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Arginase inhibition enhances angiogenesis in endothelial cells exposed to hypoxia

Lin Wang [a,b], Anil Bhatta [b], Haroldo A. Toque [b], Modesto Rojas [c], Lin Yao [b], Zhimin Xu [c], Chintan Patel [c], Ruth B. Caldwell [c], R. William Caldwell [b,*]

[a] Department of Plastic Surgery, Ren Ji Hospital, School of Medicine, Shanghai Jiao Tong University, Shanghai 200127, PR China
[b] Department of Pharmacology and Toxicology, Georgia Regents University, Augusta, GA 30912, USA
[c] Vascular Biology Center, Georgia Regents University, Charlie Norwood VA Medical Center, Augusta GA, 30912, USA

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ABSTRACT

Hypoxia-induced arginase elevation plays an essential role in several vascular diseases but influence of arginase on hypoxia-mediated angiogenesis is completely unknown. In this study, *in vitro* network formation in bovine aortic endothelial cells (BAEC) was examined after exposure to hypoxia for 24 h with or without arginase inhibition. Arginase activity, protein levels of the two arginase isoforms, eNOS, and VEGF as well as production of NO and ROS were examined to determine the involvement of arginase in hypoxia-mediated angiogenesis. Hypoxia elevated arginase activity and arginase 2 expression but reduced active p-eNOS^{Ser1177} and NO levels in BAEC. In addition, both VEGF protein levels and endothelial elongation and network formation were reduced with continued hypoxia, whereas ROS levels increased and NO levels decreased. Arginase inhibition limited ROS, restored NO formation and VEGF expression, and prevented the reduction of angiogenesis. These results suggest a fundamental role of arginase activity in regulating angiogenic function.

Introduction

Arginase, an ureohydrolase enzyme, has been shown to be involved in the several vascular diseases such as hypertension, atherosclerosis, and diabetic coronary vascular dysfunction (Yang and Ming, 2006; Sasaki et al., 2007; Romero et al., 2008). The detrimental effects of elevated arginase activity in these conditions are mostly attributed to its capability to inhibit nitric oxide (NO) synthesis by competing with endothelial NO synthase (eNOS) for their common substrate, L-arginine (Morris, 2002, 2009). When the L-arginine supply needed for eNOS activity is insufficient, NOS can become uncoupled, producing less NO and forming superoxide (O_2^-). Further, remaining NO can react with O_2^- to form the toxic oxidant peroxynitrite ($ONOO^-$) (Forstermann and Munzel, 2006). NO plays a pivotal role in vasodilation and angiogenesis (Dulak et al., 2000; Jones et al., 2004; Morris et al., 2008; Berchner-Pfannschmidt et al., 2010). Reduced NO bioavailability within the vessel wall leads to vasoconstriction, vascular remodeling and an aberrant neovasculature (Madigan and Zuckerbraun, 2013).

Many factors that trigger endothelial dysfunction also up-regulate arginase expression and activity. Hypoxia is one of these factors. Hypoxia has been reported to increase arginase-2 expression and activity in human umbilical vein and pulmonary microvascular endothelial cells (Krotova et al., 2010; Prieto et al., 2011). Also, oxidative stress due to generation of reactive oxygen species (ROS), including superoxide and peroxynitrite, contributes to elevated arginase expression/activity and eNOS uncoupling (Thengchaisri et al., 2006; Sankaralingam et al., 2010; Chandra et al., 2012). Moreover, hypoxia is a major mediator of angiogenesis. Hypoxia-mediated angiogenesis due to elevated levels of hypoxia-induced factor-1α (HIF-1α) and vascular endothelial growth factor (VEGF) has been well demonstrated (Fong, 2009; Takenaga, 2011; Ahluwalia and Tarnawski, 2012). NO also is importantly involved in regulation of hypoxia-mediated angiogenesis through an NO/HIF-1α/VEGF axis (Kimura et al., 2000, 2001; Kimura and Esumi, 2003). Hence, arginase may alter this process via its reduction of NO. However, no studies on a role of arginase in hypoxia-mediated angiogenesis have been reported. The present study examined the hypothesis that enhanced arginase activity during hypoxia limits the angiogenic process. *In vitro* network formation in endothelial cells was examined after exposure to hypoxia (2% O_2) for 12 and 24 h with or without arginase inhibition. Protein levels of the two arginase isoforms, eNOS and VEGF in addition of NO and ROS production were examined to determine the role of arginase in hypoxia-mediated angiogenesis.

* Corresponding author at: Department of Pharmacology and Toxicology, Medical College of Georgia, Georgia Regents University, Augusta, GA 30912, USA.
E-mail address: wcaldwel@gru.edu (R.W. Caldwell).

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Materials and Methods

Cell culture and hypoxia

Bovine aortic endothelial cells (BAECs, Cell Application Inc, CA, USA) were used from passages 4 to 7. Cells were cultured in normoxia (21% O_2) incubator to 70% confluence and were maintained for 2 h in low-arginine M-199 medium (Invitrogen, Carlsbad, CA, USA) containing 0.2% FBS and 50 μM L-arginine, before treatment. The cells were pretreated with the arginase inhibitor 2(S)-amino-6-boronohexanoic acid (ABH, 100 μm, a gift of Dr. Dan Berkowitz) or vehicle (1× PBS) for 1 h. Thereafter, cells were maintained with a gas mixture (5% CO_2-balanced N_2) to obtain 2% O_2 in a hypoxia chamber for 12 or 24 h. Normoxia control group cells were incubated in 21% O_2 conditions. In this concentration of ABH, 100 μM inhibits arginase activity by over 90% in endothelial cells for over 24 h (Steppan et al., 2013).

Arginase activity

BAEC were washed in PBS and incubated in lysis buffer (50 mM Tris-HCl) containing protease inhibitors. Cell lysates were centrifuged at 12,000 g for 10 min. The enzyme was activated by heating the lysate at 56 °C for 10 min in 25 mM Tris buffer (pH 7.4) containing 5 mM $MnCl_2$. L-Arginine hydrolysis was then conducted by incubating 50 μl of the activated lysate with 50 μl of 0.5 M L-arginine (pH 9.7) at 37 °C for 60 min. The reaction was stopped in acid medium (H_2SO_4:H_3PO_4:H_2O = 1:3:7 v/v). The concentration of urea was determined after adding 25 μl of 9% α-isonitrosopropiophenone. Protein concentration in the lysates was determined by a BCA assay (Thermo Scientific, IL, USA). Results were normalized to arginase activity in normoxia control group and expressed as arbitrary unit.

Western-blot

BAEC were homogenized in a RIPA Lysis Buffer (EMD Millipore, MA, USA) supplied with phosphatase inhibitor cocktail (Sigma-Aldrich, MO, USA) and protease inhibitor cocktail (Sigma-Aldrich, MO, USA). Twenty-microgram protein samples were subjected to 10% SDS–PAGE. Proteins were transferred onto a nitrocellulose membrane, and the membrane was blocked with 5% BSA and incubated with primary polyclonal chicken anti-arginase I (1:10,000, gift of Dr. S.M. Morris, Jr.), rabbit anti-arginase II (1:250, SantaCruz Biotech, Texas, USA), rabbit anti-eNOS(1:4000, Santacruz Biotech, Texas, USA), monoclonal mouse anti-eNOS phosphorylated at serine-1177 or threonine-495 (1:1000, BD Bioscience, CA, USA), human anti-VEGF antibody (LifeSpan BioScience, WA, USA), and mouse anti-β-actin (1:5000, Sigma-Aldrich, MO, USA) overnight at 4 °C. After incubation with secondary antibodies, proteins were detected using an enhanced chemiluminescence and quantified by densitometry. Results were normalized to β-actin.

Measurement of NO levels and production

Intracellular NO levels at 24 h were monitored using the NO-specific fluorescent dye 4,5-diaminofluorescein diacetate (DAF-2 DA, Calbiochem, USA). Thereafter, cells were stained with oxygenated DAF-2 DA solution (10 mM) for 30 min in the darkness at 37C, in a shaking water bath. This experimental procedure in saturated oxygen ensures that NO, rather than O_2, is the limiting factor in the reaction and the fluorescence is directly proportional to NO. Negative controls for DAF-2 DA were incubated in NOS inhibitor L-NAME (0.1 mM) throughout the experimental period. Fluorescence was monitored using fluorescence microscope (excitation, 488 nm; emission, 610 nm) at 20× magnification. Nine pictures were captured in each well (n = 3), and the fluorescent intensity was quantified using Image J software. Results were presented as mean fluorescent intensity per field and expressed as percent of control.

Cumulative production of NO by BAEC was also assessed. After 24 h exposure to normoxia, hypoxia, or hypoxia + ABH, the medium was collected. NO levels in 30 μl of media was measured using NO-specific chemiluminescence in a Sievers Nitric Oxide Analyzer as previously described (Shatanawi et al., 2011).

In vitro EC network formation assay

Cells were collected for in vitro tube formation assay. ABH (100 μM) or PBS (as control) was added to the wells of 96-well plate before 30 μl Matrigel (reduced growth factor basement membrane matrix, BD Bioscience, USA) mixed with 60 μl medium containing 1.5×10^4 cells were seeded. After 12 h and 24 h hypoxia exposure, the network-like structures were visualized and captured using microscope (Axiovert 25, Carl Zesis) at 10× magnification. Five pictures were taken for each well and five wells for each group. Network length in each field was analyzed and quantified using NIH ImageJ. Results were presented as network length in each field and expressed as percent of control.

Reactive oxygen species (ROS)

Dichlorofluorescein (DCF) is the oxidation product of the reagent 2′,7′-dichlorofluorescein diacetate (H_2DCFDA; Molecular Probes, CellRox Oxidative Stress Deep Red Reagents, Life technologies, NY, USA). It is a marker of cellular oxidation by hydrogen peroxide, peroxynitrite, and hydroxy radicals. Cells were incubated with low-arginine M199 medium containing 5 μM H_2DCFDA at 37 °C for 30 min. Fluorescence was monitored using fluorescence microscope (excitation, 640 nm; emission, 665 nm) at 20× magnification. Nine pictures were captured in each well (n = 3), and the fluorescent intensity was quantified using NIH ImageJ software. Results were presented as mean fluorescent intensity in each field and expressed as percent of control.

Statistical analysis

All the values are presented as mean ± SEM. Student's t-test or one-way analysis of variance (ANOVA) with Bonferroni post-test was used to evaluate differences between the groups as appropriate. $P < 0.05$ was considered significant. All statistical analysis was calculated by using GraphPad Prism (V5.01).

Results

In vitro tube-like formation and VEGF165 expression

We first determined the effects of hypoxia exposure on angiogenic function as shown by the alignment of ECs in tube-like networks and VEGF165 protein levels as shown by Western blot. At 12 h of exposure to hypoxia, ECs showed significantly increased VEGF165 levels. The increase in VEGF165 was accompanied by EC elongation and alignment, but network formation was not evident. Both VEGF165 levels and EC elongation/alignment in hypoxia were significantly increased by ABH treatment (Fig. 1). In normoxic conditions, EC exhibited only small spiky protrusions. After 24 h of hypoxia exposure, well-formed EC networks were observed in the normoxia control cultures. However, the network length per field in hypoxia was less than observed in normoxia control, being 63% of that level. ABH treatment partially prevented this hypoxia-induced reduction in tube-like formation (88% of control level) (Figs. 2a–d). VEGF165 protein levels in the hypoxia group were reduced to 53% of control and ABH prevented this decline (Figs. 2e, f). We therefore used the 24 h hypoxia treatment for further analyses of the role of arginase activity in this process.