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Angiotensin II limits NO production by upregulating arginase through a p38 MAPK–ATF-2 pathway

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ABSTRACT

Enhanced vascular arginase activity can impair endothelium-dependent vasorelaxation by decreasing L-arginine availability to endothelial nitric oxide (NO) synthase, thereby reducing NO production and uncoupling NOS function. Elevated angiotensin II (Ang II) is a key component of endothelial dysfunction in many cardiovascular diseases and has been linked to elevated arginase activity. In this study we explored the signaling pathway leading to increased arginase expression/activity in response to Ang II in bovine aortic endothelial cells (BAEC). Our previous studies indicate involvement of p38 mitogen activated protein kinase (MAPK) in Ang II-induced arginase upregulation and reduced NO production. In this study, we further investigated the Ang II-transcriptional regulation of arginase 1 in endothelial cells. Our results indicate the involvement of ATF-2 transcription factor of the AP1 family in arginase 1 upregulation and in limiting NO production. Using small interfering RNA (siRNA) targeting ATF-2, we showed that this transcription factor is required for Ang II-induced arginase 1 gene upregulation and increased arginase 1 expression and activity, leading to reduced NO production. Electrophoretic mobility shift assay and chromatin immunoprecipitation assay further confirmed the involvement of ATF-2. Moreover, our data indicate that p38 MAPK phosphorylates ATF-2 in response to Ang II. Collectively, our results indicate that Ang II increases endothelial arginase activity/expression through a p38 MAPK/ATF-2 pathway leading to reduced endothelial NO production. These signaling steps might be therapeutic targets for preventing vascular endothelial dysfunction associated with elevated arginase activity/expression.

1. Introduction

Arginase is the hydrolytic enzyme responsible for conversion of L-arginine into urea and L-ornithine (Demougeot et al., 2005; Yang et al., 2006). There are two distinct isoforms of arginase; arginase 1, largely found in the liver as a component of the urea cycle and arginase 2, which is predominant in kidney. Both isoforms, however, have been found in vascular tissue (Bachetti et al., 2004; Berkowitz et al., 2003).

Elevated arginase activity has been associated with cardiovascular pathologies such as hypertension, diabetes, atherosclerosis, ischemic reperfusion injury, erectile dysfunction and sickle cell anemia (Bagnost et al., 2008; Bivalacqua et al., 2007; Jeyabalan et al., 2008; Morris et al., 2005; Romero et al., 2008). In these conditions, elevation of arginase has been shown to mediate vascular dysfunction through limiting nitric oxide (NO) production or availability. Arginase can reciprocally regulate NO production in endothelial cells by competing with nitric oxide synthase (NOS) for the substrate L-arginine (Bagnost et al., 2008; Berkowitz et al., 2003; Romero et al., 2008).

Angiotensin II (Ang II) actions in endothelial cells are mostly associated with endothelial NOS (eNOS) dysfunction and uncoupling, which lead to decreased levels of NO and increased superoxide production (Satoh et al., 2008).

Elevated arginase activity also has been associated with systemic hypertension. Inhibition of arginase has been reported to decrease blood pressure and improve vascular function of resistance vessels in adult hypertensive rats (Bagnost et al., 2008; Demougeot et al., 2005). These findings thus suggest a central role

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for arginase in diseases in which vascular dysfunction is linked to elevated levels of Ang II.

However, it should be emphasized that global inhibition of arginase in the body may be dangerous. Arginase 1 is a crucial enzyme in the urea cycle for disposal of harmful ammonia and complete knock-out of the arginase 1 gene in mice is lethal by 2 weeks of age because of hyper-ammonemia (Iyer et al., 2002). Use of arginase inhibitors carries the risk of reducing its function to very low levels. Hence, identifying the steps – e.g. signaling proteins or transcription factors – that directly enhance arginase in endothelial cells may be very beneficial targets in specifically limiting vascular arginase activity without unwanted global side effects.

We have reported a role for arginase 1 upregulation in vascular dysfunction in a model of Ang II-induced vascular endothelial dysfunction and hypertension (Shatanawi et al., 2011). We have also shown that Ang II elevates arginase through a RhoA/Rho kinase (ROCK) and p38 mitogen activated protein kinase (MAPK) pathway. Others have reported that Ang II elevates arginase 1 levels in isolated rat periglomerular vessels (Hultstrom et al., 2009). Additionally, high glucose and reactive oxygen species increase arginase activity in bovine coronary endothelial cells via a RhoA/ROCK mechanism (Chandra et al., 2012; Romero et al., 2008).

Given the importance of endothelial arginase in causing eNOS dysfunction, and the link of arginase with vascular diseases associated with elevated levels of Ang II, we sought to define the transcriptional regulation of arginase 1 in response to Ang II in relation to arginase activity and NO production. Transcriptional regulation of arginase in response to thrombin in endothelial cells has been demonstrated to occur through activator protein-1 (AP-1) consensus site (Zhu et al., 2009). More specifically, two transcription factors that bind to that site were identified – activating transcription factor-2 (ATF-2) and c-Jun. Our work examined the involvement of these two transcription factors in arginase 1 elevation in endothelial cells in response to Ang II and their impact on vascular disease through limiting NO.

2. Materials and methods

2.1. Cell culture and treatments

In all cell experiments, bovine aortic endothelial cells (BAECs) were utilized. Proliferating BAECs were purchased from Cell Applications, San Diego, CA. Cells were cultured in Endothelial Growth Medium (Cell Applications, San Diego, CA) and maintained in a humidified atmosphere at 37 °C and 5% CO_2. Before starting experiments, cells were adapted to grow in M199 supplemented with 50 μM L-arginine (Invitrogen, Carlsbad, CA) for 72 h to match the normal plasma L-arginine concentration which ranges from 40 to 100 μM (Romero et al., 2006). In addition, the medium was supplemented with 10% FBS (Catalog # SH30396, hyClone, GE Healthcare Life Sciences South Logan, Utah), 1% penicillin/streptomycin, and 1% L-glutamine. When cells reached 80% confluency, they then were serum starved overnight in M199 supplemented with 50 μM L-arginine, 1% L-glutamine, 1% penicillin/streptomycin and 0.2% FBS. The p38 MAPK inhibitor, SB-202190 (2 μM) (EMD biosciences, San Diego, CA), was used in some experiments and added 2 h before the addition of angiotensin II (0.1 μM, for different time points) (Sigma-Aldrich, St. Louis, MO). All experiments were performed with cells from passage 3–7.

2.2. Luciferase activity

Luciferase constructs used were generously provided by Prof. Sydney M. Morris, Jr. of the University of Pittsburgh. The constructs were transformed in *E. Coli* competent cells (Novablue) then amplified and extracted using EndoFree® plasmid purification kit (Qiagen, Valencia, CA).

BAECS were co-transfected with one of three arginase 1 luciferase constructs and a Renilla luciferase gene (Promega) as an internal control using Lipofectin reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. After 48 h of transfection, cells were treated with Ang II. In another set of experiments the cells also received co-transfection with siRNA for ATF-2 and c-Jun or non-targeting sc-RNA. All treatments were performed in triplicate.

Firefly luciferase activity was measured for reporter expression according to the instructions provided in the Dual-Luciferase® Reporter Assay System (Promega, San Luis Obispo, CA). Transfection efficiency was corrected by co-transfection with a plasmid containing the Renilla luciferase gene (Promega). Both Firefly and Renilla luciferase activity were measured within the same sample of cell lysate sequentially using one reaction tube. All chemiluminescence readings were obtained using a microplate luminometer (POLARStar OPTIMA, BMG Labtech). Briefly, 20 μL of cell lysate were added to a microplate well followed by adding 100 μL of luciferase substrate solution (as provided in the kit). The Firefly luciferase activity (AFL) was measured immediately using 15 s as total reading time. The same well then received 100 μL of Stop & Glo reagent (contains substance to quench the enzymatic activity of Firefly and a substrate for Renilla luciferase). Renilla luciferase activity (ARL) was measured immediately using 15 s as total reading time. The corrected activity (AFL/ARL) was used to compare groups.

2.3. Arginase activity

Arginase activity was measured using a colorimetric determination of urea production from L-arginine as described previously (Corraliza et al., 1994). Cells were in Tris buffer (50 mMTris–HCl, 0.1 mM EDTA and EGTA, pH 7.5) containing protease inhibitors (Catalog # P8340, Sigma, St. Louis, MO). These mixtures were subjected to three freeze-thaw cycles and then centrifuged for 10 min at 20,000*g*. The supernatants were used for arginase activity assay.

In brief, 25 μL of supernatant was heated with $MnCl_2$ (10 mM) for 10 m at 56 °C to activate arginase. The mixture was then incubated with 50 μL L-arginine (0.5 M, pH 9.7) for one hour at 37 °C to hydrolyze the L-arginine. The hydrolysis reaction was stopped with acid and the mixture was then heated at 100 °C with 25 μL of α-isonitrosopropiophenone (9% α-ISPF in EtOH) for 45 min. The samples were kept in dark at room temperature for 10 min then absorbance was measured at 540 nm.

2.4. siRNA transfection

BAECs were transfected with siRNA targeting ATF-2 or c-Jun (Dharmacon, Lafayette, CO) using siPORT Amine (Ambion, Austin, TX), according to the manufacturer's instructions. Scrambled siRNA (non-targeting siRNA) served as control to validate the specificity of the siRNAs. In brief, cells were transfected with 50 nM of targeting or non-targeting siRNA for 48 h. Specific mRNA depletion was analyzed by Western blot.

2.5. Western blot analysis

Cells were lysed in Ripa buffer (Upstate Biotechnology, Temecula, CA) containing protease and phosphatase inhibitors (Catalog # P5726 and P0044, Sigma, St. Louis, MO). Cell lysates were centrifuged for 10 min at 20,000 *g*, and supernatants were collected for Western blotting analysis. Protein estimation was carried out in supernatants using protein assay kit (Bio Rad, Hercules, CA). Equal amounts of protein were loaded, separated