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Vasopeptidase inhibition attenuates the progression of renal injury in subtotal nephrectomized rats

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Vasopeptidase inhibition attenuates the progression of renal injury in subtotal nephrectomized rats.

Background. Vasopeptidase inhibitors are a new class of cardiovascular compounds that inhibit both angiotensin-converting enzyme (ACE) and neutral endopeptidase (NEP). The aim of the present study was to explore the effects of omapatrilat, a vasopeptidase inhibitor, on renal function and pathology in subtotally nephrectomized (STNx) rats.

Methods. STNx rats were randomized to four groups and treated for 12 weeks: no treatment ($N = 14$); omapatrilat at a low dose of 10 mg/kg (L, $N = 12$) and at a high dose of 40 mg/kg (H, $N = 10$); or an ACE inhibitor, fosinopril, at a dose of 10 mg/kg ($N = 12$). Sham-operated rats were used as control animals ($N = 12$).

Results. Elevated blood pressure in STNx rats (174 ± 9 mm Hg) was reduced by omapatrilat in a dose-dependent manner (L, 121 ± 3 mm Hg; H, 110 ± 3 mm Hg) and by fosinopril (149 ± 5 mm Hg). Proteinuria in STNx rats (246 ± 73 mg/day) was reduced by treatment with fosinopril (88 ± 21 mg/day) and was normalized by treatment with omapatrilat (L, 30 ± 4 mg/day; H, 20 ± 2 mg/day vs. control 25 ± 1 mg/day). Decreased glomerular filtration rates, elevated plasma urea and creatinine and glomerulosclerosis, and tubulointerstitial fibrosis were ameliorated by omapatrilat and fosinopril to a similar degree. Compared with fosinopril, omapatrilat treatment was associated with increased plasma renin activity and decreased renal ACE and NEP binding in a dose-dependent manner.

Conclusion. These findings suggest that vasopeptidase inhibition may provide a useful strategy for the treatment of progressive renal disease.

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Vasopeptidase inhibitors are a novel and effective strategy for treating cardiovascular diseases, including hypertension and heart failure. By simultaneously inhibiting both angiotensin-converting enzyme (ACE) and neutral endopeptidase (NEP), vasopeptidase inhibitors are associated with reduction in angiotensin II formation and prevention of atrial natriuretic peptide (ANP) degradation [1]. The vasopeptidase inhibitors, via interrupting multiple vasoactive hormone pathways, may offer advantages over currently available therapies such as renin-angiotensin system (RAS) blockade with an ACE inhibitor, in terms of greater blood pressure reduction and cardiovascular benefits, with this issue under intensive investigation [2].

Although chronic administration of the ACE inhibitors is associated with attenuation of progressive renal injury in subtotal nephrectomized (STNx) rats [3–7], progression of renal injury is not arrested. Alternative or adjunctive approaches warrant investigation. In the STNx model of renal injury, short-term treatment with an NEP inhibitor increased urinary ANP and modulated natriuresis without improving blood pressure or protein excretion [8, 9]. However, CGS 30440, a dual ACE/NEP vasopeptidase inhibitor, conferred greater reduction in proteinuria than benazepril, an ACE inhibitor, despite similar effects on blood pressure [10]. At both low dose and high dose, CGP 30440 achieved a similar reduction in blood pressure and proteinuria over a period of six weeks [10]. Whether further lowering blood pressure with a vasopeptidase inhibitor could further ameliorate proteinuria in this model of progressive renal injury remains unexplored. Furthermore, the effect of vasopeptidase inhibitor on renal ACE and NEP has not been assessed.

The aim of the present study was to explore the effects of long-term administration of omapatrilat, a vasopeptidase inhibitor, at both low dose and high dose, in retarding the progression of renal injury. In addition, the effect of omapatrilat on inhibition of renal NEP and ACE in the STNx rats was also examined.

METHODS

Experimental protocol

Experimental procedures were in accordance with the National Health and Medical Research Council of Australia guidelines for animal experimentation. STNx (N = 48) or sham surgery (control, N = 12) was performed in eight-week-old male Sprague-Dawley rats (body weight of 220 to 310 g) as described previously [6, 7]. In brief, the STNx was performed by right nephrectomy, followed by infarction of approximately two thirds of the left kidney with selective ligation of all but one extrarenal branch of the left renal artery. Anesthesia was achieved by intraperitoneal injection of pentobarbitone sodium (60 mg/kg body weight; Boehringer Ingelheim, Artarmon, NSW, Australia). Following subtotal nephrectomy, the animals were randomly allocated to an untreated group (STNx, N = 14) or the treatment with either a vasopeptidase inhibitor or an ACE inhibitor for 12 weeks. The vasopeptidase inhibitor omapatrilat (Bristol-Myers Squibb Pharmaceutical Research Institute, Princeton, NJ, USA) was administered by daily gavage at a low dose of 10 mg/kg/day (omapatrilat low dose, N = 12) or at a high dose of 40 mg/kg/day (omapatrilat high dose, N = 10). The ACE inhibitor fosinopril (Bristol-Myers Squibb Pharmaceutical Research Institute) was administered at a dose of 10 mg/kg/day by gavage (fosinopril, N = 12).

The rats had unrestricted access to water and standard rat chow. Systolic blood pressure (SBP) was measured by indirect tail-cuff plethysmography in prewarmed unanesthetized animals as previously described [11], every four weeks after surgery. At the end of the experiment, animals were anesthetized by intraperitoneal injection of pentobarbitone sodium (60 mg/kg body weight). A midline incision of the abdomen was made, and the remnant kidney was removed and weighed. A portion of kidney was fixed in 10% formalin and embedded with paraffin with standard procedure. Four-micron paraffin sections of kidney were used for histopathology. Another portion of the kidney was frozen in liquid nitrogen and stored at −80°C for the in vitro autoradiographic studies.

Assessment of renal function

Glomerular filtration rate (GFR) was measured using the ^{99m}Tc-DTPA method at the conclusion of the experiment [12]. Prior to sacrifice, animals were housed in metabolic cages for 24 hours for collection of urinary samples and measurement of urinary protein excretion using the Coomassie Brilliant Blue Method [13]. Plasma urea and creatinine concentrations were measured by autoanalyzer (Beckman Instruments, Palo Alto, CA, USA). Blood samples were collected from the tail vein of conscious rats before the animals were sacrificed for the measurement of plasma renin activity (PRA) [14].

Kidney histopathology

Assessment of glomerulosclerosis and tubulointerstitial injury was performed as described previously [7, 15]. In brief, 30 glomeruli in each kidney were graded from 0 to 4 according to the severity of the glomerular sclerosis: 0 = normal; 1 = slight glomerular damage, the mesangial matrix and/or hyalinosis with focal adhesion, involving <25% of the glomerulus; 2 = sclerosis of 25 to 50%; 3 = sclerosis of 50 to 75%; and 4 = sclerosis of >75% of the glomerulus [7, 15]. Twenty fields of tubulointerstitial area in the cortex were observed and graded as follows: 0 = normal; 1 = the area of interstitial inflammation and fibrosis, tubular atrophy, and dilation, with cast formation involving <25% of the field; 2 = lesion area between 25 and 50% of the field; and 3 = lesions involving >50% of the field [7, 15]. Indices of glomerular damage or tubulointerestitial lesion were calculated by averaging the grades assigned to all glomeruli, or tubular fields. The data were expressed relative to the control group.

In vitro autoradiography for renal ACE and NEP

Angiotensin-converting enzyme and NEP binding in the kidney (N = 5 per group) were assessed using in vitro quantitative autoradiography as previously described [16–18]. The rat kidneys (N = 5, per group) were obtained after the animals were anesthetized with an intravenous injection of pentobarbitone sodium (60 mg/kg). The kidneys were removed, bisected, and snap frozen in liquid nitrogen-cooled isopentane and stored at −20°C. Twenty-micron sections were cut on a cryostat at −20°C and then dehydrated overnight under reduced pressure at 4°C.

The sections were preincubated for 15 minutes in 10 mmol/L sodium phosphate buffer (pH 7.4). For renal ACE binding, sections were incubated in a fresh volume of the same buffer containing selective ligand for ACE, ^{125}I-MK351A (a tyrosyl derivative of enalaprilic acid) [19], at room temperature for two hours. For renal NEP binding, sections were incubated with same buffer containing selective ligand for NEP, ^{125}I-RB104, 2-[(3-iodo-4-hydroxy)-phenylmethyl]-4-N-[3-(hydroxyamino-3-oxo-1-phenylmethyl)-propyl]amino-4-oxobutanoic acid, at room temperature for two hours [20]. After incubation, the sections were washed four times for one minute each in ice-cold buffer and were air dried at room temperature. All slides, including a set of radioactivity standards, were exposed to Kodak BioMax x-ray film at room temperature for five days [9]. Following exposure, the films were processed and the optical densities quantitated by a microcomputer imaging device (MCID Imaging System, St. Catherines, Ontario, Canada) connected to an IBM AT computer. The computer program using the radioactivity standards constructed a calibration curve of optical density versus radioactivity density. Specific

Table 1. Body weight, kidney weight, and kidney to body weight ratio

Group	*N*	Body weight *g*	Mean SBP *mm Hg*	PRA angiotensin 1 *nmol/L/h*	Kidney weight *g*	Kidney:body weight *mg/g*
Control	12	589 ± 11	130 ± 2	10.3 ± 0.4	1.98 ± 0.04	3.35 ± 0.11
STNx	14	411 ± 31[a]	174 ± 9[a]	1.7 ± 0.2[a]	2.09 ± 0.39	4.51 ± 0.77[a]
Fosinopril	12	415 ± 17[a]	149 ± 5[b]	7.1 ± 1.6[b]	1.95 ± 0.14	4.61 ± 0.17
Omapatrilat						
Low dose	12	414 ± 22[a]	121 ± 3[bc]	9.1 ± 1.4[b]	1.77 ± 0.11	4.30 ± 0.17
High dose	9	411 ± 24[a]	110 ± 3[abc]	19.0 ± 2.1[abc]	1.50 ± 0.14[b]	3.68 ± 0.29[b]

Abbreviations are: SBP, systolic blood pressure; PRA, plasma renin activity; STNx, subtotal nephrectomy.
[a] $P < 0.01$ vs. control
[b] $P < 0.01$ vs. STNx
[c] $P < 0.01$ vs. fosinopril

binding densities were calculated as the difference between total and nonspecific binding densities.

Statistical analysis

Data were analyzed by analysis of variance (ANOVA) using the Statview SE program (Brainpower, Calabasas, CA, USA) on a Macintosh iMac Computer (Cupertino, CA, USA). Comparisons of group means were performed by Fisher's least significant difference method. Data are shown as mean ± SEM unless otherwise specified. PRA data were analyzed after logarithmic transformation. A *P* value of less than 0.05 was viewed as statistically significant.

RESULTS

Body weight and kidney weight

Subtotally nephrectomized rats, regardless of their treatment, gained similar weight over the period of experiment, which was much less than that observed in control animals (Table 1). Kidney weight and the ratio of kidney:body weight were increased in untreated STNx rats compared with control rats. Rats treated with the vasopeptidase inhibitor omapatrilat tended to have a lower kidney weight and kidney:body weight ratio, but omapatrilat only at the high dose was associated with reduced kidney weight and kidney:body weight ratio (Table 1).

Systolic blood pressure

Serial SBP values at weeks 4, 8, and 12 after surgery are shown in Figure 1, and the mean values of these measurements are calculated (Table 1). SBP was elevated in untreated STNx rats. The vasopeptidase inhibitor omapatrilat ameliorated the rise in SBP in a dose-dependent manner. Rats treated with low-dose omapatrilat had similar blood pressure to that observed in control rats. Rats treated with high-dose omapatrilat had lower blood pressure than rats treated with omapatrilat at low dose or the control group. The ACE inhibitor fosinopril reduced

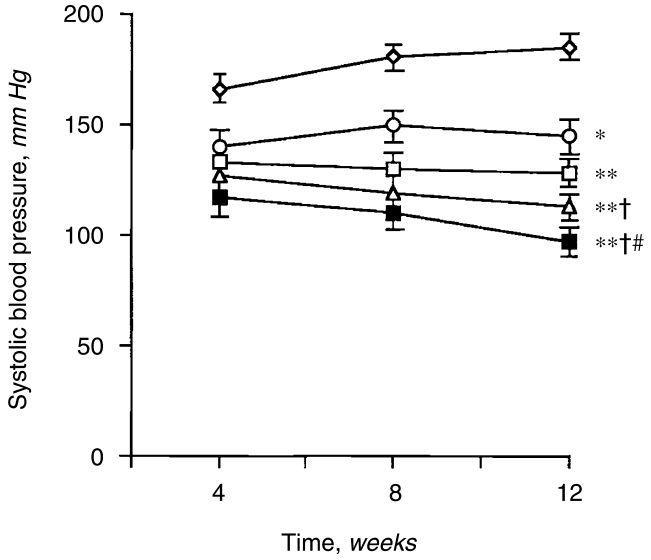

Fig. 1. Data for systolic blood pressure (SBP) are shown at weeks 4, 8, and 12 in the control (□), subtotally nephrectomized (STNx; ◇), or STNx treated with omapatrilat low dose (△), high dose (■), or fosinopril (○) groups. Data are mean ± SEM; *$P < 0.05$; **$P < 0.01$ vs STNx; †$P < 0.01$ vs fosinopril; #$P = 0.012$, Omapatrilat L vs H; ANOVA with repeated measures.

mean blood pressure by approximately 25 mm Hg, less than that achieved with low-dose omapatrilat.

Plasma renin activity

Subtotally nephrectomized rats were associated with reduced PRA when compared with control animals (Table 1). The administration of omapatrilat was associated with increased PRA in a dose-dependent manner, with the level in the lower dose being similar to and in the high dose being higher than that observed in sham animals (Table 1). PRA was increased by treatment with fosinopril to similar levels as observed in sham rats or STNx rats treated with the lower dose of omapatrilat (Table 1).

Urinary protein excretion

Urinary protein excretion was significantly increased in STNx rats (median 258 mg/day, range 106 to 637 mg/

Table 2. Glomerular filtration rate (GFR), plasma urea, and creatinine concentrations

Group	GFR *mL/min/kidney*	Plasma urea *mmol/L*	Plasma creatinine *μmol/L*	Proteinuria *mg/day*
Control	3.2 ± 0.2	7 ± 1	50 ± 2	24 ± 1
STNx	0.71 ± 0.1[a]	22 ± 2[a]	106 ± 7[a]	262 ± 73[a]
Fosinopril	1.1 ± 0.1[ab]	16 ± 1[ab]	84 ± 5[ab]	88 ± 21[ab]
Omapatrilat				
Low dose	1.1 ± 0.1[ab]	18 ± 1[ab]	83 ± 2[ab]	30 ± 4[b]
High dose	1.0 ± 0.1[ab]	18 ± 1[ab]	87 ± 5[ab]	20 ± 1[bc]

GFR is for two kidneys in sham rats and in subtotally nephrectomized (STNx) groups represents remnant kidney alone.
[a] $P < 0.01$ vs. control
[b] $P < 0.01$ vs. STNx
[c] $P < 0.01$ vs. fosinopril

day) when compared with sham rats (median 23 mg/day, range 17 to 25 mg/day; Table 2). Treatment with omapatrilat was associated with reduction in proteinuria in a dose-dependent manner (low-dose median 26 mg/day, range 9 to 55 mg/day; high-dose median 18, range 7 to 34 mg/day), with the lower dose vasopeptidase inhibitor being associated with levels of proteinuria similar to that seen in sham animals (Table 2). Proteinuria was reduced in fosinopril-treated rats (median 71mg/day, range 19 to 242 mg/day), but to a level that was still higher than in control or STNx animals treated with either dose of omapatrilat (Table 2).

GFR, plasma urea, and creatinine clearance

Subtotally nephrectomized rats had decreased GFR and increased plasma urea and creatinine levels when compared with control animals (Table 2). These parameters were reduced to similar levels with all treatments and were still higher than that observed in control animals (Table 2). There were no significant differences in GRF among STNx rats treated with either omapatrilat or fosinopril (Table 2).

Kidney histology

Subtotally nephrectomized rats developed glomerulosclerosis (Fig. 2A) and tubulointerstitial fibrosis (Fig. 2B) compared with control animals. Both omapatrilat and fosinopril reduced glomerulosclerosis and tubulointerstitial fibrosis to a similar extent.

In vitro autoradiography for renal ACE and NEP

In kidneys of control rats, ACE (Fig. 3A) and NEP (Fig. 3D) binding was noted in the cortex and inner medulla. In STNx, renal NEP was reduced compared with control rats where renal ACE trended to elevation compared with control, but did not reach significance (Table 3 and Fig. 3). Treatment with omapatrilat causes a dose-dependent inhibition of renal NEP and ACE binding. ACE binding was reduced by omapatrilat in a dose-dependent manner compared with untreated STNx rats and to a greater extent than fosinopril (Table 3).

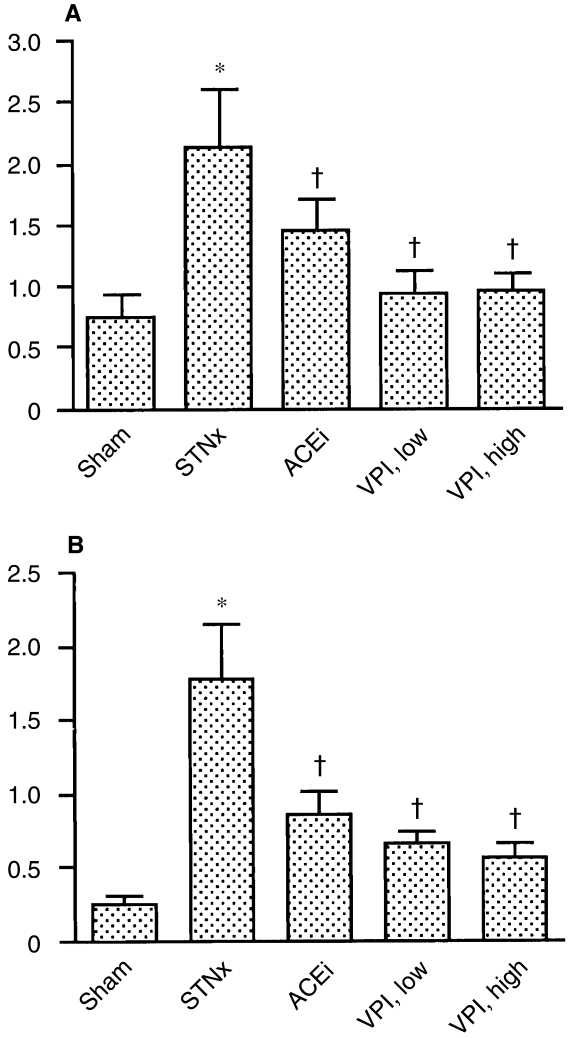

Fig. 2. Glomerulosclerosis indices (*A*) and tubular injury indices (*B*) in rats of the control, subtotally nephrectomized (STNx), or STNx treated with omapatrilat or fosinopril groups. *$P < 0.01$ vs control; †$P < 0.01$ vs STNx.

DISCUSSION

The present study demonstrates the beneficial effects of long-term administration of omapatrilat, in a dose-dependent manner, on reducing blood pressure and proteinuria and retarding glomerulosclerosis and tubulointerstitial

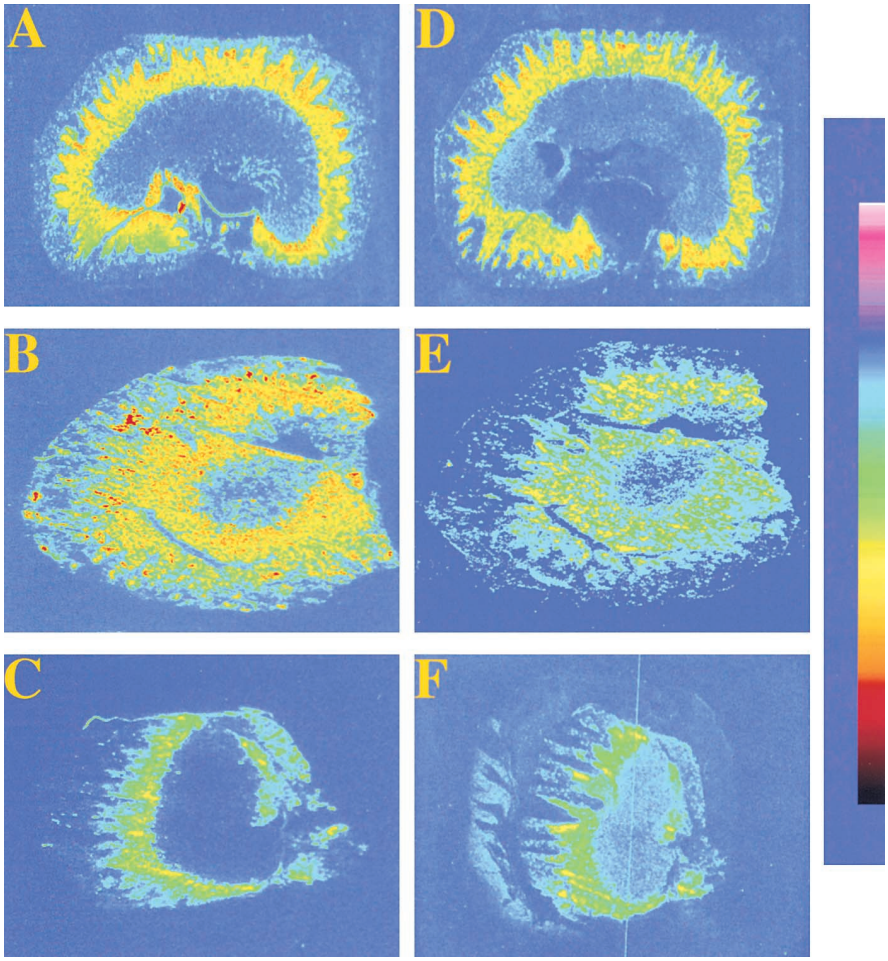

Fig. 3. Representative macroscopic autoradiographs of renal angiotensin-converting enzyme (ACE) in control (*A*), subtotally nephrectomized (STNx) (*B*) and omapatrilat high dose (*C*), and renal NEP in control (*D*), STNx (*E*), and omapatrilat high dose (*F*) groups. Reproduction of this figure in color was made possible by a grant from Bristol-Myers Squibb, Princeton, NJ, USA.

Table 3. Renal angiotensin-converting enzyme (ACE) and neutral endopeptidase (NEP) binding

Group	ACE binding	NEP binding
	%	
Control	100 ± 5	100 ± 5
STNx	110 ± 5	89 ± 4[a]
Fosinopril	82 ± 4[ab]	78 ± 4[a]
Omapatrilat		
Low dose	58 ± 9[abc]	80 ± 3[a]
High dose	30 ± 6[abcd]	62 ± 6[abcd]

Data are expressed as the percentage of the binding compared to sham.
[a] $P < 0.05$ vs. control, [b] $P < 0.01$ vs. STNx, [c] $P < 0.01$ vs. fosinopril
[d] $P < 0.01$ vs. low-dose omapatrilat

fibrosis in progressive renal injury. In the present study, the beneficial effects of omapatrilat were associated with a concomitant inhibition of renal ACE and NEP activity, as assessed by quantitative autoradiography with specific ligands for these vasoactive enzymes. These findings extend the results of previous studies showing beneficial effects of vasopeptidase inhibitor in ameliorating renal injury [10], and suggest that vasopeptidase inhibition may be a therapeutic approach for retarding progression of renal injury in which reducing both blood pressure and proteinuria are considered important targets for retarding progression of renal injury.

In the present study using STNx rats, omapatrilat reduced blood pressure in a dose-dependent manner, consistent with its hypotensive effects in other hypertensive models, including those with low, normal, and high renin models of hypertension [21, 22]. The hypotensive action of vasopeptidase inhibition has now been observed in clinical studies [23].

Since omapatrilat reduced proteinuria in a dose-dependent manner in an association with reduction in blood pressure, its effect on proteinuria may be entirely pressure related. Indeed, it has been demonstrated that with similar blood pressure reduction, blockade of the RAS with either an ACE inhibitor or an angiotensin type 1 (AT1) receptor antagonist conferred a similar degree of renoprotection [5]. This issue has been further explored by using accurate radiotelemetric assessment of blood pressure in the subtotal nephrectomy model [24]. These studies also showed the pressure-dependent nature of the benefits conferred by RAS blockers [24], as has a recent study by our group in which a combination of an ACE inhibitor and an AT1 receptor antagonist conferred

a greater reduction in proteinuria and blood pressure than single agent therapy [7].

Treatment with the vasopeptidase inhibitor was associated with preservation of renal function, as assessed by improved GFR and lower plasma urea and creatinine concentrations compared with untreated STNx rats. The effects of vasopeptidase inhibition on renal function were, however, similar to that achieved with an ACE inhibitor. Although administration of either a vasopeptidase inhibitor or an ACE inhibitor had no influence on weight gain, vasopeptidase inhibitor-treated rats had reduced kidney weight and kidney to body weight ratio compared with the ACE inhibitor-treated rats. The mechanisms underlying these differences in kidney weight are uncertain. Since vasopeptidase inhibitor or ACE inhibitor-treated rats gained similar weight over the period of experiment, a lower kidney weight and a subsequent lower kidney/body weight ratio in vasopeptidase inhibitor-treated animals may represent a specific antitrophic effect of this agent, which warrants further examination.

Vasopeptidase inhibitor therapy was associated with similar amelioration of glomerulosclerosis and tubulointerstitial fibrosis, as obtained by the ACE inhibitor alone, even though vasopeptidase inhibition conferred greater hypotensive effects. Furthermore, the effect of omapatrilat on renal pathology was not further enhanced at a higher dose. These findings are consistent with previous observations, which showed a similar relationship between blood pressure reduction conferred by the RAS blockers and amelioration of renal pathological injury in this model [7]. Specifically, we observed in that study that greater hypotensive and antiproteinuric efficacy of combined ACE inhibition and AT1 receptor blockade was not associated with a further improvement in renal pathology [7].

There appeared to be a direct relationship between blood pressure reduction and the degree of proteinuria in this study in STNx rats. The treatments associated with the greatest blood pressure reduction were most closely linked to less proteinuria. In contrast, structural indices of renal injury were not as closely linked to blood pressure reduction. This may reflect the ability of this class of agents that interrupt the RAS, even at a suboptimal dose of blood pressure reduction, to confer excellent renal protection at the structural level. Another possibility is that although blood pressure is a major determent of injury in this model, other factors such as the renal response to renal mass reduction are not influenced by blood pressure reduction and, therefore, cannot be totally abrogated by this form of treatment. Finally, one cannot exclude subtle differences in these treatments on renal morphological abnormalities that would require more sophisticated assessment of glomerular and tubulointerstitial ultrastructure. The difference in drug effects on proteinuria and renal structure may indicate that various pathological pathways are involved in the development of proteinuria and renal structural injury. Such a possibility has been recently suggested from studies using a neutralizing antibody to transforming growth factor-β [25]. This treatment in a model of diabetic nephropathy was associated with a reduction in renal fibrosis, yet no effects on albuminuria [25].

Tissue ACE measurements have been used previously as a marker of the degree of inhibition of local ACE activity in various organs including the kidney [26]. Reduced kidney ACE binding has been demonstrated after administration of various ACE inhibitors and was shown to correlate with the hypotensive efficacy of the drugs [27]. Compared with untreated rats, both fosinopril and omapatrilat inhibited renal ACE binding with the degree of inhibition correlating with their hypotensive effects. In the present study, an increase in PRA was seen with both drugs, particularly with a high dose of omapatrilat. These findings are consistent with a recent study showing more potent effects of omapatrilat compared with fosinopril in inhibiting angiotensin I hydrolysis [28].

Although kidney NEP activity was mildly decreased in untreated rats compared with control rats, omapatrilat also inhibited renal NEP activity in a dose-dependent manner, suggesting that NEP inhibition, as an additional factor, may have contributed to its efficacy as a hypotensive agent and in conferring renoprotection. Compared with the fosinopril, the vasopeptidase inhibitor omapatrilat was associated with greater inhibition on both renal ACE and NEP and increased PRA. These findings suggest that the superiority in reducing blood pressure and proteinuria with vasopeptidase inhibition involves not only NEP inhibition, but also ACE inhibition.

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APPENDIX

Abbreviations used in this article are: ACE, angiotensin-converting enzyme; ANP, atrial natriuretic peptide; AT1, angiotensin II type 1; GFR, glomerular filtration rate; NEP, neutral endopeptidase; PRA, plasma renin activity; RAS, renin-angiotensin system; SBP, systolic blood pressure; STNx, subtotally nephrectomized.

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