Association of Cardiotrophin-1 With Myocardial Fibrosis in Hypertensive Patients With Heart Failure

Begoña López,* Arantxa González,* Ramón Querejeta, Mariano Larman, Gregorio Rábago, Javier Díez

Abstract—Cardiotrophin-1 has been shown to be profibrogenic in experimental models. The aim of this study was to analyze whether cardiotrophin-1 is associated with left ventricular end-diastolic stress and myocardial fibrosis in hypertensive patients with heart failure. Endomyocardial biopsies from patients (n=31) and necropsies from 7 control subjects were studied. Myocardial cardiotrophin-1 protein and mRNA and the fraction of myocardial volume occupied by collagen were increased in patients compared with controls (P<0.001). Cardiotrophin-1 overexpression in patients was localized in cardiomyocytes. Cardiotrophin-1 protein was correlated with collagen type I and III mRNAs (r=0.653, P<0.001; r=0.541, P<0.01) and proteins (r=0.588, P<0.001; r=0.556, P<0.005) in all subjects and with left ventricular end-diastolic wall stress (r=0.450; P<0.05) in patients. Plasma cardiotrophin-1 and N-terminal pro-brain natriuretic peptide and serum biomarkers of myocardial fibrosis (carboxy-terminal propeptide of procollagen type I and amino-terminal propeptide of procollagen type III) were increased (P<0.001) in patients compared with controls. Plasma cardiotrophin-1 was correlated with N-terminal pro-brain natriuretic peptide (r=0.386; P<0.005), carboxy-terminal propeptide of procollagen type I (r=0.550; P<0.001), and amino-terminal propeptide of procollagen type III (r=0.267; P<0.05) in all subjects. In vitro, cardiotrophin-1 stimulated the differentiation of human cardiac fibroblast to myofibroblasts (P<0.05) and the expression of procollagen type I (P<0.05) and III (P<0.01) mRNAs. These findings show that an excess of cardiotrophin-1 is associated with increased collagen in the myocardium of hypertensive patients with heart failure. It is proposed that exaggerated cardiomyocyte production of cardiotrophin-1 in response to increased left ventricular end-diastolic stress may contribute to fibrosis through stimulation of fibroblasts in heart failure of hypertensive origin. (Hypertension. 2014;63:483-489.) *Online Data Supplement

Key Words: cardiotrophin I  ■  collagen  ■  fibrosis  ■  heart failure

Chronic pressure overload imposed by systemic hypertension is associated with an exaggerated deposition of collagen type I and III fibers in the interstitium and the perivascular region of the myocardium.1 Myocardial fibrosis impairs left ventricular (LV) function, thus facilitating the development of heart failure (HF) in hypertension.2 In this setting, it has been shown that, besides mechanical stress, several humoral factors are involved in the development of myocardial fibrosis.3 Cardiotrophin-1 (CT-1) is a member of the interleukin 6 superfamily, which exerts its actions through its heterodimeric glycoprotein 130/leukemia inhibitory factor receptor.4 CT-1 was originally characterized as a stress response factor inducing cardiomyocyte survival and protection in response to biomechanical stress.4 However, recent in vitro findings in rodent and canine cardiac fibroblasts5–7 and vascular smooth muscle cells8 suggest that CT-1 also behaves as a profibrotic cytokine. In fact, in vivo studies performed in rats have shown that CT-1 induces fibrosis in different organs, including the heart.9,10 Although CT-1 has been shown to be increased in the myocardium of patients with HF,11,12 there are no available data on the mechanisms involved in its overproduction and its association with myocardial fibrosis in HF.

Therefore, the aim of this study was to evaluate the potential associations of myocardial CT-1 with cardiomyocyte mechanical stress at end diastole as assessed by the estimation of LV end-diastolic wall stress (LVEDWS)13 and collagen type I and III synthesis and deposition in hypertensive patients with HF. In addition, the association between circulating CT-1 and biomarkers of myocardial fibrosis (carboxy-terminal propeptide of procollagen type I or PICP and amino-terminal propeptide of procollagen type III or PIIINP)14 was also studied in these patients. Finally, the ability of CT-1 to induce the activation of cultured human fibroblasts to myofibroblasts and to stimulate collagen synthesis was analyzed.
Methods

Subjects
All subjects gave written informed consent to participate in the study, and the institutional review committee approved the study protocol. The study conformed to the principles of the Helsinki Declaration.

The population consisted of 31 patients with hypertension with a previous clinical diagnosis of chronic stage C HF. Patients were classified as having HF with preserved LV ejection fraction (HFPEF) or HF with reduced ejection fraction (HFrEF) in accordance with the diagnostic criteria proposed by the European Society of Cardiology. Three transvenous endomyocardial biopsies were taken from the middle area of the interventricular septum from each patient during a cardiac catheterization procedure. For further details on the inclusion and exclusion criteria and the clinical characteristics of the patients, see the online-only Data Supplement.

Septal endomyocardial biopsies were obtained from autopsies of 7 age- and sex-matched subjects (5 men, 2 women; age, 58.3±5.4 years) who died of noncardiovascular causes with no macroscopic and microscopic cardiac lesions. The presence of macroscopic malignancies or microscopic chronic kidney disease in the control group was excluded after review of their autopsy files. An additional group of 20 age- and sex-matched healthy subjects (16 men, 4 women; age, 59.1±3.48 years) was used as controls for biochemical studies. The presence of cardiovascular disease was excluded after a medical examination.

Cardiac Studies
Two-dimensional echocardiographic-Doppler and pulsed-Doppler imaging was performed in all of the patients. LV mass and dimensions and mitral inflow parameters were measured. LVEDWS was calculated according to Iwanaga et al. To discard coronary artery disease (≥50% stenosis in a major epicardial coronary artery), coronary arteriography was performed in all the patients. For further details, see the online-only Data Supplement.

Histomorphological and Immunohistochemical Studies
The fraction of myocardial volume with positive staining for collagen (collagen volume fraction [CVF]) was determined by quantitative morphometry in sections stained with collagen-specific picrosirius red. The endocardium was excluded from the analysis. Immunohistochemical analysis of collagen types I and III was performed on formalin-fixed and paraffin-embedded sections, and type I CVF and type III CVF were analyzed by quantitative morphometry.

Cellular localization of CT-1 was performed by immunohistochemistry on formalin-fixed and paraffin-embedded sections. For further details, see the online-only Data Supplement.

Molecular Studies
Protein expression of collagen types I and III and CT-1 was analyzed in myocardial samples by Western blot, and data were expressed as arbitrary densitometric units relative to β-actin expression. The myocardial expression of mRNA of CT-1 and α1 chain of procollagen types I and III was analyzed by reverse transcription real-time polymerase chain reaction in unstimulated and CT-1–stimulated cells (for further details, see the online-only Data Supplement).

Biochemical Determinations
Plasma CT-1 was measured by an in-house ELISA as previously reported. Serum PICP and plasma aldosterone were measured by radioimmunoassays (Orion Diagnostica and DiaSorin, respectively). Serum PIIINP and plasma amino-terminal propeptide of brain natriuretic peptide (NT-proBNP) were measured by ELISA (MyBioSource and Roche Diagnostics, respectively).

In Vitro Study
Adult human dermal fibroblasts (adult human dermal fibroblast line) and primary cell cultures of human cardiac fibroblasts were used (for further details, see the online-only Data Supplement). Dose-response stimulation curves were performed with adult human dermal fibroblasts. Because procollagen type I and III mRNAs increased in a dose-dependent manner on CT-1 stimulation (Figure S1 in the online-only Data Supplement), a concentration of 10 ng/mL CT-1 was selected for being the dose rendering a submaximal stimulation. A minimum of 7 independent experiments were performed for each condition. The expression of α-smooth muscle actin and procollagen type I and III mRNAs was analyzed by reverse transcription real-time polymerase chain reaction in unstimulated and CT-1–stimulated cells (for further details, see the online-only Data Supplement).

Statistical Analysis
The differences between 2 groups were analyzed with a Student t test for unpaired data once normality was demonstrated; otherwise, the Mann-Whitney U test was performed. The differences in qualitative variables were evaluated with a χ2 test. The correlation between continuously distributed variables was tested by correlation coefficients and univariate regression analysis. The influence of confounding factors on correlations was excluded by partial correlation analysis for quantitative parameters. Values are expressed as mean±SEM and categorical variables as numbers and percentages. A value of P<0.05 was considered statistically significant.

Results
Clinical, Hormonal, and Cardiac Parameters
The clinical and hormonal characteristics of the patients are shown in Table S1. The group of patients was composed mainly of men, with overweight, most of them in New York Heart Association functional class III, who had been diagnosed of chronic HF for >2 years. All patients exhibited NT-proBNP levels >125 pg/mL, the cutoff value for chronic HF. Compared with control subjects, patients with HF presented elevated aldosterone levels (126.3±20.37 versus 58.67±5.74 pg/mL; P<0.005). Whereas all patients were treated with a diuretic and either an angiotensin-converting enzyme inhibitor or an angiotensin receptor blocker, none of them was receiving treatment with mineralocorticoid receptor antagonists. Table 1 and Table S2 show the echocardiographic parameters of the whole population of patients and of

<table>
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<tr>
<th>Table 1. Cardiac Parameters in Patients With Heart Failure</th>
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<tr>
<td>Parameters</td>
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<tr>
<td>LVMl, g/m²</td>
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<tr>
<td>LVEDD, mm</td>
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<tr>
<td>LVEDV, mL</td>
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<tr>
<td>LVEDVI, mL/m²</td>
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<tr>
<td>LVPWT, mm</td>
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<td>IVST, mm</td>
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<tr>
<td>LVEF, %</td>
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<td>PCWP, mmHg</td>
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<td>LVEDWS, Kdynes/cm²</td>
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Values are given as mean±SEM (n=31). CI indicates confidence interval; IVST, interventricular septum thickness; LVEDD, left ventricular end-diastolic diameter; LVEDV, left ventricular end-diastolic volume; LVEDVI, left ventricular end-diastolic volume index; LVEDWS, left ventricular end-diastolic wall stress; LVEF, left ventricular ejection fraction; LVMl, left ventricular mass index; LVPWT, left ventricular posterior wall thickness; and PCWP, pulmonary capillary wedge pressure.
the patients divided into 2 groups: those fulfilling criteria of HFPEF and those presenting with HFREF, respectively.

Myocardial and Plasma CT-1
The expression of both myocardial CT-1 mRNA and protein was higher \( (P<0.001) \) in the myocardium of patients with HF than in controls (Figure 1). In addition, plasma CT-1 was increased \( (P<0.001) \) in patients with HF compared with control subjects (Figure 1).

Myocardial CT-1 was localized mainly in cardiomyocytes but scarcely in other cell types (Figure 2). Whereas in controls CT-1 immunostaining was undetectable in 43% of subjects, mild in 28.5%, and moderate in 28.5%, in patients with HF its expression was mild in 36% of patients, moderate in 32%, and intense in 32% \( (P<0.005) \). Of note, CT-1 immunostaining seemed to be more intense in patients with HFREF than in patients with HFPEF (Figure 2). In fact, whereas in patients with HFREF CT-1 immunostaining was intense in 48% of patients, moderate in 26%, and mild in 26%, in patients with HFPERF its expression was intense only in 8% of patients, moderate in 42%, and mild in 50% \( (P<0.005) \). Furthermore, myocardial CT-1 protein was increased in patients with HFREF compared with patients with HFPEF (Figure 2). Of interest, collagen type III proteins was observed in patients with HFREF compared with controls (Table 2). In addition, increased expression of collagen type I and III proteins was found in patients with HFREF compared with patients with HFPEF.

However, the serum levels of the biomarkers of myocardial fibrosis were increased \( (P<0.01) \) in patients with HF compared with control subjects (PICP: 139.93±6.17 versus 69.77±3.95 μg/L; PIIINP: 477.07±78.45 versus 161.96±45.14 pg/mL).

Analysis of Associations
Myocardial CT-1 protein was correlated \( (P<0.05) \) with LVEDWS in patients with HF (Figure 3). In addition, myocardial CT-1 protein was directly correlated \( (P<0.01) \) with procollagen type I and III mRNAs and collagen type I and III proteins in all subjects (Figure 4). Of interest, all these correlations remained significant when we excluded the influence of several potential confounding factors (ie, age, sex, body mass index, estimated glomerular filtration rate, blood pressure, NT-proBNP, and aldosterone) in partial correlation analysis. No correlations were found between myocardial CT-1 mRNA and LVEDWS or collagen-related molecules.

Plasma CT-1 was directly correlated with serum PICP \( (r=0.550; P<0.001) \) and PIIINP \( (r=0.267; P<0.05) \) in all subjects. In addition, circulating CT-1 was directly correlated with NT-proBNP \( (r=0.386; P<0.005) \) in all subjects. These correlations remained significant when we excluded the influence of several potential confounding factors (ie, age, sex, body mass index, estimated glomerular filtration rate, blood pressure, NT-proBNP, and aldosterone) in partial correlation analysis. No other correlations were found between myocardial or plasma CT-1 and the remaining parameters measured in this study.

In Vitro Study
In both adult human dermal fibroblasts and freshly isolated human cardiac fibroblasts, stimulation with CT-1 increased α-smooth muscle actin mRNA compared with unstimulated cells \( (P<0.05) \) in both cases; Figure 5; Figure S3). Procollagen type I mRNA was increased in both cardiac \( (P<0.05) \) and dermal fibroblasts \( (P<0.001) \) exposed to CT-1 compared with control cells (Figure 5; Figure S3). Similarly, CT-1 stimulated procollagen type III mRNA \( (P<0.01) \) in both fibroblast types (Figure 5; Figure S3).

![Figure 1](image_url). Myocardial expression of cardiotrophin-1 (CT-1) mRNA (A) and protein (B) in patients with heart failure \( (n=28 and n=31, \text{respectively}) \) and control subjects \( (n=7) \). C. Plasma CT-1 levels in patients with heart failure \( (n=31) \) and control subjects \( (n=20) \). Myocardial mRNA and protein data are corrected by 18s ribosomal mRNA and β-actin, respectively. ADU indicates arbitrary densitometric units; and AU, arbitrary units.
Discussion

The main findings of this study are the following: (1) an excess of myocardial CT-1 protein is associated with LVEDWS and increased collagen type I and III mRNAs and protein expression in the fibrotic myocardium of hypertensive patients with HF, (2) an excess of plasma CT-1 is associated with increased levels of circulating biomarkers of myocardial fibrosis in hypertensive patients with HF, and (3) CT-1 induces human cardiac fibroblast differentiation to myofibroblasts and stimulates procollagen type I and III expression in these cells.

Pathophysiological Aspects

Myocardial CT-1 was found to be abnormally overexpressed at mRNA and protein levels in patients with HF, thus confirming previous data showing an excess of CT-1 in the myocardium of rats\textsuperscript{18,19} and humans\textsuperscript{12} with hypertension and HF. Based on the immunohistological localization, CT-1 seems to be localized mainly in cardiomyocytes from patients with HF. It has been previously shown that hypoxia,\textsuperscript{20} neurohumoral factors (eg, norepinephrine, angiotensin II, aldosterone),\textsuperscript{21–23} and mechanical stretch\textsuperscript{24} stimulate CT-1 expression in cardiomyocytes. In this regard, it must be noted that we ruled out the presence of coronary artery disease and microvascular ischemia in patients with HF. In addition, almost all patients from this study were treated with a β-blocker and either an angiotensin-converting enzyme inhibitor or an angiotensin receptor blocker. Furthermore, no association was found between plasma aldosterone and myocardial CT-1. However, we found an association of LVEDWS with myocardial CT-1 protein but not with mRNA, suggesting that increased diastolic stretch might stimulate post-transcriptional mechanisms upregulating CT-1 in the myocardium of patients with HF, namely in those with HFREF.

CT-1 has been shown to stimulate overall protein and collagen synthesis in cultured animal cardiac fibroblasts\textsuperscript{5,6,25} and vascular smooth muscle cells,\textsuperscript{5} as well as fibroblast growth and proliferation,\textsuperscript{6,25,26} through its receptor-triggered signaling pathways. More recently, it has been reported that chronic administration of CT-1 to normotensive rats is associated with enhanced myocardial deposition of collagen type I and III fibers, increased LV volume, and reduced LV ejection fraction.\textsuperscript{9} In addition, CT-1 knockout mice display lower arterial fibrosis than age-matched control animals.\textsuperscript{27} Collectively, these experimental data support a cardiovascular profibrotic role for CT-1.

Findings from the present study provide the first data supporting also the potential profibrotic actions of CT-1 in the human heart. In fact, we found that myocardial CT-1 protein was associated with the expression of collagen type I and III proteins. Interestingly, we found that myocardial CT-1 protein was associated with the mRNA expression of procollagen types I and III, pointing to a direct transcriptional effect of CT-1 on collagen production. The in vitro data showing that CT-1 increases the synthesis of procollagen type I and III mRNAs in human cardiac fibroblasts reinforce this possibility. In addition, we found that CT-1 stimulates the differentiation of human cardiac fibroblasts to myofibroblasts (as assessed by the increase in α-smooth muscle actin expression), which is in agreement with previous data showing that CT-1 induces myofibroblast proliferation in rat cardiac fibroblasts.\textsuperscript{25,26} Therefore, CT-1 may contribute to the development of myocardial fibrosis in human HF by turning fibroblasts into myofibroblasts, which present a highly synthetic profibrotic phenotype.

Table 2. Myocardial Collagen Parameters in Control Subjects and Patients With Heart Failure

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control Subjects (n=7)</th>
<th>Patients With HF (n=31)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total CVF, %</td>
<td>1.93±0.08</td>
<td>7.91±0.55</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Type I CVF, %</td>
<td>2.02±0.11</td>
<td>7.93±0.56</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Type III CVF, %</td>
<td>0.76±0.15</td>
<td>1.32±0.12</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td>Procollagen type I mRNA, AU</td>
<td>37.50±7.41</td>
<td>162.26±12.54</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Procollagen type III mRNA, AU</td>
<td>1.09±0.43</td>
<td>1.79±0.24</td>
<td>NS</td>
</tr>
<tr>
<td>Collagen type I protein, ADU</td>
<td>2.42±0.42</td>
<td>5.04±0.38</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td>Collagen type III protein, ADU</td>
<td>0.86±0.20</td>
<td>2.15±0.23</td>
<td>&lt;0.05</td>
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</tbody>
</table>

Data for mRNA and protein are corrected by 18s ribosomal mRNA and β-actin, respectively. Values are given as mean±SEM. ADU indicates arbitrary densitometric units; AU, arbitrary units; CVF, collagen volume fraction; HF, heart failure; and NS, nonsignificant.
Myocardial fibrosis is known to contribute to LV diastolic and systolic dysfunction. In fact, it has been proposed that whereas an excess of collagen type I tissue may increase chamber stiffness and alter LV diastolic filling, an excess of collagen type III may interfere with the transduction of the force of cardiomyocyte contraction to the ventricular chamber, thereby impairing LV ejection fraction. It thus can be hypothesized that the profibrotic effect of CT-1 may contribute to the deterioration of LV function in hypertensive patients with HF. This can be particularly relevant for patients with HFREF who exhibit both increased expression and deposition of collagen type III fibers and myocardial CT-1 protein expression compared with patients with HFPEF. This possibility does not exclude other mechanisms through which CT-1 may compromise cardiac function either directly altering cardiomyocyte growth and contractility or indirectly increasing arterial stiffness.

### Clinical Aspects

In agreement with previous data, we found that plasma CT-1 is abnormally increased in hypertensive patients with HF. It has been previously shown that the failing human heart secretes CT-1 via the coronary sinus into the peripheral circulation, therefore, circulating CT-1 may reflect cardiac CT-1 release. Interestingly, we found an association between plasma CT-1 and plasma NT-proBNP. Because circulating NT-proBNP is considered a biomarker of cardiomyocyte stress in patients with HF, it can be proposed that circulating CT-1 may also be a biomarker of the stressed cardiomyocyte in the hypertensive failing human heart.

Plasma CT-1 was also associated with serum levels of PICP and PIINP, both established biomarkers of myocardial fibrosis. In fact, it has been shown that serum PICP and PIINP are directly correlated with the amount of collagen type I and III fibers, respectively, present in the myocardium of patients with HF. Therefore, our finding reinforces the link between CT-1 and myocardial fibrosis in hypertensive patients with HF and suggests that circulating CT-1 may be an additional biomarker of myocardial fibrosis in these patients.

### Limitations

Several limitations need to be acknowledged. First, this was a study involving a relatively small number of patients. In
particular, the assessment of myocardial molecular parameters was performed on a limited number of biopsy samples. However, because of the nature of the goals under investigation, it was adequately powered. Second, we performed biopsies of the right side of the interventricular septum to assess the characteristics of collagen tissue. However, as we have shown previously,17 in terms of deposition of collagen fibers, the septum is representative of the free wall in the human hypertensive failing heart. Third, because of the methodology used for sample processing, the immunocytochemical analysis of the intracellular localization of CT-1 expression within cardiomyocytes (eg, mitochondria, endoplasmic reticulum, intercalated disks) could not be performed. Fourth, because only patients with HF of hypertensive origin were included, caution is needed before the current observations are extrapolated to the HF population at large.

Perspectives
This study shows for the first time that an excess of myocardial CT-1 protein is associated with LVEDWS and fibrosis in hypertensive patients with HF. We also show for the first time that CT-1 stimulation induces myofibroblast differentiation and collagen synthesis in human cardiac fibroblasts. These data allow us to speculate that, in the failing hypertensive human heart, increased cardiomyocyte diastolic stress produces CT-1 as a protective mechanism, which in the long-term turns to be detrimental because it promotes fibrosis acting on cardiac fibroblasts. Therefore, CT-1 emerges as a candidate pathogenic factor of myocardial remodeling in patients with HF of hypertensive origin. Furthermore, our results set the stage for further studies aimed to explore the usefulness of CT-1 as both a diagnostic biomarker and a therapeutic target of cardiomyocyte stress and myocardial fibrosis in these patients.

Acknowledgments
We thank Laura Martínez, Sonia Martínez, Ana González, and María I. González for their valuable technical assistance.

Sources of Funding
This work was funded through the Ministry of Economy and Competitiveness, Spain (RYC-2010-05797; Instituto de Salud Carlos III grants RD12/0042/0009, PS09/02234, and PI12/02563), and the European Commission FP7 Programme (MEDIA project grant HEALTH-2012–305507, and FIBRO-TARGETS project grant FP7-HEALTH-2013–602904).

Disclosures
None.

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**Novelty and Significance**

**What Is New?**

- We report the associations of an excess of myocardial cardiophrin-1 (CT-1) protein with left ventricular end-diastolic wall stress and fibrosis in patients with heart failure (HF) of hypertensive origin. We also report the associations of circulating CT-1 with established circulating biomarkers of cardiomyocyte mechanical stress and myocardial fibrosis in patients with HF. Finally, we characterize the profibrotic activity of CT-1 on human cardiac fibroblasts.

**What Is Relevant?**

- Up to now, CT-1 has been considered as a cardiomyocyte stress response factor involved in long-term alterations of cardiomyocyte growth and function associated with HF in humans. Results from the current study suggest that the excessive production of this cytokine by the mechanically stressed cardiomyocyte may also be involved in the main alteration of the cardiac extracellular matrix present in human HF (ie, myocardial fibrosis). Therefore, our findings support the notion that CT-1 is a major contributor to the global remodeling of the myocardium that occurs in the failing human hypertensive heart.

**Summary**

CT-1 emerges as a new potential pathogenic mediator, diagnostic biomarker, and therapeutic target of cardiomyocyte stress and myocardial fibrosis of hypertensive origin.