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Modulation of the vasodepressor actions of acetylcholine, bradykinin, substance P and endothelin in the rat by a specific inhibitor of nitric oxide formation

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1 The effects of the specific inhibitor of nitric oxide (NO) formation, N^G-monomethyl-L-arginine (L-NMMA), on resting systemic arterial blood pressure (BP) and on the actions of both endothelium-dependent and endothelium-independent vasodilators were investigated in the anaesthetized, normotensive rat.

2 Intravenous administration of L-NMMA (12.5–50 mg kg^{-1}; 47–188 μmol kg^{-1}) but not its enantiomer, D-NMMA, induced a dose-related increase in BP, which was reversed by the intravenous administration of L-arginine (150–600 μmol kg^{-1}), but not D-arginine.

3 The vasodepressor responses to intravenous administration of the endothelium-dependent vasodilators, acetylcholine, bradykinin and substance P were significantly inhibited by L-NMMA (94 and 188 μmol kg^{-1} i.v.), but not by D-NMMA.

4 The inhibition by L-NMMA of these vasodepressor responses was reversed by administration of L-arginine, but not D-arginine.

5 Endothelin (ET-1) induced dose-related vasodepressor responses following bolus intravenous administration, which were significantly inhibited by L-NMMA but not by D-NMMA. This inhibition was reversed by administration of L-arginine.

6 The vasodepressor effects of the endothelium-independent vasodilators, glyceryl trinitrate or prostacyclin, were not significantly inhibited by L-NMMA.

7 These findings with L-NMMA suggest that resting blood pressure in the rat is modulated by endogenous NO biosynthesis and that endothelium-dependent vasodilators act through the formation of endogenous NO to exert their actions *in vivo*.

Introduction

Acetylcholine, substance P and bradykinin induce relaxation of isolated vascular tissue by the release of an endothelium-derived relaxing factor, EDRF (Furchgott & Zawadzki, 1980; Furchgott, 1983). The requirement of this factor for such vasodilatation *in vivo* has been suggested from studies in the dog femoral artery and mouse cerebral microcirculation, where an intact endothelium was necessary for these agents to act (Angus *et al.*, 1983; Rosenblum *et al.*, 1986), and from cardiovascular studies in the rabbit with gossypol, a non-specific EDRF inhibitor (Dudel & Forstermann, 1988).

Nitric oxide (NO), formed by vascular endothelial cells from the amino acid, L-arginine, accounts for the biological actions of EDRF (Palmer *et al.*, 1987; 1988a). The formation of NO and the relaxation of the rabbit aorta induced by acetylcholine, substance P or bradykinin is specifically inhibited by the L-arginine analogue, N^G-monomethyl-L-arginine (L-NMMA) but not by its enantiomer, D-NMMA (Palmer *et al.*, 1988b; Rees *et al.*, 1989a). Furthermore, intravenous administration of L-NMMA, in doses that inhibit vascular NO production *ex vivo*, increases systemic blood pressure and attenuates the vasodepressor actions of acetylcholine in the rabbit (Rees *et al.*, 1989b).

In the present study in the anaesthetized rat, the effects of intravenous administration of L-NMMA on the vasodepressor actions of acetylcholine, substance P or bradykinin have been determined. In addition, the effects of L-NMMA on the hypotensive effects of bolus administration of endothelin (ET-1,

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Yanagisawa *et al.*, 1988a,b) a 21-residue vascular peptide that elicits endothelium-dependent vasodilatation in the rat isolated mesenteric bed (De Nucci *et al.*, 1988), has been investigated. The specificity and characteristics of these actions of L-NMMA *in vivo* have been assessed by studying its actions on the endothelium-independent vasodilators, glyceryl trinitrate and prostacyclin, by reversal of its actions by L-arginine and by the use of the enantiomers, D-NMMA and D-arginine.

Methods

Male Wistar rats (230–260 g body weight) were anaesthetized with sodium pentobarbitone (60 mg kg^{-1}, i.p.) and the trachea was cannulated to facilitate respiration. Rectal temperature was maintained at 37°C by thermistor-controlled radiant heat. Systemic arterial blood pressure was recorded from a cannula inserted into a carotid artery, and heart rate was derived from the arterial pulse. A femoral vein was cannulated, or a 25 gauge butterfly needle was inserted into tail vein, for the administration of drugs. For bolus intravenous injection, compounds were administered in randomised doses, in volumes of 1 ml kg^{-1} and flushed in with 0.25 ml of isotonic saline (0.9% w/v NaCl solution).

Drugs

Acetylcholine bromide, L-arginine, D-arginine (Sigma Chemical Co.) prepared as the acetate salts and glyceryl trinitrate (Wellcome Foundation Ltd) were freshly dissolved in isotonic saline before use. Bradykinin (Sigma), substance P (Cambridge Research Biochemicals) and endothelin (ET-1; human-porcine; Pennisula Laboratories) were dissolved in distilled water in aliquots, stored at −20°C and diluted immediately before use in isotonic saline. Prostacyclin as the sodium salt (Wellcome Foundation Ltd), was freshly dissolved in 1M Tris buffer (pH 9.6 at 4°C) immediately before use and diluted in ice-cold 1.25% w/v $NaHCO_3$ solution. Indomethacin (Sigma) was freshly dissolved in 5% w/v $NaHCO_3$ solution and diluted to 1.25% w/v with distilled water. N^G-monomethyl-L-arginine acetate and its D-enantiomer, synthesized as described previously (Patthy *et al.*, 1977) in the Department of Medicinal Chemistry, Wellcome Research Laboratories by Dr H. Hodson, were freshly dissolved in isotonic saline before use.

Statistical analysis

Results, calculated as the change in diastolic arterial blood pressure (BP) or as % inhibition of the control response to the vasodilator agent, are expressed as mean ± s.e.mean, where (n) is the number of values. The difference between groups was evaluated by Student's t test for paired or unpaired data as appropriate, with $P < 0.05$ being taken as significant.

Table 1 Vasopressor effects of intravenous administration of L-NMMA (47, 94 and 188 μmol kg^{-1}) on resting diastolic blood pressure (BP) in the anaesthetised rat, and reversal by the administration of L-arginine in a 3 fold excess (150, 300 and 600 μmol kg^{-1} i.v. respectively)

L-NMMA dose (mg kg^{-1})	*ΔBP* (mmHg) *above basal*	
	L-NMMA	*L-NMMA + L-Arginine*
12.5	18 ± 1***	8 ± 2†
25	27 ± 1***	8 ± 2†
50	28 ± 5***	8 ± 3†

Results, shown as the change in BP from its basal value (ΔBP) are the mean ± s.e.mean of 5–8 experiments in each group, where the significant increase from basal BP by L-NMMA is shown as ***$P < 0.001$, and where † indicates significant difference ($P < 0.05$) between the L-NMMA and L-arginine group and the corresponding group with L-NMMA alone.

Results

Effect of L-NMMA on resting blood pressure

Mean diastolic BP was 100 ± 2 mmHg ($n = 102$). Bolus intravenous administration of L-NMMA (12.5–50 mg kg^{-1}; 47–188 μmol kg^{-1}) induced a dose-related increase in resting BP, as shown in Table 1. The increase in BP reached its maximal level within 5 min and its duration was dose-dependent, being maintained for 45 min with L-NMMA (188 μmol kg^{-1}). The increase in BP was accompanied by a minor fall in heart rate, with L-NMMA (188 μmol kg^{-1}) inducing a fall of 34 ± 4 beats min^{-1} ($n = 16$, $P < 0.01$) from the resting value of 409 ± 16 beats min^{-1}.

Intravenous administration of L-arginine (150–600 μmol kg^{-1}) in a 3 fold molar excess, significantly reversed the L-NMMA-induced increase in BP, as shown in Table 1, whereas D-arginine (1.2 mmol kg^{-1}, i.v.) had no significant effect ($n = 4$). The enantiomer, D-NMMA (188 or 376 μmol kg^{-1}, i.v.) did not significantly affect either resting BP or heart rate ($n = 4$ for each dose).

Effect of L-NMMA on vasodepressor actions of acetylcholine

Bolus intravenous administration of acetylcholine (0.3–1.2 nmol kg^{-1}) induced a dose-related fall in

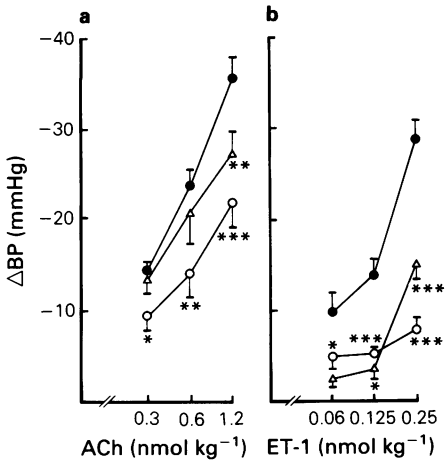

Figure 1 Inhibition of the vasodepressor responses to bolus intravenous administration of acetylcholine (●; ACh, 0.3–1.2 nmol kg^{-1}; a) or endothelin (●; ET-1, 0.06–0.25 nmol kg^{-1}; b) by L-NMMA (Δ 25 and ○ 50 mg kg^{-1}; 94 and 188 μmol kg^{-1}) in the anaesthetized rat. Results, shown as the fall in diastolic BP (ΔBP), are the mean ± s.e.mean of 6–8 experiments in each group, where significant difference from the control response is shown as $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

diastolic BP (Figure 1), with minimal changes in heart rate at these doses. Whereas pretreatment (10 min) with the low dose of L-NMMA (47 μmol kg^{-1}; $n = 4$) had no significant effect on these vasodepressor actions, pretreatment with L-NMMA (94 and 188 μmol kg^{-1}, i.v.), significantly inhibited the fall in diastolic BP induced by acetylcholine (Figure 1). Higher doses of L-NMMA (376 μmol kg^{-1}) did not further augment significantly the maximal degree of inhibition ($n = 4$). Pretreatment with D-NMMA (376 μmol kg^{-1}, i.v.) did not significantly alter the vasodepressor response to acetylcholine ($n = 3$).

Intravenous administration of L-arginine (600 μmol kg^{-1}) but not D-arginine (600 μmol kg^{-1}; $n = 4$), significantly reversed the inhibition by L-NMMA (188 μmol kg^{-1}) of the vasodepressor actions of acetylcholine (Table 2). This dose of L-arginine alone did not significantly affect either the resting BP or the vasodepressor response to acetylcholine ($n = 4$).

Effect on the vasodepressor actions of endothelin

In these preparations, with a resting diastolic BP of 112 ± 3 mmHg ($n = 30$), rapid bolus intravenous administration of endothelin (ET-1; 0.06–0.25 nmol kg^{-1}) induced a dose-dependent fall in BP (Figure 1). No consistent secondary increase in BP

Table 2 Inhibition of the vasodepressor responses to intravenous bolus administration of acetylcholine (1.2 nmol kg^{-1}), bradykinin (3.2 nmol kg^{-1}) substance P (30 pmol kg^{-1}) and endothelin (ET-1, 0.25 nmol kg^{-1}) by L-NMMA (188 μmol kg^{-1}, i.v.)

	% *inhibition*			
	L-NMMA	(n)	*L-NMMA + L-Arginine*	(n)
Acetylcholine	41 ± 6**	(8)	−10 ± 9†	(4)
Bradykinin	39 ± 11*	(4)	−38 ± 14†	(4)
Substance P	40 ± 8*	(4)	−15 ± 10†	(4)
Endothelin	72 ± 4**	(14)	19 ± 16†	(4)

Results, shown as % inhibition of the control responses to the vasodepressors, are mean ± s.e.mean of (*n*) animals, where significant inhibition is shown as $*P < 0.05$, $**P < 0.01$. L-arginine (600 μmol kg^{-1}) reversed the inhibition by L-NMMA, which led in some experiments to the potentiation of the vasodepressor responses (indicated by the negative inhibition), where † reflects significant difference ($P < 0.05$) from L-NMMA alone.

was observed following the return of BP to its resting values. Whereas the low dose of L-NMMA (47 μmol kg^{-1}, i.v.; $n = 4$) had no significant effect on these vasodepressor actions, pretreatment (10 min) with L-NMMA (94 and 188 μmol kg^{-1}, i.v.) significantly inhibited the fall in diastolic BP induced by ET-1 (Figure 1). No consistent secondary increase in BP was observed under these conditions. Higher doses of L-NMMA (376 μmol kg^{-1}) did not further augment the degree of inhibition of the responses to ET-1 ($n = 4$). D-NMMA (188 μmol kg^{-1}, i.v.) did not significantly alter the vasodepressor response to ET-1 ($n = 4$). Pretreatment (10 min) with indomethacin (5 mg kg^{-1}, i.v.), did not significantly affect the responses to ET-1 ($n = 4$), or the extent of L-NMMA (188 μmol kg^{-1})-induced inhibition of the ET-1 (0.25 nmol kg^{-1}) response (73 ± 6% inhibition, $n = 8$).

Administration of L-arginine (600 μmol kg^{-1}, i.v.), reversed the inhibition of the vasodepressor actions of ET-1 induced by L-NMMA (188 μmol kg^{-1}, i.v.) as shown in Table 2.

Effect on the vasodepressor actions of bradykinin and substance P

Bradykinin (0.4–3.2 nmol kg^{-1}, i.v.) or substance P (3.75–30 pmol kg^{-1}, i.v.) induced dose-dependent falls in diastolic BP (Figure 2). Pretreatment with L-NMMA (188 μmol kg^{-1}, i.v.) significantly inhibited the vasodepressor responses of all but the lowest

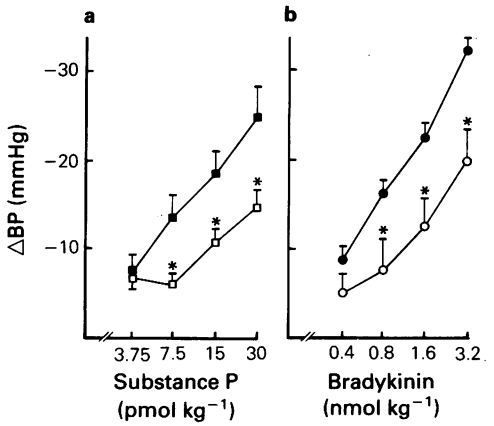

Figure 2 Inhibition of the vasodepressor responses to substance P (■, 3.75–30 pmol kg^{-1} i.v.; a) or bradykinin (○, 0.4–3.2 nmol kg^{-1} i.v.; b) by L-NMMA (□, ○, 188 μmol kg^{-1}, i.v.) in the anaesthetized rat. Results, shown as the fall in diastolic BP (ΔBP), are the mean of 4 experiments in each group, where significant inhibition is shown as $*P < 0.05$; s.e.mean shown by vertical bars.

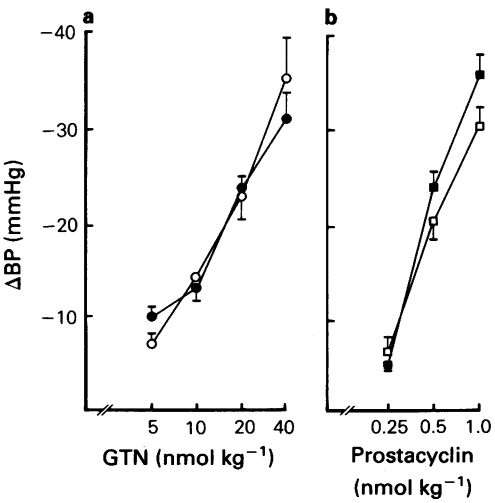

Figure 3 Lack of significant inhibition of the vasodepressor responses to glyceryl trinitrate (○, GTN, 5–40 nmol kg^{-1}, i.v.; a) or prostacyclin (■, 0.25–1.0 nmol kg^{-1}, i.v.; b) by L-NMMA (○, □, 188 μmol kg^{-1}; i.v.) in the anaesthetized rat. Results shown as the fall in diastolic BP (ΔBP), are the mean of 4 and 8 experiments respectively; s.e.mean shown by vertical bars.

dose of either agent (Figure 2). The inhibition by L-NMMA (188 μmol kg^{-1}) of these vasodepressor responses was reversed by the administration of L-arginine (600 μmol kg^{-1}) as shown in Table 2.

Effect on the vasodepressor actions of glyceryl trinitrate and prostacyclin

Glyceryl trinitrate (5–40 nmol kg^{-1}, i.v.) or prostacyclin (0.25–1 nmol kg^{-1}, i.v.) induced dose-dependent falls in diastolic BP (Figure 3). The vasodepressor actions of either glyceryl trinitrate or prostacyclin were not significantly inhibited by L-NMMA (188 μmol kg^{-1}, i.v.), as shown in Figure 3.

Discussion

In the present study in the anaesthetized rat, intravenous administration of the L-arginine analogue, L-NMMA, which specifically inhibits the formation of nitric oxide (NO) from L-arginine (Palmer *et al.*, 1988b) induced a dose-related increase in BP, with an accompanying bradycardia. The prolonged hypertensive effect of L-NMMA was rapidly reversed by the administration of a 3 fold excess of L-arginine but not D-arginine, thus confirming the recent observations with L-NMMA in the rabbit (Rees *et al.*, 1989b). The enantiomer-specific nature of the action of L-NMMA was also confirmed, since D-NMMA, which does not inhibit the synthesis of NO by endothelial cells (Palmer *et al.*, 1988b), did not affect resting BP in the rat. In a previous study in the rabbit with gossypol, a putative inhibitor of endothelium-dependent relaxation, no such effect on resting BP was observed (Dudel & Fosterman, 1988). However, that agent has a broad pharmacological profile, including inhibition of membrane enzymes involved with phospholipid metabolism and calcium transport (Reyes *et al.*, 1984; Kimura *et al.*, 1985), and its lack of specificity and possible vascular tissue disruption may therefore obscure any direct actions on the cardiovascular system.

L-NMMA inhibited the vasodepressor actions of the endothelium-dependent vasodilators, acetylcholine, bradykinin and substance P to a comparable degree, suggesting an action on a common mechanism. D-NMMA did not, however, inhibit the vasodepressor responses to these vasodilators, while L-arginine but not D-arginine reversed the inhibition by L-NMMA, as observed in the rabbit with acetylcholine-induced vasodepression (Rees *et al.*, 1989b). This rapid reversal, as well as the gradual disappearance of the inhibitory effects of L-NMMA in the absence of exogenous L-arginine, indicates that the reduction in endothelium-dependent vasodepressor responses is not just a consequence of endo-

thelial cell damage by this compound. These present findings in the rat thus support the involvement of an L-arginine-dependent mechanism in the hypotensive actions of these vasodilators.

The vasodepressor responses to the endothelium-dependent vasodilators were substantially inhibited, but not abolished, by L-NMMA in the present study. However, the mechanism by which L-NMMA interferes with either the uptake or utilisation of L-arginine by endothelial cells is not yet known, although competition with this substrate for NO synthesis is apparent. Thus, sufficiently high concentrations of L-NMMA in the microenvironment of the vascular endothelial cell may not be achieved at these doses to compete effectively with the endogenous levels of L-arginine, and hence abolish NO formation, following stimulation with exogenous agents such as acetylcholine. Knowledge of the uptake of L-NMMA into vascular tissue, its distribution and its pharmacokinetic profile, as well as its effects on endogenous L-arginine levels may, therefore, be useful in interpreting its *in vivo* profile of actions.

The failure of L-NMMA to inhibit substantially the small vasodepressor effects of the threshold doses of substance P and bradykinin is not yet understood. This may reflect a non-NO-dependent component of these agents at low doses, such as through the release of an endothelium-derived hyperpolarizing factor (Feletou & Vanhoutte, 1988; Chen *et al.*, 1988). However, since an elevation of basal BP may augment the apparent vasodepressor activity of these agents, as shown previously in the rabbit (Rees *et al.*, 1989b), such effects of L-NMMA on basal BP may lead to an underestimation of its degree of inhibition of the vasodepressor responses. Both such possibilities may also contribute to the failure of L-NMMA to abolish the responses to endothelium-dependent vasodilators. It will be of interest to compare the effects of L-NMMA on resting tone and on endothelium-dependent vasodilatation in specific vascular beds *in vivo*, to determine whether there is a differential sensitivity to this inhibitor in diverse tissues.

The selectivity of L-NMMA in the rat was confirmed by its failure to inhibit the vasodepressor responses to glyceryl trinitrate or prostacyclin, both endothelium-independent vasodilators. In studies in the rabbit, gossypol likewise did not inhibit the actions of glyceryl trinitrate or prostaglandin E, (PGE_1), but did attenuate the vasodepressor actions of acetylcholine, substance P and adenosine triphosphate (Dudel & Fostermann, 1988). Whether this reflects inhibition of NO formation or is due to other actions on the endothelial cells of gossypol is not known. In other *in vivo* studies, local infusion of methylene blue attenuated the vasodilatation induced by either acetylcholine or glyceryl trinitrate in the dog femoral artery (Sobey *et al.*, 1988). Although not fully selective, methylene blue can inhibit vascular guanylate cyclase (Martin *et al.*, 1985; 1989), and thus these latter findings could support the involvement of cyclic GMP in the mechanisms underlying endothelium-dependent and nitrate-induced vasodilatation (Rapoport & Murad 1983) and hence the actions of endogenous NO *in vivo*.

Porcine-human endothelin (ET-1) induces endothelium-independent contraction of isolated strips of vascular smooth muscle (Yanagisawa *et al.*, 1988a,b). However, in the isolated perfused mesenteric bed of the rat, low doses of ET-1 can induce vasodilatation which is inhibited by oxyhaemoglobin or removal of the endothelium, indicating the release of EDRF (De Nucci *et al.*, 1988). Studies in the rat *in vivo* with ET-1 have also demonstrated a complex cardiovascular profile, with an initial vasodepressor action preceding any vasopressor effects, these responses being dependent on the resting BP (Yanagisawa *et al.*, 1988a,b; Wright & Fozard, 1988; De Nucci *et al.*, 1988; Whittle *et al.*, 1989). In the current study, the fall in BP induced by ET-1 in the anaesthetized normotensive rat was substantially inhibited by pretreatment with L-NMMA but not D-NMMA, and this inhibition was abolished by concomitant administration of L-arginine. The inhibition of the vasodepressor actions of ET-1 by L-NMMA did not, however, lead to a consistent appearance of a vasoconstrictor phase under these conditions, while the inhibition was not further augmented by the cyclo-oxygenase inhibitor, indomethacin. However, it is pertinent that the degree of inhibition by L-NMMA of the vasodepressor responses of ET-1 was significantly greater than that of the other endothelium-dependent vasodilators. Such actions may reflect an unmasking of the inherent vasoconstrictor properties ET-1 following the inhibition of endothelium-derived NO *in vivo*, or a greater contribution of NO formation to the vasodepressor responses of this peptide.

Since EDRF has been now characterized both biologically and chemically in a variety of systems as NO (Palmer *et al.*, 1987; Hutchinson *et al.*, 1987; Radomski *et al.*, 1987; Ignarro *et al.*, 1987, Khan & Furchgott, 1987; Moncada *et al.*, 1988; Kelm *et al.*, 1988), whose synthesis can be inhibited by L-NMMA (Palmer *et al.*, 1988b; Rees *et al.*, 1989a,b) the current findings with L-NMMA in the rat further implicate endothelium-derived NO in the regulation of cardiovascular tone and blood pressure, and as a mediator of the hypotensive actions of endogenous endothelium-dependent vasodilators *in vivo*. The modulation of local substrate levels and enzymic activity involved with the biosynthesis of NO in the vascular endothelium may, therefore, make an

important contribution to the physiological regulation and pathological conditions of the cardiovascular system.

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