Arginase Reciprocally Regulates Nitric Oxide Synthase Activity and Contributes to Endothelial Dysfunction in Aging Blood Vessels

Dan E. Berkowitz, MD*; Ron White, PhD*; Dechun Li, MD, PhD; Khalid M. Minhas, MD; Amy Cernetich; Soonyul Kim, MD; Sean Burke, BS; Artin A. Shoukas, PhD; Daniel Nyhan, MD; Hunter C. Champion, MD, PhD; Joshua M. Hare, MD

Background—Although abnormal l-arginine NO signaling contributes to endothelial dysfunction in the aging cardiovascular system, the biochemical mechanisms remain controversial. l-arginine, the NO synthase (NOS) precursor, is also a substrate for arginase. We tested the hypotheses that arginase reciprocally regulates NOS by modulating l-arginine bioavailability and that arginase is upregulated in aging vasculature, contributing to depressed endothelial function.

Methods and Results—Inhibition of arginase with (S)-(2-boronoethyl)-l-cysteine, HCl (BEC) produced vasodilation in aortic rings from young (Y) adult rats (maximum effect, 46.4 ± 9.4% at 10⁻⁵ mol/L, P < 0.01). Similar vasorelaxation was elicited with the additional arginase inhibitors N-hydroxy-nor-l-arginine (nor-NOHA) and difluoromethylornithine (DFMO). This effect required intact endothelium and was prevented by 1H-oxadiazole quinoxalin-1-one (P < 0.05 and P < 0.001, respectively), a soluble guanylyl cyclase inhibitor. DFMO-elicited vasodilation was greater in old (O) compared with Y rat aortic rings (60 ± 6% versus 39 ± 6%, P < 0.05). In addition, BEC restored depressed l-arginine (10⁻⁴ mol/L)–dependent vasorelaxant responses in O rings to those of Y. Arginase activity and expression were increased in O rings, whereas NOS activity and cyclic GMP levels were decreased. BEC and DFMO suppressed arginase activity and restored NOS activity and cyclic GMP levels in O vessels to those of Y.

Conclusions—These findings demonstrate that arginase modulates NOS activity, likely by regulating intracellular l-arginine availability. Arginase upregulation contributes to endothelial dysfunction of aging and may therefore be a therapeutic target. (Circulation. 2003;108:2000-2006.)

Key Words: endothelium ■ aging ■ nitric oxide ■ cardiovascular diseases

The aging cardiovascular system undergoes characteristic changes involving both the heart and vasculature that include endothelial dysfunction. The mechanisms underlying endothelial dysfunction, defined by impaired vasodilator response to flow or agonists, likely involve multiple signaling pathways. A decrease in NO bioavailability seems to be a central mechanism for endothelial dysfunction in atherosclerosis, hypertension, and hypercholesterolemia.1-3 There are several possible mechanisms for this that include decreased endothelial NO synthase (NOS) abundance or activity, increased levels of endogenous NOS inhibitors, limited availability of l-arginine, or increased degradation or scavenging of NO.

Although intracellular concentrations of l-arginine (>100 µM/L) exceed the Kₘ of NOS (2 to 10 µM/L), exogenous l-arginine partially restores NO bioactivity in animals and human subjects, engendering the so-called arginine paradox.4 The paradox is also apparent in aging-related endothelial dysfunction, a situation in which NOS3 abundance is upregulated.5 Arginase hydrolyzes l-arginine to ornithine and urea as part of the urea cycle and has the potential to modulate l-arginine bioavailability. In this regard, arginase inhibition enhances NO production by NOS2 in macrophages.6 The purpose of this study was to test the hypotheses that arginase reciprocally regulates NOS3 activity in vascular endothelium and is upregulated in aging vasculature. This enhanced arginase activity in aging vessels could explain the arginine paradox as well as the phenomenon of enhanced NOS3 expression but decreased NOS activity and NO production in aging rat vessels.

Methods

Reagents
(S)-(2-boronoethyl)-l-Cysteine-HCl (BEC) and N-hydroxy-nor-l-arginine (nor-NOHA) were obtained from Calbiochem. Difluoro-
methylornithine (DFMO) was obtained from LKT Laboratories. 1H-Oxadiazolo quinoxalin-l-one (ODQ) and N²-monomethyl-l-arginine monooacetate (L-NMMA) were from Alexis Biochemicals. l-Arginine and phenylephrine (PE) were obtained from Sigma. Arginase I Ab was obtained from Transduction Laboratories. A polyclonal arginase II Ab was raised in rabbits.10 Arginase I Ab was obtained from Transduction Laboratories. A subset of vessel rings were deendothelialized by preincubation with the arginase inhibitors BEC and nor-NOHA. In a separate protocol, vasorelaxant responses to choline (10⁻⁶mol/L) were determined in preconstricted (10⁻⁶mol/L PE) aortic rings. To confirm that the observations were not attributable to nonspecific effects of arginine, the effect of l-arginine on vascular relaxation was tested.

Impact of Chronic Arginase Inhibition

Twenty-one-month-old Wistar rats (n=12) were randomly assigned to receive DFMO (4 mg/mL) in their drinking water or tap water. After 4 weeks, the rats were euthanized and vascular responses to l-arginine were assessed in preconstricted (10⁻⁵mol/L PE) aortic rings. To confirm that the observations were not attributable to nonspecific effects of arginine, the effect of l-arginine on vascular relaxation was tested.

NOS Activity

For determination of constitutive NOS activity (Calbiochem-Novabiochem Corporation), l-arginine to l-citrulline conversion was assayed in aortic extracts as previously described.9

Arginase Activity

Rat aortic tissue was obtained as described above, frozen in liquid nitrogen, and pulverized. Resulting tissue powder was combined 1:4 (wt:vol) with ice-cold buffer (20 mmol/L HEPE 5, MgCl2 1.5, EDTA 0.2, DTT 0.5, phenylmethylsulfonyl fluoride 0.5; pH 7.9, glycerol 26%) and incubated on ice for 30 minutes. After centrifugation twice at 15,000g at 4°C for 20 minutes, protein concentration was determined using a Bio-Rad DC protein assay and samples were stored at −70°C until use. For Western blot analysis, the supernatant was mixed with an equal volume of 2% SDS/1% β-mercaptoethanol and fractionated using 8% SDS/PAGE (70 µg/lane). Proteins were then transferred to a nitrocellulose membrane (Hybond-ECL, Amersham Life Sciences) by semidy electroblotting for 1 hour. The membranes were blocked with 5% nonfat dry milk, 0.1% Tween-20 and incubated with a primary arginine I or II antibody IgG antibody (1:500). Bound antibody was detected with labeled anti-mouse IgG secondary antibody (1:20 000) (Santa Cruz Biotechnology) and visualized using enhanced chemiluminescence.

mRNA Expression

Total RNA was isolated from rat aorta using 1 mL of TRI Reagent (Molecular Research Center) and prepared for reverse transcriptase–polymerase chain reaction (PCR). Total RNA (2 µg) was primed with oligo-dt (0.2 µg) and reverse-transcribed with MMLV RT (100 U) (Life Technologies) in the presence of RNasin (20 U; Promega) in a 20-µL total reaction volume. PCR reactions were performed in a volume of 25 µL containing 200 µmol/L dNTPs, 5 µL of RT reaction mixture, 22 U recombinant Taq DNA polymerase, and 20 µmol/L of the specific primer pairs for arginase I and arginase II. The primer pairs were chosen from the published cDNA sequences of rat arginase I (accession No. NM 017134) and rat arginase II (accession No. NM 019168). The primer sequence for arginase I corresponds to 5’-GCGAGACCCAGAAATGGACAC-3’ (sense) and 5’-CCGAGTGTGTAGCTGAGAC-3’ (antisense); PCR product size is 144 bp. The primer sequence for arginase II corresponds to 5’-TTGCTTCCTCCCAAAATC-3’ (sense) and 5’-AATGCTGTCGAAAGGTTGCC-3’ (antisense); PCR product size is 544 bp.

Quantitative PCR

Real-time quantitative PCR was used to determine relative expression of arginase I mRNA in rat aorta of O and Y rats. Total RNA was isolated using RNeasy kit (Qiagen), DNase treated (Ambion), and reverse transcribed using Superscript II (Life Technologies). PCR reactions were performed in a GeneAmp 7900 sequence detection system (Applied Biosystems) using SYBR green PCR master mix. The quantitative PCR primers are as follows: sense: CAACCTGG-GAATTTGCGAAAG; antisense: GGTCCAGTCCATCAAC-ATCAAA.

Western Blot Analysis

Aortic rings were homogenized in ice-cold buffer (in mmol/L, HEPE 5, MgCl2 1.5, EDTA 0.2, DTT 0.5, phenylmethylsulfonyl fluoride 0.5; pH 7.9, glycerol 26%) with NaCl (300 mmol/L final) and incubated on ice for 30 minutes. After centrifugation twice at 15,000g at 4°C for 20 minutes, protein concentration was determined using a Bio-Rad DC protein assay and samples were stored at −70°C until use. For Western blot analysis, the supernatant was mixed with an equal volume of 2% SDS/1% β-mercaptoethanol and fractionated using 8% SDS/PAGE (70 µg/lane). Proteins were then transferred to a nitrocellulose membrane (Hybond-ECL, Amersham Life Sciences) by semidy electroblotting for 1 hour. The membranes were blocked with 5% nonfat dry milk, 0.1% Tween-20 and incubated with a primary arginine I or II antibody IgG antibody (1:500). Bound antibody was detected with labeled anti-mouse IgG secondary antibody (1:20 000) (Santa Cruz Biotechnology) and visualized using enhanced chemiluminescence.

Immunohistochemistry

Rat aortas were washed with heparinized normal saline, and 3-mm fragments of the aorta were embedded in OCT compound (Sakura Finetek). Fresh-frozen section (8-µm-thick) were cut and mounted onto preclean glass slides (Superfrost Plus, Fisher Scientific) and then fixed in paraformaldehyde (4% wt/vol) in 0.01 mol/L PBS (pH 7.3) for 20 minutes. After washing, slides were first blocked in 10% goat serum in PBS and then incubated with monoclonal anti-arginase I or II antibody (final dilution, 1 µg/mL; Transduction Laboratories, San Diego, Calif) at 4°C overnight. After washing in PBS, the secondary goat anti-mouse IgG antibody conjugated with biotin (Vector Laboratory, Burlingame, Calif; 1:200 dilution) was applied and incubated for 1 hour at room temperature followed by incubation with ABC complex (Vector Laboratory; 1:200 dilution). A Vector DAB Kit (Vector Laboratory) was used following the manufacturer’s instructions. After washing in water, the slides were counterstained with hematoxylin and mounted with Permount (Fisher).

Statistical Analysis

Data are reported as mean±SEM. Dose-response relationships were analyzed using a 2-way ANOVA with repeated measures and Neumann-Kuels post hoc test for multiple group comparisons. Where indicated, data were analyzed using Bonferroni t test for unpaired values. P<0.05 was used as the criterion for statistical significance.
Results

Vasoreactivity Studies

To test whether arginase influences endothelial function, we exposed aortic rings to the arginase inhibitor DFMO. DFMO dose-dependently relaxed the vascular tone of endothelialized (E/H11001), preconstricted (PE, 10-6 mol/L) rings, with a maximal response of 39+6.0% at a concentration of 0.5 mmol/L (Figure 1A). Vasorelaxation to DFMO was markedly attenuated in endothelial denuded rings (E/H11002) (71% reduction versus control, P<0.001), demonstrating the endothelial dependence of this effect. Additionally, both L-NMMA (10-4 mol/L) and ODQ (10-5 mol/L) inhibited vasorelaxation (48% reduction versus control, P<0.05, and 78% reduction versus control, P<0.001, respectively), demonstrating the NOS and sGC/cyclic GMP (cGMP) dependence of this response.

We next compared vasodilatory responses to DFMO in O versus Y rat aortic rings. DFMO-induced vasorelaxation was substantially greater in rings from O compared with Y rats (60±6.0% versus 39±6.0%, n=3 to 4, P<0.05) (Figure 1A), suggesting that arginase activity is upregulated with advancing age.

Because DFMO also inhibits ornithine decarboxylase, an enzyme in the urea cycle,11 we tested the effects of additional and highly selective arginase inhibitors BEC12,13 and nor-NOHA.14 BEC (10-8 to 10-5 mol/L) (Figure 2) caused concentration-dependent vasorelaxation (maximal response of 46±9%; Figure 2A and 2B), whereas nor-NOHA also resulted in a similar vasorelaxant response (27±8%). As with the DFMO experiments, deendothelialization and ODQ markedly attenuated or abolished vasorelaxation to BEC.

Arginase Modulation of Response to L-Arginine

In aortic rings from Y rats, L-arginine (10-4 mol/L) resulted in 25±9% relaxation (Figure 3), and this was not potentiated by either BEC (30±7% relaxation, 10-5 mol/L) or nor-NOHA (10-5 mol/L). In contrast, rings from O rats failed to vasodilate after exposure to L-arginine. To test whether this was
Arginase, NOS Activity, and cGMP Levels

To provide biochemical explanations for the response to arginase inhibitors in vascular rings, we measured enzyme activities. Arginase activity was significantly elevated in aortic rings from O rats (594±26 pmol urea/min per mg protein, O versus Y, n=5 each, P<0.001) (Figure 4A).

To establish a linkage between arginase and NOS, we measured NOS activity and cGMP production in response to arginase inhibition in rings from O and Y rats. As previously reported, NOS activity was decreased in aortic rings from O rats (7.5±0.5 versus 12.6±1.6 pmol/mg per min, O versus Y, n=9, P<0.05) (Figure 4B). BEC significantly enhanced NOS activity in rings from O rats (11.6±0.8 pmol/mg per min, n=5, P<0.05) such that it was no different from that of Y rats (Figure 4B). Similarly, DFMO enhanced NOS activity to that of Y rats (9.4±0.9 pmol/mg per min).

cGMP was decreased in rings from O compared with Y rats (3.0±0.3 versus 5.0±0.2 fmol/mg protein, n=7 to 9, P<0.05) (Figure 4C) and was restored toward that of Y rats by BEC (4.7±0.4 fmol/mg protein) (Figure 4C) and DFMO (4.5±0.5 fmol/mg protein).

Arginase and NOS Expression

We next determined expression of arginase isoforms in rat aortic rings. Arginase I was the predominant isoform expressed, although arginase II was also present. Quantitative PCR demonstrated an ~7-fold increase (79.2±2.5 versus 11.6±1.1 pg/sample, O versus Y, n=5, P<0.001) in arginase I mRNA expression in aged rat aorta. Immunoblotting with antibodies specific to arginase I and II confirmed an increase in protein levels of both isoforms in O rats. Immunohistochemistry also confirmed localization of arginase I and II to the endothelium with increased expression in the endothelial cells of O rats (Figure 5).

Effect of Chronic Arginase Inhibition on Vascular Tone Response to l-Arginine

The effect of chronic arginase inhibition on l-arginine (0.1 mmol/L) responsiveness was tested in aortic rings preconstricted with PE. O control rats had little vasodilatory response to l-arginine (1±4% relaxation) (Figure 6). In contrast, addition of l-arginine resulted in a marked vasorelaxant response in PE-preconstricted rings from O, DFMO-fed rats (41±10%, P<0.01) (Figure 6). Addition of
These findings offer a potential explanation for the paradoxical observations that L-arginine administration modulates endothelial cell NO production despite intracellular concentrations of L-arginine (≈100 μmol/L) exceeding by >10-fold the $K_m$ values of purified NOS (2 to 10 μmol/L). Our findings that arginase inhibitors increase NOS enzyme activity, cGMP production, and vascular relaxation suggest that arginase competitively regulates L-arginine bioavailability for NOS3. In terms of relative enzyme affinities, Wu and Morris have pointed out that whereas the $K_m$ for arginine is several orders of magnitude higher for purified mammalian arginase (2 to 20 mmol/L) than for various NOS iso-enzymes (2 to 20 μmol/L), the $V_{max}$ of arginine at physiological pH (≈1400 μmol/L per min per mg, calculated for rat liver arginase), is more than 1000 times that of the NOS enzymes (≈1 μmol/L per min per mg), making similar rates of substrate usage possible. It is also important to note that the apparent $K_m$ of NO synthesis by intact cells is closer to 100 to 150 μmol/L, similar to the $K_m$ for the arginase transporter, which may regulate the synthetic system. Other factors may also regulate the kinetics of the complex interplay between NOS and arginase. These include endogenous NOS inhibitors (ADMA and L-NMMA) and compartmentalization of L-arginine pools. Thus, although competition for substrate may be less likely using purified enzymes, within the cell, many factors provide an environment for arginase to constrain NO synthesis, particularly if arginase is upregulated.

Our findings agree with previous demonstrations of impaired endothelial function and NO signaling associated with aging. Although several animal and human studies demonstrate age-related endothelial dysfunction associated with reduced NO signaling, potential mechanisms for this effect remain controversial. Our findings of increased arginase expression in aortic rings of aging rats help explain the paradox of increased NOS3 expression with impaired NO signaling and provide a mechanism for age-related endothelial dysfunction. Although NOS3 expression is increased, impaired bioavailability of L-arginine limits NO production, leading to impaired vasorelaxation. The enhanced vasodilator responses to arginase inhibition, as well as restored responsiveness to L-arginine administration, are congruent with this thesis.

The addition of exogenous L-arginine did not have the same effect as arginase inhibition. Rather, pretreatment with arginase inhibitors was necessary to restore L-arginine responsiveness in vascular ring preparations from O rats. This finding suggests that the enzyme kinetics clearly favor arginase when upregulated relative to NOS. However, other factors may contribute to this observation. First, L-arginine concentrations in microdomains of the cell may vary and may be available only to enzymes specifically located in these domains. In this regard, both activated macrophages and endothelial cells have L-arginine pools not freely exchangeable with the extracellular space. This pool seems to be accessible to NOS3 in endothelial cells but not to NOS2 in macrophages. Also, the L-arginine CAT transporter colocalizes with NOS3 to caveolae, and exogenous L-arginine may activate NOS3 because of its spatial association with the CAT transporter in a manner independent of intracellular...
1-arginine. In addition, caveolin-1 and -3 dissociation from caveolae occurs during aging, and NOS activity is negatively correlated with caveolin-1 levels in the cytosol.27 To the extent that aging is associated with decreased caveolar-associated NOS, the effect of exogenous 1-arginine could be additionally impaired. With arginase inhibition, 1-arginine could restore responsiveness of cytosolic NOS and thus restore vasorelaxation.

The role of arginase in modulating NO production is not limited to vascular endothelium. Recently arginase II expression was demonstrated in human corpus cavernosa, where it is upregulated in diabetes, a disease process associated with erectile dysfunction.10,13 In the airway, arginase inhibition attenuates methacholine-induced airway constriction by increasing NO production.28 Arginase may also limit NO production in macrophages and other tissue after NOS2 induction.6,29,30 Furthermore, arginase may limit NOS activity in A293 cells overexpressing NOS131 and regulate vascular smooth muscle proliferation in an NO-dependent manner.11

Several pharmacological issues warrant mention. We have used 3 chemically unique arginase inhibitors, DFMO, a fluorinated methyl substituted form of ornithine; nor-NOHA, the α-amino acid nor-NOHA, a new analogue of the endogenous intermediate in NO synthesis and arginase inhibitor (NOHA); and BEC, a boronic acid analog of 1-arginine. Use of these analogues raises concerns that they could activate NOS independent of arginase inhibition. Indeed, it was for this reason that our central observation was repeated with each of these inhibitors. Our data and those of others support the fact that DFMO is an arginase inhibitor.32,33 It is also an inhibitor of ornithine decarboxylase,11 an enzyme that is critical in polyamine synthesis and inhibition of ornithine decarboxylase, which in theory could increase 1-arginine flux through the urea cycle. Conversely, Nor-NOHA is a potent inhibitor of arginase. In contrast to NOHA, nor-NOHA is neither an intermediate in NO synthesis nor an inhibitor for NOS. It is therefore a useful compound for studying the interplay between arginase and NOS.14 Equally, BEC and the other borono-analog of 1-arginine, 2(S)-amino-6-borono-hexanoic acid (ABH), are highly selective arginase inhibitors that bind as the tetrahedral boronate anion and whose transition state structures differ significantly from those occurring in NO biosynthesis. This explains why they do not inhibit NOS.12,13 The consistency of our findings, both at biochemical and physiological levels, supports our conclusion that arginase competes with NOS and leads to impaired endothelial relaxation.

Limitations

Although both endothelial denudation and sGC inhibition inhibited most of the vasorelaxant responses (>70%) to arginase inhibition, a small component of the response was retained. These observations could be explained on the basis of incomplete mechanical endothelium removal or endothelial-independent mechanisms. Although endothelial denudation is accomplished mechanically and abolishes ∼90% of the cholinergic vasodilator response, it is possible that some endothelium is retained, accounting for the observations. It is more likely that a small endothelial-independent component may be at play. It is well-known that arginase II is found primarily in the mitochondria, where it may modulate mitochondrial reactive oxygen species production and thereby alter vascular tone. The observation that L-NMMA only inhibited DFMO-mediated vasorelaxation by ∼50% can now be easily explained on the basis of emerging literature demonstrating that 1-arginine analogues of NOS are also, not surprisingly, potent inhibitors of arginase.34

In summary, the present findings demonstrate arginase expression in endothelial cells and its role in modulating NOS activity, both biochemically and functionally. With advancing age, expression and function of arginase increases in the vasculature and contributes to endothelial dysfunction. Taken together, our data provide evidence that arginase may be a valuable therapeutic target in the treatment of endothelial dysfunction, especially as it relates to vascular aging.

Acknowledgments

This work was supported by NIH grant R01AG021523 (to Dr Berkowitz), R01HL-65455 (to Dr Hare), a Paul Beeson Physician Faculty Scholars in Aging Research Award (to Dr Hare), and a National Space Biomedical Research Institute Grant MS92-125-2015 (to Drs Berkowitz, Shoukas, Nyhan, and Hare).

References

Arginase Reciprocally Regulates Nitric Oxide Synthase Activity and Contributes to Endothelial Dysfunction in Aging Blood Vessels
Dan E. Berkowitz, Ron White, Dechun Li, Khalid M. Minhas, Amy Cernetich, Soonyul Kim, Sean Burke, Artin A. Shoukas, Daniel Nyhan, Hunter C. Champion and Joshua M. Hare

_Circulation_. 2003;108:2000-2006; originally published online September 29, 2003; doi: 10.1161/01.CIR.000092948.04444.C7

_Circulation_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2003 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/108/16/2000

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in _Circulation_ can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to _Circulation_ is online at:
http://circ.ahajournals.org/subscriptions/