Conditional Cardiac Overexpression of Endothelin-1 Induces Inflammation and Dilated Cardiomyopathy in Mice

Li L. Yang, MD, MSc; Robert Gros, PhD; M. Golam Kabir, MD; Almuktafi Sadi, MD, PhD; Avrum I. Gotlieb, MD; Mansoor Husain, MD*; Duncan J. Stewart, MD*

Background—Myocardial expression of endothelin-1 (ET-1) and its receptors ET_A and ET_B is increased in heart failure. However, the role of ET-1 and its signaling pathways in the pathogenesis of myocardial diseases is unclear.

Methods and Results—Human ET-1 cDNA was placed downstream of a promoter responsive to a doxycycline (DOX)-regulated transcriptional activator (tTA). This line (ET^+) was bred with one harboring cardiac myocyte–restricted expression of tTA (αMHC-tTA). Myocardial ET-1 peptide levels were significantly increased in binary transgenic (BT, ET^+/αMHC-tTA) compared with nonbinary transgenic (NBT, ET^+/αMHC-tTA; ET^-/αMHC-tTA; ET^-/αMHC-tTA^-) or DOX-treated BT littermates (40.1±4.7 versus 2.6±1.2 fmol/mL, P<0.003). BT mice demonstrated progressive mortality between 5 and 11 weeks after DOX withdrawal, associated with left ventricular dilatation and contractile dysfunction (peak +dP/dT, 4673±468 versus 5585±658 mm Hg/s, P<0.05). An interstitial inflammatory infiltrate, including macrophages and T lymphocytes, was evident in the myocardium of BT mice, associated with sequential increases in nuclear factor-κB translocation and expression of tumor necrosis factor-α, interferon-γ, interleukin-1 and interleukin-6. Significant prolongation of survival was observed with the combined ET_A/ET_B antagonist LU420627 (n=8, P<0.05) in BT mice but not the ET_A-selective antagonist LU135252 (n=5, P=0.9), consistent with an important role for ET_A in this model.

Conclusions—These are the first data to demonstrate that cardiac overexpression of ET-1 is sufficient to cause increased expression of inflammatory cytokines and an inflammatory cardiomyopathy leading to heart failure and death. (Circulation. 2004;109:255-261.)

Key Words: endothelin ● myocarditis ● hypertrophy ● heart failure

Endothelin-1 (ET-1) has been implicated in the progression of congestive heart failure (CHF). Plasma ET-1 levels are elevated in patients and experimental animal models of CHF. The expression of ET-1 and its receptors ET_A and ET_B is increased in myocardium of rats with postinfarction CHF and in patients with idiopathic dilated cardiomyopathy (DCM). In vitro and/or pharmacological studies have suggested that ET-1 exerts trophic effects in cardiac myocytes and stimulates collagen synthesis in fibroblasts. ET-1 also modulates leukocyte adhesion to endothelial cells and enhances production of cytokines in monocytes/macrophages.

However, the role of ET-1 in the pathogenesis of CHF remains controversial. The combined ET_A/ET_B receptor antagonist bosentan improved survival in experimental models of CHF but not in heart failure patients. Similar studies using selective ET_A antagonists have demonstrated both beneficial and deleterious effects.

Previous attempts to modulate ET-1 expression by transgenic approaches have been complicated by embryonic lethality in knockout mice and low levels of ET expression in viable progeny of gain-of-function mutants. Indeed, nontargeted and nonconditional ET-1 overexpression studies have resulted in limited numbers of founder mice, modest elevations in ET-1 levels, and no obvious cardiac phenotype. We hypothesized that this was caused by a selection bias arising from the embryonic lethal consequences of ET-1 overexpression in the heart. To overcome this limitation, we generated transgenic mice exhibiting conditional cardiac-restricted ET-1 overexpression using the α-myosin heavy chain (α-MHC) promoter-dependent cardiac-specific tetracycline-regulated gene expression system (Tet-OFF) (Figure 1).

In the present study, we demonstrate that postnatal cardiac overexpression of ET-1 induced inflammation and hypertrophy, leading to a rapid deterioration in cardiac function and death.

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255
Methods

Transgenic Mouse
Plasmid pBiGN:ET-1 carrying a full-length human ET-1 cDNA (Figure 1a) was used in pronuclei injections as described previously.20 Founders identified by polymerase chain reaction (PCR) and Southern blot were backcrossed with C57BL/6, and offspring were bred with αMHC-tTA mice. Progeny were genotyped by PCR using tTA-specific primers. 20

Reverse Transcription–PCR
Total RNA was harvested by use of TRIzol Reagent (Invitrogen Canada Inc) and reverse-transcribed with SuperScript First-Strand Synthesis System (Invitrogen). The cDNAs were subjected to either conventional reverse transcription (RT)-PCR using the human ET-1-specific primers shown above or real-time PCR with either Taqman (tumor necrosis factor (TNF)-α, interleukin (IL)-1, IL-6, interferon (IFN)-γ, and GAPDH) or SYBR Green (ET-1, β-MHC, and GAPDH) methods (Applied Biosystems). Relative standard calibration curves were generated as described previously,21 and the mRNA level of each gene (in nanograms) was normalized to that of GAPDH (in nanograms). For cytokine studies, mice were administered doxycycline (DOX) (200 mg/L) in drinking water from conception until 3 weeks of age and then withdrawn from DOX. Ventricular tissue was harvested at 0, 2, 4, 6, and 8 weeks after DOX removal. TNF-α, IL-1, IL-6, IFN-γ, and GAPDH primers were obtained as Taqman Predeveloped Assay Reagents (Applied Biosystems). Other primers used were human ET-1: 5′-GCTCGTCCCTGATGGATAAA-3′, 5′-CTGTTGCCCTTTGTTGGGAAGT-3′; β-MHC: 5′-GTGCCAACGGGCTGTAAGTGC-3′, 5′-GCAAAGGCTCCAGGTCTGA-3′; and GAPDH: 5′-TGACCCACCAACTGCTTAG-3′, 5′-GATGCAGGATGATGTC-3′.

Enzyme-Linked Immunosorbent Assay
Arterial blood (1 mL) was collected in a Vacutainer with EDTA (Becton Dickinson). Plasma was separated by centrifugation at 3000g for 5 minutes at 4°C and stored at −70°C. Organ tissues (40 mg) were homogenized in 10 times wt/vol ice-cold lysis buffer (10 mmol/L NaCl, 1% glycerol, 1% Triton X-100, 50 mmol/L HEPES, 1 mmol/L EDTA, 0.01 mmol/L PMSF, 4.8 μmol/L aprotinin, 4.8 μmol/L leupeptin) with a sonic dismembrator (Fisher Scientific Ltd). Homogenate was centrifuged at 2×10^5g for 20 minutes at 4°C, and ET-1 peptide was extracted by passing the supernatant through a YM-10 membrane (Millipore Canada Ltd), precipitated, and quantified by use of ET-1 (1–21) electroimmunoassay (APLCO Diagnostics).

β-Gal Staining
Embryos harvested at 10.5 days postcoitum were rinsed in PBS, fixed in 5% formalin for 10 minutes, and stained in filtered X-gal solution for 4 hours.22 The genotype of each was examined by genomic PCR.

Hemodynamics
Pressure tracings were recorded in animals lightly anesthetized with ketamine HCl (100 mg/kg) and xylazine (10 mg/kg IP) with a Mikro-tip transducer (1.4F sensor, 2F catheter, Millar Instruments Co.).

Electrocardiogram
Chronic ECG recordings from conscious mice were acquired with a telemetry system (Data Sciences International).

Histopathology
Perfusion-fixed cardiac tissues were paraffin-embedded, sectioned (5 μm), and stained with hematoxylin-eosin and picrosirius red. Five sections of each heart were scored for cell infiltration and necrosis by 2 blinded observers.24 Hearts from mice infected with group B coxsackieviruses were used as positive controls.24 Cardiomyocyte cross-sectional area was measured at the level of the nucleus in 60 to 80 cells from each mouse with an imaging system22 and Scion Image 4.0.1 software (Scion Corp). Longitudinal or oblique sections were excluded. Mean values for experimental groups were calculated from averaged values of individual mice.25 Length of the entire endocardial circumference was determined similarly.23

Immunohistochemistry
Cardiac sections were permeabilized with 0.125% trypsin (Zymed Laboratories Inc) at 37°C for 10 minutes and then blocked with 10% goat or rabbit serum or M.O.M mouse Ig blocking reagent (Vector Laboratories) in PBS for 30 minutes and incubated overnight at 4°C with anti-CD3 (1:100, Zymed Laboratories Inc), anti–Mac-3 (1:50;
was characterized in detail. Human ET-1 expression during development and adulthood, failure over a 6-month period of observation. The third (9 transgene overexpression and demonstrated no overt heart

Transgenic Mice
Generation of Cardiac-Restricted ET-1
Transgenic Mice
Single-cell pronuclei injections with pBGN:ET-1 produced 3 ET-1 founder mice out of 40 births (7.5%). All 3 were bred into the C57BL/6 background and transmitted the transgene in autosomal fashion. All 3 were then bred with α-MHC-tTA mice\(^{18}\) to generate binary transgenic mice (BT: ET\(^{+/\text{tTA}^-}\)). Of the 3 BT lines, one (101 ET\(^{+/\text{tTA}^-}\)) demonstrated no ET-1-β-gal expression in the heart using β-gal staining. Another (403 ET\(^{+/\text{tTA}^-}\)) showed low levels of cardiac transgene overexpression and demonstrated no overt heart failure over a 6-month period of observation. The third (9 ET\(^{+/\text{tTA}^-}\)), which demonstrated high levels of myocardial human ET-1 expression during development and adulthood, was characterized in detail.

To localize ET-1-βgal transgene overexpression during development, we performed whole-mount β-gal staining on embryonic day 10.5 embryos. The DOX-suppressible ET-βgal expression was confined to hearts of BT embryos and was not observed in non-BT embryos (NBT: ET\(^{+/\text{tTA}^-}\), ET\(^+/\text{tTA}^+\), and ET\(^{+/\text{tTA}^-}\) or noncardiac tissues of BT embryos (Figure 1b). Crossbreeding 9 ET\(^+/\text{tTA}^-\) with α-MHC-tTA mice generated significantly fewer ET\(^+/\text{tTA}^-\) offspring than expected (7% versus expected 25%, \(n=116, P<0.001\)), indicating that overexpression of ET-1 in the developing heart resulted in fetal loss. DOX administration restored the expected mendelian frequency (25%) of ET\(^+/\text{tTA}^-\) progeny (26%, \(n=160, P>NS\)). To focus on the consequences of ET-1 overexpression in adult myocardium, DOX was administered to maternal mice to suppress transgene expression during development. ET-1 overexpression was then induced by removal of DOX in the postnatal period.

Targeted and conditional expression of ET-1 in adult mice was confirmed. Human ET-1 mRNA was expressed exclusively in hearts of BT and not those of NBT littermates (Figure 1c). Total ET-1 peptide level in the heart was \(\approx\)-10-fold higher in BT than NBT littermates at 5 weeks after DOX removal (40.1±4.7 versus 2.6±1.2 fmol/mL, \(P<0.001\)). No increase in ET-1 expression was detected in plasma or any other organs examined. In BT mice, DOX treatment completely suppressed the expression of human ET-1 mRNA and brought ELISA-determined peptide levels down to background levels (3.5±1.8 fmol/mL) (Figure 1d).

Phenotype of Mice With Cardiac-Restricted Overexpression of ET-1
BT mice released from DOX at birth initially displayed normal appearance, behavior, and oral intake. However, starting at \(\approx5\) weeks of age, BT mice began to exhibit decreased activity, hunched posture, and labored breathing, which invariably led to death within days of symptom onset. Pathological examination of symptomatic BT mice revealed pulmonary and hepatic congestion not observed in asymptomatic or NBT mice (data not shown). More than half of the mice released from DOX died within 8 weeks. By contrast, BT maintained on DOX remained normal in phenotype and showed no difference in survival compared with NBT littermates (Figure 2). Invasive hemodynamic assessments made before the onset of overt morbidity or significant mortality (ie, 5 weeks after DOX) revealed decreased peak arterial systolic blood pressure in BT versus NBT littermates (83.6±7.3 versus 95.1±10.9 mm Hg, \(n=5, P<0.05\)). Peak left ventricular systolic pressure (74.3±5.9 versus 84.3±5.1 mm Hg, \(n=5, P<0.05\)) and +dp/dt (4673±468 versus 5585±658 mm Hg/s, \(n=5, P<0.05\)) were also significantly lower in BT than NBT littermates.

To determine whether arrhythmias contributed to the mortality of BT mice, ECG telemetry was monitored for 5 weeks in 8-week-old BT mice withdrawn from DOX for 5 weeks and in DOX-treated age-matched BT controls. PR intervals in BT remained unchanged until the terminal stage. However, QRS complexes lengthened progressively over time, becoming significantly wider in overtly ill BT mice compared with DOX-treated controls (31.7±3.6 versus 13.1±0.7 ms, \(n=3, P<0.05\)). Large P waves were also observed in BT but not
DOX-treated controls. Although a leftward shift in the QRS axis was noted consistently, heart block or other bradyarrhythmias or tachyarrhythmias were not observed (data not shown).

Histological examination revealed that cardiomyocytes in 6- to 8-week-old BT mice contained hyperplastic and vesicular nuclei (Figure 3, a and b) and were larger than those of NBT and DOX-treated BT littermates (290±64 versus 161±24 and 188±37 μm², n=5, P<0.01). Left ventricular endocardial circumference was also increased in BT versus NBT and DOX-treated BT littermates (4.6±0.9 versus 3.2±0.5 and 3.3±0.6 mm, n=5, P<0.01) (Figure 1e). The ratio of wet heart to body weight in ET-1-overexpressing mice was increased compared with controls (8.48±2.24 versus 4.85±0.58 and 4.92±0.59 mg/g, n=5, P<0.05), with no significant difference in body weight. Real-time RT-PCR demonstrated that GAPDH-normalized mRNA levels of inflammatory cell infiltrates included macrophages and T cells as positive controls. Histological scoring of inflammation and necrosis was performed using a blinded scoring system. Representative hematoxylin-eosin–stained sections are shown as a positive control.

β-MHC were increased in 6-week-old BT mice compared with NBT littermates (6.8±1.5 versus 2.0±1.0 ng/ng, n=3, P<0.05). Together, these data suggested the development of cardiac hypertrophy in BT mice.

Histological examination also revealed an interstitial inflammatory cell infiltrate in the right and left ventricular free walls and septum of 8-week-old BT but not NBT or DOX-treated BT littermates. Extensive serological testing and sentinel autopsies excluded the possibility of known infectious diseases. Blinded scoring of inflammation and necrosis revealed an increase in these parameters in the hearts of BT but not NBT or DOX-treated BT mice (Table). The inflammatory infiltrates included macrophages and T cells as identified by immunostaining (Figure 3, e and f). There was also a mild but appreciable increase in collagen deposition in the hearts of BT mice (Figure 3, c and d).

To understand the mechanisms involved in our model and establish whether cardiac ET-1 overexpression was a cause or a consequence of the observed inflammation, we temporally quantified ET-1 and cytokine gene expression, NF-κB activation, and histological changes in mice after withdrawal from DOX suppression. At 2 weeks after DOX removal, human ET-1 mRNA was easily detected in all BT samples, and transgene expression remained elevated at 4, 6, and 8 weeks after DOX removal (Figure 4c). Concomitant with ET-1 overexpression, nuclear NF-κB translocation was increased in cardiomyocytes (Figure 4a). Although detectable at earlier time points, mRNA levels of TNF-α, IL-1, IL-6, and IFN-γ did not increase until 6 to 8 weeks after DOX withdrawal (Figure 4d). Parallel histological analyses demonstrated a progressive inflammatory process that was widespread by 6 weeks after DOX removal (Figure 4b).

**Histopathologic Scoring**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>DOX</th>
<th>Inflammation</th>
<th>Necrosis</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>ET+/tTA- (n=5)</td>
<td>+</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>ET+/tTA+ (n=5)</td>
<td>-</td>
<td>1.9±0.8</td>
<td>0.9±0.3</td>
<td>2.7±1.1</td>
</tr>
<tr>
<td>CVB3 viral myocarditis</td>
<td></td>
<td>2.7±1.5</td>
<td>3.2±0.7</td>
<td>5.9±2.2</td>
</tr>
</tbody>
</table>

Hematoxylin-eosin–stained cardiac cross sections of ET+/tTA- mice were scored (0–4) for inflammation and necrosis by 2 blinded observers. DOX treatment completely suppressed histological evidence of cardiac inflammation and necrosis in ET+/tTA- mice. Scores from a viral model of myocarditis (CVB3 infection) are shown as a positive control.

**ET Receptor Blockade**

The involvement of ETₐ and/or ETₐ receptor pathways in the pathogenesis of this model was explored with orally active antagonists. The combined ETₐ/ETₐ antagonist LU420627 (n=8) but not the ETₐ-selective antagonist LU135252 (n=5) prolonged survival in BT compared with nontreated BT mice (P=0.025) (Figure 2).

At 4 weeks after DOX removal, myocardial tissue and plasma levels of ET-1 and the extent of NF-κB translocation were no different in LU420627-treated BT mice (n=3) and untreated BT mice (tissue, 36.5±1.4 versus 40.1±4.7 fmol/mL; plasma, 2.2±0.14 versus 3.1±2.8 fmol/mL, P=NS). However, the degree of inflammatory cell infiltration was decreased (0...
versus 1.9±0.8) and hemodynamic parameters were preserved in LU420627-treated versus nontreated BT mice (left ventricular systolic pressure, 104.0±1.0 versus 74.3±5.9 mm Hg; +dP/dT, 6002±47 versus 4394±554 mm Hg/s, P<0.05).

Discussion

Here, we report that overexpression of ET-1 in the adult mouse heart is sufficient to cause nuclear NF-κB translocation, cytokine expression, inflammation, and hypertrophy, leading to a DCM, CHF, and death as early as 5 weeks after transgene induction. NBT animals harboring either the ET1-βgal or the aMHC-tTA transgene but not both had no increase in ET-1 expression and no discernible phenotype. The finding that DOX treatment of BT animals suppressed ET-1 overexpression and completely prevented the phenotype further implicated ET-1 in the pathogenesis of our model.

Previous attempts to develop transgenic models overexpressing ET-1 have failed to demonstrate any cardiac phenotype.16,17 This may be because of a lack of efficient expression of ET-1 in the heart, developmental adaptation, or embryonic lethality of lines with high levels of cardiac ET-1 production. Indeed, ET-1 plays a pivotal role in cardiac development, and loss-of-function mutations in ET-1, ETα, and endothelin-converting enzyme-1 lead to developmental defects in structures derived from the cardiac neural crest.14,15 In the present study, temporal control of transgene expression avoided negative selection bias against mice with high levels of ET-1 expression during development.

In patients with end-stage DCM, plasma and myocardial ET-1 levels were increased 5- and 3-fold, respectively.2,5 However, it is not known whether local production of ET-1 may be higher in the early stages of disease or to what extent increased local production versus circulating ET-1 levels contribute to disease progression. In the present model, we show that an ∼10-fold increase in myocardial ET-1 expression without an increase in circulating levels of ET-1 is sufficient to induce a progressive and lethal cardiomyopathy. Our data suggest that local autocrine and paracrine actions of ET-1 within the heart may be particularly important in the pathogenesis of heart failure.

The hypothesis that many cases of DCM are a consequence of a preceding myocarditis (eg, viral, parasitic, metabolic, or toxic) has been difficult to prove.29 This is because the presumptive inflammatory insult has often disappeared before DCM is diagnosed.29 In this context, our data are the first to demonstrate the role of ET-1 in generating a myocarditis that precedes the development of DCM. Interestingly, similar inflammation in kidney and lung was observed in transgenic mice overexpressing ET-1 under control of the preproendothelin-1 promoter.16,17,30 Indeed, ET-1 is known to exert monocyte/macrophage chemoattractant activity,8 induce the release of IL-6,9,31 and act synergistically with IL-1 to mediate IL-6 expression.31 Given the biological effects of TNF-α, IL-1, IL-6, and IFN-γ,32 our demonstration that ET-1 overexpression preceded upregulation of these cytokines is consistent with a causal role of ET-1 in the initiation of myocarditis and the progression of heart failure. Of note, cardiomyocyte-specific overexpression of TNF-α in mice caused a phenotype similar to that of the present model.33

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ET-1 administration has been reported to increase activation of NF-κB in cardiac and vascular myocytes and lead to release of cytokine (IL-6) and cardiomyocyte hypertrophy.34,35 In our model, nuclear NF-κB translocation was concurrent with ET-1 overexpression and preceded the activation of cardiac cytokines, suggesting that ET-1 was sufficient to initiate an inflammatory cascade ultimately leading to cardiac dysfunction and death.
Given the lack of increase in systemic arterial pressures, cardiac hypertrophy of BT mice was probably a result of a direct effect of ET-1 on cardiomyocytes. The hyperplastic and vesiculated nuclei, β-MHC expression, and increased cardiomyocyte and ventricular size and cardiac mass of our model are consistent with the known effects of chronic ET-1 stimulation in vitro. The relative decrease in rTA-driven human ET-1 expression at 8 weeks after DOX removal (Figure 4) is also consistent with the downregulation of α-MHC promoter activity that accompanies cardiac hypertrophy.

Although ECG recordings suggested the development of atrial and ventricular enlargement, they did not demonstrate the arrhythmias noted in another α-MHC-rTA–dependent model of cardiac disease. Furthermore, concurrent histological analyses suggested that the ECG findings (ie, nonspecific conduction delays) were attributable to a generalized cardiac hypertrophy and/or inflammatory cell infiltration and collagen deposition, rather than a specific effect on cardiac conduction tissue.

The present system provided an opportunity to compare the efficacy of ET-receptor antagonists in a model dependent on increased ET-1 expression. Interestingly, ETA-selective antagonism had no effect, whereas combined ETα/ETB antagonism caused an appreciable delay in mortality. Given the levels of cardiac ET-1 overproduction in our model, coupled with the nearly irreversible interaction between ET-1 and its receptors, it may be difficult for an orally administered antagonist to compete effectively at the tissue level. Indeed, the partial response to combined ETα/ETB antagonism in our model may provide insight into the limited efficacy of this class of agents in clinical trials.

The mechanisms of how ETα/ETB antagonism ameliorated the phenotype may be complicated. Reduced inflammatory cell infiltration and improved hemodynamic function of LU420627-treated BT mice suggested that combined ETα/ETB blockade inhibited leukocyte recruitment and preserved cardiac function. However, the persistence of nuclear NF-κB translocation and eventual mortality despite LU420627 treatment suggested a partial and transient response. Because the 2 compounds tested possess similar K for ETα, the salutary effect of the combined ETα/ETB antagonism suggested a more important role for ETB. Indeed, the importance of ETB in myocardial fibrosis and inflammatory models in atherosclerosis and skin has been suggested by other studies. Future studies that use more selective ETB receptor antagonists and temporal analyses of ETα/ETB signaling pathways may directly address this question in our model.

In summary, we have generated transgenic mice with robust cardiac-specific expression of human ET-1, which was sufficient to induce cardiac inflammation, hypertrophy, dilatation, dysfunction, and death. Our study has demonstrated the potential role of ET-1 as a proinflammatory molecule in the heart and suggests that this effect may be mediated in part by ET-1/ETB interactions. Our model also provides insights into the therapeutic potential of blocking ET-1 signaling pathways in heart failure.

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References


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