Comparison of effects of chronic and acute administration of N^G-nitro-L-arginine methyl ester to the rat on inhibition of nitric oxide-mediated responses

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1 Vascular responses to acetylcholine and sodium nitroprusside *in vivo* and *in vitro*, in the isolated perfused kidney and in rings of rat thoracic aorta, were measured in rats treated chronically with N^G-nitro-L-arginine methyl ester (L-NAME; approx. 70 mg kg⁻¹) and compared to responses in age-matched control animals, and age-matched animals after the acute administration of L-NAME (3–100 µmol kg⁻¹). Parallel experiments examined alterations in responsiveness in rings of trachea and anococcygeus muscles taken from the same animals.

2 Chronic oral administration of L-NAME elevated the blood pressure in anaesthetized animals from 114 ± 5 mmHg to 153 ± 11 mmHg (*n* = 5). The hypotensive responses to both acetylcholine (1 nmol kg⁻¹) and sodium nitroprusside (10 nmol kg⁻¹) were enhanced by chronic L-NAME treatment (*n* = 5–7) whereas acute L-NAME administration enhanced only the response to sodium nitroprusside (*n* = 5).

3 After chronic treatment with L-NAME, the basal perfusion pressure in the isolated perfused kidney was elevated. However, vasodilator responses to either acetylcholine (1 nmol) or sodium nitroprusside (3 nmol) were unaltered (*n* = 5–7). The vasodilatation induced by acetylcholine was inhibited in a concentration-dependent manner by the administration of acute L-NAME (0.1–100 µM; *n* = 5), such that significant inhibition was seen at 10 µM L-NAME. The response to sodium nitroprusside was unaffected by L-NAME.

4 The relaxations of isolated rings of rat thoracic aorta induced by acetylcholine were inhibited in tissues prepared from rats treated chronically with L-NAME (*n* = 5–7). Acute administration of L-NAME (0.1–100 µM) concentration-dependently inhibited the relaxations induced by acetylcholine in this preparation, with significant inhibition occurring at 1 µM L-NAME (*n* = 5). Responses to sodium nitroprusside were unaffected by either chronic or acute exposure to L-NAME (*n* = 5–7).

5 Relaxations of precontracted anococcygeus muscles induced by electrical field stimulation, or contractions of rings of trachea induced by carbachol or endothelin-1, were unaffected by chronic oral administration of L-NAME (*n* = 4–6). Acute addition of L-NAME (0.1–100 µM) to the organ baths inhibited in a concentration-dependent manner the relaxations of anococcygeus muscles taken from control animals, with a significant effect being seen at a concentration of 10 µM (*n* = 4–6).

6 Our cardiovascular data are consistent with chronic oral administration of L-NAME inhibiting the production of nitric oxide (NO) within the vasculature, although the pattern of inhibition is not uniform between different tissues. Despite the inhibition of endothelial NO production, chronic L-NAME does not alter the vasodepressor activity of acetylcholine *in vivo* or in the isolated perfused kidney. This may be explained by an enhanced responsiveness of guanylyl cyclase pathways, the increased release of vasodilators other than nitric oxide or a decreased importance of nitric oxide in resistance vessels compared with conductance vessels. The resistance of peripheral neuronal NO responses to chronic treatment with L-NAME indicates that selective inhibition of different isoforms of NOS may be achieved *in vivo*.

Keywords: L-NAME; nitric oxide synthase; nitric oxide

Introduction

Nitric oxide (NO) is an intra- and extra-cellular signalling molecular, derived from L-arginine by the action of the enzyme NO synthase (NOS), that is formed and active throughout the body (Moncada *et al.*, 1991). In the vasculature, for instance, NO is released by the endothelium and passes to the underlying smooth muscle where it stimulates guanylyl cyclase and so promotes vasodilatation. Similarly, in non-vascular smooth muscle, NO may produce relaxation following its release from intrinsic nerve endings (Hobbs & Gibson, 1990; Moncada *et al.*, 1991; Barnes, 1993).

Arginine analogues such as N^G-monomethyl-L-arginine (L-NMMA) and N^G-nitro-L-arginine methyl ester (L-NAME)

are potent inhibitors of NOS (Moore *et al.*, 1990; Rees *et al.*, 1990). Acute administration of L-NAME, *in vivo*, elevates mean arterial blood pressure, constricts the renal, mesenteric and hind-quarters vascular beds and depresses cardiac output (Gardiner *et al.*, 1990a; Baylis *et al.*, 1990). It also reduces the glomerular filtration rate, but in these short time course experiments the accompanying hypertension is not driven by changes in the renin-angiotensin system (Lehera *et al.*, 1991; Baylis *et al.*, 1993). As may be expected, the effects of acute L-NAME treatment are reversed by administration of L-arginine, to restore the production of NO, or by agents such as glyceryl trinitrate or cyclic GMP analogues, which mimic the effects of NO (Gardiner *et al.*, 1990a; Lehera *et al.*, 1992).

In contrast to the effects of acute administration, chronic

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administration of L-NAME to rats produces a hypertension accompanied by profound changes in renal structure (Ribeiro *et al.*, 1992). This hypertension is associated with thickening of the resistance vessel media, depression of aortic cyclic GMP levels, alteration of plasma renin levels and sclerotic renal changes (Arnall *et al.*, 1992; Deng *et al.*, 1993; Pollock *et al.*, 1993; Baylis *et al.*, 1993). In this situation the hypertension produced is, at least partly, dependent upon the renin-angiotensin system (Ribeiro *et al.*, 1992; Morton *et al.*, 1993). It is therefore clear that acute and chronic administration of L-NAME produce different changes within the vasculature. To investigate these further we have compared the effects of long term (4–6 weeks) and acute administration of L-NAME on the cardiovascular responses of the rat, and on the responses of isolated vascular preparations. Furthermore, we have also investigated the activities of other tissues in which endogenously produced NO has been reported to have important regulatory functions.

Methods

Animal treatment

To examine the effects of chronic administration of L-NAME, male Wistar rats (220–250 g) were given L-NAME (approximately 70 mg kg⁻¹ day⁻¹) in the drinking water, and compared with a group of rats with normal water, for a period of 4–6 weeks. Water intake, food intake and body weight were recorded and compared with the group of age matched controls. After use in the experiments the left kidney was removed from animals after chronic administration of L-NAME for gross examination. Untreated male Wistar rats (280–320 g) were used to compare the effects of acute L-NAME administration.

Blood pressure measurement

Rats were anaesthetized with sodium thiopentone (Intraval, 4 ml kg⁻¹ i.p. of a 3% solution). The carotid artery was cannulated and mean arterial blood pressure recorded by a pressure transducer (Elcomatic 750) linked to a chart recorder (Grampotec Linearcorder WR3101). The jugular vein was also cannulated for drug administration. Blood pressure was then allowed to stabilize for 30 min before changes in mean arterial blood pressure caused by intravenous drug administration were recorded. The changes in blood pressure were allowed to return to basal levels before the next drug was given. Responses to the following agents, in the following order, were recorded; phenylephrine (10 nmol kg⁻¹), angiotensin II (50 pmol kg⁻¹), sodium nitroprusside (10 nmol kg⁻¹), acetylcholine (1 nmol kg⁻¹) and endothelin-1 (0.5 nmol kg⁻¹). Endothelin-1 was administered last because of its very long duration of action. To examine the effects of acute L-NAME, after an initial 30 min of stabilization L-NAME (3–100 µmol kg⁻¹) or vehicle was given intravenously and the blood pressure left to stabilize. Responses to acetylcholine (1 nmol kg⁻¹) or sodium nitroprusside (10 nmol kg⁻¹) were then recorded. When the blood pressure returned to a stable level the next dose of L-NAME or vehicle was administered and the cardiovascular responses to acetylcholine and sodium nitroprusside retested. Blood pressure responses were recorded, when the response to the drug reached the point of maximum change. The duration of vasodilatation was also recorded. Results are expressed as changes in mean arterial blood pressure (diastolic pressure plus one third of pulse pressure) ± standard error of the mean (s.e.mean).

Urinary bladder function

The abdomens of anaesthetized rats, prepared as above, were opened and the ureters isolated and cut. Cannulae were

passed down the ureters into the bladder for the infusion of saline (left ureter) and the measurement of intrabladder pressure (right ureter). Following a 30 min equilibration period the bladder was emptied, via the saline infusion line, and saline infused (0.25 ml min⁻¹) until the intrabladder pressure reached a plateau and micturition began. The bladder was emptied and the procedure repeated twice more.

Isolated perfused kidney preparation

After completion of the *in vivo* cardiovascular study the rats were given heparin 200 i.u. intravenously. The superior mesenteric artery was cannulated retrogradely and the cannula passed into the right renal artery. The kidney was perfused (5 ml min⁻¹) with warmed (37°C) and gassed (95% O₂:5% CO₂) Krebs buffer (composition, mM: NaCl 118, KCl 4.7, KH₂PO₄ 1.2, MgSO₄·7H₂O, 1.17, CaCl₂·6H₂O 2.5, NaHCO₃ 25, glucose 5.6, dexamethasone 0.001; pH 7.4). The rat was killed by exsanguination and the kidney excised while being continuously perfused with Krebs buffer. The perfusion rate was then increased to 10 ml min⁻¹ and changes in perfusion pressure measured by a pressure transducer (Elcomatic 750) linked to a chart recorder (Grampotec Linearcorder WR3101). The kidney was allowed to equilibrate for 30 min before drug administration. Vasoconstrictor responses to phenylephrine (1–3 nmol) or angiotensin II (1–5 pmol) were then recorded and the maximum responses measured. Vascular responses to acetylcholine (1 nmol) or sodium nitroprusside (3 nmol) were measured at the point of maximum change after precontracting the renal vasculature with an infusion of phenylephrine (1–20 µM). The duration of vasodilatation was also recorded. The response induced by endothelin-1 (1–2.5 pmol) was measured after the responses to the other agents had been recorded. In experiments examining the acute effects of L-NAME, only the vasodilatation to acetylcholine (1 nmol) or sodium nitroprusside (3 nmol) were measured. After the equilibration period L-NAME (0.1 µM) or vehicle was infused with phenylephrine (10 µM) and the vasodilator responses to acetylcholine or sodium nitroprusside measured. The perfusion pressure was allowed to return to pre-vasodilator levels and further concentrations of L-NAME (0.3–100 µM) were infused to produce a cumulative increase in L-NAME concentration. After 20 min at the new concentration of L-NAME the effects of acetylcholine and sodium nitroprusside were retested.

Isolated tissue preparations

Immediately after removal of the kidney, the thoracic aorta and the trachea were dissected free and placed into Krebs buffer gassed with 95% O₂:5% CO₂. The aorta and trachea were then dissected free of connective tissue, and rings 2–3 mm wide, were prepared. The intimal surfaces of some rings were rubbed gently to remove the endothelium or epithelium, respectively. The rings were suspended in organ baths under a tension of 1 g for the aortic rings, and 2 g for the tracheal rings, and allowed to equilibrate for 60 min, with washing every 15 min. Changes in tension were recorded with an isometric transducer (Hugo Sachs Elektronik, Germany) connected to a chart recorder (Grampotec Linearcorder WR 3101). In the rat aorta preparations, cumulative concentration-response curves to phenylephrine were then constructed in rings with and without endothelium. After washing to reverse the response to phenylephrine, the rings of thoracic aorta were precontracted with phenylephrine (10–300 nM) to produce 70% of the maximum tone. Relaxations to cumulative concentrations of acetylcholine (0.01–3 µM) or sodium nitroprusside (0.1 nM–0.1 µM) were then recorded. To study its acute effect L-NAME (0.1–100 µM) was added to the organ bath after 30 min of the stabilization period and incubated for a further 30 min, before precontracting the aorta rings with phenylephrine (0.1 µM). Responses were recorded once the constriction had

reached a plateau. In the tracheal preparations cumulative concentration-response curves were constructed to carbachol (0.1 nM – $10 \text{ }\mu\text{M}$) after which the preparations were washed and left to re-equilibrate for 30 min. Cumulative concentration curves were then constructed to endothelin-1 (10 pM – $0.1 \text{ }\mu\text{M}$).

The rat anococcygeus muscle (Gillespie, 1972) was also removed and placed in an organ bath in Krebs buffer gassed with 95% O_2 :5% CO_2 , under a resting tension of 0.5 g. Tissues were left to stabilise for 45 min with washing every 15 min. To inhibit the effects of sympathetic nerve stimulation, phentolamine ($1 \text{ }\mu\text{M}$) was present in the Krebs buffer and guanethidine ($30 \text{ }\mu\text{M}$) applied for 10 min during the incubation period. Tone was raised with carbachol ($50 \text{ }\mu\text{M}$) and field stimulation (0.3 mA , 10 Hz , 0.1 ms , 10 s every 100 s) was applied (Grass S48 stimulator; Med Lab Stimu Splitter II) to produce stable relaxations. After a washout period of 15 min, the tissues were again pre-contracted with carbachol and stimulated. When stable relaxations were achieved L-arginine (1 mM) was added to the preparation and its effect noted. In experiments investigating its acute effects, L-NAME ($0.1 \text{ }\mu\text{M}$) was added to the bath once a stable response to electrical stimulation was obtained and the tissue stimulated until the response reached a steady state, when the concentration of L-NAME was further increased (0.3 – $100 \text{ }\mu\text{M}$). After administration of $100 \text{ }\mu\text{M}$ L-NAME to the anococcygeus preparation the effects of addition of L-arginine (1 mM) were recorded.

NOS activity assay

The cerebella from both control and chronic L-NAME-treated rats were removed and frozen in liquid nitrogen. Cerebella were thawed and homogenized (ratio of 1 g of tissue to 5 ml of buffer: Tris/HCl 50 mM ; EDTA 0.1 mM ; EGTA 0.1 mM ; 2-mercaptoethanol 12 mM ; leupeptin $2 \text{ }\mu\text{M}$; pepstatin $1 \text{ }\mu\text{M}$ and phenylmethylsulphonyl fluoride 1 mM ; pH 7.4) on ice. NOS activity was measured as the ability of tissue homogenates to convert [^3H]-L-arginine to [^3H]-L-citrulline. Samples were incubated at room temperature for 30 min in the presence of NADPH (1 mM), calmodulin (2 mM) and a mixture of unlabelled and [^3H]-L-arginine ($10 \text{ }\mu\text{M}$). L-Valine (50 mM), which inhibits the conversion of L-arginine to L-citrulline by arginase and the transcarbamoylase cycle, was included in the reaction mixture to increase specificity of the assay. L-Citrulline formation was determined in the absence of NADPH as a measure of nonspecific conversion of L-arginine. Incubations were terminated by the addition of 1 ml of HEPES (20 mM , pH 5.5) containing 1 mM EGTA and EDTA. The newly formed L-citrulline was separated from L-arginine by passing the reaction mixture over Dowex columns (1 ml), and the eluted labelled material was measured with a Beckman scintillation counter. Activity is expressed as pmol citrulline mg^{-1} protein 30 min^{-1} . Protein content of the homogenates was measured by the Bradford (1976) assay. Activities were compared with the Mann-Whitney U test, significance being taken at $P < 0.05$.

Materials

L-NAME hydrochloride, phenylephrine hydrochloride, angiotensin II, sodium nitroprusside, acetylcholine chloride, phentolamine, guanethidine, carbachol, Tris/HCl, EDTA, EGTA, 2-mercaptoethanol, leupeptin, pepstatin, phenylmethylsulphonyl fluoride, NADPH, calmodulin, HEPES, Dowex, L-valine and L-arginine were purchased from Sigma Chemical Co. (Poole, Dorset). [^3H]-L-arginine was purchased from Amersham International plc. Endothelin-1 was purchased from Peptide Institute (Osaka, Japan) and dissolved in 0.9% w/v saline containing 0.1% w/v bovine serum albumin (Sigma Chemical Co.) All other drugs were dissolved in saline. The salts for the Krebs solution were

obtained from BDH (Lutterworth, U.K.). Intraval was bought from May & Baker (U.K.).

Statistical analysis

Statistical differences between data points were assessed by a paired or unpaired one- or two-tail Student's *t* test, as appropriate. Significance was taken at $P < 0.05$.

Results

General effects of chronic and acute L-NAME

Chronic administration of L-NAME resulted in a decreased weight gain over 4 weeks (control, $120 \pm 25 \text{ g}$; L-NAME, $80 \pm 13 \text{ g}$, $n = 12$) and an enhanced water intake (from 100 ± 10 to $150 \pm 25 \text{ ml}$ per box of 3 rats/day, $n = 12$). Animals became susceptible to gastroenteritis and peritonitis after 4–5 weeks of a 6 week L-NAME administration period, so animals were subsequently used after 4 weeks treatment. Chronic administration of L-NAME also caused gross changes in the renal architecture characterised by the loss of renal cortical tissue ($n = 7$). Basal heart rate was unchanged

Figure 1 Changes in mean arterial blood pressure (mmHg) following i.v. bolus injections of sodium nitroprusside (SNP, 10 nmol kg^{-1}) or acetylcholine (ACh, 1 nmol kg^{-1}). N^G -nitro-L-arginine methyl ester (L-NAME) was administered orally (approx. $70 \text{ mg kg}^{-1} \text{ day}^{-1}$ for 4–5 weeks) and the responses to SNP or ACh compared to those in control age-matched rats ($n = 5$ – 7 , a). L-NAME ($10 \text{ }\mu\text{mol kg}^{-1}$) or saline was administered acutely and the hypotensive responses to SNP or ACh (SNP: control, open column; L-NAME, stippled column; ACh: control, solid column; L-NAME, hatched column; $n = 5$) recorded (b). Values are mean $\pm$ s.e.mean; * $P < 0.05$ versus baseline (Student's unpaired *t* test). Basal blood pressure values are given in the results.

by chronic L-NAME treatment (control, 338 ± 25 ; L-NAME, 369 ± 41).

The mean arterial blood pressure of anaesthetized control rats was 114 ± 5 mmHg ($n = 5$). This was elevated in anaesthetized rats receiving L-NAME chronically to 153 ± 11 mmHg ($n = 5$, $P < 0.05$). Acute administration of L-NAME ($3\text{--}100 \mu\text{mol kg}^{-1}$) to anaesthetized control animals caused an initial increase in basal blood pressure after injection of $3 \mu\text{mol kg}^{-1}$ (21.3 ± 3.4 mmHg, $P < 0.05$, $n = 5$), but no increase with subsequent doses (data not shown, $n = 5$).

Effects of chronic and acute L-NAME on haemodynamic responses

Falls in blood pressure induced by acetylcholine (1 nmol kg^{-1}) were not inhibited by chronic treatment with L-NAME (pressure changes: control, 31.1 ± 2.3 mmHg; chronic L-NAME, 48.7 ± 4.4 mmHg, $P < 0.05$; duration changes: control, 5.4 ± 0.6 s, chronic L-NAME, 11.4 ± 3 s, $P < 0.05$; $n = 5\text{--}7$) whereas those induced by sodium nitroprusside (10 nmol kg^{-1}) were enhanced (control, 40 ± 4.9 mmHg; chronic L-NAME, 83.4 ± 10 mmHg, $P < 0.05$; Figure 1a). Intravenous injection of endothelin-1 produced a biphasic response in control animals with an initial depressor response (-41 ± 3.3 mmHg) followed by a sustained pressor response (34 ± 4 mmHg). Chronic administration of L-NAME caused an enhancement in the depressor response (72 ± 7 mmHg) but no change in the secondary pressor response (38 ± 4 mmHg). Similarly, the pressor effects of phenylephrine (10 nmol kg^{-1}) and angiotensin II (5 pmol kg^{-1}) in control animals (phenylephrine, 38 ± 3.5 mmHg; angiotensin II, 30 ± 3.5 mmHg) were unchanged by chronic L-NAME treatment (phenylephrine, 39 ± 5.7 mmHg; angiotensin II, 37 ± 2.4 mmHg) ($n = 5\text{--}7$).

Acute intravenous injection of L-NAME ($1\text{--}100 \mu\text{mol kg}^{-1}$; $n = 5$) did not affect the falls in blood pressure or the duration of the vasodilatation induced by acetylcholine (1 nmol kg^{-1} , Figure 1b), but L-NAME at doses of $10 \mu\text{mol kg}^{-1}$ or greater enhanced those in response to sodium nitroprusside (10 nmol kg^{-1}) (Figure 1b).

Effects of chronic L-NAME on bladder function

Infusion of saline into the bladders of control animals resulted in micturition after 3.49 ± 1.17 min, at which time 0.87 ± 0.29 ml of saline had been infused and the intrabladder pressure was 22.05 ± 0.59 mmHg ($n = 3$). In rats treated chronically with L-NAME, micturition began after 6.49 ± 1.23 min at which time 1.62 ± 0.31 ml of saline had been infused and the intrabladder pressure was 20.76 ± 1.58 mmHg ($n = 3$).

Effects of chronic and acute L-NAME on responses of the thoracic aorta

Contractions of isolated rings of rat aorta induced by phenylephrine were unaffected by chronic L-NAME treatment ($n = 5\text{--}7$, data not shown). Acetylcholine caused concentration-dependent relaxations of endothelium-intact preparations taken from control animals ($n = 7$) but was without effect on tissues taken from rats chronically treated with L-NAME ($n = 5$, Figure 2a). Sodium nitroprusside caused similar relaxations of rings taken from either group of animals (data not shown, $n = 5\text{--}7$).

Acute addition of L-NAME ($0.1\text{--}100 \mu\text{M}$) to the organ baths inhibited in a concentration-dependent manner relaxations of control rat aortic rings induced by acetylcholine, with a significant effect being seen at a concentration of $1 \mu\text{M}$ ($n = 5$, Figure 2b). Responses to sodium nitroprusside ($0.1 \text{ nM}\text{--}1 \mu\text{M}$) were unaffected by acute L-NAME administration ($n = 4\text{--}5$, data not shown).

Figure 2 Percentage relaxation of the rat thoracic aorta after pre-contraction with phenylephrine ($0.1 \mu\text{M}$) to increasing concentrations of acetylcholine (ACh). N^G -nitro-L-arginine methyl ester (L-NAME) was administered orally (approx. $70 \text{ mg kg}^{-1} \text{ day}^{-1}$ for 4–5 weeks) and the relaxations induced by ACh compared to responses in the aorta from control age-matched rats (ACh control with endothelium, $\square$; ACh + L-NAME with endothelium, $\blacksquare$; $n = 5\text{--}7$, a). Increasing concentrations of L-NAME ($0.1\text{--}100 \mu\text{M}$) were administered acutely in tissues from animals not receiving chronic L-NAME and the vascular relaxations induced by increasing concentrations of ACh recorded (ACh control with endothelium, $\blacksquare$; ACh + L-NAME ($0.1 \mu\text{M}$), $\square$; ACh + L-NAME ($1 \mu\text{M}$), $\circ$; ACh + L-NAME ($10 \mu\text{M}$), $\triangle$; ACh + L-NAME ($100 \mu\text{M}$), $\diamond$; $n = 5$, b). Values are mean $\pm$ s.e. mean.

Effects of chronic and acute L-NAME on responses of the isolated perfused kidney

The basal perfusion pressure of the isolated kidney was 86 ± 6.7 mmHg which was increased to 112 ± 9.1 mmHg in organs taken from rats chronically treated with L-NAME ($P < 0.05$) ($n = 5\text{--}7$).

The falls in perfusion pressure induced by acetylcholine (1 nmol) or sodium nitroprusside (3 nmol) in kidneys from L-NAME-treated rats (acetylcholine, 79.6 ± 9.4 mmHg; sodium nitroprusside, 65.8 ± 16 mmHg) were not different from those recorded in control kidneys (acetylcholine, 74.4 ± 10.0 mmHg; sodium nitroprusside, 46.7 ± 10.3 mmHg) (Figure 3a). The duration of the vasodilatation induced by acetylcholine was also unchanged by chronic L-NAME (control, 48 ± 0 ; L-NAME, 50.4 ± 1.2 s, $P < 0.05$). Conversely, vasoconstriction induced by either phenylephrine ($1\text{--}3 \text{ nmol}$) or angiotensin II ($1\text{--}5 \text{ pmol}$) were significantly enhanced by chronic L-NAME treatment (Table 1) although the response

Figure 3 Changes in the perfusion pressure (mmHg) of the isolated perfused kidney following i.v. bolus injections of sodium nitroprusside (SNP, 3 nmol) or acetylcholine (ACh, 1 nmol). N^G -nitro-L-arginine methyl ester (L-NAME) was administered orally (approx. $70 \text{ mg kg}^{-1} \text{ day}^{-1}$ for 4–5 weeks) and the responses to SNP or ACh compared to those in control age-matched rats ($n = 5$ –7, a). L-NAME ($70 \mu\text{M}$) or saline was administered acutely and the vasodilator responses to SNP or ACh recorded (SNP control, open column; SNP + L-NAME, stippled column; ACh control, solid column; ACh + L-NAME, hatched column; $n = 5$, b). Values are mean $\pm$ s.e.mean; * $P < 0.05$ versus baseline (Student's unpaired t test). Basal perfusion pressure values are given in the results.

Table 1 Changes in the perfusion pressure (mmHg) of the isolated perfused kidney caused by bolus injections of phenylephrine (1–3 nmol) or angiotensin II (1–5 pmol)

Drug	Dose	Control	L-NAME
Phenylephrine	1 nmol	10.63 ± 6.67	$69.76 \pm 16.85^{**}$
	3 nmol	51.98 ± 6.67	$111.18 \pm 17.67^*$
Angiotensin II	1 pmol	4.18 ± 2.01	$52.21 \pm 19.24^*$
	3 pmol	25.50 ± 9.51	$99.88 \pm 22.39^{**}$
	5 pmol	32.67 ± 10.84	$99.04 \pm 22.27^*$

The table shows the effect of chronic (4–6 weeks) oral administration of N^G -nitro-L-arginine methyl ester (L-NAME; approx. $70 \text{ mg kg}^{-1} \text{ day}^{-1}$) on the responses to phenylephrine or angiotensin II ($n = 5$ –7). Values are means $\pm$ s.e.mean (* $P < 0.05$; ** $P < 0.01$ versus control; Student's unpaired t test).

to endothelin-1 (2.5 pmol) was unchanged (control, $58 \pm 11.5 \text{ mmHg}$; chronic L-NAME, $51 \pm 12.3 \text{ mmHg}$).

Acute administration of L-NAME (0.1 – $100 \mu\text{M}$) inhibited in a concentration-dependent manner the falls in perfusion pressure, but did not affect the duration of vasodilatation,

induced by acetylcholine (1 nmol) in the isolated perfused kidney. L-NAME did not affect the basal perfusion pressure (control, 12 ± 3 ; $10 \mu\text{M}$ L-NAME, 11 ± 3 ; $100 \mu\text{M}$ L-NAME, $6 \pm 2\%$ fall in perfusion pressure; duration: control, 48 ± 0 , L-NAME, $48 \pm 0 \text{ s}$, $n = 5$, Figure 3b). Conversely, vasodilations induced by sodium nitroprusside (3 nmol) were unaffected by increasing concentrations of L-NAME compared to the vehicle time controls (Figure 3b).

Effects of chronic and acute L-NAME on responses of the anococcygeus and trachea

Anococcygeus muscles from control and chronically L-NAME-treated rats relaxed in a similar way in response to electrical field stimulation ($n = 5$, Figure 4a). However, acute addition of L-NAME (0.1 – $100 \mu\text{M}$) to the organ baths inhibited in a concentration-dependent manner the relaxations, but not the duration of relaxation, of tissues taken from control animals, with a significant effect being seen at a concentration of $10 \mu\text{M}$ (Figure 4b) ($n = 4$ –6).

Contractile responses to carbachol were largely unaffected by chronic treatment with L-NAME in rings of epithelium-intact or denuded trachea. For instance, in control preparations $0.3 \mu\text{M}$ carbachol caused a contraction of $1.0 \pm 0.17 \text{ g}$ ($n = 7$) in epithelium-intact rings and $0.92 \pm 0.08 \text{ g}$ ($n = 7$) in epithelium-denuded rings. In matched tissues taken from animals treated chronically with L-NAME the same concen-

Figure 4 Relaxations of the rat anococcygeus muscle pre-contracted with carbachol ($50 \mu\text{M}$) induced by electrical field stimulation (0.3 mA , 10 Hz , 0.1 ms , 10 s every 100 s). (a) Shows the effect of chronic oral administration of N^G -nitro-L-arginine methyl ester (L-NAME; approx. $70 \text{ mg kg}^{-1} \text{ day}^{-1}$) for 4–5 weeks on the electrically stimulated relaxations (control, open column; L-NAME, solid column; $n = 5$). (b) Shows the acute effects of increasing concentrations of L-NAME (0.1 – $100 \mu\text{M}$) on the relaxations induced by electrical field stimulation ($\square$; $n = 5$). Values are mean $\pm$ s.e.mean.

tration of carbachol induced contractions of 0.84 ± 0.18 (g) ($n = 4$) and 0.51 ± 0.12 (g) ($n = 5$), respectively. Similarly, endothelin-1 (30 nM) caused contractions of 0.82 ± 0.11 (g) ($n = 7$) and 0.9 ± 0.14 (g) ($n = 7$) in control preparations, respectively, with and without epithelium, and of 0.68 ± 0.17 (g) ($n = 4$) and 0.53 ± 0.1 (g) ($n = 5$) in the corresponding tissues prepared from rats chronically treated with L-NAME.

Effect of chronic L-NAME on nitric oxide synthase activity in the rat cerebellum

Cerebellar nitric oxide synthase activity was significantly reduced after treatment of rats with chronic L-NAME (control, 218.8 ± 18.8 ; L-NAME, 91.2 ± 18.0 pmol citrulline mg^{-1} protein 30 min^{-1}).

Discussion

Chronic administration of L-NAME to rats caused a marked increase in blood pressure and a complete loss of endothelial-dependent relaxations in isolated aortic rings. These data suggest that the increase in blood pressure observed was driven by a loss in NO-vasodilator tone. However, at the same time there was an enhancement in the hypotensive response to acetylcholine *in vivo*, and no reduction in the size of vasodilatations induced by acetylcholine in the isolated perfused kidney or in relaxations mediated by NO in the anococcygeus. These data suggest that chronically administered L-NAME does not produce a uniform inhibition of the activity of NO synthase. To investigate these differences further we examined the effects of the acute administration of L-NAME on the same preparations.

In isolated aortic rings, i.e. the simplest model of vascular activity used, endothelium-dependent relaxations induced by acetylcholine were significantly inhibited by L-NAME at a concentration of $1 \mu\text{M}$. This result clearly suggests that the aortic rings taken from chronically treated animals had been exposed to a concentration of L-NAME of $1 \mu\text{M}$ or higher, as they did not relax in response to acetylcholine. In contrast to the aortic rings, relaxations of the anococcygeus induced by electrical field stimulation were inhibited by L-NAME only at concentrations greater than $10 \mu\text{M}$, and tissues taken from chronically L-NAME-treated rats did not respond differently from controls. Similarly, tracheal rings, in which NO has been reported to be an inhibitory transmitter (Barnes, 1993), did not show an enhanced responsiveness to the spasmogens, carbachol and endothelin. Thus, it would appear that in animals chronically treated with L-NAME the anococcygeus had not been exposed to a high enough concentration of L-NAME, equivalent to less than $10 \mu\text{M}$. The lack of inhibition in the anococcygeus from chronically treated animals cannot be explained by metabolism of the inhibitor as L-NAME and L-NOARG inhibit responses in this tissue similarly (Hobbs & Gibson, 1990). Together these *in vitro* organ bath data suggest that ingestion by rats of approximately $70 \text{ mg kg}^{-1} \text{ day}^{-1}$ L-NAME produces an effect on individual tissues similar to the acute application of $1\text{--}10 \mu\text{M}$ of L-NAME.

Chronic treatment with L-NAME did not affect the vasodilatations of the isolated perfused kidney induced by acetylcholine or sodium nitroprusside. This may have been expected as vasodilatations induced by acetylcholine were only inhibited acutely by L-NAME administered at a concentration higher than $10 \mu\text{M}$, i.e. more than the equivalent concentration achieved *in vivo* following chronic administration of L-NAME. It is interesting, however, that endothelium-dependent relaxation of the rat aorta were inhibited by $1 \mu\text{M}$ L-NAME whereas more than 10 times this concentration was required to inhibit vasodilatation in the isolated kidney. This suggests that within the kidney vasculature, vasodilatation induced by acetylcholine is mediated by

mechanisms in addition to NO, as there is no evidence that eNOS varies between endothelial cells in different portions of the vasculature. This conclusion would be consistent with other studies that have found L-NAME to be ineffective at inhibiting the acetylcholine renal vasodilatation *in vivo* as well as the acetylcholine hypotensive response (Gardiner *et al.*, 1990b), although long term exposure to L-NAME has been associated with an attenuation of the renal vasodilator effects of acetylcholine *in vivo* (Gardiner *et al.*, 1993). There are a number of possible explanations for this relative resistance to the effects of eNOS inhibition. Firstly, the responsiveness of guanylyl cyclase to NO may be enhanced to compensate for the reduced basal activation that would follow a fall in the endogenous production of NO (Moncada *et al.*, 1991). This suggestion is supported by our finding of an enhanced response *in vivo* to sodium nitroprusside after chronic treatment with L-NAME. On the other hand, we found no such enhancement in the responses of isolated perfused kidneys or thoracic aortae prepared from animals chronically exposed to L-NAME, as is the case for both changes in arterial blood pressure and regional blood flows *in vivo* (Arnal *et al.*, 1992; Gardiner *et al.*, 1993). A second explanation may be that acetylcholine stimulates, in addition to NO, the release of endothelium-derived vasodilators such as endothelial-derived hyperpolarizing factor (Komori *et al.*, 1988). These could explain the relative resistance of acetylcholine *in vivo* and *in vitro* to the acute or chronic administration of L-NAME. Thirdly, it may be that the differences are due to variations in the tissue distribution of L-NAME, i.e. that the endothelial cells within the kidney are exposed to a lower concentration of L-NAME than the endothelium in the aorta. This appears unlikely, although it is known that while L-NAME is readily taken up into cells, it is also rapidly metabolised in blood and plasma into its active, stable metabolite, L-NOARG (Krejcy *et al.*, 1993). L-NOARG, for its part, is poorly taken up by cells, and differential cellular uptake can therefore occur (Krejcy *et al.*, 1993). However, the differences seen in experiments examining the acute effects of L-NAME cannot be explained in this way. Interestingly, the isolated perfused kidney demonstrated greatly exaggerated vasoconstrictions in response to both phenylephrine and angiotensin II, although not to endothelin-1. This marked increase may also explain the effectiveness of inhibitors of the renin-angiotensin system in reducing the hypertension caused by chronic administration of L-NAME (Riberio *et al.*, 1992; Pollock *et al.*, 1993), for an increased sensitivity of the renal vasculature to angiotensin II would lead to renal vasoconstriction which could further enhance the production of renin.

Our examination of the kidney showed gross changes similar to those noted before (Ribeiro *et al.*, 1992; Baylis *et al.*, 1993) and these appeared associated with increases in bladder content. To examine this further we investigated the differences in bladder capacity and intrabladder pressure at the time of micturition. There was a clear tendency to an increase in bladder capacity although this was not associated with an increase in intrabladder pressure. Possibly, this is an adaptive change due to the role of NO in regulating the activity of the lower urinary tract of the rat (Persson *et al.*, 1992; Parlani *et al.*, 1993). This result suggests that the changes in renal structure could in part be due to alterations in bladder emptying.

We conclude that chronic oral treatment of animals with L-NAME affects NO-dependent responses in a non-uniform manner. The possible mechanisms for these differences include alterations in the sensitivity of the guanylyl cyclase pathways or increased activity of other complementary mechanisms, such as other vasodilators. It is also interesting to note that peripheral neuronal NO responses were resistant to inhibition by chronic treatment with L-NAME, while endothelial NO responses were markedly affected, indicating that selective inhibition of different isoforms of NOS may be achieved *in vivo*.

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