Increased arginase II activity contributes to endothelial dysfunction through endothelial nitric oxide synthase uncoupling in aged mice

Woosung Shin[1], Dan E. Berkowitz[2,3] and Sungwoo Ryoo[1,4]

[1]Department of Biology
College of Natural Sciences
Kangwon National University
Chuncheon 200-701, Korea
[2]Department of Anesthesiology and Critical Care Medicine
[3]Department of Biomedical Engineering
Johns Hopkins Medical Institutions
Baltimore, MD 21287, USA
[4]Corresponding author: Tel, 82-33-250-8534;
Fax, 82-33-251-3990; E-mail, ryoosw08@kangwon.ac.kr
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Abstract

The incidence of cardiovascular disease is predicted to increase as the population ages. There is accumulating evidence that arginase upregulation is associated with impaired endothelial function. Here, we demonstrate that arginase II (ArgII) is upregulated in aortic vessels of aged mice and contributes to decreased nitric oxide (NO) generation and increased reactive oxygen species (ROS) production *via* endothelial nitric oxide synthase (eNOS) uncoupling. Inhibiting ArgII with small interfering RNA technique restored eNOS coupling to that observed in young mice and increased NO generation and decreased ROS production. Furthermore, enhanced vasoconstrictor responses to U46619 and attenuated vasorelaxation responses to acetylcholine in aged vasculature were markedly improved following siRNA treatment against ArgII. These results might be associated with increased L-arginine bioavailability. Collectively, these results suggest that ArgII may be a valuable target in age-dependent vascular diseases.

Keywords: aging; arginase II; endothelial nitric oxide synthase uncoupling; small interfering RNA; vascular diseases

Introduction

Cardiovascular disease is the leading cause of morbidity and mortality in both industrialized and developing countries. Despite effective treatments for several established cardiovascular risk factors, such as hypertension and hypercholesterolemia, the incidence of cardiovascular disease is predicted to increases as the population ages. Characteristic events in the aging cardiovascular system include vascular stiffness (Meaume et al., 2001), enhanced reactive oxygen species (ROS) production (Finkel and Holbrook, 2000; Brandes et al., 2005), and decreased nitric oxide (NO) bioavailability (Adler et al., 2003; Csiszar et al., 2008). Superoxide may react with NO to produce peroxynitrite (ONOO⁻), which is potentially detrimental to vascular cell function and viability. This nitroso-redox imbalance contributes to age- related endothelial dysfunction and vascular stiffness (Berkowitz et al., 2003).

Arginase is well demonstrated as an important enzyme in the last step of the urea cycle, which hydrolyzes L-arginine to L-ornithine and urea. There are two isoforms: arginase I (ArgI), also known as the hepatic isoform, and arginase II (ArgII), which is an extrahepatic enzyme. These proteins are encoded by different genes (Haraguchi et al., 1987; Morris et al., 1997) and are induced by a variety of factors (Meurs et al., 2003). Arginase isoforms are found in a wide variety of cells and tissues, and current reports suggest that they may be expressed in species- and tissue-specific manners (Abe and Berk, 1998; Morris et al., 1998; Chicoine et al., 2004; Ryoo et al., 2006; Nelin et al., 2007). There is increasing evidence that arginase upregulation

functionally inhibits nitric oxide synthase (NOS) activity and contributes to age-related vascular dysfunction (Berkowitz *et al.*, 2003; Ryoo *et al.*, 2006). Pharmacological inhibition and antisense knockdown of ArgI restores endothelial NO production and endothelial function *ex vivo* (Ryoo *et al.*, 2006). Arginase-mediated reciprocal regulation of NOS has been demonstrated in the majority of cell types and organs in which NO is an important signaling molecule, including cardiomyocytes (Steppan *et al.*, 2006), the penis (Bivalacqua *et al.*, 2007), the airway (Meurs *et al.*, 2003), skin (Holowatz and Kenney, 2007), inflammatory mediator cells (*e.g.*, macrophages), and endothelial cells (Berkowitz *et al.*, 2003).

Although vascular changes associated with aging have been investigated in humans and a number of other species, it is thought that the relative contributions of dysregulated mechanisms to age-related vascular pathology are species-dependent (Santhanam *et al.*, 2008). Moreover, the contribution of vascular control mechanisms in health, aging, and disease conditions is influenced by regional vascular beds and vessel type and size (Santhanam *et al.*, 2008). Therefore, we investigated which arginase isoform contributes to age-related endothelial dysfunction in mice and determined whether specific inhibition of arginase isoforms with small interfering RNA (siRNA) was able to restore vascular function. We also analyzed L-arginine (L-arg) concentrations in isolated aortic vessels to elucidate the underlying mechanism of endothelial nitric oxide synthase (eNOS) activation.

Results

ArgII is the key isoform regulating arginase activity in the aorta of aged mice

We first analyzed arginase expression in young and aged groups (Figures 1A and 1B). ArgII was strongly expressed, but we did not detect ArgI. Neither the expression level of ArgII protein (A, young vs. aged, 1.0 ± 0.07 vs. 1.05 ± 0.06, ns) nor mRNA (B, young vs. aged, 1.0 ± 0.08 vs. 1.01 ± 0.04, ns) was significantly different between groups. Next, we measured arginase activity with or without preincubation of siRNAs to specific arginase isoforms (siArgI and siArgII). siArgII incubation with young aortic vessels resulted in decrease of arginase activity (Figure 1C, [#] vs. young, 88.6 ± 3.0 vs. 100.0 ± 7.1, $P < 0.05$) that was associated with decreased protein levels (Figure 1D, [#] vs. young, 0.81 ± 0.07 vs. 1.0 ± 0.01, $P < 0.05$). Furthermore, increased arginase activity in the aged group (Figure 1C, * vs. young, 137.52 ± 6.1 vs. 100.0 ± 7.1, $P <$
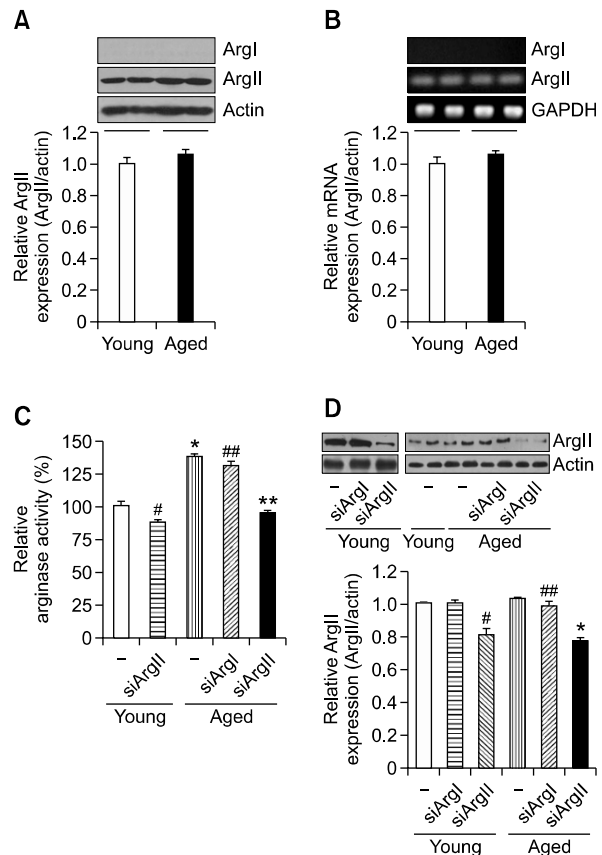

Figure 1. Upregulation of ArgII activity in aged mice aortas. (A) Western blot analysis of aortas from young and aged mice. Similar levels of ArgII were found in both groups, but ArgI protein was not detectable (upper). The lower panel shows the densitometric analysis, $n = 4$ experiments. (B) ArgI and ArgII mRNA expression levels were analyzed by RT-PCR using GAPDH as a control. Only ArgII mRNA was detected (upper). The lower panel shows the densitometric analysis, $n = 4$ experiments. (C) siArgII incubation decreased arginase activity in both young and aged aortas ([#] vs. young, $P < 0.05$; * vs. young, $P < 0.01$; ** vs. aged, $P < 0.01$). (D) siArgII effectively downregulated ArgII protein expression in both young and old aortas ([#] vs. young, $P < 0.05$; * vs. aged, $P < 0.01$), but not with siArgI ([##] vs. aged, ns).

0.01) was markedly attenuated by siArgII (Figure 1C, ** vs. aged, 137.52 ± 6.1 vs. $94.8 \pm 3.2\%$, $P < 0.01$) that resulted from downregulated ArgII protein levels (Figure 1D, * vs. aged, 0.77 ± 0.04 vs. 1.0 ± 0.01, $P < 0.01$). However, siArgI had no effect on either protein level or enzyme activity. (Figures 1C and 1D, [##] vs. aged, ns). These results imply that the ArgII isoform was the dominantly expressed enzyme in the vasculature of aged mice.

eNOS uncoupling in aged aorta was restored with siArgII

We next assessed eNOS expression in both groups. Interestingly, eNOS protein expression was significantly
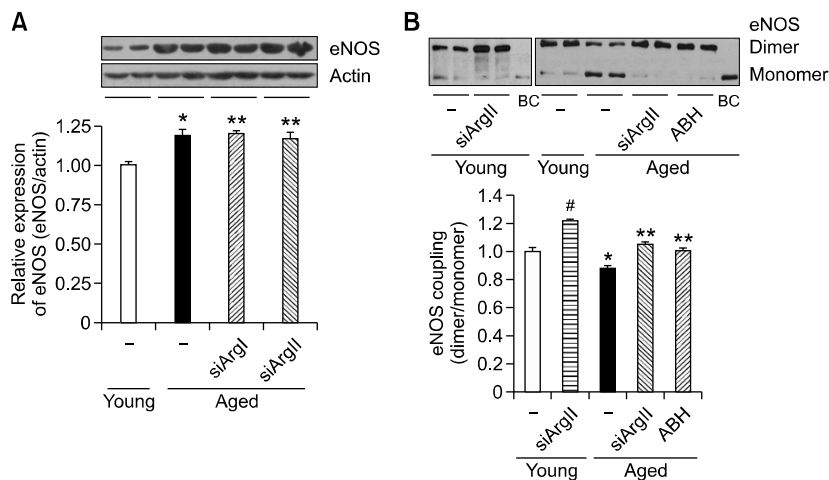
Figure 2. Increased ArgII activity was associated with eNOS uncoupling. (A) Increased eNOS expression in aged aorta was not attenuated by siArgII (* vs. young, $P < 0.01$; ** vs. aged, ns). (B) siArgII incubation induced eNOS coupling in young aorta ($^{\#}$ vs. young, $P < 0.01$). Augmented eNOS uncoupling in aged aorta was decreased by siArgII. Incubation of aged aorta with the arginase inhibitor, ABH (10 μM), enhanced eNOS dimerization (* vs. young, $P < 0.01$; ** vs. aged, $P < 0.01$). Densitometric analysis of Western blot (lower panel).

increased in the aged group (* vs. young, 1.18 ± 0.05 vs. 1.00 ± 0.02, $P < 0.01$). This observation was not affected by incubation with siArgI or siArgII (Figure 2A, ** vs. aged, ns). Therefore, we tested whether eNOS expression levels were associated with its activity by measuring eNOS dimers and found that the dimer to monomer ratio was markedly reduced in the aged group (Figure 2B, * vs. young, 0.87 ± 0.02 vs. 1.00 ± 0.02, $P < 0.05$). The decreased ratio of eNOS dimer/monomer was recovered by inhibition of arginase activity using either siRNA (** vs. aged, 1.04 ± 0.02 vs. 0.87 ± 0.02, $P < 0.05$) or a known arginase inhibitor, ABH (** vs. aged, 1.00 ± 0.02 vs. 0.87 ± 0.02, $P < 0.05$). The effect of siArgII on eNOS coupling was also found in young aorta ($^{\#}$ vs. young, 1.21 ± 0.02 vs. 1.00 ± 0.02, $P < 0.05$). These results suggest that endothelial function in aged mice is impaired despite increased eNOS expression.

siArgII affected NO production and ROS generation in aged mice

Based on the eNOS coupling data, we next measured NO production and ROS generation in endothelium from young and aged mice. As demonstrated in Figure 3A, NO production was significantly attenuated in aged mice (* vs. young, 0.58 ± 0.16 vs. 0.90 ± 0.19 change of DAF fluorescence/second, $P < 0.01$). It was restored following siArgII and ABH treatment (** siArgII vs. aged, 0.87 ± 0.11 vs. 0.58 ± 0.16, $P < 0.01$; ** ABH vs. aged, 0.81 ± 0.14 vs. 0.58 ± 0.16 change of DAF fluorescence/second, $P < 0.01$), but siArgI had no effect. Consistent with the data, siArgII induced increase in the slope of DAF fluorescence in young mice ($^{\#}$ vs. young, 1.13 ± 0.10 vs. 0.90 ± 0.19 change of DAF fluorescence/second, $P < 0.05$). On the other hand, ROS generation
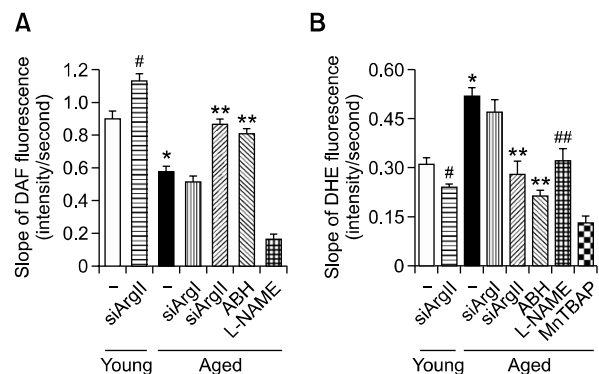

Figure 3. Inhibition of ArgII activity restored impaired endothelial function in aged mice. Isolated aortic segments were incubated with DAF-AM (5 μM) and fluorescence was measured in real-time (endothelium side up). The slope of DAF fluorescence was determined. (A) siArgII incubation with young vessels increased the slope of DAF fluorescence ($^{\#}$ vs. young, $P < 0.01$). However, DAF fluorescence was decreased in aged mouse aorta and increased following siArgII (but not siArgI) and ABH incubation. L-NAME was used as a control (* vs. young, $P < 0.01$; ** vs. aged, $P < 0.01$; $n = 4$ mice). (B) ROS production in aortic endothelium was measured with DHE (5 μM), and the slope of DHE fluorescence was determined using cumulative data. Arginase inhibition with siArgII and ABH reciprocally regulated ROS production ($^{\#}$ vs. young, $P < 0.01$; * vs. young, $P < 0.01$; ** vs. aged, $P < 0.01$; $^{\#\#}$ vs. aged, $P < 0.01$; $n = 4$ mice). MnTBAP was used as a control.

was markedly augmented in aged mice (Figure 3B, * vs. young, 0.52 ± 0.04 vs. 0.31 ± 0.03 change of DHE fluorescence/second, $P < 0.01$), but this was reduced to the level of young endothelium after siArgII and ABH incubation (**, siArgII vs. aged, 0.28 ± 0.07 vs. 0.52 ± 0.04, $P < 0.01$; **, ABH vs. aged, 0.21 ± 0.06 vs. 0.52 ± 0.04 change of DHE fluorescence/second, $P < 0.01$). siArgII incubation also reduced ROS generation in young mice ($^{\#}$ vs. young, 0.24 ± 0.04 vs. 0.31 ± 0.03 change of DHE fluorescence/second, $P < 0.05$). Interestingly, L-NG-

Figure 5. siArgII preincubation increased intracellular L-arg concentrations. Aortic vessels were incubated with or without siArgI, siArgII, and ABH and intracellular L-arg concentrations were measured with HPLC (see methods section). Treatment of siArgII to young vessels resulted in increase of L-arg amount ([#] vs. young, $P < 0.01$). The reduced L-arg level in aortas from aged mice was restored to that of young aortas by incubation of siArgII and ABH (* vs. young, $P < 0.01$; ** vs. aged, $P < 0.01$; $n = 6$).
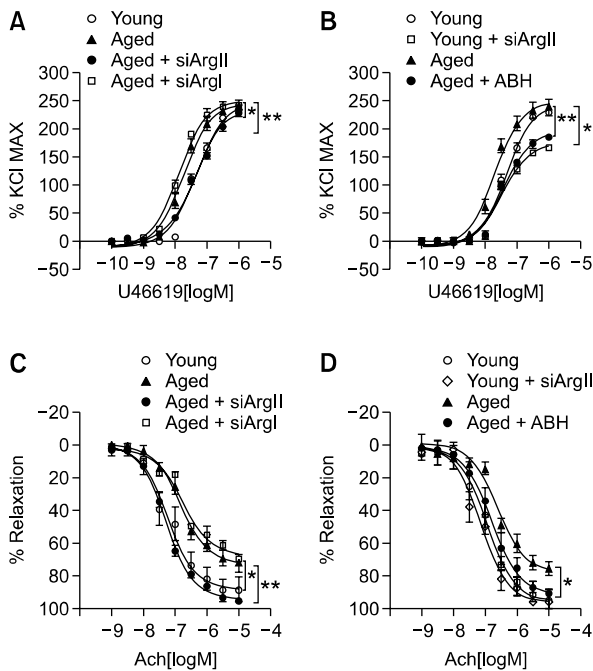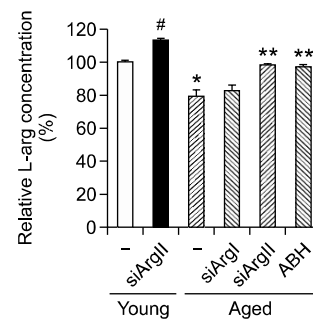
Figure 4. siArgII improved impaired vascular reactivity in aged mice. (A) Aged aortic vessels had augmented contractile responses to U46619 compared to young mice (*, young vs. aged, $P < 0.01$). siArgII restored the U46619-mediated pressor response of aged aortic vessels to levels similar to that of young aortas (*, young vs. aged, $P < 0.01$; **, aged vs. aged + siArgII, $P < 0.01$). $n = 4$ mice per each group. (B) siArgII attenuated contractile responses to U46619 in young aorta (*, young vs. young + siArgII, $P < 0.01$) and ABH incubation with old aorta significantly retarded U46619-dependent constriction (**, aged vs. aged + ABH, $P < 0.01$). (C) Endothelium-dependent relaxation responses to Ach were impaired in aged aortas (*, young vs. aged, $P < 0.01$). Impaired relaxation was recovered with siArgII pretreatment (**, aged vs. aged + siArgII, $P < 0.05$). $n = 4$ mice per each group. (D) ABH incubation augmented Ach-dependent vessel relaxation in aged aorta (*, aged vs. aged + ABH, $P < 0.01$).

nitroarginine methyl ester (L-NAME) treatment also decreased ROS production in aged mice ([#] vs. aged, 0.32 ± 0.06 vs. 0.52 ± 0.04 change of DHE fluorescence/second, $P < 0.01$). These data imply that increased eNOS uncoupling in aged mice leads to decreased NO production and increased ROS generation.

siArgII restored impaired endothelial function in aged vasculature

Because upregulated arginase activity increases ROS generation and decreases NO bioavailability, we determined whether ArgII inhibition could attenuate this dysfunction. Vasoconstrictor responses to the agonist U46619 were markedly enhanced in aorta from aged mice, as manifested by a leftward shift in dose-response curves and reduced -logEC$_{50}$ values (*, young vs. aged, 7.32 ± 0.05 M vs. 7.74 ± 0.06

M, $P < 0.01$). E$_{max}$ was not significantly changed by this treatment (young vs. aged, $245.9 \pm 7.07\%$ vs. $254.9 \pm 7.43\%$, ns) (Figure 4A). However, incubation of aortic vessels from aged mice with siArgII increased -logEC$_{50}$ upto that observed in young (**, aged vs. aged + siArgII, 7.74 ± 0.06 M vs. 7.32 ± 0.04 M, $P < 0.01$). Consistent with siArgII incubation, arginase inhibition with ABH in aged vessels attenuated vasoconstrictor responses to that of young aorta in both E$_{max}$ (**, aged vs. aged + ABH, $247.3 \pm 7.7\%$ vs. $194.9 \pm 5.4\%$, $P < 0.01$) and -logEC$_{50}$ (**, aged vs. aged + ABH, $7.70 \pm 0.07\%$ vs. $7.41 \pm 0.05\%$, $P < 0.01$) as shown in Figure 4B. On the other hand, siArgII incubation with young aortic vessels significantly decreased E$_{max}$ value (*, young vs. young + siArgII, $245.9 \pm 7.07\%$ vs. $174.2 \pm 6.43\%$, $P < 0.01$), but -logEC$_{50}$ was not changed (* young vs. young + siArgII, 7.32 ± 0.05 M vs. 7.35 ± 0.07 M, NS). Next, to determine the effects of siRNA-mediated arginase inhibition on endothelium-dependent vasorelaxation, mouse aorta were preconstricted with U46619 (10^{-8} M), and dose-response curves to the endothelium-dependent vasodilator acetylcholine (Ach) were constructed. The vasorelaxation responses in aged mice were significantly attenuated compared with those from young (Figure 4C, *, young vs. aged, $88.31 \pm 4.89\%$ vs. $72.87 \pm 2.82\%$ (E$_{max}$), $P < 0.01$; 7.23 ± 0.13 M vs. 6.81 ± 0.09 M (-logEC$_{50}$), $P < 0.01$). Consistent with the above data, incubation of aged aorta with siArgII enhanced vasorelaxation responses (Figure 4C, **, aged vs. aged + siArgII, $72.87 \pm 2.82\%$ vs. $94.36 \pm 1.93\%$ (E$_{max}$), $P < 0.01$; 6.81 ± 0.09 M vs. 7.28 ± 0.06 M (-logEC$_{50}$), $P < 0.01$). Also, inhibition of arginase activity with ABH augmented vasorelaxation responses in both E$_{max}$ (Figure 4D, *, aged vs. aged + ABH, $72.87 \pm 2.82\%$

vs. 93.37 $\pm$ 3.30%, $P < 0.01$) and -logEC$_{50}$ (*, aged vs. aged + ABH, 6.81 $\pm$ 0.09 M vs. 7.19 $\pm$ 0.06 M, $P < 0.01$). On the other hand, incubation of young vessels with siArgII increased -logEC$_{50}$ value from 7.23 $\pm$ 0.13 M to 7.44 $\pm$ 0.09 M without effect on E$_{max}$ (young vs. young + siArgII, 88.31 $\pm$ 4.89% vs. 88.93 $\pm$ 3.30%, ns). However, the responses to the endothelium-independent vasodilator sodium nitro-prusside (SNP) were not significantly changed in any group (data not shown).

ArgII inhibition increased L-arg bioavailability

Given data suggesting that arginase reciprocally regulates NOS activity by limiting L-arg bioavail-ability, we used high-performance liquid chromatog-raphy (HPLC) to determine whether intracellular L-arg concentration was affected by altered argi-nase activity. L-arg content was significantly de-creased in aged aorta but was recovered with siArgII and ABH incubation (Figure 5, * vs. young, 78.81 $\pm$ 8.10% vs. 100 $\pm$ 2.50%, $P < 0.01$; **, siArgII vs. aged, 98.1 $\pm$ 1.8% vs. 78.81 $\pm$ 8.10%, $P < 0.01$; **, ABH vs. aged, 96.8 $\pm$ 2.81 vs. 78.81 $\pm$ 8.10%, $P < 0.01$). Furthermore, siArgII incubation with young aorta resulted in increase of the L-arg amount ([#] vs. young, 113.0 $\pm$ 3.1% vs. 100 $\pm$ 2.50%, $P < 0.05$). These data suggest that aortic arginase activ-ity play a key role in regulating intracellular L-arg concentration.

Discussion

The principal findings of this study were that ArgII is the dominantly expressed arginase isoform in aortic vessels of aged mice, and specific inhibition of ArgII with siRNA restores eNOS coupling, which is associated with increased NO production and decrease in ROS generation. Arginase inhibition augments U46619-induced vasoconstriction and endothelium-dependent vasorelaxation by increasing intracellular L-arg concentration in the vasculature of aged mice.

Previous publications have demonstrated that endothelial arginase constrains eNOS activity, thereby reducing NO bioavailability and contributing to vascular diseases, including aging (Berkowitz *et al.*, 2003). In the present study, we show for the first time that increased ArgII activity plays an important role in age-related vascular endothelial dysfunction without a change at the protein level. ArgII expression has been well established in human (Ryoo *et al.*, 2006) and mice (Lim *et al.*, 2007) endothelial cells. Endothelial ArgII activation and expression can be induced by a variety of vascular insults, including oxidized low-density lipoprotein (oxLDL), peroxynitrite, lipopolysaccharide, tumor necrosis factor-α, interferon-γ, 8-bromo-cGMP, thrombin, and hypoxia (Abe and Berk, 1998; Morris *et al.*, 1998; Chicoine *et al.*, 2004; Nelin *et al.*, 2007). oxLDL (Ryoo *et al.*, 2011), thrombin (Ming *et al.*, 2004), and peroxynitrite (Chandra *et al.*, 2012) stimulate ArgII activation *via* RhoA-dependent ROCK activation without a change in mRNA and protein levels. One study reported that peroxynitrite can react with the redox-active cysteine (Cys[18]) of RhoA, which enhances GDP release from RhoA and thus modulates their activity (Brandes *et al.*, 2005). Indeed, we found peroxynitrite formation as measured by the thiobarbituric acid reactive substances assay, was significantly increased in blood from aged mice (young vs. aged, 0.28 $\pm$ 0.01 vs. 0.33 $\pm$ 0.01 μM MDA, $P < 0.05$), which is consistent with a previous publication (van der Loo *et al.*, 2000). Taken together, ArgII upregulation in aged aorta may dependent on the increase of peroxynitrite formation and RhoA activation. Although ArgI activity was upregulated by translational modification such as S-nitorsylation, ArgII was not modified by such modification (data not shown). Therefore, further study needs to find a mechanism associated with ArgII upregulation.

Vascular ROS production is enhanced in aged blood vessels (Abe and Berk, 1998; van der Loo *et al.*, 2000; Ungvari *et al.*, 2004; Brandes *et al.*, 2005). There are four enzymatic systems that contribute to increased ROS production in various pathophysio-logical states: xanthine oxidase, NADPH oxidase, eNOS, and the mitochondrial electron transport chain (Cai and Harrison, 2000). Although eNOS normally produces the vasoprotectant molecule NO, it can also produce O$_2^-$ in the absence of either L-arginine or BH$_4$. This phenomenon has been referred to as eNOS uncoupling (Stuehr *et al.*, 2001; Landmesser *et al.*, 2003). During aging, eNOS uncoupling plays a key role in increased ROS production. Our data that increased ROS production in the endothelium of aging mice was prevented with preincubation of the eNOS inhibitor L-NAME is consistent with previous observations (Kim *et al.*, 2009). Furthermore, the ratio of eNOS dimer to monomer in aged mice was significantly decreased compared with that in young. Together, these data suggest that eNOS uncoupling is an important contributor to age-related endothelial dysfunction.

Increased expression of eNOS may depend on shear stress and hemodynamic forces (Sessa *et al.*, 1994; Ranjan *et al.*, 1995) because of the presence of a shear stress-responsive element in the promoter region of the eNOS gene (Nishida *et al.*, 1992). Consistent with the demonstration, eNOS

expression was increased in aorta (Cernadas *et al.*, 1998; van der Loo *et al.*, 2000) and not changed in artery (Yang *et al.*, 2009) and decreased in arteriole (Csiszar *et al.*, 2002). Furthermore, increased expression of eNOS protein in aged aorta may be one of the compensatory mechanisms to counterbalance endothelial dysfunction by increased arginase activity.

There are a number of potential reasons why eNOS might be uncoupled and lead to increased ROS production in aging (Stuehr *et al.*, 2001). Oxidative stress may damage and deplete BH$_4$ and contribute to eNOS uncoupling (Milstien *et al.*, 1999). Limited L-arg bioavailability also promotes eNOS uncoupling (Boger *et al.*, 1995). Consistent with this concept, increased ArgII activity was associated with decreased intracellular L-arg and ArgII inhibition restored L-arg concentration in aortic vessels (Figure 5). We found that L-arg concentration was 66.6 μmol/mg protein in young mice and 51.9 μmol/mg proteins in aged. Closs and colleagues (Closs *et al.*, 2000; Simon *et al.*, 2003) have demonstrated that endothelial cells contain two pools of L-arg. Pool I is regulated by the cationic transporter and can be depleted by cationic amino acid L-lysine and pool II, which is further subdivided into IIA and IIB, is accessible to eNOS but is not freely exchangeable with extracellular L-lysine or L-arg. Mitochondrial ArgII utilizes pool IIB, which might be influenced by arginase and could modulate the local concentration of L-arg available to eNOS. Although our study did not distinguish among the L-arg pools, we demonstrated that arginase activity is involved in regulating intracellular L-arg concentration. This relationship between arginase activity and L-arg concentration was previously described in mouse plasma (Erdely *et al.*, 2010).

In summary, we demonstrated that ArgII is upregulated in aortic vessels of aged mice and plays a role in regulating NO production through eNOS uncoupling. Incubation with siArgII inhibits arginase activity, enhances NO generation, attenuates ROS production, and improves endothelial function by augmenting eNOS coupling *via* increased bioavailability of L-arg. Thus ArgII may be valuable target in age-related vascular diseases.

Methods

Materials

MnTBAP (Mn (III)tetrakis (4-benzoic acid) porphyrin Chloride) and L-NAME (N^G-nitro-L-arginine methyl ester), were obtained from Calbiochem. All other reagents were purchased from Sigma unless otherwise stated.

Animals

Young (10 ± 3 weeks) and aged (45 ± 5 weeks) mice (C57BL/6J) were used for all experiments. Mice were housed at 23°C under a 12-h light/12-h dark cycle. The dark period was from 10:00 to 22:00. All animals had access *ad libitum* to water and food (Nara Biotech.). The study protocols were in accordance with the Guide for the Care and Use of Laboratory Animals (Institutional Review Board, Kangwon National University).

siRNA incubation

Thoracic aortas were dissected from euthanized mice, cut into 1.5-mm rings, and incubated overnight in Dulbecco's modified Eagle's medium (DMEM) containing siArgI and/or siArgII (100 nM each, Santa Cruz Biotechnology, sc-29727 and sc-29730, respectively).

RT-PCR

Total RNA from aortic vessels was prepared with TRIzol reagent according to the manufacturer's protocol (Gibco). RT-PCR was performed as described previously (Ryoo *et al.*, 2006 [#13]). PCR-primer sequences of *ArgI*, *ArgII*, and *GAPDH* (Bioneer) are as follows: *ArgI* forward 5'-gga aga gac ctt cag cta cct gct gg-3', reverse 5'-ccc agt tca cag tac tct tca cc-3', 251-bp PCR-product; *ArgII* forward 5'-ggc aga ggc caa tcc acc tga gtt ttg-3', reverse 5'-ctg acc aaa act tga agc aat cac atc c-3', 251-bp PCR product; *GAPDH* forward 5'-agg ccg gtg ctg agt atg tc-3', reverse 5'-tgc ctg ctt cac cac ctt ct-3', 530-bp PCR-product.

Arginase activity assay

Aortic lysates were prepared by homogenization in lysis buffer (50 mM Tris-HCl, pH7.5, 0.1 mM ethyl-enediaminetetraacetic acid [EDTA] and protease inhibitors) at 4°C followed by centrifugation for 20 min at 14,000 × *g* at 4°C. The supernatants were used to assay arginase activity as previously described (Ryoo *et al.*, 2006).

Western blotting and eNOS dimerization analysis

Aortic segments were homogenized in the buffer (50 mM Tris-HCl, 150 mM NaCl, 1% Nonidet P-40, 1 mM EDTA, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 μg/ml aprotinin, 1 mM phenylmethylsulfonylflouride, 1 mM sodium orthovanadate, and 1 mM sodium fluoride) and centrifuged for 30 min at 14,000 × *g*. Supernatant protein concentrations were analyzed by the Bradford method. Protein samples (100 μg) were separated using sodium dodecyl sulfate polyacrylamide gel electrophoresis (10% gels) and transferred to nitrocellulose membranes (Bio-Rad). The blots were incubated with primary antibodies: monoclonal anti-ArgI (Santa Cruz Biotechnology), anti-ArgII (Santa Cruz Biotechnology), anti-eNOS (BD Biosciences), or anti-β-tubulin (BD Biosciences) antibodies, followed by an appropriate secondary antibody (Amersham). The signals were detected using an enhanced chemiluminescence detection reagent and X-ray films. eNOS dimers and monomers were separated using low-temperature SDS-PAGE as previously described (Woo *et al.*, 2010).

Estimation of NO or ROS generation using DAF-FM or DHE in isolated mice aorta

Mice aortic rings were isolated and incubated overnight at 37°C under 5% CO_2 in DMEM containing 2% fetal bovine serum and antibiotics (1×) in the presence or absence of siRNA (Ryoo et al., 2006). The fluorescence from the aortic endothelium was measured at different time intervals under microscopy (Woo et al., 2010).

Aortic vascular tension assay

Mice were anesthetized using isoflurane, and their thoracic aortas were rapidly removed, placed on ice-caged oxygenated Krebs-Ringer bicarbonate solution (in mM: NaCl 118.3, KCl 4.7, $MgSO_4$ 1.2, KH_2PO_4 1.2, $CaCl_2$ 1.6, $NaHCO_3$ 25, glucose 11.1), and cleared of adherent connective tissues. Individual mouse aortas were cut into 2.5-mm rings and suspended between two wire stirrups (150 μm) in a myograph (Multi myograph system DMT-620) in 10 ml Krebs-ringer (95% O_2 - 5% CO_2, pH7.4, 37°C). One stirrup was connected to a three-dimensional micromanipulator, and the other was attached to a force transducer. The rings were passively stretched at 10-min intervals in increments of 200 mg to reach optimal tone (600 mg). After the arterial rings had been stretched to their optimal resting tone, the contractile response to 100 mM KCl was determined. The response to a maximal dose of KCl was used to normalize the responses to agonist across vessel rings. Dose responses to the vasoconstrictor U46619 (10^{-10} - 10^{-6} M) were performed, followed by responses to the vasodilators acetylcholine (Ach, 10^{-9} - 10^{-5} M) and sodium nitroprusside (SNP, 10^{-9} - 10^{-5} M) were performed after preconstriction with U46619 (10^{-8} M). At the end of the experiments, NO-dependent vasorelaxation activity was confirmed by adding the guanylate cyclase inhibitor ODQ (1H-[1,2,4]oxadizolo[4,3-a]quinoxalin-1-one, 10^{-6} M). Data were collected online using a MacLab system and analyzed using Dose Response Software (AD Instruments).

Determination of intracellular L-arginine concentrations

Intracellular L-arg concentration was determined by HPLC using pre-column derivatization with o-phthalaldehyde (OPA) according to a modified version of a previously published method (Boger et al., 1995). L-arginine (100 μmol/L) was added to cell lysate (0.5 ml) as an internal standard. The samples were extracted on solid-phase extraction cartridges (CBA Bond Elut, Varian). Recovery rates were 87.5 ± 3.9%. Eluates were dried over nitrogen and resuspended in double-distilled water for HPLC analysis. HPLC was performed on a computer-controlled Waters chromatography system (M600E) consisting of an automatic injector (M7725i, Waters Co.) and a fluorescence detector (FP-1520, JASCO) in the Central Laboratory of Kangwon National University. Samples were incubated for exactly 1 min with OPA reagent (5.4 mg/ml OPA in borate buffer, pH8.4, containing 0.4% 2-mercaptoethanol) before automatic injection into the HPLC. The OPA derivative of L-arginine was separated on a 150 × 4.6 mm - 3.5 μm Zorbax Eclipse XDB-C18

column with the fluorescence detector set at 340 nm (excitation) and 450 nm (emission). Samples were eluted from the column with 0.96% citric acid/methanol (70:30), pH6.8, at a flow rate of 1.5 ml/min.

Statistics

All data are represented as mean ± standard deviation of at least four independent experiments. Unpaired Student's *t*-tests were used to determine significant differences. A value of $P < 0.05$ was accepted as significant.

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