Mechanisms of Combined Treatment With Celiprolol and Candesartan for Ventricular Remodeling in Experimental Heart Failure

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**Background** Both β-adrenergic blockers and angiotensin-II receptor blockers were reported to improve the prognosis of patients with heart failure, but the efficacy of combination therapy with these agents has not been fully elucidated. Also the efficacy of celiprolol, a β1-selective adrenoceptor antagonist with partial β2-agonist properties, for heart failure treatment is still controversial. We examined the cardioprotective effects and mechanisms of the therapy with celiprolol or candesartan, an angiotensin-II receptor blockers and their combination in heart failure induced by isoproterenol (ISO).

**Methods and Results** ISO 300 mg/kg was injected in rats to produce heart failure. Two months after the injection, the ISO-injected rats were divided into 4 groups (8 rats each) and treated for 4 weeks as follows: (a) vehicle; (b) celiprolol 10 mg/kg per day (BB); (c) candesartan 0.2 mg/kg per day (ARB); and (d) their combination BB + ARB. ISO significantly elevated left ventricular (LV) end-diastolic pressure, decreased peak-negative dP/dt and LV ejection fraction. BB and ARB similarly ameliorated cardiac dysfunction due to ISO, but BB + ARB were more potent than the individual therapies. Separately, ARB preserved the histological structure in LV myocardium. In contrast, BB ameliorated calcium handling, as shown by the increased ratio of SERCA2 to phospholamban protein, despite having little effect on the histology.

**Conclusion** Both celiprolol and candesartan showed cardioprotective effects in this heart failure model. The potential use of the combination treatment in heart failure might result in a synergistic effect through the different cardioprotective mechanisms of celiprolol and candesartan. (Circ J 2005; 69: 596–602)

**Key Words:** Angiotensin-II receptor blocker; Beta-blocker; Heart failure; Remodeling

In congestive heart failure, neurohumoral activation of the adrenergic nervous and the renin-angiotensin system has been reported to cause advanced cardiac remodeling.1-2 Cardiac hypertrophy, induced as an adaptive process in heart failure,3 is accompanied by a number of biochemical and molecular changes, such as increased myocardial fibrosis and alterations in intracellular calcium handling, most likely due to the impaired activity or reduced amount of the sarcoplasmic reticulum proteins.4,5 However, excessive cardiac remodeling might be involved in the progression of heart failure.

Both β-adrenergic blockers and angiotensin-II receptor blockers are known to improve cardiac dysfunction of heart failure in human and animal models through their effects on the cardiac remodeling.6,7 Kubo et al reported that treatment with a β-adrenergic blocker in heart failure normalized the abundance of myocyte Ca2+ regulatory proteins and improved Ca2+ handling.8 A clinical study showed the efficacy of combined inhibitory therapy on the adrenergic nervous and renin-angiotensin systems (RAS) in congestive heart failure.9 However, the precise mechanisms of combination therapy with β-adrenergic blocker and angiotensin-II receptor blocker has not been fully elucidated. Furthermore, β-adrenergic blockers do not constitute a homogenous drug class for cardiac protection. Recently, celiprolol, a β1-selective adrenoceptor antagonist with partial β2-agonist properties, was shown to improve insulin sensitivity or vasodilatation through the NO-dependent pathway.10,11 But the effect on cardiac dysfunction is still controversial.12

The subcutaneous injection of the β-adrenergic agonist isoproterenol (ISO) produced diffuse myocardial cell death in the rat myocardium, resulting in progressive heart failure.13 We carried out this study with the hypothesis that both celiprolol and candesartan, an angiotensin-II receptor blocker, are useful for improving cardiac dysfunction induced by ISO and the combination therapy is more potent than each treatment alone.

**Methods**

**Animals** Male Sprague–Dawley rats (3 weeks of age) were obtained from Shimizu Jikken Zairyo, Japan. All animals were kept in a specific pathogen-free facility under controlled temperature (20–24°C) and humidity (40–70%), with a 12-h lighting cycle, and were given free access to standard laboratory rat chow (MF, Oriental Yeast, Tokyo, Japan) and tap water. All procedures were in accordance

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with institutional guidelines for animal research.

Heart Failure Model and Treatment
At the age of 5 weeks, the animals received subcutaneous injection of ISO 300 mg/kg for 2 consecutive days, following the methods of the previous study.14 The rats injected with saline served as controls. At the age of 3 months, ISO rats were further subdivided into 4 groups (8 rats each) and were treated with: (a) a vehicle; (b) celiprolol 10 mg/kg per day (BB); (c) candesartan 0.2 mg/kg per day (ARB); or (d) their combination (BB + ARB) with an osmotic mini-pump placed in the peritoneal cavity for 4 weeks.

Echocardiography
Four weeks after treatment, transthoracic echocardiography was performed with a SONOS-5500 (12 MHz phased-array transducer, Phillips, Netherlands). The end-diastolic and systolic left ventricular (LV) dimension, E/A ratio in trans-mitral flow and deceleration time were measured. The ejection fraction was calculated by modifying Simpson’s method, which uses 2- and 4-chamber views.15

Hemodynamics
One day after the echocardiography, a cardiac catheterization was performed under pentobarbital anesthesia 50 mg/kg ip. A 2F catheter-tip micromanometer (Model PR-249, Millar Instruments, Houston, TX, USA) was introduced via the right carotid artery into the ascending aorta and LV cavity to measure LV systolic and end-diastolic pressure, min and max dP/dt, and heart rate. After these measurements, the heart was excised, rinsed with saline solution, blotted dry and weighed. The heart weight was evaluated using heart to body weight ratio. A part of the heart was snap frozen with liquid nitrogen for Western blotting and the rest of the heart was used for the morphological and immunohistochemical study.

Table 1  Effects of Treatment on General Parameters and Hemodynamics

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>ISO + vehicle</th>
<th>ISO + BB</th>
<th>ISO + ARB</th>
<th>ISO + BB + ARB</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (BW), g</td>
<td>524±15</td>
<td>471±10*</td>
<td>463±11*</td>
<td>443±18*</td>
<td>436±10*</td>
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<tr>
<td>Heart weight (HW), g</td>
<td>1.29±0.03</td>
<td>1.28±0.04</td>
<td>1.30±0.07</td>
<td>1.02±0.03*</td>
<td>1.00±0.07*</td>
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<tr>
<td>HW/BW×10¹</td>
<td>2.47±0.05</td>
<td>2.71±0.05*</td>
<td>2.82±0.18*</td>
<td>2.30±0.05*</td>
<td>2.29±0.07*</td>
</tr>
<tr>
<td>Heart rate, beats/min</td>
<td>410±12</td>
<td>428±10*</td>
<td>335±17**</td>
<td>410±16*</td>
<td>356±17**</td>
</tr>
<tr>
<td>LV systolic pressure, mmHg</td>
<td>94±2</td>
<td>97±2</td>
<td>87±3#</td>
<td>89±3</td>
<td>84±1##</td>
</tr>
<tr>
<td>LV end-diastolic pressure, mmHg</td>
<td>7.13±0.6</td>
<td>18.8±1.5*</td>
<td>8.0±2.2#</td>
<td>8.7±0.6#</td>
<td>3.6±0.7##</td>
</tr>
<tr>
<td>max dP/dt, mmHg/s</td>
<td>3.13±129</td>
<td>2.55±115*</td>
<td>2.65±130</td>
<td>2.63±130</td>
<td>2.78±0.174</td>
</tr>
<tr>
<td>min dP/dt, mmHg/s</td>
<td>3.36±139</td>
<td>2.04±112*</td>
<td>2.85±437</td>
<td>2.646±8,139#</td>
<td>2.840±196*</td>
</tr>
</tbody>
</table>

Values are expressed as means ± SEM. BB, celiprolol; ARB, candesartan. *p<0.05 vs control, #p<0.05 vs isoproterenol injected rats (ISO).

Fig 1. M-mode echocardiograms from control rats and isoproterenol (ISO) rats treated for 4 weeks. ISO+vehicle increased left ventricular (LV) wall thickness, LV end-diastolic dimension and LV end-systolic dimension compared with the control. Treatment with celiprolol (BB) showed no improvement, but candesartan (ARB) tended to decrease LV end-systolic dimension. The combination therapy (ISO + BB + ARB) significantly improved LV end-systolic dimension, consequently ejection fraction and decreased LV posterior wall thickness.
Histology
For the light microscopic study (LM), the specimens were fixed in 10% formaldehyde, embedded in paraffin and cut into 4-μm-thick sections. The tissue sections were stained with hematoxylin and eosin, and were examined by light microscopy. In the sections stained with Syrrius red, interstitial fibrosis was measured using a computer-assisted image analysis and the percentage of fibrosis was calculated.16,17

Beta-Adrenoreceptor Binding Assay
The membrane suspension (250 μg protein) from LV was prepared as previously described and was incubated for 180 min at 25°C with various concentrations (0.2–4 nmol/L) of 3H-CGP12177 in a total volume of 0.25 ml containing 50 mmol/L Tris-HCl (pH 7.4 at 25°C). Non-specific binding was measured by the addition of 10−5 mol/L propranolol. At the end of the incubation period, the incubation medium was immediately filtered through a GF/F glass fiber filter and washed 3 times with a 2.5 ml volume of ice-cold 50 mmol/L Tris-HCl buffer. After drying the filter, its radioactivity was counted by liquid scintillation spectrometry. Every measurement was carried out twice. The dissociation constant (Kd) and maximum binding capacity (Bmax) of specific binding were determined by Scatchard analysis.

TGF-β Gene Expression Assay System: Quantitative Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)
Cytokine mRNA concentrations were measured by a real-time quantitative RT-PCR method, with the ABI Prism 7700 sequence detection system (Perkin-Elmer Applied Biosystems).
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Biosystems, Shelton, CT, USA). The cDNA was synthesized from 100 ng of total RNA. The relative quantification and standardization were achieved using a log10 dilution series of RNA extracted from rat spleen (calibrator). Relative quantification of 18S ribosomal and transforming growth factor \( \beta \) (TGF-\( \beta \)) mRNA was calculated by the comparative cycle threshold method outlined in the user manual provided by Perkin-Elmer.

Immunohistochemistry

For the immunohistochemical light microscopic study, additional sections were obtained from the paraffin block. After incubation with normal blocking serum, the sections were incubated overnight at 4°C with monoclonal antibodies against SERCA2 (804-088-R100, Alexis Biochemicals, Alexis Co, USA) or the antibodies against SERCA regulatory protein phospholamban (PLB) (804-093-C100, Alexis Biochemicals, Alexis Co, USA)21 After incubation with a secondary antibody, the sections were allowed to react with the Vectastain Elite ABC reagent (Vector Laboratories, Burlingame, CA), then react with the peroxidase substrate solution (Vectastain 3’3’-diaminobenzidine substrate kit, Vector Laboratories, Burlingame, CA).22

Western Blotting

Quantitative immunoblotting for sarcoplasmic reticulum Ca\(^{2+}\)-ATPase (SERCA2) and PLB was performed by the methods published previously23 For the immunoreaction analysis, monoclonal antibodies to SERCA2 (Alexis Biochemicals #804-088-R100), or PLB (Alexis Biochemicals #804-093-R100) were used as the primary antibody. Following the incubation with the secondary antibody against mouse IgG, the membranes were allowed to react with ECL detection reagents (Amersham pharmacia biotech, UK #RPN2106).

Statistical Analysis

All values are expressed as means±SEM. Data were first compared using analysis of variance. When significant differences were identified, the Newman–Keuls test was applied to determine the level of significance. A p-value <0.05 was considered to be significant.

### Table 3 Effects of Treatment on Diameter of Myocytes, Bmax of Adrenergic \( \beta \)-Receptors, and TGF-\( \beta \) mRNA Expression in the LV

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>ISO + vehicle</th>
<th>ISO + BB</th>
<th>ISO + ARB</th>
<th>ISO + BB + ARB</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diameter of myocytes, ( \mu )m</td>
<td>16.80±0.54</td>
<td>24.42±0.61*</td>
<td>21.53±0.74*</td>
<td>18.99±0.34*</td>
<td>18.45±0.48*</td>
</tr>
<tr>
<td>% fibrosis</td>
<td>1.94±0.08</td>
<td>14.63±2.08*</td>
<td>9.93±0.92*</td>
<td>5.10±1.01*</td>
<td>3.73±0.25*</td>
</tr>
<tr>
<td>Bmax</td>
<td>25.35±2.13</td>
<td>18.74±2.61</td>
<td>20.83±2.93</td>
<td>16.74±1.26</td>
<td>18.04±1.17</td>
</tr>
<tr>
<td>TGF-( \beta ), Log copies/( \mu )gRNA</td>
<td>2.49±0.73</td>
<td>3.37±0.71*</td>
<td>2.90±0.43</td>
<td>3.07±0.42</td>
<td>2.70±0.44#</td>
</tr>
</tbody>
</table>

Values are expressed as means±SEM. BB, celiprolol; ARB, candesartan. \*p<0.05 vs control, \#p<0.05 vs isoproterenol injected rats (ISO).

Fig 3. Light micrographs of LV myocardium from control rats and ISO rats treated for 4 weeks (hematoxylin and eosin staining). In the control rats, the LV myocardium showed normal morphology. In ISO + vehicle rats, hypertrophied cardiomyocytes, a disarray of myofibers and increased interstitial fibrosis were observed. Treatment with either BB or ARB significantly reduced the mean diameter of the cardiomyocytes, but ARB was more potent than BB. Treatment with BB + ARB preserved the histological findings quite well, which were similar to those of the controls. Magnification; ×100.

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Results

Body and Heart Weights and Hemodynamics

Compared with the controls, ISO administration decreased body weight, increased the heart to body weight ratio and LV end-diastolic pressure, and it also significantly reduced min dP/dt.

The administration of BB, ARB and BB + ARB to ISO rats did not prevent the decrease of body weight. BB did not reduce heart weight, but decreased heart rate and LV end-diastolic pressure, and improved min dP/dt. The administration of ARB decreased heart weight, LV end-diastolic pressure, and improved min dP/dt. BB + ARB showed a more favorable reduction of LV weight and pressure, and significantly improved min dP/dt (Table 1).

Echocardiography

The administration of ISO increased LV wall thickness, and decreased LV systolic and diastolic function expressed through increased LV end-diastolic dimension, LV end-systolic dimension (Fig 1, Table 2), E/A ratio and prolonged deceleration time (Figs 1 and Table 2) and decreased LV ejection fraction compared to the controls. BB and ARB did not improve the LV dimension and wall thickness, but BB + ARB significantly improved the LV dimension, wall thickness and LV ejection fraction.

Histology, -Adrenergic Receptor Binding Assay and TGF- mRNA Expression

Hypertrophied cardiomyocytes and increased interstitial fibrosis were observed 4 months after ISO injection. BB prevented the degeneration of cardiomyocytes and decreased diameter of the cardiomyocytes and interstitial fibrosis (Table 3). The ARB reduced the diameter of cardiomyocytes and interstitial fibrosis significantly more than BB (Fig 3, Table 3). BB + ARB preserved the histological findings, which were similar to those of the controls.

The administration of ISO tended to decrease the Bmax of adrenergic -receptors compared to the controls, but all of the treatments had little effect on it. The TGF- in the LV was increased by the vehicle. The BB and ARB decreased, and BB + ARB significantly decreased TGF- in LV (Table 3).

SERCA2 and PLB

Immunohistochemically, the patchy distribution of SERCA2 in the controls was reduced by ISO (Fig 4). The reduction was prevented by BB, ARB or BB + ARB. Compared with controls, ISO increased the immunostaining of PLB and BB + ARB, but not vehicle and ARB, reduced the distribution of PLB in the LV. The results of the Western blot showed the similar trend. Although SERCA2 was not different among groups, the SERCA2/PLB ratios

Fig 4. Immunohistochemistry for sarcoplasmic reticulum Ca2+-ATPase (SERCA2) (Left panel: A, B, C, D, E) and phospholamban (PLB) (Right panel: a–e) in LV myocardium. In the control rats, the immunoreactivity against SERCA2 and PLB was observed patchily (A, a). SERCA2 was reduced in the ISO + vehicle rats, whereas PLB was increased (B, b). The reduction of SERCA2 tended to be prevented by either treatment with BB, ARB and BB + ARB (C–E). BB and BB + ARB, but not ARB, reduced PLB in the LV myocardium (c–e).
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were increased by BB and BB + ARB, but ARB had no effect on it (Fig 5).

Discussion

Ventricular remodeling is a dynamic response to an increased working load of the heart and may be a major feature of heart failure. As described previously, ISO rats significantly increased the heart to body weight ratio, dilated the LV cavity and thickened the LV wall. Histologically, hypertrophied and degenerated cardiomyocytes and increased interstitial fibrosis were seen by light microscopy 4 months after the ISO injection. Furthermore, Ca2+ handling in the cardiomyocytes was impaired by ISO. These findings might lead to cardiac dysfunction in ISO rats.

The efficacy of combination therapy with β-adrenergic blockers with angiotensin converting enzyme inhibitors or angiotensin-II receptor blockers on heart failure has not been fully elucidated and is still controversial despite the fact that each blocker of the renin – angiotensin and sympathetic nervous systems is individually known to improve cardiac dysfunction of heart failure in human and animal models.

In the present study, each treatment with BB and ARB equally and significantly improved diastolic dysfunction and LV end-diastolic pressure in ISO rats. The dilated LV dimension and thickened LV wall in ISO rats were not improved by each treatment alone. The discrepancy between hemodynamics improvement and cardiac echo findings may be explained by the fact that 4 weeks treatment was not long enough for the development of significantly favorable effects on the echocardiographic findings. Conversely, the combination treatment exhibited a more favorable improvement on hemodynamics than each individual treatment, and significantly improved the LV dimension and wall thickness compared with ISO + vehicle.

Histologically, ARB was more potent than BB in preserving cardiomyocytes and preventing fibrosis from the insults of ISO (Fig 5, Table 3). In addition to RAS activation, adrenergic activation in the progression of heart failure might increase myocardial cytokine expressions like tumor necrosis factor (TNF)-α and interleukin (IL)-1β. In the present study, we showed that ISO significantly increased TGF-β in LV myocytes. Although BB and ARB tended to decrease, BB + ARB significantly decreased the activation of TGF-β. Recently, we reported that ARB improved microangiopathy and prevented the increase of inflammatory cytokines, especially IL-1β and IL-6, in the diabetic rat heart. Prabhu et al reported that β-adrenergic blockers could suppress the activation of cytokines. BB and ARB showed similar effects on blood pressure and cytokines, but the effect on LV degeneration was less with BB than with ARB, suggesting that the RAS activation might be major role in cardiac remodeling in our ISO rats.

Heightened sympathetic nervous activity in congestive heart failure helps to maintain cardiac output and blood pressure. However, sustained sympathetic activation in congestive heart failure is thought to produce abnormalities in adrenergic signaling and cell physiology including abnormal Ca2+ handling, which might depress cardiac performance. There are several Ca2+ regulatory molecules including SERCA2 and PLB. Previous studies have shown marked variability in the abundance of SERCA2 and PLB among patients with congestive heart failure. A reduction in SERCA2/PLB ratios may explain the reduced peak and slower decay of the Ca2+ transient resulting in suppressed cardiac function. Our result showing that BB increased the SERCA2/PLB ratio may suggest that Ca2+ handling in ISO rats was more improved by BB than ARB. Kubo et al showed that β-adrenergic blocker treatments improved the decreased cardiac function and restored decreased SERCA2/PLB ratio in congestive heart failure patients. It was reported that the beneficial effect of β-adrenergic blockers on cardiomyocytes was through the blocking of
phosphorylation of ryanodine receptor 2, thus preventing a Ca^{2+} leak from sarcoplasmic reticulum. These findings suggest that restoration of cardiac function by β-adrenergic blockers may largely depend on the direct effect on myocytes through improved Ca^{2+} handling.

Some reports showed that β-adrenergic blocker treatment in congestive heart failure increased the β-adrenergic receptors. In the present study, decreased β-adrenergic receptor density was seen in ISO rats, which was not restored using BB. The treatment period in the present study was 4 weeks, which might be too short to restore β-adrenergic receptor density. A report that chronic treatment with carvedilol improved the ejection fraction in congestive heart failure patients, but did not produce the alterations in β-adrenergic receptors, support our finding.23 Also, the choice of β-adrenergic may affect the results as celiprolol, a β-selective adrenoceptor antagonist with partial β-agonist properties exhibited intrinsic sympathetic activity.

In the present study, we showed that: (1) celiprolol was potent in improving the function of myocytes through improved Ca^{2+} handling; (2) candesartan was potent in improving fibrosis and the degeneration of myocytes; and (3) the combination therapy with celiprolol and candesartan was more potent than each individual treatment through the combined beneficial mechanisms for the failing heart. Although it is still controversial whether β-adrenergic blockers or angiotensin-II receptor blockers are better for the treatment of congestive heart failure, we can conclude that a combination of celiprolol and candesartan is better than each treatment alone.

Acknowledgments

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References