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In Vitro and *ex Vivo* Inhibitory Effects of L- and D-Enantiomers of N^G-Nitro-Arginine on Endothelium-Dependent Relaxation of Rat Aorta[1]

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ABSTRACT

The *in vitro* and *ex vivo* inhibitory effects of N^G-nitro-L-arginine (L-NNA) and N^G-nitro-D-arginine (D-NNA) on endothelium-dependent relaxations were studied in rat aortic rings. L-NNA (3×10^{-7} to 3×10^{-5} M) but not D-NNA (3×10^{-6} to 3×10^{-4} M) induced contraction of resting aortic rings and potentiated phenylephrine-induced contraction in a concentration-dependent manner. In phenylephrine-preconstricted aortic rings, L-NNA (3×10^{-7} to 3×10^{-5} M) and D-NNA (3×10^{-6} to 3×10^{-4} M) concentration-dependently inhibited the rellaxation response to acetylcholine (ACh) with similar efficacies and IC_{50} values of 10^{-6} and 3.9×10^{-5} M, respectively. In addition, both L-NNA (3×10^{-5} M) and D-NNA (3×10^{-4} M) almost totally inhibited the relaxation of preconstricted rings by the calcium ionophore A 23187. The inhibitory effects of L- and D-NNA remained for at least 4 hr after the preparations were washed out. Neither the inhibitory effects of L- and D-NNA on ACh-induced relaxation nor the ACh-induced relaxation itself were affected by pretreatment with indomethacin. However, pretreatment (10 min) or post-treatment (1 hr later) with L-Arg (10^{-3} M) completely prevented or markedly reversed the inhibitory effects of L- and D-NNA. Intravenous bolus injections of L-NNA (1.6×10^{-4} mmol/kg) and D-NNA (1.6×10^{-4} mmol/kg) caused sustained increases in blood pressure in conscious, unrestrained rats *in vivo* and inhibited ACh-induced relaxation of aortic rings *ex vivo*. These findings suggest that both L- and D-NNA cause efficacious, long-lasting and reversible inhibition of endothelium-dependent relaxation, for which the L-enantiomeric form is the preferred but not essential configuration required to inhibit endothelium-dependent relaxation.

Certain analogs of N^G-substituted Arg have been shown to inhibit NO synthesis (see Moncada *et al.*, 1991). These inhibitors include L-NMMA (Palmer *et al.*, 1988; Rees *et al.*, 1989a, 1990), L-NAME (Rees *et al.*, 1990), L-NIO (Rees *et al.*, 1990), L-NNA (Ishii *et al.*, 1990; Mülsch and Busse, 1990) and L-NAA (Vargas *et al.*, 1991). L-NNA inhibited endothelium-dependent relaxations of isolated arteries (Kobayashi and Hattori, 1990; Moore *et al.*, 1990; Mülsch and Busse, 1990) as well as nonadrenergic, noncholinergic relaxations of isolated guinea pig trachea and rat anococcygeus (Hobbs and Gibson, 1990; Tucker *et al.*, 1990). L-NNA also caused pressor responses and reflex bradycardia in rats (Wang and Pang, 1990a, 1991; Wang *et al.*, 1991a) and rabbits (Humphries *et al.*, 1991). We have found recently that D-NNA is as efficacious as L-NNA in raising blood pressure in pentobarbital-anesthetized (Wang and Pang, 1990b) and conscious (Wang *et al.*, 1991b) rats; however, the D-enantiomer is less potent and the effect is slower in onset. The pressor response to both D- and L-NNA is prevented by L- but not by D-Arg (Wang *et al.*, 1991b).

Our observations are unexpected inasmuch as other investigators have reported that N^G-substituted Arg analogs exhibit stereospecificity such that the L- but not the D-enantiomers raised blood pressure (Rees *et al.*, 1989b, 1990; Gardiner *et al.*, 1990a,b; Humphries *et al.*, 1991; see Moncada *et al.*, 1991). These compounds are believed to suppress endothelium-dependent relaxations of blood vessels by inhibiting NO synthesis (see Moncada *et al.*, 1991). It is not known if D-NNA inhibits vascular relaxation *via* the same mechanism as L-NNA and if systemic administration of D-NNA is required for its constrictor action. The aim of this study was to examine 1) whether both L- and D-NNA inhibit endothelium-dependent relaxation induced by ACh and the calcium ionophore A 23187, as well as endothelium-independent relaxation induced by SNP, in preconstricted rat aortic rings *in vitro* and *ex vivo* and 2) whether L-Arg or D-Arg antagonizes the inhibitory effects of L- and D-NNA. As both L- and D-NNA cause prolonged pressor responses *in vivo* (Wang and Pang, 1990b; Wang *et al.*, 1991b),

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ABBREVIATIONS: Arg, arginine; NO, nitric oxide; NMMA, N^G-monomethyl-arginine; NAME, N^G-nitro-L-arginine methyl ester; L-NIO, N-iminoethyl-L-ornithine; NNA, N^G-nitro-arginine; L-NAA, N^G-amino-L-arginine; ACh, acetylcholine; SNP, sodium nitroprusside; PHE, phenylephrine; MAP, mean arterial pressure; EDRF, endothelium-derived relaxing factor.

and L-NIO and L-NAA have been reported to be irreversible NO synthase inhibitors (McCall *et al.*, 1991; Rouhani *et al.*, 1992), experiments were conducted to study the time course and reversibility of the inhibition of endothelium-dependent relaxation by L- and D-NNA. Lastly, the effects of L- and D-NNA on ACh-induced relaxation were investigated because it has been reported recently that cyclooxygenase inhibitors prevent L-NMMA from suppressing vasodilation induced by ACh (Rosenblum *et al.*, 1992).

Methods

Preparations. Male Wistar rats (350–450 g) were sacrificed by a blow on the head followed by exsanguination. The thoracic aorta was removed and cleared of connective tissue. Four ring segments of 0.5 cm length were prepared from one aorta and suspended randomly in separate organ baths. Each ring was connected to a Grass FT-03-C force-displacement transducer (Quincy, MA) for isometric recording. Before the study commenced, a preload of 1 g was applied, after which the rings were equilibrated for 1 hr (with three washouts) in Krebs' solution (pH 7.4) at 37°C and bubbled with a gas mixture of 95% O_2-5% CO_2. The composition of Krebs' solution was as follows (millimolar): NaCl, 118; glucose, 11; KCl, 4.7; $CaCl_2$, 2.5; $NaHCO_3$, 25; KH_2PO_4, 1.2; and $MgCl_26H_2O$, 1.2.

In the *in vitro* studies, the rings were incubated with the vehicle or drugs (see later). Afterwards, 10^{-6} M PHE (EC_{90}) was added to the baths. At the steady-state phase of the contractile response to PHE (10–20 min later), a cumulative concentration-response curve of ACh or A 23187 was constructed. Each concentration of drug was left in the bath until a plateau response was obtained. The time taken to complete each concentration-response curve was approximately 15 min. In some groups in which concentration-response curves of ACh were conducted more than once, or followed by a concentration-response curve of SNP, the preparations were washed 3 times within 30 min and given another 30 min to recover completely from the effects of PHE and ACh.

In the *ex vivo* studies, the rats were anesthetized with halothane (4% in air for induction, 1.5% in air for surgery) to allow the insertion of cannulae into the left iliac vein and artery for the injections of drugs and continuous recording of MAP by a pressure transducer (P23DB, Gould Statham, Cupertino, CA), respectively. The cannulae were tunnelled s.c. and exteriorized at the back of the neck. Afterward, the rats were given at least a 4-hr recovery from the effects of anesthesia before use and allowed free movement. After acclimatizing the rats for 20 min, the vehicle or drug was i.v. bolus injected into the rats. Forty minutes later, the rats were sacrificed and two thoracic aortic rings from each were prepared for *ex vivo* studies as described for the *in vitro* studies (the time elapsed between sacrificing the rats and application of PHE was 1 hr).

Drugs. L-Arg hydrochloride, D-Arg hydrochloride, L-NNA, PHE hydrochloride, A 23187 and ACh chloride were obtained from Sigma Chemical Co. (St. Louis, MO). D-NNA was from Bachem Bioscience Inc. (Philadelphia, PA). SNP was obtained from Fisher Scientific Co. (Springfield, NJ). L-Arg and D-Arg were dissolved in distilled water and the pH of each solution was adjusted to 7.0 with NaOH solution. A 23187 and indomethacin were dissolved in 100% dimethylsulfoxide and 80% ethanol, respectively, and diluted with normal saline (0.9% NaCl). The remaining drugs were dissolved in normal saline. The dissolution of L- and D-NNA required 20 min of sonication.

Experimental protocols. Six to seven aortic rings, each derived from a different rat, were used in each group.

Effects of L- and D-NNA on the relaxations induced by ACh, A 23187 and SNP. Concentration-response curves of ACh (10^{-8} to 3×10^{-5} M, as follows) followed by those of SNP (3×10^{-10} to 3×10^{-7} M) were performed in 11 groups of PHE-preconstricted aortic rings in the presence of vehicle, five concentrations of L-NNA (3×10^{-7} to 3×10^{-5} M) and five concentrations of D-NNA (3×10^{-6} to 3×10^{-4} M). Vehicle, L- or D-NNA were added 10 min before administration of PHE.

Three groups of aortic rings were incubated 10 min with vehicle, L-NNA (3×10^{-5} M) or D-NNA (3×10^{-4} M), followed by the construction of a concentration-relaxation response curve of A 23187 (3×10^{-10} to 10^{-6} M) in PHE-preconstricted aortic rings using the same procedure as described for ACh.

Time course of the inhibitory effect of L- and D-NNA on ACh-induced relaxation. Three groups of aortic rings were used to examine the time course of the effects of vehicle, L-NNA (3×10^{-5} M) and D-NNA (3×10^{-4} M) on the relaxation response evoked by ACh. After completing the first ACh concentration-response curve and subsequent washout and recovery, L-NNA, D-NNA or vehicle was added into the baths. This was followed 10 min later by the construction of the second curve of ACh. At 1.5 and 4 hr after the preparations were washed out, the third and fourth curves of ACh, respectively, were constructed without further adding vehicle, L- or D-NNA.

Effects of L-Arg, D-Arg or indomethacin on relaxation response of ACh. The effect of pretreatment with indomethacin (10^{-5} M) on the inhibitory effects of L-NNA (10^{-6} M) and D-NNA (3×10^{-5} M) on ACh-induced relaxation were investigated in six groups of preconstricted aortic rings. The concentration-response curves of ACh were constructed in the presence of vehicle + vehicle, indomethacin + vehicle, vehicle + L-NNA, indomethacin + L-NNA, vehicle + D-NNA and indomethacin + D-NNA. The first drug or vehicle was given 10 min before the second drug or vehicle and this was followed 10 min later by the addition of PHE.

The effects of pretreatment with L-Arg or D-Arg on ACh-induced relaxation were studied in nine groups of PHE-preconstricted aortic rings, in the presence of vehicle + vehicle, L-Arg + vehicle, D-Arg + vehicle, vehicle + L-NNA, L-Arg + L-NNA, D-Arg + L-NNA, vehicle + D-NNA, L-Arg + D-NNA and D-Arg + D-NNA. The concentrations for L- and D-NNA were 10^{-6} M and 3×10^{-5} M, respectively, and for L-Arg and D-Arg were 10^{-3} M. The first treatment (vehicle, L-Arg or D-Arg) was given 10 min before the second treatment (vehicle, L- or D-NNA) and this was followed 10 min later by the addition of PHE.

The ability of post-treatment with L-Arg to reverse the inhibitory effects of L- or D-NNA was also studied in four groups of PHE-preconstricted aortic rings. The preparations were incubated with L-NNA (10^{-6} M) or D-NNA (3×10^{-5} M) for 1 hr before adding L-Arg (10^{-3} M). After 10 min, PHE was added followed by the construction of concentration-response curves of ACh.

Effects of L- and D-NNA on MAP and relaxations induced by ACh and SNP *ex vivo*. Three groups of conscious and unrestrained rats ($n = 5$ each group) were i.v. bolus injected with vehicle, L-NNA (1.6×10^{-4} mol/kg) or D-NNA (1.6×10^{-4} mol/kg), respectively. MAP was recorded before and 40 min after the injection of a drug or vehicle. The rats were sacrificed 40 min after injections and two aortic rings were prepared from each rat for *ex vivo* concentration-relaxation response curves of ACh or single concentration of SNP (10^{-6} M).

Calculation and statistics. Responses of ACh, A 23187 and SNP were calculated as percentage of relaxation of contractile response to PHE. IC_{50} values were calculated by using average data from concentration-response curves of L- and D-NNA in inhibiting ACh-induced relaxation. All results were expressed as mean ± S.E. except in cases in which the error bars were smaller than the points (or symbols, see figures). The results were analyzed by the analysis of variance/covariance. Duncan's multiple range test was used to compare group means, with $P < .05$ selected as the criterion for statistical significance.

Results

Effects of L- and D-NNA on contraction in the presence or absence of PHE. Ten-minute incubation with L-NNA (3×10^{-7} to 3×10^{-5} M) potentiated the spontaneous contractile activity in some but not all aortae (data not shown). In addition, L-NNA caused concentration-dependent contraction in resting aortic rings (fig. 1A). D-NNA (3×10^{-6} to 3×10^{-4} M), on the

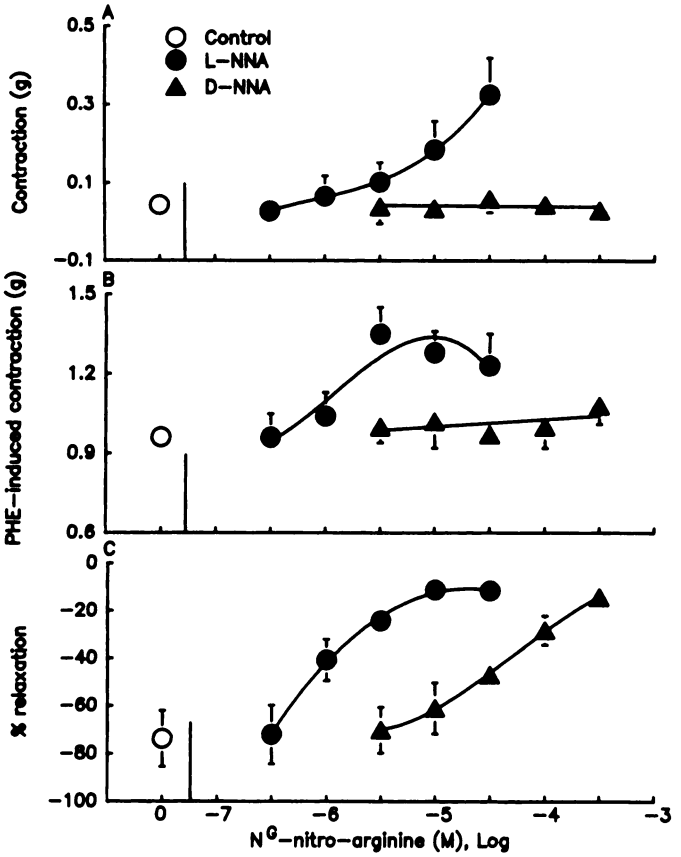

Fig. 1. Effects (mean ± S.E.) of L- and D-NNA on resting tone of aortic rings (A), on PHE (10^{-6} M)-induced contraction (B) and on ACh (3×10^{-5} M)-induced relaxation in PHE-preconstricted aortic rings (C) (n = 6–7 in each group).

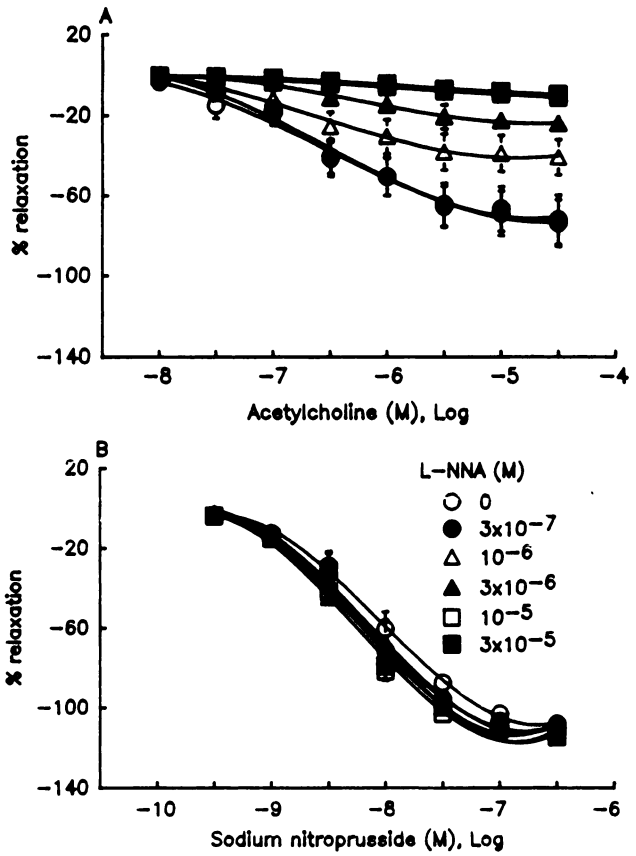

Fig. 2. Concentration-response (mean ± S.E.) of L-NNA on ACh (A)- and sodium nitroprusside (B)-induced relaxations in PHE (10^{-6} M)-preconstricted aortic rings (n = 6–7 in each group).

other hand, induces neither spontaneous nor sustained contraction in the aortae (fig. 1A). PHE (10^{-6}, EC_{90})-induced contraction reached approximately 80% maximum within 30 to 60 sec followed by a slower phase which reached plateau in 10 to 20 min. Preincubation with L-, but not with D-NNA, significantly potentiated the contraction induced by PHE (fig. 1B).

Effects of L- and D-NNA on relaxations induced by ACh, A 23187 and SNP. ACh and SNP caused concentration-dependent relaxations of PHE-preconstricted rat aortic rings, with maximum relaxation of approximately 70 and 100%, respectively (fig. 2). Incubations with L-NNA (3×10^{-7} to 3×10^{-5} M) and D-NNA (3×10^{-6} to 3×10^{-4} M) concentration-dependently and noncompetitively inhibited the relaxation responses to ACh (figs. 2A and 3A). Figure 1C illustrates the percentage of relaxation induced by 3×10^{-5} M ACh in the presence of L- or D-NNA. Whereas L- and D-NNA were equally efficacious (approximately 100%) in inhibiting ACh-induced relaxation, the IC_{50} value of L-NNA (10^{-6} M) was lower than that of D-NNA (3.9×10^{-5} M). On the other hand, neither L- nor D-NNA inhibited the relaxation response of SNP (figs. 2B and 3B).

A 23187 was as equally efficacious as ACh in causing concentration-dependent relaxation which reached a maximum of approximately 70% at 3×10^{-7} M. Incubations with both L-NNA (3×10^{-5} M) and D-NNA (3×10^{-4} M) almost inhibited completely the relaxation response induced by A 23187 (fig. 4).

Time course of the inhibitory effects of L- and D-NNA on ACh-evoked relaxation. In the control group, the concentration-relaxation response curves of ACh were repeated 4 times within 6 hr. There was time-dependent loss of relaxation response to ACh which became statistically significant at the last curve (fig. 5A). Incubations with both L-NNA (3×10^{-5} M) and D-NNA (3×10^{-4} M) completely abolished ACh-induced relaxations (fig. 5, B and C). The inhibitory effects of L- and D-NNA were still present at 1.5 as well as 4 hr after the preparations were washed out without further adding the drugs, even compared to the corresponding time controls (fig. 5).

Effects of pretreatment with L-, D-Arg or indomethacin and post-treatment with L-Arg on relaxation responses of ACh. Indomethacin (10^{-5} M) did not alter ACh-induced relaxation in the preconstricted aortic rings, compared with the vehicle group (fig. 6A). L-NNA (10^{-6} M) and D-NNA (3×10^{-5} M) inhibited the relaxation evoked by ACh (fig. 6, B and C). Pretreatment with indomethacin did not alter the inhibitory effects of L-NNA (fig. 6B) and D-NNA (fig. 6C).

Incubation with neither L-Arg (10^{-3} M) nor D-Arg (10^{-3} M) significantly altered relaxation responses to ACh (fig. 7A). Ten-minute preincubations with both L-NNA (10^{-6} M) and D-NNA (3×10^{-5} M) significantly inhibited the relaxation responses of ACh (fig. 7, B and C). The inhibitory effects of L- and D-NNA were prevented completely by 10-min pretreatment with L-Arg but not with D-Arg (fig. 7, B and C).

Figure 8 shows that the relaxation response of ACh was again inhibited by 1.5-hr incubations with L-NNA (3×10^{-5} M) and D-NNA (3×10^{-4} M). The inhibitory effects of L- or D-NNA were also markedly eliminated by post-treatment (1 hr later) with L-Arg (10^{-3} M) (fig. 8, A and B).

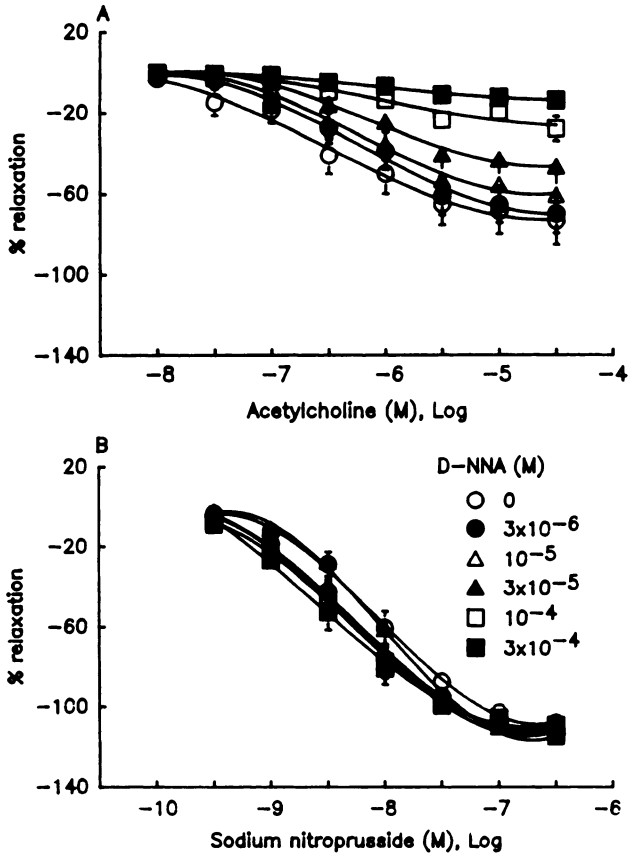

Fig. 3. Concentration-response (mean ± S.E.) of D-NNA on ACh (A)- and sodium nitroprusside (B)-induced relaxations in PHE (10^{-6} M)-preconstricted aortic rings (n = 6–7 in each group).

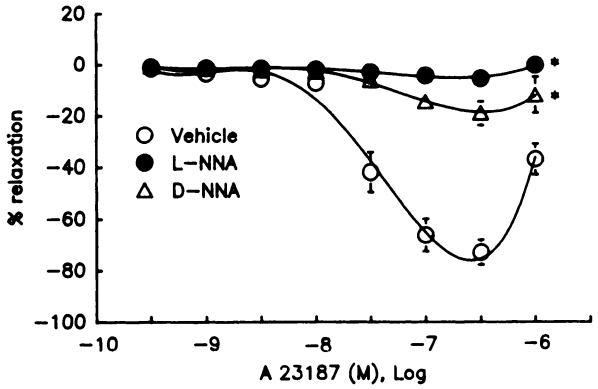

Fig. 4. Inhibitory effects on L-NNA (3×10^{-5} M) and D-NNA (3×10^{-4} M) on A 23187-induced relaxation in PHE (10^{-6} M)-preconstricted aortic rings (n = 6–7 in each group). *Significant difference from the control curve ($P < .05$).

Effects of L- and D-NNA on MAP *in vivo* and ACh-induced relaxation *ex vivo*. Base-line MAP of conscious and unrestrained rats which were i.v. bolus injected with vehicle, L-NNA (1.6×10^{-4} mol/kg) and D-NNA (1.6×10^{-4} mol/kg) were 100 ± 2, 107 ± 1 and 112 ± 2 mm Hg, respectively. Vehicle did not significantly alter MAP, whereas L- and D-NNA raised MAP to similar plateau values at 40 min after injections (fig. 9A). The relaxation responses to ACh in PHE-preconstricted aortic rings obtained from either L- or D-NNA-pretreated rats were less than in vehicle-treated rats (fig. 9B). In contrast, the relaxation response of SNP was not affected

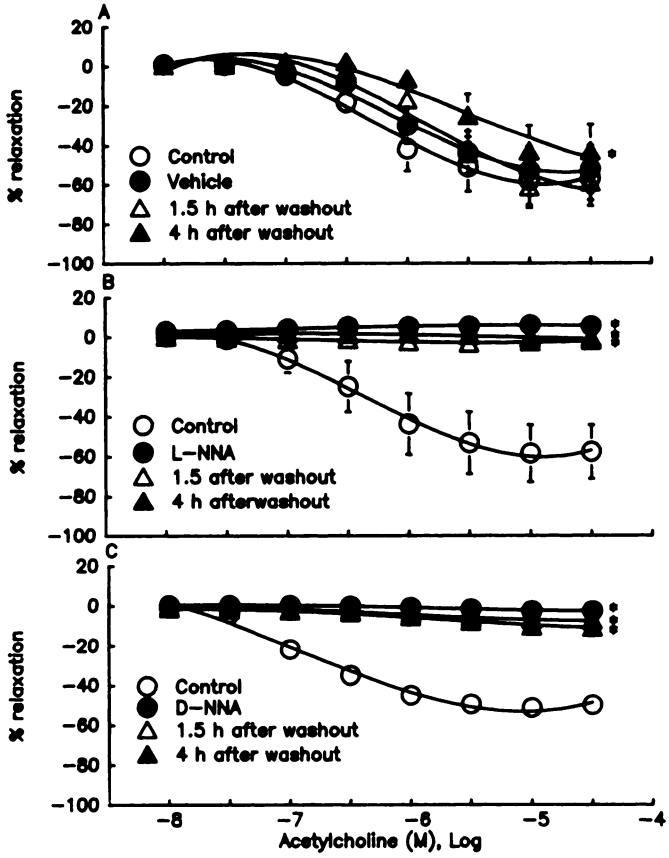

Fig. 5. Time course of the effects (mean ± S.E.) of vehicle (A), L-NNA (3×10^{-5} M, B) and D-NNA (3×10^{-4} M, C) on ACh-induced relaxation responses in PHE (10^{-6} M)-preconstricted rat aortic rings (n = 6–7 in each group). *Significant difference from the control curve ($P < .05$).

by the treatments with L- and D-NNA. Maximum relaxations in response to SNP (10^{-6} M) in vehicle-, L- and D-NNA-treated aortic rings were −107 ± 4, 99 ± 3 and 99 ± 3%, respectively.

Discussion

It has been shown that L-NMMA (Palmer *et al.*, 1988; Rees *et al.*, 1989a; Rees *et al.*, 1990; Crawley *et al.*, 1990), L-NNA (Mülsch and Busse, 1990; Lamontagne *et al.*, 1991), L-NIO (Rees *et al.*, 1990) and L-NAME (Rees *et al.*, 1990), but not the corresponding D-enantiomers, inhibited endothelium-dependent relaxations of isolated blood vessels and/or NO biosynthesis in endothelial cells (see Moncada *et al.*, 1991). L-enantiomeric specificity has also been reported to exist in other tissues or cells, *e.g.*, platelets (Radomski *et al.*, 1990a,b), macrophages (McCall *et al.*, 1991), adrenal cortex (Palacios *et al.*, 1989) and nonvascular smooth muscles (Hobbs and Gibson, 1990; Tucker *et al.*, 1990). In contrast to these findings, our results indicate that both L- and D-NNA efficaciously inhibit the relaxation response of ACh *in vitro* and *ex vivo*. Moreover, both compounds inhibit the relaxation response of the calcium ionophore A 23187. These results suggest that both L- and D-NNA inhibit endothelium-dependent relaxation induced by receptor- and nonreceptor-operated mechanisms, and that the L-enantiomeric configuration is not required for the actions of NNA.

Our present results are in accordance with our previous *in vivo* findings which show that i.v. injections of both L- and D-NNA cause pressor responses in pentobarbital-anesthetized rats (Wang and Pang, 1990b) and conscious rats (Wang *et al.*,

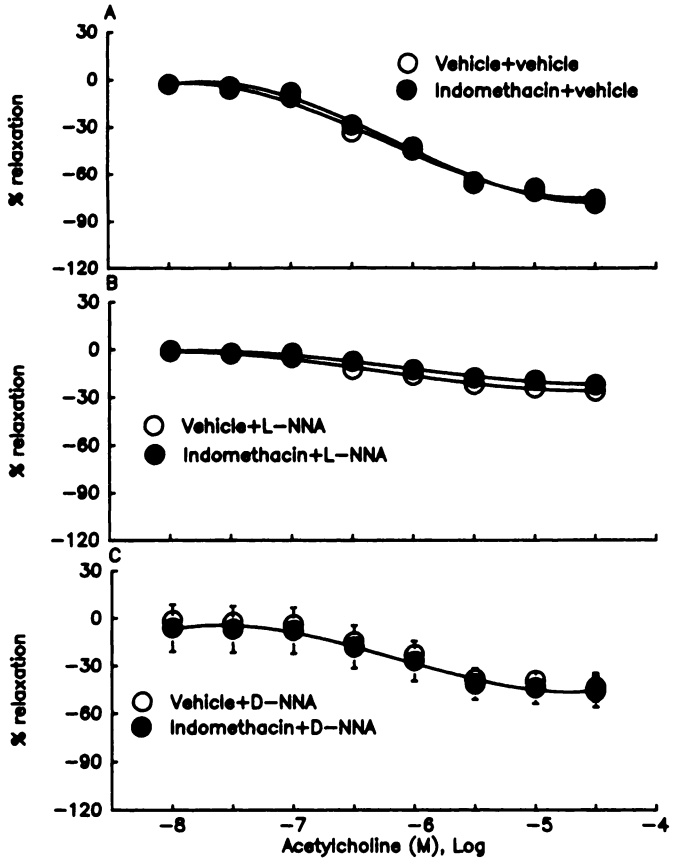

Fig. 6. Effects (mean ± S.E.) of a 10-min pretreatment with indomethacin (10^{-5} M) on ACh-induced relaxations in the absence (A) and presence of L-NNA (10^{-6} M, B) or D-NNA (3×10^{-5} M, C) in PHE (10^{-6} M)-preconstricted rat aortic rings (n = 6–7 in each group).

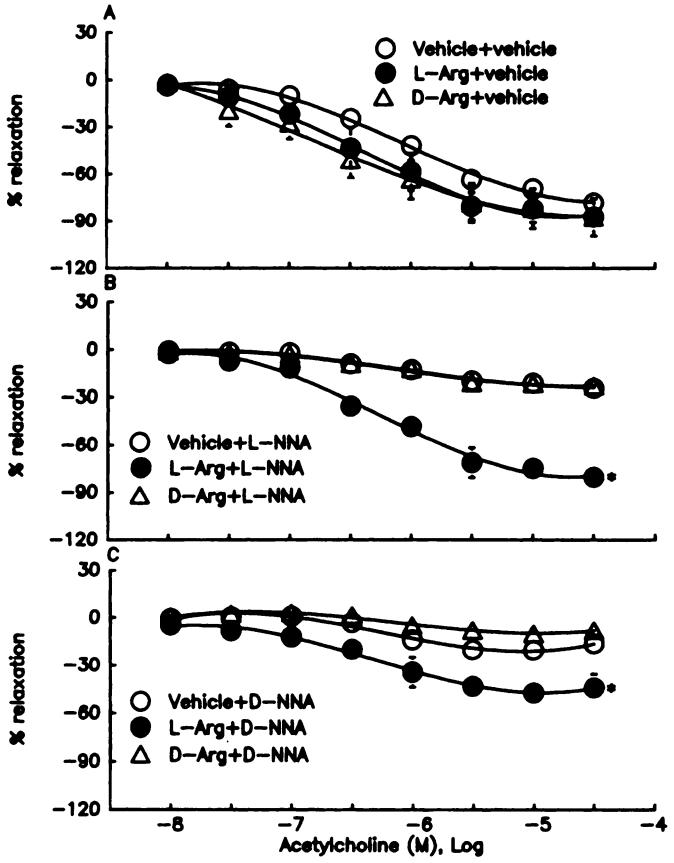

Fig. 7. Effects (mean ± S.E.) of a 10-min pretreatment with L-Arg (10^{-3} M) or D-Arg (10^{-3} M) on ACh-induced relaxations in the presence of vehicle (A), L-NNA (10^{-6} M, B) or D-NNA (3×10^{-5} M, C) in PHE (10^{-6} M)-preconstricted rat aortic rings (n = 6–7 in each group). *Significant difference from control curve ($P < .05$).

1991b). It could be argued that the effectiveness of D-NNA is due to contamination with L-NNA. However, there is no mistake about the identity of D-NNA as an independent analysis determined that the specific rotations, $[\alpha]_D$, of D- and L-NNA are −22.9° and +22.1°C, respectively (Wang *et al.*, 1991b). $[\alpha]_D$ of D-NNA from our independent analysis is consistent with the information ($[\alpha]_D$ −23.6°C) provided by the supplier, Bachem (Bubendorf, Switzerland). Moreover, other observations also indicate that the biological activities of D-NNA are not the result of contamination by L-NNA. 1) D-NNA from another drug company (Aminotech Ltd., Ontario, Canada) also exhibits similar biological activities (data not shown). We have also examined D-NNA sent to us by investigators who have reported negative results and found that the drug has activities indistinguishable from those of our supply of D-NNA (data not shown). 2) There are differences in the biological activities between L- and D-NNA (see below). 3) The onset of the pressor effect of D-NNA is markedly slower than that of L-NNA (Wang and Pang, 1990b; Wang *et al.*, 1991b). 4) L- and D-NAME also inhibit the endothelium-dependent vasodilatation evoked by ACh and/or calcitonin gene-related peptide *in vitro* and *in vivo* (Abdelrahman *et al.*, 1992; Wang *et al.*, 1992). In addition, both L- and D-NAME also cause pressor responses in conscious rats (Wang *et al.*, 1992).

The reasons for discrepancies between our results and those of others are not apparent but may be related to differences in concentrations or doses of D-NNA used, duration of observation and possibly preconceived ideas. It is well known that although the L-enantiomeric form is the main configuration of biologically active drugs, many D-enantiomers may have less or even greater biological activities than their corresponding L-enantiomers (see Ariëns, 1983). Inasmuch as the first report describing the enantiomeric specificity of L-Arg as a substrate and L-NMMA as an inhibitor, in which the same concentrations of D- and L-NMMA were used (Palmer *et al.*, 1988), the concept of L-enantiomeric specificity for activating or inhibiting NO synthase has become widely accepted (Moncada *et al.*, 1991). Due to the preconceived notion that the D-enantiomers of N^G-substituted Arg derivatives are inactive, systematic studies were not conducted with these compounds. The doses selected for the D-enantiomers of N^G-substituted Arg analogs as controls were always (without exception) the same as those of the corresponding L-enantiomers. Moreover, conclusions were usually drawn without showing data. Among the work cited in this paper the experimental conditions only in Mülsch and Busse's report (1990) are similar to ours. They found that L- but not D-NNA (both at 3×10^{-5} M) produced approximately 80% inhibition of ACh-induced relaxation in norepinephrine (EC_{60})-preconstricted rabbit femoral arteries. In the present study, L-NNA (3×10^{-5} M, supramaximal dose) almost completely inhibits ACh-induced relaxation in rat aortae. Because the *in vitro* potency of D-NNA is approximately 1/39 that of L-NNA (see later), it would be expected that D-NNA (3×10^{-5} M) should have caused considerably less response in the rabbit femoral arteries. The potencies of N^G-substituted L-Arg analogs

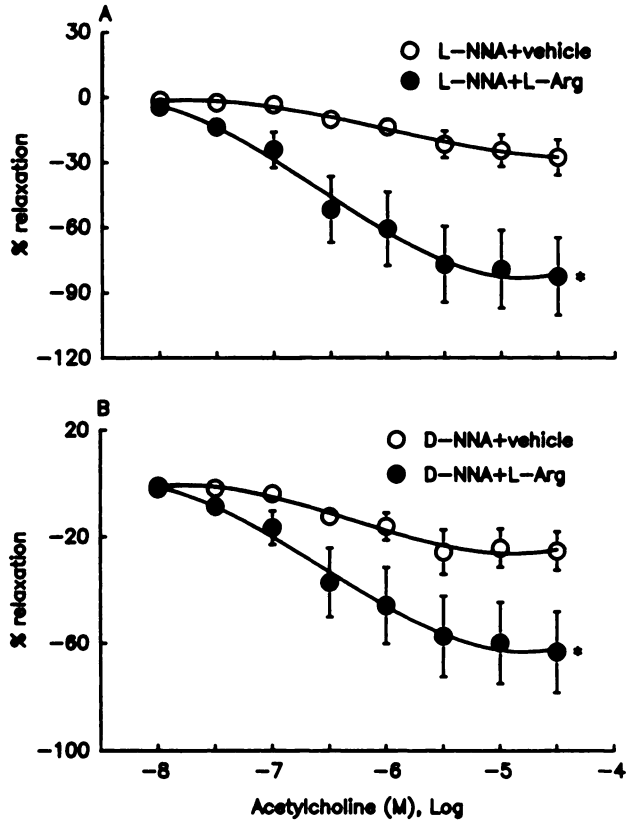

Fig. 8. Effects (mean ± S.E.) of post-treatment (1 hr) with L-Arg (10^{-3} M) on the inhibitory effects of L-NNA (10^{-6} M, A) and D-NNA (3×10^{-5} M, B) on ACh-induced relaxation in PHE (10^{-6} M)-preconstricted rat aortic rings (n = 6–7 in each group). The rings were incubated for 1 hr with L- or D-NNA followed by a 10-min treatment with L-Arg or vehicle. *Significant difference from control curve ($P < .05$).

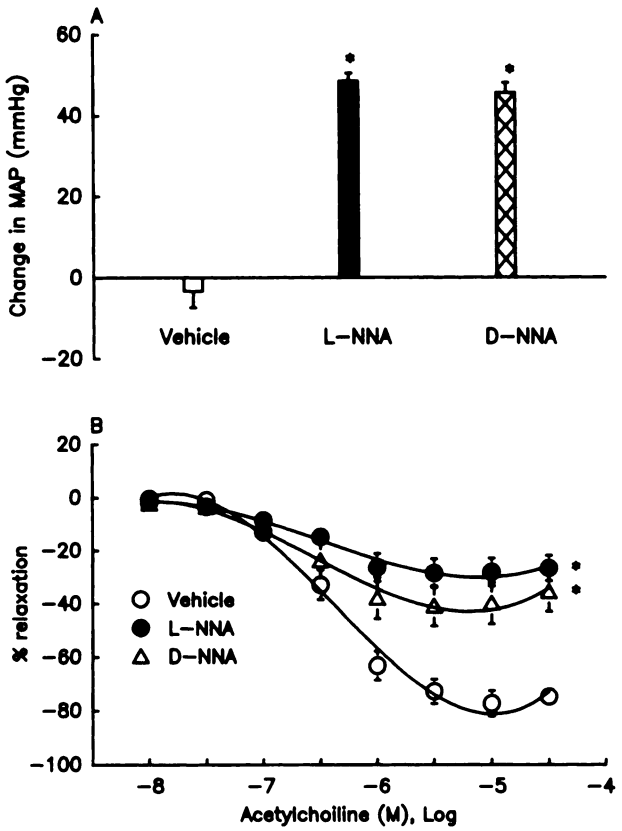

Fig. 9. Effects (mean ± S.E.) of i.v. bolus injections of vehicle, L-NNA (1.6×10^{-4} mol/kg) and D-NNA (1.6×10^{-4} mol/kg) on MAP (A) and *ex vivo* relaxation responses to ACh (B) in PHE (10^{-6} M)-preconstricted aortic rings from the treated rats (n = 5 in each group). *Significant difference from vehicle-treated group ($P<.05$).

are known to differ greatly according to particular preparations and chemical structures (see Moncada *et al.*, 1991). Therefore, the potencies of D-enantiomers should also vary with the preparations and types of compounds used. We found that L-NNA is 2-fold more potent than D-NNA in raising blood pressure (Wang *et al.*, 1991b) and 39-fold more potent than D-NNA in inhibiting endothelium-dependent relaxation (present work). L-NAME, on the other hand, is 55- and 359-fold more potent that D-NAME in raising blood pressure and inhibiting endothelium-dependent relaxation, respectively (Wang *et al.*, 1992). Moreover, pressor responses to D-NNA (Wang and Pang, 1990b; Wang *et al.*, 1991b) and D-NAME (Wang *et al.*, 1992) are substantially slower in onset than the corresponding L-enantiomers, with this difference in onset being accentuated in anesthetized rats (Wang and Pang, 1990b). Thus, it is reasonable to assume that incorrect conclusions would be derived when either the concentrations (or doses) of N^G-substituted Arg analogs were insufficient or the observation time was not long enough.

Palmer *et al.* (1988) reported that cultured endothelial cells synthesized NO from the terminal guanido nitrogen atom of L-, but not D-Arg. They also showed that L-Arg but not D-Arg produced endothelium-dependent relaxation of vascular rings and inhibited endothelium-dependent contractions induced by L-NMMA (Palmer *et al.*, 1988). More recently, it was shown that L- but not D-Arg attenuated the inhibitory effect of L-NNA (Moore *et al.*, 1990). These results are in accordance with ours which show that L- but not D-Arg prevents the inhibitory effect of L-NNA. As the effect of D-NNA is also prevented by L-Arg but not by D-Arg, the inhibitory effect of D-NNA on ACh-induced relaxation, like that of L-NNA, may also involve the inhibition of endothelial NO synthesis.

Both L- and D-NNA caused prolonged inhibition (>4 hr) of *in vitro* relaxation responses to ACh. The long-lasting inhibitory effects of L- and D-NNA are also seen in *ex vivo* studies, because the inhibitory effects on vascular preparations were tested approximately 1.5 hr after *in vivo* administrations of the drugs and after three washouts. The long duration of action of L-NNA is consistent with the report that L-NNA causes prolonged inhibition of NO synthesis in cultured endothelial cells (Mülsch and Busse, 1990). We have reported previously that both L- and D-NNA are long-lasting pressor agents (Wang and Pang, 1990b; Wang *et al.*, 1991b). Therefore, the prolonged biological effects of L- and D-NNA on endothelium-dependent relaxation may account for, at least in part, the long-lasting pressor effects of L- and D-NNA *in vivo*. Moreover, the pressor effect of L-NNA was prevented but not reversed by L-Arg (Wang and Pang, 1990b; Wang *et al.*, 1991b; Zambetis *et al.*, 1991). These observations raise the possibility that inhibitory effects of L- and D-NNA are irreversible. It has been reported that L-NIO is a long-lasting and irreversible NO synthase inhibitor in rat peritoneal neutrophils and the murine macrophage cell-line J744, inasmuch as this effect was not reversed by L-Arg but was prevented by concomitant incubations of L-NIO with L-Arg (McCall *et al.*, 1991). It was also reported recently that the inhibition of NO synthase by L-NAA was

reversible initially but became irreversible with time (Rouhani *et al.*, 1992). However, the *in vitro* inhibitory effects of L-NNA (and D-NNA) on ACh-induced relaxation, unlike those of L-NIO and L-NAA, are prevented by L-Arg and reversed by L-Arg even after the preparations were incubated for 1 hr with L- or D-NNA.

We found that indomethacin does not affect the relaxation response of ACh. This is in accordance with studies demonstrating that prostaglandins do not account for effects of EDRF/NO (Furchgott and Zawadzki, 1980). On the other hand, it was reported recently that the cyclooxygenase inhibitors indomethacin and acetylsalicylic acid, and superoxide dismutase, blocked the effects of L-NMMA on contraction and ACh- and L-Arg-induced vasodilatations of pial arterioles and platelets adhesion/aggregation in mice *in vivo*. It was suggested that L-NMMA interfered with endothelium-dependent relaxation and produced constriction by activating cyclooxygenase and producing superoxide which subsequently inactivated EDRF/NO (Rosenblum *et al.*, 1992). Such findings are contrary to previous results which showed that indomethacin does not inhibit the pressor response to L-NMMA (Rees *et al.*, 1989b). Indomethacin has been frequently added to the physiological solution in order to avoid a possible contribution by prostaglandins to endothelium-dependent relaxations (*e.g.*, Mülsch and Busse, 1990). However, our results show that indomethacin, at a concentration high enough to inhibit prostaglandin synthesis, does not alter the inhibitory effects of L- and D-NNA. These results suggest that cyclooxygenase activation and subsequent superoxide production and inactivation of EDRF/NO does not account for the inhibitory effects of L- and D-NNA on ACh-induced relaxation.

There are differences in the vasoconstrictor effects between L- and D-NNA. First, L-NNA concentration-dependently contracts aortic rings and potentiates PHE-induced contraction. Although D-NNA is as efficacious as L-NNA in inhibiting ACh-induced relaxation, it does not induce contraction of aortic rings or potentiate PHE-induced contraction. It has been reported that the concentrations of L-NNA and L-NMMA that were maximally effective at increasing tension in canine coronary arteries only caused submaximal inhibitions of ACh-induced relaxations (Cocks and Angus, 1991). In the present study, 10^{-5} M L-NNA produces maximum inhibition of ACh-induced relaxation but does not produce maximum contractile response. The contractile effect of L-NMMA was found to be endothelium-dependent and reversed by L-Arg suggesting that this response was caused by the inhibition of basal NO formation (Palmer *et al.*, 1988; Rees *et al.*, 1989a). In contrast, Cocks and Angus (1991) recently showed that the contractile response of L-NMMA in dog coronary arteries was not affected by pretreatment with hemoglobin or $FeSO_4$ in concentrations that inhibited relaxations induced by SNP and NO, suggesting that the contractile response of L-NMMA was independent of basal NO formation. Moreover, L-Arg was reported to reverse L-NAME-induced augmentation of contractions evoked by 5-hydroxytryptamine and histamine, but not L-NAME-induced inhibition of endothelium-dependent vasodilatation evoked by ACh in perfused rabbit ear preparations (Randall and Griffith, 1991). We have also found that L- but not D-NNA caused a slow and sustained contraction in denuded rat aortic rings; the effect is not affected by endothelium and L-Arg (Wang and Pang, unpublished data, 1992). These results suggest that contraction and inhibition of relaxation responses of N^G-substituted Arg derivatives may be produced by different mechanisms.

Another difference between L- and D-NNA is potency. As indicated above, although both compounds have similar efficacy, D-NNA is less potent than L-NNA in inhibiting endothelium-dependent relaxation, suggesting that the vasoconstrictor effects of NNA prefer the L-enantiomeric configuration. Moreover, the difference in potencies between D- and L-NNA *in vitro* is higher than those *in vivo*. The mechanism responsible for this discrepancy between the *in vitro* and *in vivo* potency of D- and L-NNA is not known. One possible explanation is chiral conversion. Metabolic chiral inversion has been shown to occur after the p.o. administration of stereospecific drugs (Hutt and Caldwell, 1983; Sanins *et al.*, 1991). Because D-NNA is less potent and has a slower onset of action than L-NNA *in vivo* (Wang *et al.*, 1991b), D-NNA may act *via* metabolic conversion to L-NNA *in vivo*, thus accounting for the difference in the activity ratios of D- and L-NNA between *in vivo* and *in vitro* settings. It may also be speculated that the difference is attributable to variations in affinity ratios for D- and L-NNA with respect to conductance and resistance arteries. More studies are required to resolve this puzzle.

In summary, both L- and D-NNA are selective, efficacious, long-lasting and reversible inhibitors of endothelium-dependent relaxation responses evoked by receptor-operated and non-receptor-operated mechanisms. However, D-NNA is less potent than L-NNA in inhibiting the relaxation response of ACh and, unlike L-NNA, does not produce a contractile response or potentiate PHE-induced contraction in isolated aortic rings. Our results suggest that the L-configuration of N^G-substituted Arg analogs is preferred but not essential for the inhibition of endothelium-dependent relaxation.

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References

ABDELRAHMAN, A., WANG, Y.-X., CHANG, S. D. AND PANG, C. C. Y.: Mechanism of the vasodilator action of calcitonin gene-related peptide in conscious rats. Br. J. Pharmacol. **106:** 45–48, 1992.

ARIENS, E. J.: Stereoselectivity of bioactive agents: General aspects. *In* Stereochemistry and Biological Activity of Drugs, ed. by by E. J. Ariens, W. Soudijn and P. B. M. W. M. Timmermans, pp. 11–32, Blackwell Scientific Publications, Oxford, 1983.

COCKS, T. M. AND ANGUS, J. A.: Evidence that contractions of isolated arteries by L-NMMA and NOLA are not due to inhibition of basal EDRF release. J. Cardiovasc. Pharmacol. **17:** Suppl. 3, S159–S164, 1991.

CRAWLEY, D. E., LIU, S. E., EVANS, T. W. AND BARNES, P. J.: Inhibitory role of endothelium-derived relaxing factor in rat and human pulmonary arteries. Br. J. Pharmacol. **101:** 166–170, 1990.

FURCHGOTT, R. F. AND ZAWADZKI, J. V.: The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. Nature (Lond.) **299:** 373–376, 1980.

GARDINER, S. M., COMPTON, A. M., BENNETT, T., PALMER, R. M. J. AND MONCADA, S.: Control of regional flow by endothelium-derived nitric oxide. Hypertension **15:** 486–492, 1990a.

GARDINER, S. M., COMPTON, A. M., KEMP, P. A. AND BENNETT, T.: Regional and cardiac haemodynamic effects of N^G-nitro-L-arginine methyl ester in conscious, Long Evans rats. Br. J. Pharmacol. **101:** 625–631, 1990b.

HOBBS, A. AND GIBSON, A.: L-N^G-nitro-arginine and its methyl ester are potent inhibitors of non-adrenergic, non-cholinergic transmission in the rat anococcygeus. Br. J. Pharmacol. **100:** 749–752, 1990.

HUMPHRIES, R. G., CARR, R. D., NICOL, A. K., TOMLINSON, W. AND O'CONNOR, S. E.: Coronary vasoconstriction in the conscious rabbit following intravenous infusion of L-N^G-nitro-arginine. Br. J. Pharmacol. **102:** 565–566, 1991.

HUTT, A. J. AND CALDWELL, J.: The metabolic chiral inversion of 2-arylpropionic acids: A novel route with pharmacological consequences. J. Pharm. Pharmacol. **35:** 693–704, 1983.

ISHII, K., CHANG, B., KERWIN, J. F., JR., HUANG, Z.-J. AND MUARD, F.: N^{ω}-nitro-L-arginine: A potent inhibitor of endothelium-derived relaxing factor formation. Eur. J. Pharmacol. **176:** 219–223, 1990.

KOBAYASHI, Y. AND HATTORI, K.: Nitroarginine inhibits endothelium-derived relaxation. Jpn. J. Pharmacol. **52:** 167–169, 1990.

LAMONTAGNE, D., POHL, U. AND RUSSE, R.: N^G-nitro-L-arginine antagonizes endothelium-dependent dilator responses by inhibiting endothelium-derived relaxing factor release in the isolated rabbit heart. Pflügers Arch. **418:** 266–270, 1991.

MCCALL, T. B., FEELISH, M., PALMER, R. M. J. AND MONCADA, S.: Identification of N-iminoethyl-L-ornithine as an irreversible inhibitor of nitric oxide synthase in phagocytic cells. Br. J. Pharmacol. **102:** 234–238, 1991.

MONCADA, S., PALMER, R. M. J. AND HIGGS, E.: Nitric oxide: Physiology, pathophysiology and pharmacology. Pharmacol. Rev. **43:** 109–142, 1991.

MOORE, P. K., AL-SWAYEH, O. A., CHONG, N. W. S., EVANS, R. A. AND GIBSON, A.: L-N^G-nitro arginine (L-NOARG), a novel L-arginine-reversible inhibitor of endothelium-dependent vasodilation *in vitro*. Br. J. Pharmacol. **99:** 408–412, 1990.

MÜLSCH, A. AND BUSSE, R.: N^G-nitro-L-arginine (N^5-[imino (nitroamino) methyl]-L-ornithine) impairs endothelium-dependent dilations by inhibiting cytosolic nitric oxide synthesis from L-arginine. Naunyn-Schmiedeberg's Arch. Pharmacol. **341:** 143–147, 1990.

PALACIOS, M., KNOWLES, R. G., PALMER, R. M. AND MONCADA, S.: Nitric oxide from L-arginine stimulates the soluble guanylate cyclase in adrenal glands. Biochem. Biophys. Res. Commun. **165:** 802–809, 1989.

PALMER, R. M. J., REES, D. D., ASHTON, D. S. AND MONCADA, S.: L-arginine is the physiological precursor for the formation of nitric oxide in endothelium-dependent relaxation. Biochem. Biophys. Res. Commun. **153:** 1251–1256, 1988.

RADOMSKI, M. W., PALMER, R. M. J. AND MONCADA, S.: An L-arginine to nitric oxide pathway in human platelets regulates aggregation. Proc. Natl. Acad. Sci. U.S.A. **87:** 5193–5197, 1990a.

RADOMSKI, M. W., PALMER, R. M. J. AND MONCADA, S.: Characterization of the L-arginine: Nitric oxide pathway in human platelets. Br. J. Pharmacol. **101:** 325–328, 1990b.

RANDALL, M. D. AND GRIFFITH, T. M.: Differential effects of L-arginine on the inhibition by N^G-nitro-L-arginine methyl ester of basal and agonist-stimulated EDRF activity. Br. J. Pharmacol. **104:** 743–749, 1991.

REES, D. D., PALMER, R. M. J., HODSON, H. F. AND MONCADA, S.: A specific inhibitor of nitric oxide formation from L-arginine attenuates endothelium-dependent relaxation. Br. J. Pharmacol. **96:** 418–424, 1989a.

REES, D. D., PALMER, R. M. J. AND MONCADA, S.: The role of endothelium-derived nitric oxide in the regulation of blood pressure. Proc. Natl. Acad. Sci. U.S.A. **86:** 3375–3378, 1989b.

REES, D. D., PALMER, R. M. J., SCHULZ, R., HODSON, H. F. AND MONCADA, S.: Characterization of three inhibitors of endothelial nitric oxide synthesis *in vitro* and *in vivo*. Br. J. Pharmacol. **101:** 746–752, 1990.

ROSENBLUM, W. I., NISHIMURA, H. AND NELSON, G. H.: L-NMMA in brain microcirculation of mice is inhibited by blockade of cyclooxygenase and by superoxide dismutase. Am. J. Physiol. **262:** H1343–1349, 1992.

ROUHANI, R., GROSS, S. S., MADERA, A. M., LEVI, R. AND GRIFFITH, O. W.: N-methyl substituted derivatives of N^ω-amino-L-arginine as reversible and irreversible inhibitors of nitric oxide synthase (Abstrat). Fed. Am. Soc. Exp. Biol. J. **6:** A1256, 1992.

SANINS, S. M., ADAMS, W. J., KAISER, D. G., HALSTEAD, G. W., HOSLEY, J., BARNES, H. AND BAILLIE, T. A.: Mechanistic studies on the metabolic chiral inversion of R-ibuprofen in the rat. Drug Metab. Dispos. **19:** 405–410, 1991.

TUCKER, J. F., BRAVE, S. R., CHARALAMBOUS, L., HOBBS, A. J. AND GIBSON, A.: L-N^G-nitro arginine inhibits non-adrenergic, non-cholingic relaxations of guinea-pig isolated tracheal smooth muscle. Br. J. Pharmacol. **100:** 663–664, 1990.

VARGAS, H. M., CUEVAS, J. M., IGNARRO, L. J. AND CHAUDHURI, G.: Comparison of the inhibitory potencies of N^G-methyl-, N^G-nitro- and N^G-amino-L-arginine on EDRF function in the rat: Evidence for continuous basal EDRF release. J. Pharmacol. Exp. Ther. **257:** 1208–1215, 1991.

WANG, Y.-X. AND PANG, C. C. Y.: Pressor effect of N^G-nitro-L-arginine in pentobarbital-anesthetized rats. Life Sci. **47:** 2217–2224, 1990a.

WANG, Y.-X. AND PANG, C. C. Y.: Effects of L and D enantiomers of N^G-nitro-L-arginine on blood pressure in pentobarbital-anaesthetized rats. Life Sci. Adv. **9:** 723–728, 1990b.

WANG, Y.-X. AND PANG, C. C. Y.: Possible dependence of pressor and heart rate effects of N^G-nitro-L-arginine on autonomic nerve activity. Br. J. Pharmacol. **103:** 2004–2008, 1991.

WANG, Y.-X., POON, C. I., YAU, L. C. Y. AND PANG, C. C. Y.: The *in Vivo* and *in Vitro* Vasoconstrictor Effects of L and D Enantiomers of N^G-Nitro-Arginine Methyl Ester (NAME)abstract, p. 80, The 35th Annual Meeting of Canadian Federation of Biological Societies, Victoria, Canada, 1992.

WANG, Y.-X., ZHOU, T., CHOU, T. C. AND PANG, C. C. Y.: Effects of inhalation and intravenous anaesthetic agents on pressor response to N^G-nitro-L-arginine. Eur. J. Pharmacol. **198:** 183–188, 1991a.

WANG, Y.-X., ZHOU, T. AND PANG, C. C. Y.: Pressor effects of L and D enantiomers of N^G-nitro-arginine in conscious rats are antagonized by L- but not D-arginine. Eur. J. Pharmacol. **200:** 77–81, 1991b.

ZAMBETIS, M., DUSTING, G. J., RAJANAYAGAM, S. AND WOODMAN, O. L.: Mechanism of the hypertension produced by inhibition of nitric oxide biosynthesis in rats. J. Cardiovasc. Pharmacol. **17:** Suppl. 3, S191–S197, 1991.

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