Endogenous Endothelin-1 Is Required for Cardiomyocyte Survival In Vivo

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Background—Endothelin-1 (ET-1) has potent vasoconstrictor and hypertrophic actions. Pharmacological antagonists of endothelin receptors attenuate cardiac hypertrophy, have been approved for treatment of pulmonary hypertension, and are under investigation for treatment of heart failure. To investigate the role of ET-1 in the heart, we created mice with cardiomyocyte deletion of ET-1.

Methods and Results—Mice with cardiomyocyte-specific deletion of ET-1 are phenotypically normal when young. Remarkably, as the mice age or when young animals are subjected to aortic banding, they develop an unexpected phenotype of progressive systolic dysfunction and cardiac dilation. Echocardiography, necropsy, histology, and molecular phenotype confirm a dilated cardiomyopathy. Terminal deoxynucleotidyl transferase–mediated dUTP nick-end-labeling analysis reveals greater abundance of apoptotic nuclei in the ET-1–deficient hearts. Transcriptional and Western analyses suggest enhanced tumor necrosis factor (TNF)–mediated apoptosis with increases in caspase-8 activity. These ET-1–deficient hearts also have diminished nuclear factor (NF)-κB activity, resulting in diminution of downstream inhibitors of TNF signaling.

Conclusions—Local ET-1 gene expression is necessary to maintain normal cardiac function and cardiomyocyte survival in mice with both age and hemodynamic stress. This cardiac-protective effect is mediated by paracrine ET-1 modulation of TNF-related apoptosis, in part through upregulation of NF-κB signaling. (Circulation. 2006;114:830-837.)

Key Words: apoptosis ■ cardiomyopathy ■ endothelin ■ tumor necrosis factor ■ nuclear factor-kappa B

Endothelin-1 (ET-1) was first identified as a potent vasoconstrictor peptide secreted by endothelium.1 It also is expressed in the heart and has subsequently been shown to have a direct influence on cardiac myocytes, including hypertrophic and inotropic effects.2-5 Both ET-1 receptors are found on cardiac myocytes. The ET\textsubscript{A} receptor is more abundant (90%) and has been considered more important for the cardiac effects of ET-1, but the ET\textsubscript{B} receptor may be more responsive to physiological stress.6 Remarkably, ET-1 has recently been shown to improve the survival of cardiomyocytes7 through a reduction in susceptibility to apoptosis.

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Genetic approaches have been applied to investigate the function of the endothelin axis. ET-1 and ET\textsubscript{A} gene knockout (KO) mice die after birth from asphyxia because of malformation of the hypopharynx.8,9 Endothelin-converting enzyme-1–deficient animals have a similar phenotype.10 Mice lacking ET-1, ET\textsubscript{B}, or endothelin-converting enzyme-1 also display defects of arterial formation. These genetic models help establish the function of the endothelin axis in embryonic development, but they do not produce detectable phenotypes in cardiac muscle or clues to the function of ET-1 in the adult heart. To study the isolated effects of cardiac ET-1, we have used a mouse strain with cardiomyocyte-specific deletion of the portion of the ET-1 gene encoding the mature ET-1 peptide generated with Cre-lox technology.11 This allows us to explore the isolated effects of cardiac ET-1 in an animal that has enjoyed the normal developmental functions of ET-1.

Many studies have suggested a role for the endothelin axis in congestive heart failure. In animal models of heart failure, treatment with pharmacological inhibitors of the endothelin receptors has been shown to ameliorate ventricular remodeling and to prolong life after infarction.12 This has prompted efforts to examine the effect of these agents in patients with heart failure. However, contrary to expectations, trials of inhibitors of both or specific ET receptors have not produced the anticipated clinical benefits.13 Our model allows us to examine the local paracrine effect of ET-1 in the heart and may help to explain the absence of a clinical benefit with inhibitors of the ET-1 axis. If ET-1 plays an important role in preserving cardiac myocytes exposed to stress, either that encountered chronically through life or with increased after-
load, then strategies that block the action of ET-1 in the heart may be counterproductive, particularly in patients with other stimuli for cardiac apoptosis.

**Methods**

**Reagents and Animals**

All chemicals are from Sigma Chemical Co (St Louis, Mo) unless specified. Antibodies are from Cell Signaling (Danvers, Mass). Cardiac-specific ET-1 KO mice were generated with the loxP-Cre system. Briefly, mice with loxP-bracketed ET-1 allele were mated with cardiac α-myosin heavy chain (αMHC)-Cre transgenic mice. We refer to the ET-1-lox<sup>+/−</sup> genotype as “wild type” (WT) for simplicity in this report. The only difference between this strain and true WT mice is the presence of the loxP sites bracketing exon 2 of the ET-1 gene. Excision of this exon removes the region of the gene that encodes the mature ET-1 peptide in cardiac myocytes. Aortic banding and unsedated echocardiography were performed as described.

All procedures were approved by the University of Texas Southwestern Animal Care Committee.

**Real-Time Polymerase Chain Reaction Assays**

Real-time polymerase chain reaction (PCR) was run as described. Cyclophilin A was used as an internal control.

**Electrophoretic Mobility Shift Assay**

Electrophoretic mobility shift assays (EMSAs) were performed with 100 μg nuclear protein following published methods. Nuclear factor-κB (NF-κB) consensus sequence (5′-AGT GGG GAC TTT CCC AGG C-3′; Promega, Madison, Wis) according to the manufacturer’s protocol. Counterstaining with propidium iodide indicated the location of nuclei. For tissue sections, apoptotic nuclei were counted on whole sections of both ventricles under high-power (×40) magnification. Apoptotic cardiomyocytes were counted in 3 random high-power (×40) fields. Propidium iodine–stained nuclei were counted from 6 random ×40 fields. The total number of nuclei in the section was then calculated by adjusting the average number of nuclei per field for the surface area of the section (obtained with Scion Image 1.63 software).

**Terminal Deoxynucleotidyl Transferase–Mediated dUTP Nick-End-Labeling Assays**

Antibodies are from Cell Signaling (Danvers, Mass). All chemicals are from Sigma Chemical Co (St Louis, Mo) unless specified. Antibodies are from Cell Signaling (Danvers, Mass).

**Cell Culture and Transient Gene Transfection**

Primary neonatal rat cardiomyocytes were prepared as described. After 48 hours of incubation in serum-free medium, Lipofectamine 2000 (Invitrogen, Carlsbad, Calif) was used for transfection as suggested by the manufacturer. When the transfected cells were treated with ET-1 or bosentan, the agents were added 24 hours after transfection. The culture medium was changed every 12 hours, with the inclusion of ET-1, Bosentan, or TNF, until harvest.

**Endogenous NF-κB Activity Assays**

A pNF-κB-SEAP reporter system (BD Biosciences) was used to monitor NF-κB activity in transfected cardiomyocytes in which a secreted form of the human placental alkaline phosphatase (SEAP) cDNA is driven by 4 tandem copies of NF-κB consensus sequence (κB<sub>4</sub>) fused to a basic TATA-like promoter. After endogenous NF-κB proteins bind to the κB<sub>4</sub> transcription is induced and the reporter gene is activated. The expressed protein was secreted and analyzed with the BD Great EscApe SEAP Chemiluminescence Detection Kit. CMV-Renilla luciferase reporter was used as internal control.

**Statistical Analysis**

Pairwise comparisons between groups were performed with the Student t test with homoskedasticity or heteroskedasticity according to the variance comparison results. Comparisons between multiple groups were evaluated with ANOVA. Values are reported as mean±SEM, and values of P<0.05 were considered statistically significant. The authors had full access to the data and take full responsibility for their integrity. All authors have read and agree to the manuscript as written.

**Results**

**Physiological and Histological Evaluation of Cardiac-Specific Deletion of ET-1**

Previous evaluation of the αMHC-Cre<sup>−/−</sup>, loxP<sup>−/−</sup> mice by Southern blot analysis showed cardiac-specific deletion of ET-1 with detectable excision of a reporter transgene in >80% of cardiac myocytes; transcript analyses indicated a substantial decrease in ET-1 mRNA in isolated cardiomyocytes compared with WT littermates with an intact ET-1 gene. Young ET-1 KO animals had normal mendelian distribution in litters, as well as normal growth, activity, and fertility. Cardiac function assessed by echocardiography was similar between the 2 groups of animals. Histology showed normal structure and organization of cardiac muscle fibers. Cardiac mass, indexed to body weight, was similar, and the histological surface area of cardiomyocytes was identical between ET-1 KO and WT animals.

**Aging ET-1 KO Mice Exhibited Impaired Cardiac Function**

At 2 months of age, the ET-1 KO and WT animals had indistinguishable left ventricular (LV) function by fractional shortening or area fractional shortening (Table I, available online in the accompanying Data Supplement). However, at 6 months, the ET-1–deleted mice had a significantly lower fractional shortening (although still within normal limits) compared with their WT sibs. Over the ensuing months, the left ventricles of ET-1 KO mice dilated and LV systolic function declined (Figure 1). Ultimately, the ET-1 KO mice...
died at much younger ages. The median life expectancy of ET-1 KO mice was 11 months (Figure 2), whereas WT mice live a normal lifespan of ~2 years.

**Histological Evaluation**

Histological characterization of the ET-1 KO hearts revealed dilated heart chambers with heterogeneity of myocyte size, including hypertrophy. Trichrome stains showed increased fibrosis (Figure 3).

**Heart Failure Marker Gene Expression in ET-1 KO Mice**

To characterize the molecular phenotype of the dilated cardiomyopathy, we evaluated the expression of several genes regulated in other models of heart failure. All showed the same pattern as seen in other heart failure models (Data Supplement Table II). Expression of atrial natriuretic factor, brain natriuretic peptide, and phospho-MHC expression was increased, and message for αMHC, SERCA2a, and phospholamban was less abundant in the ET-1–deleted hearts. Real-time results were obtained in triplicate on each sample of cardiac RNA from which an average expression level was derived. At least 3 hearts were analyzed for each experimental group evaluated, and the mean expression level was compared between groups to obtain the average fold change.

**Aortic Banding Accelerated the Development of Heart Failure in ET-1 KO Mice**

To observe the role of cardiomyocyte ET-1 in response to hemodynamic load, we challenged the mouse hearts with transverse aortic constriction (TAC). Aortic arch constriction to 27-gauge diameter was performed in 2-month-old mice. After they recovered from surgery, the mice were evaluated with echocardiography by an operator blinded to genotype. Strikingly, the ET-1 KO mice developed dilated heart failure in 3 weeks (Data Supplement Table III). LV function of the WT mice remained within the normal range, and LV hypertrophy developed as expected. LV dysfunction progressed in the ET-1–deleted hearts, as did hypertrophy in the nondeleted siblings (Figure 4). Once again, histological study revealed heterogeneity of cell size, cell loss, disorganization of muscle fibers, and increased fibrosis only in ET-1 KO animals (data not shown).

**ET-1 KO Mice Had Increased Apoptosis in Heart**

To explore the mechanism of development of dilated cardiomyopathy in ET-1 KO mice, we used a TUNEL assay to compare apoptosis between the hearts of ET-1 KO and WT animals. An example of this analysis is shown in Figure 5B. At the age of 6 months, the ET-1 KO animals had substantially more abundant TUNEL-positive cells in ventricles compared with their normal siblings (Figure 5A) at a time when ventricular function was still preserved. Confirmatory evidence of increased apoptosis in the ET-1 KO hearts was obtained from Western blots that showed greater cleavage of caspase-3 (Figure 5C). At 8 months of age, the ET-1 KO mouse hearts still had increased TUNEL-positive cells but fewer than at 6 months.

**FasL/TNF Signaling Pathway Involved in Increased Apoptosis in KO Hearts**

We anticipated that the increased susceptibility of the ET KO mice to cardiac apoptosis would be reflected in levels of mRNA and protein for components of apoptotic signaling. We screened key signaling molecules involved in either the TNF/FasL- or the mitochondria-mediated pathways (upstream of caspase-3 activation) by real-time PCR. We found that transcripts encoding modulators of TNF signaling were regulated in such a way to suggest enhanced responsiveness of that pathway. Caspase-8, which activates caspase-3 in the TNF pathway, as well as NF-κBp65 and TRAF1, inhibitors of this pathway, were regulated 1.6-, −30-, and −3.4-fold, respectively, compared with WT controls, in 6-month-old animals. However, the abundance of the transcript for caspase-9, which encodes the principal mediator of caspase-3 activation by the mitochondrial pathway, was similar in both mouse strains. Moreover, both the message and protein abundance of APAF-1 (1.1-fold; \( P = 0.13 \)) and Bax, proteins commonly upregulated in mitochondria-mediated apoptosis, was similar in ET-1 KO and WT animals (Figure 5C), whereas the protein abundance of caspase-8 was higher (1.7-fold; \( P = 0.0002 \)) and TRAF1 was lower (0.6-fold; \( P = 0.01 \)) in the KO hearts. Ultimately, the amount of activated caspase-3 was higher in the KO hearts (2.7-fold; \( P = 0.002 \)). In addition, transmission electron microscopy showed no gross differences between the mitochondria of

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**Figure 2.** Survival curve of WT and KO mice.

**Figure 3.** Histological evaluation of dilated cardiomyopathy. Top, Four-chamber views of hearts with premortem fractional shortening noted below. Top microscopic images (hematoxylin and eosin [H&E] staining, \( ×40 \)) shows heterogeneity and hypertrophy of cardiomyocytes in a KO heart; bottom images (trichrome staining, \( ×20 \)) show fibrosis in the KO heart. Scale bar=40 μm.
the KO and WT hearts (data not shown). Although most regulation of apoptosis is thought to occur at a posttranslational level, transcriptional regulation also plays a role\textsuperscript{22}; this may be particularly important in the chronic processes that we are examining. Overall, these data suggested that increased apoptosis in KO hearts is due predominantly to increased FasL/TNF signaling rather than the mitochondria-mediated pathway.

**Decreased NF-κB Gene Expression, Protein Phosphorylation, and Translocation in KO Mice**

To examine potential connections between ET-1 signaling and FasL/TNF-mediated apoptosis, we explored NF-κB as a downstream target of ERK1/2. Previous studies\textsuperscript{20,23} suggested that NF-κB activity is regulated by ERK1/2. The inhibitory role of NF-κB for FasL/TNF-mediated apoptosis has been established in several models.\textsuperscript{24,25} We first examined gene regulation of NF-κB by real-time PCR and identified a substantial decrease in NF-κBp65 transcript in 6-month-old KO animals compared with age-matched WT mice, as well as slightly diminished expression in 2-month-old KO hearts. Similarly, the total protein level of NF-κBp65 also is lower, albeit less strikingly, in the KO hearts (77\% to 110\% of control; \(P<0.05\)). To compare the activity of NF-κB, we examined the amount of phosphorylated NF-κBp65 by Western blot and NF-κB DNA binding activity by EMSA in cardiac nuclear proteins from animals stressed by hyperthyroidism. Both results showed
a substantial reduction in NF-κB activity in the cardiomyocytes of KO animals (Figure 6).

**Diminished Expression of NF-κB–Regulated Genes**

To extend our analysis of the diminished NF-κB activity in KO animal hearts, we looked at the expression of NF-κB–regulated genes, including cIAP1, cIAP2, IkBα, and TRAF1. Real-time PCR results showed that all of them were downregulated in 6-month-old KO mice (2.0-, 2.0-, 1.6-, and 3.4-fold). Western blot analyses also showed decreased cIAP1 and IkBα (71% and 63% of control, respectively).

**ET-1 Inhibits TNF-Induced Apoptosis in Cardiomyocytes**

To further confirm the effect of ET-1 on TNF signaling pathway, we examined cultured neonatal cardiomyocytes. After 36 hours of serum deprivation and treatment, TNF induces apoptosis of cardiomyocytes. This induction can be inhibited by ET-1. Bosentan, a competitive antagonist of ET-1 receptors, reverses the inhibition of ET-1. We also measured NF-κB binding activity by EMSA. ET-1 slightly increased NF-κB binding activity in nuclei, and bosentan blocked this effect. TNF substantially increased NF-κB binding activity in nuclei; ET-1 further increased this activity; and again, the effect was blocked by bosentan (Figure 7). Similar results were
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obtained with the pNF-κB–SEAP reporter system (compared with control, ET-1 induced a 2.1±0.19-fold increase in SEAP, P<0.05; bosentan brought the amount of SEAP back to 0.87±0.09-fold, P=0.6 versus control, P<0.01 versus ET-1).

Deletion of ET-1 Gene in Mouse Heart Impairs Its Ability to Increase NF-κB Binding Activity in Response to Aortic Banding

We further examined the effect of 27-gauge TAC on NF-κB binding activity in the hearts of 2-month-old mice. After 2 days of 27-gauge TAC, the cardiac function of both WT and KO mice was unchanged. However, the increase in NF-κB binding activity in WT mouse hearts was significantly inhibited in KO mouse hearts (WT versus KO in phosphorimager counts: before TAC, 1.7×10^5±10% versus 1.5×10^5±11%, P>0.05; after TAC, 3.7×10^5±11% versus 2.7×10^5±3%, P<0.05; n=3 for each).

Discussion

In this study, we showed that cardiomyocyte-specific deletion of ET-1 leads to dilated cardiomyopathy either spontaneously with aging or with the extrinsic challenge of aortic banding. We identified increased apoptosis that correlated with the development of cardiomyopathy in these animals and suggested that this apoptosis is likely to be mediated by the FasL/TNF signaling pathway. We went on to support a role for NF-κB signaling in connecting the ET-1 axis with FasL/TNF-mediated apoptosis. These results indicate that cardiomyocyte-derived ET-1 is essential for these cells to survive in response to age or hemodynamic stress.

Several previous investigations have shown that ET-1 has direct effects on myocardial cells, including hypertrophy and increased contractility. Such effects presumably are mediated by the 2 types of Gq-coupled endothelin receptors present on cardiomyocytes, ETα and ETβ. However, the plasma concentration of endothelin ranges from 10^{-12} to 10^{-11} mol/L, which is much lower than the concentrations required to obtain physiological effects on cardiomyocytes in cell culture experiments (nanomoles per liter). This also is probably too low to exert an effect on receptors, with a Kd of 20 pmol/L, and suggests that ET-1 acts in a paracrine or autocrine fashion in the heart.

Elucidation of such a paracrine role for ET-1 in the heart has been limited. In rat studies, pharmacological blockade of ET receptors has produced benefits in heart failure, but these studies are limited by the inevitable systemic effects of the agents on blood pressure, volume status, and peripheral resistance. Conventional deletion of the endothelin gene produces a lethal phenotype, with suffocation at birth resulting from developmental defects in pharyngeal structures. Cardiac-specific overexpression of ET-1 also has been difficult, and until recently, published reports have been limited to only modest overexpression with negligible phenotype. Substantial cardiomyocyte overexpression of ET-1 in the heart recently was reported to produce a dilated cardiomyopathy, but the marked overexpression of a human transgene and inflammatory myocarditis might have contributed to this finding. Concomitant treatment with ET receptor blocking agents only partially improved the phenotype. A cardiac-specific ETα-deficient model also has been created. These mice are viable and exhibit a normal phenotype at 6 weeks, similar to our young ET-1-deficient mice. They also exhibited normal hypertrophic and contractile responses to 10-day infusion of angiotensin II or isoproterenol. Interestingly, they have upregulation of the ETα receptor, possibly compensating for the reduction in ETα.

One prediction of our findings is that these mice, lacking the putative protective effects of ET-1 signaling, would show increased apoptosis in response to stress if the ETα receptor is mediating this effect.

Our cardiac ET-1-deficient mice were normal when young. They developed heart failure only with aging or increased afterload. ET-1 normally is upregulated in the heart in response to a variety of stresses, including hyperthyroidism, increased afterload, hormones such as angiotensin II and adrenaline, and various cytokines. In the absence of ET-1 peptide, the hypertrophic and inotropic influences of this molecule would be missing, possibly leading to inadequate compensation to the stress and, for example, greater-than-appropriate dilation, generating increased wall stress and tissue hypoperfusion, with a resulting augmentation of TNF-mediated apoptosis. It also is possible that the paracrine effects of cardiomyocyte ET-1 on other cell types such as cardiac fibroblasts might affect remodeling of the heart in response to stress.

In our study, the prolonged exposure, over many months, to Cre recombinase might raise the concern that the recombinase itself could exert toxic effects. However, an echocardiogram of a 15-month-old Creα mouse (lackingloxP sites in the ET-1 gene) showed normal ventricular function (data not shown). The young ET-1 KO mice subjected to aortic banding recapitulate the dilated cardiomyopathy phenotype, but Creα mice did not in 3 weeks (data not shown). In a recent report, using the same αMHC-Cre allele, normal ventricular function was observed in a large number of 8-month-old mice.

An antiapoptotic activity of ET-1 has recently been recognized in cancer cells, endothelial cells, human smooth muscle cells, and cardiomyocytes. As in most tissues, there are 2 principal pathways for apoptosis in cardiomyocytes, the FasL/TNF extrinsic pathway and the mitochrondrial-related route. From the degree of regulation seen in components of both pathways, we suggest that ET-1 is exerting an antiapoptotic effect in the heart predominantly through downregulation of the FasL/TNF pathway. Our data do not exclude the involvement of the mitochondrial pathway, which others have suggested is important in cardiomyocyte apoptosis. However, the components of this pathway were similarly abundant in both ET-1 KO and WT animals. In contrast, in the FasL/TNF pathway, decreased TRAF1, decreased NF-κB, decreased cIAPs, and increased caspase-8 expression all suggest that there is enhanced FasL/TNF receptor pathway activity in 6-month-old ET-1 KO hearts. Several studies have shown that NF-κB strongly inhibits the apoptotic activation of TNF. We propose that the antiapoptotic effect of NF-κB would be diminished in the ET-1 KO animal in which NF-κB activity is downregulated. Both ET-1 and TNF increase in the stressed heart, and cardiac-specific expression of TNF has previously been shown to produce dilated cardiomyopathy, presumably through intact TNF-related apoptosis. In our study, we found that ET-1 can increase NF-κB activity in cultured cardiomyocytes and thereby inhibit TNF-induced apoptosis. Similarly, we observed a significant increase in NF-κB activity in stressed (TAC or T3) WT mouse hearts, but this increase was diminished in KO mouse hearts. Therefore, we conclude that paracrine/autocrine ET-1 signaling is needed to modulate TNF activation and to preserve cardiomyocytes in the face of various stresses and that NF-κB is at least one of the connections between
these 2 signaling pathways. A schematic of the putative inhibitory role of ET-1 on TNF signaling is presented in Figure 8.

Our observation may have important clinical ramifications. The nonspecific antagonist of ET-1 receptors, bosentan, is already used widely to treat primary pulmonary hypertension. Such treatment is anticipated to be long term, with no obvious limitation on therapeutic course discussed in the literature.19 However, on the basis of this study, we suggest that reduced ET-1 signaling may increase the vulnerability of the heart to stress, including the pulmonary hypertension itself. Although we are unaware of evidence for acceleration of right ventricular or LV dysfunction with bosentan treatment, we would recommend that a careful assessment be obtained in patients treated chronically with this drug. Moreover, in several recent studies of endothelin receptor antagonists in heart failure,13,50 the disappointing results could be due to this increased vulnerability of the cardiomyocyte to apoptosis, despite the likely beneficial effect of such agents on afterload and myocardial oxygen consumption. Perhaps an agent that distributed poorly to myocardium would be more effective, isoform-specific receptor modulation would be more successful, or patients with low levels of TNF would obtain greater benefit.

Conclusions
Our study demonstrates that cardiomyocyte ET-1 is crucial in maintaining normal cardiac remodeling and cardiomyocyte survival in response to age and increased afterload. We further show that this cardioprotective effect of local ET-1 may be mediated by inhibition of FasL/TNF signaling–related apoptosis, influenced at least in part by NF-κB.

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Disclosures
None.

References
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