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RESEARCH PAPER

The vascular effects of different arginase inhibitors in rat isolated aorta and mesenteric arteries

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Background and purpose: Arginase and nitric oxide (NO) synthase share the common substrate L-arginine, and arginase inhibition is proposed to increase NO production by increasing intracellular levels of L-arginine. Many different inhibitors are used, and here we have examined the effects of these inhibitors on vascular tissue.
Experimental approach: Each arginase inhibitor was assessed by its effects on isolated rings of aorta and mesenteric arteries from rats by: (i) their ability to preserve the tolerance to repeated applications of the endothelium-dependent agonist acetylcholine (ACh); and (ii) their direct vasorelaxant effect.
Key results: In both vessel types, tolerance (defined as a reduced response upon second application) to ACh was reversed with addition of L-arginine, (*S*)-(2-boronethyl)-L-cysteine HCl (BEC) or N^G-Hydroxy-L-arginine (L-NOHA). On the other hand, N^ω-hydroxy-nor-L-arginine (nor-NOHA) significantly augmented the response to ACh, an effect that was partially reversed with L-arginine. No effect on tolerance to ACh was observed with L-valine, nor-valine or D,L, α-difluoromethylornithine (DFMO). BEC, L-NOHA and nor-NOHA elicited endothelium-independent vasorelaxation in both endothelium intact and denuded aorta while L-valine, DFMO and nor-valine did not.
Conclusions and implications: BEC and L-NOHA, but not nor-NOHA, L-valine, DFMO or nor-valine, significantly reversed tolerance to ACh possibly conserving L-arginine levels and therefore increasing NO bioavailability. However, both BEC and L-NOHA caused endothelium-independent vasorelaxation in rat aorta, suggesting that these inhibitors have a role beyond arginase inhibition alone. Our data thus questions the interpretation of many studies using these antagonists as specific arginase inhibitors in the vasculature, without verification with other methods.
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Keywords: arginase; L-arginine; aorta; mesenteric arteries; nitric oxide; vasorelaxation

Abbreviations: BEC, (*S*)-(2-boronethyl)-L-cysteine HCl; DFMO, D,L, α-difluoromethylornithine; L-NAME, N^G-nitro-L-arginine-methyl ester; L-NOHA, N^G-hydroxy-L-arginine; nor-NOHA, N^ω-hydroxy-nor-L-arginine; ODQ, 1H-[1,2,4]-oxadiazolol[4,3-1]quinoxaline-1-one

Introduction

Nitric oxide (NO) bioavailability is compromised in many cardiovascular disease states. As L-arginine is rate limiting in NO production, this amino acid precursor has been postulated as a factor contributing to NO availability. Also, because the enzyme arginase catalyses the catabolism of L-arginine to form ornithine and urea, many argue that arginase can be manipulated to influence NO bioavailability. In this context, increased expression and/or activity of arginase has been demonstrated in many vascular pathologies such as pulmonary hypertension (associated with sickle cell disease) (Morris *et al.*, 2003), primary pulmonary arterial hypertension (Xu *et al.*, 2004), ischaemia-reperfusion (Hein *et al.*, 2003), uraemia (Thuraisingham *et al.*, 2002) as well as in animal models of arterial hypertension (Johnson *et al.*, 2005), aging (Berkowitz *et al.*, 2003; Santhanam *et al.*, 2007), sexual arousal (Berkowitz *et al.*, 2003; Cama *et al.*, 2003a), diabetes (Romero *et al.*, 2008) and atherosclerosis (Ming *et al.*, 2004; Ryoo *et al.*, 2006; 2008). Furthermore, inhibition of arginase has been shown to stimulate NO production (Chicoine *et al.*, 2004), while over-expression of arginase I or II decreases intracellular L-arginine concentrations and suppresses NO synthesis (Li *et al.*, 2001). However, because N^G-hydroxy-L-arginine (L-NOHA), a reaction intermediate of NO synthase (NOS), is also a potent intracellular inhibitor of arginase (Boucher *et al.*, 1994; Daghigh *et al.*, 1994) some of the effects observed may not confined to substrate competition alone.

Both vascular endothelial and smooth muscle cells express arginase I and II, but their distribution appears to be vessel- and species-dependent (Buga *et al.*, 1996; Zhang *et al.*, 2001;

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Bachetti *et al.*, 2004; Ming *et al.*, 2004). In addition to the production of urea, arginase is also involved in the biosynthesis of polyamines and the amino acids, ornithine, proline and glutamate (Cederbaum *et al.*, 2004). As such it is not surprising that amino acids such as ornithine, leucine, valine, lysine, isoleucine and nor-valine inhibit arginase (Hunter and Downs, 1945). Among them, ornithine is the most potent of the competitive amino acids, with nor-valine, a non-competitive inhibitor, demonstrating similar potency (Hunter and Downs, 1945). Another commonly used inhibitor is the indirectly acting, irreversible inhibitor of ornithine decarboxylase, D,L, α-difluoromethylornithine (DFMO), which increases ornithine levels endogenously (Selamnia *et al.*, 1998). Despite the relatively high concentrations required to inhibit arginase, the use of nor-valine, L-valine and DFMO have recently been reported in the context of studying NO function (Ming *et al.*, 2004; Santhanam *et al.*, 2007; Lewis *et al.*, 2008).

As L-NOHA also acts as a substrate for NO production, its utility as a specific arginase inhibitor is limited, although it is still used as a specific arginase inhibitor (Sakai *et al.*, 2004; Holan *et al.*, 2006). Due to the complications with the use of L-NOHA, N^{ω}-hydroxy-nor-L-arginine (nor-NOHA) was synthesized and reported to be more potent than L-NOHA, but not a substrate for NO (Tenu *et al.*, 1999). Aside from these inhibitors, several boron-based inhibitors have been designed. Among these are (*S*)-(2-boronethyl)-L-cysteine HCl (BEC) and 2(*S*)-amino-6-boronohexanoic acid (ABH) (Colleluori and Ash, 2001), both of which have been shown to effectively inhibit arginase (Khangulov *et al.*, 1995).

Due to the potential for manipulating intracellular L-arginine stores, and possibly increasing NO bioavailability in disease states, the interest in this field of research has escalated, resulting in the recent use of many of these inhibitors (Santhanam *et al.*, 2007; Bagnost *et al.*, 2008; Lewis *et al.*, 2008). To address which of the arginase inhibitors is most specific and appropriate for studying functional NO effects in the vasculature, a comparative assessment of these arginase inhibitors was performed.

Methods

Animals

All animal procedures and the study protocol was approved by the Alfred Medical Research and Education Precinct (AMREP) Animal Ethics Committee (applications E/0323/2003/B, E/0238/2004/2004B and E/0352/2004/B) that adheres to the National Health and Medical Research Council (NHMRC) of Australia Code of Practice for the Care and Use of Animals for Scientific Purposes. Male Sprague-Dawley rats were housed under standard laboratory conditions with access to food and water *ad libitum*. Animals were killed by decapitation and exsanguination following overexposure to 80% CO_2 and 20% O_2.

Vascular reactivity

The thoracic aorta and mesenteric arteries were excised and placed into ice-cold modified Krebs solution (composition in mmol·L^{-1}: NaCl 119, KCl 4.7, $MgSO_4 \cdot 7H_20$ 1.17, $NaHCO_3$ 25, KH_2PO_4 1.18, $CaCl_2$ 2.5, glucose 11 and EDTA 0.03). The adipose and connective tissue were removed. Rat aorta was sectioned into eight rings of 3 mm length and mesenteric arteries into eight rings of 2 mm length with the aid of a dissecting microscope (Olympus, Tokyo). In some of the vessels, endothelium denudation of thoracic aortic rings was performed by gently rubbing the lumen of the aorta against a wire. For mesenteric arteries, this procedure was achieved by pulling a strand of human hair backwards and forwards through the lumen of the vessel.

Aortic rings and mesenteric arteries were mounted in organ baths and on a wire-myograph as previously described (Lewis *et al.*, 1997; Kimura *et al.*, 2002). Once the vessels were mounted and incubated for an equilibration period of 30 min, all vessels were subjected to an oxygenated and pre-warmed (37°C) high potassium physiological salt solution (KPSS in mmol·L^{-1}; KCl 123, $MgSO_4 \cdot 7H_20$ 1.17, $NaHCO_3$ 25, KH_2PO_4 1.18, $CaCl_2$ 2.5, glucose 6.05 and EDTA 0.03) until a plateau contractile response was observed. The vessel was rinsed three times with oxygenated and pre-warmed (37°C) modified Krebs' solution. Endothelium integrity or successful denudation was confirmed by pre-constricting the vessel with a constrictor agonist followed by addition of acetylcholine (ACh) (1 μmol·L^{-1}). Rat aorta was pre-constricted with noradrenaline (10 nmol·L^{-1}), while for mesenteric arteries, the modified Krebs solution was replaced with 40 mmol·L^{-1} potassium salt solution (40 mmol·L^{-1} KCl, composition in mmol·L^{-1}; NaCl 84, KCl 40, $MgSO_4 \cdot 7H_2O$ 1.17, $NaHCO_3$ 25, KH_2PO_4 1.18, $CaCl_2$ 2.5, glucose 11 and EDTA 0.03). Potassium was chosen as the constrictor for mesenteric arteries since at the concentration used, any effect of endothelial-derived hyperpolarizing factor (EDHF) would be negated, unmasking the contribution of NO to vasodilatation (Chen and Suzuki, 1990). Where applicable, for intact (i.e. non-denuded) vessels, a relaxation response of >80% in rat aorta and >60% in mesenteric arteries was set as the inclusion criteria upon addition of 1 μmol·L^{-1} ACh. Vessels were deemed denuded when relaxation was less than 10% in either aorta or mesenteric arteries.

Experimental design

Effect of L-arginine supplementation and arginase inhibitors on NO function

Cumulative full concentration–response curves in half log increments to the endothelium- and NO-dependent dilator, ACh, (1 nmol·L^{-1}–10 μmol·L^{-1}), were obtained and repeated 30 min apart. Between each curve, vessels were washed, and a total of three concentration–response curves to ACh were obtained successively, 30 min apart, in the absence of any treatment and used as the comparative time control experiment.

In separate baths and myograph chambers, vessels were incubated with L-arginine (1 μmol·L^{-1} or 10 μmol·L^{-1}) or the arginase inhibitors: BEC (100 μmol·L^{-1}), nor-NOHA (10 μmol·L^{-1}), L-NOHA (10 μmol·L^{-1}), DFMO (10 μmol·L^{-1}), L-valine (10 μmol·L^{-1}) or nor-valine (10 μmol·L^{-1}) for 30 min before and during the repeat concentration–response curve to ACh. Therefore, matched controls were obtained for each compound, such that (–) and (+) denotes the ACh concentration–response curve performed before and after

addition of L-arginine or the arginase inhibitor as indicated. The concentration of the arginase inhibitor used during the incubation period was determined from pilot studies. Vasorelaxant responses in rat aorta were performed on vessels pre-constricted with noradrenaline and in mesenteric arteries with 40 mmol·L^{-1} KCl.

The role of the endothelium on the direct vasodilatory effect of arginase inhibitors

Full cumulative concentration–response curves in half log increments to the arginase inhibitors: BEC (aorta and mesenteric arteries: 0.1 μmol·L^{-1}–3 mmol·L^{-1}), nor-NOHA (aorta and mesenteric arteries: 0.1 μmol·L^{-1}–3 mmol·L^{-1}), L-NOHA (aorta: 1 μmol·L^{-1}–1 mmol·L^{-1}, mesenteric arteries: 0.1 μmol·L^{-1}–3 mmol·L^{-1}), DFMO, L-valine or nor-valine (aorta: 1 μmol·L^{-1}–3 mmol·L^{-1}, mesenteric arteries: 10 μmol·L^{-1}–3 mmol·L^{-1}) were obtained in endothelium intact and denuded vessels. Experiments were performed in rat aortic rings pre-constricted with noradrenaline and in mesenteric arteries pre-constricted with 40 mmol·L^{-1} KCl. Concentration–response curves were also obtained to L-NOHA, nor-NOHA and BEC in the presence of the soluble guanlylyl cyclase (sGC) inhibitor, 1H-[1,2,4]-oxadiazolol[4,3-1]quinoxaline-1-one (ODQ, 10 μmol·L^{-1}) or the NOS inhibitor, N^G-nitro-L-arginine-methyl ester (L-NAME, 100 μmol·L^{-1}).

Data analysis and statistics

All vasorelaxation responses were expressed as percentage relaxation from the pre-constriction response to noradrenaline in aortic rings, or to 40 mmol·L^{-1} KCl in mesenteric arteries. R_{max} depicts the maximum relaxation response obtained. Variable slope sigmoidal concentration–response curves to each agonist were fitted and graphed, and the potency [–log EC_{50} (M) i.e. the concentration giving 50% of the maximum response] calculated for individual curves by using GraphPad Prism (V. 4.01, USA). Results were analysed by Student's *t*-test (paired or unpaired, as appropriate). Where three curves were compared, comparisons were made by using a one-way ANOVA. Statistical analysis was performed by using GraphPad Prism where $P < 0.05$ was considered statistically significant. All data are presented as mean ± SEM.

Drugs and reagents

Arginase inhibitors, L-NOHA (N^G-hydroxy-L-arginine monoacetate salt), nor-NOHA (N^ω-hydroxy-nor-L-arginine diacetate salt) and BEC, were purchased from Calbiochem, USA. DFMO, L-NAME, L-valine, nor-valine, ODQ, noradrenaline bitartrate, ACh chloride, sodium nitroprusside dihydrate and L-arginine were purchased from Sigma-Aldrich, St. Louis, MO, USA. Drug stock solutions were made in milliQ water and stored at –20°C. All drugs were diluted in modified Krebs solution, with the exception of ODQ that was made up in 100% ethanol on the day of experiment and stored on ice until ready for use. Salts used in modified KPSS, modified Krebs solution and 40 mmol·L^{-1} KCl were all purchased from Merck P/L, Kilsyth, Victoria, Australia. Drug/molecular target nomenclature conforms to the British Journal of Pharmacology Guide to Receptors and Channels (Alexander *et al.*, 2008).

Results

L-arginine and tolerance to ACh in aorta

As shown in Figure 1A and consistent with previous findings (Hogan *et al.*, 2005), a successive reduction in both potency (rightward shift in concentration–response curve) and maximal efficacy, R_{max}, was observed with each successive construction of a full concentration–response curve to ACh. Upon addition of either 1 μmol·L^{-1} or 10 μmol·L^{-1} of L-arginine, 30 min prior to performing the second ACh concentration–response curve, the shift or 'tolerance' to ACh was no longer present (Fig. 1B), suggesting that depletion of intracellular L-arginine, over time plays a role in the observed tolerance.

Arginase inhibitors and tolerance to ACh in aorta

The ability of six different arginase inhibitors to reverse ACh tolerance was investigated in rat isolated aortic rings (Fig. 2). In the presence of BEC and L-NOHA, tolerance to ACh was not observed, that is, there was no significant difference in either the EC_{50} or R_{max} values in ACh in the presence of the arginase inhibitor ($P > 0.05$; Fig. 2A,B). In contrast, nor-NOHA enhanced the shift to the right substantially and reduced the maximum of the second ACh concentration–response curve when compared with the second control ACh concentration–response curve (EC_{50}, 0.3 ± 0.1 vs. 0.09 ± 0.02 μmol·L^{-1}; R_{max},

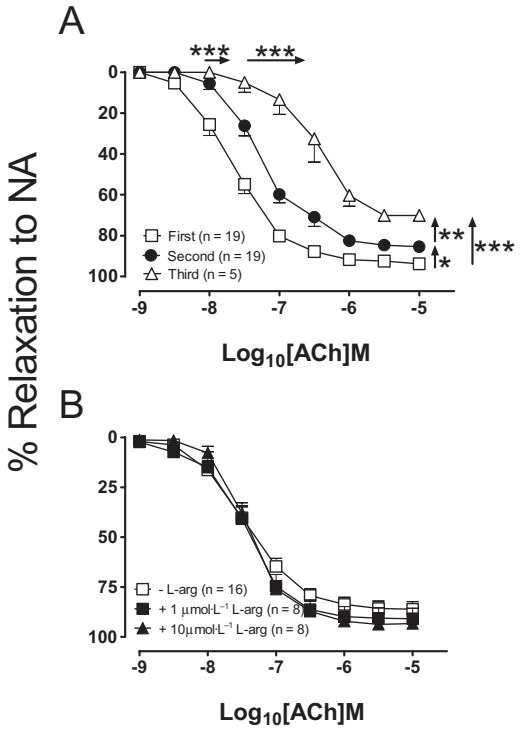

Figure 1 (A) Successive concentration–response curves to acetylcholine (ACh), repeated 30 min apart. In separate experiments, (B) the second application of ACh was performed in the presence of either 1 μmol·L^{-1} or 10 μmol·L^{-1} of L-arginine (L-arg). All data are presented as mean ± SEM. The horizontal and vertical arrows refer to changes in EC_{50} and R_{max} respectively; *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ by using a one-way ANOVA with Tukey's post hoc analysis. NA, noradrenaline.

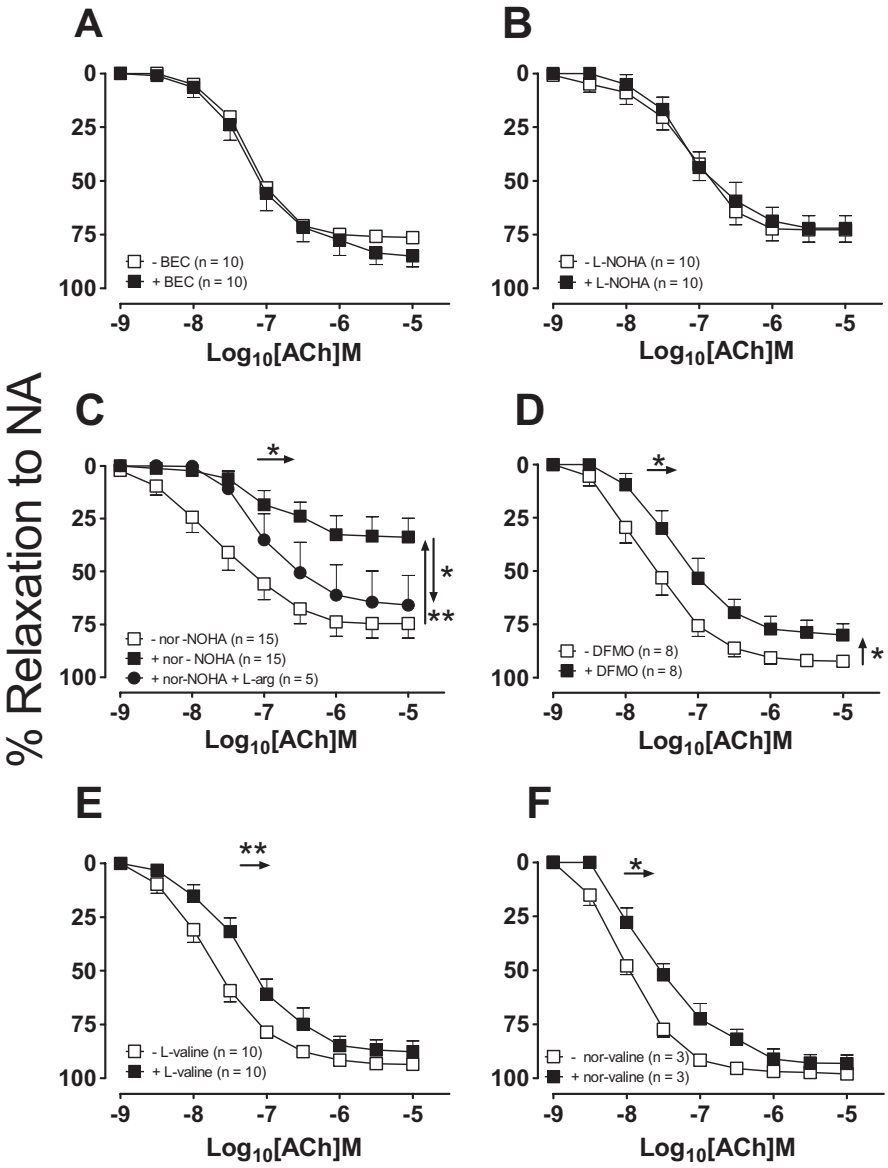

Figure 2 Concentration–response curves to ACh were repeated 30 min after the addition of either (A) 100 μmol·L^{-1} BEC, (B) 10 μmol·L^{-1} L-NOHA, (C) 10 μmol·L^{-1} nor-NOHA, (D) 10 μmol·L^{-1} DFMO, (E) 10 μmol·L^{-1} L-valine or (F) 10 μmol·L^{-1} nor-valine. All data are presented as mean ± SEM where *$P < 0.05$ and **$P < 0.01$ by using a paired Student's *t*-test comparison of the EC_{50} (horizontal arrows) or R_{max} (vertical arrows) before and after the addition of an arginase inhibitor. ACh, acetylcholine; BEC, (*S*)-(2-boronethyl)-L-cysteine HCl; DFMO, D,L, α-difluoromethylornithine; L-NOHA, N^G-hydroxy-L-arginine; NA, noradrenaline; nor-NOHA, N^{ω}-hydroxy-nor-arginine.

34 ± 9 vs. 85 ± 2%; $n = 15$–19; $P < 0.05$; Fig. 2C), an effect that was partially restored by L-arginine. DFMO and the equipotent (to DFMO) competitive and non-competitive arginase inhibitors, L-valine and nor-valine had no significant effect on the EC_{50} of the ACh-induced tolerance, albeit in the presence of L-valine and nor-valine there was no longer a significant difference in the maximal response (Fig. 2D–F).

Arginase inhibitors as vasodilators in aorta

Since arginase expression has been reported in both endothelial and vascular smooth muscle cells (Berkowitz *et al.*, 2003; Buchwalow *et al.*, 2004; Johnson *et al.*, 2005), the effects of the arginase inhibitors were examined in both endothelium-intact and denuded vessels. As shown in Figure 3A, BEC elicited concentration-dependent vasorelaxation which was non-endothelium-dependent (EC_{50} and R_{max}, intact vs. denuded rings: 13 ± 4 μmol·L^{-1} and 80 ± 6% vs. 62 ± 26 μmol·L^{-1} and 67 ± 8%, $n = 11$–15; $P > 0.05$). Similarly, L-NOHA and nor-NOHA induced vasorelaxation in both intact and denuded vessels with comparable potencies (Fig. 3B,C). In endothelium-denuded aorta, vasorelaxation to BEC, L-NOHA and nor-NOHA, was significantly attenuated in the presence of the sGC inhibitor, ODQ (10 μmol·L^{-1}) suggesting a cGMP-dependent mechanism. Responses to L-NOHA were attenuated by the NOS inhibitor L-NAME (100 μmol·L^{-1}) in both intact and denuded aorta ($P < 0.05$) while those to BEC were unaffected. DFMO, L-valine and nor-valine did not induce significant vasorelaxation (see Fig. 3D–F) when compared with their

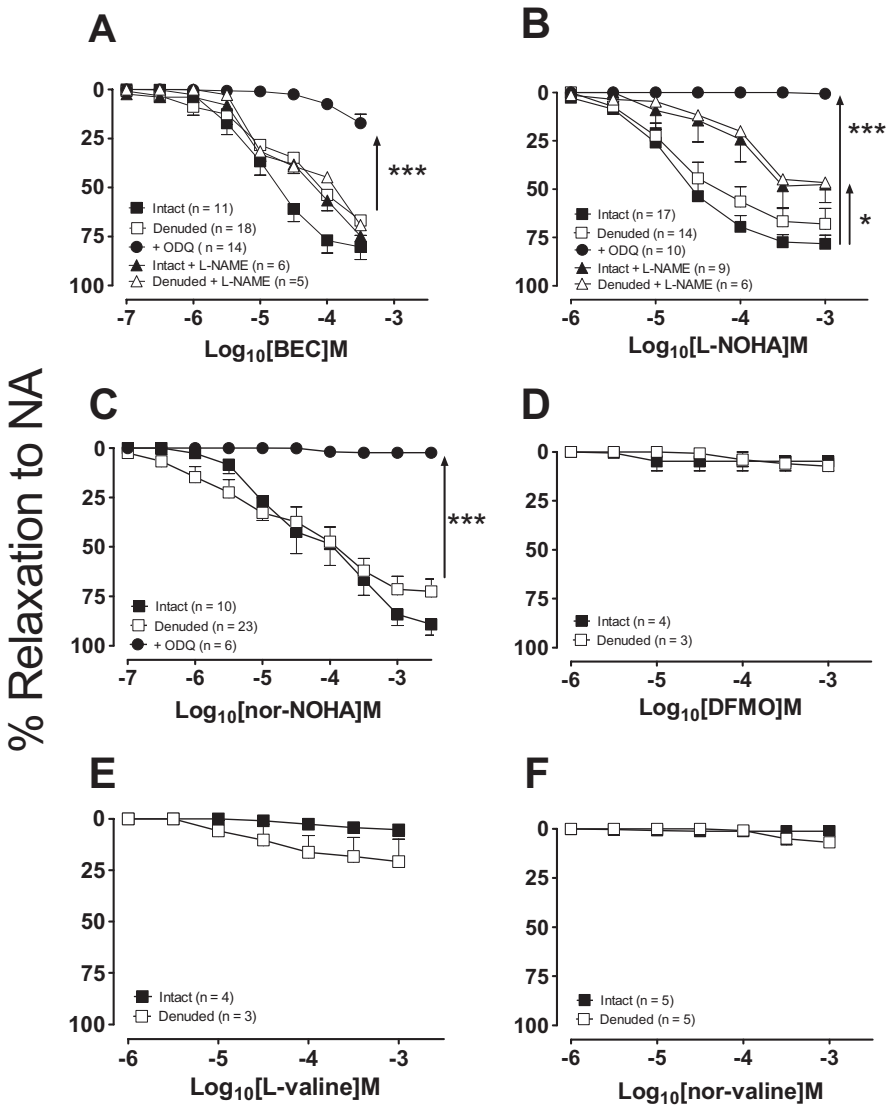

Figure 3 Concentration–response curves to the arginase inhibitors: (A) BEC, (B) L-NOHA, (C) nor-NOHA, (D) DFMO, (E) L-valine and (F) nor-valine were performed in endothelium-intact and denuded aortic rings pre-constricted with NA. Responses to L-NOHA, nor-NOHA and BEC were also performed in the presence of the cGMP inhibitor, ODQ (10 μmol·L^{-1}) in endothelium-denuded vessels or the NOS inhibitor L-NAME (100 μmol·L^{-1}) in endothelium-intact and denuded vessels. All responses are expressed as % relaxation to NA and as mean ± SEM where *P < 0.05 (–nor-NOHA vs. +nor-NOHA) and ***P < 0.001 by using an unpaired Student's t-test. BEC, (*S*)-(2-boronethyl)-L-cysteine HCl; DFMO, D,L, α-difluoromethylornithine; L-NAME, N^G-nitro-L-arginine-methyl ester; L-NOHA, N^G-hydroxy-L-arginine; NA, noradrenaline; nor-NOHA, $N^{(\omega)}$-hydroxy-nor-arginine; ODQ, 1H-[1,2,4]-oxadiazolol[4,3-1]quinoxaline-1-one.

time controls (data not shown), which coincided with their reduced ability to reverse tolerance to ACh.

L-arginine and tolerance to ACh in mesenteric arteries

While NO is thought to play a significant role in the vasodilatory profile in conduit vessels, it has been frequently reported to play only a minor role in resistance vessels such as mesenteric arteries, where a larger contribution by other vasodilators such as EDHF is reported (Wu *et al.*, 1993; Shimokawa *et al.*, 1996; Chataigneau *et al.*, 1999). Therefore, to negate the effects of EDHF, which dilates via K^+ channels, all experiments were performed by using a high K^+ solution (40 mmol·L^{-1}) as the vasoconstrictor. Hence, under these conditions, ACh-induced relaxation is NOS-dependent and is abolished in the presence of L-NAME (data not shown). When similar experiments to those described above, but this time in mesenteric arteries were performed, tolerance to ACh was also observed (EC_{50} values, first vs. second control concentration–response curves, 0.1 ± 0.01 vs. 0.4 ± 0.2 μmol·L^{-1}; n = 13; P < 0.05; Fig. 4A) but without an effect on the maximal response to ACh (P > 0.05). As observed in aortic rings, supplementation with either 1 μmol·L^{-1} or 10 μmol·L^{-1} L-arginine abolished the rightward shift in the concentration–response curve to ACh (P > 0.05; Fig. 4B).

Arginase inhibitors and tachyphylaxis to ACh in mesenteric arteries

As we had observed in aortic rings, there was no tolerance to ACh in rings of mesenteric arteries in the presence of BEC or

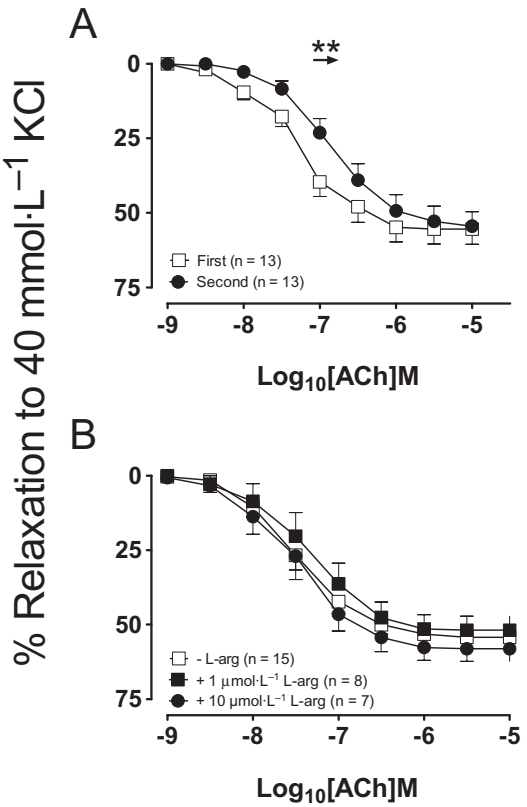

Figure 4 (A) Concentration–response curves to acetylcholine (ACh) were repeated 30 min apart in mesenteric artery rings pre-constricted with 40 mmol·L^{-1} KCl. The second application of ACh was also performed (B) in the presence of either 1 µmol·L^{-1} or 10 µmol·L^{-1} of L-arginine (L-arg). All data are presented as mean ± SEM where $^{**}P < 0.01$ by using a paired Student's *t*-test.

L-NOHA (Fig. 5A,B). Again, nor-NOHA significantly enhanced the tolerance to ACh, a finding, which again was partially reversed with addition of 100 µmol·L^{-1} of L-arginine (Fig. 5C). DFMO, L-valine and nor-valine, likewise, did not reduce tolerance to Ach (Fig. 5D–F).

The endothelium and arginase inhibitors in mesenteric arteries

In contrast to the finding in aortic rings, BEC did not induce significant vasorelaxation in mesenteric artery rings (Fig. 6A). In mesenteric arteries, L-NOHA and nor-NOHA induced concentration-dependent vasorelaxation, similar to those observed in the aorta, and both were abolished in the presence of the sGC inhibitor, ODQ (see Fig. 6B,C). There were no vasorelaxant responses to L-valine, DFMO or nor-valine in intact or denuded mesenteric arteries ($P > 0.05$; two-way ANOVA, when compared with time control; Fig. 6D–F).

Discussion

Several studies have reported that arginase inhibition restores endothelial function in various animal models of disease including hypertension (Rodriguez *et al.*, 2004; Zhang *et al.*, 2004; Demougeot *et al.*, 2005; Johnson *et al.*, 2005; Bagnost *et al.*, 2008), diabetes (Romero *et al.*, 2008), atherosclerosis (Ming *et al.*, 2004; Ryoo *et al.*, 2008) and aging (Berkowitz *et al.*, 2003; Santhanam *et al.*, 2007). This data are consistent with the hypothesis that arginase competes with NOS for the catabolism of L-arginine and that the inhibition of arginase allows increased production of NO. Many of these papers use the commonly available arginase inhibitors with the assumption of arginase and endothelial specificity. The current paper, however, shows that these arginase inhibitors each have differing effects in the vasculature and that some caution should be exercised with their use and in the subsequent interpretation of the data obtained particularly in diseased models.

An *in vitro* method of inducing decreased NO bioavailability by repeated applications of ACh so that a significant shift to the right in the concentration–response curve to ACh indicated decreased potency and a reduction in the response to 10 µmol·L^{-1} ACh indicated decreased maximal effect, in the rat aorta. In the mesenteric arteries, a modest decrease in potency was observed without changes to the maximal effect. ACh is a well-known endothelium-dependent dilator in conduit vessels, where it releases NO via a NOS-dependent mechanism (Furchgott and Zawadzki, 1980). However, in resistance vessels, such as the mesenteric arteries utilized in this study, the majority of the relaxation to ACh is mediated via EDHF, rather than NO, as we and others (Hogan *et al.*, 2005) have shown relaxation is only partially reduced by NOS inhibitors. In both the current study and in bovine intrapulmonary arterial vessels (Gold *et al.*, 1989), reduced responses to ACh suggest that depletion of intracellular L-arginine over time plays a role in tolerance, as L-arginine supplementation prevented this loss of response. To examine functionally whether arginase inhibition increased intracellular L-arginine and could therefore prevent tolerance to ACh, several arginase inhibitors were examined by using a protocol, similar to that used for L-arginine in both the aorta and the mesenteric arteries. Interestingly, only two of the arginase inhibitors examined, BEC and L-NOHA, effectively prevented ACh tolerance in aortic and mesenteric artery preparations. This suggests that indeed some but not all arginase inhibitors can conserve intracellular L-arginine stores to the level required to allow the preservation of ACh responses, mediated by NO.

Based on the published inhibitory constants of the six commercially available arginase inhibitors utilized in this study, it was anticipated that BEC would be the most potent, followed by nor-NOHA, L-NOHA and collectively least potent: DFMO, L-valine and nor-valine (Hunter and Downs, 1945; Daghigh *et al.*, 1994; Custot *et al.*, 1997; Cama *et al.*, 2003b). While BEC and L-NOHA were able to prevent tolerance to ACh, DFMO and the valine amino acids failed to inhibit the ACh-induced shift to the right in the concentration–response curves or reductions in the maximum response. These findings fit with the notion that these compounds are comparatively poorer inhibitors of arginase and as such are not as effective in the vasculature, in relation to improving L-arginine levels to a level where a functional advantage is observed. It has been reported that the synthetic derivative of the intermediate L-NOHA, nor-NOHA, is not a substrate for or inhibitor of NOS unlike L-NOHA itself (Daghigh *et al.*, 1994) and as such it has been proposed to be a more specific inhibitor of arginase than L-NOHA (Tenu *et al.*, 1999). However, in

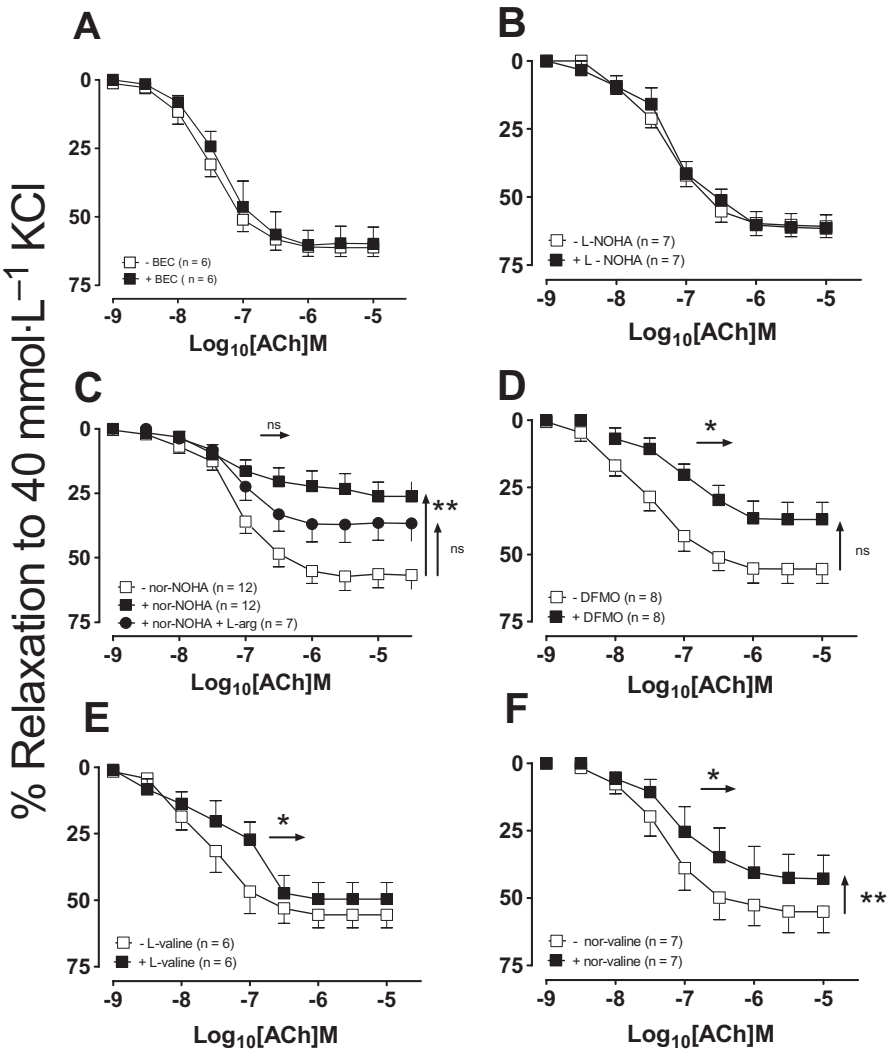

Figure 5 The second concentration–response curve to ACh was repeated in mesenteric artery rings after a 30 min incubation with either (A) 100 μmol·L^{-1} BEC, (B) 10 μmol·L^{-1} L-NOHA, (C) 10 μmol·L^{-1} nor-NOHA, (D) 10 μmol·L^{-1} DFMO, (E) 10 μmol·L^{-1} L-valine or (F) 10 μmol·L^{-1} nor-valine. All data are presented as mean ± SEM where $*P < 0.05$ and $**P < 0.001$ by using a paired *t*-test comparison of the EC_{50} and R_{max} before and after the addition of an arginase inhibitor. ACh, acetylcholine; BEC, (*S*)-(2-boronethyl)-L-cysteine HCl; DFMO, D,L, α-difluoromethylornithine; L-NOHA, N^G-hydroxy-L-arginine; nor-NOHA, N^ω-hydroxy-nor-arginine.

both aorta and mesenteric arteries, nor-NOHA caused further inhibition of responses to ACh, an effect that was partially restored by L-arginine supplementation. This result was unexpected because nor-NOHA is 40 times more potent than L-NOHA as an arginase inhibitor and, unlike L-NOHA, is neither a substrate nor inhibitor of NOS (Tenu *et al.*, 1999). As L-arginine supplementation in the presence of nor-NOHA improved the response to ACh, it is possible that nor-NOHA may in fact inhibit or compete with NOS in contrast to previous findings (Tenu *et al.*, 1999). When used at the same concentration as L-NOHA, DFMO that has been reported to inhibit arginase with a similar potency (Selamnia *et al.*, 1998) or the competitive and non-competitive arginase inhibitors, L-valine and nor-valine, did not prevent tolerance to ACh. Taken together, our results suggest that in the vasculature, caution should be used when assessing the effect of arginase by the use of these inhibitors alone, and that other methods should be used to verify the results obtained.

As arginase has been identified in both endothelial and vascular smooth muscle cells (Berkowitz *et al.*, 2003; Buchwalow *et al.*, 2004; Johnson *et al.*, 2005), we examined the direct effect of the inhibitors as vascular relaxants. L-NOHA, nor-NOHA and BEC all caused concentration-dependent vasorelaxation in the aorta and L-NOHA and nor-NOHA in small mesenteric arteries, which was not endothelium-dependent. The relaxant effect of all three of these compounds is cGMP-dependent, because treatment with the sGC inhibitor, ODQ, in denuded aortic vessels abolished the responses. The guanididium groups of L-NOHA and nor-NOHA are thought to bind to arginase by displacing the metal-bridging hydroxide ion of the native enzyme and asymmetrically joining to the binuclear manganese cluster (Cox *et al.*, 2001). Thus, it is possible that rather than binding to the hydroxyl group of arginase, the guanididium group of L-NOHA and nor-NOHA may bind to other active enzymes. Certainly, as an intermediate of NO production, this is not the

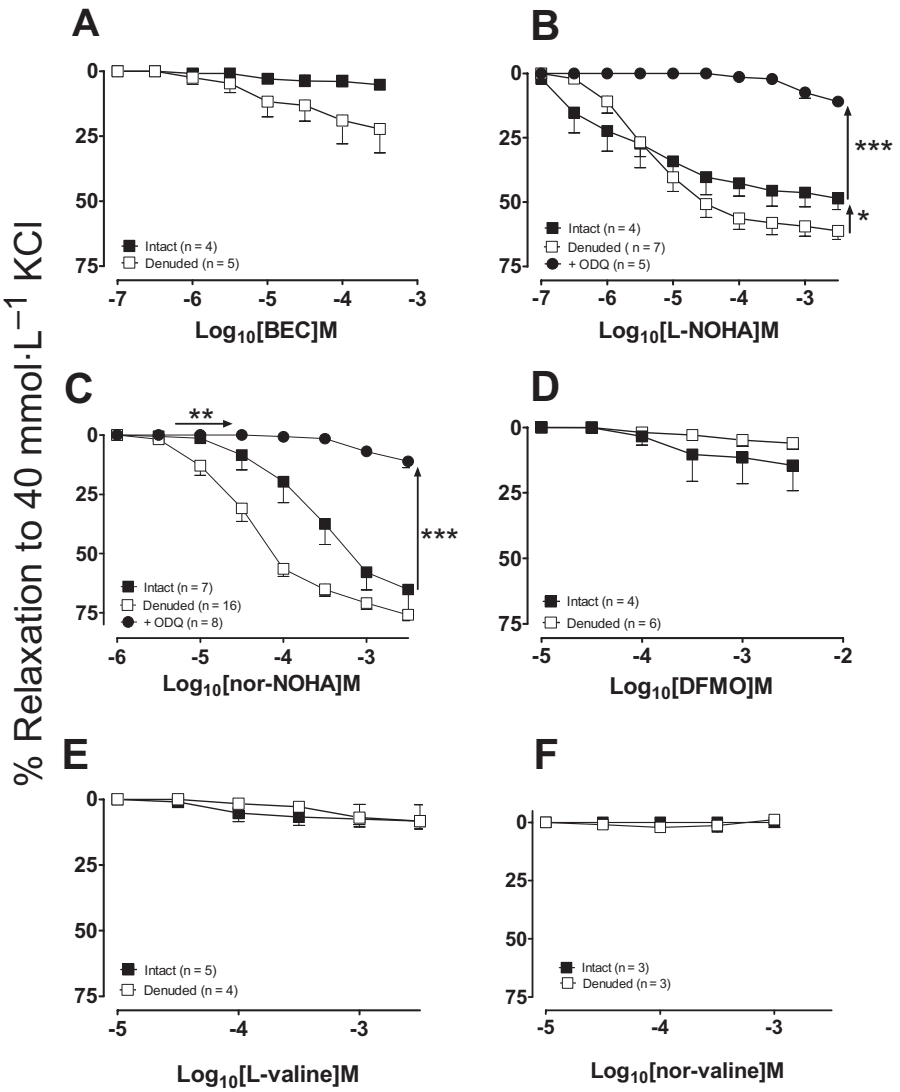

Figure 6 Concentration–response curves to the arginase inhibitors: (A) BEC, (B) L-NOHA, (C) nor-NOHA, (D) L-valine, (E) DFMO and (F) nor-valine were performed in endothelium-intact and denuded mesenteric arteries pre-constricted with 40 mmol·L^{-1} KCl. Concentration–response curves to L-NOHA and nor-NOHA were also performed in the presence of sGC inhibitor, ODQ (10 μmol·L^{-1}), in endothelium-denuded vessels. All responses are presented as mean ± SEM, where *P < **P < 0.01 and ***P < 0.001 by using an unpaired Student's t-test. BEC, (*S*)-(2-boronethyl)-L-cysteine HCl; DFMO, D,L, α-difluoromethylornithine; L-NOHA, N^{G}-hydroxy-L-arginine; nor-NOHA, N^{ω}-hydroxy-nor-arginine; ODQ, 1H-[1,2,4]-oxadiazolol[4,3-1]quinoxaline-1-one; sGC, soluble guanlylyl cyclase.

first time that L-NOHA has been demonstrated to cause both endothelium-dependent (NOS- and sGC-dependent) and independent relaxation (proposed to be via an NO-dependent but NOS-independent mechanism) (Wallace *et al.*, 1991; Abdul-Hussain *et al.*, 1996; Vetrovsky *et al.*, 2002). However, this is the first report of BEC inducing endothelium-independent vasorelaxation, as a previous report (Berkowitz *et al.*, 2003) suggested it was endothelium-dependent (NOS- and sGC-dependent) in aortas from Wistar Kyoto rats. The vasorelaxation response was sensitive to ODQ but not L-NAME, suggesting BEC may be able to directly activate sGC in an NOS-independent manner. Future studies utilizing purified sGC may be able to identify its mechanism of action. While arginase activity was not measured in the current study, BEC has been shown to effectively decrease arginase activity (Kim *et al.*, 2001; Berkowitz *et al.*, 2003; Steppan *et al.*, 2006). Interestingly, the NOS inhibitor L-NAME blunted the dilatory responses of L-NOHA in both intact and denuded vessels suggesting that the effects of L-NOHA are, in part, dependent on smooth muscle NOS.

In summary, the current study demonstrates that the commonly used arginase inhibitors differ in their potency in the vasculature when assessed by their ability to prevent tolerance to ACh. BEC and L-NOHA appear to be effective inhibitors of endothelial arginase in the aorta but also have direct, non-endothelium-dependent, cGMP-sensitive and vasorelaxant actions in aorta. In both aorta and mesenteric arteries, nor-NOHA may in fact compete for NOS. The amino acids, DFMO, L-valine and nor-valine were ineffective at preventing tolerance to ACh, suggesting they did not sufficiently increase L-arginine levels to an adequate level to have an effect functionally. Caution should thus be

exercised in the interpretation and use of these antagonists in vascular tissue, without verification with other methods.

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Conflict of interest

None.

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