Selective Upregulation of Cardiac Endothelin System in Patients With Ischemic but Not Idiopathic Dilated Cardiomyopathy

Endothelin-1 System in the Human Failing Heart

Gian Gastone Neri Serneri, Ilaria Cecioni, Simone Vanni, Rita Paniccia, Brunella Bandinelli, Annamaria Vetere, Xiao Janning, Iacopo Bertolozzi, Maria Boddi, Gian Franco Lisi, Guido Sani, Pietro Amedeo Modesti

Abstract—Only scarce information is available on the activity and modifications of the cardiac endothelin (ET)-1 system in heart failure due to ischemic (ICM) or idiopathic dilated (DCM) cardiomyopathy. The activity of the ET-1 system was investigated by measuring cardiac ET-1 and big ET-1 formation and quantifying cardiac mRNA for prepro–ET-1 (ppET-1), ET-converting enzyme-1, and ETA and ETB receptors both in myocardium and in isolated myocytes using Northern blot, reverse transcription–polymerase chain reaction, and in situ hybridization in 22 patients with DCM and 20 with ICM who underwent cardiac transplantation and in 7 potential heart transplant donors (nonfailing hearts).

Notwithstanding a similar increase of plasma ET-1 in the 2 groups, cardiac ET formation, mRNA levels for ppET-1, and ETA and ETB receptors were higher on both the myocardium and isolated myocytes from ICM than on those from DCM hearts (P<0.001 for all). ppET-1 and ET-converting enzyme-1 mRNAs were expressed on myocytes and endothelial and interstitial cells in ICM, whereas in DCM and nonfailing hearts they were mainly expressed on nonmyocyte cells. In both ICM and DCM, the ETA mRNA signal was expressed on both myocytes and nonmyocyte cells, whereas ETB mRNA was almost exclusively localized on nonmyocyte cells. ETA- and ETB-specific receptor binding was increased on both myocytes and cardiac membranes, showing a positive correlation with left ventricular ejection fraction in ICM (r=0.78 and 0.70) but not in DCM patients. The present results show that human ventricular myocytes express all of the components of the ET-1 system, which is selectively upregulated in ICM patients and appears to be functionally important in the maintenance of cardiac function.

Key Words: endothelin • heart failure • myocytes • receptors • RNA

Endothelin (ET)-1 is a multifunctional peptide that exerts pleiotropic activities, including arterial and venous constriction, direct positive inotropic and chronotropic effects on isolated heart, and growth effects on vascular smooth muscle cells, fibroblasts, and isolated cardiomyocytes. Prepro–ET-1 (ppET-1) mRNA is expressed by both rat and human cardiac myocytes and interstitial cells that synthesize and secrete mature ET-1. The protease that catalyzes the conversion (ET-converting enzyme, ECE) from the 38-residue inactive intermediate big ET-1 to achieve ET-1 is expressed in the endocardium and myocardium. The differing biological activities of ET-1 appear to be mediated through 2 receptor subtypes (ETA and ETB), which are both present in the human myocardium and that of other species. Thus, a complete ET-1 system is represented in human myocardium.

Cardiac ppET-1 mRNA expression and ET-1 synthesis have been found to be increased in experimental hypertrophy by pressure overload and in experimental models of congestive heart failure (CHF), thus suggesting that the cardiac ET-1 system may be involved in cardiac diseases.

There have been very few investigations of the cardiac ET-1 system in human hypertrophy and heart failure. Expression of mRNA for ppET-1 and cardiac ET-1 formation have been found to be increased in hypertrophy with parietal wall stress <90 kilodyne/cm² because of aortic valve stenosis. In situ hybridization has shown that ppET-1 mRNA is expressed in myocytes and to a lesser degree also in vascular and interstitial cells. Several studies have investigated cardiac ET-1 receptors. In endomyocardial biopsy specimens from patients with hypertrophic cardiomyopathy, mRNA expres-
tion for ET\textsubscript{A} receptors was not different from controls.\textsuperscript{14} ET-1 receptor subtypes have recently been investigated in myocardial homogenates of hearts from patients with end-stage idiopathic dilated cardiomyopathy (DCM)\textsuperscript{15-17} or ischemic cardiomyopathy (ICM),\textsuperscript{18} with conflicting results. Thus, despite the great potential pathophysiological and clinical implications of the cardiac ET-1 system,\textsuperscript{19} information about its functional activity in heart failure is lacking. Two factors might be critical in the study of the ET-1 system in this setting, as follows: first, a separate analysis of ET-1 receptors for myocytes and nonmyocyte myocardial cells, and second, investigation of the cardiac ET-1 system in relation to the etiology and severity of cardiac failure. The present study was therefore designed to investigate the functional activity of the cardiac ET-1 system in patients with DCM and ICM at different stages of heart failure severity. To this end, cardiac ET formation and receptor binding were evaluated and mRNA levels of ppET-1, ECE-1, ET\textsubscript{A}, and ET\textsubscript{B} receptor subtypes were quantified separately in isolated cardiomyocytes and in homogenated hearts.

**Materials and Methods**

We investigated 42 patients with heart failure afflicted with DCM (n=22) or ICM (n=20) who were scheduled to undergo cardiac transplantation. Patients with arterial hypertension, a recent history (6 months) of myocardial infarction, or echocardiographic evidence of valve or congenital heart disease were not considered for the study. The control group was made up of 20 normotensive patients with atypical chest pain in whom angiography and routine diagnostic procedures did not reveal any abnormalities. Diagnosis of DCM or ICM was based on clinical and echocardiographic examination, cardiac catheterization, and coronary angiography.

Myocardial ventricular tissue was collected from patients with DCM and ICM during cardiac transplantation. Cardiac specimens were also obtained from 7 donors with no histories or signs of heart disease, whose hearts could not be transplanted because of surgical reasons or blood group incompatibility (nonfailing [NF] hearts) (Table 1). The protocol of this study complies with the principles of the Helsinki declaration. All patients gave their informed, written consent to participate and to have their heart and blood samples used for the study. Echocardiographic and hemodynamic measurements were performed prospectively, as previously described.\textsuperscript{4}

The estimation of cardiac ET-1 formation was performed by measuring the aorta-coronary sinus gradient of ET-1 and big ET-1 corrected for coronary flow and cardiac mass.\textsuperscript{4} Cardiomyocytes were isolated and membrane suspension was prepared from a noninfarcted portion of left ventricle free wall.\textsuperscript{19} Binding studies were performed at equilibrium using selective ET\textsubscript{A} (BMS-182874, a gift of Bristol-Myers-Squibb) or ET\textsubscript{B} (BQ-788) antagonists.\textsuperscript{19} mRNAs for ppET-1, ECE-1, ET\textsubscript{A}, and ET\textsubscript{B} receptors, extracted with phenol-chloroform from transmural myocardial specimens, were quantified with Northern blots, using \( \alpha \)-\textsuperscript{32}P-labeled gel-purified specific cDNA probes. Levels of ppET-1, ECE-1, ET\textsubscript{A}, and ET\textsubscript{B} messengers were also quantified both in the myocardium and in isolated myocytes by reverse transcription–polymerase chain reaction (RT-PCR) using specific primers.\textsuperscript{17} The densitometric ratio was calculated using GAPDH as internal standard and expressed as the percentage of the values obtained in NF hearts.\textsuperscript{4} The in situ hybridization procedure was performed as previously described\textsuperscript{4} using specific cDNA photobiotin-labeled (Vector) probes. Myocardial cell types were identified by immunohistochemical methods using specific monoclonal antibodies. The average myocyte diameter was obtained by measuring the short-axis length of 200 myocytes using a computerized image-analysis system (Qwin, Leica).

Data are expressed as mean±SD. Comparisons between groups were performed using 1-way ANOVA. For multivariate re-evaluation of univariate correlations, left ventricular end-diastolic diameter index, left ventricular mass index, left ventricular ejection fraction (LVEF), mean midwall velocity of circumferential fiber shortening (Vcf), mean pulmonary artery pressure, pulmonary capillary wedge pressure, average myocyte diameter, ET-1 and big ET-1 cardiac production, and concentration of ET-1 in venous blood were entered in a stepwise multiple regression analysis as independent variables considering ET\textsubscript{A} and ET\textsubscript{B} receptor densities on cardiomyocytes and membranes as dependent variables.

An expanded Materials and Methods section is available online at http://www.circresaha.org.

**Results**

**Plasma Assays of Cardiac ET-1 and Big ET-1 Formation**

The clinical characteristics of investigated subjects are reported in Table 1.

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**Table 1. Main Echocardiographic and Hemodynamic Characteristics of Subjects Investigated**

<table>
<thead>
<tr>
<th>Age, y</th>
<th>Sex, M/F</th>
<th>Body surface area, m(^2)</th>
<th>LVEDDI, mm/m(^2)</th>
<th>LVMI, g/m(^2)</th>
<th>Ejection fraction, %</th>
<th>Vcf, m/s</th>
<th>Cardiac index, L/m(^2)×min(^{-1})</th>
<th>MPAP, mm Hg</th>
<th>PCWP, mm Hg</th>
</tr>
</thead>
<tbody>
<tr>
<td>58±12</td>
<td>16/4</td>
<td>1.87±0.17</td>
<td>26.7±2.3</td>
<td>107±15</td>
<td>63±4.5</td>
<td>1.16±0.17</td>
<td>2.96±0.36</td>
<td>15.2±2.3</td>
<td>5.7±1.8</td>
</tr>
<tr>
<td>5±2</td>
<td>15/5</td>
<td>1.86±0.18</td>
<td>26.4±3.1</td>
<td>104±18</td>
<td>61±3.9</td>
<td>1.09±0.23</td>
<td>2.04±0.45</td>
<td>32.3±10.1*</td>
<td>21.6±7.8*</td>
</tr>
<tr>
<td>14/8</td>
<td></td>
<td>1.85±0.17</td>
<td>39.1±4.5*</td>
<td>213±35*</td>
<td>22.2±6.5*</td>
<td>0.48±0.19*</td>
<td>2.03±0.40</td>
<td>33.7±13.6*</td>
<td>21.6±9.3*</td>
</tr>
<tr>
<td>56±11</td>
<td></td>
<td>1.83±0.26</td>
<td>44.1±6.7*</td>
<td>266±49*†</td>
<td>19±7.5*</td>
<td>0.46±0.16*</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

NYHA indicates New York Heart Association; LVEDDI, left ventricular end diastolic diameter index; LVMI, left ventricular mass index; Vcf, mean midwall velocity of circumferential fiber shortening; MPAP, mean pulmonary artery pressure; and PCWP, pulmonary capillary wedge pressure.

\( \frac{\alpha}{\beta} P<0.01 \) vs controls; † \( \frac{\alpha}{\beta} P<0.01 \) vs ICM.
ET-1 and big ET-1 plasma concentrations in peripheral veins of ICM (3.1 ± 1 and 15.2 ± 3.3 pg/mL) and DCM patients (2.8 ± 0.9 and 17.2 ± 4.1 pg/mL) were higher than in controls (0.79 ± 0.4 and 4.7 ± 1.6 pg/mL, *P < 0.001 for both), with no significant differences between the 2 patient groups.

The mean aorta-coronary sinus concentration gradient of ET-1 was not significantly different among the 3 groups. Conversely, the concentration gradient of big ET-1 was significantly higher in ICM (1.7 ± 1.1 pg/mL, *P < 0.001) than in DCM patients (−1.4 ± 1.8 pg/mL, *P < 0.001) or controls (−0.04 ± 0.23 pg/mL) (Figure 1A). The mean highest concentration gradient of big ET-1 was found in ICM and the lowest in DCM patients, as well as the amount of big ET-1 formed per minute per gram of tissue (Figure 1B).

**Radioligand Binding Studies**

The density of ET-1 binding sites (B<sub>max</sub>) on myocytes isolated from the left ventricle of NF hearts was 42 ± 6 fmol/mg protein with 86% of ET<sub>A</sub> subtype (Table 2). Cardiac membranes from NF hearts had an ET-1 binding site density (B<sub>max</sub>) of 195 ± 25 fmol/mg with balanced proportions of ET<sub>A</sub> (63%) and ET<sub>B</sub> (37%) (Table 2 and Figure 2A).

The density of ET-1 binding sites on myocytes was significantly higher in ICM than DCM or NF hearts (Table 2). Both ET<sub>A</sub> and ET<sub>B</sub> subtypes were increased in the same proportion (+45% and +50%, respectively) (Figure 2B). The affinities of both subtypes were not significantly different among the various groups (Table 2).

Membranes from ICM hearts showed significantly higher binding site density than DCM (*P < 0.01) or NF (*P < 0.01) hearts (Table 2) without any differences in the affinity with a proportional increase of both ET<sub>A</sub> (+62%, *P < 0.01) and ET<sub>B</sub> (+59%, *P < 0.01) (Table 2). The individual data of the receptor densities are shown in Figure 2B.

Multivariate stepwise analysis revealed that ET<sub>A</sub> and ET<sub>B</sub> receptor density on both myocytes and membranes from ICM hearts was positively correlated with LVEF, whereas in DCM hearts only ET<sub>B</sub> receptors demonstrated this positive correlation (Table 3 and Figure 2C). ET<sub>A</sub> receptor density was positively correlated with myocyte diameter in both ICM (r = 0.80, *P < 0.01) and DCM (r = 0.66, *P < 0.01) hearts. No correlation was found among ET<sub>A</sub> and ET<sub>B</sub> myocyte or membrane receptor density and plasma concentration or cardiac production of ET-1 and big ET-1.

**Quantification of ppET-1, ECE-1, ET<sub>A</sub>, and ET<sub>B</sub> mRNA Levels in the Myocardium and in Isolated Cardiomyocytes**

RT-PCR revealed that ppET-1, ECE-1, ET<sub>A</sub>, and ET<sub>B</sub> receptor genes were expressed on myocytes isolated from all the ventricles. Levels of mRNA for ppET-1, ECE-1, ET<sub>A</sub>, and ET<sub>B</sub> receptors were significantly increased in myocytes from ICM hearts (P < 0.001 versus NF hearts for all) but were not in myocytes from DCM hearts. No significant differences were found between DCM and NF hearts (Figure 3A).

RT-PCR performed on homogenated hearts showed a lower ET<sub>A</sub>/ET<sub>B</sub> mRNA ratio than in isolated myocytes, with an increased expression of both ET<sub>A</sub> and ET<sub>B</sub> mRNAs in ICM hearts (Figure 3A). mRNA for ppET-1 was also increased in ICM hearts, whereas no differences were found for ECE-1 mRNA expression in comparison with DCM and NF hearts (Figure 3A). Northern blots performed in myocardial samples confirmed the increased expression of mRNA for ppET-1, ET<sub>A</sub>, and ET<sub>B</sub> in ICM as compared with DCM and NF hearts (Figure 3B).
In Situ Hybridization Studies

The average myocyte diameter was larger in ICM (20.1 ± 1.8 μm) than in DCM (17.2 ± 0.9 μm, P < 0.001) and NF (15.2 ± 1.4 μm, P < 0.001) hearts. Negative and positive controls for hybridization showed that the hybridization signal was specific for mRNA, and mRNA in the biopsies was intact (Figure 4A and 4B).

In NF (Figure 4C and 4D) and DCM (Figure 4G and 4H) hearts, ppET-1 and ECE-1 mRNAs were mainly expressed on the interstitial and endothelial cells, whereas in ICM specimens a positive signal was also present in myocytes (Figures 4E and 4F). In NF hearts, ET<sub>A</sub> receptor mRNA was expressed on both myocyte and nonmyocyte cells (Figure 5A), whereas ET<sub>B</sub> receptors were almost exclusively localized on nonmyocyte cells (Figure 5B). In ICM specimens, mRNA both for ET<sub>A</sub> (Figure 5C) and ET<sub>B</sub> (Figure 5D) subtypes had the same localization as observed in DCM specimens (Figure 5E and 5F) and NF hearts. The intensity of the signal was higher in ICM specimens than in the other groups.

Discussion

The present study establishes the following for the first time in humans: (1) the presence, distribution, and characterization of ET-1 receptor subtypes in ventricular myocytes from NF hearts; (2) the different pattern of ET-1 receptors on myocytes and ventricular membranes in ICM versus DCM; and (3) the selective activation of the cardiac ET-1 system in ICM and its changes in relation to the progressive worsening of heart failure.

Myocyte ET-1 Subtype Receptors

Binding studies and densitometric analysis for mRNA indicated that both ET<sub>A</sub> and ET<sub>B</sub> receptors are represented in ventricular myocytes from NF hearts, with a marked predominance of ET<sub>A</sub> (86% versus 14%). The absolute density of ET-1 receptors was higher in membranes than in myocytes, with a more balanced proportion between ET<sub>A</sub> and ET<sub>B</sub> (63% versus 37%). The different ratio of ET<sub>A</sub> and ET<sub>B</sub> receptors in membranes and isolated myocytes may point to different functions of the 2 receptor subtypes. There is evidence that ET<sub>A</sub> receptors mediate the positive inotropic and growth-promoting effects of ET-1. The close relationship found in the present study between myocyte ET<sub>A</sub> receptor density and myocyte diameter corroborates this role of ET<sub>A</sub>. The function of ET<sub>B</sub> receptors is not yet completely clear. Several studies have suggested that ET<sub>B</sub> receptors may act as clearance receptors for ET-1, and the observation that ET-1 binding to ET<sub>B</sub> receptors is less stable than its binding to ET<sub>A</sub> receptors supports the above hypothesis. Because the total density of ET<sub>B</sub> receptors is higher in ventricular membranes than in myocytes, it may be speculated that ET<sub>B</sub> receptors may contribute to preventing excessive ET-1 myocardial concentrations.

The modifications of ventricular ET-1 binding sites occurring in CHF, particularly in DCMs, have been studied on ventricular homogenates. The different densities of ET-1 binding sites and the different proportion between ET<sub>A</sub> and ET<sub>B</sub> receptors on myocytes versus ventricular membranes may be the cause of the discrepancies among the various studies. Indeed, the densities of ET-1 binding sites in membranes from ICM and DCM hearts have not been found to differ significantly from those in NF hearts. Other studies performed in DCM hearts have reported either increased ET<sub>A</sub> density and ET<sub>B</sub> downregulation without any significant changes in the total density, or enhanced total density due to ET<sub>B</sub> increase without any modifications of ET<sub>A</sub> receptors.

The separate analyses for myocytes and membranes, as performed in the present study, demonstrate that ET-1 binding sites are increased only in ICM hearts, with a proportional
increase in ET\textsubscript{A} and ET\textsubscript{B} receptors both on myocytes and membranes and with changes correlated to LVEF.

**Upregulation of Cardiac ET-1 System and Its Functional Meaning**

The increased density of ET\textsubscript{A} and ET\textsubscript{B} receptors on myocytes and ventricular membranes and the overexpression of mRNA for ppET-1 and ECE-1 on myocytes indicate an upregulation of the cardiac ET-1 system in ICM patients, resulting in an accelerated cardiac synthesis of ET-1, as shown also by the increased big ET-1 aorta-coronary sinus gradient. The absence of an accompanying increase in the ET-1 aorta-coronary sinus gradient does not contradict this statement, because it may be due to the increased ET-1 degradation, to the enhanced binding of ET-1 to its receptors, or to both. Because ET-1 degradation was not investigated, this hypothesis cannot be excluded. However, the increased density of ET\textsubscript{A} and ET\textsubscript{B} receptors both on myocytes and ventricular membranes suggests an enhanced capture of ET-1 by binding sites rather than an increase in ET-1 degradation. This mechanism would be consistent with the increased ET-1 concentration in cardiac tissue reported in failing myocardium\textsuperscript{15,23,24} and with the finding that DCM patients, who showed neither enhanced cardiac ET-1 formation by myocytes nor increased receptor density, had instead a significantly lower coronary sinus gradient for big ET-1 than controls. In these patients, whose mRNA for ECE-1 was not significantly different from NF hearts, the negative gradient for big ET-1 was associated with an ET-1 gradient that did not differ from that of controls or ICM patients, suggesting an increased conversion of plasma big ET-1 to ET-1 during the transcardiac passage. Upregulation of mRNA for ppET-1, increased peptide ET-1 level, and enhanced ET-1 receptor density were found in the myocardium from rats with the coronary artery ligation model of CHF.\textsuperscript{25–28} Conversely, ventricular ET-1 receptor density and myocyte ET-1 production in pigs with pacing-induced CHF were not different from those in control pigs.\textsuperscript{12} The present results are thus consistent with experimental observations and emphasize the different pattern of the cardiac ET-1 system in ICM versus DCM patients.

ET-1 binding sites on cultured myocytes are downregulated by pretreatment with ET-1,\textsuperscript{29} and the level of ET\textsubscript{B} receptor mRNA is downregulated by ET-1 through decreasing the intracellular stability of mRNA molecules.\textsuperscript{30} In ICM patients, ET\textsubscript{A} and ET\textsubscript{B} receptors on both myocytes and membranes were not downregulated, despite the elevated

**TABLE 3. Correlation Between ET\textsubscript{A} and ET\textsubscript{B} Receptors and Clinical, Hemodynamic, and Neurohormonal Variables (Stepwise Regression Analysis)**

<table>
<thead>
<tr>
<th></th>
<th>ICM</th>
<th>DCM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ET\textsubscript{A} r</td>
<td>ET\textsubscript{B} r</td>
</tr>
<tr>
<td>Isolated cardiomyocytes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age, y</td>
<td>0.24</td>
<td>0.16</td>
</tr>
<tr>
<td>Plasma ET-1, pg/mL</td>
<td>−0.35</td>
<td>−0.49</td>
</tr>
<tr>
<td>Cardiac ET-1 production, pg/min×g\textsuperscript{−1}</td>
<td>0.19</td>
<td>0.19</td>
</tr>
<tr>
<td>Cardiac big ET-1 production, pg/min×g\textsuperscript{−1}</td>
<td>0.60</td>
<td>0.67</td>
</tr>
<tr>
<td>Mean myocyte diameter, μm</td>
<td>0.80*</td>
<td>0.71</td>
</tr>
<tr>
<td>LVMI, g/m\textsuperscript{2}</td>
<td>−0.22</td>
<td>−0.19</td>
</tr>
<tr>
<td>LVEDDI, mm/m\textsuperscript{2}</td>
<td>−0.24</td>
<td>−0.33</td>
</tr>
<tr>
<td>MPAP, mm Hg</td>
<td>0.05</td>
<td>0.09</td>
</tr>
<tr>
<td>PCWP, mm Hg</td>
<td>−0.16</td>
<td>0.03</td>
</tr>
<tr>
<td>LVEF, %</td>
<td>0.78*</td>
<td>0.84*</td>
</tr>
<tr>
<td>Vcf, s\textsuperscript{−1}</td>
<td>0.72</td>
<td>0.22</td>
</tr>
<tr>
<td>Cardiac membranes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age, y</td>
<td>0.12</td>
<td>0.13</td>
</tr>
<tr>
<td>Plasma ET-1, pg/mL</td>
<td>−0.14</td>
<td>−0.24</td>
</tr>
<tr>
<td>Cardiac ET-1 production, pg/min×g\textsuperscript{−1}</td>
<td>−0.07</td>
<td>0.06</td>
</tr>
<tr>
<td>Cardiac big ET-1 production, pg/min×g\textsuperscript{−1}</td>
<td>0.62</td>
<td>0.58</td>
</tr>
<tr>
<td>LVMI, g/m\textsuperscript{2}</td>
<td>−0.32</td>
<td>−0.27</td>
</tr>
<tr>
<td>LVEDDI, mm/m\textsuperscript{2}</td>
<td>−0.68*</td>
<td>−0.55</td>
</tr>
<tr>
<td>MPAP, mm Hg</td>
<td>0.09</td>
<td>0.01</td>
</tr>
<tr>
<td>PCWP, mm Hg</td>
<td>0.07</td>
<td>−0.03</td>
</tr>
<tr>
<td>LVEF, %</td>
<td>0.70*</td>
<td>0.65*</td>
</tr>
<tr>
<td>Vcf, s\textsuperscript{−1}</td>
<td>0.65</td>
<td>0.64</td>
</tr>
</tbody>
</table>

LVMI indicates left ventricular mass index; LVEDDI, left ventricular end-diastolic diameter index; MPAP, mean pulmonary artery pressure; PCWP, pulmonary capillary wedge pressure; LVEF, left ventricular ejection fraction; and Vcf, mean midwall velocity of circumferential fiber shortening.

*Independent variable at stepwise regression.
ET-1 plasma levels. Only in patients with very low ejection fraction was the receptor density in the range of that of NF hearts. The precise mechanism determining the lack of ET-1 receptor downregulation is not clear. Cardiac ET-1 formation may be induced by different stimuli, including mechanical forces, hypoxia, and angiotensin II, which may be operating in ICM patients and may also upregulate the ET-1 receptors. Thus, it may be speculated that when the cardiac ET-1 synthesis is activated by locally acting stimuli, the local mechanisms of regulation predominate over the downregulation induced by plasma ET-1 levels. However, specially designed studies are needed to investigate this issue.

The upregulation of the cardiac ET-1 system seems to be important in the maintenance of the cardiac function in ICM patients, as indicated by the positive correlation of ETA receptor density with LVEF and Vcf and, conversely, the negative correlation between ET A receptors and left ventricular end-diastolic diameter index. The functional importance of cardiac ET-1—system activation seems to be confined to the normally functioning myocytes or to providing short-term support to failing myocardium. The favorable activity of ET-1 on cardiac function does not rule out the possibility that in the long term it may have adverse effects, because it has been shown to depress myocardial contractility and relaxation in pacing-induced CHF regardless of its effect on arterial load.

Mechanisms of Cardiac ET-1 Activation

The present investigation extends current knowledge regarding the role of ET-1 in the pathophysiology of severe heart failure. The widely different pattern of the ET-1 system in ICM and DCM patients is not attributable to apparent clinical, echocardiographic, and hemodynamic differences, because the 2 groups of patients did not differ either for these parameters or for the drugs administered. Thus, it is reasonable to conclude that heart failure is not in itself responsible for cardiac ET-1 system activation, which is probably instead due to the primary disease underlying the heart failure. There is evidence that mechanical forces selectively induce cardiac growth factor synthesis in relation to pressure or volume overload. Although ICM consists of a complex mixture of ischemia, stress due to myocardial scar, and often a component of volume overload, myocytes are substantially subjected to stretch because of pressure overload, given that the residual healthy myocytes are forced to face a greater workload to compensate for the loss of neighboring contractile elements resulting from myocardial infarction and chronic myocardial ischemia. Experimental and human pressure-overload hypertrophy is associated with an increased ET-1 formation. In addition, in experimental severe heart failure induced by coronary artery ligation, an increased expression of ppET-1 mRNA was reported in nonischemic myocardial areas. Hypoxia and acute experimental ischemia have each been reported to induce ET-1 forma-
tion in isolated myocytes\textsuperscript{43} or ventricular myocardium.\textsuperscript{44} However, ischemia does not seem to be a major mechanism in the increased ET-1 formation in ICM patients, because an ischemic component is present in the heart of DCM patients\textsuperscript{45,46} and myocardial perfusion is impaired both at rest and in response to vasodilating stimuli in DCM patients.\textsuperscript{47} Therefore, pressure overload seems to be a major mechanism of cardiac ET-1 system activation in ICM.

The inciting factor of DCM is unknown, but this condition is characterized by ventricular remodeling producing chamber dilation with normal or decreased wall thickness. The resulting eccentric hypertrophy\textsuperscript{45,48} suggests volume overload as a prevalent hemodynamic mechanism. Patients with predominant volume-overload hypertrophy, like those with aortic regurgitation, show no evidence of increased myocyte ET-1 formation.\textsuperscript{4}

Hence, although we cannot exclude other mechanisms, including the incapacity of myocytes from DCM patients to produce ET-1 in response to mechanical forces or cardiac and humoral factors, the different type of hemodynamic overload would seem to be the major mechanism responsible for the different patterns of the cardiac ET-1 system in cardiomyopathies.

In conclusion, the cardiac ET-1 system is selectively upregulated in ICM patients, whereas it is not in DCM.
patients, and it appears to be functionally important for the maintenance of cardiac function in the former.

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References


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MATERIALS AND METHODS

Subjects investigated
We investigated 42 patients with heart failure affected by idiopathic dilated cardiomyopathy (DCM, n=22) or ischemic cardiomyopathy (ICM, n=20) who were scheduled to undergo cardiac transplantation. Patients with diastolic blood pressure above 90 mmHg, a recent history (less than 6 months) of myocardial infarction, and echocardiographic evidence of valve or congenital heart disease were not considered for the study. The control group was made up of 20 normotensive patients with atypical chest pain in whom angiography and routine diagnostic procedures did not reveal any abnormalities. Diagnosis of DCM or ICM was based on clinical and echocardiographic examination, cardiac catheterization and coronary angiography. Myocardial ventricular tissue was collected from the patients with dilated cardiomyopathy during cardiac transplantation. Cardiac specimens were also obtained from 7 donors with no histories or signs of heart disease, whose hearts could not be transplanted because of surgical reasons or blood group incompatibility (non failing hearts, NF). The characteristics of subjects investigated are reported in Table 1. The protocol of this study complies with the principles of the Helsinki declaration (1). After a full explanation of the purposes of the study, all patients gave their written consent to participate and to have their heart and blood samples used for the study. Echocardiographic and hemodynamic measurements were performed in a prospective way, as previously described (2).

Estimation of the cardiac production of ET-1 and big ET-1
The estimation of cardiac ET-1 formation was evaluated by measuring the aorta-coronary sinus gradient of ET-1 and big ET-1 indexed by coronary flow and cardiac mass. Furthermore, ppET-1 mRNA levels were quantified both in the myocardium and in isolated myocytes by Northern and RT-PCR studies. Blood withdrawal, plasma extraction and radioimmunoassay of ET-1 and big ET-1 were performed as previously described (2).
Cardiomyocyte isolation and membrane preparation

Cardiomyocytes were isolated from a non-infarcted portion of left ventricle free wall, as previously reported (3). Cell purity (over 99% cardiomyocytes) was assessed by using specific monoclonal antibodies against human myosin (SIGMA, M8421), vimentin (SIGMA, clone n.V9, V6630) or von Willebrand factor (SIGMA, F3520) and a second fluorescein labeled sheep antibody (F 4143 SIGMA). Membrane suspension was prepared as previously described (3).

Binding studies and identification of ET receptor subtypes.

Binding studies were performed at equilibrium on isolated cardiomyocytes (10⁵ cells/mL) or cardiac membranes (300 µg/mL) incubated with [¹²⁵I]-ET-1 (100 pmol/L, 2000 Ci/mmol, Amersham) and increasing concentrations of unlabeled ET-1 (0 to 1 µmol/L) or selective ETₐ (BMS-182874) (a kind gift of Dr. GB Leproux, Bristol-Myer-Squibb, Italy) or ETₐ (BQ-788) antagonists (0 to 100 µmol/L) for 120 min at 22°C, as previously reported (3).

Quantification of ppET-1, ETₐ and ETₐ and ECE-1 mRNA levels

RT-PCR studies

Levels of ppET-1, ECE-1 and ETₐ and ETₐ transcripts in isolated cardiomyocytes were quantified with RT-PCR using GAPDH as internal standard as previously described (2). All PCR primers were designed according to Zolk et al (4) and purchased from Pharmacia. The densitometric ratio (mRNA for ppET-1, ECE-1, ETₐ, and ETₐ / mRNA for GAPDH) was calculated and expressed as percent of the values obtained in NF hearts.

Northern blot analysis

mRNA for ppET-1, ECE-1, ETₐ and ETₐ receptors were quantified in transmural myocardial specimens obtained from approximately the same region of the left ventricular free wall. Total RNA was extracted by phenol-chloroform extraction (5). Thirfty-five micrograms of RNA per lane was separated by electrophoresis on a 1.2% agarose-formaldehyde gel and transferred to
a nylon membranes (Hybond-N+, Amersham) using 10x SSC as transfer buffer, and baked at 80 °C for 2 hours. Membranes were prehybridized for 1 hour and hybridized overnight at 65 °C in 10 mL of Church & Gilbert solution with the following \([a-^{32}P]\)-labeled gel purified cDNA probes: 0.85-kb EcoRI for ppET-1, a 1.23-kb BamH-I and HIND-III KpnI for ECE-1, a 0.45-kb PstI EcoRV for \(E_{T_A}\) receptor, a 0.5-kb EcoRI KpnI for \(E_{T_B}\) receptor, and a 1-kb for GAPDH (2) as the internal control. Autoradiographs were quantified by densitometry and the ratios of the ppET-1, ECE-1, \(E_{T_A}\) and \(E_{T_B}\) mRNA levels by the GAPDH mRNA level were calculated.

**Localization of mRNA for ppET-1, ECE-1, \(E_{T_A}\) and \(E_{T_B}\) in the myocardium**

The in situ hybridization procedure was performed as previously described (6) using cDNA photobiotin labeled (Vector) probes reported above for human ppET-1, ECE-1, \(E_{T_A}\) and \(E_{T_B}\) receptor subtypes and GAPDH. Myocardial cell types were identified by immunohistochemical methods using specific monoclonal antibodies against human myosin (SIGMA M8421), vimentin (SIGMA, clone n.V9, V6630) or von Willebrand factor (SIGMA, F3520) and a second fluorescein labeled sheep antibody (F 4143 SIGMA). The average diameter of the myocytes was obtained by measuring the short-axis length of 200 myocytes. The sections in which myocardial fibers were longitudinally or obliquely cut were selected from the hematoxylin and eosin stained sections, and the short axes of the fibers that contained nuclei were measured, using a computerized image-analysis system (Qwin, Leica).

**Statistical analysis**

Data are expressed as mean ± SD. Comparisons between groups was performed using a one-way ANOVA and Student t test, followed by the Tukey multiple-range comparison test, as appropriate. Univariate linear relations were analyzed with the Pearson correlation. A stepwise multiple regression analysis was used for multivariate re-evaluation of univariate correlations. \(E_{T_A}\) and \(E_{T_B}\) receptor densities on cardiomyocytes and membranes were included as dependent variables and independent variables were: age, left ventricular end-diastolic diameter index (LVEDDI), left ventricular mass index (LVMI), left ventricular ejection fraction
(LVEF), mean midwall velocity of circumferential fiber shortening (Vcf), mean pulmonary artery pressure (MPAP), pulmonary capillary wedge pressure (PCWP), average myocyte diameter, cardiac production of ET-1 and big ET-1, and concentration of ET-1 in venous blood. The significance level for univariate and multivariate testing was set at 0.05. All calculations were performed using BMDP statistical software.

REFERENCES


