Endothelin A Receptor Antagonism in Experimental Congestive Heart Failure Results in Augmentation of the Renin-Angiotensin System and Sustained Sodium Retention

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Background—While both the endothelin-1 (ET-1) and renin-angiotensin systems (RAS) are activated in congestive heart failure (CHF), the temporal sequence of this activation remains unclear. Understanding this pattern of neurohumoral activation may aid in understanding the significance of ET-1 in CHF and provide strategies for ET-1 antagonism. Although acute endothelin (ET) receptor antagonism improves systemic hemodynamics in CHF, clinical trials with chronic ET receptor antagonism report worsening CHF symptoms.

Methods and Results—In a canine model of progressive left ventricular dysfunction, we demonstrated activation of myocardial and plasma ET-1 without activation of the RAS during transition to overt CHF, suggesting that ET-1 contributes to this transition. We next evaluated the effects of chronic oral ET-A receptor antagonism on neurohumoral function, renal hemodynamics, and sodium excretion in pacing-induced CHF. After 7 days of treatment (n=7) with ET-A receptor antagonism (with LU135252), sodium excretion did not improve in treated versus untreated CHF (n=6). Furthermore, both plasma renin activity and plasma ET-1 increased with ET-A receptor blockade.

Conclusions—Activation of the myocardial and plasma ET-1 systems precedes activation of the myocardial and plasma RAS in CHF. ET-A receptor antagonism in experimental CHF further activates the RAS without improving sodium excretion. These findings suggest an important role for ET-1 in the progression of CHF and a potential mechanism for the exacerbation of CHF symptoms observed in clinical trials with chronic ET receptor antagonism. Further studies with combined modulation of the ET and other neurohumoral systems in CHF are required.

Key Words: endothelin • heart failure • receptors • sodium

The activation of tissue and circulating endothelin-1 (ET-1) in experimental and human congestive heart failure (CHF) is well established. Positive correlations in the magnitude of increases in plasma ET-1 with the severity of CHF and with increased mortality rates in human CHF have been reported. Although ET receptor blockade in experimental and human CHF improves hemodynamic function, recent clinical trials with chronic ET receptor antagonists in CHF report increased congestive symptoms, raising questions regarding the biological significance of ET-1 activation during progression of CHF. In contrast to ET receptor blockade, antagonism of the renin-angiotensin-aldosterone system (RAS) initiated during symptomatic CHF improves morbidity and mortality rates, Angiotensin-converting enzyme inhibitors (ACEI) in asymptomatic left ventricular (LV) dysfunction, however, does not improve mortality rates, suggesting that other neurohumoral systems, such as the ET system, contribute to progression to overt symptomatic CHF. A pathophysiological role for ET-1 in CHF is supported by reports that infusion of ET-1 at concentrations observed in CHF results in systemic and renal vasoconstriction, whereas ET receptor antagonism in human CHF results in systemic and regional vasodilation. However, the failure of chronic ET receptor antagonism to produce long-term benefits prompts the need to further understand the role of ET-1 during the evolution of CHF.

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Recently, studies have attempted to better define the contribution of neurohumoral mechanisms in the transition from early to overt CHF. However, the relative temporal activation of myocardial and circulating ET-1 and the RAS systems during progression of CHF remains poorly defined. We therefore attempted to characterize the temporal activation of the ET-1 and RAS systems in myocardial tissue and plasma during transition to overt CHF, characterized by
decreased sodium excretion, in a canine model of progressive LV dysfunction.18 We found that ET-1 but not the RAS is activated during this transition. Given the early activation of the ET system in CHF, we next characterized the effect of the ET system in CHF, we next characterized the effect of early activation of chronic ET-A receptor antagonism without the confounding effects of other therapeutic agents on neurohumoral function, renal hemodynamics and sodium excretion in experimental CHF. We hypothesized that ET-A receptor antagonism increases sodium excretion in addition to improving systemic hemodynamics.

Methods

Temporal Activation of ET-1 and the RAS

Studies were conducted in two groups of male mongrel dogs (weight, 18 to 23 kg) in accordance with the Animal Welfare Act and with approval of the Mayo Clinic Institutional Animal Care and Use Committee (IACUC). The transition group (n=5) underwent rapid ventricular pacing at 180 bpm and 210 bpm and dogs were then euthanized (Sleepaway, Fort Dodge Labs) for rapid tissue harvesting.

After a 2-week recovery period, dogs underwent pacing with a stepwise increase of stimulation frequencies over 31 days. Dogs were fed a fixed sodium diet (58 mEq/d, Hill’s ID), allowed water ad libitum, and kept in metabolic cages for 24-hour urine collections to determine sodium excretion. For 10 days, animals were paced at 180 bpm. As previously described,19 this pacing protocol results in early LV dysfunction, as defined by significant systolic dysfunction with decreased cardiac output, cardiac enlargement, and increased filling pressures but maintained systemic perfusion pressure and renal sodium excretion and no clinical signs of heart failure. The pacing rate was then increased weekly to 200, 210, and 220 bpm, and early LV dysfunction evolved to a transition phase, which we have defined as the onset of decreased renal sodium excretion.20

At baseline and at the end of the protocol, mean arterial pressure (MAP) was measured in the conscious state through the femoral port catheter, and arterial blood was drawn. Cardiac filling pressures and output by thermodilution (American Edwards Laboratories, model 9570-A) were measured in the conscious state at baseline and at transition phase through a percutaneously placed flow-directed balloon-tip catheter (American Edwards, model 93131). Systemic vascular resistance (SVR) was calculated according to the standard formula. Arterial blood was collected in EDTA tubes and placed on ice. Twenty-four-hour urine samples were collected before pacing, at the end of pacing at 180 bpm and 210 bpm, and daily during the 7 days of pacing at 220 bpm. Twenty-four-hour urinary sodium excretion was significantly reduced (see Results section) at the end of pacing at 180 bpm and 210 bpm and dogs were then euthanized (Sleepaway euthanasia solution iv, Fort Dodge Labs) for rapid tissue harvesting. Left ventricular weight was obtained, normalized to baseline body weight, and expressed as LV mass index (LVMi, gm/kg). Hearts were rapidly trimmed, weighed, and snap-frozen in liquid nitrogen, and stored at −80°C until further processing. Blood was centrifuged at 2500 rpm and 4°C, and the plasma was stored at −20°C until analysis. All pacemakers were checked at the time of programming and then weekly and at the day of death for proper pacing.

A control group of 5 normal dogs served as tissue donors. These dogs had chronic femoral artery catheters placed as described above. Dogs were fed a fixed sodium diet (58 mEq/d, Hill’s ID) and allowed water ad libitum for 1 week. Invasive hemodynamic measurements were obtained to assess cardiac function, and arterial blood was drawn before animals were euthanized and tissue was rapidly harvested and deep-frozen.

Hormonal and Electrolyte Analysis

After extraction, plasma atrial natriuretic peptide (ANP) and ET-1 levels and PRA were measured by radioimmunoassay, as previously described.20–22

Tissue Analysis

Tissue for hormone analysis was snap-frozen in liquid nitrogen before storage at −80°C until processing. For extraction of tissue, samples were pulverized frozen, boiled for 5 minutes in 10 vol of acetic acid (1 mol/L)/HCl (20 mmol/L), and homogenized (PT 1200, Polytron). The homogenate was then ultracentrifuged at 27,000g at 4°C, and the supernatant was stored at −20°C until radioimmuno assay. Before centrifugation, a sample of the homogenate was taken for measurement of tissue protein content.23 ET-1 and angiotensin (Ang) II protein concentrations (pg/mg) were determined in the supernatant from atrial and ventricular homogenates.

Northern Blot Analysis

Messenger RNA (mRNA) was isolated from atrial and ventricular tissue with the use of the FastTrack 2.0 kit (Invitrogen). The tissue was lysed in detergent-based buffer containing RNase/protein degrader, incubated at 45°C, and applied directly to oligo(dt) cellulose for adsorption. DNA, degraded proteins, and cellular debris were washed from the resin with a high salt buffer. Nonpolyadenylated RNA was washed off with a low salt buffer, and the poly(A) RNA was then eluted in the absence of salt (elution buffer). Purity and quality of isolated mRNA was assessed by reading optical densities at 260 and 280 nm and by electrophoresis in 1.2% denaturing agarose gel. For Northern blot analysis, 4 μg of mRNA from cardiac tissue was loaded on a 1.2% agaroseigel and electrophoresed for 3 hours at 70 V. The gel was transferred downward onto a nylon membrane (Maximum Strength Nytran Membrane, Schleicher & Schuell) overnight using Turboblotter (Schleicher & Schuell) and cross-linked with using UV Stratalinker (Stratagene). Canine prepro–ET-1 cDNA fragment restricted by EcoRI containing 162 bp gene for canine prepro–ET-1 was labeled with P-dCTP by a random-priming labeling kit (Megaprime DNA labeling system, Amersham) and purified with G-50 Quick Spin Columns (Boehringer Mannheim). Labeled canine prepro–ET-1 cDNA probe was hybridized to the membranes at 68°C overnight in Quick-hyb hybridization solution (Stratagene). The membranes were then washed in 2×SSC, 0.1% SDS at 22°C for 15 minutes, then in 0.2×SSC, 0.1% SDS at 22°C for 15 minutes, and 0.2×SSC, 0.1% SDS at 55°C for 20 minutes. Autoradiography was carried out with Kodak X-ray film at −80°C overnight. To standardize loading conditions and mRNA transfer onto membranes, blots were rehybridized with a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe.

ET-A Receptor Antagonism in Heart Failure

Experiments were performed in three groups of male mongrel dogs (weight, 20 to 25 kg) maintained on a fixed sodium diet of 58 mEq/d. Experimental CHF was produced by ventricular pacing at 245 beats per minute for 10 days. Programmable pacemakers were implanted 10 days before initiation of pacing to produce CHF as described above and previously.4 The dogs recovered for 14 days and received prophylactic antibiotic treatment as described above. Pacemakers were then programmed to 245 beats per minute, and pacing was continued for 10 days and through the acute experiment. Dogs were fasted from the night before the experiment but permitted to drink water ad libitum. At the day of the acute experiment, dogs were anesthetized with sodium pentobarbital. The animals were intubated and mechanically ventilated with supplemental oxygen at 4 L/min. A femoral artery was cannulated for monitoring of arterial pressure and blood sampling. A balloon-tipped, flow-directed pulmonary artery catheter was placed through the right internal jugular vein for measurement of cardiac filling pressures and determination of cardiac output. A flank incision was performed to gain exposure to the left kidney in all groups. The ureter was cannulated for urine collection, and a
Results

Cardiovascular Hemodynamics, Sodium Excretion, and Neurohumoral Function at Transition to Overt CHF

Cardiovascular hemodynamics, sodium excretion, and plasma neurohumoral profiles at baseline and during the transition phase with a reduction in urinary sodium excretion are reported in Table 1. The decrease in urinary sodium excretion occurred during the fourth week of progressive CHF, as characterized by a lower daily urinary sodium excretion compared with the prepacing baseline (67 ± 15 to 22 ± 2 µEq/24 h, P < 0.05). Additionally, cardiac output (CO) was reduced and pulmonary capillary wedge pressure (PCWP), right atrial pressure (RAP), and systemic vascular resistance were increased, whereas MAP was unchanged from baseline. Plasma ET-1 and ANP were increased in the absence of activation of the RAS when compared with baseline.

Figure 1 illustrates atrial and ventricular tissue ET-1 and Ang II concentrations at the transition phase and in controls. Ventricular (0.5 ± 0.1 to 2.0 ± 0.4 pg/mg protein, P < 0.05) and atrial (0.7 ± 0.1 to 4.0 ± 0.8 pg/mg protein, P < 0.05) concentrations of ET-1 were increased when compared with controls. No increases in atrial or ventricular Ang II concentrations were noted. The increase in myocardial ET-1 was associated with an increase in ventricular and atrial prepro-ET-1 mRNA, determined by Northern blot analysis (Figure 2). Figure 3 illustrates the individual ET-1 and PRA concentrations in the two groups, emphasizing again the significant elevation of plasma ET-1 but not PRA at this transition stage.

Cardiorenal and Neurohumoral Function in the Presence and Absence of Chronic Oral ET-A Receptor Antagonism in CHF

Table 2 reports cardiovascular, renal, and neurohumoral function in CHF as compared with normal. GFR and renal

Statistical Analysis

Results are expressed as mean ± SEM. The Student paired t test for single comparison within the same group and the Student unpaired t test for single comparison between any two groups, using GraphPad Prism software, were used. Statistical significance was accepted as P < 0.05.
blood flow (RBF) were preserved in CHF as compared with normal, whereas UNaV was decreased. MAP and CO decreased in CHF, whereas SVR and PCWP were increased. Plasma ANP, ET-1, and PRA were increased in CHF.

Sodium excretion with chronic oral ET-A receptor antagonism is illustrated in Figure 4a. There was no change in sodium excretion with ET-A receptor antagonism compared with the untreated group in CHF (7±3 μEq/min untreated versus 4±2 μEq/min treated). As shown in Table 2, there was no change in GFR (31±10 versus 41±16 mL/min), RBF (123±22 versus 120±12 mL/min), or RVR (1.1±0.2 versus 0.9±0.1 resistance units [RU]) with ET-A receptor antagonism compared with the untreated group.

Figure 4b illustrates PRA in the normal group and in CHF with and without chronic oral ET-A receptor antagonism. PRA was increased with ET-A receptor antagonism in CHF compared with the untreated group. ET-1 increased with ET-A receptor antagonism compared with the untreated group in CHF (14±3 versus 30±7 pg/mL, P<0.05). Plasma ANP, which was elevated in CHF, was unchanged in a subgroup (n=3) analyzed in CHF treated with chronic oral ET-A receptor antagonism as compared with untreated CHF.

Additionally, as shown in Table 2, CO (2.1±0.1 versus 2.7±0.2 L/min, P<0.05) in CHF was greater with ET-A receptor antagonism when compared with the untreated group. SVR (51±4 versus 42±7 RU) tended to decrease with ET-A antagonism in CHF compared with the untreated group.

**Discussion**

In the current study, we demonstrate that myocardial and plasma ET-1 is selectively activated before the RAS in the transition stage to overt CHF in a large animal model of ventricular dysfunction. Second, we report that early initiation of ET-A receptor antagonism in experimental CHF has deleterious neurohumoral and renal actions that include further activation of the RAS with continued sodium retention.

The first objective of our study was to define the temporal activation of the ET-1 and RAS systems in myocardial tissue and plasma at the transition from compensated ventricular dysfunction to overt CHF. With the use of a canine model of pacing-induced progressive ventricular dysfunction, the current investigation demonstrates that myocardial and circulating ET-1 are activated before the activation of the myocardial and circulating RAS. We determined plasma and tissue ET-1 and components of the RAS during the critical phase of progression to overt CHF, when there is a reduction in urinary sodium excretion, as determined by a decrease in daily 24-hour urinary sodium excretion. In our study, sodium excretion decreased during the fourth week of pacing and was associated with increases in plasma and myocardial ET-1 concentrations without increases in tissue Ang II. Furthermore, PRA was not increased. This is consistent with findings in the SOLVD trial that showed that subjects with asymptomatic LV dysfunction and not taking diuretics did not have increases in PRA.12 The increase in myocardial ET-1 was also associated with increases in ventricular and atrial prepro–ET-1 gene expression. Of interest, there was no increase in renal ET-1 gene expression (data not shown). Thus, in this model of experimental CHF, ET-1 is activated before the activation of RAS during the transition to overt CHF, suggesting a possible pathophysiological role for ET-1 at this stage of CHF. Indeed, blockade of the ET-A receptor early during the development of experimental CHF prevents hypertrophy.26 In addition, previous studies suggested beneficial renal effects with ET-A receptor antagonism.27 Further,
Serneri et al report that in compensated ventricular hypertrophy in humans with aortic stenosis, there is activation of myocardial ET and not Ang II, which may be the clinical correlate to the current observation in experimental CHF.\textsuperscript{28} These studies and our findings support the concept of early activation of ET-1 in ventricular dysfunction raising the question of the response to early initiation of an ET-A receptor antagonist in CHF.

Our second objective was to investigate the effects of chronic oral ET-A receptor antagonism early in experimental CHF. Consistent with previous studies with selective and nonselective ET receptor blockade in both human and experimental CHF,\textsuperscript{4-10} we found that chronic oral administration of LU 135252 resulted in increased cardiac output and a trend to decreased MAP and SVR. In the absence of a natriuretic effect with ET-A receptor antagonism, pulmonary capillary wedge pressure remained unchanged with treatment. Evidence for the potential beneficial effects of ET-A receptor antagonism was suggested by one report of improved survival in experimental CHF.\textsuperscript{28} Recently, however, clinical trials have reported an exacerbation of congestive symptoms in patients with CHF treated chronically with endothelin receptor antagonist.\textsuperscript{9,10} In our study, we report that chronic oral ET-A receptor antagonism, despite improving cardiac output, does not improve urinary sodium excretion and actually tends to increase sodium retention. This may in part be explained by the increase in PRA and the absence of any increase in ANP as observed in the treated group. Interestingly, these findings are consistent with previous studies reporting increased PRA in normal dogs administered endothelin receptor antagonist and with in vitro studies reporting that ET-A receptor antagonist attenuates ET-1 mediated release of ANP from atrial myocytes.\textsuperscript{29,30}

Spinale et al, consistent with previous studies in normal dogs, reported an increase in PRA in normal rabbits treated with an ET-A receptor antagonist.\textsuperscript{5} However, in contrast to our study, in a rabbit model of pacing-induced CHF, these authors report no change in PRA with ET-A receptor antagonism. It should be noted that these respective findings occur in different models of CHF and in different species of animals. An alternative explanation for the increase in PRA might be that decreased MAP and a trend toward a decrease in SVR resulted in an activation of arterial baroreceptors, which in turn activated renin release.

Previous studies\textsuperscript{4} in a model of pacing-induced canine heart failure reported sodium excretion sustained at normal levels with chronic ET-A receptor antagonism in the context of decreased plasma ANP in dogs treated with a different ET-A receptor antagonist. Although not significantly decreased, it should be noted that sodium excretion in the treated group in this study was still approximately 50% below the normal level of sodium excretion and thus not inconsistent with the current studies. Blockade was also initiated before the onset of CHF, in contrast to the current study, in which blockade was started 3 days into pacing. Furthermore, consistent with our findings, these authors report no change in RBF with ET-A receptor antagonism. The increased levels of PRA that we report in the absence of increases in a counter-regulatory system such as the natriuretic peptides could account for the absence of improvement in RBF and sodium excretion. The mechanism for the increase in PRA with ET-A receptor antagonism may be due to a direct effect of increased ET-1 plasma levels, as previous studies report that at pharmacological doses, ET-1 increases PRA,\textsuperscript{31} as well as to possible activation of arterial baroreceptors, as discussed above.

The relative imbalance of sodium retaining to natriuretic neurohumoral factors we report with chronic oral ET-A receptor antagonism suggests the need for further studies combining endothelin receptor antagonism with other pharmacological agents, such as inhibitors to the RAS. Indeed, future studies combining a vasopeptidase inhibitor (to attenuate the RAS and augment the natriuretic peptide system) and ET-A receptor antagonism would be interesting. Alternatively, it would also be interesting for future clinical studies to evaluate the effects of ET-A receptor antagonism at significantly lower doses than the recent clinical trials to minimize adverse reactions, in the absence of attenuation of the RAS or β-blockade.

**Clinical Implications**

The pacing model of CHF that we used has clinical relevance. It is characterized by increased SVR, decreased CO, and increased PCWP, as well as sodium retention and neurohumoral activation, all consistent with human CHF. Indeed, this model has been used to evaluate a variety of therapeutic agents including β-blockers, neutral endopeptidase inhibitors, ACE inhibitors, and vasopeptidase inhibitors.\textsuperscript{32} Moreover, the model allows for an integrated view of the cardiovascular, renal, and neurohumoral systems in a large animal model of CHF. CHF is a complex and progressive disease process that involves vasoconstriction, sodium retention, alterations in myocardial structure and function, and activation of numerous neurohumoral systems that effect these processes. Some, such as the sympathetic nervous system (SNS), the renin-angiotensin-aldosterone system (RAAS), and the ET system promote vasoconstriction, ventricular hypertrophy, fibrosis, and sodium retention. The natriuretic peptide system, on the other hand, promotes vasorelaxation, natriuresis, and lusitropic and antifibrotic actions.\textsuperscript{33,34} Additionally, the natriuretic peptide system attenuates the RAAS.\textsuperscript{21,35} Modulation of some of these neurohumoral systems are well established as therapeutic strategies to attenuate the progression of CHF or to affect mortality. Our findings provide new insights into the ET-1 system in experimental CHF and may aid in better understanding how to modulate this system from a therapeutic perspective.

In summary, based on the early activation of the ET-1 system in CHF, we investigated the effects of early chronic oral ET-A receptor antagonism in CHF on sodium retention, neurohumoral function, and renal hemodynamics. We found that despite improving cardiac output, chronic oral ET-A receptor antagonism further activates the RAS and results in sustained sodium retention in experimental CHF. These findings provide important insight into recent clinical trials in which subjects had exacerbation of congestive symptoms with chronic endothelin receptor antagonism and suggest future studies combining ET-A receptor antagonism with
therapeutic modalities that modulate other neurohumoral and renal systems in CHF.

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