rapidly excised under sodium pentobarbital (50 mg/kg body weight, IP) and further used fresh or snap frozen in liquid nitrogen. All the animal experiment protocols were approved by the Institutional Animal Ethical Committee and were in accordance with the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Environment and Forest, Government of India.

2.3. Estimation of arginase activity

Arginase activity was estimated in freshly collected plasma and heart tissue of all the animals by Arginase assay kit (Abnova Corp., Taiwan) as per manufacturer's protocol. Briefly, plasma and 5% tissue homogenates were passed through 10 kDa cut-off membranes and arginase activity was measured by comparing absorbance at 430 nm with respective standards. Arginase activity was expressed as units/mg of protein.

2.4. Estimation of oxidative stress parameters

2.4.1. ROS measurement

ROS levels were measured with dichlorofluorescein diacetate (DCFHDA) method [19]. Briefly, 10 μl of 10 μM DCFHDA was added to the 150 μl of cardiac homogenate (10% in 0.15 M KCl) and incubated for 40 min at 37 °C in dark. Fluorescence was measured at 488 nm excitation and 525 nm emission (LS45 luminescence spectrophotometer, PerkinElmer) and converted to arbitrary fluorescent units per milligram of protein.

2.4.2. Estimation of lipid peroxides

The myocardial lipid peroxides were estimated using OxiSelect™ HNE-His Adduct ELISA Kit (Cell Biolabs, CA, USA) according to manufacturer's instructions. In brief, myocardial tissue lysates (10 μg/ml) as well as HNE-BSA standards were adsorbed onto a 96-well protein binding plate overnight at 4 °C. The HNE adducts present in the lysates or standard were probed with anti-HNE antibody, followed by a horseradish peroxidase (HRP)-conjugated secondary antibody. The HNE–protein adduct content in an unknown sample was determined by comparing with a standard curve.

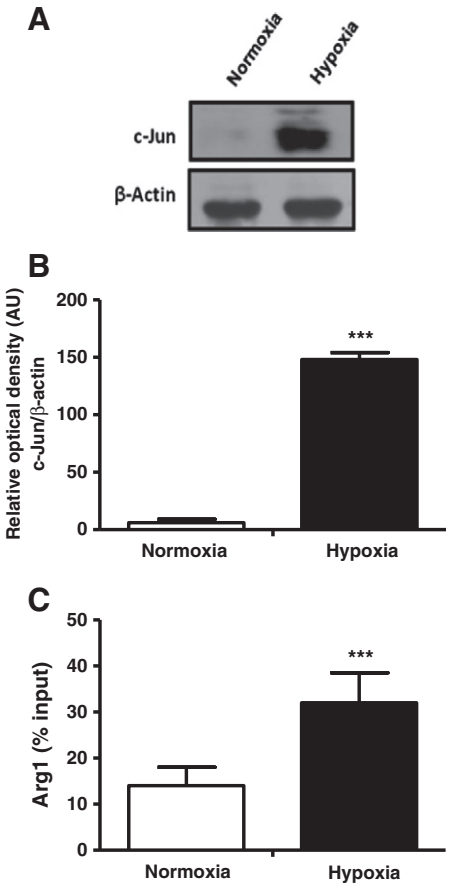

Fig. 3. Regulation of arginase expression during hypoxia. c-Jun group of proteins are co-factors which bind to AP-1 site on ARG1 promoter to facilitate its transcription. c-Jun expression was estimated by Western blotting (A). Densitometry results revealed that hypobaric hypoxia increases c-Jun expression in the heart (B). Regulation of ARG1 expression was studied using chromatin immunoprecipitation (ChIP) assay. Chromatin was immunoprecipitated with c-Jun antibody and the eluted DNA was quantified with EpiTectChIP qPCR primer for rat ARG1 ($n = 3$ rats per group; *** $p < 0.001$ with respect to normoxia).

2.4.3. Estimation of protein carbonyl content

The protein carbonyl content in myocardial tissues was determined using the Protein Carbonyl Assay Kit (Cayman Chemicals, MI, USA) according to the manufacturer's instructions. In brief, protein carbonyls present in nucleic acid free protein samples were derivatized to DNP hydrazone, analyzed spectrophotometrically and expressed as nanomoles per milligram of protein.

2.4.4. Estimation of nitrotyrosine content

The cardiac nitrotyrosine content was estimated using OxiSelect™ Nitrotyrosine ELISA Kit (Cell Biolabs, CA, USA) according to manufacturer's instructions. In brief, the 3-nitrotyrosine levels in heart homogenates were determined by comparing absorbance at 450 nm with nitrated BSA standard curve and expressed as picomoles per milligram of protein.

2.5. RNA isolation and real-time PCR

RNA was isolated from liquid nitrogen frozen heart tissues using TRI reagent and further treated with RNase free DNase I. After purification with RNeasy spin columns, spectrophotometric quantitation was done by recording $OD_{260/280}$ ratio. The purity and integrity of isolated RNA was assessed on 1% agarose gel. cDNA was synthesized from 1 μg total RNA by using high-capacity cDNA Reverse Transcription kit with random hexamer primers. Semi-quantitative PCR was performed in 20 μl volume containing 10 μl 2× PCR master mix using 100 nM of primers for ARG1 (5′-GTTCCCAGATGTACCAGGATTCTC-3′ and 5′-CGGT GGTTTA AGGTAGTCAGTCTC-3′) and ARG2 (5′-CCACCTGAGCTTTGACATAGAT GC-3′ and 5′-CTAGGAGTAGGAAGGTGGTCATAG-3′). Amplification conditions were standardized for each primer pairs.

2.6. Protein extraction and Western blotting

Frozen heart tissues were homogenized by Polytron™ homogenizer in RIPA buffer containing Tris—50 mM pH 7.4, NaCl—150 mM, 0.5% sodium deoxycholate, NP-40—1%, PMSF 0.05 mM, protease inhibitor cocktail for mammalian tissue extract and phosphatase inhibitor cocktail 2. Protein quantification was done with Bradford assay. 30–50 μg protein samples were separated by 10–12% SDS-PAGE and transferred onto nitrocellulose membrane. The nitrocellulose membrane was incubated in 5% BSA overnight at 4 °C to block non-specific binding of proteins. Further, blots were probed with primary antibodies against ARG1 (1:1000), eNOS (1: 4000), c-Jun (1: 1000) and β-actin (1:2000). Subsequently, the membrane was washed thrice with PBST (5 min each) and incubated with their respective secondary antibodies for 1 h at room temperature on rocking platform. Luminescent signals were developed using chemiluminescence peroxidase substrate and captured on X-ray films. Quantification of blots was done by ImageJ Software (National Institute of Health, USA). All the biochemical and protein expression experiments were repeated by persons blinded with the protocol.

2.7. Immunohistochemical analysis for arginase expression

In situ ARG1 expression was measured by performing immunohistochemistry. In brief, the heart and aorta from all the animals were perfusion-fixed with 4% formaldehyde (in PBS, pH 7.4) and further fixed overnight in formaldehyde. The fixed tissues were processed, embedded in paraffin and cut into 5 μM serial sections. Sections were mounted on glass slides, deparaffinized and rehydrated. Endogenous peroxidase activity was blocked by pre-treatment with 0.3% H_2O_2/methanol solution and sections were incubated with blocking buffer (BSA 5%). Sections were probed with rabbit polyclonal anti-ARG1 antibody at dilutions of 1:100 at 4 °C. Presence of antigen was visualized by developing the sections with 3,3-diaminobenzidine tetrahydrochloride (DAB) and sections were further counterstained with hematoxylin. Immunostained slides were viewed and photomicrographs were obtained with Olympus BX51 microscope at 200× magnification. For each image six different fields were analyzed independently.

2.8. Chromatin immunoprecipitation (ChIP) assay

ChIP assay was performed to evaluate the transcriptional regulation of ARG1 gene though c-Jun (AP-1) during hypobaric hypoxia. Briefly, heart tissues were grind using liquid nitrogen and tissue homogenates were cross linked with 1% formaldehyde solution. Subsequently, tissue suspension was lysed and chromatin was fragmented by using Micrococcal Nuclease (MNase). Digested chromatin was immunoprecipitated with 10 μg c-Jun antibody along with anti-RNA polymerase II antibody as positive control and normal rabbit IgG as negative control, by incubating overnight on rocking platform at 4 °C. Chromatin:antibody complex was captured onto agarose resin beads followed by incubation with elution buffer at 65 °C for 30 min. Elutes were further incubated at 65 °C for 1.5 h to separate DNA:protein complex along with Proteinase K and 5 M NaCl. Obtained DNA was quantified using EpiTectChIP qPCR primer for rat ARG1 [NM_017134.1(−)04Kb: GPR1056044(−)04A] along with positive and negative controls in a StepOnePlus PCR system (Applied Biosystems, USA).

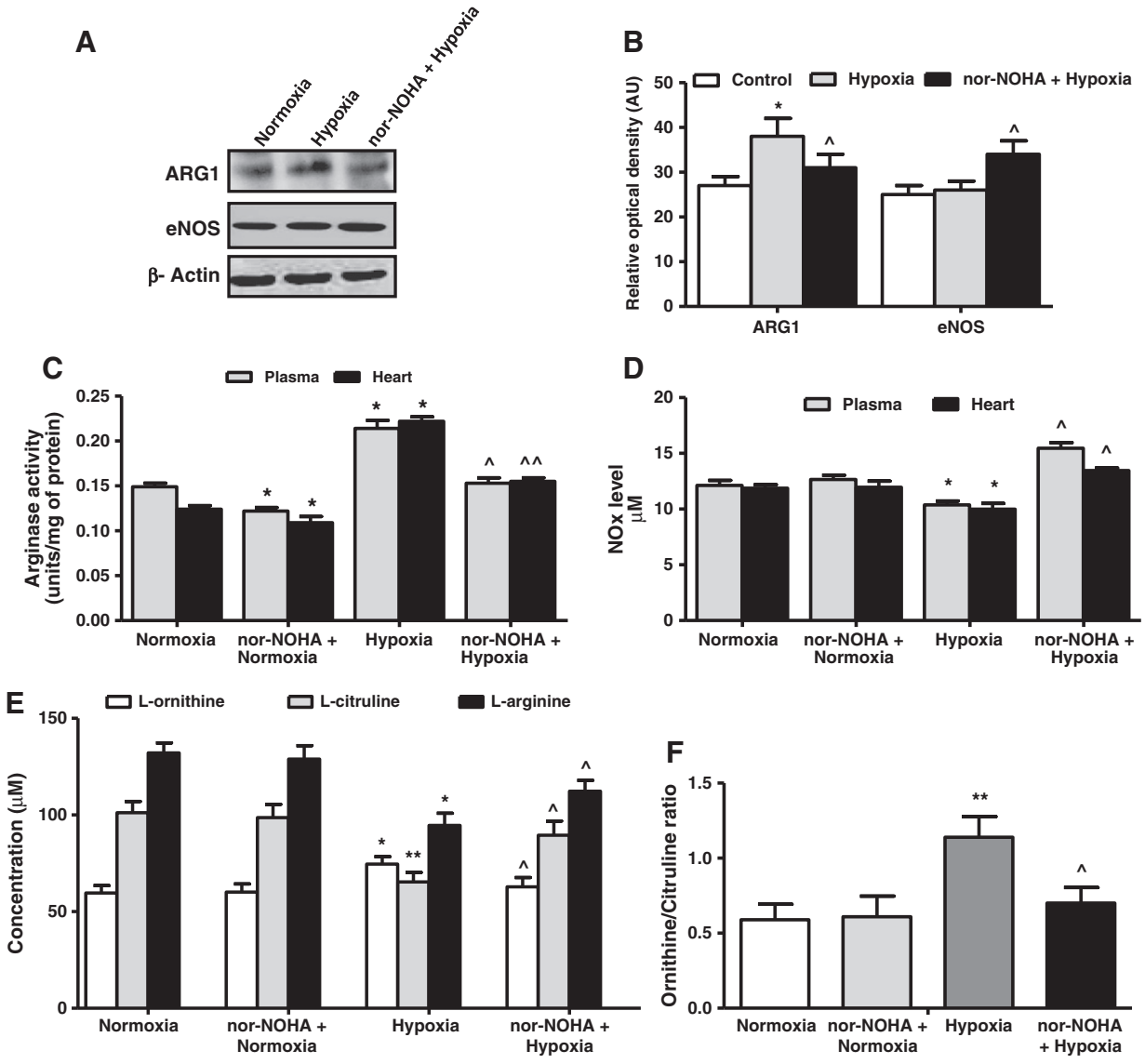

Fig. 4. Arginase inhibition increases cardiac NO availability during hypoxia. Arginase inhibition by nor-NOHA during hypoxia upregulates cardiac eNOS expression in contrast to hypoxia-mediated eNOS downregulation (A, B). Similarly, arginase inhibition also resulted in lower levels of arginase activity (C) and in higher levels of nitrite and nitrate levels both in plasma and in cardiac tissue (D). Determination of amino acids by HPLC also revealed increased levels of L-citrulline and decreased levels of L-ornithine levels in arginase inhibited hypoxic hearts as compared to hypoxic hearts (E), and representative figure of L-ornithine/L-citrulline ratio for all the exposure groups (F) suggesting that arginase blockade results in better NO availability during hypoxia (n = 6 rats per group; * $p < 0.05$, ** $p < 0.01$ with respect to normoxia, ^ $p < 0.05$, ^^ $p < 0.01$ with respect to hypoxia).

2.9. Estimation of amino acids by HPLC analysis

Plasma of all the animals was analyzed for L-arginine, L-citrulline and L-ornithine levels by HPLC system (Waters 600E dual piston pump system controller, Waters 717 Plus auto sampler and a Waters 474 fluorescence detector) using a method previously described [20]. Briefly, all the plasma samples were hydrolyzed using hydrochloric acid. Further, pre-column derivatization of all the samples was done using phenylisothiocyanate (PITC). Derivatized amino acids were washed with hexane by centrifuging at 15,000 ×*g* for 10 min and supernatant was separated on Waters C18 column (250 × 4.6 mm, 0.5 μm particle size) at room temperature. The separation was done by two phase mobile system. Mobile phase A consists of 30 mM potassium dihydrogen phosphate buffer with 0.4% tetrahydrofuran (pH 7.0). However, mobile phase B consists of 50% HPLC grade acetonitrile in HPLC grade water. Throughout the analysis flow rate of mobile phase was 1 ml/min. Detection was done fluorimetrically at excitation and emission wavelength of 340 nm and 450 nm respectively. All the standards were prepared in similar ways as of samples to cover the normal physiological range.

2.10. Statistical analysis

All the values are presented as mean ± SEM of at least three independent experiments. Student's *t*-test or two-way analysis of variance (ANOVA) with Bonferroni post-test was used to evaluate differences between the groups as appropriate. A p-value less than 0.05 was considered significant. All statistical analysis was calculated by using GraphPad Prism (V5.01).

3. Results

3.1. Effect of hypobaric hypoxia on ARG1 expression

To measure ARG1 expression in cardiac tissue during hypoxia, we measured both mRNA and protein levels of ARG1 (Fig. 1). The expression analysis revealed that hypobaric hypoxia upregulated ARG1 mRNA expression by 2.7 fold (Fig. 1A,B, $p < 0.01$) and protein expression by 2.1 fold (Fig. 1C and D, $p < 0.01$) as compared to normobaric hearts. Furthermore, we observed increased arginase activity in hypoxic

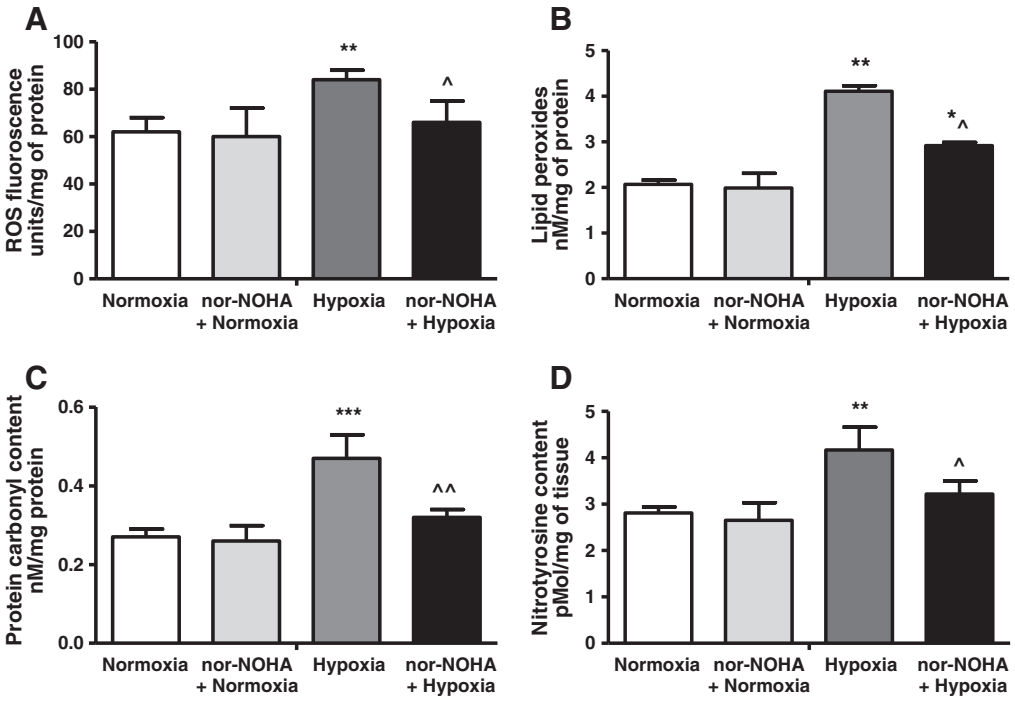

Fig. 5. Arginase inhibition decreases hypoxia-induced cardiovascular stress. Cardiac oxidative stress was measured by estimating ROS levels (A), lipid peroxides (B), protein carbonyl levels (C) and nitrotyrosine content (D) in normoxic, hypoxic as well as arginase inhibited hypoxic hearts. Arginase inhibition significantly reduced hypoxia-induced ROS, lipid peroxides and protein carbonyl levels. Similarly significant decrease in nitrotyrosine content was also observed ($p < 0.05$) suggesting reduced levels of oxidative–nitrosative stress ($n = 8$ rats per group; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ with respect to normoxia, ^ $p < 0.05$, ^^ $p < 0.01$ with respect to hypoxia).

heart (1.9 fold, $p < 0.01$) and plasma (Fig. 1E, 1.6 fold, $p < 0.05$). However, we could not detect both ARG2 mRNA and protein expression in hypoxia exposed cardiac tissue. The in situ ARG1 expression studies also revealed increased DAB stained cells in the inner linings of small capillaries in the heart and aorta of hypoxia exposed animals (Fig. 1F) suggesting hypoxia-induced increased arginase expression.

3.2. Effect of hypoxia on NO availability and signaling

To assess cardiac NO availability, we measured eNOS and phosphorylated eNOS (p-eNOS^{Ser1177}) protein levels in both normoxic and hypoxic heart homogenates. Densitometry analysis revealed significant decrease (2.6 fold, $p < 0.01$) in p-eNOS^{Ser1177} levels in hypoxia exposed hearts (Fig. 2A,B). We also estimated total content of bioactive NO metabolites nitrate and nitrites (NOx) as well as cGMP levels in cardiac homogenates. Our results demonstrate a 20% reduction in NOx levels ($p < 0.05$) along with 36% reduction in cGMP levels ($p < 0.05$) in hypoxia exposed hearts further suggesting limited NO availability and downstream signaling during hypoxia (Fig. 2C and D).

3.3. Regulation of ARG1 expression by hypobaric hypoxia

Transcription factor AP-1 regulates arginase activity by binding to its consensus site present at −3296 to −3111 bp on the promoter region of ARG1 gene [21]. We monitored c-Jun (AP1) expression in heart tissue by Western blot analysis (Fig. 3A,B). Our results suggest that hypoxia exposure significantly upregulates c-Jun expression in cardiac tissue. We further immunoprecipitated the DNA fragments with anti-c-Jun antibody and performed PCR with EpiTectChIP qPCR primers (assay position −3276) for ARG1. Quantitative estimation revealed 2.5 fold increased expression ($p < 0.001$) in hypoxia exposed cardiac tissue as compared to normoxia (Fig. 3C) suggesting c-Jun/AP1-mediated transcription regulation of arginase during hypoxia.

3.4. Effect of arginase inhibition on cardiac NO bioavailability

To establish the role of arginase in regulating NO availability during hypoxia, we inhibited arginase activity by injecting nor-NOHA prior to hypoxia (Fig. 4). This resulted in inhibition of both arginase activity and expression during hypoxia with concomitant increase in eNOS expression (1.5 fold, $p < 0.05$) as compared to hypoxia (Fig. 4, A–C). Estimation of NO metabolites also revealed increased availability of both nitrite and nitrate suggesting better NO availability (Fig. 4D). To further establish the role of arginase in regulating hypoxic NO availability, we measured amino acid levels by HPLC (Fig. 4E). Hypoxia exposure significantly increased L-ornithine levels with concomitant decrease in L-citrulline levels suggesting higher arginase activity. In contrast, we observed significant increase in L-citrulline levels (89.52 ± 7.3 μM) and decreased L-ornithine levels (62.84 ± 4.8 μM) in nor-NOHA treated groups as compared to hypoxia exposure [(65.37 ± 4.9 μM) and (74.56 ± 3.88 μM) respectively] suggesting restoration of NO pathway. Hypoxia exposure also significantly decreased L-arginine levels (94.6 ± 6.25 μM) as compared to normoxia (132.1 ± 5.2 μM) which was subsequently increased in nor-NOHA treated groups during hypoxia (112.3 ± 5.6 μM). These results further substantiate the role of hypoxia-induced arginase in regulating NO availability in the heart.

3.5. Effect of arginase inhibition on cardiac oxidative stress

We studied the cardiac oxidative stress in nor-NOHA treated cardiac tissue during hypoxia by measuring ROS, MDA, protein carbonyl level and nitrotyrosine content (Fig. 5). As shown in the figure, arginase inhibition significantly reduced hypoxia-induced both ROS ($p < 0.05$) and tissue MDA levels ($p < 0.05$). We also observed lower levels of protein carbonyls ($p < 0.01$) in arginase inhibited hypoxic hearts (Fig. 5C). Similarly, we also observed significant lower levels of peroxynitrite ($p < 0.05$) in nor-NOHA injected hearts as compared to hypoxic hearts. These cumulative results suggest that vascular oxidative stress during hypoxia is partly mediated by arginase.

4. Discussion

Endothelium-derived NO is well established as a critical mediator for vascular functions and homeostasis. Studies in high altitude natives as well as systematic human acclimatization studies to high altitude have reported the importance of NO and bioactive nitrogen oxides in hypoxic adaptation. However, regulation of endothelial NO production and availability during hypoxia is complex and depends on multiple factors. The purpose of the present study was to elucidate the molecular mechanism of eNOS regulation by arginase and downstream signaling in the heart during hypoxia. Specifically, we sought to determine the expression of arginase during hypoxia and its transcriptional regulation for hypoxic NO availability.

Endothelial production of NO is an oxygen dependent process and hypoxic conditions limit eNOS activity [22]. This compromised eNOS activity redirects the substrate L-arginine towards arginase, the competitive inhibitor of eNOS. This increased arginase activity further reduces NO availability by increased ROS generation, depleting BH4 and L-arginine as well as eNOS uncoupling [23]. Several studies have reported overexpression of ARG1 in hypoxic heart and aorta promotes vascular stress leading to pulmonary hypertension, vascular stiffening and endothelial dysfunction [24–26]. In the present study, we observed higher arginase activity in the heart and aorta of hypoxia exposed animals. We also observed concomitant decrease in eNOS phosphorylation, NO availability and cGMP levels in hypoxic heart. Since NO availability largely depends on L-arginine concentration and enhanced expression of arginase reduces global arginine concentration [27], we estimated amino acid levels by HPLC. Hypoxia exposure increased L-ornithine concentrations and decreased L-citrulline concentrations suggesting substrate redirection towards arginase instead of eNOS further supporting increased arginase expression during hypoxia. Inhibition of arginase by nor-NOHA increased eNOS activity and NO availability during hypoxia as well as redirected available substrates towards eNOS–NO pathway. Arginase inhibition is a promising therapeutic target in cardiovascular diseases [13,28,29]. Arginase inhibition promotes cardioprotection during ischemia–reperfusion (I/R) injury by an eNOS–NO pathway and shifting arginase utilization from arginase to eNOS [28,30]. Since arginase can inhibit L-arginine transport in endothelial cells further limiting NO availability [22] inhibition of arginase may facilitate substrate availability for eNOS promoting NO bioavailability. In a recent study, Yang et al. have demonstrated that arginase reciprocally regulates eNOS-derived NO formation in red blood cells and arginase blockade significantly improved post-ischemic functional recovery in an ex vivo model of myocardial ischemia–reperfusion injury [11]. Our present results along with the previous studies suggest that hypoxia exposure induces arginase expression which further limits NO synthesis and availability by directing the substrate towards arginase.

Along with hypoxia, several cardiovascular risk factors including hyperinsulinemia, hyperglycemia, aging, angiotensin-II, thrombin, oxidized LDL, oxidants like hydrogen peroxide and peroxynitrite upregulate arginase expression through p38MAPK, mTORC1-S6K, Rho/ROCK and JAK/STAT6 signaling pathways [13]. However, there is limited information available regarding the upstream regulatory mechanisms involved in gene expression and activity of arginase in vascular cells [11,31]. Studying thrombin induced arginase expression in rat aortic endothelial cells (RAECs), Zhu et al. have demonstrated that the activating protein-1 (AP-1) consensus site located at −3157 bp in the arginase I promoter was a thrombin responsive element. Upon thrombin stimulation, both c-Jun and activating transcription factor-2 (ATF-2) bind to the AP-1 site and initiate transactivation [21]. Using immunoblot analysis, we observed increased expression of c-Jun in hypoxic hearts as compared to normoxic hearts. Further, using quantitative PCR analysis we observed 1.7 fold higher AP-1 transactivation consensus site fragments in c-Jun immunoprecipitated chromatin fragments of hypoxic heart. Our current results reveal that after hypoxia exposure c-Jun is recruited to the AP-1 consensus sequence in the arginase promoter and arginase 1 transcription is regulated.

Oxidative stress is a hallmark of hypoxia and contributes to endothelial dysfunction. Paucity of molecular oxygen as terminal electron acceptor at complex IV of electron transport chain results in formation of superoxide anion (O_2^-). Enzyme systems including NADPH oxidase, cytochrome P450, xanthine oxidase and uncoupled eNOS also produce superoxide anion which can combine with NO to form peroxynitrite ($ONOO^-$) and further reduce NO bioavailability [4]. Peroxynitrite is known to oxidize and reduce tetrahydrobiopterin (BH_4), a co-factor necessary for production of eNOS-mediated NO. Under these conditions eNOS uncoupling may occur [32] which results in eNOS-mediated superoxide formation and further increases peroxynitrite levels. Arginase upregulation contributes to superoxide formation and endothelial oxidative stress in preeclamptic women [33] whereas arginase inhibition reduces superoxide levels [16,34]. In the present study, hypoxia exposure resulted in generation of higher levels of free radicals and oxidative stress. However, arginase inhibition resulted in lower levels of cardiac peroxynitrite levels suggesting lower levels of superoxide formation. Concomitantly, we also observed higher levels of bioactive NO products suggesting limited NO quenching by peroxynitrite. These results demonstrate that pharmacological inhibition of arginase can promote better NO availability in the heart during hypoxia.

There are limitations in the present study which need to be considered. Since both arginase I and II are expressed in vascular endothelium and compete with eNOS for L-arginine, we could not detect arginase II in rat heart. Since arginase I is the predominant isoforms in the rat endothelium and arginase II is the major isoforms present in human aortic endothelial cells and human umbilical vein endothelial cells (HUVECs) [16,31,34–36], differential transcriptional regulation may limit the application of present findings in humans. Secondly, arginase activation though other transcriptional pathways should be investigated. Since transcription factors like STAT-6, C/EPBβ, PU1, PPARγ and δ have been reported to regulate arginase expression [13], their role in promoting arginase expression during hypoxia is warranted.

5. Conclusion

In conclusion, the present study demonstrates that hypobaric hypoxia increases cardiac arginase expression by transcriptional regulation mediated through c-Jun and AP-1 interaction on ARG1 promoter. The increased expression of arginase redirects substrate availability towards arginase and limits eNOS function and NO availability. This also leads to cardiac oxidative stress and peroxynitrite formation. Inhibition of arginase restores the L-arginine–eNOS–NO pathway and limits cardiac oxidative stress during hypoxia. These results suggest that pharmacological inhibition of arginase is a promising therapeutic strategy for hypoxia-induced cardiovascular disorders.

Conflict of interest

The authors declare no conflict of interest.

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References

[1] B. Basnyat, D.R. Murdoch, High-altitude illness, Lancet 361 (2003) 1967–1974.
[2] C.J. Boos, P. Hodkinson, A. Mellor, N.P. Green, D.R. Woods, The effects of acute hypobaric hypoxia on arterial stiffness and endothelial function and its relationship to changes in pulmonary artery pressure and left ventricular diastolic function, High Alt. Med. Biol. 13 (2012) 105–111.

[3] D.M. Bailey, C. Dehnert, A.M. Luks, E. Menold, C. Castell, G. Schendler, V. Faoro, M. Gutowski, K.A. Evans, S. Taudorf, P.E. James, J. McEneny, I.S. Young, E.R. Swenson, H. Mairbaurl, P. Bartsch, M.M. Berger, High-altitude pulmonary hypertension is associated with a free radical-mediated reduction in pulmonary nitric oxide bioavailability, J. Physiol. 588 (2010) 4837–4847.
[4] C.M. Beall, D. Laskowski, S.C. Erzurum, Nitric oxide in adaptation to altitude, Free Radic. Biol. Med. 52 (2012) 1123–1134.
[5] D.Z. Levett, B.O. Fernandez, H.L. Riley, D.S. Martin, K. Mitchell, C.A. Leckstrom, C. Ince, B.J. Whipp, M.G. Mythen, H.E. Montgomery, M.P. Grocott, M. Feelisch, The role of nitrogen oxides in human adaptation to hypoxia, Sci. Rep. 1 (2011) 109.
[6] A.J. Janocha, C.D. Koch, M. Tiso, A. Ponchia, A. Doctor, L. Gibbons, B. Gaston, C.M. Beall, S.C. Erzurum, Nitric oxide during altitude acclimatization, N. Engl. J. Med. 365 (2011) 1942–1944.
[7] H. Duplain, R. Burcelin, C. Sartori, S. Cook, M. Egli, M. Lepori, P. Vollenweider, T. Pedrazzini, P. Nicod, B. Thorens, U. Scherrer, Insulin resistance, hyperlipidemia, and hypertension in mice lacking endothelial nitric oxide synthase, Circulation 104 (2001) 342–345.
[8] C.M. Beall, D. Laskowski, K.P. Strohl, R. Soria, M. Villena, E. Vargas, A.M. Alarcon, C. Gonzales, S.C. Erzurum, Pulmonary nitric oxide in mountain dwellers, Nature 414 (2001) 411–412.
[9] S.C. Erzurum, S. Ghosh, A.J. Janocha, W. Xu, S. Bauer, N.S. Bryan, J. Tejero, C. Hemann, R. Hille, D.J. Stuehr, M. Feelisch, C.M. Beall, Higher blood flow and circulating NO products offset high-altitude hypoxia among Tibetans, Proc. Natl. Acad. Sci. U. S. A. 104 (2007) 17593–17598.
[10] I.M. Corraliza, G. Soler, K. Eichmann, M. Modolell, Arginase induction by suppressors of nitric oxide synthesis (IL-4, IL-10 and PGE2) in murine bone-marrow-derived macrophages, Biochem. Biophys. Res. Commun. 206 (1995) 667–673.
[11] J. Yang, A.T. Gonon, P.O. Sjoquist, J.O. Lundberg, J. Pernow, Arginase regulates red blood cell nitric oxide synthase and export of cardioprotective nitric oxide bioactivity, Proc. Natl. Acad. Sci. U. S. A. 110 (2013) 15049–15054.
[12] A.T. Gonon, C. Jung, A. Katz, H. Westerblad, A. Shemyakin, P.O. Sjoquist, J.O. Lundberg, J. Pernow, Local arginase inhibition during early reperfusion mediates cardioprotection via increased nitric oxide production, PLoS ONE 7 (2012) e42038.
[13] J. Pernow, C. Jung, Arginase as a potential target in the treatment of cardiovascular disease: reversal of arginine steal? Cardiovasc. Res. 98 (2013) 334–343.
[14] J.C. Lopez-Ramos, R. Martinez-Romero, F. Molina, A. Canuelo, E. Martinez-Lara, E. Siles, M.A. Peinado, Evidence of a decrease in nitric oxide-storage molecules following acute hypoxia and/or hypobaria, by means of chemiluminescence analysis, Nitric Oxide 13 (2005) 62–67.
[15] Y. Jin, B. Chen, T.J. Calvert, L.G. Chicoine, Y. Liu, L.D. Nelin, Chronic hypoxia decreases arterial and venous compliance in isolated perfused rat lungs: an effect that is reversed by exogenous L-arginine, Am. J. Physiol. Heart Circ. Physiol. 304 (2013) H195–H205.
[16] J.H. Kim, L.J. Bugaj, Y.J. Oh, T.J. Bivalacqua, S. Ryoo, K.G. Soucy, L. Santhanam, A. Webb, A. Camara, G. Sikka, D. Nyhan, A.A. Shoukas, M. Ilies, D.W. Christianson, H.C. Champion, D.E. Berkowitz, Arginase inhibition restores NOS coupling and reverses endothelial dysfunction and vascular stiffness in old rats, J. Appl. Physiol. 107 (2009) 1249–1257.
[17] M. Singh, A. Arya, R. Kumar, K. Bhargava, N.K. Sethy, Dietary nitrite attenuates oxidative stress and activates antioxidant genes in rat heart during hypobaric hypoxia, Nitric Oxide 26 (2012) 61–73.
[18] H. Wang, R. Jiang, Q. He, Y. Zhang, Y. Li, R. Zhuang, Y. Luo, J. Wan, Y. Tang, H. Yu, Q. Jiang, J. Yang, Expression pattern of peroxisome proliferator-activated receptors in rat hippocampus following cerebral ischemia and reperfusion injury, PPAR Res. 2012 (2012) 596394.
[19] H. Wang, J.A. Joseph, Quantifying cellular oxidative stress by dichlorofluorescein assay using microplate reader, Free Radic. Biol. Med. 27 (1999) 612–616.
[20] M.P. Frank, R.W. Powers, Simple and rapid quantitative high-performance liquid chromatographic analysis of plasma amino acids, J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 852 (2007) 646–649.
[21] W. Zhu, U.M. Chandrasekharan, S. Bandyopadhyay, S.M. Morris Jr., P.E. DiCorleto, V.S. Kashyap, Thrombin induces endothelial arginase through AP-1 activation, Am. J. Physiol. Cell Physiol. 298 (2010) C952–C960.
[22] C.P. Prieto, B.J. Krause, C. Quezada, R. San Martin, L. Sobrevia, P. Casanello, Hypoxia-reduced nitric oxide synthase activity is partially explained by higher arginase-2 activity and cellular redistribution in human umbilical vein endothelium, Placenta 32 (2011) 932–940.
[23] Y.C. Luiking, G.A. Ten Have, R.R. Wolfe, N.E. Deutz, Arginine de novo and nitric oxide production in disease states, Am. J. Physiol. Endocrinol. Metab. 303 (2012) E1177–E1189.
[24] C. Zhang, T.W. Hein, W. Wang, M.W. Miller, T.W. Fossum, M.M. McDonald, J.D. Humphrey, L. Kuo, Upregulation of vascular arginase in hypertension decreases nitric oxide-mediated dilation of coronary arterioles, Hypertension 44 (2004) 935–943.
[25] L. Santhanam, D.W. Christianson, D. Nyhan, D.E. Berkowitz, Arginase and vascular aging, J. Appl. Physiol. 105 (2008) 1632–1642.
[26] D.L. Michell, K.L. Andrews, J.P. Chin-Dusting, Endothelial dysfunction in hypertension: the role of arginase, Front. Biosci. (Schol. Ed.) 3 (2011) 946–960.
[27] S. Elms, F. Chen, Y. Wang, J. Qian, B. Askari, Y. Yu, D. Pandey, J. Iddings, R.B. Caldwell, D.J. Fulton, Insights into the arginine paradox: evidence against the importance of subcellular location of arginase and eNOS, Am. J. Physiol. Heart Circ. Physiol. 305 (2013) H651–H666.
[28] C. Jung, A.T. Gonon, P.O. Sjoquist, J.O. Lundberg, J. Pernow, Arginase inhibition mediates cardioprotection during ischaemia–reperfusion, Cardiovasc. Res. 85 (2010) 147–154.
[29] Y. Tratsiakovich, A.T. Gonon, A. Krook, J. Yang, A. Shemyakin, P.O. Sjoquist, J. Pernow, Arginase inhibition reduces infarct size via nitric oxide, protein kinase C epsilon and mitochondrial ATP-dependent K^+ channels, Eur. J. Pharmacol. 712 (2013) 16–21.
[30] Y. Tratsiakovich, J. Yang, A.T. Gonon, P.O. Sjöquist, J. Pernow, Arginase as a target for treatment of myocardial ischemia-reperfusion injury, Eur J Pharmacol 720 (1–3) (2013 Nov 15) 121–123, http://dx.doi.org/10.1016/j.ejphar.2013.10.040(Epub 2013 Oct 29).
[31] X.F. Ming, C. Barandier, H. Viswambharan, B.R. Kwak, F. Mach, L. Mazzolai, D. Hayoz, J. Ruffieux, S. Rusconi, J.P. Montani, Z. Yang, Thrombin stimulates human endothelial arginase enzymatic activity via RhoA/ROCK pathway: implications for atherosclerotic endothelial dysfunction, Circulation 110 (2004) 3708–3714.
[32] U. Forstermann, W.C. Sessa, Nitric oxide synthases: regulation and function, Eur. Heart J. 33 (2012) 829–837(837a–837d).
[33] S. Sankaralingam, H. Xu, S.T. Davidge, Arginase contributes to endothelial cell oxidative stress in response to plasma from women with preeclampsia, Cardiovasc. Res. 85 (2010) 194–203.
[34] M.J. Romero, D.H. Platt, H.E. Tawfik, M. Labazi, A.B. El-Remessy, M. Bartoli, R.B. Caldwell, R.W. Caldwell, Diabetes-induced coronary vascular dysfunction involves increased arginase activity, Circ. Res. 102 (2008) 95–102.
[35] D.E. Berkowitz, R. White, D. Li, K.M. Minhas, A. Cernetich, S. Kim, S. Burke, A.A. Shoukas, D. Nyhan, H.C. Champion, J.M. Hare, Arginase reciprocally regulates nitric oxide synthase activity and contributes to endothelial dysfunction in aging blood vessels, Circulation 108 (2003) 2000–2006.
[36] A.R. White, S. Ryoo, D. Li, H.C. Champion, J. Steppan, D. Wang, D. Nyhan, A.A. Shoukas, J.M. Hare, D.E. Berkowitz, Knockdown of arginase I restores NO signaling in the vasculature of old rats, Hypertension 47 (2006) 245–251.