Differential Effects of Angiotensin II Versus Endothelin-1 Inhibitions in Hypertrophic Left Ventricular Myocardium During Transition to Heart Failure

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Background—In view of their mutual crosstalk, the roles of angiotensin II (Ang II) and endothelin-1 (ET-1) in the myocardium are assumed to be synergistic and supplemental.

Methods and Results—In the phase of compensated left ventricular (LV) hypertrophy of Dahl salt-sensitive rats, Ang II peptide and the ACE mRNA in the LV were increased by 1.6- and 3.8-fold, respectively. In contrast, ET-1 peptide and the preproET-1 mRNA remained unchanged. In subsequent congestive heart failure (CHF), Ang II and ACE mRNA did not show further increases. But ET-1 and the mRNA were increased de novo by 5.3- and 4.1-fold, respectively. In ascending aorta–banded rats, the local activations of Ang II and ET-1 also showed a differential time course between LV hypertrophy and CHF. Long-term treatments of Dahl salt-sensitive rats with temocapril (an ACE inhibitor) and with bosentan (a mixed ET receptor blocker) equally improved long-term survival. Temocapril reduced the LV/body weight ratio and ameliorated LV fractional shortening. Conversely, although bosentan equally improved fractional shortening, it did not reduce the increase in LV mass. Combined treatment with these 2 drugs further ameliorated the animal’s survival without additional decreases in systolic pressure.

Conclusions—The pathophysiological roles in the myocardium during the transition to CHF differ qualitatively between Ang II and ET-1. Thus, long-term therapy with a combination of ACE inhibition and ET antagonism may provide a new approach for heart failure in humans. (Circulation. 2001;104:606-612.)

Key Words: angiotensin n endothelin n hypertension n hypertrophy n ventricles

Both angiotensin II (Ang II) and endothelin-1 (ET-1) are potent vasoactive and growth-promoting peptides that show ubiquitous and extensive tissue distributions. On the basis of myocardial expression of their receptors, precursors, and converting enzymes, cardiac growth and function are believed to be significantly regulated by the local systems in an autocrine/paracrine manner. In studies using cultured neonatal myocytes, exogenous and stretch-released endogenous Ang II induced hypertrophic responses by endogenous release of ET-1. Exogenous administration of ET-1 also produced myocyte hypertrophy in culture. Thus, these 2 peptides might compose a mutual reciprocal signal network in the myocardium. Furthermore, as the predominant receptors on myocytes, AT1 and ET4 receptors share common subcellular signaling pathways. Because of these local positive feedback loops and signal redundancy, their individual and specific roles in chronic processes, such as in vivo hypertrophy and cardiac failure, have remained elusive. Clarification of these processes, however, should be invaluable for establishment of a multiple-pharmaceutical approach with endothelin receptor blockers in addition to the established ACE inhibitors for the treatment of patients with chronic heart failure.

In previous studies using a heart-failure-transition model of Dahl salt-sensitive hypertensive (DS) rats, ET-1 started to accumulate in myocardial tissue only when the heart reached the failing stage, and hypertrophy per se was not accompanied by a local increase of ET-1. This observation suggested that Ang II and ET-1 might essentially play different pathophysiological roles in states of chronic hypertrophy and subsequent failure. In the present study, we further evaluated the time course of activation of these 2 local systems and their modulation by specific blockers in these animals.

Methods

Animals and Study Protocols
Male inbred DS and Dahl salt-resistant (DR) rats were fed an 8% NaCl (high-salt) diet from 6 weeks of age. In protocol 1, 11-week-
old DS (left ventricular hypertrophy, LVH), 15-week-old DS (pre-congestive heart failure, CHF), and 17-week-old DS (CHF) and age-matched DR rats (n=6, respectively) were used for measurement of myocardial Ang II and ET-1 activities. In vivo hemodynamic and LV geometry were assessed by transesophageal echocardiography as previously reported.\(^1\) In protocol 2, 11-week-old LVH-DS rats with established concentric LVH were randomized into 4 groups and treated with vehicle (Cont group, n=27); bosentan (F. Hoffmann-La Roche, Ltd)\(^9\) at a dose of 100 mg · kg body wt \(^{-1}·d^{-1}\) (Bos group, n=32); temocapril (Sankyo Co Ltd), an ACE inhibitor,\(^10\) at 20 mg · kg body wt \(^{-1}·d^{-1}\) (Temo group, n=20); or a combination of Bos and Temo (Temo+Bos group, n=16). These drugs were administered by gastric gavage once a day from 11 weeks of age. Animals were monitored on a day-by-day basis. The in vivo blood pressure and echocardiograms were recorded at weeks 11, 15, and 17. In protocol 3, 60 male Sprague-Dawley rats were banded at the ascending aorta (AOB) according to the methods of Litwin et al.\(^11\) At 18 to 20 weeks after banding, 38 animals were alive and were subjected to echocardiographic study and the subsequent assay of LV Ang II and ET-1.

**Measurement of Plasma and Myocardial Ang II and ET-1 Levels**

Under anesthesia, blood was collected from the abdominal aorta, and the heart was quickly excised. The LV tissue was homogenized, centrifuged, and stored at \(-80^\circ\text{C}\) until use. Ang II and ET-1 were extracted from the plasma and the ventricular tissue according to the method of Kitamura et al.\(^12\) Ang II content was determined with a radioimmunoassay kit (Nichols Institute) according to the manufacturer's recommendations. ET-1 levels were measured with the specific enzyme immunoassay kit (Wako Pure Chemical) originally developed by Suzuki et al.\(^13\)

**Quantitative RT-PCR for Angiotensinogen, ACE, preproET-1, and ECE mRNA**

Total RNA was isolated from the LV tissue by the acid guanidinium thiocyanate–phenol-chloroform method. Quantitative reverse transcription–polymerase chain reaction (RT-PCR) was carried out as previously described in detail.\(^14\) After synthesis of the first-strand cDNA, a constant amount of cDNA was amplified by PCR with a serially diluted nonhomologous DNA fragment containing primer template sequences as an internal control according to the instructions of the PCR MIMIC Construction kit (Clontech). Sense primers (S) and antisense primers (AS) for rat angiotensinogen (S: 5'-CTGACCGACTTCTGCTGCC-3' [position 698 to 717]), ACE (S: 5'-CTGACCGACTTCTGCTGCC-3' [position 698 to 717]), and AS: 5'-CTGACCGACTTCTGCTGCC-3' [position 698 to 717]) were synthesized by use of the published cDNA sequences.\(^15–19\) A portion of the PCR reaction product was then resolved by electrophoresis on a 5% polyacrylamide gel and analyzed with a FUJIX bioimaging analyzer BAS2000.

**Statistical Analysis**

All data are expressed as mean±SEM. The significance of the difference between group means was analyzed by 1-way ANOVA with post hoc comparisons by Fisher’s protected least-significant-difference test. The main effects of the drug were tested by 2-factor ANOVA for repeated measures, and differences at specific time points between the groups were assessed by 1-factor ANOVA with post hoc comparisons by Fisher’s protected least significant difference test. Relationships between 2 variables were tested by linear regression analysis. Survival was analyzed by the standard Kaplan-Meier analysis with log-rank test. In all tests, a value of \(P<0.05\) was considered statistically significant.

**Results**

**Blood Pressure, LV Weight, and Hemodynamic Measures in Dahl Rats**

At the LVH stage, the DS rats showed systemic hypertension and typical concentric LVH, whereas LV fractional shortening (FS) and LV systolic wall stress (WS) remained within the normal range (Table). The DS rats showed the initial signs of transition to heart failure at 15 weeks of age, as indicated by an increase in WS and a decrease in FS. At the CHF stage, a marked increase in LV diameter was associated with a substantial increase in WS and a decrease in FS. Hence, consistent with our previous reports,\(^2,3\) the DS rats presented the transition from established LVH to CHF.

**Ang II Peptide, Angiotensinogen mRNA, and ACE mRNA Levels**

Figure 1, A and B, shows plasma Ang II and LV Ang II levels at the 3 distinct stages. At the LVH stage, although the plasma Ang II level was within the normal range, the LV Ang II level was increased by 1.6-fold compared with that in the age-matched DR rats. This elevated tissue level was sustained even before (15 weeks) and after the transition to CHF (17 weeks). The mRNA levels of angiotensinogen increased equally at the LVH stage and the CHF stage (Figure 1C). ACE mRNA was similarly increased at the LVH stage, the pre-CHF stage, and the CHF stage (Figure 1D), but these...
increases were greater than those of angiotensinogen mRNA. Thus, the local angiotensin system might be critically regulated at the converting enzyme level, whereas the angiotensin-system activation is maintained to the same extent during the transition from LVH to CHF.

**ET-1 Peptide, preproET-1 mRNA, and ECE mRNA Levels**

Figure 2, A and B, shows the plasma and LV tissue ET-1 levels at the 3 stages. At the LVH stage, both the plasma and LV ET-1 levels did not change from the levels in the age-matched DR rats. At 15 weeks of age, however, although the plasma level remained unchanged, the LV ET-1 started to increase by 1.9-fold. After the transition to CHF, both the plasma and LV ET-1 levels were markedly increased, by 3.8- and 5.4-fold, respectively. The mRNA levels of preproET-1 and ECE changed in parallel to the peptide levels (Figure 2, C and D). The magnitude of increase at the CHF stage, however, was much higher for the preproET-1 mRNA than for the ECE mRNA, and the quantitative change of preproET-1 mRNA more closely followed the changes in the corresponding peptide levels. In summary, the production of preproET-1, the ET-1 precursor, is a critical regulation step for its local activation, and this step was activated de novo during the transition to CHF.

**Relationship of Activation Levels of the Local Angiotensin and ET-1 Systems to LVH and Function**

As demonstrated above, ACE should critically determine activation of the local angiotensin system, whereas the preproET-1 would be limiting for activation of the myocardial ET-1 system. Figure 3 shows the relationships of these mRNA levels to LV/body weight (BW) ratio (LVH), as well as to LV FS (LV function). The values of LV/BW showed a strong and positive correlation with the ACE mRNA levels throughout these 3 stages ($r^2=0.981$, $P <0.001$). At the same time, they were not related to the preproET-1 mRNA levels ($r^2=0.646$, $P =0.055$). Conversely, the levels of LV FS showed no relationship to the ACE mRNA levels ($r^2=0.525$, $P =0.103$), whereas they presented a close and inverse correlation with the preproET-1 mRNA levels ($r^2=0.987$, $P <0.001$). Thus, activation of the local angiotensin system was correlated with the extent of the LVH. The ET-1 system, however, was more closely correlated with the LV dysfunction, but not with the LVH per se.

**Effects of Long-Term Administration of Temocapril and Bosentan From the LVH Stage**

All rats of the vehicle-treatment (Cont) group died of pulmonary congestion with LV dysfunction between 15 and 18 weeks (mean±SEM 16.6±0.4 weeks, Figure 4). In contrast, the survival of the temocapril-treatment (Temo) and the bosentan-treatment (Bos) groups showed marked but equivalent prolongation ($P <0.001$ compared with the Cont group). There was no difference in survival between the Temo and
Bos groups (19.3 ± 0.9 and 18.7 ± 0.8 weeks, respectively). In addition, combination therapy with Temo and Bos (Temo + Bos group) further prolonged animal survival, half of them surviving to 22.1 ± 1.4 weeks (P < 0.001 versus other groups). The survival rates at 17 weeks were 35% in the Cont group, 90% in the Temo and the Bos groups, and 100% in the Temo + Bos group. As illustrated in Figure 5A, there was a similar decline of systolic blood pressure in the Temo and Bos groups. Figure 5B shows that at 15 weeks, suppression of LVH occurred in the Temo group (P < 0.01 versus other groups). This suppression was still observed at 17 weeks. LV/BW in the Bos group, however, did not differ from that in the Cont group at 15 and 17 weeks. The FS change (Figure 5C) demonstrated that temocapril, even at 15 weeks, ameliorated the progression of LV dysfunction. Although bosentan did not affect LV/BW, it inhibited the progression of LV dysfunction at 17 weeks. As a result, as shown in Figure 5D, both drugs suppressed increases in systolic WS at 17 weeks to a similar extent (P < 0.01 versus Cont). These parameters stayed at the baseline levels at 17 weeks in the Temo + Bos group, whereas the systolic blood pressure remained at a level similar to those in the Temo and Bos groups.

Local Ang II Versus ET-1 Peptides in AOB Rats
At 18 to 20 weeks after AOB, the rats were divided into 3 groups according to the following criteria: (1) modest LVH (n = 15): LV/BW < 3.5 mg/g and LV FS > 45%, (2) LVH (n = 12): LV/BW > 3.5 mg/g and LV FS > 45%, and (3) CHF (n = 11): LV/BW > 3.5 mg/g and LV FS < 45%. As shown in Figure 6, A and B, a significant increase in ET-1 was observed between the LVH and CHF groups, whereas Ang II showed a gradual increase along with hypertrophy before the heart failure transition. Although these animals tended to show increases in both Ang II and ET-1 in the compensatory stage, ET-1 but not Ang II showed a further increase when the animal reached the failing stage. The Ang II versus ET-1 relationship of each animal is plotted in Figure 6C. These 2 parameters showed a nonsignificant (r = 0.57, P = 0.08) concave curvilinear relationship.

Discussion
In the present study, we used DS rats, which provided an opportunity to observe the distinctive transition from LVH to CHF. At the stage of compensatory LVH, the myocardial Ang II system showed a remarkable activation, whereas the ET-1 system remained in the basal state. During the transition to CHF, the Ang II system maintained the same activation state, whereas the ET-1 system showed de novo activation. The local Ang II activation showed a positive correlation with the extent of LVH, whereas it was not significantly related to concomitant LV function. In contrast, the local ET-1 activation did not show any relation to the extent of LVH, but it was closely correlated with the progress of LV dysfunction. Furthermore, long-term treatment with an ACE inhibitor blunted the LVH, with subsequent improvement in the survival rate. This result was in contrast to long-term ET receptor blockade, which ameliorated LV dysfunction during the transition to CHF with no reduction in the progression of LVH but resulted in a similar improvement in the prognosis. In addition, their combined treatment further maintained LV function, with a marked improvement in survival. In AOB
rats, activation of cardiac Ang II and ET-1 occurred differentially during the period of transition from LVH to CHF. Taken together, this set of observations indicates that, although both the cardiac Ang II and ET-1 systems play critical roles in the progression of heart failure, their local regulations may be largely independent.

The local Ang II system not only activates the hypertrophy process but also upregulates itself to induce a positive feedback loop; these observations have clarified the mechanistic background by showing that ACE inhibitors both lower the degree of hypertrophy and improve cardiac function to a greater extent than other unloading regimens.\(^\text{20}\) In the present study, we also confirmed the relationship of the cardiac angiotensin system to the extent of myocardial hypertrophy. Temocapril treatment blunted LVH and maintained a smaller LV cavity, resulting in the improvement of LV function. Litwin et al\(^\text{11}\) showed that ACE inhibition delayed the transition from pressure-overload LVH to CHF in the AOB rat model. This is thoroughly compatible with our present findings in the DS rats. We have clarified for the first time, however, that the cardiac angiotensin system was not further activated during the transition to heart failure and showed no direct relationship with the change in function of the hypertrophic LV myocardium. Hence, in ACE inhibition, the suppression of the hypertrophic process appeared to be the central effect causing amelioration of the animal’s prognosis.

ET-1, in combination with Ang II, has been postulated to be a factor involved in the cardiac hypertrophy process in vitro as well as in animal models in vivo.\(^\text{6,21}\) In heart failure, the level of ET-1 has been shown to be elevated in the plasma; however, its local significance in the hypertrophic and failing myocardium remains unresolved.\(^\text{22}\) In contrast to its potential as a cardiac growth factor and to its presumed concordant activation with the cardiac angiotensin system,\(^\text{23,24}\) we observed that the cardiac ET-1 system was not activated in the established LVH stage. This observation was consistently supported by long-term bosentan treatment, which, in contrast to temocapril, did not diminish the progression of LVH. These results may not be surprising, because in spontaneously hypertensive rats and Ren2 rats, long-term treatments with bosentan did not affect cardiac or vascular hypertrophy.\(^\text{25,26}\) We further demonstrated, however,
that the cardiac ET-1 system showed de novo activation during the transition to CHF in a manner parallel to the progression of myocardial dysfunction. The bosentan treatment ameliorated LV contraction without reducing LV mass. Therefore, local activation of the cardiac ET-1 system may directly disturb the function of the hypertrophic myocytes rather than affecting them in a growth-promoting way.

In vitro studies have reported crosstalk between the angiotensin system and the ET-1 system. In an in vivo study, Clavell et al. reported that activation of the renin-angiotensin system contributed to elevations of circulating and local ET-1 peptides in dogs with vena caval constrictions. On the basis of our observations in the hypertrophic myocardium from a model with low circulating renin, however, the activation time courses as well as the pathophysiological roles of Ang II and ET-1 differed qualitatively. A similar observation was reported recently in rats with hypertension due to genetically high renin. Therefore, distinctive roles between the tissue Ang II and ET-1 may not be specific to the low-renin condition in our animals. Our findings do not exclude crosstalk between the 2 systems. Actually, in the Temo group, the tissue ET-1 level was decreased modestly but significantly, by 27%, compared with that in the control group (ET-1 peptide/LV myocardium at 17 weeks [pg/g] was 464±22 in DR, 2123±411 in vehicle-treated DS, 1674±215 in Temo, and 2001±404 in Temo+ Bos; n=6, respectively). The data rather suggest that the major part of the tissue ET-1 activation was independent of the Ang II system. Touyz et al. reported that in isolated myocytes from the hypertrophic heart of spontaneously hypertensive rats, there was a difference in intracellular Ca2+ modulation between Ang II and ET-1 that was not found in cells from normal rats. Several reports also suggested agonist-induced desensitization of ET-1 or Ang II signaling in pathological conditions. Taken together, care should be taken when extrapolating findings relative to signaling cascades and their potential roles obtained in the intact myocardium to disease conditions such as heart failure.

ACE inhibitors are established pharmacological tools in the treatment of hypertensive heart disease and chronic heart failure. Morbidity and mortality from CHF still remain unacceptably high, however, even in patients receiving this regimen. Because the present study indicates temporally different activation and regulation as well as qualitatively different pathophysiological roles of the cardiac angiotensin and ET-1 systems, it is conceivable that long-term treatment with ET-1 receptor blockers, in addition to the prerequisite ACE inhibitor therapy, would produce beneficial effects on cardiac hypertrophy and failure in clinical settings. In the study by Sutsch et al, bosentan was given in addition to conventional treatment, including ACE inhibitors, to patients with severe heart failure, and improvements in LV function were observed after 2 weeks. Although our experimental settings should not be extrapolated directly to chronic heart failure in humans, our findings support the rationale that long-term therapy with a combination of ACE inhibition and ET antagonism may provide an alternative strategy beyond the current renin-angiotensin system inhibition.

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