Dual ECE/NEP Inhibition on Cardiac and Neurohumoral Function During the Transition From Hypertrophy to Heart Failure in Rats

Noriaki Emoto, Sunu Budhi Raharjo, Daiji Isaka, Shigeru Masuda, Suko Adiarto, Arco Y. Jeng, Mitsuhiro Yokoyama

Abstract—CGS 26303 is a vasopeptidase inhibitor that simultaneously inhibits endothelin-converting enzyme (ECE) and neutral endopeptidase (NEP). We compared the effects of chronic treatment with CGS 26303 to the selective inhibition of angiotensin-converting enzyme (ACE) and NEP during the transition from left ventricular hypertrophy (LVH) to congestive heart failure (CHF) in hypertensive rats. LV geometry and function were assessed in Dahl salt-sensitive rats placed on a high-salt diet from age 6 weeks (hypertensive rats) and in control rats fed a low-salt diet. The hypertensive rats were randomized into groups that received no treatment or were treated with an ACE inhibitor (temocapril), an ECE/NEP inhibitor (CGS 26303), or a NEP inhibitor (CGS 24592) from the LVH stage (11 weeks) to the CHF stage (17 weeks). All treatments decreased the systolic blood pressure equally and significantly improved LV fractional shortening. Both temocapril and CGS 26303 ameliorated LV perivascular fibrosis, reduced mRNA levels of types I and III collagen, and decreased the heart weight/body weight ratio. CHF rats had increased plasma ET-1 levels compared with control rats. Only CGS 26303 reduced ET-1 to normal levels. ET-1 levels were found to correlate with heart/body weight, right ventricle/body weight and perivascular fibrosis ratios. During the transition to CHF, CGS 26303 produces effects that are comparable to temocapril and superior to CGS 24592. The beneficial effects of CGS 26303 are likely caused in part by the greater reduction of plasma ET-1. Dual ECE/NEP inhibitor may provide a new strategy for the treatment of human heart failure. (Hypertension. 2005;45:1145-1152.)

Key Words: endothelin ■ heart failure ■ hypertension ■ hypertrophy

Neurohumoral factors are important in the pathophysiology of congestive heart failure (CHF). Inhibitors of angiotensin-converting enzyme (ACE) are standard treatments for CHF, preventing the formation of angiotensin II (Ang II), attenuating blood pressure, and improving cardiac function and survival.1 However, despite the well-proven benefits of therapy with ACE inhibitors, a significant proportion of patients continue to be symptomatic and are at high risk for mortality. Thus, the development of new drugs that act on neurohumoral systems other than the renin-angiotensin system may be advantageous for the treatment of CHF.

Plasma endothelin-1 (ET-1) levels are elevated in patients with heart failure and predict adverse clinical outcomes including mortality.2,3 Circulating ET-1 levels correlate with the hemodynamic severity and prognosis.4 Therefore, strategies to antagonize the effects of ET-1 may be a therapeutic approach for CHF. In fact, several ET-receptor antagonists (selective for ET₁ or nonselective for ET₁ and ET₂) are undergoing evaluation in the treatment of CHF. Unfortunately, the favorable hemodynamic and neurohumoral effects obtained with these compounds in animal studies have failed to translate into improved patient outcomes.5,6 In patients with CHF, treatment with either nonselective or selective ET-receptor antagonists, increased circulating levels of ET-1.5,7 This side effect may blunt their efficacy with long-term clinical use. Therefore, blocking the generation of ET-1 by inhibiting endothelin-converting enzyme (ECE) is an attractive alternative to the use of ET-receptor antagonists.

Atrial natriuretic peptide (ANP) has direct vasodilator effects, antagonizes the renin-angiotensin system, inhibits endothelin secretion, and reduces sympathetic activity.8 Furthermore, increased levels of ANP in plasma is a hallmark of ventricular remodeling secondary to heart failure.9 One approach to potentiate the endogenous effects of ANP is to inhibit its enzymatic degradation by neutral endopeptidase 24.11 (NEP). Many studies show that NEP inhibitors have modest beneficial effects in animal models10,11 and in patients with heart failure.12,13
Additional evidence supports the hypothesis that combined inhibition of ET-1 formation by ECE inhibition and degradation of vasodilatory peptides by NEP inhibition may be beneficial for the treatment of heart failure. Recently, the combined inhibition of ECE/NEP inhibition has been shown to have additive antihypertensive and cardiac antihypertrophic effects in normotensive diabetic rats. Whether long-term dual inhibition of ECE and NEP offers any advantage over selective ACE or NEP inhibition in CHF remains unknown. In the current study, CGS 26303, which simultaneously inhibits ECE and NEP, was compared with the ACE inhibitor, temocapril, and the NEP inhibitor, CGS 24592.

**Materials and Methods**

**Animals and Experimental Design**

Experimental procedures were conducted according to the Guidelines for Animal Experimentation at Kobe University Graduate School of Medicine. Male Dahl salt-sensitive (DS) rats were purchased from Eisai Co, Ltd. (Tokyo, Japan). The rats were fed an 8% NaCl (high-salt) diet from 6 weeks of age (DSH rats) (n=44). Age-matched DS rats fed a low-salt diet (0.3% NaCl) were used as controls (DSL rats) (n=16). At 11 weeks of age, DS rats with established concentric left ventricular hypertrophy were randomized into 4 groups of treatments as shown in Figure 1. All treatments were administered subcutaneously using osmotic minipump (ALZA Corp). Temocapril was provided by Sankyo Co, Ltd. (Tokyo, Japan). CGS 26303 and CGS 24592 were synthesized at the Novartis Institutes for BioMedical Research (East Hanover, NJ). The IC50 values of these compounds toward ACE, ECE, and NEP have been previously described. The doses were selected based on the equipotential effect to decrease the systolic blood pressure (SBP) in preliminary experiments.

**Hemodynamic and Echocardiographic Studies**

The SBP was measured using tail-cuff method (Muramachi Kikai, Japan). In vivo hemodynamic state and LV geometry were assessed by transthoracic echocardiography as previously reported. Under pentobarbital anesthesia, blood was collected from the abdominal aorta in a polypropylene tube containing aprotinin (300 kallikrein-inhibiting units/mL) and heparin and immediately centrifuged at 1800g for 15 minutes at 4°C. Plasma was stored at −80°C until assayed. The plasma ET-1 levels were measured using enzyme immunoassay, plasma ANP, and Ang II levels by radioimmunoassay. These measurements were performed by SRL, Inc (Tokyo, Japan).

**Histological Examination and Evaluation of Myocardial Remodeling**

Light microscopy of the heart was performed on 5-μm sections stained by Sirius red as previously described. Collagen deposition was evaluated in the endocardium of left ventricular free wall. We analyzed 5 sites from each ventricle in all rats. The perivascular fibrosis was determined as the ratio of the area of fibrosis surrounding the vessel wall to the total area of the vessel.

**Gene Expression Studies**

Northern blot analysis was performed with [32P]-dCTP cDNA probes for collagen type I, III, ANP, and preproET-1 and standardized with GAPDH probe. The identity of the probes was confirmed by DNA sequencing. The density of individual mRNA bands was measured using a bioimaging analyzer (BAS-2000; Fuji Photo Film, Tokyo, Japan).

**Statistical Analysis**

Results are expressed as mean±SEM. The significance of the difference among groups at specific stages was assessed using 1-way
ANOVA with post-hoc comparisons by Fisher’s protected least significant difference test. Relationship between 2 variables was tested by linear regression analysis. \( P<0.05 \) was considered statistically significant.

Results

Global Parameters

DS rats fed a high-salt diet (8% NaCl) (DSH rats) beginning at 6 weeks of age had systemic hypertension (\( >200 \text{ mm Hg} \)) developed at 11 weeks, which continued through 17 weeks of age (CHF period). DSH rats showed a markedly higher SBP (\( P<0.0001 \)) compared with DSL rats at 8 weeks and remained significantly higher thereafter. All drug treatments reduced blood pressure of DSH rats to a comparable degree (Figure 2).

We observed cardiac hypertrophy and RV hypertrophy in rats with congestive heart failure (DSH) at 17 weeks of age (Figure 3). Treatment with either temocapril or CGS 26303 attenuated the increase in heart/body weight ratio observed in DSH rats (\( P<0.05 \) for DSH-A and \( P<0.01 \) for DSH-D). Interestingly, only dual ECE/NEP inhibitor CGS 26303 decreased the increase in the ratio of right ventricle/body weight (\( P=0.01 \) versus DSH).

Echocardiographic Changes

At 11 weeks, the LV posterior wall thickness (PWT) increased in the DSH rats compared with DSL rats (\( P<0.01 \)), indicating the presence of LV concentric hypertrophy (Table). The increase in PWT was then lowered when these DSH rats reached 17 weeks of age (\( P<0.01 \) versus 11 weeks, DSH rats). Furthermore, at 17 weeks, although there was no difference in LV end-diastolic dimension between DSH and DSL animals, we observed that the LV motion in DSH rats was significantly lower than in DSL rats with a larger LV end-systolic dimension and less LV percent fractional shortening (FS) (Table).

The increased LV diameters and reduced percentage of FS in DSH rats were improved by all active treatments. However, the percent FS of DSH rats treated with CGS 24592 (DSH-N) was still significantly lower than with either ACE inhibitor or dual ECE/NEP inhibitor treatment (\( P<0.05 \) versus DSH-A; \( P<0.01 \) versus DSH-D) (Figure 4).

Perivascular Fibrosis

Perivascular fibrosis was evaluated using Sirius red staining (Figure 5). The ratio of perivascular fibrosis was 1.7-fold higher in the LV of DSH rats compared with DSL rats (\( P<0.0001 \)). DSH rats treated with ACE inhibitors showed a significantly reduced perivascular fibrosis ratio (\( P<0.01 \)). Interestingly, dual ECE/NEP inhibitor treatment led to a greater decrease than ACE inhibitors alone (\( P<0.05 \) versus DSH-A; \( P<0.0001 \) versus DSH) even though NEP inhibitors alone did not significantly decrease the perivascular fibrosis ratio.

LV Collagens, PreproET-1, and ANP mRNA Levels

LV collagens type I and III, preproET-1 (ppET-1), and ANP mRNA levels were increased in CHF rats and significantly reduced in both ACE inhibitor and dual ECE/NEP inhibitor

<table>
<thead>
<tr>
<th>Groups</th>
<th>PWT, mm</th>
<th>LVdd, mm</th>
<th>LVds, mm</th>
<th>FS, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>6-wk DS (n=8)</td>
<td>1.3±0.02</td>
<td>6.9±0.11</td>
<td>3.2±0.12</td>
<td>54.2±1.32</td>
</tr>
<tr>
<td>11-wk DSL (n=8)</td>
<td>1.7±0.03</td>
<td>7.4±0.11</td>
<td>3.4±0.12</td>
<td>53.6±1.15</td>
</tr>
<tr>
<td>11-wk DSH (n=8)</td>
<td>2.2±0.03*</td>
<td>7.3±0.16</td>
<td>3.2±0.16</td>
<td>57.2±0.73</td>
</tr>
<tr>
<td>17-wk DSL (n=8)</td>
<td>1.7±0.07</td>
<td>8.2±0.22†</td>
<td>4.2±0.20§</td>
<td>48.2±1.83§</td>
</tr>
<tr>
<td>17-wk DSH (n=12)</td>
<td>1.6±0.03†</td>
<td>8.3±0.13†</td>
<td>5.5±0.17†</td>
<td>34.3±1.19†</td>
</tr>
</tbody>
</table>

*Values are given as mean±SEM.

FS indicates left ventricular fractional shortening; LVdd, left ventricular end-diastolic dimension; LVds, left ventricular end-systolic dimension; PWT, left ventricular posterior wall thickness at end-diastole.

\( ^{*} P<0.0001 \) vs age-matched DSL.

\( ^{†} P<0.01 \) vs 11-wk DSL.

\( ^{‡} P<0.01 \) vs 11-wk DSL.

\( ^{§} P<0.05 \) vs 11-wk DSL.
groups (Figure 6). Although NEP inhibitors also reduced the level of ANP and ppET-1 mRNAs, it only slightly decreased the upregulation of LV collagens. It is important to note that the changes in ppET-1 mRNA levels correlated significantly with parameters of cardiac function, ie, percent FS (R²=0.919; P=0.01) and LV end-systolic dimension (R²=0.811; P<0.05).

Plasma Neurohumoral Concentrations

CHF is characterized by a marked increase in plasma neurohumoral concentrations.22 As shown in Figure 7, at 17 weeks of age plasma ET-1 levels were higher in DSH rats than in DSL rats and were significantly decreased by dual ECE/NEP inhibition, whereas ACE inhibitors and NEP inhibitors reduced ET-1 levels in CHF rats to a lesser extent. Plasma Ang II and ANP levels were also elevated in DSH rats and were significantly lowered by all drug treatments. Interestingly, the changes in plasma ET-1 concentrations were strongly corre-

lated with the heart/body weight (R²=0.850, P<0.05), right ventricle/body weight (R²=0.784, P<0.05), and LV perivascular fibrosis ratios (R²=0.856, P<0.05) (Figure 8).

Discussion

Although the effects of CGS 26303 as well as CGS 24592 have been recently reported using a rat model of heart failure induced by coronary artery ligation,19 we believe that our current study using the model of transition from left ventricular hypertrophy to heart failure in hypertensive rats demonstrated the following major novel findings. During the transition to CHF, CGS 26303 produces effects that are comparable to temocapril and superior to CGS 24592. The increase in the level of plasma ET-1 and cardiac ppET-1 associated with CHF was prevented to the greatest extent by the dual inhibitor. The changes in plasma ET-1 concentration significantly correlated with cardiac structure, whereas the changes in ppET-1 mRNA levels correlated with cardiac function in rats with CHF. These beneficial effects were obtained despite the fact that long-term dual ECE/NEP inhibitor treatment did not reduce blood pressure to a greater extent than the other active treatments.

Neurohumoral Activation

Consistent with previous reports,23,24 we found that plasma ANP, Ang II, and ET-1 concentrations were significantly higher in DS rats with cardiac dysfunction. It is unclear whether the changes in plasma levels of Ang II and ANP observed after treatment with the enzyme inhibitors reflect a direct effect of the drugs on neurohormone production or whether the production of Ang II and ANP is modulated by a change in hemodynamic status.

ET-1 levels correlate with the severity of heart failure2 and is a prognostic indicator of CHF.3 Although all active treatments reduced circulating Ang II and ANP, only CGS 26303 prevented the increase in plasma ET-1. Moreover, the level of plasma ET-1 significantly correlated with cardiac hypertrophy and perivascular fibrosis ratio, suggesting that the reduction in plasma ET-1 may be crucial to the greater beneficial effect of CGS 26303. In this study it is unlikely that the protective effects of the dual inhibitor are attributed to the increase activity of ANP because all active treatments reduced ANP to similar extent at the transcriptional levels (Figure 6) and circulating peptide levels (Figure 7).

In this study, NEP inhibition by CGS 24592 did not change plasma ET-1, yet in heart failure caused by acute myocardial infarction and in DOCA-salt hypertension NEP inhibition using ONO-9902 and candoxatril decreased plasma ET-1.25,26 In contrast, the NEP inhibitor thiorphan increased plasma ET-1 levels in normal rats.27 Consequently, data from studies of NEP inhibitors need to be viewed with caution, because the evidence shows that different NEP inhibitors have different effects on ET-1 levels. A possible reason for the complex results obtained with NEP inhibitors is that NEP is a ubiquitous ecto-enzyme present not only in the kidney but also in the heart, lung, brain, intestine, spleen, endothelial cells, and neutrophils.28 Also, NEP in addition to metabolizing different vasoactive peptides is active toward natriuretic peptides and ET-1. NEP is involved in the enzymatic con-
version of big ET-1 to the vasoconstrictor ET-1.29 Hence, the overall effect of NEP inhibition will depend on whether the predominant substrates degraded are vasodilators or vasoconstrictors and on the extent of NEP involvement in the processing of big endothelin-1.

Because CGS 24592 has similar NEP inhibitory activity as CGS 26303 but does not inhibit ECE activity,30 the decreased levels of plasma ET-1 obtained with CGS 26303 is likely caused by ECE inhibition. This is in agreement with the findings reported by Pelletier et al.31 However, we feel that selective inhibition of ECE may not be sufficient to reduce the circulating levels of ET-1 because substantial levels of mature ET-1 are still found in ECE-1−/− or double ECE-1−/−/ECE-2−/− knockout mice32,33 as well as in rats with heart failure treated with selective ECE inhibitor.34 These results not only underscore the superiority of this new compound to selective NEP inhibitor in the treatment of experimental heart failure but also indicate the potential of using circulating ET-1 levels as a biomarker of improvement of CHF.

Figure 5. Left ventricular perivascular fibrosis ratio (A) and representative Sirius red staining (B) in the 5 experimental groups. Other abbreviations are the same as in Figures 3 and 4. Results are expressed as mean±SEM. *P<0.0001, †P<0.001 vs DSL; ‡P<0.001 vs DSH; #P<0.05 vs DSH-A.

Figure 6. Types I and III collagen mRNA levels, preproET-1, and ANP gene expression in the left ventricle of the 5 experimental groups. The expression of types I and III collagen, preproET-1, and ANP mRNA levels were corrected for GAPDH mRNA levels. Mean±SEM, n=4 to 6 per group. #P<0.05, *P<0.01, **P<0.0001 vs DSL; †P<0.05, †P<0.01, ††P<0.001 vs DSH.
LV Perivascular Fibrosis

Another important observation is that both the dual ECE/NEP inhibitor and ACE inhibitor, but not the NEP inhibitor, significantly lowered the LV perivascular fibrosis ratio and completely prevented the activation of collagen type I and III gene expression. Of these, the dual ECE/NEP inhibitor showed the greater reduction in LV perivascular fibrosis.

Previous reports have shown that ET-1 activates the procollagen I promoter, increases cardiac fibroblast collagen synthesis, and enhances the expression of collagen III, TGF-β, fibronectin, and laminin in the cardiac matrix. Considering that in this study only the dual enzyme inhibitor reduced the levels of plasma ET-1, it is tempting to speculate that the more marked improvement of LV perivascular fibrosis by dual ECE/NEP inhibition may be partly mediated by the decreased expression levels of plasma ET-1, leading to amelioration of not only collagen I and collagen III induction but also other molecules such as TGF-β, fibronectin, and/or laminin.

Cardiac Hypertrophy

CHF is associated with the development of myocardial hypertrophy as a response to chronic volume overload. In this study, we observed an increased ratio of heart/body weight and right ventricle/body weight together with a decreased percentage of fractional shortening in DSH rats as compared with DSL rats, consistent with a state of decompensated heart failure. Surprisingly, although both temocapril and CGS 26303 reduced cardiac hypertrophy, complete prevention of right ventricular hypertrophy was observed only in CGS 26303-treated rats. Although chronic treatment with the NEP inhibitor reduced blood pressure to the same level as ACE or ECE/NEP inhibition, it failed to improve the ratio of heart/body weight and right ventricle/body weight, indicating only a minor contribution to the afterload reduction in the cardiac geometry.

It is worth noting that only CGS 26303 caused regression of right ventricular hypertrophy, an indicator of the elevation of LV end-diastolic pressure and the existence of pulmonary congestion. This could suggest that circulating ET-1 may mediate the structural remodeling of the failing heart. This
finding is particularly important in light of the disappointing results of clinical trials using ET receptor antagonists, which exacerbated the congestive symptoms of heart failure. The fact that ET receptor antagonists caused elevated ET-1 levels, sodium retention, and increased renin release may have contributed to the worsening of heart failure. In addition, our regression analysis demonstrates an intimate relationship between the levels of plasma ET-1 and the right ventricular hypertrophy in all 5 animal groups. Interestingly, a recent study demonstrated that a major contributor to the elevation of plasma ET-1 with the progression of heart failure was increased pulmonary synthesis of ET-1 by ECE-1. Consequently, dual ECE/NEP inhibition may represent an attractive approach to lowering ET-1 levels in the treatment of heart failure.

Study Limitations
In this study, although the LV percent FS of DSH rats was significantly lower than that of DSL rats, the dilatation in LV end-diastolic dimension at 17 weeks were observed in both DSL rats and the age-matched control DSL rats. Therefore, these changes are considered as growth-related changes as reported by Doi et al. Also, the fact that even when fed normal diets the DS rats (DSL) were mildly hypertensive (Figure 2; \( P<0.01 \) versus 6 weeks, DSL) supports this hypothesis. It is well known that the sympathetic nervous system contributes significantly for the development of heart failure. Therefore, we cannot exclude a possible role of sympathetic nervous system in this model. In addition, considering the limited numbers of rats in each group this study was not designed to evaluate long-term survival.

Perspectives
This study and previous reports\(^2\),\(^4\),\(^3\) have demonstrated that selective ACE inhibitors provide beneficial effects on LV hemodynamics (ie, preservation of LV fractional shortening and prevention of LV dilatation) in this model of experimental heart failure. Nevertheless, in practice, ACE inhibitors are often combined with diuretics to relieve congestive symptoms. Diuretics cause further activation of the RAS and sympathetic nervous system, which may limit their long-term efficacy. Therefore, the ability of CGS 26303 to reduce pulmonary congestion could reduce the need to administer diuretics.

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References


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