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# Stimulus-specific blockade of nitric oxide-mediated dilatation by asymmetric dimethylarginine (ADMA) and monomethylarginine (L-NMMA) in rat aorta and carotid artery

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ABSTRACT

Previous work on female rat aorta has shown that although monomethylarginine (L-NMMA) and asymmetric dimethylarginine (ADMA) each enhance submaximal phenylephrine-induced tone, consistent with blockade of basal nitric oxide activity, neither agent has any major effect on acetylcholine-induced relaxation. The aim of this study was to adopt a variety of different experimental approaches to test the hypothesis that these methylarginines block basal but not agonist-stimulated activity of nitric oxide. Basal activity of nitric oxide was assessed by observing the rise in submaximal phenylephrine-induced tone produced by nitric oxide synthase (NOS) inhibitors in male and female aorta and female carotid artery, and by monitoring the vasodilator actions of superoxide dismutase (SOD) or the PDE 5 inhibitor, T-0156. Agonist-stimulated activity of nitric oxide was assessed by observing the relaxant actions of acetylcholine or calcium ionophore A23187. L-NMMA, ADMA and L-NAME (100 μM) each enhanced submaximal phenylephrine-induced tone and inhibited SOD- or T-0156-induced relaxation, consistent with each NOS inhibitor blocking basal nitric oxide activity. In contrast, L-NMMA and ADMA had little effect on acetylcholine- or A23187-induced relaxation, while L-NAME produced powerful blockade. These observations provide support for the hypothesis that L-NMMA and ADMA selectively block basal over agonist-stimulated activity of nitric oxide in rat vessels.



## 1. Introduction

A large number of guanidino ($N^G$)-substituted analogues of L-arginine, including $N^G$-monomethyl-L-arginine (L-NMMA), asymmetric $N^G$, $N^G$-dimethyl-L-arginine (ADMA), $N^G$-nitro-L-arginine (L-NOARG) and $N^G$-nitro-L-arginine methyl ester (L-NAME) are well recognised inhibitors of nitric oxide synthase (NOS) (Hobbs et al., 1999; Leiper and Vallance, 2006; Moore et al., 1990; Rees et al., 1989; Rees et al., 1990; Vallance et al., 1992). As a group, they have proved valuable tools for investigating the role of the L-arginine-nitric oxide system in a diverse range of biological processes. Moreover, the methylarginines, L-NMMA and ADMA, are endogenously produced, and the latter in particular is known to accumulate in renal failure (Vallance et al., 1992) and in a wide range of pathological conditions associated with vascular dysfunction, including systemic hypertension, pulmonary hypertension, atherosclerosis, diabetes mellitus and pre-eclampsia (Leiper and Vallance, 2006; Siroen et al., 2006).

It is generally believed that the guanidino-substituted analogues are not substrates for NOS, but act as classical competitive inhibitors, whose actions can be either prevented or reversed by an excess of the enzyme substrate, L-arginine. Despite this, numerous studies with the methylarginines suggest they exhibit properties that deviate from classical competitive behaviour on all three isoforms of NOS [i.e., endothelial (eNOS), neuronal (nNOS) and inducible (iNOS)]. Specifically, L-NMMA acts as an alternative substrate and mechanism-based suicide inhibitor of iNOS in murine macrophages (Olken and Marletta, 1993). Furthermore, unlike L-NOARG and L-NAME, L-NMMA fails to inhibit nitrergic nerve (nNOS) mediated relaxation in the bovine penile and ciliary arteries and retractor penis muscle (Liu et al., 1991; Martin et al., 1993; Overend and Martin, 2007). In addition, while L-NMMA and ADMA powerfully block the basal nitric oxide activity that suppresses vasoconstrictor tone in rat aorta, they have no effect on maximal acetylcholine-induced relaxation, although they do produce a modest (~2–3-fold) reduction in sensitivity to acetylcholine (AL-Zobaidy et al., 2010; Frew et al., 1993).

The aim of this study was to explore further the seemingly paradoxical ability of L-NMMA and ADMA to block basal activity of nitric oxide but have little effect on acetylcholine-stimulated activity. Specifically, since this action has thus far been reported only on female rat aorta, we have examined whether it occurs also in male rat aorta and in female rat carotid artery. Furthermore, we wished to determine if the endothelium-dependent relaxation to calcium ionophore

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A23187, which stimulates nitric oxide production by a receptor-independent mechanism, was also insensitive to blockade by L-NMMA or ADMA. In addition, we wished to determine the effects of L-NMMA and ADMA on the endothelium-dependent relaxation induced by superoxide dismutase (SOD) or the PDE5 inhibitor, T-0156, which occur through the potentiation of basal nitric oxide activity by scavenging destructive superoxide anions and by suppressing cyclic GMP hydrolysis, respectively (Mian and Martin, 1995; Mochida et al., 2002).

## 2. Material and methods

### 2.1. Preparation of rat aortic and carotid artery rings for tension recording

The preparation of rat aortic and carotid artery rings for tension recording was essentially similar to previous studies (AL-Zobaidy et al., 2010; Frew et al., 1993). Most experiments were conducted with tissues from female Wistar rats weighing 150–200 g, but where specified, male rats of similar size were used for comparison. Following killing by $CO_2$ overdose, the aorta and carotid arteries were removed, cleared of adhering fat and connective tissue and cut into 2.5 mm wide transverse rings using a device with parallel razor blades. Endothelial cells were removed from some rings by gently rubbing the intimal surface with a moist wooden stick for 30 s. Successful removal of the endothelium was confirmed by the inability of acetylcholine (1 μM) to elicit relaxation. Aortic rings and carotid artery rings were mounted under 10 mN and 5 mN resting tension, respectively, on stainless steel hooks in 10 ml tissue baths, and bathed at 37 °C in Krebs solution containing (mM): NaCl 118, KCl 4.8, $CaCl_2$ 2.5, $MgSO_4$ 1.2, $KH_2PO_4$ 1.2, $NaHCO_3$ 24, glucose 11, and gassed with 95% $O_2$ and 5% $CO_2$. Tension was recorded isometrically with Grass FTO3C transducers and displayed on a PowerLab (ADInstruments, Hastings, UK).

### 2.2. Experimental protocols with the rat aorta and carotid artery

The ability of NOS inhibitors to block the basal activity of nitric oxide, which exerts a tonic depression of vasoconstriction, was assessed from the enhancement of phenylephrine-induced tone they produce in endothelium-containing rings of rat aorta or carotid artery (AL-Zobaidy et al., 2010; Frew et al., 1993). This involved obtaining a low-level contraction (~1–3 mN) to phenylephrine (10–30 nM) and then measuring the maximal enhancement of tone produced following the addition of ADMA, L-NMMA or L-NAME (100 μM for each). In some experiments, a second protocol was used to assess blockade of basal nitric oxide activity; this involved pretreating endothelium-containing rings for 1 h with L-NAME (100 μM) before constructing cumulative concentration–response curves to phenylephrine (1 nM–10 μM) and comparing the contractions obtained with those of time-matched controls.

All experiments involving endothelium-dependent, nitric oxide-mediated relaxation of aortic and carotid artery rings were conducted following contraction with concentrations of phenylephrine (30–100 nM) producing ~50% of the maximal response. Following stabilisation of tone, relaxation was induced by the cumulative addition of acetylcholine (1 nM–10 μM) or calcium ionophore A23187 (1 nM–1 μM). Some experiments were also conducted with superoxide dismutase (SOD, 0.1–300 U $ml^{-1}$), which produces endothelium-dependent relaxation through potentiation of basal nitric oxide activity by scavenging destructive superoxide anions (MacKenzie et al., 1999; Mian and Martin, 1995). All experiments with SOD were conducted in the presence of catalase (3600 U $ml^{-1}$) to prevent the accumulation of hydrogen peroxide. The endothelium-dependent component of relaxation resulting from the potentiation of basal nitric oxide activity by the phosphodiesterase isoform 5 inhibitor, T-0156 (1–300 nM) (Mochida et al., 2002), was also examined.

When the effects of the NOS inhibitors, L-NAME, L-NMMA or ADMA (each at 100 μM) were to be examined on endothelium-dependent relaxation to acetylcholine, A23187, SOD or T-0156, they were added for 1 h before contracting the tissues with phenylephrine. In all cases, the level of phenylephrine-induced tone was matched with that of the control experiments (*i.e.* contracted to ~50% of the maximum response); these tissues required less phenylephrine because of the enhancement of tone resulting from blockade of basal nitric oxide activity.

In some experiments, the effects of a 1 h pretreatment with the calmodulin inhibitor, calmidazolium (10 μM) (Illiano et al., 1992), or the PI-3 kinase inhibitor, wortmannin (100 nM) (Zeng and Quon, 1996), were examined on basal nitric oxide activity, assessed by the enhancement of phenylephrine-induced contraction induced by L-NAME (100 μM), and on the endothelium-dependent relaxation to acetylcholine, A23187, SOD or T-0156.

### 2.3. Drugs and chemicals

Acetylcholine chloride, ADMA (asymmetric $N^G$, $N^G$-dimethyl-L-arginine dihydrochloride), calmidazolium chloride, L-NAME ($N^G$-nitro-L-arginine methyl ester), L-NMMA ($N^G$-monomethyl-L-arginine acetate), papaverine hydrochloride, phenylephrine hydrochloride and superoxide dismutase (from bovine erythrocytes), were all obtained from Sigma, UK. Calcium ionophore A23187 and wortmannin were obtained from Enzo Life Sciences, UK. Catalase (bovine liver) was obtained from Calbiochem, UK. T-0156 (2-(2-methylpyridin-4-yl)methyl-4-(3,4,5-trimethoxyphenyl)-8-(pyrimidin-2-yl)methoxy-1,2-dihydro-1-oxo-2,7-naphthyridine-3-carboxylic acid methyl ester hydrochloride) was obtained from Tocris, UK. All drugs were dissolved and diluted in 0.9% saline, with the exceptions of calmidazolium, T-0156, ionophore A23187 and wortmannin, which were dissolved in DMSO (all 10 mM stocks).

### 2.4. Data analysis

Contractions were measured in milliNewtons. Vasodilator responses to agonists were calculated as a percentage of the maximum dilatation achieved by papaverine (300 μM), added at the end of each experiment. Data are expressed as the mean ± S.E.M. of n separate observations, each from a separate tissue. Graphs were drawn and statistical comparisons made using one-way analysis of variance and Bonferroni's post-test with the aid of a computer program, Prism (GraphPad, San Diego, USA). A probability (P) less than or equal to 0.05 was considered significant.

## 3. Results

### 3.1. Effects of ADMA on male and female aortic rings

Following induction of a low level of contraction (~3 mN) using phenylephrine (10–30 nM), subsequent addition of ADMA (100 μM) led to similar increases in tone in male and female endothelium-containing rings of rat aorta, consistent with inhibition of basal nitric oxide activity (Fig. 1a).

Following induction of ~50% maximal phenylephrine (30–100 nM)-induced tone, acetylcholine (1 nM–10 μM) produced concentration-dependent relaxation in male ($E_{max}$ 95.2 ± 1.9%; $pEC_{50}$ 7.15 ± 0.03) and female ($E_{max}$ 77.5 ± 7.8%; $pEC_{50}$ 7.30 ± 0.03) endothelium-containing aortic rings (Fig. 1b). These tissues were then washed, treated with ADMA (100 μM, 1 h) and then constricted to the same level of tone as before by using lower concentrations of phenylephrine (10–30 nM). In the presence of ADMA, maximal relaxation was unaffected ($E_{max}$ 86.5 ± 2.2% and 77.3 ± 3.7% for male and female, respectively), but there was a ~2-fold depression in sensitivity to

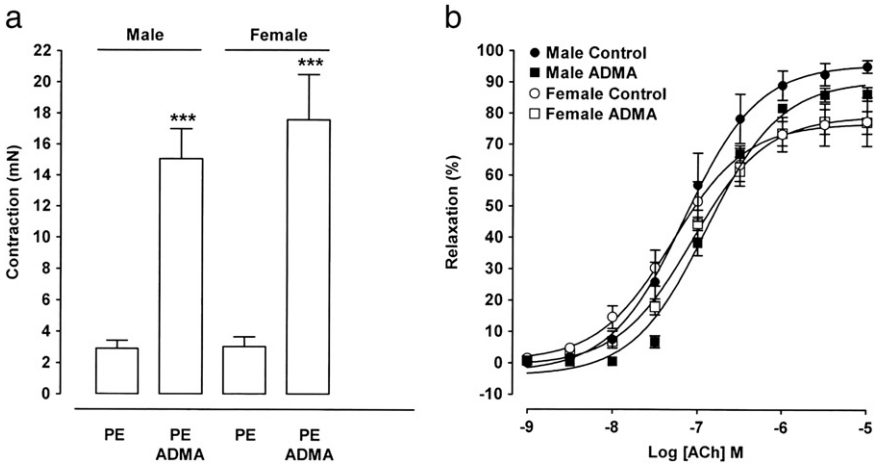


**Fig. 1.** (a) ADMA (100 μM) enhanced submaximal phenylephrine-induced tone in rings of both male and female rat aorta, consistent with inhibition of basal nitric oxide activity. (b) ADMA (100 μM) had no effect on maximal relaxation, but produced a modest (~2-fold) reduction in sensitivity to acetylcholine in rings of both male and female rat aorta. Data are the mean ± S.E.M. of 6–8 observations. *** $P<0.001$ indicates a significant enhancement of phenylephrine-induced tone by ADMA.

acetylcholine ($pEC_{50}$ 6.89 ± 0.08 and 7.06 ± 0.04 for male and female, respectively).

### 3.2. Effects of L-NAME, L-NMMA and ADMA on female carotid artery rings

Following induction of a low level of contraction (~1 mN) in female carotid artery rings using phenylephrine (10–30 nM), subsequent addition of L-NAME, L-NMMA or ADMA (each 100 μM), led to similar increases in tone, consistent with inhibition of basal nitric oxide activity (Fig. 2a).

Following induction of ~50% maximal phenylephrine (30–100 nM)-induced tone in carotid artery rings, acetylcholine (1 nM–10 μM) produced concentration-dependent relaxation ($E_{max}$ 82.9 ± 2.3%; $pEC_{50}$ 6.98 ± 0.03; Fig. 2b). In experiments conducted at the same level of phenylephrine-induced tone as before, L-NAME (100 μM) powerfully blocked acetylcholine-induced relaxation ($E_{max}$ 15.2 ± 5.1%; $pEC_{50}$ 6.91 ± 0.37), but L-NMMA (100 μM) or ADMA (100 μM) had little effect ($E_{max}$ 82.8 ± 5.1% and $pEC_{50}$ 6.73 ± 0.07; $E_{max}$ 79.6 ± 5.3% and $pEC_{50}$ 6.90 ± 0.07 for L-NMMA and ADMA, respectively). Increasing the concentration of L-NMMA or ADMA to 1 mM also failed to block acetylcholine-induced relaxation significantly (data not shown).

The PI-3 kinase inhibitor, wortmannin (100 nM), had no effect on acetylcholine (1 nM–10 μM)-induced relaxation, but the calmodulin inhibitor, calmidazolium (10 μM), produced powerful blockade (Fig. 2c).

### 3.3. Effects of L-NAME, L-NMMA and ADMA on relaxation to SOD or T-0156 in female aortic rings

SOD (1–300 U $ml^{-1}$) induced concentration-dependent relaxation in endothelium-containing but not in endothelium-denuded rings of female rat aorta (Fig. 3a). The NOS inhibitors, L-NAME, L-NMMA or ADMA (all 100 μM) each powerfully inhibited SOD-induced relaxation (Fig. 3b). Neither wortmannin (100 nM) nor calmidazolium (10 μM) alone had any effect on SOD-induced relaxation, but the combination of the two produced a small, but significant inhibition (Fig. 3c).

The PDE 5 inhibitor, T-0156 (1–300 nM), induced concentration-dependent relaxation that was greater in endothelium-containing than in endothelium-denuded rings of female rat aorta (Fig. 4a). L-NAME, L-NMMA or ADMA (all 100 μM) each powerfully inhibited the endothelium-dependent component of relaxation to T-0156 (Fig. 4b). Neither wortmannin (100 nM) nor calmidazolium (10 μM) alone had any effect on the endothelium-dependent component of

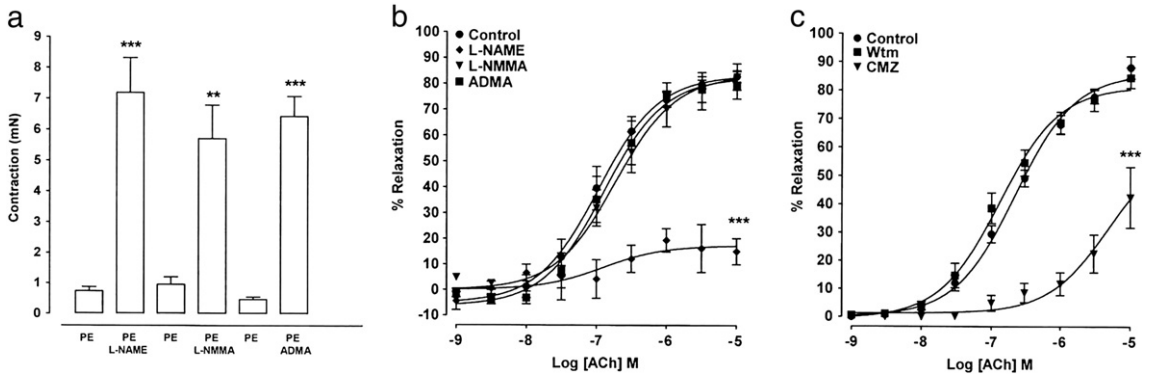


**Fig. 2.** (a) L-NAME, L-NMMA and ADMA (all 100 μM) each enhanced submaximal phenylephrine-induced tone in rings of female carotid artery, consistent with inhibition of basal nitric oxide activity. (b) L-NAME (100 μM) powerfully blocked acetylcholine-induced relaxation in rings of female carotid artery, but L-NMMA (100 μM) or ADMA (100 μM) had little effect. (c) Wortmannin (Wtm; 100 nM) had little effect on acetylcholine-induced relaxation, but calmidazolium (CMZ; 10 μM) produced powerful blockade. Each point is the mean ± S.E.M. of 5–10 observations. ** $P<0.01$ and *** $P<0.001$ indicate significant differences from respective controls.

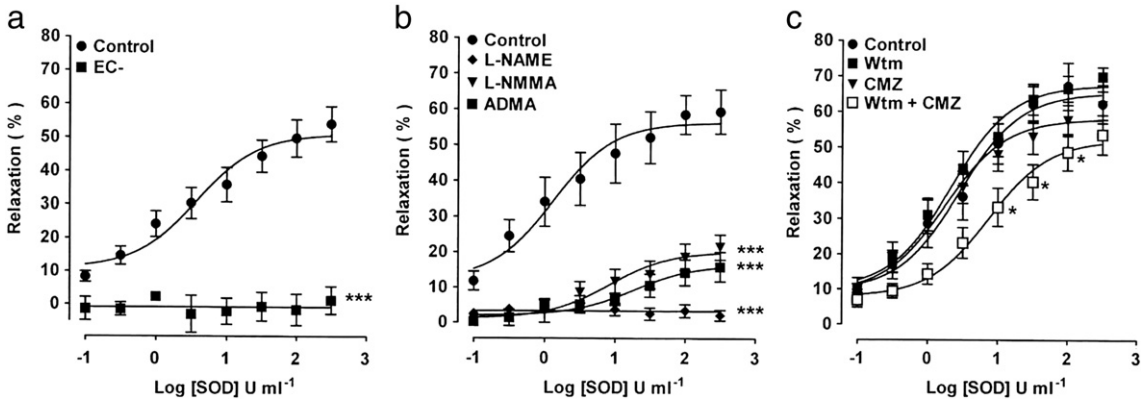


**Fig. 3.** (a) Superoxide dismutase (SOD; 1–300 U $ml^{-1}$) induced concentration-dependent relaxation in endothelium-containing (control) but not in endothelium-denuded (EC-) rings of female rat aorta. (b) L-NAME, L-NMMA and ADMA (all 100 μM) each produced powerful blockade of SOD-induced relaxation. (c) Neither wortmannin (Wtm; 100 nM) nor calmidazolium (CMZ; 10 μM) alone had any effect on SOD-induced relaxation, but the combination of the two produced significant blockade. Each point is the mean ± S.E.M. of 5–6 observations. * $P<0.05$ and *** $P<0.001$ indicate significant differences from respective controls.

relaxation to T-0156, but the combination of the two produced a small, but significant inhibition (Fig. 4c).

### *3.4. Effects of wortmannin and calmidazolium on the suppression of phenylephrine-induced tone by basal nitric oxide activity in female aortic rings*

Phenylephrine (1 nM–10 μM) induced concentration-dependent contraction of endothelium-containing rings of female rat aorta (Fig. 5). Treatment with L-NAME (100 μM) powerfully enhanced both the sensitivity to phenylephrine and the maximal contraction obtained, consistent with inhibition of basal nitric oxide activity. Wortmannin (100 nM; Fig. 5a) or calmidazolium (10 μM; Fig. 5b) alone each also induced a significant enhancement of phenylephrine-induced tone, but these were smaller in magnitude to that seen with L-NAME. In the combined presence of wortmannin and calmidazolium, the enhancement of phenylephrine-induced tone was still smaller than that seen with L-NAME (Fig. 5c). The ability of wortmannin or calmidazolium either alone or in combination to enhance phenylephrine-induced tone was lost in tissues treated with L-NAME.

### *3.5. Effects of L-NAME, L-NMMA and ADMA on relaxation to A23187 in female aortic rings*

The calcium ionophore, A23187 (1 nM–1 μM), induced concentration-dependent relaxation of female aortic rings (Fig. 6a). L-NAME (100 μM) powerfully blocked A23187-induced relaxation, but L-NMMA (100 μM) or ADMA (100 μM) had little effect. Wortmannin (100 nM) had no effect on A23187-induced relaxation, but calmidazolium (10 μM) produced powerful blockade (Fig. 6b).

## 4. Discussion

The new observations provided by this study support and extend the previous seemingly anomalous findings that the methylarginines, L-NMMA and ADMA, powerfully block basal activity of nitric oxide in rat aorta, but have little effect on acetylcholine-stimulated activity (AL-Zobaidy et al., 2010; Frew et al., 1993). These unexpected actions contrast with those of the more typical NOS inhibitors, L-NOARG and L-NAME, which block both basal and agonist-stimulated activity.

In many isolated blood vessels, a basal nitric oxide activity is present which exerts a tonic endothelium-dependent depression of vasoconstriction and is typically uncovered by observing a rise in submaximal vasoconstrictor-induced tone produced upon addition of an agent that inhibits either the action or synthesis of nitric oxide (Martin et al., 1986b; Moore et al., 1990; Rees et al., 1989). In rat aorta, this vasodepressant action of basal nitric oxide suppresses constriction to a wide range of agents including phenylephrine, 5-hydroxytryptamine and prostaglandin $F_{2\alpha}$. Previous work demonstrating the ability of the methylarginines to inhibit selectively basal over acetylcholine-induced activity of nitric oxide was conducted on female rat aorta (AL-Zobaidy et al., 2010; Frew et al., 1993), but

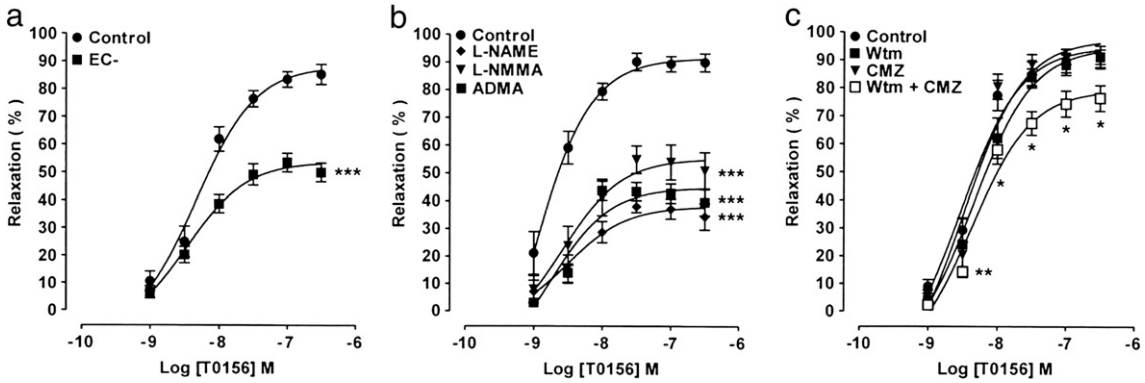


**Fig. 4.** (a) The PDE 5 inhibitor, T-0156 (1–300 nM), induced greater concentration-dependent relaxation in endothelium-containing (control) than in endothelium-denuded (EC-) rings of female rat aorta. (b) L-NAME, L-NMMA and ADMA (all 100 μM) each produced powerful blockade of the endothelium-dependent component of T-0156-induced relaxation. (c) Neither wortmannin (Wtm; 100 nM) nor calmidazolium (CMZ; 10 μM) alone had any effect on T-0156-induced relaxation, but the combination of the two produced significant blockade. Each point is the mean ± S.E.M. of 6 observations. * $P<0.05$, ** $P<0.01$ and *** $P<0.001$ indicate significant differences from respective controls.

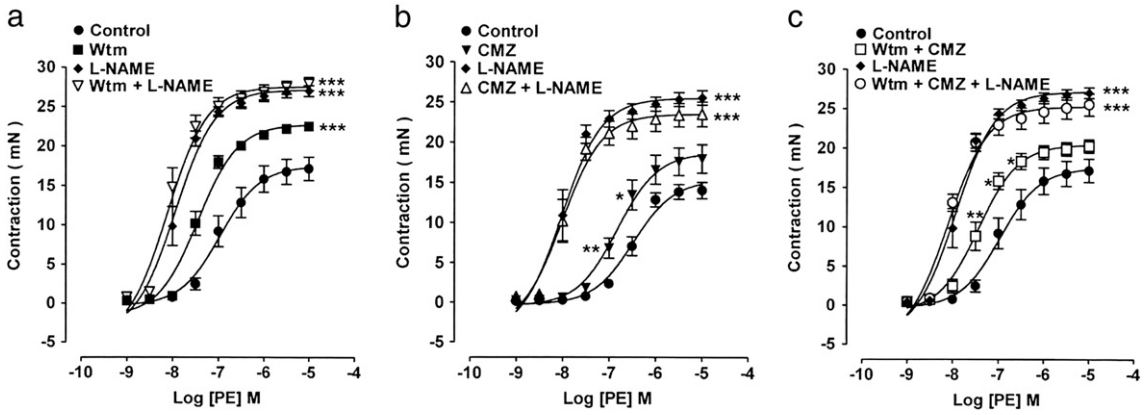


**Fig. 5.** The concentration-dependent contractile effect of phenylephrine (1nM–10 μM) in rings of female rat aorta was enhanced following pretreatment with L-NAME (100 μM), consistent with blockade of basal nitric oxide activity. Wortmannin (Wtm; 100 nM) and calmidazolium (CMZ; 10 μM), either alone or in combination, enhanced phenylephrine-induced contraction in control tissues, but not in those treated with L-NAME. The magnitude of the enhancement of phenylephrine-induced tone by wortmannin and calmidazolium, either alone or in combination was smaller than that seen with L-NAME. Each point is the mean ± S.E.M. of 5–7 observations. * $P<0.05$, ** $P<0.01$ and *** $P<0.001$ indicate significant differences from respective controls.

the present study shows that this holds also for male rat aorta. In the female rat carotid artery too, L-NMMA and ADMA both enhance submaximal phenylephrine-induced tone, consistent with blockade of basal nitric oxide activity, but have almost no effect on relaxation to acetylcholine, whereas L-NAME inhibits both. Our previous reports showed that the poor ability of L-NMMA or ADMA to block acetylcholine-induced relaxation did not result from use of too low a concentration, because in each case 1 mM was just as ineffective as 100 μM (AL-Zobaidy et al., 2010; Frew et al., 1993), and this was confirmed in the present study. Thus, the anomalous actions of these methylarginines in rat vessels appear not to be gender- or site-specific.

Although basal nitric oxide activity is most commonly observed by the ability of NOS inhibitors to elevate submaximal vasoconstrictor tone (Martin et al., 1986b; Moore et al., 1990; Rees et al., 1989), it can also be uncovered using a variety of different approaches. For example, SOD and PDE 5 inhibitors induce endothelium-dependent relaxation, not by stimulating nitric oxide production in the manner used by agonists such as acetylcholine, but by enhancing the activity of basal nitric oxide. Specifically, SOD induces concentration-dependent, endothelium-mediated dilatation by potentiating basal nitric oxide activity (MacKenzie et al., 1999; Mian and Martin, 1995; Ohlstein and Nichols, 1989) through removal of destructive superoxide anions (Gryglewski et al., 1986; Rubanyi and Vanhoutte, 1986). While selective PDE 5 inhibitors, such as zaprinast (M&B 22948), T-1032 and T-0156, can induce relaxation in endothelium-denuded vessels, they are much more effective in endothelium-containing vessels where they potentiate basal nitric oxide activity by slowing the hydrolysis of its second messenger, cyclic GMP (Martin et al., 1986a; Mochida et al., 2002, 2004). Using these alternative approaches in the present study, we found that the endothelium-dependent relaxant actions of SOD and T-0156 were powerfully inhibited by the methylarginines, L-NMMA and ADMA, as well as by L-NAME. Thus, regardless of whether basal activity of nitric oxide in rat aorta is assessed through its ability to suppress vasoconstrictor tone or from the relaxant actions of SOD or PDE 5 inhibitors, its synthesis is clearly blocked by L-NMMA and ADMA.

In stark contrast to their ability to block basal nitric oxide activity, L-NMMA and ADMA have little effect on relaxation to acetylcholine, so long as experiments are conducted at similar levels of submaximal tone used in control experiments (AL-Zobaidy et al., 2010; Frew et al., 1993). If, however, similar concentrations of vasoconstrictor agents are used in L-NMMA- or ADMA-treated preparations as in control tissues, the resulting elevation of tone through inhibition of basal nitric oxide activity in the former group produces an apparent blockade of acetylcholine-induced relaxation as a consequence of over-constriction, *i.e.* through physiological antagonism (AL-Zobaidy et al., 2010). It is therefore possible that previous reports of acetylcholine-induced relaxation being blocked by these methylarginines in rat

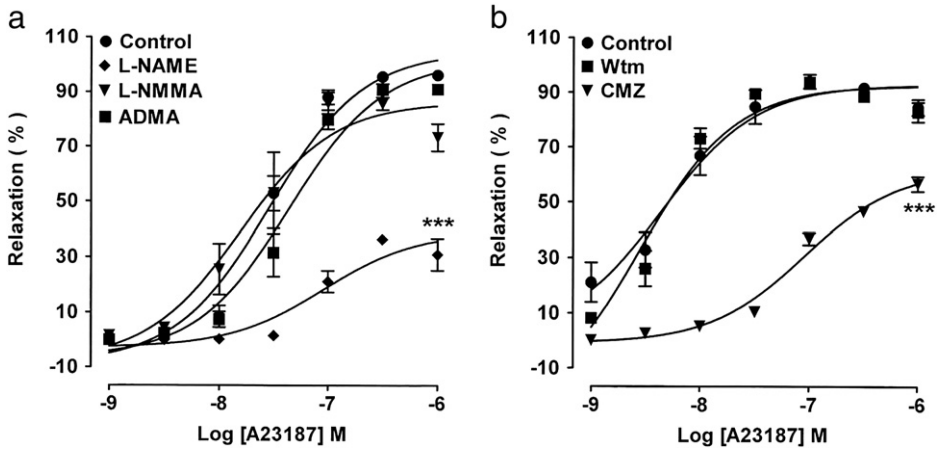


**Fig. 6.** Calcium ionophore, A23187 (1 nM–1 μM) induced concentration-dependent relaxation in endothelium-containing rings of female rat aorta. (a) L-NAME (100 μM) powerfully blocked A23187-induced relaxation, but L-NMMA (100 μM) or ADMA (100 μM) had little effect. (b) Wortmannin (Wtm; 100 nM) had no effect on A23187-induced relaxation, but calmidazolium (CMZ; 10 μM) produced powerful blockade. Each point is the mean ± S.E.M. of 5–7 observations. *** $P<0.001$ indicates a significant difference from respective controls.

aorta arose from a failure to match the levels of tone in control and treated preparations (Feng et al., 1998; Jin and D'Alecy, 1996; Vallance et al., 1992).

Acetylcholine is not the only endothelial agonist whose ability to promote relaxation appears largely unaffected by these methylarginines; ATP-induced relaxation is similarly refractory (Frew et al., 1993), and the present study extends this to calcium ionophore A23187, which induces endothelium-dependent relaxation by a receptor-independent mechanism. Thus, regardless of the endothelial activator used (acetylcholine, ATP, A23187), agonist-stimulated activity of nitric oxide in rat vessels is little affected by L-NMMA or ADMA, whereas basal activity is powerfully blocked.

An explanation for the seemingly anomalous ability of L-NMMA and ADMA to block selectively basal over agonist-stimulated activity of nitric oxide in rat vessels is not immediately apparent. We are confident that interference by the other endothelium-derived vasodilators, prostacyclin and EDHF, is not involved, because inhibitors of these agents, indomethacin (cyclooxygenase blocker) and the combination of apamin and TRAM-34, respectively, have no effect in rat aorta (unpublished observations). Other workers, employing aorta from eNOS or nNOS knockout mice, or studying the effects in intact rats or human volunteers of S-methyl-L-thiocitrulline (SMTC), a putatively selective inhibitor of nNOS, have proposed that basal nitric oxide is derived from nNOS, whereas that induced by agonists is produced by eNOS (Melikian et al., 2009; Nangle et al., 2004; Seddon et al., 2009; Wakefield et al., 2003). Thus, the ability of L-NMMA and ADMA to block selectively basal nitric oxide activity could potentially be explained if these agents powerfully blocked the nNOS responsible for this source, but had less effect on the eNOS responsible for agonist-stimulated activity.

An alternative explanation for the anomalous effects of L-NMMA and ADMA might arise, however, from differences in the transduction pathways governing basal and stimulated production of nitric oxide by NOS. It is well established that agonists such as acetylcholine, ATP and A23187 stimulate nitric oxide production via the calcium-calmodulin pathway (Archer and Cowan, 1991; Schini and Vanhoutte, 1992), whereas flow-mediated dilatation resulting from shear stress does so through activation of the phosphatidylinositol-3 (PI-3) kinase/Akt pathway (Dimmeler et al., 1999; Fulton et al., 1999). Our finding that the calmodulin inhibitor, calmidazolium, inhibits acetylcholine and A23187-induced relaxation in rat vessels, but the PI-3 kinase inhibitor, wortmannin, does not, is consistent with these previous observations. The mechanisms regulating basal production of nitric oxide are less well understood, however. Those originally describing the existence of a basal nitric oxide activity opposing vasoconstrictor tone believed it to arise from the spontaneous, *i.e.* unstimulated release of nitric oxide from the endothelium (Martin et al., 1986b; Moore et al., 1990; Rees et al., 1989). Indeed, this conclusion is supported by the finding that in the absence of any stimulating agent, the cyclic GMP content of endothelium-containing rings of rat aorta is 2–3-fold higher than endothelium-denuded rings (Rapoport and Murad, 1983). Others have suggested that basal nitric oxide activity might actually reflect indirect stimulation of the endothelial cells to release nitric oxide. For example, the elevated level of calcium associated with smooth muscle contraction may spill over into the endothelium through myo-endothelial gap junctions, thus stimulating nitric oxide production (Dora et al., 2000; Jackson et al., 2008). Alternatively, it has been suggested that the mechanical stress resulting from isometric contraction can activate eNOS by a calcium-independent, PI-3 kinase/Akt-dependent mechanism analogous to that underlying flow-mediated dilatation (Fleming et al., 1999). We therefore made use of calmidazolium and wortmannin to investigate the origins of basal nitric oxide activity in rat aorta. Indeed, we found that both agents enhanced submaximal phenylephrine-induced tone significantly, indicating that each blocks basal nitric oxide activity, but the magnitude of these effects was very small compared to the powerful effects of L-NAME. Furthermore, neither calmidazolium nor wortmannin alone had any effect on SOD- or T-0156-induced relaxation which occur by potentiating basal nitric oxide activity, but the combination of the two transduction inhibitors did produce a small degree of blockade. Thus, in rat aorta, it would appear that the calcium–calmodulin and PI-3 kinase/Akt pathways each make a minor contribution to basal nitric oxide activity, with the greater part occurring either through other as yet indentified pathways or from tonic, unstimulated activity of NOS. Differences in the mechanisms controlling basal and agonist-stimulated production of nitric oxide might therefore account for the selective actions of the methylarginines.

## 5. Conclusions

Our new observations with calcium-ionophore A23187, SOD and T-0156 support the previous conclusions that the methylarginines, L-NMMA and ADMA, preferentially block basal over agonist-stimulated activity of nitric oxide in rat aorta (AL-Zobaidy et al., 2010; Frew et al., 1993). Moreover, they show that this seemingly anomalous phenomenon originally observed in female rat aorta extends also to the male aorta and to the carotid artery.

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