VASCULAR PHARMACOLOGY OF NITRIC OXIDE SYNTHASE INHIBITORS

by

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A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF

THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

in

THE FACULTY OF GRADUATE STUDIES

(Department of Pharmacology & Therapeutics, Faculty of Medicine)

We accept this thesis as conforming

to the required standard

THE UNIVERSITY OF BRITISH COLUMBIA

SEPTEMBER, 1993

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Date April 27, 1994

DE-6 (2/88)

Abstract

This dissertation examined the effects of nitric oxide synthase (NOS) inhibitors, N^G-substituted arginine analogues (NSAAs) including N^G-nitro-arginine (NNA) and its methyl ester (NAME), as well as diphenyleneiodonium (DPI), on endothelium-dependent vasodilatation and pressor response in rats.

All NSAAs caused complete, prolonged and "noncompetitive" inhibition of acetylcholine (ACh)-, the calcium ionophore A 23187- and/or bradykinin-induced relaxations *in vitro* and *ex vivo*, with lower potencies for the D-enantiomers. The Hill coefficient (*n*)s for the inhibition of NSAAs, were not significantly different from 1. The inhibition of NSAAs was antagonized by either pre- or post-treatment with L-arginine (L-Arg). L-NAME partially inhibited ACh-induced depressor response *in vivo*. NSAAs caused long-lasting pressor responses in conscious rats. The *n*s of the dose-pressor response curves of L-NNA, L-NAME and D-NAME were 2 or more. The pressor response to L-NNA or L-NAME was not attenuated by pithing, or treatment with mecamylamine, reserpine, phentolamine, captopril, or indomethacin, while those to NSAAs were attenuated by L-Arg; L-Arg competitively antagonized the pressor response to L-NAME at $n = 2$.

DPI caused complete, long-lasting and "noncompetitive" inhibition of ACh- and A 23187-induced vasodilatation *in vitro* and partial inhibition of ACh-induced vasodilatation *in vivo*. The inhibition was prevented by pretreatments with nicotinamide adenine dinucleotide (NADPH) and flavin adenine dinucleotide (FAD), but was slightly reversed by post-treatment with NADPH. DPI caused transient

pressor response with *n* of 3 or 4, as well as raised plasma catecholamines in rats. The pressor response to DPI was attenuated by pithing and spinal cord transection, as well as treatment with tetrodotoxin, reserpine, mecamylamine, guanethidine or phentolamine.

Therefore, both NSAAs and DPI inhibit endothelium-dependent relaxations, the inhibiton of NSAAs is reversible whereas that of DPI is irreversible. The L-configuration is preferred but not essential for NSAAs to inhibit endothelium-dependent relaxation. The mechanism of NSAAs involves antagonizing the NOS substrate L-Arg, whereas that of DPI involves interfering with the NOS cofactors FAD and NADPH. The pressor responses to NSAAs are competitively antagonized by L-Arg and are not dependent on the integrity of the central and autonomic nervous, angiotensin or prostaglandin system. The pressor response to DPI is due to sympathetic activation.

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Abbreviations

ACh	acetylcholine
ADMA	dimethylarginine
Arg	arginine
C	concentration
cAMP	cyclic adenosine 3',5'-monophosphate
cGMP	cyclic guanosine 3',5'-monophosphate
CO	cardiac output
cpm	counts per minute
D	dose
DPI	diphenyleneiodonium
DR	dose ratio
EC_{50}	half-effective concentration
ED_{50}	half-effective dose
EDHF	endothelium-derived hyperpolarizing factor
EDRF	endothelium-derived relaxing factor
EGTA	ethylene glycol-*bis*(ß-amino-ethyl ether) N,N,N',N'-tetraacetic acid
E_{max}	maximal effect
E_{min}	minimal effect
eNOS	endothelium nitric oxide synthase
FMN	flavin mononucleotide
GTP	guanosine 5'-triphosphate
HR	heart rate
IC_{50}	half-inhibitory concentration
iNOS	inducible nitric oxide synthase
L-NAA	N^G-amino-L-arginine
LPS	lipopolysaccharide

MAP	mean arterial pressure
MCFP	mean circulatory filling pressure
n	Hill coefficient
NAME	N^G-nitro-arginine methyl ester
NANC	nonadrenergic, noncholinergic
NIO	N-iminoethyl-ornithine
NMDA	N-methyl-D-aspartate
NMMA	N^G-monomethyl-arginine
NNA	N^G-nitro-arginine
nNOS	neuron nitric oxide synthase
NO	nitric oxide
NOS	nitric oxide synthase
NSAAs	N^G-substituted arginine analogues
6-OH-DA	6-hydroxydopamine
PHE	phenylephrine
SNP	sodium nitroprusside
SOD	superoxide dismutase
TPR	total peripheral resistance
TTX	tetrodotoxin

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Acknowledgements

I thank all members of this department who have helped complete this dissertation. I would like to acknowledge my gratitude especially to:

Dr. Catherine C.Y. Pang, my research supervisor, for sharing her knowledge and experience, for her friendship and encouragement, constructive criticism, as well as wise guidance throughout the study, and for her generous financial support during the first stage of the Ph.D. program from her MRC grant.

Dr. David M.J. Quastel for his invitation to work as a post-doctoral research fellow during my first year in Canada, his help in analyzing dose-response curves by the use of his computer program, and his valuable advice in my work.

Drs. Morley C. Sutter, Michael J.A. Walker, Jack Diamond, James M. Wright, Richard A. Wall, Sultan Karim and Reza Tabrizchi for their valuable advice and critical evaluations of my work.

Ms. Su Lin Lim, Miss Christina C.I. Poon, Dr. Ting Zhou, Dr. Aly Abdelrahman, Miss Lorraine C.Y. Yau and Mr. Ken S. Poon for their technical assistance.

My wife, Ji-Ping Huang, and my mother for their understanding, encouragement and help throughout my study.

The Medical Research Council of Canada for its generous financial support during the last stage of the Ph.D. program by awarding me a Post-doctoral Research Fellowship; the Canadian Pharmacological Society and Canadian Hypertension Society for their encouragement by awarding me the Annual (1992) Graduate Student Presentation Award and Annual (1993) Trainee Award, respectively; the University of British Columbia, American Pharmacological Society and British Columbia Government for their financial support for me to attend scientific conferences.

Preface

This dissertation was based on the results from the following list of my publications which were referred to by Roman numerals in the appendices, and some unpublished data which were presented in the contents. Permission to reproduce these publications were kindly provided by the individual copyright holders.

I. 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.

II. Wang, Y.-X., Zhou, T. and Pang, C.C.Y.: Pressor effects of L and D enantiomers of N^G-nitro-L-arginine in conscious rats are antagonized by L- but not D-arginine. Eur. J. Pharmacol. 200: 77-81, 1991.

III. Wang, Y.-X., Poon, C.I. and Pang, C.C.Y.: *In vitro* and *ex vivo* inhibitory effects of L and D enantiomers of N^G-nitro-arginine on endothelium-dependent relaxation of rat aorta. J. Pharmacol. Exp. Ther. 265: 112-119, 1993.

IV. Wang, Y.-X. and Pang, C.C.Y.: Functional integrity of the central and sympathetic nervous systems is a prerequisite for pressor and tachycardic effects of diphenyleneiodonium, a novel inhibitor of nitric oxide synthase. J. Pharmacol. Exp. Ther. 265: 263-272, 1993.

V. Wang, Y.-X. and Pang, C.C.Y.: Halothane inhibits pressor effect of diphenyleneiodonium. Br. J. Pharmacol. 109: 1186-1191, 1993.

VI. Wang, Y.-X., Abdelrahman, A. and Pang, C.C.Y.: Selective inhibition of pressor and haemodynamic effects of N^G-nitro-L-arginine by halothane. J. Cardiovasc. Pharmacol. 22: 571-578, 1993.

VII. Wang, Y.-X., Poon, C.I., Poon, K.S. and Pang, C.C.Y.: Inhibitory actions of diphenyleneiodonium on endothelium-dependent vasodilatations *in vitro* and *in vivo*. Br. J. Pharmacol. 110: 1232-1238, 1993.

VIII. Wang, Y.-X., Poon, C.I. and Pang, C.C.Y.: Vascular pharmacodynamics of N^G-nitro-L-arginine methyl ester *in vitro* and *in vivo*. J. Pharmacol. Exp. Ther. 267: 1091-1099, 1993.

The following publications related to this study were also referred to in the introduction and discussion sections, and were listed in the bibliography.

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, 1990.

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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.

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Wang, Y.-X. and Pang, C.C.Y.: Effects of adrenalectomy and chemical sympathectomy on the pressor and tachycardic responses to diphenyleneiodonium. J. Pharmacol. Exp. Ther. in press, 1994.

Wang, Y.-X. and Pang, C.C.Y.: N^G-nitro-L-arginine contracts vascular smooth muscle *via* a non-nitric oxide synthesis mechanism. J. Cardiovasc. Pharmacol. in press, 1994.

1. Introduction

1.1. EDRF and NO

1.1.1. Endothelium-dependent relaxation and EDRF

The investigation of the actions of acetylcholine (ACh) in the vasculature was initiated over 80 years ago. It was found that while ACh always caused depressor responses in whole animals (Hunt, 1915), the actions of ACh in isolated vascular preparations were rather inconsistent. Dale (1914) observed that ACh dilated rabbit ear arteries whereas Hunt (1915) observed that ACh had either no effect or vasoconstriction in a similar preparation. This paradox was reported by many authors in many vascular preparations (see review by Furchgott, 1955). The controversial results were intensely studied during the subsequent more than half-century, and some hypotheses were put forward, such as excess ACh hypothesis (Burn and Robinson, 1951), adrenaline release hypothesis (Kottegoda, 1953) and two receptor type hypothesis (see review by Furchgott, 1955). The cause for the differing responses, however, was not determined until the last decade.

In 1980, Furchgott and Zawadzki for the first time found that if care was taken to preserve the intimal surface to prepare the isolated aortic rings or strips, the preparations were always relaxed by ACh. They therefore concluded that ACh stimulated muscarinic receptors on vascular endothelial cells to release a nonprostanoid substance which then caused relaxation of smooth muscles (Furchgott and Zawadzki, 1980). The substance was later termed endothelium-derived relaxing factor (EDRF) (Cherry *et al.*, 1982). Since then, many other vasodilator agents were also found to require the presence of endothelial cells to produce partial or complete relaxation of arteries, veins and microvessels. Among these are the calcium ionophore A 23187, adenosine 5'-triphosphate (ATP) and

adenosine 5'-diphosphate (ADP), substance P, arachidonic acid, bradykinin (for pig, dog and human but not rabbit or cat arteries), histamine (for rat aorta and guinea pig pulmonary artery), thrombin (for canine arteries) and calcitonin gene-related peptide (for rat aorta) (see review by Furchgott, 1983; see review by Moncada *et al.*, 1988; see review by Lüscher and Vanhoutte, 1990a). Other stimuli, such as hypoxia, sheer stress and electrical stimulation, also induce endothelium-dependent relaxation of vasculatures *in vitro* (see review by Moncada *et al.*, 1988; see review by Lüscher and Vanhoutte, 1990a). Certain differences among species and vasculatures in the dependence of the endothelium for vasodilatation have also been identified (see review by Furchgott, 1983; see review by Moncada *et al.*, 1988; see review by Lüscher and Vanhoutte, 1990b).

There are also many compounds which cause relaxation of isolated arterial preparations in an endothelium-independent manner. Among these are the nitrovasodilators which include sodium nitroprusside (SNP) and nitroglycerin, bovine retractor-penis inhibitor, and prostacyclin (see review by Moncada *et al.*, 1988; see review by Lüscher and Vanhoutte, 1990a). ß-Adrenoceptor agonists are widely believed to cause endothelium-independent vasodilatation *via* the production of cyclic adenosine 3',5'-monophosphate (cAMP) (see review by Furchgott and Vanhoutte, 1989; see review by Lüscher and Vanhoutte, 1990; see review by Moncada *et al.*, 1991). However, $ß_2$-adrenoceptors have been detected in cultured or naive (Molenaar *et al.*, 1988) human and bovine endothelial cells (Howell *et al.*, 1988; Ahmad *et al.*, 1990). Moreover, mechanical removal of the endothelium partially prevented salbutamol ($ß_2$-adrenoceptor agonist)-induced relaxation of preconstricted rat aortae, suggesting that endothelial cells contribute to $ß_2$-adrenoceptor-induced vascular relaxation (Wang *et al.*, 1993a).

The role of the endothelium in the actions of vasodilators from traditional Chinese medicinal plants has been studied. It was reported that the relaxant effect of magnolol was dependent on the endothelium. Removal of the

endothelium, on the other hand, did not alter the vasorelaxant effects of dicentrine, berberine, tetramethylpyrazine and norathyriol; these compounds may have antagonistic activities on the calcium channels or intracellular calcium release (see review by Sutter and Wang, 1993).

EDRF was reported to be a labile humoral factor with a half-life of less than 5 seconds in physiological preparations (Palmer *et al.*, 1987). It has also been suggested that EDRF is continuously released (Martin *et al.*, 1985). In addition to relaxing vascular preparations, EDRF also inhibits platelet aggregation (Azuma *et al.*, 1986; Radomski *et al.*, 1987a,c) and platelet adhesion (Radomski *et al.*, 1987d). A rise in the level of cyclic guanosine 3',5'-monophosphate (cGMP) in smooth muscles or platelets, which is a consequence of the stimulation of soluble guanylyl cyclase (Ignarro *et al.*, 1986; Russe, 1987), was found to accompany endothelium-dependent relaxation (Holzmann, 1982; Rapoport and Murad, 1983; Diamond and Chu, 1983; Ignarro *et al.*, 1984) and the inhibition of platelet aggregation (Rapoport and Murad, 1983). Endothelium-dependent relaxation and the inhibition of platelet aggregation are potentiated by M & B 22948 (zaprinast) and MY 5545, two selective inhibitors of cGMP phosphodiesterase (Kukovetz *et al.*, 1982; Martin *et al.*, 1986a; Radomski *et al.*, 1987a,c,d) and are inhibited by the soluble guanylyl cyclase inhibitor methylene blue (Martin *et al.*, 1985). Moreover, endothelium-dependent relaxation, inhibition of platelet aggregation and rise in cGMP are all inhibited by haemoglobin and other reduced haemoproteins which bind to EDRF (Ignarro *et al.*, 1984; Martin *et al.*, 1986b; Radmoski *et al.*, 1987a).

1.1.2. The pharmacology of NO

Nitric oxide (NO) is a very small lipophilic molecule that rapidly diffuses through biological membranes and reaches intracellular compartments of nearby

cells with diverse functions. Interestingly, much like oxygen, NO is a gas that is sparingly soluble in aqueous solution and it functions biologically as a molecule in solution (see review by Ignarro, 1990). However, NO is unstable, with an ultrashort half-life of probably less than 5 seconds in oxygen-containing solution or biological tissues (Palmer *et al.*, 1987).

Before the discovery of EDRF, authentic NO was found to stimulate guanylyl cyclase (Schultz *et al.*, 1977; Katsuki *et al.*, 1977; Craven and DeRubertis, 1978; Gruetter *et al.*, 1979) and to relax vascular smooth muscles (Gruetter *et al.*, 1979). Later, NO was reported to prevent platelet aggregation (Mellion *et al.*, 1981, 1983; Azuma *et al.*, 1986; Radmoski *et al.*, 1987a) and adhesion (Radmoski *et al.*, 1987d). The inhibitory effects of NO on vascular smooth muscle tone and platelet adhesion/aggregation were inhibited by oxyhaemoglobin (Martin *et al.*, 1985; Radomski *et al.*, 1987a,b). The interaction between NO and haemoprotein of the cytosolic guanylyl cyclase results in the formation of the labile nitrosyl-haeme-enzyme complex, the activated state of guanylyl cyclase, which markedly increases the velocity of conversion of guanosine 5'-triphosphate (GTP) to cGMP, leading to smooth muscle relaxation and the inhibition of platelet aggregation/adhesion (see review by Ignarro, 1989; 1990). In contrast to atrial natriuretic peptide which stimulates particulate guanylyl cyclase (Winquist *et al.*, 1984), NO activates the soluble form of the enzyme (Gruetter *et al.*, 1979). Therefore, the NO-induced activation of soluble guanylyl cyclase, and subsequent relaxation as well as inhibition of platelet aggregation/adhesion are inhibited by methylene blue (Gruetter *et al.*, 1979, 1981; Mellion *et al.*, 1981, 1983; Martin *et al.*, 1985; Radomski *et al.*, 1987a).

Organic nitrate and nitrite esters as well as nitroso compounds react with free thiols to generate labile S-nitrosothiols which liberates NO (Ignarro *et al.*, 1981). The NO donors activate soluble guanylyl cyclase, elevate tissue levels of cGMP, relax vascular smooth muscles, decrease systemic blood pressure and

inhibit platelet aggregation (Gruetter *et al.*, 1979; Kukovetz *et al.*, 1979; see review by Ignarro and Kadowitz, 1985; Ignarro *et al.*, 1987). It has been proposed that S-nitrosothiols are active intermediates which mediate the vasorelaxant effects of the nitrovasodilators; however, the final common mediator that stimulates cGMP and causes relaxation is NO (see review by Ignarro, 1992).

1.1.3. EDRF is NO or a labile nitroso precursor

Soon after the discovery of EDRF, it was postulated that EDRF might be a labile free radical formed as an intermediate from arachidonic acid, *via* the lipooxygenase pathway (Furchgott and Zawadzki, 1980; Cherry *et al.*, 1982; see review by Furchgott, 1983; Singer and Peach, 1983; Forstermann and Neufang, 1984), or an unknown compound with a carbonyl group near its active site (Griffith *et al.*, 1984), or a product of the cytochrome P-450 enzyme system (Pinto *et al.*, 1986; Macdonald *et al.*, 1986). However, these hypotheses were soon disputed (see review by Moncada *et al.*, 1991).

In 1986, Furchgott and Ignarro *et al.* independently postulated that EDRF was NO or a closely-related derivative of NO. This was based on the similar pharmacological profiles between NO and EDRF as discussed above, *i.e.*, both compounds are unstable and are inhibited by haemoglobin and stabilized by superoxide dismutase (SOD). Also, both agents exert biological actions by stimulating soluble guanylyl cyclase (Furchgott, 1988; Ignarro *et al.*, 1988). Within a year, several laboratories provided more detailed chemical evidence that EDRF is NO. (1) Both EDRF and NO are similar in their effects on vascular relaxation (Palmer *et al.*, 1987; Hutchinson *et al.*, 1987) as well as inhibition of platelet aggregation and adhesion (Radomski *et al.*, 1987a). (2) Both agents have the same chemical stability under different conditions (Palmer *et al.*, 1987). (3) Endothelial cells in culture release NO in amounts sufficient to account for the

activities of EDRF on vascular strips (Palmer *et al.*, 1987) and platelet aggregation (Radomski *et al.*, 1987c) as well as adhesion (Radomski *et al.*, 1987d). Ignarro *et al.* (1987a,b) also showed that NO or a labile nitroso species was released from the bovine pulmonary artery. Subsequently, the release of NO from endothelial cells was confirmed by using a spectrophotometric assay (Kelm *et al.*, 1988). It was also demonstrated that the amount of NO released from the perfused aorta (Chen *et al.*, 1989) or the isolated perfused heart (Amezcua *et al.*, 1988; Kelm and Schrader, 1988) was sufficient to cause the vasodilatations observed.

Although NO has been identified as an EDRF, questions have arisen on the specificity of chemical procedures employed to draw such a conclusion. It is clear that the chemical techniques which involve chemiluminescence, diazotization, and nitrosation of haemoglobin are not selective for NO, as labile nitroso compounds that spontaneously decompose to liberate NO are also readily detected by all these chemical procedures. Therefore, the chemical evidence that EDRF is identical to NO is not so definite. It has been reported that the activity and potency of EDRF more closely resemble those of a nitrosylated compound, S-nitrocysteine, than to those of NO (Myers *et al.*, 1990). EDRF is also postulated to be either a labile nitroso precursor which releases NO at the smooth muscle cell, or a mixture of nitroso compounds plus NO (see review by Ignarro, 1992). However, it is generally agreed that the existence of such a precursor does not detract from the fact that the biological effects of EDRF are mediated ultimately by NO (Myers *et al.*, 1990; see review by Moncada *et al.*, 19991; see review by Ignarro, 1992).

Palmer *et al.* (1988a) reported that endothelial cells incubated in an L-arginine (L-Arg)-free medium for 24 h have reduced capacity to release NO. This capacity was restored by the addition of L-Arg to the medium prior to stimulation with bradykinin. The effect of L-Arg was enantiomer specific as it was not shared with D-Arg. These findings suggest that NO formation is dependent on the availability of free L-Arg. Furthermore, mass spectrometric analysis demonstrated

the formation of ^{15}NO and L-citrulline from L-Arg, which was previously labeled with ^{15}N at the terminal guanido nitrogen atoms. These results provide conclusive evidence that NO is derived from the terminal guanido nitrogen atom(s) of L-Arg (Palmer *et al.*, 1988a). Moreover, N^G-monomethyl-L-arginine (L-NMMA), an analogue of L-Arg, inhibited endothelial NO synthesis (Palmer *et al.*, 1988b). All these findings, which are supported by other groups (Schmidt *et al.*, 1988a,b; Sakuma *et al.*, 1988; Marletta *et al.*, 1988) are very exciting, though not entirely surprising. In fact, one year earlier Hibbs *et al.* (1987a,b) have reported that L-Arg is required for the expression of the activated macrophage cytotoxic effector mechanism that inhibits mitochondrial respiration. They also demonstrated that murine cytotoxic activated macrophages synthesized L-citrulline and nitrite in the presence of L-Arg but not D-Arg, and that L-citrulline and nitrite biosynthesis by cytotoxic activated macrophages was inhibited by L-NMMA, which also inhibited the cytotoxic effector mechanism. Furthermore, the imino nitrogen was removed from the guanido group of L-Arg. Iyengar *et al.* (1987) also reported that NO_2^- and NO_3^- produced by cultured macrophages, were exclusively derived from the terminal guanidino nitrogens of L-Arg.

It is now generally believed that the NO synthetic pathway involves the interaction of L-Arg with O_2, yielding NO and L-citrulline, with N^G-hydroxy-L-arginine as an intermediate (Stuehr *et al.*, 1991c). NO is then rapidly broken down in biological tissues into NO_2^- and NO_3^-, both of which have only weak biological activities.

1.2. NOS

The synthesis of NO was demonstrated in endothelial homogenates by the formation of citrulline from L-Arg *via* a mechanism which was dependent on reduced nicotinamide adenine dinucleotide (NADPH) and inhibited by L-NMMA

(Palmer and Moncada, 1989). The enzyme which synthesizes NO was subsequently named NO synthase (NOS) and found to be Ca^{2+}-dependent and located in the soluble fraction of endothelial homogenates (Moncada and Palmer, 1990). Through several years of study, NOSs are now well-known to be present in many organs, tissues and cells, such as endothelial cells (Palmer *et al.*, 1988a,b; Rees *et al.*, 1990; Kilbourn and Belloni, 1990), platelets (Radomski *et al.*, 1990a,b,d), brain neurons (Garthwaite *et al.*, 1988; Knowles *et al.*, 1989, 1990a; Bredt and Snyder, 1989, 1990; Bredt *et al.*, 1990; Schmidt *et al.*, 1989, 1992), macrophages and neutrophils (Marletta *et al.*, 1988; Hibbs, *et al.*, 1988; Stuehr *et al.*, 1989a,b; McCall *et al.*, 1991a,b), Kupffer cells and hepatocytes (Curran *et al.*, 1990), smooth muscle cells (Kilbourn *et al.*, 1992), nonadrenergic, noncholinergic (NANC) neurons (Bredt *et al.*, 1990, 1991; Young *et al.*, 1992) and the cortex and the medulla of the adrenal gland (Palacios *et al.*, 1989).

Therefore, the interaction between NO and the haeme group of guanylyl cyclase represents a novel and widespread signal transduction process that links extracellular stimuli to the biosynthesis of the second messenger cGMP in adjacent cells. Due to its wide distribution, NO modulates not only the functions of vasculatures and platelets, but also macrophage and neutrophil cytotoxicity (Hibbs *et al.*, 1987a,b), long-term potentiation in the hippocampus (Böhme *et al.,* 1991), long-term synaptic depression in the cerebellum (Shibuki and Okada 1991) and nociceptive activity in the brain (Moore *et al.,* 1991), as well as NANC relaxation of smooth muscles such as guinea pig isolated tracheal smooth muscle and rat anococcygeus (Tucker *et al.*, 1990; Hibbs and Gibson, 1990).

Whereas many oxidative enzymes use a single electron donor, the oxidation of Arg to NO by NOS involves multiple oxidative cofactors with associated binding sites (Bredt *et al.*, 1991; Xie *et al.*, 1992; Lowenstein *et al.*, 1992; Lyons *et al.*, 1992; Lamas *et al.*, 1992; Marsden *et al.*, 1992; see review by Dinerman *et al.*, 1993). These cofactors are NADPH (Mayer *et al.*, 1989; Stuehr *et al.*, 1989b,

1990, 1991a,b; Bredt *et al.*, 1991; see review by McCall and Vallance, 1992; Marsden *et al.*, 1992), flavin adenine dinucleotide (FAD) (Stuehr *et al.*, 1989b, 1990, 1991a,b; Hevel. *et al.*, 1991; Yui *et al.*, 1991; Mayer *et al.*, 1991; Bredt *et al.*, 1991, 1992; Hiki *et al.*, 1992; White and Marletta, 1992; Lowenstein *et al.*, 1992; Marsden *et al.*, 1992), flavin mononucleotide (FMN) (Bredt *et al.*, 1991; Marsden *et al.*, 1992), iron-protoporphyrin IX haeme (McMillan *et al.*, 1992; White and Marletta, 1992), tetahydrobiopterin (Tayer and Marletta, 1989; Kwon *et al.*, 1989) and calcium/calmodulin (Knowles *et al.*, 1989; Bredt *et al.*, 1991; Marsden *et al.*, 1992).

NOS has at least two isoforms in the same or different organs, tissues or cells.

1.2.1. Constitutive NOS

Constitutive NOS is present in brain neurons, platelets, endothelial cells as well as NANC neurons. The enzyme is Ca^{2+}/calmodulin-dependent (Bredt and Snyder, 1990; Bredt *et al.*, 1991, 1992) and not affected by L-canavanine, a guanidinooxy structural analogue of L-Arg (Palmer and Moncada, 1989; Mayer *et al.*, 1989). However, cloning studies of human endothelial NOS predicted the enzyme to consist of 1203 amino acids which were identical to those of the bovine endothelial NOS by 95%, but which shared only 60% identity with those of the brain NOS isoform (Bredt *et al.*, 1991; Marsden *et al.*, 1992). Moreover, the sequence of the endothelial NOS contains a site at the N-terminus for myristoylation that probably accounts for the association of endothelium NOS with membranes, which is absent in the macrophage and cerebellar NOS isoforms (Lamas *et al.*, 1992). Therefore, although brain and endothelial NOS share many common characteristics, they can be divided into two isoforms. *i.e.*, e (endothelium) NOS and n (neuron) NOS.

1.2.2. Inducible NOS

Inducible NOS differs from the constitutive form in that it is not detectable in the "rest condition", *i.e.*, prior to activation by an inducing agent such as lipopolysaccharide (LPS) alone, or combination with interferon-γ (Stuehr and Marletta, 1985, 1987a,b), and that it requires protein synthesis for its expression (Marletta *et al.*, 1988). It takes hours before NO_2^- and NO_3^- synthesis can be detected. The synthesis of these products then continues until either no more substrate is available or the cell dies (Stuehr and Marletta, 1987a,b). Therefore, the enzyme is referred to as i (inducible) NOS. iNOS is Ca^{2+}-independent and inhibited by L-canavanine (Iyengar *et al.*, 1987; McCall *et al.*, 1989). The induction of iNOS is inhibited by glucocorticoids (Rees *et al.*, 1990a; Knowles *et al.*, 1990b; McCall *et al.*, 1991b). Its clone and expression have also been reported (Xie *et al.*, 1992; Lowenstein *et al.*, 1992; Lyons *et al.*, 1992). iNOS is present in vascular smooth muscle cells, neutrophils, macrophages, Kupffer cells, hepatocytes, endothelial cells and cardiac myocytes.

1.3. NOS inhibitors

There are at least 2 classes of NOS inhibitors, of which the N^G-substituted arginine analogues (NSAAs) are more studied. Endogenous NOS inhibitors have also been reported.

1.3.1. NSAAs

1.3.1.1. Structures

Both isoforms of NOS are reported to be inhibited in many tissues in an enantiomerically specific manner by L-NMMA (Palmer *et al.*, 1988b; Rees *et al.*, 1989a, 1990b), N^G-nitro-L-arginine (L-NNA) (Ishii *et al.*, 1990a,b; Mülsch and Busse, 1990), N^G-nitro-arginine methyl ester (L-NAME) (Rees *et al.*, 1990b), N-iminoethyl-L-ornithine (L-NIO) (Rees *et al.*, 1990b), and N^G-amino-L-arginine (L-NAA) (Gross *et al.*, 1990; Kilbourn *et al.*, 1992). The activities or potencies of NSAAs on different isoforms of NOS may vary (see review by Moncada *et al.*, 1991). For example, L-canavanine inhibits iNOS (Iyengar *et al.*, 1987; McCall *et al.*, 1989) but not eNOS (Palmer and Moncada, 1989; Mayer *et al.*, 1989), although it at high concentration was shown to inhibit endothelium-dependent relaxation (Schmidt *et al.*, 1988a, 1990), but probably by another mechanism (see review by Moncada *et al.*, 1991). The chemical structures of NSAAs frequently used were shown in Fig. 1 in Appendix IV.

It was also reported that N^G,N^G-dimethylarginine (asymmetrical dimethylarginine, ADMA) inhibited NO synthesis in J744 murine macrophages, potentiated the contraction and inhibited the relaxation of preconstricted rat endothelium-intact aorta (Vallance *et al.*, 1992). Moreover, aminoguanidine is structurally similar to L-Arg and NSAAs in that these compounds contain two chemically equivalent guanidino nitrogen groups. Aminoguanidine was shown to be equipotent to L-NMMA as an inhibitor of the cytokine-induced iNOS but to be 10-100 fold less potent as an inhibitor of eNOS (Corbett *et al.*, 1992; Misko *et al.*, 1993). These results may suggest that the guanidino nitrogen group is essential for NSAAs to inhibit NO biosynthesis, although NSAAs and aminoguanidine inhibit different isoforms of NOS with different potencies.

1.3.1.2. Competitive inhibition

Since NSAAs are analogues of L-Arg and since L-Arg blocks the actions of NSAAs, it is reasonable to assume that interactions between NSAAs and L-Arg are competitive. It was reported that L-NMMA, L-NNA and L-NIO inhibited brain NOS in competitive manner (Knowles *et al.*, 1990a), however, detailed pharmacodynamic study to elucidate the type of antagonism between L-Arg and NSAAs in the vasculature has not been performed.

Interestingly, L-NMMA also inhibited endothelium-dependent relaxation in aortic rings but not in pulmonary arterial rings, and L-NMMA enhanced, rather than reduced, NO synthesis in pulmonary artery and aortic rings. In contrast, L-NNA did not stimulate NO synthesis and inhibited A 23187-induced relaxation in vascular rings. Therefore, it has been concluded that L-NMMA is a partial agonist for NO synthesis (Archer and Hampl, 1992). This conclusion is confirmed by Martin *et al.*'s findings (1993) which showed that L-NMMA did not block NANC relaxation in bovine retractor penis but inhibited the blockade induced by L-NNA or L-NAME with greater potency than did the substrate L-Arg.

1.3.1.3. Reversibility

It has been reported that NSAAs inhibit iNOS for a long period of time after washouts (Rees *et al.*, 1989a; Mülsch *et al.*, 1990). L-NIO has been identified as an irreversible inhibitor for iNOS since post-treatment with L-Arg did not reverse the inhibition by L-NIO (McCall *et al.*, 1991a). In contrast, L-NMMA, L-NNA and L-NAME are reversible inhibitors of iNOS since post-treatment with L-Arg is as effective as pretreatment to reverse their inhibitory effects on macrophage NO synthesis (McCall *et al.*, 1991a).

1.3.1.4. Stereospecificity

NOS has been extensively claimed to be stereospecific, since L- but not D-enantiomers of NMMA (Palmer *et al.*, 1988b; Rees *et al.*, 1989a, 1990b), NNA (Ishii *et al.*, 1990a,b; Mülsch and Busse, 1990; Rees *et al.*, 1990b), NAME (Rees *et al.*, 1990b), NIO (Rees *et al.*, 1990b) have been found to inhibit NO synthesis.

1.3.1.5. Specificity

NSAAs are frequently used as tool drugs to elucidate the biological effects of the inhibition of NO biosynthesis, on the basis of the assumption that NSAAs are specific inhibitors of NOS. However, it has been reported that L-NAME, but not L-NNA or L-NMMA, has weak antimuscarinic effects (Buxton *et al.*, 1993). Therefore, caution must be exercised in the interpretation of results from studies using NSAAs.

1.3.2. DPI and other iodonium compounds

A group of iodonium compounds have been reported to be another class of NOS inhibitors in the macrophage (Stuehr *et al.*, 1991b; Kwon *et al.*, 1991; Keller *et al.*, 1992). These compounds include diphenyleneiodonium (DPI), iodoniumdiphenyl and di-2-thienyliodonium, all of which have similar chemical structures and are distinct from those of NSAAs. The chemical structure of DPI was shown in Fig. 1 in Appendix IV.

DPI was initially found to be a potent hypoglycaemic agent (Stewart and Hanly, 1969; Gatley and Martin, 1979) which, by inhibiting gluconeogenesis from lactate and aspartate, suppressed the oxidation of NADH-linked substances (Holland *et al.*, 1973). It was later shown that DPI, iodoniumdiphenyl and di-2-thienyliodonium suppressed the activities of neutrophil and macrophage NADPH-dependent oxidase (Cross and Jones, 1986; Hancock and Jones, 1987; Ellis *et al.*,

1988, 1989). All these actions of DPI are due to its inhibition of a flavoprotein which is coupled to NADPH-dependent enzymes (Holland *et al.*, 1973; Hancock and Jones, 1987; Ellis *et al.*, 1989; Stuehr *et al.*, 1991b; O'Donnell *et al.*, 1993). Therefore, it is not surprising that DPI and its analogues inhibit NO biosynthesis, as NADPH and FAD are cofactors of NOS (Bredt *et al.*, 1991; Xie *et al.*, 1992; Lowenstein *et al.*, 1992; Lyons *et al.*, 1992; Lamas *et al.*, 1992; see review by Dinerman *et al.*, 1993). However, the inhibitory effects of DPI and its analogues on eNOS and nNOS have not been reported.

1.3.3. Endogenous NOS inhibitors

It has been known for a long time that methylated Arg such as L-NMMA and ADMA are naturally occurring agents (Kakimoto and Akazawa, 1970; Nakajima *et al.*, 1970; Park *et al.*, 1988). Vallance *et al.* (1992) confirmed that ADMA was detected in human plasma and urine, where more than 10 mg is excreted in the urine over 24 hours. Moreover, in patients with chronic renal failure with little or no urine output, circulating concentration of the inhibitor rose to a level sufficient to inhibit NO synthesis *de vivo*. These investigators suggested that the accumulation of endogenous ADMA may lead to an impaired NO synthesis which, in turn, may lead to the development of hypertension and immune dysfunction, conditions often associated with chronic renal failure.

It was also reported that an inhibitor of endothelium-dependent relaxation existed in the rabbit brain although its chemical structure was not known but might be a large peptide or a protein (Moore *et al.*, 1990).

1.4. Actions of NOS inhibitors on the vasculature

1.4.1. Inhibition of endothelium-dependent vasodilatation

In addition to eliciting contraction and potentiating the contractile effects of vasoconstrictor agents, NSAAs, *e.g.*, L-NMMA (Palmer *et al.*, 1988b; Sakuma *et al.*, 1988; Rees *et al.*, 1989a, 1990b; Crawley *et al.*, 1990), L-NNA (Moore *et al.*, 1990; Mülsch and Busse, 1990; Kobayashi and Hattori, 1990), L-NAME (Rees *et al.*, 1990b), L-NIO (Rees *et al.*, 1990b) and L-NAA (Fukuto *et al.*, 1990; Vargas *et al.*, 1991), inhibit endothelium-dependent relaxation in many isolated vascular preparations *in vitro*. The inhibition of endothelium-dependent relaxation by NSAAs are prevented by L-Arg but not D-Arg (Palmer *et al.*, 1988b; Rees *et al.*, 1990b; Moore *et al.*, 1990; Mülsch *et al.*, 1990) and are long-lasting. However, it is not yet known whether the sustained inhibition of relaxation by NSAAs is reversible.

It was also reported that DPI effectively inhibited ACh- but not SNP-induced relaxation of preconstricted aortae of the rabbit (Stuehr *et al.*, 1991b) and rat (Rand and Li, 1993).

1.4.1.1. Discrepancy between *in vitro* and *in vivo*

While NSAAs effectively inhibit ACh- or bradykinin-induced vasodilatation in isolated preconstricted vascular preparations, or regional vascular beds, *e.g.*, coronary (Woodman and Dusting, 1991), hindquarter (Bellan *et al.*, 1991), renal (Gardiner *et al.*, 1990c, 1991; Lahera *et al.*, 1990), pulmonary (Fineman *et al.*, 1991), mesentery (Fortes *et al.*,, 1990; Gardiner *et al.*, 1990c) and carotid (Gardiner *et al.*, 1990c, 1991) beds, there are discrepancies in reports of their abilities to interfere with the depressor effect of ACh in whole animals. The inhibition of ACh- or bradykinin-induced depressor response has been shown by L-NMMA (Whittle *et al.*, 1989; Rees *et al.*, 1990; Aisaka *et al.*, 1989b), L-NAME (Rees *et al.*, 1990) and L-NIO (Rees *et al.*, 1990). However, an absence of

inhibition, or even potentiation, of the depressor response to ACh has been reported by L-NMMA (Yamazaki and Nagao, 1991), L-NNA (Treshman *et al.*, 1991; Wang *et al.*, 1992) and L-NAME (Gardiner *et al.*, 1990c, 1991; van Gelderen *et al.*, 1991).

1.4.1.2. Stereospecificity of NSAAs

It is extensibly reported that D-enantiomers of NSAAs have no effect on endothelium-dependent relaxation *in vitro*. L-NMMA but not D-NMMA (Rees *et al.*, 1989a,b; Whittle *et al.*, 1989; Crawley *et al.*, 1990; Persson *et al.*, 1990; Rees *et al.*, 1990b), L-NAME but not D-NAME (Rees *et al.*, 1990b) and L-NIO but not D-NIO (Rees *et al.*, 1990b) contracted isolated arterial preparations, or reduced microvascular diameters *in vivo*. It was also reported that L-NMMA but not D-NMMA, L-NAME but not D-NAME, and L-NIO but not D-NIO enhanced human platelet aggregation induced by ADP, arachidonic acid and thrombin (Radomski *et al.*, 1990c). L-NMMA but not D-NMMA potentiated the aggregation of platelets and white cells in rabbits (Persson *et al.*, 1990). Moreover, it has been reported that L-NNA but not D-NNA prevented EDRF release from endothelial cells and inhibited the dilator effects of ACh on rabbit femoral arteries (Mülsch and Busse, 1990). L-NNA but not D-NNA inhibited NANC relaxation of guinea pig isolated tracheal smooth muscle and rat anococcygeus (Tucker *et al.*, 1990; Hobbs and Gibson, 1990). Therefore, it appears that all biological actions of NSAAs are enantiomerically specific. However, it is well-known that although the L-enantiomeric form is a main configuration for biologically active drugs, many D-enantiomers have less or even greater biological activities than their corresponding L-enantiomers (see review by Ariëns, 1983). Since the concentrations of D-enantiomers used in all the above studies were never more than those of L-enantiomers, the activities of D-enantiomers may not be properly assessed. It is

reasonable to construct dose-response curves to elucidate the potencies and efficacies of both enantiomers of NSAAs.

1.4.2. Vasoconstriction *in vivo*

The administration of L-NMMA, L-NNA, L-NAME, L-NIO or L-NAA into intact animals caused sustained pressor responses and bradycardia (see review by Moncada *et al.*, 1991). Although it was reported that L-NAME caused pressor response in pentobarbitone-anaesthetized rats but not in pentobarbitone-anaesthetized cats (van Gelderen *et al.*, 1991), the pressor responses to NSAAs were generally observed in all species tested, such as mice (Moore *et al.*, 1991), anaesthetized rats (Rees *et al.*, 1989b; Wang and Pang, 1990a,b), conscious normotensive Sprague-Dawley (Wang *et al.*, 1993b), Wistar and Wistar-Kyoto rats (Wang *et al.*, 1992), as well as spontaneously hypertensive rats (Yamazaki *et al.*, 1991; Wang *et al.*, 1992), guinea pigs (Aisaka *et al.*, 1989a,b), rabbits (Humphries *et al.*, 1991), cats (Bellan *et al.*, 1991), dogs (Chu *et al.*, 1990; Woodman and Dusting, 1991; Toda et al., 1993), sheep (Fineman *et al.*, 1991; Tresham *et al.*, 1991; Garcìa *et al.*, 1992), monkeys (Peterson *et al.*, 1993), and human (Petros *et al.*, 1991). The pressor response to NSAAs are attenuated by L-Arg but not D-Arg (Rees *et al.*, 1989b; Whittle *et al.*, 1989; Gardiner *et al.*, 1990b; Persson *et al.*, 1990; Wang and Pang, 1990b). It was also reported that the pressor response to L-NMMA was accompanied by the inhibition of NO synthesis *ex vivo* (Rees *et al.*, 1989b) and *in vivo* (Suzuki *et al.*, 1992).

It should be pointed out that the pressor responses to NSAAs are dependent on the conscious or anaesthetic condition of the experimental animals. Variable effects of inhalation and intravenous anaesthetic agents on the pressor responses to L-NNA and L-NMMA have been reported (Wang *et al.*, 1991; Aisaka *et al.*, 1991). Surgically anaesthetic doses of halothane totally and reversibly abolished

the pressor response to L-NNA (Wang *et al.*, 1991). Therefore, it is important to evaluate the influence of anaesthetic agents on the pressor responses to vasoactive agents (Abdelrahman *et al.*, 1992b), particularly to NSAAs (Wang *et al.*, 1991). Moreover, the effects of the vehicles for the drugs on the pressor responses to NSAAs must also be considered. It has been reported that ethanol, a frequently used vehicle for drugs, dose-dependently and "noncompetitively" inhibited the pressor response to L-NNA (Wang and Pang, 1993). Furthermore, interactions of drugs should also be considered. It has been reported that while SNP "noncompetitively" but selectively inhibited the pressor response to L-NNA but not to noradrenaline or to angiotensin II, nifedipine (L type of calcium channel antagonist) nonselectively and "noncompetitively" attenuated the pressor responses to L-NNA and noradrenaline and angiotensin. On the hand, pinacidil (ATP-sensitive K^+ channel agonist) did not inhibit the pressor response to L-NNA nor noradrenaline or angiotensin II (Wang *et al.*, 1993b).

L- but not D-enantiomers of NSAAs are generally reported to cause hypertensive responses (Rees *et al.*, 1989b, 1990b; Humphries *et al.*, 1991). However, D-NNA has also been found to cause pressor responses in pentobarbitone-anaesthetized rats (Wang and Pang, 1990a) and urethane-anaesthetized rats (Raszkiewicz *et al.*, 1992). The stereospecificity of NSAAs to cause vasoconstriction *in vivo* is not at all clear.

It was also reported that aminoguanidine raised blood pressure in anaesthetized rats, but it was only approximately 1/40 as potent as L-NMMA (Corbett *et al.*, 1992). There are as yet no reports on the *in vivo* effects of DPI.

1.5. Mechanisms of the pressor responses to NSAAs

The pressor response to NSAAs are generally interpreted to be due to the inhibition of NO synthesis and subsequent endothelium-dependent vasodilatation. However, other mechanisms are possible and have been explored.

1.5.1. The endothelial L-Arg/NO pathway

The hypothesis that the pressor effects of NSAAs are attributed to the inhibition of endothelial NO biosynthesis, and subsequently that endothelial-derived NO modulates vascular tone and blood pressure (Aisaka *et al.*, 1989a; Rees *et al.*, 1989b, 1990b; Moncada *et al.*, 1991), is based on the following major evidence which was discussed previously in more detail. (1) NO is endogenously released and causes vasodilatation, therefore, the inhibition of NO biosynthesis *in vivo* would lead to the suppression of endothelium-dependent dilator tone and elevation of blood pressure. (2) L-Arg is the precursor of NO and the pressor responses to NSAAs are attenuated by L-Arg. However, since all the evidence obtained are from the results by using NSAAs, other classes of NOS inhibitors with structures different from NSAAs should be used to confirm the above hypothesis.

1.5.2. Endothelial membrane L-Arg transport

It is known that NO synthesis is absolutely dependent on the availability of extracellular L-Arg and that L-Arg enters cells *via* the L-Arg transporter (Palmer *et al.*, 1988a; Bogle *et al.*, 1992a). Therefore, it is possible that NSAAs may produce pressor response by inhibiting the L-Arg transporter in the endothelial membrane thereby restricting the availability of intracellular L-Arg to produce NO. However, this possibility is unlikely in the light of the report that L-Arg uptake is inhibited by L-NMMA and L-NIO but not by L-NNA or L-NAME (Bogle *et al.*, 1992b), whereas all these compounds are similarly effective in causing

hypertensive responses. Moreover, bradykinin elevated L-Arg transporter and NO synthesis in endothelial cells (Bogle *et al.*, 1991). While L-NNA inhibited bradykinin-induced NO biosynthesis, it had negligible effects on the basal/stimulated L-Arg transporter.

1.5.3. The central nervous system

It has been reported that intravenous injection of L-NMMA increased post-ganglionic renal sympathetic nerve activity both in intact rats and denervated rats. Spinal transection at C_1-C_2 level attenuated the pressor and elevated renal sympathetic nerve activity in response to intravenous injection of L-NMMA (Sakuma *et al.*, 1992). Furthermore, it was reported that intracisternal injection of L-NMMA elicited a small pressor response accompanied by a marked increase in sympathetic renal nerve activity. The increases in the sympathetic renal nerve activity and blood pressure elicited by L-NMMA were abolished by spinal transection at the C_1-C_2 level and by intravenous administration of L-Arg. When administered intracisternally, L-Arg also abolished the increase in the sympathetic renal nerve activity in response to intravenous injection of L-NMMA and significantly attenuated its pressor response (Togashi *et al.*, 1992). These results indicated that L-NMMA may raise blood pressure and increase sympathetic discharge partially *via* the central nervous system, and that L-NMMA centrally stimulates the sympathetic nerve activity by an Arg-reversible mechanism in anaesthetized rats (Sakuma *et al.*, 1992; Togashi *et al.*, 1992). These findings are also supported by those of Harada *et al.* (1993) in which microinjection of L-NMMA into the rabbit nucleus tractus solitarius caused increases in blood pressure and renal sympathetic nerve activity, which were prevented by microinjection of L-Arg into the nucleus tractus solitarius. On the other hand, it was reported that intracerebroventricular injection of L-NAME decreased blood pressure and heart

rate of LPS-treated rats but not the control rats. Furthermore, the pressor response to intracerebroventricular injection of N-methyl-D-aspartate (NMDA) was enhanced by L-Arg or LPS, and in both cases the potentiation was blocked by L-NAME. These results suggested that in some experimental conditions, such as the activation of NMDA receptors or LPS pretreatment, the L-Arg/NO pathway may interfere with blood pressure and heart rate regulation (Mollace *et al.*, 1992).

There is evidence that a central mechanism is not responsible for the pressor response to NSAAs administered especially by peripheral routes. It was reported that the pressor response to intravenous injections of L-NAME was not influenced by pithing (Pegoraro *et al.*, 1992). Moreover, intracerebroventricular injections of L-NAME caused antinociceptive activity but not pressor response while intraperitoneal injections of L-NAME caused both antinociceptive and pressor response in mice (Moore *et al.*, 1991). It was also reported that intravenous injection of L-NMMA caused pressor response in pithed rats and that L-NAME dose-dependently and frequency-dependently potentiated the pressor response to electrical stimulation of the sympathetic chain in pithed rats and the potentiation was greater in spontaneously hypertensive rats than in Wistar-Kyoto rats (Tabrizchi and Triggle, 1991, 1992). Therefore, it remains to be resolved whether a central mechanism contributes to the pressor responses elicited by peripherally administered NSAAs.

1.5.4. The autonomic nervous system

The vascular endothelium has been shown to inhibit the release of noradrenaline from the sympathetic nerves which innervate canine pulmonary artery and vein, by comparing the efflux of ^{14}C-noradrenaline induced by transmural nerve stimulation between endothelium-intact and denuded preparations. These results suggest that endothelial cells, *via* EDRF/NO release,

may act as an endogenous inhibitor of transmitter release from the sympathetic nerve terminals (Greenberg *et al.*, 1989, 1990, 1991). Moreover, L-NMMA was found to increase the sympathetic nerve activity in rats (Sakuma *et al.*, 1992; Togashi *et al.*, 1992). It is therefore possible that the pressor responses to NSAAs are partially produced by activating ganglionic transmission and/or sympathetic nervous system.

Indeed, it was reported that the ganglionic blockade with pentolinium or hexamethonium significantly reduced or abolished the hypertensive effect of L-NMMA in urethane-anaesthetized rats (Vargas *et al.*, 1990) or the pressor response to L-NNA in pentobarbitone-anaesthetized dogs (Toda *et al.*, 1993). The ganglion blocker chlorisondamine has also been shown to abolish L-NNA-induced increases in blood pressure and renal vasoconstrictor response, as well as to attenuate the increases in mesenteric and hindquarter resistances (Lacolley *et al.*, 1991). In contrast to these findings, it was reported that pentolinium and hexamethonium potentiated the pressor response to L-NAME in urethane-anaesthetized rats (Chyu *et al.*, 1992). The pressor responses to L-NMMA and L-NAME were not influenced by treatment with hexamethonium in anaesthetized rats (Pegoraro *et al.*, 1992). Moreover, the pressor and renal vasoconstrictor responses to L-NNA were not impaired by chlorisondamine in pentobarbitone-anaesthetized rats (Pucci *et al.*, 1992). Hence, the role of ganglionic transmission on the pressor responses to NSAAs needs to be examined in conscious animals to avoid the influence of anaesthesia and surgery on haemodynamic responses.

On the other hand, it was reported that phentolamine, prazosin and labetolol, as well as atropine and atenolol, did not inhibit the pressor response to L-NMMA, L-NNA or L-NAME (Rees *et al.*, 1989b; Aisaka *et al.*, 1989a; Pucci *et al.*, 1992; Widdop *et al.*, 1992; Toda *et al.*, 1993). Moreover, L-NNA did not elevate plasma noradrenaline in conscious sheep (Tresham *et al.*, 1991; Elsner *et al.*, 1992). These results suggest that the pressor responses to NSAAs are not

due to the release of catecholamines or the activation of α-adrenoceptors in the vascular smooth muscle.

1.5.5. Afferent nerve transmitters

Pretreatment with capsaicin did not alter the pressor response to L-NAME (Tepperman and Whittle, 1992; Wang and Pang, unpublished observation, 1993), suggesting that afferent nerve transmitters do not contribute to the pressor responses to NSAAs.

1.5.6. The renin-angiotensin system

It was reported that EDRF/NO inhibited the release of renin, and L-NMMA increased renin concentration in rat renal cortical slices *in vitro* (Beierwaltes and Carretero, 1992). It may be reasonable to hypothesize that NSAAs elevate blood pressure directly, by suppressing the synthesis and release of EDRF and indirectly, by elevating the activities of the renin-angiotensin system. In fact, it was reported that chronic treatment with L-NAME caused a sustained pressor response which was accompanied by elevated plasma renin activity and that the pressor response was prevented by the angiotensin II (AT-1) antagonist losartan (Ribeiro *et al.*, 1992). A81989, DuP753 and enalapril were also reported to block the pressor response induced by the chronic administration of L-NAME in conscious rats (Polakowski *et al.*, 1993). These results suggest that activation of the renin-angiotensin system may contribute to the vasoconstrictor activity of L-NAME administered chronically. However, it was reported that the angiotensin converting enzyme inhibitor captopril did not alter the pressor response to acute administration of L-NNA in rats (Pucci *et al.*, 1992). It appears that the roles of

the renin-angiotensin system on the pressor responses to acute or chronic administration of NSAAs are different.

1.5.7. The prostaglandin system

Although it is widely-accepted in the scientific community that EDRF/NO biosynthesis does not involve the cyclooxygenase metabolism, discrepancies in the actions of the cyclooxygenase inhibitors on the pressor responses to NSAAs have been reported. Indomethacin did not inhibit the pressor response to L-NMMA or L-NAME in anaesthetized rats (Rees *et al.*, 1989b; Tepperman and Whittle, 1992). It was also reported that the combination of chlorisondamine, captopril and indomethacin did not impair pressor and renal vasoconstrictor responses to L-NNA in anaesthetized rats (Pucci *et al.*, 1992). In contrast, indomethacin was reported to block the regional vasoconstrictor actions of L-NMMA in urethane-anaesthetized mice (Rosenblum *et al.*, 1992). Moreover, indomethacin was also reported to abolish the pressor but not systemic vasoconstrictor and cardiac depressant effects of L-NMMA in anaesthetized dogs (Klabunde *et al.*, 1991). Since all these studies were performed in anaesthetized animals without the construction of dose-response curves of NSAAs, the effects of cyclooxygenase inhibitors on dose-pressor response curves to NSAAs should be performed in conscious animals to elucidate the role of prostanoids in the pressor responses to NSAAs.

1.5.8. Vasopressin

It was reported that oral administration of L-NMMA or L-NAME caused pressor and regional vasoconstrictor responses in conscious Brattleboro rats with diabetes insipidis which were vaspopressin deficient (Gardiner *et al.*, 1990a). Moreover, it was reported that the pressor and renal vasoconstrictor effects of L-

NNA in anaesthetized rats were not impaired by pretreatment with a V1 vasopressin antagonist (Pucci *et al.*, 1992), and that the combination of pentolinium, captopril and vasopressin V1 antagonist did not inhibit the pressor response to L-NAME (Gardiner *et al.*, 1990c). These results suggest that vasopressin is not involved in the pressor responses to NSAAs.

1.6. The aims of this study

This project was started in the middle of 1990 when the *in vivo* pharmacology of NOS inhibitors was rather novel. L-NNA was the first drug we studied due to its availability, potency and lower cost, relative to those of L-NMMA. As the experiments progressed, we also studied L-NAME due to its substantially higher solubility in water than L-NNA. DPI was later tested because it is a new inhibitor of NOS with chemical structure distinct from NSAAs. Our aims were to systematically and comprehensively investigate the vascular pharmacology of NOS inhibitors in order to elucidate the role of EDRF/NO in blood pressure and haemodynamic regulation as well as the pharmacodynamics of NOS inhibitors. During the course of the study, our acceptance of the hypothesis that NSAAs cause *in vivo* vasoconstriction by the inhibition of endogenous endothelial NO biosynthesis has been weakened; this was reflected in our publications. Our research primarily focused on the following areas.

1.6.1. Stereospecificity of NSAAs.

It is generally shown that only L-enantiomers of NSAAs are biologically active in inhibiting NO biosynthesis and endothelium-dependent relaxation, as well as in causing pressor responses. However, from our preliminary studies to examine the effect of L-NNA on blood pressure, we found that D-NNA, initially

used as a negative control, also caused pressor response in pentobarbitone-anaesthetized rats (Wang and Pang, 1990a). It was also later reported that D-NNA caused pressor response in urethane-anaesthetized rats (Raszkiewicz *et al.*, 1992). Therefore, studies were conducted to examine whether stereospecificity exists for NSAAs, in their effects on the inhibition of endothelium-dependent relaxations *in vitro* and *ex vivo*, and in their ability to raise blood pressure in conscious rats.

1.6.2. Effects of NSAAs and DPI on ACh-induced vasodilatations *in vitro* and *in vivo*

Although it is known that NOS inhibitors abolished *in vitro* endothelium-dependent relaxation, their *in vivo* effects on ACh-induced depressor response are not clear. The information is crucial for the understanding of the pharmacology of NOS inhibitors and the role of EDRF/NO in blood pressure regulation. In order to resolve the question of whether NOS inhibitors suppress endothelium-dependent vasodilatation *in vivo*, the effects of NSAAs were assessed along with those of DPI. The effect of L-NAME on the depressor response to ACh was studied in the vehicle-treated rats as well as in phenylephrine (PHE)-treated rats. PHE was utilized as a second control for L-NAME (and D-NAME) in order to raise blood pressure to the same level as that produced by L-NAME (and D-NAME), since depressor responses are generally greater at a higher baseline blood pressure (Rees *et al.*, 1990b; van Gelderen *et al.*, 1991; Chyu *et al.*, 1992).

1.6.3. Pharmacodynamics of NSAAs and DPI

It is generally assumed that NSAAs are competitive inhibitors of NOS since they are structurally-related to L-Arg and their actions are antagonized by L-Arg.

However, there is little pharmacodynamic data to establish such a model in the vasculature, especially *in vivo*. There is also no report regarding the pharmacodynamics of DPI. Hence, experiments were carried out to investigate the *in vitro* and *in vivo* pharmacodynamics of L-NAME and DPI, *via* the use of modified dose (concentration)-response model and modified Schild plots, by a computer program.

1.6.4. Mechanisms of the pressor responses to NSAAs and DPI

The inhibitory effects of NSAAs in inhibiting NO synthesis *in vitro*/*in vivo* and endothelium-dependent vasodilatation are antagonized by L-Arg. The prolonged pressor responses to these compounds are also antagonized by L-Arg. Therefore, it is logical to assume that the pressor responses to NSAAs are due to the inhibition of endothelial NO biosynthesis and subsequent endothelium-dependent vasodilatation. However, there are reports of other mechanisms *(e.g.*, inhibition of the central and ganglionic NO biosynthesis) contributing to the pressor effects of NSAAs. Part of this dissertation examined the mechanisms by which NSAAs cause pressor responses, *e.g.*, whether the central and autonomic nervous system, renin-angiotensin system and prostanoid system, in addition to the L-Arg pathway, are involved. Furthermore, the effect of NSAAs were compared with those of DPI whose chemical structure is distinct from that of NSAAs, in order to elucidate whether the inhibition of endothelial NO biosynthesis alone necessarily lead to a rise in blood pressure in whole animals. During the course of this study, it was found that DPI, unlike NSAAs which cause prolonged pressor responses, only produced transient elevation of blood pressure. Therefore, *in vivo* experiments were also carried out with DPI to find out the mechanism by which DPI causes this transient pressor response.

2. Materials and methods

2.1. Materials

2.1.1. Animals

Male Sprague-Dawley rats (280-420 g) were used in this study. All the animals were from the Animal Care Center of the University of British Columbia. The recommendation from the Canada Council of Animal Care and internationally accepted principles in the care and use of experimental animals have been adhered to.

2.1.2. Drugs

The following drugs were purchased from Sigma Chemical Co. (MO, U.S.A.): mecamylamine hydrochloride, atropine sulfate, D-L-propranolol hydrochloride, N^{ω}-nitro-L-arginine (L-NNA), N^{ω}-nitro-L-arginine methyl ester (L-NAME) hydrochloride, L-arginine (L-Arg) hydrochloride, guanethidine sulfate, rauwolscine hydrochloride, prazosin hydrochloride, acetylcholine (ACh) chloride, A 23187, phenylephrine (PHE) hydrochloride, flavin adenine dinucleotide (FAD) disodium, ß-nicotinamide adenine dinucleotide phosphate (reduced form) (NADPH), indomethacin, bradykinin and tyramine hydrochloride. The following drugs were also used: diphenyleneiodonium (DPI) sulfate (Colour Your Enzyme, Ont., Canada), phentolamine hydrochloride (CIBA Pharmaceutical Co., NJ, U.S.A.), captopril (E. R. Squibb & Sons Inc., NJ, U.S.A.), reserpine injection (CIBA Pharmaceutical Co., Quebec., Canada) and tetrodotoxin (TTX) (Sankyo Co. Ltd., Tokyo, Japan), sodium nitroprusside (SNP) (Fisher Scientific Co., NJ, U.S.A.), halothane (Laboratories Ayerst, Montreal, Canada). All the powder drugs were solubilized in

normal saline (0.9% NaCl solution) except for DPI which was dissolved in 5% glucose solution, and prazosin, indomethacin as well as A 23187 which were dissolved in 100% 30% and 10% dimethyl sulfoxide, respectively. L-NNA and D-NNA were dissolved in normal saline by 10 min of sonication.

2.2. Methods

2.2.1. Isolated aortic rings

The rats 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 (Grass Instrument Co., Quincy, MA, U.S.A.) for isometric recording with a preload of 1 g. The rings were equilibrated for 1 h (with 3 washouts) in normal Krebs solution (pH 7.4) at 37°C and bubbled with a gas mixture of 95% O2 and 5% CO2. The Krebs solution had the following composition (1x10-3 mol/L): NaCl, 118; glucose, 11; KCl, 4.7; CaCl2, 2.5; NaHCO3, 25; KH2PO4, 1.2; MgCl26H2O, 1.2.

The rings were first incubated with a vehicle or drugs followed by PHE (1x10-6 mol/L, EC_{90}). After 15-20 min, at the steady-state phase of the contractile response to PHE, a cumulative concentration-response curve of ACh, A 23187, bradykinin or SNP was constructed. Each concentration of drug was left in the bath for usually 1-3 min until a plateau response was reached. The time taken to complete each concentration-response curve was approximately 20 min. In groups where more than one concentration-response curve of ACh was constructed, the preparations were washed three times within 30 min and given another 30 min to completely recover from the effects of the previous applications

of PHE and ACh. Afterwards, PHE was again added followed by the construction of ACh concentration-response curves.

2.2.2. Surgery

The rats were anaesthetized with sodium pentobarbitone (2.6×10^{-4} mol/kg, i.p.) or briefly with halothane, in the studies using conscious rats (4% in air for induction and 1.2% in air for surgery). A polyethylene cannula (PE50) was inserted into the left iliac artery, for the measurement of mean arterial pressure (MAP) by a pressure transducer (Model P23DB) (Gould Statham, Cupertino, CA, U.S.A). Heart rate (HR) was determined electronically from the upstroke of the arterial pulse pressure using a tachograph (Model 7P44G) (Grass Instrument Co., Quincy, MA, U.S.A.). One or two PE50 cannulae was/were also inserted into the left or both iliac vein(s) for the administration of drugs. In some rats, another PE50 cannula was inserted into the right iliac artery to collect blood samples. The cannulae were filled with heparinized (25 IU/ml) normal saline. For anaesthetized rats, their body temperature was maintained at 37°C with a heat lamp connected to a thermostat (Model 73A) (Yellow Springs Instruments, Yellow Springs, Ohio, U.S.A.).

Tetrodotoxin (TTX)-pretreated, pithed, spinal cord-transected rats and the corresponding control rats were anaesthetized with pentobarbitone sodium. Tracheostomy was performed to allow artificial ventilation with 100% oxygen at 54 strokes/min and a stroke volume of 3-4 ml (1 ml/100 g body weight). Pithing was performed through the orbit with a 3 mm-diameter stainless steel rod and spinal transection was performed at the T_1 level by a pair of sharp scissors. All experiments were conducted 20 min after surgery.

In conscious rats, the cannulae were tunnelled subcutaneously along the back and exteriorized at the back of the neck. The rats were then put into small cages

allowing free movement and given more than 6 h's recovery from the effects of surgery and halothane before use.

2.2.3. Microsphere technique (Pang, 1983)

For haemodynamic experiments, additional cannulae were inserted into the left ventricle *via* the right carotid artery, for the injections of radioactively-labelled microspheres into the left iliac artery for blood withdrawal. A well-stirred suspension of 30,000-40,000 microspheres (15 μm in diameter), labelled with either ^{57}Co or ^{113}Sn (Du Pont Canada Inc., Ontario, Canada), was injected into the left ventricle in the control period and after the administration of a drug or a vehicle. The order of administration of the microspheres was reversed in half of each group of rats. At the end of the experiments, blood samples, whole organs of lungs, heart, liver, stomach, intestine, caecum and colon (presented as colon in the table and figures), kidneys, spleen, testes and brain, as well as 30 g each of skeletal muscle and skin, were removed for the counting of radioactivity (count per minute, cpm) using a 1185 Series Dual Channel Automatic Gamma Counter (Nuclear-Chicago, Illinois, U.S.A.) with a 3 inch NaI crystal at energy settings of 80-160 keV and 330-480 keV for ^{57}Co and ^{113}Sn, respectively. At these energy settings, the "spill-overs" from ^{57}Co to the ^{113}Sn channel was negligible (0.03%) and from ^{113}Sn to the ^{57}Co channel was 16%. A correction was made for the ^{113}Sn "spill-over". MAP and HR were continuously recorded. Cardiac output (CO), total peripheral resistance (TPR) and organ blood flow were calculated as follows. (1) CO (ml/min) = [rate of withdrawal of blood (ml/min) x total injected cpm]/cpm in withdrawn blood. (2) Organ blood flow (ml/min) = [rate of withdrawal of blood (ml/min) x organ cpm]/cpm in withdrawn blood. (3) TPR = MAP/CO.

2.2.4. Measurement of plasma catecholamines (Passon and Peuler, 1973)

Plasma catecholamines were measured by a catecholamine assay kit (Amersham Canada Ltd., Ont., Canada). Blood samples (0.5 ml) were immediately inserted into precooled tubes containing ethylene glycol-*bis*(ß-amino-ethyl ether) N,N,N',N'-tetraacetic acid (EGTA) and reduced glutathione and centrifuged at 1,200 g at 4^{o} C. Afterwards, the plasma was removed and stored at -70^{o} C until assayed within a month. Duplicate assays were run for the standard, plasma or diluted plasma samples (50 μl each sample), with distilled water used as a blank control for each run. The catechol-O-methyltransferase was used to catalyze the transfer of a ^{3}H-methyl group from S-adenosyl-L-[methyl-^{3}H]-methionine to the hydroxyl group in the 3-position of noradrenaline, adrenaline and dopamine. The resultant products were separated by thin layer chromatography, eluted if necessary, and counted by a 1600 TR liquid scintillation analyzer (Packard Instrument Co., CT, U.S.A.). The standard curves for noradrenaline, adrenaline and dopamine (0.01, 0.03, 0.1, 0.3, 1, 3 and 10 ng/ml for each standard solution) were prepared with the control rat plasma. Two standard curves were constructed at two separate occasions and were found to be indistinguishable from each other. The data were combined to formulate the following linear regression equations for noradrenaline, adrenaline and dopamine: $Y=8.03x+29.5$ ($r=0.998$, $P<0.05$), $Y=2.49x-14.1$ ($r=0.999$, $P<0.05$) and $Y=2.99x+10.2$ ($r=0.999$, $P<0.05$), respectively. The sensitivity of the catecholamine assay was 0.005 ng/ml.

2.3. Calculations and statistical analyses

2.3.1. Dose (concentration)-response curve (Kenakin, 1987)

The parameters, *i.e.*, minimal effect (E_{min}), maximal effect (E_{max}), half-effective (inhibitive) dose or concentration (ED_{50}, EC_{50} or IC_{50}) and Hill coefficient (n) were calculated from individual dose-MAP and dose-HR curve of testing drugs by using a program written by Dr. David M.J. Quastel in this department and executed on an IBM compatible microcomputer. To determine these parameters, values of response (Y, rise in MAP or HR) at various doses (D) or concentrations (C) were fitted by non-linear least-squares to the relation $Y = a + bx$, where Y = response and $x = [D]^n/(ED_{50}^n + [D]^n)$ or $x = [C]^n/(EC_{50}^n + [C]^n)$ with n fixed at integral values (1, 2, 3, 4 and 5), and repeated with n "floating" to obtain a best-fit (Quastel and Saint, 1988). This gave the value of ED_{50} or EC_{50} yielding a minimal residual sum of squares of deviations from the theoretical curve. This was preferred to the more usual fit to $Y = bx$, in order to take into account the possible systematic underestimate or overestimate of MAP or HR corresponding to [D] or [C] = 0; the data set was augmented by 20 points with $Y = 0$ at [D] or [C] = 0. Usually, the reduction in minimal residual sum of squares obtained by "floating" n was not significant in the sense that the reduction (from that obtained with the nearest integral value of n) was no more than expected from the reduction in degrees of freedom (by F test). With this fitting, the maximal response to [D] or [C] is given by b; values of a at the best-fitting were never significantly different from 0.

2.3.2. Modified Schild plot (Kenakin, 1987)

In principle, a competitive inhibitor (I) shifts the dose-response curve to an agonist [A] in parallel to the right, with the ED_{50} increasing by the factor $(1 + [I]/K_i)$ when there is a one-to-one competition of I with A on the receptor. This arises from the well-known relationship: $[AR]/[R_t] = [A]/\{[A] + K_a(1 + [I]/K_i)\}$ where $[AR]/[R_t]$ is the fraction of receptors occupied by A and K_a and K_i are the

dissociation constants for A and I, respectively. It can readily be shown that the same relationship holds when A is an antagonist and I is an agonist.

Using the standard assumption that equal responses, with and without I, represent equal $[AR]/[R_t]$, it follows that $K_a(1+[I]/K_i)/[A]_I = K_a/[A]_o$, where $[A]_I$ is the [A] that gives the same response in the presence of I as did $[A]_o$ in the absence of I. Therefore "dose-ratios" (DR) $([A]_I/[A]_o$, which are the same as ratios of ED_{50}s), follows the equation: $DR = [A]_i/[A]_o = 1+[I]/K_i$ and, therefore, log (DR-1) = log[I]-log K_i. That is, the "Schild plot" - a graph of log (DR-1) *vs* log [I] - has a slope of unity and K_i is given by the (extrapolated or interpolated) value of [I] at DR = 2.

However, if the response depends upon a form of the receptor bound to more than one (n_1) molecules of agonist while more than one (n_2) molecules of I are necessary to block action of the receptor, the above equation does not hold. Instead, the above equations become, at the simplest (assuming high positive cooperativity in binding of A to R):

$$[A^{n1}R]/[R_t] = [A]^{n1}/\{[A]^{n1} + Ka^{n1}(1+[I]^{n2}/K_i)\}$$

$$DR^{n1} = (\text{dose ratio})^{n1} = ([A]_i/[A]_o)^{n1} = 1 + [I]^{n2}/K_i$$

$$DR^{n1}-1 = [I]^{n2}/K_i$$

Thus, one must plot log (DR^{n1}-1) *vs* n_2 x log [I] to obtain a slope of unity.

2.3.3. Statistics (Zar, 1984)

The animals and aortic rings were randomly assigned into groups within each experimental design. Within each experimental design, all experiments were performed one block at a time. Six rats or 6 aortic rings (each derived from a different animal) were usually used except in cases where indicated.

All results were expressed as mean ± standard error (S.E.), or as geometric mean and 95% confidence range in cases where the results were transformed to

obtain homogeneity of variances. The results were analyzed by the analysis of variance followed by Duncan's multiple range test, by Number Cruncher Statistical System Program (by J. L. Hintze, Kaysville, Utah, U.S.A.), with $P<0.05$ selected as the criterion for statistical significance.

3. Results and discussion I

3.1. Results

3.1.1. Effects of L-NNA and D-NNA on contractile responses of the isolated aorta

A 10 minute incubation with L-NNA ($3x10^{-7}$ to $3x10^{-5}$ mol/L) caused a spontaneous contractile effect in some but not all aortae (data not shown). In addition, L-NNA caused concentration-dependent contraction of resting aortic rings (Fig. 1A in appendix III). D-NNA ($3x10^{-6}$ to $3x10^{-4}$ mol/L), on the other hand, induced neither spontaneous nor sustained contraction of the aortae (Fig. 1A in appendix III).

PHE ($1x10^{-6}$ mol/L)-induced contraction reached approximately 80% of maximal force within 30-60 s followed by a slower phase which reached plateau (maximum) in 10-20 min. Preincubation with L-NNA, but not with D-NNA, significantly potentiated the contraction induced by PHE (Fig. 1B in appendix III).

3.1.2 Effects of NSAAs on endothelium-dependent relaxation *in vitro*

3.1.2.1. Concentration-responses

In the 7 groups of aortic rings used to study concentration-response relationship of L-NAME, PHE caused contraction of 0.77 ± 0.04 g in the control rings. L-NAME (1, 2, 4, 8, 18 and $32x10^{-7}$ mol/L) slightly potentiated PHE-induced contraction to values of 1.10 ± 0.15, 0.86 ± 0.04, 0.94 ± 0.08, 0.99 ± 0.07, 0.94 ± 0.08 and 1.13 ± 0.09 g, respectively. All the values of contraction were significantly different from the control except for $2x10^{-7}$ mol/L L-NAME. In another 7 groups of rings to study the concentration-response of D-

NAME, PHE caused contraction of 0.92±0.15 g in the control rings. D-NAME (0.5, 1, 2, 4, 8, $16x10^{-4}$ mol/L) did not potentiate the effect of PHE, which caused contractions of 0.85±0.17, 0.86±0.13, 1.03±0.13, 0.96±0.17, 0.98±0.07 and 1.26±0.23 g, respectively.

ACh ($1x10^{-8}$ to $3x10^{-5}$ mol/L) caused concentration-dependent relaxation of PHE-preconstricted aortae, with maximal relaxation of approximately 60%. Incubation with L-NAME ($1x10^{-7}$ to $3.2x10^{-6}$ mol/L) and D-NAME ($5x10^{-5}$ to $1.6x10^{-3}$ mol/L) concentration-dependently and "noncompetitively" inhibited ACh-induced relaxation (Fig. 1A, 1B). Analysis of the concentration-inhibition curves of L-NAME and D-NAME at 3x10-5 mol/L ACh gave best-fitted *n*s of 0.86 and 0.88 (not significantly different from 1.0), with maximal inhibition of 87% and 95% as well as IC_{50}s of $3.5x10^{-7}$ mol/L ($pD_2=6.5$) and $1.4x10^{-4}$ mol/L ($pD_2=3.8$), respectively. As shown in Fig 2A the theoretical curve with *n* of 1 fitted the observed data very much better than that with *n* of 2. Correspondingly, a Hill plot of the inhibition by L-NAME and D-MAME of 3x10-5 mol/L ACh-induced relaxation yielded slopes of 0.81±0.06 and 0.79±0.04 (both $P<0.05$), respectively (Fig. 2B). The dose-ratio of IC_{50}s for D-NAME *vs* L-NAME was 337:1.

SNP ($1x10^{-8}$-$3x10^{-6}$ mol/L) also caused concentration-dependent relaxation of PHE-preconstricted aortic rings with maximal relaxation of approximately 100%. The relaxation was not significantly altered by $3.2x10^{-6}$ mol/L L-NAME or by $1.6x10^{-3}$ mol/L D-NAME, which were approximately 10-fold the IC_{50} for these compounds to inhibit ACh-induced relaxation (Fig. 3).

In another study in Appendix III, incubations with L-NNA ($3x10^{-7}$ to $3x10^{-5}$ mol/L) and D-NNA ($3x10^{-6}$ to $3x10^{-4}$ mol/L) also concentration-dependently and "noncompetitively" inhibited the relaxant response to ACh (Fig. 2A, 3A in Appendix III). Fig. 1C in Appendix III illustrates the relaxation induced by $3x10^{-5}$ mol/L ACh in the presence of L-NNA or D-NNA, with average best-fitted *n*s of 0.80 and 1.15, IC_{50}s of $1x10^{-6}$ mol/L ($pD_2=6.0$) and $3.9x10^{-5}$ mol/L

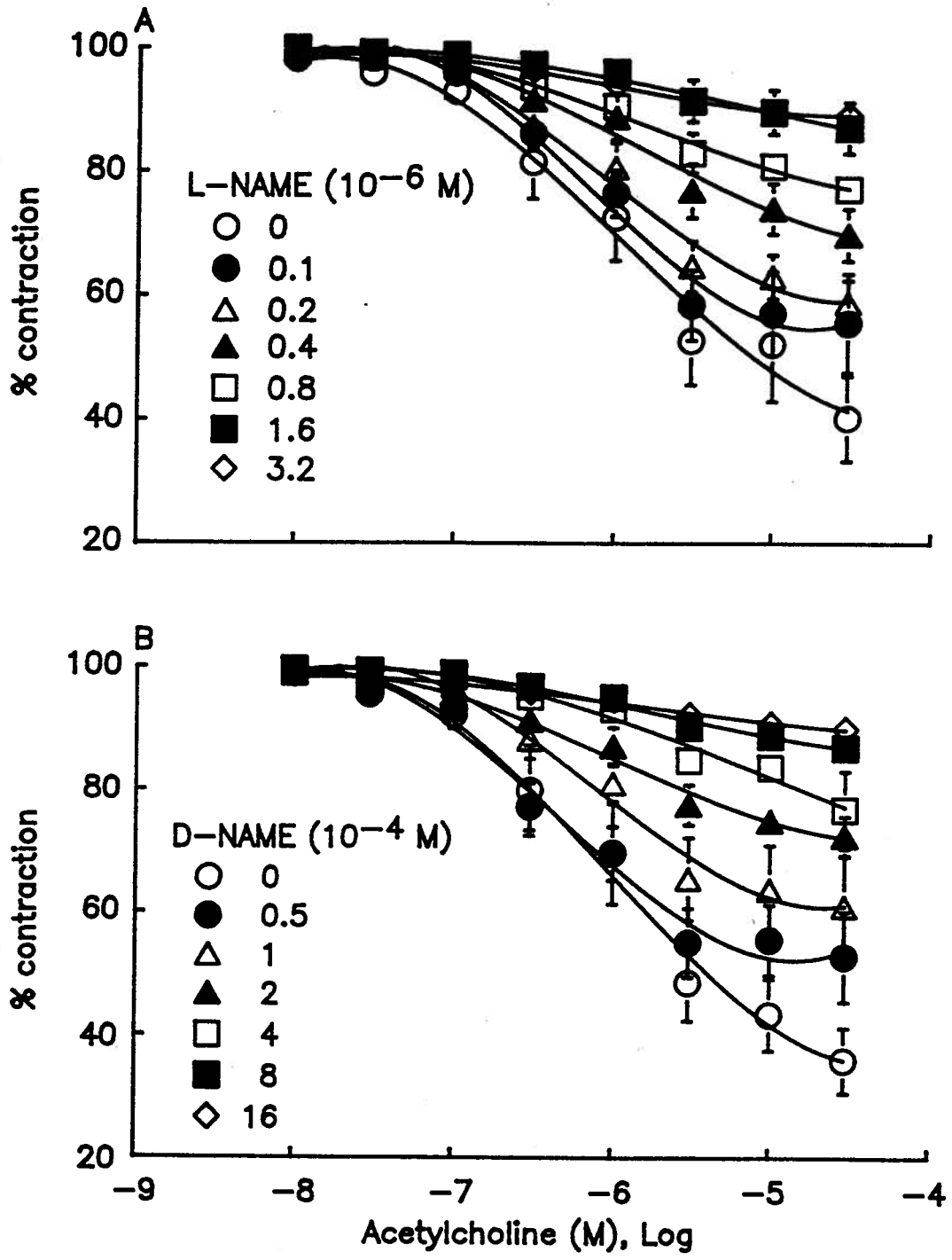

Fig. 1. Inhibitory effects (mean ± S.E.) of NG-nitro-L-arginine methyl ester (L-NAME, **A**) and D-NAME (**B**) on acetylcholine-induced relaxation in phenylephrine (1x10-6 mol/L)-preconstricted aortic rings (N = 5-6 each group).

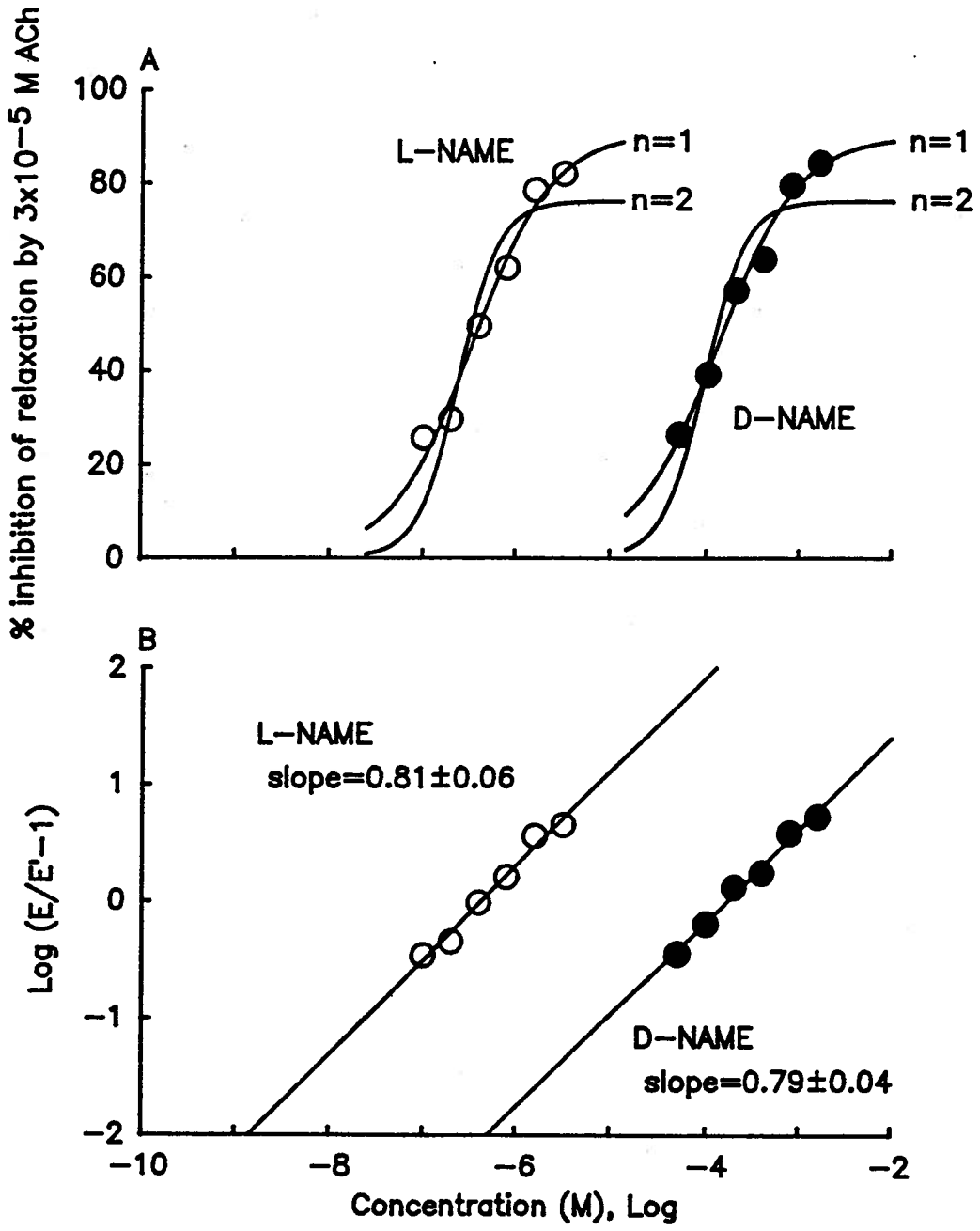

Fig. 2. Analyses of the inhibition by NG-nitro-L-arginine methyl ester (L-NAME, 1x10-7 to 3.2x10-6 mol/L) and D-NAME (5x10-5 to 1.6x10-3 mol/L) of acetylcholine (ACh, 3x10-5 mol/L)-evoked relaxation in preconstricted aortic rings. The data were the average values from 5-6 aortic rings. **(A)** Concentration-response curve of L-NAME and D-NAME. The theoretical curves were plotted with Hill coefficient *n* of 1 and 2. **(B)** Hill Plot of the concentration-response curve of L-NAME and D-NAME. E and E' represented the relaxations at 3x10-5 mol/L ACh in the absence and presence, respectively, of different concentrations of L-NAME or D-NAME.

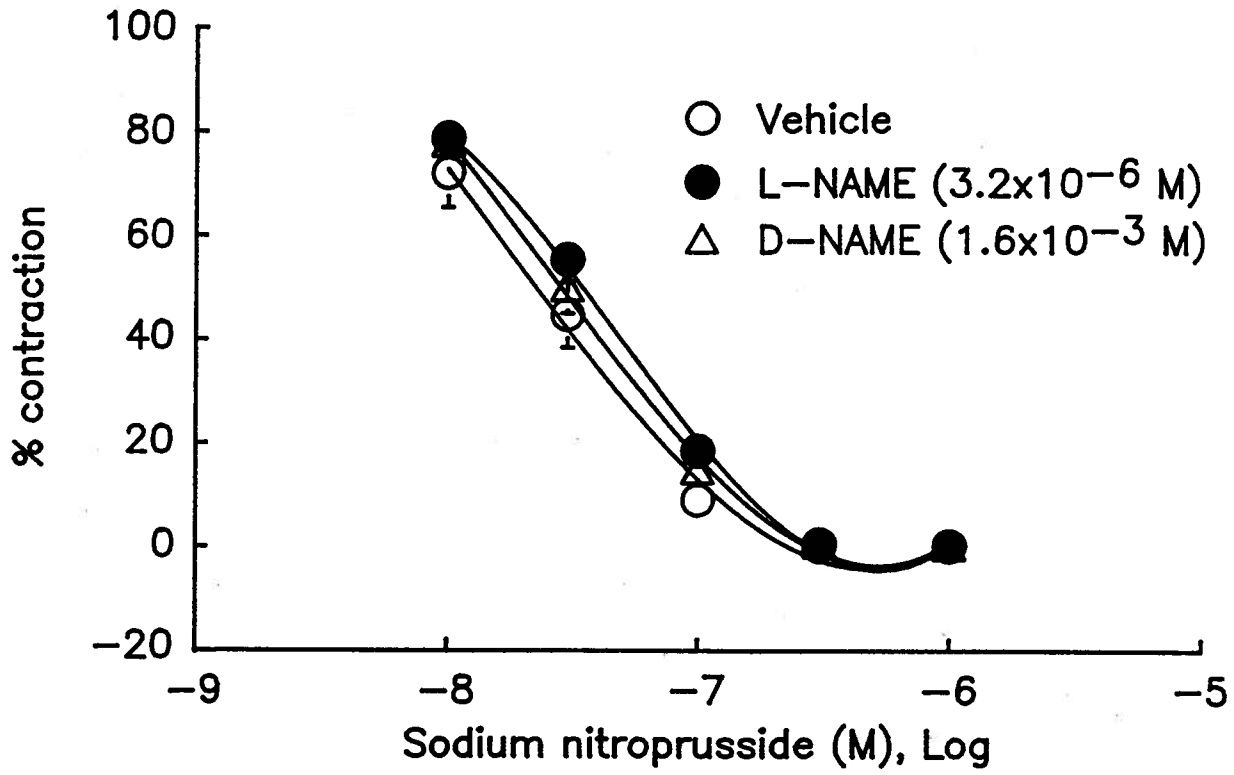

Fig. 3. Effects (mean ± S.E.) of NG-nitro-L-arginine methyl ester (L-NAME, 3.2x10-6 mol/L) and D-NAME (1.6x10-3 mol/L) on sodium nitroprusside-induced relaxation in phenylephrine (1x10-6 mol/L)-preconstricted aortic rings (N = 5-6 each group).

($pD_2 = 4.4$), and E_{max}s of 89% and 104%, respectively. The dose-ratio of IC_{50}s for D-NNA *vs* L-NNA was 39:1. On the other hand, neither L-NNA nor D-NNA inhibited the relaxant response to SNP (Fig. 2B, 3B in Appendix III).

A 23187 was as equally efficacious as ACh in causing concentration-dependent relaxation which reached a maximum of approximately 70% at $3x10^{-7}$ mol/L. Incubations with both L-NNA ($3x10^{-5}$ mol/L) and D-NNA ($3x10^{-4}$ mol/L) almost completely inhibited the relaxant response to A 23187 (Fig. 4 in Appendix III).

Furthermore, bradykinin caused a slight but dose-dependent relaxation of PHE-preconstricted aortic rings with relaxation of $30 \pm 5\%$ at $3x10^{-7}$ mol/L. Both incubation with L-NNA ($3x10^{-5}$ mol/L) and D-NNA ($3x10^{-4}$ mol/L) attenuated the relaxant response to bradykinin. The relaxation of bradykinin at $3x10^{-7}$ mol/L in the presence of L-NNA and D-NNA was reduced to $9 \pm 2\%$ and $12 \pm 5\%$ (both $P < 0.05$), respectively.

3.1.2.2. Mechanisms

Incubation with neither L-Arg ($1x10^{-3}$ mol/L) nor D-Arg ($1x10^{-3}$ mol/L) significantly altered the relaxant response to ACh (Fig. 7A in Appendix III). Ten minute preincubations with both L-NNA ($1x10^{-6}$ mol/L) and D-NNA ($3x10^{-5}$ mol/L) significantly inhibited the relaxant effect of ACh (Fig. 7B, 7C in Appendix III). The inhibitory effects of L-NNA and D-NNA were eliminated by 10 min pretreatment with L-Arg but not with D-Arg (Fig. 7B, 7C in Appendix III). Pretreatment with L-Arg ($3x10^{-7}$ mol/L) but not D-Arg ($3x10^{-7}$ mol/L) also abolished the inhibitory effects of L-NAME ($3.2x10^{-6}$ mol/L) and D-NAME ($1.6x10^{-3}$ mol/L) on ACh-induced relaxation (Fig. 4A).

Indomethacin ($1x10^{-5}$ mol/L) did not alter ACh-induced relaxation in the preconstricted aortic rings, compared with the response in the vehicle group (Fig.

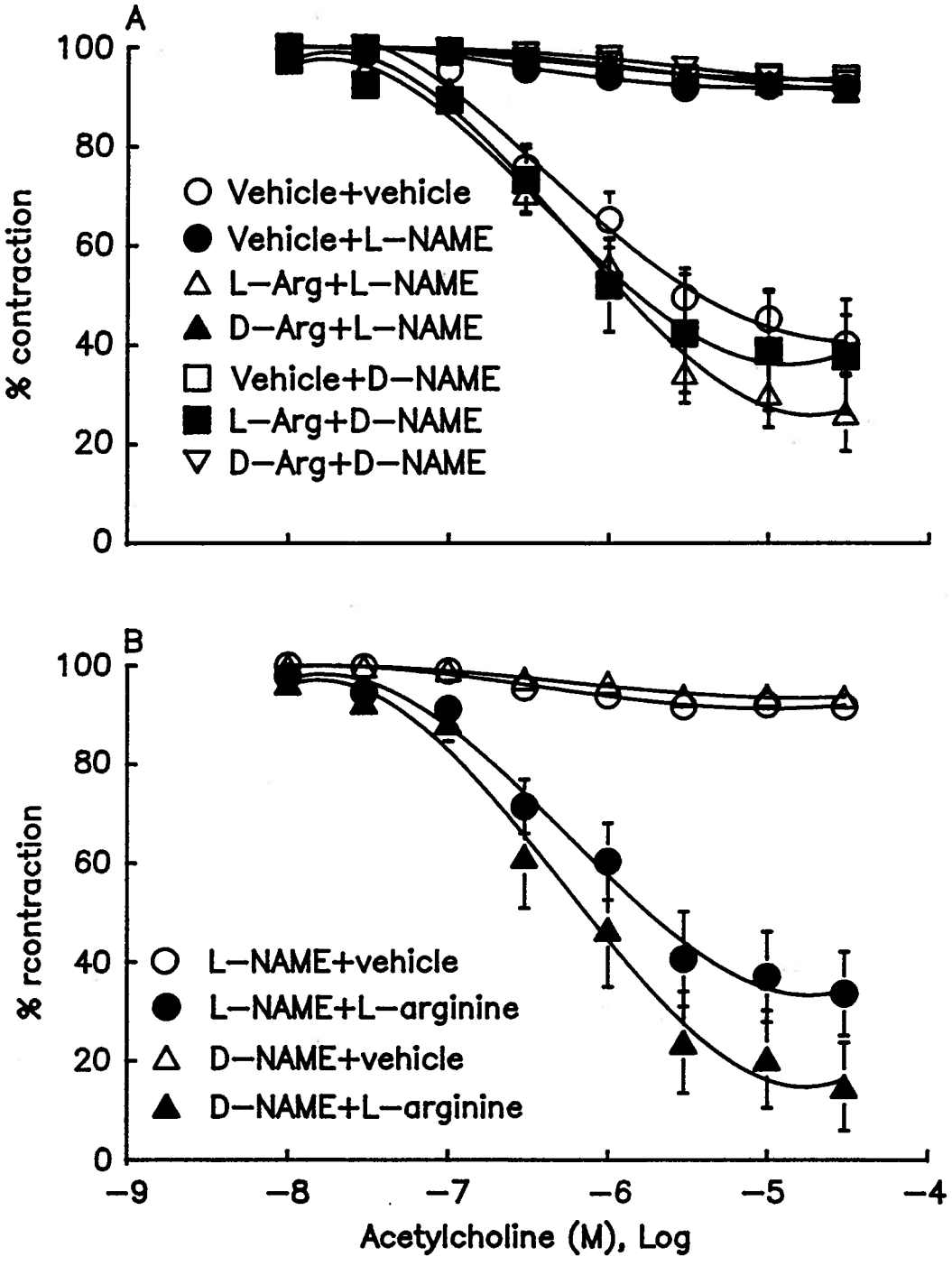

Fig. 4. Time courses of the effects (mean ± S.E.) of the vehicle (**A**), N^G-nitro-L-arginine methyl ester (L-NAME, 3.2×10^{-6} mol/L, **B**) and D-NAME (1.6×10^{-3} mol/L, **C**) on acetylcholine-induced relaxation in phenylephrine (1×10^{-6} mol/L)-preconstricted rat aortic rings (N = 5-6 each group). The various times represent the times after the preparations were washed without further adding the vehicle, L-NAME or D-NAME.

6A in Appendix III). L-NNA ($1x10^{-6}$ mol/L) and D-NNA ($3x10^{-5}$ mol/L) inhibited the relaxation evoked by ACh (Fig. 6B, 6C in Appendix III). Pretreatment with indomethacin did not alter the inhibitory effect of L-NNA (Fig. 6B in Appendix III) or D-NNA (Fig. 6C in Appendix III) on ACh-induced relaxation.

PHE caused contractions of 1.46 ± 0.12 and 1.67 ± 0.13 g in the presence of vehicle + L-NNA ($1x10^{-6}$ mol/L) and NADPH ($5x10^{-3}$ mol/L) + L-NNA ($1x10^{-6}$ mol/L), respectively. L-NNA markedly inhibited ACh-induced relaxation; pretreatment with NADPH did not affect the inhibitory effect of L-NNA (Fig. 4 in Appendix VII).

3.1.2.3. Time courses and reversibility

In the control group, concentration-relaxant response curves of ACh were repeated 4 times within 6 h. There was a time-dependent loss of the relaxant response to ACh which became statistically significant at the last curve (Fig. 5A in Appendix III). Incubations with both L-NNA ($3x10^{-5}$ mol/L) and D-NNA ($3x10^{-4}$ mol/L) abolished ACh-induced relaxation (Fig. 5B, 5C in Appendix III). The inhibitory effects of L-NNA and D-NNA were still present at 1.5 and 4 h after the preparations were washed out without further adding the L-NNA or D-NNA, even when compared to the corresponding time controls (Fig. 5A, 5B, 5C in Appendix III). The inhibitory effects of both L-NAME ($3.2x10^{-6}$ mol/L) and D-NAME ($1.6x10^{-3}$ mol/L) were also long-lasting and remained for at least 4 h after washouts (Fig. 5). The results at 9 h after washout showed that the inhibitory effect of L-NAME lasted longer than that of D-NAME (Fig. 5B, 5C).

Fig. 8 in Appendix III showed that the relaxant response to ACh was again inhibited by 1.5 h incubations with L-NNA ($3x10^{-5}$ mol/L) and D-NNA ($3x10^{-4}$ mol/L). The inhibitory effects of L-NNA and D-NNA were also markedly eliminated by post-treatment (1.5 h later) with L-Arg ($1x10^{-3}$ mol/L) (Fig. 8A, 8B in Appendix

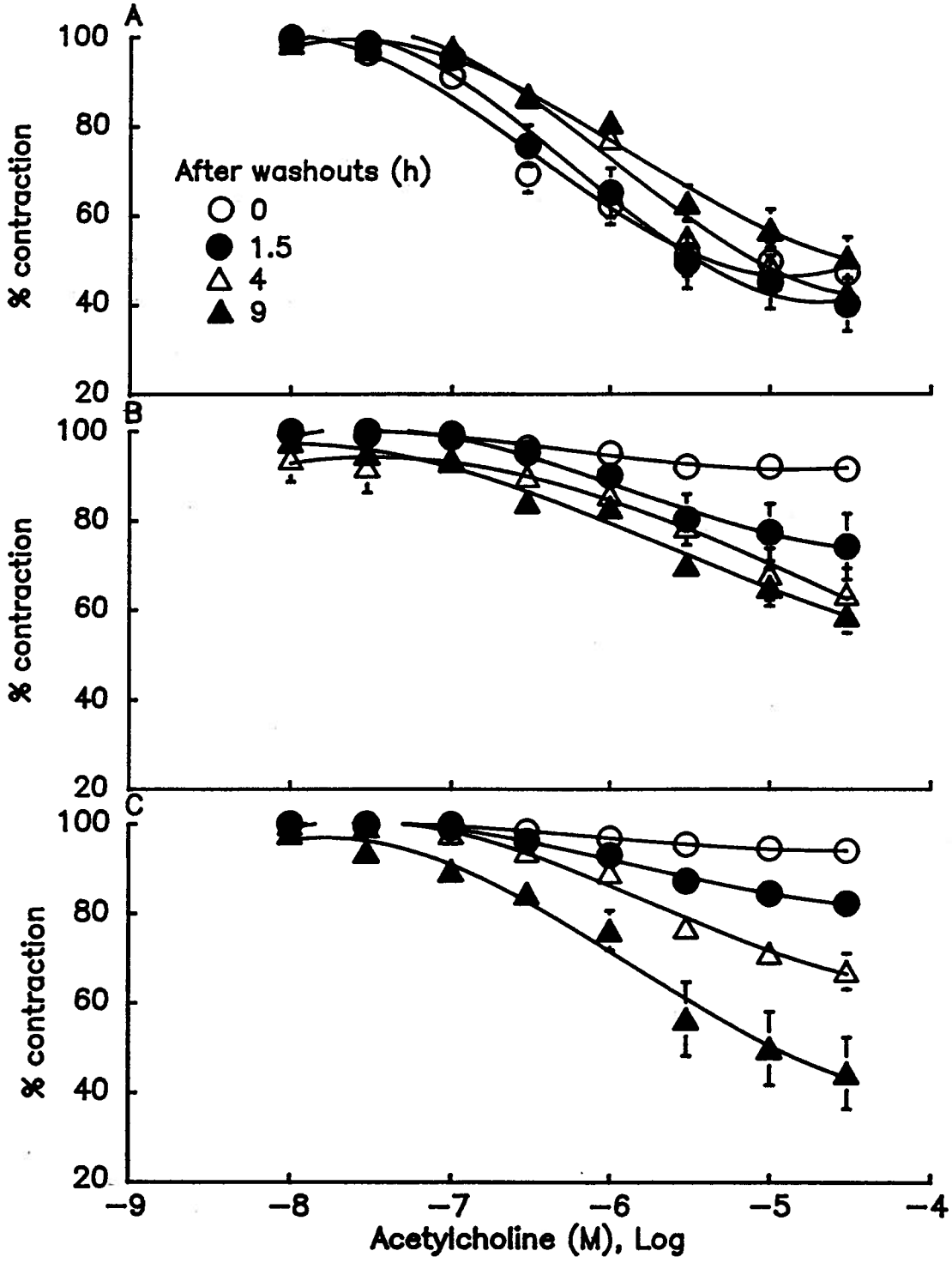

Fig. 5. Effects (mean ± S.E.) of pretreatment (10 min earlier, **A**) and post-treatment (4 h later, **B**) with L-arginine (L-Arg, 3×10^{-4} mol/L) or D-Arg (3×10^{-4} mol/L) on inhibitory effects of N^G-nitro-L-arginine methyl ester (L-NAME, 3.2×10^{-6} mol/L) and D-NAME (1.6×10^{-3} mol/L) on acetylcholine (ACh)-induced relaxation in phenylephrine (1×10^{-6} mol/L)-preconstricted aortic rings (N = 5-6 each group).

III). Moreover, post-treatment (1.5 h later) with L-Arg ($3x10^{-4}$ mol/L) also completely reversed the inhibitory effects of L-NAME ($3.2x10^{-6}$ mol/L) and D-NAME ($1.6x10^{-3}$ mol/L) on ACh-induced relaxation (Fig. 4B).

3.1.3. Effects of L-NNA and D-NNA on ACh- and SNP-induced relaxations *ex vivo*

Baseline MAPs of the conscious rats which were i.v. bolus injected with the vehicle, L-NNA ($1.6x10^{-4}$ mol/kg) and D-NNA ($1.6x10^{-4}$ mol/kg) were 100 ± 2, 107 ± 1 and 112 ± 2 mmHg, respectively. The vehicle did not significantly alter MAP while L-NNA and D-NNA raised MAP to similar plateau values at 40 min after injections (Fig. 9A in Appendix III). The relaxant response to ACh in PHE-preconstricted aortic rings obtained from either L-NNA- or D-NNA-pretreated rats were less than those in the vehicle-treated rats (Fig. 9B in Appendix III). In contrast, the relaxant response to SNP was not inhibited by treatment with L-NNA or with D-NNA. Maximal relaxations in response to SNP ($1x10^{-6}$ mol/L) in the vehicle-, L-NNA- and D-NNA-treated aortic rings were $107 \pm 4\%$, $99 \pm 3\%$ and $99 \pm 3\%$, respectively.

3.1.4. Effects of L-NAME and D-NAME on the depressor responses to ACh and SNP *in vivo*

Baseline MAPs in the four groups of conscious rats were not significantly different from each other (Table 1). I.V. infusions of ACh ($5.5x10^{-8}$ to $8.8x10^{-7}$ mol/kg per min, each dose for 4 min) or SNP ($4x10^{-8}$ to $5.6x10^{-7}$ mol/kg per min, each dose for 4 min) dose-dependently decreased MAP (expressed as % baseline MAP) (Fig. 6). The infusion of PHE ($2x10^{-8}$ mol/kg per min), which raised MAP to the same extent as that by a single bolus injection of $4.8x10^{-5}$ mol/kg L-NAME or

Table 1. Values (mean ± S.E.) of mean arterial pressure (MAP) before (a) and 10 (b), 20 (40 for D-NAME, c), or 100 (120 for D-NAME, d) min after i.v. administrations of the vehicle, L-arginine (L-Arg, 1.2-4.8x10^{-5} mol/kg per min), D-Arg (4.8x10^{-5} mol/kg per min), phenylephrine (PHE, 2x10^{-8} mol/kg per min), N^G-nitro-L-arginine methyl ester (L-NAME, 4.8x10^{-5} mol/kg) and D-NAME (1.2x10^{-3} mol/kg) in conscious rats (N = 5-6 each group).

Treatment	Dose (mol/kg or mol/kg per min)	MAP (mmHg)			
		a	b	c	d
Section 3.1.3					
Vehicle	-	115 ± 5	-	124 ± 9	-
PHE	2.0x10^{-8}	109 ± 4	-	160 ± 4*	-
L-NAME	4.8x10^{-5}	112 ± 5	-	160 ± 5*	-
D-NAME	1.2x10^{-3}	115 ± 5	-	165 ± 4*	-
Section 3.1.3					
Vehicle	-	108 ± 5	-	111 ± 5	108 ± 4
L-Arg	4.8x10^{-5}	113 ± 4	-	115 ± 4	110 ± 3
PHE	2.0x10^{-8}	103 ± 4	-	162 ± 5*	156 ± 3*
L-NAME	4.8x10^{-5}	113 ± 4	-	159 ± 5*	156 ± 4*
D-NAME	1.2x10^{-3}	96 ± 2	-	137 ± 4*	143 ± 4*
Section 3.1.5.3.					
Vehicle	-	107 ± 5	106 ± 4	-	-
L-Arg	1.2x10^{-5}	103 ± 6	102 ± 7	-	-
L-Arg	2.4x10^{-5}	114 ± 2	114 ± 3	-	-
L-Arg	4.8x10^{-5}	105 ± 5	106 ± 5	-	-
D-Arg	4.8x10^{-5}	112 ± 6	112 ± 6	-	-
Section 3.1.5.3.					
Vehicle	-	105 ± 3	108 ± 3	-	-
L-Arg	4.8x10^{-5}	112 ± 7	113 ± 7	-	-

* denotes significant difference from baseline MAP ($P < 0.05$).

$1.2x10^{-3}$ mol/kg D-NAME (Table 1), enhanced the depressor responses to various doses of ACh and SNP by average values of 140±53% and 94±31%, respectively, with greater potentiation at the lower doses of ACh and SNP (Fig. 6). L-NAME and D-NAME markedly inhibited the depressor response to ACh, by 53±1% and 54±1% respectively, when the ACh-response was compared to that in the rats treated with PHE (Fig. 6). On the other hand, L-NAME and D-NAME potentiated depressor responses to all doses of SNP, by an average of 142±53% and 92±29%, respectively, compared to the responses in the vehicle-treated rats. At the lowest two doses of SNP, the potentiation by L-NAME but not by D-NAME was significantly greater than that by PHE which gave a similar increase in baseline MAP as did L-NAME and D-NAME (Fig. 6).

Baseline MAPs in another 5 groups of conscious rats were also not significantly different from each other (Table 1). PHE, L-NAME and D-NAME raised MAP to similar levels but L-Arg did not alter MAP. ACh ($3x10^{-10}$ to $8.8x10^{-8}$ mol/kg) was i.v. bolus injected into all groups of rats. The first three doses of ACh caused significantly less reduction in MAP in the rats treated with L-Arg ($4.8x10^{-5}$ mol/kg per min) than in the control rats given the vehicle (Fig. 7A). In the animals given PHE ($2x10^{-8}$ mol/kg per min), L-NAME ($4.8x10^{-5}$ mol/kg) or D-NAME ($1.2x10^{-3}$ mol/kg), the magnitude of the depressor response to ACh was not significantly different from that in the control rats given the vehicle. However, the durations (expressed as modified half-recovery time, *i.e.*, half-recovery time/baseline MAP$x10^{4}$) of the (transient) response to ACh was shortened by L-NAME and D-NAME (Fig. 7B), by 23±3% and 9±2%, respectively, when compared to that in the vehicle-treated rats ($P<0.05$ at the last 2 doses of ACh for L-NAME), and by 26±3% and 13±3%, respectively, when compared to that in PHE-treated rats ($P<0.05$ at the last 4 doses of ACh for L-NAME, and the last dose of ACh for D-NAME).

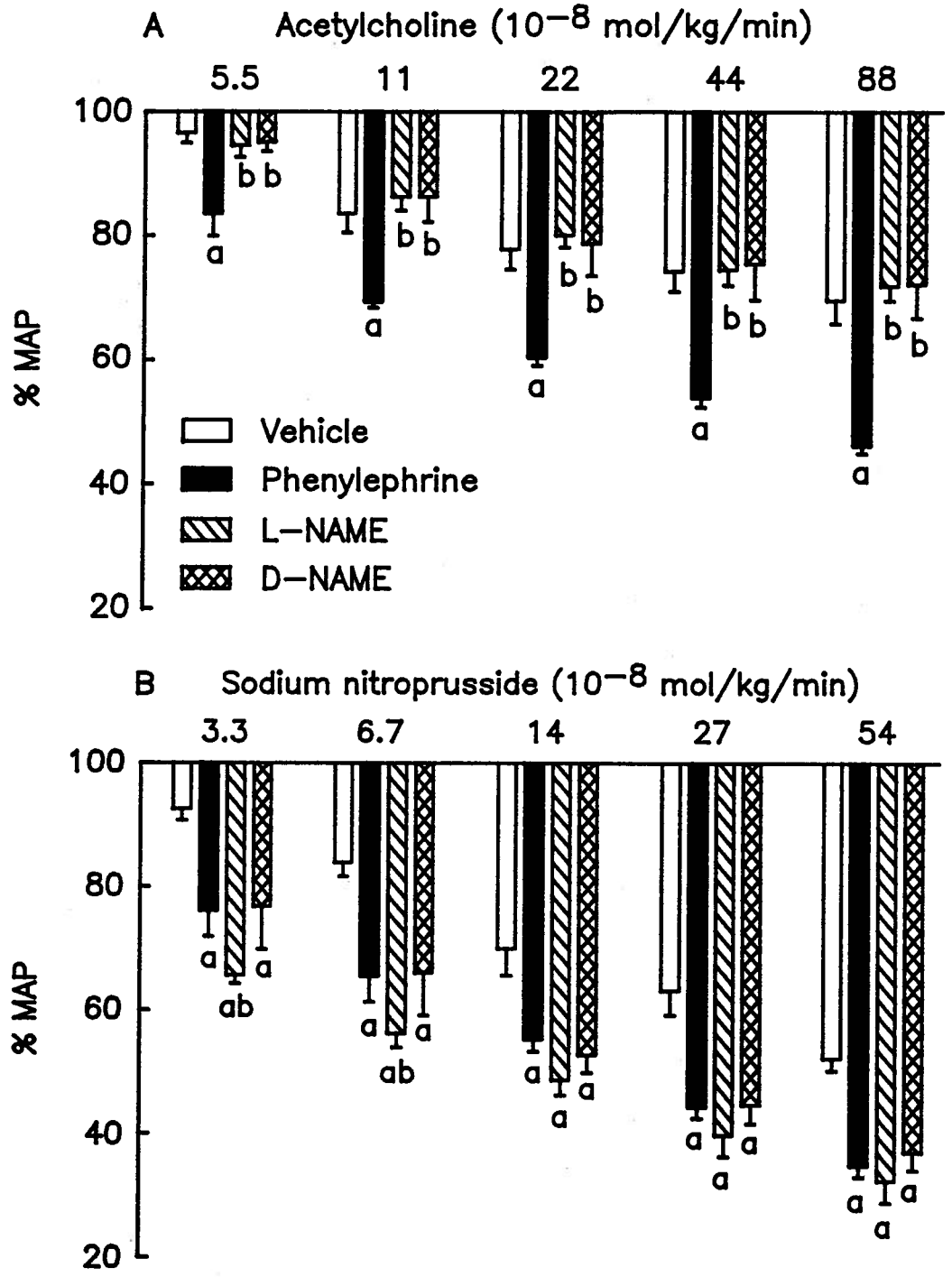

Fig. 6. Effects (mean ± S.E.) of i.v. infusions (4 min for each dose) of acetylcholine (**A**) and sodium nitroprusside (**B**) on the mean arterial pressure (MAP, expressed as % baseline MAP) in conscious rats (N = 6 each group) pretreated with the vehicle, phenylephrine (2x10-8 mol/kg per min), NG-nitro-L-arginine methyl ester (L-NAME, 4.8x10-5 mol/kg) or D-NAME (1.2x10-3 mol/kg). a denotes significant difference from the vehicle-treated group; b denotes significant difference from phenylephrine-treated group.

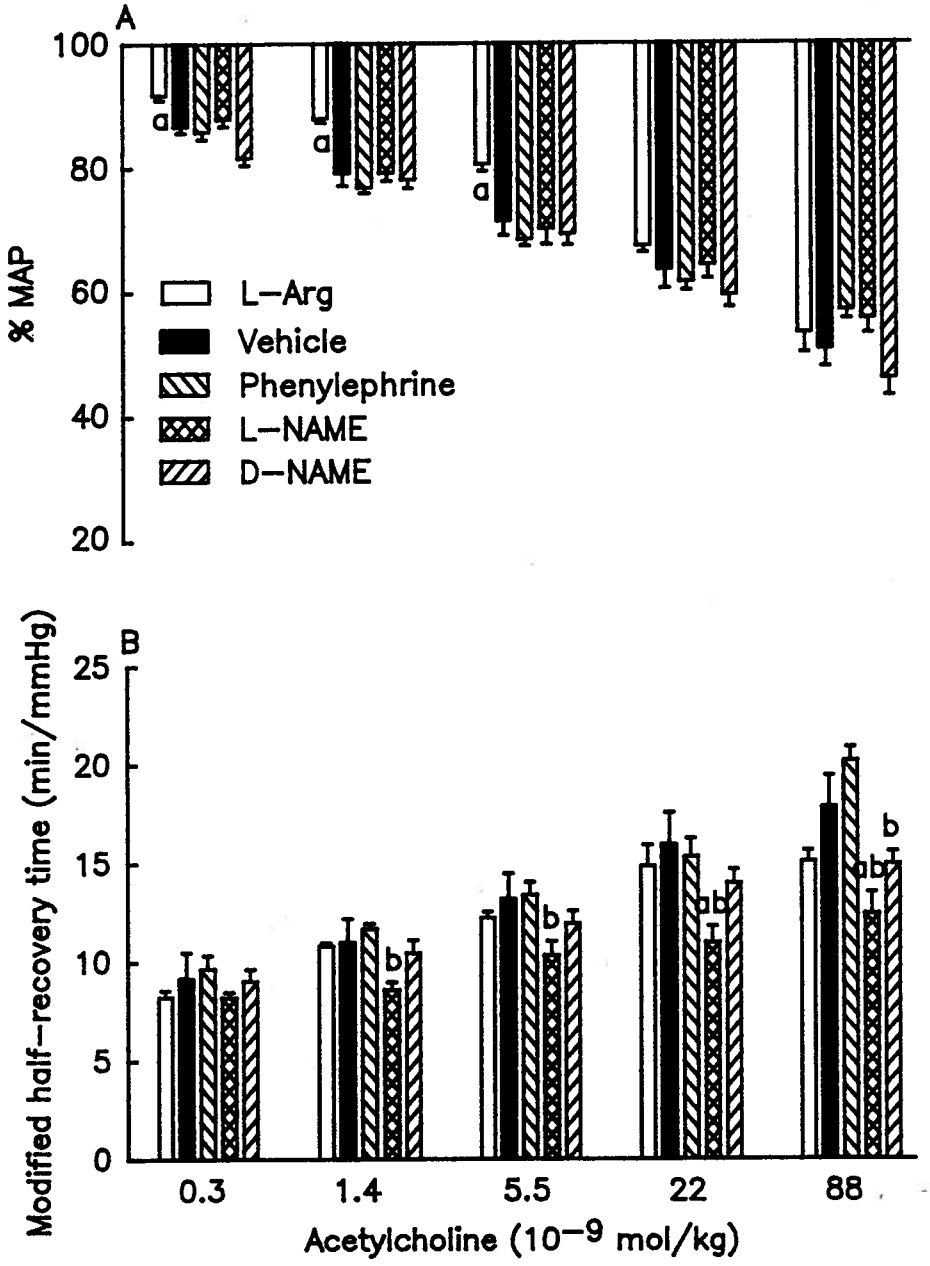

Fig. 7. Effects (mean ± S.E.) of i.v. bolus injections of acetylcholine on the mean arterial pressure (MAP, expressed as % baseline MAP, **A**) and modified half-recovery time (**B**) in conscious rats (N = 6 each group) pretreated with L-arginine (L-Arg, 4.8×10^{-5} mol/kg per min), the vehicle (1 ml/kg), phenylephrine (2×10^{-8} mol/kg per min), N^G-nitro-L-arginine methyl ester (L-NAME, 4.8×10^{-5} mol/kg) or D-NAME (1.2×10^{-3} mol/kg). a denotes significant difference from the vehicle-treated group; b denotes significant difference from phenylephrine-treated group. Modified half-recovery time was normalized as half-recovery time/baseline MAP $\times 10^4$.

In contrast, in the rats treated with bolus injections of SNP ($1.5x10^{-9}$ to $4.4x10^{-7}$ mol/kg) instead of ACh to cause transient depressions of MAP, the vasodilator response was somewhat potentiated by L-NAME or D-NAME, with significance at the lower two doses of SNP for L-NAME and at all the doses except the third one for D-NAME, even when comparison was made with that the rats infused with PHE (which itself slightly and non-significantly increased SNP response) (Fig. 8A). Decrease in the magnitude of SNP-induced depressor response by L-Arg was also observed, similar to those seen with ACh, but was significant ($P<0.05$) only at the third dose of SNP (Fig. 8A). The duration of the depressor response to SNP was unaltered by PHE, somewhat prolonged by L-Arg, as compared to the vehicle group ($P<0.05$ at the second dose of SNP), and somewhat prolonged by L-NAME ($P<0.05$ at the second dose of SNP compared to the vehicle group and the lowest three doses of SNP compared to PHE group) and D-NAME ($P<0.05$ at the lowest three doses of SNP as compared to those of the vehicle and PHE groups) (Fig. 8B).

3.1.5. Haemodynamic effects of L-NNA

Table 1 (Groups IX and X) in Appendix VI showed that baseline MAPs, HRs, COs and TPRs were similar in the two groups of conscious rats. I.V. bolus injection of L-NNA ($8x10^{-5}$ mol/kg) significantly increased MAP and TPR, and decreased HR and CO, as compared to the corresponding values in the vehicle group (Fig. 3 in Appendix VI).

Table 2 (Groups IX and X) in Appendix VI showed that baseline values of regional blood flows and vascular conductances in the two groups of conscious rats were not significantly different from each other. Compared with the vehicle, L-NNA significantly decreased blood flow to all organs or tissues except the liver and spleen (Fig. 4A in Appendix VI). However, conductance values showed that

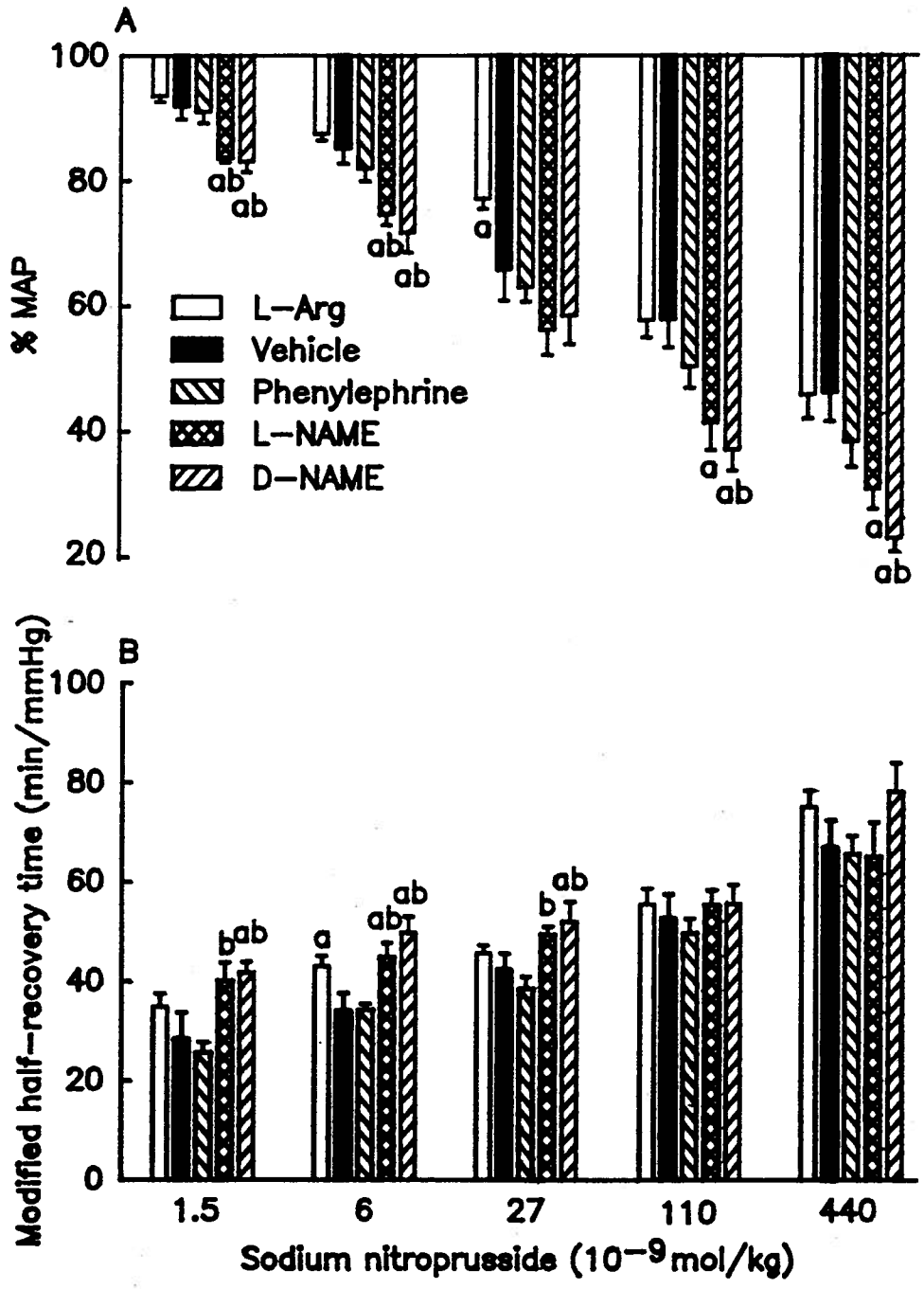

Fig. 8. Effects (mean ± S.E.) of i.v. bolus injections of sodium nitroprusside on the mean arterial pressure (MAP, expressed as % baseline MAP, **A**) and modified half-recovery time (**B**) in conscious rats (N = 6 each group) pretreated with L-arginine (L-Arg, 4.8x10-5 mol/kg per min), the vehicle (1 ml/kg), phenylephrine (2x10-8 mol/kg per min), NG-nitro-L-arginine methyl ester (L-NAME, 4.8x10-5 mol/kg) or D-NAME (1.2x10-3 mol/kg). a denotes significant difference from the vehicle-treated group; b denotes significant difference from phenylephrine-treated group. Modified half-recovery time was normalized as half-recovery time/baseline MAPx104.

L-NNA vasoconstricted all beds (Fig. 4B in Appendix VI). Changes in conductances were also expressed as % control to reflect the magnitudes of vasoconstriction in each organ/tissue in response to L-NNA (Fig. 6 in Appendix VI). The results showed that, while L-NNA reduced vascular conductances in all beds in conscious rats, the greatest influence was in the lungs while the least was in the liver.

3.1.6. Pressor responses to NSAAs

3.1.6.1. Time courses

In the three groups of conscious rats subsequently treated with the vehicle (1 ml/kg), L-NAME (4.8×10^{-5} mol/kg) and D-NAME (1.2×10^{-3} mol/kg), baseline MAPs (106 ± 3, 112 ± 3 and 117 ± 3 mmHg) and HRs (339 ± 8, 363 ± 8 and 368 ± 11 beats/min) were not significantly different from each other. I.V. bolus injection of L-NAME and D-NAME (but not the vehicle) caused slow-developing and prolonged increases in MAP. Plateau MAP responses to L-NAME and D-NAME were attained approximately 10 and 40 min after i.v. bolus and lasted at least 2 h, with half-rise phases of 2.3 ± 0.4 and 7.3 ± 1.6 min ($P < 0.05$) (Fig. 9A), respectively. Both L-NAME and D-NAME initially caused bradycardia. The HR response to L-NAME or D-NAME, however, slowly returned to or inclined to return to the baseline levels, even when the pressor responses were still maintained for the period of 2 h observed (Fig. 9B).

In the study presented in Appendix II, baseline MAPs and HRs in the three groups of conscious rats (Groups I-III) were similar (Table 1 in Appendix II). I.V. bolus injection of L-NNA (1.6×10^{-4} mol/kg), but not the vehicle, caused a sustained increase in MAP which reached plateau response at 10 min after injection, with average (geometric mean) half-rise phase of 5 min (95% confidence range of 2-12 min) (Fig. 1A in Appendix II). MAP at 80-160 min was lower than

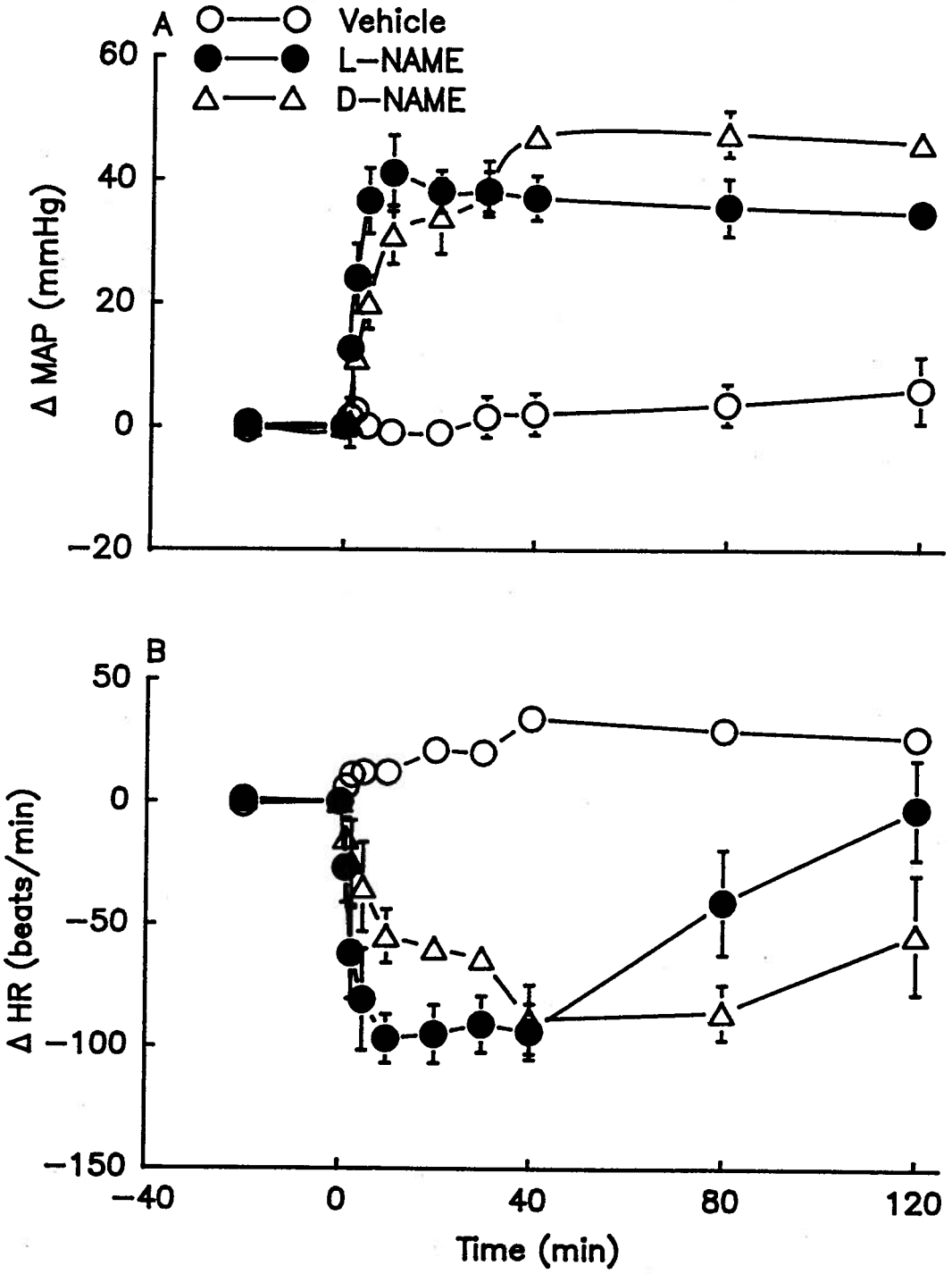

Fig. 9. Time courses (mean ± S.E.) of the mean arterial pressure (MAP) responses to i.v. bolus injections of the vehicle, N^G-nitro-L-arginine methyl ester (L-NAME, 4.8×10^{-5} mol/kg) and D-NAME (1.2×10^{-3} mol/kg) in conscious rats (N = 5 each group).

that at 40 min, but was still significantly higher than the baseline MAP. I.V. bolus injection of D-NNA ($1.6x10^{-4}$ mol/kg) also increased MAP to a similar plateau, but the onset of the response was significantly slower than that of L-NNA and plateau MAP was reached at 40 min after injection, with average (geometric mean) rise phase $t_{1/2}$ of 27 min (95% confidence range of 15-48 min) ($P<0.05$, compared with that of L-NNA). Both L-NNA and D-NNA caused bradycardia during the rise in MAP. A biphasic HR response was observed in L-NNA- but not in D-NNA-treated rats even when MAP was significantly elevated above the control level (Fig. 1B in Appendix II). The inability of D-NNA to cause tachycardiac response might be due to a markedly longer duration of action of D-NNA.

3.1.6.2. Dose-pressor response

I.V. bolus injections of cumulative doses of L-NAME ($1.5x10^{-6}$ to $4.8x10^{-5}$ mol/kg) in a group of conscious rats dose-dependently increased MAP (Fig. 10A) from baseline MAP of 106 ± 4 mmHg. Analysis of dose-response curves with *n* "floating" gave average best-fitted $n=2.01\pm0.32$ and it is evident that the theoretical curve with *n* of 2 but not 1 or 3 fitted the observed data best well. In each of the individual curves, *n* was not significantly different from 2. Using "floating" *n* for each individual curve, average values of ED_{50} and E_{max} for the pressor effect of L-NAME were $5.0\pm1.1x10^{-6}$ mol/kg and 50 ± 7 mmHg, respectively. These ED_{50}s and/or E_{max}s were not significantly different from those calculated using $n=2$ for analysis of each curve, or those from the observed data (Table 2). I.V. bolus injection of cumulative doses ($4x10^{-5}$ to $1.2x10^{-3}$ mol/kg) of D-NAME also caused a dose-dependent increase in MAP (Fig. 10B), with average best-fitted *n* of 2.65 ± 0.14, ED_{50} of $2.6\pm0.4x10^{-4}$ mol/kg and E_{max} of 50 ± 2 mmHg (Table 2). The dose ratio of ED_{50}s for D-NAME *vs* L-NAME was 52:1.

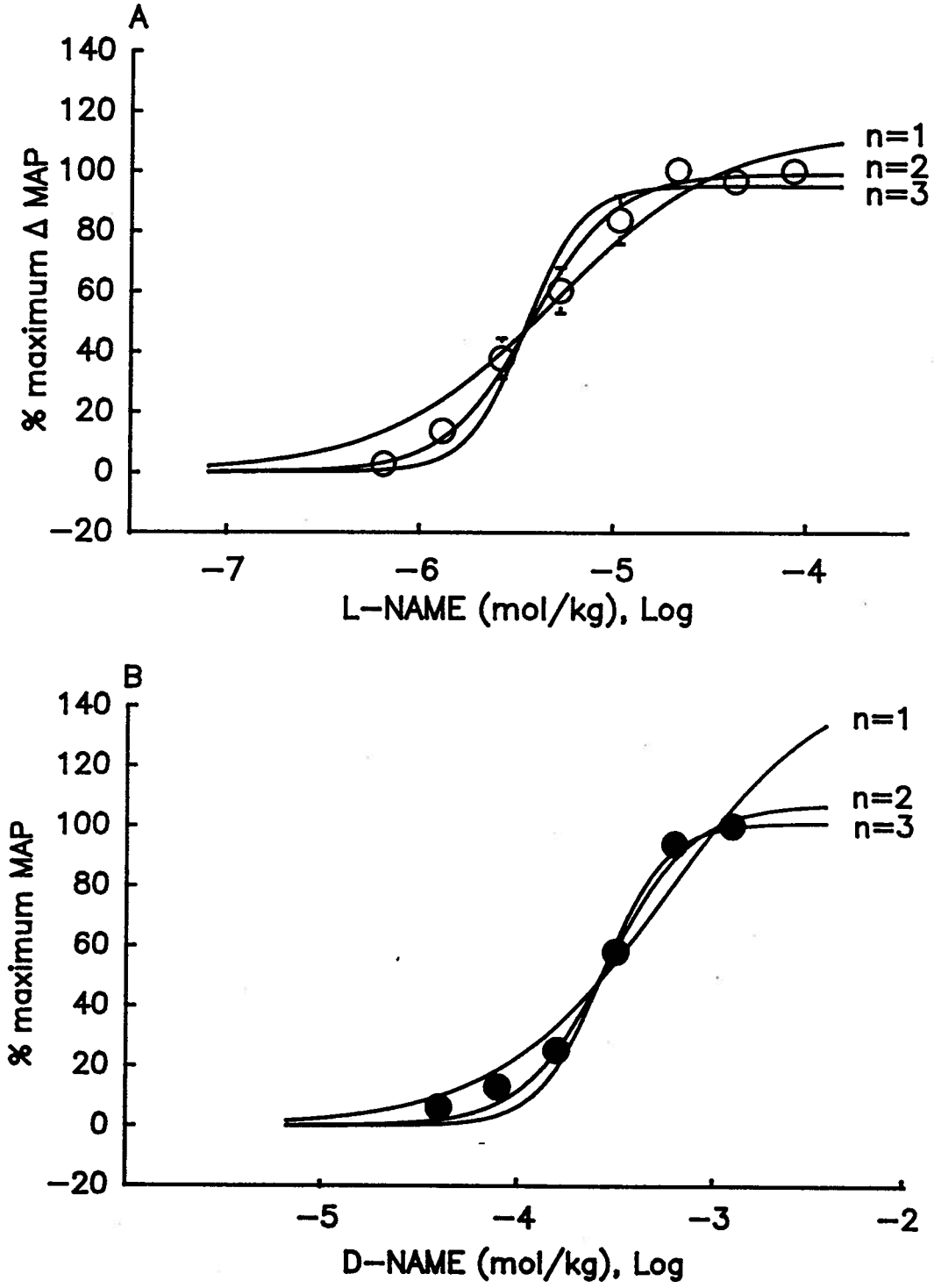

Fig. 10. Cumulative dose-response curves (mean ± S.E.) of NG-nitro-L-arginine methyl ester (L-NAME, **A**) and D-NAME (**B**) in conscious rats (N = 5 each group). Theoretical lines were drawn using *n* of 1, 2 and 3.

Table 2. Values (mean ± S.E.) of Hill coefficient (*n*), ED_{50} and E_{max} calculated by best-fitted or by specific *n*, as well as observed E_{max} of N^G-nitro-L-arginine methyl ester (L-NAME) and D-NAME in the presence of the vehicle, L-arginine (L-Arg) and D-Arg in conscious rats (N = 5 each group).

	Calculations			
	n	ED_{50} (1×10^{-6} mol/kg)	E_{max} (mmHg)	Observed E_{max} (mmHg)
L-NAME				
Best-fitted				
Vehicle	2.01 ± 0.32	5.0 ± 1.1	49.7 ± 6.7	50.0 ± 5.8
L-Arg (1×10^{-5} mol/kg per min)				
1.2	1.87 ± 0.31	19.4 ± 2.8*	52.0 ± 2.8	52.5 ± 2.6
2.4	1.99 ± 0.23	32.0 ± 5.0*	54.6 ± 2.2	53.0 ± 1.3
4.8	2.53 ± 0.30	40.9 ± 7.3*	52.1 ± 1.9	52.5 ± 2.0
D-Arg (4.8×10^{-5} mol/kg per min)				
	2.26 ± 0.31	4.1 ± 0.5	49.7 ± 2.9	49.5 ± 2.6
Specified, *n* = 2				
Vehicle	2	4.7 ± 1.1	48.4 ± 6.4	50.0 ± 5.8
L-Arg (1×10^{-5} mol/kg per min)				
1.2	2	19.2 ± 2.7*	51.1 ± 2.7	52.5 ± 2.6
2.4	2	31.1 ± 4.6*	53.7 ± 1.3	53.0 ± 1.3
4.8	2	42.2 ± 8.4*	52.6 ± 2.1	52.5 ± 2.0
D-Arg (4.8×10^{-5} mol/kg per min)				
	2	4.1 ± 0.5	49.4 ± 2.0	49.5 ± 2.6
D-NAME				
Best-fitted				
Vehicle	2.26 ± 0.35	273 ± 64	53.4 ± 3.8	49.0 ± 0.9
L-Arg (1.2×10^{-5} mol/kg per min)				
	1.73 ± 0.27	753 ± 185*	50.2 ± 6.5	47.0 ± 6.3
Specified, *n* = 2				
Vehicle	2	241 ± 39	51.2 ± 2.0	49.0 ± 0.9
L-Arg (1.2×10^{-5} mol/kg per min)				
	2	612 ± 182*	47.2 ± 7.0	47.0 ± 6.3

* denotes significant difference from the vehicle-treated groups ($P < 0.05$).

In another study in Appendix II, the baseline MAPs and HRs were similar between the two groups of conscious rats (Groups IV and V) and were summarized in Table 1 in Appendix II. I.V. bolus injections of both L-NNA ($1x10^{-5}$ to $3.2x10^{-4}$ mol/kg) and D-NNA ($2x10^{-5}$ to $3.2x10^{-4}$ mol/kg) dose-dependently increased MAP (Fig. 2A in Appendix II), with average best-fitted *n*s of 2.50 ± 0.15 and 1, ED_{50}s of $1.6x\pm0.4x10^{-5}$ mol/kg and $3.4x10^{-5}$ mol/kg, as well as E_{max}s of 55 ± 4 and 53 mmHg, for L-NNA and D-NNA respectively. The dose-ratio of ED_{50} for D-NNA *vs* L-NNA was 2:1. Both L-NNA and D-NNA caused dose-dependent bradycardia (Fig. 2B in Appendix II).

3.1.6.3. Effects of L-Arg and D-Arg on the pressor responses to NSAAs

Mean values of MAP in the 5 groups of conscious rats prior to, and 10 min after the start of infusion of the vehicle, L-Arg or D-Arg were not significantly different from each other (Table 1). Continuous i.v. infusions of L-Arg (1.2, 2.4, and $4.8x10^{-6}$ mol/kg per min) but not D-Arg ($4.8x10^{-6}$ mol/kg per min) dose-dependently shifted the dose-pressor response curve of L-NAME to the right, without significantly changing E_{max} or *n* (Fig. 6A in Appendix VIII and Table 2). The theoretical curves in Fig. 6A in Appendix VIII were plotted using *n* as 2 and these fitted the observed data well. The modified Schild plots in Fig. 6B in Appendix VIII for the apparently competitive block by L-Arg were drawn using "dose-ratio" (DR) of the ED_{50} in the presence of L-Arg divided by the ED_{50} in the absence of L-Arg. Plots were essentially linear whether n_1 was chosen as 1, 2 or 3 when n_2 was chosen as 1. However, with $n_1=2$ the slope was 1.17 ± 0.16, while with n_1 chosen as 1 or 3, the slopes were 0.68 ± 0.1 and 1.71 ± 0.22, respectively, both significantly different from 1.0. On the other hand, if the plots were to use Log (DR-1) (*i.e.*, $n_1=1$) against n_2xLog (L-Arg), where n_2 was 1, 2 and 3, they were also essentially linear, but the slopes were 0.66 ± 0.14,

0.33±0.07 and 0.22±0.05, respectively. Thus, the data fitted a model in which 1 molecule (n_2) of L-Arg effectively competes with 2 molecules (n_1) of L-NAME. The calculated half-blocking infusion dose for L-Arg to antagonize the pressor response to L-NAME was at a rate of $1x10^{-6}$ mol/kg per min (with $n_1=2$).

Continuous i.v. infusions of L-Arg ($1.2x10^{-6}$ mol/kg per min) also shifted the dose-pressor response curve of D-NAME to the right without significantly changing E_{max} or n (Fig. 11 and Table 2).

In another study in Appendix II, the baseline MAPs in the 6 groups of conscious rats were similar and were summarized in Table 1 (Groups VI to XI) in Appendix II. Although continuous i.v. infusion of L-Arg ($4.8x10^{-6}$ mol/kg per min) and D-Arg ($4.8x10^{-6}$ mol/kg per min) did not alter MAP in rats (Table 1 in Appendix II), L-Arg significantly attenuated the pressor effects of L-NNA ($4x10^{-5}$ mol/kg, i.v. bolus injection) and D-NNA ($4x10^{-5}$ mol/kg, i.v. bolus injection) (Fig. 3 in Appendix II), but not the pressor effects of noradrenaline ($1.2x10^{-8}$ mol/kg, i.v. bolus injection) nor angiotensin II ($9.1x10^{-11}$ mol/kg, i.v. bolus injection) (Table 2 in Appendix II). D-Arg, however, altered neither the pressor effect of L-NNA or D-NNA (Fig. 3 in Appendix II), or that of noradrenaline or angiotensin II (Table 2 in Appendix II).

3.1.6.4. Effects of pithing and of pharmacological antagonists on the pressor and HR responses to NSAAs

Baseline MAPs and HRs in the two groups of pithed rats i.v. bolus injected with the vehicle and L-NAME were 48±3 and 50±4 mmHg, and 308±22 and 335±22 beats/min, respectively. I.V. bolus injection of cumulative doses ($1.5x10^{-6}$ to $4.8x10^{-5}$ mol/kg) of L-NAME, but not the vehicle, dose-dependently increased MAP, but not HR, in pithed rats, with E_{max} in MAP similar to those in the intact rats (Fig. 8 in Appendix IV, ref. Fig. 10A).

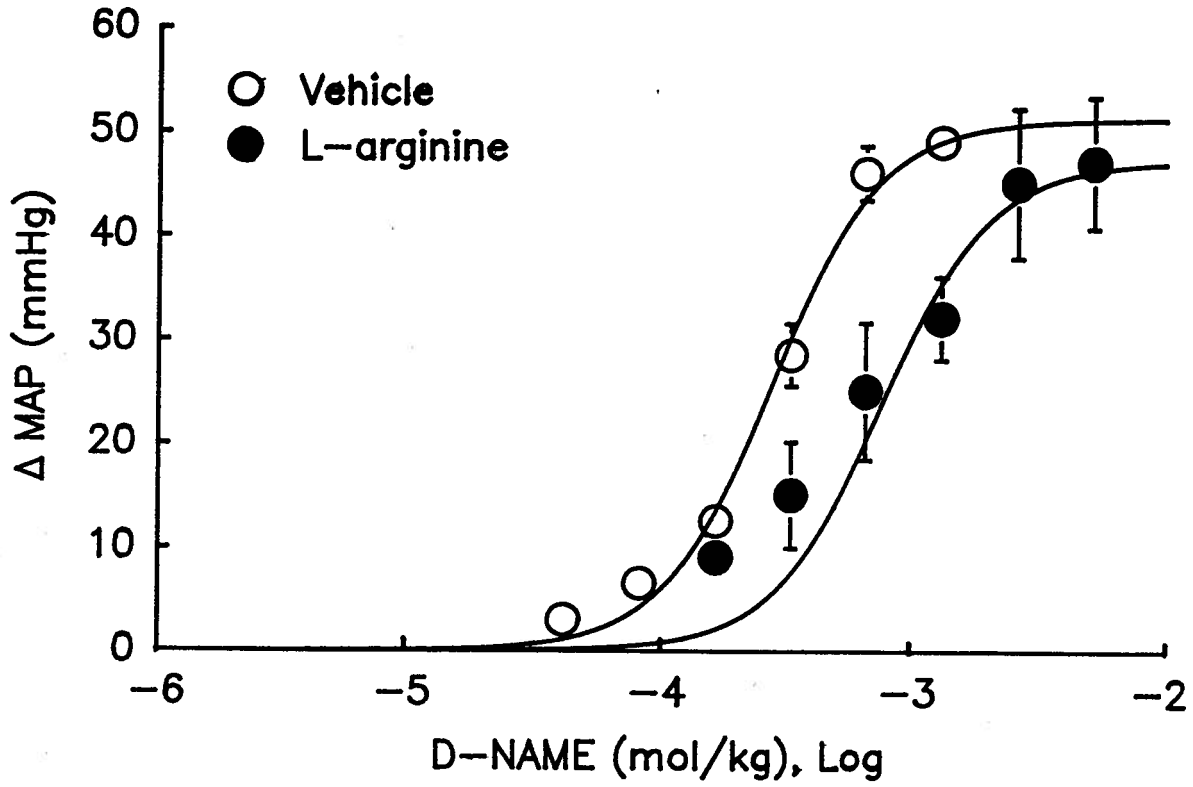

Fig. 11. Effect (mean ± S.E.) of continuous i.v. infusions of L-arginine (L-Arg) on the mean arterial pressure (MAP) response to i.v. bolus injections of cumulative doses of NG-nitro-D-arginine methyl ester (D-NAME) in conscious rats (N = 5 each group).

Fig. 1 in Appendix I showed the dose-pressor response curves of L-NNA in the conscious rats pretreated with the vehicle, phentolamine (9.4×10^{-7} mol/kg per min), propranolol (i.v. bolus injection at 3.4×10^{-6} mol/kg followed by an i.v. infusion at 5.4×10^{-9} mol/kg per min), reserpine (7.7×10^{-6} mol/kg, i.p. 26 h prior to the experiments), mecamylamine (i.v. bolus injection at 3.8×10^{-5} mol/kg followed by i.v. infusion at 1.5×10^{-6} mol/kg per min), atropine (i.v. bolus injection at 1.5×10^{-5} mol/kg followed by i.v. infusion at 1.2×10^{-8} mol/kg per min) and captopril (9.2×10^{-5} mol/kg, i.v. bolus injection), respectively. All the doses of the antagonists and inhibitor were shown to be effective in blocking their corresponding receptors or enzymes (Table 2 in Appendix I).

I.V. bolus injections of cumulative doses (5×10^{-6} to 1.6×10^{-4} mol/kg) of L-NNA in the vehicle-treated rats caused a dose-dependent increase in MAP (Fig. 1 in I), with n of 2.6 ± 0.2, ED_{50} of $2.1 \pm 0.4 \times 10^{-5}$ mol/kg and E_{max} of 52 ± 2 mmHg (Table 3 in Appendix I). Treatment with either mecamylamine or phentolamine markedly potentiated the pressor response to L-NNA with ED_{50}s of $9.5 \pm 1.0 \times 10^{-6}$ and $1.0 \pm 0.1 \times 10^{-5}$ mol/kg, and E_{max}s of 86 ± 5 and 87 ± 5 mmHg, respectively (Fig. 1 and Table 3 in Appendix I). The other antagonists, namely, atropine, propranolol, reserpine and captopril, did not significantly alter the dose-MAP response curves of L-NNA (Fig. 1 and Table 3 in Appendix I).

Fig. 2 in Appendix I showed the relationship between HR and MAP in these rats. In the vehicle-treated rats, the MAP effects of L-NNA was negatively correlated with its HR effect. Significant correlation of MAP with HR was also obtained in the rats pretreated with phentolamine, propranolol, atropine and captopril, but not with reserpine or mecamylamine. The slope of the curve was not altered by atropine, significantly decreased by propranolol, and increased by phentolamine and captopril. Intercept was decreased by propranolol and increased by phentolamine, atropine and captopril (Table 4 in Appendix I).

In the time course study in Appendix I, baseline MAPs and HRs of the rats pretreated with the vehicle, mecamylamine (i.v. bolus injection at 3.8×10^{-5} mol/kg followed by i.v. infusion at 1.5×10^{-6} mol/kg per min) and phentolamine (9.4×10^{-7} mol/kg per min) were summarized in Table 1 (Protocol 2) in Appendix I. The time course of an i.v. bolus injection of a single dose of L-NNA (1.6×10^{-4} mol/kg) on MAP and HR were shown in Fig. 4 in Appendix I. The MAP response had an average (geometric mean) half-rise phase of 4.8 min (95% confidence range of 2.0-11.6 min), which were similar to that of the previous time course study in Appendix II.

Treatments with both mecamylamine and phentolamine potentiated peak MAP response to L-NNA (Fig. 4A in Appendix I). Mecamylamine did not alter the average half-rise phase (geometric mean) of L-NNA (5.5 min and 95% confidence range of 3.2-9.4) but phentolamine reduced it (1.5 min and 95% confidence range of 1.0-2.3). Moreover, mecamylamine abolished the biphasic effects of L-NNA on HR; phentolamine, on the other hand, markedly potentiated L-NNA-induced bradycardia and abolished L-NNA-induced tachycardia (Fig. 4 in Appendix I).

In an unpublished study, MAPs at the pre-drug condition (112 ± 6 *vs* 109 ± 3 mmHg) and 20 min after the vehicle or indomethacin (112 ± 6 *vs* 110 ± 3 mmHg) in the two groups of conscious rats were not significantly different from each other. I.V. bolus injections of cumulative doses (2×10^{-6} to 6×10^{-5} mol/kg) of L-NAME caused dose-dependent pressor responses in the vehicle (1 ml/kg)-pretreated rats. Pretreatment (20 min earlier) with indomethacin (1.4×10^{-5} mol/kg, i.v. bolus injection) did not alter the dose-pressor response curve of L-NAME (Fig. 12).

3.1.6.5. Effects of L-NNA on plasma catecholamines

Baseline values of plasma catecholamines, MAP and HR were summarized in Table 4 in Appendix IV. Compared to the control group, L-NNA (8×10^{-5} mol/kg)

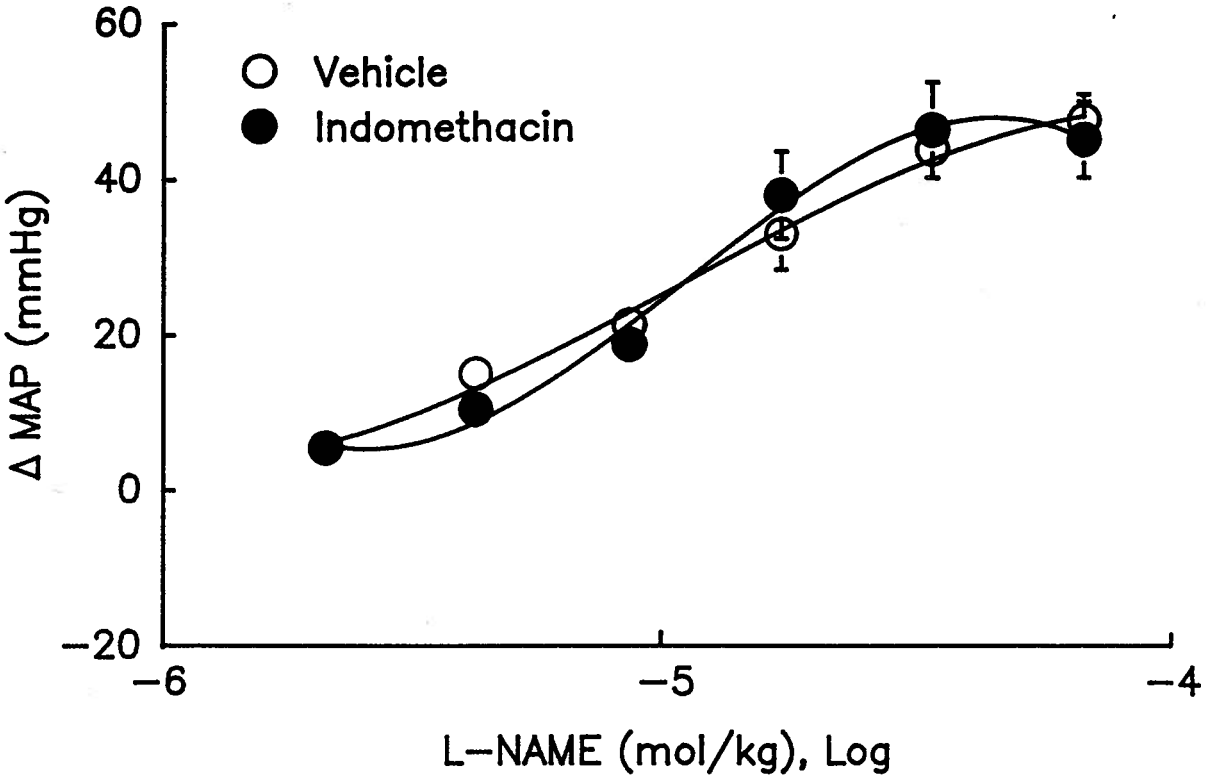

Fig. 12. Effect (mean ± S.E.) of i.v. bolus injection of indomethacin (1.4x10-5 mol/kg) on the mean arterial pressure (MAP) response to i.v. bolus injections of cumulative doses of N^G-nitro-L-arginine methyl ester (L-NAME) in conscious rats (N = 6 each group).

increased MAP, decreased HR and slightly decreased plasma dopamine, but did not alter plasma noradrenaline or adrenaline (Fig. 9 in Appendix IV).

3.2. Discussion

3.2.1. Effects of NSAAs on endothelium-dependent relaxation *in vitro*

Both L-NNA and L-NAME completely inhibit the relaxant responses to ACh, the calcium ionophore A 23187 and bradykinin, but not to SNP, in preconstricted aortic rings. The inhibitory effects of L-NNA and L-NAME are prevented completely by L-Arg but not by D-Arg. Our results are in accordance with those of Palmer *et al.* (1988b), which showed that cultured endothelial cells synthesized NO from a terminal guanido nitrogen atom of L-Arg but not D-Arg, and that L-Arg but not D-Arg produced endothelium-dependent relaxation of vascular rings. Our results suggest that the inhibitory effects of L-NNA and L-NAME are due to the inhibition of NO biosynthesis in endothelial cells of the aorta.

The inhibitory effects of L-NNA and L-NAME on ACh-induced relaxation are long-lasting (>4 h). The prolonged duration of inhibitory effects of L-NNA is also observed in the *ex vivo* studies, since the inhibitory effects of L-NNA on the vascular preparations were tested approximately 1.5 h after *in vivo* administrations of the drugs and after three washouts in baths. The long duration of action of L-NNA is consistent with the report that L-NNA caused prolonged inhibition of NO synthesis in cultured endothelial cells (Mülsch and Busse, 1990). Moreover, the pressor effect of L-NNA was prevented by pretreatment with L-Arg but not reversed by post-treatment with L-Arg (Wang and Pang, 1990a; Zambetis *et al.*, 1991). These observations raise a possibility that the inhibitory effects of L-NNA and D-NNA are irreversible. It has been reported that L-NIO is a long-lasting and irreversible NOS inhibitor in rat peritoneal neutrophils and murine macrophages,

since its effects were not reversed by L-Arg but was prevented by concomitant incubations of L-NIO with L-Arg (McCall *et al.*, 1991a). It was also reported that the inhibition of NOS by L-NAA was reversible initially but became irreversible with time (Rouhani *et al.*, 1992). However, the inhibitory effects of L-NNA and L-NAME 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 1.5 h with L-NNA and L-NAME. This also suggests that different mechanisms may be involved in the pressor and inhibition of ACh-induced relaxation by L-NNA and L-NAME.

We found that indomethacin does not affect the relaxant response to ACh. This is in accordance with studies showing that prostaglandins do not contribute to the effects of endothelium-derived relaxing factor or (EDRF)/NO (Furchgott and Zawadzki, 1980; see review by Lüscher and Vanhoutte, 1990a). On the other hand, it was recently reported that indomethacin, acetylsalicylic acid and SOD blocked the effects of L-NMMA on contraction, on ACh- as well as L-Arg-induced vasodilatations of pial arterioles and, on platelets adhesion/aggregation in mice *in vivo*. It was suggested that L-NMMA interfered with endothelium-dependent relaxation and it also produced constriction by activating cyclooxygenase and producing superoxide which subsequently inactivated EDRF/NO (Rosenblum *et al.*, 1992). Indomethacin is frequently added to the physiological solution in order to avoid a possible contribution by prostaglandins to endothelium-dependent relaxation (*e.g.*, Mülsch and Busse, 1990). However, our results show that indomethacin, at a concentration sufficient to inhibit prostaglandin synthesis, does not alter the inhibitory effects of L-NNA. These results suggest that cyclooxygenase activation and subsequent superoxide production leading to the inactivation of EDRF/NO are not involved in the inhibitory effects of L-NNA on ACh-induced relaxation.

3.2.2. Effects of NSAAs on endothelium-dependent vasodilatation *in vivo*

While it has been shown by many laboratories that ACh-induced relaxation *in vitro* is inhibited completely by NSAAs, there are uncertainties with respect to the efficacies of these compounds in suppressing ACh-induced vasodilatation *in vivo*. Inconsistent modifications (*e.g.*, inhibition, no effect or even potentiation) of ACh-induced depressor response by L-Arg analogues *in vivo* cast doubts upon the role of NO in ACh-induced vasodilatation in whole animals. The discrepancies in the effectiveness of NOS inhibitors in suppressing ACh-induced depressor response are unlikely related to the species of animals used or the conscious or anaesthetized state. Instead, the inconsistencies are most likely due to the comparison of ACh-elicited responses at different baseline MAPs (higher MAP caused by NSAAs), the different modes of administrations (bolus injection *vs* infusion) of ACh, as well as the way of expression of data. Vasodilator drugs are known to cause greater hypotension at higher baseline MAPs (Rees *et al.*, 1990b; van Gelderen *et al.*, 1991; Chyu *et al.*, 1992).

Therefore, PHE was used as a control for L-NAME (and D-NAME), and the depressor responses were calculated as % baseline MAP and modified half-recovery time, in order to eliminate difficulties associated with the comparison of responses at different baseline MAPs. Indeed, the depressor responses to ACh and SNP were potentiated by 140% and 95%, respectively, by PHE-induced hypertension, as compared to those in the vehicle-treated rats. With the use of appropriate controls, it was unequivocally clear that L-NAME (and D-NAME) interfered with the depressor response to ACh. This inhibition by L-NAME (or D-NAME) of vasodepressor response has also been seen for calcitonin gene-related peptide (Abdelrahman *et al.*, 1992a) and salbutamol (Wang *et al.*, 1993a). However, similar to the cases of calcitonin gene-related peptide and salbutamol, the depressor response to infused ACh was only partially (by 50%) suppressed by

L-NAME (and D-NAME). The lack of *in vivo* effectiveness of L-NAME (and D-NAME) in eliminating response to ACh is not due to insufficient doses, since a supramaximal pressor dose (4.8×10^{-5} mol/kg, 10 times ED_{50} for pressor response) of L-NAME was used. In our preliminary studies, even at a dose as high as 3.8×10^{-3} mol/kg, L-NAME still only partially inhibited ACh-induced depressor response in conscious rats (N=2, data not shown). Moreover, the incomplete inhibition of ACh-induced depressor response by L-NAME is unlikely due to the activation of nicotinic receptors by ACh, since ACh-induced activation of ganglionic nicotinic receptors are known to produce pressor response, rather than depressor response. In addition, we found that following effective ganglionic blockade with mecamylamine, L-NAME (3.8×10^{-4} mol/kg) still failed to completely inhibit ACh-induced fall in MAP in pentobarbitone-anaesthetized rats (N=6) (Wang *et al.*, unpublished data, 1993). Gardiner *et al.* (1990c) also reported similar findings. These results suggest that there may be a difference between ACh-induced responses in conduit vessels (*e.g.*, the aorta) and resistance blood vessels.

Further analysis of the depressor response to bolus injections of ACh showed that L-NAME decreased the duration, but not the magnitude, of the response to ACh by 26%, which is in contrast to the depressor response to salbutamol in which L-NAME reduced the magnitude but not the duration (Wang *et al.*, 1993a). The results that L-NAME reduced the duration but not the magnitude of the depressor response to ACh are in accordance with those of Aisaka *et al.* (1989b) using L-NMMA. Aisaka *et al.* (1989b) also showed that exogenous L-Arg prolonged the duration of the depressor response to ACh and suggested that the availability of L-Arg determined the duration of response to ACh. In contrast, we found that L-Arg, at a dose 48-fold its half-block dose to antagonize the pressor response to L-NAME, did not affect the duration but reduced the magnitude of the depressor response to the lower doses of bolus injected ACh. Van Gelderen *et al.*

(1991) also found that L-Arg did not reduce the duration of ACh-induced depressor response in anaesthetized rats. The mechanism by which L-Arg suppressed ACh-induced depressor response is not known but it is likely nonspecific, since the dose of L-Arg used was relatively high, and L-Arg also reduced the magnitudes of the depressor responses to the lower doses of SNP.

It is unclear why L-NAME shortened but L-Arg did not prolong the duration of the depressor response to bolus injected ACh. It has been suggested that the endogenous concentration of L-Arg is sufficient to saturate NOS (Rees *et al.*, 1989a). This hypothesis may explain why L-NAME but not L-Arg influenced the duration of the depressor response to ACh. An additional mechanism, besides NO biosynthesis, must be responsible for the establishment of the magnitude of the depressor response to bolus injected ACh, which was neither reduced by L-NAME nor prolonged by L-Arg. ACh is shown to release endothelium-derived hyperpolarizing factor (EDHF) in addition to endothelium-derived relaxing factor (EDRF)/NO (Chen *et al.*, 1988; Chen and Suzuki, 1990; see review by Suzuki and Chen, 1990). EDHF vasodilates some vascular preparations (Garland and McPherson, 1992) and may contribute to the depressor response to ACh *in vivo*. Consistent with this hypothesis, it was reported that the Ca^{2+}-activated K^{+}-channel (which leads to hyperpolarization) blocker charybdotoxin attenuated the depressor response to ACh in rats (Watkins *et al.*, 1993).

It is likely that L-NAME caused a redistribution of blood flow in rats. L-NNA (present study) and other NSAAs (Gardiner *et al.*, 1990b,d) have been shown to cause pressor response by increasing total peripheral resistance *via* systemic vasoconstriction but their degrees of influence vary with different beds. It was also shown that the renal, internal carotid, common carotid and mesenteric but not hindquarter vasodilator effects of ACh were partially attenuated by L-NAME (Gardiner *et al.*, 1990c, 1991). The varying ability of L-NAME to inhibit vasodilator response to ACh in different beds suggests that part of the vasodilator

response to ACh is mediated *via* mechanism insensitive to the inhibition of NO synthesis by L-NAME.

3.2.3. Stereospecificity of NSAAs *in vitro* and *in vivo*

It has been shown that L-NMMA (Palmer *et al.*, 1988b; Rees *et al.*, 1989a; Rees *et al.*, 1990b; Crawley *et al.*, 1990), L-NNA (Mülsch and Busse, 1990; Lamontagne *et al.*, 1991), L-NIO (Rees *et al.*, 1990b) and L-NAME (Rees *et al.*, 1990b), but not the corresponding D-enantiomers, inhibited endothelium-dependent relaxation of isolated blood vessels and/or NO biosynthesis in endothelial cells (see review by 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.*, 1991a), adrenal cortex (Palacios *et al.*, 1989) and non-vascular smooth muscles (Hobbs and Gibson, 1990; Tucker *et al.*, 1990). In contrast to these findings, our results indicate that both L-NNA and D-NNA, as well as L-NAME and D-NAME, efficaciously inhibit the relaxant response to ACh *in vitro* and *ex vivo*. Moreover, both L-NNA and D-NNA inhibit the relaxant responses to the calcium ionophore A 23187 and bradykinin. These results suggest that both L-NNA and D-NNA as well as both L-NAME and D-NAME inhibit endothelium-dependent relaxation induced by receptor- and non-receptor-operated mechanisms, and that the L-enantiomeric configuration is not required for the actions of NNA and NAME. As the inhibitory effects of D-NNA and D-NAME are also reversed by L-Arg, the results suggest that the actions of D-NNA and D-NAME are also involved in the inhibition of NO synthesis.

It could be argued that the effectiveness of D-NNA and D-NAME are due to contamination with L-NNA and L-NAME, respectively. However, there is no mistake about the identity of D-NNA, as an independent analysis determined that the specific rotations, $[\alpha]_D$, of D-NNA and L-NNA are -22.9^o and $+22.1^o$,

respectively (data shown in Appendix II). $[\alpha]_D$ of D-NNA from our independent analysis is consistent with the information ($[\alpha]_D$ of -23.6^o) provided by the supplier, Bachem Bioscienca Inc. (Philadelphia, PA, U.S.A.). Moreover, other observations also indicate that the biological activities of D-NNA or D-NAME are not the result of contamination by L-NNA or D-NAME. (1) D-NNA from another drug company (Aminotech Ltd., Ont., Canada) also exhibited 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-NNA and D-NNA as well as L-NAME and D-NAME (see below). (3) The onsets of the pressor effects of D-NNA and D-NAME are markedly slower than those of L-NNA and L-NAME.

The reasons for the discrepancy between our results and those of others are not apparent but may be related to differences in concentrations or doses of D-NNA and D-NAME 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 review by Ariëns, 1983). Since 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-NMMA and L-NMMA were used (Palmer *et al.*, 1988b), the concept of L-enantiomeric specificity for activating or inhibiting NOS has become widely accepted (see review by Moncada *et al.*, 1991). Due to the preconceived notion that D-enantiomers of NSAAs are inactive, systematic studies have been not conducted with these compounds. The doses or concentrations selected for D-enantiomers of NSAAs as controls were always (without exception) the same as those of the corresponding L-enantiomers. Moreover, conclusions were usually drawn without showing detailed data. Among the work cited in this dissertation,

only the experimental conditions in Mülsch and Busse's report (1990) are similar to ours. They found that L-NNA but not D-NNA (both at $3x10^{-5}$ mol/L) produced approximately 80% inhibition of ACh-induced relaxation in noradrenaline (EC_{60})-preconstricted rabbit femoral arteries. In our study, L-NNA ($3x10^{-5}$ mol/L, supramaximal dose) almost completely inhibited ACh-induced relaxation in rat aortae. Since the *in vitro* potency of D-NNA is approximately 1/39 that of L-NNA, it would be expected that D-NNA ($3x10^{-5}$ mol/L) should have caused considerably less response in the rabbit femoral arteries. The potencies of NSAAs are known to differ greatly according to particular preparations and chemical structures (see review by Moncada *et al.*, 1991). Therefore, the potencies of D-enantiomers would be expected to also vary with the preparations and types of NSAAs used. Indeed, we found that L-NNA is two-fold more potent than D-NNA in raising blood pressure and 39-fold more potent than D-NNA in inhibiting endothelium-dependent relaxation. L-NAME, on the other hand, is 52- and 337-fold more potent than D-NAME in raising blood pressure and inhibiting endothelium-dependent relaxation, respectively. Moreover, the pressor responses to D-NNA and D-NAME are substantially slower in onset than the corresponding L-enantiomers. This difference in the onsets between D- and L-enantiomers are accentuated in anaesthetized rats (Wang and Pang, 1990a). Therefore, it is reasonable to assume that incorrect conclusions cannot be avoided when either the concentrations/doses of NSAAs were insufficient or the observation time was not longer enough.

There are differences in the vasoconstrictor effects between L-NNA and D-NNA as well as L-NAME and D-NAME. Firstly, 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

inhibition of ACh-induced relaxation (Cocks and Angus, 1991). In the present study, $1x10^{-5}$ mol/L L-NNA produces maximal inhibition of ACh-induced relaxation but does not produce maximal 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.*, 1988b; Rees *et al.*, 1989a). In contrast, Cocks and Angus (1991) showed that the contractile response to L-NMMA in dog coronary arteries was not affected by pretreatment with haemoglobin or $FeSO_4$ in concentrations which inhibited the relaxations induced by SNP and NO, suggesting that the contractile response to 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-NNA but not D-NNA caused a slow and sustained contraction in endothelium-intact and denuded rat aortic rings; the effect was not affected by L-Arg (Wang and Pang, 1994b). In addition, L-NAME but D-NAME potentiated contraction induced by PHE. These results suggest that contraction and inhibition of the relaxant responses by NSAAs may be produced by different mechanisms.

Another difference between L- and D- enantiomers of NNA and NAME is potency. Although these four compounds have similar efficacies, D-NNA and D-NAME are less potent than L-NNA and L-NAME in inhibiting endothelium-dependent relaxation, suggesting that the vasoconstrictor effects of NNA and NAME prefer the L-enantiomeric configuration. Moreover, the differences in potencies between D-NNA and L-NNA as well as D-NAME and L-NAME *in vitro* are greater than those *in vivo*. The mechanism responsible for this discrepancies between the *in vitro* and *in vivo* potencies of D-NNA and L-NNA as well as D-NAME and L-NAME are not known. One possible explanation is chiral conversion.

A metabolic chiral inversion has been shown to occur after the oral administration of stereospecific drugs (Hutt and Caldwell, 1983; Sanins *et al.*, 1991). Since D-NNA and D-NAME are less potent and have slower onset of actions than L-NNA and L-NAME *in vivo*, D-NNA and D-NAME may act *via* metabolic conversion to L-NNA and L-NAME *in vivo*, respectively. Metabolic conversion may account for the differences in the activity ratios of D- and L- enantiomers of NNA and NAME in *in vivo* and *in vitro* settings.

3.2.4. Pharmacodynamic analyses of the vascular actions of NSAAs

A Hill coefficient (*n*) of 0.9 for L-NAME-induced inhibition of ACh-induced relaxation was derived from our *in vitro* results. This value is the same as that of L-NNA (0.9). These results suggest that one molecule of L-NAME or L-NNA competes with one molecule of endogenous L-Arg to inhibit NO biosynthesis. Our *in vivo* results, on the other hand, show that the *n* for L-NAME to cause pressor response is 2.0, and this value is not significantly affected by L-Arg which causes a rightward displacement of the dose-response curve of L-NAME. The *n* for L-NAME *in vivo* is also consistent with the *n* of 2.5 for L-NNA. These results imply that the presssor responses to L-NNA, L-NAME and D-NAME require the "positive cooperation" of probably 2 molecules of the compounds (see review by Rang, 1971; Pennefather and Quastel, 1982). For comparison, the *n* for the pressor effects of angiotensin II is 1.0 (unpublished calculation from Wang *et al.*, 1993b) and DPI is 3.3 in conscious rats (Results and discussion II).

The nature of the difference between *n* values for NSAAs obtained *in vivo* (pressor response) and *in vitro* (inhibition of vascular relaxation) is not known but may suggest that the mechanism involve in raising MAP (in resistance blood vessels) is different from that in inhibiting vascular relaxation (in large arteries). It is possible that L-NAME and L-NNA raise MAP by an unknown mechanism which,

although it is reversed by the administration of L-Arg, is different from the inhibition of endogenous endothelial NO synthesis. An alternative explanation is that there is a difference between stimulated NO synthesis (ACh-induced relaxation) and basal NO synthesis (endogenous dilator tone) (Chyu *et al.*, 1992). It was reported that the endothelium-dependent contractions elicited by L-NMMA and L-NNA in the dog coronary artery were not a consequence of the suppression of basal NO synthesis (Cocks and Angus, 1991). It was also reported that thimerosal, a acetyl-CoA lysolecithin acyltransferase inhibitor, blocked ACh-, substance P-, bradykinin- and A 23187-induced relaxations but did not suppress L-NNA-evoked contraction in the dog isolated coronary artery (Crack and Cocks, 1992). Moreover, although both L-NNA and D-NNA inhibited ACh-induced relaxation in aortic rings, only L-NNA elicited contraction and potentiated PHE-induced contraction (see above).

3.2.5. Mechanisms of the pressor responses to NSAAs

3.2.5.1. Pressor responses to NSAAs are due to vasoconstriction

Our results show that L-NNA increased MAP in conscious rats by elevating TPR, since both CO and HR were reduced. Reduced CO by L-NNA may be the resultant effects of reduced HR/cardiac contractility and increased flow resistance (TPR). The raised TPR is secondary to systemic vasoconstriction (reduced conductance) in all the beds. Humphries *et al.* (1991) reported that intravenous infusion of L-NNA in conscious rabbits raised MAP (by 11 mmHg) and TPR, reduced HR and CO, and caused significant vasoconstrictions in the brain, heart, kidneys, duodenum, but not in the muscle, skin, stomach, ileum or colon. The greater extent of vasoconstriction in response to L-NNA in our study is likely due to the use of a higher dose of L-NNA. Other NSAAs such as L-NMMA and L-

NAME were also reported to reduce CO and decrease renal, mesenteric, hindquarters or internal carotid blood flows in rats (Gardiner *et al.*, 1990a,b,c,d).

However, the extent of vasoconstriction in response to L-NNA, as revealed by % conductance changes, is not uniform; the greatest influence is in the lungs and the least is in the liver. The lungs receive circulations from the bronchial artery, the pulmonary artery and arteriovenous anastomoses. Counts in the lungs reflect primarily circulations from the bronchial artery and arteriovenous anastomoses since microspheres are virtually completely trapped within one circulation (Pang, unpublished observation, 1983). Likewise, due to the entrapping of microspheres in the splanchnic area, it is expected that liver blood flow represents primarily hepatic arterial flow. It has been postulated that the hepatic arterial flow is controlled by the hepatic arterial buffer response such that decreases in portal venous flows are associated with increases in hepatic arterial flows, thereby maintaining the constancy of total hepatic blood flows (Lautt, 1980). This hypothesis is in accordance with our findings that reduced splanchnic and consequently portal venous flows occurred concurrently with increased hepatic arterial flow. Therefore, the lesser vasoconstrictor effect of L-NNA (less reduced arterial conductance) in the hepatic bed may be due to the hepatic arterial buffer response. Moreover, L-NNA caused a marked coronary vasoconstriction in conscious rats. NSAAs have been shown to cause coronary vasoconstriction in conscious rabbits (Amezcua *et al.*, 1989) and dogs (Chu *et al.*, 1990), as well as a sustained increase in the rabbit coronary perfusion pressure *in vitro* (Palmer *et al.*, 1989). In contrast, Klabunde *et al.* (1991) reported that L-NMMA and L-NNA did not reduce HR or coronary flow in pentobarbitone-anaesthetized dogs. Their inability to show coronary constrictor effect of NOS inhibitors may be due to the influence of pentobarbitone.

3.2.5.2. Antagonism of L-Arg on the pressor responses to NSAAs

The pressor responses to L-NNA, D-NNA, L-NAME and D-NAME are prevented by L-Arg but not D-Arg. More detailed analysis shows that L-Arg dose-dependently shifted the dose-pressor response curves of L-NAME and D-NAME to the right without changing E_{max} or *n*. A modified Schild plot demonstrates that L-Arg competitively antagonizes the pressor response to L-NAME, with half-blocking dose at $1x10^{-6}$ mol/kg per min when *n* is chosen as 2. It should be pointed out that the antagonism by L-Arg of the pressor responses to NSAAs is specific as L-Arg does not modify the pressor effect to noradrenaline nor angiotensin II in conscious rats. These results are consistent with reports that L-Arg did not attenuate the pressor effects of noradrenaline nor angiotensin II in pentobarbitone-anaesthetized guinea pigs (Aisaka *et al.*, 1989a) nor vasopressin in conscious rats (Gardiner *et al.*, 1990b).

3.2.5.3. Effects of impairment of the central nervous, ganglionic, sympathetic, angiotensin or prostanoid systems on the pressor responses to NSAAs

It has been shown that pithing does not alter the pressor response to intravenous injection of L-NAME (Pegoraro *et al.*, 1992) and that intravenous injection of L-NMMA causes a pressor response in pithed rats (Tabrizchi and Triggle, 1992). On the other hand, spinal transection has been reported to attenuate the pressor response to intravenous injection of L-NMMA (Sakuma *et al.*, 1992; Togashi *et al.*, 1992). It is difficult to explain these controversial findings but our results, which showed that the intravenous injection of L-NAME into pithed rats caused a similar dose-pressor response to that in intact rats, suggest that the pressor responses to peripherally administered NSAAs are not dependent on the integrity of the central nervous system. However, our results do not exclude the possibility that central NO biosynthesis modulates sympathetic nerve activity and subsequently blood pressure and heart rate, as suggested by many

authors (Sakuma *et al.*, 1992; Togashi *et al.*, 1992; Mollace *et al.*, 1992; Harada *et al.* 1993).

We have found that the ganglion blocker mecamylamine does not inhibit, but instead potentiates, the pressor response to L-NNA in conscious rats. Moreover, mecamylamine did not inhibit the pressor response to L-NAME in conscious rats (Wang and Pang, unpublished data, 1993). These results are supported by those of other investigators using pentolinium or chlorisondamine (Chyu *et al.*, 1992; Pucci *et al.*, 1992; Pegoraro *et al.*, 1992). However, there are reports which showed that chlorisondamine, pentolinium or hexamethonium abolished or attenuated the pressor response to L-NMMA or L-NNA in anaesthetized rats or dogs (Vargas *et al.*, 1990; Lacolley *et al.*, 1991; Toda *et al.*, 1993), and suggested that there were nitroxidergic vasodilator nerves innervating the arterial wall (Toda *et al.*, 1993). The difference in these findings also cannot be explained. Nevertheless, the dose-response curves of our results performed in conscious animals suggest that ganglionic transmission is not a prerequisite for the pressor responses to NSAAs. It should be pointed out that potentiation of the effects of NSAAs by ganglion blockers is not because of the lower baseline MAP, since rats treated with pentolinium and PHE to maintain similar baseline MAP as those in the control rats also had larger pressor responses to L-NAME than the controls. The potentiation may suggest that the pressor response to L-NNA in the control rats were limited by hypertension-induced reflex withdrawal of sympathetic tone to the vasculature (Pucci *et al.*, 1992).

Neither the blockers of the autonomic nervous system (phentolamine, reserpine, propranolol and atropine), nor the angiotensin converting enzyme inhibitor captopril attenuated the pressor effects of L-NNA or L-NAME. These results are supported by many reports (Rees *et al.*, 1989b; Aisaka *et al.*, 1989a; Pucci *et al.*, 1992; Widdop *et al.*, 1992; Toda *et al.*, 1993). Also, the prostaglandin synthesis inhibitor indomethacin does not alter the dose-pressor

response curve to L-NNA or L-NAME. The results of indomethacin are in accordance with reports which showed that the pressor response to L-NMMA or L-NAME was not attenuated by indomethacin in anaesthetized rats (Rees *et al.*, 1989b; Tepperman and Whittle, 1992). However, indomethacin was reported to block the pressor but not the systemic vasoconstrictor or cardiac depression response to L-NMMA (Klabunde *et al.*, 1991). It should be pointed out that the pressor response in the latter study was small (10 mmHg). Our results suggest that the acute pressor responses to NSAAs do not rely on the integrity of the sympathetic, parasympathetic, angiotensin or prostanoid system. Our conclusion does not exclude the possibility that the renin-aniotensin system may modulate the hypertension caused by chronic administration of NSAAs (Ribeiro *et al.*, 1992; Polakowski *et al.*, 1993). However, it should be noted that captopril and other angiotensin system inhibitors or antagonists also prevent the development of many kinds of hypertensions, such as in spontaneously hypertensive rats (Jonsson *et al.*, 1991; Chillon *et al.*, 1992; Wu and Berecek, 1993) and in rats harboring pheochromocytoma (Hu *et al.*, 1990). Therefore, the exact mechanism by which the inhibitors of the renin-angiotensin system prevent hypertension caused by chronic administration of NSAAs should be further studied.

3.2.6. Mechanisms of the HR responses to NSAAs

The bradycardiac response to L-NNA was abolished by the ganglion blockade while that to L-NAME was abolished by pithing. These results, which are also supported by another study in which mecamylamine also abolished the bradycardiac response to L-NAME (Wang and Pang, unpublished data, 1993), suggest that the bradycardiac responses to NSAAs are reflex-mediated. It is of special interest that reserpine but not atropine reduced the bradycardia. The

results suggest that inhibition of the sympathetic nerve activity rather than potentiation of the parasympathetic nerve activity is responsible for the reflex-mediated HR response to elevation in MAP. These results are consistent with the observation that the bradycardia caused by L-NMMA was associated with reduced renal sympathetic nerve activity (Sakuma *et al.*, 1992). Our results also showed that phentolamine increased the slope of the curve. This suggests that the enhanced reflex bradycardiac activities may be a consequence of the elevated background sympathetic nerve activity in the presence of phentolamine. Indeed, HR was elevated after treatment with phentolamine. Phentolamine has been shown to markedly elevate plasma levels of adrenaline and noradrenaline suggesting that it increases activities of the sympathetic nervous system (Tabrizchi *et al.*, 1988). However, it was reported that the bradycardia caused by L-NMMA in pentobarbitone-anaesthetized guinea pigs (Aisaka *et al.*, 1989a) and the bradycardia caused by L-NAME in conscious, Long Evans rats (Widdop *et al.*, 1992) were blocked by atropine. The difference between our results and other's may be due to varying sympathetic and/or parasympathetic activities of the experimental animals.

L-NNA also caused a tachycardiac response following a bradycardiac phase. Biphasic HR responses to L-NNA and L-NMMA were also observed in pentobarbitone-anaesthetized rats (Wang and Pang, 1990b), and chloralose/urethane-anaesthetized rats (Sakuma *et al.*, 1992). The inability to see the tachycardiac component of the HR response in rats given cumulative doses of L-NNA may be due to the shorter observation period (15 to 20 min). We found that mecamylamine also abolished the tachycardiac response to L-NNA. Sakuma *et al.* (1992) reported that the biphase HR responses to L-NMMA were associated with the biphase renal sympathetic nerve responses. Therefore, the tachycardiac responses to NSAAs may be due to the activation of the sympathetic nervous system.

3.3. Summary

1. L-NNA and D-NNA, as well as L-NAME and D-NAME, cause efficacious, long-lasting and reversible inhibition of endothelium-dependent relaxations *in vitro* and *ex vivo*. The inhibitory effects of NSAAs are antagonized by L-Arg, but not by D-Arg, indomethacin or NADPH. These results suggest that the inhibitory effects of NSAAs are due to the inhibition of NO biosynthesis by antagonizing the substrate L-Arg. The L-enantiomer form of NSAAs is a preferred but not essential configuration required to inhibit endothelium-dependent relaxation. Since the ratios of the potencies of L-NNA *vs* D-NNA as well as L-NAME *vs* D-NAME are greater in *in vitro* than *in vivo* settings and since D-NNA and D-NAME are slower in onset of action than L-NNA and L-NAME, respectively, it is possible that the D-enantiomers of NNA and NAME are converted to the corresponding L-enantiomers *in vivo*.

2. L-NNA and D-NNA, as well as L-NAME and D-NAME cause slow-developing and long-lasting pressor and bradycardiac responses in conscious rats, as a consequence of generalized vasoconstriction of all beds. The bradycardiac responses to NSAAs are baroreflex-mediated. The pressor responses to NSAAs are not dependent on the integrity of the central/autonomic nervous, angiotensin and prostaglandin systems. L-Arg, but not D-Arg, competitively antagonizes the pressor responses to NSAAs. These results suggest that the *in vivo* vasoconstriction elicited by NSAAs is due to a mechanism which is related to the L-Arg pathway.

3. In contrast to the effectiveness in inhibiting relaxation *in vitro,* L-NNA and L-NAME do not entirely block ACh-induced depressor responses *in vivo*. The *n*s for L-NNA and L-NAME to inhibit endothelium-dependent relaxation are 1 *in vitro* and the *n*s for L-NNA and L-NAME to produce pressor response are 2 or more *in vivo*. Our results appear to be consistent with the hypothesis that the pressor responses

to NSAAs result from the inhibition of NOS and suppression of endothelial basal NO synthesis only if (1) NSAAs blocks basal NO synthesis more effectively than stimulated NO synthesis, and (2) stimulated NO synthesis is inhibited by NSAAs in a one to one antagonistic manner (NSAAs *vs* L-Arg) while basal NO synthesis is inhibited by NSAAs in a two (or more) to one antagonistic manner (NSAAs *vs* L-Arg).

4. Therefore, the mechanisms responsible for NSAAs to cause inhibition of endothelium-dependent relaxation and pressor responses may be different, although both effects of NSAAs are antagonized by L-Arg. Either one of the following explanations are consistent with our observations: (1) pressor response to NSAAs is due to the activation of an "L-Arg receptor" with signal transduction different from NO biosynthesis; (2) the inhibition of NO biosynthesis alone is not sufficient to cause vasoconstriction *in vivo*.

5. It is obvious that the commonly used NOS inhibitors (*i.e.*, NSAAs) which have structures related to Arg cannot be used to provide further information on the question - whether the inhibition of NOS leads to the elevation of blood pressure. Hence, use of other NOS inhibitors (*e.g.*, DPI) with different chemical structures and mechanism of action would be of value.

4. Results and discussion II

4.1. Results

4.1.1. Effects of DPI on endothelium-dependent relaxation *in vitro*

4.1.1.1. Concentration-responses

All five concentrations of DPI (3x10-8, 1x10-7, 3x10-7, 1x10-6 and 3x10-6 mol/L) slightly potentiated PHE-induced contraction from the baseline value of 0.99±0.10 g to 1.09±0.15, 1.27±0.11, 1.32±0.10, 1.31±0.10 and 1.18±0.14 g, respectively. However, only the effects of the third and fourth doses of DPI were statistically significant ($P<0.05$).

In the vehicle-treated group, ACh (1x10-8 to 3x10-5 mol/L) concentration-dependently relaxed the preconstricted aorta with maximal relaxation of approximately 60% (Fig. 1A in Appendix VII). DPI concentration-dependently and "noncompetitively" inhibited ACh-induced relaxation. At 3x10-5 mol/L ACh, the *n*, IC50 and E_{max} of DPI were 1.4, 1.8x10-7 mol/L and 92%, respectively (Fig. 1B in Appendix VII).

In another two vehicle-treated groups, A 23187 (1x10-9 to 3x10-6 mol/L) and SNP (3x10-10 to 1x10-7 mol/L) also concentration-dependently relaxed the preconstricted aorta, with maximal relaxations of approximately 60% and 100%, respectively (Fig. 2 in Appendix VII). DPI (3x10-6 mol/L) completely inhibited A 23187-induced relaxation but did not affect the relaxant response to SNP.

4.1.1.2. Mechanisms

Baseline contractions elicited by PHE in the presence of the vehicle or DPI (3x10-7 mol/L) were 1.29±0.07 and 1.67±0.12 g, respectively. In 10 different groups of aortae, treatments with NADPH (1.5 and 5x10-3 mol/L), FAD (5x10-4 and 5x10-6 mol/L) and L-Arg (2x10-3 mol/L) did not significantly affect PHE-induced contractions in the presence of either the vehicle (1.04±0.06, 1.04±0.11, 1.16±0.06, 1.33±0.11, 1.23±0.08 g, respectively) or DPI (1.43±0.10, 1.37±0.14, 1.59±0.06, 1.52±0.13, 1.44±0.04 g, respectively).

DPI inhibited ACh-induced relaxation (Fig. 3A in Appendix VII). Treatment with L-Arg did not affect either ACh-induced relaxation or the inhibitory effect of DPI on ACh-induced relaxation (Fig. 3B in Appendix VII). Although the lower concentration (5x10-6 mol/L) of FAD also did not alter either ACh-induced relaxation or the inhibitory effect of DPI on ACh, the higher concentration (3x10-4 mol/L) of FAD suppressed the relaxant effect of ACh and prevented further inhibition by DPI of ACh-induced relaxation (Fig. 3C in Appendix VII). Although both concentrations of NADPH did not significantly affect ACh-induced relaxation, the higher concentration (5x10-3 mol/L) but not the lower concentration (1.5x10-3 mol/L) of NADPH completely prevented the inhibitory effect of DPI (Fig. 3D in Appendix VII). The effectiveness of pretreatment with NADPH (5x10-3 mol/L) in inhibiting the effect of DPI, expressed as the ratio of the relaxant effect of 1x10-5 mol/L ACh in the presence of NADPH (67%) to that in the absence of NADPH (32%), was 209%.

4.1.1.3. Time courses and reversibility

The PHE-induced contractions in the presence of the vehicle or DPI did not change with passage of time (data not shown). ACh-induced maximal relaxation was not altered until at least 4 h after washouts. Maximal relaxation at 9 h was 48±7%, which was significantly less than that (69±6%) at 0 h (Fig. 5 in

Appendix VII). DPI at 3x10-7 and 3x10-6 mol/L inhibited ACh-induced relaxation by approximately 50 and 100%, respectively (Fig. 5A in Appendix VII). The inhibitory effect of DPI remained at least 4 h after washouts (Fig. 5B, 5C in Appendix VII). At 9 h after washouts, the relaxation of DPI-preconstricted rings was still less, though insignificantly, than those of the vehicle-pretreated rings (Fig. 5D in Appendix VII).

Maximal relaxation of ACh after 1.5 h exposure to 3x10-7 mol/L DPI (38.3±3.1%, Fig. 6 in Appendix VII) was similar to that after a 10 min exposure to DPI (32.2±4.8%, Fig. 3A in Appendix VII). Post-treatment (1.5 h later) with NADPH (5x10-3 mol/L) significantly but slightly suppressed the inhibitory effect of DPI. The effectiveness of post-treatment with NADPH (5x10-3 mol/L) in inhibiting the effect of DPI, expressed as a ratio of the relaxant effect of 1x10-5 mol/L ACh in the presence of NADPH (51%) to that in the absence of NADPH (38%), was 134% (Fig. 6 in Appendix VII).

4.1.2. Effects of DPI on the depressor responses to ACh and SNP *in vivo*

In conscious rats, i.v. bolus injection of DPI caused an immediate and transient pressor response (see later) and did not cause additional pressor responses during 2 h observation (Fig. 7 in Appendix VII). Baseline MAPs of the conscious rats before and 20 min after treatment with the vehicle were 109±2 and 112±3 mmHg, respectively, which were similar to those (118±5 and 114±5 mmHg) of DPI (1x10-5 mol/kg)-treated rats (1x10-5 mol/kg, i.v. bolus injection). I.V. infusions of ACh (6x10-8 to 1.8x10-6 mol/kg per min, each dose for 4 min) and SNP (3x10-8 to 4.8x10-7 mol/kg per min, each dose for 4 min) caused dose-dependent depressor responses. Pretreatment with DPI significantly attenuated the depressor response to ACh but not to SNP (Fig. 8 in Appendix VII).

4.1.3. Pressor and tachycardiac responses to DPI

I.V. bolus injections of the vehicle did not alter MAP or HR in pentobarbitone-anaesthetized rats (data not shown). I.V. bolus injections of DPI ($1.5x10^{-7}$ to $5x10^{-6}$ mol/kg) caused immediate and transient pressor and tachycardiac responses as shown in a typical experimental tracing from a rat (Fig. 2 in Appendix IV). The duration of the pressor response lasted approximately 1-2 min while that of tachycardiac response was slightly longer (Fig. 2 in Appendix IV). At $2.5x10^{-6}$ mol/kg, the half-rise time for the pressor and tachycardiac responses were 2.9 ± 0.2 s and 4.9 ± 0.7 s ($P < 0.05$) while the corresponding half-fall time were 31 ± 3 s and 60 ± 9 s ($P < 0.05$), respectively.

Pooled ($N = 12$) baseline MAP and HR from the above group and the control group in another protocol to be described later were 104 ± 3 mmHg and 347 ± 10 beats/min, respectively. The pressor and tachycardiac response curves of DPI were dose-dependent and notably "steep", with negligible effect at 1.5 and $3x10^{-7}$ mol/kg, large increases in MAP at $6x10^{-7}$ and $1.2x10^{-6}$ mol/kg and maximal effect at $2.5x10^{-6}$ mol/kg (Fig. 3 in Appendix IV). The best-fitted *n* for MAP and HR were 3.6 ± 0.3 and 4.2 ± 0.6, respectively. These two values were not significantly different from each other or from 4, but different from 1, 2, 3 and 5, though all values of *n*, best-fitted (3.6, 4.2) or selected (1, 2, 3, 4 and 5), were statistically significant ($P < 0.05$). The theoretical dose-response curves for integral values of *n* are shown in Fig. 3 in Appendix IV. E_{min} and E_{max} calculated from dose-MAP and dose-HR curves at the best-fitted *n* were not significantly different from those observed; other values of *n* gave significant differences of calculated parameters from those observed (E_{min} and E_{max}) or derived by averaging parameters obtained using best-fitted *n* for each dose-response curve (Table 1 in Appendix IV). Correlation between observed data points and

theoretical curves, expressed as 1000x(1-r), was the greatest when $n=3$ and 4, and the least when $n=1$.

4.1.4. Mechanisms of the pressor and tachycardiac responses to DPI

4.1.4.1. Effects of pharmacological antagonists on the pressor and tachycardiac responses to DPI

Compared to the vehicle (1 ml/kg), treatments with mecamylamine (3.8×10^{-5} mol/kg) and reserpine (7.7×10^{-6} mol/kg, i.p. 26 h earlier) reduced both MAP and HR; phentolamine (3.2×10^{-5} mol/kg), propranolol (3.4×10^{-6} mol/kg) and captopril (9.2×10^{-5} mol/kg) reduced MAP but did not affect HR while guanethidine (2.0×10^{-5}mol/kg) did not alter MAP but increased HR. On the other hand, atropine (1.5×10^{-5} mol/kg), prazosin (2.4×10^{-6} mol/kg), rauwolscine (2.6×10^{-6} mol/kg) and L-Arg (1.9×10^{-3} mol/kg) did not alter either MAP or HR (Table 2 in Appendix IV).

The dose-MAP and dose-HR response curves of DPI (1.5×10^{-7} to 5×10^{-6} mol/kg) in the presence of the vehicle or the antagonists, and the corresponding ED_{50}s and E_{max}s at the best-fitted *n*s were shown in Figs. 4-6 and Table 3 in Appendix IV, respectively. Reserpine, guanethidine and mecamylamine attenuated the MAP as well as HR responses to DPI (Fig. 4 in Appendix IV) with either a decrease in E_{max} or an increase in ED_{50} of DPI (Table 3 in Appendix IV). Phentolamine and prazosin, but not rauwolscine, reduced the MAP response by decreasing E_{max} of DPI. The HR response and the corresponding E_{max}, on the other hand, were increased by phentolamine and rauwolscine but not prazosin (Fig. 5 and Table 3 in Appendix IV). Propranolol abolished the HR response with a decrease in E_{max}, but potentiated the MAP response to DPI with an increase in E_{max} (Fig. 5 and Table 3 in Appendix IV). Atropine potentiated both the MAP (by decreasing ED_{50} and increasing in E_{max}) and HR (by decreasing ED_{50}) responses

to DPI (Fig. 6 and Table 3 in Appendix IV). Captopril markedly potentiated the MAP (by increasing E_{max}) but not HR response while L-Arg did not affect either the MAP or HR response to DPI (Fig. 6 and Table 3 in Appendix IV).

4.1.4.2. Effects of TTX, pithing or spinal cord (T_1) transection on the pressor and tachycardiac responses to DPI

Baseline MAPs in TTX (3.1×10^{-8} mol/kg)-pretreated rats (53 ± 2 mmHg), pithed rats (45 ± 2 mmHg) and spinal cord-transected (T_1) rats (52 ± 2 mmHg) were lower than those of the ventilated control rats (105 ± 5 mmHg). Baseline HRs in TTX-pretreated rats (318 ± 9 beats/min) and pithed rats (333 ± 10 beats/min) were similar to, while those of spinal cord-transected rats (412 ± 16 beats/min) were higher than those of the ventilated control rats (312 ± 10 beats/min). DPI (1.5×10^{-7} to 5×10^{-6} mol/kg) also caused dose-dependent pressor and tachycardiac responses in the ventilated control rats; pretreatment with TTX and pithing totally abolished both the MAP and HR responses to DPI (Fig. 7 in Appendix IV). However, noradrenaline (3.9×10^{-8} mol/kg) still caused increases in MAP and HR in TTX-pretreated and pithed rats; their increases were 109 ± 1 and 44 ± 2 mmHg, and 92 ± 6 and 57 ± 6 beats/min, respectively. On the other hand, spinal cord transection (T_1) markedly suppressed the dose-MAP and dose-HR responses to DPI compared to the responses in the intact rats; the suppression by spinal cord transection, however, was less than that by pithing (Fig. 7 in Appendix IV).

4.1.4.3. Effects of DPI on plasma catecholamines in intact, pithed or reserpinized rats.

Baseline levels of plasma catecholamines were similar among the two groups of intact rats to be treated with the vehicle and DPI (Table 4 in Appendix IV). Compared to the pooled data, pithing did not alter circulating catecholamines. Pretreatment with reserpine significantly decreased plasma noradrenaline but increased plasma adrenaline.

Compared to the vehicle, DPI ($5x10^{-6}$ mol/kg) caused large increases in plasma noradrenaline and adrenaline (more than 1 ng/ml), and moderate increase in plasma dopamine (more than 0.1 ng/ml), as well as increases in MAP and HR (Fig. 9 in Appendix IV). The increases in MAP and HR were significantly greater than those caused by the same dose of DPI in the multiple dose regimen (70 ± 2 *vs* 53 ± 5 mmHg and 123 ± 7 *vs* 43 ± 5 beats/min, respectively). Reserpine markedly reduced DPI-induced increases in plasma noradrenaline, adrenaline and dopamine by 91%, 93% and 74%, respectively, and attenuated the pressor and tachycardiac responses by 56% and 68%, respectively (Fig. 10 in Appendix IV). In reserpinized rats, the pressor and tachycardiac responses to a single dose of DPI were also greater than those of the multiple dose regimen (31 ± 10 *vs* 18 ± 1 mmHg and 39 ± 10 *vs* 22 ± 1 beats/min, respectively). Pithing totally abolished the effects of DPI on plasma catecholamines, as well as on MAP and HR (Fig. 10 in Appendix IV).

Concentration-response and linear regression models were used to examine the relationships between plasma noradrenaline or adrenaline and MAP or HR in intact, pithed and reserpinized rats i.v. bolus injected with DPI or the vehicle ($N = 25$). The linear regression model gave significant correlation between plasma noradrenaline and MAP ($r = 0.83$) or HR ($r = 0.87$), as well as between adrenaline and MAP ($r = 0.78$) or HR ($r = 0.81$). The concentration-response model, however, gave better fits. Fig. 11 and 12 in Appendix IV showed the concentration-response relationships between individual plasma noradrenaline or adrenaline and MAP or HR response caused by DPI. Correlation coefficient between plasma

noradrenaline and MAP or HR were 0.97 or 0.96, respectively; correlation coefficient between plasma adrenaline and MAP or HR were 0.94 or 0.94, respectively.

4.1.3. Inhibitory effect of halothane on the pressor response to DPI

In conscious rats, DPI caused an immediate (approximately 15 s in onset) and transient (1-2 min in duration) pressor response, similar to those in pentobarbitone-anaesthetized rats. The pressor response was dose-dependent (Fig. 1 in Appendix V), with ED_{50} of $2.2 \pm 0.3 \times 10^{-7}$ mol/kg and maximal MAP reached at 59 ± 2 mmHg, based on the best-fitted calculations (Table 1 in Appendix V). Hill coefficient (n) of 3.3 ± 0.5 was significantly different from 1, 2 and 5 but not from 3 or 4. DPI also caused tachycardia at the lower doses (7.5×10^{-8} to 3×10^{-7} mol/kg), bradycardia at higher doses (6×10^{-7} to 5×10^{-6} mol/kg) and movements following the onset of the pressor response (data not shown).

Halothane (0.5-1.25%) reduced baseline MAP in a dose-dependent manner (Table 1 in Appendix V). Halothane dose-dependently reduced the maximal effect of DPI and shifted the dose-pressor response curve of DPI to the right (Fig. 2 in Appendix V). ED_{50}s were linearly correlated while E_{max}s were inversely correlated with the doses of halothane (Fig. 3 in Appendix V). None of the doses of halothane affected the *n* of the curves (Table 1 in Appendix V). Halothane also inhibited DPI-induced tachycardiac and bradycardiac responses, as well as movements (data not shown).

Baseline MAP in halothane (1.25%)-anaesthetized rats was lower than that in conscious rats (Table 2 in Appendix V). Baseline plasma level of adrenaline but not noradrenaline or dopamine in halothane-anaesthetized rats was also significantly lower than that in conscious rats (Table 2 in Appendix V). In conscious rats, i.v. bolus injection of DPI (5×10^{-6} mol/kg) caused immediate and

large increases (more than 1 ng/ml) in plasma noradrenaline and adrenaline and a smaller increase (0.1 ng/ml) in plasma dopamine (Fig. 4A in Appendix), as well as immediate pressor response (Fig. 4B in Appendix V). Halothane markedly attenuated DPI-induced increases in plasma catecholamines (Fig. 4A in Appendix V) and in MAP (Fig. 4B in Appendix V); the reductions of plasma noradrenaline, adrenaline and MAP were 86%, 81% and 95%, respectively.

4.2. Discussion

4.2.1. Inhibitory effects of DPI on endothelium-dependent vasodilatation *in vitro* and *in vivo,* and their mechanisms

Our *in vitro* results show that DPI selectively and completely inhibits endothelium-dependent relaxation induced by receptor-mediated (ACh) or non-receptor-mediated (A 23187) mechanisms. These results are consistent with the report that DPI inhibits ACh-induced relaxation in the rabbit and rat aortae (Stuehr *et al.*, 1991b; Rand and Li, 1993). DPI also attenuates ACh- but not SNP-induced decreases in MAP in conscious rats, and ACh- but not SNP-induced vasodilatation in the perfused rat hindquarter and mesenteric preparations (Wang *et al.*, 1993; unpublished data, 1993). These results suggest that DPI inhibits endothelium-dependent vasodilatation in both conductance and resistance blood vessels. Therefore, the *in vitro* inhibitory effects of DPI on endothelium-dependent vasodilatation are similar to those of NSAAs. These results are in accordance with the hypothesis that the inhibition of NO synthesis suppresses endothelium-dependent vasodilatation.

It has been known since 1973 that DPI suppresses the oxidation of NADH-like substrates thereby inhibiting mitochondrial oxidation (Holland *et al.*, 1973). It was later shown that DPI inhibits NADPH-dependent oxidase of neutrophils and

macrophages (Cross and Jones, 1986; Hancock and Jones, 1987; Ellis *et al.*, 1988, 1989), and macrophage NOS (Stuehr *et al.*, 1991b), by specifically binding to and inhibiting the action of a plasma membrane polypeptide which may be a component of flavoprotein (Cross and John, 1986; Hancock and Jones, 1987; Ellis *et al.*, 1989). This suggests that flavin is the site of attack by DPI and that a protein is associated with FAD (O'Donnell *et al.*, 1993). Isoenzymes of NOS are known to be flavoproteins which contain FAD as a cofactor in the endothelial cells (Marsden *et al.*, 1992), macrophage (Stuehr *et al.*, 1989b, 1990, 1991a,b; Hevel. *et al.*, 1991; White and Marletta, 1992), neutrophil (Yui *et al.*, 1991), brain (Mayer *et al.*, 1991; Lowenstein *et al.*, 1992; Bredt *et al.*, 1991, 1992; Hiki *et al.*, 1992) and liver (Evans *et al.*, 1992). There is, however, no functional documentation of a role of FAD as a cofactor of eNOS. Our *in vitro* results demonstrate that FAD interferes with both ACh-induced relaxation and the inhibitory effect of DPI on ACh-induced relaxation. The latter result, which is consistent with Stuehr *et al.*'s report (1991b) that FAD antagonizes the inhibitory effect of DPI on macrophage NO synthesis, suggests that FAD and DPI may inhibit endothelial NO synthesis by a mechanism similar to that in macrophages. The former result is puzzling, since as a cofactor, FAD should facilitate rather than interfere with endothelium-dependent relaxation. FAD was indeed reported to facilitate macrophage NO synthesis (Stuehr *et al.*, 1990; Hevel. *et al.*, 1991). The mechanism by which FAD inhibits ACh-induced relaxation is not clear at the moment, however, the effect may not be specific as FAD also inhibits SNP-induced relaxation (Wang and Pang, unpublished observation, 1993).

Our *in vitro* results also show that the inhibitory effect of DPI is not affected by L-Arg, at a concentration found to reverse the inhibitory effects of L-NNA and L-NAME on endothelium-dependent relaxation in aortic rings. The results are in accordance with those of Stuehr *et al.* (1991b), in which L-Arg did not prevent the inhibitory effect of DPI in NO biosynthesis in macrophages, and with those of Rand

and Li (1993), in which L-Arg did not attenuate the inhibitory effect of DPI on endothelium-dependent relaxation in the isolated rabbit aorta. Moreover, NADPH interferes with the inhibitory effect of DPI on ACh-induced relaxation. The antagonism of DPI by NADPH is specific since the same concentration of NADPH does not alter the inhibitory effect of L-NNA. Our functional results with NADPH and DPI are consistent with those which show that both the constitutive (*e.g.*, brain and endothelial) and inducible (*e.g.*, macrophage and smooth muscle) NOS are dependent on NADPH as an essential cofactor (Mayer *et al.*, 1989; Stuehr *et al.*, 1989b, 1990, 1991a,b; see review by McCall and Vallance, 1992; Marsden *et al.*, 1992; see review by Dinerman *et al.*, 1993). These results suggest that the mechanism for the inhibitory effects of DPI and NSAAs are rather different. Regarding the nature of the interaction between NADPH and FAD, it has been suggested that NADPH suppresses the binding of DPI to the flavoprotein in neutrophil oxidase by preventing the attachment of DPI to a site in close proximity to the NADPH-binding site (Cross and Jones, 1986). It is very likely that NADPH may interfere with the action of DPI on endothelial NOS by the same mechanism.

DPI was found to inhibit ACh-induced relaxation in aortic rings for at least 4 h after washout and to suppress ACh-induced vasodilatation for at least 2 h after intravenous bolus injection. Therefore, our *in vitro* and *in vivo* results are supportive of a prolonged inhibitory effect of DPI on endothelium-dependent vasodilatation. DPI has been reported to irreversibly inhibit macrophage NOS (Stuehr *et al.*, 1991b); the mechanism may involve the formation of a covalent bond with components of flavoprotein (Ragan and Bloxham, 1977; O'Donnell *et al.*, 1993). However, our results show that post-treatment (1.5 h later) with NADPH still attenuates the effect of DPI, although the response is significantly less than that following pretreatment (10 min earlier). These results imply that fresh synthesis of NO occurs in endothelial cells.

4.2.2. Pressor and tachycardiac responses to DPI, and their mechanisms *in vivo*

Detailed analyses of the dose-MAP and dose-HR response curves of DPI show that the Hill coefficients for the MAP and HR effects of DPI are 3.6±0.3 and 4.2±0.6, respectively, in conscious and pentobarbitone-anaesthetized rats. These results suggest that the cardiovascular effects of DPI involve "positive cooperation" of probably 3 or 4 molecules of DPI (see review by Rang, 1971; Pennefather and Quastel, 1982) and the mechanism by which DPI causes pressor response is different from that of NSAAs, as L-NAME cause pressor response with a Hill coefficient of 2.

Moreover, the similar transient time course and pharmacodynamics suggest a common causative factor for both pressor and tachycardiac responses to DPI in pentobarbitone-anaesthetized rats. Since captopril markedly potentiated the pressor response and did not alter the tachycardiac response to DPI, it is safe to conclude that the renin-angiotensin system is not responsible for the response to DPI, although the mechanism of the potentiation by captopril is not known.

By the use of sympatholytic drugs, we also investigated whether the sympathetic nervous system is responsible for the pressor and tachycardiac effects of DPI. Reserpine markedly attenuates DPI-induced increases in MAP and HR. The results suggest that DPI causes cardiovascular effects by activating the peripheral sympathetic nerve terminals and adrenal medullae, resulting in the releases of noradrenaline and adrenaline. This activation is dependent on the functional integrity of the central and autonomic nervous systems, as pithing abolishes while spinal cord transection (T_1) attenuates the pressor and tachycardiac effects of DPI. The indirect activation of the sympathetic nervous system by DPI is further supported by the observations that TTX abolishes while guanethidine and mecamylamine attenuate the effects of DPI. TTX has been shown to block conductances of the central and peripheral nerves (Gage, 1971)

but not those of the vascular smooth muscle (see review by Hirst and Edwards, 1989) or the myocardium (Abraham *et al.,* 1989), *via* selective blockade of voltage-dependent sodium channels. Guanethidine has been shown to be a specific adrenergic neuron blocker (Shand *et al.,* 1973; Kirpekar and Furchgott, 1972).

Furthermore, we also used bilateral adrenalectomy and chemical sympathectomy by 6-hydroxydopamine (6-OH-DA) to examine the contributions of the adrenal medullae and sympathetic nerve terminals for the pressor and tachycardiac responses to DPI in pentobarbitone-anaesthetized rats. We found that neither bilateral adrenalectomy nor pretreatment (26 h earlier) with 6-OH-DA (4.9×10^{-4} mol/kg, i.p.) significantly affected the dose-MAP response curve of DPI although 6-OH-DA but not adrenalectomy slightly and significantly shifted the dose-HR curve to the right without affecting the maximum. However, the combination of both bilateral adrenalectomy and 6-OH-DA markedly reduced the maximal MAP and maximal HR responses to DPI by 71% and 35%, respectively. These results show that both sympathetic nerve terminals and sympathoadrenals play important and overlapping roles in the pressor and tachycardiac responses to DPI (Wang and Pang, 1994a).

DPI causes immediate and large increases in plasma noradrenaline and adrenaline, with the same time-course as the pressor and tachycardiac responses. Pithing totally abolishes and reserpinization attenuates DPI-induced increases in plasma catecholamines as well as MAP and HR. Further analysis shows that positive correlations exist between DPI-induced changes in MAP and HR with plasma noradrenaline as well as adrenaline. Taken together, the above results indicate that DPI activates the sympathetic nerve terminals and adrenal medullae to release noradrenaline and adrenaline in the rats with the functionally intact central and autonomic nervous systems. Since DPI releases large quantities of catecholamines, repetitive injections would lead to tachyphylaxis. Our results

indeed show that the MAP and HR responses to a single dose of DPI are greater than those elicited by the same dose in a multiple injection regimen. We have also observed that multiple injections of high doses of DPI eventually produce negligible pressor and tachycardiac responses (data not shown).

Noradrenaline and adrenaline released by DPI would be expected to cause vasoconstriction, *via* the activation of α_1-adrenoceptors, and tachycardia, *via* the activation of $ß_1$-adrenoceptors. Indeed, we found that the pressor effect of DPI is suppressed by the phentolamine and prazosin (selective α_1-adrenoceptor antagonist) but not rauwolscine (selective α_2-adrenoceptor antagonist). Moreover, rauwolscine and phentolamine but not prazosin enhanced the tachycardiac effect of DPI; this potentiation is likely due to the blockade of the central and/or peripheral prejunctional α_2-adrenoceptors which mediate inhibition of noradrenaline release (Berthelsen and Pettinger, 1977). This hypothesis may also explain why rauwolscine caused a small potentiation of the pressor effect of DPI. Our results also show that the tachycardiac but not pressor effect of DPI is abolished by propranolol. The inability of propranolol to affect the MAP effect of DPI suggests that the pressor effect of DPI is not due to tachycardia or cardiac inotropy. The slight potentiation of the pressor response to DPI by propranolol may be due to the blockade of vasodilator $ß_2$-adrenoceptors which are prominent in skeletal muscle beds (Abdelrahman *et al.,* 1990).

The mechanism by which DPI activates the sympathetic nervous system is not known, however, the following possible mechanisms could be excluded. Firstly, it is logical to expect that DPI increases sympathetic discharge by inhibiting NOS. There have been extensive work done indicating that NO synthesis and release take place in the brain (Garthwaite *et al.*, 1988; Knowles *et al.*, 1989, 1990a; Bredt and Snyder, 1989, 1990; Bredt *et al.*, 1990; Schmidt *et al.*, 1989, 1992). NO synthesis is reported to be responsible for long-term potentiation in the hippocampus (Böhme *et al.,* 1991), long-term synaptic depression in the

cerebellum (Shibuki and Okada 1991) and nociceptive activity in the brain (Moore *et al.,* 1991). Moreover, endothelium-derived relaxing factor (EDRF)/NO has been shown to inhibit noradrenaline release from isolated sympathetic nerves innervating the canine pulmonary artery and vein (Greenberg *et al.*, 1989, 1990, 1991) and other preparations (see the introduction of Greenberg *et al.,* 1990). However, our results do not support this hypothesis. In contrast to DPI, L-NNA, at a dose which caused a maximal pressor response did not increase plasma catecholamines. These results suggest that DPI-induced sympathetic activation is unlikely due to the inhibition of the central NO synthesis. Secondly, the effect of DPI on the sympathetic nervous system is not due to its hypoglycemic effect, since DPI causes immediate and transient increases in blood pressure and plasma catecholamines, while it causes hypoglycemic effect slowly and reaches the plateau at 4 h after administration (Gatley and Martin, 1979). Thirdly, the pressor response to DPI is not likely because of its possible activation of "pain receptor", as we found that pretreatment with capsaicin ($3.3x10^{-4}$ mol/kg, s.c. in 2 d) blocked DPI-induced limb kicking movements but not the pressor response in pentobarbitone-anaesthetized rats (N=6, unpublished observation). More studies are needed to elucidate if the inhibition of other flavoproteins or NADPH-dependent enzymes account for the actions of DPI.

The site(s) of the actions of DPI is not known but the central nervous system is unlikely a primary or major site for the actions of DPI, although we can not exclude this possibility in view of the suppression of DPI's effects by pithing and spinal cord transection. If the site of action of DPI is in the central nervous system, local injection of the drug then would produce greater effects than intravenous administration. However, results from our preliminary studies show that intracarotid and intravertebral injections of DPI caused similar pressor responses as intravenous injections into the same rats (N=3). As well, intracerebroventricular injection of DPI into the third cerebroventricle at doses up

to $3x10^{-7}$ mol/kg (ED_{50} of $6.9x10^{-7}$ mol/kg by intravenous injection) did not cause any pressor or tachycardiac response (N=3). There is uncertainty about the accessibility of DPI to the central nervous system. Although ^{125}I was detected in the brain 10 min after intravenous injection of [^{125}I] DPI (Gatley and Martin, 1979), DPI, being a charged molecule, may not adequately access the central nervous system within 0.5-1 min after intravenous injection. Moreover, it is difficult to explain why pithing is more effective than spinal transection in attenuating the cardiovascular effects of DPI if the central nervous system is the only site of action of DPI. On the other hand, we cannot rule out the possible involvement of the peripheral sympathetic nervous system in the actions of DPI, since even the action of indirectly-acting sympathomimetic agents rely on a functional amine-uptake system and therefore, sympathetic tone. Therefore, though the integrity of the central nervous system is a prerequisite for the actions of DPI, its primary site(s) of actions is not clear. Further studies are required to identify the site(s) of actions of DPI in the central, efferent or even afferent nervous systems.

4.2.3. Mechanisms of inhibitory effect of halothane on the pressor response to DPI

Our results demonstrate that halothane dose-dependently and "noncompetitively" inhibits the pressor and increases in plasma catecholamines responses to DPI. It should be pointed out that the inhibition by halothane is not due to its hypotensive effects, as DPI caused greater pressor response in the rats anaesthetized with chloralose, urethane or ethanol, where baseline blood pressures were either similar to or lower than those in conscious rats (data shown in Appendix V). Halothane markedly inhibits DPI-induced release of catecholamine. These results suggest that the inhibitory effect of halothane on the pressor

response to DPI is primarily due to suppression of the sympathetic activation. Halothane has been shown to depress the activities of the sympathetic nervous system at different levels: (1) areas of the central nervous system controlling the sympathetic nerve activity (Price *et al.*, 1963; Miller *et al.*, 1969; Larach *et al.*, 1987; Bazil and Minneman, 1989), (2) the sympathetic ganglia (Skovsted *et al.*, 1969; Bosnjak *et al.*, 1982; Christ, 1977; Seagard *et al.*, 1982), and (3) the sympathetic nerve endings located in the walls of blood vessels (Muldoon *et al.*, 1975; Lunn and Rorie, 1984; Rorie *et al.*, 1990).

In addition, a small component of nonspecific inhibition by halothane may also be responsible for its effect on the pressor response to DPI. Halothane, at 1.25%, inhibited the maximal pressor response to DPI by 95%, and inhibited the increases in plasma noradrenaline and adrenaline by 86% and 81%, respectively. In another study, the same concentration of halothane reduced the pressor response produced by exogenous noradrenaline and angiotensin II by 18% (data shown in Appendix VI). Therefore, the suppression by halothane of the pressor response to DPI is mainly (approximately 80%) attributable to its inhibition of sympathetic transmission, the remainder of the inhibition (approximately 20%) is due to its nonspecific inhibition of vascular smooth muscle contraction.

4.3. Summary

1. DPI is an efficacious and "irreversible" inhibitor of endothelium-dependent vasodilatation *in vivo* and *in vitro*; the mechanism of the inhibition may involve the antagonism of the effects of FAD and NADPH, cofactors of NOS.
2. DPI causes immediate and transient pressor and tachycardiac responses, as well as increases in plasma catecholamines, in conscious and pentobarbitone (but not halothane)-anaesthetized rats. These effects are inhibited by maneuvers which interfere with the activities of the central or sympathetic nervous systems, namely,

pithing and spinal cord transection, as well as pretreatments with TTX, reserpine, mecamylamine and guanethidine. Moreover, the pressor but not tachycardiac effect of DPI is attenuated by phentolamine and prazosin; while the tachycardiac but not pressor effect of DPI is inhibited by propranolol. However, the pressor and tachycardiac responses to DPI are not antagonized by L-Arg.

3. These results demonstrate that DPI causes inhibition of *in vitro* and *in vivo* endothelium-dependent vasodilatation, and pressor and tachycardiac responses. However, the pressor response to DPI, unlike those of NSAAs, is *via* the indirect activation of the sympathetic nervous systems, rather than the inhibition of endothelium-dependent vasodilatation.

5. General discussion and conclusions

5.1. General discussion

It is well-known that all NSAAs which inhibit endothelium-dependent relaxation *in vitro* and *in vivo* cause long-lasting pressor responses in whole animals. The pressor responses to NSAAs are a consequence of generalized vasoconstriction. There are at least three existing hypotheses on the mechanism by which NSAAs cause pressor responses, namely, (1) the inhibition of endothelial NO biosynthesis (Aisaka *et al.*, 1989a; Rees *et al.*, 1989b, 1990b; see review by Moncada *et al.*, 1991); (2) the inhibition of brain NO synthesis (Sakuma *et al.*, 1992; Togashi *et al.*, 1992); (3) the inhibition of autonomic nerve NO synthesis (Toda *et al.*, 1993). Among them, the first hypothesis is best-accepted by the scientific community and it leads to the postulation that endogenous endothelial NO biosynthesis regulates vascular tone and blood pressure.

Our results clearly show that the pressor response to L-NAME is not blocked by the impairment of the central nervous system by pithing, and that the pressor responses to L-NNA and L-NAME are not attenuated by the blockade of ganglionic transmission with mecamylamine. Moreover, we also found that the pressor responses to L-NNA are not attenuated by impairing the sympathetic nervous, angiotensin and prostanoid systems. These results, taken together, suggest that the pressor responses to peripherally administered NSAAs are not dependent on the functional integrity of the central and autonomic nervous systems, and are therefore in disagreement with hypotheses 2 and 3, which postulate that the pressor responses to NSAAs are due to the inhibition of brain and autonomic nerve NO synthesis. On the other hand, L-Arg but not D-Arg competitively antagonizes the pressor response to L-NAME and L-NNA. Therefore, NSAAs cause pressor responses *via* a novel mechanism which involves the L-Arg pathway.

The L-Arg/NO pathway is well-established in endothelial cells and other organs, tissues and cells (see review by Moncada *et al.*, 1991). It is logical to conclude that NSAAs produce pressor responses by the inhibition of endogenous endothelial NO biosynthesis. However, our results and others' show that NSAAs only partially inhibit endothelium-dependent vasodilatation in whole animals, whereas they completely inhibit endothelium-dependent relaxation in isolated vascular preparations. Moreover, the pharmacodynamics of NSAAs in inhibiting endothelium-dependent relaxation and causing pressor response are different. Since NSAAs are chemically-related to L-Arg, the interaction of NSAAs and L-Arg could be simply at the membrane "receptor" level, and the signal transduction process may be distinct from that associated with the inhibition of NO synthesis. It is of ultimate importance to investigate the *in vitro* and *in vivo* pharmacology of other NO synthase inhibitors with structures unrelated to L-Arg.

As an "irreversible" inhibitor of NOS, DPI causes prolonged inhibition of endothelium-dependent vasodilatation *in vitro* and *in vivo*, but, unlike NSAAs, it does not cause a prolonged pressor response. Instead, the intravenous bolus injections of DPI only produce immediate and transient increases in MAP. The pressor response to DPI is blocked by maneuvers which impair the activities of the central or sympathetic nervous systems, namely, pithing, spinal cord transection and the administrations of TTX, reserpine, guanethidine, phentolamine and prazosin. Moreover, the pressor response to DPI but not to L-NNA is accompanied by elevations of plasma noradrenaline and adrenaline. These results show that the transient pressor response to DPI, unlike that of NSAAs, is solely dependent on the activation of the sympathetic nervous system. Therefore, DPI does not elicit NO synthesis-dependent sustained rise in blood pressure as do the NSAAs.

Although one may postulate that the inability of DPI to cause a sustained rise in blood pressure is due to inadequate accumulation of drug *in situ* to inhibit NO synthesis, this is unlikely true. DPI was shown to rapidly and adequately distribute

to all organs or tissues (Gatley and Martin, 1979). Moreover, its peak hypoglycemic effect was reached at 1.5 h (Holland *et al.*, 1973) or 4 h (Gatley and Martin, 1979) after intraperitoneal injection, suggesting a long duration of action. Our present results also show that DPI irreversibly inhibits endothelium-dependent relaxation *in vitro* for more than 4 h, and partially inhibits ACh-induced vasodilatation even at 2 h after intravenous injection.

Therefore, although it is generally accepted that NSAAs produce pressor response by the inhibition of endothelial NO synthesis and endothelium-dependent vasodilatation *in situ*, and that endogenous NO modulates vascular tone and blood pressure (Aisaka *et al.*, 1989a; Rees *et al.*, 1989b, 1990; see review by Moncada *et al.*, 1991), our data with DPI suggest otherwise, namely, the inhibition of NO synthesis and endothelium-dependent vasodilatation do not always cause vasoconstriction *in vivo*. Our hypothesis is supported by other publications which show that methylene blue does not produce a pressor response (Loeb and Longnecker, 1992; Pang and Wang, 1993), although it inhibits endothelium-dependent vasodilatation *in vitro* (Pang and Wang, 1993) and *in vivo* (Loeb and Longnecker, 1992). L-NNA also caused much longer inhibition of endothelium-dependent vasodilatation than elevation of blood pressure in conscious rabbits, suggesting that the suppression of NO synthesis alone does not result in hypertension (Cocks *et al.*, 1992). Therefore, the hypotheses that NSAAs produce pressor response by the inhibition of endothelial NO biosynthesis and that endogenous NO modulates vascular tone could be challenged. Accordingly, it is possible that NSAAs produce pressor response by activating an "L-Arg receptor", with subsequent signal transduction distinct from the inhibition of NO biosynthesis. This hypothesis does not exclude another possibility that the inhibition of endothelial NO synthesis is a necessary but not a sufficient requirement to cause vasoconstrictions *in vivo*. One requirement for the actions of NOS inhibitors may be the presence of chemical structures similar to those of

NSAAs or Arg. Another necessary condition may be a particular type of vasculature. It has been shown that NSAAs inhibit endothelium-dependent relaxation in both isolated arterial and venous preparations (Gold *et al.*, 1990; Miller, 1991; Nagao and Vanhoutte, 1991; Pawloski and Chapnick, 1991; Martin *et al.*, 1992; Elmore *et al.*, 1992). Although L-NMMA has no effect on the basal tone of dorsal hand veins, it inhibits ACh-elicited venodilatation, however, L-NMMA induces direct vasoconstriction and inhibits vasodilatations induced by ACh and bradykinin in the brachial artery (Vallance *et al.*, 1989a,b). Moreover, L-NAME causes a marked increase in arterial pressure but not in mean circulatory filling pressure (MCFP, an index of body venous tone) in intact or ganglion-blocked conscious rats (Wang and Pang, unpublished data, 1993), or a low increase in MCFP (by approximately 12%) in anaesthetized cats (Bower and Law, 1993). These results again suggest that the inhibition of endothelium-dependent relaxation alone is insufficient to cause vasoconstriction *in vivo*.

5.2. Conclusions

1. Both NSAAs and DPI inhibit endothelium-dependent vasodilatations, completely *in vitro*, and partially *in vivo*. However, the inhibition by NSAAs is reversible whereas that by DPI is irreversible. The inhibitory mechanism of NSAAs involves limiting the availability of the NOS substrate L-Arg, whereas that of DPI involves antagonizing the actions of the NOS cofactors, FAD and NADPH.

2. Although both NSAAs and DPI cause pressor responses, the time course of the responses are different. The responses to NSAAs are slowly-developing and long-lasting whereas that of DPI is immediate and transient. Moreover, the pressor responses to NSAAs are accompanied by bradycardia and that of DPI is accompanied by tachycardia.

3. The pressor responses to NSAAs are competitively antagonized by L-Arg and are not dependent on the integrity of the central/autonomic nervous, angiotensin and prostanoid systems. Therefore, the pressor responses to NSAAs are not due to the inhibition of NO biosynthesis in the brain or autonomic nervous system. However, the pressor and tachycardiac responses to DPI are entirely due to sympathetic activation, rather than the inhibition of endothelium-dependent vasodilatation.

4. Although the mechanism by which NSAAs produce pressor responses is novel and is antagonized by L-Arg, it may not be related to the inhibition of NO biosynthesis. This hypothesis is derived on the basis of the following evidence. (1) DPI, as an effective inhibitor of *in vitro* and *in vivo* endothelium-dependent vasodilatations, does not cause NO-mediated pressor response. (2) NSAAs and DPI do not completely inhibit ACh-induced depressor responses *in vivo*. (3) the *n* of NSAAs for producing pressor response is different from that for inhibiting endothelium-dependent relaxation. (4) methylene blue, a putative inhibitor of guanylyl cyclase, inhibits ACh-induced relaxation in preconstricted aortic rings but does not cause pressor response. It may be reasonable to postulate that NSAAs produce pressor response *via* interaction with "L-Arg receptors", with subsequent signal transduction different from that associated with the inhibition of NO biosynthesis.

5. Alternatively, the pressor response produced by the NSAAs may involve the inhibition of NOS, however, this only condition may not be sufficient to cause vasoconstriction *in vivo*. Pressor response by NOS inhibitors may require: (1) the inhibition of endogenous endothelial NO biosynthesis in arteries and arterioles, and (2) chemical structures of NOS inhibitors to be similar to those of NSAAs or Arg.

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APPENDICES

Paper I

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. The reproduction of this paper was kindly permitted by the copyright holder, Macmillan Press Ltd., Hampshire, U.K.

Br J Pharmacol. (1991), 103, 2004–2008

Possible dependence of pressor and heart rate effects of N^G-nitro-L-arginine on autonomic nerve activity

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1 The effects of N^G-nitro-L-arginine (L-NNA) on mean arterial pressure (MAP) and heart rate (HR) were investigated in conscious rats.

2 Intravenous bolus cumulative doses of L-NNA (1–32 mg kg^{-1}) dose-dependently increased MAP. Both mecamylamine and phentolamine increased MAP responses to L-NNA, angiotensin II and methoxamine. Propranolol, reserpine, atropine and captopril did not affect MAP response to L-NNA.

3 A significant negative correlation of HR and MAP responses to L-NNA was obtained in control rats but not in rats pretreated with reserpine or mecamylamine. Significant negative correlations also occurred in the presence of atropine, propranolol, phentolamine or captopril.

4 A single i.v. bolus dose of L-NNA (32 mg kg^{-1}) raised MAP to a peak value of 53 ± 3 mmHg and the effect lasted more than 2 h; the rise and recovery of MAP were accompanied by significant decrease and increase in HR, respectively. While both phentolamine and mecamylamine increased peak MAP response to L-NNA, mecamylamine abolished the biphasic HR response and phentolamine potentiated the bradycardic component of HR.

5 Blockade of the autonomic nervous and renin-angiotensin systems did not attenuate the pressor effects of L-NNA. However, the biphasic HR response to L-NNA is mediated via modulation of autonomic nerve activities.

Keywords: N^G-nitro-L-arginine (L-NNA); vasopressor; autonomic ganglion; sympathetic and parasympathetic nervous system; renin-angiotensin system

Introduction

There is evidence that endothelium-derived relaxing factor (EDRF) released by vascular endothelial cells is nitric oxide (NO) (Palmer *et al.*, 1987; Ignarro *et al.*, 1987) which is formed from the precursor L-arginine (L-Arg) (Palmer *et al.*, 1988; Sakuma *et al.*, 1988). It has been shown that NO synthase and endothelium-dependent vascular relaxation responses in isolated arteries are inhibited by N^G-substituted L-Arg analogues which include N^G-monomethyl-L-arginine (L-MMA) (Palmer *et al.*, 1988; Rees *et al.*, 1989a; 1990), N^G-nitro-L-arginine methyl ester (Moore *et al.*, 1990; Rees *et al.*, 1990), N-iminoethyl-L-ornithine (Rees *et al.*, 1990) and N^G-nitro-L-arginine (L-NNA) (Moore *et al.*, 1990; Mülsch & Busse, 1990; Kobayashi & Hattori, 1990; Ishii *et al.*, 1990). *In vivo* studies show that L-MMA (Rees *et al.*, 1989b; 1990; Aisaka *et al.*, 1989; Whittle *et al.*, 1989; Gardiner *et al.*, 1990b,c), N^G-nitro-L-arginine methyl ester (Gardiner *et al.*, 1990a,c,d), N-iminoethyl-L-ornithine (Rees *et al.*, 1990) and L-NNA (Wang & Pang, 1990) cause pressor responses and bradycardia.

Although it is likely that the pressor effects of L-Arg analogues are caused by the inhibition of NO production from vascular endothelial cells (Aisaka *et al.*, 1989; Rees *et al.*, 1989b; Wang & Pang, 1990), other vasopressor systems may contribute to the response. It has been shown that vascular endothelium inhibits the release of noradrenaline from sympathetic nerves which innervate canine pulmonary artery and vein suggesting that the endothelium, in part via endothelium-derived relaxing factor (EDRF) release, acted as an endogenous inhibitor of sympathetic transmitter release (Greenberg *et al.*, 1990). Togashi *et al.* (1990) showed that L-MMA increased postganglionic sympathetic nerve activities in intact and bilateral sino-aortic- and vagal-denervated rats and preganglionic adrenal nerve activity in sino-aortic and vagal-denervated rats. It has also been reported that EDRF inhibited renin release (Vidal *et al.*, 1988). Therefore it is logical to postulate that the haemodynamic effects of L-Arg analogues are partially mediated via potentiation of activities of the autonomic nervous and/or renin-angiotensin system(s).

The aims of this study were: (1) to assess the contribution of the autonomic nervous and renin-angiotensin systems on pressor response to L-NNA; (2) to examine whether the bradycardia produced in response to L-NNA is mediated via reflex activation of the autonomic nervous system.

Methods

Surgical preparation

Sprague-Dawley rats (240–400 g) were anaesthetized with halothane (4% in air for induction, 2% in air for surgical preparation). A polyethylene cannula (PE50) was inserted into the left iliac artery to allow recordings of mean arterial pressure (MAP). PE50 cannulae were also inserted into the right (or both) iliac vein(s) for the administration of drugs. The cannulae were filled with heparinized saline (25 iu ml^{-1}) and tunnelled s.c. along the back, exteriorized at the back of the neck and secured. The rats were given 6–7 h recovery of the effects of halothane and surgery before further use.

Experimental protocol

The indwelling arterial catheter from each rat was connected to a pressure transducer (P23DB, Gould Statham, CA, U.S.A.) for the recordings of MAP and heart rate (HR) which was derived electronically from the upstroke of the arterial pulse pressure by a tachograph (Grass, Model 7P4G). The conscious rats were allowed to wander freely in a small cage for 1 h before the administrations of drugs. MAP and HR were continuously monitored. The rats were killed by an overdose of pentobarbitone at the end of each experiment. Two main studies were conducted:

Dose-response curves for N^G-nitro-L-arginine Rats, randomly divided into seven groups ($n = 6$ each), were pretreated with: (I) normal saline (0.9% NaCl); (II) phentolamine (i.v. infusion,

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Table 1 Baseline values of mean arterial pressure (MAP) and heart rate (HR) (mean ± s.e.mean) in conscious rats prior to and 40 min after the administration of normal saline, phentolamine, propranolol, reserpine, mecamylamine, atropine and captopril

	MAP (mmHg)		*HR* (beats min^{-1})	
Antagonists	*Before*	*After*	*Before*	*After*
Protocol 1				
Normal saline	103 ± 5	104 ± 5	403 ± 10	410 ± 8
Phentolamine	106 ± 4	69 ± 7*	442 ± 12	518 ± 18*
Propranolol	104 ± 4	104 ± 4	364 ± 10	334 ± 8*
Reserpine	—	80 ± 5†	—	295 ± 11†
Mecamylamine	113 ± 3	88 ± 4*	377 ± 9	313 ± 13*
Atropine	116 ± 2	118 ± 3	370 ± 23	432 ± 18*
Captopril	105 ± 6	103 ± 5	372 ± 19	418 ± 16*
Protocol 2				
Normal saline	106 ± 3	104 ± 3	343 ± 13	344 ± 13
Mecamylamine	108 ± 4	69 ± 2*	431 ± 10	308 ± 8*
Phentolamine	101 ± 5	72 ± 3*	401 ± 13	443 ± 8*

* Denotes significant difference from corresponding control values within the same group ($P < 0.05$); † Denotes significant difference from normal saline group ($P < 0.05$). $n = 6$ per group.

300 μg kg^{-1} min^{-1}); (III) propranolol (i.v. bolus at 1 mg kg^{-1} followed by infusion at 1.6 μg kg^{-1} min^{-1}); (IV) reserpine (5 mg kg^{-1}, i.p., 24 h prior to the study); (V) mecamylamine (i.v. bolus at 10 mg kg^{-1} followed by infusion at 300 μg kg^{-1} min^{-1}); (VI) atropine (i.v. bolus at 10 mg kg^{-1} followed by infusion at 8 μg kg^{-1} min^{-1}) and (VII) captopril (20 mg kg^{-1}, i.v. bolus). With the exception of reserpine and captopril, all antagonists were continuously infused for approximately 160 min, i.e., to the end of the experiment. Cumulative doses of L-NNA (1–32 mg kg^{-1}, i.v. bolus) were given 40 min after the administration of the vehicle or blockers at dose-intervals of 15–20 min, the period required to obtain steady state MAP responses. A single dose of angiotensin I (1 μg kg^{-1}), methoxamine (20 or 30 μg kg^{-1}), acetylcholine (1 μg kg^{-1}) or isoprenaline (1 μg kg^{-1}) was injected as an i.v. bolus prior to and 20 min after the start of administration of captopril, phentolamine, atropine or propranolol, respectively, and again 2 h after giving L-NNA to assess the degrees of inhibition at the start and completion of the studies. In rats pretreated with reserpine and vehicle, tyramine (200 μg kg^{-1}) was injected as an i.v. bolus 20 min prior to and 2 h after giving L-NNA. Excluding the equilibration time, the duration of each study was approximately 3 h.

Time course of responses to a single dose of N^G-nitro-L-arginine Another three groups of rats ($n = 6$ each) were pretreated with: (VIII) normal saline; (IX) mecamylamine; (X) phentolamine, at the same doses as those described previously. In phentolamine and mecamylamine groups, angiotensin II was injected as an i.v. bolus prior to and 20 min after the administration of a blocker. In Groups VIII, IX and X, a single dose of L-NNA (32 mg kg^{-1}) was injected as an i.v. bolus 40 min after the start of administration of a blocker. MAP and HR were continuously monitored for 2 h.

Drugs

The following drugs were obtained from Sigma Chemical Co. (MO, U.S.A.): N^G-nitro-L-arginine (L-NNA), mecamylamine hydrochloride, atropine sulphate, Des-Asp^1-angiotensin I acetate, angiotensin II acetate, acetylcholine hydrochloride, (±)-propranolol hydrochloride, (−)-isopropylnoradrenaline hydrochloride and tyramine hydrochloride. The following drugs were also used: phentolamine hydrochloride (Ciba Pharmaceutical Co., NJ, U.S.A.), methoxamine hydrochloride (B.W. & Co. Ltd., Quebec, Canada), captopril (E.R. Squibb & Sons Inc., NJ, U.S.A.) and reserpine (Ciba Pharmaceutical Co., Quebec, Canada). All drugs were dissolved in normal saline.

Calculation and statistical analysis

The ED_{50} and maximum response (E_{max}) values of L-NNA were obtained from individual dose-response curves. Correlation coefficient (r), slope and intercept were calculated from individual HR versus MAP curves at various doses of L-NNA. Rise phase $t_{1/2}$ of L-NNA were obtained from time-course curves. To obtain normal distribution of rise phase $t_{1/2}$, the data were logarithmically-transformed prior to statistical analysis. All data were analyzed by the analysis of variance followed by Duncan's multiple range test with $P < 0.05$ selected as the criterion for statistical significance. All results are expressed as mean ± standard error (s.e.mean) except for rise phase $t_{1/2}$ which is expressed as geometric mean and 95% confidence range.

Results

Effects of antagonists on mean arterial pressure and heart rate

Table 1 shows baseline MAP and HR (protocol 1: Groups I to VII; protocol 2: Groups VIII to X) in rats prior to and 40 min after pretreatment with normal saline, phentolamine, propranolol, reserpine, mecamylamine, atropine or captopril. Normal saline (protocols 1 and 2) affected neither MAP nor HR. MAP was not affected by propranolol, atropine or captopril but significantly decreased by phentolamine, reserpine and mecamylamine. HR was decreased by reserpine, mecamylamine and propranolol and increased by phentolamine, atropine and captopril.

Table 2 shows the effects of the antagonists on responses to several agonists. Phentolamine (Group II) completely blocked

Table 2 The effects of antagonists on mean arterial pressure (MAP) and heart rate (HR) responses to several agonists in conscious rats

		Change in MAP (mmHg)			*Change in HR* (beats min^{-1})		
Blocker	*Agonist*	a	b	c	a	b	c
Phent	Mtx1	40 ± 6	0	0	−106 ± 14	0	0
Reserp	Tyram	46 ± 3[a]	17 ± 5	17 ± 5	−108 ± 11*	−16 ± 10	−21 ± 9
Prop	Isop	−3* ± 3	1 ± 1	17 ± 8	116 ± 13	2 ± 2	2* ± 9
Mecam	Mtx2	55 ± 2	101 ± 5	43 ± 8	−169 ± 17	0	0
Atrop	ACh	−44 ± 2	0	0	47 ± 2	0	0
Capt	AI	43 ± 4	0	15 ± 1	−83 ± 13	0	−28 ± *

The effects (mean ± s.e.mean) of isoprenaline (Isop, 1 μg kg^{-1}), tyramine (Tyram, 200 μg kg^{-1}), methoxamine (Mtx1 20 μg kg^{-1}; Mtx2, 30 μg kg^{-1}), acetylcholine (ACh, 1 μg kg^{-1}) and angiotensin I (AI, 1 μg kg^{-1}) on MAP and HR were obtained before (a), 20 min after (b) the administrations of: phentolamine (Phent), propranolol (Prop), reserpine (Reserp), mecamylamine (Mecam), atropine (Atrop) or captopril (Capt) and, 2 h after (c) an i.v. bolus injection of N^G-nitro-L-arginine.
All results in (b) and (c) are significantly different from the corresponding control values (a) within the same group ($P < 0.05$). [a] The data were from the vehicle-treated rat group. $n = 6$ per group.

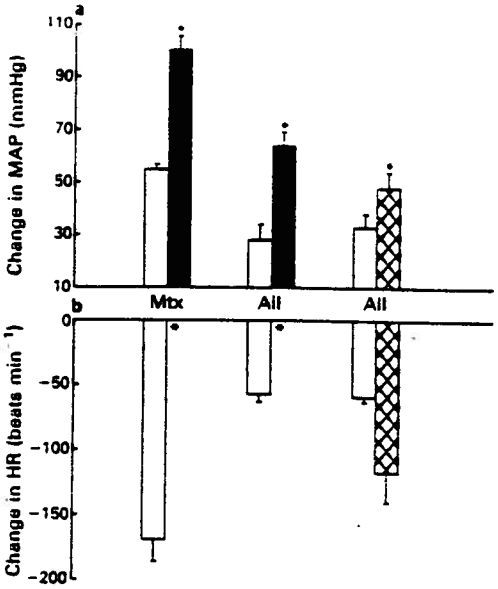

Figure 1 Effects of methoxamine (Mtx) and angiotensin II (AII) on mean arterial pressure (MAP) (a) and heart rate (HR) (b) before (open columns) and 20 min after the administrations of mecamylamine (filled columns) and phentolamine (cross hatched column). Values are means with s.e.mean shown by vertical bars; $n = 6$ in each group. * Represents significant difference from corresponding control values prior to the administration of an antagonist ($P < 0.05$).

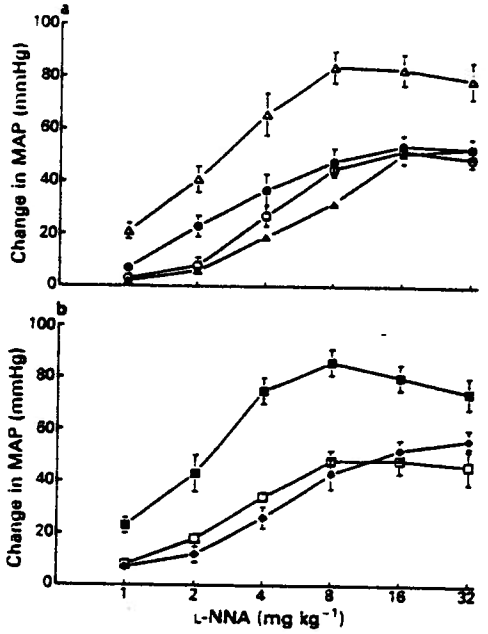

Figure 2 Dose-response curves (mean ± s.e.mean) of the effects of i.v. bolus doses of N^G-nitro-L-arginine (L-NNA) on mean arterial pressure (MAP) in groups ($n = 6$ each) of conscious rats pretreated with normal saline (○ in a), reserpine (● in a), phentolamine (△ in a), propranolol (▲ in a), atropine (□ in b), mecamylamine (■ in b) and captopril (◆ in b).

pressor effects and bradycardia induced by methoxamine throughout the study period. The pressor response to methoxamine was enhanced by mecamylamine (Group V) at 20 min but not altered at 2 h following the administration of L-NNA. The reflex bradycardia induced by methoxamine was abolished by mecamylamine throughout the experiments. Intravenous bolus doses of tyramine in vehicle-treated rats (Group I) increased MAP and decreased HR; in Group IV rats pretreated with reserpine tyramine caused markedly less pressor and bradycardic responses than in control rats. Isoprenaline (Group III) caused depressor and tachycardic responses; both were almost totally abolished at 20 min after the injection of propranolol and remained markedly attenuated 2 h after the administration of L-NNA. Atropine (Group VI) completely abolished the depressor and reflex tachycardic response of acetylcholine throughout the study period. At 20 min after the injection of captopril (Group VII), the pressure and bradycardic effects of angiotensin I were abolished. Both responses to angiotensin I remained attenuated 2 h after the injections of L-NNA.

Pressor responses to angiotensin II (Groups IX and X) and methoxamine (Group V) were potentiated by mecamylamine or phentolamine (Figure 1). Reflex bradycardia in response to angiotensin II and methoxamine was totally blocked by mecamylamine but reflex bradycardia to angiotensin II was unaffected by phentolamine.

Dose-response curves for N^G-nitro-L-arginine

Intravenous bolus doses of L-NNA in vehicle-treated rats dose-dependently increased MAP (Figure 2). Pretreatment with either mecamylamine or phentolamine potentiated the pressor response to L-NNA by reducing ED_{50} and increasing E_{max} values (Figure 2, Table 3). Pretreatment with the other antagonists used in this study did not significantly alter the dose-MAP response curves for L-NNA (Figure 2, Table 3).

Figure 3 shows the relationships between HR and MAP for rats in Groups I to VII. In vehicle-treated rats (Group I), MAP after L-NNA was negatively correlated with HR. Significant correlations of MAP with HR were also obtained in rats pretreated with phentolamine, propranolol, atropine or captopril but not with reserpine or mecamylamine (Table 4). The slope of the curve was not significantly altered by atropine, propranolol or captopril but was significantly increased by phentolamine. The intercept was decreased by propranolol.

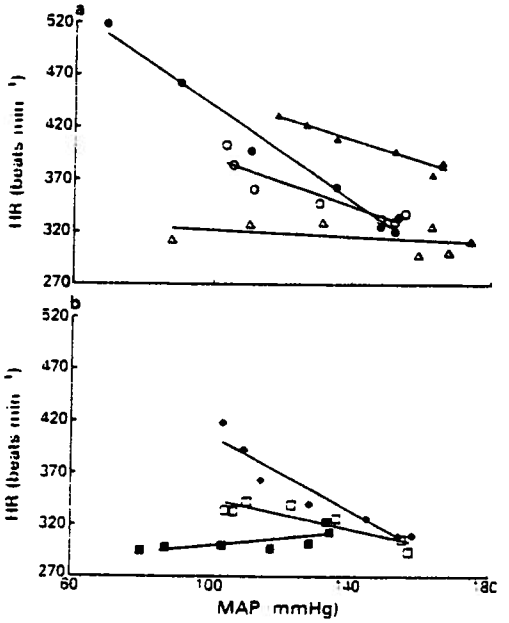

Figure 3 Relationship of heart rate (HR) (a) to mean arterial pressure (MAP) (b) after injection of N^G-nitro-L-arginine (L-NNA, 1–32 mg kg^{-1}, i.v. bolus) in conscious rats pretreated with normal saline (○ in a), phentolamine (● in a), mecamylamine (△ in a), atropine (▲ in a), propranolol (□ in b), reserpine (■ in b) and captopril (◆ in b). Each point represents mean values from six rats given the same dose of L-NNA.

Table 3 ED_{30} values and maximum effects (E_{max}) of N^G-nitro-arginine (L-NNA) on mean arterial pressure in conscious rats pretreated with normal saline, phentolamine, propranolol, reserpine, mecamylamine, atropine or captopril

Antagonist	ED_{30} (mg kg^{-1})	E_{max} (mmHg)
Normal saline	4.3 ± 0.8	52 ± 2
Phentolamine	2.1 ± 0.2*	87 ± 6*
Propranolol	6.3 ± 0.6	54 ± 3
Reserpine	3.1 ± 0.6	56 ± 4
Mecamylamine	1.9 ± 0.2*	86 ± 5*
Atropine	2.7 ± 0.3	51 ± 5
Captopril	5.0 ± 1.1	56 ± 4

All values represent mean ± s.e.mean. $n = 6$ per group. * Denotes significant difference from normal saline-treated group ($P < 0.05$).

Table 4 Slope, intercept and correlation coefficient (r) of the heart rate vs mean artery pressure curves of N^G-nitro-L-arginine (L-NNA, 1–32 mg kg^{-1}, i.v. bolus) in conscious rats ($n = 6$ per group) pretreated with normal saline, phentolamine, propranolol, reserpine, mecamylamine, atropine or captopril

Group	r	*Slope*	*Intercept*
Normal saline	0.85 ± 0.03*	−1.17 ± 0.24	509 ± 32
Phentolamine	0.96 ± 0.01*	−2.29 ± 0.08*	669 ± 23*
Propranolol	0.80 ± 0.05*	−0.75 ± 0.20	419 ± 20*
Reserpine	0.72 ± 0.06	b	b
Mecamylamine	0.46 ± 0.12	b	b
Atropine	0.83 ± 0.04*	−1.16 ± 0.22	557 ± 51
Captopril	0.89 ± 0.02*	−1.68 ± 0.14	570 ± 17

Slope (beats min^{-1} mmHg^{-1}), intercept (beats min^{-1}) and r values represent mean ± s.e.mean. * Denotes significance of r ($P < 0.05$); b values were not obtained due to insignificant correlation coefficient; * denotes significant difference from respective values in normal saline group ($P < 0.05$).

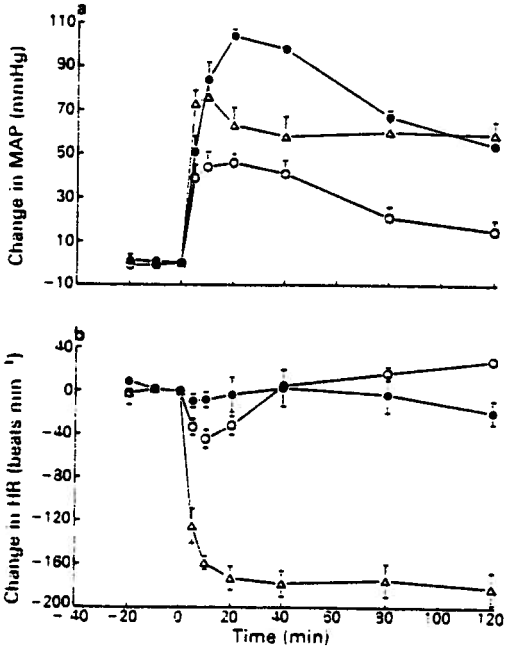

Figure 4 Time course of the effects of N^G-nitro-L-arginine (L-NNA, 32 mg kg^{-1}) on mean arterial pressure (MAP) (a) and heart rate (HR) (b) in groups ($n = 6$ each) of conscious rats pretreated with normal saline (○), mecamylamine (●) and phentolamine (△). Values are means with s.e.mean shown by vertical bars.

increased by phentolamine but not significantly altered by captopril and atropine.

Time course of the effects of N^G-nitro-L-arginine

In control rats given normal saline the MAP response to a single dose of L-NNA started almost immediately and reached a plateau 10 min after injection (Figure 4a). The rise phase $t_{1/2}$ was 4.8 min (geometric mean, 95% confidence limit: 2.0–11.6); MAP at 40 min was not different from MAP at 10 min and remained elevated 2 h after injection. Mecamylamine and phentolamine potentiated the peak MAP response to L-NNA (Figure 4a). Mecamylamine did not alter the rise phase $t_{1/2}$ (5.5 min, 95% confidence limit: 3.2–9.4) but phentolamine reduced it (to 1.5 min, 95% confidence limit: 1.0–2.3).

The pressor response to L-NNA was accompanied by initial significant decreases of HR at 5, 10, 20 min after injection followed by a recovery of HR and continual significant increases of HR at 80 and 120 min even when MAP was still above the control level (Figure 4b). Mecamylamine abolished the biphasic effects of L-NNA on HR. Phentolamine, on the other hand, potentiated and prolonged the bradycardia.

Discussion

Our results show that L-NNA is a potent and long-lasting pressor agent in conscious rats. Captopril and blockers of the autonomic nervous system, namely, mecamylamine, phentolamine, reserpine, propranolol and atropine, did not attenuate the pressor responses to L-NNA. This indicates that the pressor effect of L-NNA does not rely on the integrity of these two vasopressor systems. It has been reported that the pressor effects of L-MMA (Rees *et al.*, 1989b; Aisaka *et al.*, 1989) and L-NNA (Wang & Pang, 1990) are antagonized by L-Arg suggesting that N^G-substituted L-Arg analogues raise MAP via inhibiting NO synthase.

Pretreatment with mecamylamine potentiated MAP responses to L-NNA, angiotensin II and methoxamine. Phentolamine increased pressor responses to L-NNA and angiotensin II. Phentolamine but not mecamylamine, however, reduced the rise time $t_{1/2}$. This non-specific enhancement of the pressor effects of vasopressor agents after ganglionic or α-adrenoceptor blockade is consistent with well-known observations that acute pressor responses in intact animals are buffered by the simultaneous withdrawal of sympathetic tone to vascular smooth muscles (Lum & Rashleigh, 1961; Mawji & Lockett, 1963; Minson *et al.*, 1989).

MAP response to the injection of a single dose of L-NNA was associated with significant initial bradycardia (0 to 40 min) followed by tachycardia. A biphasic HR response to L-NNA was also observed in pentobarbitone-anaesthetized rats (Wang & Pang, 1990). Biphasic HR responses to L-MMA in chloralose and urethane anaesthetized rats have also been described (Togashi *et al.*, 1990). Mecamylamine abolished HR responses to L-NNA, suggesting that the biphasic HR response is mediated via reflex changes in the activities of the autonomic nervous system.

The tachycardic component was not seen in rats given cumulative doses of L-NNA, presumably due to the shorter observation time given to each dose of L-NNA. The results from cumulative dose-response relationships to L-NNA in the absence of an antagonist, show that the MAP effects of L-NNA are negatively correlated with HR. Treatment with mecamylamine or reserpine abolished the reflex changes in HR following alterations in MAP. The slope of the HR-MAP curve was slightly reduced by propranolol but unaffected by atropine. The lack of a correlation of HR to MAP after treatment with reserpine suggests that in conscious rats, inhibition of sympathetic nerve activity rather than potentiation of parasympathetic nerve activity is involved in reflex changes in HR. These results are consistent with the observation that bradycardia induced by L-MMA was associated with reduced renal

sympathetic nerve activity (Togashi *et al.*, 1990). Our results also show that phentolamine increased the slope of the curve. Phentolamine has been shown to increase markedly plasma levels of adrenaline and noradrenaline (Tabrizchi *et al.*, 1988). Therefore, enhanced reflex bradycardia in response to L-NNA in the presence of phentolamine may have been a consequence of elevated background sympathetic nerve activities as baseline HR was elevated by phentolamine.

In conclusion, the pressor effect of L-NNA does not rely on the integrity of the autonomic nervous system or the renin-angiotensin system. The biphasic changes of HR induced by L-NNA are attributable to reflex alterations in the activities of the autonomic nervous system.

This work was supported by the Medical Research Council of Canada.

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(Received November 19, 1990
Revised April 22, 1991
Accepted April 23, 1991)

Paper II

Wang, Y.-X., Zhou, T. and Pang, C.C.Y.: Pressor effects of L and D enantiomers of N^G-nitro-L-arginine in conscious rats are antagonized by L- but not D-arginine. Eur. J. Pharmacol. 200: 77-81, 1991. The reproduction of this paper was kindly permitted by the copyright holder, Elsevier Science Publishers B.V., Amsterdam, The Netherlands.

European Journal of Pharmacology, 200 (1991) 77–81

ADONIS 001429999100482P

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Pressor effects of L and D enantiomers of N^G-nitro-arginine in conscious rats are antagonized by L- but not D-arginine

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Received 13 December 1990, revised MS received 22 March 1991, accepted 23 April 1991

The effects of N^G-nitro-L-arginine (L-NNA) and N^G-nitro-D-arginine (D-NNA) on mean arterial pressure (MAP) were studied in conscious, unrestrained rats. I.v. bolus of either L-NNA (1-64 mg/kg) or D-NNA (2-64 mg/kg) dose dependently increased MAP to similar maximum values of 55 ± 7 and 52 ± 4 mm Hg and with ED_{50} values of 4.0 ± 0.9 and 8.9 ± 1.2 mg/kg ($P < 0.05$), respectively. The time course of the MAP response to a single dose (32 mg/kg i.v. bolus) of L-NNA and D-NNA were also obtained. The pressor effects of L-NNA and D-NNA each lasted > 2 h with the rise phase $t_{1/2}$ of 5 and 27 min ($P < 0.05$), respectively. I.v. infusions (10 mg/kg per min) of L-arginine (L-Arg) and D-arginine (D-Arg) did not alter the pressor response to noradrenaline nor angiotensin II. L-Arg but not D-Arg attenuated the pressor responses to both L-NNA and D-NNA. Therefore, both L-NNA and D-NNA are efficacious and long-lasting pressor agents: the pressor effects of both can be antagonized by L-Arg but not D-Arg. Our results suggest that the pressor effects of both L-NNA and D-NNA involve the L-Arg/nitric oxide pathway.

N^G-Nitro-L-arginine; N^G-Nitro-D-arginine; L-Arginine; D-Arginine; Pressor effects

1. Introduction

It is generally accepted that endothelium-derived relaxing factor (EDRF) is nitric oxide (NO) or NO-containing compound(s) (Ignarro et al., 1987; Palmer et al., 1987; Myers et al., 1990). NO is enzymatically synthesized from L-arginine (L-Arg) (Palmer et al., 1988a; Sakuma et al., 1988; Schmidt et al., 1988) and spontaneously released to maintain vascular tone (Kelm and Schrader, 1990). NO synthase and/or endothelium-dependent vascular relaxation response in isolated arterial preparations can be inhibited by L-Arg analogues which include N^g-monomethyl-L-arginine (L-MMA) (Palmer et al., 1988b; Rees et al., 1989a, 1990), N^G-nitro-L-arginine methyl ester (L-NAME) (Moore et al., 1990), N-iminoethyl-L-ornithine (L-NIO) (Rees et al., 1990) and N^G-nitro-L-arginine (L-NNA) (Ishii et al., 1990; Kobayashi and Hattori, 1990; Moore et al., 1990; Mülsch and Busse, 1990). In vivo studies show that L-MMA (Aisaka et al., 1989; Rees et al., 1989b; 1990; Whittle et al., 1989; Gardiner et al., 1990b,c; Kilbourn et al., 1990), L-NAME (Gardiner et al., 1990a,c,d), L-NIO (Rees et al., 1990) and L-NNA (Wang and Pang, 1990) caused pressor effects and bradycardia.

It has been reported that endothelial NO synthase is inhibited in an enantiomerically specific manner by Arg analogues. L but not the D enantiomers of MMA (Rees et al., 1989a; Crawley et al., 1990; Rees et al., 1990), NAME (Rees et al., 1990) and NIO (Rees et al., 1990) inhibited endothelial NO formation and/or endothelium-dependent relaxation. As well, L but not D enantiomers of MMA (Rees et al., 1989a,b; Whittle et al., 1989; Crawley et al., 1990; Persson et al., 1990; Rees et al., 1990), NAME (Rees et al., 1990) and NIO (Rees et al., 1990) contracted isolated arterial preparations or raised blood pressure in intact animals. It has also been reported that L but not D stereoisomers of MMA, NAME and NIO enhanced platelet aggregation (Radomski et al., 1990a,b; Persson et al., 1990). Moreover, L-NNA but not D-NNA prevented EDRF release from endothelial cells and inhibited the dilator effect of acetylcholine on rabbit femoral arteries (Mülsch and Busse, 1990) and, L-NNA but not D-NNA inhibited non-adrenergic, non-cholinergic relaxation of guinea pig isolated tracheal smooth muscle and rat

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anococcygeus (Hobbs and Gibson, 1990; Tucker et al., 1990). In contrast, results from our preliminary studies show that i.v. bolus of D-NNA into pentobarbital-anaesthetized rats also raised arterial pressure. It is not clear if the pressor response to D-NNA is related to the NO/EDRF system. In this study, we compare the effects of L-NNA and D-NNA on arterial pressure in conscious rats and examine the ability of L- and D-Arg to antagonize the pressor effects of L- and D-NNA.

2. Materials and methods

2.1. Measurement of optical rotation of Arg and NNA

L-Arg, D-Arg, L-NNA and D-NNA were dissolved in 2 M HCl. The specific rotation of the compounds were measured at 586 nm (D line of sodium) by a polarimeter (Optical Activity Polarimeter, model AA-1000).

2.2. Surgical preparations

Sprague-Dawley rats (250–400 g) were anesthetized with halothane (4% in air for induction and 2% in air for maintenance). A polyethylene cannula (PE50) was inserted into the left iliac artery for continuous measurement of mean arterial pressure (MAP) by a pressure transducer (P23DB, Gould Statham, CA, U.S.A.). Heart rate (HR) was determined electronically from the upstroke of the arterial pulse pressure using a tachograph (Grass, Model 7P4G). A cannula was inserted into the left iliac vein for the administration of test drugs. In some experiments, a cannula was also inserted into the right iliac vein for the infusion of L- or D-Arg. The rats were given 6 h recovery from the effects of surgery and anesthesia before further use.

2.3. Experimental protocols

2.3.1. Time course of L-NNA and D-NNA

The rats in Group I, II and III (n = 6 each) were given i.v. bolus of normal saline (0.9% NaCl, 8 ml/kg) or a single dose (32 mg/kg, 8 ml/kg) of L-NNA or D-NNA, respectively. MAP and HR were continuously monitored for approximately 3 h.

2.3.2. Dose-responses of L-NNA and D-NNA

Cumulative dose-response curve of L-NNA was constructed in Group IV (n = 6) at dose intervals of 10-15 min (time required to obtain plateau MAP response). With D-NNA, it was difficult to construct a complete dose-response curve in a single rat since the time required to obtain peak MAP varied between 20-90 min, with generally longer onsets of action for low doses of D-NNA. Therefore, 30 rats (Group V) were used to obtain a dose-response curve of D-NNA, with only one dose injected into each rat (n = 6 per dose). The MAP and HR responses to each dose were followed for 2 h.

2.3.3. Antagonistic effects of L-Arg and D-Arg on pressor response to L-NNA, D-NNA, noradrenaline and angiotensin II

Normal saline (0.9% NaCl), D-Arg (10 mg/kg per min) and L-Arg (10 mg/kg per min) were continuously i.v. infused into rats in Groups VI, VII and VIII, respectively. Prior to and 20 min after the start of the infusion of D-Arg in Group VII, noradrenaline (2.5 μg/kg) was i.v. bolus injected into the rats. While in Group VIII, angiotensin II (100 ng/kg) was i.v. bolus injected prior to and 20 min after the start of the infusion of L-Arg. In all three groups (VI, VII and VIII), a single dose of L-NNA (8 mg/kg) was i.v. bolus injected at 40 min after start of normal saline, D-Arg and L-Arg infusions, respectively. In Groups IX, X and XI, the protocol was similar to those in VI, VII and VIII except that angiotensin II (same dose as before) was given to Group XI, noradrenaline (5 μg/kg) was given to Group X and D-NNA (8 mg/kg) instead of L-NNA was given to all three groups. MAP was continuously monitored for 2 h after the injection of L-NNA or D-NNA.

2.4. Drugs

L-Arg HCl, D-Arg HCl, L-NNA, angiotensin II acetate and noradrenaline HCl were from Sigma Chemical Co. (MO, U.S.A.). D-NNA was from Bachem Bioscience Inc. (PA, U.S.A.). L-Arg and D-Arg were dissolved in distilled water and the pH of each solution was adjusted to 7.0 with 4 N NaOH solution. L- and D-NNA were solubilized in normal saline by 30 min sonication. The rest of the drugs were also dissolved in normal saline.

2.5. Calculations and statistics

MAP and HR readings at peak MAP responses to each dose of a pressor agent were noted. The ED_{50} value and maximum response (E_{max}) of L-NNA were obtained from individual dose-response curves. ED_{50} and E_{max} of D-NNA were obtained from the entire group of 30 rats by the method of Litchfield and Wilcoxon (1949). Rise phase $t_{1/2}$ values were obtained from individual time course curves. To obtain normal distribution of rise phase $t_{1/2}$, data were logarithmically transformed prior to statistical analysis. Results were analyzed by analysis of variance followed by Duncan's multiple range test with $P < 0.05$ selected as the criterion for statistical significance. All results were expressed as means ± S.E.M. except for the rise phase

$t_{1/2}$ which was expressed as mean ± 95% confidence range.

3. Results

3.1. Optical rotation of Arg and NNA

The specific rotations [α]D of L-NNA and D-NNA were +22.1° and −22.9° while those of L-Arg and D-Arg were +20.2° and −21.4°, respectively. Thus, the compounds are optically active with D-NNA and D-Arg levorotatory and L-NNA and L-Arg dextrorotatory.

3.2. Time course of L-NNA and D-NNA

The control values of MAP and HR in Groups I, II and III were summarized in table 1. I.v. bolus of L-NNA (32 mg/kg) into conscious rats caused a sustained increase in MAP which reached plateau response at approximately 10 min after injection, with rise phase $t_{1/2}$ of 5 min (95% confidence range of 2-12 min). MAP at 80-160 min was lower than that at 40 min, but was still significantly higher than control MAP. I.v. bolus of D-NNA (32 mg/kg) also increased MAP to a similar plateau value, but the onset of the response was significantly slower than that of L-NNA. Plateau MAP was reached approximately 50 min after injection and remained at the steady state level for > 120 min (fig. 1A). The rise phase $t_{1/2}$ was 27 min (95% confidence range of 15-48 min), which was significantly longer than that of L-NNA. Both L-NNA and D-NNA caused bradycardia during the rise in MAP. A biphasic HR response was only observed with L-NNA whereby during the recovery of MAP, HR continued to rise to a level above control HR even when MAP was still elevated above control level (fig. 1B). I.v. bolus of the same volume of normal saline did not significantly alter MAP nor HR throughout the entire study period.

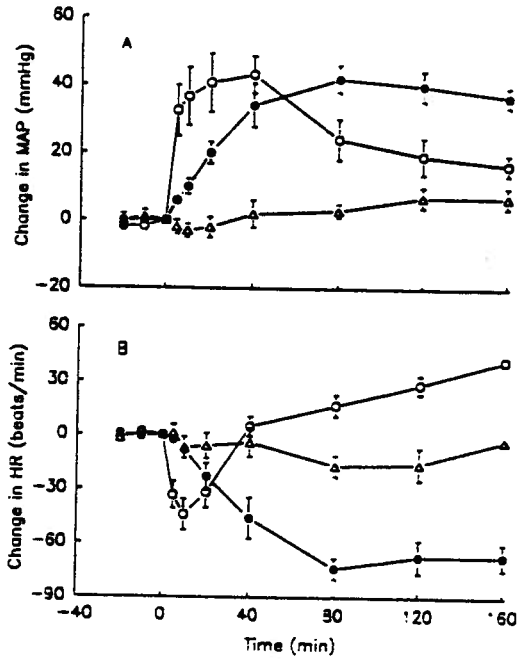

Fig. 1. The effects (means ± S.E.M.) of i.v. bolus of normal saline (0.9% NaCl, open triangles), N^G-nitro-L-arginine (open circles, 32 mg/kg) and N^G-nitro-D-arginine (closed circles, 32 mg/kg) on mean arterial pressure (MAP) and heart rate (HR) in conscious rats. N = 6 per group.

TABLE 1

Baseline values (means ± S.E.M.) of mean arterial pressure (MAP) and heart rate (HR) in rats from Group I to XI. MAP was also measured 20 min after start of i.v. infusion of normal saline (Groups VI and IX), D-Arg (10 mg/kg per min, Groups VII and X) or D-Arg (10 mg/kg per min, Groups VIII and XI)

Group	n	MAP (mm Hg)		HR (beats/min)
		Before	After	
I	6	103 ± 3	-	370 ± 9
II	6	108 ± 4	-	338 ± 11
III	6	103 ± 2	-	391 ± 6
IV	6	111 ± 4	-	400 ± 10
V	30	107 ± 1	-	386 ± 5
VI	6	103 ± 3	102 ± 3	-
VII	6	111 ± 3	109 ± 2	-
VIII	6	108 ± 3	111 ± 2	-
IX	6	113 ± 5	112 ± 3	-
X	6	104 ± 4	103 ± 3	-
XI	6	104 ± 4	104 ± 2	-

3.3. Dose-response of L-NNA and D-NNA

The control MAP and HR values in Groups IV and V are summarized in table 1. I.v. bolus of both L-NNA and D-NNA dose dependently increased MAP to similar maximum values (fig. 2A). The time taken for different doses of L-NNA to reach peak MAP response ranged from 10 to 20 min while time (min) taken to reach peak effect in response to D-NNA were: 56 ± 8 (for 4 mg/kg), 49 ± 5 (8 mg/kg), 71 ± 8 (16 mg/kg), 53 ± 9 (32 mg/kg) and 38 ± 4 (64 mg/kg). The ED_{50} of L-NNA (4.0 ± 0.9 mg/kg) was significantly less than that of D-NNA (8.9 ± 1.2 mg/kg). The E_{max} of L-NNA (55 ± 7 mm Hg) and D-NNA (52 ± 4 mm Hg) were similar. Both L-NNA and D-NNA reduced HR at the rise phase of the pressor responses.

3.4. Antagonist effects of L-Arg and D-Arg on the pressor responses to L-NNA and D-NNA

Control MAP in Groups VI–XI are shown in table 1. I.v. infusions of normal saline, L-Arg (10 mg/kg per min) and D-Arg (10 mg/kg per min) did not alter

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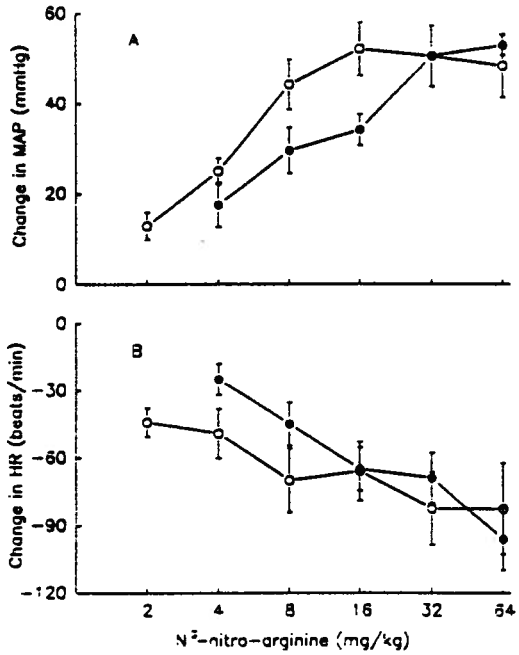

Fig. 2. Dose-response curves (means ± S.E.M.) of i.v. bolus of N^G-nitro-L-arginine (open circles) and N^G-nitro-D-arginine (closed circles) on mean arterial pressure (MAP) and heart rate (HR) in conscious rats. N = 6 each point.

TABLE 2

Mean arterial pressure (MAP) responses (means ± S.E.M.) to i.v. bolus of noradrenaline (NA) and angiotensin II (AII) before (a) and 20 min after (b) i.v. infusion (10 mg/kg per min) of L-arginine (L-Arg) or D-arginine (D-Arg) in conscious rats (n = 6 per group)

	Increase in MAP (mm Hg)	
	a	b
L-Arg		
NA (2.5 μg/kg)	30 ± 4	32 ± 1
AII (100 ng/kg)	48 ± 3	42 ± 3
D-Arg		
NA (5 μg/kg)	58 ± 4	58 ± 3
AII (100 ng/kg)	39 ± 3	40 ± 4

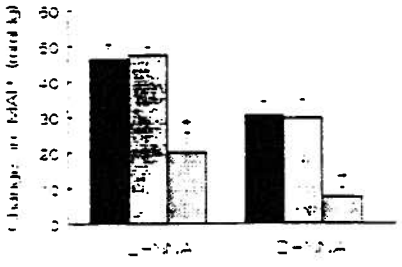

Fig. 3. Effects (means ± S.E.M.) of i.v. infusions of D-arginine (10 mg/kg per min; filled columns), normal saline (0.9% NaCl, shaded columns) and L-arginine (10 mg/kg per min; cross-hatched columns) on pressor responses to i.v. bolus N^G-nitro-L-arginine (L-NNA, 8 mg/kg) and N^G-nitro-D-arginine (D-NNA, 8 mg/kg) in conscious rats. N = 6 per group. * Denotes significant difference from the normal saline group ($P < 0.05$).

baseline MAP (table 1). L-Arg and D-Arg also did not alter MAP effects of noradrenaline or angiotensin II (Table 2). L-Arg attenuated the pressor effects of L-NNA and D-NNA while D-Arg did not alter the MAP effect of L-NNA nor D-NNA (fig. 3).

4. Discussion

Our results show that both L-NNA and D-NNA raise MAP in conscious rats. Pharmacokinetic differences (rise phase $t_{1/2}$ and ED_{50}) which exist between the MAP effects of L- and D-NNA show that D-NNA is a slower and less potent pressor agent than L-NNA. The results here are in contrast with those of in vitro studies which show D-NNA to be ineffective in causing contraction of smooth muscles (Mülsch and Busse, 1990; Hobbs and Gibson, 1990; Tucker et al., 1990). It has also been shown that other D-Arg analogues, namely D-MMA (Rees et al., 1989a,b; Whittle et al., 1989; Crawley et al., 1990; Persson et al., 1990; Rees et al., 1990). D-NAME (Rees et al., 1990) and D-NIO (Rees et al., 1990) are ineffective in causing contractions in both in vitro and in vivo preparations.

L-Arg did not modify the pressor effect of noradrenaline nor angiotensin II in conscious rats. These results are consistent with those which show that L-Arg did not attenuate the pressor effects of noradrenaline nor angiotensin II in pentobarbital-anesthetized guinea pigs (Aisaka et al., 1989) nor vasopressin in conscious rats (Gardiner et al., 1990b). We have previously reported that i.v. bolus of L-Arg attenuated the pressor effect of L-NNA in pentobarbital-anesthetized rats (Wang and Pang, 1990). In the present study, i.v. infusion of L-Arg but not D-Arg significantly attenuated the pressor effect of L-NNA. This is consistent with reports that L-NNA is antagonized by L-Arg but not D-Arg in vitro (Moore et al., 1990; Mülsch et al., 1990). The results are also consistent with observations that L-MMA can be antagonized by L-Arg but not D-Arg in vitro (Rees et al., 1989a) and in vivo (Rees et al., 1989b; Whittle et al., 1989; Gardiner et al., 1990a; Persson et al., 1990). In contrast to the results which show stereospecificity of L-Arg to antagonize the pressor effect of L-NNA, the pressor effect of D-NNA is antagonized by L-Arg but not D-Arg. Therefore, although pharmacokinetic differences in onset and potency of actions exist between the pressor effects of L- and D-NNA, the pressor responses of both compounds are antagonized by L-Arg. It is of great interest that L-Arg, NO pathway can be inhibited enantiomerically non-specifically by both L and D enantiomers of NNA.

We conclude here that both L-NNA and D-NNA are efficacious pressor agents in conscious rats. D-NNA has slower onset and longer duration of action and less potency than L-NNA. The pressor responses to L-NNA

and D-NNA can be specifically attenuated by L-Arg but not D-Arg. Our results suggest that the pressor effects of both L-NNA and D-NNA involve the L-Arg NO pathway.

Acknowledgements

The authors appreciate the assistance of Dr. Kenneth Curry (Department of Physiology, University of British Columbia) for the measurement of optical rotations of L-NNA, D-NNA, L-Arg and D-Arg. This work was supported by the Medical Research Council of Canada.

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Paper III

Wang, Y.-X., Poon, C.I. and Pang, C.C.Y.: *In vitro* and *ex vivo* inhibitory effects of L and D enantiomers of N^G-nitro-arginine on endothelium-dependent relaxation of rat aorta. J. Pharmacol. Exp. Ther. 265: 112-119, 1993. The reproduction of this paper was kindly permitted by the copyright holder, Willams & Wilkins, Baltimore, U.S.A.

0022-3565/93/2651-0112$03.00/0
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Vol. 265, No. 1
Printed in U.S.A.

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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Accepted for publication November 30, 1992

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),

Received for publication January 24, 1992.
[1] This work was supported by a grant from the Medical Research Council of Canada (MRC) and a MRC fellowship to Y.-X. W.

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_{50}) 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^{-6} 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^{-6} 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^{-4} M) and D-NNA (3×10^{-4} 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^{-4} 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^{-4} M) or D-NNA (3×10^{-4} 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

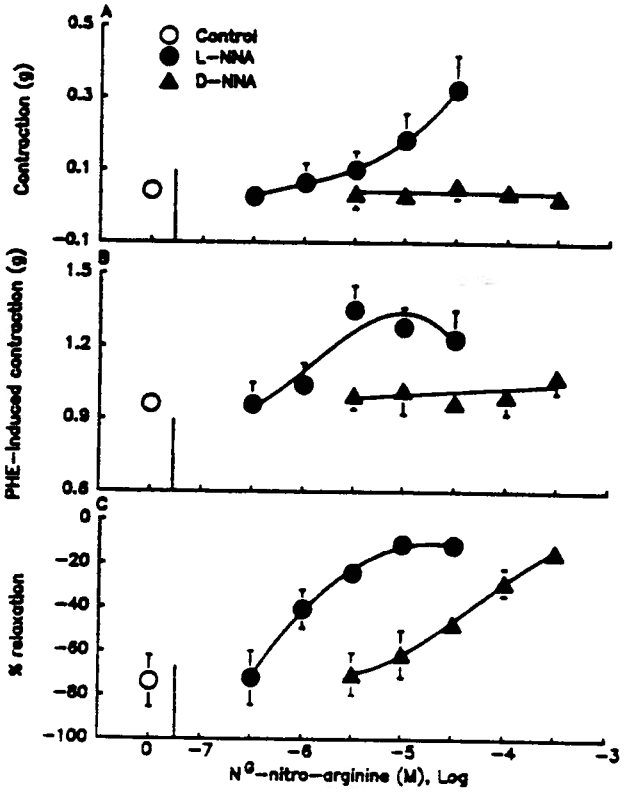

Fig. 1. Effects (mean ± S.E.) of L- and D-NNA on resting tone of aortic rings (A), on PHE (10^{-6} M)-induced contraction (B) and on ACh (3×10^{-5} M)-induced relaxation in PHE-preconstricted aortic rings (C) (n = 6–7 in each group).

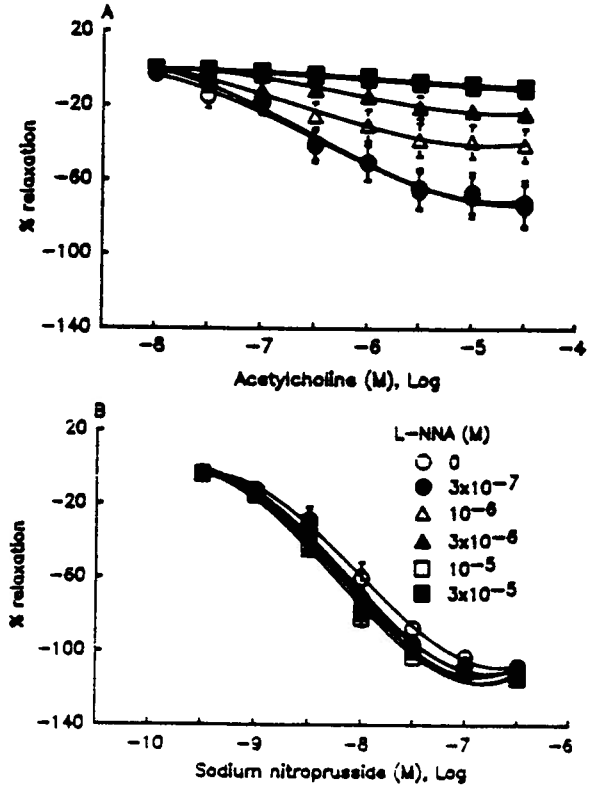

Fig. 2. Concentration-response (mean ± S.E.) of L-NNA on ACh (A)- and sodium nitroprusside (B)-induced relaxations in PHE (10^{-6} M)-preconstricted aortic rings (n = 6–7 in each group).

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^{-6} 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^{-6} 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).

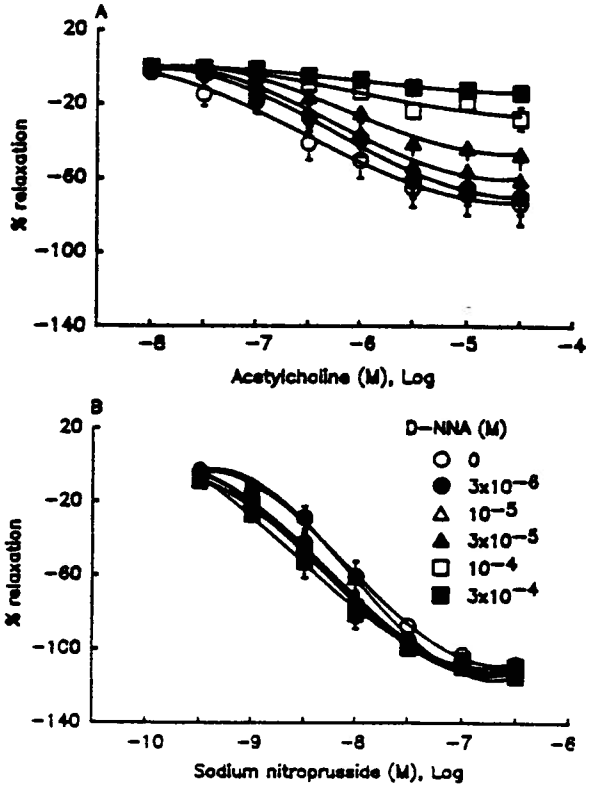

Fig. 3. Concentration-response (mean ± S.E.) of D-NNA on ACh (A)- and sodium nitroprusside (B)-induced relaxations in PHE (10^{-6} M)-preconstricted aortic rings (n = 6–7 in each group).

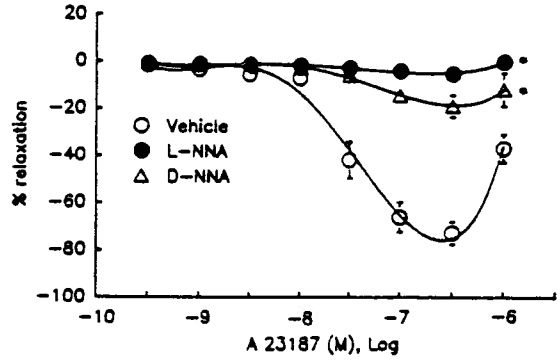

Fig. 4. Inhibitory effects on L-NNA (3×10^{-5} M) and D-NNA (3×10^{-4} M) on A 23187-induced relaxation in PHE (10^{-6} M)-preconstricted aortic rings (n = 6–7 in each group). *Significant difference from the control curve ($P < .05$).

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

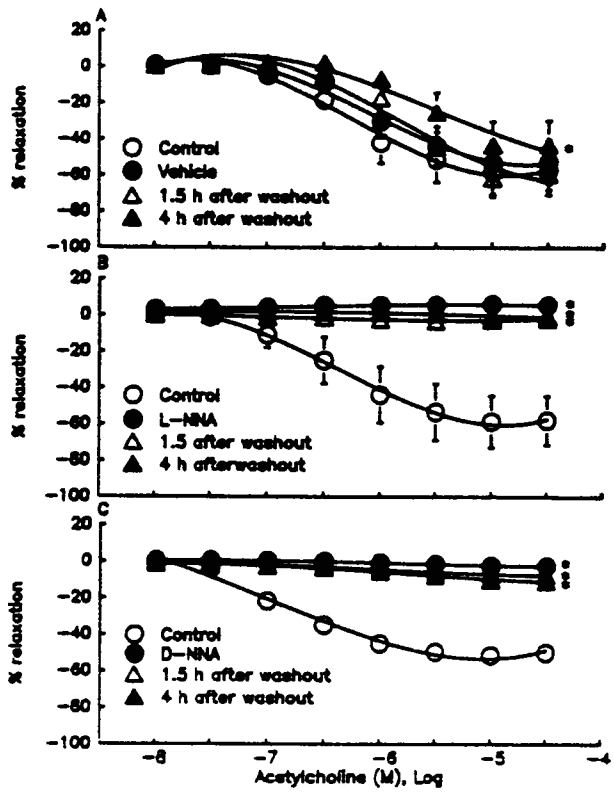

Fig. 5. Time course of the effects (mean ± S.E.) of vehicle (A), L-NNA (3×10^{-5} M, B) and D-NNA (3×10^{-4} M, C) on ACh-induced relaxation responses in PHE (10^{-6} M)-preconstricted rat aortic rings (n = 6–7 in each group). *Significant difference from the control curve ($P < .05$).

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.*,

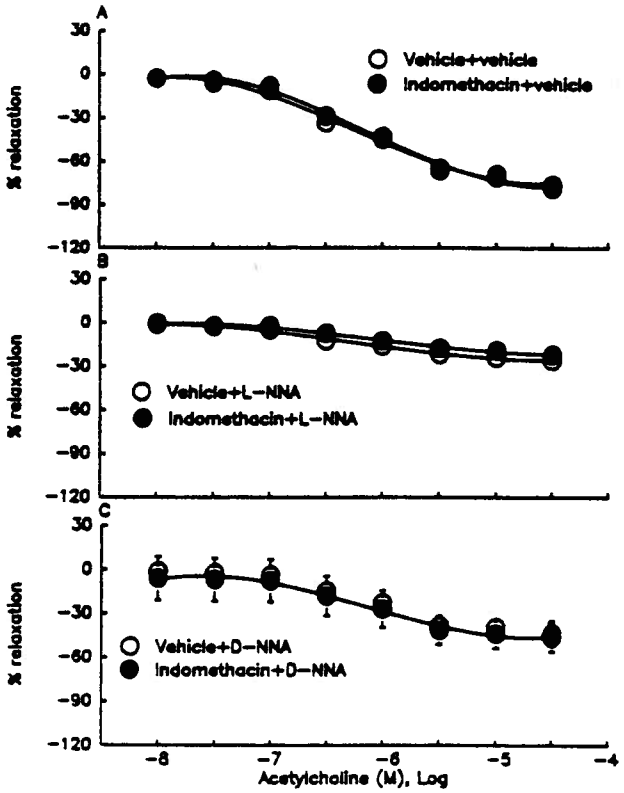

Fig. 6. Effects (mean ± S.E.) of a 10-min pretreatment with indomethacin (10^{-5} M) on ACh-induced relaxations in the absence (A) and presence of L-NNA (10^{-6} M, B) or D-NNA (3×10^{-5} M, C) in PHE (10^{-6} M)-preconstricted rat aortic rings (n = 6–7 in each group).

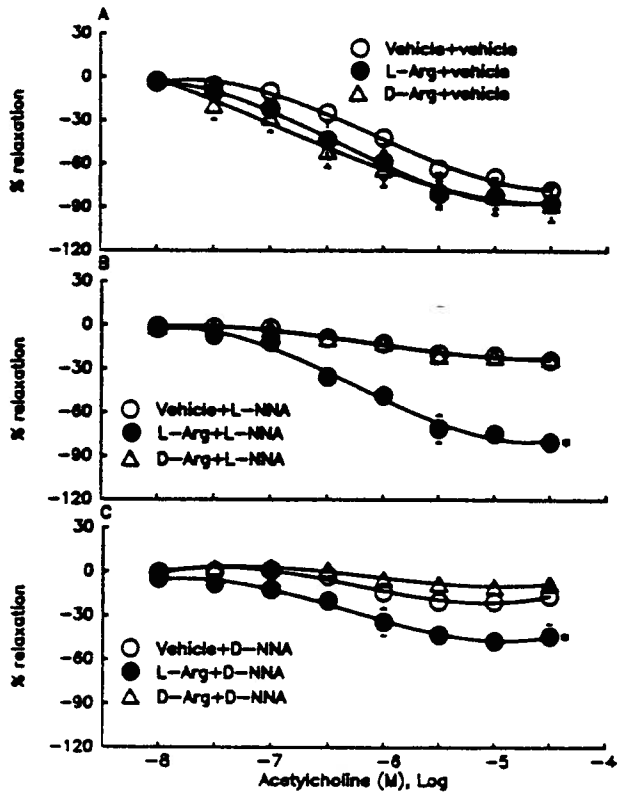

Fig. 7. Effects (mean ± S.E.) of a 10-min pretreatment with L-Arg (10^{-3} M) or D-Arg (10^{-3} M) on ACh-induced relaxations in the presence of vehicle (A), L-NNA (10^{-6} M, B) or D-NNA (3×10^{-5} M, C) in PHE (10^{-6} M)-preconstricted rat aortic rings (n = 6–7 in each group). *Significant difference 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_{80})-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^{-4} M) should have caused considerably less response in the rabbit femoral arteries. The potencies of N^G-substituted L-Arg analogs

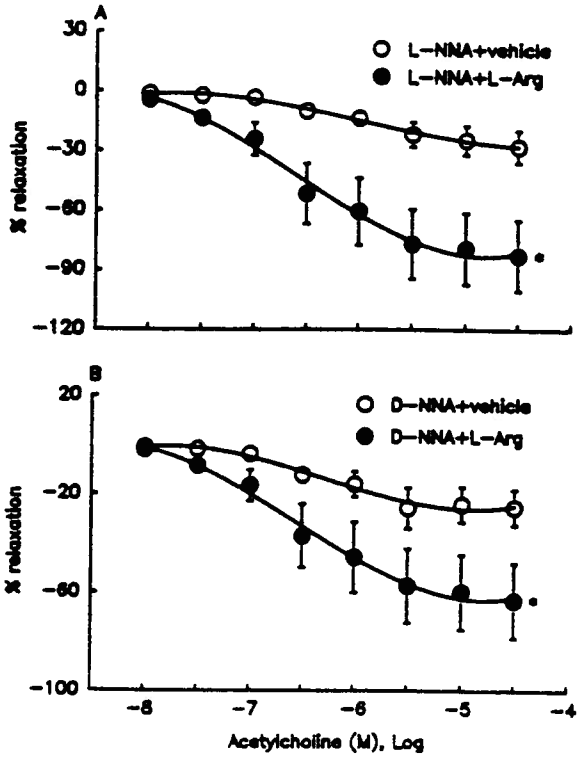

Fig. 8. Effects (mean ± S.E.) of post-treatment (1 hr) with L-Arg (10^{-3} M) on the inhibitory effects of L-NNA (10^{-4} M, A) and D-NNA (3×10^{-4} M, B) on ACh-induced relaxation in PHE (10^{-6} M)-preconstricted rat aortic rings (n = 6–7 in each group). The rings were incubated for 1 hr with L- or D-NNA followed by a 10-min treatment with L-Arg or vehicle. *Significant difference from control curve ($P < .05$).

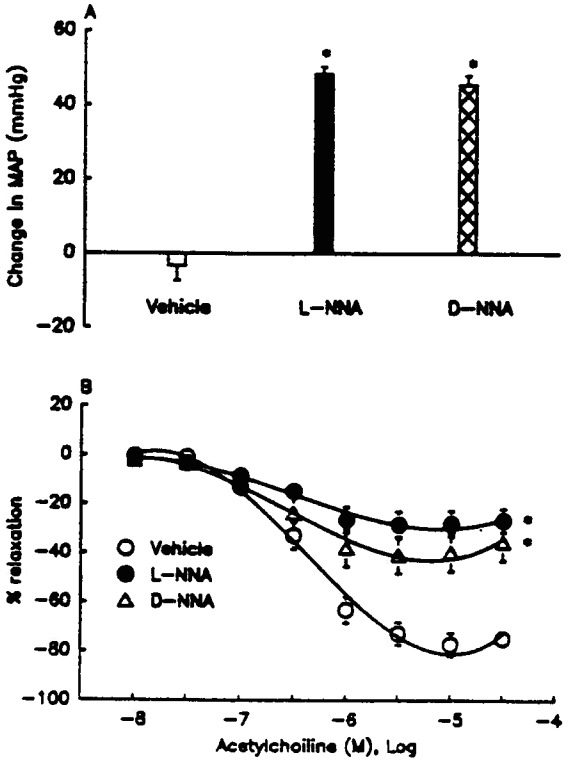

Fig. 9. Effects (mean ± S.E.) of i.v. bolus injections of vehicle, L-NNA (1.6×10^{-4} mol/kg) and D-NNA (1.6×10^{-4} mol/kg) on MAP (A) and *ex vivo* relaxation responses to ACh (B) in PHE (10^{-6} M)-preconstricted aortic rings from the treated rats (n = 5 in each group). *Significant difference from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

Acknowledgments

The authors would like to thank Ms. Su Lin Lim for her skilled technical assistance.

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Paper IV

Wang, Y.-X. and Pang, C.C.Y.: Functional integrity of the central and sympathetic nervous systems is a prerequisite for pressor and tachycardic effects of diphenyleneiodonium, a novel inhibitor of nitric oxide synthase. J. Pharmacol. Exp. Ther. 265: 263-272, 1993. The reproduction of this paper was kindly permitted by the copyright holder, Willams & Wilkins, Baltimore, U.S.A.

0022-3565/93/2661-0263$03.00/0
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Vol. 265, No. 1
Printed in U.S.A.

Functional Integrity of the Central and Sympathetic Nervous Systems is a Prerequisite for Pressor and Tachycardic Effects of Diphenyleneiodonium, a Novel Inhibitor of Nitric Oxide Synthase[1]

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Accepted for publication November 23, 1992

ABSTRACT

The pressor and tachycardic effects of diphenyleneiodonium (DPI), a novel inhibitor of endothelial nitric oxide synthase with chemical structure different from those of N^G-substituted Arg analogs, were studied in pentobarbital-anesthetized rats. Bolus injections of DPI (0.05–1.6 mg/kg i.v.) caused transient (1–2 min in duration) and dose-dependent increases in mean arterial pressure (MAP) with ED_{50} of 0.22 ± 0.02 mg/kg and maximum effect (E_{max}) of 58 ± 3 mm Hg, and heart rate (HR) with ED_{50} of 0.26 ± 0.03 mg/kg and E_{max} of 60 ± 5 beats/min. Pretreatments with tetrodotoxin, reserpine, guanethidine, mecamylamine, but not atropine, rauwolscine, captopril nor L-Arg, attenuated the MAP and HR responses to DPI. Phentolamine and prazosin attenuated the MAP but not HR response whereas propranolol attenuated the HR but not MAP response of DPI. Pithing abolished, whereas spinal cord transection reduced, the MAP and HR responses to DPI. Pithing did not alter the pressor response but blocked the reflex bradycardic response to N^G-nitro-L-arginine methyl ester, an inhibitor of nitric oxide synthase. Bolus injection of a single dose of DPI (1.6 mg/kg i.v.) or N^G-nitro-L-arginine increased MAP, but only DPI caused immediate and large increases (>1 ng/ml) in plasma norepinephrine, epinephrine and moderate increase in dopamine; pretreatment with reserpine attenuated, whereas pithing abolished these increases. The increases in plasma norepinephrine and epinephrine by DPI were positively correlated to increases in MAP and HR. The results demonstrate that DPI, unlike N^G-substituted Arg analogs, produces pressor and tachycardic effects *via* indirect activation of the sympathetic nervous system.

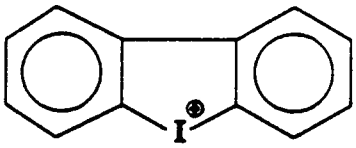
The first class of inhibitors of nitric oxide (NO) synthase are the N^G-substituted Arg analogs, which include L-NMMA, L-NNA, L-NAME, L-NIO and L-NAA (see Moncada *et al.*, 1991). These compounds suppress *in vitro* endothelium-dependent relaxation and produce prolonged pressor and bradycardic responses in whole animals (Aisaka *et al.*, 1989; Rees *et al.*, 1989, 1990; Wang and Pang, 1990a, b; Wang *et al.*, 1991a, b; Wang *et al.*, 1992, 1993a,b). The pressor responses induced by these agents are antagonized by L-Arg (Aisaka *et al.*, 1989; Rees *et al.*, 1989; Wang *et al.*, 1990b, 1991b, 1992) but are insensitive to inhibitions of activities of the central nervous system (Tabrizchi and Triggle, 1992), sympathetic nervous system (Wang and Pang, 1991; Aisaka *et al.*, 1989; Rees *et al.*, 1989) and renin-angiotensin system (Wang and Pang, 1991). The pressor response has been attributed to the inhibition of the L-Arg/NO pathway which leads to endothelium-dependent relaxation (see Moncada *et al.*, 1991); the bradycardic response is shown to be mediated by baroreflex mechanisms (Wang and Pang, 1991; Widdop *et al.*, 1992).

DPI is a bivalent iodine compound. Its chemical structure is distinct from those of N^G-substituted Arg analogs (fig. 1). DPI was first reported to be a potent hypoglycemic agent (Stewart and Hanly, 1969; Gatley and Martin, 1979). It was later shown to suppress activities of neutrophil and macrophage NADPH-dependent oxidase (Cross and Jones, 1986; Ellis *et al.*, 1989), probably by the inhibition of a flavoprotein (Hancock and Jones, 1987; Ellis *et al.*, 1989). Recently, DPI and its analogs were reported to inhibit macrophage NO synthase (Stuehr *et al.*, 1991). This is not surprising because NO synthase is also a NADPH-dependent enzyme which requires FAD as a cofactor (Stuehr *et al.*, 1989, 1990). DPI caused long-lasting suppression of endothelium-dependent relaxation in rabbit (Stuehr *et al.*, 1991) and rat (Poon *et al.*, 1993) aortae and inhibition of endothelium-dependent vasodilatation *in vivo* (Poon *et al.*, 1993).

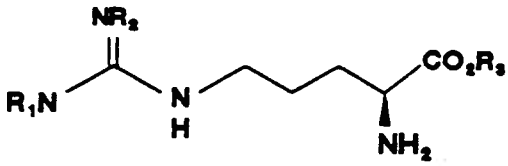
Although the *in vivo* cardiovascular effects of N^G-substituted Arg analogs have been intensely investigated (see Moncada *et*

Received for publication July 13, 1992
[1] This work was supported by a grant from the Medical Research Council of Canada (MRC) and a MRC Postdoctoral Fellowship to Y.-X.W.

ABBREVIATIONS: NO, nitric oxide; L-NMMA, N^G-monomethyl-L-arginine; L-NNA, N^G-nitro-L-arginine; L-NAME, N^G-nitro-L-arginine methyl ester; L-NIO, L-iminoethylornithine; L-NAA, N^G-amino-L-arginine; DPI, diphenyleneiodonium; MAP, mean arterial pressure; TTX, tetrodotoxin; E_{min}, minimum effect; E_{max}, maximum effect; n_H, Hill coefficient; HR, heart rate; D, dose; C, concentration.



diphenyleneiodonium



R_1=H_2, R_2=H, R_3=H: L-Arg
R_1=HCH_3, R_2=H, R_3=H: L-NMMA
R_1=H_2, R_2=NO_2, R_3=H: L-NNA
R_1=H_2, R_2=NO_2, R_3=CH_3: L-NAME
R_1=H_2, R_2=NH_2, R_3=H: L-NAA

N^G-substituted arginine analogues

Fig. 1. Chemical structures of DPI and N^G-substituted arginine analogs. L-NMMA, L-NNA, L-NAME, L-NIO and L-NAA.

al., 1991), those of DPI have not been studied for the past 20 years or more. As an inhibitor of NO synthase, DPI is expected to inhibit endothelium-dependent vasodilatation thereby causing pressor response and reflex bradycardia *in vivo*. The aim of this study was to examine the effects of DPI on blood pressure and heart rate, to elucidate its mechanism of actions and, to compare its actions with those of N^G-substituted Arg analogs.

Materials and Methods

Surgical Preparation

Sprague-Dawley rats (300–350 g) were anesthetized with sodium pentobarbital (65 mg/kg i.p.). A polyethylene cannula (PE50) was inserted into the left iliac artery for the measurement of MAP by a P23DB pressure transducer (Gould Statham, CA). HR was determined electronically from the upstroke of the arterial pulse pressure using a tachograph (Grass, model 7P4G). Another PE50 cannula was inserted into the left iliac vein for the administration of drugs. In some rats, a PE50 cannula was also inserted into the right iliac artery to collect blood samples. The body temperature of the rats was maintained at 37°C with a heating lamp connected to a thermostat (73A, Yellow Springs Instruments).

In TTX-pretreated, pithed, spinal cord-transected rats and their control rats, tracheostomy was also performed to allow artificial ventilation with 100% oxygen at 54 strokes/min and a stroke volume of 3 to 4 ml (1 ml/100 g b.w.). Pithing was performed through the orbit with a 3-mm diameter stainless steel rod. The spinal cord was severed by a pair of sharp scissors at the T_1 level. All experiments were conducted 20 min after surgery.

Measurement of Plasma Catecholamines

Plasma catecholamines were measured by a catecholamine assay kit (Amersham Canada Ltd., Ontario, Canada). The principles of the assay was described by Passon and Peuler (1973). Briefly, catechol-O-methyltransferase was used to catalyze the transfer of a [^{3}H]methyl group from *S*-adenosyl-L-[methyl-^{3}H]-methionine to the hydroxyl group in the 3-position of norepinephrine, epinephrine and dopamine. The resultant products were separated by thin layer chromatography, eluted if necessary, and counted by a 1600 TR liquid scintillation analyzer (Packard Instrument Co., CT).

Blood samples (0.5 ml) were immediately inserted into precooled tubes containing EGTA and reduced glutathione and centrifuged at 1,200 × *g* at 4°C. Afterward, the plasma was removed and stored at −70°C until assayed within a month. Duplicate assays were run for the standard, plasma or diluted plasma samples (50 μl each sample) with distilled water used as a blank control for each run. The standard curves for norepinephrine, epinephrine and dopamine (0.01, 0.03, 0.1, 0.3, 1, 3 and 10 ng/ml for each standard solution) were prepared with control rat plasma. Two standard curves were constructed at two separate occasions and were found to be indistinguishable from each other. The data were combined to formulate the following linear regression equations for norepinephrine, epinephrine and dopamine: $Y = 8.03x + 29.5$ ($r = 0.998$; $P < .05$); $Y = 2.49x - 14.1$ ($r = 0.999$; $P < .05$) and $Y = 2.99x + 10.2$ ($r = 0.999$; $P < .05$), respectively. The sensitivity of the catecholamine assay was 0.005 ng/ml.

Drugs

The following drugs were purchased from Sigma Chemical Co. (St. Louis, MO): mecamylamine hydrochloride, atropine sulfate, D-L-propranolol hydrochloride, L-NNA, L-NAME hydrochloride, L-Arg hydrochloride, guanethidine hydrochloride, rauwolscine hydrochloride and prazosin hydrochloride. The following drugs were also used: DPI sulfate (Colour Your Enzyme Ltd., Ontario, Canada), phentolamine hydrochloride (CIBA Pharmaceutical Co., NJ), captopril (E. R. Squibb & Sons Inc., NJ), reserpine injection (CIBA Pharmaceutical Co., Quebec, Canada) and TTX (Sankyo Co. Ltd., Tokyo, Japan). The powder drugs were dissolved in normal saline (0.9% NaCl solution) except for DPI and L-NNA which were solubilized in 5% glucose solution by 10 min of sonication and prazosin which was dissolved in 100% dimethyl sulfoxide.

Experimental Protocol

The rats ($n = 6$ in each group except for one group with $n = 7$ as indicated) were randomly assigned into groups. The MAP and HR responses were continuously monitored throughout the experiments.

Protocol 1: Dose-responses of DPI on MAP and HR. Effects of i.v. bolus injections of vehicle (5% glucose solution, up to 2 ml/kg) and DPI (0.05–1.6 mg/kg) on MAP and HR were examined in one group of rats. The intervals of doses were 3 to 6 min, which was required for the rats to recover completely from the effects of the previous dose.

Protocol 2: Effects of antagonists on the MAP and HR responses of DPI. Eleven groups of rats were pretreated with vehicle (normal saline, 1 ml/kg), reserpine (5 mg/kg), mecamylamine (10 mg/kg), guanethidine (10 mg/kg), phentolamine (10 mg/kg), prazosin (1 mg/kg), rauwolscine (1 mg/kg), propranolol (1 mg/kg), atropine (10 mg/kg), captopril (20 mg/kg) or L-Arg (400 mg/kg). The doses selected for the antagonists (including TTX, see protocol 3) were those previously shown to block effectively the corresponding receptors, enzymes or ionic channels (Abraham *et al.*, 1989; Tabrizchi and Pang, 1986; Wang and Pang, 1990a; Wang and Pang, 1991). The drugs were i.v. bolus injected 10 min before the construction of the dose-response curve of DPI except for reserpine which was i.p. injected 24 h earlier.

Protocol 3: Effects of TTX, pithing and spinal cord transection (T_1) on the MAP and HR responses of DPI. Dose-response curves of i.v. bolus injections of DPI (0.05–1.6 mg/kg) were constructed in four groups of rats, namely, ventilated control, TTX-pretreated (10 μg/kg), pithed and spinal cord-transected (T_1) rats. TTX was i.v. bolus injected 10 min before the construction of the dose-response curve of DPI. A single dose of norepinephrine (8 μg/kg) was also i.v. bolus injected into the TTX-pretreated and pithed rats 5 min after the last dose of DPI.

Another two groups of pithed rats were i.v. bolus injected with cumulative doses of L-NAME (0.2–12.8 mg/kg) or equal volumes of normal saline at dose-intervals of 15 min, the time required to attain plateau responses of L-NAME.

Protocol 4: Effects of DPI on plasma catecholamines. Three groups of intact rats were i.v. bolus injected with vehicle (5% glucose solution, 1 ml/kg), DPI (1.6 mg/kg) or L-NNA (16 mg/kg). One group of pithed rats and one group of reserpinized (5 mg/kg i.p., 24 h previously) rats (n = 7) were also i.v. bolus injected with DPI (1.6 mg/kg). Blood samples (0.5 ml) were withdrawn over 15 s from the iliac arterial catheter into 1-ml syringes 20 min before and 30 s after the injection of vehicle or DPI or 40 min after the injection of L-NNA. All blood samples removed were replaced with i.v. injection of an equal volume of normal saline.

Calculation and Statistical Analysis

The parameters, *i.e.*, E_{min}, E_{max}, ED_{50} or EC_{50} and n_H were calculated from individual dose-MAP and dose-HR curve of DPI using a program executed on an IBM-compatible microcomputer. To determine these parameters, values of response (Y, rise in MAP or HR) at various D or C were fitted by nonlinear least-squares to the relation $Y = a + bx$, where Y = response and $X = [D]^{n_H}/(ED_{50}^{n_H} + [D]^{n_H})$ or $X = [C]^{n_H}/(EC_{50}^{n_H} + [C]^{n_H})$ with n_H fixed at integral values (1, 2, 3, 4 and 5), and repeated with n_H "floating" to obtain a best fit (Quastel and Saint, 1988). This gave the value of ED_{50} or EC_{50} yielding a minimum residual sum of squares of deviations from the theoretical curve. This was preferred to the more usual fit to $Y = bx$, in order to take into account the possible systematic underestimate or overestimate of MAP or HR corresponding to [D] or [C] = 0; the data set was augmented by 20 points with Y = 0 at [D] or [C] = 0. Usually, the reduction in minimum residual sum of squares obtained by floating n_H was not significant in the sense that the reduction (from that obtained with the nearest integral value of n_H) was no more than expected from the reduction in degrees of freedom (by Fisher's test). With this fitting the maximum response to [D] or [C] is given by b; values of a at the best fit were never significantly different from 0.

For linear least-squares regression to fit response $Y = a + bx$ where x = [C], the data set was also augmented by 20 points with response Y = 0 at [C] = 0.

All results were expressed as mean ± S.E. and analyzed by the analysis of variance followed by Duncan's Multiple Range test by Number Cruncher Statistical System Program (Dr. J. L. Hintze, Kaysville, UT), with $P < .05$ selected as the criterion for statistical significance.

Results

Effects of DPI on MAP and HR. The i.v. bolus injections of vehicle in rats did not alter MAP or HR (data not shown). Bolus injections of DPI (0.05–1.6 mg/kg i.v.) caused immediate and transient pressor and tachycardic responses as shown in a typical experimental tracing from a rat (fig. 2). The duration of the pressor response lasted approximately 1 to 2 min, whereas that of tachycardic response was slightly longer (fig. 2). At 0.8 mg/kg, half-rise time for the pressor and tachycardic responses were 2.9 ± 0.2 and 4.9 ± 0.7 s ($P < .05$), whereas the corresponding half-fall times were 31 ± 3 and 60 ± 9 s ($P < .05$), respectively.

Pooled (n = 12) base-line MAP and HR from the above group and the control group in protocol 2 were 104 ± 3 mm Hg and 347 ± 10 beats/min, respectively. The pressor and tachycardic response curves of DPI were dose-dependent and notably "steep," with negligible effect at 0.05 to 0.1 mg/kg, large increases in MAP at 0.2 and 0.4 mg/kg and maximum effect at 0.8 mg/kg (fig. 3). The best-fitted n_H for MAP and HR were 3.6 ± 0.3 and 4.2 ± 0.6, respectively. These two values were not significantly different from each other or from 4, but different from 1, 2, 3 and 5, though all values of n_H, best-fitted (3.6, 4.2) or selected (1, 2, 3, 4 and 5), were statistically significant ($P < .05$). The theoretical dose-response curves for integral values of n_H are shown in figure 3. E_{min} and E_{max} calculated from dose-MAP and dose-HR curves at the best-fitted n_H were not significantly different from those observed; other values of n_H gave significant differences of calculated parameters from those observed (E_{min} and E_{max}) or derived by averaging parameters obtained using best-fitting n_H for each dose-response curve (table 1). Correlation between observed data points and theo-

Fig. 2. Typical tracings of blood pressure (BP) and HR responses after i.v. bolus injections (shown by arrows) of DPI (0.2, 0.4 and 0.8 mg/kg) in a pentobarbital-anesthetized rat.

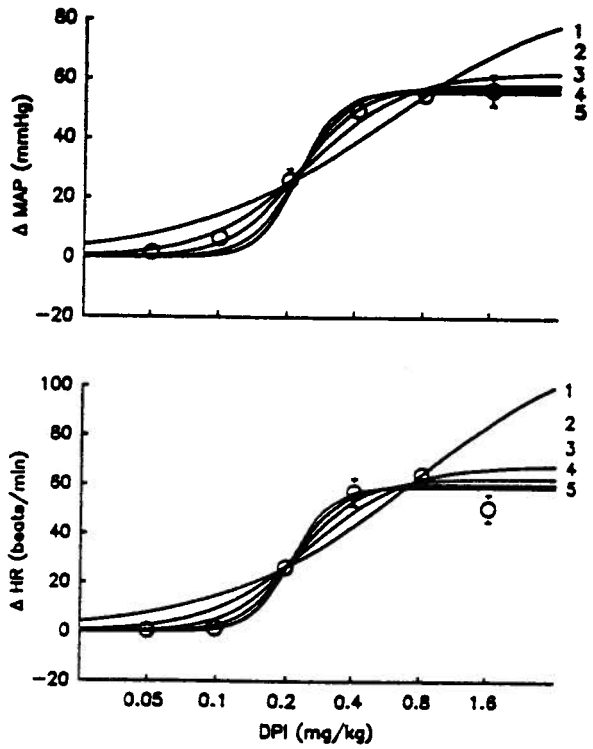


Fig. 3. Dose-response curves (mean ± S.E.) of MAP and HR after i.v. bolus injections of DPI in pentobarbital-anesthetized rats (n = 12). The theoretical lines were calculated using $Y = E_{max}*([D]^{n_H}/(ED_{50})^{n_H} + [D]^{n_H})$ with n_H = 1, 2, 3, 4 or 5 as shown in the figure. The corresponding ED_{50} and E_{max} for the curves are shown in table 1.

TABLE 1

Values (mean ± S.E.) of n_H, correlation coefficient (r) expressed as 1000 $(1 - r)$, E_{min}, E_{max} and ED_{50} of the dose-MAP and dose-HR response curves after i.v. bolus injections of DPI (0.05–1.6 mg/kg)) in pentobarbital-anesthetized rats (n = 12). The parameters were calculated from individual dose-response curves using the formula $Y = a + bx$ where $x = [D]^{n_H}/(ED_{50}^{n_H} + [D]^{n_H})$ (see text)

	n_H	$1000(1-r)$	E_{min}	E_{max}	ED_{50} DPI mg/kg
MAP (mm Hg)					
Observed data			0 ± 0	59 ± 4	
Best-fitted	3.6 ± 0.4	3.4 ± 1.0	0.01 ± 0.04	58 ± 3	0.22 ± 0.02
n_H Specified	1*	26.3 ± 3.6*	−0.37 ± 0.09*	91 ± 8*	0.52 ± 0.09*
	2*	9.7 ± 2.1*	−0.21 ± 0.04*	63 ± 3	0.24 ± 0.02
	3*	6.0 ± 1.2	−0.03 ± 0.04	58 ± 3	0.22 ± 0.02
	4	6.7 ± 1.2	0.18 ± 0.04*	56 ± 3	0.22 ± 0.02
	5*	6.9 ± 1.5	0.25 ± 0.05*	56 ± 3	0.22 ± 0.02
HR (beats/min)					
Observed data			0 ± 0	63 ± 4	
Best-fitted	4.2 ± 0.6	4.8 ± 1.6	0.03 ± 0.05	62 ± 4	0.23 ± 0.02
n_H Specified	1*	31.1 ± 4.1*	−0.47 ± 0.06*	124 ± 22*	0.74 ± 0.10*
	2*	13.1 ± 2.4*	−0.21 ± 0.08*	68 ± 5	0.25 ± 0.03
	3*	8.2 ± 2.0	−0.04 ± 0.07	63 ± 4	0.22 ± 0.02
	4	7.6 ± 2.1	0.09 ± 0.13	60 ± 4	0.22 ± 0.02
	5*	8.3 ± 4.4	0.14 ± 0.07	59 ± 4	0.21 ± 0.02

* Significant difference from the best-fitted data ($P < .05$).

retical curves, expressed as 1000 $(1 - r)$, was the greatest for n = 3 and 4, and least for n = 1.

Effects of antagonists on the MAP and HR responses of DPI. Compared to vehicle, mecamylamine and reserpine reduced both MAP and HR; phentolamine, propranolol and captopril reduced MAP but did not affect HR, whereas guanethidine did not alter MAP but increased HR. On the other hand, atropine, prazosin, rauwolscine and L-Arg did not alter either MAP or HR (table 2).

The dose-MAP and dose-HR curves of DPI in the presence of vehicle or antagonists and the corresponding ED_{50} and E_{max} at the best-fitted n_H of DPI were shown in figures 4 to 6 and table 3, respectively. Reserpine, guanethidine and mecamylamine attenuated the MAP as well as HR responses of DPI (fig. 4) with either a decrease in E_{max} or an increase in ED_{50} of DPI (table 3). Phentolamine and prazosin but not rauwolscine reduced the MAP responses by decreasing E_{max} of DPI. The HR responses and the corresponding E_{max}, on the other hand, were increased by phentolamine and rauwolscine but not prazosin (fig. 5 and table 3). Propranolol abolished the HR responses with a decrease in E_{max}, but potentiated the MAP responses of DPI with an increase in E_{max} (fig. 5 and table 3). Atropine potentiated both the MAP (by decreasing ED_{50} and increasing in E_{max}) and HR (by decreasing ED_{50}) responses of DPI (fig. 6 and table 3). Captopril markedly potentiated the MAP (by increasing E_{max}) but not HR response, whereas L-Arg did not

TABLE 2

Base-line values (mean ± S.E.) of MAP and HR at 10 min after i.v. bolus injections of vehicle, mecamylamine (10 mg/kg), atropine (10 mg/kg), phentolamine (10 mg/kg), propranolol (1 mg/kg), prazosin (1 mg/kg), rauwolscine (1 mg/kg), guanethidine (10 mg/kg), captopril (20 mg/kg) and L-arginine (400 mg/kg) or 24 hr after i.p. injection of reserpine (5 mg/kg) in pentobarbital-anesthetized rats (n = 6 each group)

Antagonists	MAP mm Hg	HR beats/min
Vehicle	102 ± 6	353 ± 10
Reserpine	66 ± 2*	240 ± 30*
Mecamylamine	71 ± 2*	283 ± 20*
Guanethidine	100 ± 5	418 ± 14*
Phentolamine	82 ± 4*	329 ± 22
Prazosin	97 ± 5	362 ± 13
Rauwolscine	89 ± 5	354 ± 11
Propranolol	86 ± 6*	322 ± 14
Atropine	91 ± 2	351 ± 8
Captopril	82 ± 6*	319 ± 8
L-Arginine	96 ± 3	358 ± 8

* Significant difference from vehicle-pretreated group ($P < .05$).

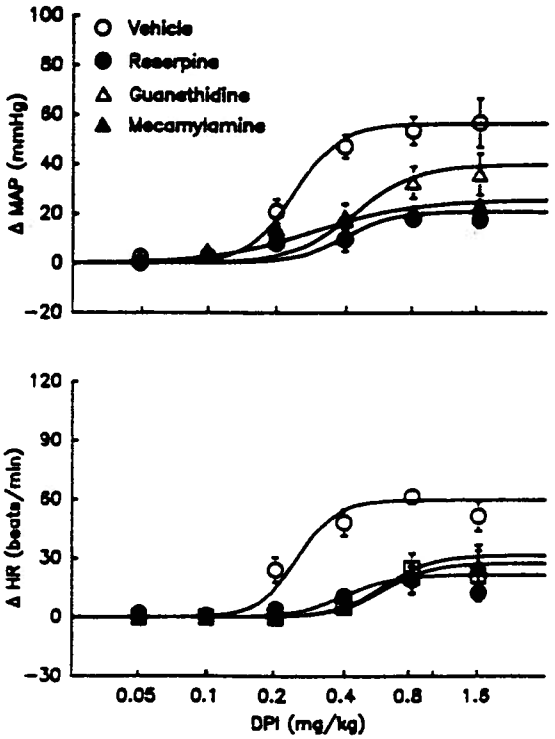


Fig. 4. Dose-response curves (mean ± S.E.) of i.v. bolus injections of DPI on MAP and HR in pentobarbital-anesthetized rats (n = 6 each group) pretreated with vehicle, reserpine (5 mg/kg), guanethidine (10 mg/kg) and mecamylamine (10 mg/kg). All the pretreatment drugs were i.v. bolus injected 10 min before the construction of the dose-response curve of DPI except for reserpine which was i.p. injected 24 hr previously. The lines represent theoretical curves using the formula $Y = E_{max}[D]^{n_H}/(ED_{50}^{n_H} + [D]^{n_H})$ and best-fitted n_H, ED_{50} and E_{max} shown in table 3.

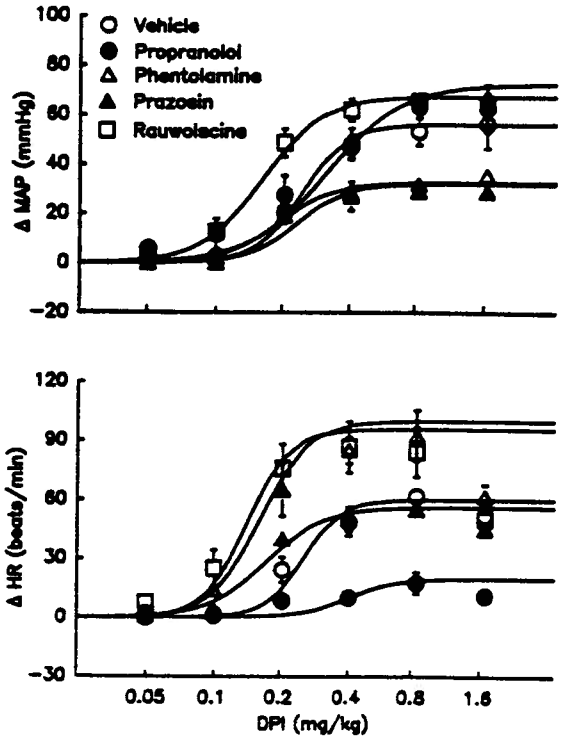


Fig. 5. Dose-response curves (mean ± S.E.) of i.v. bolus injections of DPI on MAP and HR in pentobarbital-anesthetized rats (n = 6 each group) pretreated with vehicle, propranolol (1 mg/kg), phentolamine (10 mg/kg), prazosin (1 mg/kg) and rauwolscine (1 mg/kg). All the pretreatment drugs were i.v. bolus injected 10 min before the construction of a dose-response curve of DPI. The lines represent theoretical curves using the formula $Y = E_{max}([D]^{n_H}/(ED_{50}^{n_H} + [D]^{n_H})$ and best-fitted n_H, ED_{50} and E_{max} shown in table 3.

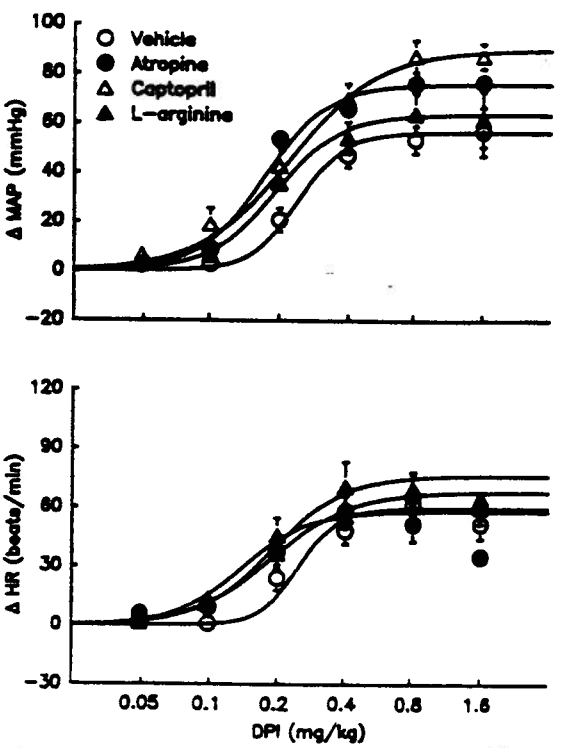


Fig. 6. Dose-response curves (mean ± S.E.) of i.v. bolus injections of DPI on MAP and HR in pentobarbital-anesthetized rats (n = 6 each group) pretreated with vehicle, atropine (10 mg/kg), captopril (20 mg/kg) and L-arginine (400 mg/kg). All the pretreatment drugs were i.v. bolus injected 10 min before the construction of a dose-response curve of DPI. The lines represent theoretical curves using the formula $Y = E_{max}([D]^{n_H}/(ED_{50}^{n_H} + [D]^{n_H})$ and best-fitted n_H, ED_{50} and E_{max} shown in table 3.

affect either the MAP or HR response of DPI (fig. 6 and table 3).

Effect of TTX-pretreatment, pithing and spinal cord (T_1) transection on the MAP and HR responses of DPI. Base-line MAP in TTX-pretreated rats (53 ± 2 mm Hg), pithed rats (45 ± 2 mm Hg) and spinal cord-transected (T_1) rats (52 ± 2 mm Hg) was lower than that of ventilated control rats (105 ± 5 mm Hg). Base-line HR in TTX-pretreated rats (318 ± 9 beats/min) and pithed rats (333 ± 10 beats/min) was similar to, whereas that of spinal cord-transected rats (412 ± 16 beats/min) was higher than that of ventilated control rats (312 ± 10 beats/min). DPI (0.05–1.6 mg/kg) also caused dose-dependent pressor and tachycardic responses in ventilated control rats; pretreatment with TTX and pithing totally abolished both the MAP and HR responses of DPI (fig. 7). However, norepinephrine (8 μg/kg) still caused increases in MAP and HR in TTX-pretreated and pithed rats; their increases were 109 ± 1 and 44 ± 2 mm Hg, and 92 ± 6 and 57 ± 6 beats/min, respectively. On the other hand, spinal cord transection (T_1) markedly suppressed the dose-MAP and dose-HR responses of DPI compared to the responses in control rats; the suppression by spinal cord transection, however, was less than that by pithing (fig. 7).

The base-line MAP and HR in another two groups of pithed rats i.v. bolus injected with vehicle or L-NAME were 48 ± 3 and 50 ± 4 mm Hg, and 308 ± 22 and 335 ± 22 beats/min. Bolus (i.v.) injections of cumulative doses of L-NAME but not vehicle dose-dependently increased MAP, but not HR in pithed rats (fig. 8).

Effect of DPI on plasma catecholamines in intact, pithed and reserpinized rats. Base-line levels of plasma catecholamines were similar among the three intact rat groups to be treated with vehicle, DPI and L-NNA (table 4). Compared to the pooled data, pithing did not alter circulating catecholamines. Pretreatment with reserpine significantly decreased plasma norepinephrine but increased plasma epinephrine. Base-line MAP and HR of these five groups of rats were similar to those of the corresponding groups in protocol 2 and 3 (data not shown).

Compared to the vehicle, DPI (1.6 mg/kg) caused large increases in plasma norepinephrine and epinephrine, and moderate increase in plasma dopamine, as well as increases in MAP and HR (fig. 9). The increases in MAP and HR were significantly greater than those caused by the same dose of DPI in the multiple dose regimen in protocol 2 (70 ± 2 *vs.* 53 ± 5 mm Hg and 123 ± 7 *vs.* 43 ± 5 beats/min, respectively). In contrast to DPI, L-NNA (16 mg/kg) increased MAP, decreased HR and slightly decreased plasma dopamine, but did not alter plasma norepinephrine or epinephrine (fig. 9). Reserpine markedly reduced DPI-induced increases in plasma norepinephrine, epinephrine and dopamine by 91, 93 and 74%, respectively, and attenuated the pressor and tachycardic responses by 56 and 68%, respectively (fig. 10). In reserpinized rats, the pressor and tachycardic responses to a single dose of DPI were also greater

TABLE 3

Values (mean ± S.E.) of n_H, ED_{50} and E_{max} of the dose-MAP and dose-HR response curves of DPI (0.05–1.6 mg/kg) in pentobarbital-anesthetized rats pretreated with vehicle, mecamylamine (10 mg/kg), atropine (10 mg/kg), phentolamine (10 mg/kg), propranolol (1 mg/kg), prazosin (1 mg/kg), rauwolscine (1 mg/kg), reserpine (5 mg/kg), guanethidine (10 mg/kg), captopril (20 mg/kg) and L-arginine (400 mg/kg) (n = 6 each group)

	MAP			HR		
	n_H	ED_{50}	E_{max}	n_H	ED_{50}	E_{max}
		mg/kg	mm Hg		mg/kg	mm Hg
Vehicle	4.2 ± 0.7	0.24 ± 0.03	56 ± 6	5.0 ± 0.8	0.25 ± 0.03	60 ± 5
Reserpine			21 ± 3*#			22 ± 6*#
Mecamylamine	2.0 ± 0.4*	0.29 ± 0.10	26 ± 3*	3.4 ± 0.4	0.35 ± 0.05	28 ± 8*
Guanethidine	3.1 ± 0.2	0.44 ± 0.10*	39 ± 8	4.1 ± 0.4	0.35 ± 0.04	32 ± 9*
Phentolamine	3.5 ± 0.4	0.19 ± 0.03	33 ± 2*	4.6 ± 0.4	0.14 ± 0.04	96 ± 12*
Prazosin	3.9 ± 0.7	0.23 ± 0.04	32 ± 5*	3.4 ± 0.5	0.17 ± 0.01	56 ± 5
Rauwolscine	3.2 ± 0.4	0.16 ± 0.02*	68 ± 4	4.0 ± 1.1	0.16 ± 0.04	100 ± 13*
Propranolol	2.6 ± 0.3*	0.31 ± 0.07	73 ± 5*			19 ± 5*#
Atropine	3.1 ± 0.6	0.17 ± 0.02*	76 ± 4*	3.0 ± 0.5*	0.14 ± 0.02*	58 ± 8
Captopril	2.2 ± 0.1*	0.22 ± 0.02	90 ± 5*	2.6 ± 0.3*	0.19 ± 0.01	68 ± 7
L-arginine	3.0 ± 0.3	0.19 ± 0.02	64 ± 8	2.8 ± 0.1*	0.19 ± 0.03	76 ± 13

* Significant difference from the corresponding values in vehicle-pretreated group ($P < .05$).
# Observed data.

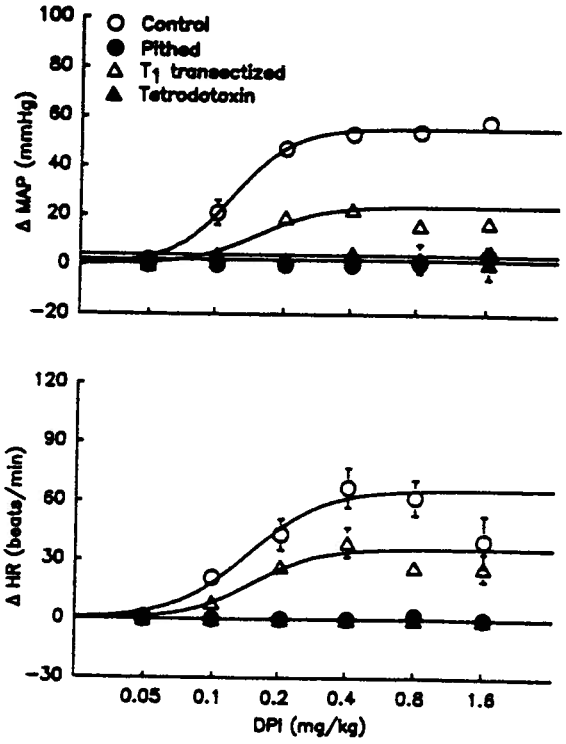


Fig. 7. Dose-response curves (mean ± S.E.) of i.v. bolus injections of DPI on MAP and HR in pentobarbital-anesthetized control rats. TTX (10 μg/kg) pretreated rats, pithed rats and spinal cord-transected (T_1) rats (n = 6 each group). TTX was i.v. bolus injected 10 min before the construction of a dose-response curve of DPI. The lines represent theoretical curves using the formula $Y = E_{max}[D]^{n_H}/(ED_{50}^{n_H} + [D]^{n_H})$ and best-fitted n_H, ED_{50} and E_{max} (values not shown).

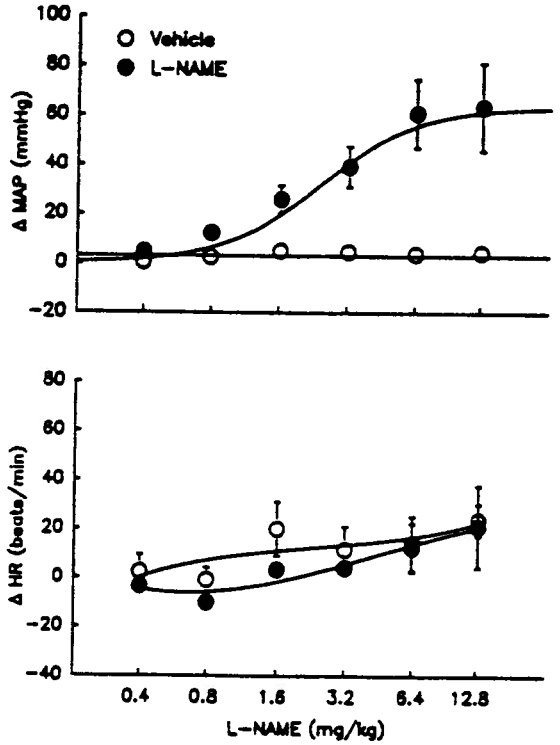


Fig. 8. Cumulative dose-response curves (mean ± S.E.) of i.v. bolus injections of L-NAME or equal volume of vehicle on MAP and HR in pithed rats (n = 6 each group). The lines represent theoretical curves using the formula $Y = E_{max}[D]^{n_H}/(ED_{50}^{n_H} + [D]^{n_H})$ and best-fitted n_H, ED_{50} and E_{max} (values not shown).

than those of the multiple dose regimen in protocol 2 (31 ± 10 *vs.* 18 ± 1 mm Hg and 39 ± 10 *vs.* 22 ± 1 beats/min, respectively). Pithing totally abolished the effects of DPI on plasma catecholamines, as well as MAP and HR (fig. 10).

Concentration-response and linear regression models were used to examine the relationships between plasma norepinephrine or epinephrine and MAP or HR in intact, pithed and reserpinized rats i.v. bolus injected with DPI or vehicle (n = 25). The linear regression model gave significant correlation between plasma norepinephrine and MAP (r = 0.83) or HR (r = 0.87), as well as between epinephrine and MAP (r = 0.78) or HR (r = 0.81). The concentration-response model, however, gave better fits. Correlation coefficient between plasma norepinephrine and MAP or HR were 0.97 or 0.96, respectively; correlation coefficient between plasma epinephrine and MAP or HR were 0.94 or 0.94, respectively. Figures 11 and 12 showed the concentration-response relationships between individual

TABLE 4

Base-line levels (mean ± S.E.) of plasma catecholamines in pentobarbital-anesthetized intact, pithed and reserpinized rats i.v. bolus injected with vehicle, DPI or L-NNA

In each group, $n = 6$ except for reserpinized group, in which $n = 7$.

	Catecholamines		
	Norepinephrine	Epinephrine	Dopamine
	ng/ml		
Intact (vehicle)	0.374 ± 0.049	0.042 ± 0.010	0.082 ± 0.020
Intact (DPI)	0.324 ± 0.053	0.054 ± 0.022	0.102 ± 0.014
Intact (L-NNA)	0.410 ± 0.048	0.050 ± 0.011	0.097 ± 0.012
Pooled	0.369 ± 0.030	0.049 ± 0.009	0.094 ± 0.009
Pithed (DPI)	0.414 ± 0.047	0.048 ± 0.009	0.095 ± 0.009
Reserpinized (DPI)	0.206 ± 0.018*	0.081 ± 0.011*	0.078 ± 0.007

* Significant difference from the control group ($P < .05$).

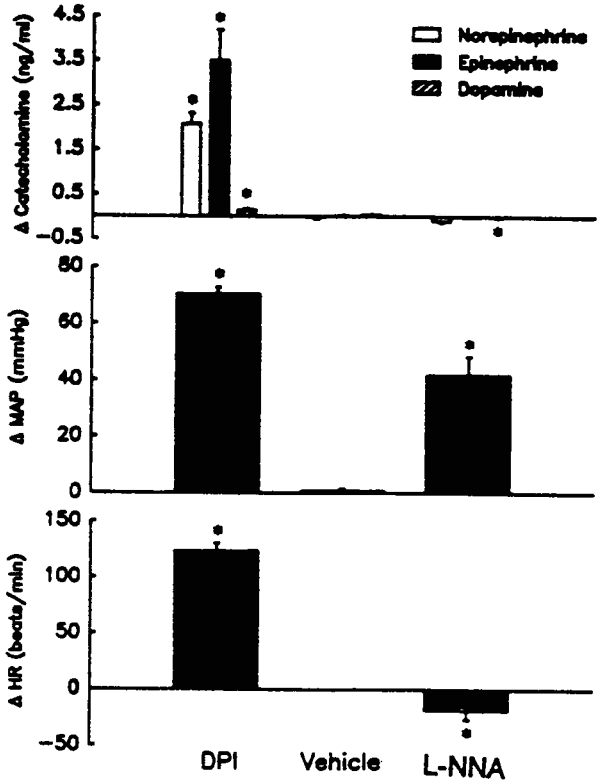


Fig. 9. Effects (mean ± S.E.) of i.v. bolus injections of a single dose of vehicle, DPI (1.6 mg/kg) or L-NNA (16 mg/kg) on plasma catecholamines, MAP and HR in pentobarbital-anesthetized rats ($n = 6$ each group). Blood samples and MAP and HR measurements were obtained 20 min before and 30 sec after injection of vehicle or DPI and 40 min after injection of L-NNA. * Significant difference from vehicle group ($P < .05$).

plasma norepinephrine or epinephrine and MAP or HR response caused by DPI.

Discussion

To our knowledge, this study is the first to show the *in vivo* cardiovascular effects of DPI. The similar transient time course and pharmacodynamics suggest a common cause for both pressor and tachycardic responses of DPI. Because captopril markedly potentiated the pressor response and did not alter the tachycardic response of DPI, it is safe to conclude that the renin-angiotensin system is not responsible for the responses

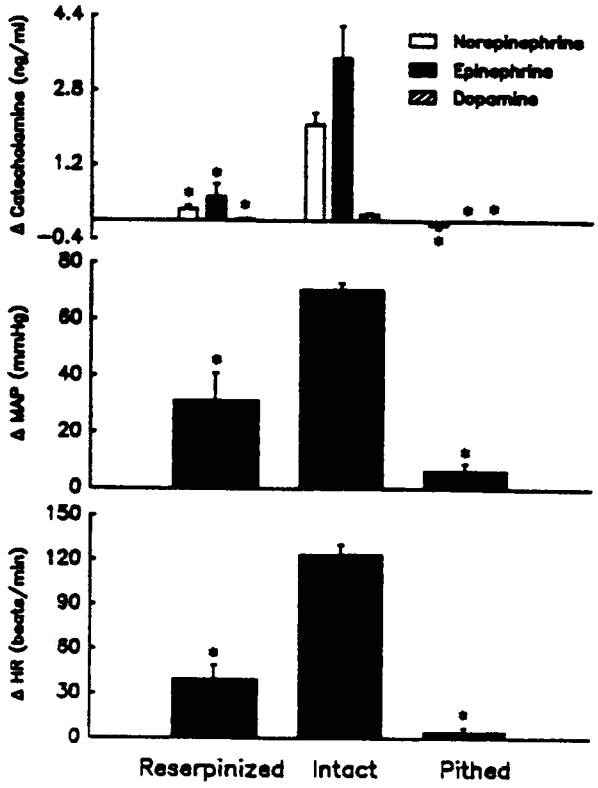


Fig. 10. Effects (mean ± S.E.) of i.v. bolus injection of a single dose of DPI (1.6 mg/kg) on plasma catecholamines, MAP and HR in pentobarbital-anesthetized intact, pithed and reserpinized rats. In each group $n = 6$ except for reserpinized group, in which $n = 7$. Blood samples and MAP and HR measurements were obtained 20 min before and 30 sec after DPI injection. * Significant difference from intact rats ($P < .05$).

of DPI although the mechanism of the potentiation by captopril is not known.

By the use of sympatholytic drugs, we investigated whether the sympathetic nervous system was responsible for the pressor and tachycardic effects of DPI. Reserpine markedly attenuated the DPI-induced increases in MAP and HR. The results suggest that DPI causes cardiovascular effects by activating the peripheral sympathetic nerve terminals and adrenal medullae causing releases of norepinephrine and epinephrine. This activation is dependent on the functional integrity of the central and autonomic nervous systems, as pithing abolishes, whereas spinal cord transection (T_1) attenuates the pressor and tachycardic effects of DPI. The indirect activation of the sympathetic nervous system by DPI is further supported by the observations that TTX abolishes, whereas guanethidine and mecamylamine attenuate, the effects of DPI. TTX has been shown to block conductances of the central and peripheral nerves (Gage, 1971) but not those of vascular smooth muscle (see Hirst and Edwards, 1989) or the myocardium (Abraham *et al.*, 1989) *via* selective blockade of voltage-dependent sodium channels. Guanethidine has been shown to be a specific adrenergic neuron blocker (Shand *et al.*, 1973; Kirpekar and Furchgott, 1972).

DPI caused immediate and large increases in plasma norepinephrine and epinephrine with the same time course as the pressor and tachycardic responses. Pithing totally abolished and reserpinization attenuated DPI-induced increases in plasma catecholamines as well as MAP and HR. Further analy-

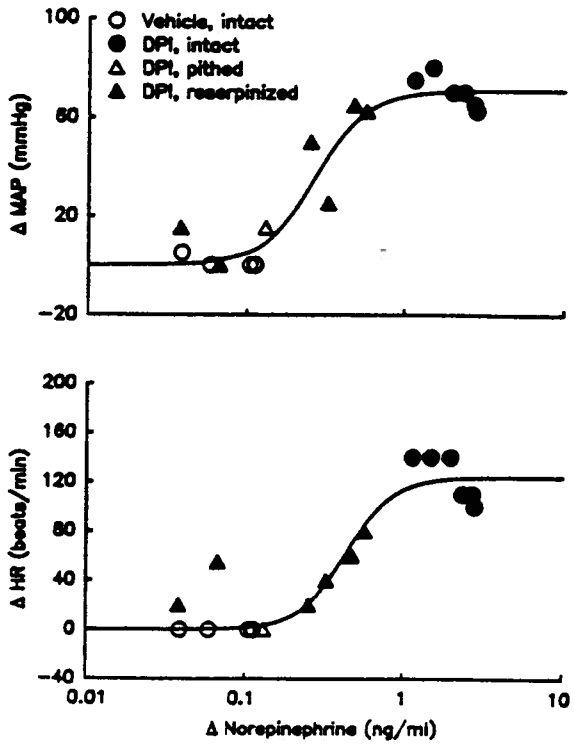


Fig. 11. Concentration-response curves of changes in plasma norepinephrine and MAP as well as HR after i.v. bolus injections of vehicle in intact rats and DPI (1.6 mg/kg) in intact, pithed and reserpinized rats. In each group $n = 6$ except for reserpinized group, in which $n = 7$. The line represents the theoretical curve calculated using the formula $Y = E_{max}([C]^{n_H}/(EC_{50}^{n_H} + [C]^{n_H})$ and best-fitted n_H, EC_{50} and E_{max} (values not shown).

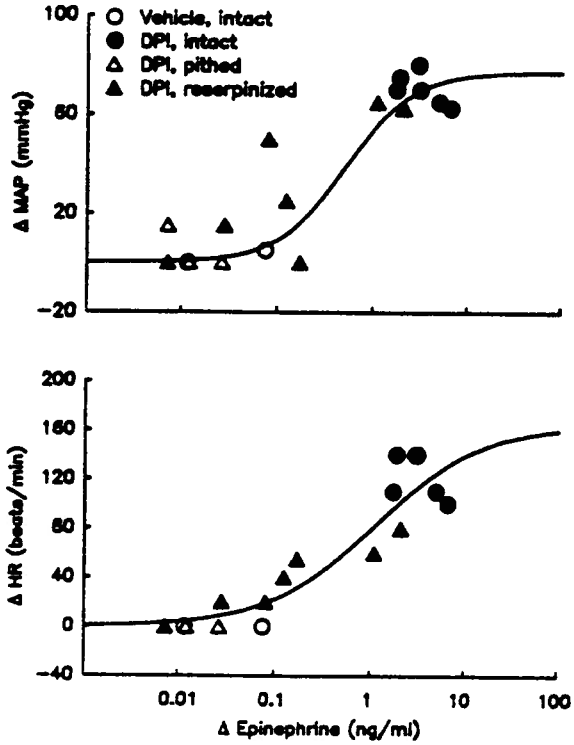


Fig. 12. Concentration-response curves of plasma epinephrine and MAP as well as HR after i.v. bolus injections of vehicle in intact rats and DPI (1.6 mg/kg) in intact, pithed and reserpinized rats. In each group $n = 6$ except for reserpinized group, in which $n = 7$. The line represents the theoretical curve calculated using the formula $Y = E_{max}([C]^{n_H}/(EC_{50}^{n_H} + [C]^{n_H})$ and best-fitted n_H, EC_{50} and E_{max} (values not shown).

sis shows that positive correlations exist between DPI-induced changes in MAP and HR with plasma norepinephrine as well as epinephrine. Taken together, the above results indicate that DPI activates the sympathetic nerve terminals and adrenal medullae to release norepinephrine and epinephrine in rats with functional intact central and autonomic nervous systems. Because DPI releases large quantities of catecholamines, repetitive injections should lead to tachyphylaxis. Our results indeed show that the MAP and HR responses of a single dose of DPI are greater than those elicited by the same dose in a multiple injection regimen. We have also observed that multiple injections of high doses of DPI eventually produce negligible pressor and tachycardic responses (data not shown).

Norepinephrine and epinephrine released by DPI would be expected to cause vasoconstriction, *via* the activation of *alpha*-1 adrenoceptors, and tachycardia, *via* the activation of *beta*-1 adrenoceptors. We found that the pressor effect of DPI is suppressed by the nonselective *alpha*-adrenoceptor antagonist phentolamine and selective *alpha*-1 adrenoceptor antagonist prazosin but not the selective *alpha*-2 adrenoceptor antagonist rauwolscine. Moreover, rauwolscine and phentolamine, but not prazosin, enhanced the tachycardic effect of DPI; this potentiation is likely due to the blockade of the central and/or peripheral prejunctional *alpha*-2 adrenoceptors which mediate inhibition of norepinephrine release (Berthelsen and Pettinger, 1977). This hypothesis may also explain why rauwolscine caused a small potentiation of the pressor effect of DPI. Our results also show that the tachycardic but not pressor effect of DPI is abolished by the *beta* adrenoceptor antagonist propranolol. The inability of propranolol to affect the MAP effect of DPI suggests that the pressor effect of DPI is not due to tachycardia or cardiac inotropy. The slight potentiation of the pressor response to DPI by propranolol may be due to the blockade of vasodilator *beta*-2 adrenoceptors which are prominent in skeletal muscle beds (Abdelrahman *et al.*, 1990).

The mechanism and primary site(s) of actions for DPI are unclear at the moment. It is logical to expect that DPI increases sympathetic discharge by inhibiting NO synthase. Extensive evidences indicate that NO synthesis and release take place in the brain (Garthwaite *et al.*, 1989; Knowles *et al.*, 1989, 1990). NO synthesis is reported to be responsible for long-term potentiation in the hippocampus (Böhme *et al.*, 1991), long-term synaptic depression in the cerebellum (Shibuki and Okada 1991) and nociceptive activity in the brain (Moore *et al.*, 1991). Moreover, endothelium-derived relaxing factor/NO has been shown to inhibit norepinephrine release from isolated sympathetic nerves innervating the canine pulmonary artery and vein (Greenberg *et al.*, 1990) and other preparations (see "Discussion" of Greenberg *et al.*, 1990). However, our results do not support this hypothesis. In contrast to DPI, L-NNA at a dose that caused maximal pressor response (Wang *et al.*, 1991b), did not increase plasma catecholamines. These results suggest that DPI-induced sympathetic activation is unlikely due to the inhibition of NO synthesis. More studies are needed to elucidate

whether the inhibitions of other flavoproteins or NADPH-dependent enzymes account for the actions of DPI.

The central nervous system is unlikely a primary or major site for the actions of DPI although we cannot exclude this possibility in view of the suppression of DPI's effects by pithing and spinal cord transection. If the site of DPI is in the central nervous system, local injection of the drug should then produce greater effects than i.v. administration. However, results from our preliminary studies show that intracarotid and intravertebral injections of DPI caused similar pressor responses as i.v. injections into the same rats ($n = 3$). As well, intraventricular injection of DPI into the third cerebroventricle at doses up to 0.1 mg/kg (ED_{50} of 0.22 mg/kg by i.v. injection) did not cause any pressor or tachycardic responses ($n = 3$). There is uncertainty about the accessibility of DPI to the central nervous system. Although ^{125}I was detected in the brain 10 min after i.v. injection of [^{125}I]DPI (Gatley and Martin, 1979), DPI, being a charged molecule, may not adequately access the central nervous system within 0.5 to 1 min after injection. Moreover, it is difficult to explain why pithing is more effective than spinal transection in attenuating the cardiovascular effects of DPI if the central nervous system is the only site of action of DPI. On the other hand, we cannot rule out the possible involvement of the peripheral sympathetic nervous system in the actions of DPI, because even the action of indirectly acting sympathomimetic agents rely on a functional amine-uptake system and therefore, sympathetic tone. Therefore, although the integrity of the central nervous system is a prerequisite for the actions of DPI, its primary site(s) of actions is not clear. Further studies are required to identify the site(s) of actions of DPI in the central, efferent or even afferent nervous systems.

It was reported that DPI appeared to cause respiratory difficulties leading to deaths of rats or mice (Gatley and Martin, 1979) and, chronic administration of DPI caused fatigue of the skeletal muscle (Hayes *et al.*, 1985; Cooper *et al.*, 1988). We also observed that higher doses (0.8–1.2 mg/kg) of DPI occasionally caused respiratory difficulty. Therefore, it is possible that the cardiovascular effects of DPI are secondary to respiratory dysfunction. However, this is unlikely the situation as DPI caused similar pressor and tachycardic responses with or without artificial ventilation.

Detailed analyses of the dose-MAP and dose-HR response curves of DPI show that the n_H for the MAP and HR effects of DPI are 3.6 ± 0.3 and 4.2 ± 0.6, respectively. These results suggest that the cardiovascular effects of DPI involve "positive co-operation" of probably 4 molecules of DPI (see Rang, 1971 and "Discussion" in Pennefather and Quastel, 1982). It should be noted that even if n_H is not exactly 4, the 4th root of either MAP or HR responses should be linearly correlated to the doses of DPI.

Much has been published on the effects of N^G-substituted Arg analogs on the *in vitro* and *in vivo* endothelium-dependent vasodilatation or MAP responses (Aisaka *et al.*, 1989; Rees *et al.*, 1989, 1990; Wang and Pang, 1990a, b; Wang *et al.*, 1991a, b; Wang *et al.*, 1992, 1993a,b; see Moncada *et al.*, 1991). DPI also inhibits endothelium-dependent vasodilatation both *in vitro* (Stuehr *et al.*, 1991; Poon *et al.*, 1993) and *in vivo* (Poon *et al.*, 1993) and causes transient pressor response. However, the mechanism of the pressor response is different from that of N^G-substituted Arg analogs. The pressor responses of N^G-substituted Arg analogs are susceptible to inhibition by L-Arg but not the impairments of the central nervous, autonomic nervous and renin-angiotensin systems (Wang and Pang, 1990b, 1991; Wang *et al.*, 1991b; Wang *et al.*, 1992; Tabrizchi and Triggle, 1992; see Moncada *et al.*, 1991). These drugs also caused reflex bradycardia (Wang and Pang, 1991). The pressor and tachycardic actions of DPI, on the other hand, are entirely dependent on the functional integrity of the central and sympathetic nervous systems, but are not affected by L-Arg. The latter observation is consistent with the report that L-Arg does not antagonize DPI-induced inhibition of NO synthase activity (Stuehr *et al.*, 1991).

In conclusion, DPI but not L-NNA concomitantly causes immediate and transient pressor and tachycardic responses, as well as immediate increases in plasma norepinephrine and epinephrine. These effects are inhibited by maneuvers which interfere with the activities of the central or sympathetic nervous systems, namely, pithing, spinal cord transection and pretreatments with TTX, reserpine, mecamylamine and guanethidine. Moreover, the pressor but not tachycardic effect of DPI is attenuated by phentolamine and prazosin. The tachycardic but not pressor effect of DPI is inhibited by propranolol. Our results suggest that DPI, unlike the N^G-substituted Arg analogs, produces pressor and tachycardic effects *via* the indirect activation of the sympathetic nervous system.

Acknowledgments

We would like to express our appreciations to Dr. David M. J. Quastel for his help in analyzing the DPI dose-response curves by the use of his dose-response curve analysis program; to Drs. Morley C. Sutter, Michael J.A. Walker and Reza Tabrizchi for their critical evaluations of our work and to Dr. Gerald S. Marks (Queen's University) for his advice. We also thank Ms. Su Lin Lim for her skilled technical assistance.

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Paper V

Wang, Y.-X. and Pang, C.C.Y.: Halothane inhibits pressor effect of diphenyleneiodonium. Br. J. Pharmacol. 109: 1186-1191, 1993. The reproduction of this paper was kindly permitted by the copyright holder, Macmillan Press Ltd., Hampshire, U.K.

Br. J. Pharmacol. (1993), **109**, 1186 - 1191

Halothane inhibits the pressor effect of diphenyleneiodonium

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1 We have recently found that diphenyleneiodonium (DPI), a novel inhibitor of nitric oxide (NO) synthase, causes pressor and tachycardic responses in pentobarbitone- but not halothane-anaesthetized rats. The present study investigated the mechanism by which halothane suppresses the pressor response of DPI. The effects of halothane on the pressor response of DPI were also compared with those of other anaesthetic agents.

2 In conscious rats, i.v. bolus injections of DPI (0.025– 1.6 mg kg^{-1}) caused dose-dependent increases in mean arterial pressure (MAP), with ED_{50} of 0.07 ± 0.01 mg kg^{-1} and maximal rise of MAP (E_{max}) of 59 ± 2 mmHg. While ketamine potentiated E_{max} without altering the ED_{50} and pentobarbitone increased the ED_{50} without changing E_{max} of the pressor response to DPI, chloralose, urethane and ethanol displaced the curve to the right and potentiated E_{max}. In contrast, halothane (0.5–1.25%) dose-dependently and non-competitively reduced the pressor responses to DPI.

3 Intravenous bolus injection of a single dose of DPI (1.6 mg kg^{-1}) caused immediate and large increases in plasma noradrenaline and adrenaline, as well as MAP in conscious rats. Halothane (1.25%) almost completely inhibited these increases.

4 The results suggest that DPI causes a pressor response in conscious rats by activating the sympathetic nervous system and halothane abolishes this pressor response by inhibiting activities of the sympathetic nervous system. The results also show that influences of anaesthetics must be taken into consideration when evaluating pressor response of vasoactive agents.

Keywords: Diphenyleneiodonium (DPI); nitric oxide synthase inhibitor; anaesthetics; halothane; pentobarbitone; ketamine; urethane; ethanol; chloralose; pressor; sympathetic nervous system; noradrenaline; adrenaline; endothelium-dependent vasodilatation

Introduction

Diphenyleneiodonium (DPI), a bivalent iodine compound, was first reported to be a potent hypoglycaemic agent (Stewart & Hanly, 1969; Gatley & Martin, 1979). It was later shown to suppress the activities of neutrophil and macrophage NADPH-dependent oxidase (Cross & Jones, 1986), as well as macrophage nitric oxide (NO) synthase (Stuehr *et al.*, 1991), probably via the inhibition of a flavoprotein (Hancock & Jones, 1987; Ellis *et al.*, 1989; Stuehr *et al.*, 1990). DPI was also found to inhibit endothelium-dependent vasodilatation *in vitro* (Stuehr *et al.*, 1991; Poon *et al.*, 1993) and *in vivo* (Poon *et al.*, 1993). Recently, we found that DPI caused pressor and tachycardic effects in pentobarbitone-anaesthetized rats; the cardiovascular effects were due to the modulation of sympathetic nerve activities (Wang & Pang, 1993). In our preliminary experiments, we also found that halothane markedly suppressed the pressor effect of DPI.

Halothane has been shown to inhibit sympathetic nervous transmission at several levels (Seagard *et al.*, 1982; Larach *et al.*, 1987; Rorie *et al.*, 1990). Halothane was also found to alter endothelium-dependent vasodilatation (Muldoon *et al.*, 1988; Blaise, 1991), and to abolish the pressor responses of other NO synthase inhibitors, namely N^G-nitro-L-arginine (L-NNA) and its methyl ester (L-NAME) (Wang *et al.*, 1991a; Pang *et al.*, 1992). The aim of this study was to (1) investigate the characteristics of the inhibitory effect of halothane on the pressor response to DPI; (2) to examine whether halothane suppresses the pressor response of DPI by inhibiting activities of the sympathetic nervous system; (3) to compare the effect of halothane with those of intravenous and inhalation anaesthetic agents on the pressor response of DPI.

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Methods

Surgical preparation

Sprague-Dawley rats (300–360 g) were used in this study. Cannulae (PE50) were inserted into the left iliac vein for the administration of drugs, and into the left iliac artery for the recordings of mean arterial pressure (MAP) by a P23DB pressure transducer (Gould Statham, CA, U.S.A.) and heart rate which was determined electronically from the upstroke of the arterial pulse pressure using a tachograph (Grass, Model 7P4G). In some rats, another PE50 cannula was also inserted into the right iliac artery to collect blood samples. The body temperature of the anaesthetized rats was maintained at 37°C with a heating lamp connected to a thermostat (73A, Yellow Springs Instruments). In halothane-anaesthetized rats in Protocol 2 and 3 (see later), tracheostomy was also performed with a PE160 catheter. All anaesthetized rats were equilibrated for 20 min at the appropriate anaesthetic doses before the commencement of studies. The conscious rats in all the protocols were first anaesthetized with halothane (1.5% in air), to allow surgical preparation, and were allowed to recover for at least 6 h from the effects of anaesthesia before further use. The catheters were tunnelled subcutaneously and exteriorized at the back of the neck.

Measurement of plasma catecholamines

Plasma catecholamines levels were determined by a catecholamine Assay kit (Amersham Canada Ltd., Ont., Canada) (Passon & Peuler, 1974). The radioactivity (^{3}H) was detected by a 1600 TR liquid scintillation analyzer (Packard Instrument Co., CT, U.S.A.). Blood samples (0.5 ml) were immediately placed in prechilled tubes containing EGTA and reduced glutathione and centrifuged at 1,200 *g* at 4°C. Afterwards, the plasma was removed and stored at − 70°C until assayed within two weeks. Duplicate assays were run for the

standard (in blank plasma) and plasma samples (50 μl each) using distilled water as a blank control for each run. The sensitivity of the catecholamine assay was 0.005 ng ml^{-1} for each catecholamine.

Drugs

Halothane and diphenyleneiodonium (DPI) sulphate were obtained from Ayerst Lab. (Quebec, Canada) and Colour Your Enzyme Ltd. (Ont., Canada), respectively. Urethane ethyl carbamate and ketamine hydrochloride were from Sigma Chemical Co. (MO, U.S.A.). The following anaesthetics were also used: α-chloralose (BDH Chemical Ltd., Poole, England), sodium pentobarbitone (M.T.C. Pharmaceuticals, Cambridge, Ontario, Canada) and anhydrous ethyl alcohol (Stanchem Co., Quebec, Canada). The powder drugs were dissolved in normal saline (0.9% NaCl) except for DPI which was solubilized in 5% glucose solution by 10 min sonication.

Experimental protocol

The rats ($n = 6$ each group) were randomly assigned into groups. MAP and HR were continuously monitored throughout the experiments.

(1) *Dose-MAP response curves to DPI in conscious and anaesthetized rats* Six groups of rats were used: conscious rats and rats anaesthetized with sodium pentobarbitone (65 mg kg^{-1}), ketamine (140 mg kg^{-1}), urethane (2 g kg^{-1}), ethanol (4 g kg^{-1}) and chloralose (150 mg kg^{-1}). Dose-MAP response curves to i.v. bolus injections of DPI (0.025–1.6 mg kg^{-1}) were constructed at dose-intervals of 3–6 min, the time required to recover from the effects of the previous dose.

(2) *Dose-MAP response curves of DPI in conscious and halothane-anaesthetized rats* Five groups of rats were used: conscious rat and rats anaesthetized with different concentrations of halothane (0.5, 0.75, 1 and 1.25% in air) at flow rates of 1500 ml min^{-1}. The dose-MAP response curves of i.v. bolus injections of DPI (0.025–6.4 mg kg^{-1}) were constructed at dose-intervals of 3–6 min as above.

(3) *Effects of DPI on plasma catecholamine levels in conscious and halothane-anaesthetized rats* One group of conscious rats and one group of halothane (1.25%)-anaesthetized rats were injected i.v. with a single bolus dose of DPI (1.6 mg kg^{-1}). Blood samples were collected 20 min prior to and 30 s after the injections of DPI. MAP was continuously monitored.

Calculation and statistical analysis

Maximum effect (E_{max}), half-effective dose (ED_{50}) and Hill coefficient (n), from individual dose-response curves were fitted by a computer programme using non-linear least-squares to the relation $Y = a + bx$, where Y = response and $x = [D]^n/(ED_{50}^n + [D]^n)$ (Quastel & Saint, 1988; Wang & Pang, 1993). All results were expressed as mean ± standard error (s.e.mean) and analyzed by the analysis of variance followed by Duncan's multiple range test with $P<0.05$ selected as the criterion for statistical significance.

Results

Effects of anaesthetics on DPI-induced MAP response

Baseline MAP in rats anaesthetized with urethane, ethanol and chloralose, but not pentobarbitone or ketamine, was less than that in conscious rats (Table 1).

In conscious rats, DPI caused an immediate (approximately 15 s in onset) and transient (1–2 min in duration) pressor response. The pressure response was dose-dependent (Figure 1) with ED_{50} of 0.07 ± 0.01 mg kg^{-1} and maximal MAP reached at 59 ± 2 mmHg, based on the best-fitted calculations (Table 1). The Hill coefficient n of 3.3 ± 0.5 was significantly different from 1, 2 and 5 but not from 3 or 4. DPI also caused tachycardia at lower doses (0.025–0.1 mg kg^{-1}), bradycardia at higher doses (0.2–1.6 mg kg^{-1}) and movements following the onset of the pressor response (data not shown).

Pentobarbitone, chloralose, urethane and ethanol but not ketamine displaced the dose-MAP curve of DPI to the right (Figure 1) by increasing ED_{50}s (Table 1). On the other hand, ketamine, chloralose, urethane and ethanol but not pentobarbitone potentiated the maximal MAP response to DPI (Figure 1 and Table 2). None of the anaesthetic agents significantly altered the n value of the dose-response curves of DPI (Table 1). Under the influence of all anaesthetics, the hindlimbs and occasionally the forelimbs displayed kicking motion immediately following injections of DPI.

Inhibitory effect of halothane on DPI pressor response

Halothane (0.5–1.25%) reduced baseline MAP in a dose-dependent manner (Table 1). In conscious rats, i.v. bolus injections of DPI also caused similar pressor responses as in protocol (1) (Figure 2 and Table 1). Halothane dose-dependently reduced the maximal effect of DPI and shifted the DPI curve to the right (Figure 2). ED_{50} values were linearly correlated while E_{max} values were inversely correlated with the

Table 1 Values of baseline mean arterial pressure (MAP), as well as parameters (Hill coefficient n, ED_{50} and E_{max}) of the dose-pressor response curves of diphenyleneiodonium (DPI) in conscious rats and rats anaesthetized with sodium pentobarbitone (65 mg kg^{-1}), ketamine (140 mg kg^{-1}), urethane (2 g kg^{-1}), ethanol (4 g kg^{-1}), chloralose (150 mg kg^{-1}) and halothane (0.5–1.25%)

Anaesthetics	*Baseline MAP* mmHg	*Dose-response curve to DPI* n	ED_{50} (mg kg^{-1})	E_{max} (mmHg)
Protocol 1				
Conscious	118 ± 2	3.3 ± 0.5	0.07 ± 0.01	59 ± 2
Ketamine	107 ± 7	3.2 ± 0.4	0.08 ± 0.01	72 ± 2*
Pentobarbitone	111 ± 6	3.3 ± 0.7	0.22 ± 0.04*	64 ± 4
Chloralose	89 ± 3*	3.7 ± 0.4	0.12 ± 0.01*	90 ± 4*
Urethane	72 ± 7*	2.9 ± 0.6	0.20 ± 0.03*	71 ± 4*
Ethanol	57 ± 6*	2.5 ± 0.4	0.26 ± 0.03*	78 ± 4*
Protocol 2				
Conscious	113 ± 4	2.9 ± 0.5	0.07 ± 0.01	59 ± 2
0.5% Halothane	96 ± 10	2.6 ± 0.2	0.48 ± 0.09*	62 ± 4
0.75% Halothane	83 ± 4*	2.7 ± 0.5	0.57 ± 0.08*	47 ± 4*
1% Halothane	80 ± 7*	3.8 ± 0.7	0.77 ± 0.15*	34 ± 3*
1.25% Halothane	69 ± 2*	–	–	13 ± 4*[a]

Values are mean ± s.e.mean $n = 6$ for each group
*denotes significant difference from the conscious rat groups ($P<0.05$). [a]represents observed data.

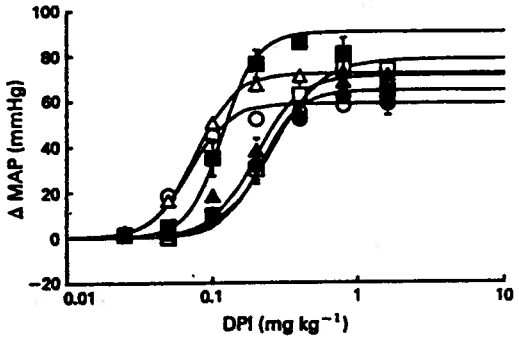

Figure 1 Dose-response (mean ± s.e.mean) curves of i.v. bolus injections of diphenyleneiodonium (DPI) on mean arterial pressure (MAP) in conscious rats (○), and rats anaesthetized with pentobarbitone (65 mg kg^{-1}) (●), ketamine (140 mg kg^{-1}) (△), urethane (2 g kg^{-1}) (▲), ethanol (4 g kg^{-1}) (□) and chloralose (150 mg kg^{-1}) (■). $n = 6$ each group. The lines represent theoretical curves using the formula $Y = E_{max}([D]^n/ED_{50}^n + [D]^n)$ and best-fitted n, ED_{50} and E_{max} shown in Table 1.

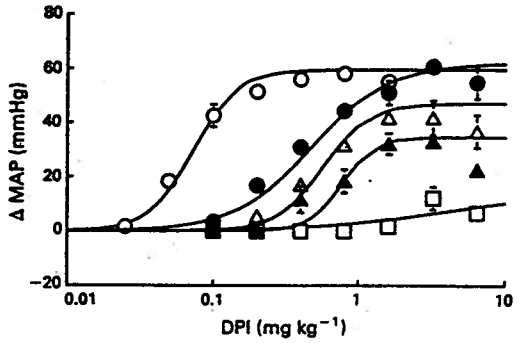

Figure 2 Dose-response (mean ± s.e.mean) curves of i.v. bolus injections of diphenyleneiodonium (DPI) on mean arterial pressure (MAP) in conscious rats and halothane (0.5–1.25%)-anaesthetized rats ($n = 6$ each group). Halothane: 0 (○); 0.5% (●); 0.75% (△); 1% (▲); 1.25% (□). The lines represent theoretical curves using the formula $Y = E_{max}([D]^n/ED_{50}^n + [D]^n)$ and best-fitted n, ED_{50} and E_{max} shown in Table 1.

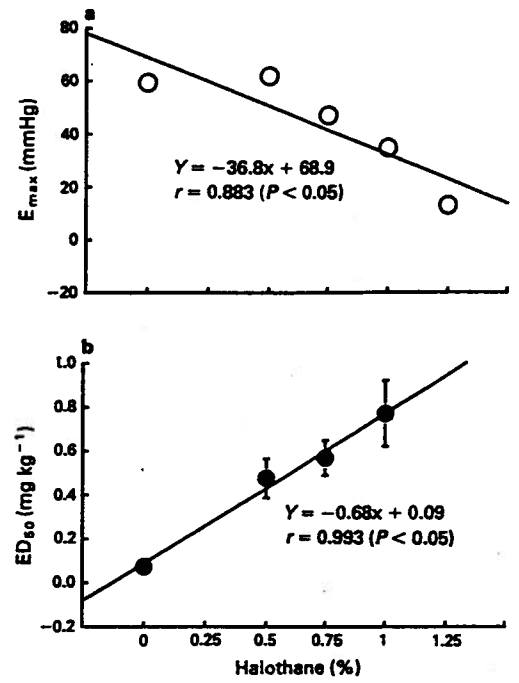

Figure 3 Effects of halothane (0.5–1.25%) on the maximal effect (E_{max}, a) and half-effective dose (ED_{50}, b) of the dose-mean arterial pressure response curves of i.v. bolus injections of diphenyleneiodonium (0.025–6.4 mg kg^{-1}) in rats ($n = 6$ each group).

Table 2 Baseline values of plasma catecholamines and mean arterial pressure (MAP) in conscious and halothane (1.25%)-anaesthetized rats

	Plasma catacholamine (ng ml^{-1})			
	Noradrenaline	*Adrenaline*	*Dopamine*	*MAP*
Conscious	0.26 ± 0.05	0.12 ± 0.01	0.10 ± 0.01	119 ± 4
Halothane	0.25 ± 0.03	0.06 ± 0.01*	0.10 ± 0.01	76 ± 2*

Values are mean ± s.e.mean. $n = 6$ each group.
*denotes significant difference from conscious rats ($P < 0.05$).

concentration of halothane (Figure 3). None of the doses of halothane affected the n value of the curves (Table 1). Halothane also inhibited DPI-induced tachycardia, bradycardia as well as movements (data not shown).

Effect of halothane on DPI-induced catecholamine release

Baseline MAP in halothane (1.25%)-anaesthetized rats was lower than that in conscious rats (Table 2). Baseline plasma level of adrenaline but not noradrenaline or dopamine in halothane-anaesthetized rats was also significantly lower than that in conscious rats (Table 2).

In conscious rats, i.v. bolus injection of DPI (1.6 mg kg^{-1}) caused immediate and large increases (more than 1 ng ml^{-1}) in plasma noradrenaline and adrenaline and a smaller increase (0.1 ng ml^{-1}) in plasma dopamine (Figure 4a), as well as immediate pressor response (Figure 4b). Halothane markedly attenuated DPI-induced increases in plasma catecholamines (Figure 4a) and in MAP (Figure 4b); the reductions of plasma noradrenaline, adrenaline and MAP were 86%, 81% and 95%, respectively.

Discussion

As in previous experiments with pentobarbitone-anaesthetized rats (Wang & Pang, 1993), DPI caused transient and dose-dependent increases in MAP in conscious rats with a Hill coefficient not significantly different from 3 or 4. Moreover, none of the anaesthetics affected the Hill coefficient value. The results suggest that the pressor effects of DPI in conscious and anaesthetized rats are due to the positive cooperation of 3 or 4 molecules of DPI with the corresponding 'receptors' (see Rang, 1971; see discussion of Pennefather & Quastel, 1982; Wang & Pang, 1993).

Although DPI inhibits endothelium-dependent vasodilatation *in vitro* and *in vivo* (Stuehr *et al.*, 1991; Poon *et al.*, 1993), the pressor response to DPI is not a consequence of this inhibition. In a previous study, we found that DPI caused pressor and tachycardic responses by sympathetic stimulation as reflected by concurrent elevations of blood pressure and plasma noradrenaline and adrenaline (Wang & Pang, 1993). Moreover, the pressor response of DPI was attenuated by manoeuvres which impair sympathetic nerve transmission,

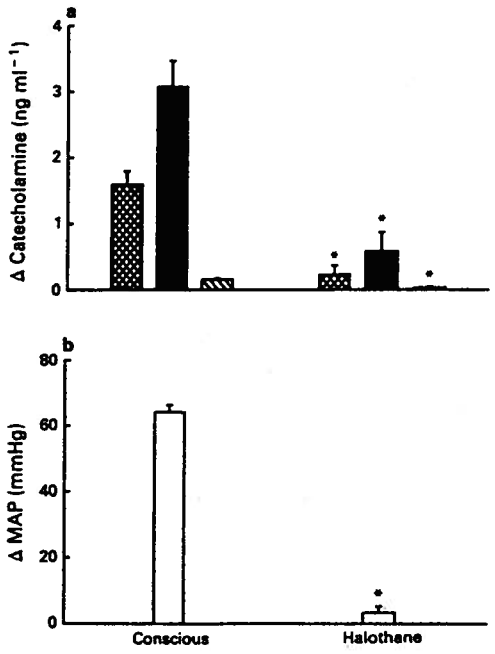

Figure 4 Effects (mean ± s.e.mean) of i.v. bolus injection of diphenyleneiodonium (1.6 mg kg^{-1}) on plasma catecholamines (a) and mean aterial pressure (MAP, b) in conscious and halothane (1.25%)-anaesthetized rats ($n = 6$ each group). In (a), noradrenaline: cross hatched columns; adrenaline: solid columns; dopamine; hatched columns.

namely, pithing, spinal cord transection, tetrodotoxin, mecamylamine, guanethidine, reserpine and phentolamine (Wang & Pang, 1993). In accordance with our previous results, the pressor responses of DPI in conscious rats in the present study were also accompanied by large increases in plasma noradrenaline and adrenaline. Although it is not clear how DPI activates sympathetic nerve activities, the following mechanisms are unlikely to be involved. Firstly, the effect of DPI on sympathetic nerve activity is unrelated to its hypoglycaemic action since the blood pressure and sympathetic stimulatory effects of DPI are immediate and transient (present study) while its hypoglycaemic effect is slow in onset (plateau at 4 h after i.p. administration) and prolonged in action (Gatley & Martin, 1979). Secondly, the pressor response to DPI is unlikely due to the activation of 'pain receptors' as pretreatment with capsaicin (100 mg kg^{-1} s.c. for 2 d) blocked DPI-induced limb kicking but not pressor responses in pentobarbitone-anaesthetized rats ($n = 6$, unpublished observations). Thirdly, the possible inhibitory effect of DPI on brain NO synthesis is unlikely to be responsible for the increase in sympathetic outflow (see discussion of Wang & Pang, 1993).

DPI caused tachycardia in pentobarbitone-anaesthetized rats, with the same time course and pharmacodynamics (ED_{50} and Hill coefficient) as the pressor response (Wang & Pang, 1993). It should be pointed out that the pressor and tachycardic responses of DPI are not interdependent, as propranolol blocked tachycardia but not the pressor response while phentolamine attenuated the pressor but potentiated the tachycardic response (Wang & Pang, 1993). In conscious rats in the present study, DPI produced tachycardia at low doses and bradycardia at high doses. In pentobarbitone-anaesthetized rats, the latter response was absent (Wang & Pang, 1993). The abilities of pentobarbitone anaesthesia to inhibit bradycardic responses to high doses of DPI and phentolamine to potentiate tachycardic responses suggest that bradycardia in response to high doses of DPI in conscious rats was secondary to hypertension-induced baroreflex activation. Different anaesthetic agents have variable influences on baroreflex activity. Due to the difficulty in separating variable direct and indirect effects of DPI on heart rate in different anaesthetic conditions, detailed kinetic analyses of the heart rate effect on DPI were not performed.

Our results also show that halothane dose-dependently and non-competitively inhibited the pressor responses of DPI. It should be emphasized that the inhibitory effect of halothane is not due to its hypotensive action as DPI caused greater pressor responses in rats anaesthetized with chloralose, urethane or ethanol where baseline blood pressures were either similar or lower than those in halothane-anaesthetized rats. Therefore, anaesthetics, at standard anaesthetic doses, have differential effects on the potency and/or efficacy of the pressor response to DPI, and this can be classified as follows: (1) no change in ED_{50} but potentiation of E_{max} of the pressor response to DPI – ketamine; (2) increase in ED_{50} and no change in E_{max}-pentobarbitone; (3) increase in ED_{50} and potentiation of E_{max} - urethane, ethanol and chloralose; (4) increase in ED_{50} and reduction of E_{max} - halothane. The results suggest that the variable influence of anaesthetic agents must be taken into consideration when evaluating the pressor effects of DPI and other pressor agents (Wang *et al.*, 1991a; Abdelrahman *et al.*, 1992).

Halothane has been shown to potentiate (Blaise, 1991) or reduce (Muldoon *et al.*, 1988) endothelium-dependent vasodilatation in different vascular preparations. We have also found that halothane (1.25%) abolishes the pressor effects of L-NNA and L-NAME but not those of noradrenaline or angiotensin II (Wang *et al.*, 1991a; Pang *et al.*, 1992). Although it appears that halothane has a selective inhibitory effect on NO synthase inhibitors, the mechanism by which it suppresses pressor responses to L-NNA and L-NAME is different from that of DPI. L-NNA and L-NAME cause pressor responses by inhibiting NO synthase and subsequent endothelium-dependent relaxation (Wang *et al.*, 1993a; see Moncada *et al.*, 1991). The pressor responses of L-NNA and L-NAME are L-arginine-sensitive (Wang & Pang, 1990; Wang *et al.*, 1991b; 1992; see Moncada *et al.*, 1991) and are independent of the integrity of the central and sympathetic nervous systems (Wang & Pang, 1991; Wang & Pang, 1993). The mechanism by which halothane inhibits pressor responses to L-NNA may involve selective increase in NO synthesis and or release, or potentiation of the effect of NO (Wang *et al.*, 1991b; Pang *et al.*, 1992) and, this may be analogous to the inhibitory effect of sodium nitroprusside on the pressor effect of L-NNA (Wang *et al.*, 1993b).

Halothane markedly inhibited DPI-induced increases in both plasma catecholamines and MAP. The results suggest that the inhibitory effect of halothane on the pressor response to DPI is primarily due to the suppression of sympathetic activation rather than inhibition of endothelium-dependent vasodilatation. Halothane has been shown to depress activities of the sympathetic nervous system at different levels (1) areas of the central nervous system controlling sympathetic nerve activity (Price *et al.*, 1963; Millar *et al.*, 1969; Larach *et al.*, 1987; Bazil & Minneman, 1989), (2) sympathetic ganglia (Skovsted *et al.*, 1969; Christ, 1977; Bosnjak *et al.*, 1982; Seagard *et al.*, 1982), and (3) sympathetic nerve endings located in the walls of blood vessels (Muldoon *et al.*, 1975; Lunn & Rorie, 1984; Rorie *et al.*, 1990). In addition, a small component of non-specific inhibition by halothane may also be responsible for its effect on the pressor response to DPI. Halothane, at 1.25%, inhibited the maximal pressor response to DPI by 95%, and the increases in plasma noradrenaline and adrenaline by 86% and 81%, respectively. These results are consistent with those of our other study in which the same concentration of halothane reduced the pressor response produced by exogenous noradrenaline or angiotensin II by 18% (Pang *et al.*, 1992). Therefore, the inhibition by halothane of the pressor response to DPI is primarily (approximately 80%)

attributable to the inhibition of sympathetic transmission and secondarily (approximately 20%) due to non-specific inhibition of vascular smooth muscle contraction.

The influence of ketamine and pentobarbitone on sympathetic transmission may also account for their effects on the pressor response to DPI. Ketamine activates the sympathetic nervous system (Traber & Wilson, 1969) by inhibiting neuronal noradrenaline uptake (Nedergaard, 1973; Clanachan & McGrath, 1976). The blockade of uptake$_1$ by ketamine may cause potentiation of the pressor effect of DPI. This interpretation is consisent with our unpublished observation that the uptake$_1$ inhibitor, cocaine also potentiates pressor and tachycardic responses of DPI ($n = 6$). The inhibitory effect of pentobarbitone may involve the suppression of catecholamine release since pentobarbitone has been shown to inhibit the release of noradrenaline from the peripheral sympathetic nerve terminals in rabbit and chicken isolated hearts (Gothert & Rieckesmann, 1978; see Richter & Holtman, 1982), as well as releases of noradrenaline and adrenaline from the perfused cow adrenal glands (see Richter & Holtman, 1982).

In summary, i.v. bolus injections of DPI caused immediate and transient pressor responses in conscious rats; the pressor response was accompanied by large increases in plasma noradrenaline and adrenaline. Among the various anaesthetics (ketamine, urethane, ethanol, chloralose) studied, halothane is the only one which dose-dependently and non-competitively inhibits DPI-induced pressor response and the rises in plasma noradrenaline and adrenaline. The results suggest that (1) DPI causes a pressor response by stimulating sympathetic nerve activities in conscious rats; (2) halothane inhibits the pressor response of DPI by suppressing sympathetic nerve activities rather than interfering with endothelium-dependent vasodilatation; (3) the variable effects of anaesthetics must be taken into consideration when assessing the pressor and heart rate responses of DPI and other vasoactive agents.

This work was supported by a grant from the Medical Research Council of Canada (MRC) and a MRC post-doctoral fellowship to Y.-X. Wang. We would like to express our appreciation to Ms Su Lin Lim for her skilled technical assistance.

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(Received November 26, 1992
Revised February 23, 1993
Accepted April 1, 1993)

Paper VI

Wang, Y.-X., Abdelrahman, A. and Pang, C.C.Y.: Selective inhibition of pressor and haemodynamic effects of N^G-nitro-L-arginine by halothane. J. Cardiovasc. Pharmacol. 22: 571-578, 1993. The reproduction of this paper was kindly permitted by the copyright holder, Raven Press, Ltd., New York, U.S.A.

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Selective Inhibition of Pressor and Haemodynamic Effects of N^G-Nitro-L-Arginine by Halothane

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Summary: We investigated the characteristics of inhibition by halothane of the pressor responses to N^G-substituted L-arginine derivatives, nitric oxide (NO) synthase inhibitors. Intravenous (i.v.) bolus injections of N^G-nitro-L-arginine (L-NNA, 1-32 mg/kg), N^G-nitro-L-arginine methyl ester (L-NAME, 0.4–12.8 mg/kg), norepinephrine (NE, 0.25–8 μg/kg) and angiotensin II (AII, 0.02–0.64 μg/kg) each caused dose-dependent pressor responses in conscious rats. Halothane attenuated responses to the highest dose of NE and AII by ~18% but completely abolished responses to L-NNA and L-NAME. The haemodynamic effects of L-NNA were further examined by the microsphere technique in two groups of conscious rats and two groups of halothane-anaesthetized rats. An i.v. bolus injection of L-NNA (16 mg/kg) in conscious rats increased mean arterial pressure (MAP) and total peripheral resistance (TPR) and reduced heart rate (HR) and cardiac output (CO). These changes were associated with reduced conductance in all vascular beds, with the greatest reduction in the lungs and the least in the liver. In halothane-anaesthetized rats, L-NNA caused significant but markedly less change in MAP, HR, TPR, and CO as compared with those in conscious rats. The vasoconstrictor effects of L-NNA were attenuated by halothane in all beds except liver and spleen, with the greatest inhibition in heart. Our results suggest that NO plays a role in maintenance of peripheral vascular resistance and that halothane selectively and "noncompetitively" inhibits the vasoconstrictor effects of NO synthase inhibitors.
Key Words: Halothane—N^G-Nitro-L-arginine—N^G-Nitro-L-arginine methyl ester—Blood pressure—Haemodynamics—Vascular conductance—Nitric oxide—Rat.

Synthesis and release of nitric oxide (NO) or endothelium-derived relaxing factor (EDRF) from cultured and native endothelial cells has been reported to be inhibited by N^G-nitro-L-arginine (L-NNA) (1,2). In vitro studies, L-NNA suppressed endothelium-dependent vascular relaxation (2–4). L-NNA also caused pressor responses in pentobarbital- (5) and urethane-anaesthetized (6,7) and conscious rats (8). The pressor effect of L-NNA is antagonized by L-arginine but not by D-arginine (9) or blockers of the autonomic nervous system or renin-angiotensin system (RAS) (8).

In a study that examined the effects of intravenous (i.v.) and inhalation anaesthetic agents on pressor response to L-NNA, we showed that halothane abolished the pressor effect of L-NNA; this inhibition is reversible on discontinuation of halothane anaesthesia (10). Halothane nonselectively inhibits the pressor and vasoconstrictor effects of vasopressor agents (11–13). Whether halothane selectively abolishes pressor responses of N^G-substituted L-arginine derivatives or nonselectively interferes with the final common pathway for contraction is not known.

We investigated whether halothane causes selective inhibition of the pressor effects of NO synthase inhibitors, L-NNA and N^G-nitro-L-arginine methyl ester (L-NAME). The pressor responses to L-NNA and L-NAME were compared with those to norepinephrine (NE) and angiotensin II (AII) in conscious and halothane-anaesthetized rats. In the second part of the study, we examined the haemodynamic effects of L-NNA in conscious and halothane-anaesthetized rats to determine whether inhibition

Received November 20, 1993; revision accepted June 7, 1993.
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of L-NNA by halothane is caused by reduction in cardiac output (CO) and/or regional vascular resistance.

METHODS

Surgical preparations

Sprague-Dawley rats weighing 320–400 g were anaesthetized with halothane (4% for induction, 2% for surgical preparation, and 1.2% for maintenance) in air at a flow rate of 1,500 ml/min. Cannulas filled with heparinized normal saline (25 IU/ml) were inserted in the right iliac artery for recording of mean arterial pressure (MAP) by a pressure transducer (P23DB, Gould Statham, CA, U.S.A.) and into the right femoral vein for drug administration. For haemodynamic experiments, additional cannulas were also inserted in the left ventricle through the right carotid artery for injections of radioactively labeled microspheres and in the left iliac artery for blood withdrawal. From the upstroke of the arterial pulse pressure, heart rate (HR) was determined electronically by a tachograph (7P4G Grass). In the conscious rat experiments, the rats were allowed >4 h to recover from the effects of halothane before further use. The body temperature of halothane-anaesthetized rats was kept at 37°C with a heating lamp connected to a 73A thermostat (Yellow Springs Instruments), and the rats were used 30 min after operation.

Microsphere technique

The reference sample microsphere technique was described previously in detail (14): 30,000–40,000 microspheres (15-μm diameter) labeled with either ^{57}Co or ^{113}Sn (Du Pont Canada, Ontario, Canada) were injected in the left ventricle in the control period and after administration of a drug or vehicle. The order of administration of the microspheres was reversed in half of each group of rats. At the end of the experiments, blood samples, whole organs of lungs, heart, liver, stomach, intestine, caecum, and colon (presented as colon in Table 1 and Figs. 1–6), kidneys, spleen, testes and brain, as well as 30 g each skeletal muscle and skin, were removed for counting of radioactivity with an 1185 Series Dual Channel Automatic Gamma Counter (Nuclear, Chicago, IL. U.S.A.).

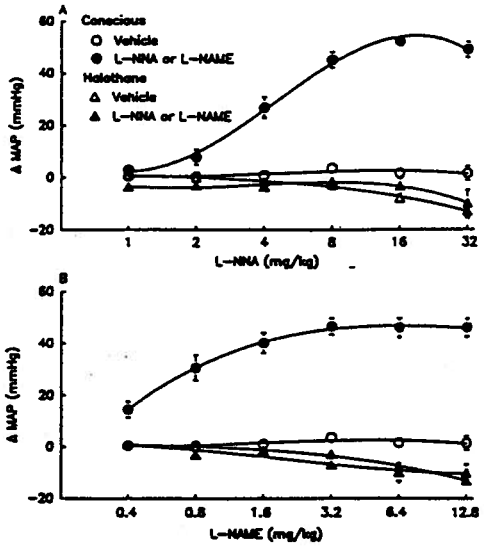

FIG. 1. Dose–mean arterial pressure (MAP) response (means ± SE) curves of cumulative intravenous bolus injections of N^G-nitro-L-arginine (L-NNA (A) and N^G-nitro-L-arginine methyl ester (L-NAME) (B) or equivalent volume of vehicle (0.9% NaCl) in conscious and halothane-anaesthetized rats (n = 5 per group). Values represent changes from pretreatment values.

TABLE 1. *Baseline values (means ± SE) of MAP, HR, CO, and TPR in conscious (groups I–IV, IX–X) and halothane-anaesthetized (groups V–VIII, XI–XII) rats*

Group	n	MAP (mm Hg)	HR (beats/min)	CO (ml/min)	TPR (mm Hg/min/ml)
Protocol 1					
I	5	110 ± 5			
II	5	108 ± 7			
III	5	114 ± 4			
IV	5	121 ± 4			
Pooled	20	113 ± 3			
V	5	79 ± 1			
VI	5	78 ± 2			
VII	5	85 ± 2			
VIII	5	79 ± 1			
Pooled	20	80 ± 1[a]			
Protocol 2					
IX	6	123 ± 3	368 ± 11	102 ± 3	1.20 ± 0.0
X	6	132 ± 3	392 ± 16	110 ± 8	1.20 ± 0.0
Pooled	12	128 ± 2	380 ± 10	106 ± 4	1.20 ± 0.0
XI	6	73 ± 2	319 ± 8	84 ± 5	0.89 ± 0.0
XII	6	79 ± 2	298 ± 7	82 ± 4	0.98 ± 0.0
Pooled	12	76 ± 2[a]	309 ± 6[a]	309 ± 6[a]	0.94 ± 0.0

MAP, mean arterial pressure; HR, heart rate; CO, cardiac output; TPR, total peripheral resistance.
[a] Significant difference from pooled data in conscious rats ($p < 0.05$).

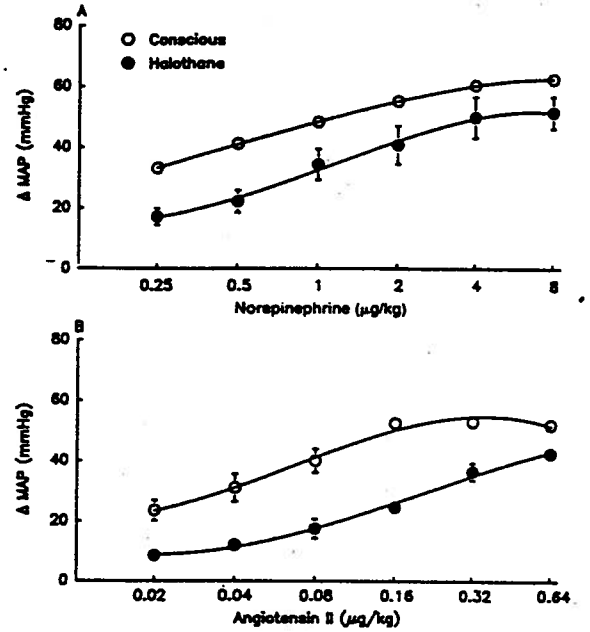

FIG. 2. Dose–mean arterial pressure (MAP) response (means ± SE) curves of intravenous bolus injections of norepinephrine (A) and angiotensin II (B) in conscious and halothane-anaesthetized rats (n = 5 per group). Values represent changes from pretreatment values.

Drugs

L-NNA, L-NAME hydrochloride, AII acetate, and NE hydrochloride were obtained from Sigma Chemical, (St. Louis, MO, U.S.A.) and dissolved in normal saline (0.9% NaCl). Dissolution of L-NNA required 20-min sonication.

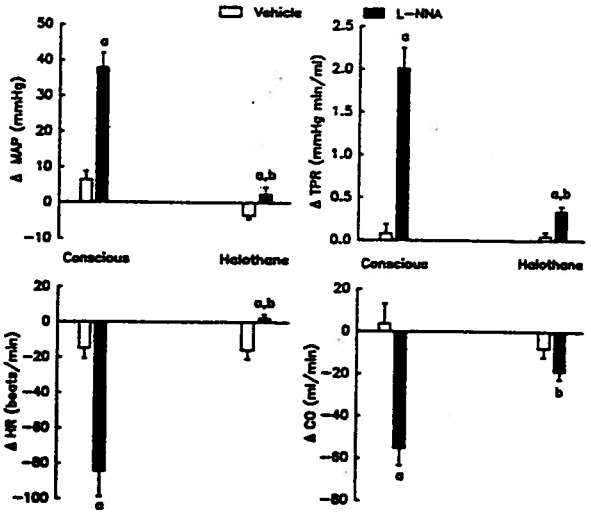

FIG. 3. Effects (means ± SE) of intravenous bolus injections of vehicle (0.9% NaCl) and N^G-nitro-L-arginine (L-NNA 16 mg/kg) on mean arterial pressure (MAP), heart rate (HR), cardiac output (CO), and total peripheral resistance (TPR) in four groups of conscious and halothane-anaesthetized rats (n = 6 per group). Values represent changes from pretreatment values. [a]Significant difference from vehicle group ($p < 0.05$). [b]Significant difference from conscious rat group ($p < 0.05$).

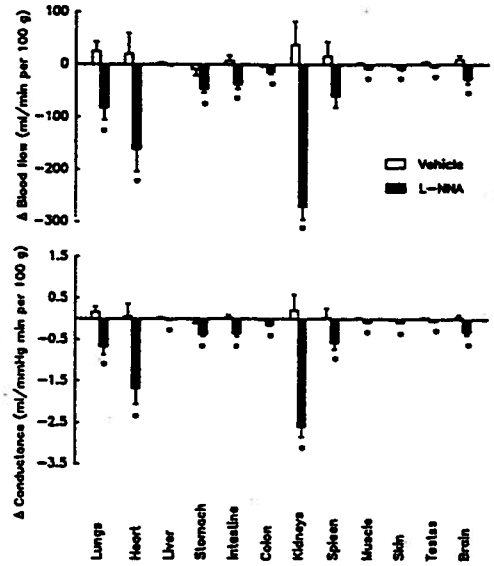

FIG. 4. Effects (means ± SE) of intravenous bolus injections of vehicle (0.9% NaCl) and N^G-nitro-L-arginine (L-NNA, 16 mg/kg) on blood flow and vascular conductance in conscious rats (n = 6 per group). Values represent changes from pretreatment values. *Significant difference from vehicle group ($p < 0.05$).

Halothane was obtained from Ayerst Laboratory (Montreal, Canada).

Experimental protocol

In the first study, eight groups of rats (n = 5 each group) were used to construct the dose–MAP response curves of L-NNA, L-NAME, NE, AII, and vehicle in conscious rats (groups I–IV) and halothane-anaesthetized rats (groups V–VIII). After 30-min equilibration to obtain stable baseline readings, vehicle was injected as an i.v. bolus in groups I and V. L-NNA (1–32 mg/kg) was cumulatively injected as an i.v. bolus in groups II and VI, and L-NAME (0.4–12.8 mg/kg) was administered to groups III

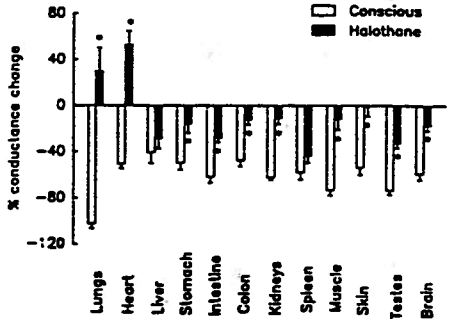

FIG. 5. Percentage of change in vascular conductance (means ± SE) after intravenous bolus injection of N^G-nitro-L-arginine (L-NNA 16 mg/kg) in conscious and halothane-anaesthetized rats (n = 6 per group). Values were calculated by subtracting vehicle-induced conductance changes from corresponding L-NNA–induced conductance changes, dividing this difference by absolute vascular conductance value before L-NNA injection (predrug control), and then multiplying the resultant number by 100%. *Significant difference from conscious rats ($p < 0.05$).

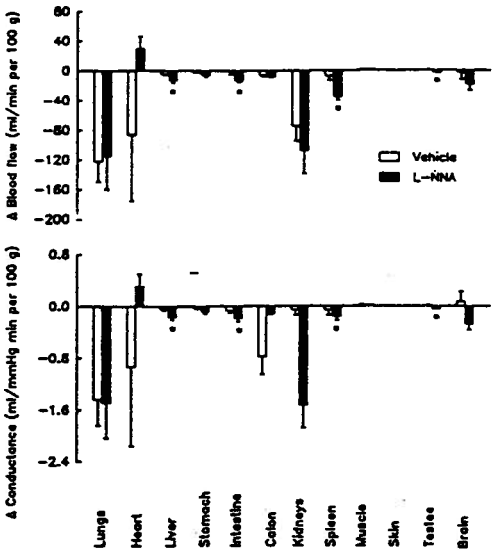

FIG. 6. Effects (means ± SE) of intravenous bolus injections of vehicle (0.9% NaCl) and N^G-nitro-L-arginine (L-NNA 16 mg/kg) on blood flow and vascular conductance in halothane-anaesthetized rats (n = 6 per group). Values represent changes from pretreatment values. *Significant difference from vehicle group ($p < 0.05$).

and VII. Ten-minute dose intervals were allowed between injections for MAP to reach plateau values. In groups IV and VIII, i.v. bolus injections of doses of NE (0.25–8 μg/kg) and AII (0.02–0.64 μg/kg) were given at dose intervals of 5 min to allow recovery from the effects of the previous dose. The sequence of the injections of NE and AII was reversed in half of the experiments in each group.

In the second study, four groups of rats (n = 6 each group) were used to investigate the haemodynamic effects of L-NNA in conscious (groups IX and X) and halothane-anaesthetized rats (groups XI and XII). One minute after the first set of microspheres was injected, vehicle was injected as an i.v. bolus in groups X and XII and L-NNA (16 mg/kg) was injected as an i.v. bolus in groups IX and XI. After 20 min more, the second set of microspheres was injected.

Calculations and statistical analysis

Total peripheral resistance (TPR), CO, blood flow, and vascular conductance (blood flow/MAP) were calculated as described (14). Blood flow and vascular conductance are given as values per 100 g tissue. Changes in vascular conductance by L-NNA in conscious and halothane-anaesthetized rats are also determined as percentage of conductance change to compare the magnitudes of vasoconstriction in the two states; this was obtained by subtracting the vehicle-induced organ/tissue conductance change in the time-control group from the corresponding change by L-NNA, dividing this difference by the absolute conductance value before L-NNA injection (predrug control), and multiplying the resultant value by 100%. All results are mean ± SE and were determined by analysis of variance followed by Duncan's multiple-range test to compare group means; $p < 0.05$ was preselected as the criterion for statistical significance.

RESULTS

Effects of halothane on MAP responses to L-NNA, L-NAME, NE, and AII

There were no significant differences in baseline values of MAP among conscious rats in groups I–IV and among halothane-anaesthetized rats in groups V–VIII (Table 1). Comparison of pooled values shows that MAP of halothane-anaesthetized rats was lower than that of conscious rats.

Vehicle did not alter MAP in conscious rats (Fig. 1A and B). Bolus injections of i.v. cumulative doses of L-NNA (Fig. 1A) and L-NAME (Fig. 1B) in conscious rats caused dose-dependent increases in MAP, with maximum increases of ~50 mm Hg. In halothane-anaesthetized rats, vehicle, L-NNA, and L-NAME each caused similar slight decreases in MAP with passage of time (Fig. 1A and B).

Bolus i.v. injections of NE (Fig. 2A) and AII (Fig. 2B) caused dose-dependent increases in MAP in conscious rats with maximum increases of ~50–60 mm Hg. Halothane "noncompetitively" inhibited the pressor responses of NE and AII, but the degrees of inhibition caused by halothane at the highest doses of NE and AII were 17.6 and 18.0%, respectively.

Haemodynamic effects of L-NNA in conscious and halothane-anaesthetized rats

Table 1 shows that baseline values of MAP, HR, CO, and TPR were similar in the two groups of conscious rats (IX and X) and the two groups of halothane-anaesthetized rats (XI and XII). Pooled values of MAP, HR, CO, and TPR in halothane-anaesthetized rats were significantly less than those of conscious rats.

In conscious rats, i.v. bolus injection of L-NNA significantly increased MAP and TPR and decreased HR and CO as compared with changes in the vehicle-treated group (Fig. 3). In halothane-anaesthetized rats, i.v. bolus injection of L-NNA caused slight but significant increases in MAP and TPR, significant increase in HR, and insignificant decrease in CO (Fig. 3). However, as compared with the changes in conscious rats, the effects of L-NNA on MAP, TPR, HR, and CO were significantly less in halothane-anaesthetized rats (Fig. 3).

Table 2 shows baseline values of regional blood flow and vascular conductance in conscious and halothane-anaesthetized rats. There were no significant differences in baseline values of blood flow and conductance in any organs/tissues between the two groups of conscious rats (IX and X) and between the two groups of halothane-anaesthetized rats (XI and XII). Pooled data showed that halothane increased blood flow to lungs, liver, testes and brain, decreased blood flow to stomach, intestine, kidney, spleen, muscle, and skin, but did not alter blood flow to heart, caecum, and colon. Blood flow was also normalized by MAP to yield vascular

TABLE 2. *Baseline values (means ± SE) of blood flow and vascular conductance in conscious (groups IX–X) and halothane-anaesthetized (groups XI–XII) rats (n = 6 per group)*

Parameter	Conscious			Halothane		
	Group IX	Group X	Pooled	Group XI	Group XII	Pooled
Blood flow in each organ (ml/min/100 g)						
Lung	72 ± 14	111 ± 24	91 ± 15	267 ± 39	184 ± 59	225 ± 37[a]
Heart	269 ± 12	431 ± 63	350 ± 40	309 ± 67	201 ± 18	255 ± 38
Liver	14 ± 1	15 ± 3	14 ± 2	34 ± 2	33 ± 4	34 ± 2[a]
Stomach	71 ± 7	72 ± 6	71 ± 5	41 ± 6	32 ± 3	36 ± 4[a]
Intestine	81 ± 4	79 ± 8	80 ± 4	55 ± 7	56 ± 6	56 ± 5[a]
Colon	31 ± 1	35 ± 5	33 ± 3	34 ± 3	30 ± 3	32 ± 2
Kidney	459 ± 54	591 ± 24	525 ± 35	422 ± 29	470 ± 23	446 ± 20[a]
Spleen	143 ± 22	127 ± 20	135 ± 15	79 ± 10	75 ± 7	77 ± 6[a]
Muscle	16 ± 2	16 ± 1	16 ± 1	6.9 ± 0.4	6.6 ± 0.8	6.7 ± 0.4
Skin	19 ± 2	19 ± 1	19 ± 1	6.5 ± 0.4	6.9 ± 0.4	6.7 ± 0.3
Testes	11 ± 1	14 ± 1	12 ± 1	17 ± 1	17 ± 2	17 ± 1[a]
Brain	67 ± 6	80 ± 12	74 ± 7	163 ± 5	167 ± 12	165 ± 7[a]
Vascular conductance in each organ (ml/mm Hg min/100 g)						
Lung	0.57 ± 0.1	0.84 ± 0.19	0.71 ± 0.1	3.65 ± 0.56	2.28 ± 0.7	2.97 ± 0.50
Heart	2.19 ± 0.1	3.27 ± 0.50	2.73 ± 0.3	4.15 ± 0.86	2.55 ± 0.2	3.35 ± 0.50
Liver	0.11 ± 0.0	0.12 ± 0.03	0.11 ± 1.0	0.47 ± 0.03	0.42 ± 0.0	0.44 ± 0.03
Stomach	0.58 ± 0.0	0.54 ± 0.05	0.56 ± 0.0	0.55 ± 0.07	0.40 ± 0.0	0.47 ± 0.04
Intestine	0.66 ± 0.0	0.60 ± 0.07	0.63 ± 0.0	0.75 ± 0.08	0.71 ± 0.0	0.73 ± 0.06
Colon	0.26 ± 0.0	0.26 ± 0.04	0.26 ± 0.0	0.46 ± 0.04	0.38 ± 0.0	0.42 ± 0.03
Kidney	3.76 ± 0.4	4.48 ± 0.24	4.12 ± 0.2	5.73 ± 0.30	5.98 ± 0.3	5.86 ± 0.24
Spleen	1.17 ± 0.1	0.97 ± 0.16	1.07 ± 0.1	1.10 ± 0.16	0.94 ± 0.0	1.02 ± 0.09
Muscle	0.13 ± 0.0	0.12 ± 0.01	0.13 ± 0.0	0.09 ± 0.01	0.08 ± 0.0	0.09 ± 0.01
Skin	0.15 ± 0.0	0.14 ± 0.01	0.14 ± 0.0	0.09 ± 0.01	0.09 ± 0.0	0.09 ± 0.01
Testes	0.09 ± 0.0	0.10 ± 0.01	0.01 ± 0.0	0.24 ± 0.01	0.21 ± 0.0	0.22 ± 0.01
Brain	0.55 ± 0.0	0.61 ± 0.09	0.58 ± 0.0	2.24 ± 0.11	2.13 ± 0.1	2.18 ± 0.11

[a] Significant difference from pooled data of conscious rats ($p < 0.05$).

conductance values to reflect active changes in vascular tone. Comparison of pooled conductance values showed that halothane increased vascular conductance of lungs, liver, caecum and colon, kidney, testes, and brain, decreased conductance of muscle and skin, but did not affect conductance of other organs/tissues.

In conscious rats, i.v. bolus injection of L-NNA as compared with vehicle decreased blood flow to all organs or tissues except liver and spleen (Fig. 4A). Conductance values show that L-NNA vasoconstricted all beds (Fig. 4B). Changes in conductance were also expressed as percentages of conductance change to reflect the magnitudes of vasoconstriction in each organ/tissue response to L-NNA (Fig. 5). The results show that although L-NNA reduced vascular conductance in all beds in conscious rats, the greatest influence was in lung and the least was in liver.

In halothane-anaesthetized rats, i.v. bolus injection of L-NNA as compared with vehicle decreased both blood flow and conductance in liver, intestine, spleen, and testes but not in other vascular beds (Fig. 6A and B). Comparison of percentage of conductance change in response of L-NNA in conscious and halothane-anaesthetized rats showed that halothane reduced the vasoconstrictor effects of L-NNA in every organ or tissue; significant reductions were obtained in all beds except liver and spleen (Fig. 5). The greatest inhibitory effect of halothane was in heart and lung, in which the effect of L-NNA was completely reversed from vasoconstriction to vasodilatation.

DISCUSSION

Our results show that L-NNA increased MAP in conscious rats by increasing TPR, since both CO and HR were reduced. Reduction in CO caused by L-NNA may be due to the effects of reduced HR, reduced cardiac contractility, and increased flow resistance (TPR). The bradycardic response to L-NNA was previously shown to be the result of hypertension-induced activation of baroreflex activity (8).

L-NNA vasoconstricted all beds, suggesting that the endogenous EDRF/NO system modulates vascular conductance in all organs/tissues in rats. Humphries and colleagues reported that intravenous infusion of L-NNA in conscious rabbits increased MAP (by 11 mm Hg) and TPR, reduced HR and CO, and caused significant vasoconstriction in brain, heart, kidney, and duodenum, but not in muscle, skin, stomach, ileum, or colon (15). The greater extent of vasoconstriction in response to L-NNA in our study may be due to use of a higher

dose of L-NNA. Other analogues of N^G-substituted L-arginine, N^G-monomethyl-L-arginine (L-NMMA) and L-NAME were also reported to reduce CO and decrease renal, mesenteric, hindquarters, or internal carotid blood flow in rats (16–19).

The extent of vasoconstriction in response to L-NNA, as determined from percentage of conductance changes, is not uniform, however; i.e., the greatest influence is in lung and the least is in liver. The lungs receive circulation from the bronchial artery, pulmonary artery, and arteriovenous anastomoses. Counts in the lungs primarily reflect circulation from the bronchial artery and arteriovenous anastomoses since microspheres are almost completely trapped in one circulation (unpublished observations). Likewise, owing to the entrapping of microspheres in the splanchnic area, liver blood flow is expected to represent primarily hepatic arterial flow. Hepatic arterial flow has been postulated to be controlled by the hepatic arterial buffer response so that decreases in portal venous flow are associated with increases in hepatic arterial flow, thereby maintaining the constancy of total hepatic blood flow (20). This hypothesis is in accordance with our findings that reduced splanchnic and consequently portal venous flow occurred concurrently with increased hepatic arterial flow. Therefore, the absence of vasoconstrictor effect of L-NNA in the hepatic bed may be due to the hepatic arterial buffer response. L-NNA caused marked coronary vasoconstriction in conscious rats. NO synthase inhibitors cause coronary vasoconstriction in conscious rabbits (21) and dogs (22), as well as sustained increase in rabbit coronary perfusion pressure in vitro (21). In contrast, Klabunde and co-workers reported that L-NMMA and L-NNA did not reduce HR or coronary flow in pentobarbital-anaesthetized dogs (23); their inability to show coronary constrictor effect of NO synthase inhibitors may have been due to the influence of pentobarbital.

Halothane reduced MAP, HR, CO, and TPR. The hypotensive effect of halothane in this study probably is a consequence of reduced regional vascular resistances as well as myocardial depression as reflected by reduced HR. Halothane reduces HR and cardiac contractility by interfering with baroreflex activity (24). The greatest vasodilatation effects of halothane were in lung, liver, and brain, where three- to fourfold increases in conductance values were obtained. Smaller degrees of vasodilatation were also observed in caecum/colon, kidney, and testes. In contrast to our results, halothane was reported to decrease MAP, HR, and CO, but to have little effect on TPR (25).

Our study shows that halothane noncompetitively and completely inhibited pressor responses of L-NNA and L-NAME. Greenblatt and associates reported that halothane did not affect the haemodynamic effects of L-NMMA in rats pretreated with indomethacin 5 mg/kg intravenously (i.v.) (26). However, in our experiments, halothane also abolished the pressor response of L-NNA in rats pretreated with the same dose of indomethacin (data not shown). The results of Greenblatt and associates with halothane (26) are not characteristic because the anaesthetic nonselectively inhibits the vasoconstrictor effects of drugs [e.g., phenylephrine (12), azepexole (11), potassium, prostanoids (27), and serotonin (13)]. Therefore, it is also important to rule out the nonspecific component of the inhibitory effects on L-NNA and L-NAME. Our results show that halothane noncompetitively inhibited pressor effects of NE and AII by ~18% but completely abolished the response of L-NNA and L-NAME. Nonselective inhibition of smooth muscle contractility by halothane may result from interference with calcium mobilization, since halothane was shown to decrease tension developed in saponin-skinned rabbit aortic strips by depleting calcium in the sarcoplasmic reticulum (28). Because only a small component of the abolition of the MAP response to L-NNA or L-NAME is nonselective, our results show that halothane selectively inhibits the vasoconstrictor effects to L-NNA and L-NAME.

The mechanism by which halothane selectively inhibits the vasoconstrictor effects of NO synthase inhibitors is not clear. Blaise reported that halothane potentiated acetylcholine-induced coronary arteriolar dilatation in isolated perfused rabbit hearts; the results suggested that halothane may release or potentiate the action of EDRF (29). Because L-NAME did not affect the relaxation responses of halothane and enflurane in canine middle cerebral arteries (30), release of NO may be caused by an alternative mechanism distinct from the well-known L-arginine/NO synthetic pathway. We noted that the NO donor (31,32) sodium nitroprusside and ethanol, like halothane, also selectively and noncompetitively attenuated maximal pressor response to L-NNA (33,34). Therefore, we postulate that halothane inhibits the vasoconstrictor effects of NO synthase inhibitors by releasing NO or potentiating the vasodilator effect of NO. In contrast to the above observations, Muldoon and associates reported that halothane attenuated endothelium-dependent vasodilatation evoked by ACh and bradykinin but not endothelium-independent vasodilatation produced by nitroglycerin in isolated rabbit arteries, suggesting that halothane inhibits release and/or action of EDRF (35). The different effects of halothane on endothelium-dependent relaxation may result from different animal species, vascular preparations, and concentrations of halothane.

In the haemodynamic studies, halothane almost completely inhibited the effects of L-NNA on MAP, HR, CO, and TPR. Neither were the effects of halothane, as reflected by percentage of change in con-

ductance, uniform. Halothane significantly reduced the vasoconstrictor effects of L-NNA in all beds except the liver and spleen. The greatest inhibitory effect of halothane was in heart. Although halothane alone did not alter coronary conductance, it reversed the effect of L-NNA in the coronary bed from vasoconstriction to vasodilatation.

L-NNA is an efficacious pressor agent which increases MAP by increasing TPR. TPR is increased through generalized vasoconstriction, but the degree of influence is not uniform in all beds. Halothane causes selective and "noncompetitive" inhibition of vasoconstrictor and pressor responses to L-NNA and L-NAME. The results suggest that endogenous NO biosynthesis contributes to vascular tone and blood pressure; halothane may suppress the vasoconstrictor effects of NO synthase inhibitors through release of NO or potentiation of the vasodilator action of NO.

Acknowledgment: This work was supported by a grant from the Medical Research Council (MRC) of Canada and an MRC Fellowship to Y.-X.W.

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Paper VII

Wang, Y.-X., Poon, C.I., Poon, K.S. and Pang, C.C.Y.: Inhibitory actions of diphenyleneiodonium on endothelium-dependent vasodilatations *in vitro* and *in vivo*. Br. J. Pharmacol. 110: 1232-1238, 1993. The reproduction of this paper was kindly permitted by the copyright holder, Macmillan Press Ltd., Hampshire, U.K.

Br. J. Pharmacol. (1993), 110, 1232-1238

Inhibitory actions of diphenyleneiodonium on endothelium-dependent vasodilatations *in vitro* and *in vivo*

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1 This study examined the *in vitro* and *in vivo* inhibitory effects of diphenyleneiodonium (DPI), a novel inhibitor of nitric oxide (NO) synthase, on endothelium-dependent vasodilatations.

2 DPI (3×10^{-8}–3×10^{-6} M) concentration-dependently inhibited acetylcholine (ACh)-induced relaxation in preconstricted rat thoracic aortic rings, with an IC_{50} of 1.8×10^{-7} M and a maximal inhibition of nearly 100%. DPI (3×10^{-6} M) also completely inhibited the relaxation induced by the calcium ionophore, A23187 but not by sodium nitroprusside (SNP). The inhibitory effect of DPI (3×10^{-7} M) on ACh-induced relaxation was prevented by pretreatment with NADPH (5×10^{-3} M) and FAD (5×10^{-4} M) but not L-arginine (L-Arg, 2×10^{-3} M). Pretreatment with NADPH did not alter the inhibitory effect of N^G-nitro-L-arginine on ACh-induced relaxation.

3 The inhibitory effect of DPI on ACh-induced relaxation in the aortae lasted >4 h after washout. In contrast to pretreatment, post-treatment (1 h later) with NADPH (5×10^{-3} M) reversed only slightly the inhibitory effect of DPI.

4 In conscious rats, DPI (10^{-5} mol kg^{-1}) inhibited the depressor response to i.v. infused ACh, but not SNP. However, it caused only a transient pressor response which was previously shown to be due completely to sympathetic activation.

5 Thus, DPI is an efficacious and 'irreversible' inhibitor of endothelium-dependent vasodilatation *in vivo* and *in vitro*. The mechanism of the inhibition may involve antagonism of the effects of FAD and NADPH, co-factors of NO synthase. However, unlike the N^G-substituted arginine analogues (another class of NO synthase inhibitors), DPI-induced suppression of endothelium-dependent vasodilatation *in vivo* does not lead to a sustained rise in blood pressure.

Keywords: Diphenyleneiodonium (DPI); nitric oxide synthase inhibitor; endothelium-dependent relaxation; blood pressure; acetylcholine (ACh); A23187; sodium nitroprusside (SNP); FAD

Introduction

A group of iodonium compounds have been reported to be a new class of nitric oxide (NO) synthase inhibitors in the macrophage (Stuehr *et al.*, 1991b; Kwon *et al.*, 1991; Keller *et al.*, 1992). These compounds include diphenyleneiodonium (DPI), iodoniumdiphenyl and di-2-thienyliodonium, all of which have chemical structures distinct from those of N^G-substituted arginine (Arg) analogues. DPI was initially found to be a potent hypoglycaemic agent (Stewart & Hanley, 1969; Gatley & Martin, 1979) which, by inhibiting gluconeogenesis from lactate and aspartate, suppressed the oxidation of NADH-linked substances (Holland *et al.*, 1973). It was later shown that DPI, iodoniumdiphenyl and di-2-thienyliodonium suppressed the activities of neutrophil and macrophage NADPH-dependent oxidase (Cross & Jones, 1986; Hancock & Jones, 1987; Ellis *et al.*, 1988; 1989), probably *via* inhibition of a flavoprotein (Cross & Jones, 1986; Hancock & Jones, 1987; Ellis *et al.*, 1989; O'Donnell *et al.*, 1993).

The pharmacology of N^G-substituted Arg analogues, which include N^G-monomethyl-L-Arg (L-NMMA), N^G-nitro-L-Arg (L-NOARG), N^G-nitro-L-Arg methyl ester (L-NAME), L-iminoethyl-ornithine (L-NIO) and N^G-amino-L-Arg (L-NAA) (see Moncada *et al.*, 1991), has been extensively studied. These compounds cause sustained inhibition of endothelium-dependent relaxation *in vitro* and produce prolonged pressor responses in whole animals (Aisaka *et al.*, 1989; Rees *et al.*, 1989; 1990; Wang & Pang, 1990; Wang *et al.*, 1991; 1992; 1993; Pang & Wang, 1993). The pressor effects of these compounds have been attributed to the inhibition of the L-Arg/NO pathway and endothelium-dependent vasodilatation *in situ* (Aisaka *et al.*, 1989; Rees *et al.*, 1989; see Moncada *et al.*, 1991). Accordingly, DPI being an inhibitor of NO synthase, would be expected to cause a pressor response in whole animals. Indeed, i.v. injections of DPI produced transient pressor responses in pentobarbitone-anaesthetized and conscious rats (Wang & Pang, 1993a,b). However, unlike that of the N^G-substituted Arg analogues (Wang & Pang, 1991), the pressor effect of DPI was attenuated or abolished by blockers of the sympathetic nervous system (Wang & Pang, 1993a,b).

The reasons for the differences in the causative factor and time course of the pressor responses elicited by these two classes of NO synthase inhibitors are unclear. One possibility is that DPI does not produce prolonged inhibition of endothelium-dependent vasodilatations *in vitro* and/or *in vivo*. Another possibility is that inhibition of endothelium-dependent vasodilatation by DPI is not the cause of the elevation of blood pressure. Hence, the first aim of this study was to find out if DPI causes prolonged *in vitro* and *in vivo* inhibition of endothelium-dependent vasodilatations, as DPI has been shown to cause prolonged inhibition of NO biosynthesis in macrophages (Stuehr *et al.*, 1991b). This was assessed by studying the effects of DPI on relaxations induced by the endothelium-dependent vasodilators, acetylcholine (ACh) and A23187 (calcium ionophore), and the endothelium-independent vasodilator sodium nitroprusside (SNP) in preconstricted rat aorta. In addition, the effects of DPI on depressor responses to ACh and SNP were studied in conscious, unrestrained rats. The second aim was to determine if slower onset pressor responses (other than the initial transient rise in blood pressure) followed the administration of DPI. The third aim was to examine if the NO synthase co-factors NADPH and FAD, as well as the NO synthase substrate L-Arg, reversed the inhibitory effect of DPI on endothelium-dependent relaxation.

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Methods

Male Sprague-Dawley rats (350–420 g) were used in this study.

Isolated aortic rings

The rats were killed 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 in random order in separate organ baths. Each ring was connected to a Grass FT-03-C force-displacement transducer for isometric recording with a preload of 1 g. The rings were equilibrated for 1 h (with 3 washouts) in Krebs solution (pH 7.4) at 37°C and bubbled with a gas mixture of 95% O_2 and 5% CO_2. The Krebs solution had the following composition (mM): NaCl 118, glucose 11, KCl 4.7, $CaCl_2$ 2.5, $NaHCO_3$ 25, KH_2PO_4 1.2, $MgCl_26H_2O$ 1.2.

The rings were first incubated with vehicle or drugs (see later) followed by phenylephrine (PE, 10^{-6} M, EC_{90}). After 15–20 min, at the steady-state phase of the contractile response to PE, a cumulative concentration-response curve to ACh, A23187 (calcium ionophore) or SNP was obtained. Each drug concentration was left in the bath until a plateau response was reached. The time taken to complete each concentration-response curve was approximately 20 min. In groups where more than one concentration-response curve of ACh was constructed, the preparations were washed three times within 30 min and given another 30 min to recover completely from the effects of the previous applications of PE and ACh. Afterwards, PE was again added followed by the construction of ACh concentration-response curves.

Conscious rats

The rats were anaesthetized with halothane (4% in air for induction and 1.2% in air for surgery). Polyethylene cannulae (PE50) were inserted into the left iliac artery for the measurement of mean arterial pressure (MAP) by a pressure transducer (P23DB, Gould Statham, CA, U.S.A.) and into the left iliac vein for the administration of drugs. The cannulae were filled with heparinized (25 iu ml^{-1}) normal saline, tunnelled s.c. along the back and exteriorized at the back of the neck. The rats were put into small cages allowing free movement and given >6 h recovery from the effects of surgery and halothane before use.

Drugs

The following drugs were purchased from Sigma Chemical Co. (MO, U.S.A.): acetylcholine (ACh) chloride, A23187, phenylphrine (PE) hydrochloride, flavin adenine dinucleotide (FAD) disodium, β-nicotinamide adenine dinucleotide phosphate, reduced form (NADPH), N^{ω}-nitro-L-arginine (L-NOARG) and L-arginine (L-Arg) hydrochloride. Diphenyleneiodonium (DPI) sulphate and sodium nitroprusside (SNP) were obtained from Colour Your Enzyme Ltd. (Ontario, Canada) and Fisher Scientific Co. (N.J., U.S.A.), respectively. All drugs were dissolved in normal saline (0.9% NaCl) except for DPI and A23187 which were dissolved in 5% glucose solution and 10% dimethyl sulphoxide, respectively.

Experimental protocols

Each experiment included 6–8 aortic rings or 6 conscious, unrestrained rats.

Protocol 1: Effects of DPI on ACh-, A23187- and SNP-induced relaxations in the aorta Six groups of aortic rings were incubated with the vehicle or DPI (3×10^{-8}–3×10^{-6} M) followed by PE 10 min later. After another 10–15 min, concentration-response curves to ACh (10^{-8}–3×10^{-5} M) were obtained. Only one concentration of DPI was studied in each group. Another two groups were treated with the vehicle or DPI (3×10^{-6} M) followed by the application of PE and construction of concentration-response curve to A23187 (10^{-9}–10^{-6} M). The last two groups were treated in the same way as the previous two groups except that SNP (3×10^{-10}–10^{-7} M) was used in place of A23187.

Protocol 2: Effects of pretreatment with NADPH, FAD or L-Arg on the inhibitory effect of DPI Twelve groups of aortic rings were treated with vehicle + vehicle, NADPH (1.5×10^{-3} M) + vehicle, NADPH (5×10^{-3} M) + vehicle, FAD (5×10^{-6} M) + vehicle, FAD (5×10^{-4} M) + vehicle and L-Arg (2×10^{-3} M) + vehicle, vehicle + DPI, NADPH (1.5×10^{-3} M) + DPI, NADPH (5×10^{-3} M) + DPI, FAD (5×10^{-6} M) + DPI, FAD (5×10^{-4} M) + DPI, and L-Arg (2×10^{-3} M) + DPI, with 3×10^{-7} M DPI added in all cases. Another two groups of aortic rings were treated with vehicle + L-NOARG (10^{-6} M) and NADPH (5×10^{-3} M) + L-NOARG (10^{-6} M). The first treatments were given 10 min prior to the second treatments. Afterward, the rings were preconstricted with PE and relaxed with ACh as described in Protocol 1.

Protocol 3: Time course and reversibility of the inhibitory effect of DPI on relaxation response of ACh The time course of the inhibitory effect of DPI was studied in 3 groups of aortic rings. After completing the first concentration-response curve to ACh in PE-preconstricted rings in the presence of vehicle or DPI (3×10^{-7} or 3×10^{-6} M), the preparations were washed out without further addition of drug or vehicle. Second, third and fourth ACh curves were constructed in preconstricted rings at 1.5, 4 and 9 h after the preparations were washed out. In another two groups of aortic rings, the effect of post-treatment with NADPH on the inhibitory effect of DPI was studied. These rings were incubated with DPI (3×10^{-7} M) or vehicle for 1 h, followed by the application of NADPH (5×10^{-3} M) and the construction of ACh concentration-response curves in PE-preconstricted conditions.

Protocol 4: Effect of DPI on resting MAP and depressor response to ACh and SNP In one group of rats, DPI (10^{-5} mol kg^{-1}) was injected (i.v. bolus) and blood pressure was continuously monitored for 2 h.

Two groups of rats were injected (i.v. bolus) with the vehicle or DPI (10^{-5} mol kg^{-1}) 20 min prior to i.v. infusions of ACh (6×10^{-8}–1.8×10^{-6} mol kg^{-1} min^{-1}, each dose for 4 min) and SNP (3×10^{-8}–4.8×10^{-7} mol kg^{-1} min^{-1}, each dose for 4 min). The sequence of ACh and SNP administrations was reversed in half of the studies with 20 min recovery after the completion of the first dose-response curve. The time taken to complete the experiment was approximately 2 h after i.v. injection of DPI or the vehicle.

Calculation and statistical analysis

IC_{50} and E_{max} were calculated from individual concentration-response curves (see Wang & Pang, 1993a). All results are expressed as mean ± standard error (s.e.mean) except for the points where the error bars were smaller than the symbols (see figures). The results were analysed by the analysis of variance/co-variance followed by Duncan's multiple range test with $P<0.05$ selected as the criterion for statistical significance.

Results

Effects of DPI on ACh, A23187- and SNP-induced relaxations

All five concentrations of DPI (3×10^{-8}, 10^{-7}, 3×10^{-7}, 10^{-6} and 3×10^{-6} M) slightly potentiated PE-induced contraction

from the baseline value of 0.99 ± 0.10 g to 1.09 ± 0.15, 1.27 ± 0.11, 1.32 ± 0.10, 1.31 ± 0.10 and 1.18 ± 0.14 g, respectively. However, only the effects of the third and fourth concentrations of DPI were statistically significant.

In the vehicle-treated group, ACh relaxed the preconstricted aorta concentration-dependently with maximum relaxation of approximately 60% (Figure 1a). DPI inhibited concentration-dependently the ACh-induced relaxation. At 3×10^{-5} M ACh, the IC_{50} of DPI was 1.8×10^{-7} M with a maximum inhibition of 96% (Figure 1b).

In another two vehicle groups, A23187 and SNP also relaxed concentration-dependently the preconstricted aortae, with maximal relaxations of approximately 60 and 100%, respectively. DPI (3×10^{-6} M) completely inhibited A23187-induced relaxation (Figure 2a) but did not affect the relaxation response of SNP (Figure 2b).

Influences of NADPH, FAD and L-Arg on the inhibitory effects of DPI and, the effect of L-NOARG on ACh-induced relaxation

Baseline contractions elicited by PE in the presence of vehicle or DPI (3×10^{-7} M) were 1.29 ± 0.07 and 1.67 ± 0.12 g, respectively. Treatment with NADPH (1.5 and 5×10^{-3} M), FAD (5×10^{-4} and 5×10^{-6} M) and L-Arg (2×10^{-3} M) did not significantly affect PE-induced contractions in the presence of either the vehicle (1.04 ± 0.06, 1.04 ± 0.11, 1.16 ± 0.06, 1.33 ± 0.11, 1.23 ± 0.08 g, respectively) or DPI (1.43 ± 0.10, 1.37 ± 0.14, 1.59 ± 0.06, 1.52 ± 0.13, 1.44 ± 0.04 g, respectively).

DPI inhibited ACh-induced relaxations (Figure 3a). Treatment with L-Arg did not affect either the ACh-induced

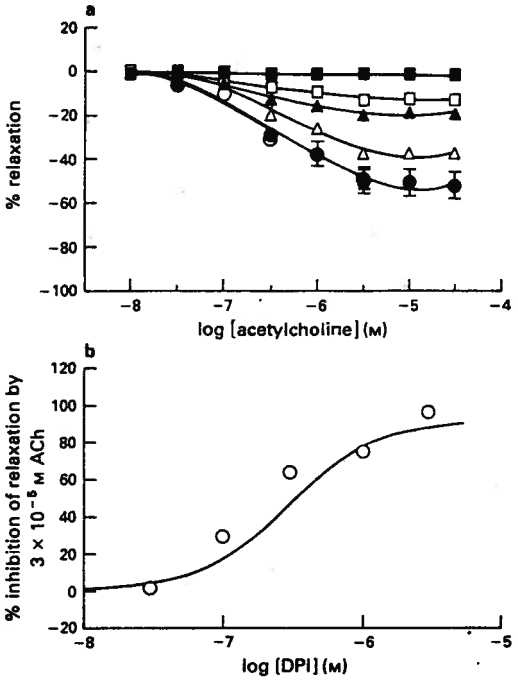

Figure 1 (a) Concentration-response (mean ± s.e.mean) curves of diphenyleneiodonium (DPI) on acetylcholine-induced relaxation in phenylephrine (10^{-6} M)-preconstricted aortic rings ($n = 7$ each group). Concentrations of DPI were as follows: vehicle (○); 3×10^{-8} M (●); 10^{-7} M (△); 3×10^{-7} M (▲); 10^{-6} M (□); 3×10^{-6} M (■). (b) Percentage inhibition by DPI of 3×10^{-5} M acetylcholine-induced relaxation in the aorta. The data were derived from the mean values of (a).

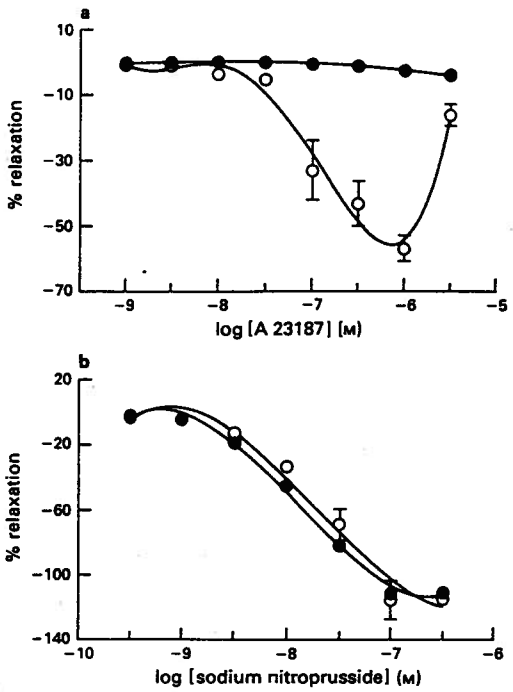

Figure 2 Effects (mean ± s.e.mean) of vehicle (○) or diphenyleneiodonium (DPI, 3×10^{-6} M, ●) on A23187 (a) and sodium nitroprusside (b) induced relaxations in the phenylephrine (10^{-6} M) preconstricted aortic rings ($n = 6$ each group). *Significant difference from vehicle-pretreated control curve ($P < 0.05$).

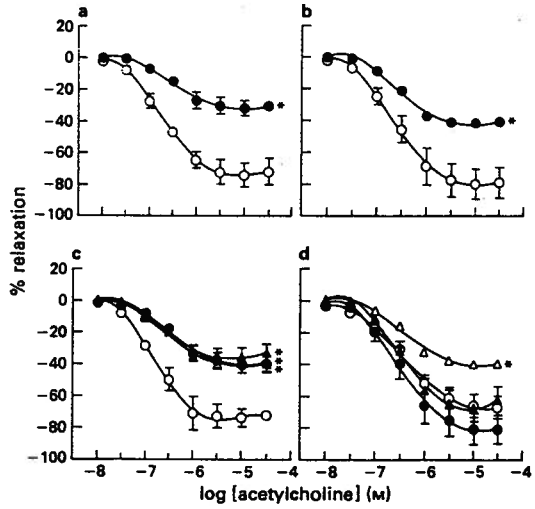

Figure 3 Effects (mean ± s.e.mean) of vehicle, NADPH, FAD and L-arginine on acetylcholine-induced relaxation and on the inhibitory influence of diphenyleneiodonium (DPI, 3×10^{-7} M) on relaxation in phenylephrine (10^{-6} M), preconstricted aortic rings ($n = 6$ each group). The first treatments were performed 10 min before the second treatments. (a) Vehicle + vehicle (○); vehicle + DPI (●). (b) L-Arginine (2×10^{-3} M) + vehicle (○); L-arginine (2×10^{-3} M) + DPI (●). (c) FAD (5×10^{-6} M) + vehicle (○); FAD (5×10^{-4} M) + vehicle (●); FAD (5×10^{-6} M) + DPI (△); FAD (5×10^{-4} M) + DPI (▲). (d) NADPH (1.5×10^{-3} M) + vehicle (○); NADPH (5×10^{-3} M) + vehicle (●); NADPH (1.5×10^{-3} M) + DPI (△); NADPH (5×10^{-3} M) + DPI (▲). *Significant difference from control curve ($P < 0.05$).

relaxation or the inhibitory effect of DPI on ACh-induced relaxation (Figure 3b). Although the lower concentration (5×10^{-6} M) of FAD also did not alter either ACh-induced relaxation or the inhibitory effect of DPI on ACh, the higher concentration (5×10^{-4} M) of FAD suppressed the relaxant effect of ACh and prevented further inhibition by DPI on ACh-induced relaxation (Figure 3c). Although neither concentration of NADPH significantly affected ACh-induced relaxation, the higher (5×10^{-3} M) but not the lower (1.5×10^{-3} M) concentration completely prevented the inhibitory effect of DPI (Figure 3d). The effectiveness of pretreatment with NADPH (5×10^{-3} M) in inhibiting the effect of DPI, expressed as the ratio of the relaxation effect of 10^{-5} M ACh in the presence of NADPH (−67%) to that in the absence of NADPH (−32%), was 209%.

PE caused contractions of 1.46 ± 0.12 and 1.67 ± 0.13 g in the presence of vehicle + L-NOARG (10^{-6} M) and NADPH (5×10^{-3} M) + L-NOARG (10^{-6} M), respectively. Compared to the pooled vehicle control derived from Figures 1a and 3a, L-NOARG markedly inhibited ACh-induced relaxation. Pretreatment with NADPH did not affect the inhibitory effect of L-NOARG (Figure 4).

Time course and reversiblity of the inhibitory effect of DPI on ACh-induced relaxation

The PE-induced contractions in the presence of vehicle or DPI did not change with the passage of time (data not shown). The ACh-induced maximal relaxation was not altered until at least 4 h after washout. Maximal relaxation at 9 h was $-48 \pm 7\%$, which was significantly less than that ($-69 \pm 6\%$) at 0 h (Figure 5). DPI at 3×10^{-7} and 3×10^{-6} M inhibited ACh-induced relaxation by approximately 50 and 100%, respectively (Figure 5a). The inhibitory effect of DPI remained at least 4 h after washout (Figure 5b,c). At 9 h after washout, the relaxations of DPI-pretreated rings were still less, though insignificantly, than those of vehicle-pretreated rings (Figure 5d).

Maximum relaxation to ACh after 1 h exposure to 3×10^{-7} M DPI ($-38.3 \pm 3.1\%$, Figure 6) was similar to that after a 10 min exposure to DPI ($-32.2 \pm 4.8\%$, Figure 3a). Post-treatment (1 h later) with NADPH (5×10^{-3} M) slightly but significantly, suppressed the inhibitory effect of DPI. The effectiveness of post-treatment with NADPH (5×10^{-3} M) in inhibiting the effect of DPI, expressed as a ratio of the relaxation effect of 10^{-5} M ACh in the presence of NADPH (−51%) to that in the absence of NADPH (−38%), was 134% (Figure 6).

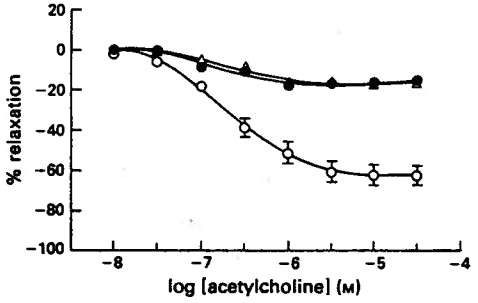

Figure 4 Effect (mean ± s.e.mean) of pretreatments (10 min earlier) of NADPH (5×10^{-3} M) on the inhibitory effect of N^G-nitro-L-arginine (L-NOARG, 10^{-6} M) on acetylcholine-induced relaxation in the phenylephrine (10^{-6} M) preconstricted aortic rings ($n = 8$ each group except for the pooled control rings where $n = 13$). Vehicle (○); vehicle + L-NOARG (●); NADPH + L-NOARG (△). *Significant difference from vehicle-pretreated control curve ($P < 0.05$).

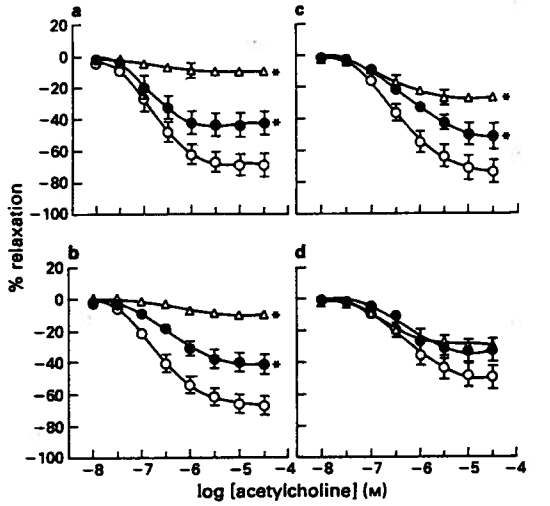

Figure 5 The time course of the effects (mean ± s.e.mean) of vehicle (○), diphenyleneiodonium (DPI, 3×10^{-7} M, ●) and DPI (3×10^{-6} M, △) on acetylcholine-induced relaxations in phenylephrine (10^{-6} M) preconstricted aortic rings ($n = 6$ each group). (a), (b), (c) and (d) represent responses at 0, 1.5, 4 and 9 h after washout without further addition of the vehicle or DPI. *Significant difference from vehicle-pretreated control curve ($P < 0.05$).

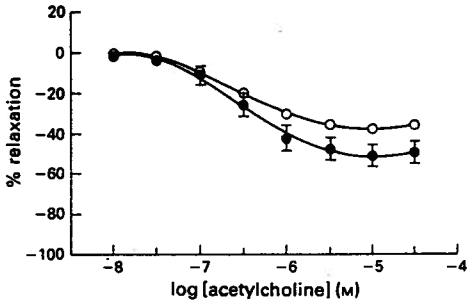

Figure 6 Effects (mean ± s.e.mean) of post-treatment (1 h later) with vehicle (○) or NADPH (5×10^{-3} M, ●) on the inhibitory effect of diphenyleneiodonium (DPI, 3×10^{-7} M) on acetylcholine-induced relaxation in the phenylephrine (10^{-6} M) preconstricted aortic rings ($n = 6$ each group). *Significant difference from vehicle-pretreated control curve ($P < 0.05$).

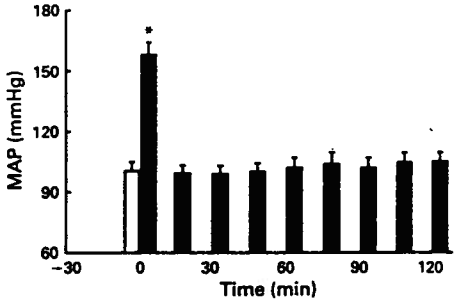

Figure 7 Effect (mean ± s.e.mean) of i.v. bolus injections of diphenyleneiodonium (DPI, 10^{-5} mol kg^{-1}) on mean arterial pressure (MAP) in conscious rats ($n = 6$). Open and solid columns represent pre- and post-administration with DPI. *Significant difference from pre-administration with DPI ($P < 0.05$).

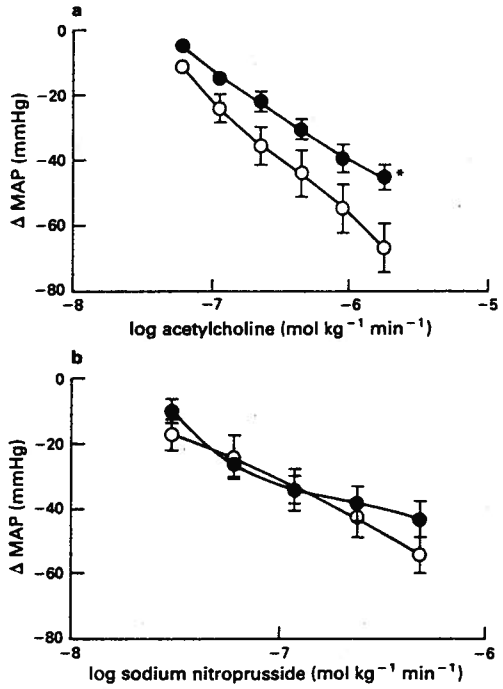

Figure 8 Dose-response curves (mean ± s.e.mean) of i.v. infusions of acetylcholine (a) and sodium nitroprusside (b) on mean arterial pressure (MAP) in conscious rats ($n = 6$ each group) pretreated with i.v. bolus injection of vehicle (○) or diphenyleneiodonium (DPI, 10^{-5} mol kg^{-1}, ●). *Significant difference from vehicle-pretreated control curve ($P < 0.05$).

Effects of DPI on resting blood pressure and depressor responses to ACh and SNP

Intravenous bolus injections of DPI (10^{-5} mol kg^{-1}) in conscious rats caused immediate and transient increases in MAP which were similar to the responses in pentobarbitone-anaesthetized and conscious rats (Wang & Pang, 1993a,b). MAP returned to the baseline level approximately 4 min after the injection of DPI and remained there during the 2 h observation period (Figure 7).

Baseline MAPs of rats before and 20 min after treatment with vehicle were 109 ± 2 and 112 ± 3 mmHg, respectively, which were similar to those of DPI-treated rats (10^{-5} mol kg^{-1}, i.v. bolus) (118 ± 5 and 114 ± 5 mmHg). Intravenous infusions of ACh and SNP caused dose-dependent depressor responses. Pretreatment with DPI significantly attenuated the depressor responses to ACh but not to SNP (Figure 8).

Discussion

Our *in vitro* results show that DPI selectivity inhibits endothelium-dependent relaxation induced by receptor-mediated (ACh) or non-receptor-mediated (A23187) mechanisms. The results are consistent with the report that DPI inhibits ACh-induced relaxation in the rabbit aorta (Stuehr *et al.*, 1991b) and further suggest there is no species difference for the actions of DPI. DPI also attenuates ACh- but not SNP-induced decreases in MAP in conscious rats, and ACh- but not SNP-induced vasodilatation in the perfused rat hindquarter preparation (unpublished observation). The results suggest that DPI inhibits endothelium-dependent vasodilatations in both conductance and resistance vessels. Therefore, the *in vitro* inhibitory effects of DPI on endothelium-dependent vasodilatations are similar to those of the N^G-substituted Arg analogues. These results are in accordance with the hypothesis that inhibition of NO synthesis causes the suppression of endothelium-dependent vasodilatation.

It has been known since 1973 that DPI suppresses the oxidation of NADH-like substrates thereby inhibiting mitochondrial oxidation (Holland *et al.*, 1973). It was later shown that DPI inhibits NADPH-dependent oxidase of neutrophils and macrophages (Cross & Jones, 1986; Hancock & Jones, 1987; Ellis *et al.*, 1988; 1989), and macrophage NO synthase (Stuehr *et al.*, 1991b), by specifically binding to and inhibiting the action of a plasma membrane polypeptide which may be a component of flavoprotein (Cross & Jones, 1986; Hancock & Jones, 1987; Ellis *et al.*, 1989). This suggests that flavin is the site of attack by DPI and that a protein is associated with FAD (O'Donnell *et al.*, 1993). Isoenzymes of NO synthase are known to be flavoproteins which contain FAD as a cofactor in the macrophage (Stuehr *et al.*, 1980; 1990; 1991a; Hevel *et al.*, 1991; White & Marletta, 1992), neutrophil (Yui *et al.*, 1991), brain (Mayer *et al.*, 1991; Lowenstein *et al.*, 1992; Bredt *et al.*, 1991; 1992; Hiki *et al.*, 1992) and liver (Evans *et al.*, 1992). There is, however, no functional documentation of a role for FAD as a cofactor of NO synthase in endothelial cells. Our *in vitro* results demonstrate that FAD interferes with both ACh-induced relaxation and the inhibitory effect of DPI on ACh-induced relaxation. The latter result, which is consistent with Stuehr *et al.*'s observation (1991b) that FAD antagonizes the inhibitory effect of DPI on macrophage NO synthesis, suggests that FAD and DPI may inhibit endothelial NO synthesis by a mechanism similar to that in macrophages. The former result is puzzling, since as a cofactor, FAD should facilitate rather than interfere with endothelium-dependent relaxation. FAD was indeed reported to facilitate macrophage NO synthesis (Stuehr *et al.*, 1990; Hevel *et al.*, 1991). The mechanism by which FAD inhibits ACh-induced relaxation is not clear at the moment, however, the effect may not be specific as FAD also inhibits SNP-induced relaxation (unpublished observation).

Our *in vitro* results also show that NADPH interferes with the inhibitory effect of DPI on ACh-induced relaxation. The antagonism of DPI by NADPH was specific since the same concentration of NADPH did not alter the inhibitory effect of L-NOARG. The inhibitory effect of DPI was also not affected by L-Arg, at a concentration previously found to reverse the inhibitory effects of L-NOARG and L-NAME on endothelium-dependent relaxations in aortic rings (Wang *et al.*, 1992; 1993). Our results with NADPH are consistent with those which show that both the constitutive (e.g. brain and endothelial) and inducible (e.g. macrophage and smooth muscle) NO synthases are dependent on NADPH as an essential cofactor (Mayer *et al.*, 1989; Stuehr *et al.*, 1989; 1990; 1991a; see McCall & Vallance, 1992). Regarding the nature of the interaction between NADPH and FAD, it has been suggested that NADPH suppresses the binding of DPI to the flavoprotein in neutrophil oxidase by preventing the attachment of DPI to a site in close proximity to the NADPH-binding site (Cross & Jones, 1986). It is very likely that NADPH may interfere with the action of DPI on endothelial NO synthase by the same mechanism.

Pretreatment with DPI was found to inhibit ACh-induced relaxation in aortic rings for at least 4 h after washout and to suppress ACh-induced vasodilatation for at least 2 h after intravenous bolus injection. Therefore, our *in vitro* and *in vivo* results are supportive of a prolonged inhibitory effect of DPI on endothelium-dependent vasodilatations. DPI has been reported to inhibit irreversibly macrophage NO synthase (Stuehr *et al.*, 1991b); the mechanism may involve the formation of a covalent bond with components of a flavoprotein (Ragan & Bloxham, 1977; O'Donnell *et al.*, 1993). However, our results show that post-treatment (1 h later) with NADPH still attenuates the effect of DPI, although the response is significantly less than that following pretreatment

(10 min earlier). These results may imply that fresh synthesis of NO occurs in endothelial cells.

It is well-known that all N^G-substituted Arg analogues which inhibit endothelium-dependent relaxation *in vitro* cause long-lasting pressor effects in whole animals. The pressor response of N^G-substituted Arg analogues is not blocked by the impairment of the central nervous system (Tabrizchi & Triggle, 1992; Wang & Pang, 1993a), sympathetic nervous system (Wang & Pang, 1991), renin-angiotensin system (Wang & Pang, 1991), or prostaglandin system (Rees *et al.*, 1989; Wang & Pang, unpublished data, 1993), but is inhibited by L-Arg (Aisaka *et al.*, 1989; Rees *et al.*, 1989; Wang & Pang, 1990; Wang *et al.*, 1991b; 1992). These observations have been accepted as evidence of a role of NO in the regulation of blood pressure (Rees *et al.*, 1989; Aisaka *et al.*, 1989; see Moncada *et al.*, 1991). As an 'irreversible' inhibitor of NO synthase, DPI should also cause a prolonged pressor response. However, unlike the N^G-substituted Arg analogues, intravenous bolus injections of DPI produced only immediate and transient increases in MAP. The pressor response of DPI was blocked by procedures which impair the activities of the central or sympathetic nervous systems, namely, pithing, spinal cord transection and the administration of tetrodotoxin, reserpine, guanethidine, phentolamine or prazosin (Wang & Pang, 1993a). Moreover, the pressor response to DPI, but not to L-NOARG, was accompanied by elevations of plasma noradrenaline and adrenaline (Wang & Pang, 1993a,b). These results show that the transient pressor response of DPI, unlike that of the N^G-substituted Arg analogues, is solely dependent on the activation of the sympathetic nervous system, i.e., DPI does not elicit a NO-dependent sustained rise in blood pressure as do the other NO synthase inhibitors.

Although one may postulate that the lack of effect of DPI is due to inadequate accumulation of drug *in situ* to inhibit NO synthesis, this is unlikely. DPI was shown to distribute rapidly and adequately to all organs or tissues (Gatley & Martin, 1979); moreover, its peak hypoglycaemic effect was reached at 1.5 h (Holland *et al.*, 1973) or 4 h (Gatley & Martin, 1979) after intraperitoneal injections, suggesting a long duration of action. Our present results also show that DPI inhibits irreversibly endothelium-dependent relaxation *in vitro* for more than 4 h, and partially inhibits ACh-induced vasodilatation *in vivo* even at 2 h after intravenous injection. It should be noted that a lack of complete inhibition of ACh-induced relaxation is a typical observation with NO synthase inhibitors since N^G-substituted Arg analogues, at maximal pressor doses, also cause partial inhibition of ACh-induced vasodilatation – this suggests that either the depressor/vasodilatation response of ACh *in vivo* is only partially due to the release of NO (Rees *et al.*, 1990; Wang *et al.*, 1992) or it is entirely independent of the biosynthesis and/or release of NO (Pang & Wang, 1993).

Although it is generally accepted that endogenous NO modulates vascular tone and blood pressure and that N^G-substituted Arg analogues produce pressor response by inhibition of endothelial NO synthesis and endothelium-dependent vasodilatations *in situ* (see Moncada *et al.*, 1991), our data with DPI suggest otherwise, i.e. inhibition of NO synthesis and endothelium-dependent vasodilatations do not always cause vasoconstriction *in vivo*. This hypothesis is supported by the recent publications which show that methylene blue does not produce a pressor response (Loeb & Longnecker, 1992; Pang & Wang, 1993) although it inhibits endothelium-dependent vasodilatation *in vitro* (Pang & Wang, 1993) and *in vivo* (Loeb & Longnecker, 1992). L-NOARG was also shown to cause much longer inhibition of endothelium-dependent vasodilatation than elevation of blood pressure in conscious rabbits, suggesting that the suppression of NO synthesis alone does not result in hypertension (Cocks *et al.*, 1992). Therefore, the hypothesis that endogenous NO modulates vascular tone and Arg analogues produce pressor response by inhibition of endothelial NO biosynthesis may need re-examination.

In summary, DPI efficaciously and 'irreversibly' inhibits endothelium-dependent vasodilatation *in vitro* and *in vivo* by a mechanism involving the suppression of the actions of FAD and NADPH. Unlike the N^G-substituted Arg analogues, DPI does not cause NO-mediated sustained pressor response. Instead, DPI causes immediate and transient pressor responses which are solely due to the activation of the sympathetic nervous system (Wang & Pang, 1993a,b). These results suggest that inhibition of NO synthesis *in situ* does not necessarily cause a pressor response.

This work was supported by a research grant from the Medical Research Council of Canada (MRC), an MRC Post-doctoral Fellowship to Y.-X.W. and a Heart & Stroke Foundation of B.C. & Yukon Research Traineeship to C.I.P.

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(Received May 11, 1993
Revised July 16, 1993
Accepted July 20, 1993)

Paper VIII

Wang, Y.-X., Poon, C.I., Yau, L.C.Y. and Pang, C.C.Y.: Vascular pharmacodynamics of N^G-nitro-L-arginine methyl ester *in vitro* and *in vivo*. J. Pharmacol. Exp. Ther. 267: 1091-1099, 1993. The reproduction of this paper was kindly permitted by the copyright holder, Willams & Wilkins, Baltimore, U.S.A.

0022-3565/93/2673-1091$03.00/0
THE JOURNAL OF PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS
Vol. 267, No. 3
Printed in U.S.A.

Vascular Pharmacodynamics of N^G-Nitro-L-Arginine Methyl Ester *in Vitro* and *in Vivo*[1]

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Accepted for publication August 9, 1993

ABSTRACT

The inhibitory effects of N^G-nitro-L-arginine methyl ester (L-NAME) on endothelium-dependent vasodilatation were studied in conscious rats and isolated rat aortic rings. In phenylephrine (PE, ED_{90})-preconstricted aortae, L-NAME caused prolonged and complete inhibition of acetylcholine (ACh)-induced relaxation with IC_{50} of 4×10^{-7} M and Hill coefficient (n) of 1. The inhibition was abolished by L-arginine (L-Arg), independently of whether it was applied 10 min earlier or 4 hr later than L-NAME. Intravenous bolus injection of L-NAME caused prolonged increases in mean arterial pressure (MAP), with E_{max} of 50 ± 7 mm Hg, ED_{50} of 5 ± 1×10^{-6} mol/kg and n of 2. Intravenous infusion of L-Arg shifted the dose-MAP curve of L-NAME to the right without changing E_{max} or n. A modified Schild plot (n = 2) for the action of L-NAME gave a slope not different from unity, suggesting that L-Arg inhibits competitively the MAP response of L-NAME. Intravenous infusion of ACh decreased MAP in rats treated with L-NAME (4.8×10^{-5} mol/kg) or PE. Compared to PE-treated rats, L-NAME inhibited the depressor response to ACh by 50%. Thus, a dose of L-NAME 10 times its ED_{50} in raising MAP only partially blocked the depressor responses to ACh. Our results are consistent with the hypothesis that pressor response to L-NAME results from the inhibition of NO synthase only if 1) basal NO synthesis is more effectively blocked by L-NAME than stimulated NO synthesis and 2) stimulated NO synthesis is inhibited by L-NAME in a one to one antagonist manner whereas basal NO synthesis is inhibited by L-NAME in a two to one antagonist manner.

Analogs of N^G-substituted Arg have been shown to suppress NO synthesis as well as to interfere with endothelium-dependent relaxations of vascular preparations (see Moncada *et al.*, 1991). These inhibitors, including L-NMMA (Palmer *et al.*, 1988a,b; 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), cause prolonged pressor responses which are antagonized by the NO donor sodium nitroprusside (Wang *et al.*, 1993c) and by L-Arg (but not by D-Arg) (Aisaka *et al.*, 1989b; Rees *et al.*, 1989b; Wang and Pang, 1990; Wang *et al.*, 1991).

It is generally assumed that N^G-substituted Arg analogs are competitive inhibitors of NO synthase because they are structurally related to L-Arg and because their actions are antagonized by L-Arg. However, there is little pharmacodynamic evidence to establish such a mechanism, particularly in *in vivo* studies. Moreover, whereas N^G-substituted Arg analogs effectively inhibit ACh-induced vasodilatations in isolated precontricted vascular preparations (Palmer *et al.*, 1988b; Crawley *et al.*, 1990; Rees *et al.*, 1990; Wang *et al.*, 1993b), or regional vascular beds, *e.g.*, coronary (Woodman and Dusting, 1991), hindquarter (Bellan *et al.*, 1991), renal (Gardiner *et al.*, 1990a, 1991; Lahera *et al.*, 1990), pulmonary (Fineman *et al.*, 1991), mesentery (Fortes *et al.*, 1990; Gardiner *et al.*, 1990a) and carotid (Gardiner *et al.*, 1990a, 1991) beds, there are discrepancies in reports of their abilities to interfere with the depressor effects of ACh in whole animals. Inhibition of ACh-induced depressor responses has been shown for L-NMMA (Whittle *et al.*, 1989; Rees *et al.*, 1990), L-NAME (Rees *et al.*, 1990) and L-NIO (Rees *et al.*, 1990). However, an absence of inhibition, or even potentiation, of depressor responses to ACh has been reported with L-NMMA (Aisaka *et al.*, 1989a; Yamazaki and Nagao, 1991), L-NNA (Wang *et al.*, 1992) and L-NAME (Gardiner *et al.*, 1990a, 1991; van Gelderen *et al.*, 1991). It is therefore unclear whether the pressor responses of these compounds are secondary to the inhibition of endogenous endothelial NO synthesis and release.

To resolve the above questions, we compared the *in vivo* and *in vitro* activities of L-NAME. L-NAME was selected from N^G-substituted Arg analogs due to its solubility in water, low cost and high potency. *In vitro*, the concentration-relaxant re-

Received for publication March 15, 1993.
[1] This work was supported by a grant from the Medical Research Council of Canada (MRC), a MRC Postdoctoral Fellowship awarded to Y.-X. Wang and a Canadian Hypertension Society Summer Research Studentship awarded to C. I. Poon.

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

sponses of ACh and SNP were determined in isolated endothelium-intact aortic rings, in the presence of varied concentrations of L-NAME. *In vivo*, conscious and unrestrained rats were used to examine the effects of L-NAME on blood pressure and depressor responses to ACh and SNP.

Methods

Surgery and preparations. Sprague-Dawley rats (from The University of British Columbia) (300–400 g) were used for all experiments. Each group consisted of five to six rats *in vivo* or five to six aortic rings *in vitro*, with each ring derived from a different rat.

In *in vitro* studies, the rats were sacrificed by a blow on the head followed by exsanguination. The thoracic aorta was removed and cleared of connective tissues. Four ring segments of 0.5 cm length were prepared from one aorta and suspended in separate organ baths. Each ring was connected to a Grass (Quincy, MA) FT-03-C force-displacement transducer for isometric recording with a preload of 1 g and was equilibrated for 1 hr (with three washouts) in normal Krebs' solution (pH 7.4) at 37°C with a gas mixture of 95% O_2-5% CO_2. Krebs' solution had the following composition (10^{-3} M): 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.

The rings were incubated with the vehicle or drugs (see later). Afterwards, 10^{-6} M PE (EC_{80}, data not shown) was added to the baths and 15 to 20 min later, at the steady-state phase of the contractile response to PE, a cumulative concentration-response curve of ACh (10^{-9} to 3×10^{-6} M) or SNP (10^{-9} to 10^{-6} M) was constructed by using sequential doubling of drug concentration. Each concentration of ACh or SNP was left in the bath for 1 to 3 min to ensure a plateau response. The time taken to complete each concentration-response curve was approximately 20 min. In some groups in which the concentration-response curve of ACh was conducted more than once, the preparations were washed 3 times in 30 min and given another 30 min to recover from the effects of PE and ACh, before constructing the next concentration-response curve.

In the *in vivo* studies, the rats were anesthetized with halothane (4% in air for induction and 1.5% in air for surgery). A polyethylene cannula (PE-50) was inserted into the left iliac artery for the measurement of MAP by a pressure transducer (P23DB, Gould Statham, Oxnard, CA). PE-50 cannulae were also inserted into both iliac veins for the administration of the vehicle or drugs. The rats were then put into a small cage and allowed to move freely before as well as during the experiments. The rats were given > 6 hr to recover from the effect of anesthesia before use.

Drugs. L-NAME, ACh hydrochloride, PE hydrochloride, L- and D-Arg hydrochloride were obtained from Sigma Chemical Co. (St. Louis, MO). SNP was obtained from Fisher Scientific Co. (Springfield, NJ). All drugs were dissolved in 0.9% saline (vehicle) except for L- and D-Arg, which were dissolved in distilled water and pH was adjusted to 7.0 with NaOH solution.

Experimental protocols. 1) Concentration-response curves of L-NAME on relaxation responses of ACh and SNP were studied in nine groups of aortic rings. The first seven groups of aortae were incubated with vehicle or L-NAME (10^{-7} to 3.2×10^{-6} M), followed 10 min later by the administration of PE (10^{-6} M) and determination of concentration-relaxation response curves for ACh (10^{-9} to 3×10^{-6} M). Only one concentration of L-NAME was used in each group. In another two groups of aortae pretreated with L-NAME or vehicle, SNP (10^{-9} to 10^{-6} M) was used instead of ACh and L-NAME was used at only one concentration (3.2×10^{-6} M).

2) The time course of the inhibition by L-NAME of the relaxation response to ACh was studied in two groups of aortic rings. After completing the first concentration-response curve of ACh in the presence of vehicle or L-NAME (3.2×10^{-6} M), the preparations were washed without further addition of L-NAME. The 2nd, 3rd and 4th curves of ACh were repeated at 1.5, 4 and 9 hr later.

3) The effects of L- and D-Arg on the inhibitory effects of L-NAME on ACh-induced relaxation were studied in six groups of aortic rings. In the first four groups, the concentration-response curves for ACh (with 10^{-6} M PE) were determined in the presence of vehicle + vehicle, vehicle + L-NAME (3.2×10^{-6} M), L-Arg (3×10^{-4} M) + L-NAME (3.2×10^{-6} M) and D-Arg (3×10^{-4} M) + L-NAME (3.2×10^{-6} M), with the first treatments applied 10 min before L-NAME ("pre-exposure"). The other two groups were first incubated with L-NAME (3.2×10^{-6} M) for 4 hr before adding vehicle or L-Arg (3×10^{-4} M) ("postexposure"); PE was added 10 min later, followed by determination of the concentration-response curve of ACh.

4) The time course of the pressor response to L-NAME was studied in two groups of conscious rats. The rats were i.v. bolus injected with vehicle (1 ml/kg) or L-NAME (4.8×10^{-6} mol/kg), and MAP was recorded continuously for 2 hr.

5) Dose-pressor response curves for L-NAME in the absence or presence of L- or D-Arg were obtained in five groups of rats, i.v. infused continuously with vehicle (0.025 ml/kg/min), L-Arg (1.2, 2.4 and 4.8×10^{-6} mol/kg/min) or D-Arg (4.8×10^{-6} mol/kg/min). Cumulative doses of L-NAME (1.5×10^{-6} to 4.8×10^{-5} mol/kg) were i.v. bolus injected beginning 10 min after starting the infusions of vehicle, L- or D-Arg at dose-intervals of 10 to 15 min, the time required to obtain plateau effect.

6) The effects of L-NAME on the magnitude and duration of depressor responses to i.v. infusions or i.v. bolus injections of ACh or SNP were studied in seven groups of rats. The first three groups were treated with vehicle (1 ml/kg i.v. bolus injection), PE (2×10^{-6} mol/kg/min, i.v. infused continuously) or L-NAME (4.8×10^{-6} mol/kg, i.v. bolus injection) followed 20 min later by i.v. infusions of ACh (5.5×10^{-6} to 8.8×10^{-7} mol/kg/min) or SNP (4×10^{-6} to 5.6×10^{-7} mol/kg/min). Each dose of ACh or SNP was infused for 4 min before the next higher dose. The sequence of administrations of ACh and SNP was reversed in one-half of the rats in each group with an interval of 20 min. The other four groups were treated with vehicle (1 ml/kg i.v. bolus injection), L-Arg (4.8×10^{-6} mol/kg/min, i.v. infusion), PE (2×10^{-6} mol/kg/min, i.v. infusion) or L-NAME (4.8×10^{-6} mol/kg, i.v. bolus injection). At 20 min later, ACh (3×10^{-10} to 8.8×10^{-5} mol/kg) and SNP (1.5×10^{-9} to 4.4×10^{-7} mol/kg) were i.v. bolus injected into these rats, at intervals between injections of 2 to 5 min. The sequence of administrations of ACh and SNP was also reversed in one-half of the rats in each group. To facilitate the measurement of the time course of the transient depressor responses to ACh and SNP, the chart was sped up from 10 mm/min to 10 mm/sec.

Calculation and Statistical Analysis

Analysis of dose (concentration)-response curves. To determine the parameters (ED_{50} or EC_{50}, maximum and Hill coefficient, n) of dose-response or concentration-response curves, values of response (R, rise in MAP or relaxation in the aortae) were fitted (by using an IBM-compatible microcomputer) by nonlinear least-squares curves to the relation $R = a + bx$, where $x = [D]^n/(ED_{50}^n + [D]^n)$ or $x = [C]^n/(EC_{50}^n + [C]^n)$ (see Kenakin, 1987), to give the value of ED_{50} or EC_{50} and b (maximum response) yielding a minimum residual sum of squares of deviations from the theoretical curve. The detailed analysis was published elsewhere (Wang and Pang, 1993a).

Modified Schild plots. In principle, a competitive inhibitor (I) shifts the dose-response curve to an agonist [A] in parallel to the right, with the ED_{50} increasing by the factor $(1 + [I]/K_i)$ when there is a one-to-one competition of I with A on the receptor. This arises from the well-known relationship: $[AR]/[R_t] = [A]/\{[A] + K_a(1 + [I]/K_i)\}$ where $[AR]/[R_t]$ is the fraction of receptors occupied by A and K_a and K_i are the dissociation constants for A and I, respectively. It can readily be shown that the same relationship holds when A is an antagonist and I is an agonist.

By using the standard assumption that equal responses, with and without I, represent equal $[AR]/[R_t]$, it follows that $K_a(1 + [I]/K_i)/[A]_I = K_a/[A]_o$, where $[A]_I$ is the [A] that gives the same response in the presence of I as did $[A]_o$ in the absence of I. Therefore "dose-ratios" ($[A]_I/[A]_o$, which are the same as ratios of ED_{50} values), follows the equation: DR = dose ratio = $[A]_I/[A]_o = 1 + [I]/K_i$ and, therefore, log

$(DR - 1) = \log [I] - \log K_i$. That is, the "Schild plot," a graph of log $(DR - 1)$ *vs.* log $[I]$, has a slope of unity and K_i is given by the (extrapolated or interpolated) value of $[I]$ at $DR = 2$.

However, if the response depends upon a form of the receptor bound to more than one (n_1) molecule of agonist whereas more than one (n_2) molecule of I is necessary to block action of the receptor, the above equation does not hold. Instead, the equations become, at the simplest (assuming high positive cooperation in binding of A to R):

$$[A^{n1}R]/[R_t] = [A]^{n1}/([A]^{n1} + K_a^{n1}(1 + [I]^{n2}/J))$$

$$DR^{n1} = (\text{dose ratio})^{n1} = ([A]_I/[A]_o)^{n1} = 1 + [I]^{n2}/K_i$$

$$DR^{n1} - 1 = [I]^{n2}/K_i$$

Thus, one must plot log $(DR^{n1} - 1)$ *vs.* $n_2 *$ log $[I]$ to obtain a slope of unity (see Kenakin, 1987).

Expression of data. The relaxant response of ACh or SNP *in vitro* was calculated as percentage of contractile response to PE and the decrease in MAP response to ACh or SNP *in vivo* was calculated as percentage of base-line MAP. The duration of each depressor response to ACh or SNP *in vivo* was expressed as "modified half-recovery time" (minutes per millimeter of mercury), which was obtained by dividing half-recovery time by base-line MAP and then multiplying by 10^4. All results were expressed as mean ± S.E. and analyzed by analysis of variance followed by Duncan's multiple range test, with $P < .05$ selected as the criterion for statistical significance.

Results

Concentration-response of L-NAME on relaxations evoked by ACh and SNP. PE (10^{-6} M) caused an average contraction of 0.84 ± 0.09 g in two groups of control aortic rings to be treated with ACh or SNP. PE-induced contractions were significantly increased by L-NAME (10^{-7} to 3.2×10^{-6} M) to a pooled value of 1.05 ± 0.10 g, with potentiating effects similar for all doses of L-NAME. ACh caused concentration-dependent relaxation of PE-preconstricted aortae (fig. 1A). Preincubation with L-NAME concentration-dependently inhibited ACh-induced relaxation, with equal effect at all concentrations of ACh (fig. 1A). Analysis of the inhibition-concentration curve for L-NAME gave a best-fitting n of 1.28 (not significantly different from 1.0) with maximal inhibition of 87% and IC_{50} of 3.5×10^{-7} M ($pD_2 = 6.5$). With n set at 1, the maximum inhibition was 96% and IC_{50} was 4.3×10^{-7} M ($pD_2 = 6.4$). As shown in figure 2A, the theoretical curve with n of 1 fits the observed data very much better than the theoretical curve with $n = 2$. Correspondingly, a Hill plot of the inhibition by L-NAME of ACh-induced relaxations had a slope of 0.97 ± 0.06 (fig. 2B).

SNP also caused concentration-dependent relaxations of PE-preconstricted aortic rings with maximal relaxation of approximately 100%; relaxations were not significantly altered by 3.2×10^{-6} M L-NAME (fig. 1B).

Time course of inhibitory effect of L-NAME on ACh-evoked relaxation of aortic rings. Repeated PE-induced contractions were consistent at 1.5, 4 and 9 hr after a first test (0.99 ± 0.10 g), with contractions averaging 1.07 ± 0.09, 1.14 ± 0.13 and 0.81 ± 0.07 g, respectively. On the other hand, the potentiation of PE-induced contraction by 3.2×10^{-6} M L-NAME (to 1.42 ± 0.15 g at the first test) slowly declined, contractions at 1.5, 4 and 9 hr after the first test averaged 0.95 ± 0.12 ($P < .05$), 0.87 ± 0.06 ($P < .05$) and 0.63 ± 0.06 g ($P < .05$), respectively. The concentration-relaxation response curves of ACh in aortic rings were repeated 4 times within 9 hr in the presence of vehicle or L-NAME. In the controls, the

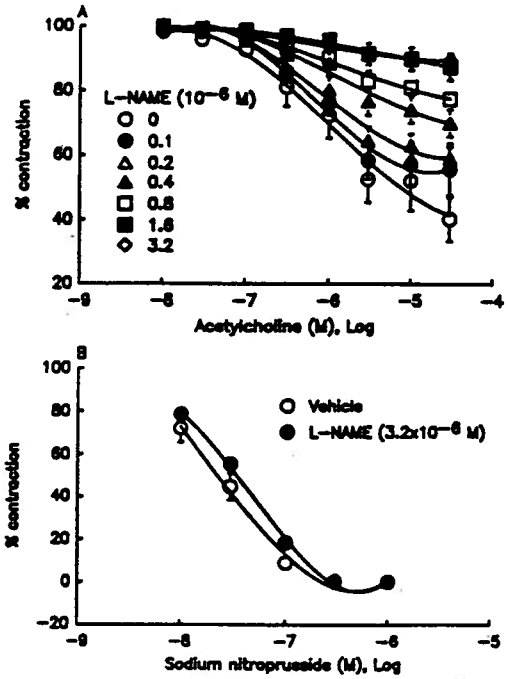

Fig. 1. Effects of L-NAME on ACh (A)- and SNP (B)-induced relaxations in PE (10^{-6} M)-preconstricted aortic rings (n = 5–6 each group).

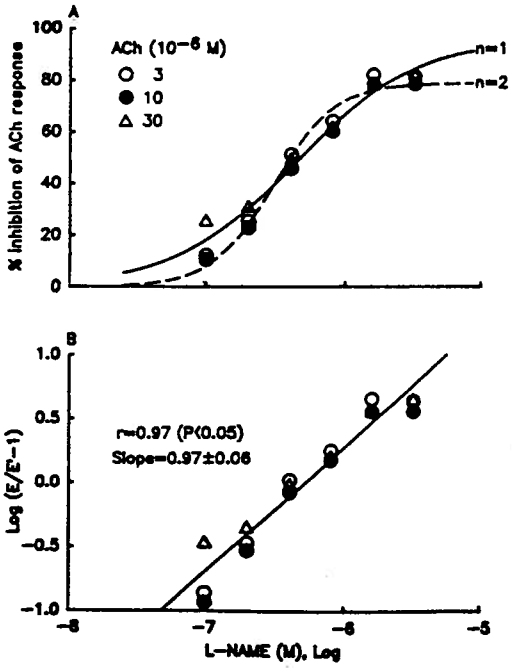

Fig. 2. Inhibition by L-NAME (10^{-7} to 3.2×10^{-6} M) of ACh (3×10^{-8} to 3×10^{-6} M)-evoked relaxation. The data were the average values from five to six aortic rings. A, concentration-response curve of L-NAME. The theoretical curves are plotted with Hill coefficient n_1 of 1 and 2 (see "Methods" for details). B, Hill Plot of the concentration-response curve of L-NAME. E and E', the relaxations at 3×10^{-8} to 3×10^{-6} M ACh in the absence or presence of different concentrations of L-NAME.

relaxation-concentration curves of ACh were slightly but time-dependently shifted to the right (fig. 3A). L-NAME (3.2×10^{-6} M) almost abolished ACh-induced relaxation completely. The inhibitory effect of the single exposure to L-NAME declined gradually at 1.5 to 9 hr after the preparations were washed out between tests (fig. 3B).

Effects of pre- and postexposure of L- and D-Arg on the inhibitory effects of L-NAME. In these groups of aortic rings, PE caused contractions of 1.07 ± 0.09 and 1.22 ± 0.10 g in the presence of vehicle + vehicle and vehicle + L-NAME (3.2×10^{-6} M), respectively. Pre-exposure (10 min earlier) to L-Arg (but not D-Arg) insignificantly reduced PE-caused contraction to 0.84 ± 0.17 and 0.98 ± 0.12 g, respectively. With L-Arg (3×10^{-4} M), but not D-Arg, the effect of L-NAME (3.2×10^{-6} M) to abolish ACh-induced relaxation was prevented totally (fig. 4A).

In another series, L-Arg was applied after L-NAME. After a 4-hr incubation of aortic rings with 3.2×10^{-6} M L-NAME, PE caused an average contraction of 1.22 ± 0.10 g. Addition of L-Arg (3×10^{-4} M) (postexposure) reduced the mean PE-induced contraction to 0.85 ± 0.13 g ($P < .05$), and restored totally the relaxation response of ACh (fig. 4B).

Pressor response to L-NAME *in vivo*. Base-line values of MAP in conscious rats treated with vehicle or L-NAME were not significantly different from each other (106 ± 3 and 112 ± 3 mm Hg, respectively). Intravenous bolus injections of L-NAME (4.8×10^{-5} mol/kg), but not the vehicle, caused a slow-developing and prolonged increase in MAP. Plateau MAP response to L-NAME, which was attained approximately 10 min after injection, with half-rise at 2.3 ± 0.4 min, lasted at least 2 hr (fig. 5A).

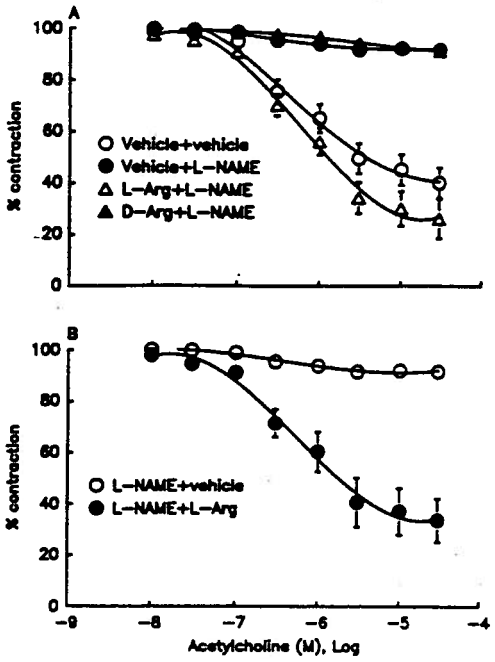

Fig. 4. Effects (mean ± S.E.) of pre- or postexposure with L- (3×10^{-4} M) or D-Arg (3×10^{-4} M) on inhibitory effect of L-NAME (3.2×10^{-6} M) on ACh-induced relaxation in PE (10^{-6} M)-preconstricted aortic rings (n = 5–6 each group). A, pre-exposure (10 min earlier) with vehicle, L- or D-Arg. B, postexposure (4 hr later) with vehicle or L-Arg.

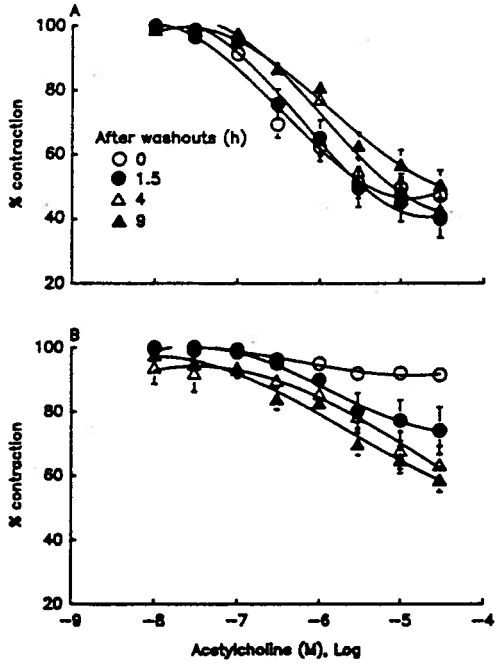

Fig. 3. Time course of the effects (mean ± S.E.) of vehicle (A) and L-NAME (3.2×10^{-6} M, B) on ACh-induced relaxation in PE (10^{-6} M)-preconstricted rat aortic rings (n = 5–6 each group). The various times represent the times after the preparations were washed without further adding the vehicle or L-NAME.

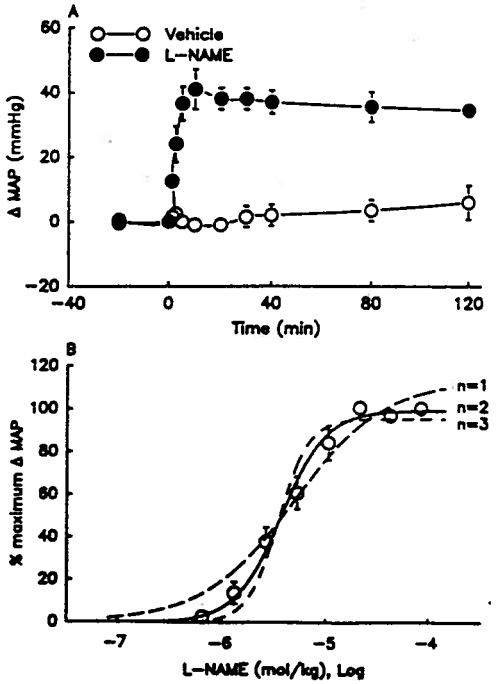

Fig. 5. Time course and cumulative dose-response curves of the MAP response (mean ± S.E.) to i.v. bolus injections of L-NAME in conscious rats (n = 5 each group). A, the time course of vehicle or L-NAME (4.8×10^{-5} mol/kg). B, cumulative dose-response of L-NAME. Theoretical lines were drawn by using n of 1, 2 and 3 (see "Methods" for details).

In another group of rats, with base-line MAP of 106 ± 4 mm Hg (table 1), i.v. bolus injections of cumulative doses of L-NAME increased MAP in a dose-dependent manner (fig. 5B). Analysis of dose-response curves with n "floating" gave average best-fitting n = 2.01 ± 0.32; for in no individual curve was n significantly different from 2 and it is evident that the theoretical curve with n of 2 but not 1 or 3 fitted the observed data best. By using floating n for each individual curve, average values of ED_{50} and E_{max} for the pressor effects of L-NAME were 5.0 ± 1.1 × 10^{-6} mol/kg and 50 ± 7 mm Hg, respectively. The calculated E_{max} values with n = 2 for each curve were not significantly different from observed E_{max} data (table 2).

Effects of L- or D-Arg on the pressor dose-response curve of L-NAME. Before and 10 min after the start of infusion of vehicle, L- or D-Arg, mean values of MAP were not significantly different from each other (table 1). Intravenous infusion of L- but not D-Arg dose-dependently shifted the pressor dose-response curve of L-NAME to the right, without significantly changing E_{max} or n_1 (fig. 6A; table 2). The theoretical curves in figure 6A were plotted by using n_1 as 2 and indeed fitted the observed data well. The modified Schild plots in figure 6B (see "Methods") for the apparently competitive block by L-Arg were drawn by using, as each dose-ratio, the ED_{50} value in the presence of L-Arg divided by the ED_{50} in the absence of L-Arg. Plots were essentially linear whether n_1 was chosen as 1, 2 or 3 when n_2 was chosen as 1. However, with n_1 = 2 the slope was 1.17 ± 0.16, whereas with n_1 chosen as 1 or 3 the slope was 0.68 ± 0.1 or 1.71 ± 0.22, respectively, both significantly different from 1.0. On the other hand, if the plots were to use log ($DR-1$) (*i.e.*, n_1 = 1) against n_2 * log (L-Arg), where n_2 was chosen as 1, 2 and 3, they were also essentially linear, but the slopes were 0.66 ± 0.14, 0.33 ± 0.07 and 0.22 ± 0.05, respectively. Thus, the data fit a model in which 1 molecule (n_2) of L-Arg competes effectively with 2 molecules (n_1) of L-NAME. The calculated half-blocking infusion dose for L-Arg to antagonize the pressor response to L-NAME was at a rate of 10^{-6} mol/kg/min (with n_1 = 2).

Effects of L-NAME on the depressor responses to ACh and SNP. In conscious rats, i.v. infusions of ACh or SNP dose-dependently decreased MAP (expressed as percentage of base-line MAP); infusion of PE (2 × 10^{-6} mol/kg/min), which raised MAP to the same extent as a single bolus injection of 4.8 × 10^{-5} mol/kg L-NAME (table 1), enhanced the depressor responses to various doses of ACh and SNP by average values of 140 ± 53 and 94 ± 31%, respectively, with greater potentiation at low doses of ACh and SNP (fig. 7, A and B). L-NAME inhibited the depressor response to ACh markedly, by 53 ± 4%, when the ACh-responses were compared to those in rats treated with PE (fig. 7A). On the other hand, L-NAME potentiated the depressor responses to all the doses of SNP, by an average of 142 ± 53%; at the lowest two doses of SNP, the potentiation was significantly ($P < .05$) greater than with PE infusion which gave the same increase in base-line MAP (fig. 7B).

In another four groups of rats, i.v. bolus injections of ACh were given (fig. 8). The first three doses of ACh caused significantly less reductions of MAP in rats treated with L-Arg (4.8 × 10^{-5} mol/kg/min) than in control rats given vehicle (fig. 8A). In animals given PE (2 × 10^{-6} mol/kg/min) or L-NAME (4.8 × 10^{-5} mol/kg), the magnitudes of depressor responses to ACh

TABLE 1

Values (mean ± S.E.) of MAP before (a) and 10 (b), 20 (c) or 100 (d) min after i.v. administrations of vehicle, L-Arg (1.2 to 4.8 × 10^{-5} mol/kg/min), D-Arg (4.8 × 10^{-5} mol/kg/min), PE (2 × 10^{-6} mol/kg/min) or L-NAME, 4.8 × 10^{-5} mol/kg) in conscious rats (n = 5–6 each group)

Treatment	Dose	MAP (mm Hg)			
		a	b	c	d
	mol/kg or mol/kg/min				
Vehicle		107 ± 5	106 ± 4		
L-Arg	1.2 × 10^{-5}	103 ± 6	102 ± 7		
L-Arg	2.4 × 10^{-5}	114 ± 2	114 ± 3		
L-Arg	4.8 × 10^{-5}	105 ± 5	106 ± 5		
D-Arg	4.8 × 10^{-5}	112 ± 6	112 ± 6		
Vehicle		115 ± 5		124 ± 9	
PE	2 × 10^{-6}	109 ± 4		160 ± 4*	
L-NAME	4.8 × 10^{-5}	112 ± 5		160 ± 5*	
Vehicle		108 ± 5		111 ± 5	108 ± 4
L-Arg	4.8 × 10^{-5}	113 ± 4		115 ± 4	110 ± 3
PE	2 × 10^{-6}	103 ± 4		162 ± 5*	156 ± 3*
L-NAME	4.8 × 10^{-5}	113 ± 4		159 ± 5*	156 ± 4*

* Significant difference from base-line MAP ($P < .05$).

TABLE 2

Values (mean ± S.E.) of Hill coefficient (n), ED_{50} and E_{max} calculated from by best fitting or by specific n, as well as observed E_{max} of L-NAME in the presence of vehicle, L-Arg and D-Arg in conscious rats (n = 5 each group)

	Calculations			Observed E_{max}
	n	ED_{50}	E_{max}	
		10^{-6} *mol/kg*	*mm Hg*	*mm Hg*
Best-fitted				
Vehicle	2.0 ± 0.3	5.0 ± 1.1	49.7 ± 6.7	50.0 ± 5.8
L-Arg (10^{-5} mol/kg/min)				
1.2	1.8 ± 0.3	19.4 ± 2.8*	52.0 ± 2.8	52.5 ± 2.6
2.4	2.0 ± 0.2	32.0 ± 5.0*	54.6 ± 2.2	53.0 ± 1.3
4.8	2.5 ± 0.3	40.9 ± 7.3*	52.1 ± 1.9	52.5 ± 2.0
D-Arg (10^{-5} mol/kg/min)				
4.8	2.2 ± 0.3	4.1 ± 0.5	49.7 ± 2.9	49.5 ± 2.6
Specified, n = 2				
Vehicle	2	4.7 ± 1.1	48.4 ± 6.4	50.0 ± 5.8
L-Arg (10^{-5} mol/kg/min)				
1.2	2	1.92 ± 2.7*	51.1 ± 2.7	52.5 ± 2.6
2.4	2	31.1 ± 4.6*	53.7 ± 1.3	53.0 ± 1.3
4.8	2	42.2 ± 8.4*	52.6 ± 2.1	52.5 ± 2.0
D-Arg (10^{-5} mol/kg/min)				
4.8	2	4.1 ± 0.5	49.4 ± 2.0	49.5 ± 2.6

* Significant difference from vehicle-treated groups ($P < .05$).

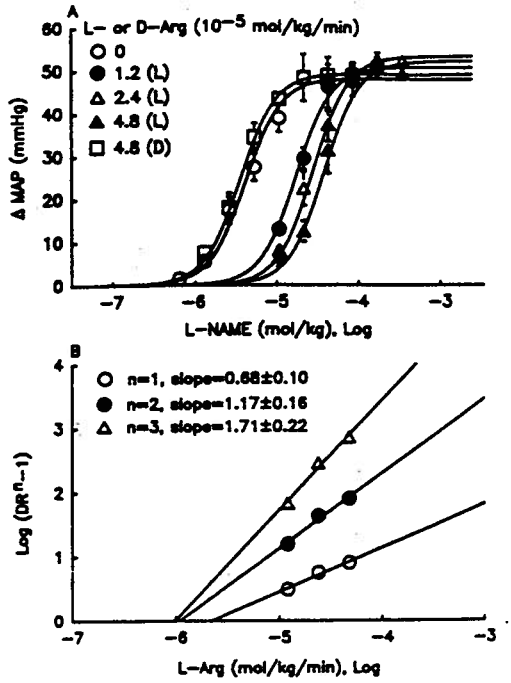

Fig. 6. A, effects (mean ± S.E.) of i.v. infusions of L and D-Arg on the MAP response to i.v. bolus injections of L-NAME in conscious rats (n = 5 each group). B, modified Schild plot (see "Methods" for details) for L-Arg to antagonize L-NAME (i.v. bolus injections). The lines were drawn with n of 1, 2 or 3.

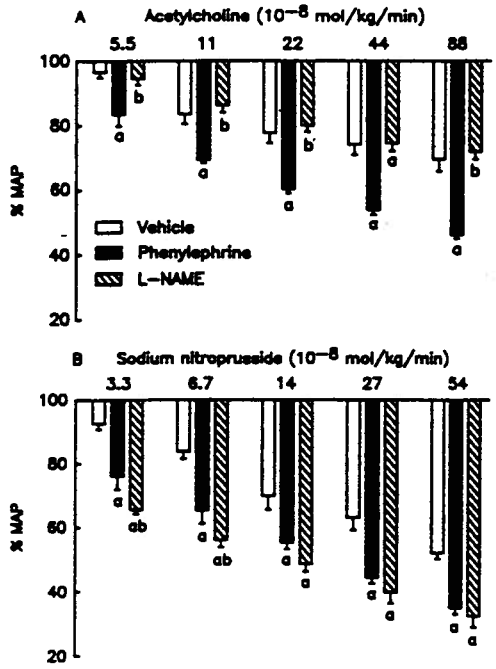

Fig. 7. Effects (mean ± S.E.) of i.v. infusions of ACh (5.5×10^{-8} to 8.8×10^{-7} mol/kg/min, 4 min for each dose, A) and SNP (3.3×10^{-8} to 5.4×10^{-7} mol/kg/min, 4 min for each dose, B) on the MAP (expressed as percentage of base-line MAP) in conscious rats (n = 6 each group) pretreated with vehicle, PE (2×10^{-6} mol/kg/min) or L-NAME (4.8×10^{-5} mol/kg). a, significant difference from vehicle-treated group; b, significant difference from PE-treated group.

were not significantly different from those in control rats. However, the duration (expressed as modified half-recovery time) of the (transient) responses to ACh was shortened by L-NAME (fig. 8B), by 23 ± 3% when the value was compared to that in vehicle-treated rats ($P < .05$ for the last 2 doses of ACh) and by 26 ± 3% when compared to that in PE-treated rats, with significance at the last 4 doses of ACh.

In contrast, in the groups of rats treated in the same way but with bolus injections of SNP to cause transient depressions of MAP, responses were somewhat potentiated by L-NAME (fig. 8, C and D), with significance at the lower two doses of SNP even by comparison with PE infusion (which increase responses slightly and nonsignificantly). A decrease in the magnitude of MAP response to L-Arg, similar to that seen with ACh, was significant ($P < .05$) only at the third dose of SNP. The duration of depressor response to SNP was unaltered by PE, somewhat prolonged by L-Arg ($P < .05$ at the second dose of SNP) and somewhat prolonged by L-NAME at the lower doses of SNP.

Discussion

There is a large body of information, which is derived primarily from *in vitro* studies, to support the concept that ACh produces vascular relaxation *via* NO biosynthesis (*e.g.*, Palmer *et al.*, 1988b; Crawley *et al.*, 1990; Rees *et al.*, 1990). Whereas it is generally shown that the ACh-induced relaxation response *in vitro* is inhibited completely by N^G-substituted Arg derivatives, there are uncertainties with respect to the efficacies of these compounds in suppressing ACh-induced vasodilatation *in vivo* (see Introductory section). Inconsistent modifications (*e.g.*, inhibition, no effect or even potentiation) of ACh-induced depressor response by L-Arg analogs *in vivo* cast doubts upon the role of NO in ACh-induced vasodilatation in whole animals. The discrepancies in the effectiveness of NO synthase inhibitors in suppressing ACh-induced depressor responses are unlikely related to the species of animals used or the conscious or anesthetized state. Instead, the inconsistencies are most likely due to comparison of ACh responses at different base-line MAPs (in the absence or presence of N^G-substituted L-Arg derivatives), the different modes of administration (bolus injection *vs.* infusion) of ACh as well as the method of expression of data. Vasodilator drugs are known to cause greater hypotension at higher base-line MAPs (Rees *et al.*, 1990; van Gelderen *et al.*, 1991; Wang *et al.*, 1992; Chyu *et al.*, 1992).

Therefore, in this study PE was used as a base-line MAP control for L-NAME and the depressor responses were calculated as percentage of base-line MAP and modified half-recovery time, in order to eliminate difficulties associated with the comparison of responses at different base-line MAPs. Indeed, depressor responses to ACh and SNP were potentiated by 140 and 95%, respectively, in PE-treated rats, as compared to those in vehicle-treated rats. With the use of appropriate controls, it was unequivocally clear that L-NAME interfered in part with the depressor responses of ACh. The depressor response to infused ACh was decreased by approximately 50%. The lack of *in vivo* effectiveness of L-NAME in eliminating responses of ACh is not due to insufficient dose, since a supramaximal pressor dose (4.8×10^{-6} mol/kg, 10 times ED_{50} for pressor response) of L-NAME was used. In our preliminary studies, even a dose of L-NAME as high as 3.8×10^{-3} mol/kg in

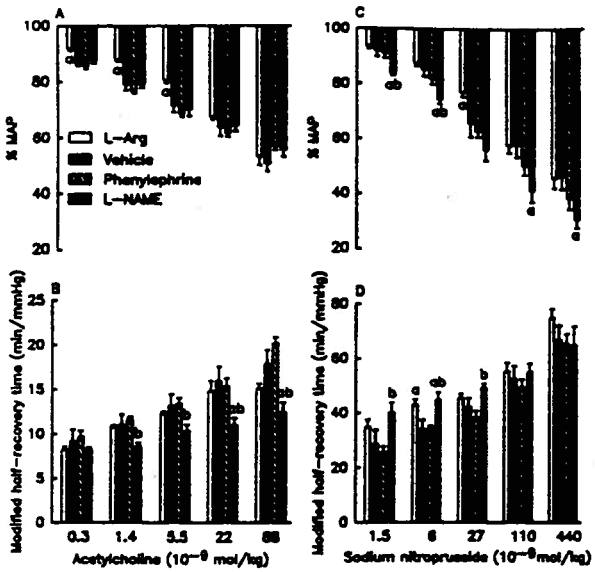

Fig. 8. Effects (mean ± S.E.) of i.v. bolus injections of ACh (3×10^{-10} to 8.8×10^{-6} mol/kg) or SNP (1.5×10^{-9} to 4.4×10^{-7} mol/kg) on the MAP (expressed as percentage of base-line MAP) and modified half-recovery time in conscious rats ($n = 6$ each group) pretreated with L-Arg (4.8×10^{-5} mol/kg/min), vehicle (1 ml/kg), PE (2×10^{-6} mol/kg/min), L-NAME (4.8×10^{-5} mol/kg). a, significant difference from vehicle-treated group; b, significant difference from PE-treated group. Modified half-recovery time was normalized as half-recovery time $\times 10^4$ divided by base-line MAP.

conscious rats only inhibited in part ACh-induced depressor response ($n = 2$, data not shown). Moreover, the incomplete inhibition of ACh-induced depressor response by L-NAME is unlikely due to the activation of nicotinic receptor by ACh, because ACh-induced activation of ganglionic nicotinic receptors are known to produce pressor responses. In addition, we found that after effective ganglionic blockade with mecamylamine, L-NAME (3.8×10^{-4} mol/kg) still failed to inhibit completely ACh-induced fall in MAP in pentobarbital-anesthetized rats ($n = 6$) (Y.-X. Wang, C. J. Poon and C. C. Y. Pang, unpublished data). Gardiner *et al.* (1990a) also reported similar findings. These results suggest that there may be a difference between ACh-induced responses in conduit vessels (*e.g.*, the aorta) and resistance blood vessels.

Further analysis of the depressor response to bolus injections of ACh shows that L-NAME decreased the duration, but not the magnitude, of the responses to ACh by 26%. These results are in accordance with those of Aisaka *et al.* (1989a) by using L-NMMA. Aisaka *et al.* (1989a) also showed that exogenous L-Arg prolonged the duration of the depressor response to ACh and suggested that the availability of L-Arg determined the duration of response to ACh. In contrast, we found that L-Arg, at 48 times its half-block dose to antagonize the pressor response of L-NAME, did not affect the duration but reduced the magnitude of the depressor response to low doses of bolus injected ACh. van Gelderen *et al.* (1991) also found that L-Arg did not reduce the duration of the ACh-induced depressor response in anesthetized rats. The mechanism by which L-Arg suppressed ACh-induced depressor response is not known but it is likely nonspecific, because the dose of L-Arg used was relatively high and L-Arg also reduced the magnitudes of the depressor responses to low doses of SNP.

It is unclear why L-NAME shortened but L-Arg did not prolong the duration of the depressor response to bolus injected ACh. It has been suggested that endogenous concentration of L-Arg is sufficient to saturate NO synthase (Rees *et al.*, 1989b). This hypothesis may explain why L-NAME but not L-Arg influenced the duration of the depressor response to ACh. An additional mechanism, besides NO biosynthesis, must be responsible for the establishment of the magnitude of the depressor response to bolus injected ACh, which was neither reduced by L-NAME nor prolonged by L-Arg. ACh is shown to release endothelium-derived hyperpolarizing factor in addition to endothelium-derived relaxing factor/NO (Chen *et al.*, 1988; see Suzuki and Chen, 1990). Endothelium-derived hyperpolarizing factor vasodilates some vascular preparations (Garland and McPherson, 1992) and may contribute to the depressor response to ACh *in vivo*. Consistent with this hypothesis, it was reported recently that the Ca^{++}-activated K^+-channel (which leads to hyperpolarization) blocker charybdotoxin attenuated the depressor response to ACh in rats (Watkins *et al.*, 1993).

It is likely that L-NAME had caused a redistribution of blood flow in the rats. N^G-substituted L-Arg analogs have been shown to cause pressor response by increasing total peripheral resistance *via* systemic vasoconstriction (Gardiner *et al.*, 1990b,c; Wang *et al.*, 1993a), but their degree of influence vary with different beds (Wang *et al.*, 1993a). It was also shown that the renal, internal carotid, common carotid and mesenteric, but not hindquarter vasodilator effects of ACh, were attenuated in part by L-NAME (Gardiner *et al.*, 1990a, 1991). The varying ability of L-NAME to inhibit vasodilator responses of ACh in different beds suggests that part of the vasodilator response to ACh is mediated *via* a mechanism insensitive to the inhibition by L-NAME.

A Hill coefficient (n) of 1.3 for L-NAME-induced inhibition of ACh-induced relaxation was derived from our *in vitro* results. This value is similar to the n of 1.2 for L-NNA (unpublished result) calculated from data of a previous study (Wang *et al.*, 1993b). These results suggest that one molecule of L-NAME or L-NNA competes with one molecule of endogenous L-Arg to inhibit NO biosynthesis. Our *in vivo* results, on the other hand, show that the n for L-NAME to cause pressor response is 2.0 and this value is not affected by L-Arg which caused a rightward displacement of the dose-response curve of L-NAME. The n for L-NAME *in vivo* is also consistent with the n of 2.2 and 2.3 for L-NNA (unpublished results) calculated from the data of intact and ganglionic-blocked conscious rats, respectively (Wang and Pang, 1991). For comparison, the n for the pressor effects of angiotensin II is 1.0 (unpublished calculation from Wang *et al.*, 1993c) and of diphenyleneiodonium, a novel inhibitor of NO synthase (Stuehr *et al.*, 1991) is 3.3 in conscious rats (Wang and Pang, 1993b).

The nature of the difference between n values for N^G-substituted Arg analogs obtained *in vivo* (pressor response) and *in vitro* (inhibition of vascular relaxation) is not known, but may suggest that the mechanism involved in raising MAP (in resistance blood vessels) is different from that in inhibiting vascular relaxation (in large arteries). It is possible that L-NAME raises MAP by an unknown mechanism (which, nevertheless, is L-Arg-reversible) distinct from the inhibition of endogenous endothelial NO synthesis. This hypothesis is consistent with the observation that diphenyleneiodonium pro-

duced pressor responses by sympathetic activation rather than the inhibition of NO synthesis (Wang and Pang, 1993a,b), even though diphenyleneiodonium caused prolonged inhibition of endothelium-dependent vasodilatations *in vitro* and *in vivo* (Stuehr *et al.*, 1991; Poon *et al.*, 1993). Moreover, methylene blue suppressed ACh-induced relaxations *in vitro* but did not cause a pressor response *in vivo* (Pang and Wang, 1993). Therefore, the inhibition of endogenous endothelial NO synthesis does not necessarily cause vasoconstriction *in vivo*. An alternative explanation is that there is a difference between stimulated NO synthesis (ACh-induced relaxation) and basal NO synthesis (vasodilatation) (Chyu *et al.*, 1992). It was reported that the endothelium-dependent contractions elicited by L-NMMA and L-NNA in the dog coronary artery were not a consequence of the suppression of basal NO synthesis (Cocks and Angus, 1991). It was also shown that, although both L-NNA and D-NNA inhibited ACh-induced relaxation in aortic rings, only L-NNA elicited contraction and potentiated PE-induced contraction (Wang *et al.*, 1993b). Moreover, it has been suggested recently that N^G-substituted L-Arg analogs cause pressor responses by inhibiting central (Togashi *et al.*, 1992) or ganglionic (Toda *et al.*, 1993) NO synthesis rather than endothelial NO production. However, most authors reported that the pressor responses to N^G-substituted Arg analogs remain (or are potentiated) after impairments of the central (Wang and Pang, 1993a) or autonomic (Rees *et al.*, 1989b; Wang and Pang, 1991; Chyu *et al.*, 1992) nervous systems. Regardless of the exact mechanism or site of the pressor response of L-NAME, this study is the first to show that L-Arg antagonizes competitively the pressor response of L-NAME, with half-blocking dose at 10^{-6} mol/kg/min.

In summary, L-NAME is an efficacious and long-lasting inhibitor of ACh-induced relaxation of the aortae, with a Hill coefficient of 1 and maximal inhibition of nearly 100%. In conscious rats, L-NAME causes partial inhibition of the vasodilatation response to ACh. This is characterized as a shortened duration (but not magnitude) of action of i.v. bolus injected ACh and a reduced magnitude of i.v. infused ACh. L-NAME causes a dose-dependent pressor response with a Hill coefficient of 2, which can be antagonized competitively by L-Arg (but not by D-Arg), without changing the Hill coefficient. Differences in Hill coefficients and effectiveness of inhibition of ACh-induced vasodilatation *in vitro* and *in vivo* for L-NAME suggest that either one of the following hypotheses may be true: 1) that the pressor response of NO synthase inhibitors in whole animals is not due entirely to the inhibition of endogenous endothelial NO biosynthesis or 2) that basal NO synthesis is blocked more effectively by L-NAME than stimulated NO synthesis.

Acknowledgments

The authors are deeply indebted to Dr. David M. J. Quastel in this department for his help in pharmacodynamic analyses of our data and Ms. Lorraine C. Y. Yau for her technical assistance.

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