Activation of cardiorenal and pulmonary tissue endothelin-1 in experimental heart failure

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Endothelin-1 (ET-1) is a cardiovascular peptide whose circulating concentrations are increased in congestive heart failure (CHF) (10, 15, 35). This increase has been demonstrated to have functional importance as Kłosowski et al. (8) elegantly demonstrated that acute administration of a dual ET\textsubscript{A} and ET\textsubscript{B}-receptor antagonist reduces systemic and pulmonary vascular resistance as well as cardiac filling pressures in human CHF (8). More recently, we and others reported that chronic ET receptor antagonism also has beneficial actions in rapid ventricular pacing-induced CHF and leads to sustained decreases in cardiac filling pressures, systemic, renal and pulmonary vascular resistance, increased cardiac output, and attenuated myocyte remodeling (1, 16, 18, 27).

Despite the emerging role of ET-1 for CHF, the activation of ET-1 in local tissues during CHF remains poorly defined. This is particularly relevant as ET-1 is thought to be predominantly an autocrine and paracrine rather than a circulating hormone. Indeed, circulating concentrations of ET-1 in CHF increase to a significantly lesser extent compared with other neurohormones (28). To better understand ET-1 in the pathophysiology of CHF, it is therefore essential to assess local activation in important target organs of the disease, such as the heart, kidney, and lung.

Although previous studies focused primarily on left ventricular activation of the prepro-ET-1 gene in models of cardiac dysfunction or hypertrophy (7, 24, 31) and only two studies have addressed cardiac and pulmonary prepro-ET-1 gene expression (6, 32), a combined assessment of atrial, ventricular, pulmonary, and renal prepro-ET-1 gene expression, together with local accumulation of ET-1, has so far not been carried out in a well-documented model of overt experimental CHF.

It was therefore the objective of the present study to assess regional accumulation of ET-1 in atrial, ventricular, renal, and pulmonary tissue in a large-animal model of CHF. To further determine whether regional activation of ET-1 reflects local production or accumulation, we also defined regional expression of the prepro-ET-1 gene. The utilized model of progressive rapid ventricular pacing-induced left ventricular dysfunction shares many similarities with human CHF and results in overt CHF with severe cardiac dysfunction and atrial and ventricular hypertrophy as well as pulmonary hypertension, systemic hypotension, marked neurohormonal activation, and avid renal sodium retention (14, 28). On the basis of recent findings of functional improvement of renal and pulmonary vascular as well as cardiac function (1, 18) and selective pulmonary and atrial prepro-ET-1 gene activation in a
vena caval constriction model of cardiac low output in the dog (34), we hypothesized that progressive rapid ventricular pacing-induced canine CHF would be characterized by cardiac, renal, and pulmonary activation of ET-1.

To address our hypothesis, we assessed ET-1 tissue concentrations and prepro-ET-1 gene expression in atrial and ventricular myocardium as well as in renal and pulmonary tissue in normal dogs and dogs with overt CHF after 38 days of progressive rapid ventricular pacing.

METHODS

Study Protocol

Sixteen male mongrel dogs were used for the study. Overt CHF was induced in nine animals by rapid right ventricular pacing (hemodynamics were assessed in 5 of the 9 dogs), and seven animals served as the control group. Implantation of a programmable cardiac pacemaker (Medtronic, Minneapolis, MN) was carried out under pentobarbital sodium anesthesia (30 mg/kg iv) and artificial ventilation (Harvard respirator, Harvard Apparatus, Millis, MA). The heart was exposed via a small left lateral thoracotomy and pericardiotomy, and a screw-in epicardial pacemaker lead was implanted into the right ventricle. The pacemaker was implanted subcutaneously into the left chest wall and connected to the pacemaker lead. In addition, a chronic indwelling catheter (model GPV Vascular-Access Port, Access Technologies, Skokie, IL) was implanted into the left femoral artery and subcutaneously connected to a port above the left upper hindlimb. All dogs were allowed to recover for at least 10 days after surgery.

For the induction of CHF, dogs underwent pacing with a stepwise increase of stimulation frequencies over 38 days. As previously described, this pacing regimen results in overt CHF, as characterized by severe cardiac dysfunction, increased left ventricular mass, impaired systemic and regional hemodynamics, broad neurohumoral activation, and avid renal fluid and sodium retention (14, 28). All pacemakers were checked for proper pacing at the time of programming and then weekly and on completion of the study. At baseline (control) and at the end of the protocol (overt CHF), cardiac filling pressures and cardiac output by thermodilution (model 9510-A, American Edwards Laboratories) were measured in the conscious dog via a Swan-Ganz catheter, and mean arterial pressure was measured via the arterial port catheter. In addition, urine was collected over a 24-h period from animals in a metabolic cage for assessment of renal sodium excretion, and a two-dimensional guided M-mode echocardiogram was obtained. Arterial blood was collected in EDTA tubes, centrifuged at 2,500 rpm and 4°C, and the plasma was stored at −20°C until measurement of ET-1. After the dogs were euthanized (Sleepaway solution iv, Fort Dodge Laboratories, Fort Dodge, IA), tissue was rapidly harvested. Hearts were quickly trimmed, and atria and left ventricles were weighed for the calculation of mass indices. Left ventricular, left atrial, renal, and pulmonary tissues were snap-frozen in liquid nitrogen and stored at −80°C until further processing. A second group of normal dogs served as tissue donors for the control group. All studies were approved by the Institutional Animal Care and Use Committee of the Mayo Clinic and conducted in accordance with the Animal Welfare Act.

Analytic Methods

Plasma and tissue ET-1 were measured by using a sensitive and specific radioimmunoassay (15). For the extraction of tissue ET-1, samples were pulverized frozen, boiled for 5 min in 10 vol of 1 M acetic acid/20 mM HCl, and homogenized at high speed (Polytron PT 1200). The homogenate was then ultracentrifuged at 27,000 g and 4°C, and the supernatant was stored at −20°C until radioimmunoassay. Before centrifugation, a sample of the homogenate was taken for measurement of tissue protein content according to the method by Lowry et al. (13). Immunoreactive ET-1 in tissue was measured as picograms per milliliter homogenate and either normalized for protein content and expressed as picograms ET-1 per milligram tissue protein or expressed as picograms ET-1 per gram tissue wet weight. Immunoreactive ET-1 in plasma was expressed as picograms per milliliter.

For analysis of prepro-ET-1 gene expression, mRNA was extracted from tissues by utilizing a commercially available kit (Fasttrack, Invitrogen). Briefly, tissue was homogenized (Polytron PT 1200) in a detergent-based buffer containing RNase/protein degrader and incubated in a slowly shaking waterbath. DNA was precipitated and sheared, and oligo(dT) cellulose was added for adsorption of polyadenylated mRNA. DNA, proteins, cell debris, and nonpolyadenylated RNA were washed off, and RNA was eluted off the oligo(dT) cellulose. The yield of mRNA was determined in a spectrophotometer by absorption of 260-nm ultraviolet light. mRNA was loaded on a 1.2% agarose-formaldehyde gel and electrophoresed for 2–3 h at 75 V. The gel was blotted downward overnight (Turbo-Blotter, Schleicher & Schuell) onto a nylon membrane (maximum strength nitryl membrane, Schleicher & Schuell). A bovine endothelial prepro-ET-1 full-length cDNA (34) or a 283-base pair partial cDNA were random primed with P32-dCTP (Random Primed DNA Labeling Kit, Boehringer Mannheim Biochemical) and column purified. Membranes were prehybridized (QuickHyb Hybridization Solution, Stratagene, La Jolla, CA) for 10 min at 68°C and then hybridized with the labeled probe for 80 min at 68°C. Membranes were then washed stringent [2× standard sodium citrate (SSC)/0.1% SDS at 22°C for 5 min, then 0.2× SSC/0.1% SDS at 22°C for 5 min, then 0.2× SSC/0.1% SDS at 55°C for 20 min] and exposed to X-ray film for autoradiography. To control for loading conditions and mRNA transfer onto the membranes, blots were rehybridized with a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe. The respective autoradiographic bands for prepro-ET-1 and GAPDH were quantitated with a scanning spectrophotometer, and prepro-ET-1 mRNA was expressed in arbitrary units as the ratio of autoradiographic densities of the prepro-ET-1 band and the GAPDH band.

Urinary sodium was measured by using ion-selective electrodes (Beckman Instruments, Brea, LA).

Echocardiography

The echocardiogram (Toshiba) was performed by an expert echocardiographer from the right parasternal window. Left ventricular end-diastolic (LVEDd) and end-systolic (LVESd) dimensions were determined from three repeated two-dimensional guided M-mode tracings. From those measurements, the ejection fraction (EF) was calculated as (22)

\[ EF = \left( \frac{LVEDd^2 - LVESd^2}{LVEDd^2} \right) \]

Statistical Analysis

Results of the quantitative studies were expressed as means ± SE. Comparisons between control and overt CHF
variables were performed by t-test. Comparisons within groups were performed by ANOVA with Bonferroni correction. Statistical significance was defined as P < 0.05.

RESULTS

Functional Characteristics and Cardiac Mass

Compared with control, overt CHF was characterized by significantly decreased cardiac output and mean arterial pressure, whereas right and left ventricular filling pressures as well as pulmonary artery pressures were significantly increased (Table 1). Echocardiographic analysis demonstrated significant systolic left ventricular dysfunction and eccentric left ventricular remodeling in overt CHF, and post mortem analysis revealed that hearts from overt CHF dogs were characterized by significantly increased atrial (+166%, P < 0.01) and left ventricular mass index (+12%, P < 0.02). Twenty-four-hour urinary sodium excretion was markedly and significantly reduced in overt CHF dogs, and all dogs had clinical signs of fluid retention.

Circulating and Tissue ET-1

Plasma ET-1 concentrations were significantly increased in overt CHF compared with control (+66%, P < 0.01) (Fig. 1). Immuno-reactive ET-1 was detected in all tissues, and statistical comparison of tissue ET-1 normalized to protein content demonstrated that ET-1 concentrations were highest in the lung under control conditions (P < 0.0083 vs. left ventricle, ANOVA). In overt CHF, significant increases of tissue ET-1 concentrations were observed in pulmonary (+122%, P < 0.04 vs. control), left ventricular (+71%, P < 0.04 vs. control), and renal tissue (+385%, P < 0.01 vs. control), whereas a modest increase in left atrial ET-1 did not reach statistical significance. The highest ET-1 concentrations in overt CHF were detected in pulmonary (P < 0.0083 vs. left ventricle and atrium, ANOVA) and renal tissue (P < 0.0083 vs. left ventricle, ANOVA).

Expression of Prepro-ET-1 mRNA

Under control conditions, the relatively strongest expression of prepro-ET-1 mRNA was detected in pulmonary tissue, whereas all other issues showed only weak expression. In overt CHF, expression of prepro-ET-1 mRNA was significantly increased in pulmonary and left atrial tissues compared with control dogs. This increase was 208 and 118%, respectively, and representative autoradiographies are depicted in Figs. 2 and 3. A tendency toward increased expression of prepro-

Table 1. Functional characteristics and cardiac mass in overt CHF

<table>
<thead>
<tr>
<th>Variable</th>
<th>Control</th>
<th>Overt CHF</th>
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<tbody>
<tr>
<td>CO, l/min</td>
<td>5.4 ± 0.5</td>
<td>2.5 ± 0.2*</td>
</tr>
<tr>
<td>MAP, mmHg</td>
<td>125.2 ± 6.3</td>
<td>84.8 ± 2.9*</td>
</tr>
<tr>
<td>RAP, mmHg</td>
<td>4.2 ± 0.4</td>
<td>11.0 ± 1.8†</td>
</tr>
<tr>
<td>PCWP, mmHg</td>
<td>7.0 ± 0.5</td>
<td>21.2 ± 4.8*</td>
</tr>
<tr>
<td>PAP, mmHg</td>
<td>18.2 ± 0.9</td>
<td>27.8 ± 6.6*</td>
</tr>
<tr>
<td>EF, %</td>
<td>57.8 ± 2.8</td>
<td>21.3 ± 3.0*</td>
</tr>
<tr>
<td>LVEDd, mm</td>
<td>38.9 ± 0.5</td>
<td>47.1 ± 1.2*</td>
</tr>
<tr>
<td>AMI, g/kg</td>
<td>0.64 ± 0.04</td>
<td>1.7 ± 0.2*</td>
</tr>
<tr>
<td>LVMd, g/kg</td>
<td>4.0 ± 0.2</td>
<td>4.5 ± 0.1†</td>
</tr>
<tr>
<td>U_{Na^+}, meq/24 h</td>
<td>38.2 ± 1.9</td>
<td>92.4 ± 4.4*</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 5 dogs. CHF, congestive heart failure; CO, cardiac output; MAP, mean arterial pressure; RAP, right atrial pressure; PCWP, pulmonary capillary wedge pressure; PAP, pulmonary artery pressure; EF, ejection fraction; LVEDd, left ventricular end-diastolic diameter; AMI, atrial mass over body mass index; LVMd, left ventricular mass over body mass index; U_{Na^+}, urinary 24-h sodium excretion. *P < 0.01 vs. control. †P < 0.05 vs. control.
pulmonary tissue is not only a target for ET-1 in CHF finding extends previous reports and suggests that chronic ET-receptor antagonists (1, 8). The present study associates with CHF on the basis of a reduction in pulmonary tissue ET-1 in association with increased pulmonary vasoconstriction associated with CHF, as reported (20), and studies utilizing chronic ET receptor antagonists have demonstrated an improvement in myocardial structure and function in CHF (1, 27). The present study clearly does not provide functional data. It does, however, support activation of left ventricular ET-1 in the absence of significant activation of prepro-ET-1 gene expression, which has been advanced by studies in rodents with myocardial infarction and pressure-overload hypertrophy (7, 24, 31), as it suggests that increased circulating ET-1 contributes to increased local ET-1 in the left ventricle during CHF.

Recent studies in vitro have suggested that the positive inotropic actions of ET-1 on the myocyte may be attenuated (21, 27) or even reversed in CHF (30), and it has been suggested that ET-1-induced coronary vasoconstriction and increased afterload may offset or even reverse residual positive inotropic effects of ET-1 on the myocyte in CHF. Indeed, negative effects of endogenous ET-1 in CHF on total left ventricular contractile performance and relaxation have been reported (20), and studies utilizing chronic ET receptor antagonists have demonstrated an improvement in myocardial structure and function in CHF (1, 27). The present study clearly does not provide functional data. One way to accomplish this is to use a left atrial myocyte in CHF, and suggests that local mechanisms participate in the detrimental effects of ET-1 on the left ventricle in CHF.

The observed significant gene activation in atrial but not ventricular myocardium during CHF underscores the role of atrial myocytes in cardiac endocrine function. Such a role is also supported by the attenuated increase in local ET-1 in left atrial tissue despite the twofold increase in gene transcription, which is consistent with findings by Tsumamoto et al. (33), who have reported an increase in plasma ET-1 between the main pulmonary artery and the pulmonary capillary wedge region in patients with CHF.

With respect to cardiac ET-1 in CHF, our results demonstrate differential activation in atrial and ventricular myocardium. Although ET-1 was significantly increased in left ventricular tissue in the absence of significant alterations in prepro-ET-1 gene expression, left atrial tissue ET-1 was unchanged, despite significantly increased gene expression. The observation of increased left ventricular ET-1 in the absence of significant activation of prepro-ET-1 gene expression confirms a previous report in a rabbit model of CHF (12) and supports the concept that local gene activation is not a necessary prerequisite for increased accumulation of ET-1 in the left ventricle. Indeed, this finding challenges the concept of a predominantly autocrine-paracrine function of left ventricular ET-1 in CHF, which has been advanced by studies in rodents with myocardial infarction and pressure-overload hypertrophy (7, 24, 31), as it suggests that increased circulating ET-1 contributes to increased local ET-1 in the left ventricle during CHF.

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tent with secretion of ET-1 into the circulation. In addition to an endocrine role of left atrial tissue to generate and release ET-1, local activation of ET-1 may also have an important regulatory function for the secretion of atrial natriuretic peptide (ANP) in CHF. Indeed, a permissive effect of ET-1 for ANP secretion has previously been suggested by studies in vitro and in vivo (2-5, 26, 29). Furthermore, a blunted increase in circulating ANP during experimental heart failure has been reported in the presence of ET receptor antagonism (1).

To our knowledge, the present studies are the first to address alterations of local ET-1 in the kidney during overt CHF with avid renal sodium retention. We observed a significant increase in renal ET-1 concentrations that was even more pronounced compared with those in pulmonary or cardiac tissue and almost achieved a concentration equivalent to pulmonary ET-1 during overt CHF. This finding further underscores an important role of ET-1 in the pathogenesis of renal dysfunction associated with CHF, which has been suggested by studies that reported significantly increased renal vascular resistance, decreased glomerular filtration, and increased tubular reabsorption of sodium in response to the infusion of exogenous ET-1 (9, 17) and which were then partially reversed by ET\(_A\) receptor blockade (1, 19). In addition to renal ET-1 concentrations, the present study also assessed whether renal prepro-ET-1 gene expression is regulated in overt CHF. The potential for regulation of renal prepro-ET-1 gene expression has previously been demonstrated by studies that reported activated gene expression in association with renal artery stenosis and hypoxia (23) as well as cytokine stimulation (25). Our present observations extend and complement these studies as they demonstrate that renal prepro-ET-1 gene expression is not activated in a model of overt CHF and sodium retention and that increased renal ET-1 during overt CHF, similar to left ventricular ET-1, must therefore represent local accumulation rather than synthesis.

In summary, the present studies report ET-1 and prepro-ET-1 gene expression in cardioenal and pulmonary tissues in CHF produced by progressive rapid ventricular pacing. Significantly increased ET-1 concentrations in the left ventricle, lung, and kidney were associated with enhanced gene expression of prepro-ET-1 only in the left atrium and the lung. The relatively strongest increases in tissue ET-1 were observed in lung and kidney, and the relatively strongest increase in prepro-ET-1 gene expression was observed in the lung. These studies therefore suggest differential mechanisms by which ET-1 is activated in experimental CHF.

Perspectives

ET-1 has emerged as a neurohumoral system that is activated in CHF and contributes to multiorgan dysfunction associated with this syndrome. The focus of the present study is on patterns of local activation of ET-1 in CHF with respect to expression of the prepro-ET-1 gene as well as accumulation of mature peptide. Our findings demonstrate that both prepro-ET-1 gene expression as well as ET-1 concentrations are regulated in CHF. Although increased prepro-ET-1 gene expression could be detected in lung and left atrium, increased local ET-1 in left ventricle and kidney was independent of activated gene expression. These findings challenge the traditional concept of ET-1 as a predominantly autocrine-paracrine system, as they suggest that increased circulating ET-1 must contribute to increased local ET-1 in left ventricle and kidney during CHF. Furthermore, the finding of strong activation of local prepro-ET-1 gene expression suggests that increased circulating ET-1 may be recruited from pulmonary and atrial tissue during overt CHF, and the magnitude of relative increases of ET-1 in lung and kidney suggests a particular importance of ET-1 for dysfunction of these organs in CHF.

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