Expression of Endothelin-1, Endothelin-Converting Enzyme, and Endothelin Receptors in Chronic Heart Failure

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**Background**—Elevated plasma levels of endothelin (ET)-1 have been reported in association with heart diseases, including heart failure. Furthermore, it has been suggested that ET-1 acts as a local autocrine/paracrine factor with biological activities such as vasoconstriction, mitogenesis, and inotropic effects on the heart. This study investigated alterations of ET-1, ET receptor, and endothelin-converting enzyme (ECE) expression in left ventricular myocardium from patients with end-stage heart failure.

**Methods and Results**—mRNA concentrations of ET A and ET B receptors, prepro-ET-1 (ppET-1), and ECE in left ventricles from nonfailing donors hearts (NF) and from patients with end-stage chronic heart failure (NYHA functional class IV) due to dilated cardiomyopathy (DCM) were compared by use of a competitive reverse transcription–polymerase chain reaction technique. There was no significant difference in mRNA expression for ppET-1, ECE-1, and ET A receptors, whereas a significant reduction of ET B-receptor mRNA was observed in DCM hearts. 125 I-labeled ET-1 radioligand binding studies demonstrated a significant downregulation of ET B receptors, whereas ET A-receptor density was increased in membranes from DCM hearts. Phosphoramidon-sensitive ECE activity and immunodetectable amounts of ECE protein in left ventricular membrane preparations did not differ between NF and DCM hearts. Finally, immunoreactive ET-1 concentrations were increased in DCM hearts.

**Conclusions**—The present study demonstrates changes in the ET-receptor expression pattern in favor of the ET A receptor in human end-stage heart failure. Furthermore, activation of the cardiac ET system with increased tissue ET-1 concentrations in the failing myocardium is observed. This is more likely due to decreased clearance than to increased synthesis, because ppET-1 gene expression and ECE activity are unchanged. (Circulation. 1999;99:2118-2123.)

**Key Words:** endothelin, heart failure, enzymes, RNA, receptors

Many studies have shown that plasma concentrations of the vasoconstrictor peptide endothelin-1 (ET-1) are increased 2- to 3-fold in patients with heart failure without respect to the underlying cause. As with norepinephrine, plasma levels of ET-1 and big ET-1, the inactive precursor of ET-1, are of prognostic significance, predicting worsening heart failure, need for hospitalization, and death. Although the precise physiological and pathophysiological roles played by ET in heart failure remain uncertain, evidence suggests that ET-1 acts as a local autocrine and paracrine factor rather than a circulating hormone. Cardiac myocytes express prepro-ET-1 (ppET-1) mRNA and synthesize and secrete mature ET-1, as shown by in vitro experiments. ET-1 is produced from the 38-residue inactive intermediate big ET-1 via a specific cleavage at Trp 21 and Val 22 . The protease that catalyzes the conversion, endothelin-converting enzyme (ECE), is expressed in the endocardium and myocardium and constitutes a potential regulatory site for the production of the active peptide. The diverse biological activities of ET-1, such as positive inotropic and chronotropic effects or growth-promoting effects, are mediated through 2 receptor subtypes, the ET A and ET B receptors. Both receptor subtypes are expressed on cardiac myocytes.

Although the cardiac ET system has been investigated in several animal models of cardiac hypertrophy and heart failure, the precise pattern of ET receptor, ppET-1, and ECE expression in the human failing heart is unknown. Therefore, we studied the mRNA expression of ppET-1, ET A and ET B receptors, and ECE in the left ventricular (LV) myocardium from patients with end-stage heart failure compared with nonfailing (NF) control subjects. Furthermore, we investigated the density of ET binding sites, the concentration of ET-1, and the activity of ECE.

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Methods

Myocardial Tissue

Failing hearts were obtained from patients undergoing orthotopic heart transplantation due to end-stage heart failure (NYHA functional class IV) resulting from idiopathic dilated cardiomyopathy (DCM). Patients with ischemic cardiomyopathy (by coronary angiography) were excluded. Medical therapy of the patients had consisted of digitalis, diuretics, nitrates, and ACE inhibitors. None of the patients had received catecholamines. All patients gave written informed consent before operation. Myocardial tissue from 9 NF hearts that could not be transplanted because of surgical reasons or blood group incompatibility was studied for comparison. The major source of donor hearts was persons dying of spontaneous intracerebral or subarachnoidal bleeding. Neither the donor patient histories nor 2-dimensional echocardiography had revealed signs of heart disease. Furthermore, β-adrenergic receptor density was not altered in NF donor hearts and corresponded to β-adrenergic receptor densities found in previous studies.10 Hearts were transported on ice-cold cardioplegia (modified Bretschneider solution). The average time from cross-clamping to tissue arrival in the laboratory was 1 hour 30 minutes, and tissue samples from failing and NF hearts were snap-frozen in liquid nitrogen not later than 2 hours after explantation.

Generation of Internal Standard RNA for Quantitative Polymerase Chain Reaction

Total cellular RNA was isolated from LV myocardium with RNA-Clean (AGS) according to the manufacturer’s protocol. Total RNA (2 μg) was reverse-transcribed with Moloney murine leukemia virus reverse transcriptase. One microliter of the RT reaction mixture was subsequently amplified by Taq DNA polymerase in a reaction mixture containing 10 mmol/L Tris-HCl (pH 8.3), 1.5 mmol/L MgCl₂, 50 mMol/L KCl, 0.2 mmol/L each deoxyribonucleotide triphosphate, and 12.5 pmol of each primer specific for the human ET₁a receptor, ET₁b receptor, ppET-1, and ECE-1, respectively. Polymerase chain reaction (PCR) products were cloned into the Bluescript SK− plasmid vector (Stratagene) or PCRII plasmid vector (Invitrogen). The cDNA was digested and ligated to obtain a plasmid with an insert lacking 10% to 20% of the original length. PCR products were cloned into the Bluescript SK− plasmid vector (Stratagene) or PCRII plasmid vector (Invitrogen). The cDNA was digested and ligated to obtain a plasmid with an insert lacking 10% to 20% of the original length. The plasmids were linearized, and deletion-mutated mRNA fragments for ET₁a receptor, ET₁b receptor, ppET-1, and ECE-1 respectively, were synthesized by in vitro transcription with the Megascript-Kit (Ambion).

Quantitative RT-PCR Analysis of ET Receptor, ppET-1, and ECE mRNA Expression

Two micrograms of the total RNA isolated from left ventricle together with 10 μg of mutated ET₁a, mutated ET₁b, mutated ppET-1, or mutated ECE mRNA, respectively, were reverse-transcribed with random primers, and PCR amplification of single-stranded cDNA was performed (for primer sequences, see the Table). PCR products were separated on 1.5% (wt/vol) agarose gels and transferred onto nylon membranes (Hybond N, Amersham). Southern blot hybridization was performed with the hybridization buffer containing 50% formamide, 100 mg/mL salmon sperm DNA, 6× SSC, and 0.5% SDS. 32P-labeled probes were prepared by use of the Prime-it II kit from Ambion. The membranes were washed twice with 2× SSC at room temperature and once with 2× SSC/0.1% SDS at 50°C for 60 minutes. Autoradiography was performed, and autoradiograms were quantified by laser densitometry (ImageQuant software, Molecular Dynamics). PCR amplification has been shown to be linear within a range of 28 to 40 cycles. Noncompetitive amplification of GAPDH was used to demonstrate equivalence of RNA loading in RT-PCR reactions.

RT-PCR for Analysis of ECE-1a and ECE-1b/c iso-mRNA Expression

ECE-1a and ECE-1b/c mRNA expression in DCM hearts compared with NF hearts was investigated with a standard RT-PCR technique.

Seasons of Upstream and Downstream Oligonucleotide Primers

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<th>mRNA</th>
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Northern Blot Analysis of Atrial Natriuretic Peptide

Northern blots were prepared from 10 μg total RNA as described previously.11 A 32P-labeled 411-bp fragment of human atrial natriuretic peptide (ANP) cDNA was used as a specific probe.

Immunoblot Analysis

Various tissues and cells were homogenized in 10 volumes of ice-cold homogenization buffer (5 mmol/L Tris-HCl [pH 7.5], 5 mmol/L MglCl, 5 μg/mL leupeptin, 5 μg/mL aprotinin, and 0.1 mL/mm PMSF). The homogenates were spun at 800g for 10 minutes, and the supernatants were further centrifuged (100 000 g for 30 minutes) and washed by resuspension in 10 volumes of homogenization buffer. The membrane fractions thus obtained were resuspended in 25 mmol/L Tris-HCl (pH 7.5)/20 mmol/L MglCl. SDS-PAGE was carried out with a 6% gel. Separated proteins were transferred to a nitrocellulose membrane and probed with anti-ECE antibody AEC32-236 (IgG1κ). The immunoreactive bands were visualized with the ECL detection system (Amersham). The monoclonal antibody AEC32-236, originally raised against purified rat ECE, also recognizes human ECEs.12

Radioligand Binding Studies

Membrane fractions were prepared from the left ventricles as described.10 Membranes (100 μg protein) were incubated with 50 pmol/L 125I-labeled ET-1 (NEN) and 15 different concentrations of the nonselective ET₁a/ET₁b-receptor antagonist bosentan or the selective ET₁a-receptor antagonist BQ-123 ranging from 10⁻¹¹ to 10⁻⁴ mol/L in a total volume of 250 μL incubation buffer including 0.5% BSA, 0.5 μg/mL leupeptin, and 0.3 mmol/L PMSF. Tubes were incubated for 60 minutes at 37°C, and the precipitates were then filtered through Whisman GF/C filters coated with 0.3% CHAPS followed by washing with 3× 6 mL ice-cold incubation buffer. Nonspecific binding, defined as binding not displaced by 10 μmol/L bosentan, was <10% of the total binding at a concentration of 50 pmol/L 125I-labeled ET-1. Myocardial β-adrenergic receptors were studied as described.10

ECE Activity

LV membrane preparations (25 μg protein) were incubated in the presence of big ET-1. The standard enzyme reaction was carried out in 50 μL of assay buffer (50 mmol/L Tris-HCl [pH 7.0], 100 mmol/L NaCl, 5 mg/mL BSA) containing 10 μmol/L big ET-1 at 37°C. After an incubation period of 3 hours, the reaction was stopped by addition of 50 μL of 10 mmol/L EDTA, the amount of ET-1 formed was determined by a high-sensitivity ET-1 ELISA (Amersham, which
exhibits cross-reactivity to big ET-1 of \( \approx 0.001 \% \). Samples were diluted 1:1000, and the ELISA assay was performed according to the manufacturer’s protocol. ECE activity was determined in the absence and presence of the ECE inhibitor phosphoramidon (100 \( \mu \text{mol/L} \)).

### Tissue Levels of Immunoreactive ET-1
LV tissue (~200 mg) was homogenized in 10 volumes of 1 mol/L acetic acid, and the homogenate was boiled for 10 minutes in a water bath. The homogenates were then centrifuged at 15,000 \( \text{g} \) for 30 minutes at 4°C. These samples were lyophilized and reconstituted with 250 \( \mu \text{L} \) buffer. Aliquots (100 \( \mu \text{L} \)) were assayed in duplicate with an ET-1 ELISA kit (Amersham). This kit exhibits cross-reactivity with other ET peptides as follows: ET-2, 100%; ET-3, \( < 0.001 \% \); human big ET, 0.07%; and human big ET 22-38, \( < 0.001 \% \).

### Statistics
Data are expressed as mean±SEM. Statistical significance was estimated with Student’s \( t \) test for unpaired observations and ANOVA. A probability value, \( P < 0.05 \) was considered significant.

### Results

#### \( \beta \)-Adrenergic Receptor Density and ANP mRNA Expression
The \( \beta \)-adrenergic receptor density was significantly decreased in DCM hearts (38 ± 9 fmol/mg) compared with NF myocardium (71 ± 13 fmol/mg) (not shown). These data correlate closely with those reported from previous studies on failing and NF human myocardium. Chronic exposure of the NF hearts to endogenous or exogenous catecholamines before explantation is unlikely, because \( \beta \)-adrenergic receptor densities determined in the present study are not decreased in NF hearts.

Because cardiac ANP expression has been shown to be positively related to clinical status and the degree of LV impairment and dilatation,\(^{12}\) the level of ANP gene expression was determined. ANP mRNA concentrations were significantly higher in DCM myocardium than in NF hearts (ratio for ANP mRNA to GAPDH mRNA: for NF hearts, 0.27±0.07; for DCM hearts, 1.00±0.30; \( P < 0.05 \)) (not shown).

### Tissue ET-1 Levels
To evaluate whether the cardiac ET system is activated in end-stage heart failure, tissue ET-1 levels were determined. Figure 1 illustrates a 1.6-fold increase in ET-1 concentrations in LV myocardium from failing hearts (64.3±6.4 pg ET-1/100 mg wet wt tissue, \( n = 11, P = 0.011 \) versus NF) compared with NF hearts (41.1±3.7, \( n = 9 \)).

### ET Receptors
In LV membranes, total ET-receptor density as assessed by \( ^{125} \text{I} \)-labeled ET-1 binding amounted to 101±7.7 fmol \( ^{125} \text{I} \)-ET-1 specifically bound/mg protein in NF hearts (\( n = 8 \)) and 105±9.2 fmol/mg protein in DCM hearts (\( n = 10 \)) (Figure 2A). Inhibition of \( ^{125} \text{I} \)-ET-1 binding by the highly selective ET\(_{A}\)-receptor antagonist BQ-123 resulted in both tissues in biphasic competition curves (Figure 2B). From these curves, an \( \text{ET}_{A}/\text{ET}_{B} \) ratio of 1:1.4 in NF hearts and 1:0.6 in DCM hearts was calculated. The \( K_{i} \) values for BQ-123 were as follows: ET\(_{A}\) receptor, 2.2 nmol/L and ET\(_{B}\) receptor, 4.6 \( \mu \text{mol/L} \) in membranes from NF hearts; and ET\(_{A}\) receptor, 1.3 nmol/L and ET\(_{B}\) receptor, 2.1 \( \mu \text{mol/L} \) in membrane preparations from failing left ventricles. Inhibition of ET-1 binding by the nonspecific ET\(_{A}/\text{ET}_{B} \) antagonist bosentan resulted in a monophasic competition curve as shown in Figure 3; the \( K_{d} \) value for bosentan was calculated to be 140 \( \text{nmol/L} \) and 21 \( \text{nmol/L} \) for NF and DCM hearts, respectively.

### RT-PCR Analysis
The expression of ppET-1, ET\(_{A}\) receptor, ET\(_{B}\) receptor, and ECE mRNA in the left ventricle of DCM and NF hearts was determined with quantitative RT-PCR analysis. Typical

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**Figure 1.** LV tissue ET-1 concentrations in NF hearts (\( n = 9 \)) and failing human hearts with DCM (\( n = 11 \)).

**Figure 2.** A, ET\(_{A}\)-, ET\(_{B}\)-, and total ET-receptor density in LV membranes from NF hearts (\( n = 8 \)) and failing human hearts with DCM (\( n = 10 \)). *\( P < 0.05 \) vs NF. B, Inhibition of \( ^{125} \text{I} \)-labeled ET-1 binding by BQ-123 and bosentan in LV membrane preparations from NF and failing human hearts with DCM. Specific binding remaining in presence of BQ-123 and bosentan at indicated concentrations was expressed as percentage of maximal binding in absence of ET-receptor antagonists. Graph shows representative binding experiments performed in triplicate.
Figure 3. A, Expression of ET<sub>A</sub>, ET<sub>B</sub>, ppET-1, and ECE mRNA in left ventricle from donor hearts and DCM hearts. A, Representative RT-PCR experiments. B, Bar graph shows relative changes in mRNA expression as percent of expression in donor hearts, n=8 for NF, n=10 for DCM left ventricles. *P<0.05 vs NF.

ECE Activity

Ventricular membrane conversion of exogenous big ET-1 proved to be time-dependent (linear up to 4 hours) and largely inhibited by phosphoramidon pretreatment (≈79±9% inhibition). Conversion of big ET-1 to mature ET-1 in the membrane fraction of LV myocardium was determined in the absence and presence of the ECE inhibitor phosphoramidon. The difference between the 2 values was used as a measure of ECE activity. No significant difference was observed between DCM (76.1±11.1 ng ET-1·h<sup>-1</sup>·mg<sup>-1</sup>, n=6) and NF hearts (69.6±19.9 ng ET-1·h<sup>-1</sup>·mg<sup>-1</sup>, n=6) (Figure 5A).

Immunoblot Analysis

Immunoblot analysis was performed with the monoclonal ECE antibody AEC 32-236 (Figure 5B). High levels of ECE expression were observed in membrane fractions of rat lung, human lung, and human umbilical vein endothelial cells (HUVECs). Membrane fractions of human LV myocardium from DCM and NF hearts showed very weak expression. ECE appeared as a 120- to 130-kDa protein. The electrophoretic mobility was slightly different between rat lung ECE and ECE from human tissues and cells.

Discussion

Previous investigations have demonstrated increased circulating ET levels in chronic heart failure and correlations between elevated plasma ET and LV dysfunction in humans.
Furthermore, tissue ET seems to be increased in left ventricles but unchanged in atria and right ventricles from congestive heart failure patients compared with NF control myocardium. However, no work to date has established the expression pattern of the main components of the ET system in left ventricles from patients with severe congestive heart failure compared with NF controls.

In contrast to the well-characterized increase in ANP and its analogues or alterations of the β-adrenergic system in human heart failure, regulation of cardiac genes encoding for the ET systems seems to be more complex. In failing left ventricles, steady-state mRNA expression of the ET₄ receptor, ppET-1, and ECE was unchanged, whereas expression of the ET₆ receptor was significantly decreased. Quantitative changes on the mRNA level in general paralleled changes in protein content or function. Nevertheless, myocardial ET-1 content was increased, and the ETA receptor subtype was significantly upregulated in the failing left ventricle.

Few studies have investigated alterations in ET₄/ET₆-receptor density in pathophysiological conditions in the human myocardium. In agreement with the present data, Pieske et al. reported an increase in ETA-receptor density in end-stage heart failure due to dilated cardiomyopathy. Although no change in ET₄-receptor binding sites had been observed, their data as well as the present study support the notion that a relative shift in ET receptor expression in favor of the ET₄ subtype occurs. Similarly, Pönöcke et al. found that ETA receptor density tends to be increased in LV myocardium from patients with chronic heart failure. This alteration was much more pronounced in dilated than in ischemic cardiomyopathy. Although ET₄ receptor number is increased (previous and present findings), ET₁-induced inositol phosphate (IP) formation is unchanged in the failing LV myocardium. These findings indicate that the ET₄-receptor/phospholipase C/IP₃ pathway is desensitized in end-stage heart failure. The precise mechanisms are not known. Although the immunoreactive amount of Gq/11, the G protein most likely coupled to the phospholipase C/IP₃/diacylglycerol system, is not changed in chronic heart failure, the ET₄ receptor–G-protein coupling might be impaired. A candidate mechanism is the agonist-induced phosphorylation of the ET₄ receptor protein by G-protein–coupled receptor kinases (GRKs), as recently shown by Freedman et al. They demonstrated that especially GRK2 is involved in the desensitization process of human ETA and ET₄ receptors.

The observations that ET₄ receptors are downregulated but ET₄-receptor density is markedly increased in the failing human myocardium indicate that additional mechanisms of ET receptor regulation besides agonist-induced (homologous) downregulation are involved. Indeed, angiotensin II and the intracellular second messenger cAMP have been reported to induce a heterologous upregulation of ET-receptor expression.

The present study shows a significant, 1.6-fold increase in immunoreactive ET-1 (irET-1) content in LV myocardium of DCM hearts. In contrast, previous reports by Beyermann et al. indicate a much more pronounced (~2- to 4-fold) increase in irET-1 tissue concentration in left ventricles of patients with chronic heart failure. However, this elevation of tissue irET might represent predominantly big ET, whereas irET-1 as determined in the present study represents exclusively cardiac concentration of mature ET-1, because the predominant form of cardiac ET is ET₁. The ET-1 immunobassay system used in the present study exhibits negligible cross-reactivity to big ET-1.

The source of elevated tissue ET-1 in congestive heart failure is unclear. Several mechanisms have been discussed. One possibility is an increased production of ET-1 via enhanced synthesis of ppET-1. Ventricular hypertrophy and congestive heart failure have been associated with increased myocardial expression of ppET-1 mRNA in experimental animal models. Over time, however, a normalization of ET-1 mRNA expression is observed in rat models of norepinephrine-induced cardiac hypertrophy and congestive heart failure due to myocardial infarction, although cardiac dysfunction persists. In human LV myocardium, ppET-1 mRNA expression is unchanged in DCM hearts compared with NF donor hearts (present study). These results, taken together, suggest that induction of ET-1 is rather an early, transient response to acute alterations of heart function.

In addition to ppET-1 expression, enhanced conversion of ET precursor peptides to ET-1 may result in increased tissue ET-1. Between the 2 ECEs identified, the membrane-bound metalloprotease ECE-1 is the major ET-1-forming enzyme in the cardiovascular system. The present study shows that immunoreactive ECE-1 protein is expressed in human LV myocardium. Moreover, phosphoramidon-sensitive ECE activity in LV membrane preparations did not differ significantly between failing hearts and donor hearts. These data correlate with findings on mRNA level indicating unchanged ECE-1 mRNA expression. Recently, 3 different human ECE-1 isoforms, ECE-1a, -1b, and -1c, which differ only in their N-terminal regions through alternative splicing of 1 ECE-1 gene, have been identified. The observation that the promoter region of ECE-1a contains a CAAT box and potential binding sites for various transcription factors suggests the regulatory expression of the ECE-1a isoform. However, no significant difference was observed in ECE-1a and ECE-1b/c mRNA expression between DCM and NF donor hearts, indicating that ECE isoenzyme expression is not differentially regulated in human heart failure.

Alternatively to altered ET-1 production, decreased clearance of ET-1 within the myocardium may also occur. Several in vivo and in vitro data raise the possibility of a local clearance mechanism mediated by ET₄ receptors. Because ET₄ receptors are downregulated in LV myocardium from patients with end-stage heart failure, this mechanism may contribute to elevated tissue ET-1 levels observed in these patients.

As is the case for any investigation of human myocardial tissue samples from explanted donor hearts, some potential limitations may have influenced our results. The major source of donor hearts is patients dying of head trauma or spontaneous intracranial hemorrhage. The sudden increase in intracranial pressure leads to a series of pathophysiological changes referred to as “the autonomic storm.” The massive sympathetic discharge results in acute-type desensitization phenomena of the β-adrenergic receptor pathways, which
have been suggested to be the reason for myocardial dysfunction observed in 10% to 20% of cardiac donors with no previous cardiac history.\textsuperscript{30} However, this desensitization process is observed primarily in hearts with echocardiographically apparent LV dysfunction,\textsuperscript{30} and such hearts were excluded from our study. Furthermore, prolonged exposure of the NF hearts to catecholamines before explantation can be excluded, because \( \beta \)-adrenergic receptor densities were not decreased. Therefore, it is unlikely that autonomic disorders associated with brain death influenced our observations concerning the expression pattern of the ET system.

In conclusion, the present study demonstrates alterations of the tissue ET system in severe chronic heart failure. Elevated endogenous ET-1 concentrations due to decreased cardiac clearance and changes in the ET-receptor expression pattern in favor of the ET\(_4\) receptor may have important implications for the pathophysiology of heart failure.

Acknowledgments

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References


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