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Chronic Oral Administration of the Arginase Inhibitor 2(*S*)-amino-6-boronohexanoic Acid (ABH) Improves Erectile Function in Aged Rats

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ABSTRACT: Arginase expression and activity have been noted to be heightened in conditions associated with erectile dysfunction, including aging. Previously, arginase inhibition by chronic administration of the arginase inhibitor 2-(*S*)-amino-6-boronohexanoic acid (ABH) has been shown to improve endothelial dysfunction in aged rats. The objective of this study was to assess whether chronic oral ABH administration affects cavernosal erectile function. Rats were divided into 4 groups: young control, young treated with arginase inhibitor, aged control, and aged treated with arginase inhibitor. Arginase activity was measured and presented as a proportion of young untreated rats. In vivo erectile responses to cavernous nerve stimulation were measured in all cohorts. The cavernous nerve was stimulated with a graded electrical stimulus, and the intracavernosal/mean arterial pressure ratios and total intracavernosal pressure were recorded. Arginase activity was elevated in the aged rats compared with young controls; however, arginase activity was significantly decreased in aged rats treated with ABH. With the addition of ABH, erectile responses improved in the aged rats ($P < .05$). Oral inhibition of arginase with ABH results in improved erectile function in aged rats, resulting in erectile hemodynamics similar to young rats. This represents the first documentation of systemic arginase inhibition positively affecting corporal cavernosal function.

Key words: Erectile dysfunction, nitric oxide, penile erection, aging.
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Erectile dysfunction (ED) is a serious health concern for men that has major quality-of-life implications, particularly among aging men (Feldman et al, 1994; Albersen et al, 2012). There are multiple risk factors for ED, including arteriosclerosis, diabetes mellitus, hypertension, hypogonadism, depression, and advanced age (Feldman et al, 1994; Ahn et al, 2007; Teles et al, 2008). Aging itself has been shown to be associated with endothelial dysfunction, which in the absence of other risk factors contributes to declining erectile function (Musicki et al, 2005; Figueiredo et al, 2011).

Penile erection is mediated by the production of nitric oxide (NO), which is synthesized by NO synthase (NOS) from L-arginine (Burnett et al, 1992). Arginase is an enzyme of the urea cycle whose function is the catalysis of L-arginine to form L-ornithine and urea. There are two isoforms: arginase I, found predominantly in the liver, and arginase II, a mitochondrial enzyme (Masuda, 2008), although both have been localized to the vascular endothelium, smooth muscle in general as well as human corpus cavernosum (Cox et al, 1999; Wei et al, 2000; Bivalacqua et al, 2001b; Zhang et al, 2001; Berkowitz et al, 2003). Given that arginine is the common substrate for both NOS and arginase, arginase activity can inhibit endothelium-derived NO production by depleting the available substrate needed for its biosynthesis (Berkowitz et al, 2003; White et al, 2006). Penile arginase expression and activity have been shown to be elevated in a variety of conditions known to be associated with ED, namely, smoking (Imamura et al, 2007), diabetes mellitus (Bivalacaqua et al, 2001b; Jelodar et al, 2007), Peyronie disease (Bivalacqua et al, 2001a), hypertension (Toque et al, 2010), and aging (Bivalacqua et al, 2007; Numao et al, 2007). Furthermore, reduction of arginase activity in the penis via gene knockout or arginase inhibition in vitro and in vivo results in improvement in endothelial cell function and restored erectile responses

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(Bivalacqua et al, 2007; Kim et al, 2009; Toque et al, 2011).

Arginase inhibitors have been developed for the treatment of vascular disorders related to impaired endothelial cell function. 2(*S*)-amino-6-boronohexanoic acid (ABH) is a highly selective competitive inhibitor of arginase (Baggio et al, 1999), and has proven useful for assessing the role of arginase in various organ systems. In addition to its expression in the corpus cavernosum and influence on erectile function, arginase has also been localized to the pancreas (Stickings et al, 2002), spinal cord (Xu et al, 2003), and lungs (Maarsingh et al, 2011). Inhibition of arginase using the selective inhibitor ABH has proven to be therapeutic, at least experimentally, in animal models of diabetes mellitus type 1, autoimmune encephalitis, and chronic asthma, respectively, as well as age-related vascular stiffness (Stickings et al, 2002; Xu et al, 2003; Kim et al, 2009; Maarsingh et al, 2011). Therefore, the aims of our study were to determine whether systemic administration of ABH could restore impaired erectile function in aging rats. Second, we sought to determine whether ABH therapy could significantly reduce arginase activity in the penile vasculature of aged rats.

Materials and Methods

Animals

Old (age ~22–24 months) and young (age 3 months) male Fischer 344 rats were used in the present study. The Fischer 344 strain has been widely used in aging studies and has been shown by Miller et al (2007) to have a progressive rather than an abrupt development of age-related pathophysiology. All of the surgical procedures and experimental protocols were approved by the institutional animal care and use committee.

Chronic Arginase Inhibition

Rats were equally divided into 4 groups: young control, young rats treated with arginase inhibitor, old (aged) control, and old (aged) treated with arginase inhibitor ABH (Baggio et al, 1997; Di Costanzo et al, 2005; Kim et al, 2009). Each rat consumed ~400 μg of ABH in water daily for 25 days. Control groups were given normal tap water. There was no obvious difference in the consumption of water between the treatment and control groups. ABH (K_d = 5 nM against human Arg1; Di Costanzo et al, 2005) was synthesized as previously reported (Baggio et al, 1997; Xu et al, 2003).

Measurement of Erectile Response

To measure the erectile response in vivo, after the 25 days of either ABH treatment or control diet administration, the rats were anesthetized with pentobarbital (30 mg/kg intraperitoneally) and placed on a thermoregulated surgical table. Prior to performing in vivo experiments, there was a 24- to 48-hour washout period of ABH. A carotid artery was cannulated (PE-50 tubing) to measure the mean systemic arterial pressure (MAP). As previously described, a 25-Gauge needle filled with 250 U/mL heparin and connected to PE-50 tubing was inserted into the right crura and connected to a pressure transducer to permit continuous measurement of intracavernosal pressure (ICP; Bivalacqua et al, 2003, 2004). The right major pelvic ganglion and cavernosal nerve (CN) were identified posterolaterally to the prostate on one side, and an electrical stimulator with a stainless-steel bipolar hook was placed around the CN. MAP and ICP were measured with a pressure transducer connected to a data acquisition system (Biopac Systems, Santa Barbara, California). The CN was stimulated with a square-pulse stimulator (Grass Instruments, Quincy, Massachusetts) at a frequency of 15 Hz and pulse width of 30 seconds. The application of 2, 4, 6, and 8 V was used in the present protocol to achieve a significant and consistent erectile response. The duration of stimulation was 1 minute, with rest periods of 2 to 3 minutes between subsequent stimulations. Total erectile response or total ICP was determined by the area under the erectile curve (AUC; mm Hg*s) from the beginning of CN stimulation until the ICP returned to baseline or prestimulation pressures. During stimulation, the peak ICP was registered from the level of the basal ICP. The ratio between the maximum ICP and MAP obtained at the peak of the erectile response was calculated to normalize for variations in systemic blood pressure. The Johns Hopkins Hospital Animal Care and Use Committee approved all procedures used in the present study.

Arginase Activity Assay

Following ICP/MAP measurements, penes were excised and immediately frozen. Arginase activity was measured by determining levels of urea production by colorimetric analysis (QuantiChrom Arginase Assay Kit, BioAssay Systems, Hayward, California) as previously described (White et al, 2006; Kim et al, 2009). Penes were homogenized in extraction buffer (50 mM potassium phosphate buffer, pH 7.0; 1 mM EDTA; 1 mM ethylene glycol tetraacetic acid; 0.2 mM phenylmethanesulfonylfluoride; 1 μg/mL pepstatin; 0.5 μg/mL leupeptin; 10 mM NaF; 2 mM Na_3VO_4; and 10 mM β-mercaptoethanol), and centrifuged for 30 minutes at 14 000 × *g* at 4°C. Protein concentration was determined by the BCA kit (Pierce, Rockford, Illinois), and 40 μg of penile lysates from each sample was used for the measurement of arginase activity. The hydrolysis reaction of L-arginine by arginase was performed by incubating the mixture containing activated arginase and was stopped by adding acid solution. For calorimetric determination of urea, α-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. Arginase activity was expressed as a percentage of the Young activity to account for differences between the activity assays performed.

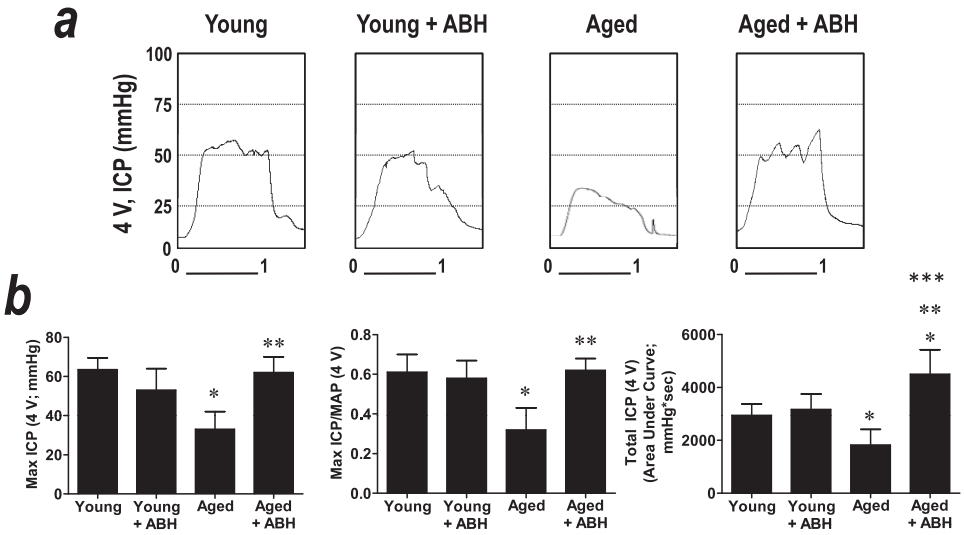


Figure 1. **(a)** Representative intracavernosal (ICP) tracing to cavernous nerve stimulation (CNS; 4 V) in young and aged rats with and without treatment with 2-(*S*)-amino-6-boronohexanoic acid (ABH). **(b)** Maximum ICP, ICP/mean systemic arterial pressure (ICP/MAP), and total ICP to CNS (4V) in all experimental rat cohorts. n indicates number of experiments; *, $P < .05$, response significantly different compared with young; **, $P < .05$, response significantly different compared with aged; ***, $P < .05$, response significantly different compared with young, young + ABH, and aged.

Statistics

Data were expressed as mean ± SEM. Differences between multiple groups were compared by analysis of variance (ANOVA) followed by Newman-Keuls multiple comparisons test. Two-group analysis was performed by *t* test (paired or unpaired as appropriate). Serial studies were tested by repeated measures ANOVA. *P* value of less than .05 was used as criteria for statistical significance.

Results

Chronic Arginase Inhibition Restores Erectile Function in Aged Rats

We have previously shown that aging impairs erectile function in rodent models of ED (Bivalacqua et al, 2007). Therefore, the effect of systemic administration of the arginase inhibitor ABH on in vivo erectile responses to CNS was evaluated in young and aged rats. These data are summarized in Figures 1 and 2. Figure 1 represents maximum ICP, maximum ICP/MAP, and total ICP (AUC) for a 4-V stimulation of the CN with representative ICP tracings for each group. The maximum ICP, ICP/MAP, and total ICP were significantly reduced ($P < .05$) in aged rats compared with erectile responses of young rats at 4 V (Figure 1). With ABH administration, no change was seen in the erectile response of young rats (Figure 1). However, in aged rats treated with ABH, a significant ($P < .05$) increase in the maximum penile hemodynamic parameters was found at 4 V, approaching that seen in young rats for maximum ICP and ICP/MAP (Figure 2b). For the total ICP response at 4 V, aged rats treated with ABH had total erectile responses that were significantly greater than aged and young rats treated with or without ABH (Figure 1b).

To evaluate the effect of ABH inhibition on young and aged rat penile erectile hemodynamics, in vivo erectile response to CNS at increasing voltage settings was studied. These data are summarized in Figure 2. There was a significant ($P < .05$) decline in erectile function in aged rats compared with young rats (Figure 2). The ratio of ICP/MAP and total ICP (AUC; mm Hg*s) after CNS in aged rats were significantly lower ($P < .05$) than that of young animals at all levels of stimulation (Figure 2). In aged rats treated with ABH, the ICP/MAP was significantly increased ($P < .05$) at all voltage settings to values similar to those for young rats (Figure 2). The most dramatic change in erectile hemodynamic indices was evident in the total ICP of rats treated with ABH. There was a significant increase in total ICP in aged rats treated with ABH to values greater than those found in young rats treated with or without ABH (Figure 2). We saw no change in erectile function in young rats treated with ABH (Figure 2). These data demonstrate that severe ED occurring in aged rats is reversed with the selective arginase inhibitor ABH.

There were no differences in baseline ICP or MAP in the groups, with the exception of the young rats treated with ABH. This group showed a small but statistically significant elevation in MAP ($P < .05$; Figure 3).

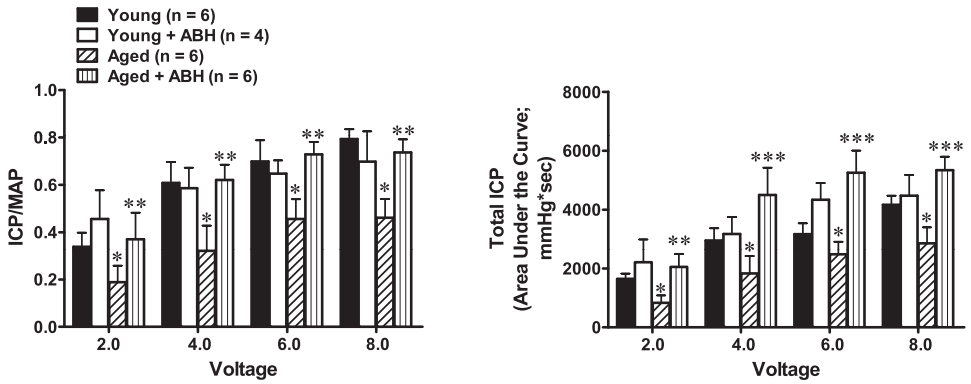
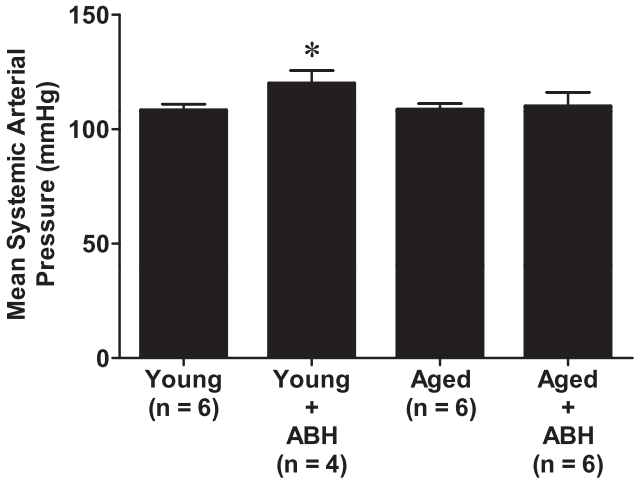


Figure 2. Bar graph depicting the voltage-dependent erectile response as measured by the intracavernosal pressure/mean systemic arterial pressure (ICP/MAP) ratio, and total ICP (area under the erectile curve) after cavernous nerve stimulation for 1 minute in young and aged rats with and without treatment with 2-(*S*)-amino-6-boronohexanoic acid (ABH). n indicates number of experiments; *, $P < .05$, response significantly different compared with young; **, $P < .05$, response significantly different compared with aged; ***, $P < .01$ response significantly different compared with aged.



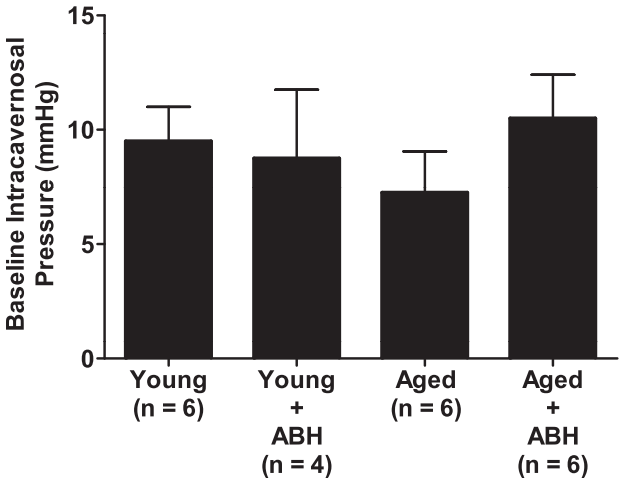


Figure 3. Mean systemic arterial blood pressure and baseline intracavernosal pressure in young and aged anesthetized rats with and without treatment with the arginase inhibitor 2-(*S*)-amino-6-boronohexanoic acid (ABH). n indicates number of tissue samples; *, $P < .05$ when compared with young.

Arginase Activity

Total arginase activity was measured in young and aged penes with and without treatment with ABH, and these data are summarized in Figure 4. Total penile arginase activity levels were significantly higher ($P < .05$) in the aged rat penes compared with young activity levels (Figure 4). The arginase inhibitor ABH significantly decreased ($P < .05$) the total arginase activity in aged rat penes to values similar to those of young rats (Figure 4).

Discussion

The present study demonstrates that chronic systemic arginase inhibition with ABH markedly improves

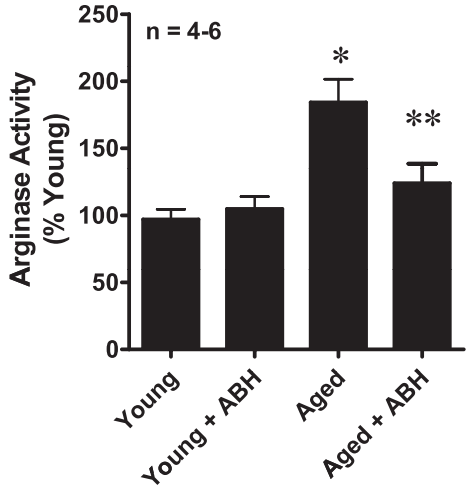


Figure 4. Total arginase activity in young and aged rat penes with and without treatment with the arginase inhibitor 2-(*S*)-amino-6-boronohexanoic acid (ABH). n indicates number of tissue samples; *, $P < .05$ compared with young and young + ABH; **, $P < .05$ compared with aged.

erectile hemodynamics in aged rats. Treatment with ABH reduced total arginase activity in the aged penis and resulted in significant improvements in erectile responses in aged rats. The fact that there is no significant difference in ICP/MAP ratios in young rats treated with the selective arginase inhibitor further supports the notion that corporal arginase activity is significantly increased in aging and contributes to ED in aged rats in the absence of other known risk factors.

Vascular disease remains a significant complication of aging, and it is central to the development of a number of age-related pathologies. Aging results in damage to the small blood vessels (microvascular disease), neuropathy, and vascular stiffness, which are the hallmark features of peripheral vascular disease. There are a number of cellular and molecular changes that occur in the systemic vasculature with aging that cause endothelial dysfunction. L-Arginine is the required substrate for the generation of NO by NOS. It is also metabolized by arginase, which results in the generation of ornithine and urea. Arginase has been localized to the human corpus cavernosum (Cox et al, 1999; Bivalacqua et al, 2001b), and its physiologic role has been postulated to be maintenance of intrinsic corporal smooth muscle tone (Masuda et al, 2004). This mechanism likely occurs as a result of NOS substrate depletion, which decreases the amount of NO generated, with resultant smooth muscle contraction. Up-regulation of arginase has as such been associated with a variety of conditions known to be risk factors for ED (Bivalacaqua et al, 2001a,b; Imamura et al, 2007; Jelodar et al, 2007), and is considered to be a major molecular mechanism that impairs penile endothelial cell function. In particular, aging itself has been linked to heightened arginase expression and activity (Numao et al, 2007), which is localized to the penile endothelium and smooth muscle (Bivalacqua et al, 2007), and aging is known to be associated with ED (Feldman et al, 1994). In the present study we demonstrate an increase in total arginase activity in the penis with associated impaired in vivo erectile function, suggesting that an elevation in arginase expression and activity contributes to corporal vascular dysfunction in aging.

Although inhibition of arginase via gene knockout or gene therapy techniques has been shown to improve erectile function (Bivalacqua et al, 2003; Toque et al, 2011) in rats and mice, this has not been demonstrated with an orally administrated arginase inhibitor, a factor that is critical for practical ED pharmacotherapy. Therefore, the purpose of this study was to assess whether arginase inhibition with a potent, selective arginase inhibitor (Baggio et al, 1999) administered via chronic oral supplementation would have an impact on penile vascular hemodynamics in an animal model of aging. We show that aged rats have significantly impaired erectile responses to CNS. In vivo erectile parameters are restored in aged rats treated with the arginase inhibitor ABH and compare with those of young rats, without a systemic effect on mean arterial blood pressure. We found no change in erectile function in young rats treated with ABH, suggesting that arginase inhibition only influences penile vascular function in the presence of elevated arginase expression and activity.

Several complex mechanisms are thought to contribute to the pathogenesis of age-related penile vascular dysfunction. Natural aging results in significant fibrosis of the corpora cavernosa (Ferrini et al, 2001). One potential mechanism of increased collagen deposition and fibrosis of the penis in aging may be associated with increased arginase signaling in the penis. Increased production of urea and ornithine by arginase leads to vascular hyperplasia and fibrosis (Alef et al, 2011) through increased production of proline synthesis. Thus, the improved erectile responses in rats treated with ABH may be associated with a reduction in collagen deposition and fibrosis, thus improving corporal vascular resistance and blood flow.

Further study is required to elucidate the exact mechanism by which systemic arginase inhibition results in improved erectile function. Although depletion of NOS substrate is certainly plausible, other mechanisms may have a critical role. Endothelial dysfunction manifested by endothelial NOS (eNOS) uncoupling and higher oxidative stress were shown to be significantly elevated in the aged rat penile vasculature (Bivalacqua et al, 2007; Kim et al, 2009; Johnson et al, 2011). Therefore, arginase inhibition may improve endothelial cell function by reversing eNOS uncoupling and reducing oxidative stress. We have previously shown that in human corporal cavernosal tissue obtained from diabetic patients, inhibition of arginase in vitro improved constitutive NOS activity, supporting the concept that arginase inhibition improves endothelial cell function in the penis (Bivalacqua et al, 2001a). Furthermore, there are multiple other molecular mechanisms that have been ascertained to be responsible for age-related ED in both preclinical and human studies that may be implicated. These include differential phosphorylation of eNOS (Musicki et al, 2005), altered corporal body structure (Bakirciorglu et al, 2001; Costa and Vendeira, 2008), increased endogenous NOS inhibitors (Imamura et al, 2007), corporal cavernosal extracellular matrix production and resultant fibrosis (Castela et al, 2011), and increased smooth muscle contractility as a result of increased RhoA/Rho-kinase activity (Jin et al, 2006). A major limitation to this study is the lack of direct NO measurement in the rats treated

with the arginase inhibitor. Therefore, further studies are warranted to delineate the compensatory molecular changes, such as improved NO biosynthesis, that occur in the penis and which influence erectile physiology and ultimate functional recovery of penile erections.

In conclusion, this is the first study to demonstrate an in vivo effect on penile vascular function in aged rats treated with the oral arginase inhibitor ABH. The results further support the notion that the arginase pathway may be exploited for the treatment of ED associated with aging.

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