Differential sensitivity of basal and acetylcholine-induced activity of nitric oxide to blockade by asymmetric dimethylarginine (ADMA) in the rat aorta

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Summary

Background and purpose: Previous work has shown that N^G-monomethyl-L-arginine (L-NMMA) paradoxically inhibits basal but not acetylcholine-stimulated activity of nitric oxide in rat aorta. The aim of this study was to determine if the endogenously produced agent, asymmetric N^G, N^G-dimethyl-L-arginine (ADMA), also exhibits this unusual selective blocking action.

Experimental approach: The effect of ADMA on basal nitric oxide activity was assessed by examining its ability to enhance phenylephrine-induced tone in endothelium-containing rings. Its effect on acetylcholine-induced relaxation was assessed both in conditions where ADMA greatly enhanced phenylephrine tone and where tone was carefully matched with control tissues at a range of different levels.

Key results: ADMA (100 μM) potentiated phenylephrine-induced contraction, consistent with inhibition of basal nitric oxide activity. Higher concentrations (300-1000 μM) had no greater effect. Although ADMA (100 μM) also appeared to block acetylcholine-induced relaxation when it enhanced phenylephrine tone to maximal levels, virtually no block was seen at intermediate levels of tone in the presence of ADMA. Even ADMA at 1000 μM had no effect on the maximal relaxation to acetylcholine, although it produced a small (2-3-fold) reduction in sensitivity. ADMA and L-NMMA, like L-arginine (all at 1000 μM), protected acetylcholine-induced relaxation against blockade by L-NAME (30 μM).

Conclusions and implications: In the rat aorta, ADMA, like L-NMMA, blocks basal activity of nitric oxide but has little effect on that stimulated by acetylcholine. Further studies are required to explain these seemingly anomalous actions of ADMA and L-NMMA.

Abbreviations: ADMA, asymmetric N^G, N^G-dimethyl-L-arginine; L-NAME, N^G-nitro-L-arginine methyl ester; L-NMMA, N^G-monomethyl-L-arginine; NOS, nitric oxide synthase; SDMA, symmetric N^G, N'^G-dimethyl-L-arginine.

Introduction

It is well established that one of the two equivalent guanidino nitrogens of L-arginine is utilised by nitric oxide synthase (NOS) in the synthesis of nitric oxide (Palmer et al., 1988). Since this discovery, an extensive range of guanidino (N^G)-substituted analogues of L-arginine have been developed as inhibitors of NOS (Rees et al., 1989; Moore et al., 1990; Rees et al., 1990; Hobbs et al., 1999) and these have become valuable tools for investigating the role of the L-arginine-nitric oxide system in biological processes. Amongst the earliest examples developed were N^G-monomethyl-L-arginine (L-NMMA), N^G-nitro-L-arginine (L-NOARG) and N^G-nitro-L-arginine methyl ester (L-NAME). These remain the most commonly used investigational tools in experimental biology, probably because they are effective blockers of all three isoforms of NOS, i.e. the endothelial (eNOS), neuronal (nNOS) and inducible (iNOS) forms. In the vast majority of cases, these three agents are not metabolised by NOS, but act as classical competitive inhibitors, whose actions can be either prevented or reversed by an excess of the substrate, L-arginine. Of these three agents, however, L-NMMA alone has frequently been reported to exhibit properties that are inconsistent with it acting as a simple competitive inhibitor. For example, L-NMMA acts as an alternative substrate and mechanism-based suicide inhibitor of iNOS in murine macrophages (Olken & 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 & Martin, 2007). Most surprisingly of all, however, is that L-NMMA selectively blocks basal but not agonist (acetylcholine or ATP)-induced activity of nitric oxide in rat aorta (Frew et al., 1993). Furthermore, this study showed that pretreatment with L-NMMA protects agonist-induced nitric oxide activity against blockade by L-NOARG in a manner similar to L-arginine, suggesting it might act as an alternative substrate for eNOS. Indeed, chemiluminescence detection has confirmed that L-NMMA, like L-arginine, but unlike L-NOARG, can fuel nitric oxide synthesis in rat aorta and pulmonary artery (Archer & Hampl, 1992).

Renewed interest in the biological actions of methylarginines was sparked by the discovery that L-NMMA, asymmetric N^G, N^G-dimethyl-L-arginine (ADMA) and symmetric N^G, N'^G-dimethyl-L-arginine (SDMA) are produced endogenously during the proteolytic digestion of methylated proteins (Vallance et al., 1992; Leiper & Vallance, 2006). These authors showed also that ADMA, like L-NMMA, inhibits NOS but SDMA does not. Moreover, while the plasma concentration of L-NMMA is low, levels of ADMA and SDMA are around 10-times higher and accumulate further in renal failure (Vallance et al., 1992) and a wide range of pathological conditions associated with vascular dysfunction, including hypertension, pulmonary hypertension, atherosclerosis, diabetes mellitus and preeclampsia (Siroen et al., 2006; Leiper & Vallance, 2006).

In view of the growing evidence for the involvement of ADMA in cardiovascular disease, the aim of this study was to determine if this agent shared with L-NMMA the

ability to block selectively basal but not acetylcholine-stimulated activity of nitric oxide in rat aorta.

Methods

Preparation of aortic rings and tension recording

The preparation of rat aortic rings for tension recording was essentially similar to a previous study (Frew et al., 1993). Briefly, female Wistar rats weighing 150-200 g were killed by stunning and exsanguination. The aorta was 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. The aortic rings were mounted under 10 mN resting tension 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).

Experimental protocols with the rat aorta

The ability of ADMA to block basal activity of nitric oxide was assessed from its enhancement of agonist-induced contraction in endothelium-containing aortic rings (Frew et al., 1993). This was performed in two ways. The first, by obtaining a low-level contraction (~2-5 mN) to phenylephrine (30-60 nM) and then measuring the enhancement of tone produced by a range of ADMA concentrations (0.3-300 μM),

each for 1 h. The ability of L-arginine (10 mM, 1 h) to either protect against or reverse the enhancement by ADMA (100 μM) was also assessed. The effects of ADMA were also examined on phenylephrine-induced tone in endothelium-denuded rings. We also determine if submaximal tone induced by 5-hydroxytryptamine (3 μM) or by prostaglandin $F_{2\alpha}$ (3 μM) in endothelium-containing rings was enhanced when subsequently treated with ADMA (100 μM). The second protocol for examining blockade of basal nitric oxide activity involved pretreating endothelium-containing rings for 1 h with ADMA (100, 300 or 1000 μM) and then constructing full concentration-response curves to phenylephrine (1 nM-10 μM), together with time-matched controls.

Agonist-stimulated activity of nitric oxide was assessed by measuring the relaxation to acetylcholine (1 nM-10 μM) in endothelium-containing rings. When the effects of ADMA or other NOS inhibitors were to be examined, they were added for 1 h before construction of the acetylcholine concentration-response curve. As will be seen in the Results, the level of phenylephrine tone was critical in determining the magnitude of acetylcholine-induced relaxation both in control and ADMA-treated tissues. In some experiments with ADMA, its effects on relaxation were examined after it had enhanced phenylephrine-induced tone, whereas in others, the tone was carefully matched with those of control tissues at low, intermediate or maximal levels. In other experiments, the ability of ADMA (100 μM) to affect acetylcholine-induced relaxation was also assessed on sub-maximal tone induced by 5-hydroxytryptamine or prostaglandin $F_{2\alpha}$.

In some experiments, L-NAME (30 μM, 1h) was used to inhibit maximal acetylcholine (1 nM-10 μM)-induced relaxation by ~ 50 %. In these, the ability of L-arginine, L-NMMA, ADMA or SDMA (all at 1000 μM; pre-treatment or post-treatment for 1h) either to protect against or reverse this inhibition by L-NAME was also assessed.

Drugs and chemicals

Acetylcholine chloride, ADMA (asymmetric N^G, N^G-dimethyl-L-arginine dihydrochloride), L-arginine hydrochloride, 5-hydroxytryptamine, L-NAME (N^G-nitro-L-arginine methyl ester), L-NMMA (N^G-monomethyl-L-arginine acetate), papaverine hydrochloride, phenylephrine hydrochloride and prostaglandin $F_{2\alpha}$ were all obtained from Sigma, UK. SDMA (symmetric N^G, N'^G-dimethyl-L-arginine dihydrochloride) was obtained from Enzo Life Sciences, UK. All drugs were dissolved and diluted in 0.9% saline.

Data analysis

Contractions were measured in milliNewtons. Papaverine (300 μM) was added at the end of each experiment to fully relax tissues, and acetylcholine-induced relaxations were calculated as % relaxation of phenylephrine-induced tone with respect to this baseline. Data are expressed as the mean ± s.e.mean 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.

Results

Effects of ADMA on basal nitric oxide activity

Following induction of a low level of contraction (3.1 ± 0.6 mN) of endothelium-containing rings of rat aorta using phenylephrine (30-60 nM), subsequent addition of ADMA (0.3-300 μM) led to an immediate further elevation of tone, consistent with inhibition of basal nitric oxide activity (Figure 1 and Figure 2b). The threshold concentration was 0.3-1 μM and the maximum was obtained at 100 μM. The enhancement of phenylephrine-induced contraction by ADMA (100 μM) was both prevented and reversed by treatment with L-arginine (10 mM). ADMA did not enhance phenylephrine-induced contraction in endothelium-denuded rings. In contrast to ADMA, SDMA (100 μM) had no effect on phenylephrine-induced contraction in endothelium-containing rings. Sub-maximal contraction induced by 5-hydroxytryptamine (3 μM) or prostaglandin $F_{2\alpha}$ (3 μM) was also enhanced by ADMA (100 μM).

A full concentration-response curve to phenylephrine (1 nM-10 μM) in endothelium-containing rings gave an E_{max} of 14.4 ± 1.5 mN and a pEC_{50} of 6.75 ± 0.07 (Figure 2). Pretreatment with ADMA (100 μM) for 1 h enhanced both the E_{max} (22.2 ± 0.8 mN) and sensitivity (pEC_{50} 7.88 ± 0.04) to phenylephrine. Increasing the concentration of ADMA to 300 or 1000 μM produced little additional enhancement.

Effects of ADMA on acetylcholine-induced relaxation

Following induction of intermediate phenylephrine-induced tone (11.9 ± 2.1 mN) on endothelium-containing rings, acetylcholine (1 nM-10 μM) produced concentration-dependent relaxation (E_{max} 77.5 ± 7.8 %; pEC_{50} 7.30 ± 0.03; Figure 3a and b). When

ADMA (100 μM, 1 h) was added on top of this intermediate phenylephrine-induced contraction, raising tone to a higher level (18.8 ± 1.5 mN), acetylcholine-induced relaxation was depressed (E_{max} 22.1 ± 3.1 %; pEC_{50} 6.91 ± 0.04). However, when these same tissues were washed, with restoration of intermediate phenylephrine-induced tone (7.6 ± 0.9 mN) in the presence of ADMA, the blockade of acetylcholine-induced relaxation was almost completely abolished: maximal relaxation was similar to controls, but there was a 1.7-fold reduction in sensitivity (E_{max} 77.6 ± 4.0 %; pEC_{50} 7.06 ± 0.04). Concentrations of ADMA below 100 μM at sub-maximal phenylephrine-induced tone had no effect on acetylcholine-induced relaxation. ADMA (100 μM) at intermediate levels of tone induced by 5-hydroxytryptamine or prostaglandin $F_{2\alpha}$ also failed to affect maximal acetylcholine-induced relaxation, but produced modest reductions in sensitivity of 2.6- and 2.3-fold (data not shown).

These findings prompted us to examine the effect the level of tone had on the magnitude of acetylcholine induced relaxation in control and ADMA (100 μM)-treated rings. In control rings, acetylcholine (1 nM-10 μM)-induced relaxation was similar at two different levels of submaximal phenylephrine-induced tone (3.7 ± 0.4 and 10.3 ± 0.5 mN; Figure 4a). Relaxation was, however, significantly depressed at a maximal level of tone (14.1 ± 0.5 mN). The ability of ADMA (100 μM) to enhance phenylephrine-induced contraction permitted examination of a wider range of tone levels in the presence of this agent. At levels of phenylephrine-induced tone of 4.1 ± 0.6 or 10.2 ± 0.5 mN in the presence of ADMA, maximal acetylcholine-induced relaxation was not significantly different from controls at similar levels of tone (Figure 4a and b). However at 15.7 ± 0.5 mN tone, acetylcholine-induced relaxation was depressed, but to the same extent as control tissues at the same level of tone. At

20.3 ± 0.7 mN and 24.7 ± 0.6 mN tone, which cannot be achieved in control tissues, acetylcholine-induced relaxation was even more depressed.

With a higher concentration of ADMA (1000 μM, 1 hr) at intermediate phenylephrine-induced tone, the E_{max} to acetylcholine was unaffected, but there was a 2.0-fold decrease in sensitivity when compared to controls (Figure 5; Table 1). Testing these tissues again at 3 h and 4.5 h showed a slight progressive decrease in sensitivity to acetylcholine in controls, but in ADMA-treated tissue the E_{max} remained unchanged when compared to time-matched controls, with a roughly similar decrease in sensitivity (3-fold) to that seen at 1 h.

Effects of ADMA on the blockade of acetylcholine-induced relaxation by L-NAME

In the presence of intermediate phenylephrine-induced tone, L-NAME (30 μM, 1 h) blocked maximal acetylcholine (1 nM-10 μM)-induced relaxation by ~50 % (Figure 6). This blockade was both largely prevented and reversed by treatment with L-arginine (1000 μM; Figure 6a). L-NMMA (1000 μM) induced a 2.6-fold reduction in sensitivity to acetylcholine without affecting the E_{max} and, like L-arginine, both prevented and reversed the blockade induced by L-NAME (Figure 6b; Table 1). ADMA (1000 μM) too produced significant protection but did not reverse the blockade induced by L-NAME (Figure 6c). SDMA (1000 μM) had no effect by itself on acetylcholine-induced relaxation and did not prevent or reverse the blockade induced by L-NAME (Figure 6d; Table 1).

Discussion

The main new finding in this study is that while ADMA blocks basal activity of nitric oxide in rat aorta, it has little effect on that stimulated by acetylcholine. These seemingly anomalous findings parallel the actions of L-NMMA in rat aorta (Frew et al., 1993), but are in stark contrast to those of L-NOARG and L-NAME which, as expected of typical NOS inhibitors, uniformly abolish both basal and agonist (acetylcholine or ATP)-stimulated activity of nitric oxide.

Basal nitric oxide activity exerts a tonic endothelium-dependent vasodilator influence in many isolated blood vessels and is typically assessed by observing the rise in submaximal vasoconstrictor-induced tone produced by agents that inhibit either the action or synthesis of nitric oxide (Martin et al., 1986; Rees et al., 1989; Moore et al., 1990). These authors, working on rat and rabbit aorta and rat mesentery concluded that this basal vasodilator influence opposing vasoconstriction resulted from the spontaneous (i.e. unstimulated) release of nitric oxide from the endothelium. In these tissues there was no evidence that the constricting adrenoceptor agonists used had stimulated release of nitric oxide through activation of endothelial α_2-adrenoceptors as occurs in canine and porcine coronary and pulmonary arteries (Cocks & Angus, 1983; Miller & Vanhoutte, 1985). Others, have instead suggested that vascular smooth muscle contraction may indirectly stimulate endothelial cells to release nitric oxide via a signal transmitted either through myoendothelial gap junctions (Jackson et al., 2008; Dora et al., 2000) or by mechanical stress (Fleming et al., 1999). However, in endothelium-containing untreated rings of rat aorta, cyclic GMP levels are 2-3-fold higher than in endothelium-denuded rings (Rapoport & Murad, 1983), thus demonstrating that the tonic influence of basal nitric oxide is present in the complete

absence of smooth muscle activation. Nevertheless, regardless of the mechanisms governing the basal activity of nitric oxide, there is general agreement that NOS inhibitors enhance vasoconstrictor tone by abolishing its inhibitory influence. In keeping with a previous report (Vallance et al., 1992), we found that the endogenously produced NOS inhibitor, ADMA, also enhances vasoconstrictor tone induced by a number of agents (phenylephrine, 5-hydroxytryptamine or prostaglandin $F_{2\alpha}$) in rat aorta. Both studies report a threshold effect for ADMA at 0.3-1 μM, consistent with concentrations found in the plasma in a number of disease states (Vallance et al., 1992; Siroen et al., 2006), and a maximal effect at ~100 μM. They also show that the inactive isomer, SDMA, lacks the ability of ADMA to enhance vasoconstrictor tone. Moreover, the ability of the endogenous substrate, L-arginine, to both protect against and reverse blockade of basal nitric oxide activity by ADMA shows it occurs by simple competitive antagonism.

Our most striking new finding is that ADMA, at a concentration of 100 μM which abolishes basal nitric oxide activity, has virtually no effect on acetylcholine-induced relaxation. Although ADMA appeared to produce blockade under conditions where it had potentiated phenylephrine-induced tone to near maximal levels, this was almost certainly due to physiological antagonism rather than blockade of NOS, since matching this increased tone in control tissues with additional phenylephrine produced a similar degree of blockade. When phenyleprine-induced tone in the presence of ADMA was held at intermediate levels, virtually no blockade of acetylcholine-induced relaxation was seen; there was a modest 1.7-fold reduction in sensitivity without any effect on the maximal relaxation. ADMA was similarly ineffective against acetylcholine-induced-relaxation in tissues contracted

submaximally with 5-hydroxytryptamine or prostaglandin $F_{2\alpha}$. Even increasing both the concentration of ADMA to 1000 μM and the time of exposure to 4.5 h at intermediate levels of phenylephrine tone failed to produce any greater blockade of acetylcholine-induced relaxation. These findings are clearly in conflict with previous studies on rat aorta which report blockade of acetylcholine-induced relaxation by ADMA (Vallance et al., 1992; Jin & D'Alecy, 1996; Feng et al., 1998). Since these authors made no mention of matching the tone in control and ADMA-treated tissues, it is likely that the blockade of acetylcholine-induced relaxation they reported resulted from physiological antagonism, due to over-contraction, rather than to blockade of NOS.

Our finding that ADMA blocks basal but not acetylcholine-induced nitric oxide activity in rat aorta mirrors what we reported previously for L-NMMA (Frew et al., 1993). Furthermore, just like the endogenous substrate, L-arginine, both ADMA and L-NMMA, protect acetylcholine-induced relaxation against blockade by the NOS inhibitor, L-NAME; L-arginine and L-NMMA also reversed established blockade by L-NAME, but ADMA failed to do this after an hour of treatment, perhaps due to lower potency than the other agents. Whether or not longer treatments with ADMA would have reversed the blockade by L-NAME was not investigated. Thus, although ADMA and L-NMMA both act like conventional competitive inhibitors of basal nitric oxide synthesis, they paradoxically appear to behave more like substrates for acetylcholine-induced and ATP-induced (Frew et al., 1993) synthesis of nitric oxide. Indeed, chemiluminescence detection has revealed that L-NMMA, just like L-arginine, fuels the synthesis of nitric oxide in rat aorta and pulmonary artery, whereas L-NOARG inhibits it (Archer & Hampl, 1992). It is unlikely that demethylation

reactions to form L-arginine explains the abilities of ADMA and L-NMMA to behave as alternative substrates for agonist-stimulated eNOS, because the blockade they produce of basal NO activity is sustained, suggestive of on-going stability of these agents.

At present we can only speculate about the mechanisms that govern why ADMA and L-NMMA block basal but have little effect on agonist-stimulated activity of nitric oxide. It is possible that different isoforms of eNOS, with different structural requirements for substrates and inhibitors, might be responsible for basal and agonist-stimulated production of nitric oxide, but there is no evidence in the literature to support this. Alternatively, it is known that activation of eNOS by acetylcholine or ATP via Ca^{2+}-calmodulin binding induces a conformational change that permits dissociation of the enzyme from its inhibitory anchor protein, caveolin-1 (Dudzinski & Michel, 2007). It is therefore possible that a concomitant conformational change in the eNOS substrate binding site occurs such that it now recognises ADMA and L-NMMA as substrates rather than inhibitors. In this context, there is good evidence that flow-mediated dilatation, which occurs through the Ca^{2+}-independent activation of eNOS via phosphorylation by the phosphatidylinositol 3-kinase/Akt pathway (Fulton et al., 1999; Gallis et al., 1999), is inhibited by ADMA in humans (Boger et al., 1998; Vladimirova-Kitova et al., 2008). A direct comparison of the differential sensitivity to blockade by ADMA and L-NMMA of stimuli operating via Ca^{2+}-calmodulin-stimulated and phosphorylation-stimulated activation of eNOS in a single tissue will be required to explore this more thoroughly.

Although ADMA and L-NMMA block basal but not agonist-induced activity of nitric oxide in rat aorta, both elevate blood pressure when infused into anaesthetised rats (Rees et al., 1990; Jin & D'Alecy, 1996; De Gennaro Colonna et al., 2007). It would therefore be interesting to explore *in vivo* the sensitivity to blockade by ADMA and L-NAME of the components of nitric oxide activity (basal vs flow-stimulated vs agonist-stimulated) that contribute to the regulation of blood pressure. Indeed, since ADMA and L-NMMA both elevate vascular tone in human volunteers (Vallance et al., 1989; Vallance et al., 1992) and the former accumulates in a number of disease states (Vallance et al., 1992; Siroen et al., 2006; Leiper & Vallance, 2006), it will be valuable to re-assess the effects of ADMA and L-NMMA on isolated blood vessels from a range of species, including humans, to determine if the differential sensitivity of basal and agonist-stimulated activity of nitric oxide seen in the rat occurs elsewhere. We would caution that such studies should be conducted at submaximal tone with matched controls to ensure the outcomes are not compromised by physiological antagonism, resulting from over-contraction of inhibitor-treated tissues.

In conclusion, our findings demonstrate that ADMA, like L-NMMA, preferentially blocks basal activity of nitric oxide in rat aorta, but has little effect on that stimulated by acetylcholine. Previous reports suggesting blockade of acetylcholine-induced relaxation of this tissue by ADMA are likely to have been due to physiological antagonism rather than blockade of NOS. Further studies will be required to determine the mechanism by which ADMA and L-NMMA produce this paradoxically selective action.

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Conflict of interest - none

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Figure Legends

Figure 1. ADMA (100 μM) enhances tone induced by phenylephrine (PE), 5-hydroxytryptamine (5-HT) or prostaglandin $F_{2\alpha}$ ($F_{2\alpha}$) in endothelium-containing but not endothelium-denuded (-EC) rings of rat aorta. The enhancement of PE-induced tone is both prevented and reversed by treatment with L-arginine (10 mM). In contrast to ADMA, SDMA (100 μM) does not enhance tone in endothelium-containing rings. In the trace (a) concentrations are given in log molar units. In the histogram (b) the order of listing of drugs (PE, ADMA, L-arg; L-arg, PE, ADMA etc) reflects their order of addition to tissues; data are the mean ± s.e.mean of 6 observations. *** $P<0.001$ indicates a significant enhancement by ADMA; $^{###}$ $P<0.001$ indicates a significant difference in the presence of L-arginine.

Figure 2. (a) Concentration-response curves showing the contractile effects of phenylephrine (PE) on endothelium-containing rings of rat aorta and the enhancement of this tone following pretreatment for 1 h with ADMA (100, 300 and 1000 μM). (b) Concentration-response curve showing the ability of ADMA to enhance low level contraction to phenylephrine (30-60 nM); the response to each concentration of ADMA was measured after 1h. Each point is the mean ± s.e.mean of 6-15 observations. *** $P<0.001$ indicates a significant difference from control.

Figure 3. Experimental traces (a) and concentration-response curves (b) showing that when ADMA (100 μM, 1 h) enhances phenylephrine (PE, 0.1-0.3 μM)-induced tone to a high (H) level, acetylcholine (Ach)-induced relaxation appears powerfully inhibited. However, when these same tissues are washed, with intermediate PE (10-30 nM)-induced tone (I) re-established in the presence of ADMA, the blockade of

acetylcholine-induced relaxation is almost entirely abolished. Each point is the mean ± s.e.mean of 6 observations. *** P<0.001 indicates a significant difference from control.

Figure 4. Concentration-response curves showing acetylcholine (Ach)-induced relaxation in aortic rings taken to different levels of phenylephrine tone (indicated in mN) in (a) control tissues and (b) in tissues treated with ADMA (100 μM, 1 h). Both control and ADMA treated tissues exhibit depression of relaxation but only at high levels of tone. Each point is the mean ± s.e.mean of 6-9 observations. * P<0.05, ** P<0.01 and *** P<0.001 indicate significant differences from the first control.

Figure 5. Concentration-response curves showing acetylcholine (Ach)-induced relaxation at intermediate phenylephrine tone in control tissues (Control 1) and tissues treated with ADMA (1000 μM) for 1 hr (ADMA 1). These same tissue were tested again at 3h (Control 2; ADMA 2) and at 4.5 h (Control 3; ADMA 3), but there was little evidence of progressive blockade with time using ADMA. Each point is the mean ± s.e. mean of 5-6 observations.

Figure 6. Concentration-response curves showing acetylcholine (Ach)-induced relaxation at intermediate phenylephrine tone and blockade of this relaxation with L-NAME (30 μM, 1 h). The ability of (a) L-arginine, (b) L-NMMA, (c) ADMA and (d) SDMA (all at 1000 μM) to either protect against or reverse blockade by L-NAME is also shown. Note that the order of listing of drugs reflects their order of addition to tissues. Each point is the mean ± s.e.mean of 6-9 observations. *** P<0.001 indicates

significant blockade by L-NAME; $^{\#\#}$ P<0.001 and $^{\#\#\#}$ P<0.001 indicate significant protection against or reversal of blockade induced by L-NAME.

Table 1. Effects of NOS inhibitors on acetylcholine-induced relaxation of rat aorta, together with time-matched controls. All experiments were conducted at submaximal levels of phenylephrine-induced tone.

Treatment	*Concentration*	E_{max} *(%)*	pEC_{50}
Control	-	81.3 ± 5.9	7.61 ± 0.02
ADMA	1000 μM (1 h)	83.8 ± 2.8	7.31 ± 0.07 **
Control	-	87.9 ±2.5	7.33 ± 0.04 $^{\#\#}$
ADMA	1000 μM (4.5 h)	81.9 ± 2.3	6.85 ± 0.04 ***
Control	-	93.3 ±2.8	7.51 ± 0.03
SDMA	1000 μM (1 h)	91.2 **±** 4.4	7.52 ± 0.04
Control	-	86.0 ± 5.8	7.54 ± 0.05
L-NMMA	1000 μM (1 h)	89.0 ± 4.3	7.12 ± 0.04 ***

Data are expressed at mean ± s.e.mean of n=6-9 observations. ** P<0.01 and *** P<0.001 indicate significant differences from respective controls. $^{\#\#}$ P<0.01 indicates a difference from the control at 1 h.

Figure 1

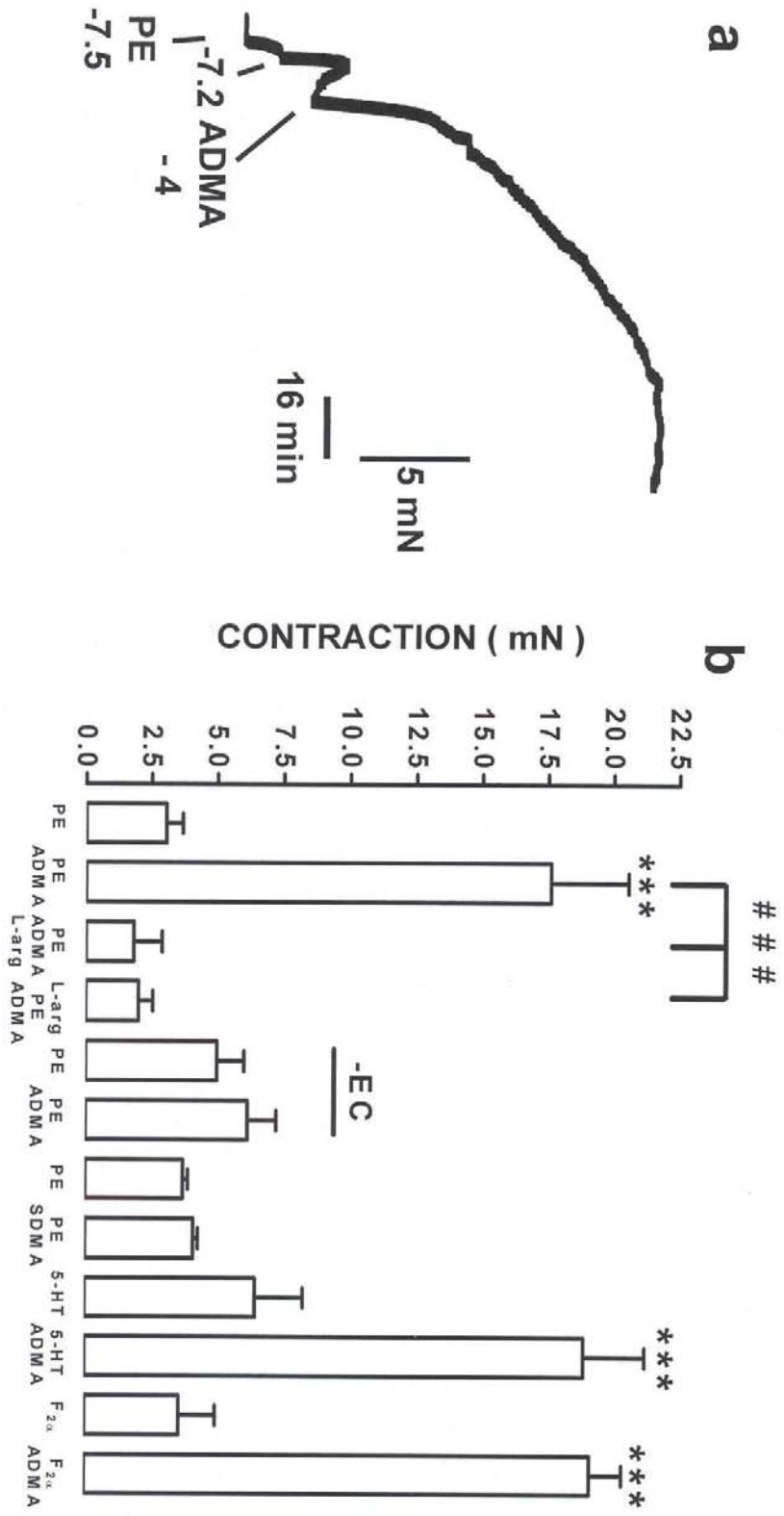

a
-7.2 ADMA
-4
PE
-7.5
16 min
5 mN
b
CONTRACTION (mN)
0.0
2.5
5.0
7.5
10.0
12.5
15.0
17.5
20.0
22.5
-EC
###
***
***
***
PE
PE ADMA
PE ADMA L-arg
L-arg PE ADMA
PE
PE ADMA
PE
PE SDMA
5-HT
5-HT ADMA
F2α
F2α ADMA

Figure 2

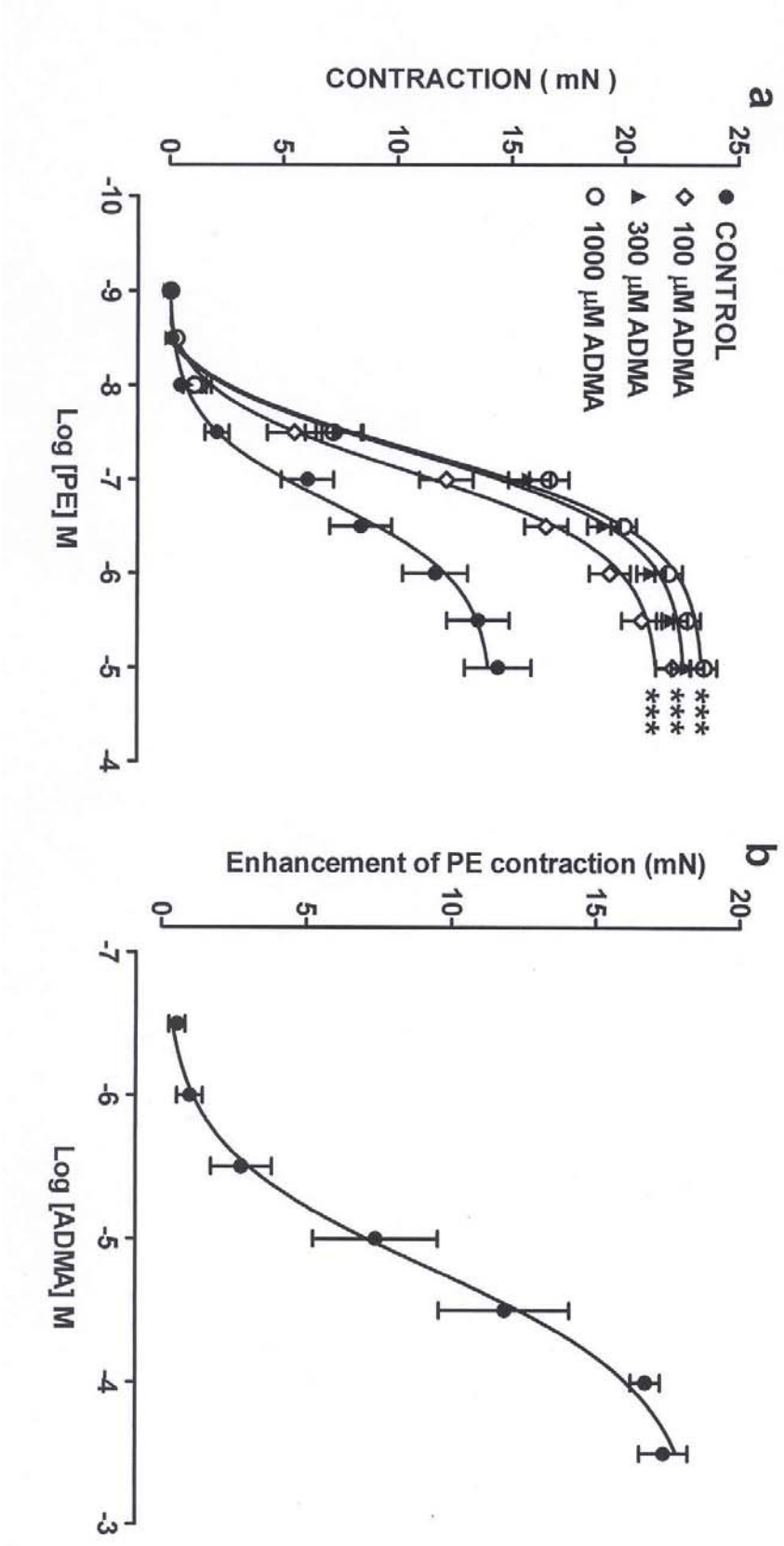

a
CONTRACTION (mN)
0
5
10
15
20
25
-10
-9
-8
-7
-6
-5
-4
Log [PE] M
● CONTROL
◇ 100 μM ADMA
▲ 300 μM ADMA
○ 1000 μM ADMA
***
***
***
b
Enhancement of PE contraction (mN)
0
5
10
15
20
-7
-6
-5
-4
-3
Log [ADMA] M

Figure 3

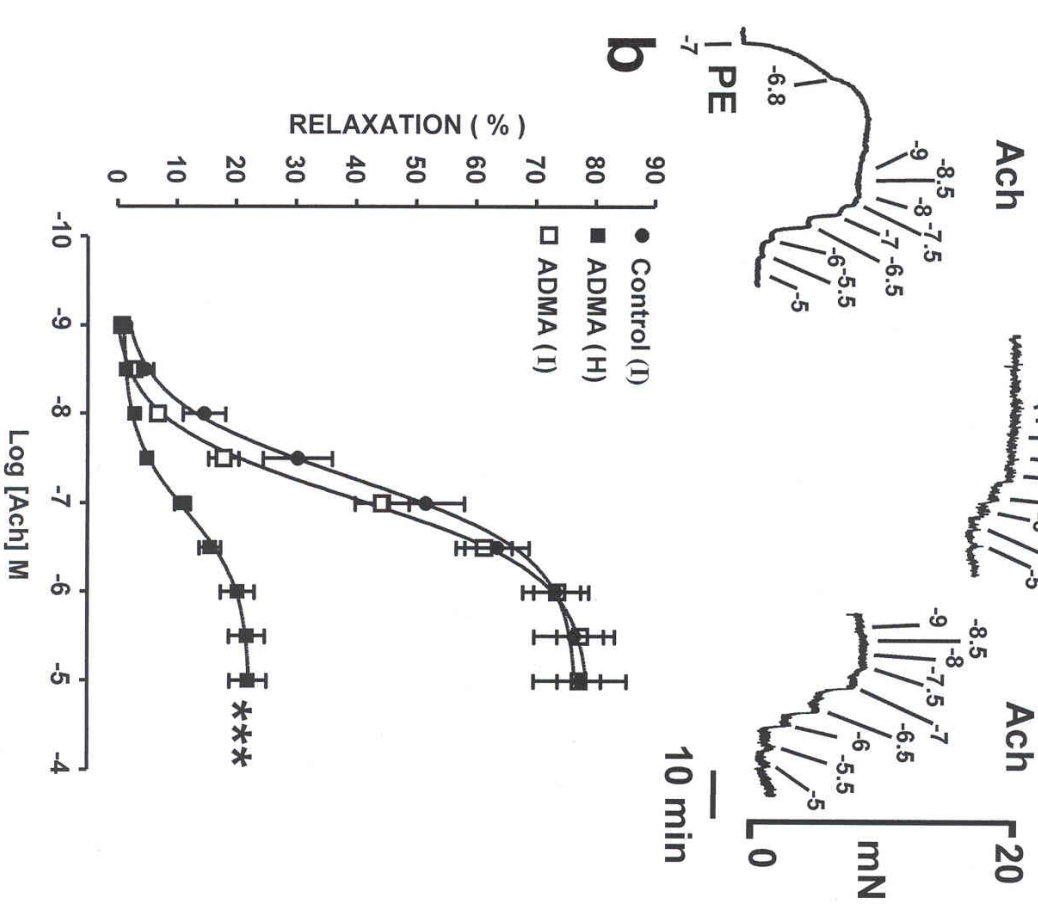

a
Control (I)
ADMA (H)
ADMA (I)
Ach
-9
-8.5
-8
-7.5
-7
-6.5
-6
-5.5
-5
PE
-7
-6.8
20
0
mN
10 min
b
RELAXATION (%)
Control (I)
ADMA (H)
ADMA (I)
Log [Ach] M
***

Figure 4

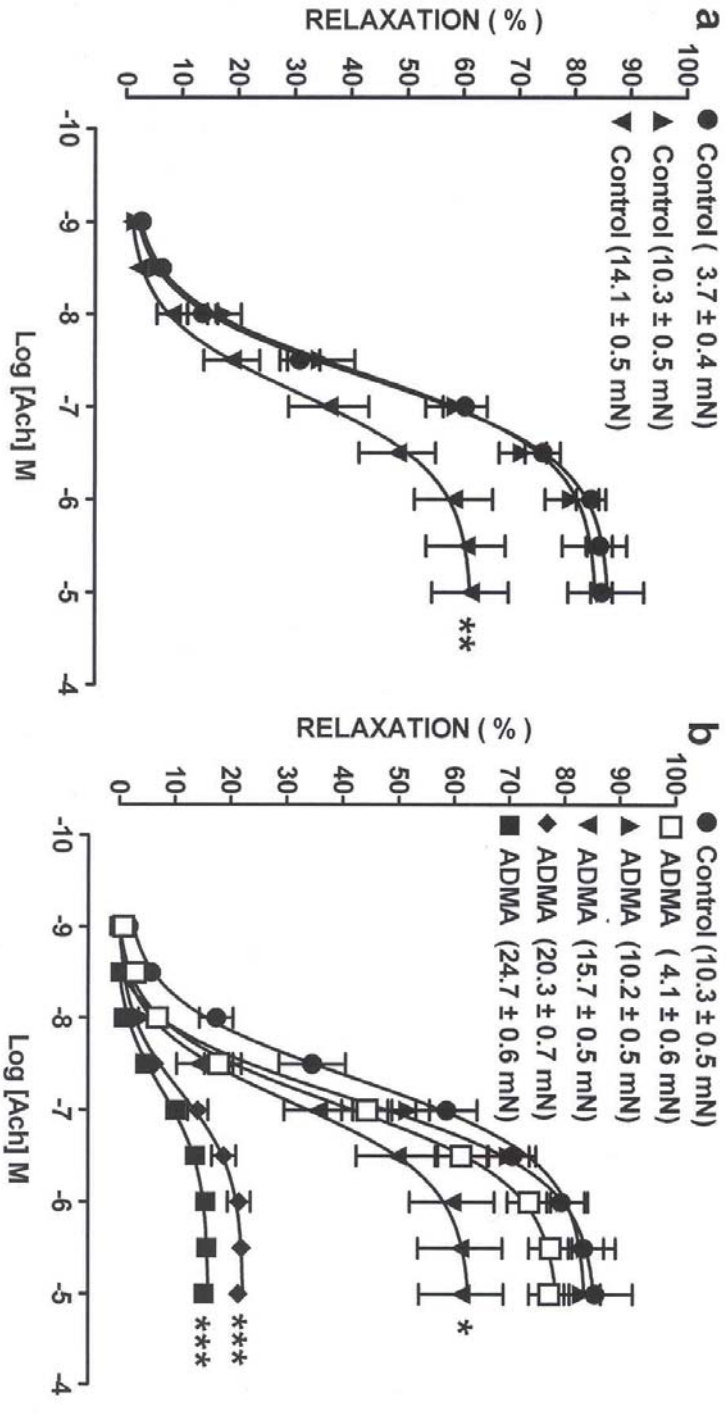

a
RELAXATION (%)
Log [Ach] M
● Control (3.7 ± 0.4 mN)
▲ Control (10.3 ± 0.5 mN)
▼ Control (14.1 ± 0.5 mN)
**
b
RELAXATION (%)
Log [Ach] M
● Control (10.3 ± 0.5 mN)
□ ADMA (4.1 ± 0.6 mN)
▲ ADMA (10.2 ± 0.5 mN)
▼ ADMA (15.7 ± 0.5 mN)
◆ ADMA (20.3 ± 0.7 mN)
■ ADMA (24.7 ± 0.6 mN)
*
***
***

Figure 5

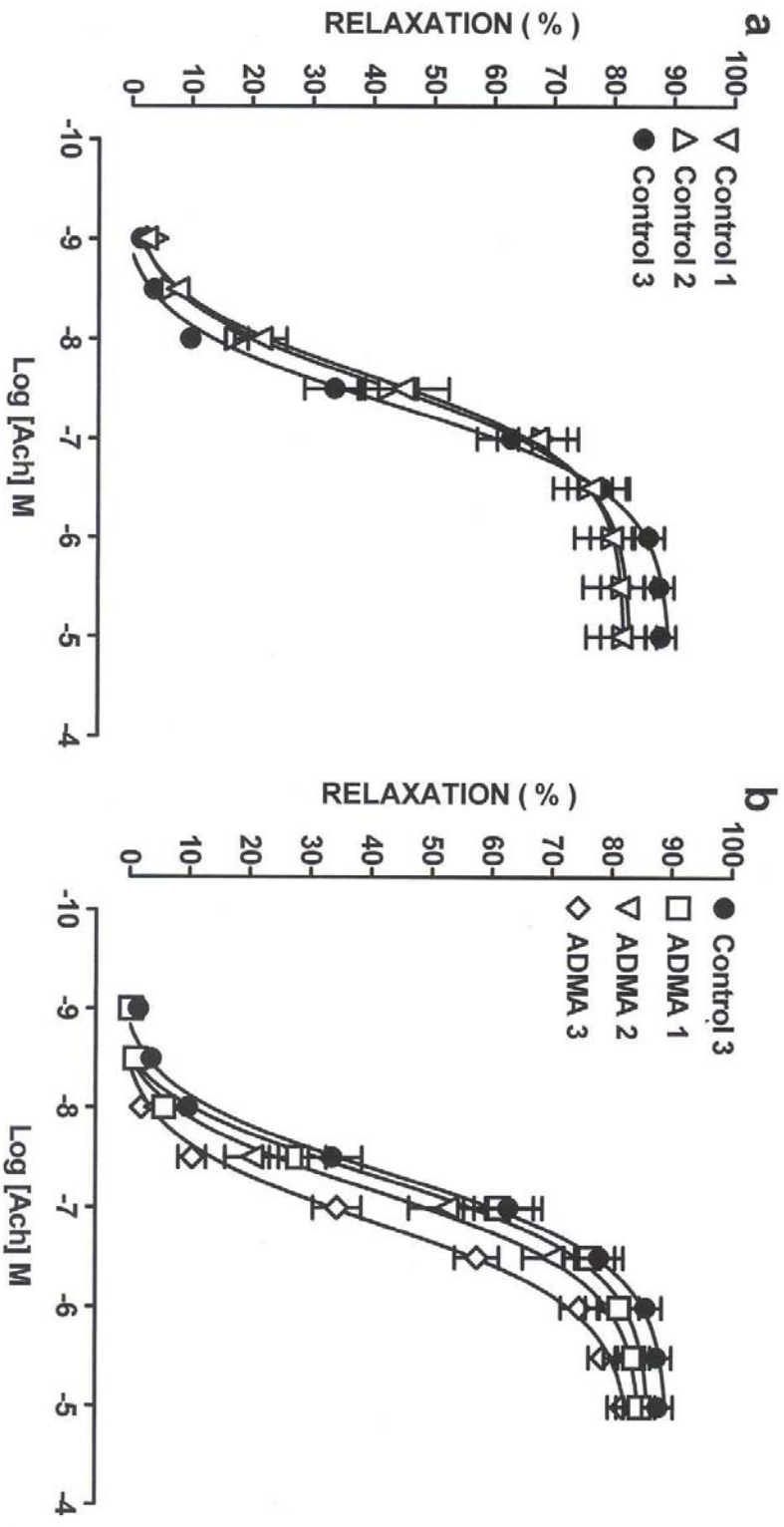
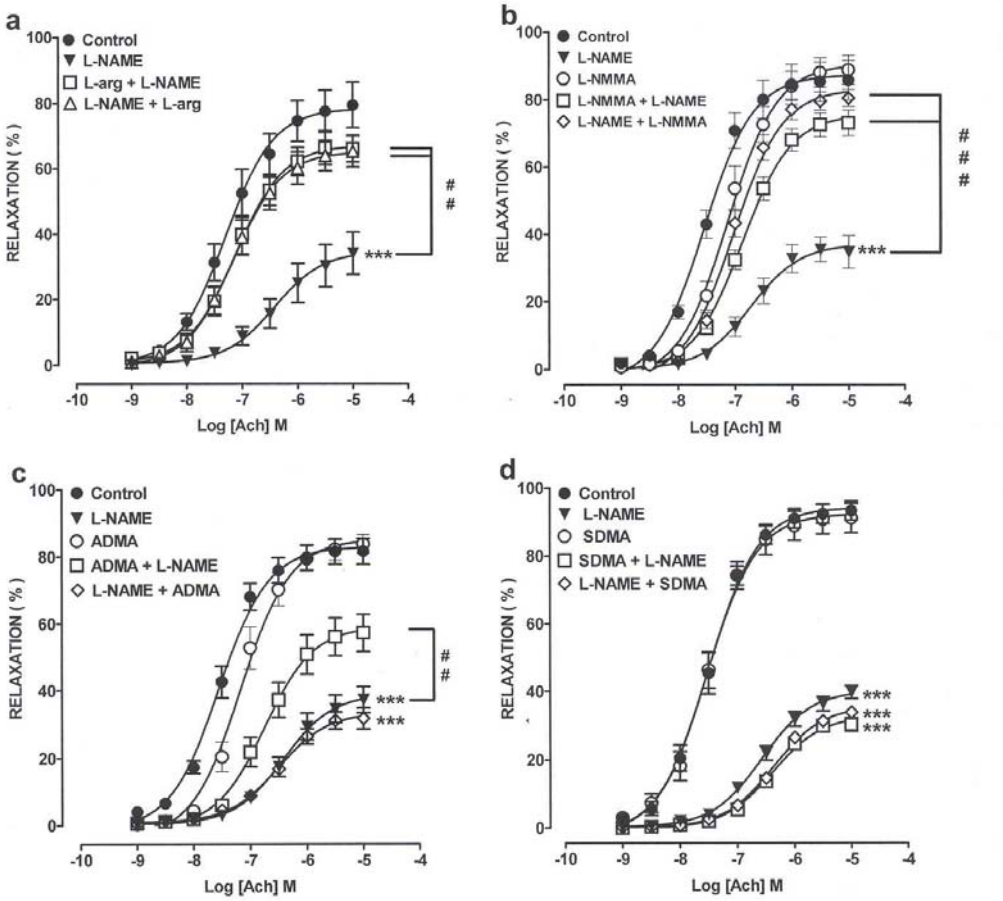

a
RELAXATION (%)
0
10
20
30
40
50
60
70
80
90
100
Control 1
Control 2
Control 3
Log [Ach] M
-10
-9
-8
-7
-6
-5
-4
b
RELAXATION (%)
Control 3
ADMA 1
ADMA 2
ADMA 3
Log [Ach] M

Figure 6


a
Control
L-NAME
L-arg + L-NAME
L-NAME + L-arg
RELAXATION (%)
Log [Ach] M
b
Control
L-NAME
L-NMMA
L-NMMA + L-NAME
L-NAME + L-NMMA
RELAXATION (%)
Log [Ach] M
c
Control
L-NAME
ADMA
ADMA + L-NAME
L-NAME + ADMA
RELAXATION (%)
Log [Ach] M
d
Control
L-NAME
SDMA
SDMA + L-NAME
L-NAME + SDMA
RELAXATION (%)
Log [Ach] M