Combined angiotensin receptor blocker and ACE inhibitor on myocardial fibrosis and left ventricular stiffness in dogs with heart failure

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CONGESTIVE HEART FAILURE (CHF) is characterized by left ventricular (LV) pump dysfunction, chamber dilatation, and exercise intolerance related to LV diastolic dysfunction. The renin-angiotensin system plays a central role in this process (10, 12). Over the past decade, clinical and laboratory studies have reported that angiotensin-converting enzyme (ACE) inhibitor (ACEI) prevents myocardial fibrosis as a result of inhibition of angiotensin II (ANG II) production (12, 15). However, ACEI does not always suppress concentrations of ANG II in patients with CHF, presumably a reflection of the existence of other enzyme pathways (e.g., chymase) that escape ACE inhibition (17, 28). ANG II receptor blocker (ARB) appears to be an obvious choice as an alternative therapy to ACEI in CHF. Recently, bradykinin antagonist has been shown to prevent the decrease in collagen deposition associated with ACEI, suggesting that prevention of myocardial fibrosis by ACEI is due to a bradykinin-mediated mechanism as well as inhibition of ANG II (13). These studies suggest that ARB + ACEI is beneficial in preventing myocardial fibrosis in CHF compared with ARB or ACEI alone. However, the usefulness of combined therapy for the prevention of cardiac fibrosis and LV stiffness has not been documented in animals with a non-ACE pathway.

Chronic rapid pacing produces time-dependent changes in LV function, hemodynamic compromise, and hormonal activation that are similar to the clinical spectrum of CHF (8, 21, 22). Accordingly, a model of pacing-induced CHF in dogs with additional pathways in the heart for generating ANG II as well as ACE, A model of pacing-induced congestive heart failure (CHF) was used to test the central hypothesis that ARB + ACEI prevents myocardial fibrosis and decreases LV stiffness to a greater extent than ARB or ACEI alone. Thirty-five dogs were assigned to the following treatment protocols on the 8th day of a 4-wk pacing schedule: 1) rapid ventricular pacing, 2) ARB (candesartan cilexetil, 1.5 mg·kg−1·day−1) with pacing, 3) ACEI (enalapril, 1.9 mg·kg−1·day−1) with pacing, 4) ARB (candesartan cilexetil, 0.75 mg·kg−1·day−1) + ACEI (enalapril, 0.95 mg·kg−1·day−1) with pacing, and 5) sham operation. The LV stiffness coefficient was significantly increased after rapid pacing but was significantly lower with ARB + ACEI than with ARB or ACEI alone. The collagen volume fraction and mRNA levels of collagen I and III, which were increased by rapid pacing, were significantly lower with ARB + ACEI than with ARB or ACEI alone. Thus ARB + ACEI prevents myocardial fibrosis and decreases LV stiffness during the progression of CHF compared with ARB or ACEI alone.

MATERIALS AND METHODS

This study was approved by the Animal Research Committee of Mie University School of Medicine. Specific attention was given to the appropriateness of the animal model, the welfare of the animals, the adequacy of anesthesia, and the methods of instrumentation.

Dose-selection studies. In a preliminary study, we tested five doses of an ARB, candesartan cilexetil, and an ACEI, enalapril, to establish the maximum doses that did not significantly affect systemic blood pressure but did sufficiently suppress the pressor effects of ANG I or ANG II. A catheter was positioned in the femoral artery to measure systemic blood pressure under anesthesia in six healthy male dogs (20–26 kg body wt). First, basal pressor responses to ANG II (0.1 μg/kg; Sigma) were obtained in six dogs (6, 32). After the dogs recovered from the pressor-response study, candesartan cilexetil (0.5 mg·kg−1·day−1) was administered for 3 days, and pressor responses to ANG II were obtained again. Pressor responses after the administration of higher doses of candesartan cilexetil (1.0, 1.5, and 2.0 mg·kg−1·day−1) were obtained in a similar fashion (23). We found that candesartan at 1.5 mg·kg−1·day−1 completely eliminated pressor responses to ANG II without significantly affecting resting blood pressure. Next, pressor responses to ANG I (0.1 μg/kg) were obtained before and after the administration of enalapril (0.9, 1.3, 1.9, and 2.5 mg·kg−1·day−1 for 3 days) in a manner similar to that used to obtain responses to ANG II (26, 32). At 1.9 mg·kg−1·day−1, enalapril significantly suppressed the pressor response to ANG I without significantly affecting resting blood pressure. Finally, we chose half doses of candesartan cilexetil and enalapril as the combined therapy to
avoid a significant effect on resting blood pressure (20). The pressor responses to ANG I and ANG II were obtained as described previously (21, 32). We confirmed that candesartan cilexetil at 0.75 mg·kg\(^{-1}\)·day\(^{-1}\) and enalapril at 0.95 mg·kg\(^{-1}\)·day\(^{-1}\) completely eliminated the pressor responses to ANG II and significantly suppressed the pressor responses to ANG I without significantly affecting resting blood pressure.

**Experimental design.** The rapid-pacing model of CHF in dogs has been described previously (8, 21, 22). Briefly, to induce CHF by rapid right ventricular pacing, a modified multiprogrammable pacemaker (model 8329, Medtronic) was implanted in 38 healthy adult male mongrel dogs (20–26 kg body wt). After full recovery from the instrumentation (10–14 days after surgery), the animals were assigned to the following treatment protocols: 1) rapid ventricular pacing (240 beats/min, \(n = 7\)), 2) concomitant ARB (candesartan cilexetil, 1.5 mg·kg\(^{-1}\)·day\(^{-1}\)) and rapid pacing (\(n = 7\)), 3) concomitant ACEI (enalapril, 1.9 mg·kg\(^{-1}\)·day\(^{-1}\)) and rapid pacing (\(n = 7\)), 4) concomitant combined ARB (candesartan cilexetil, 0.75 mg·kg\(^{-1}\)·day\(^{-1}\)) and ACEI (enalapril, 0.95 mg·kg\(^{-1}\)·day\(^{-1}\)) and rapid pacing (\(n = 7\)), and 5) sham operation (\(n = 7\)). The drug treatment protocols were begun on the 8th day of a 4-wk pacing protocol. Two dogs in the rapid-pacing-only group and one in the group treated with ACEI only were excluded from the study because of unsuccessful pacing or unexpected sudden death.

**LV function and hemodynamics.** After 4 wk of pacing, the pacemaker was turned off and the animals were allowed to equilibrate for 30–40 min. After 20 ml of arterial blood were drawn for assay, animals were anesthetized with α-chloralose (50 mg/kg) and urethane (600 mg/kg). To measure LV pressures and volumes, a 7-F micromanometer-tipped catheter (Millar Instruments) and a 6-F conductance catheter (Webster Labs) were positioned at the LV apex as previously described (8, 21). The femoral vein was cannulated with a balloon-tipped catheter to transiently obstruct venous inflow from the inferior vena cava. We collected three sets of steady-state and variably loaded P-V loops generated by transient occlusion of the inferior vena cava over a 15-s period. After the hemodynamic studies were completed, the dogs were killed with an overdose of anesthetic, and the LV free wall was cut into four to five pieces and stored at \(-80^\circ\text{C}\).

**Data processing and analysis.** End-systolic elastance (\(E\text{es}\)), representing LV end-systolic stiffness, was calculated as described in the literature. \(E\text{es}\) is sensitive to changes in contractile state but is relatively insensitive to changes in loading conditions (8, 22).

The monoexponential-based time constant of the isovolumic fall of LV pressure (\(τ\)) was calculated with the assumption that pressure decayed to a nonzero asymptote (8, 21). Total systemic resistance was calculated as LV-end systolic pressure divided by cardiac output. Effective arterial elastance (\(E\text{sa}\)) was calculated as LV-end systolic pressure divided by stroke volume (SV). Coupling of the LV and arterial system was quantified as \(E\text{sa}/E\text{sa}\) (8, 21).

To estimate passive LV chamber stiffness, multiple P-V loops were obtained for each caval occlusion. The combined diastolic data from the mid-to-late filling portion of each loop were fitted to the following monoexponential equation

\[
P = α[e^{β(V_0 - ⟨V⟩)} - 1]
\]

where \(V_0\) is the equilibrium volume, \(β\) is the chamber stiffness coefficient, and \(α\) is a stiffness and a scaling coefficient (26).

**Neurohormonal assays.** Plasma ANG II and plasma aldosterone levels were determined by RIA as previously described (8, 13, 21). Plasma norepinephrine (NE) concentration was measured using high-performance liquid chromatography (8, 21). Myocardial tissue samples were suspended in acetic acid (1 mol/L), homogenized, and centrifuged at 15,000 rpm for 30 min at 4°C (2). Myocardial tissue ANG II levels were measured by RIA using a specific antibody directed against synthetic ANG II (34).

**Histomorphometry.** Specimens from the LV free wall were stained with picrosirius red stain to evaluate the degree of fibrosis (13).

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**Table 1. LV systolic function and systemic hemodynamics with chronic rapid pacing: effects of ARB, ACEI, and ARB + ACEI**

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>CHF</th>
<th>ARB</th>
<th>ACEI</th>
<th>ARB + ACEI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart rate, beats/min</td>
<td>107±3</td>
<td>135±10*</td>
<td>126±12*</td>
<td>126±6*</td>
<td>128±6*</td>
</tr>
<tr>
<td>End-systolic LVP, mmHg</td>
<td>105±9</td>
<td>122±6*</td>
<td>113±3*</td>
<td>114±4*</td>
<td>101±3*</td>
</tr>
<tr>
<td>End-diastolic LVP, mmHg</td>
<td>7±2</td>
<td>26±1*†</td>
<td>26±2*†</td>
<td>26±2*†</td>
<td>22±2*‡†$</td>
</tr>
<tr>
<td>End-diastolic LVV, ml</td>
<td>70±5</td>
<td>44±8*</td>
<td>32±4*†</td>
<td>32±4*†</td>
<td>24±3*‡$</td>
</tr>
<tr>
<td>SV, ml</td>
<td>43±5</td>
<td>5.8±0.7*</td>
<td>4.1±0.6†</td>
<td>4.0±0.4*‡</td>
<td>3.0±0.4*†$</td>
</tr>
<tr>
<td>Total systemic resistance, mmHg·l·es−1·min</td>
<td>2.5±0.2</td>
<td>1.1±0.1*</td>
<td>1.3±0.2*</td>
<td>1.3±0.2*</td>
<td>1.6±0.1*†‡§</td>
</tr>
<tr>
<td>(E\text{es}/E\text{a})</td>
<td>1.6±0.5</td>
<td>0.2±0.1*</td>
<td>0.3±0.1*</td>
<td>0.3±0.1*</td>
<td>0.6±0.1*†‡§</td>
</tr>
</tbody>
</table>

Values are means ± SD. Left ventricular systolic function and systemic hemodynamics in sham-operated animals (Cont). Animals with pacing-induced congestive heart failure (CHF), CHF animals treated with candesartan cilexetil [ANG II receptor blocker (ARB)], CHF animals treated with angiotensin-converting enzyme inhibitor (ACEI), and CHF animals treated with ACEI + ARB. LVP, left ventricular (LV) pressure; LVV, LV volume; SV, stroke volume; \(E\text{es}\), arterial elastance; \(E\text{sa}\), end-systolic elastance. *\(P < 0.05\) vs. Cont. †\(P < 0.05\) vs. CHF. ‡\(P < 0.05\) vs. ARB. §\(P < 0.05\) vs. ACEI.

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Fig. 1. Left ventricular (LV) relaxation (\(τ\)) and stiffness coefficient (\(β\)) in sham-operated animals (Cont) and animals with pacing-induced congestive heart failure (CHF), CHF treated with candesartan cilexetil [ANG II receptor blocker (ARB)], CHF treated with enalapril [angiotensin-converting enzyme inhibitor (ACEI)], and CHF treated with candesartan cilexetil + enalapril (Combi). *\(P < 0.05\) vs. Cont. †\(P < 0.05\) vs. CHF. ‡\(P < 0.05\) vs. ARB. §\(P < 0.05\) vs. ACEI.
Twenty microscope fields were randomly selected and digitized at \times 100 magnification. Any fields containing vessels, minor scars, or artifacts in the LV were excluded from the study.

**Quantitative RT-PCR.** Expression of collagen I and III in the heart was examined by RT-PCR (13). The total RNA (2 \mu g) isolated from LV tissue using TRIzol reagent (GIBCO BRL) was converted to cDNA, and PCR was performed using oligonucleotide primers complementary to dog collagen I cDNA. Collagen I transcripts were amplified using the forward primer 5'-ATGGGTCCCTCTGGTCCTCG-3' and the reverse primer 5'-ACCAGTAGCACCATCATT-3'. Collagen III transcripts were amplified using the forward primer 5'-TCCGTTCTCTGCGATGACATA-3' and the reverse primer 5'-AGCTGGACCTTTGATACCTGG-3'. Each of the PCR products was separated on a 2% agarose gel and visualized by ethidium bromide staining. Gels were analyzed using an ATTO Densitograph. RNA expression was quantified after scanning and by comparison with internal-control  \beta-actin.

For the analysis of myocardial canine endothelial nitric oxide (NO) synthase (eNOS) gene expression, mRNA levels were evaluated by RT-PCR. Briefly, first-strand cDNA was synthesized from 5 \mu g of total RNA, and 1-\mu g aliquots of cDNA were amplified as previously described (13). Canine eNOS-specific primer pairs were synthesized according to the published literature (13, 14). The PCR products were then resolved by electrophoresis and quantified.

**Statistical analysis.** Multiple comparisons were performed using ANOVA. When a significant overall effect was present, intergroup comparisons were performed using paired t-tests and Bonferroni's correction for multiple comparisons. Significance was accepted at \( P < 0.05 \). Values are means \( \pm \) SD.

**RESULTS**

**LV function and hemodynamics.** At baseline, hemodynamic variables in all dogs were within the normal limits for our laboratory and were similar among the five study groups (data not shown).

In all pacing groups, end-diastolic pressure and volume were increased and accompanied by a significant decrease in LV elastance (\( E_{es} \); Table 1). In the pacing-only group, \( E_{es} \) and total systemic resistance were significantly increased and SV was decreased. In the groups treated with ARB or ACEI, total systemic resistance, \( E_{es} \), and end-diastolic volume were significantly decreased and \( E_{es}/E_{es} \) and SV were significantly increased. Comparisons among treatment groups showed that ARB + ACEI significantly decreased end-diastolic pressure and volume, total systemic resistance, and \( E_{es} \) and significantly increased SV and \( E_{es}/E_{es} \) relative to ARB or ACEI alone.

The index of LV relaxation (\( \tau \)) was significantly prolonged and \( \beta \) was increased after 4 wk of chronic rapid pacing (Fig. 1). There were no significant changes in \( \tau \) and \( \beta \) with ARB or ACEI alone. However, with ARB + ACEI, \( \tau \) was significantly shorter and \( \beta \) was significantly lower than in the rapid-pacing-only groups, indicating an improvement in LV relaxation and stiffness.

**Neurohormonal activity.** Plasma NE levels were significantly increased in the rapid-pacing-only and ARB groups...
compared with the sham-operated group, whereas the increase in plasma NE levels was significantly attenuated in the ACEI and ARB + ACEI groups (Fig. 2). Plasma ANG II levels were significantly increased in all rapid-pacing groups (P < 0.05). Myocardial ANG II levels in the rapid-pacing-only group were significantly increased (P < 0.05), but there were no significant differences in myocardial ANG II levels in the sham-operated group or any of the treated groups (Fig. 3). Plasma aldosterone levels increased by >10-fold in the rapid-pacing-only group. In the groups treated with ARB and ACEI alone, plasma aldosterone fell significantly from rapid-pacing-only values but remained higher than control values. Plasma aldosterone levels in the combined-therapy group were similar to those in the sham-operated group and significantly lower than those in rapid-pacing-only group and the groups treated with ARB and ACEI alone. Myocardial eNOS levels were significantly decreased in the rapid-pacing-only and ARB groups compared with the sham-operated group. In contrast, ACEI alone and ARB + ACEI increased eNOS levels.

Effects on histomorphometry. Representative photomicrographs of histological sections of the LV from each group stained with picrosirius red are shown in Fig. 4. Long-term rapid pacing resulted in accumulation of a diffuse collagen network and increased the amount of interstitial fibrosis, expressed as an increase in the collagen volume fraction. Collagen density was significantly lower in all treated groups (Fig. 4). Collagen density was significantly lower in the ARB + ACEI group than in the groups treated with ARB or ACEI alone.

Effects on collagen I and III gene expressions. Expression of collagen I and III mRNA significantly increased in all pacing groups compared with control values (Fig. 5). In all treated groups, the increase in collagen I and III was significantly suppressed. Collagen I and III were significantly lower in the ARB + ACEI group than in the groups treated with ARB or ACEI alone.

DISCUSSION

The major finding of the present study was that ARB + ACEI synergistically prevented myocardial fibrosis and decreased LV stiffness during the progression of CHF in animals that have additional pathways in the heart for generating ANG II as well as ACE.

Consistent with past reports (9, 31), LV fibrosis accompanied an increase in LV stiffness in CHF. ANG II stimulates fibroblasts via the AT1 receptor to increase collagen I and III synthesis and to reduce matrix metalloproteinase-1 (interstitial collagenase) activity (4, 5). A change in collagen architecture, with abnormal alignment and/or endomyocardial fibrosis, ap-
pears to increase myocardial stiffness. ACEI does not always suppress ANG II in patients or dogs with CHF because of the existence of other enzyme pathways (e.g., chymase) that escape ACE inhibition (17, 28). Consistent with past reports (25), we found a significant increase in mRNA levels of chymase in the LV of dogs with CHF compared with normal dogs (data not shown). Accordingly, ARB may be an obvious choice as an alternative therapy to ACEI in CHF because of the effective blockade of ANG II.

Aldosterone, which is increased in CHF (18, 30), has been implicated in myocardial interstitial and perivascular fibrosis (33, 36). ACEI partly, but not completely, attenuated an increase in plasma aldosterone levels in the present study, which may be attributable to ANG II generation via non-ACE pathways. ARB also failed to completely block the formation of aldosterone, probably because of the ANG II-independent stimuli of aldosterone production, such as intravascular volume depletion, potassium, corticosterone, and endothelin (29, 35). We demonstrated that combined therapy synergistically decreased plasma aldosterone, which may contribute to the prevention of myocardial fibrosis.

On the other hand, we demonstrated that ACEI alone or ARB + ACEI significantly increased myocardial eNOS, which may be due to an increase in local bradykinin levels. Bradykinin decreases mRNA and protein levels for fibronectin and collagen I and III (13, 19). In bradykinin B2 receptor knockout mice, perivascular and reparative fibrosis were observed in the LV (11, 24).

Consequently, ARB + ACEI has the advantage of preventing myocardial fibrosis and decreasing LV stiffness in CHF compared with ACEI or ARB alone. These results may be related to the fact that, in experimental and clinical studies, ARB + ACEI has favorable effects on hemodynamic indexes, LV remodeling, and neurohumoral activity in CHF compared with ACEI or ARB alone (3, 21, 27, 32, 37). ARB + ACEI also increases exercise capacity and improves New York Heart Association function class (16).

Consistent with past reports, chronic rapid pacing also caused a prolongation of LV relaxation (6, 8). Cheng et al. (7) reported that ANG II prolonged myocyte relaxation in CHF and that these effects are reversed by ARB. On the other hand, captopril shortened LV relaxation in isolated ejecting guinea pig hearts, and a B2 receptor antagonist and an NO scavenger inhibited the effects of captopril on LV relaxation (1). These effects may cause the synergistic improvement of LV relaxation in CHF treated with ARB + ACEI.

We measured LV stiffness using a monoexponential fitting of mid-to-late filling portions of each P-V loop. However, this technique is limited, because unless the diastolic data are perfectly monoexponential, simply elevating the same P-V curve or restricting analysis to a slightly different range of volumes and pressures can influence the derived parameters (26).

We did not address the time course of the progression of LV fibrosis. Spinale et al. (32) reported in swine that LV end-diastolic pressure was increased after 1 wk of rapid pacing, and an increase in LV fibrosis was obvious after 3 wk of pacing. Furthermore, we did not measure the time course of blood pressure during the development of heart failure, which may affect myocardial fibrosis and LV stiffness. Further work is necessary to determine whether the timing of the prevented effects on cardiac fibrosis is different among the treatment groups and to define the mechanisms involved.

We examined the expression of collagen I and III in the heart by RT-PCR, which is not more quantitative than Northern blot or real-time PCR. Further work may be necessary to clarify the direct relation between the changes in collagen mRNA levels and LV stiffness.

Conclusions. ARB + ACEI in CHF suppressed the renin-angiotensin-aldosterone system and the activation of the bradykinin-NO system and, thereby, decreased the expression of collagen I and III mRNA and prevented myocardial fibrosis. Combined therapy also decreased LV stiffness as estimated using LV P-V loops. These findings suggest that ARB + ACEI has the advantage of preventing myocardial fibrosis and decreasing LV stiffness in CHF compared with ARB or ACEI alone.

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