ET-receptor antagonism, myocardial gene expression, and ventricular remodeling during CHF in rats

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 Øie, Erik, Reidar Bjørnerheim, Haakon K. Grøgaard, Heidi Kongshaug, Otto A. Smiseth, and Håvard Attramadal. ET-receptor antagonism, myocardial gene expression, and ventricular remodeling during CHF in rats. Am. J. Physiol. 275 (Heart Circ. Physiol. 44): H868–H877, 1998.—Both myocardial and plasma endothelin-1 (ET-1) are elevated in congestive heart failure (CHF). However, the role played by endogenous ET-1 in the progression of CHF remains unknown. The aim of the present study was to investigate and correlate myocardial gene expression programs and left ventricular (LV) remodeling during chronic ET-receptor antagonism in CHF rats. After ligation of the left coronary artery, rats were randomized to oral treatment with a nonselective ET-receptor antagonist (bosentan, 100 mg·kg−1·day−1, n = 11) or vehicle (saline, n = 13) for 15 days, starting 24 h after induction of myocardial infarction. Bosentan substantially attenuated LV dilatation during postinfarction failure as evaluated by echocardiography. Furthermore, bosentan decreased LV systolic and end-diastolic pressures and increased fractional shortening. Myocardial expression of preproET-1 mRNA and a fetal gene program characteristic of myocardial hypertrophy were increased in the CHF rats and were not affected by bosentan. Consistently, right ventricular-to-body weight ratios, diameters of cardiomyocytes, and echocardiographic analysis demonstrated a sustained hypertrophic response and a normalized relative wall thickness after intervention with bosentan. Thus the modest reduction of preload and afterload provided by bosentan substantially attenuates LV dilatation, causing improved pressure-volume relationships. However, the compensatory hypertrophic response was not altered by ET-receptor antagonism. Therefore, ET-1 does not appear to play a crucial role in the mechanisms of myocardial hypertrophy during the early phase of postinfarction failure.

Plasma ET-1 is usually present at levels lower than those reported to exert significant biological effects and is regarded as spillover into the plasma from the tissues (5). According to the prevailing opinion, ET-1 acts as an autocrine/paracrine factor rather than as a hormone (25). However, increased levels of plasma ET-1 during congestive heart failure (CHF) have been demonstrated in both humans (24) and experimental animal models (2). Furthermore, plasma ET-1 has been shown to correlate closely to the functional classes of heart failure (4, 24) and has also been shown to be a strong and independent predictor of mortality in both acute (18) and chronic heart failure (19).

We and others have recently shown that myocardial preproendothelin-1 (ppET-1) mRNA is substantially upregulated during CHF (17a, 27) and that cardiomyocytes appear to synthesize ET-1 (1, 32). Recent evidence also suggests that such endogenous elevations of ET-1 may contribute to maintenance of cardiac function during CHF in rats (27). However, to what extent endogenous ET-1 plays a significant role in the pathophysiology of CHF still remains to be elucidated. Currently, a number of ET-receptor antagonists with different receptor subtype selectivities have become available. Such antagonists now offer possibilities to investigate both the biological and pathophysiological actions of ET-1. Indeed, in animal models and in patients with CHF, acute administration of ET-receptor antagonists have been shown to exert favorable hemodynamic responses (12, 31). Thus ET-receptor antagonists may represent a novel pharmacotherapeutic modality in the treatment of CHF. Two recent studies have demonstrated a substantial reduction of mortality after chronic administration of ET-receptor antagonists during ischemic heart failure in rats (16, 26). In a recent study, long-term administration of the nonselective ET-receptor antagonist bosentan attenuated left ventricular (LV) dilatation in postinfarction failure in rats (7). However, the mechanisms of these beneficial effects of ET-receptor antagonism remain to be determined.

Thus the aim of the present study was to investigate to what extent the beneficial effects of ET-receptor antagonism on LV remodeling could be attributed to a decrease in myocardial wall stress secondary to reduced preload and afterload, and to what extent ET-mediated autocrine/paracrine growth regulatory mechanisms are involved in the remodeling process. To address these mechanisms, a quantitative assessment of the alterations in LV dimensions and hemodynamics was correlated to myocardial gene expression of phenotypic markers of hypertrophy and to ppET-1 and angiotensinogen mRNA levels during the early phase of severe

endothelin; hypertrophy; ischemic heart failure

ENDOTHELIN-1 (ET-1) is a 21-amino acid peptide with diverse cardiovascular effects including potent vasoconstrictor properties on resistance and capacitance vessels (for review, see Refs. 14 and 25) as well as positive inotropic effects (11, 15). Furthermore, ET-1 has been shown to act as a potent growth factor in several cells in vitro, including fibroblasts (30) and cardiomyocytes (29). ET-1 has also been shown to induce several phenotypic markers of hypertrophy in isolated cardiomyocytes (29). However, the physiological and pathophysiological roles played by ET-1 in vivo are still poorly understood.

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ET-receptor antagonism and remodeling during CHF

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ischemic heart failure in rats after chronic treatment with bosentan, a nonpeptidic ET<sub>1</sub>-ET<sub>2</sub>-receptor antagonist.

**MATERIALS AND METHODS**

Animal preparation. We used the left coronary artery-ligated rat model of CHF according to the method of Selye et al. (28) and the modifications of Pfeffer and colleagues (21). Male Wistar rats (300–350 g) were subjected to ligation of the left coronary artery, resulting in infarction of the LV free wall. Briefly, the rats were anesthetized with halothane and ventilated with the use of a rodent ventilator with a mixture of 30% O<sub>2</sub>–70% N<sub>2</sub>O and 1% halothane. A left thoracotomy was performed, the heart was exteriorized, and the proximal portion of the left coronary artery was rapidly ligated by an intramural 6-0 silk suture. The heart was subsequently returned to its normal position, and the thoracotomy was closed. Except for ligation of the coronary artery, sham-operated rats underwent an identical procedure. Surgical mortality was ~30% in the rats with myocardial infarction. The animal experiments, procedures, and housing were approved by the Hospital Board for Animal Research in accordance with the Norwegian Council for Animal Research.

Study protocol. After ligation of the left coronary artery, rats were randomized to treatment with bosentan (Ro 47–0203, Hoffmann-La Roche, Basel, Switzerland) or saline, starting 24 h after induction of myocardial infarction. Bosentan (100 mg·kg<sup>−1</sup>·day<sup>−1</sup>) was prepared fresh every day in saline and administered by gavage once daily for 15 days. It has previously been shown that this oral dose of bosentan blocks the pressor actions of ET-1 for more than 24 h (3, 7, 13). It was previously shown that this oral dose of bosentan blocks the pressor actions of ET-1 for more than 24 h (3, 7, 13). The treatment protocol was started 24 h after induction of myocardial infarction to minimize the possibility of a direct effect of bosentan on infarct size. Infarct size was determined by assessing the segmental length of the scar tissue relative to the circumference of the LV immediately after excision of the heart and by weighing the scar tissue. All rats had infarct size >40% of the LV circumference. A group of sham-operated rats received no treatment.

Hemodynamic measurements. Sixteen days after the induction of myocardial infarction or sham operation, and 24 h after the last dose of bosentan or saline, the rats were anesthetized and ventilated with the use of a rodent ventilator as described above. A 2-F micromanometer-tipped catheter (model SPR-407, Millar Instruments, TX) was inserted through the right carotid artery and advanced into the LV. LV end-diastolic pressure (LVEDP), LV systolic pressure (LVSP), and peak positive first derivative of the LV pressure (dP/dt<sub>max</sub>) were subsequently recorded (CardioMed Flowmeter CM 4008, Medi-Stim, Oslo, Norway). The time constant of isovolumic relaxation (τ) was calculated according to the logarithmic method (34).

Echocardiographic examination. Immediately after the hemodynamic measurements, echocardiography was performed using the fully digital Vingmed System FiVe (Vingmed Sound, Horten, Norway), with a 7.5-MHz linear array transducer. The system software was modified to allow for high-frame-rate recordings with an area of interest <20 mm from the surface of the transducer. Images were obtained by placing the transducer directly on the chest with the rat in the supine position. All recordings were digitally transferred to an on-line computer and stored on magnetic optical disks for subsequent analysis using the EchoPac software (Vingmed Sound). Two-dimensional long-axis and short-axis views of the LV were recorded with a typical frame rate of 196 s<sup>−1</sup>. The short-axis dimensions were recorded at the level of the papillary muscles. After gain settings were optimized, M-mode tracings were recorded at the same level, and septal and posterior wall thickness as well as LV internal dimensions were measured in both the M-mode and the two-dimensional tracings. LV internal dimensions were recorded as the largest anteroposterior diameter. In all cases, this diameter was recorded outside the infarcted area. The tracings were analyzed by one observer (R. Bjønerheim), who had no knowledge of the study group. Examples of the two-dimensional and M-mode recordings are shown in Fig. 1.

Tissue sampling and RNA extraction. The rats were killed by exsanguination from the heart. The atria were removed, and the right and left ventricles were dissected, separated, and weighed. The infarcted area (scar tissue) was excised from the noninfarcted LV, carefully avoiding contamination of viable myocardial tissue with necrotic tissue. The scar tissue was weighed to estimate infarct size. The tissues were snap frozen in liquid N<sub>2</sub> and stored at −70°C. Total RNA from the myocardial tissues was prepared by acid-phenol extraction in the presence of chaotropic salts (TRIzol, GIBCO-BRL, Gaithersburg, MD) and subsequent isopropanol-ethanol precipitation.

Histochemistry and morphometric analysis. Frozen myocardial tissue from nonischemic LV free wall was cut into 5-µm sections in a cryostat microtome. The sections were fixed in acetone for 10 min and subsequently stained with hematoxylin and eosin. Individual myocytes were subjected to morphometric analysis using a micrometric system (Olympus BX50, Olympus Optical, Tokyo, Japan) at a magnification of ×400. The cell diameter was measured at the site of the nucleus, and the recordings were restricted to myocytes containing nuclei in the center of the fiber in longitudinal sections. A total of 30 myocytes per animal were analyzed.

cDNA cloning and plasmid vector constructs. A 390-bp Sac I/Pvu II restriction fragment of the rat ppET-1 cDNA was subcloned into the pBluescript SK<sup>+</sup> + vector (Stratagene, La Jolla, CA) between the restriction sites Sac I and Smal I. With the use of DNA sequence information published for the rat angiotensinogen DNA (GenBank accession nos. L00093 and L00094), a 294-bp fragment of the angiotensinogen cDNA [nucleotides (nt) 51–157, exon 4, and nt 61–249, exon 5] was

![Fig. 1. Examples of echocardiographic M-mode and 2-dimensional short-axis (SAX) recordings of left ventricle (LV) in a sham-operated rat, a congestive heart failure (CHF) rat treated with vehicle (saline), and a CHF rat treated with bosentan at end of the 16-day protocol. There was symmetrical LV wall thickness in sham-operated control rats. In contrast, the LV cavity was dilated in the nonischemic interventricular septum (IVS) was thinned, and the posterior wall (PW) was hypokinetic in CHF-saline rats. LV dilatation was substantially reduced and IVS thickness increased in CHF-bosentan rats compared with CHF-saline rats.](http://aphpnet.org/photography.org)
amplified by RT-PCR from mRNA isolated from the liver, subcloned into pBluescript SK+, and sequenced by the dideoxy chain-termination technique. Similarly, the rat glycerolaldehyde-3-phosphate dehydrogenase (GAPDH), cardiac α-actin, atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), myosin light chain-2 (MLC-2), and troponin I cDNA were amplified by RT-PCR from rat myocardial tissues, and the skeletal α-actin DNA was amplified from rat skeletal muscle. The DNA fragments were characterized by DNA sequence analysis using the dideoxy chain-termination method and subcloned into pBluescript SK+. [GAPDH, nt 458–994 (GenBank accession no. M17701); cardiac α-actin, nt 453–986 (GenBank accession no. X80130); ANP, nt 1–635 (GenBank accession no. X00665); BNP, nt 180–365 (GenBank accession no. M25297); MLC-2, nt 81–525 (GenBank accession no. M11851); troponin I, nt 214–559 (GenBank accession no. M11851); troponin I, nt 2581–3037 (GenBank accession no. J00692)].

Synthesis of radiolabeled RNA probes. The pBluescript SK+/ppET-1 vector was linearized at the SacI site and blunt ended with T4 DNA polymerase to allow the synthesis of an antisense ppET-1 RNA probe from the T7 promoter site (protected fragment 390 bp). For angiotensinogen riboprobe synthesis, pBluescript SK+/angiotensinogen was linearized at a unique Bst XI site (protected fragment 275 bp). GAPDH riboprobe was synthesized by linearizing pBluescript SK+/GAPDH at a unique Eco1019 I site (protected fragment 128 bp).

Continuously radiolabeled antisense RNA probes for RNAse protection assay of ppET-1, angiotensinogen, and GAPDH mRNAs were generated by in vitro transcription using T3 or T7 RNA polymerase and [α-32P]CTP (3,000 Ci/mmOL, NEN, Boston, MA). Generally, linearized template DNA was transcribed in the presence of 40 mmol/l Tris·HCl, pH 7.5, 6 mmol/l MgCl2, 2 mmol/l spermidine, 10 mmol/l NaCl, 10 mmol/l diethyretrol, 500 µmol/l of each of the unlabelled ribonucleotides (ATP, GTP, and UTP), 3 µmol/l of [α-32P]CTP (specific activity ~800 Ci/mmOL), 20 units of the RNase inhibitor RNasin (Promega, Madison, WI), and 20 units of either T3 or T7 RNA polymerase. After the transcription reaction was completed, the template DNA was removed by digestion with DNase I. Under the conditions described, the RNA probes were labeled with high specific activity (~0.5 × 10⁶ counts/min (cpm)/µg).

Synthesis of radiolabeled cDNA probes. Continuously labeled cDNA probes for Northern blot analysis of ANP, BNP, skeletal α-actin, cardiac α-actin, MLC-2, troponin I, and GAPDH mRNA expression were radiolabeled with the random priming method in the presence of [α-32P]dCTP (specific activity ~6,000 Ci/mmOL).

RNAse protection assay. Total RNA (20 µg per assay) from the nonischemic myocardial tissue of the LV and from the right ventricle were mixed with the antisense ppET-1, angiotensinogen, and GAPDH RNA probes, coprecipitated with ethane, and dissolved in hybridization buffer (80% deionized formamide, 100 mmol/l sodium citrate, 300 mmol/l sodium acetate, and 1 mmol/l EDTA, pH 6.4). The hybridization reaction was performed overnight at 43°C. The samples were subsequently treated with RNase (RNase A-RNase T1) at 37°C for 30 min. This reaction was terminated with a commercial RNase inactivation-precipitation mixture (RPA II kit, Ambion, Austin, TX). The precipitated RNA was dissolved in 80% formamide, 0.1% xylene cyanol, 0.1% bromphenol blue, and 2 mmol/l EDTA and subjected to electrophoresis on a 5% denaturing polyacrylamide gel. Autoradiography of the gel was performed with the use of storage phosphor plates and a scanning PhosphorImager (445 Si, Molecular Dynamics, Sunnyvale, CA). Densitometric analysis of the bands was performed with the Image-Master software package (PharMeica Biotech, Piscataway, NJ). The data were subsequently corrected for variations in RNA loading by normalizing the ppET-1 mRNA and angiotensinogen mRNA expression to the GAPDH mRNA levels.

Northern blot analysis. Total RNA (20 µg/lane) from the noninfarcted part of the LV and from the right ventricle of CHF-bosentan rats (n = 11), CHF saline rats (n = 12), and sham-operated rats (n = 6) were denatured in 50% formamide and 6.5% formaldehyde and size fractionated with formaldehyde-agarose gel electrophoresis, transferred onto nylon filter membranes by capillary blotting, and successively hybridized with radiolabeled cDNA probes for ANP, BNP, skeletal α-actin, cardiac α-actin, MLC-2, and troponin I. The nylon membranes were hybridized at 42°C for 18 h in a hybridization buffer containing 5× SSC (sodium-sodium dolate), 5× Denhardt’s solution, 50% formamide, 50 mmol/l sodium phosphate, pH 6.5, 125 µg/ml sonicated salmon sperm DNA, and 2–3 × 10⁶ cpm/ml cDNA probe (specific activity 1–2 × 10⁸ cpm/µg). After the hybridization, the filters were washed in 2× SSC-0.1% SDS at room temperature and, finally, washed in 0.1× SSC-0.1% SDS at 60°C. Autoradiography and densitometric analysis of the bands were performed as described in RNAse protection assay. To normalize for variations of the amount of total RNA loaded on the gel and for variations of transfer efficiencies, the same membranes were rehybridized with the GAPDH cDNA as internal control.

RT-PCR of α- and β-myosin heavy chain cDNA. The relative amounts of cardiac α- and β-myosin heavy chain (MHC) mRNAs were determined by simultaneous amplification of the two mRNA species by “hot” (radioactively labeled) RT-PCR using assay conditions recently described (9). One common pair of oligonucleotide primers was used to amplify both the α- and β-MHC cDNA fragments. With the assumption that this set of primers will anneal to identical sequences in the α- and β-MHC transcripts with equal efficiencies and that the length of elongation is identical, the amplified fragments should be representative of the endogenous levels of mRNA for these two transcripts. Determination of the relative proportions of the amplified DNA fragments was done by quantitative restriction endonuclease digestion with an enzyme for which a restriction site is found only in the β-MHC cDNA fragment. cDNA was synthesized from 1 µg of total RNA isolated from the noninfarcted part of the left and right ventricles. cDNA synthesis conditions and PCR conditions were the same as previously described (9). The PCR amplifications were performed with the following oligonucleotides identical to sequences of both α- and β-MHC: forward primer, 5'-GCA GAC CAT CAA GGA CCT (nt 5,401–5,418 for α-MHC, GenBank accession no. X15938; and nt 5,371–5,388 for β-MHC, GenBank accession no. X15939), and reverse primer, 5'-TGG TGC ACC TTG CGG AAC TTT (nt 5,742–5,752 for α-MHC and nt 5,712–5,722 for β-MHC, GenBank accession no. X15939).
Effects of bosentan on infarct size and myocardial hypertrophy. As shown in Table 1, the ratios of ventricular weight to body weight were significantly increased in both CHF groups compared with the sham-operated group (P < 0.05), demonstrating compensatory ventricular hypertrophy after myocardial infarction in rats. Sixteen days after myocardial infarction, the LV weight-to-body weight ratio was increased by 13 and 24% in the CHF-saline group and the CHF-bosentan group, respectively, compared with that in the sham-operated controls (P < 0.05). The right ventricular weight-to-body weight ratio was increased by 65% in both the CHF-saline group and the CHF-bosentan group compared with that in the sham-operated group (P < 0.05). Thus, no significant differences in right ventricular weight-to-body weight ratios between the two treatment groups were observed, indicating sustained hypertrophic response during intervention with the ET-receptor antagonist bosentan. Furthermore, the diameter of the cardiomyocytes in sections of LV tissue was similar in the CHF-bosentan and CHF-saline groups (13.23 ± 0.1 and 13.30 ± 0.1 μm, respectively), demonstrating maintained cellular hypertrophy in the CHF-bosentan group.

Table 1. Cardiac chamber weights and hemodynamic measurements in rats 16 days after myocardial infarction or sham operation

<table>
<thead>
<tr>
<th>n</th>
<th>Sham</th>
<th>CHF-Saline</th>
<th>CHF-Bosentan</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>13</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td>BW</td>
<td>328 ± 3</td>
<td>324 ± 3</td>
<td>331 ± 3</td>
</tr>
<tr>
<td>BW</td>
<td>347 ± 4</td>
<td>312 ± 6</td>
<td>327 ± 7</td>
</tr>
<tr>
<td>SCA</td>
<td>1.3 ± 0.05</td>
<td>1.4 ± 0.04</td>
<td></td>
</tr>
<tr>
<td>LVW/BW</td>
<td>0.175 ± 0.04</td>
<td>0.170 ± 0.05†</td>
<td></td>
</tr>
<tr>
<td>RWW/BW</td>
<td>0.51 ± 0.02</td>
<td>0.83 ± 0.04*</td>
<td>0.85 ± 0.05*</td>
</tr>
<tr>
<td>HR</td>
<td>405 ± 15</td>
<td>408 ± 8</td>
<td>395 ± 10</td>
</tr>
<tr>
<td>LVSP</td>
<td>131.5</td>
<td>107.3 ± 3</td>
<td>99 ± 2†</td>
</tr>
<tr>
<td>LVEDP</td>
<td>2.5 ± 0.6</td>
<td>23.2 ± 0.7*</td>
<td>19.8 ± 1.6*</td>
</tr>
<tr>
<td>+dP/dtmax</td>
<td>7.750 ± 250</td>
<td>4.631 ± 243*</td>
<td>4.280 ± 260*</td>
</tr>
<tr>
<td>τ, ms</td>
<td>8.8 ± 0.7</td>
<td>16.3 ± 0.6*</td>
<td>15.4 ± 1.0*</td>
</tr>
</tbody>
</table>

Values are means ± SE. CHF, congestive heart failure; BW, body weight; LVW, left ventricular weight; RWW, right ventricular weight; HR, heart rate; LVSP, LV systolic pressure; LVEDP, LV end-diastolic pressure; +dP/dtmax, peak rate of LV pressure increase; τ, time constant of isovolumic relaxation. †P < 0.05 vs. sham-operated rats; ‡P < 0.05 vs. CHF-saline rats.

Statistical analysis. All data are presented as means ± SE. Statistical analysis was assessed by two-tailed, unpaired Student’s t-test. The data groups were tested for equality of variances using Levene’s test. P values <0.05 were considered to be statistically significant.
mm, P < 0.05). However, IVSEDT in the bosentan-treated CHF group (2.2 ± 0.08 mm) was not statistically different from that in the sham-operated control group. Less prominent reductions of LV posterior wall end-diastolic thickness (PWEDT) were observed in the CHF-saline group compared with the sham-operated controls (1.9 ± 0.06 vs. 2.1 ± 0.05 mm). PWEDT in the CHF-bosentan group (2.1 ± 0.05 mm) was not statistically different from that in the sham-operated group. With regard to the substantial attenuation of LV dilatation in the bosentan-treated CHF rats, relative wall thickness ([IVSEDT + PWEDT]/LVEDD) was almost normalized in the CHF-bosentan group compared with the CHF-saline group (Table 3).

Effects of bosentan on LV systolic function during CHF. Peak +dP/dt was decreased in both untreated and bosentan-treated CHF rats compared with sham-operated rats (P < 0.05, Table 1). However, +dP/dt max in CHF rats treated with bosentan was not statistically different from that in CHF rats treated with saline. As demonstrated in Fig. 2A, coronary artery ligation caused a rightward and downward displacement of the systolic pressure-diameter relationship. However, bosentan shifted the systolic pressure-diameter relationship leftward, indicating sustained or even improved LV contractility after treatment with bosentan. Furthermore, LV fractional shortening ([LVEDD − LVESD]/LVEDD) was markedly decreased in the CHF groups. However, treatment with bosentan improved systolic function, causing a 50% increase in fractional shortening compared with untreated CHF rats (18 ± 1% in the CHF-bosentan group vs. 12 ± 1% in the CHF-saline group, P < 0.05).

Effects of bosentan on LV diastolic function during CHF. +dP/dt was significantly increased after ligation of the left coronary artery (Table 1). However, no significant effect on +dP/dt was observed after intervention with bosentan. As shown in Fig. 2B, induction of severe CHF was associated with a substantial rightward and upward shift of the end-diastolic pressure-diameter relationship. Chronic treatment with bosentan during CHF attenuated the rightward and upward displacement of the end-diastolic pressure-diameter relationship, demonstrating improved diastolic function.

Effects of bosentan on myocardial expression of ppET-1 mRNA and angiotensinogen mRNA. RNase protection assays were performed to assess regulation of myocardial ppET-1 and angiotensinogen mRNA levels in the nonischemic regions of the left and right ventricles at the end of the treatment protocol 16 days after induction of myocardial infarction. In concordance with previous reports (17a, 27), significant elevations of myocardial ppET-1 mRNA levels were found in the left (Fig. 3) and right ventricles (Fig. 6) of the CHF-saline group compared with the sham-operated group (4-fold and 3-fold elevations, respectively, P < 0.05). However, the myocardial ppET-1 mRNA levels of the left and the right ventricles of the bosentan-treated rats were not statistically different from those of the CHF-saline group, indicating that ET-receptor blockade does not affect induction of ppET-1 mRNA. As shown in Fig. 3, angiotensinogen mRNA levels in the left ventricle were found to be expressed at very low levels in sham-operated rats. Myocardial angiotensinogen mRNA levels did not appear to be regulated in the CHF rats 16 days after induction of myocardial infarction, irrespective of treatment protocol.

Effects of bosentan on induction of a hypertrophic gene program during CHF. Myocardial ANP, BNP, β-MHC, and skeletal α-actin mRNAs are expressed at very low levels in the normal adult rat ventricle. However, these genes are reactivated in the process of hypertrophy of the myocardium associated with CHF. Thus these mRNAs are frequently used as molecular markers of the hypertrophic phenotype. Northern blot analysis and hot RT-PCR were performed to investigate the expression of these mRNA markers. Characteristically, myocardial ANP, BNP, and skeletal α-actin mRNA expression were substantially induced in both the left (Fig. 4) and the right ventricles (Fig. 6) in untreated CHF rats. In the LV, myocardial expression of ANP, BNP, and skeletal α-actin mRNAs were increased 16-, 5-, and 2-fold, respectively, in the untreated CHF group compared with the sham-operated controls (P < 0.05). Similar elevations of the mRNA levels of ANP, BNP, and skeletal α-actin were observed in the right ventricle of untreated CHF rats (3-fold, 7-fold, and 2-fold, respectively) compared with the sham-operated group.
Fig. 3. RNase protection assay of myocardial preproendothelin-1 (ppET-1), angiotensinogen, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels in LV of sham-operated and CHF rats after 15 days of intervention with either bosentan or saline. Treatment was started 24 h after induction of CHF. Twenty micrograms of total RNA were used in each hybridization reaction. A: autoradiograph was exposed to a high-resolution storage phosphorimaging plate for 48 h and analyzed with a PhosphorImager. B: histograms show densitometric analysis of scanning data, presented as ratios of levels of ppET-1 and angiotensinogen mRNA to levels of GAPDH mRNA to normalize for variations in RNA loading. Data are presented as means ± SE. *P < 0.05 vs. sham-operated group.

Fig. 4. Northern blot analyses showing myocardial mRNA expression of muscle-specific and fetal genes in LV of sham-operated rats and CHF rats treated with bosentan or saline for 15 days. Twenty micrograms of total RNA were used in each lane. A: autoradiographs were exposed for 24 h and analyzed with a PhosphorImager. B: densitometric analysis of scanning data, presented as ratios of mRNA expression levels of muscle-specific and fetal genes to levels of GAPDH mRNA to normalize for variations of amount of total RNA loaded on gel and for transfer efficiencies. Data are presented as means ± SE in sham-operated (open bars), CHF-saline (solid bars), and CHF-bosentan rats (shaded bars). ANP, atrial natriuretic peptide; BNP, brain natriuretic peptide; MLC-2, myosin light chain-2. *P < 0.05 vs. sham-operated group.
(P < 0.05). However, no significant differences of myocardial ANP, BNP, and skeletal α-actin mRNA levels were observed between the CHF-bosentan group and the CHF-saline group. The ratios of β-MHC to α-MHC mRNA (β-MHC/α-MHC) were dramatically elevated in the CHF rats compared with the sham-operated controls (Figs. 5 and 6), indicating induction of β-MHC mRNA. In the LV, β-MHC/α-MHC was increased 22-fold in the CHF-bosentan group and 11-fold in the CHF-saline group compared with that in sham-operated rats (P < 0.05). The difference in β-MHC/α-MHC between bosentan-treated CHF rats and untreated CHF rats was not statistically significant (P = 0.082). Similar increments in myocardial β-MHC/α-MHC were observed in myocardial tissues from the right ventricles of both bosentan-treated CHF rats and untreated CHF rats. Overall, myocardial expression of the mRNA markers of a hypertrophic ventricular phenotype was not attenuated by bosentan during the early phase of ventricular remodeling after myocardial infarction in rats. Thus the hypertrophic response during CHF in rats clearly did not appear to be hampered by ET-receptor blockade.

Effects of bosentan on expression of constitutive myocardial genes. Expression of the genes encoding the contractile proteins MLC-2, troponin I, and cardiac α-actin was investigated using Northern blot analysis. High levels of these constitutive mRNAs were observed in both the left (Fig. 4) and the right ventricles (Fig. 6) of sham-operated rats. MLC-2, troponin I, and cardiac α-actin mRNA levels did not appear to be regulated during CHF and were not affected by treatment with bosentan when analyzed at the end of the intervention 16 days after induction of myocardial infarction, indicating that these genes are not regulated by ET.

**DISCUSSION**

Ligation of the left coronary artery in rats is associated with induction of myocardial hypertrophy in the noninfarcted regions of the heart. This process is considered a beneficial adaptation compensating at least partially for the loss of contractile tissue. As demonstrated in the present study, both gross myocardial hypertrophy of the viable myocardium as well as induction of several phenotypic markers of hypertrophy were not affected by the treatment with bosentan. Despite improved hemodynamic responses, the ventricular-to-body weight ratios, the cardiomyocyte diameters, and the echocardiographic analysis of LV wall thickness as well as the hypertrophic gene program all consistently demonstrate that the hypertrophic response during CHF is not affected by bosentan. These findings may seem at odds with the expected outcome. However, it is important to bear in mind that the CHF rats treated with bosentan still exhibited significantly impaired hemodynamics, indicating that the compensatory mechanisms leading to hypertrophy would remain activated. Furthermore, treatment with bosentan did not affect myocardial expression of ppET-1 mRNA. The ppET-1 mRNA levels remained elevated during the treatment, indicating increased tissue ET-1. However, such elevations of tissue ET-1 cannot play a crucial role in the mechanism of hypertrophy during ischemic heart failure in rats because the high dose of bosentan employed in the present study would effectively block all the actions of ET-1 (3, 7, 13). In contrast to our data, Sakai et al. (26) recently reported amelioration of LV hypertrophy in CHF rats after ET-receptor antagonism. Although their study did not provide a quantitative assessment of the effects of ET-receptor antagonism on LV hypertrophy, several reasons may account for the apparent differences observed. First of all, the different findings may be due to different study protocols. Our study was designed to investigate the mechanisms of ET-receptor antagonism during the early phase of ventricular remodeling after myocardial infarction. Thus the effects of a 15-day treatment protocol with a mixed ETA/ETB-receptor antagonist were recorded at day 16 after induction of myocardial infarction to enable correlation of structural changes to regulation of gene expression programs. On the other hand, Sakai and colleagues (26) performed a 12-wk treatment protocol with BQ-123, a selective ETA-receptor antagonist. Thus it could be argued that the amelioration of LV hypertrophy after ETA-receptor antagonism observed by Sakai and colleagues could be due to compensatory activation of myocardial ETβ receptors. However, ETβ receptors have been reported (17) to mediate hypertrophic signals in cardiomyocytes in vitro. Thus the role of the ETβ receptor in the process of myocardial remodeling needs to be addressed in future studies. Furthermore, administration of BQ-123...
was started at day 10 after induction of myocardial infarction. Thus it is conceivable that the different findings of the two studies are due to modulation of different adaptive processes operating at various stages of myocardial remodeling. Consequently, it is important to keep in mind that the present study addresses the effects of ET-receptor antagonism at a single point in time, i.e., the first 16 days after myocardial infarction.

Apoptotic cardiomyocyte death has been shown to take place after myocardial infarction (10). Therefore, it could be hypothesized that apoptosis was more prominent in the CHF-saline rats than in CHF-bosentan rats as a result of ET-induced apoptosis through the ET receptors. Thus enhanced apoptosis could have counterbalanced and masked an increase in myocardial hypertrophy in the CHF-saline group. However, the diameters of the cardiomyocytes were similar in the CHF groups, indicating no significant alterations of hypertrophic response after intervention with bosentan. Thus the small but significant increase in LV-to-body weight ratio in bosentan-treated CHF rats could be explained by lower levels of apoptosis in the LV after ET-receptor antagonism. Although apoptosis has been shown to occur in failing myocardial tissue, the significance of this process as to the loss of myocardial tissue still needs to be established.

LV cavity size has been shown to be a major predictor of mortality in patients with CHF (35). The substantial attenuation of LV dilatation observed in the present study after intervention with bosentan may therefore explain the beneficial effects of ET-receptor antagonism on mortality in rats. Mulder and colleagues (16) recently reported a minor decrease in LV cavity size in CHF rats treated with bosentan. However, in the latter study, intervention with bosentan was initiated 7 days after ligation of the left coronary artery. In this context, the first few weeks after induction of ischemic heart failure in rats are associated with critical and substantial alterations in LV geometry (20). Dilatation of the LV may be a result of slippage and elongation of the cardiomyocytes (33). This process of slippage and elongation has been shown (20) to have already started within 2 days after myocardial infarction and rapidly progresses during the first weeks. Our findings indicate that early intervention with an ET-receptor antagonist after myocardial infarction may prove to be particularly beneficial. As shown in the present study, LV cavitary volume-to-mass ratio was reduced after treatment with bosentan. Previous evidence from other
treatment protocols indicates that intervention leading to attenuation in LV volume without a proportional reduction in cardiac mass is associated with a more favorable ventricular performance and prolongation in survival (22).

Because the molecular markers of myocardial hypertrophy were similar in the CHF-bosentan and the CHF-saline groups, it seems unlikely that the beneficial effects of bosentan on LV geometry are caused by a direct action of bosentan on the myocardial tissue. However, due to the capacity of bosentan to elicit favorable hemodynamic responses during CHF, it seems most likely that the attenuation of LV dilatation is due to reduced preload and afterload and a subsequent decrease in myocardial wall stress. As indicated by the modest decrease in LVSP and the marked decrease in LVESD and LVEDD, systolic and diastolic wall tensions were markedly reduced. In the bosentan-treated CHF group, wall thickness of the viable myocardium was preserved, whereas the untreated CHF group showed considerable LV wall thinning in the nonischemic region. Therefore, not only wall tension but also wall stress was markedly reduced in the bosentan-treated CHF rats. Although we have not calculated wall stress because of asymmetry of the LV after myocardial infarction, all parameters determining wall stress were significantly reduced in the CHF rats after ET-receptor antagonism, implying decreased wall stress. The reduction in wall stress implies that bosentan decreased the load on the individual myocardial fibers and therefore reduced the stimulus to further dilatation. However, myocardial wall stress in the bosentan-treated CHF rats was presumably still significantly increased compared with that in sham-operated rats. Thus the stimulus to myocardial hypertrophy would still be present in the bosentan-treated CHF rats. The decrease in myocardial wall stress in the bosentan-treated CHF rats may exert an additional beneficial effect. A decrease in wall stress subsequently leads to reduced myocardial oxygen demand, which is a favorable effect in the setting of ischemic heart failure. Furthermore, it is conceivable that the beneficial effects of ET-receptor antagonism on coronary blood flow may have increased oxygen supply of the noninfarcted myocardium and indirectly contributed to improve cardiac function.

Previous evidence indicates that two separate properties of ET-receptor antagonists may mediate the capacity to reduce LVEDP and preload. First, ET-receptor antagonists have been shown to cause venodilation in addition to arterial vasodilation (6, 8). Second, it has recently been shown (23) that the ET, receptor antagonist BQ-123 significantly enhances early LV relaxation and lowers LVEDP in normal guinea pigs without altering systolic performance. However, in the present study, treatment with bosentan did not affect τ, indicating that ET-receptor antagonism does not improve active ventricular relaxation during severe CHF. Thus the mechanisms of the decrease of preload after intervention with bosentan appears to be due to venodilation rather than a direct effect on myocardial relaxation during diastole.

Endothelin-1 has been shown to elicit a powerful inotropic response in isolated myocardial fibers (11). Thus ET-receptor antagonism could, at least theoretically, adversely affect LV function in vivo. Consequently, the putative beneficial effects of ET-receptor antagonism during CHF would depend on the capacity of ET-receptor blockers to reduce pulmonary and systemic vascular tone. Despite the anticipation that bosentan might impair myocardial contractility, the present study clearly demonstrates that systolic function was maintained or even improved. First, +dP/dt max was not significantly altered after intervention with bosentan. Second, fractional shortening was vastly improved in the CHF-bosentan group. Third, the plot of LVSP vs. LVEDD is consistent with maintained or improved contractility. Thus, although we were not able to measure cardiac output in the present study, the structural and dynamic data strongly indicate that systolic function was maintained or even improved with bosentan.

In conclusion, in the present study, early intervention with the nonselective ET-receptor antagonist bosentan during postinfarction failure in rats markedly attenuated LV dilatation and improved LV pressure-volume relationships. In addition, fractional shortening was increased and myocardial contractility maintained. The mechanism of these beneficial effects of bosentan appeared to be a substantial decrease in myocardial wall stress, attributed to decreases in both preload and afterload and LV diameter as well as to the preservation of myocardial wall thickness. ET-receptor antagonism by bosentan did not reduce gene expression markers of myocardial hypertrophy. Therefore, the favorable effects of bosentan during ischemic heart failure were caused by a reduction in the hemodynamic load, leading to ventricular dilatation without altering compensatory myocardial hypertrophy. Thus the data do not support an autocrine/paracrine role of ET-1 in the mechanisms of myocardial hypertrophy in the early phase of postinfarction failure.

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REFERENCES
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