Endothelin in Human Congestive Heart Failure

Chi-Ming Wei, MD, PhD; Amir Lerman, MD; Richard J. Rodeheffer, MD; Christopher G.A. McGregor, MD; Roland R. Brandt, MD; Scott Wright, MD; Denise M. Heublein; Pai C. Kao, PhD; William D. Edwards, MD; John C. Burnett, Jr, MD

Background Although recent investigations report the elevation of plasma endothelin (ET) in congestive heart failure (CHF), it remains unclear if this elevation is that of the biologically active peptide ET-1 or of its precursor big-ET. Furthermore, it is unclear if such elevation is associated with increased myocardial ET and if the molecular form from cardiac tissue is altered ET. Last, it remains to be established whether circulating ET is increased at the earliest stage of CHF in patients with asymptomatic left ventricular dysfunction and correlates with the magnitude of ventricular dysfunction. Methods and Results The present study was designed to investigate concentrations and molecular forms of ET in plasma and cardiac tissue in healthy subjects and CHF patients with New York Heart Association (NYHA) class I through IV using cardiac radionuclide angiogram, cardiac myocardial biopsy, radioimmunoassay, gel permeation chromatography (GPC), and immunohistochemical staining (IHCS). Plasma ET was increased only in patients with moderate (NYHA class III) or severe (NYHA class IV) CHF compared with healthy subjects and individuals with asymptomatic (NYHA class I) or mild (NYHA class II) CHF. The elevation of circulating ET in CHF showed a negative correlation with left ventricular ejection fraction and cardiac index and a positive correlation with functional class and left ventricular end-diastolic volume index. GPC demonstrated that immunoreactive plasma ET was ET-1 in healthy subjects and both mature ET-1 and its precursor big-ET in severe CHF patients, with big-ET the predominant molecular form. Cardiac tissue concentrations and IHCS revealed ET presence in healthy atrial and ventricular tissue, which were not different in severe CHF. GPC revealed that the molecular form of cardiac ET was ET-1 in both healthy and CHF hearts. Conclusions The present study establishes for the first time that the elevation of plasma ET in severe human CHF represents principally elevation of big-ET. Second, ET is present in healthy and failing myocardia, and its activity by both immunohistochemistry and radioimmunoassay is not changed in CHF. Furthermore, the elevated plasma ET is characteristic of severe CHF and not asymptomatic or mild CHF. In addition, the degree of plasma elevation of ET correlates with the magnitude of alterations in cardiac hemodynamics and functional class. The present study confirms and extends previous investigations of ET in human CHF and establishes the evolution of circulating and local cardiac ET in the spectrum of human CHF. (Circulation 1994;89:1580-1586)

Key Words • endothelin • congestive heart failure • vasoconstriction • chromatography, gel permeation

Endothelin (ET) is a potent endothelial cell-derived venous and arterial vasoconstrictor peptide that functions as both a circulating hormone and a paracrine factor in the regulation of vascular tone.1-4 Studies have also established that ET may modulate the renin-angiotensin-aldosterone system, augment myocardial inotropic function, and stimulate vascular smooth muscle proliferation and cardiac hypertrophy.2,5-7 These various biological actions have implicated the ET system in a spectrum of cardioregional disease states such as atherosclerosis, hypertension, congestive heart failure (CHF), acute coronary ischemic syndromes, coronary vasospasm, and acute and chronic renal failure.8-19

With the repeated demonstration of increases in circulating ET in experimental13 and clinical CHF20,21 and the known actions of ET, which mimic cardiorenal adaptations of CHF,2,22 major questions emerge regarding ET in human CHF. These include whether the elevation of immunoreactive ET in CHF represents the mature biologically active peptide, ET-1, or the less biologically active precursor big-ET. Second, as ET may augment myocardial contractility and stimulate myocardial cell hypertrophy,5,7 the presence, localization, distribution, and cardiac tissue molecular forms of ET require elucidation. Finally, although previous work has established that plasma ET is elevated in severe CHF, it remains to be established whether circulating ET is increased at the earliest stage of CHF in patients with asymptomatic left ventricular dysfunction (ALVD) and thus be a marker and/or participant in early and/or late CHF.

The current investigation was therefore undertaken in human heart failure to address the following hypotheses. (1) Human heart failure results in elevation of both mature ET-1 and the less biologically active precursor big-ET. (2) ET is present in healthy and failing human atrial and ventricular myocardia. (3) Circulating ET is increased only in advanced CHF and not in asymptomatic patients and parallels alterations in cardiac hemodynamics. To address these hypotheses, we used cardiac catheterization, myocardial biopsy, radioimmunoassay (RIA) analysis, immunohistochemical staining (IHCS), and gel permeation chromatography (GPC) to define the extent and molecular forms of
plasma and cardiac ET in humans with ALVD and chronic CHF, including end-stage CHF.

Methods

Subject Selection

Forty subjects (30 men and 10 women; average age, 64±5 years) were investigated. CHF patients were classified by New York Heart Association (NYHA) functional class criteria according to their cardiac symptoms after a complete physical examination and laboratory evaluation at the Mayo Clinic. The following class distributions resulted: NYHA class I (n=14; mean age, 61±3 years), NYHA class II (n=5; mean age, 62±3 years), NYHA class III (n=7; mean age, 69±2 years), NYHA class IV (n=8; mean age, 65±4 years), and six control subjects (mean age, 61±4 years). The etiologies of CHF included idiopathic dilated cardiomyopathy and ischemic cardiomyopathy. Exclusion criteria included individuals with renal failure (serum creatinine >176 μmol/L), abnormal liver function tests, significant valvular heart disease, atrial fibrillation, and frequent premature ventricular contractions. All severe CHF patients were on drug treatment, including digoxin, diuretics, and/or vasodilators. Left ventricular ejection fraction (LVEF) was determined by echocardiography or radionuclide angiograms. Exclusion criteria included individuals with severe CHF patients were on drug treatment, including digoxin, diuretics, and/or vasodilators. Left ventricular ejection fraction (LVEF) was determined by echocardiography or radionuclide angiography. Left ventricular end-diastolic volume was determined as well in those patients who underwent radionuclide angiography. All studies were performed with the patient in the supine position. Three ECG leads were monitored continuously. For radionuclide imaging, patient's red blood cells were labeled using 30 mCi of 99mTc and using the modified in vivo procedure of Callahan et al. Cardiac imaging was obtained with a small-field gamma camera (Picker Dyna Mo) in the left anterior oblique view by conventional consecutive R-wave gating and collected at 20 frames per cardiac cycle. A blood sample was obtained for cardiac volume determinations. Radionuclide data were processed using a commercially available computer and software (Medical Data Systems) and previously reported techniques. The left ventricular region of interest was identified in each frame using a second derivative technique. A background region was chosen 5 pixels lateral to the left ventricular systolic region. LVEF was calculated from the background-corrected left ventricular counts-versus-time curve. End-diastolic and end-systolic volumes were determined using a count-based method and a previously reported regression equation from the Mayo Clinic Diagnostic Nuclear Medical Laboratory. Correlation coefficients for end-diastolic and end-systolic volumes determined by radionuclide angiograms compared with contrast ventriculography have been previously reported as 0.85 and 0.94, respectively. The end-diastolic volume index was determined by dividing end-diastolic volume by body surface area. For LVEF by echocardiography, the LVEF at rest was calculated by means of the left ventricular systolic and diastolic dimensions measured on the two-dimensional derived M-mode tracing in the majority of patients. When no adequate measurements could be obtained, a visual estimation of the LVEF was used. Techniques and methods have been previously reported at this institution. Venipuncture for measurement of ET was performed while each patient was in the supine position. Six individuals with a resting ejection fraction of ≥50% and with no detectable cardiac disease served as the control group.

Healthy and CHF Subjects for Cardiac Tissue Characterization

Cardiac tissue was obtained from six patients (five men and one woman; average age, 49±2 years) with end-stage heart failure undergoing cardiac transplantation at the Mayo Clinic. Venous plasma samples from these patients were obtained before the transplantation procedure. Cardiac tissue included both atria and ventricles. Tissue samples were immediately placed in liquid nitrogen and stored at −70°C until further processing occurred. Cardiac tissue from both atria was harvested from healthy donor hearts at the time of transplantation and processed in an identical manner.

Quantitation of Plasma and Tissue ET

Plasma and tissue ET was determined by the ET-1-[125I] assay system (Amersham International, Amersham, UK) as previously described. Briefly, plasma was taken from chilled potassium EDTA tubes after centrifugation at 2500 RPM at 4°C and frozen at −20°C until assay. Cardiac tissue was immediately placed in liquid nitrogen and stored at −70°C until further processing was performed. Cardiac tissue was pulverized, boiled for 5 minutes in 10 vol of 1 mol/L acetic acid/20 mmol/L hydrochloric acid solution to abolish intrinsic proteolytic activity and then homogenized. The homogenate was centrifuged for 30 minutes at 15000 rpm at 4°C. The supernatant was then stored at −20°C and analyzed by a specific RIA. The recovery of the extraction procedure was 81%, as determined by addition of synthetic ET to plasma, and interassay and intra-assay variabilities were 9% and 5%, respectively. The cross-reactivity of the assay to big-ET is 37%; ET-2, 100%; and ET-3, <1%.

Gel Permeation Chromatography Analysis

ET was characterized from nonextracted plasma by a P-6 (Bio-Rad Laboratories, Richmond, Calif) gel filtration column (1×13 cm). Plasma (500 μL) was applied to the column and eluted with 0.5 mol/L acetic acid buffer. Fractions of 0.5 mL were collected and dried by savant speedvac. The concentration of ET in each fraction was determined by RIA as previously described. The P-6 column was calibrated with synthetic 125I ET-1 and 125I big-ET (Peninsula Laboratory, Belmont, Calif). Fig 1 represents the calibration of the P-6 column with 125I ET-1 and 125I big-ET. Big-ET was eluted in 35 mL fractions of 8 mL (peak at fraction 6). ET-1 was eluted in fractions 7 to 17 (peak at fraction 12). Total ET recovery was determined by adding the concentrations of each fraction (5 through 17) after subtraction of the background ET (2 pg per fraction). The mean column recovery of total ET was 86%.

Immunohistochemical Staining

IHCS for ET was performed in cardiac tissue taken from healthy and severe CHF subjects. IHCS was performed in atrial and ventricular samples from healthy cardiac tissue and CHF patients. Atrial sections were taken from the appendages and free walls of both atria. Full-thickness sections of human ventricular myocardium were obtained from the middle third of the free wall of both the left and right ventricles from failing hearts. The healthy human right ventricular endocardial tissues were obtained from healthy donor heart during cardiac transplantation. Tissue was dehydrated and embedded in paraffin, and sections were cut to a 5-μm thickness. The presence of cardiac ET was assessed using a previously described immunohistochemical technique that is a modification of the Chapeau technique. Slides from the cardiac tissue were counterstained with hematoxylin to enhance nuclear
healthy and CHF subjects. As shown in this figure, plasma ET was modestly increased in patients with moderate CHF (NYHA class III) and markedly increased in patients with severe CHF (NYHA class IV). In contrast, patients with asymptomatic and mild left ventricular dysfunction (NYHA class I and II) did not demonstrate an elevation in circulating ET compared with healthy subjects. There was no difference in ET levels between idiopathic dilated cardiomyopathy and ischemic cardiomyopathy in each NYHA classification.

Fig 4 is a representative GPC of plasma from a healthy subject (Fig 4A) and from a patient with severe CHF (NYHA class IV) (Fig 4B). This figure demonstrates that the only ET isoform in healthy subjects is mature ET-1, the 21-amino-acid biologically active peptide, whereas both mature ET-1 and its precursor big-ET, the 38-amino-acid prohormone, are present in plasma in patients with severe CHF. Fig 5 demonstrates the absolute values of plasma ET-1 and big-ET in healthy and CHF subjects (n=4 in each group). As shown in this figure, no circulating big-ET was detected by GPC in healthy subjects (n=4). However, big-ET represented 62±7% (14.6±2.4 pg/mL) and ET-1 represented 38±5% (8.6±1.2 pg/mL) of total immunoreactive plasma ET among the severe heart failure group (n=4).

Table 1 and Fig 6 report significant clinical correlates with total circulating immunoreactivity of ET. As reported, functional NYHA classification, LVEF, cardiac index, and left ventricular end-diastolic volume index all significantly correlated with plasma ET.

IHCS of atrial and ventricular myocardia for ET from a representative healthy heart and a severe failing heart is illustrated in Fig 7. ET immunoreactivity was present at similar intensities in both healthy and failing hearts in atrial and ventricular myocardia and located less predominantly in the perinuclear region of cardiac myocytes. ET IHCS scores, percentage distribution (percent positive staining area), and tissue concentrations are reported in Table 2. No differences were observed in these measured parameters between healthy and CHF hearts. Fig 8 illustrates a similar pattern for myocardial tissue concentrations in healthy and failing myocardia. Furthermore, gel permeation (n=4) for ET from both healthy and failing atrial tissue revealed that the predominant molecular form of ET in myocardium was mature ET-1.

Discussion
Although previous investigators have demonstrated increased circulating ET in CHF and correlations be-
between ET and left ventricular dysfunction and regional vascular resistance in CHF,20,21 no work to date has established the circulating forms of plasma ET in CHF, the activity of local cardiac ET in CHF, and whether asymptomatic CHF (ALVD) is characterized by elevated plasma ET. The present investigation extends current knowledge regarding circulating and local cardiac ET activity in CHF by establishing for the first time that (1) the elevation of plasma ET in CHF represents predominantly big-ET (62%), (2) immunoreactive ET is present in both healthy and failing human atrial and ventricular tissue and its presence is unaltered in severe CHF; (3) cardiac ET is ET-1, whereas plasma ET represents both ET-1 and big-ET, (4) total plasma ET is elevated in severe CHF (NYHA class III and IV) but not in ALVD and mild CHF (NYHA class II), and (5) increased circulating ET is a late phenomenon in human CHF and correlates with function class and cardiac dysfunction only in moderately and severely symptomatic CHF.

Table: Correlation of Endothelin and Clinical Variables

<table>
<thead>
<tr>
<th>Variable</th>
<th>R</th>
<th>P</th>
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<tbody>
<tr>
<td>New York Heart Association classification</td>
<td>.858</td>
<td>&lt;.05</td>
</tr>
<tr>
<td>Left ventricular ejection fraction, %</td>
<td>.857</td>
<td>&lt;.05</td>
</tr>
<tr>
<td>Cardiac index, L·min⁻¹·m⁻²</td>
<td>.822</td>
<td>&lt;.05</td>
</tr>
<tr>
<td>Left ventricular end-diastolic volume index, mL/m²</td>
<td>.710</td>
<td>&lt;.05</td>
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The observation of a predominance of big-ET immunoreactivity in plasma in severe CHF significantly extends previous studies and provides new insight into ET in the pathophysiology of human CHF. Several important speculations may be put forward based on this observation. First, the presence of big-ET in human plasma in severe CHF suggests accelerated synthesis and release of ET. Therefore, the predominance of big-ET in plasma may suggest that circulating ET-1 may not be a major humoral participant in the pathophysiological adaptations of CHF but, more important, may represent spillover of locally produced ET-1 in CHF. This would be consistent with a role for ET-1 in CHF as functioning by paracrine and autocrine mechanisms. Such a paracrine role is supported by in vivo and in vitro observations that ET-1 augments myocardial contractility; promotes myocardial cell hypertrophy; stimulates release of atrial natriuretic peptide, aldosterone, and catecholamines; and is antinatriuretic in the kidney.2,5,7,18 In addition, an alternative mechanism that must be explored is the possibility that in CHF there is decreased conversion of big-ET to ET-1 by reduced ET-converting enzyme activity. Indeed, the current observation regarding the predominance of big-ET in plasma underscores the importance of defining the structure and activity of ET-converting enzyme in the human in the absence and presence of CHF. A limitation of the present investigation is that these alternative mechanisms, which may mediate the increase in big-ET in CHF, are not fully elucidated and will require further investigation.

In the present study, IHCS demonstrates for the first time that ET is present in atrial and ventricular myocardium of both healthy and failing human hearts. This may underscore the importance of ET as a possible paracrine regulator of cardiac function in both healthy and pathophysiological states. Specifically, the presence of immunoreactive ET by both immunohistochemistry and tissue RIA is consistent with synthesis and release from endothelial cells or the myocardium with interaction with known receptors localized to cardiac myocytes.42 The functional significance of myocardial ET in the presence and absence of CHF can only be speculated. However, the presence of myocardial ET and the known cardiac actions, which include enhanced inotropic action, mito-
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Fig 6. Plots of correlations of plasma endothelin and human subject characteristics. A, Plasma endothelin and New York Heart Association classification; B, plasma endothelin and left ventricular ejection fraction; C, plasma endothelin and cardiac index; and D, plasma endothelin and left ventricular end-diastolic volume index (LVEDVI).

generation, and coronary vasoconstriction,\textsuperscript{2,5,14-18,18} have clear relevance to the pathophysiology of CHF. One may also speculate that the lack of big-ET in atrial tissue as determined by RIA may suggest that the heart is a target organ for ET-1 regardless of whether ET-1 is secreted by the overlying endocardial or coronary endothelium in a paracrine manner or is released from systemic vascular endothelium in a humoral manner. Further studies defining the presence of big-ET in ventricular myocardium and the possible synthesis of ET by ventricular myocytes are required. It should be noted that in the present study, we used the ET-1,2 immunoassay system, which indicated that cross-reactivity of the assay to big-ET was 37%; ET-2, 100%; and ET-3, <1%. As ET-2 has not been detected in human plasma, we would not expect any of the increased immunoreactivity to be due to ET-2. As the assay essentially does not cross-react with ET-3, we do not believe that this isoform contributes to the elevation of total immunoreactive plasma ET as observed in the present study.

The demonstration that increased circulating ET occurs late in the severity of CHF and is not a marker for ALVD extends previous reports.\textsuperscript{20,21,30} Lerman et al\textsuperscript{32} demonstrated in an animal model that ET at pathophysiological plasma concentrations as encountered in severe CHF produced by exogenous ET has biological action. This observation supports a functional role for endogenous ET as a pathophysiological vasoconstrictor. This observation is consistent with the growing data in experimental pathophysiological states that demonstrate that elevated circulating ET conventionally is associated with states of severe cardiovascular stress such as cardiogenic shock\textsuperscript{31} and endotoxin shock\textsuperscript{32} or associated with known sequegues such as angiotensin II\textsuperscript{33} and cyclosporin.\textsuperscript{34} Previous work that reported elevated circulating ET in CHF suggested that ventricular dysfunction was the cardiovascular stress that results in increased plasma ET. Although in the present investigation a significant correlation exists between ET and ejection fraction, normal plasma ET concentrations were also observed in both asymptomatic and mild CHF subjects despite significant impairment of ventricular function. Thus, other clinical correlates such as NYHA class, cardiac index, and left ventricular end-diastolic volume index may be additional factors that must be considered in determining stimuli that may be associated with ET activation.

Of interest, Lerman et al\textsuperscript{30} found persistence of elevated plasma ET concentrations after cardiac transplantation in a group of patients despite restoration of normal cardiac function, even at 1 year of follow-up. Our observations coupled with this recent report suggest that elevations in plasma ET may be related more to generalized endothelial cell dysfunction than purely secondary to generalized ventricular dysfunction. This hypothesis is supported by a number of previous observations that suggest generalized endothelial cell dysfunction in CHF.\textsuperscript{35} In addition, the hypothesis of plasma ET elevation secondary to endothelial cell dysfunction...
is supported by the observations of elevated ET in association with atherosclerosis\textsuperscript{9,36} and sepsis,\textsuperscript{32,37} which are also states of generalized endothelial cell dysfunction. Further longitudinal studies nonetheless are needed to provide further insight into mechanisms of elevated ET as such investigations would permit evaluation of the impact of drug therapy-mediated improvement in those clinical correlates found to be significant. This is underscored by the fact that in the present study, conventional cardiovascular medications were continued. The potential significance of these medications is unclear. Based on the present study, one may view elevation of total circulating ET in CHF as a marker for generalized endothelial cell dysfunction and/or as activation of a more pathophysiologically important local autocrine or paracrine system in symptomatic CHF with plasma spillover. Furthermore, the observation of preserved local cardiac ET presence in symptomatic CHF underscores the potential role of ET as a paracrine modulator of cardiac function in evolving CHF. Indeed, circulating, paracrine, and autocrine mechanisms have emerged as playing a fundamental mechanistic role in the progression of CHF.\textsuperscript{43}

In conclusion, the present investigation demonstrates for the first time that the elevation of plasma ET in severe CHF is primarily related to elevated circulating big-ET. The present investigation reveals for the first time the presence of atrial and ventricular ET-1 of similar activity in both the presence and absence of CHF without any big-ET activity, perhaps suggesting that the heart is a target of ET and not the source of

\begin{figure*}[h]
\centering
\includegraphics[width=\textwidth]{fig7.png}
\caption{Immunohistochemical staining (IHCS) for endothelin (ET) from human right and left atrial (RA and LA) and right and left ventricular (RV and LV) myocardia from representative normal and severe failing heart (CHF). NRS indicates nonimmunohistochemical staining.}
\end{figure*}

\begin{table}[h]
\centering
\caption{Cardiac Tissue Endothelin Staining Score and Area}
\begin{tabular}{|l|l|l|l|l|}
\hline
 & \multicolumn{2}{|c|}{ET Stain Score} & \multicolumn{2}{|c|}{ET Stain Area, \%} \\
 & Healthy Subject & CHF Patient & Healthy Subject & CHF Patient \\
\hline
RA & 1.9±0.1 & 2.0±0 & 60±6 & 70±6 \\
LA & 2.0±0 & 1.9±0.1 & 60±6 & 65±5 \\
RV & 2.0±0 & 2.0±0 & 70±11 & 75±10 \\
LV & NA & 2.0±0 & NA & 70±10 \\
\hline
\end{tabular}
\end{table}

ET indicates endothelin; CHF, congestive heart failure; RA, right atrium; LA, left atrium; RV, right ventricle; LV, left ventricle; and NA, not available. Values are mean±SEM.

\begin{figure*}[h]
\centering
\includegraphics[width=\textwidth]{fig8.png}
\caption{Bar graph of cardiac tissue endothelin (ET) concentrations in healthy (normal) and severe congestive heart failure (CHF-New York Heart Association [NYHA] IV) subjects. Values are mean±SEM. NA indicates not available; RA, right atrium; LA, left atrium; RV, right ventricle; and LV, left ventricle.}
\end{figure*}
increased circulating ET concentrations. The present study also demonstrates that ALVD is characterized by normal circulating ET concentrations and that only with severe CHF do plasma ET concentrations rise. We also demonstrate that elevated circulating total ET correlates significantly with functional class and alterations in cardiac hemodynamics. The present investigation confirms and extends previous investigations regarding ET in CHF and establishes the evolution of circulating and local cardiac ET in the spectrum of human CHF.

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