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Investigation of the inhibitory effect of N^G-nitro-L-arginine methyl ester on the antihypertensive effect of the angiotensin AT_1 receptor antagonist, GR138950

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1 The effect of systemic administration of the nitric oxide synthase inhibitor, N^G-nitro-L-arginine methyl ester (L-NAME) on the antihypertensive effects of the angiotensin AT_1 receptor antagonist, GR138950, the angiotensin-converting enzyme (ACE) inhibitor, enalapril, or hydralazine has been evaluated in unrestrained, conscious renal artery ligated hypertensive (RALH) rats. The effect of the phosphodiesterase type V inhibitor, zaprinast on the antihypertensive effect of GR138950 in RALH rats was also examined. The effect of GR138950 on blood pressure, and plasma and urine cyclic GMP levels was compared to that of zaprinast in conscious RALH rats.

2 GR138950, enalapril or hydralazine caused marked reductions in blood pressure associated with immediate tachycardia in conscious RALH rats. L-NAME pretreatment attenuated the antihypertensive effects of GR138950 or enalapril but not that of hydralazine in conscious RALH rats. The initial tachycardia caused by GR138950 or enalapril but not hydralazine was attenuated by L-NAME pretreatment. L-NAME alone caused a transient (20 min) pressor response and a prolonged (6 h) bradycardia in conscious RALH rats.

3 Pretreatment with indomethacin did not affect the cardiovascular effect of GR138950 in conscious RALH rats. Indomethacin alone did not significantly change basal blood pressure or heart rate in RALH rats.

4 Zaprinast pretreatment did not affect the antihypertensive effect of GR138950 in conscious RALH rats but potentiated the depressor response to sodium nitroprusside. Zaprinast alone caused a small reduction in basal blood pressure but did not change basal heart rate in RALH rats.

5 The antihypertensive effect of GR138950 was not associated with an increase in plasma or urine cyclic GMP levels in conscious RALH rats, whereas zaprinast caused a small fall in blood pressure associated with increases in plasma and urine cyclic GMP.

6 The ability of L-NAME to inhibit the antihypertensive action of GR138950 or enalapril suggests that these agents release nitric oxide (NO) and/or enhance the cardiovascular effects of NO as part of their mechanism of action. However, the inability of zaprinast to potentiate the antihypertensive effects of GR138950 and the finding that GR138950 did not increase urine and plasma cyclic GMP levels are not consistent with this view. Attenuation of the response to GR138950 or enalapril, but not hydralazine, suggests a selective interaction between L-NAME and inhibitors of the renin-angiotensin system, although the nature of this interaction is unknown.

Keywords: Conscious rats; renal hypertension; blood pressure; angiotensin AT_1 receptors; GR138950; enalapril; L-NAME; zaprinast; cyclic GMP; nitric oxide

Introduction

GR138950 is a potent and selective non-peptide antagonist at angiotensin AT_1 receptors *in vitro* and *in vivo* (Hilditch *et al*., 1995). GR138950 causes a marked antihypertensive response in conscious rats in which blood pressure has been elevated following activation of the renin-angiotensin system (RAS; Hilditch *et al*., 1995). The antihypertensive action of GR138950 and other such compounds has mainly been attributed to blocking the effects of angiotensin II (A II) at vascular AT_1 receptors. Interestingly, however, the time course of the antihypertensive effect of GR138950 in renal hypertensive rats does not coincide with that of its antagonist profile against A II-induced pressor responses in normotensive rats (Akers *et al*., 1991; Hilditch *et al*., 1995). This lack of a temporal relationship between the antihypertensive and angiotensin AT_1 receptor blocking actions of GR138950 has also been described for other angiotensin AT_1 receptor antagonists, including GR117289 (Drew, 1993; Hilditch *et al*., 1994) and losartan (Akers *et al*., 1991; Ohlstein *et al*., 1992; Drew, 1993).

A recent study from our laboratory has shown that the antihypertensive activity of GR138950 in renal hypertensive rats coincides better with its blockade of responses to exogenously administered A I than A II, suggesting that A II generated locally in the vasculature, as well as plasma borne A II, contributes to the cardiovascular profile of GR138950 (Hilditch *et al*., 1996). Despite these findings, it is still apparent that the maximum fall in blood pressure in renal hypertensive rats occurs approximately 5–7 h after administration of GR138950, whereas the maximal blockade of angiotensin AT_1 receptors (as described by the rightward shift of the dose-pressor response to A I or A II) occurs at 1 h (Hilditch *et al*., 1995; 1996). Similar observations were made for losartan (Ohlstein *et al*., 1992) and GR117289 (Hilditch *et al*., 1994) as well as enalapril (Hilditch *et al*., 1996).

The reason for this disparate cardiovascular profile of GR138950 and other angiotensin AT_1 receptor antagonists is unknown but several mechanisms are possible (see Hilditch *et al*., 1994; 1995). It is also possible that angiotensin AT_1 antagonists reduce blood pressure via additional mechanisms distinct from vascular angiotensin AT_1 receptor block (see Ohlstein *et al*., 1992).

Recently, inhibitors of nitric oxide (NO) synthase (N^G-nitro-L-arginine methyl ester (L-NAME) or N^G-monomethyl-L-arginine (L-NMMA), Rees *et al*., 1990) have been shown to attenuate the antihypertensive effects of angiotensin convert-

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ing enzyme (ACE) inhibitors as well as the angiotensin receptor antagonists, saralasin, losartan or its structurally related analogue, L-158,809 in conscious renal artery ligated hypertensive (RALH) rats (Hegde *et al*., 1993) and spontaneously hypertensive rats (Cachofeiro *et al*., 1992; 1995). A possible explanation for these observations is that ACE inhibitors and angiotensin AT_1 receptor antagonists release NO and/or enhance the cardiovascular effects of NO as part of their mechanism of action. In this respect, ACE inhibitors, in addition to the prevention of formation of A II, have been shown to reduce the rate of degradation of bradykinin and related kinins. The enhanced levels of bradykinin result in a NO-dependent vasodilatation which can contribute to the antihypertensive response of ACE inhibitors (Carretero *et al*., 1981; Carbonell *et al*., 1988; Bao *et al*., 1988; Wiemer *et al*., 1991; Cachofeiro *et al*., 1992). However, this mechanism does not explain the ability of L-NAME to inhibit the antihypertensive effect of losartan which, like GR138950, is devoid of ACE inhibitor activity (Chiu *et al*., 1989; Hilditch *et al*., 1996). The involvement of NO in the response to the angiotensin AT_1 receptor antagonists is implied by this inhibitory action of L-NAME or L-NMMA but relies on the assumption that these compounds selectively inhibit NO synthase. In conscious normotensive rats, either L-NAME or L-NMMA causes hypertension due to a wide-spread vasoconstriction. Bradycardia and a reduction in cardiac output are also observed (Gardiner *et al*., 1990a,b; Widdop *et al*., 1992). The main mechanism of the hypertensive effects of L-NAME is believed to result from the removal of the vasodilatator influence of NO. However, NO-independent mechanisms may be involved (see Nafrialdi *et al*., 1996). Since L-NAME has cardiovascular effects which may not be due simply to blockade of NO synthase, it would be prudent to gain supporting evidence for the release of NO or enhancement of the cardiovascular effects of NO by the angiotensin AT_1 receptor antagonists.

In the present study, the involvement of NO in the antihypertensive effect of the AT_1 receptor antagonist, GR138950, was investigated in conscious, unrestrained RALH rats. Initially, the effect of the NO synthase inhibitor, L-NAME on the antihypertensive response to GR138950, enalapril or the vasodilatator, hydralazine, was examined. It is well established that NO, released from the endothelium activates soluble guanylate cyclase causing an increase in guanosine 3′:5′-cyclic monophosphate (cyclic GMP) turnover leading to the cellular effect, in this case relaxation of vascular smooth muscle (see Murad *et al*., 1992). Therefore, to gain further evidence for the participation of NO in the response to GR138950, the antihypertensive effect of GR138950 has also been correlated with its ability to affect plasma and urine cyclic GMP levels in conscious RALH rats. Furthermore, the effect of zaprinast, (M&B 22,948; a relatively selective phosphodiesterase type V (cyclic GMP specific) enzyme inhibitor, Lugnier *et al*., 1986; see Murray, 1993), which has been shown to potentiate responses mediated by NO release or NO donors (Harris *et al*., 1989; Dundore *et al*., 1990; Merkel *et al*., 1992), on the antihypertensive effects of GR138950 was examined. A preliminary account of these observations has been presented to the British Pharmacological Society (Anderson *et al*., 1995).

Methods

Experiments were performed in male Allen Hanbury/Albino (AH/A) rats (250–350 g). Rats were individually housed and maintained under a 12:12 h light-dark cycle (lights on at 06h 00min) with free access to food and water. Renal hypertension was induced in all animals. Surgery was performed in 2 stages with 5 days between each stage. After each surgical stage wounds were closed and dusted with chlorotetracycline and the animals were also given an injection of procaine penicillin and dihydrostreptomycin (7 mg kg^{-1}, i.m.).

Renal artery ligated hypertensive rats

RALH rats were produced as described previously (Hilditch *et al*., 1995). Briefly, anaesthesia was induced and maintained in normotensive rats with isoflurane (5% and 2–3%, respectively) in oxygen (0.4 l min^{-1}) and nitrous oxide (0.8 l min^{-1}). A laparotomy was performed and the left renal artery was separated from surrounding connective tissue, adjacent to the abdominal aorta. The artery was ligated with a cotton ligature and the incision was closed with suture (Ethicon 3/0, 2 metric). The skin was closed (Autoclips, 9 mm) and the animal was allowed to recover. Four days later, systolic blood pressure was measured by use of an indirect tail cuff method (Ugo Basile BP Recorder 8006). Only those rats that developed a systolic blood pressure greater than 160 mmHg were subsequently progressed to experimentation.

Chronic implantation of vascular catheters

Five days after renal artery ligation (1 day before experimentation), RALH rats were anaesthetized with sodium methohexitone (40 mg kg^{-1}, 1.3 ml kg^{-1}, i.p.). Two intravenous catheters were implanted in the right jugular vein for drug administration and an intra-arterial catheter was placed in the distal abdominal aorta, via the caudal artery (for blood pressure and heart rate recordings). The vascular catheters were tunnelled subcutaneously and exteriorized at the back of the neck. To maintain patency, when not in use, the arterial cannula was continuously infused (0.3 ml h^{-1}) with heparin-treated saline (15 U ml^{-1}) by use of an infusion pump (Razzel).

In one group of RALH rats, blood and urine samples were taken for subsequent cyclic GMP analysis. These animals underwent a surgical procedure similar to that described above except that the intra-arterial cannula was placed in the carotid artery (for blood sampling and arterial pressure measurement). The carotid arterial cannula was not infused with heparin-treated saline.

Experimental design

Experiments were performed with the animals in their home cages and they were given free access to food and water. Each animal wore a specially designed harness which was attached to a counterbalanced spring. The vascular catheters led out of the cage through the spring (for protection) and the arterial catheter connected via a water-tight swivel (Instech, 375/22) to a pressure transducer (SensoNor 840). Arterial blood pressure was recorded and heart rate derived electronically from the blood pressure signal (Lectromed ECG amplifier).

The RALH rats from which blood and urine samples were taken (for subsequent cyclic GMP analysis) were placed in metabolism cages for urine collection. Blood pressure and heart rate were monitored (as described above) at intervals before the removal of a blood sample (0.5 ml). Urine was collected 24 h before and 24 h after vehicle or drug administration.

Drug or vehicle solutions were administered intravenously either by bolus injection (0.5 ml kg^{-1} over 1 min) or by intravenous infusion (0.3 ml h^{-1}). Injections of vehicle or drug were made when the animals were settled and when cardiovascular variables were stable.

Experimental protocols

On the morning of each experiment (6 days after ligation), the rats were allowed to settle and baseline cardiovascular parameters were established. The patency of the intravenous catheters was ensured following a bolus injection of saline (0.2 ml, i.v.). Experimental protocols began 10 min after this injection.

Group 1: effect of L-NAME or D-NAME on the response to GR138950, enalapril or hydralazine A single dose of either

GR138950 (1 mg kg^{-1}), enalapril (1 mg kg^{-1}) or hydralazine (1 mg kg^{-1}) was administered i.v. and flushed in with a saline bolus (0.2 ml, i.v.). In each rat the cardiovascular response to a single dose of the test drug was followed for 6 h.

In separate animals, L-NAME (10 mg kg^{-1} bolus, then 1 mg kg^{-1} h^{-1} infusion, i.v.) was administered 30 min before the administration of GR138950 (1 mg kg^{-1}, i.v.), enalapril (1 mg kg^{-1}, i.v.) or hydralazine (1 mg kg^{-1}, i.v.). L-NAME infusion was continued following administration of these compounds. Time matched control experiments were performed to evaluate the cardiovascular effects of L-NAME alone, over a 6.5 h period. In a further 5 rats, D-NAME (10 mg kg^{-1} bolus, then 1 mg kg^{-1} h^{-1} infusion, i.v.) was given 30 min before GR138950 (1 mg kg^{-1}; 0.5 ml kg^{-1}, i.v.). The infusion of D-NAME was continued following administration of GR138950.

Group 2: effect of indomethacin on the response to GR138950 Indomethacin (10 mg kg^{-1} bolus, then 3 mg kg^{-1} h^{-1} infusion, i.v.) was administered 30 min before the administration of GR138950 (1 mg kg^{-1}, i.v.). The infusion of indomethacin was continued following administration of GR138950.

Group 3: effect of zaprinast on the response to GR138950 A single dose of GR138950 (0.1 mg kg^{-1}) was administered i.v. and flushed in with a saline bolus (0.2 ml, i.v.). The cardiovascular response to GR138950 was followed for 6 h. In separate rats, an infusion of zaprinast (3 mg kg^{-1} bolus, then 3 mg kg^{-1} h^{-1} infusion, i.v.) was given 1 h before the administration of GR138950 (0.1 mg kg^{-1}, i.v.). The infusion of zaprinast was continued for 6 h following administration of GR138950. Time matched control experiments were performed to evaluate the cardiovascular effects of zaprinast alone, over a 7 h period.

Group 4: effect of vehicle, GR138950 or zaprinast on plasma and urine cyclic GMP levels A single dose of either vehicle (0.5 ml kg^{-1}), GR138950 (1 mg kg^{-1}) or zaprinast (3 mg kg^{-1}) was administered i.v. and flushed in with a saline bolus (0.2 ml, i.v.). In each rat the cardiovascular response to a single dose of vehicle or drug was followed for up to 24 h (3 h for zaprinast treatment). Arterial blood samples (0.5 ml) were taken before and 0.5, 3, 6 and 24 h after vehicle or drug administration (0.5 and 3 h for zaprinast). The volume was replaced with heparin-treated saline (15 u ml^{-1}, 0.7 ml). Urine samples were collected 24 h before and 24 h after administration of vehicle or drug (urinary volumes were inadequate to collect and analyse at intermediate times).

Cyclic GMP analysis

Blood samples were collected into tubes containing ice-cold EDTA (100 μl; 10% w/v in distilled water), were centrifuged (2000 *g* for 15 min at 4°C) and plasma (the supernatant) was removed. The plasma samples were frozen for up to 1 week before protein extraction before assay for cyclic GMP. Plasma cyclic GMP concentration has previously been shown to be a good marker for aortic cyclic GMP levels and is correlated to the change in blood pressure caused by zaprinast-induced vasodilatation (Dundore *et al*., 1993). The extraction efficiency was 74%. Urine samples were collected and frozen before assay for cyclic GMP (protein extraction was not required).

Plasma samples were thawed and 200 μl was transferred to a centrifuge tube containing 0.5 ml ice-cold ethanol to precipitate the plasma proteins. The samples were centrifuged (2000 *g* for 15 min at 4°C) and the supernatant was extracted and placed into a glass vial. The extracts were evaporated under a stream of nitrogen at 60°C (for approximately 20 min). The dried extracts were dissolved in 0.3 ml of cyclic GMP assay buffer. Urine samples were thawed and diluted (1 : 10) in cyclic GMP assay buffer.

Plasma and urine sample cyclic GMP levels were assayed in duplicate by use of a cyclic GMP ^{125}I scintillation proximity assay (SPA) system (RPA 540; Amersham International plc, Amersham, U.K.). Results are expressed as the amount of cyclic GMP present in 1 ml of plasma or urine (pmol ml^{-1}).

Analysis of results

Baseline cardiovascular values were taken 1 min before the addition of drug or vehicle. Post-pretreatment baseline values were taken just before the administration of GR138950, enalapril or hydralazine in drug pretreated animals. Results are expressed as changes from baseline or post-pretreatment values. The area over the curve (AOC; mmHg.min) for mean arterial pressure or area under the curve (AUC; beats) for heart rate was determined for the responses to GR138950, enalapril or hydralazine in the absence and presence of a pretreatment. The inhibitory effects of L-NAME on the antihypertensive treatments (AHT) were described by calculating the percentage inhibition from the following equation:-

$$\%\ \text{inhibition}_{(\text{NAME})} = \frac{[(\text{AOC}_{(\text{NAME+AHT})} - \text{mean AOC}_{(\text{AHT})})}{\text{mean AOC}_{(\text{AHT})})] \times 100$$

The mean ± s.e.mean percentage inhibition values were given in the text. The effects of GR138950 in the absence or presence of the various pretreatments were compared by one way analysis of variance and Dunnett's test. The effects of hydralazine and enalapril in the absence and presence of L-NAME were compared by use of Student's *t* test for unpaired data. Post-pretreatment baseline values were compared to the baseline values by Student's *t* test for paired data. AOC and AUC values were determined for the cardiovascular response caused by L-NAME, D-NAME, indomethacin and zaprinast pretreatment and statistical significance was tested by use of Student's *t* test for single samples.

In experiments in which plasma and urine cyclic GMP concentrations were determined, absolute blood pressure values are shown. Drug and vehicle treatments were compared to baseline values by one way analysis of variance and Dunnett's test, except for urine cyclic GMP levels which were compared to pre-dose baseline values by use of Student's *t* test for paired data. All values are expressed as the mean ± s.e.mean; differences in the mean were considered significant when $P < 0.05$.

Drugs used

The drugs were were N^G-nitro-L-arginine methyl ester HCl (L-NAME; Sigma, Poole, Dorset, U.K.); N^G-nitro-D-arginine methyl ester HCl (D-NAME; Sigma, Poole, Dorset, U.K.); chlorotetracycline HCl (Aureomycin; Cyanamid, Wayne, New Jersey, U.S.A.); enalapril maleate (Sigma, Poole, Dorset, U.K.); hydralazine HCl (Sigma, Poole, Dorset, U.K.); isoflurane (Abbott Labs, Queensborough, Kent, U.K.); indomethacin (Sigma, Poole, Dorset, U.K.); methohexitone sodium (Eli Lilly, Basingstoke, U.K.); procaine penicillin/dihydrostreptomycin (Duphar + Strep; Solvay-Duphar, South Normington, Derbyshire, U.K.); sodium nitroprusside (Sigma, Poole, Dorset, U.K.); zaprinast (M&B 22,948; Sigma, Poole, Dorset, U.K.).

GR138950 (1-[[3-bromo-2-[2-[(trifluoromethyl)sulphonyl]amino]phenyl] - 5 - benzofuranyl]methyl] - 4-cyclopropyl-2-ethyl-1*H*-imidazole-5-carboxamide), in the form of the amphoteric neutral compound, was prepared in the Chemistry Research Division of Glaxo Wellcome Research & Development Ltd (Ware, U.K.).

All drugs for intravenous administration were dissolved in sterile 0.9% w/v saline with the following exceptions. GR138950 was dissolved in sodium bicarbonate (1 M; 5%) ethanol (absolute, 5%) in distilled water (90%; v/v). Zaprinast was dissolved in sodium hydroxide (1 M; 5%; v/v) in saline. All drug solutions were prepared on the day of the experiment and

the doses given represent the amount of biologically active compound.

Results

Baseline values

Baseline values and, where appropriate, post-pretreatment baselines values for mean arterial blood pressure and heart rate in RALH rats for the various treatment groups are shown in Table 1.

Effect of L-NAME and D-NAME in RALH rats

L-NAME (10 mg kg^{-1} bolus, then 1 mg kg^{-1} h^{-1} infusion; $n=5$) caused an immediate significant ($P<0.05$) increase in blood pressure (maximum increase 25 ± 5 mmHg after 5 min). Blood pressure had returned to baseline levels 30 min after administration (Figure 1), and remained close to baseline values during a further 6 h infusion of L-NAME (Figure 2). L-NAME also caused a significant bradycardia which was immediate in onset and maximal after 30 min (maximum fall 104 ± 27 beats min^{-1}; Figure 1). Heart rate remained below baseline levels for up to 6 h after administration. The initial (30 min) cardiovascular effects of L-NAME in the groups subsequently treated with GR138950, enalapril or hydralazine were not significantly different from those obtained for L-NAME alone (data not shown). Since blood pressure had returned to baseline values 30 min after administration of L-NAME (see Table 1), subsequent administration of GR138950, enalapril or hydralazine was made at this time.

D-NAME (10 mg kg^{-1} bolus, then 1 mg kg^{-1} h^{-1} infusion; $n=4$) did not significantly change blood pressure or heart rate up to 30 min after administration (Figure 1). GR138950 was administered 30 min after administration of D-NAME.

Effect of GR138950, alone and in the presence of L-NAME or D-NAME

GR138950 (1 mg kg^{-1}; $n=9$) caused an antihypertensive effect which was immediate in onset. Mean arterial blood pressure decreased rapidly during the first 30 min following administration, after which a further slow decline was observed which reached maximum (-96 ± 6 mmHg) 6 h after administration (Figure 2). Pretreatment with D-NAME (10 mg kg^{-1} bolus, then 1 mg kg^{-1} h^{-1} infusion; $n=4$) did not significantly effect the response to GR138950 (Figure 2). However, in animals

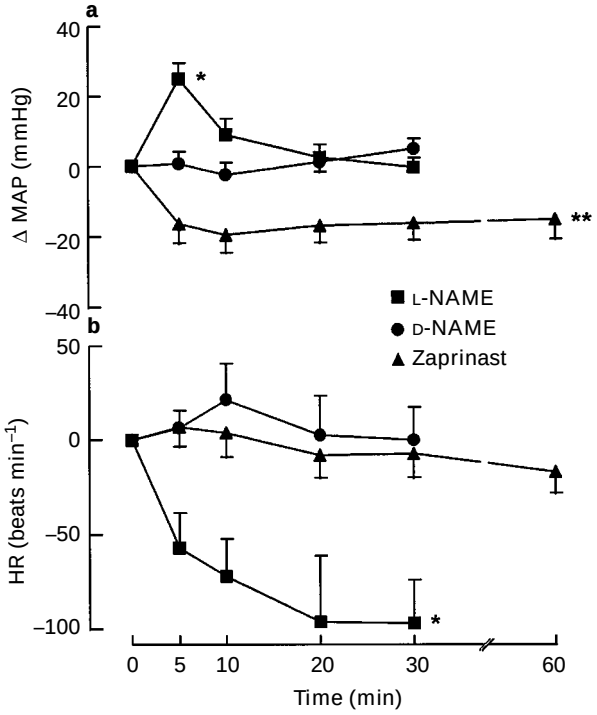

Figure 1 Conscious RALH rats. A comparison of the changes from baseline values over time (min) caused by L-NAME (10 mg kg^{-1} bolus, then 1 mg kg^{-1} h^{-1} infusion i.v.; $n=5$), D-NAME (10 mg kg^{-1} bolus, then 1 mg kg^{-1} h^{-1} infusion i.v.; $n=4$) and zaprinast (3 mg kg^{-1} bolus, then 3 mg kg^{-1} h^{-1} infusion i.v.; $n=8$) in (a) mean arterial pressure (MAP) and (b) heart rate (HR). Each point represents the mean value and the vertical lines show the s.e.mean. Statistical significance was tested with Student's t test for single samples by use of AOC and AUC values for MAP and HR as appropriate. *$P<0.05$; **$P<0.01$.

Table 1 Baseline values and post-pretreatment baseline values (where appropriate) for mean arterial blood pressure (MAP) and heart rate (HR) in the conscious RALH rats

Experimental group			*Baseline*		*Post-pretreatment baseline*	
Pretreatment	*Treatment*	n	*MAP* (mmHg)	*HR* (beats min^{-1})	*MAP* (mmHg)	*HR* (beats min^{-1})
Group 1						
–	GR138950	9	173±4	366±13	–	–
–	Enalapril	7	180±4	372±18	–	–
–	Hydralazine	4	168±6	373±17	–	–
L-NAME	–	5	173±3	422±20	172±4	325±17*
L-NAME	GR138950	7	169±8	385±25	180±4	312±19**
L-NAME	Enalapril	7	167±3	378±21	165±5	317±14*
L-NAME	Hydralazine	4	168±10	418±29	168±6	338±26*
D-NAME	GR138950	4	160±11	410±38	165±8	410±26
Group 2						
Indomethacin	GR138950	4	180±7	413±32	182±4	398±43
Group 3						
–	GR138950	8	176±5	391±23	–	–
Zaprinast	–	8	172±4	399±26	157±8**	382±21
Zaprinast	GR138950	7	175±7	412±33	160±5*	403±25
Group 4						
–	Vehicle	5	171±6	376±26	–	–
–	GR138950	8	169±5	391±21	–	–
–	Zaprinast	4	171±4	405±10	–	–

All drug doses are given in the text. RALH rats were pretreated with L-NAME, D-NAME or indomethacin for 30 min; zaprinast pretreatment time was 60 min. For L-NAME, D-NAME, indomethacin or zaprinast-treated animnals, post-pretreatment values were compared to baseline values by use of Student's t test for paired data. *$P<0.05$; **$P<0.01$.

pretreated with L-NAME (10 mg kg^{-1} bolus, then 1 mg kg^{-1} h^{-1} infusion; $n=7$) the antihypertensive effect of GR138950 was significantly attenuated (maximum fall 42±6 mmHg 30 min after administration; AOC values for blood pressure were reduced by 63±5% compared to that for GR138950 alone) (Table 2, Figure 2).

The fall in blood pressure caused by GR138950 was accompanied by an immediate tachycardia. Maximum tachycardia (146±20 beats min^{-1}) was observed 10 min following administration and heart rate remained elevated for the duration of the experiment (6 h). Pretreatment with D-NAME did not significantly effect the heart rate response to GR138950. In animals treated with L-NAME the immediate (0–30 min) tachycardia caused by GR138950 was significantly attenuated although a slowly developing increase in heart rate was observed (Table 2, Figure 2).

Effect of enalapril, alone and in the presence of L-NAME

Enalapril (1 mg kg^{-1}; $n=7$) caused a marked fall in blood pressure which was immediate in onset, reaching its nadir (−85±3 mmHg) after 3 h. The antihypertensive response was sustained for at least 6 h following administration (Figure 3). In L-NAME (10 mg kg^{-1} bolus, then 1 mg kg^{-1} h^{-1} infusion; $n=7$) pretreated animals, the antihypertensive response to enalapril was significantly attenuated (maximum fall in blood pressure 22±5 mmHg; AOC values for blood pressure were reduced by 88±3% compared to that for enalapril alone; Table 2, Figure 3).

Enalapril also caused tachycardia which was maximal after 10 min (143±25 beats min^{-1}) and was maintained above baseline levels for at least 6 h. In rats treated with L-NAME the immediate (0–30 min) tachycardia caused by enalapril was significantly attenuated after which there was a slowly developing increase in heart rate (Table 2, Figure 3).

Effect of hydralazine, alone and in the presence of L-NAME

Hydralazine (1 mg kg^{-1}; $n=4$) caused an immediate fall in blood pressure reaching its nadir (−63±10 mmHg) 10 min after administration. Blood pressure remained below baseline levels for up to 6 h. The antihypertensive response was associated with an immediate tachycardia (maximum increase 136±20 beats min^{-1} after 3 min) which was sustained for 2 h after administration. Pretreatment with L-NAME (10 mg kg^{-1} bolus, then 1 mg kg^{-1} h^{-1} infusion; $n=4$) did not significantly affect the cardiovascular response to hydralazine (Table 2, Figure 4).

Effect of GR138950 in the presence of indomethacin

Indomethacin (10 mg kg^{-1} bolus, then 3 mg kg^{-1} h^{-1} infusion, i.v., $n=4$) did not significantly affect blood pressure or heart rate in RALH rats (Table 1) and did not significantly change the blood pressure or heart rate response caused by GR138950 (1 mg kg^{-1}, i.v.; $n=4$; Table 2).

Effect of zaprinast in RALH rats

Figure 1 shows the immediate effect of zaprinast (3 mg kg^{-1} bolus, then 3 mg kg^{-1} h^{-1}, i.v.; $n=8$) on blood pressure and heart rate in RALH rats. Zaprinast caused a significant fall in blood pressure which was maximal (−20±5 mmHg) after 10 min. The fall in blood pressure was maintained during a 7 h infusion of zaprinast. Zaprinast caused little change in heart rate for up to 7 h after administration. The initial (60 min) fall in blood pressure caused by zaprinast in the group subsequently treated with GR138950 was similar to that for zaprinast alone (Table 1).

In three animals the depressor effect of i.v. infusion of sodium nitroprusside (3 μg kg^{-1} min^{-1}) was compared before, and 1 and 4 h after the addition of zaprinast (3 mg kg^{-1} bolus, then 3 mg kg^{-1} h^{-1}, i.v.). The depressor response to sodium nitroprusside was significantly ($P<0.05$, Student's t test for paired data) greater 1 and 4 h after administration of zaprinast (maximum change before zaprinast = −12±2 mmHg, compared with −22±3 mmHg and −29±4 mmHg 1 and 4 h, respectively, after zaprinast). In a control animal that did not receive zaprinast the responses to sodium nitroprusside were unchanged.

Effect of GR138950 in the absence and presence of zaprinast

GR138950 (0.1 mg kg^{-1}, i.v., $n=8$) caused a fall in blood pressure which was immediate in onset. Mean arterial blood pressure decreased rapidly in the first 30 min following administration, after which a further slow decline was observed which reached maximum (−59±7 mmHg) 6 h after administration (Figure 5). In rats pretreated with zaprinast ($n=4$), GR138950 caused an antihypertensive effect similar to that

Table 2 AOC values for changes in mean arterial blood pressure (MAP) and AUC values for changes in heart rate (HR) in conscious RALH rats in the various treatment groups

Experimental group			*MAP* $AOC_{0-360\ min}$	*HR* $AUC_{0-30\ min}$
Pretreatment	*Treatment*	n	(mmHg.min)	(beats)
L-NAME	–	5	135±78	320±169
–	GR138950	9	29928±2048	3808±554
L-NAME	GR138950	7	11041±1416**	1127±387**
D-NAME	GR138950	4	22999±1968NS	3404±858NS
Indomethacin	GR138950	4	31481±3320NS	2036±1096NS
–	Enalapril	7	27456±1467	3870±679
L-NAME	Enalapril	7	3426±844**	1346±362**
–	Hydralazine	4	15516±3097	3212±1087
L-NAME	Hydralazine	4	11453±2802NS	2486±1122NS
–	GR138950	8	15480±2048	1712±358
Zaprinast	GR138950	7	12260±2963NS	650±181*

The effect of GR138950 (1 mg kg^{-1}) alone in RALH rats was compared to that in the presence L-NAME, D-NAME or indomethacin by one way ANOVA and Dunnett's test. The effect of enalapril (1 mg kg^{-1}) or hydralazine (1 mg kg^{-1}) alone were compared to those in animals pretreated with L-NAME by use of Student's t test for unpaired data. The effect of GR138950 (0.1 mg kg^{-1}) alone was compared to that in animals pretreated with zaprinast by means of Student's t test for unpaired data. *$P<0.05$; **$P<0.01$; NS not significant. There was no significant difference in the HR response to any antihypertensive agent over 360 min, the HR AUC values shown are up to 30 min after administration.

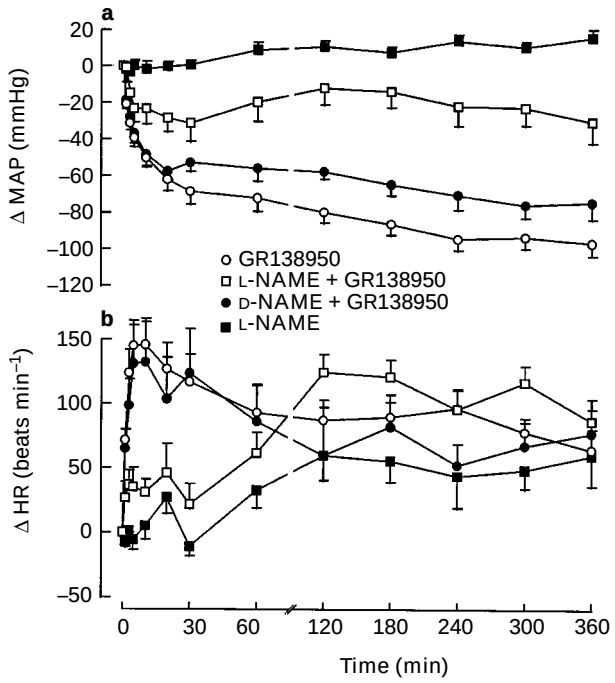

Figure 2 Conscious RALH rats. A comparison of the changes from baseline or post-pretreatment values over time (min) caused by GR138950 1 mg kg^{-1}, i.v., in the absence ($n=9$) and presence of L-NAME (10 mg kg^{-1} bolus, then 1 mg kg^{-1} h^{-1} infusion, i.v.; $n=7$) or D-NAME (10 mg kg^{-1} bolus, then 1 mg kg^{-1} h^{-1} infusion, i.v.; $n=4$) in (a) mean arterial pressure (MAP) and (b) heart rate (HR). The effects of L-NAME alone over the same time period are shown ($n=5$). Each point represents the mean value and the vertical lines show the s.e.mean (See Table 2 for statistical analysis).

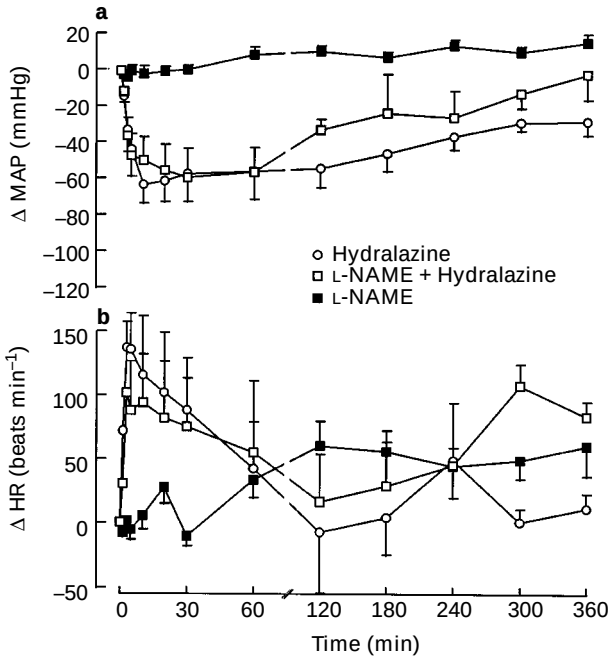

Figure 4 Conscious RALH rats. A comparison of the changes from baseline or post-pretreatment values over time (min) caused by hydralazine 1 mg kg^{-1}, i.v., in the absence ($n=4$) and presence of L-NAME (10 mg kg^{-1} bolus, then 1 mg kg^{-1} h^{-1} infusion i.v.; $n=4$) in (a) mean arterial pressure (MAP) and (b) heart rate (HR). The effects of L-NAME alone over the same time period are shown ($n=5$). Each point represents the mean value and the vertical lines show the s.e.mean. There was no significant difference comparing the AOC or AUC values (for MAP and HR, respectively) for hydralazine in non-pretreated rats and rats pretreated with L-NAME (see Table 2).

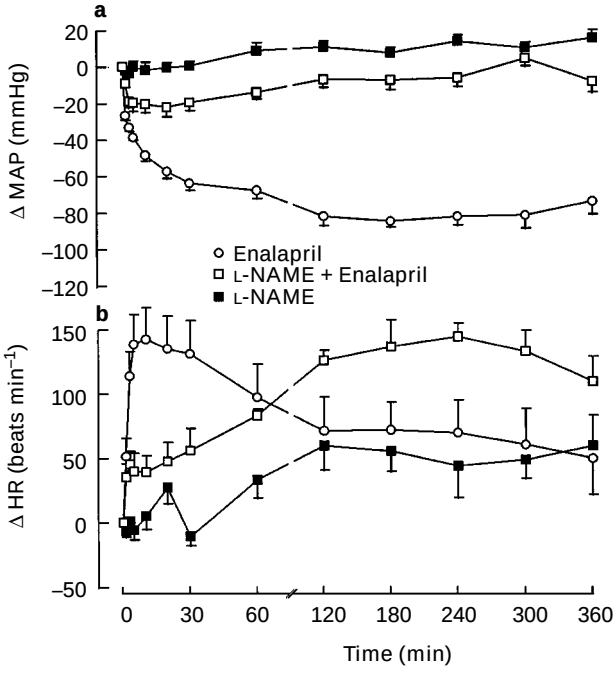

Figure 3 Conscious RALH rats. A comparison of the changes from baseline or post-pretreatment values over time (min) caused by enalapril 1 mg kg^{-1}, i.v., in the absence ($n=7$) and presence of L-NAME (10 mg kg^{-1} bolus, then 1 mg kg^{-1} h^{-1} infusion, i.v.; $n=7$) in (a) mean arterial pressure (MAP) and (b) heart rate (HR). The effects of L-NAME alone over the same time period are shown ($n=5$). Each point represents the mean value and the vertical lines show the s.e.mean (see Table 2 for statistical analysis).

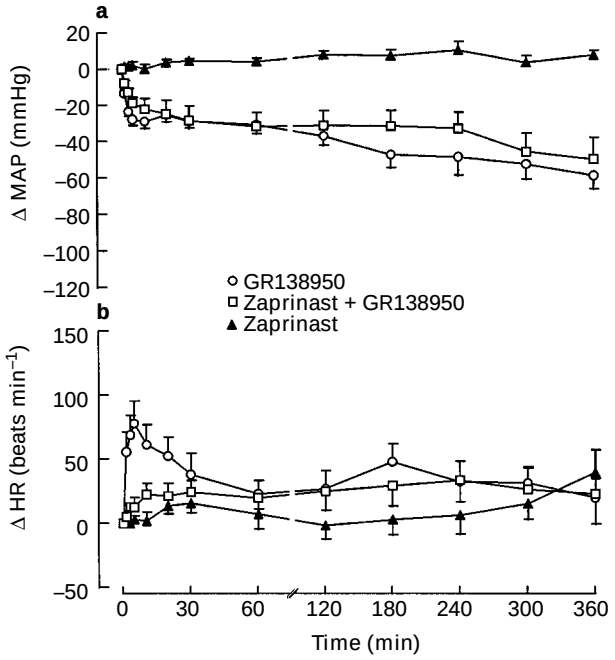

Figure 5 Conscious RALH rats. A comparison of the changes from baseline or post-pretreatment values over time (min) caused by GR138950 0.1 mg kg^{-1}, i.v., in the absence ($n=8$) and presence of zaprinast (3 mg kg^{-1} bolus, then 3 mg kg^{-1} h^{-1} infusion i.v.; $n=7$) in (a) mean arterial pressure (MAP) and (b) heart rate (HR). The effects of zaprinast alone over the same time period are shown ($n=7$). Each point represents the mean value and the vertical lines show the s.e.mean (See Table 2 for statistical analysis).

produced by GR138950 alone (maximum -50 ± 70 mmHg; Table 2, Figure 5).

The fall in blood pressure caused by GR138950 was accompanied by an immediate tachycardia. Maximum tachycardia (78 ± 18 beats min^{-1}) was observed 5 min following administration; heart rate had returned close to baseline levels after 60 min (Figure 5). In zaprinast pretreated animals, the initial tachycardia (0–30 min) caused by GR138950 was significantly attenuated (Table 2, Figure 5).

Effect of vehicle, GR138950 or zaprinast on cyclic GMP levels

In conscious RALH rats, baseline values for mean arterial blood pressure, plasma cyclic GMP concentration and urine cyclic GMP concentration were similar in vehicle, GR138950 and zaprinast treatment groups (Figure 6). Vehicle (0.5 ml kg^{-1}, i.v.; $n=5$) treatment did not significantly affect blood pressure, or cyclic GMP levels in plasma or urine up to 24 h after administration. Administration of GR138950 (1 mg kg^{-1}, i.v.; $n=8$) did not significantly change plasma or urine cyclic GMP concentrations up to 24 h after administration despite causing a marked fall in blood pressure (Figure 6). Zaprinast (3 mg kg^{-1}, i.v.; $n=4$) caused a small but significant fall in blood pressure which was associated with a significant increase in plasma cyclic GMP concentration 30 min after administration. Cyclic GMP concentration in urine also increased significantly after zaprinast administration (Figure 6).

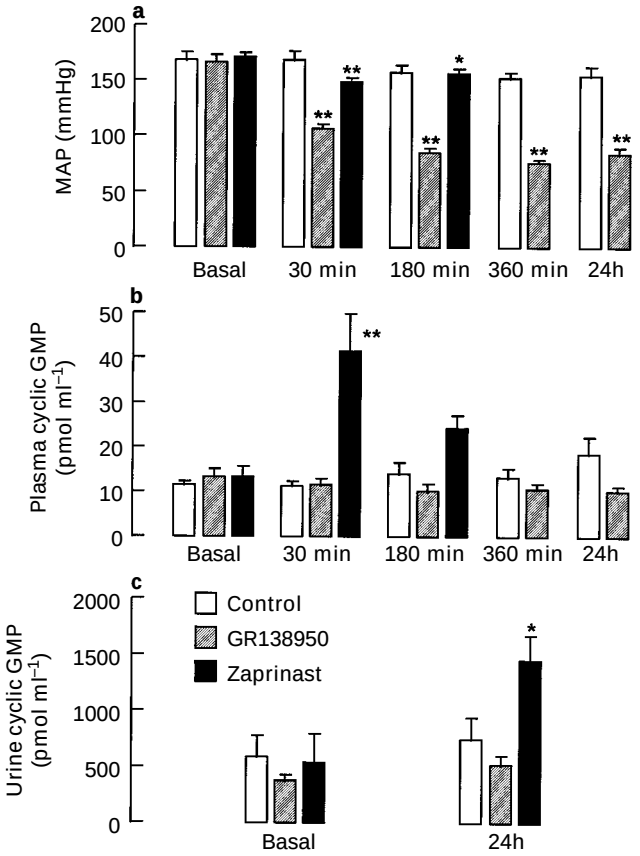

Figure 6 Conscious RALH rats. Histograms showing changes over time (min or h) caused by vehicle (0.5 ml kg^{-1}, i.v.; $n=5$), GR138950 (1 mg kg^{-1}, i.v.; $n=8$) and zaprinast (3 mg kg^{-1}, i.v.; $n=4$) in (a) mean arterial blood pressure (MAP), and (b) plasma and (c) urine cyclic GMP concentrations. Each column represents the mean value and the vertical lines show s.e.mean. Drug and vehicle treatments were compared to baseline values by one way ANOVA and Dunnett's test except for urine cyclic GMP levels which were compared to pre-dose baseline by use of Student's t test for paired data. $^*P<0.05$ and $^{**}P<0.01$.

Discussion

In the present study, the angiotensin AT_1 receptor antagonist, GR138950, or the angiotensin converting enzyme inhibitor, enalapril, caused a marked and prolonged antihypertensive effect in rats with renin-dependent hypertension. The fall in blood pressure caused by GR138950 or enalapril was markedly attenuated following pretreatment with L-NAME, at a dose that has previously been used to inhibit NO synthase *in vivo* (Gardiner *et al*., 1990b). In contrast, the antihypertensive effect of hydralazine was not affected by L-NAME pretreatment. Furthermore, D-NAME, the inactive stereoisomer of the NO synthase inhibitor did not affect the antihypertensive effects of GR138950. Similar findings were obtained by Hegde *et al*. (1993), who demonstrated that the antihypertensive effects of losartan, L-158,809, saralasin or captopril were attenuated by L-NAME (10 mg kg^{-1} bolus, 5 mg kg^{-1} min^{-1} infusion) in RALH rats, whereas the effects of hydralazine and nifedipine were unchanged. Attenuation of the response to the angiotensin AT_1 receptor antagonists or ACE inhibitors, but not hydralazine or nifedipine, suggests that a selective interaction exists between NO synthase and the renin-angiotensin system.

An obvious deduction from these findings is that angiotensin AT_1 receptor antagonists and ACE inhibitors are dependent upon the functional integrity of the vascular NO synthase system for the expression of a major part of their antihypertensive activity in RALH rats. Implicit in this is the assumption that L-NAME and L-NMMA (see below) exert their effects specifically via inhibition of NO synthase.

Enalapril (and other ACE inhibitors) have been shown to enhance bradykinin levels resulting in a NO-dependent vasodilatation which contributes to its antihypertensive response and is sensitive to inhibition by NO synthase inhibitors and bradykinin receptor antagonists (see Introduction, Carbonell *et al*., 1988; Cachofeiro *et al*., 1992). This mechanism does not explain the ability of L-NAME to inhibit the antihypertensive effects of GR138950 because this compound (like losartan) is devoid of ACE inhibitor activity (Chiu *et al*., 1989; Hilditch *et al*., 1996). In this respect, it is interesting to note that L-NAME had a significantly greater inhibitory effect on the antihypertensive response to enalapril compared with that to GR138950 ($88 \pm 3\%$ vs $63 \pm 5\%$, respectively; $P<0.01$, Student's t test). This may suggest that enalapril has a greater NO-dependent component to its antihypertensive action compared to GR138950 and this is presumably due to enhanced levels of bradykinin. However, this needs to be confirmed with a bradykinin B_2-receptor antagonist.

In conscious, normotensive rats, L-NAME increases blood pressure due to widespread vasoconstriction, offset by bradycardia and a reduction in cardiac output (Gardiner *et al*., 1990a,b; Widdop *et al*., 1992). The main mechanism responsible for the haemodynamic effects of L-NAME is believed to be the removal of the tonic vasodilator influence of NO generated locally within the vascular endothelium. Thus, in order to explain the observations made in the present experiments, and in a previous study (Hegde *et al*., 1993), it would be necessary to postulate that AII, through the activation of AT_1 receptors, inhibits the production of NO. Thus, under normal circumstances, GR138950 and similar compounds, would withdraw this inhibition, leading to the unrestrained synthesis of NO and consequently to a widespread vasodilatation and a fall in blood pressure. Prior blockade of NO synthase, by L-NAME, would reduce this effect, as seen in these, and previous, experiments.

The main objection to the hypothesis outlined above is that there is no evidence to support the proposal that A II inhibits endothelial NO synthase activity. In fact, evidence to the contrary exists; A II has recently been shown to stimulate constitutive NO synthase activity in the vessel wall *in vitro* (Boulanger *et al*., 1995; Seyedi *et al*., 1995). In contrast, there is evidence that A II can inhibit cytokine-stimulated expression of inducible NO synthase and nitrite production in vascular smooth muscle cells, via an action mediated by AT_1 receptors

(Nakayama *et al*., 1994). It is possible that hypertension in some way increases expression of inducible NO synthase (perhaps as a result of increases in circulating concentrations of cytokines, or as a compensatory mechanism following vascular tissue damage), but that it is kept in check by A II, possibly generated locally within the vascular smooth muscle. This hypothesis could also explain why the antihypertensive effects of losartan and captopril were attenuated by L-NAME or L-NMMA in spontaneously hypertensive rats (Cachofeiro *et al*., 1992; 1995; Kumagai *et al*., 1993). Although circulatory levels of A II are not elevated in these animals (unlike in RALH rats), the activity of the local vascular renin-angiotensin system *is* elevated (Inada *et al*., 1988). Furthermore, there is evidence that the antihypertensive activity of GR138950 in RALH rats is attributable, at least in part, to blockade of locally generated, rather than circulating, A II (Hilditch *et al*., 1996). In support of this proposal is the finding by Xiao and Pang (1994) that vascular smooth muscle cells from adult spontaneously hypertensive rats produce higher amounts of NO than those from corresponding normotensive rats, implying a general activation of inducible NO synthase. However, in contrast are the findings of Malinski *et al*. (1993) who showed that generation of NO, by constitutive NO synthase, in endothelial cells, and by inducible NO synthase in vascular smooth muscle cells, was suppressed in tissues from spontaneously hypertensive, compared with normotensive, rats. Dubois (1996) also found that the nitric oxide generating pathway was decreased in vascular smooth muscle cells from spotaneously hypertensive, compared with normotensive rats. Thus, the role of inducible NO synthase, and the influence of A II on it in spontaneously hypertensive rats is far from clear. Furthermore, we are unaware of any such data from renal hypertensive rats. An alternative hypothesis that could account for our findings is that enalapril and GR138950 might somehow directly enhance NO synthesis in vascular smooth muscle. However, this seems unlikely since Ikeda and Shimada (1994) observed no effect of three ACE inhibitors on basal or interleukin-1β (IL-1β)-induced increases in nitrite production in rat aortic vascular smooth muscle.

Implicit in the hypotheses raised above is the assumption that L-NAME (and L-NMMA) exert their effects simply by inhibiting NO synthase activity (constitutive or inducible). However, NO-independent mechanisms might be involved as well as, or instead of, NO synthase inhibition (see Nafrialdi *et al*., 1996). Therefore, to gain evidence to support the role of NO in the antihypertensive effects of GR138950, we attempted to correlate this effect with increases in plasma and urine levels of cyclic GMP (the effector molecule of NO; eg. see Murad *et al*., 1992). Responses mediated through the release of endothelium-derived NO have previously been shown to produce falls in blood pressure that correlate with an increase in aortic or urinary cyclic GMP (Tolins *et al*., 1990). However, in the present experiments, GR138950 markedly lowered blood pressure but failed to cause an increase in plasma or urine cyclic GMP concentrations. In contrast, the phosphodiesterase type V inhibitor, zaprinast (Lugnier *et al*., 1986), caused a significant decrease in blood pressure associated with a 3–4 fold increase in plasma and urine cyclic GMP concentration in RALH rats. It is possible that any rise in cyclic GMP levels caused by GR138950 was restricted to the vasculature, and failed to reach the plasma or urine and thus were not detected. To evaluate this possibility, in separate experiments, the effect of zaprinast on the antihypertensive effect GR138950 and sodium nitroprusside (as a positive control) was examined. The dose of zaprinast used has previously been shown to enhance the depressor response to the NO donor sodium nitroprusside (Dundore *et al*., 1990; Merkel *et al*., 1992). Thus, zaprinast would be expected to potentiate the antihypertensive effect of GR138950 assuming that the AT_1 antagonist caused the release of NO, which in turn elevated vascular cyclic GMP levels resulting in vasodilatation. However, zaprinast failed to potentiate the fall in blood pressure caused by GR138950, despite enhancing the depressor response to sodium nitroprusside. These data are not consistent with an action of GR138950 to release or enhance the effects of NO although it is possible that phosphodiesterase V inhibition did not occur in the vascular bed in which GR138950 was active.

It is difficult to attribute the attenuation of the antihypertensive effects of the inhibitors of RAS to inhibition of NO synthase by L-NAME or L-NMMA in the absence of corroborative evidence. Alternatively, the attenuation of the antihypertensive effects of inhibitors of the RAS by NO synthase inhibitors may be explained by other mechanisms. One possibility is that L-NAME attenuated the A II-dependence of the hypertension in RALH rats and this could have occurred at the level of the vasculature. L-NAME causes a marked constriction of several vascular beds including the renal, mesenteric and hindquarter vascular regions (Gardiner *et al*., 1990b). Is it possible, therefore, that differences in the haemodynamic profiles of the individual antihypertensive agents could explain the differential effects that L-NAME had on them? Hydralazine is a potent, relatively non-selective arteriolar dilator that decreases vascular resistance in the femoral, renal, mesenteric and carotid vascular beds; peripheral vasodilatation is accompanied by an increase in cardiac output (Chevillard *et al*., 1981). Acute blockade of the RAS, with ACE-inhibitors or AT_1 receptor antagonists, produces a broadly comparable haemodynamic profile, with decreases in renal, mesenteric and hindquarters resistances; increases in cardiac output, albeit modest, are also seen (Muller *et al*., 1990; Batin *et al*., 1991; Widdop *et al*., 1993). Clinically, hydralazine causes an appreciable tachycardia, although ACE-inhibitors and AT_1 receptor antagonists do not. However, acute administration of all agents in the present experiments caused tachycardia of comparable magnitudes and durations. Thus, although cardiac output was not measured in these experiments, it seems likely that the gross haemodynamic effects of these agents were similar and are unlikely to account for the differential effect of L-NAME on their antihypertensive effects.

As pointed out above, one shortcoming of the present experiments is that cardiac output was not measured. L-NAME reduced heart rate, stroke volume and, thus, cardiac output. The decrease in cardiac output seems to be attributable to the increase in peripheral resistance (Widdop *et al*., 1992). One way in which L-NAME might reduce the fall in blood pressure caused by enalapril and GR138950 is if they increase cardiac output much more in the presence, than in the absence, of L-NAME, thus offsetting the decrease in vascular resistance caused by these agents. Whilst we cannot exclude this possibility, it seems unlikely that such an effect would be restricted to enalapril and GR138950.

Recent studies have shown that losartan can increase production of vasodilator prostaglandins (Jaiswal *et al*., 1991) which contribute to the antihypertensive action of this AT_1 receptor antagonist (Cachofeiro *et al*., 1995). In the present study, the antihypertensive response to GR138950 was not affected by indomethacin, suggesting that prostaglandin production was not required for the response of GR138950 in rats with renin-dependent hypertension. Furthermore, administration of indomethacin alone did not modify blood pressure, suggesting that prostaglandins do not play a role in the control of basal blood pressure in RALH rats.

The antihypertensive effects of GR138950 and enalapril were associated with a marked and sustained tachycardia. This has previously been demonstrated in RALH rats for these compounds as well as for losartan. Furthermore, the tachycardia induced by GR138950 appeared to be mediated by sympathetic stimulation and inhibition of vagal tone and was likely to be reflex in origin (Anderson & Hilditch, 1994). In the present study, the tachycardia caused by GR138950 or enalapril was attenuated by pretreatment with L-NAME. This would be expected following reduced stimulation of the baroreceptor reflex, as a consequence of the smaller blood pressure fall resulting from a smaller decrease in vascular resistance. This attenuation is not likely to be due to a direct cardiac action of L-NAME, because the tachycardia caused by

hydralazine was not affected by this NO synthase inhibitor. Thus, in this study, as in a previous study (Gardiner *et al*., 1990b), L-NAME (alone) caused a marked bradycardia. Gardiner *et al*. (1990b) also demonstrated a reduction in cardiac function (including cardiac output, stroke volume and contractility) with L-NAME treatment. It is possible that the blood pressure rise caused by L-NAME in RALH rats was transient as a consequence of buffering mediated by a reduction in cardiac function.

This investigation has demonstrated that L-NAME inhibits the antihypertensive responses elicited by GR138950 and enalapril, which suggests that these agents release nitric oxide (NO) and/or enhance the cardiovascular effects of NO as part of their mechanism of action. However, the inability of zaprinast to potentiate the antihypertensive effects of GR138950 and the finding that GR138950 did not increase plasma and urine cyclic GMP levels are not consistent with this view. Attenuation of the response to GR138950 or enalapril, but not hydralazine, suggests a selective interaction between L-NAME and inhibitors of the renin-angiotensin system, although the nature of this interaction is unknown.

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