ET\textsubscript{A}-receptor blockade prevents matrix metalloproteinase activation late postmyocardial infarction in the rat

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AFTER MYOCARDIAL INFARCTION (MI), the left ventricle (LV) undergoes “remodeling,” a chronic process marked by chamber dilation, myocardial hypertrophy, and alterations in the extracellular matrix (3, 8, 22). Plasma levels of endothelin (ET)-1 are elevated in patients with chronic heart failure (4). Furthermore, the expression of ET and its receptors is increased in the myocardium late post-MI in the rat (30, 34). These observations have raised the possibility that ET plays a role in chronic myocardial remodeling post-MI (7). This thesis is supported by the demonstration that chronic treatment with ET-receptor antagonists decreases chamber dilation, improves LV function, and increases survival post-MI in the rat (12, 25, 26, 33).

ETF binds to two receptor subtypes, ET\textsubscript{A} and ET\textsubscript{B} (36). In the vasculature, ET\textsubscript{A} receptors predominate on vascular smooth muscle cells, where they mediate constriction. ET\textsubscript{B} receptors primarily mediate relaxation via an endothelium-dependent relaxing factor-dependent mechanism. In the myocardium, ET\textsubscript{A} receptors predominate on myocytes (24), where they stimulate hypertrophic growth (37). ET\textsubscript{A} and ET\textsubscript{B} receptors are present on cardiac fibroblasts (18). In cardiac fibroblasts in vitro, ET receptors appear to mediate both collagen synthesis and degradation (14).

MMP activity is increased in the myocardium of patients with heart failure (41) and in animal models of heart failure (6). Increased MMP activity can lead to degradation of fibrillar collagen that is important for the structural integrity of the ventricle. These observations have led to the suggestion that increased MMP activity contributes to pathological LV remodeling by promoting chamber dilation (23). We tested the hypothesis that ET\textsubscript{A} receptors regulate MMP activity in the myocardium late post-MI. Accordingly, beginning on day 3 post-MI, rats were treated with sitaxsentan, an ET-receptor antagonist that is ~6,000-fold selective for the ET\textsubscript{A} subtype (43). Six weeks post-MI, LV chamber volume and contractile function were measured using the isovolumic (balloon-in-LV) Langendorff technique. The activity of myocardial MMPs was measured by in-gel zymography, and protein levels of tissue inhibitors of metalloproteinases (TIMPs) were measured by immunoblotting in myocardium remote from the area of infarction.

METHODS

MI protocol. MI was caused in adult male Wistar rats (250–300 g; Charles River) by ligation of the left coronary artery by the technique of Pfeffer et al. (29), as previously described.

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described (13). Briefly, rats were anesthetized with pentobarbital sodium (25 mg/kg ip), intubated with a fine polyethylene tube, and ventilated mechanically with room air (70 min⁻¹) using a rodent ventilator (Harvard Apparatus, Boston, MA).

After local anesthesia with lidocaine, a lateral thoracotomy was performed, the heart was exteriorized, and a ligature was placed around the proximal portion of the left coronary artery. The heart was returned to its normal position, and the chest was closed. Sham-operated hearts were treated similarly, except no suture was placed around the coronary artery. All rats were kept in single animal cages and had free access to standard rat chow and water. Perioperative mortality in the first 48 h was ~40%.

**Treatment protocol.** A fresh solution of sitaxsentan was prepared each day by adding the soluble salt to distilled water. The treatment group received this solution as drinking water for 6 wk, starting on day 3 post-MI. The amount of drinking water was progressively increased from 50 to 100 ml/day over the course of the study. However, the total daily dose of sitaxsentan was held constant at 90 mg/kg body wt. Control rats received equivalent amounts of water without drug. At 6 wk, arterial blood (1 ml) was withdrawn from the right carotid artery and centrifuged, and the plasma was frozen at ~70°C for measurement of sitaxsentan. The mean plasma concentration of sitaxsentan was 55 ± 9 µg/ml in the treatment group.

**In vivo hemodynamic measurements.** Six weeks post-MI, resting hemodynamics were measured under light anesthesia with pentobarbital sodium (10 mg/kg ip), which allowed spontaneous respiration. A 2-F Millar micropipet catheter was advanced via the right carotid artery into the LV. After measurement of LV pressures, the catheter was withdrawn to the aorta.

**In vitro myocardial function.** After in vivo measurements were completed, rats were heparinized (200 IU iv), the chest was opened, and the beating heart was rapidly excised and placed on the Langendorff apparatus within 10 s, as described previously (10), using an isolated, erythrocyte-perfused, isovolumically beating preparation. The perfusate consisted of a Krebs-Henseleit buffer with bovine red blood cells at a hematocrit of 40%. The buffer contained (mmol/l) 118 NaCl, 4.7 KCl, 2.0 CaCl₂, 1.2 MgSO₄, 1.2 KH₂PO₄, 25 NaHCO₃, 5.5 glucose, 1.0 lactate, and 0.4 palmitic acid and 4 g/100 ml BSA (Sigma Chemical, St. Louis, MO). A collapsed thin-walled polyvinylchloride film balloon attached to a catheter was placed in the LV via the left atrium and secured in place by sutures. The balloon was connected to a pressure transducer (Statham P23 dB, Spectramed) for constant measurement of coronary venous effluent. The MMP activity (per 100 µg of protein) was measured by in-gel zymography with gelatin (10 mg/ml, type A from porcine skin; Sigma Chemical) or α-casein (15 mg/ml; Sigma Chemical) as the substrate, as described previously (14). Clear, digested regions representing MMP activity were quantified using an imaging densitometer (model GS700, Bio-Rad), and MMP levels were estimated using prestained molecular weight markers.

**Tissue plasminogen activator (tPA) and urokinase plasminogen activator (uPA) activity.** Aliquots of frozen remote LV (~100 mg) were homogenized with the use of a Teflon dounce homogenizer and sonicated in 50 mM Tris-HCl (pH 7.6), 0.2 M NaCl, 5 mM CaCl₂, 0.02% (wt/vol) Brij 35, and 0.02% (wt/vol) NaN₃ at 4°C. An aliquot of crude homogenate was frozen for spectrophotometric quantitation of hydroxyproline content by the method of Bergman and Lozley (1). The remaining homogenate was centrifuged at 10,000 g for 20 min. Protein content of the supernatant was quantified by the Bradford method. Extracts were stored at ~80°C.

**Assessment of hydroxyproline content.** MMP, tissue and tissue plasminogen activator activity, and TIMP protein levels. Aliquots of frozen remote LV (~100 mg) were homogenized with the use of a Teflon dounce homogenizer and sonicated in 50 mM Tris-HCl (pH 7.6), 0.2 M NaCl, 5 mM CaCl₂, 0.02% (wt/vol) Brij 35, and 0.02% (wt/vol) NaN₃ at 4°C. An aliquot of crude homogenate was frozen for spectrophotometric quantitation of hydroxyproline content by the method of Bergman and Lozley (1). The remaining homogenate was centrifuged at 10,000 g for 20 min. Protein content of the supernatant was quantified by the Bradford method. Extracts were stored at ~80°C.
diluted 1:5,000 in 4% nonfat dry milk in TBST and incubated overnight at 4°C. The membranes were washed in TBST for 1 h with six changes of buffer. The secondary antibody (horseradish peroxidase-conjugated goat anti-rabbit IgG or donkey anti-sheep IgG; Santa Cruz) was diluted 1:100,000 in 4% nonfat dry milk in TBST for 1 h at room temperature. The membranes were again washed in TBST for 1 h with six changes of buffer. Signals were detected using enhanced chemiluminescence with a luminal substrate (Super Signal, Pierce) and exposed to film. Signals were quantified using a densitometer (model GS-700, Bio-Rad), and molecular weights were estimated using prestained molecular weight markers and human TIMP-1 and TIMP-2 standards (Chemicon).

Statistical methods. Values are means ± SE. Comparison of single parameters was performed by ANOVA. Comparison of pressure-volume relationships between experimental groups was performed by two-way ANOVA. If ANOVA indicated a significant difference or interaction, values at specific points were tested by the method of least significant differences. P < 0.05 was considered to be significant. All statistical procedures were performed using the StatView statistical software package (version 5.0, SAS Institute, Cary, NC).

RESULTS

Survival post-MI. Twenty-nine infarcted rats survived the initial 48-h post-MI period and were randomized to control or sitaxsentan treatment beginning on day 3. One MI control rat died on day 4. Of the remaining 28 infarcted rats, 12 control and 12 sitaxsentan-treated animals survived to the 6-wk terminal study, as did 4 of 4 sham-operated animals.

In vivo hemodynamics. Six weeks post-MI, heart rate was similar in the control and sitaxsentan-treated groups (Table 1). LV end-diastolic pressure (LVEDP) was increased to a similar degree in the control and sitaxsentan-treated groups (vs. sham-operated). LV systolic pressure was similar in all three groups. LV developed pressure tended to be lower in the control MI group, which was not different from the sitaxsentan-treated group. Maximal and minimal rate of pressure development (dP/dt) were decreased to a similar degree in the control and sitaxsentan-treated groups.

Morphometrics. Infarct size was similar in the control MI and sitaxsentan-treated groups (Table 2). Body weight and tibial length were similar in the three groups. In the infarcted animals, the LV weight-to-body weight ratio was decreased in the sitaxsentan-treated versus the control MI group, as was the RV weight-to-body weight ratio. In the infarcted animals, ascites was noted in two control rats but in no sitaxsentan-treated animals. Pleural effusions were noted in two sitaxsentan-treated animals and one control animal.

ANP and SERCA2 mRNA levels. The level of mRNA for ANP was increased 192% in the sitaxsentan-treated animals (vs. sham-operated animals) and was increased to a similar degree in sitaxsentan-treated versus the control MI group, as was the RV weight-to-body weight ratio. In the infarcted animals, ascites was noted in two control rats but in no sitaxsentan-treated animals. Pleural effusions were noted in two sitaxsentan-treated animals and one control animal.

Effects of sitaxsentan on LV volume and contractile function in vitro. At 6 wk post-MI, the LVEDP-to-volume ratio was shifted rightward (P < 0.01, control vs. sham-operated; Fig. 1). This rightward shift was prevented in the sitaxsentan-treated group, which did not differ from the sham-operated group.

The LV systolic pressure-to-volume ratio was shifted rightward in the control MI group (P < 0.01 vs. sham-operated; Fig. 2). In the sitaxsentan-treated group, the LV systolic pressure-to-volume ratio was shifted less than in the control MI group and differed from the sham-operated group only at the lowest three volumes.

Hydroxyproline content. Collagen content, as reflected by hydroxyproline, increased in remote LV myocardium from the MI control animals (vs. sham-operated animals) and was increased to a similar degree in sitaxsentan-treated animals (184% increase, P = nonsignificant [NS] vs. MI control animals). SERCA2 mRNA levels were similar in all three groups (data not shown).

MMP activity. Total gelatinase activity was increased by 65% in the control MI group (P = 0.044 vs. sham-operated), and this increase was prevented in

Table 1. In vivo hemodynamics 6 wk post-MI

<table>
<thead>
<tr>
<th>MI</th>
<th>Sham-Operated (n = 12)</th>
<th>Control (n = 12)</th>
<th>Sitaxsentan (n = 12)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart rate, beats/min</td>
<td>320 ± 88</td>
<td>373 ± 14</td>
<td>383 ± 17</td>
</tr>
<tr>
<td>Aortic systolic pressure, mmHg</td>
<td>113 ± 6</td>
<td>100 ± 7</td>
<td>116 ± 9</td>
</tr>
<tr>
<td>Aortic diastolic pressure, mmHg</td>
<td>80 ± 3</td>
<td>78 ± 8</td>
<td>89 ± 9</td>
</tr>
<tr>
<td>LVSP, mmHg</td>
<td>98 ± 9</td>
<td>97 ± 5</td>
<td>101 ± 7</td>
</tr>
<tr>
<td>LVEDP, mmHg</td>
<td>1 ± 0.4</td>
<td>17 ± 3†</td>
<td>13 ± 2†</td>
</tr>
<tr>
<td>LV developed</td>
<td>98 ± 7</td>
<td>81 ± 4</td>
<td>88 ± 7</td>
</tr>
<tr>
<td>pressure, mmHg</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LV dP/dt, mmHg/s</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Maximum</td>
<td>3,800 ± 510</td>
<td>2,800 ± 100m</td>
<td>3,000 ± 323</td>
</tr>
<tr>
<td>Minimum</td>
<td>3,500 ± 430</td>
<td>2,489 ± 107m</td>
<td>2,486 ± 282m</td>
</tr>
</tbody>
</table>

Values are means ± SE. MI, myocardial infarction; LV, left ventricular; LVSP, LV systolic pressure; LVEDP, LV end-diastolic pressure; dP/dt, rate of pressure development. *P < 0.05 vs. sham. †P < 0.01 vs. sham.
the sitaxsentan-treated animals (20 ± 12%, \( P = 0.037 \) vs. control MI and \( P = \text{NS vs. sham-operated}; \) Fig. 3). Likewise, total caseinase activity was increased by 76 ± 17% in the control MI group (\( P = 0.029 \) vs. sham-operated), and this increase was prevented in the sitaxsentan-treated animals (18 ± 20%, \( P = 0.038 \) vs. control MI and \( P = \text{NS vs. sham-operated}; \) Fig. 3). The specific activities of MMP-1 (57/48 kDa), MMP-2 (72/66 kDa), MMP-9 (95/88 kDa), and 92-kDa caseinase were increased in the control MI group, and all the increases were prevented in the sitaxsentan-treated group (Fig. 4). The activities of tPA and uPA were similar in all three groups (Fig. 5).

TIMP protein levels. The level of TIMP-1 protein as determined by Western blotting was decreased in the control MI group (vs. sham-operated), and the decrease was prevented in the sitaxsentan-treated group (Fig. 6). The levels of TIMP-2 and TIMP-4 did not differ in the control MI and sham-operated groups and were not affected by sitaxsentan treatment.

**DISCUSSION**

**Effect of sitaxsentan on post-MI remodeling.** Post-MI remodeling was associated with a rightward shift in the LV diastolic pressure-volume relationship, indicating dilation of the LV chamber. This rightward shift was markedly attenuated by sitaxsentan treatment. A similar antiremodeling effect has been reported for other ET-receptor antagonists, including bosentan (12,
26), BQ-123 (33), and LU-135252 (25). In contrast, when an ET-receptor antagonist was initiated early, within the first 24 h post-MI, there was adverse remodeling with more LV dilation (15, 27), suggesting that the timing of anti-ET treatment post-MI may be critical. Sitaxsentan treatment had no apparent effect on MI size, suggesting that by **day 3**, when treatment was started, MMP activity may not have been critical to scar formation. It is interesting that this temporal pattern appears to differ from that observed with angiotensin-converting enzyme inhibitors or angiotensin-receptor antagonists, which reduce scar size and collagen content even when given in the first 24 h post-MI (9, 40, 44).

Post-MI remodeling was also associated with myocardial hypertrophy, as indicated by increases in LV and RV mass and the expression of ANP mRNA. Sitaxsentan treatment prevented RV and LV hypertrophy but did not inhibit the expression of ANP mRNA. Although ANP expression is commonly associated with myocardial hypertrophy (2), it is recognized that the signaling pathways for myocyte hypertrophy and ANP induction may diverge with regard to the effects of ET (19). This result differs from that of Sakai et al. (35),

who found that the increase in ANP mRNA 3 mo post-MI was prevented by treatment with the ET-receptor blocker BQ-123, but is similar to that of Oie et al. (28), who found no effect of bosentan on ANP mRNA at 2 wk post-MI. In our animals, ANP mRNA was only modestly increased at 6 wk, and therefore it is possible that the increase in ANP would be prevented at a later time.

**Effects on LV contractile function.** Sitaxsentan did not exert measurable effects on resting LV hemodynamics measured in vivo. In particular, the post-MI changes in LVEDP, developed pressure, and maximum and minimum dP/dt were not affected by sitaxsentan. A decrease in LVEDP has been observed in some (33), but not all (12), studies of ET-receptor blockers post-MI.

The LV systolic pressure-volume relationship was shifted rightward post-MI, indicating that these hearts were able to generate a comparable systolic pressure, albeit at a higher end-diastolic volume. Sitaxsentan treatment attenuated the rightward shift in this relationship. Similar beneficial effects on LV contractile function late post-MI have been observed with bosentan (12, 25) and BQ-123 (33). An advantage of the isovolumic Langendorff approach is that it allows contractile function to be assessed over a wide range of controlled loading conditions and, thus, may detect changes in contractile function that are not apparent under basal in vivo conditions.

**Metalloproteinase activation post-MI.** A new finding of this study is that late post-MI there was increased MMP lytic activity by in vitro zymography in LV myocardium remote from the infarct. Although increased MMP lytic activity has been demonstrated early post-MI in the infarct region (32), there is little information available regarding MMP activation in remote myocardium late post-MI. Our data demonstrate increases in total MMP activity, as well as activation of several specific MMPs, including MMP-1, MMP-2, and MMP-9. This activation was associated with a decrease in TIMP-1 protein expression. MMPs may be activated by tPA and uPA, but neither was elevated in our post-MI animals.
An increase in lytic activity has been shown in the myocardium of patients with end-stage heart failure (21, 41) and in animal models of myocardial failure, including the rapid-paced pig (6). These observations have been interpreted to indicate an increase in MMP activity. Since TIMPs are not active during in vitro zymography, the observation of increased MMP lytic activity per se need not be associated with increased MMP activity in vivo. However, since we also observed decreased expression of TIMP-1 post-MI, the finding of increased lytic activity suggests that MMP activity is increased in vivo and, furthermore, raises the possibility that a decrease in TIMP-1 contributes to increased MMP activity in vivo. A decrease in TIMP-1 activity has been demonstrated in failing human myocardium (21).

Effect of sitaxsentan on MMP activation post-MI. A second new finding of this study is that chronic treatment with sitaxsentan abolished MMP activation and prevented the decrease in TIMP-1 activity. It is possible that sitaxsentan decreased MMP activity by a direct action on cardiac fibroblasts, which are a major source of interstitial MMPs. However, because it has been shown that ET<sub>A</sub> receptor stimulation decreases MMP activity in cardiac fibroblasts in vitro (14), the anticipated effect of ET<sub>A</sub> receptor blockade would be an increase in MMP activity. An alternative explanation is that sitaxsentan caused a decrease in MMP activity indirectly through its vasodilator effects, leading to a decrease in myocardial wall stresses and/or the activity of other pathways that are involved in the regulation of collagen balance.

There is increased expression of ET mRNA and protein in remote myocardium late post-MI (20). Therefore, it is possible that MMPs play a role in the anti-remodeling effect of sitaxsentan. In this regard, it is noteworthy that Spinale et al. (38) showed that a specific MMP inhibitor can reduce the extent of LV dilation in the paced-pig model of heart failure. Likewise, Rohde et al. (31) showed that another MMP inhibitor can decrease the extent of early LV dilation in the first 3 days post-MI in the mouse. Thus our data are consistent with the thesis that increased ET pathway signaling in the remote myocardium late post-MI leads to MMP activation. A corollary of this thesis is that the beneficial effect of sitaxsentan on LV dilation is mediated, at least in part, by a decrease in ET-stimulated MMP activity. ET can stimulate myocyte hypertrophy (16, 37) and exert profound effects on gene expression (37) that might also contribute to the anti-remodeling effects of sitaxsentan.

Collagen content post-MI. Hydroxyproline content was increased in remote myocardium post-MI, suggesting increased collagen content. This finding is similar to that of Cleutjens et al. (3). We further observed that treatment with sitaxsentan tended to increase hydroxyproline content and, therefore, consistent with the observed inhibition of MMP activity by sitaxsentan. However, this finding should be interpreted with caution, since collagen content per se does not reflect other important properties, such as the type and quality of the collagen. For example, increased MMP activity may increase net collagen content while decreasing the abundance of fine collagen struts (42). Further elucidation of the effects of ET and ET blockade on collagen architecture is needed to address this issue.

In conclusion, these findings demonstrate that ET, acting via the ET<sub>A</sub> receptor, plays an important role in chronic post-MI remodeling. The data further show that chronic post-MI remodeling is associated with activation of MMPs, which can be prevented by treatment with an ET<sub>A</sub>-receptor antagonist. This later finding suggests that ET is involved in MMP activation and is consistent with the thesis that MMP activation plays a pathophysiological role in chronic post-MI remodeling. Finally, the data suggest that sitaxsentan may prevent chronic post-MI remodeling, at least in part, by preventing the activation of MMPs.

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