Knockdown of Arginase I Restores NO Signaling in the Vasculature of Old Rats

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Abstract—Arginase, expressed in endothelial cells and upregulated in aging blood vessels, competes with NO synthase (NOS) for L-arginine, thus modulating vasoreactivity and attenuating NO signaling. Moreover, arginase inhibition restores endothelial NOS signaling and L-arginine responsiveness in old rat aorta. The arginase isoform responsible for modulating NOS, however, remains unknown. Because isoform-specific arginase inhibitors are unavailable, we used an antisense (AS) oligonucleotide approach to knockdown arginase I (Arg I). Western blot and quantitative PCR confirmed that Arg I is the predominant isoform expressed in endothelialized aortic rings and is upregulated in old rats compared with young. Aortic rings from 22-month-old rats were incubated for 24 hours with sense (S), AS oligonucleotides, or medium alone (C). Immunohistochemistry, immunoblotting, and enzyme assay confirmed a significant knockdown of Arg I protein and arginase activity in AS but not S or C rings. Conversely, calcium-dependent NOS activity and vascular metabolites of NO was increased in AS versus S or C rings. Acetylcholine (endothelial-dependent) vasorelaxant responses were enhanced in AS versus S or C treated rings. In addition, 1H-oxadiazolo quinoxalin-1-one (10 μmol/L), a soluble guanylyl cyclase inhibitor, increased the phenylephrine response in AS compared with S and C rings suggesting increased NO bioavailability. Finally, L-arginine (0.1 mmol/L)-induced relaxation was increased in AS versus C rings. These data support our hypothesis that Arg I plays a critical role in the pathobiology of age-related endothelial dysfunction. AS oligonucleotides may, therefore, represent a novel therapeutic strategy against age-related vascular endothelial dysfunction. (Hypertension. 2006;47:245-251.)

Key Words: atherosclerosis • nitric oxide • endothelium • aging

NO is a key regulator of multiple cardiovascular functions including vasoregulation and myocardial contractility. NO is produced by NO synthase (NOS)-induced catalysis of the amino acid substrate L-arginine. Arginase (Arg), an enzyme of which the primary function is the deamination of L-arginine in the urea cycle, also uses L-arginine as its substrate. We have demonstrated recently that Arg is expressed in rat vascular endothelial cells and constrains NOS activity by limiting L-arginine availability. Furthermore, we have demonstrated that Arg I expression is upregulated in aging blood vessels and contributes to the endothelial dysfunction of aging.

The primary function of Arg is to catalyze the last step of the urea cycle whereby toxic ammonia is converted to less-toxic urea in the liver. However, Arg exists in 2 isoforms: liver-type Arg I and the nonhepatic type II. Arg I is located in the cytosol, whereas Arg II is thought to be confined to the mitochondrial matrix. Tissue locations of Arg activity and expression are summarized elsewhere. A postulated function of extrahepatic Arg may be the production of the polyamine and proline precursor L-ornithine, known to be important in tissue repair and cell growth. Another emerging and potentially important role of extrahepatic Arg is the regulation of NOS. This phenomenon was originally demonstrated with regard to inducible NOS. Specifically, Arg I and II were coinduced in macrophages with inducible NOS after endotoxin (lipopolysaccharide) administration, leading to the hypothesis that Arg may limit sustained overproduction of NO. Recently, Arg I and II expression have been demonstrated in the rat lung where they modulate cholinergic airway responses and NO activity.

The reciprocal regulation of NOS has also been demonstrated with regard to constitutive NOS isoforms. For example, it has been demonstrated recently in the penis and in A293 cells overexpressing NOS-I that there exists reciprocal regulation of Arg and constitutive NOS-I. Our previously published data, as well as that of others, support the notion that Arg isoforms are expressed constitutively in the vascular endothelium and may perform a function quite different from that in the liver, that is, they modulate NOS...
activity by regulating l-arginine availability. Furthermore, up-regulation of Arg activity has been implicated as a contributory mechanism in the pathophysiology of age-related vascular dysfunction, hypertension, pulmonary hypertension, atherosclerosis, and pregnancy-induced hypertension.

The specific Arg isoform responsible for regulating l-arginine bioavailability/NOS activity in the endothelium is unknown. Because of the lack of isoform-specific antagonists, we have used a molecular antisense (AS) knockdown approach to determine whether the Arg I isoform is responsible for the endothelial-specific effects observed in aging rat blood vessels and whether knockdown of Arg I could restore endothelial l-arginine responsiveness and NO signaling in the vasculature of old rats.

Methods

Animals
Ten old (23.0±0.89-month-old) male Wistar rats and 10 young male Wistar rats (3.0±0.4-month-old) were used in the study. All of the surgical procedures and experimental protocols were approved by the Institutional Animal Care and Use Committee and are fully accredited by the American Association for Accreditation in Laboratory Animal Care. Their retention and use is in compliance with federal, state, and local laws and regulations and in accordance with the National Research Council Guide.

AS Incubation and Knockdown of Arg I
The thoracic aorta was dissected from euthanized rats, cut into 5-mm rings, and incubated overnight in DMEM containing phosphorothislated AS (100 nM), sense (S; 100 nM), or no oligonucleotides (oligos). The oligos were fluorescent and tagged with 6-FAM (100 nM) so that they could be visualized on histological sections.

PCR and Quantitative PCR
Total RNA from rat aorta was prepared by homogenization in the presence of Trizol reagent (Gibco). To exclude contamination of genomic DNA, total RNA was treated with RNase-free DNase (Roche). PCR reaction was performed in iCycler optical system (Bio-Rad) with or without SYBR green PCR master mix.

Immunohistochemistry
Frozen 8-mm sections of the aorta (n=3) were used for the detection of Arg I protein and S or AS oligos in the vessels. For the visualization of the Arg I oligos labeled with 6-FAM, the slides were washed with PBS and examined under fluorescent microscope (Leitz Fluovert) linked with a Sony DX5000 digital camera. Vector Elite kit (mouse) and diaminobenzidine were used for the detection of the positive signals in the vessels.

Immunoblotting
The animal aorta from old and young rats was homogenized and centrifuged for 30 minutes at 14 000g. The protein content of the supernatant was analyzed by the method of Bradford. Protein (100 µg) from the aorta homogenates was electrophoresed. The blots were incubated with a monoclonal anti-Arg I antibody followed by a goat anti-mouse horseradish peroxidase–labeled secondary antibody. Signals were detected using chemiluminescence.

NOS Activity
For determination of NOS 3 enzyme activity (Calbiochem-Novabiochem Corporation), l-arginine to l-citrulline conversion was assayed in aorta extracts. This assay is selective for NOS 3.

Metabolites of NO Measurement
NO production was evaluated by measuring nitrite levels, a major stable breakdown product of NO, by specific light absorbance (Calbiochem).

Arg Activity
The rat aortic vessels were homogenized in lysis buffer and centrifuged for 30 minutes at 14 000g at 4°C. Briefly, aortic lysates were added to Tris-HCl. The hydrolysis reaction of l-arginine by Arg was performed by incubating the mixture containing activated Arg and was stopped by adding acid solution. For calorimetric determination of urea, 1,5-isonitrosopropiophenone was added, and the mixture was heated at 100°C for 45 minutes. After placing the sample in the dark for 10 minutes at room temperature, the urea concentration was determined spectrophotometrically by the absorbance at 550 nm.

In Vitro Isometric Tension Measurements in Aortic Rings
Vessel rings were suspended for isometric tension recording in 25-ml organ chambers. Vasorelaxant responses to acetylcholine were tested in phycocerythrin (PE) preconstricted rings. NO bioavailability was tested by determining the increase in tension in PE preconstricted rings induced by the addition of the soluble guanylyl cyclase inhibitor 1H-oxadiazolo quinoxalin-1-one (ODQ). The direct effect of l-arginine was also tested in preconstricted rings. Data were collected online using a MacLab system and analyzed using Dose Response Software (AD Instruments). A more detailed methods section can be found in an online supplement available at http://www.hypertensionaha.org.

Statistical Analysis
Data are reported as mean±SEM. Where indicated, data were analyzed using Bonferroni t test for unpaired values. A P value of <0.05 was used as the criterion for statistical significance. Between 2 and 8 rings were used for each protocol. Dose responses were analyzed using 2-way ANOVA and post hoc t test.

Results
We first examined the mRNA and protein abundance of each Arg isoform in old and young rat aorta (Figure 1). As is demonstrated using conventional RT-PCR (Figure 1A), Arg I mRNA expression is present in endothelialized (E+) vascular rings from old and young rats. On the other hand, there is no observable Arg I signal in rings in which the endothelium has been removed (E−). Furthermore, as we have demonstrated previously, there is an increase in the expression of Arg I in the E+ aortic rings of old rats. There is, however, no detectable Arg II signal in either old or young E+ or E− rings (positive control for Arg II is rat kidney). We next performed quantitative PCR for both Arg I and Arg II in E+ and E− rings in old and young rats (Figure 1B). We demonstrated that although Arg II expression is present in rat rings and is increased in the rings of old rats (P<0.001), the expression of Arg II is ~30-fold lower than that of Arg I. Furthermore, whereas removal of the endothelium markedly decreases Arg I mRNA abundance (P<0.001), this effect appears to be less so in the case of Arg II, suggesting that Arg II may be expressed in cells other than the endothelium. Figure 1C confirms that Arg I protein is expressed in rat aorta and that its abundance is significantly greater in old versus young rats (P<0.01). Furthermore, given the same protein loading and film exposure time as Arg I, Arg II is almost undetectable. Thus, we conclude that Arg I is the predominant isoform present in the endothelium of rats and that it is upregulated in old rats.
We next tested whether Arg I is the isoform important in reciprocal regulation of NOS using an AS oligonucleotide technique. Both S and AS oligos were taken up predominantly by the endothelial cells as demonstrated by bright immunofluorescence in rings incubated with oligos versus control (Figure 2). Immunohistochemical staining for Arg I demonstrated endothelial staining in C and S rings but a marked reduction in endothelial staining in rings preincubated with AS. These data suggest that AS oligos selectively knock down Arg I in aortic endothelium. To confirm this, we performed Western blots in aortic rings from old C vessels, young C, and old rats preincubated with S, C, and AS (Figure 2B). Consistent with our previous data, there was a significant increase in the abundance of Arg I in old C versus young C rat rings (100% ± 7 versus 148 ± 13%; P < 0.05). There was
a trend toward a difference in the abundance of Arg II in old C versus young C rat rings consistent with previous immunohistochemical data and our quantitative PCR data. Incubation with AS oligos resulted in a significant reduction (114%±10% versus 144±6% and 148±13% P<0.001 AS versus S and C, respectively) in the protein abundance of Arg I, such that the Arg I was not significantly different from that of Y rat rings (100±7.4%). There was, however, no reduction in the amount of Arg II protein consistent with the selective knockdown of Arg I.

We next assessed the biochemical consequence of Arg I knockdown by measuring Arg, NOS activity, and NOx in aortic rings preincubated with AS, S, and oligos and in C (Figure 3A and 3B). Again, consistent with our previous findings, there was a significant increase in Arg activity in old C compared with Y rings (11.8±1.2 versus 6.9±0.6 mmol urea/mg protein per minute; P<0.0001). There was, however, a significant decrease in Arg activity in aortic rings preincubated with AS versus S or C. The AS oligos reduced the Arg activity so that it was not significantly different from young C rings (7.4±0.4 versus 6.9±0.6 mmol urea/mg protein per minute). We have demonstrated previously that NOS and Arg I are reciprocally regulated in the aorta from old rats. Measurements of NOS activity revealed a significant increase in NOS activity (Figure 3B) and NOx production (Figure 3C) in AS preincubated rings compared with either S or C. Furthermore, there was no significant difference in Ca²⁺-dependent NO bioavailability in AS versus S and C, respectively) in the protein abundance of Arg I and NOS activity and NOx production in young C rings and old AS treated rings. These data support our previous observations regarding reciprocal regulation of Arg I and NOS and demonstrate the important role of Arg I in the dysregulation of NO production.

We next investigated the functional consequences of Arg I knockdown by determining vascular endothelial responses of aortic rings in organ chambers. As demonstrated previously, there is a significant attenuation of the acetylcholine responses in old C versus young C rings (maximal vasodilation 79.9±8.6% versus 99.1±4.9; P<0.001). However, vasorelaxant responses to acetylcholine were significantly enhanced in rings preincubated with AS compared with C. The AS oligos reduced the Arg activity so that it was not significantly different from young C rings (7.4±0.4 versus 6.9±0.6 mmol urea/mg protein per minute). We have demonstrated previously that NOS and Arg I are reciprocally regulated in the aorta from old rats. Measurements of NOS activity revealed a significant increase in NOS activity (Figure 3B) and NOx production (Figure 3C) in AS preincubated rings compared with either S or C. Furthermore, there was no significant difference in Ca²⁺-dependent NO bioavailability in AS versus S and C, respectively) in the protein abundance of Arg I and NOS activity and NOx production in young C rings and old AS treated rings. These data support our previous observations regarding reciprocal regulation of Arg I and NOS and demonstrate the important role of Arg I in the dysregulation of NO production.

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We have demonstrated previously that both acute and chronic inhibition of Arg restore the vasodilatory effect of 1-arginine in aortic rings of old rats. Here we determined the effect of AS knockdown on 1-arginine responses in aortic rings (Figure 4C). Vasodilatory responses were significantly enhanced in rings preincubated with AS compared with C treated rings (n=5; P<0.01). A similar trend was seen in AS versus S treated rings (79.4±10.2 versus 40.9±13.4% relaxation; n=5; P<0.05).

**Discussion**

There is emerging evidence that Arg may be a key regulator of NO signaling, counteracting NOS activity by limiting their shared substrate. Although 2 isoforms of Arg have been cloned and characterized, the specific isoform present in endothelial cells responsible for limiting NOS activity has not been determined. Because of the lack of availability of isoform-specific inhibitors, we have used an AS molecular approach to knockdown Arg I. We have demonstrated that Arg I knockdown can enhance NOS activity and restore 1-arginine responsiveness and endothelial NO signaling in aging rat vessels.
The intracellular concentration of L-arginine exceeds by 2- to 3-fold its Michaelis constant for the enzyme NOS-3 (or endothelial NOS; eNOS), indicating that L-arginine availability should not limit eNOS activity or NO production. Yet, in certain conditions (diabetes, hypertension, and hypercholesterolemia) the addition of extracellular L-arginine does enhance NO-dependent relaxation. This is known as the “arginine paradox” and suggests that factors other than L-arginine concentration also influence L-arginine bioavailability to the enzyme eNOS. One such factor is the enzyme Arg, which competes with eNOS for L-arginine. Increased Arg expression and activity in pathological conditions may limit L-arginine bioavailability for eNOS. Our previous study and our current results, whereby nonspecific Arg inhibition and Arg I knockdown, respectively, restore endothelial function and L-arginine responsiveness in vessels from old animals, are entirely consistent with this thesis. Additional factors that could influence L-arginine bioavailability include: (1) spatial confinement of eNOS and a caveolar complex between it and the cationic amino acid–transported (L-arginine) transporter; and (2) the presence of distinct intracellular L-arginine pools available to eNOS and Arg. Based on their results, Closs et al suggest that there are 2 intracellular pools of L-arginine, both accessible to eNOS, but only 1 of which is exchangeable with extracellular L-arginine. Our results, whereby the addition of extracellular L-arginine alone has minimal success in restoring endothelial function, whereas in contrast, L-arginine plus Arg inhibition is strikingly effective, suggest that Arg has access to at least the exchangeable L-arginine pool. Our results with Arg inhibition alone and with Arg I knockdown could indicate that Arg may modulate the nonexchangeable L-arginine pool.

It is increasingly recognized that Arg is constitutively expressed in endothelial cells of multiple vascular beds. Bachetti et al demonstrated recently that both isoforms are constitutively expressed in human umbilical vein endothelial cells where they regulate cell cycling (inhibition of Arg leads to growth inhibition). The predominant isoform in this cell population appears to be Arg I. In contrast, in a porcine coronary artery model, Zhang et al have shown that the Arg I isoform is mainly responsible for constraining endothelial-dependent relaxation. Moreover, bovine pulmonary endothelial cells express both Arg I and II where upregulation can be induced by cytokines and Arg inhibition accentuates NO release. We have previously demonstrated constitutive expression of both Arg I and II in rat aortic endothelium where Arg I is the predominant isoform.

Although these studies provide indirect evidence that NOS and Arg are reciprocally regulated in endothelial cells, the results of this study are the first to address the role of a specific Arg isoform in this process. Specifically, Arg I knockdown restores L-arginine responsiveness, NOS activity, and NO signaling. This supports the notion that Arg I is the predominant functional isoform in rat vascular endothelial cells. This is consistent with the putative subcellular locale of the specific isoforms: Arg I is thought to be confined to the cytosol, whereas Arg II, with its N-terminal mitochondrial leader sequence, is confined to the mitochondrion. The functional distinction between these 2 isoforms may be overly simplistic in that recent data suggest that, under certain pathophysiologic circumstances, different isoforms may be expressed that have overlapping function. Furthermore, Arg II–specific knockout mice demonstrate no apparent phenotypic change from their littermate controls suggesting that this could result from Arg I upregulation, which compensates for Arg II. This is in marked contrast to Arg I knockout mice, which exhibit a severe debilitated phenotype, succumbing to hyperammonemia at 10 to 12 days of life.
The critical role of Arg in the biology and pathobiology of NO signaling continues to emerge. Endogenous Arg activity in erectile tissue endothelium modulates corpus cavernous tone in an NO-dependent manner, and Arg upregulation is associated with impotence in diabetic patients. NO is also an important modulator of bronchial smooth muscle tone and is regulated by Arg, which appears to be upregulated in asthma. 1-arginine depletion, secondary to Arg upregulation, appears to contribute to the pathophysiology of poor placental perfusion seen in preeclampsia. Furthermore, upregulation of Arg activity has been implicated as a contributory mechanism in the pathophysiology of aging-related vascular dysfunction, hypertension, pulmonary hypertension, and atherosclerosis where an increase in Arg activity appears to be dependent on Rho and Rho kinase. Our findings also support an important role for Arg I in vascular endothelial tissue in that it regulates substrate availability for eNOS. In addition, increased Arg I expression represents an important pathophysiological mechanism in age-related endothelial dysfunction.

The decrease in eNOS activity that we have demonstrated in old-rat aorta despite an increase in protein abundance is consistent with the idea that enhanced Arg expression constrains the activity by limiting 1-arginine availability. There remains, however, some controversy regarding eNOS activity and abundance in aging rat aorta. Whereas van der Loo et al demonstrated a significant increase in expression of eNOS and a corresponding increase in Ca2+-dependent NOS activity, our findings are consistent with those of Cernadas et al, who demonstrated both an increase in eNOS expression and a significant decrease in Ca2+-dependent NOS activity. Whereas Zieman et al did, indeed, show both an increase in expression and activity of eNOS, the samples were from myocardium rather than aorta. A comparison, therefore, is difficult given the complexity of NOS and its isoforms and spatial confinement in the heart, as well as the contribution of coronary vessels to the signal. One possible explanation for the differences observed among our work, Cernadas, and the work of van der Loo is the differences in rat strains used. In the van der Loo study, Fisher 344 rats were used. On the other hand, in our study and the study by Cernadas, Wistar rats were used. It is well established that there are significant differences in the vascular biology of these different rat strains. Furthermore, there are differences in the vascular phenotype expression with aging between the strains.

One of the seminal features of the aging vasculature is an increase in arterial stiffness, which is additionally exacerbated by hypertension and diabetes. This decreased arterial compliance is the primary cause of increased central pulse pressure and increased pulse wave velocity in the elderly. It is increasingly being recognized that an increase in pulse pressure associated with vascular stiffening (and associated ventricular stiffness) is an important risk factor for cardiovascular disease. Arterial compliance is determined by mean pressure, vessel tone, structure, and endothelial function. Endothelial vasodilator function and NO signaling are impaired, and ROS production is increased in the aging vessels. These mechanisms play a major etiologic role in the observed vascular changes. The role of NO in regulating vascular compliance is well established. Moreover, ROS inhibition with antioxidants can reduce arterial stiffness in humans. However, few studies have been performed in humans examining the therapeutic efficacy of L-arginine in altering vascular stiffness. Chauhan et al studied patients with normal coronary anatomy and demonstrated age-related endothelial-dependent dysfunction, which was reversed with acute intracoronary administration of L-arginine. However, chronic oral L-arginine has little effect on cardiovascular hemodynamics. This is consistent with our observations and supports the concept that Arg upregulation limits the efficacy of long-term L-arginine administration. Arg inhibition, specifically Arg I inhibition, could be a therapeutic target for the treatment of age-related endothelial dysfunction and resultant vascular stiffness. This strategy may have a significant impact on cardiovascular disease outcome. Enthusiasm for Arg inhibition as a therapeutic strategy for age-related vascular dysfunction needs to be tempered by the potential for significant inhibition of Arg in vivo and its consequences. However, based on our experiments, we have only been able to reduce Arg activity in endothelial cells by 30% to 40%. It is unlikely that, given this degree of reduction in Arg activity in the liver, this would lead to significant hyperammonemia. This is supported by data in Arg I knockout mice. As predicted, the homozygous knockout mice as Arg I–knockout mice completely lacked liver Arg activity, exhibited severe symptoms of hyperammonemia, and died between postnatal days 10 and 14. On the other hand, heterozygous mice with some reduction in Arg activity showed no significant phenotypic changes. Despite this, however, therapy in vivo targeted to the inhibition of endothelial-specific Arg appears to be a more rational goal for the treatment of age-related endothelial function.

Perspectives
As so eloquently argued by Najjar et al, vascular aging is no longer considered an “immutable cardiovascular risk.” We and others continue to demonstrate discrete but related alterations in signaling pathways that contribute to altered vascular structure and function with aging. We have demonstrated that knockdown of a specific enzyme, Arg I, can restore endothelial function in old rat blood vessels. Whereas in vitro experiments are critical in defining dysregulated pathways, our next order of business is to define whether attenuation of this pathway can alter in vivo measures of vascular health that have been shown to predict cardiovascular outcomes, such as vascular stiffness.

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White et al. Arginase and Endothelial Dysfunction


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