Overexpression of arginase in the aged mouse penis impairs erectile function and decreases eNOS activity: influence of in vivo gene therapy of anti-arginase

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Bivalacqua TJ, Burnett AL, Hellstrom WJ, Champion HC.

Overexpression of arginase in the aged mouse penis impairs erectile function and decreases eNOS activity: influence of in vivo gene therapy of anti-arginase. Am J Physiol Heart Circ Physiol 292: H1340–H1351, 2007. First published October 27, 2006; doi:10.1152/ajpheart.00121.2005.—Since both increased nitric oxide (NO) synthase (NOS) abundance and diminished NO signaling have been reported in the aging penis, the role of NO in the adaptations of aging remains controversial. Here we tested the hypothesis that arginase, an enzyme that competes with NOS for the substrate l-arginine, contributes to erectile dysfunction with advanced age in the B6/129 mouse strain. Arginase protein abundance, mRNA expression, and enzyme activity were elevated in aged compared with young penile endothelial cells. In addition, endothelial NOS (NOS3) protein abundance was greater in aged versus young penile endothelial cells, whereas NO activity and cGMP levels were reduced. Calcium-dependent l-arginine-to-l-citrulline conversion and cGMP formation increased significantly in aged mouse penes in the presence of the arginase inhibitor 2(S)-amino-6-boronohexanoic acid (ABH). However, there was no effect on l-arginine-to-l-citrulline conversion or cGMP accumulation in the endothelium from young mouse penes. To assess the functional role of arginase in the inhibition of NO pathway responsiveness in the penis, we evaluated the effects of ABH and an adeno-associated virus encoding an antisense sequence to arginase I (AAVanti-arginase) on erectile function in vivo. ABH and AAVanti-arginase enhanced endothelium-dependent erectile responses in the aged mice without altering endothelium-independent responses. Paralleling our in vitro observations, ABH or AAVanti-arginase did not affect vascular responses in the young mice. Inhibition of the arginase pathway improves endothelial function in the aging penile circulation, suggesting that the arginase pathway may be exploited to improve erectile dysfunction associated with aging.

Erectile dysfunction (ED) is defined as the inability to achieve and/or maintain penile erection sufficient to permit satisfactory sexual intercourse. Pathological alteration in the anatomy of the penile vasculature or impairment of any combination of neurovascular processes can result in ED (1, 8). Although a number of pathophysiological processes are recognized to lead to abnormal function and responsiveness of the penile vascular bed, the natural aging process is also associated with ED with elevated vasconstrictor tone and decreased endothelium- and neurogenic-mediated relaxation of the corpus cavernosum (1, 8, 23, 25, 33, 37). Aging-associated ED involves aberrancies at multiple levels of the NO/cGMP cascade in the penis, decreased constitutive NOS activity, impaired endothelial-dependent smooth muscle relaxation, and diminished NO bioavailability (9, 15, 16, 24).

It has been shown that dietary l-arginine supplementation as well as acute infusion of l-arginine results in improved NO release and enhanced endothelium-dependent vasodilation in the penis (26, 38). The basis for which l-arginine supplementation can improve endothelial function and NO release remains controversial. It has been reported in animal models of penile vascular aging that constitutive NOS expression is reduced (8). Alternatively, it has been reported that NOS3 expression is upregulated with advanced age in the penis and in the peripheral vasculature but that constitutive NOS activity is idealized to lead to abnormal function and responsiveness of the corpus cavernosum (1, 8, 23, 25, 33, 37). Aging-associated ED involves aberrancies at multiple levels of the NO/cGMP cascade in the penis, decreased constitutive NOS activity, impaired endothelial-dependent smooth muscle relaxation, and diminished NO bioavailability (9, 15, 16, 24).

Arginase is a metalloenzyme that converts l-arginine to urea and l-ornithine in a number of cell types. In endothelial cells, l-arginine is used as a substrate by both NOS3 and arginase. Because both NOS and arginase use l-arginine as a common substrate, arginase may downregulate NO biosynthesis by competing with NOS for l-arginine. Thus NO production may be linked to the regulation of arginase activity (39). Arginase exists in two isoforms: the hepatic type, arginase I; and the extrahepatic type, arginase II. Although arginase I is traditionally thought to exist exclusively in the liver, it has recently been reported that significant expression of arginase exists in vascular endothelium and smooth muscle cells (5, 34, 52). Recently, both arginase isoforms have been shown to exist in human corpus cavernosum, and inhibition of this enzyme resulted in facilitation of corporal smooth muscle relaxation (7,
Additionaly, arginase activity was shown to be increased in human diabetic corpus cavernosum (7). Since arginase activity is upregulated in diabetes, a condition known to be associated with reduced endothelial-derived NO, this may be interpreted to suggest that the diminished erectile response to decreased NO production found in men with diabetes may be due to a combination of increased expression of arginase and decreased amounts of NOS nerve fibers. This is a similar vascular phenomenon that occurs in the aged penile vascular bed.

Therefore, we hypothesized that impaired endothelial-derived NO bioactivity or signaling by increased expression of arginase contributes to the reduced endothelium-dependent responses in the penis of aging mice via the scavenging of intracellular l-arginine. To test this hypothesis, we established that the aging B6/129 mouse ages in a manner consistent with aging rats and humans with a reduction in erectile responses to cavernous nerve stimulation and endothelium-dependent erectile responses. We then measured the arginase and NOS3 concentrations and activity in corporal endothelial cells isolated from penes of B6/129 mice. Moreover, we established that the pharmacological and genetic inhibition of arginase in the mouse penis abrogates the reduction of neurogenic- and endothelium-dependent erectile responses in the aged mouse. These results demonstrate that arginase signaling is substantially elevated in penile vascular endothelium of the aged mouse, which results in significant reductions in NOS3 and ED.

MATERIALS AND METHODS

Animal Model

B6/129 hybrid mice were used in the present study. For initial studies on characterization of the aging-associated reduced erectile responses in the mouse strain, mice ranging from 4 to 26 mo were used (n = 35). For grouped comparisons between young and aged mice, we studied aged animals (n = 87), defined as 22 to 26 mo old, the age at which there is ~45% mortality of the colony, and young adults (n = 42), 4 to 8 mo old, that have reached sexual maturity.

Isolation of Murine Penile Endothelial Cells

Magnetic cell sorting of labeled endothelial cells was performed as previously described by using a miniMACS separation unit (Cat. No. 421-01, Miltenyi Biotec, Bisley, Surrey, UK), and confirmation of the vascular endothelial cell population was performed by flow cytometry (18). Briefly, penes were excised and rinsed with PBS to remove blood and diced in a petri dish using sterile crossed scalpels. The resulting pieces were washed twice in PBS, subjected to low-speed centrifugation (210 g, 1 min) and incubated in a solution of collag- enase (0.5 mg/ml) for 1 h at 37°C in a humid incubator. Following incubation, the slurry was passed through a cell strainer to remove undigested blocks, washed twice in PBS supplemented with 2.5% FCS, and incubated for a further 10 min in trypsin-EDTA solution to obtain single cell suspension. The suspension was further incubated for 30 min at 4°C with murine immunoglobulins to block Fc receptors, followed by being washed twice in cold PBS supplemented with 2.5% FCS. The suspension was then incubated for 30 min at 4°C with rat anti-mouse CD31 and subsequently washed twice in cold PBS supplemented with 0.5% FCS. The cells were counted and then incubated with PBS 0.5% FCS (200 µL, 2.5 x 107 cells), rat anti-mouse Ig (25 µg/ml, 2.5 x 107 cells)- and streptavidin-conjugated microbeads (25 µg/ml, 2.5 x 107 cells) for 15 min at 4°C (total volume, 250 µl). Columns were then loaded onto the separation unit (one column for every 1–2.5 x 107 cells) and washed with 500 µL PBS 0.5% FCS as per manufacturer’s instructions, followed by the loading of each column with 250-µL cell suspension. The magnetically labeled cells are retained in the column(s), whereas unlabeled cells pass through. The columns were then unloaded from the magnet, and the magnetically retained cells were eluted with PBS 0.5% FCS. Labeled cells were incubated with MACS magnetic goat anti-mouse IgG (H+L) (Miltenyi Biotec) MicroBeads and streptavidin (Cat. No. 481-01, Miltenyi Biotec). MicroBeads were then separated by using a high-gradient magnetic separation column 1 (MS columns, Cat. No. 422-01, Miltenyi Biotec).

Adeno-Associated Viral Vectors

A plasmid encoding an antisense sequence for arginase I linked to a cytomegalovirus (CMV) promoter was designed as described previously (5, 50). Adeno-associated viruses (AAVs) encoding β-galactosidase (AAVβgal) and antisense for arginase I (AAVanti-arginase), both driven by a CMV promoter, were prepared according to standard procedures. The arginase I oligonucleotide antisense sequence used in the present study was 5'-ATGTTGCGCATTCACAGTCAC-3' (50). Briefly, fragments of anti-arginase and β-galactosidase, containing the open reading frame sequence, were cleaved and subcloned at the corresponding sites in the plasmid. The insert was cut at EcoRI sites and cloned into corresponding sites in recombinant AAV backbone containing the CMV promoter and the bovine growth hormone polyan- denylation signal flanked by the AAV-inverted terminal repeat sequences. Packaging, propagation, and purification of AAV viral particles were carried out by standard procedures (47).

Localization and Measurement of Penile Endothelial Arginase

Quantitative PCR. Real-time quantitative PCR was used to determine relative expression of arginase I mRNA in whole mouse penes and penile endothelial cells isolated from young and aged mouse penes. 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 as previously described (5). To amplify specific gene products, the following intron-spanning primers were used: 1) arginase I: sense, 5'-CAAGCTGGGGAATTTGCGAAAG-3'; antisense, 5'-GGTTGACGTCATGCACTATCAAAATCCC-3'; and 2) NOS3: sense, 5'-AAGACCAAGCGCAGTGGGAA-3'; antisense, 5'-GCAGCGCAAGCAGAAATAGTT-3'. Mouse GAPDH was coamplified as an internal control, using primer sequences: GAPDH: sense, 5'-CATCACCATTCTTCCAGGAGG-3'; antisense, 5'-GAGGGCCCATCCACGTCTCTC-3'. Standard curves were performed with GAPDH to ensure appropriate RNA loading per sample.

Western blot analysis. Penile vascular endothelial cells were pelleted by centrifugation and then resuspended in a hypotonic buffer containing 50 mM Tris·HCl (pH 7.4), 0.1 mM EGTA, 2 mM β-mercaptoethanol, and protease inhibitors (1 µg/ml leupeptin, 1 µg/ml pepstatin, and 1 mM PMSF). After centrifugation twice at 15,000 g for 4°C for 20 min, protein concentration was determined by using a Bio-Rad DC protein assay (Bio-Rad, Hercules, CA), and the 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, Ghent, Belgium) by semidy electroblotting for 1 h. The membranes were blocked 1 h at room temperature with blooto-Tween (5% nonfat dry milk, 0.1% Tween-20) and incubated with a primary mouse monoclonal arginase I IgG antibody (1:500) and primary rabbit monoclonal NOS3 IgG (1:250; Transduction Laboratories, Lexington, KY). Bound antibody was detected with labeled anti-mouse IgG secondary antibody (1:20,000; AJP-Heart Circ Physiol • VOL 292 • MARCH 2007 • www.ajpheart.org)

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In vivo Erectile Responses in the Mouse

In vivo erectile function to cavernous nerve stimulation (CNS) and intracavernous injection of vasoactive agents were studied in the young and aged anesthetized mice. Induction of anesthesia was achieved by placing the animal in a jar containing gauze soaked with isoflurane. The mice were then intubated and placed on a thermoregulated surgical table. The animals were ventilated with 95% O2-5% CO2 and 2% isoflurane using a custom-designed, constant-flow mouse ventilator with tidal volume set to 6.7 μl/g at 140 breaths/min. A carotid artery was cannulated for measurement of mean systemic arterial pressure (MAP), which was measured continuously with a Viggo-Spectramed transducer (Viggo-Spectramed, Oxnard, CA) attached to a data acquisition system (Biopac, Santa Barbara, CA). Heart rate was determined from the systolic pressure pulses with a tachometer (Biopac).

The shaft of the penis was freed of skin and fascia, and the removal of part of the overlying ischiocavernous muscle exposure of the right crus was performed. A 27-gauge needle filled with 250 U/ml of heparin and connected to polyethylene-50 tubing was inserted into the right crura and connected to a pressure transducer to permit continuous measurement of intracavernosal pressure (ICP). The bladder and prostate were exposed through a midline abdominal incision. The right major pelvic ganglion and cavernous nerve were identified posterolateral to the prostate on one side, and an electrical stimulator with a stainless steel bipolar hook was placed around the cavernosal nerve. MAP and ICP were measured with a pressure transducer connected to a data acquisition system (Biopac) for continuous measurement of MAP and ICP pressures. The cavernous nerve was stimulated with a square-pulse stimulator (Grass Instruments, Quincy, MA). Each mouse underwent CNS at a frequency of 15 Hz and pulse width of 30 μs. The application of 1–8 V was used in the current protocol to achieve a significant and consistent erectile response. The duration of stimulation was ∼1 min with rest periods of 2 to 3 min between subsequent stimulations. This procedure has been previously described (19, 45). Johns Hopkins University Animal Care and Use Committees have approved all procedures used in the present study.

In experiments in which ACh (Sigma), an endothelium-dependent vasodilator, and CGRP (Phoenix Pharmaceuticals), an endothelium-independent vasodilator, were utilized, intracavernosal administration via a 30-gauge needle inserted into the left corpus cavernosum was performed, as described previously (19). In all experiments, injections of agonists were made when intracavernosal pressure was at baseline value. The erectile effects of a single injection of ACh (13 and 44 nmol) and CGRP (0.01 and 0.03 nmol) on intracavernosal pressure were monitored until intracavernosal pressure returned to preinjection level. The next injection was made after a 10- to 15-min period from the end of the preceding response to ensure a stable baseline. In experiments in which ABH was injected, all erectile responses were evaluated 20 min after injection of ABH. There was only a transient increase in intracavernosal pressure after injection of ABH (∼8–10 min), which returned to baseline before cavernous nerve stimulation or intracavernous injection of pharmacological agents. These methods have been previously described (19).

Drugs

ABH was synthesized as previously described (5). Preliminary experiments were performed to determine the final concentrations and doses used in the present study based on selective inhibition of arginase in the mouse. These concentrations of ABH inhibited recombinant arginase I but failed to inhibit recombinant NOS3 activity in an assay in vitro. ACh bromide (Sigma Chemical) and CGRP (Phoenix Pharmaceuticals) were dissolved in 0.9% NaCl. The solvents for the agonists used in this study did not alter vascular intracavernosal pressures or responses to the agonists. The stock solutions were stored in a freezer in 1-ml opaque tubes, and working solutions were prepared daily and kept on crushed ice during the course of the experiment.
Statistical Analysis

Data are presented as means ± SE. Comparisons between baseline variables in ABH- and vehicle-treated penes were performed by using paired or unpaired t-tests, as appropriate. Comparisons between groups were by using ANOVA with repeated-measures and Newman-Keuls post hoc test for multiple group comparisons. Statistical calculations were performed using Systat software.

RESULTS

Influence of Age on Erectile Responses in the Mouse

The influence of age on erectile responses to CNS and to intracavernosal injection of the endothelium-dependent vasodilator ACh and the endothelium-independent vasodilator CGRP were studied in B6/129 mice, and these data are summarized in Fig. 1. There was an age-related decrease in erectile responses to CNS and intracavernosal injections of ACh with a strong positive correlation ($r^2 = 0.8376$ for CNS, and $r^2 = 0.6832$ for ACh). In contrast, there was no significant correlation between age and responses to intracavernosal injection of the endothelium-independent vasodilator CGRP (Fig. 1). Although the maximal response to ICP was not different in young and aged mice in response to CGRP, the area under the curve was lower in the aged group ($P < 0.05$; data not shown).

The influence of L-arginine supplementation on erectile responses to CNS and ACh injection was studied in this aging model to validate this animal model. Dietary supplementation of L-arginine via a high L-arginine chow for 2 wk resulted in increased erectile responses to CNS and intracavernosal injection.
of ACh compared with aged mice fed regular chow (Fig. 2). L-Arginine supplementation did not alter erectile responses to CNS or ACh injection in young mice (data not shown).

**Arginase Expression and Activity: Biochemical Assessment**

Mouse penile endothelial cells were isolated as described (18). The purity of the penile endothelial cell population was confirmed by flow cytometry (data not shown) as well as by Western blot analysis, confirming the presence of caveolin 1, platelet endothelial cell adhesion molecule (PECAM; CD31), and the absence of α-smooth muscle actin (Fig. 3A). Cell lysate from mouse pulmonary vascular endothelial cells from primary culture served as a positive control for endothelial cells. In addition, the expression of arginase I and NOS3 was evaluated in this endothelial cell lysate. Arginase I mRNA expression and protein abundance were ~60% higher in penile vascular endothelial cells from aged mice compared with those in young adult mice, and these data are summarized in Fig. 3B. Moreover, the mRNA and protein concentrations of NOS3 were significantly higher in penile endothelial cells from aged mice compared with those in young mice (Fig. 3B).

The higher concentration of arginase protein was associated with higher enzyme activity in aged mouse penile endothelial cells compared with endothelial cells isolated from young mouse penes (Fig. 4A). In some cases, additional immunostaining was seen below the 38-kDa location of arginase I. Pretreatment of the membrane with a blocking peptide for the monoclonal arginase I antibody resulted in a loss of all staining, suggesting that the additional immunostaining is likely secondary to degradation products of arginase I (data not shown). In

Fig. 3. A: Western blot analysis of the endothelium-selective protein caveolin-1 (Cav1) and endothelium-specific protein platelet endothelial cell (EC) adhesion molecule (PECAM; CD31) in isolated mouse pulmonary vascular ECs and isolated penile ECs. Western blot analysis for α-smooth muscle actin (α-sm-actin) confirmed no expression of this smooth muscle selective protein in isolated penile ECs. Positive control (+C) is mouse vascular smooth muscle cell extract. B: Western blot analysis demonstrating expression of arginase I (Arg I; left) and nitric oxide synthase 3 (NOS3; right) protein in isolated penile ECs from young (lanes 1 and 2) and aged (lanes 3 and 4) mice penes. Bar graph demonstrates the densitometry analysis of Arg I and NOS3 protein normalized as the gel units of Arg I/GAPDH and NOS3/GAPDH in young and aged mice penile ECs (n = 7 animals/group). Additional bar graph demonstrates quantitative PCR for Arg I and NOS3 mRNA (in pg/sample) in young and aged mice penile ECs (n = 7 animals/group). RNA samples were normalized for GAPDH RNA expression to confirm RNA loading. *P < 0.05, significantly different than young mice.
The effect of in vitro administration of ABH (1–30 \mu M) on cGMP accumulation in mouse penile homogenate is shown in Fig. 4C. Baseline cGMP concentrations in penes from aged mice were significantly lower (952 ± 68 fmol/mg protein) compared with those in young mice (2,008 ± 174 fmol/mg protein; \( P < 0.05 \)). In both young and aged mouse penes, ABH produced significant concentration-dependent increases in cGMP concentrations (Fig. 4C). The magnitude of the increase in cGMP in response to ABH was significantly higher in the penes from aged mice compared with young mice (\( P < 0.05; \) Fig. 4C).

In Vivo Physiological Erectile Response to ABH in the Mouse

The effect of the arginase inhibitor ABH (18.2 nmol/kg ic) on baseline erectile function was evaluated in young and aged mice, and these data are summarized in Fig. 5. Intracavernosal injection of ABH resulted in a small, but significant, increase in intracavernosal pressure in aged mice (3.9 ± 0.2 mmHg) compared with vehicle in the aged mice (0.2 ± 0.1 mmHg; \( P < 0.05 \)). Intracavernosal pressure was not altered in response to intracavernosal injection of ABH in the young mice (data not shown). In aged mice, intracavernosal pressure returned to baseline pressures over the course of 8–10 min and erectile responses were evaluated 20 min after injection of ABH. The transient increase in intracavernosal pressure after administration of ABH did not alter the ratio of ICP to MAP (\( P > 0.05 \)). In aged mice treated with ABH, erectile responses to CNS were significantly increased at all voltages studied (Fig. 5A, left). In contrast to the increase in erectile responses to CNS observed in the presence of ABH in the aged mice, responses to CNS were not altered in young mice when compared before and after injection of ABH (18.2 nmol/kg ic; Fig. 5A, right). In addition to intracavernosal pressures, MAP were measured in young (102 ± 4.7 mmHg) and aged (112 ± 4.6 mmHg; \( P = 0.047 \)) mice, and there was a statistical increase in baseline MAP in aged mice. Whereas there was a statistically significant difference in MAP in young and aged mice, the ICP-to-MAP ratio was not statistically different at baseline (\( P = 0.068 \)). Moreover, intracavernosal injection of ABH resulted in a nonsignificant reduction in MAP in young (101.9 ± 5.1 mmHg; \( P > 0.05 \)) or aged (108 ± 4.9 mmHg; \( P > 0.05 \)) mice.

Erectile responses to the endothelium-dependent vasodilator ACh and the endothelium-independent vasodilator CGRP were evaluated in aged and young mice before and after injection of the arginase inhibitor ABH (18.2 nmol/kg ic; Fig. 5, B and C). Erectile responses to ACh and CGRP were determined ~20 min after intracavernous injection of ABH. Responses to ACh were enhanced in aged mice (Fig. 5B, left) treated with ABH, whereas there was no difference in erectile responses to ACh in young mice (Fig. 5B, right). In contrast to the enhanced responses after treatment with ABH, erectile responses to the endothelium-independent vasodilator CGRP were unaltered by ABH in young or aged mice (Fig. 5C).

In Vivo Gene Transfer of Anti-Arginase on Erectile Function in the Mouse

To further evaluate the role of arginase I on age-associated erectile function, the influence of gene transfer of an antisense sequence for arginase I was studied, and these data are sum-

Fig. 4. Effect of aging on arginase and endothelial NOS activity as well as penile cGMP levels. A: arginase enzyme activity as determined by the conversion of \( L \)-arginine to \( L \)-ornithine in young and aged (old) EC lysates in the absence and presence of the arginase inhibitor \( 2(5) \)-amino-6-boronoheptanic acid (ABH, 5 \mu M; \(* P < 0.05 \) compared with young; \( ** P < 0.05 \) compared with aged). B: endothelial NOS activity as determined by the conversion of \( L \)-arginine to \( L \)-citrulline in the absence and presence of the arginase inhibitor ABH (5 \mu M) in EC lysates (*\( P < 0.05 \) compared with young; **\( P < 0.05 \) compared with aged). C: concentration-dependent influence of the arginase inhibitor ABH (1–30 \mu M) on cGMP concentrations in young and aged mice penes (*\( P < 0.05 \) compared with baseline; **\( P < 0.05 \) compared with young), \( n \), Number of experiments.
marized in Fig. 6. One month after injection of an AAV vector encoding an antisense sequence for arginase I (AAVanti-arginase), mRNA and protein expression of arginase I in penile homogenates were significantly lower compared with those in vehicle-treated aged mice (Fig. 6A). Arginase I protein and mRNA expression were significantly higher in aged mice penes treated with vehicle or AAVβgal (Fig. 6A). Treatment of aged mice with AAVβgal did not significantly alter arginase I protein expression (Fig. 6A, right) or mRNA expression and arginase activity compared with treatment of aged mice with vehicle (data not shown). This increase in arginase I was associated with a significant decrease in constitutive NOS activity, the conversion of L-arginine to L-citrulline in the presence of calcium, and cGMP levels in aged mice penes treated with vehicle (Fig. 6B and C) or AAVβgal. Constitutive NOS activity was significantly increased in the penes of aged mice after AAVanti-arginase transfection compared with vehicle-treated (Fig. 6B) and AAVβgal-transfected aged mice (data not shown). cGMP concentrations in penes of aged mice transfected with AAVanti-arginase were significantly higher compared with those in vehicle-treated (Fig. 6C) or AAVβgal-transfected aged mice (data not shown).

Erectile responses to CNS and vasoactive agents were compared in young and aged mice 1 mo after transfection with AAVβgal and AAVanti-arginase. One month after transfection with AAVanti-arginase, erectile responses to CNS were significantly improved compared with mice treated with AAVβgal (a gene product that has no measurable effect on erectile function; Fig. 7A). In results that paralleled the acute studies with ABH, responses to ACh were improved in AAVanti-arginase-treated mice (Fig. 7B), whereas responses to CGRP (data not shown) were not different from AAVβgal-treated mice.

DISCUSSION

The results of the present study demonstrate for the first time a mouse model of age-related ED and that arginase is upregulated in endothelial cells isolated from the penis of aged mice where it directly influences and decreases eNOS activity. Moreover, these data are the first to identify a physiological role for arginase in modulating erectile function in vivo by reducing eNOS activity, thus decreasing penile cGMP levels. Additionally, this study demonstrates that the pharmacological inhibitor of arginase, ABH, and AAV gene transfer of anti-arginase to the aged mouse penis decrease arginase I protein and mRNA and restore endothelial and erectile function in vivo as a direct result of an increase in penile constitutive NOS.
activity and elevated penile cGMP levels. Furthermore, these data provide pharmacological and gene transfer evidence for a role of the arginase system in mediating at least a portion of age-related endothelial and erectile dysfunction in the penile vascular bed. These findings implicate that arginase may influence erectile function through attenuation of endothelial-derived NO in the penis.

The decline in erectile function that occurs with aging can be attributed to decreased NOS expression/activity, phosphorylation, and bioavailability of NO in the penile vasculature (8, 9, 40, 41, 46, 49). Overexpression of eNOS by adeno viral gene transfer to the penis of aged and diabetic rats restores endothelial and erectile function, suggesting that decreased production or bioavailability of NO from the endothelium plays a significant role in mediating penile vascular dysfunction (6, 10–12, 17). In the present study, we have established a mouse model of age-associated ED. The B6/129 strain of mouse has significant impairments in endothelial and erectile function as determined by CNS and endothelium-dependent vasodilator responses to ACh with advancing age. These in vivo physiological parameters parallel a significant reduction in penile endothelial NOS activity and cGMP concentrations. These defining characteristics support the use of this aging mouse model for the study of age-associated penile vascular dysfunction.

In the present study we found a significant increase in eNOS protein expression in isolated penile endothelial cells from aged mice, which is consistent with previous reports in aged rat penes (4). Bakircioglu and colleagues (4) have shown that corporal eNOS protein expression is significantly upregulated in the aging rat penis due to a reduction in caveolin-1 protein in the penises (4). Caveolin-1 tightly regulates eNOS in the
vascular endothelium. Therefore, it is possible that the upregulation of eNOS protein expression in mouse penile endothelial cells is a result of reduced caveolin-1 expression. Although eNOS protein expression is upregulated in the aged mouse endothelium, there is significant decline in eNOS activity, suggesting that the increased protein is not biologically active. Data to support this hypothesis are drawn from the effect of AAV gene transfer of anti-arginase on improving eNOS activity after inhibition of arginase and thus restoring endothelial vascular responses in the aging mouse penis. This biochemical process occurs by increasing the amounts of eNOS enzyme available for synthesis of NO, thus allowing more eNOS to be available for l-arginine. Additionally, adenoviral gene transfer of eNOS to the aging rat penis improves erectile function via increases in corporal eNOS activity and improved cGMP levels in the penis by increasing endothelium-derived NO synthesis (12).

Endothelial dysfunction has been shown to be a major mechanism that contributes to ED (31, 42). Activation of NOS3 plays an important role in the relaxation of corporal smooth muscle to endogenous agonists such as ACh and shear stress (14, 28, 40, 41). Because impaired endothelium-dependent corporal smooth muscle relaxation in vitro and erectile function in vivo are restored by supplementation with l-arginine, this suggests that a reduction of l-arginine availability for NOS3 may be involved (26, 38, 44). Therefore, we postulated that arginase, if expressed in endothelial cells of the corpus cavernosum, might play a significant role in the regulation of NO-mediated vasodilation in vivo by reducing l-arginine availability to NOS3 in the aging penile vascular bed. Arginase is responsible for the hydrolysis of l-arginine to ornithine and urea, thus playing a critical role in urea synthesis from ammonia in the urea cycle. However, l-arginine is a common substrate for both arginase and NOS, and thus arginase plays a significant role in regulating NOS and NO synthesis by modulating l-arginine bioavailability. Recently, both arginase isoforms have been identified in rabbit and human corpus cavernosum, and the arginase inhibitor ABH enhanced the nonadrenergic noncholinergic corporal smooth muscle relaxation, suggesting that arginase inhibition may enhance the substrate pool of l-arginine that is available for NOS (7, 22, 32). Whereas inhibition of arginase activity has been shown to enhance NO production by NOS2, in macrophages there is a relative paucity of information about the role of arginase in regulating the function of NOS3 in the aging penile vascular bed (20, 44, 48). Recently, a role for arginase in the aging rabbit penis in vitro has been elucidated (44). In this study, endothelium-dependent corporal smooth muscle relaxation in vitro was improved after pretreatment with a selective arginase inhibitor (44). However, in vivo erectile responses were not determined.

Data from the present study show arginase I mRNA and protein are constitutively expressed in the mouse penile endothelium with an age-associated increase in the B6/129 mouse penis. Arginase activity in penile endothelial homogenates was present and could be suppressed with the arginase inhibitor ABH. These data suggest that arginase expression is not limited to the liver and macrophages and imply that extrahepatic expression of arginase exists in the penile endothelium. Moreover, enzyme activity studies in the penile endothelial cells suggest that endothelial arginase is active as evidenced by the ability to convert l-arginine to urea and l-ornithine. Whereas there is constitutive expression of arginase in young mouse penile endothelial cells and the arginase inhibitor ABH in vitro can inhibit this arginase activity, arginase inhibition had no significant effect on erectile responses to CNS or intracavernous ACh in the young mouse. Moreover, the inhibition of arginase by ABH did not alter the calcium-dependent conversion of l-arginine to l-citrulline in the young penile endothelial homogenates. The reason for the lack of effect of ABH on the calcium-dependent conversion of l-arginine to l-citrulline in young mouse endothelial cells is uncertain. It is possible that there is a specific concentration of...
arginase is required to have an effect on the ability of NOS to convert l-arginine to l-citrulline in the presence of calcium and that the concentration of arginase is not sufficient in the young mouse endothelial cells. However, in aged mouse endothelial cells, the overexpression of arginase may reach this threshold to influence NOS3 and constitutive NOS activity. These findings are consistent to what was observed in isolated corporal strips from young and aged rabbits (44). There may be a constitutive role for arginase in the young mouse penis other than influencing corporal smooth muscle reactivity, such as the control of corporal smooth muscle proliferation or other signaling pathways yet undetermined (30). The concentration of ABH required to selectively inhibit arginase activity was lower than in our previous report in the diabetic human corpus cavernosum (7). The reasons for the differences are uncertain but may reflect the differences in species or in the magnitude of the increase in arginase expression/activity in the aging process compared with that in diabetes. The doses chosen for the present study were based on preliminary experiments to determine a concentration range of ABH that would effectively inhibit arginase but not NOS3 in experiments in which NOS3 protein was purified by immunoprecipitation.

In the present study, arginase I was the molecular target investigated in young and aged mice penile vasculature in vitro and in vivo. All biochemical and physiological analyses were determined after pharmacological inhibition with ABH, a potent nonselective arginase inhibitor with higher affinity for arginase II (3). It is possible that some of the physiological effects observed after ABH therapy may be in part due to inhibition of both isoforms; however, when arginase I was selectively inhibited using the AAV encoding for anti-arginase I, similar physiological effects in vivo and significant reductions in arginase activity and improvements in eNOS activity were observed, suggesting that selectively inhibiting one isoform of arginase (arginase I) can improve neurogenic- and endothelium-dependent erectile responses via improvements in endothelial-derived NO/cGMP signaling. The role of arginase II in the pathophysiology of age-associated ED is uncertain and warrants further investigation.

To further establish a link between arginase, NOS, and NO/cGMP interactions, we measured the effect of arginase inhibition on cyclic nucleotide (cGMP) production in the mouse penis. Inhibition of arginase resulted in increases in cGMP concentrations in penile homogenates from aged mice, supporting the hypothesis that inhibiting arginase results in increased NOS activity, enhanced NO production, and elevation of cGMP concentrations. The observation that ABH increased cGMP concentrations in the young mouse penile homogenates suggests that the assay for studying cGMP may be more sensitive than the l-arginine-to-l-citrulline assay in measuring small changes in arginase activity/concentrations. Alternatively, it is possible that ABH, by an unknown mechanism, increases cGMP in a direct manner. Regardless of the mechanism by which ABH increases cGMP in young mouse penes, there was no measurable change in physiological erectile responses.

It has previously been shown that chronic administration of L-arginine enhances erectile function in models of age-associated ED (38, 44). Additionally, oral administration of L-arginine in high doses caused significant subjective improvement in sexual function in men with organic ED (21). The mechanism by which L-arginine enhances NO signaling despite apparently adequate intracellular levels of this substrate remains controversial but has been widely documented in the setting of endothelial reactivity. It has been suggested that although total intracellular L-arginine content is relatively high, the pool available to NOS may be reduced or compartmentalized within the cell and sequestered away from NOS in that it would limit the function of NOS (2, 34). Moreover, intracellular arginase can be methylated, thus rendering it useless to NOS. When taken together, these data suggest that intracellular L-arginine may be tightly controlled by a number of factors. These findings may suggest that by reducing the available supply of free L-arginine in the endothelial cells of the penis, NOS activity can be reduced. By inhibiting arginase, we hypothesize that the concentration of L-arginine that is available for use by NOS3 is increased, resulting in increased NO release and subsequent increases in penile cGMP levels. The specific contribution of the NO/cGMP signaling pathway to erectile function is further supported by our findings that the arginase inhibitor ABH and AAV gene transfer of anti-arginase to the penis, like L-arginine, improves erectile function to CNS and the endothelium-dependent vasodilator ACh.

In the current study, we did not investigate other potential alterations in L-arginine availability that could lead to decreased NO production. Among the possibilities are changes in L-arginine transport and/or an increase in levels of other endogenous NOS inhibitors with aging, which can be overcome with increased L-arginine (35, 36). Yet, regardless of any other age-associated alterations in precursor availability/metabolism or downstream NO inactivation, arginase inhibition, like L-arginine administration, appears to overcome these secondary mechanisms. Thus the response to arginase inhibition may have important therapeutic implications with regard to erectile function in aging or other disease states (diabetic and vasculogenic ED).

It has been suggested that the mechanisms associated with the impairment of endothelium-dependent erectile responses with advancing age may be multifactorial. Thus the inability of arginase inhibition either pharmacologically or genetically to fully restore normal erectile function could be related to additional mechanisms besides increased arginase activity. Potential mechanisms that may be intimately related to impaired endothelial and erectile function postulated are 1) increased levels of reactive oxygen species, in particular, superoxide anion, in the aging penis could directly inactivate NO; 2) downregulation of NOS1 in the cavernous nerves innervating the penis; and 3) diminished levels of NOS3 cofactors and downregulation of eNOS activity (8, 9, 15, 46, 51). Although these mechanisms could contribute to the impairment of endothelium-derived NO-mediated dilation in the penis of the aged mouse, the predominant mechanism in the present model appears to be related to the upregulation of arginase. Previous reports in the literature vary on the effect of age on penile constitutive NOS activity (8, 24, 46). In the present study, endothelial cells isolated from aging mouse penes have a significant decline in eNOS activity. Others have shown that increasing age reduces constitutive NOS activity in the penis (46). However, this is not uniformly constant, depending on the age of the experimental animals (8, 24, 46). Results of the present study demonstrate that isolated endothelial cells from aging mice have reduced eNOS activity, whereas this differ-
ence from previous reports shows that the disparity may be a result of species difference and/or cell type used (pure endothelial cells) versus whole penes (24).

In conclusion, the results of the present study provide the first evidence, at both the molecular and functional level, for a biological role of arginase in regulating erectile function in the aging penis vascular bed. With advancing age, expression and function of arginase increase in the penis vasculature and contribute to endothelial and erectile dysfunction. Penile endothelial cells isolated from the aged mouse penis overexpress arginase and, as a result, decreased NOS3 activity and impaired vascular function. Inhibition of arginase via AAV gene transfer of anti-arginase in the aged mouse penis enhanced penile eNOS activity and cGMP levels, thus restoring endothelial-derived NO vasodilation and erectile function. Taken together, our data demonstrate that arginase is an endogenous competitor of NOS for their common substrate L-arginine in the penis, and, consequently, an aged-related upregulation of arginase may compromise NO-mediated vasodilation in the penis via a reduction in substrate availability. Therefore, arginase may represent a novel molecular therapeutic target for the treatment of age-associated ED (vasogenic ED).

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