Nephrol Dial Transplant (2001) 16: 1176–1182

Nephrology Dialysis Transplantation

Original Article

Chronic vasopeptidase inhibition restores endothelin-converting enzyme activity and normalizes endothelin levels in salt-induced hypertension

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Abstract

Background. Vasopeptidase inhibition (VPI) represents a new therapeutic principle including both inhibition of angiotensin-converting enzyme (ACE) and neutral endopeptidase (NEP). The present study investigated the effect of the vasopeptidase inhibitor omapatrilat on endothelin-1 (ET-1)-mediated vascular function in salt-induced hypertension.

Methods. Dahl salt-sensitive rats ($n=6$/group) on standard or salt-enriched (4% NaCl) chow were treated for 8 weeks with either omapatrilat (36 ± 4 mg/kg/day), captopril (94 ± 2 mg/kg/day) or placebo. Aortic and renal artery segments were isolated and suspended in organ chambers for isometric tension recording. Functional endothelin-converting enzyme (ECE) activity was assessed in native segments and after preincubation with omapatrilat. Furthermore, vascular ECE protein levels as well as plasma and tissue ET-1 levels were determined.

Results. The increase in systolic blood pressure of salt-fed rats was prevented by omapatrilat and captopril to a comparable degree. In salt-induced hypertension, functional ECE activity (calculated as the ratio of the contraction to big ET-1 divided by the contraction to ET-1) in renal arteries (0.46 ± 0.05) and in aorta (0.68 ± 0.05) was reduced as compared with control animals (0.9 ± 0.05 and 0.99 ± 0.04, respectively; $P<0.05$). While omapatrilat *in vitro* blunted the response to big endothelin-1 (big ET-1) and diminished ECE activity further ($P<0.01$ *vs* native segments), chronic treatment with omapatrilat *in vivo* restored contractions to ET-1 ($120\pm6\%$) and big ET-1 ($98\pm9\%$) in renal arteries, and therefore normalized renovascular ECE activity. In addition, omapatrilat normalized plasma ET-1 concentrations (12.9 ± 1.2 *vs* 16.6 ± 1.4 pg/ml on high salt diet; $P<0.05$) and renovascular ECE protein levels.

Conclusions. In salt-induced hypertension, vasopeptidase inhibition restores alterations in the endothelin system, such as renovascular ECE activity and responsiveness to ET-1 and big ET-1 with chronic but not acute *in vitro* application. Thus, the beneficial effects of vasopeptidase inhibition may reflect a resetting of cardiovascular control systems and therefore may be particularly suited to treat hypertension and heart failure.

Keywords: endothelin; endothelium; hypertension; neutral endopeptidase; nitric oxide

Introduction

Vasopeptidase inhibition represents a new therapeutic principle in hypertension [1] and heart failure [2], which includes inhibition of both neutral endopeptidase (NEP) and angiotensin-converting enzyme (ACE). NEP catalyses the degradation of a number of endogenous vasodilator peptides, including atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), C-type natriuretic peptide (CNP), substance P and bradykinin, as well as vasoconstrictor peptides, including endothelin-1 (ET-1) and angiotensin II [3]. Hence, the effects of NEP inhibition on vascular tone will result from its overall effects on different vasoactive substances. In particular, the effects of NEP inhibition on the endothelin system are rather complex and will depend on its influence on production and breakdown of ET-1.

Vasopeptidase inhibition may be particularly useful in the treatment of hypertension [4] and heart failure [5] as the neurohumeral dysregulation appears to be more effectively corrected [6]. Omapatrilat is a new vasopeptidase inhibitor which induces long lasting antihypertensive effects in experimental hypertension greater than those elicited by selective inhibition of either enzyme alone [7]. Meanwhile, first clinical data are available, demonstrating haemodynamic benefits

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of treatment with omapatrilat in patients with heart failure [5] and a positive effect of omapatrilat on vessel stiffness [8], vascular remodelling [9] and renal function [10]. Despite obvious clinical benefits of vasopeptidase inhibitors in hypertension and heart failure, their mechanism of action is still poorly understood. Neurohumoral influences of vasopeptidase inhibitors on the renal circulation may substantially alter renal haemodynamics and therefore contribute to their beneficial systemic effects in both hypertension and chronic heart failure. As it is an extensive debate, whether and how alterations of the endothelin system may contribute to hypertension [11], and since local differences in the regulation of the endothelin system appear to be important [12], the role of the renal endothelin system becomes a target of major interest.

Elevated ET-1 plasma and tissue levels have been demonstrated to contribute to hypertrophic remodelling in resistance arteries, particularly in salt-sensitive hypertension [13], and therefore may play a role in the maintenance of hypertension. Through activation of ET_A-receptors, ET-1 exerts vasoconstriction and proliferation in this model of hypertension. Correspondingly, the endothelin system provides a wide range of possible interactions with the combination of ACE and NEP inhibition. As vasopeptidase inhibitors exert complex neurohumoral as well as haemodynamic changes in the cardiovascular system, their acute and chronic effects on the endothelin system may differ substantially.

Therefore, the present study was designed to investigate the effects of long-term treatment with the vasopeptidase inhibitor omapatrilat *vs* its acute *in vitro* effects on the activity of the endothelin-converting enzyme (ECE) in salt-induced hypertension.

Methods

Animals

Male Dahl salt-sensitive rats of 12 weeks of age were obtained from Charles River WIGA GmbH (Sulzfeld, Germany) and randomly assigned to one of four treatment regimens: (i) standard chow (control); (ii) salt-enriched (4% NaCl) chow (Harlan Teklad, Madison, WI, USA) given alone (salt diet); (iii) chow given together with omapatrilat (salt + O); or (iv) chow given with captopril (salt + C). Omapatrilat and captopril were provided by Bristol-Myers Squibb Pharmaceutical Research Institute (Princeton, NJ, USA). The rats were treated for 8 weeks, and chow and drug intake was monitored during the entire study. Systolic arterial blood pressure (SBP) and heart rate (HR) were measured by the tail-cuff method using a pulse transducer (model LE 5000, Letica, Barcelona, Spain). The study design and the experimental protocols were approved by the institutional animal care committee (Kommission für Tierversuche des Kantons Zürich, Switzerland).

Tissue harvesting

Animals were anaesthetized with pentobarbital (50 mg/kg i.p.) after 8 weeks of treatment, and blood samples were collected through puncture of the right ventricle. The aorta and the renal arteries were removed and placed immediately into cold (4°C) modified Krebs–Ringer bicarbonate solution (in mmol/l): NaCl, 118.6; KCl, 4.7; $CaCl_2$, 2.5; $MgSO_4$, 1.2; KH_2PO_4, 1.2; $NaHCO_3$, 25.1; EDTA, 0.026; glucose, 10.1. Under a microscope (Leica Wild M3C, Heerbrugg, Switzerland), vessels were cleaned of adherent tissue and cut into segments 4 and 3 mm long for aorta and renal artery, respectively.

Organ chamber experiments

Vessel segments were suspended to fine tungsten stir-ups (diameter 50 μM), placed in an organ bath filled with 25 ml Krebs solution for isometric tension recording as described previously [14]. Cumulative concentration-response curves to ET-1 (10^{-10}–10^{-7} mol/l) and big endothelin-1 (big ET-1; 10^{-9}–10^{-7} mol/l) were obtained in quiescent preparations. In additional experiments, vessel segments of control animals and animals on the high-salt diet were preincubated with omapatrilat 10^{-7} mol/l for 30 min. ET-1 and big endothelin were purchased from Novabiochem/Calbiochem AG (La Jolla, CA, USA), whereas AT I and AT II were obtained from Sigma Chemical Co. (Buchs, Switzerland).

Renovascular ECE protein levels

ECE protein levels were determined in renal artery tissue. After homogenization of renal artery segments, equal amounts of protein was used for electrophoresis and comparable loading was confirmed by silver staining. The protein was transferred onto ImmobilonTM-P filter papers (Millipore AG, Volketswil, Switzerland) with a semi-dry transfer unit. The membranes were subsequently blocked by using 2% skimmed milk in phosphate-buffered saline (PBS)–Tween buffer (0.1% Tween 20; pH 7.5) for 1 h and incubated with 2.5 μg/ml ECE-1-B61-104 mouse monoclonal antibody (BASF, Mannheim, Germany). The immunoreactive bands were detected by an enhanced chemiluminescence system (Amersham, Zürich, Switzerland). Optical density of ECE protein bands was detected by NIH imaging software, and optical density in control cells was regarded as 100%.

Plasma ET-1 levels

After puncture of the right ventricle, blood was immediately transferred to a tube containing EDTA and centrifuged at 4°C for 10 min. Plasma was separated at 4°C and kept at −80°C until assay. Plasma ET-1 levels were determined as described in detail elsewhere [15]. The radioimmunoassay of plasma ET-1 was carried out using synthetic human/porcine ET-1 (Sigma Chemical Co.), a rabbit antibody against synthetic ET-1 (Peninsula Laboratories) and ^{125}I-ET-1 (Amersham).

Aortic ET-1 tissue levels

Aortic tissue was snap-frozen in liquid nitrogen and kept at−80°C until assayed. ET-1 was extracted as described previously [13]. Eluates were dried in a speed-vac and reconstituted in working assay buffer for radioimmunoassay. Measurements of ET-1 were verified by reverse-phase high performance liquid chromatography and related to wet tissue weight (pg/ng).

Calculations and statistical analysis

The contractions in isolated vessels were expressed as a percentage of 100 mmol/l KCl-induced contractions, which were obtained at the beginning of each experiment. Results are presented as mean ± SEM. Functional ECE activity was calculated as the ratio of the contraction to big ET-1 (10^{-7}mol/l) divided by the contraction to ET-1 (10^{-7}mol/l). In all experiments, *n* equals the number of rats per experiment. For statistical analysis, the sensitivity of the vessels to the drugs was expressed as the negative logarithm of the concentration that caused half-maximal relaxation or contraction (pD_2). Maximal relaxation (expressed as a percentage of pre-contraction) or contraction was determined for each individual concentration–response curve by non-linear regression analysis with the use of MatLab software. For comparison between two values, the unpaired Student's *t*-test or the non-parametric Mann–Whitney test were used when appropriate. For multiple comparisons, results were analysed by analysis of variance (ANOVA) followed by Bonferroni's correction [16]. Pearson's correlation coefficients were calculated by linear regression. A value of $P < 0.05$ was considered significant.

Results

Characteristics of animals

Systolic blood pressure increased after chronic administration of a high-salt diet (4% NaCl) in salt-sensitive Dahl rats as compared with rats on a standard chow after 2, 4 and 8 weeks after introduction of the diet (Table 1). Treatment with either omapatrilat or captopril prevented the salt-induced blood pressure rise ($P < 0.05$ *vs* rats on high salt diet alone). Omapatrilat, at a mean daily dose of 36.2 ± 4 mg/kg was equipotent in lowering blood pressure as captopril at 94.1 ± 2 mg/kg. Changes in heart rate during treatment and differences in heart rate among the treatment groups did not reach statistical significance (407 ± 8 bpm for controls *vs* 417 ± 9 bpm for salt diet *vs* 398 ± 6 bpm for salt + O *vs* 400 ± 4 bpm for salt + C after 8 weeks of treatment; n.s.).

Table 1. Systolic blood pressure (mmHg) of salt-sensitive Dahl rats during 56 days of treatment with different Regimens (day 0 indicates blood pressure before treatment)

	Day			
	0	14	28	56
Control	143 ± 5	146 ± 5	148 ± 6	148 ± 9
Salt diet (4%)	143 ± 4	177 ± 6*	197 ± 6*	196 ± 8*
Salt + omapatrilat	140 ± 7	151 ± 6**	156 ± 6**	162 ± 8**
Salt + captopril	144 ± 5	149 ± 5**	157 ± 6**	164 ± 7**

*$P < 0.01$ *vs* control rats (ANOVA and Bonferroni's correction); **$P < 0.05$ *vs* rats on salt diet.
Data are given as mean ± SEM of six rats in each group.

ACE inhibition

The effectiveness of ACE inhibition as assessed by determination of functional ACE activity and expressed as the ratio of the contraction to angiotensin I 10^{-7} mol/l divided by the contraction to angiotensin II 10^{-7} mol/l, did not differ between omapatrilat and captopril (0.28 ± 0.04 *vs* 0.33 ± 0.06 respectively; n.s.) but ACE activity was significantly reduced by either captopril or omapatrilat as compared with the control group (0.74 ± 0.08; $P < 0.01$).

Acute in vitro *effects of omapatrilat on contractions to ET-1 and big ET-1*

Preincubation of vascular segments *in vitro* with omapatrilat (10^{-7} mol/l) affected neither contractions to ET-1 in rats on salt-enriched chow nor in control animals, and neither in renal arteries nor in the aorta (Table 2; n.s.). In contrast, in aortic segments as well as in renal artery segments, contractions to big ET-1 were blunted by preincubation with omapatrilat (Table 2; both $P < 0.01$ in comparison with native vessels for rats on a high-salt diet and control animals, respectively). Thus, functional ECE activity was markedly diminished in both renal arteries (Figure 1A; $P < 0.01$ *vs* native segments) and aortic segments (Figure 1B; $P < 0.01$ *vs* native segments).

Vascular contractions and functional ECE activity during chronic treatment

Contractions of renal arteries to ET-1 were reduced in Dahl rats on a high salt diet (Figure 2A; $P < 0.05$) and were normalized by long-term administration of omapatrilat or captopril, respectively ($P < 0.05$ *vs* placebo-treated, salt-fed Dahl rats for maximal response; Figure 2A). In addition, renal artery contractions to big ET-1 were markedly reduced in salt

Table 2. Concentration-dependent contractions to ET-1 (10^{-7} mol/l) and big ET-1 (10^{-7} mol/l) in aortic and renal artery segments of salt-sensitive Dahl rats

	Renal artery		Aorta	
	Controls	Salt diet	Controls	Salt diet
ET-1				
without OMA	128 ± 5	98 ± 5	138 ± 5	110 ± 7
with OMA 10^{-7} mol/l	139 ± 8	105 ± 7	140 ± 8	111 ± 7
Big ET-1				
without OMA	116 ± 7	47 ± 6*	137 ± 9	75 ± 11*
with OMA 10^{-7} mol/l	12 ± 4**	9 ± 4**	29 ± 6**	10 ± 4**

Experiments were performed with and without preincubation with omapatrilat (OMA) 10^{-7} mol/l. Contractions are expressed as percentage of 100 mmol/l KCl.
Data are given as mean ± SEM of six rats in each group.
*$P < 0.05$ *vs* control rats.
**$P < 0.01$ *vs* vessel segments without preincubation with omapatrilat (ANOVA and Bonferroni's correction).

sensitive hypertension (Figure 2B; $P<0.05$ for sensitivity (pD_2 value) and maximal response). Treatment with omapatrilat but not with captopril ($P<0.05$ for maximal contractions *vs* omapatrilat) normalized contractions to big ET-1 (Figure 2B).

In the aorta, reduced contractions to ET-1 in salt-induced hypertension were improved on treatment with both captopril and omapatrilat (Figure 3A; $P<0.05$ *vs* high-salt diet). Even though the improvement in maximal contractions by omapatrilat was not significant in the aorta, there were no statistically significant differences to captopril (Figure 3A, n.s.).

Both captopril and omapatrilat significantly improved contractions to big ET-1 ($P<0.05$ *vs* placebo-treated, salt-fed Dahl rats for maximal response; Figure 3B), but were not able to normalize contractile responses to big ET-1 completely ($P<0.05$ *vs* controls for maximal response).

Therefore, functional ECE activity in renal arteries was significantly lowered in salt-sensitive hypertension (Table 3; $P<0.05$ *vs* controls). ECE activity was normalized by omapatrilat (Table 3; $P<0.05$ *vs* salt diet) but was not significantly affected by captopril.

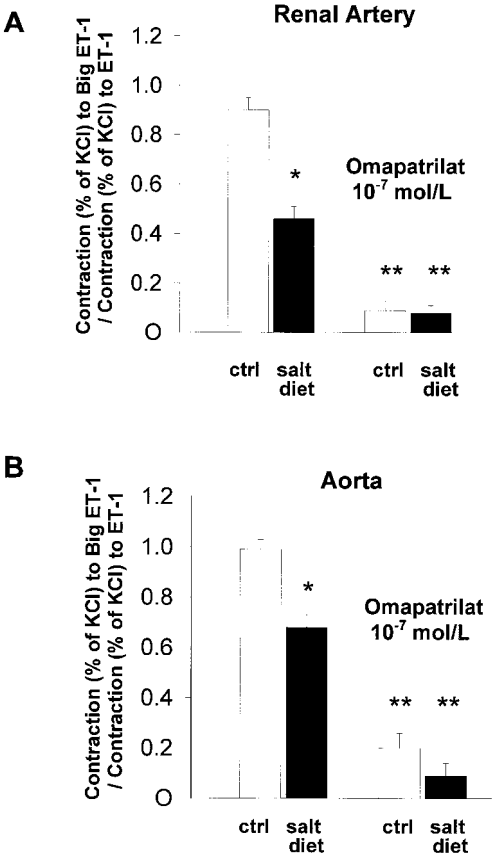

Fig. 1. Functional ECE activity (given as contraction to big ET-1 (10^{-7} mol/l) divided by contraction to ET-1 (10^{-7} mol/l)) in renal artery segments (**A**) and aortic segments (**B**) of salt-sensitive Dahl rats on standard or salt-enriched chow, with and without preincubation with omapatrilat 10^{-7} mol/l. Results are shown as mean ± SEM ($n=6$ per group). *$P<0.01$ *vs* control rats; **$P<0.01$ *vs* segments without preincubation.

ECE activity in the aorta was impaired too (Table 3; $P<0.05$ *vs* controls) and was improved by treatment with omapatrilat, even though the beneficial effects were pronounced in renal arteries.

Renovascular ECE protein levels

ECE protein levels were significantly reduced in salt-induced hypertension as compared with controls and were restored by omapatrilat but not by captopril (Table 3).

Plasma ET-1 levels

Plasma ET-1 levels were significantly elevated in hypertensive animals as compared with controls (16.6 ± 1.4 *vs* 9.4 ± 1.2 pg/ml, $P<0.05$; Figure 4A). Omapatrilat significantly lowered elevated plasma ET-1 levels ($P<0.05$ *vs* high-salt diet) whereas captopril did not ($P<0.05$ *vs* omapatrilat and $P<0.05$ *vs* controls).

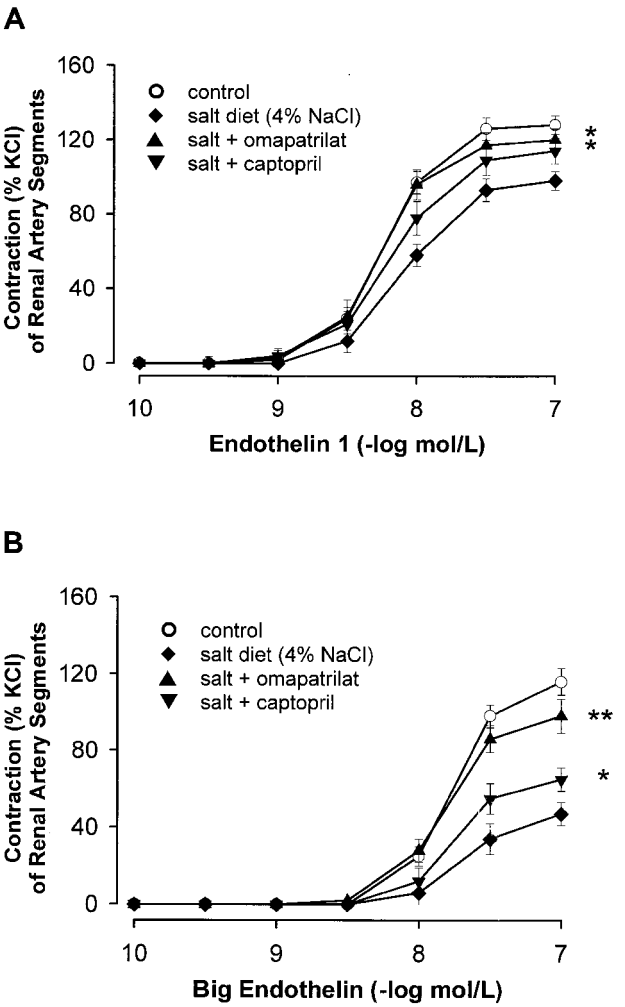

Fig. 2. Concentration-dependent contractions to ET-1 (**A**) and big ET-1 (**B**) in renal artery segments of salt-sensitive Dahl rats after 8 weeks of treatment with different regimens. Contractions are expressed as percentage of 100 mmol/l KCl. Results are shown as mean ± SEM ($n=6$ per group). *$P<0.05$ *vs* rats on a salt diet; **$P<0.05$ *vs* rats on captopril treatment.

Aortic ET-1 tissue levels

Aortic ET-1 levels were significantly elevated in hypertensive animals as compared with controls (191 ± 40 *vs* 104 ± 30 pg/ng wet weight, respectively, $P < 0.05$; Figure 4B). Neither omapatrilat (225 ± 38 pg/ng) nor captopril (190 ± 30 pg/ng) was able to influence elevated ET-1 levels significantly.

Discussion

The present study demonstrates that long-term treatment with the vasopeptidase inhibitor omapatrilat restores alterations in the endothelin system, which occur in salt-induced hypertension. Indeed, treatment with omapatrilat—unlike captopril—normalized renovascular ECE activity and lowered elevated ET-1 plasma levels. In contrast, if administered *in vitro*,

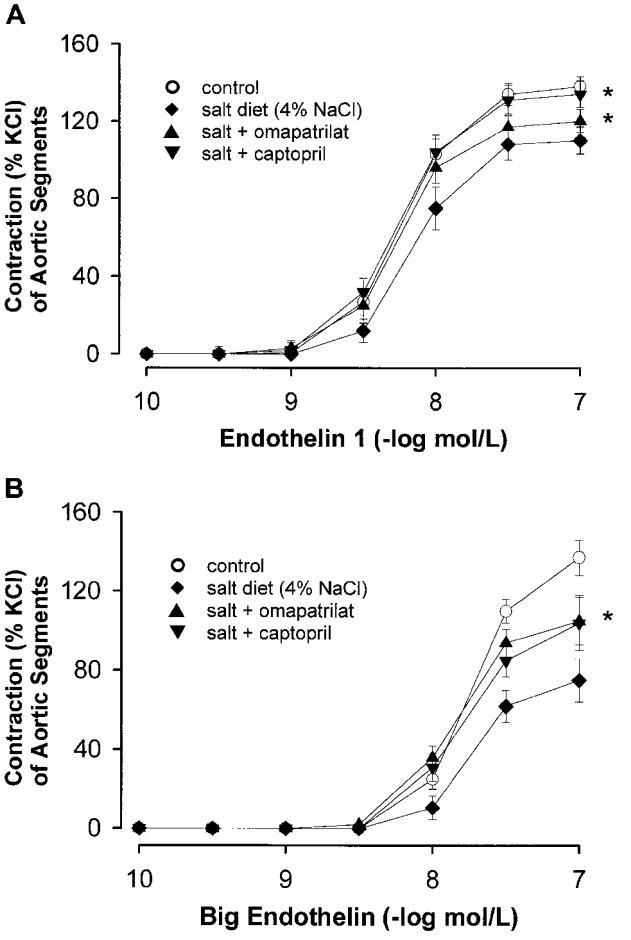

Fig. 3. Concentration-dependent contractions to ET-1 (**A**) and big ET-1 (**B**) in aortic segments of salt-sensitive Dahl rats after 8 weeks of treatment with different regimens. Contractions are expressed as percentage of 100 mmol/l KCl. Results are shown as mean ± SEM ($n = 6$ per group). $^{*}P < 0.05$ *vs* rats on a salt diet.

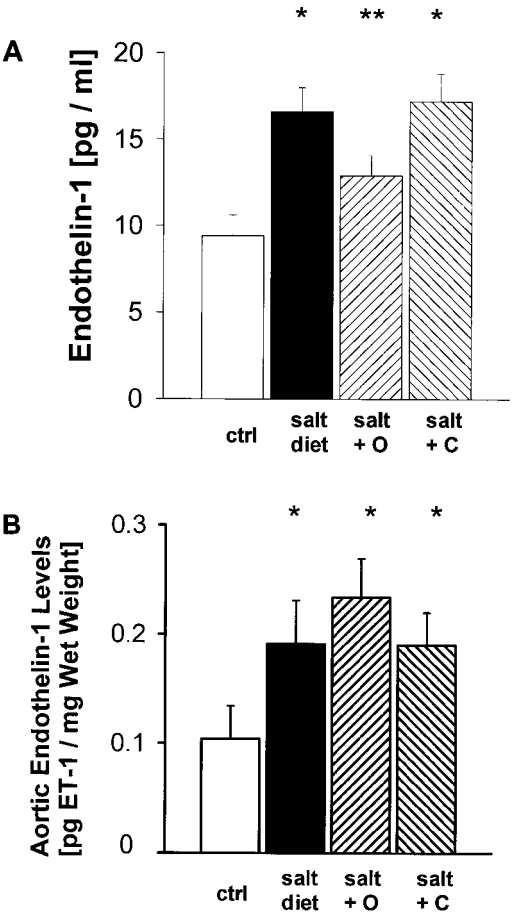

Fig. 4. Plasma ET-1 levels (**A**) and aortic ET-1 levels (**B**) in salt-sensitive Dahl rats after 8 weeks of treatment with different regimens. Results are shown as mean ± SEM ($n = 6$ per group). $^{*}P < 0.05$ *vs* control rats; $^{**}P < 0.05$ *vs* rats on a salt diet and *vs* rats on captopril treatment.

Table 3. Functional ECE activity and ECE protein levels (western blot analysis) of salt-sensitive Dahl rats after 8 weeks of treatment with different regimens

	Controls	Salt diet	Salt + O	Salt + C
ECE activity in renal artery (big ET-1/ET-1)	0.90 ± 0.05	0.46 ± 0.05*	0.81 ± 0.04**	0.56 ± 0.04*
ECE activity in aorta (big ET-1/ET-1)	0.99 ± 0.04	0.68 ± 0.05*	0.83 ± 0.05**	0.73 ± 0.05*
Renovascular ECE protein (arbitrary units)	100 ± 9	47 ± 6*	114 ± 12	45 ± 5*

$^{*}P < 0.05$ *vs* control rats; $^{**}P < 0.05$ *vs* rats on a salt diet.
Results are given as mean ± SEM of six rats in each group. Functional ECE activity is given as contraction (% of KCl) to big ET-1/contraction (% of KCl) to ET-1. Quantification of optical density of eNOS protein bands was performed with NIH imaging software. Optical density is given in arbitrary units.

omapatrilat inhibited the conversion of big ET-1 to ET-1.

It is well established that salt-sensitive hypertension is associated with impaired endothelial function [13]. In this study, we documented reduced contractile responses to ET-1 and big-ET-1 as well as impaired ECE activity in aorta and renal arteries of salt-sensitive Dahl rats. On the other hand, both plasma and tissue levels of endothelin were elevated. Elevated ET-1 levels exert vasoconstriction and proliferation via ET_A receptors, contribute to hypertrophic remodelling in resistance arteries, and therefore may play a role in the maintenance of hypertension [13].

Long-term treatment with the ACE-inhibitor captopril improved vascular responsiveness to ET-1 to a degree comparable to omapatrilat, but omapatrilat was superior in increasing contractions to big ET-1, and therefore increased ECE activity and lowered ET-1 plasma levels. Lowering of elevated ET-1 plasma levels in combination with enhanced conversion of big ET to ET-1 appears to be paradoxical at first glance. It certainly reflects the complex influence of vasopeptidase inhibition on the endothelin system, including both generation of ET-1 from big ET-1 and inhibition of ET-1 degradation [17]. However, it is of note that both the lowering of plasma ET-1 and elevation of ECE activity normalizes pathological parameters in this model of hypertension.

Since in placebo-treated animals, responsiveness of renal arteries to big ET-1 is even more impaired than the contractions to ET-1, salt-sensitive hypertension is characterized by decreased functional ECE activity and impaired ET-1 degradation at the same time.

While we demonstrated an increase in ECE activity in salt-induced hypertension by long-term vasopeptidase inhibition, omapatrilat inhibited the conversion of big ET-1 to ET-1 when acutely added *in vitro*. This may be explained with direct interactions of the compound with the ECE, which may be counterbalanced by neurohumoral and haemodynamic regulation on chronic administration *in vivo*. Inhibition of ECE has indeed been described previously for inhibitors of neutral endopeptidase [18].

On chronic *in vivo* treatment, long-term effects of omapatrilat may come in to play, probably due to resetting of local and vascular control mechanisms, of which only a few are known so far and require further investigation.

Salt-sensitive hypertension is known for only limited response to ACE inhibitors. Correspondingly, vasopeptidase inhibition has recently been demonstrated to lower blood pressure more effectively than ACE inhibition in salt-sensitive, hypertensive patients [19]. To avoid effects of different blood pressure levels among the groups, preliminary experiments were performed and appropriate equipotent doses of captopril and omapatrilat were chosen. Furthermore, to assess effectivity and comparability of ACE inhibition during treatment, functional ACE activity was shown to be comparable in the two treatment groups, which was confirmed by biochemical methods. It is noteworthy that other authors described effective ACE inhibition even with lower doses of captopril [20]. Hence, the different effects of the two drugs on the endothelin system must be related to properties of omapatrilat other than ACE inhibition.

When given chronically, vasopeptidase inhibition induced decrease in ET-1 levels simultaneously with normalization of ECE activity and vascular reactivity to ET-1 and big ET-1. This implies that the chronic presence of vasopeptidase inhibitors *in vivo* induces multiple effects on the endothelium, which may include alterations in the bioavailability of nitric oxide as well as in the metabolism of natriuretic peptides. Indeed, we recently demonstrated that in salt-sensitive hypertension, vasopeptidase inhibition increases vascular eNOS protein and nitrate levels, and improves endothelium-dependent relaxations in the aorta [21] in parallel with an increase in plasma ANP levels [22]. Therefore, normalization of the endothelin system may be one additional constituent that contributes to the beneficial effects of vasopeptidase inhibitors in salt-sensitive hypertension.

In conclusion, this study demonstrates that acute effects of omapatrilat on ECE activity *in vitro* are distinct from its long-term effects *in vivo*. Prevention of salt-sensitive hypertension with omapatrilat restored the vascular response to ET-1 and big ET-1, and normalized ECE activity as well as ET-1 plasma levels. Since the beneficial effects of omapatrilat were particularly pronounced in the renal circulation as compared with the aorta, vasopeptidase inhibition may represent an interesting new approach, especially in the treatment of renovascular disease. A number of large clinical studies, already under way in part, will be necessary to evaluate further the future clinical role of vasopeptidase inhibitors in the treatment of cardiovascular and renovascular disease.

Acknowledgements. This work was supported by grants from the Swiss National Research Foundation (grant no. 32–51069.97), the Deutscher Akademischer Austauschdienst (to T.Q.), the ADUMED Foundation and Novartis Foundation (to L.V.d'U.), and Bristol-Myers Squibb Pharmaceutical Research Institute, Princeton, NJ, USA.

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Received for publication: 7.8.00
Accepted in revised form: 9.1.01