Endothelin-Converting Enzyme Inhibition Ameliorates Angiotensin II–Induced Cardiac Damage


Abstract—We tested the hypothesis that endothelin-converting enzyme (ECE) inhibition ameliorates end-organ damage in rats harboring both human renin and human angiotensinogen genes (dTGR). Hypertension develops in the animals, and they die by age 7 weeks of heart and kidney failure. Three groups were studied: dTGR (n = 12) receiving vehicle, dTGR receiving ECE inhibitor (RO0687629; 30 mg/kg by gavage; n = 10), and Sprague-Dawley control rats (SD; n = 10) receiving vehicle, all after week 4, with euthanasia at week 7. Systolic blood pressure was not reduced by ECE inhibitor compared with dTGR (205±6 versus 206±6 mm Hg at week 7, respectively). In contrast, ECE inhibitor treatment significantly reduced mortality rate to 20% (2 of 10), whereas untreated dTGR had a 52% mortality rate (7 of 12). ECE inhibitor treatment ameliorated cardiac damage and reduced left ventricular ECE activity below SD levels. Echocardiography at week 7 showed reduced cardiac hypertrophy (4.8±0.2 versus 5.7±0.2 mg/g, P<0.01) and increased left ventricular cavity diameter (5.5±0.3 versus 3.1±0.1 mm, P<0.001) and filling volume (0.42±0.04 versus 0.16±0.06 mL, P<0.05) after ECE inhibitor compared with untreated dTGR. ECE inhibitor treatment also reduced cardiac fibrosis, tissue factor expression, left ventricular basic fibroblast growth factor mRNA levels, and immunostaining in the vessel wall, independent of high blood pressure. In contrast, the ECE inhibitor treatment showed no renoprotective effect. These data are the first to show that ECE inhibition reduces angiotensin II–induced cardiac damage. (Hypertension. 2002;40:1296–1302.)

Key Words: angiotensin II ■ enzymes ■ fibrosis ■ hypertrophy

Angiotensin (Ang) II–related vascular effects are partially mediated by endothelin-1 (ET-1). Long-term Ang II infusion induces preproendothelin mRNA expression.1 In rats transgenic for both the human renin and human angiotensinogen genes (dTGR), hypertension as well as severe heart and kidney damage develop, largely independent of blood pressure elevation. The rats die by age 7 weeks.2 The ET-1 A and B (ETA/B) receptor blocker bosentan inhibits the activation of both nuclear factor-kappa B (NF-kB) and transcription factor activator protein (AP)-1 in the kidney and the heart, independent of blood pressure reduction in these rats.3 Bohlender et al4 studied the same rat strain and showed that a specific ETA receptor blocker is effective, particularly when combined with an Ang II receptor blocker. ET-1 is a 21–amino acid peptide that was first isolated from porcine endothelial cells.5 Two structurally related peptides differing by 2 (ET-2) and 6 (ET-3) amino acids were subsequently identified. The endothelin precursors are processed by 2 proteases that create mature active forms, termed preproendothelins. The preproendothelins are cleaved at dibasic sites by furin-like endopeptidases to produce inactive intermediates termed big endothelins. Big endothelins are cleaved to form the final products. A family of membrane-bound zinc metalloproteases from the nephrilysin superfamily conducts the last processing step. These enzymes are the endothelin-converting enzymes (ECEs). ET-1 is found in endothelial cells and has a sharp activity peak at neutral pH. The enzyme processes big ETs both intracellularly and on the cell surface. The enzyme structure has recently been elucidated in detail.6 Big ET-1 and ET-1 levels are strongly related to survival in patients with congestive heart failure.7 Because selective enzymatic processing of ET-1 formation appears to be an important therapeutic target for heart failure, we tested the utility of ECE inhibition in our model.

Methods

Four-week-old male age-matched and body weight–matched dTGR and Sprague Dawley (SD) rats (RCC Ltd, Füllinsdorf, Switzerland) were used as described elsewhere.8,9 All procedures were done according to guidelines from the American Physiological Society (permit No. G408/97). We compared vehicle-treated dTGR (n = 12),
dTGR treated with the ECE inhibitor RO0687629 (30 mg/kg for 3 weeks once per day by gavage; n=10), and SD vehicle-treated control rats (n=10). The ECE inhibitor was provided from Hoffmann-La Roche. Systolic blood pressure was measured at weeks 5, 6, and 7 by the tail-cuff method under light ether anesthesia 20 hours after the last drug dose. Urine was collected over a 24-hour period once per week. Echocardiography (M-mode tracings and short axis; n=5 to 6 per group at week 7) was performed with the use of a commercially available system equipped with a 7-MHz phased-array transducer under light thiopental anesthesia. Three measurements per heart were determined, averaged, and statistically analyzed. Rats were killed at age 7 weeks. The kidneys and hearts were washed with ice-cold saline, blotted dry, and weighed. For immunohistochemistry, organs were frozen in isopentane (−35°C) and stored at −80°C. Urinary albumin was determined with an ELISA kit (CellTrend).

Tissue preparation and immunohistochemical techniques were performed as described before in detail. The sections were incubated with primary antibodies against rat monocytes/macrophages (surface marker ED1, Serotec), collagen I (South Bio ASS), fibronectin (Paesel), basic fibroblast growth factor (bFGF; Transductions Laboratories), and tissue factor (gift of Dr. Th. Luther, TU Dresden, Germany). Scoring of ED1–positive cells was performed with the use of the program KS 300 3.0 (Zeiss). Fifteen different areas of each heart and kidney (n=5 in all groups) were analyzed without knowledge of rat identification. Collagen IV expression and fibronectin expression were assessed semiquantitatively by two independent observers who were blind to the treatments. The data are expressed in arbitrary units (0 to 5), based on the staining intensity.

For RT-PCR, RNA of the left ventricle was isolated by means of the TRIZOL protocol (Gibco Life Technology). Real-time quantitative RT-PCR was performed by using the TaqMan system (PE Biosystems), as described earlier in detail. The sequences were GAPDH: F: AAGCTGGTCATCAATGGGAAAC; GAPDH-R: ACCCCATTGTGTAGCGG; GAPDH-P: CATCACCACCTCTCCAGAGCCGCAGGT; and bFGF-R: GGAGTTGTGTCCATCAAGGGA; bFGF-R: AGCAAGCGTCCATCTTCTTCT; bFGF-P: TGTGTTGCGACCGGTACCTGGCT. Each sample was performed in duplicate. For quantification, the target sequence was normalized to the GAPDH gene.

ECE activity of total kidney and left ventricular extracts were measured as described earlier. The NEP assay was based on a method described by Carvahlo et al. The ACE assay is based on the method described by Carmel et al. Incubations were carried out in 96-well microplates in triplicate at 4 to 6 concentrations ranging from 100 μmol/L to 10 nmol/L. IC50 was calculated after logit/log transformation of the percentage inhibition data with a best-fit regression model. For both assays, the inhibitors were tested for their fluorescence or quenching properties and values were corrected accordingly. ECE inhibitors were measured in triplicate at 5 to 7 concentrations in the range of 10 μmol/L to 10 nmol/L. IC50 values were calculated after logit/log transformation of the percent inhibition data with a best-fit regression model. All assays were calibrated with phosphoramidon as internal standard inhibitor. Under our assay conditions, the IC50 of phosphoramidon was 1.0 ± 0.2 μmol/L (n=30). IC50 values of unknown compounds were accepted when the IC50 (×) measured for phosphoramidon in the assay was 0.85 ± 1.2 μmol/L.

Data are presented as mean ± SEM. Statistically significant differences in mean values were tested by ANOVA, blood pressure by repeated ANOVA, and the Scheffé test. Mortality rate was examined with Kaplan-Meier analysis. A value of P<0.05 was considered statistically significant. The data were analyzed with Statview statistical software.

Results

To test the hypothesis that ECE inhibition ameliorates end-organ damage, we treated dTGR with the ECE inhibitor (2S,4R)-4-Acetylsulfanyl-2-(2,4,5-trifluoro-benzoyl)methyl)-pyrrolidine-1-carboxylic acid 2-methoxycarbonyl-phenyl ester RO0687629.
Untreated dTGR showed a mortality rate $>50\%$ at the end of the study (Figure 1C). The remaining animals in each group were killed at week 7. By this time, only 2 of 10 (20\%) ECE inhibitor–treated dTGR had died ($P<0.05$). ECE inhibitor treatment did not lower blood pressure in dTGR (Figure 1D). RO0687629 treated and untreated dTGR were severely hypertensive. Systolic blood pressure of both groups was $\sim 100$ mm Hg higher compared with nontransgenic controls, namely 205$\pm$6 versus 206$\pm$6 versus 110$\pm$3 mm Hg at week 7 for dTGR, ECE inhibitor, and SD, respectively.

Left ventricular ECE activity was significantly higher in untreated dTGR compared with both other groups (Figure 2A). RO0687629 treatment reduced ECE activity below nontransgenic SD levels as follows: 1445$\pm$126 versus 885$\pm$40 versus 1046$\pm$81 ng/g tissue per hour, for dTGR, dTGR+RO0687629, and SD, respectively. Cardiac function and structure were significantly improved by ECE inhibitor treatment. Left ventricular hypertrophy and diminished left ventricular cavity dimensions were observed in untreated dTGR by M-mode echocardiography measured at week 7 and markedly improved by RO0687629 (Figure 2B). Cavity diameter of untreated dTGR (3.1$\pm$1.0 mm) was significantly lower compared with ECE inhibitor–treated dTGR (5.5$\pm$0.3 mm) and nontransgenic SD (5.8$\pm$0.1 mm) (Figure 2C, $P<0.05$). Left ventricular systolic filling volume was similar in RO0687629-treated dTGR and SD control rats, both significantly more compared with untreated dTGR. The data were $0.42\pm0.04$ versus $0.43\pm0.03$ versus $0.16\pm0.06$ mL, $P<0.05$ for dTGR+RO0687629, SD and dTGR, respectively. Cardiac hypertrophy index (Figure 2D), expressed as heart weight–to–body weight ratio, was 5.7$\pm$0.2 versus 4.8$\pm$0.2 versus 3.6$\pm$0.1 mg/g, $P<0.01$ for dTGR, dTGR+RO0687629, and SD, respectively. Body weights between untreated and ECE inhibitor–treated dTGR were not different. However, SD rats were heavier than dTGR.

ECE inhibitor treatment reduced extracellular matrix production. Collagen I (Figure 3A) and fibronectin (Figure 3B) were most prominently deposited around blood vessels, in the vascular adventitia, and focally around fibrotic areas of scarring. Collagen I (grade 3$\pm$1 for RO0687629 treated dTGR versus 5$\pm$1 for vehicle-treated dTGR versus 1$\pm$1 for SD rats) and fibronectin staining in RO0687629-treated dTGR were reduced toward SD level (grade 2$\pm$1 for RO0687629 treated dTGR versus 5$\pm$1 for vehicle-treated dTGR versus 1$\pm$1 for SD rats). Figure 3C shows a representative section of a dTGR heart with increased tissue staining in untreated and ECE inhibitor–treated dTGR as well as infiltrated cells. TF expression was markedly reduced by ECE inhibitor treatment (grade 2$\pm$1 for RO0687629 treated dTGR versus 5$\pm$1 for vehicle-treated dTGR versus 1$\pm$1 for SD rats).

Basic FGF plays an important role in cardiac damage. Immunohistochemistry for bFGF shows immunostaining in the endothelium and media of arterial blood vessels as well as perivascular infiltrated cells and infiltration between the cardiomyocytes (grade 1$\pm$1 for RO0687629 treated dTGR versus 5$\pm$1 for vehicle-treated dTGR versus 1$\pm$1 for SD rats; Figure 4). We also analyzed basic FGF mRNA expression in the left ventricle. The bFGF expression was significantly increased in vehicle-treated dTGR compared with SD rats. ECE inhibitor almost normalized bFGF mRNA (Figure 4).

Surprisingly, long-term ECE inhibitor treatment did not ameliorate kidney damage. Albuminuria occurred in ECE inhibitor–treated dTGR to a degree no different than in untreated dTGR (Figure 5A). Furthermore, plasma creatinine and urea elevations as well as renal fibronectin staining in untreated and ECE inhibitor–treated dTGR.
were not different. Instead, these parameters were significantly increased compared with SD (data not shown). In contrast to ECE activity in the heart, long-term ECE inhibitor treatment did not reduce ECE activity in the kidney. The results were 5665 ± 1167 versus 5486 ± 2006 versus 709 ± 554 ng/g tissue per hour for dTGR, dTGR + RO0687629, and SD, respectively. Untreated and RO0687629-treated dTGR both showed significantly increased ECE activity (Figure 5B). We considered the possibility that the dose we used was not sufficient to inhibit ECE in the kidney. Therefore, we measured both the prodrug RO0687629 and thiol RO0677447 in the kidney. We could not detect any acetyl sulfanyl RO0687629, indicating that the prodrug was completely cleaved to its metabolite RO0677447. We found 8-fold more thiol ECE inhibitor in the kidney compared with heart. The amounts present were >800-fold and >100-fold above the IC50, respectively.

**Discussion**

This study is the first showing that ECE inhibitor treatment can ameliorate Ang II–induced end-organ damage. The pro-

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**Figure 3.** Untreated dTGR show cardiac fibrosis. RO0687629 treatment reduced collagen I (A) and fibronectin (B). C, Representative section of a dTGR heart with increased TF expression in vessel wall and adventitia as well as infiltrated cells. TF expression was markedly reduced by RO0687629 treatment.
tection was relatively confined to the heart. Reduction of cardiac hypertrophy, improved cardiac function, and reduced matrix formation as well as tissue factor expression was obtained despite the fact that long-term ECE inhibitor treatment did not lower blood pressure. In contrast to long-term ET A/B receptor blockade in dTGR rats, the ECE inhibitor that we used failed to protect the kidney. Long-term ECE inhibitor treatment reduced left ventricular ECE activity, whereas renal ECE activity was unchanged. The findings document the observation that Ang II–induced cardiac injury is in part mediated by ET-1–related mechanisms.3,4 We were surprised that long-term ECE inhibitor treatment did not ameliorate renal damage. We showed earlier that the ET A/B receptor blocker bosentan effectively reduced cardiac and renal damage.3 First, we considered the possibility that other ET-1–forming enzymes such as neutral endopeptidase (NEP) might have lessened the renal effect of the ECE inhibitor. We therefore measured renal ECE and NEP activity. In contrast to the reduction in left ventricular ECE activity below nontransgenic levels, renal ECE activity was not reduced and cardiac and renal NEP activity was unchanged in all 3 groups. Thus, the lack of ECE inhibitor action on renal ECE activity may be the reason for the lack of effectiveness in this organ.

To exclude the possibility that the dose used in the present study was not sufficiently high to inhibit renal ECE activity, we measured the inhibitor levels in the tissue. We found a 100-fold excess of the IC50 in the heart and an 800-fold excess in the kidney. Therefore, both concentrations should have been sufficient to completely inhibit the enzyme. The lack of effectiveness in the kidney is not clear and remains speculative. We showed earlier that untreated dTGR exhibit oxidative burst in the kidney and that antioxidant treatment improves the renal damage.16,17 Thus, under conditions of high oxidative stress, the thiol ECE inhibitor is oxidized to the inactive dimer. In addition, ECE and NEP activity were several-fold higher in the kidney compared with the heart, suggesting that higher levels of active inhibitor are necessary to achieve enzyme inhibition in that organ.

Much interest has been generated by the possibility that Ang II exerts its effects largely through endothelin. Serneri et al18 studied cardiac Ang II formation in the clinical course of heart failure and its relation with left ventricular function. They found that the clinical course of heart failure was associated with a progressive increase in cardiac Ang II formation. Failing myocytes were unable to synthesize IGF-I and ET-1 in response to Ang II stimulation. Nonfailing myocytes, on the other hand, synthesized both IGF-I and ET-1 in response to Ang II. Thus, the heart can apparently synthesize these components during volume or pressure overload, and their production is directly correlated with increased contractility. With advancing heart failure, these adaptations are no longer possible.
ET-mediated effects are not confined to Ang II–induced models. Schiffrin19 has emphasized the important role of ET-1 in hypertension, particularly in desoxycorticosterone acetate (DOCA)-salt hypertension. Pollock et al20 studied the combined effects of AT1 and ETA receptor antagonists in DOCA-salt hypertensive rats. They found that both compounds together were required to effectively decrease blood pressure. However, they were unable to prevent renal fibrosis. This result is puzzling because NF-κB activation has also been found in the DOCA-salt rat. Beswick et al21 found that antioxidants attenuate systolic blood pressure, suppress renal NF-κB DNA binding activity, and partly alleviate renal monocyte/macrophage infiltration in DOCA-salt hypertension. Furthermore, in the aldosterone-infused rat, Park and Schiffrin22 found that ETA receptor blockade prevented blood pressure elevation and vascular remodeling. Conceivably, the common ground may be the mineralocorticoid receptor that is involved in both Ang II and DOCA-mediated hypertensive models. In dTGR, the mineralocorticoid receptor plays a dominant signaling role in both heart and kidney. We showed earlier that spironolactone could inhibit NF-κB and AP-1 transcription factor activation in this model. We did not examine transcription factor activation in this study because the general anesthesia required for the echocardiographic measurements interferes with reliable determinations.

We believe that our findings have therapeutic implications. The role of endothelin antagonists in the prevention of end-organ damage has been recently reviewed.23,24 We showed earlier that renin-angiotensin system inhibition is highly effective in this model. Whether or not a combined Ang II–ET inhibition would have particular utility is not known for certain. A representative study examining blockade of renin-angiotensin and endothelin systems in a model of progressive renal injury was conducted by Cao et al.25 They relied on the subtotal nephrectomy rat model. The animals were randomized to control, ACE inhibitor treatment, AT1 receptor blocker treatment, ETA receptor blocker treatment, or bosentan. Treatment with the AT1 receptor blocker or the ACE inhibitor was associated with an improved glomerular filtration rate and reductions in blood pressure, urinary protein excretion, glomerulosclerosis, and tubular injury in association with reduced gene expression of TGF-β and matrix protein type IV collagen. No beneficial effects of either the ETA blocker or combined blockade with bosentan were noted. Furthermore, the addition of ETA blockade to AT1 receptor blockade did not confer any additional benefits. The authors suggest that the renin-angiotensin system but not the endothelin system is the major mediator of progressive renal injury after renal mass reduction. We were interested to observe that ECE inhibition was more effective in ameliorating cardiac compared with renal damage. Conceivably, Ang II–related ET-1–mediated effects are more prominent in the heart. Cao et al25 focused on the kidney and did not report whether or not combined treatment had a positive effect on cardiac hypertrophy in their model.

**Perspectives**

ECE inhibition is feasible in an animal model and provides protection against Ang II–induced cardiac damage. The recent clarification of ECE structure will facilitate the development of other novel ECE inhibitors. These compounds may add an additional dimension to cardiovascular treatment. They may be particularly useful as an adjunct to other therapies.

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