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# L-NAME inhibits $Mg^{2+}$-induced rat aortic relaxation in the absence of endothelium

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**1** L-$N^G$-nitro-arginine methyl ester (L-NAME; 100 $\mu$M), a nitric oxide synthase (NOS) inhibitor, reversed the relaxation induced by 3 $\mu$M acetylcholine (ACh) and 2–10 mM $Mg^{2+}$ in endothelium-intact (+E) rat aortic rings precontracted with 1 $\mu$M phenylephrine (PE). In PE-precontracted endothelium-denuded (−E) rat aorta, 3 $\mu$M ACh did not, but $Mg^{2+}$ caused relaxation which was reversed by L-NAME, but not by D-NAME.

**2** The concentration response profiles of L-NAME in reversing the equipotent relaxation induced by 5 mM $Mg^{2+}$ and 0.2 $\mu$M ACh were not significantly different.

**3** L-NAME (100 $\mu$M) also reversed $Mg^{2+}$-relaxation of −E aorta pre-contracted with 20 mM KCl or 10 $\mu$M prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$). L-$N^G$-monomethyl-arginine (L-NMMA; 100 $\mu$M) was also effective in reversing the $Mg^{2+}$-relaxation.

**4** Addition of 0.2 mM $Ni^{2+}$, like $Mg^{2+}$, caused relaxation of PE-pre-contracted −E aorta, which was subsequently reversed by 100 $\mu$M L-NAME.

**5** Reversal of the $Mg^{2+}$-relaxation by 100 $\mu$M L-NAME in PE-precontracted −E aorta persisted following pre-incubation with 1 $\mu$M dexamethasone or 300 $\mu$M aminoguanidine (to inhibit the inducible form of NOS, iNOS).

**6** Pretreatment of either +E or −E aortic rings with 100 $\mu$M L-NAME caused elevation of contractile responses to $Ca^{2+}$ in the presence of 1 $\mu$M PE.

**7** Our results suggest that L-NAME exerts a direct action on, as yet, unidentified vascular smooth muscle plasma membrane protein(s), thus affecting its reactivity to divalent cations leading to the reversal of relaxation. Such an effect of L-NAME is unrelated to the inhibition of endothelial NOS or the inducible NOS.

**Keywords:** L-NAME; magnesium; calcium; endothelium; nitric oxide; vascular smooth muscle; aorta

**Abbreviations:** ACh, acetylcholine; AG, aminoguanidine; DXM, dexamethasone; EDRF, endothelium-derived relaxing factors; L-NAME, L-$N^G$-nitro-arginine methyl ester; L-NMMA, L-$N^G$-monomethyl-arginine; L-NOARG, L-nitro-arginine; NOS, nitric oxide synthase; PE, phenylephrine; $PGF_{2\alpha}$, prostaglandin $F_{2\alpha}$

## Introduction

A number of pharmacological agents indirectly mediate vascular relaxation by stimulating the release of endothelium-derived relaxing factor (EDRF) from endothelial cells (Furchgott & Zawadski, 1980; Furchgott, 1983; Palmer *et al.*, 1988). Subsequent studies have identified EDRF to be nitric oxide (NO) synthesized from the terminal guanidino nitrogen atom(s) of the amino acid, L-arginine, in the endothelial cell (Furchgott *et al.*,1981; Van de Voorde & Leusen, 1983; Palmet *et al*). Since NO has a very short half-life and its formation is difficult to measure directly in biological tissues a number of arginine analogues, such as L-nitro-monomethyl arginine (L-NMMA), L-nitro-arginine (L-NOARG) and L-nitro-arginine methyl ester (L-NAME) which stereoselectively compete with the substrate, L-arginine, and so inhibit the formation of NO and thus its biological effects, are commonly used to demonstrate indirectly the presence of NO synthase (Rees *et al*., 1989; 1990). These analogues have recently been reported to elicit actions other than endothelial NO inhibition (Buxton *et al*., 1993, Wang & Pang, 1994). For example, L-NMMA blocks the vasodilation caused by amiloride and dibutyryl cyclic AMP, which are not endothelium-dependent (Thomas & Ramwell, 1992). L-NAME and L-NMMA have been shown to interact with iron-containing enzymes. Peterson *et al*. (1992) concluded that it cannot be assumed that the hemodynamic effects of the arginine analogues are exclusively due to the inhibition of NO synthesis. They further suggested that the inhibition of endothelial NO formation by arginine analogues could be *via* an effect on electron transfer (Peterson *et al*., 1992). In this work, we investigate the possibility that the vascular effects of L-NAME in isolated rat aortic rings are not confined to inhibition of NOS in endothelial cells, but that L-NAME may act directly on smooth muscle.

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## Methods

### *Animals and vascular tissues*

For this study, 3 months old male Sprague-Dawley rats, weighing 250–300 g were used. All rats were maintained at the University facility for experimental animals under standard conditions conforming to government and university regulations. The rats were sacrificed by stunning followed by decapitation. The thoracic aorta was excised and placed in a Petri dish containing physiological salt solution aerated with a mixture of 95% $O_2$ and 5% $CO_2$, cleared of adherent connective tissue and cut into rings 3–4 mm in length. Rings from main pulmonary arteries from male adult Sprague-Dawley rats and rings of femoral veins

as well as the superior mesenteric arteries from mongrel dogs of either sex (unknown age) were also similarly trimmed as described above and used for contractility studies. Dogs were obtained from an authorized dog pond *via* the experimental animal facility of the University of Hong Kong and McMaster University and were killed with an over-dose of pentobarbital by qualified personnel in the animal facility.

### *Measurement of isometric contraction*

The vascular rings were mounted on hooks which were placed in 10 ml organ baths that contained normal Krebs' solution with the following composition in (mM): NaCl 133, KCl 5, $NaHCO_3$ 12, $NaH_2PO_4$ 1, $MgSO_4$ 1.2, $CaCl_2$ 2.5 Glucose 11 (pH 7.2 at 37°C) and were bubbled continuously with a mixture of 95% $O_2$ and 5% $CO_2$. One of the hooks was connected to the organ chamber and the other was connected to a Grass FT 03 force displacement transducer. The rat aortic and pulmonary rings were placed under a stable resting tension of 1.5 and 1.0 g, respectively, which yielded optimal active tension in response to 60 mM KCl. For the canine femoral vein and mesenteric artery, the optimal resting tension was 2 and 3 g, respectively. The vascular rings were allowed to equilibrate for a minimum period of 90 min after which time isometric contractions were recorded with a Grass Polygraph. We prepared endothelium-denuded aortic rings by adding saponin (0.3 mg $ml^{-1}$) to the organ baths for a period of 3 min (Samata *et al*., 1986). ACh (3 $\mu$M) was used to test the completeness of functional endothelial denudation in rings pre-contracted with 1 $\mu$M phenylephrine. This short exposure of aortic rings to saponin totally eliminated the relaxation induced by ACh and occasionally caused a small elevation of the resting tension (Guan *et al*., 1988) without any significant effects on the contraction induced by PE or KCl. Specific experimental protocols and representative tracings of contractile responses are as described in the corresponding Figure legends. For each experimental protocol, the following time-control responses were obtained. The plateau responses of rat aortic rings contracted with KCl, PE or $PGF2_{2\alpha}$ remained stable over at least 60 min. The vascular relaxation induced by a given concentration of $Mg^{2+}$ or $Ni^{2+}$ reached maximum in about 10 min and remained stable for at least 20 min. For each aortic ring, control values of KCl (60 mM)-contraction were obtained immediately before and soon after the end of the experimental protocol. The ratio of these control KCl-contractions was usually >90% suggesting that the contractile function of these vascular preparations did not deteriorate over time throughout the experiment. The resting tension of the aortic rings was not affected by any of the L-arginine analogues over a period of at least 30 min.

### *Statistics*

The data are expressed as means±s.e.means. Tests of significance were made using unpaired Student's *t*-test between control and test groups or ANOVA test for multiple comparisons. $P<0.05$ was considered statistically significant. The *n* value used for statistical analyses represents the number of separate experiments.

### *Materials*

All inorganic chemicals ($MgSO_4$, $NiCl_2$, $CaCl_2$, KCl, and NaCl) were purchased from Merck (ON, Canada). PE, ACh, L-NAME, D-NAME, L-NMMA, $PGF_{2\alpha}$, dexamethasone,

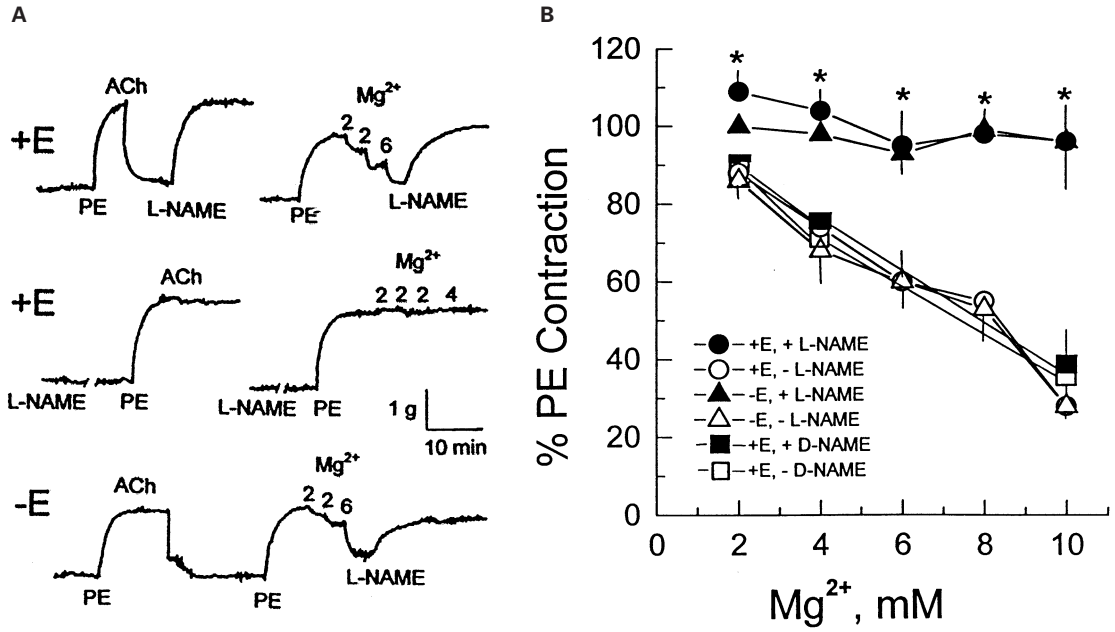


**Figure 1** (A) Typical tracings (left) and data summary (right) of the effects of L-NAME on aortic contraction, Top tracings: In endothelium-intact (+E) aortic rings precontracted with 1 $\mu$M PE, 100 $\mu$M L-NAME reversed the relaxation induced by 3 $\mu$M ACh or cumulatively added 10 mM $Mg^{2+}$; Middle tracings: Pre-incubation with 100 $\mu$M L-NAME, inhibited the relaxation in response to 3 $\mu$M ACh or 10 mM $Mg^{2+}$. Bottom tracings: In endothelium-denuded (−E) rings precontracted with 1 $\mu$M PE, 3 $\mu$M ACh had no effect but 10 mM $Mg^{2+}$ (added cumulatively) induced concentration-dependent relaxation, which was reversed by 100 $\mu$M L-NAME. (B) The data summary shows that 100 $\mu$M L-NAME inhibits the concentration-dependent relaxation induced by added $Mg^{2+}$ (2–10 mM) in +E and −E rat aortic rings. D-NAME (100 $\mu$M) did not affect the relaxation induced by 2, 4 and 10 mM $Mg^{2+}$ in −E or +E aortic rings (only the data for +E rings are shown for clarity). Values are expressed as mean±s.e.mean; $n=6-8$. *$P<0.001$, comparison between corresponding values, in the presence and absence of L-NAME.

aminoguanidine and saponin were obtained from Sigma (St. Louis, MO, U.S.A.).

## Results

Typical tracings of the effects of ACh, $Mg^{2+}$ and L-NAME on the contractile responses of rat aortic rings to 1 $\mu$M PE are shown in Figure 1. At the plateau phase of the contraction in endothelium-intact preparations (+E), 3 $\mu$M ACh or cumulatively added (2–10 mM) $Mg^{2+}$ caused relaxation of intact rings precontracted with PE. Subsequent addition of 100 $\mu$M L-NAME fully reversed this relaxation (top two tracings). When the rat aortic rings were preincubated with 100 $\mu$M L-NAME for 1 h prior to the addition of 1 $\mu$M PE to induce contraction, addition of 3 $\mu$M ACh or cumulatively added $Mg^{2+}$ (2–10 mM) at the plateau phase of PE-induced contraction elicited no relaxation (middle two tracings). To test the possibility that $Mg^{2+}$, like ACh, may induce NO release from the endothelium resulting in inhibition of PE-induced contraction, we also examined the effect of L-NAME in endothelium-denuded aortic preparations (−E). The bottom tracing shows quite characteristically the lack of ACh-induced relaxation in endothelium-denuded aorta precontracted with 1 $\mu$M PE. However, $Mg^{2+}$ still caused concentration-dependent (2–10 mM) relaxation and addition of 100 $\mu$M L-NAME at the steady level of relaxation to $Mg^{2+}$ reversed the relaxation. The quantitative results obtained from 6–8 separate experiments under the above experimental conditions shown in Figure 1A are summarized in Figure 1B. The results establish that the inhibition by L-NAME of $Mg^{2+}$-induced relaxation is not endothelium-dependent. In contrast to L-NAME, 100 $\mu$M D-NAME had no effect on the vascular relaxation induced by 2–10 mM $Mg^{2+}$ (Figure 1B) or 3 $\mu$M ACh (not shown), in intact as well as endothelium-denuded aortic preparations. We have also examined the concentration dependence of L-NAME on the reversal of ACh- and $Mg^{2+}$-induced relaxation in rat aortic rings with intact endothelium (Figure 2). For a proper comparison in this experiment, 5 mM $Mg^{2+}$ or 0.2 $\mu$M ACh was applied to produce an equipotent relaxation (about 50%) in rings precontracted with 1 $\mu$M PE, and L-NAME was subsequently added cumulatively from 0.01–100 $\mu$M. The reversal effects of L-NAME on ACh- and $Mg^{2+}$-induced relaxation were not statistically significant. The concentration of L-NAME required to reverse completely the relaxation was about 10 $\mu$M, i.e., similar to the findings of Rees *et al.* (1990).

To investigate whether the inhibitory action of L-NAME on $Mg^{2+}$-induced relaxation is dependent on the agent used to induce vascular contraction, we also studied the effect of $Mg^{2+}$ on KCl- and $PGF_{2\alpha}$-induced contraction and the effect of the subsequent addition of L-NAME. The results are shown in Figure 3. The addition of 2–8 mM $Mg^{2+}$ to the endothelium-intact or -denuded rings precontracted with 60 mM KCl elicited little or no relaxant response (data not shown). However, stimulation of the aortic rings with submaximal doses of KCl (20 mM) resulted in contraction (25–30% of that induced by 60 mM KCl), which was relaxed by the cumulative addition of $Mg^{2+}$ at the plateau phase of this contraction. Subsequent addition of 100 $\mu$M L-NAME significantly reversed the relaxation. Similarly, in endothelium-denuded rat aortic rings precontracted with 10 $\mu$M $PGF_{2\alpha}$, 2 mM $Mg^{2+}$ caused about 55% relaxation, and this relaxation was reverted to 80% of the maximal PE contraction by 100 $\mu$M L-NAME.

We also considered the possibility that the inhibitory effect of L-NAME on $Mg^{2+}$-induced relaxation may not be confined to L-NAME. We demonstrated in Figure 4 that 10 $\mu$M L-NMMA, which is also widely used NOS inhibitor, elicited a similar inhibitory effect as 100 $\mu$M L-NAME (see Figure 1) on

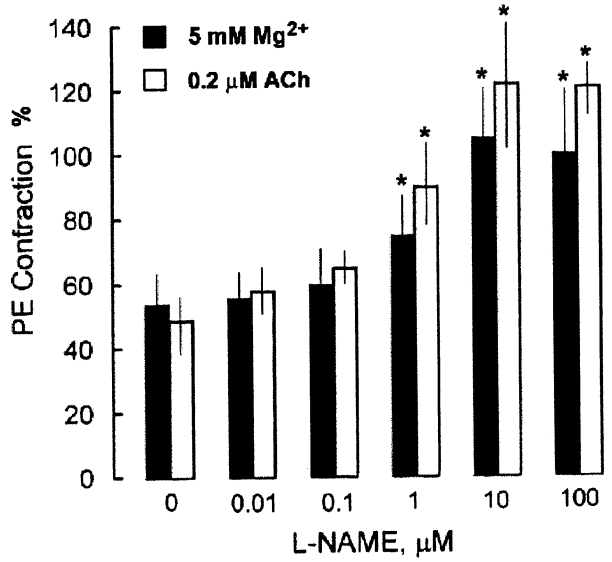


**Figure 2** The concentration response profiles of the reversal effect of L-NAME on the relaxation by 5 mM $Mg^{2+}$ and 0.2 $\mu$M ACh, which are equipotent in inhibitling the contraction to 1 $\mu$M PE by about 50% in the presence of L-NAME. Data are expressed as mean ± s.e.mean in four separate experiments. $*P<0.05$. significantly different from the corresponding control value in the absence of L-NAME.

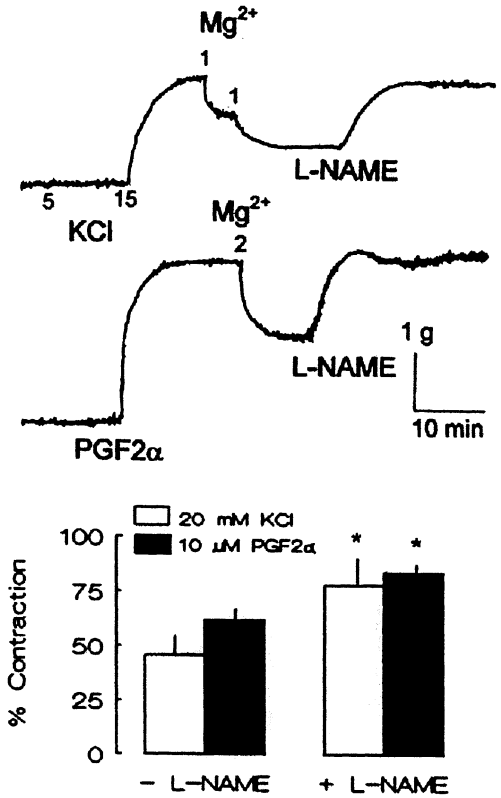


**Figure 3** In endothelium-denuded aortic rings contracted with added 20 mM KCl or 10 $\mu$M $PGF_{2\alpha}$, 100 $\mu$M L-NAME reversed the relaxation induced by 2 mM $Mg^{2+}$. The time controls for KCl or $PGF_{2\alpha}$-induced contraction remained sustained for the entire duration of the experiments (not shown). +L-NAME values are significantly different from −L-NAME values in 4–6 separate experiments ($*P<0.05$; as mean ± s.e.mean).

$Mg^{2+}$-induced relaxation of aortic rings (with endothelium intact or denuded) precontracted with 1 $\mu$M PE.

Like $Mg^{2+}$, $Ni^{2+}$ is known to behave like a $Ca^{2+}$ antagonist and inhibit vascular contraction (Blackburn & Highsmith, 1990), we also studied the effect of L-NAME on the relaxation of PE-contracted aorta by $Ni^{2+}$. It was demonstrated in Figure 5 that addition of 0.2 mM $Ni^{2+}$ at the plateau phase of PE-induced contraction resulted in sustained relaxation, and a full contraction could be restored by subsequent addition of 100 $\mu$M L-NAME.

If the vascular relaxant effect of increasing concentration of extracellular $Mg^{2+}$ is attributed to reduce availability of $Ca^{2+}$ for contraction by competing for the same binding sites in the cell membrane, it is conceivable that L-NAME may reverse the relaxant effect by restoring the availability of $Ca^{2+}$. To test this hypothesis, we studied the effect of extracellularly added $Ca^{2+}$ on the aortic contraction elicited by 1 $\mu$M PE in the presence and absence of 100 $\mu$M L-NAME. Figure 6 shows that the contractile response to $Ca^{2+}$ was augmented by preincubation with 100 $\mu$M L-NAME, thus lending support to the above hypothesis.

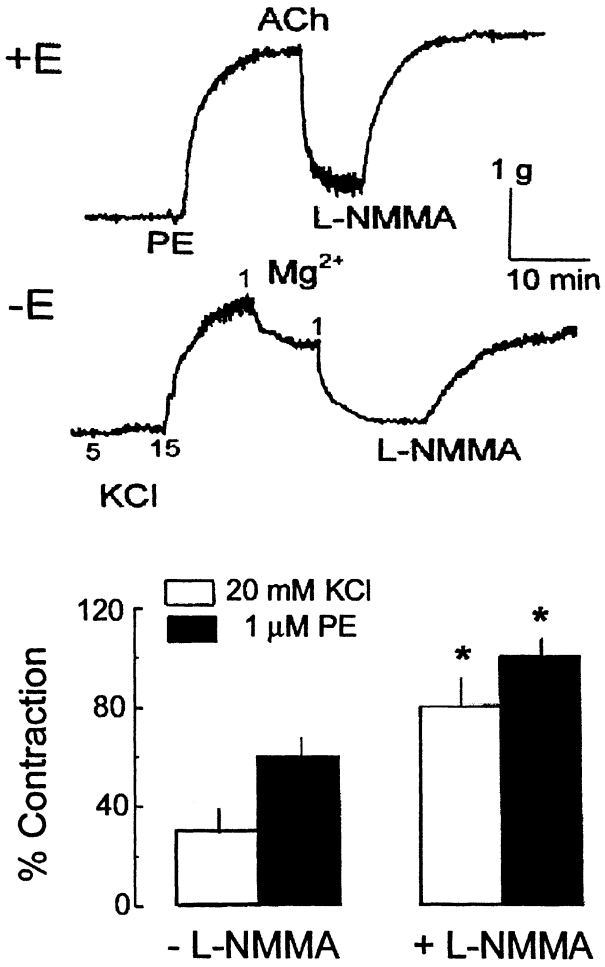


**Figure 4** Top tracing, as a positive control, shows that 100 $\mu$M L-NMMA completely reversed the relaxation induced by 3 $\mu$M ACh of endothelium-intact (+E) rings precontracted with 1 $\mu$M PE. Lower tracing shows that in endothelium-denuded (−E) rings precontracted with 20 mM KCl, L-NMMA also reversed the relaxation induced by 2 mM $Mg^{2+}$. The bar graph shows that similar effect of L-NMMA on $Mg^{2+}$ (2 mM)-induced relaxation was also observed in rings precontracted with 1 $\mu$M PE. +L-NMMA values are significantly different from −L-NMMA values (*$P<0.05$; mean±s.e.mean; $n=3-4$).

We next considered the possibility of $Mg^{2+}$ being able to induce nitric oxide synthase (iNOS) in smooth muscle, thus causing L-NAME-sensitive vasorelaxation. As shown in Figure

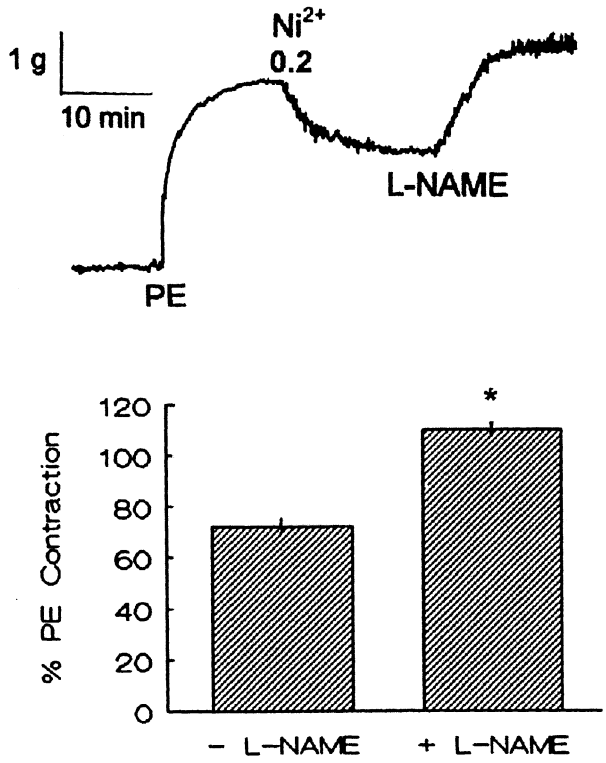


**Figure 5** The effect of 100 $\mu$M L-NAME on the relaxation induced by 0.2 mM $Ni^{2+}$ of endothelium-denuded aortic rings precontracted with 1 $\mu$M PE. +L-NAME values are significantly different from −L-NAME values (*$P<0.001$; mean±s.e.mean; $n=5$).

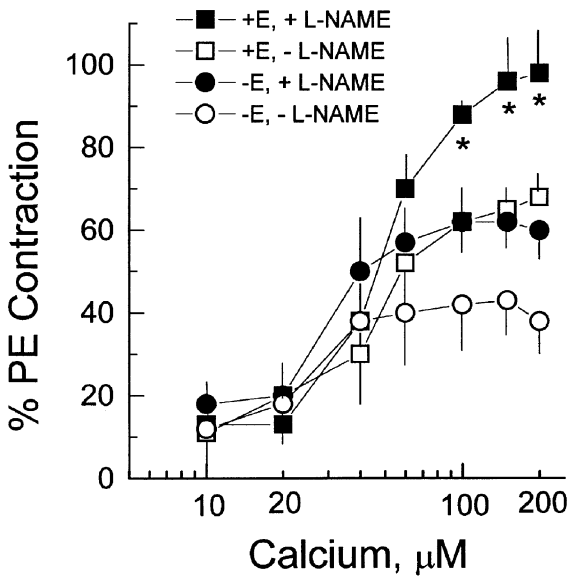


**Figure 6** Effect of L-NAME on the contractile responses to added $Ca^{2+}$ in the presence of PE in endothelium-intact (+E) and -denuded (−E) aortic rings. Rings were incubated in nominally $Ca^{2+}$-free medium with (+) or without (−) 100 $\mu$M L-NAME for 1 h prior to the addition of 1 $\mu$M PE, aliquots of $Ca^{2+}$ were subsequently added in cumulative manner to initiate contractions. *$P<0.05$ (mean±s.e.mean; $n=6$) compared to values in the absence of L-NAME in +E rings. **$P<0.05$ (mean±s.e.mean; $n=4$) compared to values in the absence of L-NAME in −E rings.

7, pretreatment of rat aortic rings with the selective inhibitors of iNOS activity, aminoguanidine (300 $\mu$M for 20 min) or expression, dexamethasone (1 $\mu$M for 5 h) did not eliminate the relaxation induced by $Mg^{2+}$ and L-NAME remained effective in reversing $Mg^{2+}$-induced relaxation.

Finally, we determined whether the inhibitory effect of L-NAME on $Mg^{2+}$-induced vasorelaxation is unique in rat aorta or it is a more generalized observation in vascular smooth muscle. We studied endothelium-denuded rings isolated from dog femoral veins, dog mesenteric artery and rat pulmonary arteries. The results are shown in Table 1. Reversal of $Mg^{2+}$ (4 mM)-induced relaxation by 100 $\mu$M L-NAME was observed in all three blood vessels.

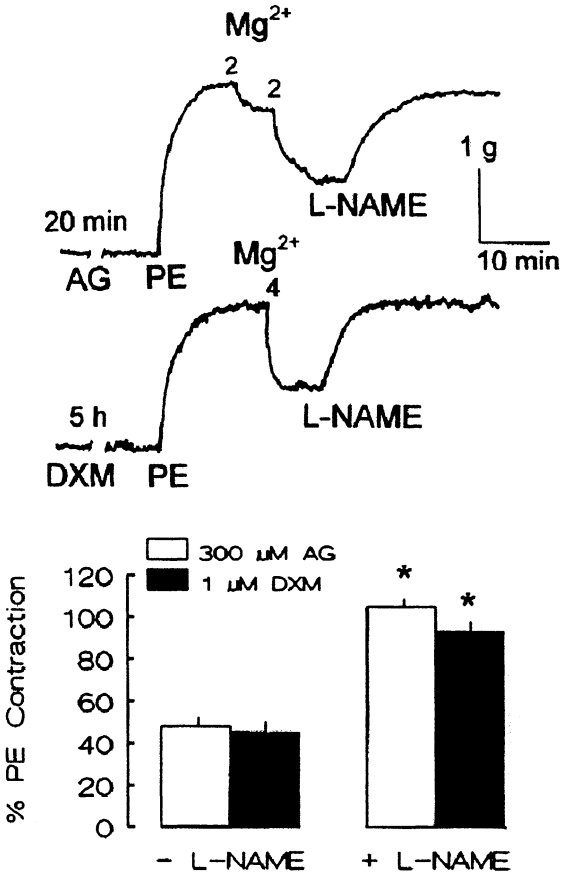


**Figure 7** Preincubation with 300 $\mu$M aminoguanidine (AG) for 20 min or with 1 $\mu$M dexamethasone (DXM) for 5 h did not modify the resting tension or the relaxation induced by 4 mM $Mg^{2+}$ in endothelium-denuded rings precontracted with 1 $\mu$M PE. Subsequent addition of 100 $\mu$M L-NAME still exerted significant reversal effect on $Mg^{2+}$-induced relaxation in both cases (*$P<0.01$; mean$\pm$s.e.mean; $n=3-4$).

**Table 1** Effect of 100 $\mu$M L-NAME on $Mg^{2+}$ (4 mM)-induced relaxation of blood endothelium-denuded vessels precontracted with 1 $\mu$M PE

| *Vessels* | n | *After 4 mM $MgCl_2$* | *After 100 $\mu$M L-NAME* |
|---|---|---|---|
| Rat aorta | 7 | 65$\pm$4 | 144$\pm$15* |
| Rat pulmonary artery | 7 | 81$\pm$3 | 110$\pm$4* |
| Dog femoral vein | 11 | 60$\pm$4 | 94$\pm$7* |
| Dog mesenteric artery | 4 | 55$\pm$5 | 98$\pm$6* |

Endothelium-denuded ring preparations were used for all vessels. Vascular rings were contracted with 1 $\mu$M PE. At the plateau phase of the PE-contraction, 4 mM $Mg^{2+}$ was added to cause relaxation and 100 $\mu$M L-NAME was subsequently added when the relaxation reached maximum. Data are expressed as mean$\pm$s.e.mean of the per cent contraction induced by 1 $\mu$M PE prior to $Mg^{2+}$-induced relaxation. *$P<0.05$ compared to the value obtained after addition of 4 mM $Mg^{2+}$.

## Discussion

### *L-NAME-sensitive, $Mg^{2+}$-induced relaxation in rat aorta is not endothelium-dependent*

Guanidine-substituted L-arginine analogues have been reported to inhibit the endothelium-dependent relaxation of vascular smooth muscle and this inhibition can be overcome by L-arginine, which is the physiological substrate for nitric oxide synthase, NOS (Rees *et al*., 1990; Mulsch & Busse, 1990). These results are consistent with the accepted mechanism of action of these L-arginine analogues, i.e., inhibition of NOS in the endothelial cells.

The fact that in endothelium-intact rat aortic rings, L-NAME fully reversed the ACh-induced relaxation and that preincubation of the intact rings with L-NAME completely inhibited the relaxation induced by ACh are in accordance with the contention that L-NAME inhibits NOS and, so, NO formation. However, this is not the case with $Mg^{2+}$-induced relaxation of PE-precontracted aortic preparations. Since $Mg^{2+}$-induced relaxation is not endothelium-dependent (Figure 1), the reversal or prevention of $Mg^{2+}$-induced relaxation by the addition of L-NAME, either at the maximum of $Mg^{2+}$-induced relaxation or prior to the contraction with PE, respectively, suggests that the effect of L-NAME is not mediated *via* inhibition of endothelial NOS. Furthermore, since rat aorta is not innervated by non-adrenergic, non-cholinergic nerves, which contains NOS, it is unlikely that the reversal of $Mg^{2+}$-induced relaxation by L-NAME is nerve-mediated. These findings suggest that L-NAME, in addition to its known action on the inhibition of the production of endothelium-derived NO, also exerts its effect directly on the rat aortic smooth muscle cells. The novel observation of L-NAME-sensitive, $Mg^{2+}$-induced relaxation has not been previously documented. The effect of L-NAME is independent of the stimulant used for contraction (PE, low KCl and $PGF_{2a}$) and the divalent cations used for induction of relaxation ($Mg^{2+}$ and $Ni^{2+}$), but it is stereospecific and observable with another L-arginine analogue (L-NMMA) and is not affected by inhibitors of iNOS activity (aminoguanidine) or synthesis (dexamethasone).

### *The effect of L-NAME is unlikely to be due to inhibition of iNOS in rat aorta*

In our study, thoracic aortic rings were denuded of endothelium to preclude the involvement of endothelial NOS following which they were preincubated with dexamethasone for 5 h prior to constriction with PE to assess the role of iNOS in $Mg^{2+}$-induced, L-NAME-sensitive vasorelaxation. Subsequent addition of $Mg^{2+}$ induced relaxation, which was significantly reversed by L-NAME. Glucocorticoids are potent inhibitors of the expression of iNOS in smooth muscle cells (Rees *et al*., 1990; Kanno *et al*., 1993). Dexamethasone presumably reduces the iNOS mRNA by inhibiting transcription (Kanno *et al*., 1993). Adeagbo & Triggle (1993) also concluded from their studies that dexamethasone acts by inhibiting iNOS production in aortic smooth muscle rather than exerting direct effect on receptors as suggested by Dural *et*

*al*. (1977) and Kornel *et al*. (1981). However, not all forms of iNOS are dexamethasone-sensitive. The induction of a $Ca^{2+}$-dependent, dexamethasone-insensitive iNOS by endotoxin or interleukin-1 has been reported in rabbit articular chondrocytes (Palmer *et al*., 1992) and activated rodent macrophages (Hiki *et al*., 1991; Hecker *et al*., 1992). Our contention that the ability of L-NAME to reverse $Mg^{2+}$-induced relaxation in endothelium-denuded aortic rings under our experimental conditions is unrelated to L-NAME-sensitive iNOS in vascular smooth muscle is further supported by the inability of aminoguanidine pretreatment, which preferentially inhibits iNOS activity (Corbett *et al*., 1992; Griffiths *et al*., 1993; Joly *et al*., 1994; Yen *et al*., 1995), to counteract the reversal effect of L-NAME on $Mg^{2+}$-induced relaxation. To date, there has been no precedence to demonstrate that $Mg^{2+}$ or any divalent cations could induce iNOS in smooth muscle of any type.

### *The action of L-NAME is not due to direct chelation of $Mg^{2+}$*

The non-specific action of L-arginine analogues has been attributed to their chelation with metal cations, such as $Fe^{2+}$ (Peterson *et al*., 1992). Therefore, it is possible that L-NAME may prevent or reverse $Mg^{2+}$-induced relaxation by direct binding with $Mg^{2+}$. This possibility is unlikely, because, in the first place, amino acids do not have strong affinity for $Mg^{2+}$ (Martell & Smith, 1974), secondly, the concentration of $Mg^{2+}$ used in the experiments is much in excess of that of L-NAME; and, thirdly, Peterson *et al*. (1992) reported that high concentration of L-NAME (>2.5 mM) was necessary to inhibit the iron-containing enzymes and D-NAME was as potent as the L-isomers in binding metal ions, whereas our work has shown that D-NAME had no effect on the relaxation induced by $Mg^{2+}$. Therefore, removal of $Mg^{2+}$ by direct binding does not seem to explain the ability of L-NAME to reverse $Mg^{2+}$ relaxation. The selective action of L-NAME compared to D-NAME further implies that it may interact, stereoselectively, with plasma membrane proteins that are involved in the control of binding or mobilization of $Mg^{2+}$ (and/or $Ca^{2+}$) which ultimately leads to muscle contraction (Altura & Altura, 1974; Kravtsov & Kwan, 1995).

Both $Mg^{2+}$ and $Ca^{2+}$ regulate vascular muscle tone at the cell membranes in a reciprocal manner (Altura & Altura, 1974). For example, $Ca^{2+}$ influx into the cytosol causes smooth muscle contraction which can be potentiated by withdrawal of extracellular $Mg^{2+}$ ($[Mg^{2+}]_0$), whereas elevated levels of $[Mg^{2+}]_0$ inhibit the spontaneous vascular tone. These effects of changes in $[Mg^{2+}]_0$ are due to the influence of $Mg^{2+}$ on $Ca^{2+}$ permeability, binding, translocation and membrane stability (Altura & Altura, 1974; Turlapaty & Altura, 1983). Such a reciprocal relationship also exists between $Mg^{2+}$ and $K^+$ (Schini *et al*., 1969; Kravtsov & Kwan, 1995), thus accounting for the observation that $Mg^{2+}$ is more effective in relaxing the rat aorta precontracted with sub-optimal concentration of KCl (20 mM) compared to higher KCl concentration (>60 mM). Gold *et al*. (1990) reported that excess $[Mg^{2+}]_0$ (>1.2 mM) elicits marked endothelium-independent and cyclic GMP-independent vasorelaxation that is unaltered by oxyhaemoglobin, methylene blue or indomethacin and offered the interpretation that excess $Mg^{2+}$ competes with and overrides the contractile effects of $Ca^{2+}$ on smooth muscle (Gold *et al*., 1990). Our observation that the effects of L-NAME, including the stereospecific structural stringency (L-NAME vs D-NAME), and the attenuation of relaxation to $Mg^{2+}$, but the augmentation of contraction to $Ca^{2+}$, are consistent with the hypothesis that L-NAME acts by affecting the availability of these divalent cations at the smooth muscle cell membranes, perhaps involving some specific membrane proteins.

Our study has shown for the first time that, in addition to inhibiting NOS and NO production in the endothelial cells, L-NAME also acts directly on vascular smooth muscle of rat aorta and other blood vessels, by reversing the relaxant effect of $Mg^{2+}$ thereby restoring the contractile response in the presence of the stimulant. Our results are consistent with the contention that L-NAME acts specifically with smooth muscle membranes, probably on the protein moieties that affect the binding and mobilization of $Mg^{2+}$ or $Ca^{2+}$. The present results, however, do not rule out the possibility that L-NAME acts on a novel NOS constitutively present in the rat aortic smooth muscle. Although a functional constitutive NOS has not been identified in any vascular smooth muscle, a functional myogenic NOS has recently been suggested to be associated with the plasma membranes of canine lower esophageal sphincter (Salapatek *et al*., 1998). If this novel myogenic NOS is also present in vascular smooth muscle and represents the protein moiety that interacts with L-NAME, it appears to be activated non-selectively by high concentration of divalent cations added extracellularly. This suggestion also receives support from the fact that L-NAME elicited a similar potency in reversing the effect of 5 mM $Mg^{2+}$ or 0.2 $\mu$M ACh (due to inhibition of endothelial NOS), which causes equipotent (50%) relaxation of PE-precontracted rat aortic rings (Figure 2). This interesting possibility warrants future investigations.

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