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Regional Hemodynamic Effects of Neutral Endopeptidase Inhibition and Angiotensin (AT_1) Receptor Antagonism Alone or in Combination in Conscious Spontaneously Hypertensive Rats

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ABSTRACT

We tested the hypothesis that angiotensin (AT_1) receptor antagonism (with losartan) would enhance the cardiovascular actions of neutral endopeptidase (NEP) inhibition [with candoxatrilat or (2*S*)-2-{[1-({[(1*S*)-1-carboxy-2-(5-phenyl-1,3-oxazol-2-yl)ethyl]amino}carbonyl)cyclopentyl]methyl}-4-methoxybutanoic acid (UK-489,329)] in conscious spontaneously hypertensive rats (SHR). Four-day continuous intravenous infusion of candoxatrilat (1.9 μg kg^{-1} min^{-1}) or UK-489,329 (0.15 μg kg^{-1} min^{-1}) had no significant cardiovascular effects, whereas candoxatrilat (6.4 μg kg^{-1} min^{-1}) had a modest antihypertensive effect (−10.9 mm Hg on day 4) but no significant sustained effects on regional hemodynamics. Losartan caused a fall in blood pressure (maximum −29.2 mm Hg on day 4) that was associated with renal, mesenteric, and, to a lesser extent, hindquarters vasodilatation. The combination of losartan with either dose of candoxatrilat had no greater antihypertensive or vasodilator effects than losartan alone, with the exception of the increase in renal vascular conductance, which was greater with the combination of the drugs than with either drug alone (significant only in the lower dose study). Losartan combined with UK-489,329 showed a greater antihypertensive effect than losartan alone (−14.6 mm Hg greater on day 4), although the effects of the combination were not significantly greater than the sum of the effects of both agents administered separately. However, losartan combined with UK-489,329 caused increases in renal and hindquarters vascular conductance that were significantly greater with the combination than with either agent given alone. Thus, in conscious SHR, the renin-angiotensin system may act to oppose a vasodilator action of NEP inhibition, particularly in the renal vascular bed.

Neutral endopeptidase 24.11 (NEP) is a zinc metalloprotease responsible for the breakdown of a number of short linear or cyclic peptides, such as the natriuretic peptides, bradykinin, angiotensin II, and endothelin. Other members of the zinc metalloprotease family that may be involved in the metabolism of biologically active peptides include endothelin-converting enzyme and soluble secreted endopeptidase (SEP) (Ikeda et al., 1999). Although NEP inhibitors were developed as antihypertensive agents, their effectiveness has turned out to be limited, probably because of their short half-life in the circulation, together with the fact that the breakdown of not only vasodilator/natriuretic peptides, but also vasoconstrictor peptides, such as angiotensin II and endothelin, is reduced (Richards et al., 1993; McDowell et al., 1997). In fact, some studies have found predominant vasoconstrictor effects of NEP inhibition in humans (Ferro et al., 1998). In animal studies, NEP inhibition with, for example, candoxatrilat, has only consistently been shown to exert antihypertensive effects in salt-sensitive models of hypertension (Shepperson et al., 1991; Hirata et al., 1994), and in human essential hypertension, candoxatril is reported to have either no clinically relevant effect on blood pressure (Bevan et al., 1992) or a modest antihypertensive effect (Richards et al., 1993), with evidence for activation of the renin-angiotensin system and sympathetic nervous system offsetting the blood pressure-lowering effect (Richards et al., 1993).

The development of "vasopeptidase" inhibitors, which simultaneously inhibit the two zinc metallopeptidases angiotensin-converting enzyme (ACE) and NEP, was based on the

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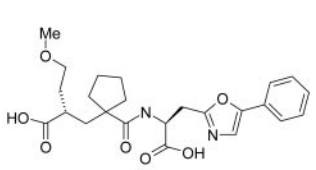
ABBREVIATIONS: NEP, neutral endopeptidase; SEP, soluble secreted endopeptidase; ACE, angiotensin-converting enzyme; AT_1, angiotensin type 1; SHR, spontaneously hypertensive rats; HDAS, hemodynamics data acquisition system; UK-489329, (2*S*)-2-{[1-({[(1S)-1-carboxy-2-(5-phenyl-1,3-oxazol-2-yl)ethyl]amino}carbonyl)cyclopentyl]methyl}-4-methoxybutanoic acid; EXP 3174, 2-butyl-4-chloro-1-{[2′-(2*H*-tetrazol-5-yl)biphenyl-4-yl]methyl}-1*H*-imidazole-5-carboxylic acid; i.v., intravenous.

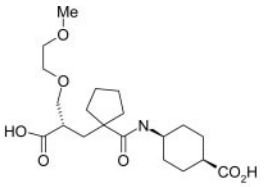
premise that such drugs would combine the vasodilator/natriuretic effects of NEP inhibition, with inhibition of angiotensin II formation by ACE (for reviews, see Weber, 2001; Molinaro et al., 2002; Wells and Little, 2002). Indeed, preclinical, and early clinical studies with the vasopeptidase inhibitor omapatrilat showed beneficial effects in hypertension and in congestive heart failure. However, more recent, larger clinical trials have revealed a problematic incidence of angioedema with omapatrilat (Coats, 2002; Zanchi et al., 2003). Both ACE and NEP inhibit bradykinin degradation, and because bradykinin has been implicated in the angioedema associated with ACE inhibition (Cugno et al., 2002), perhaps the higher incidence of angioedema with dual ACE/NEP inhibition is not surprising (Campbell, 2003).

Angiotensin (AT_1) receptor antagonism is another approach to inhibiting the vasoconstrictor effects of the renin-angiotensin system, which differs from ACE inhibition in several respects. First, although AT_1 receptor antagonists are not necessarily devoid of effects on bradykinin metabolism (e.g., Campbell et al., 2005), such effects are likely to be less than with ACE inhibitors and dependent on NEP (Walther et al., 2002). Second, the AT_1 receptor-mediated actions of angiotensin, formed via pathways independent of ACE, are inhibited. Since the incidence of angioedema with the use of angiotensin receptor antagonists is substantially less than with ACE inhibitors (Irons and Kumar, 2003), another logical approach to optimizing the effects of NEP inhibition would be to combine it with AT_1 receptor antagonism.

To our knowledge, the integrated cardiovascular effects of combined NEP inhibition and angiotensin receptor antagonism have not been studied. Hence, the aim of the present study was to evaluate the regional hemodynamic effects of continuous NEP inhibition, using candoxatrilat (McDowell and Nicholls, 2000) or UK-489,329, a potent novel NEP inhibitor (Fig. 1), with or without concomitant administration of a low dose of the angiotensin receptor antagonist losartan, in conscious, spontaneously hypertensive rats (SHR). We chose this model because it is reported to be relatively resistant to the antihypertensive effects of NEP inhibition (Koepke et al., 1990; Sybertz et al., 1990; Seymour et al., 1991; Pham et al., 1993, 1995; Sala et al., 1994; Tikkanen et al., 1998) but susceptible to the effects of inhibition of the renin-angiotensin system, either by ACE inhibition (for reviews, see Rubin and Antonaccio, 1980; Unger et al., 1990) or by AT_1 receptor antagonism (Wong et al., 1990; Bunkenburg et al., 1991; Li and Widdop, 1996).



UK-489329



Candoxatrilat

		IC_{50} (nM)[1]	
Enzyme	Species/ source	Candoxatrilat	UK-489,329
NEP	Human kidney Rat kidney	6.4 (4.2-9.9) 2.3 (2.0-2.8)	0.29 (0.24-0.34) 0.19 (0.12-0.29)
SEP	Human recombinant	25.2 (22.8-27.8)	17.7 (13.7-22.6)
ACE	Human kidney	>10,000	271 (244-300)
ECE-1	Human recombinant	ND	>10000

NEP = neutral endopeptidase (EC 3.4.24.11); SEP = soluble secreted endopeptidase; ACE = angiotensin converting enzyme; ECE-1 = endothelin converting enzyme-1 ;.
[1] All IC_{50}s were obtained using substrate concentrations less than 1/3rd Km, where IC_{50} approximates to K_i for competitive inhibitors.
ND = not determined.

Fig. 1. Structures of candoxatrilat and UK-489,329, together with IC_{50} values for inhibition of neutral endopeptidase (EC 3.4.24.11) and other related peptidase enzymes. IC_{50} values are geometric mean ($n \geq 3$, except where indicated).

Materials and Methods

All procedures were approved by the University of Nottingham Ethical Review Committee and were performed under Home Office Project License authority.

Experiments were carried out on male SHR (Charles River, Margate, Kent, UK), weighing between 260 and 380 g (i.e., between 20 and 22 weeks of age) at the time of study. Animals were housed in a temperature-controlled environment (20–22°C) with a 12-h light/dark cycle (lights on at 6:00 AM), with free access to food (Beekay Rat and Mouse Diet No. 1, sodium 0.18%; B&K Universal Limited, Hull, UK) and water throughout.

Surgical Preparation. Surgery was performed under general anesthesia (fentanyl and medetomidine; 300 $\mu g\ kg^{-1}$ of each i.p.) in two stages. First, miniaturized pulsed Doppler flow probes were sutured around the left renal artery, the superior mesenteric artery, and the distal abdominal aorta (below the level of the ileocecal artery, to monitor flow to the hindquarters). Second, catheters were implanted in the distal abdominal aorta (via the caudal artery) to monitor arterial blood pressure and heart rate, and in the right jugular vein for drug administrations. After each surgical stage, anesthesia was reversed, and analgesia was provided with atipamezole and nalbuphine, respectively (1 mg kg^{-1} of each subcutaneously). The two surgical stages were separated by at least 10 days. Before the second stage, the fitness of all animals was certified by the named veterinary surgeon.

After catheterization, animals were fitted with custom-designed harnesses that were attached to counterbalanced spring systems. The catheters ran through the spring and were connected to double-channel, fluid-filled swivels to allow overnight i.v. infusion of drugs or saline (0.4 ml h^{-1}) and intra-arterial infusion of heparinized (15 U ml^{-1}; 0.4 ml h^{-1}) saline to maintain catheter patency. Experiments began 24 h after catheterization, when the animals were fully conscious, freely moving, and had access to food and water ad libitum.

Cardiovascular Recordings. Cardiovascular variables were monitored using a customized, computer-based system [Hemodynamics Data Acquisition System (HDAS), University of Limburg, Maastricht, The Netherlands] connected to the transducer amplifier (model 13-4615-50; Gould Instrument Systems Inc., Cleveland, OH) and the Doppler flowmeter [Crystal Biotech VF-1 mainframe (pulse repetition frequency 125 kHz) fitted with high-velocity (HVPD-20) modules]. Raw data were sampled by HDAS every 2 ms, averaged every cardiac cycle, and stored to disc at 5-s intervals. Data were analyzed off-line using software (Datview; University of Limburg) that interfaced with HDAS.

Experimental Protocol. Three series of experiments were run, each involving four groups of 9 to 10 animals. In experiment 1, rats were randomized to receive candoxatrilat (1.9 $\mu g\ kg^{-1}\ min^{-1}$), losartan (8.5 $\mu g\ kg^{-1}\ min^{-1}$), candoxatrilat plus losartan (doses as described above), or vehicle (isotonic saline adjusted to pH ~8.0 with

Na_2CO_3). Experiments 2 and 3 involved the same groupings, but in experiment 2, the dose of candoxatrilat was increased to 6.4 μg kg^{-1} min^{-1}, and in experiment 3, the NEP inhibitor UK-489,328 (0.15 μg kg^{-1} min^{-1}) was used.

After a control period of at least 90 min of baseline recording on day 1, drug or vehicle infusions were begun, and they were continued for the following 4 days. Cardiovascular data were collected for 7 h after the onset of drug administration on day 1 and for periods of 7 h on days 2 to 4.

Arterial blood samples were collected into tubes containing EDTA (as anticoagulant) before any intervention on day 1 and after the recording period of each experimental day. Plasma was prepared and stored frozen at −80°C, before analysis for drug and metabolite concentrations.

Cardiovascular Data Analysis. The three experiments were run as separate experimental blocks over several months. Each experimental block ran over several weeks, and in each week, typically, four animals were used such that data for one rat in each treatment group were collected. The baseline was taken as the 30- to 45-min period before drug administration on day 1, when the animals were settled. For graphical representation, postdosing data are expressed as three sequential averages (~140 min) on day 1 and as four sequential averages (~105 min) on days 2 to 4 relative to the original baseline. A repeated measures analysis of covariance was performed on these data (displayed in panel a of subsequent figures), and the consistency of the treatment effects across time was assessed (a treatment × time interaction). For the majority of the responses across all three studies, we found a significant treatment × time interaction, indicating that the treatment effects may not be consistent across all 4 days. To investigate this further, the average response for each day (data averaged across the entire 7-h recording period) was analyzed. For each day, mean heart rate and blood pressure for each animal were subjected to analysis of covariance, allowing for potential week-to-week differences, and for differences at baseline. Likewise, analysis of percentage of change in Doppler shift, and percentage of change in conductance was performed for each day using analysis of variance, again allowing for potential week to week differences. The possibility of a statistical interaction between losartan and candoxatrilat/UK-489,329 was assessed using the models described. This interaction can be considered as a comparison of whether the combined action of the two compounds is greater than the sum of the individual compound effects.

The estimated treatment differences presented reflect the differences between each treated group and the vehicle group on each day. An additional comparison on each day reflecting the difference between losartan alone and the combination with losartan is also presented under *Results*. The estimates used in these comparisons arise naturally from these methods of analysis and compensate for

TABLE 1

Free concentrations (Conc.) of candoxatrilat, UK-489,329, and EXP 3174 (the active metabolite of losartan) in plasma averaged across 4 days infusion

Values are geometric mean with 95% confidence interval in parentheses.

Study	Treatment Group	Compound	Free Conc[a]	*n*
			nM	
Low-dose candoxatrilat	Candoxatrilat	Candoxatrilat	244 (208–286)	10
	Candoxatrilat + losartan	Candoxatrilat	221 (212–230)	9
	Candoxatrilat + losartan	EXP 3174	34.4 (28.7–41.3)	9
	Losartan	EXP 3174	33.3 (30.7–36.2)	9
High-dose candoxatrilat	Candoxatrilat	Candoxatrilat	432 (397–469)	9
	Candoxatrilat + losartan	Candoxatrilat	389 (341–443)	8
	Candoxatrilat + losartan	EXP 3174	39.2 (32.3–46.7)	8
	Losartan	EXP 3174	40.1 (32.6–49.3)	8
UK-489,329[b]	UK-489,329	UK-489,329	4.4 (3.7–5.3)	8
	UK-489,329 + losartan	UK-489,329	4.2 (3.3–5.3)	8

[a] Free concentration = total concentration × free fraction in plasma (candoxatrilat, 0.80; UK-489,329, 0.10; and EXP 3174, 0.016).
[b] Concentrations of EXP 3174 were not determined in this study.

TABLE 2

Resting heart rate, mean blood pressure, and renal, mesenteric, and hindquarters Doppler shift and vascular conductance values before any intervention in conscious spontaneously hypertensive rats

Values are mean ± S.E.M. Units for vascular conductance are (kHz mm Hg^{-1}) 10^3. Experiment 1: group 1, candoxatrilat (1.9 μ g kg^{-1} min^{-1}), n = 10; group 2, losartan (8.5 μ g kg^{-1} min^{-1}), n = 9; group 3, candoxatrilat + losartan (doses as described above), n = 9; and group 4, vehicle, n = 10. Experiment 2: group 5, candoxatrilat (6.4 μ g kg^{-1} min^{-1}), n = 9; group 6, losartan (8.5 μ g kg^{-1} min^{-1}), n = 8; group 7, candoxatrilat + losartan (doses as described above), n = 8; and group 8, vehicle, n = 9. Experiment 3: group 9, UK-489,329 (0.15 μ g kg^{-1} min^{-1}), n = 9; group 10, losartan (8.5 μ g kg^{-1} min^{-1}), n = 9; group 11, UK-489,329 + losartan (doses as described above), n = 9; and group 12, vehicle, n = 8.

Group	1	2	3	4	5	6	7	8	9	10	11	12
Heart rate (beats min^{-1})	315 ± 10	315 ± 11	300 ± 5	327 ± 7	319 ± 9	319 ± 8	339 ± 11	318 ± 10	326 ± 9	303 ± 6	327 ± 5	318 ± 10
Mean blood pressure (mm Hg)	162 ± 7	163 ± 3	164 ± 4	169 ± 5	170 ± 5	161 ± 5	171 ± 4	164 ± 4	160 ± 3	152 ± 3	167 ± 4	158 ± 7
Renal Doppler shift (kHz)	8.5 ± 0.5	6.8 ± 0.4	7.1 ± 0.8	6.2 ± 0.6	6.2 ± 0.5	8.9 ± 1.1	6.6 ± 0.5	7.2 ± 0.7	6.5 ± 0.5	6.8 ± 0.6	5.8 ± 0.4	6.2 ± 0.6
Mesenteric Doppler shift (kHz)	8.6 ± 0.5	8.8 ± 0.6	8.4 ± 0.5	8.4 ± 0.8	9.0 ± 0.8	8.8 ± 0.8	8.0 ± 0.6	8.0 ± 0.7	7.4 ± 0.5	7.8 ± 0.8	8.2 ± 0.6	8.5 ± 0.8
Hindquarters Doppler shift (kHz)	3.9 ± 0.4	4.3 ± 0.5	3.5 ± 0.5	4.4 ± 0.3	3.8 ± 0.4	4.2 ± 0.4	4.2 ± 0.4	3.9 ± 0.3	5.2 ± 0.5	5.2 ± 0.5	5.6 ± 0.6	5.5 ± 0.4
Renal vascular conductance (U)	53 ± 4	42 ± 2	44 ± 6	37 ± 4	37 ± 3	55 ± 7	39 ± 3	44 ± 5	41 ± 3	45 ± 4	35 ± 3	41 ± 5
Mesenteric vascular conductance (U)	54 ± 4	54 ± 4	51 ± 3	50 ± 5	53 ± 5	54 ± 4	47 ± 4	49 ± 4	46 ± 4	52 ± 6	49 ± 3	56 ± 7
Hindquarters vascular conductance (U)	24 ± 3	27 ± 3	21 ± 3	26 ± 2	23 ± 2	26 ± 2	25 ± 3	24 ± 2	33 ± 3	34 ± 4	34 ± 5	36 ± 3

differences at baseline and week-to-week differences; 95% confidence intervals are presented with the estimated differences, and these show the range of values within which the true treatment differences are likely to lie. All analyses were carried out using GenStat for Windows, version 6.1. A P value ≤ 0.05 was taken as significant.

Drugs and Plasma Analyses. Fentanyl citrate was from Janssen-Cilag (High Wycombe, UK); medetomidine hydrochloride (Domitor) and atipamezole hydrochloride (Antisedan) were from Pfizer Central Research (Sandwich, Kent, UK); and nalbuphine hydrochloride (Nubain) was from Bristol-Myers Squibb (Hounslow, UK). Candoxatrilat, UK-489,329 and losartan were supplied by Pfizer Central Research. Drugs and vehicle were infused at a rate of 0.4 ml h^{-1}.

Concentrations of candoxatrilat, UK-489,328, and EXP 3174, the active metabolite of losartan, were determined in plasma samples using liquid chromatography/mass spectrometry. Plasma protein binding of test compounds was determined by equilibrium dialysis essentially as described by Walker et al. (2005) using control rat plasma to which test compounds were added to give 1 μg/ml. After dialysis, concentrations of drug in plasma and buffer were determined by liquid chromatography/mass spectrometry and the free (unbound) fraction of compound in plasma calculated from the ratio of the concentration in buffer to plasma. Free concentrations of compounds present in plasma during in vivo studies were calculated by multiplying the measured total concentrations by the free fraction.

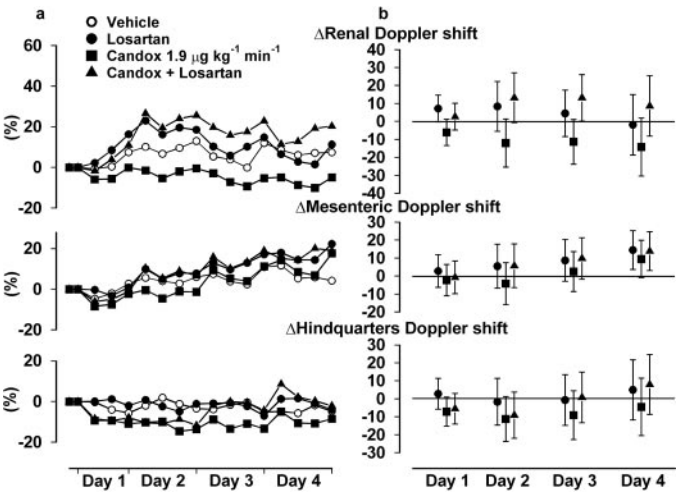


Fig. 3. Changes in regional Doppler shift over a 4-day continuous infusion of vehicle (n = 10), candoxatrilat (1.9 μg kg^{-1} min^{-1}; n = 10), losartan (8.5 μg kg^{-1} min^{-1}; n = 9), or candoxatrilat together with losartan (doses as described above; n = 9). a, values averaged over 105 min during the 7-h monitoring period on each day. b, estimated differences between each treatment group and vehicle with 95% confidence intervals. Treatment effects are significantly different from vehicle ($P <$ 0.05) where the confidence interval bar does not cross the zero line.

Results

Plasma Concentrations of Compounds. Plasma concentrations of candoxatrilat, UK-489,329, and EXP 3174 showed a high degree of between-day and between-animal reproducibility. Table 1 shows the overall geometric mean free (unbound) concentrations in each treatment group. The free concentrations of EXP 3174 ranged from 33.3 to 40.1 nM, equating to 4- to 5-fold the IC_{50} for inhibition of angiotensin II binding to the human angiotensin AT_1 receptor (9 nM; Inada et al., 1999), and 40- to 50-fold the ED_{50} for inhibition of angiotensin II-induced pressor responses in conscious rats (0.9 nM; Wong et al., 1996). Free candoxatrilat in the low-dose group ranged from 96- to 106-fold IC_{50} for inhibition of rat kidney NEP (IC_{50} = 2.3 nM) and that in the high dose group ranged from 170- to 190-fold IC_{50}. Free UK-489,329 reached 22- to 23-fold IC_{50} for 0.19 nM NEP. Thus, the infusions of candoxatrilat and UK-489,329 would have been expected to provide near complete inhibition of NEP, whereas candoxatrilat would also have inhibited SEP (Fig. 1), although any functional consequences of SEP inhibition have not been reported.

Baseline Cardiovascular Variables. Resting cardiovascular variables before drug or vehicle administration in the 12 groups of rats from the three experiments are shown in Table 2. Any differences between the average baseline responses for the four treatment groups in each experiment were adjusted for in subsequent statistical analysis by the use of analysis of covariance (see *Materials and Methods*).

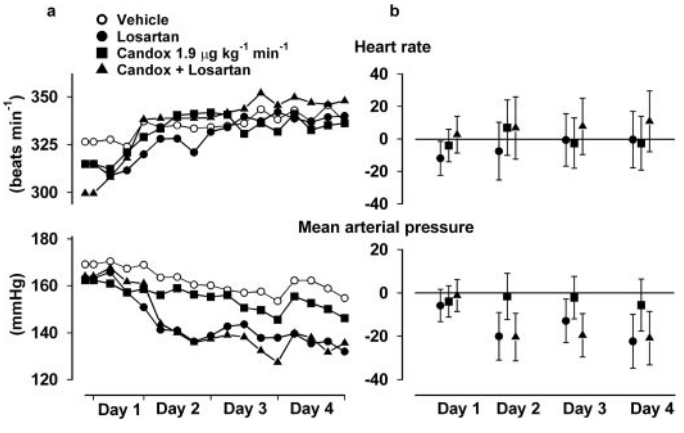


Fig. 2. Heart rate and mean arterial blood pressure over a 4-day continuous infusion of vehicle (n = 10), candoxatrilat (1.9 μg kg^{-1} min^{-1}; n = 10), losartan (8.5 μg kg^{-1} min^{-1}; n = 9), or candoxatrilat together with losartan (doses as described above; n = 9). a, values averaged over 105 min during the 7-h monitoring period on each day. b, estimated differences between each treatment group and vehicle with 95% confidence intervals. Treatment effects are significantly different from vehicle ($P <$ 0.05) where the confidence interval bar does not cross the zero line.

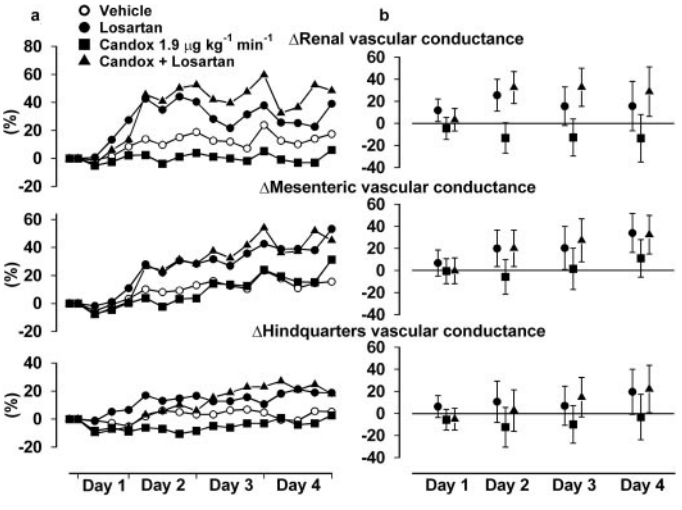


Fig. 4. Changes in regional vascular conductance over a 4-day continuous infusion of vehicle (n = 10), candoxatrilat (1.9 μg kg^{-1} min^{-1}; n = 10), losartan (8.5 μg kg^{-1} min^{-1}; n = 9), or candoxatrilat together with losartan (doses as described above; n = 9). a, values averaged over 105 min during the 7-h monitoring period on each day. b, estimated differences between each treatment group and vehicle with 95% confidence intervals. Treatment effects are significantly different from vehicle ($P <$ 0.05) where the confidence interval bar does not cross the zero line.

Figures 2 to 4 show the data from experiment 1 [lower dose (1.9 μg kg^{-1} min^{-1}) candoxatrilat and/or losartan], Figs. 5 to 7 show the data from experiment 2 [higher dose (6.4 μg kg^{-1} min^{-1}) candoxatrilat and/or losartan], and Figs. 8 to 10 show the data from experiment 3 [UK-489,329 (0.15 μg kg^{-1} min^{-1}) and/or losartan]. The changes in mean blood pressure and heart rate (Figs. 2a, 5a, and 8a), percentage of changes in Doppler shift (Figs. 3a, 6a, and 9a), and percentage of changes in vascular conductances (Figs. 4a, 7a, and 10a) across the entire experiment are shown for illustrative purposes, but statistical analyses were performed on the corresponding treatment effects (i.e., adjusted mean differences from vehicle; Figs. 2b–10b).

Heart Rate. There were no significant changes in heart rate in any experimental group relative to the corresponding vehicle effects (Figs. 2, 5, and 8), except for the group receiving losartan alone in experiment 3, in which there was a significant tachycardia on days 2 and 3 (Fig. 8).

Blood Pressure. In experiment 1, there were no changes in mean blood pressure in rats treated with the lower dose of candoxatrilat (1.9 μg kg^{-1} min^{-1}) relative to vehicle, whereas losartan alone, and in combination with candoxatrilat, caused significant falls in blood pressure on days 2 to 4 of the study, up to a maximum difference from vehicle of −22.3 and −20.8 mm Hg, respectively (Fig. 2). There was no evidence of interaction between the effects of losartan and candoxatrilat on blood pressure, i.e., the effects of the combination were not significantly different from the sum of effects of each compound administered separately.

In experiment 2, the higher dose of candoxatrilat (6.4 μg kg^{-1} min^{-1}) caused significant falls in mean blood pressure relative to vehicle on days 2 to 4 of the study, up to a maximum difference of −10.9 mm Hg (Fig. 5). Losartan alone, and in combination with high-dose candoxatrilat, also caused falls in mean blood pressure; the effect of losartan was significant from day 1 onward (maximum difference −23.4 mm Hg), and the effect of the combination of losartan and candoxatrilat was significant from day 2 onward (maximum difference −30.8 mm Hg) (Fig. 5). Although there was a trend for blood pressure to be lower in the combined treatment group than in the losartan alone group on study days 3 and 4, this did not reach statistical significance, and there was no evidence of interaction between the effects of losartan and candoxatrilat on blood pressure (Fig. 5).

In experiment 3, relative to vehicle, UK-489,329 had no significant effects on blood pressure. However, there was significant hypotension with losartan alone (days 2–4), and in combination with UK-489,329 (days 1–4), up to maxima of −29.2 and −43.8 mm Hg differences from vehicle, respectively (Fig. 8). The effects of combined treatment on mean blood pressure were significantly greater than those of losartan alone on day 4; however, there was no significant inter-

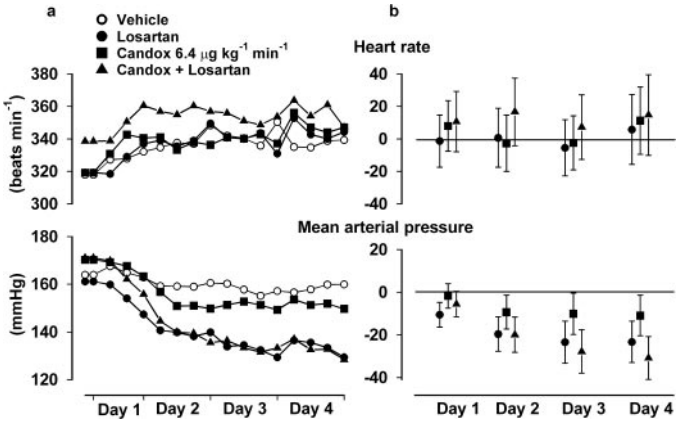


Fig. 5. Heart rate and mean arterial blood pressure over a 4-day continuous infusion of vehicle (n = 9), candoxatrilat (6.4 μg kg^{-1} min^{-1}; n = 9), losartan (8.5 μg kg^{-1} min^{-1}; n = 8), or candoxatrilat together with losartan (doses as described above; n = 8). a, values averaged over 105 min during the 7-h monitoring period on each day. b, estimated differences between each treatment group and vehicle with 95% confidence intervals. Treatment effects are significantly different from vehicle (P < 0.05) where the confidence interval bar does not cross the zero line.

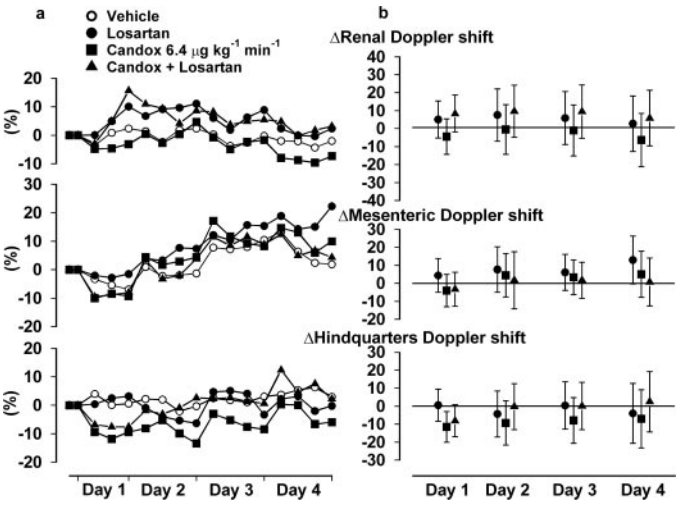


Fig. 6. Changes in regional Doppler shift over a 4-day continuous infusion of vehicle (n = 9), candoxatrilat (6.4 μg kg^{-1} min^{-1}; n = 9), losartan (8.5 μg kg^{-1} min^{-1}; n = 8), or candoxatrilat together with losartan (doses as described above; n = 8). a, values averaged over 105 min during the 7-h monitoring period on each day. b, estimated differences between each treatment group and vehicle with 95% confidence intervals. Treatment effects are significantly different from vehicle (P < 0.05) where the confidence interval bar does not cross the zero line.

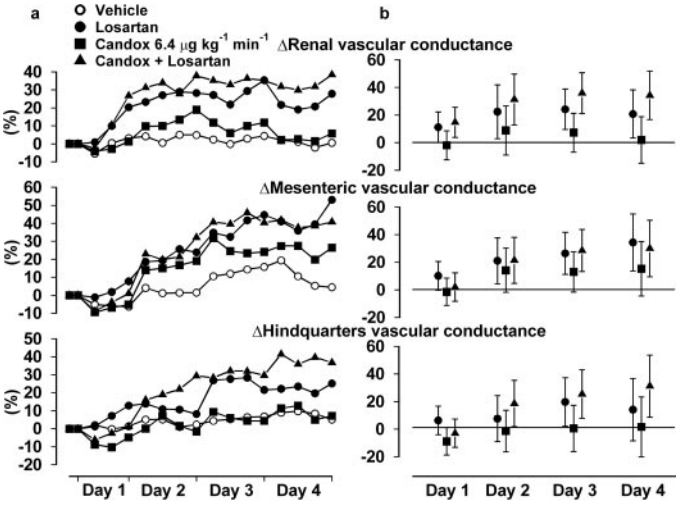


Fig. 7. Changes in regional vascular conductance over a 4-day continuous infusion of vehicle (n = 9), candoxatrilat (6.4 μg kg^{-1} min^{-1}; n = 9), losartan (8.5 μg kg^{-1} min^{-1}; n = 8), or candoxatrilat together with losartan (doses as described above; n = 8). a, values averaged over 105 min during the 7-h monitoring period on each day. b, estimated differences between each treatment group and vehicle with 95% confidence intervals. Treatment effects are significantly different from vehicle (P < 0.05) where the confidence interval bar does not cross the zero line.

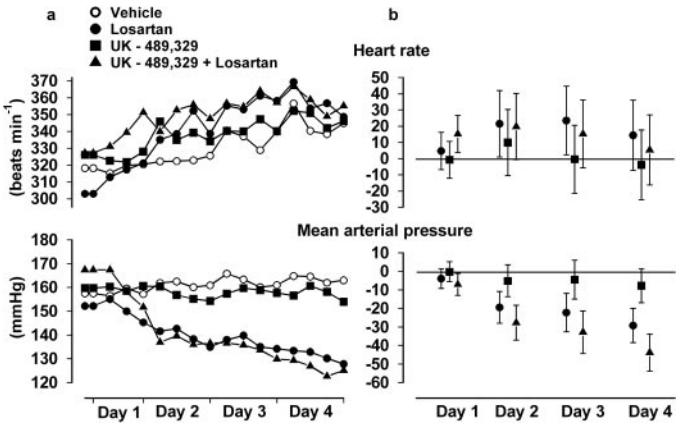


Fig. 8. Changes in heart rate and mean arterial pressure over a 4-day continuous infusion of vehicle ($n = 8$), UK-489,329 (0.15 μg kg^{-1} min^{-1}; $n = 9$), losartan (8.5 μg kg^{-1} min^{-1}; $n = 9$), or UK-489,329 together with losartan (doses as described above; $n = 9$). a, values averaged over 105 min during the 7-h monitoring period on each day. b, estimated differences between each treatment group and vehicle with 95% confidence intervals. Treatment effects are significantly different from vehicle ($P < 0.05$) where the confidence interval bar does not cross the zero line.

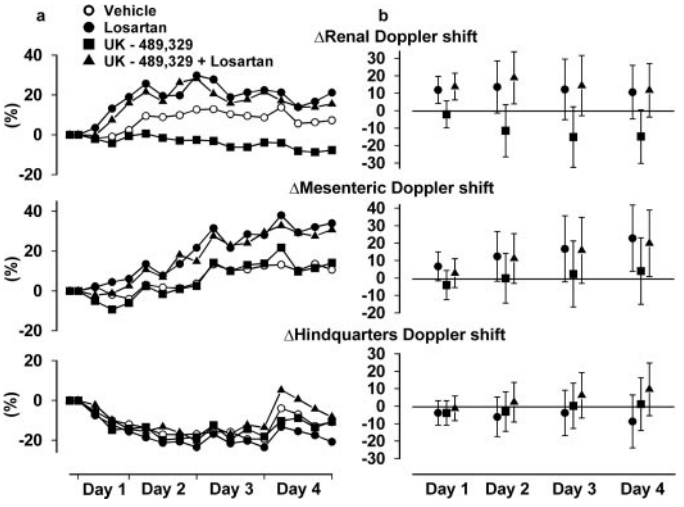


Fig. 9. Changes in regional Doppler shift over a 4-day continuous infusion of vehicle ($n = 8$), UK-489,329 (0.15 μg kg^{-1} min^{-1}; $n = 9$), losartan (8.5 μg kg^{-1} min^{-1}; $n = 9$), or UK-489,329 together with losartan (doses as described above; $n = 9$). a, values averaged over 105 min during the 7-h monitoring period on each day. b, estimated differences between each treatment group and vehicle with 95% confidence intervals. Treatment effects are significantly different from vehicle ($P < 0.05$) where the confidence interval bar does not cross the zero line.

action between the effects of losartan and UK-489,329, i.e., the effect of the combination was no greater than the sum of the individual effects of the drugs.

Renal Doppler Shift and Vascular Conductance. In experiment 1, the lower dose of candoxatrilat tended to cause reductions in renal Doppler shift and vascular conductance relative to the changes seen with the vehicle, although the differences were not significant (Figs. 3 and 4). Losartan alone had no significant effect on renal Doppler shift compared with the vehicle effect (Fig. 3), although it caused a significant increase in renal vascular conductance (Fig. 4). The renal vasodilator effects of losartan seemed to be maintained, but they were only significant on days 1 and 2 (11.9 and 25.6% difference, respectively), because of increased variability toward the end of the experiment (Fig. 4). In rats

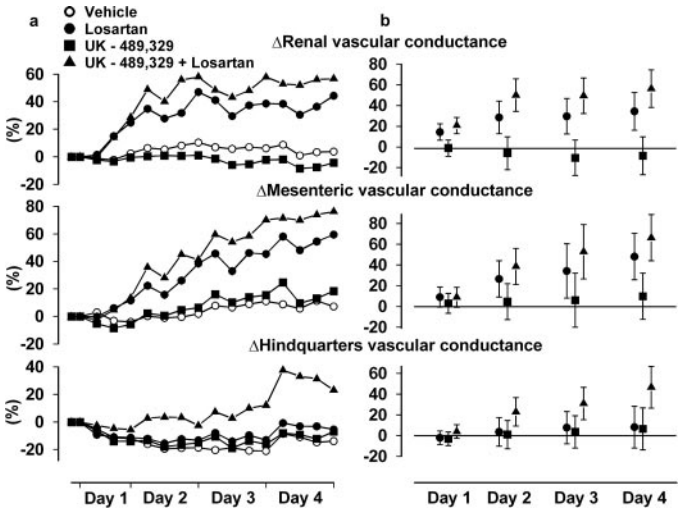


Fig. 10. Changes in regional vascular conductance over a 4-day continuous infusion of vehicle ($n = 8$), UK-489,329 (0.15 μg kg^{-1} min^{-1}; $n = 9$), losartan (8.5 μg kg^{-1} min^{-1}; $n = 9$), or UK-489,329 together with losartan (doses as described above; $n = 9$). a, values averaged over 105 min during the 7-h monitoring period on each day. b, estimated differences between each treatment group and vehicle with 95% confidence intervals. Treatment effects are significantly different from vehicle ($P < 0.05$) where the confidence interval bar does not cross the zero line.

treated with the combination of losartan and the lower dose of candoxatrilat, there was a tendency toward an increase in renal Doppler shift (significant on day 3) (Fig. 3) and marked, sustained increases in renal vascular conductance (significant on days 2–4; maximum difference 32.8%) (Fig. 4). There was evidence for interaction between the effects of losartan and candoxatrilat on renal vascular conductance (significant on days 2 and 3; $P < 0.05$), because the drugs given in combination caused an effect that was greater than the sum of their individual effects. However, because this interaction was influenced by an apparent decrease in conductance in the candoxatrilat alone group, the effect of the combination of candoxatrilat and losartan was not significantly greater than that of losartan alone.

In experiment 2, the higher dose of candoxatrilat had no significant effects on renal Doppler shift or vascular conductance relative to vehicle (Figs. 6 and 7). As in experiment 1, losartan had no effect on renal Doppler shift, but it caused an increase in renal vascular conductance, and in this group of animals there was less variability such that the renal vasodilator effects of losartan were significant on all experimental days (maximum 24.2% difference from vehicle). Rats given the combination of losartan and the higher dose of candoxatrilat also showed marked, and sustained, increases in renal vascular conductance (significant on days 1–4; maximum difference 35.9%). However, although the effects of the combination tended to be greater than the sum of the individual effects, the difference did not reach significance, and there was no evidence for interaction.

In experiment 3, UK-489,329 had no significant effects on renal Doppler shift or vascular conductance relative to vehicle, although there was a tendency for these variables to be reduced. In contrast, losartan alone caused a significant increase in renal Doppler shift (day 1) and vascular conductance (days 1–4; maximum difference 34.4%) (Figs. 9 and 10). In rats treated with the combination of losartan and UK-489,329, there was an increase in renal Doppler shift (days 1

and 2) (Fig. 9) and in renal vascular conductance (days 1–4, maximum difference 56.2%) (Fig. 10). Furthermore, there was evidence for interaction between the effects of losartan and UK-489,329 on renal vascular conductance (significant on days 2–4; $P < 0.05$), because the drugs given in combination caused an effect that was greater than the sum of the individual drug effects. Furthermore, the effect of the combination of UK-489,329 and losartan on days 2 and 4 was significantly greater than that of losartan alone by a maximum of 21.8%.

Mesenteric Doppler Shift and Vascular Conductance. In experiment 1, candoxatrilat (1.9 μg kg^{-1} min^{-1}), given alone, had no effects on mesenteric Doppler shift (Fig. 3) or vascular conductance (Fig. 4) relative to the vehicle. Losartan given alone, or in combination with candoxatrilat, increased the mesenteric Doppler shift (significant on day 4) (Fig. 3) and mesenteric vascular conductance (significant on days 2–4) (Fig. 4). The maximum effect on mesenteric vascular conductance of losartan alone (34.0% difference) was similar to the maximum effect of the combined treatments (32.5% difference); hence, there was no evidence for interaction between the effects of the drugs on mesenteric hemodynamics.

In experiment 2, the higher dose of candoxatrilat (6.4 μg kg^{-1} min^{-1}) was also devoid of significant effects on mesenteric Doppler shift and vascular conductance relative to the vehicle. As in the first experimental series, losartan caused sustained increases in mesenteric vascular conductance (significant on days 2–4; maximum 34.4% difference), although in this group there were no significant effects on mesenteric Doppler shift. Likewise, the combination of losartan and candoxatrilat caused increases in mesenteric vascular conductance (significant on days 2–4; maximum 30.0% difference), with no evidence for interaction between the effects of the drugs (Figs. 6 and 7).

In experiment 3, UK-489,329 given alone had no significant effects on mesenteric Doppler shift (Fig. 9) or vascular conductance (Fig. 10) relative to the vehicle. However, losartan alone increased the mesenteric Doppler shift (significant on day 4) (Fig. 9) and mesenteric vascular conductance (significant on days 1–4; maximum 48.2%) (Fig. 10). Losartan combined with UK-489,329 also increased the percentage of change in mesenteric Doppler shift (day 4) and vascular conductance (days 1–4; maximum 66.4% difference), but these effects were not significantly different from those of losartan alone, and there was no evidence for interaction between the effects of losartan and UK-489,329.

Hindquarters Doppler Shift and Vascular Conductance. In experiment 1, there were no changes in hindquarters Doppler shift in any treatment group that differed from the vehicle (Fig. 3). Losartan alone, or in combination with the low dose of candoxatrilat, tended to cause an increase in hindquarters vascular conductance on the last experimental day (Fig. 4), although the effect was only significant in the group given the combined treatment (22.2% difference).

In experiment 2, the group given the higher dose of candoxatrilat showed a small, but significant, reduction in the percentage of change in hindquarters Doppler shift on day 1 only; otherwise, there were no changes in hindquarters Doppler shift relative to the vehicle (Fig. 6). In this group of animals, losartan alone caused some increase in hindquarters vascular conductance, which was significant on day 3 (19.8% difference). Losartan in combination with candoxatrilat also caused a delayed increase in hindquarters vascular conductance (Fig. 7), which was significant on days 2–4 (maximum 31.3% difference). Although the effects of the combined treatment tended to be greater than the sum of the individual effects, the difference was not significant; hence, there was no evidence for interaction.

In experiment 3, UK-489,329 alone, and losartan alone, had no significant effects on hindquarters Doppler shift or vascular conductance, relative to vehicle (Figs. 9 and 10). However, the combination of losartan and UK-489,329 produced significant increases in hindquarters vascular conductance (significant on days 2–4; maximum 46.8% difference) (Fig. 10), although this did not result in significant effects on hindquarters Doppler shift as a consequence of the greater decrease in blood pressure in the combination group (Figs. 8 and 9). The effect of the combination on hindquarters vascular conductance was significantly greater than that of losartan alone on days 2–4, and there was evidence for an interaction between the effect of losartan and UK-489,329 (significant on day 4), because the combination showed a significantly greater effect than the sum of effects of each drug administered alone.

Discussion

Combined ACE/NEP inhibition as a therapeutic approach to treating hypertension has proven to be problematic due to a high incidence of angioedema, which has been attributed, at least in part, to the dual effects of ACE and NEP inhibition on bradykinin metabolism (Campbell, 2003). Because the incidence of angioedema is less with angiotensin receptor antagonists than with ACE inhibitors (Irons and Kumar, 2003), we reasoned that combined NEP inhibition with angiotensin receptor antagonism could provide an interesting alternative therapeutic strategy. To our knowledge, this is the first study to examine any possible interaction between the cardiovascular effects of angiotensin AT_1 receptor antagonism (with losartan) and NEP inhibition (with candoxatrilat or UK-489,329) in an in vivo setting. The experiments were performed in conscious SHR—a model that generally shows little or no hypotensive response to NEP inhibition (Koepke et al., 1990; Sybertz et al., 1990; Seymour et al., 1991; Pham et al., 1993, 1995; Sala et al., 1994; Tikkanen et al., 1998) but robust and reproducible antihypertensive responses to inhibition of the renin-angiotensin system, either by ACE inhibition (for reviews, see Rubin and Antonaccio, 1980; Unger et al., 1990) or by AT_1 receptor antagonism (Wong et al., 1990; Bunkenburg et al., 1991; Li and Widdop, 1996). Overall, the results provide no evidence for interaction between the antihypertensive effects of AT_1 receptor antagonism and NEP inhibition, although the renal vasodilator effects of combined treatment were generally greater than the sum of the individual effects.

We, like others (see above), found that NEP inhibition alone had only modest antihypertensive effects in SHR, but because none of the above-mentioned studies included regional hemodynamic measurements of the sort obtained here, we have extended these earlier observations. Thus, our findings, which show no significant regional vascular effects of candoxatrilat or UK-489,329, are novel and indicate that there are no underlying, regionally selective vasodilator ac-

tions of NEP inhibition being offset by vasoconstrictions in other vascular beds. Hence, the modest blood pressure reduction seen with the higher dose of candoxatrilat is likely to have been due to a fall in cardiac output (Sybertz et al., 1990; Pham et al., 1995), secondary to drug-induced natriuresis (Hirata et al., 1991), although some studies have failed to show any actions of NEP inhibition on indices of renal function in SHR (Sala et al., 1994).

The short half-life of NEP inhibitors in the circulation has been offered as one possible explanation for their modest cardiovascular effects (Weber, 2001). In all the above-mentioned studies in rats, NEP inhibitors have either been given by acute i.v. injection or chronically, in oral dosing regimes. Thus, it seems this is the first study to administer the drug continuously by i.v. infusion for longer than a few hours. But, even under such conditions, where the pharmacokinetic data indicate near-complete inhibition of NEP, no marked hemodynamic effects of NEP inhibition were seen.

One interpretation of the lack of a substantial blood pressure response to NEP inhibition in the SHR could be that increased angiotensin II levels, resulting from NEP inhibition (see Introduction) (Yamamoto et al., 1992), prevented the fall in blood pressure. If this was the case, then an interaction between the effects of losartan and candoxatrilat, or losartan and UK-489,329, on blood pressure might have been expected; however, this was not found. Thus, even though the higher dose of candoxatrilat had some antihypertensive effects itself, combined administration with losartan had no greater effect than the sum of the individual effects of the drugs given alone. Nevertheless, there was a trend for blood pressures to be lower in the groups receiving losartan in combination with either the high-dose candoxatrilat or UK-489,329 than in the corresponding groups receiving losartan alone, and the difference with UK-489,329 was statistically significant and biologically relevant (−14.6 mm Hg). Thus, combined angiotensin (AT_1) receptor antagonism with NEP inhibition may resemble combined ACE/NEP inhibition in providing a greater antihypertensive effect than angiotensin pathway antagonism alone.

We know of no other in vivo studies in which NEP inhibition has been combined with AT_1 receptor antagonism, but several studies have examined the effects of combined ACE and NEP inhibition on blood pressure in SHR, with variable results. Seymour et al. (1991) and Pham et al. (1993) both found greater antihypertensive effects of NEP inhibition when given in combination with ACE inhibition, although the former study did not test for statistical interaction between the effects of the drugs, and, in the latter study, the enhancement was most apparent in the first 30 min after the onset of drug treatment, with little or no difference at the end of a 2-h recording period. Indeed, in a later study by Pham et al. (1995) the fall in blood pressure with combined ACE and NEP inhibition tended to be less than the expected sum of the individual effects, although, statistically, the antihypertensive effects of combined treatment did not differ from those of ACE alone. Likewise, Tikkanen et al. (1998) found that, in nondiabetic SHR, combined ACE and NEP inhibition was no more effective at lowering blood pressure than ACE inhibition alone.

It has been suggested that the lack of positive interaction between the effects of ACE and NEP on blood pressure is due to a greater vasodilatation being offset by an increase in cardiac output, consequent upon the reduction in afterload (Seymour et al., 1993; Pham et al., 1995). However, in the present study, a positive interaction between the effects of candoxatrilat and losartan was only apparent in the renal vascular bed, and only significant at the lower dose of candoxatrilat. A positive interaction between the effects of UK-489,329 and losartan was also seen in the renal vascular bed, and this combination of drugs additionally augmented hindquarters vasodilatation, consistent with angiotensin II opposing the vasodilator actions of NEP inhibition. The interactive effects of UK-489,329 and losartan on renal and hindquarters hemodynamics are consistent with the greater blood pressure-lowering effect of this combination. The reason for the differences observed between candoxatrilat and UK-489,329 are unclear, although it is notable that only the former would have inhibited SEP. Although the cardiovascular consequences of SEP inhibition are unknown, it is feasible that inhibition of the breakdown of vasoconstrictor peptides was more effective in the presence of candoxatrilat, due to inhibition of SEP in addition to NEP.

Antihypertensive effects of losartan (or its metabolite EXP 3174) in SHR have been reported previously (Wong et al., 1990; Bunkenburg et al., 1991; Li and Widdop, 1996), but ours is the first study to measure the regional hemodynamic effects of continuous administration of the drug over several days. Here, we showed that the vasodilator effects of losartan were more pronounced in the renal and mesenteric vascular beds than in the hindquarters. This regional hemodynamic pattern is consistent with the effects of administration of exogenous angiotensin II, which causes much less vasoconstriction in the hindquarters than in the renal or mesenteric circulations (Gardiner et al., 1993). We have recently reported the regional hemodynamic responses to ACE inhibition in conscious SHR, using the same experimental paradigm as in the present study, i.e., continuous i.v. infusion over 4 days in chronically instrumented animals (Gardiner et al., 2004, 2005). In those studies, an antihypertensive dose of enalaprilat was shown to be associated with widespread vasodilatation, although the magnitude of effect was greater in the renal and mesenteric vascular beds than in the hindquarters. Preferential renal vasodilator actions of AT_1 receptor antagonism have been reported in SHR (Li and Widdop, 1996), but that study used a bolus i.v. dose of the antagonist, and measurements were only made over a 6-h period.

In conclusion, the present results show clearly that chronic AT_1 receptor antagonism with losartan has more marked, sustained, antihypertensive effects in conscious SHR than does NEP inhibition with either candoxatrilat or UK-489,329. Furthermore, the antihypertensive effect of losartan is associated with vasodilatation, whereas the NEP inhibitors used were both devoid of regional vasodilator effects. There was a trend for the combination of either NEP inhibitor and losartan to reduce blood pressure to a greater extent than losartan alone, but there was no evidence that the antihypertensive effect of losartan was enhanced in a supra-additive manner by simultaneous NEP inhibition. Although combined AT_1 receptor antagonism and NEP inhibition generally caused greater renal vasodilatation than the sum of the individual drug effects, whether this would provide added clinical benefit remains to be explored. In SHR, an antihypertensive dose of losartan has no effect on plasma levels of bradykinin (Campbell et al., 1995), but whether

angiotensin receptor antagonists affect any NEP-induced influence on bradykinin metabolism is unknown. We did not measure circulating bradykinin concentrations in the present study, but this would be an interesting area for further research.

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