Vasopeptidase Inhibition Exhibits Endothelial Protection in Salt-Induced Hypertension

Thomas Quaschning, Livius V. d’Uscio, Sidney Shaw, Thomas F. Lüscher

Abstract—Omapatrilat represents a new class of drugs capable of inhibiting both ACE and neutral endopeptidase 24.11. The overall effect of NEP inhibition on vascular tone will result from the addition of its effect on different vasoactive substances and is, especially in combination with inhibition of another enzyme, difficult to predict. Nevertheless, vasopeptidase inhibitors effectively lower blood pressure in salt-dependent and volume-dependent as well as in renin-dependent forms of hypertension.20,21 Furthermore, the combination of ACE and NEP inhibition may be particularly useful in the treatment of hypertension9,11,22,23 and heart failure.13,14,24,25 Inhibitors of both ACE and NEP, the so-called vasopeptidase inhibitors, lower blood pressure in a broader range of conditions than inhibition of ACE or NEP alone, independent of the activity of the renin-angiotensin system or the degree of salt retention.26,27 Omapatrilat is a new vasopeptidase inhibitor that induces long-lasting antihypertensive effects in certain forms of experimental hypertension20,23,28 greater than those elicited by selective inhibition of either enzyme alone.21 Furthermore, omapatrilat lowers blood pressure and attenuates cardiac hypertrophy in diabetic hypertensive rats.29 Besides the antihypertensive effect of combined NEP/ACE inhibition, the vascular protective effects of this new therapeutic principle on endothelial function are of
interest. This study was designed to investigate the effects of long-term treatment with the vasopeptidase inhibitor omapatrilat compared with the ACE inhibitor captopril on endothelial function in a low-renin model of hypertension.

Methods

Animals
Male Dahl salt-sensitive rats 6 weeks of age were obtained from Charles River WIGA GmbH (Sulzfeld, Germany) and randomly assigned to 1 of 5 treatment regimens: (1) standard chow (control), (2) standard chow plus omapatrilat (control+O), and 3 groups on salt-enriched (4% NaCl) chow (Harlan Teklad), which was given (3) alone (S), (4) together with omapatrilat (S+O), or (5) with captopril (S+C).

Tissue Harvesting
Animals were anesthetized with pentobarbital (50 mg/kg IP) after 8 weeks' treatment, and blood samples were collected through puncture of the right ventricle. The aorta and the renal arteries were removed, dissected free from adherent connective tissue, and placed immediately into cold (4°C) modified Krebs-Ringer bicarbonate solution (in mmol/L): NaCl 118.6, KCl 4.7, CaCl2 2.5, MgSO4 1.2, KH2PO4 1.2, NaHCO3 25.1, EDTA 0.026, and glucose 10.1. Under a microscope (Leica Wild M3C), vessels were cleaned of adherent tissue and cut into rings 4 mm long for aorta and 3 mm for renal artery.

Organ Chamber Experiments
Vessel rings were suspended to fine tungsten stir-ups (diameter, 50 μm) in organ baths filled with 25 mL Krebs solution, and connected to force transducers (UTC 2, Gould Statham) for isometric tension recording as described before.2 After an equilibration period of 60 minutes, the rings were progressively stretched to their optimal passive tension (3.0±0.2 g for aorta and 2.0±0.2 g for renal artery) as assessed by the response to 100 mmol/L KCl in modified Krebs solution.32 Rings were preconstricted with norepinephrine (NE, ~70% of KCl 100 mmol/L) and relaxation to acetylcholine (ACH; 10−6 to 10−3 mol/L) or sodium nitroprusside (SNP; 10−5 to 10−3 mol/L) were obtained. Relaxation to ACH was assessed with and without preincubation of the nitric oxide synthase (NOS) inhibitor L-NAME (preincubation for 30 minutes, 3×10−4 mol/L). In additional experiments, cumulative concentration–response curves to NE (10−10 to 10−4 mol/L), ET-1 (10−10 to 10−7 mol/L), and big ET-1 (10−9 to 10−7 mol/L) were obtained in quiescent preparations. Because of the rapid development of tachyphylaxis, only single concentrations of Ang I and Ang II were used (10−7 mol/L). All drugs used in the organ bath were obtained from Sigma Chemical Co apart from ET-1 and big ET, which were purchased from Novabiochem/Calbiochem AG.

Aortic eNOS Protein Content
After incubation with collagenase for 15 minutes at 37°C, the aortic endothelium was scraped off with a surgical blade. Cells were suspended in Krebs-Ringer bicarbonate solution and centrifuged at 5000 rpm at 4°C. The pellet was resuspended with Tris-SDS buffer (Tris-HCl 0.0635 mol/L, pH 6.8, SDS 2%), boiled for 1 minute, and then subjected to 8% SDS-PAGE gel for electrophoresis. Equal amounts of protein were used for electrophoresis, and comparable loading was confirmed by silver staining. The protein was then transferred onto ImmobilonTM-P filter papers (Millipore AG) with a semidy transfer unit. The membranes were subsequently blocked by using 2% skim milk in PBS-Tween buffer (0.1% Tween 20; pH 7.5) for 1 hour and incubated with a 1:1000 dilution of rabbit anti-eNOS 3 IgG antibody (Santa Cruz Biotechnology Inc). Immunoreactive bands were detected by an enhanced chemiluminescence system (Amersham). Optical density of eNOS protein bands was detected by NIH imaging software, and optical density in control rats was regarded as 100%.

Aortic Nitrite and Nitrate Levels
Homogenized aortic tissue was diluted 1:4 in sterile distilled water and deproteinized (Millipore 10 ultrafiltration membranes). Nitrites and nitrates, the stable end products of NO oxidation,33 were quantified by reverse-phase high-performance liquid chromatography (RP-HPLC) on an ECE250/4.5 Supersil 100 RP column (Machery & Nagel) by ion-pairing chromatography with photodiode array detection at 210, 215, and 220 nm, as described before.34

Aortic ET-1 Levels
Aortic tissue was snap-frozen in liquid nitrogen and kept at −80°C until assayed. ET-1 was extracted as previously described.32,35 Eluates were dried in a speed-vac and reconstituted in working assay buffer for radioimmunoassay. Measurements of ET-1 were verified by RP-HPLC and related to wet tissue weight (pg×mg−1).

Plasma ET-1 Levels
Plasma was separated at 4°C and kept at −80°C until assay. Plasma ET-1 levels were determined as described in detail elsewhere.36,37 Briefly, extraction was performed by absorption on 500-mg SepPak Vac C18 cartridges (Millipore). After the eluate was dried and redissoled in assay buffer, the radioimmunoassay of plasma ET-1 was carried out with synthetic human/porcine ET-1 (Sigma Chemical Co), a rabbit antibody against synthetic ET-1 (Peninsula Laboratories), and 125I–ET-1 (Amersham).

Calculations and Statistical Analysis
Relaxation to agonists in isolated arteries is given as percent precontraction in rings precontracted with NE to ~70% of contraction induced by KCl (100 mmol/L). The contractions 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. 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 (pD2). Maximal relaxation (expressed as a percentage of precontraction) or contraction was determined for each individual concentration–response curve by nonlinear regression analysis with the use of MatLab software. For comparison between two values, the unpaired Student’s t test or the nonparametric Mann-Whitney test was used when appropriate. For multiple comparisons, results were analyzed by ANOVA followed by Bonferroni’s correction.38 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 long-term administration of a high-salt diet (4% NaCl) in salt-sensitive Dahl rats as compared with rats on a standard chow at weeks 2, 4, and 8 after introduction of the diet (P<0.05, Figure 1). Treatment with either omapatrilat or captopril prevented the salt-induced blood pressure rise (P<0.05 versus 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 94.1±2 mg/kg of captopril.

**Vascular Relaxation**

In hypertensive animals, endothelium-dependent relaxation to ACH in the aorta was markedly impaired compared with control rats (Figure 2A, P<0.05). The sensitivity of the concentration-response curve to ACH was reduced in salt-fed animals (pD₂, 6.8±0.1) compared with control animals (pD₂, 7.4±0.2, P<0.05). Both omapatrilat and captopril improved endothelium-dependent relaxation (Fig 2A, P<0.05 and P<0.01, respectively), but the maximal relaxation achieved by omapatrilat was significantly higher than by captopril (Fig 2A, P<0.05). In renal arteries, differences between omapatrilat and captopril in maximal endothelium-dependent relaxation also reached statistical significance (Figure 2B, P<0.05). Preincubation with the NOS inhibitor L-NAME blunted relaxation to ACH completely in both aorta (Figure 3A) and renal arteries (Figure 3B). In contrast to endothelium-dependent relaxation, maximal endothelium-independent relaxation to the NO donor SNP was comparable in all groups in aortic rings as well as in renal arteries (data not shown). Preincubation with indomethacin (10⁻⁷ mol/L) did not alter maximal relaxation or sensitivity (pD₂ value) to either ACH or SNP.

**Vascular Contractions**

Contractions to NE were reduced in Dahl rats on a high-salt diet (Figure 4, P<0.01) and were almost normalized by long-term administration of omapatrilat or captopril (P<0.05 versus untreated salt-fed Dahl rats for maximal response, Figure 3). In addition, contractions to both ET-1 and big ET-1 were reduced in salt-induced hypertension (P<0.05 versus untreated salt-fed Dahl rats).

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**Figure 1.** Systolic blood pressure of salt-sensitive Dahl rats during 8 weeks of treatment with different regimens. Week 0 indicates blood pressure before treatment. Data are mean±SEM of 6 rats.

**Figure 2.** Endothelium-dependent relaxation to ACH in aortic rings (A) and renal artery rings (B) of salt-sensitive Dahl rats after 8 weeks of treatment with different regimens. Results are shown as mean±SEM (n=6 per group) and are expressed as percent relaxation of contraction to NE (3×10⁻⁷ mol/L).

**Figure 3.** Endothelium-dependent relaxation to ACH in aortic rings (A) and renal artery rings (B) of salt-sensitive Dahl rats after 8 weeks of treatment with different regimens after preincubation with L-NAME (3×10⁻⁴ mol/L). Results are shown as mean±SEM (n=6 per group) and are expressed as percent relaxation of contraction to NE (3×10⁻⁷ mol/L).

**Figure 4.** Contraction to NE in rat aortic rings of salt-sensitive Dahl rats after 8 weeks of treatment with different regimens. Contraction is expressed as percentage of 100 mmol/L KCl. Results are shown as mean±SEM (n=6 per group).
control rats, Table). Vascular responses to ET-1 and big ET-1 increased on treatment with captopril and omapatrilat, respectively, but only omapatrilat was able to completely normalize contractions to ET-1, whereas the response to ET-1 on captopril treatment was still distinct from control rats ($P<0.05$).

The effectiveness of ACE inhibition, assessed by determination of functional ACE activity, did not differ between omapatrilat and captopril (0.28±0.04 versus 0.33±0.06 respectively, NS), but ACE activity was significantly reduced by either captopril or omapatrilat compared with the control group (0.74±0.08, $P<0.01$).

### Aortic eNOS Protein Content

eNOS protein content of thoracic aorta decreased in salt-fed animals compared with control animals (Figure 5, 46.6±4% versus 100±6%, $P<0.01$) as measured by detection of optical density of eNOS protein bands by NIH imaging software ($n=4$). The increase of eNOS protein with omapatrilat (96±6%, $P<0.05$ versus S) tended to be more pronounced than with captopril (84±5%, $P<0.05$ versus S), but the difference between the two treatment regimens did not reach statistical significance.

### Aortic Nitrite and Nitrate Levels

Aortic tissue levels of nitrite ($\text{NO}_2^-$), and nitrate ($\text{NO}_3^-$) in hypertensive animals were reduced compared with control animals (Figure 6, $P<0.05$). Treatment with either omapatrilat or captopril restored aortic nitrite and nitrate levels completely. Differences among the treatment groups and in comparison to control animals were not statistically significant.

### Aortic and Plasma ET-1 Levels

Aortic ET-1 levels were significantly elevated in hypertensive animals as compared with control animals (0.19±0.04 versus 0.11±0.03 pg/mg wet wt, respectively, $P<0.05$). Neither omapatrilat (0.23±0.035 pg/mg) nor captopril (0.19±0.03 pg/mg) significantly altered elevated aortic ET-1 levels. In contrast, elevated plasma ET-1 levels in salt-induced hypertension (16.6±1.4 versus 9.4±1.2 pg/mL in control animals) were restored by omapatrilat (12.9±1.2 pg/mL; $P<0.05$ versus salt diet) but not by captopril (17.2±1.6 pg/mL, NS).

### Maximal Response and Sensitivity ($pD_2$) of Contractions to Endothelin-1 and Big Endothelin-1 in the Aorta and Endothelin-1 Plasma Levels of Salt-Sensitive Dahl Rats

<table>
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<th>S+C</th>
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<td>12.9±1.2*</td>
<td>17.2±1.6</td>
<td>16.6±1.4</td>
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CTRL indicates rats on standard chow; CTRL+O, rats on standard chow plus omapatrilat; S+O, rats on salt-enriched chow plus omapatrilat; S+C, rats on salt-enriched chow plus captopril; S, rats on salt-enriched chow without treatment; Max (%), maximal response; $pD_2$, $-\log\text{ mol/L of substance causing half-maximal response.}$ Data are given as mean±SEM of 6 rats in each group.

* $P<0.05$ vs S (ANOVA and Bonferroni’s correction), † $P<0.05$ vs CTRL.

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**Figure 5.** Western blot analysis of eNOS protein levels in thoracic aorta of salt-sensitive Dahl rats after 8 weeks of treatment with different regimens. Quantification of optical density of eNOS protein bands was performed with NIH imaging software. Results are shown as mean±SEM ($n=4$ per group). *$P<0.05$ vs control rats and vs treatment groups.

**Figure 6.** Aortic nitrite and nitrate levels in salt-sensitive Dahl rats after 8 weeks of treatment with different regimens. Results are shown as mean±SEM ($n=4$ per group). *$P<0.05$ vs control rats and vs treatment groups.
Discussion

This study demonstrates that endothelial dysfunction, which accompanies salt-sensitive hypertension, can be improved by long-term treatment with the ACE inhibitor captopril but almost normalized by the vasopeptidase inhibitor omapatrilat. It is well established that salt-sensitive hypertension is associated with impaired endothelial function. In this study, we documented impaired endothelium-dependent relaxation to ACh as well as reduced contractile responses to NE, ET-1, and big ET-1 in both aorta and renal artery. Long-term treatment with the ACE inhibitor captopril improved vascular relaxation but did not restore endothelial function to a comparable degree as omapatrilat, even though blood pressure was reduced similarly. Analogous improvement of vasorelaxation by omapatrilat in rat mesenteric arteries has most recently been reported. Because the modest effects of captopril in this model contrast with that of other antihypertensive drugs such as diuretics, the degree of ACE inhibition during captopril treatment was assessed and confirmed to be comparable in the two treatment groups, both functionally and biochemically. Other authors described effective ACE inhibition even with lower doses of captopril. Hence, the different effects of the two drugs on endothelial function must be related to other properties of omapatrilat.

Vasopeptidase inhibition is a new concept in cardiovascular therapy. It is based on the simultaneous inhibition of two key enzymes, ACE and NEP, which are involved in the regulation of cardiovascular function in many ways. This includes the metabolism of several vasoactive peptides such as angiotensin, natriuretic peptides, bradykinin, and ET-1 and their clearance from the circulation. Therefore, the effects of additional NEP inhibition on vascular tone are dependent on whether the predominant substrates degraded by NEP are vasodilators or vasoconstrictors.

Because relaxation to SNP was not altered whereas endothelium-dependent relaxation was markedly diminished and blunted after preincubation with the NOS inhibitor L-NAME, reduced endothelial production of NO contributes to the reduced endothelium-dependent reactivity in hypertensive salt-sensitive Dahl rats. In accordance with recent findings, we demonstrated a decrease in eNOS protein levels as well as in aortic nitrate and nitrate levels in salt-sensitive hypertension, which was normalized by concomitant treatment with both omapatrilat and captopril. Regarding the lack of difference between omapatrilat and captopril in eNOS levels and aortic nitrates, influence on the NO metabolism may not account for superiority of omapatrilat in improving endothelium-dependent relaxation. Omapatrilat appears to predominantly reduce the breakdown of natriuretic peptides. Therefore, ameliorated aortic and renal artery relaxation may at least in part be due to the inhibition of the degradation of atrial natriuretic peptide. The influence of omapatrilat on other vasoactive peptides such as the endothelin system must be taken into account as well. Indeed, conditions with impaired NO production usually are associated with increased ET release because NO exerts a negative feedback on the expression and production of the peptide. In salt-induced hypertension, we have previously shown activation of the vascular ET system. In the present study, we confirmed our previous finding that vascular ET levels are markedly increased in this model. However, we found that neither omapatrilat nor captopril was able to lower the increased vascular tissue levels of ET-1 in animals on a high-salt diet. In contrast, omapatrilat was capable of reducing elevated ET-1 plasma levels. Thus, modulation of the endothelin system by vasopeptidase inhibitors appears to be rather complex and requires further investigation. The lack of association between vascular ET-1 levels and treatment of hypertension has been previously demonstrated in Ang II-induced hypertension.

Conclusions

This study demonstrates that long-term prevention of salt-sensitive hypertension with equipotent dosages of omapatrilat or captopril markedly improves endothelium-dependent relaxation. Because endothelial effects were less pronounced with captopril than with omapatrilat, combined NEP/ACE inhibition may represent an interesting new approach in the treatment of hypertensive vascular disease, even though the underlying mechanism of the beneficial effects is not fully understood. Vasopeptidase inhibitors also may be useful in the treatment of heart failure and coronary artery disease, in which improvement of endothelial function is important as well. A number of large clinical studies—many already under way—will be necessary to further evaluate the future clinical role of vasopeptidase inhibitors in the treatment of cardiovascular diseases.

Acknowledgments

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References


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