Probucol Attenuates Left Ventricular Dysfunction and Remodeling in Tachycardia-Induced Heart Failure
Roles of Oxidative Stress and Inflammation

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Background—Oxidative stress and inflammation are potentially involved in the pathogenesis of heart failure (HF). We examined whether antioxidant and antiinflammatory treatment with probucol decreases myocardial oxidative stress and inflammation and attenuates the progression of left ventricular (LV) dysfunction and remodeling (dilatation) in tachycardia-induced HF.

Methods and Results—We studied 3 groups of dogs: a sham-operated control group and 2 other groups that underwent ventricular pacing at 240 bpm with and without probucol treatment (100 mg/kg IP per week) for 4 weeks. Dogs that underwent ventricular pacing for 4 weeks developed signs of HF, such as a reduction in the LV ejection fraction and increases in the LV end-diastolic dimension and LV end-diastolic pressure. Myocardial oxidative stress, as measured by electron spin resonance spectroscopy with 4-hydroxy-2,2,6,6-tetramethyl-piperidine-N-oxyl (hydroxy-TEMPO), was significantly increased. There was an increase in myocardial monocyte infiltration, monocyte chemoattractant protein-1 expression, and renin-angiotensin system and matrix metalloproteinase activity. Probucol treatment prevented increases in oxidative stress, inflammation, and matrix metalloproteinase activity and attenuated LV dysfunction and remodeling.

Conclusions—Probucol attenuated LV dysfunction and remodeling, possibly through its antioxidant and/or antiinflammatory effects in ventricular pacing–induced HF. These data suggest that inflammatory disorders, which cause an abnormal interaction between failing myocardium and activated monocytes, have an important role in the progression of HF. (Circulation. 2002;106:362-367.)

Key Words: heart failure ■ stress ■ remodeling ■ inflammation ■ leukocytes

Heart failure (HF) is a serious clinical syndrome with progressive myocardial dysfunction and a poor clinical outcome. Changes in the ventricular geometry (remodeling) are recognized to be a major determinant for the development of impaired ventricular function and a poor prognosis. The mechanisms responsible for the progression of HF and remodeling, however, remain to be elucidated.

Recent evidence suggests that oxidative stress might be involved in the pathogenetic processes in HF. Myocardial oxidative stress and/or inflammation increase in hypertrophied or failing myocardium. There is ample evidence that oxidative stress activates redox-sensitive transcription factors and induces transcription of proinflammatory cytokine (tumor necrosis factor-α, interleukin-6) and chemokine (monocyte chemoattractant protein [MCP]-1, interleukin-8) genes. In particular, recent evidence suggests that the infiltration/activation of monocytes contributes to the development of left ventricular (LV) remodeling and failure. For example, increased monocyte infiltration into failing myocardium, MCP-1 expression, and increased serum concentration of MCP-1 are observed in animals and humans with HF. Elevated circulating levels of MCP-1 correlate with the severity of HF. Transgenic mice overexpressing MCP-1 in the myocardium develop myocarditis and subsequent LV remodeling and HF. The role of oxidative stress and/or inflammation during HF progression, however, has not been fully addressed. Elucidation of the mechanisms of the interaction between activated monocytes and myocardium is important, because activated monocytes are a major source of oxidative stress, proinflammatory cytokines, and matrix metalloproteinases (MMPs). Previous studies reported increased myocardial MMP activity in animal and human models of HF. An MMP inhibitor or absence of MMP limits LV remodeling after myocardial infarction. Angiotensin II, which is central to the pathogenesis of HF, also induces oxidative stress and MCP-1 gene expression in vitro and in vivo.

Recent studies demonstrated that myocardial oxidative stress increases in noninfarcted myocardium after myocardial infarction in mice and that antioxidant treatment with dimeth-

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ithiourea attenuates LV remodeling and failure.\textsuperscript{17} Sia et al\textsuperscript{18} reported that antioxidant treatment with probucol improved survival in rats with large myocardial infarction associated with reduced cardiac fibrosis and expression of inflammatory cytokines. To the best of our knowledge, however, no previous study has addressed the role of oxidative stress in the development of LV dysfunction and remodeling in tachycardia-induced HF. Tachycardia-induced HF serves as a useful experimental model for study of the pathogenesis of human dilated cardiomyopathy, because it produces a well-defined syndrome of biventricular HF and activation of neurohormonal factors, including the renin-angiotensin system, which mimics human dilated cardiomyopathy.\textsuperscript{19} In the present study, we examined whether treatment with probucol attenuates myocardial oxidative stress and inflammation (monocyte infiltration, MCP-1 expression) and MMP activity and thereby inhibits the progression of LV dysfunction and remodeling in a canine model of tachycardia-induced HF.

## Methods

### Animal Model of Pacing-Induced HF

This study was approved by the Committee for the Ethics of Animal Experiments and was conducted according to the animal care guidelines of the American Physiological Society. A part of this study was performed at the Kyushu University Station for Collaborative Research.

Experiments were performed in adult mongrel dogs (18 to 22 kg body weight; Kyuda, Fukuoka, Japan). Under general anesthesia and fluoroscopic guidance, a bipolar pacing lead (1452T/58, Pacesetter body weight; Kyuda, Fukuoka, Japan). Under general anesthesia and ventilated with a respirator. After thoracotomy, aortic arch was cannulated and occluded and the LV was perfused with saline solution during cardiopulmonary bypass. The mitral valve was incised and the LV was inspected for evidence of infarction and scar.

### Echocardiography and Hemodynamic Measurements

Before and after 1, 2, 3, and 4 weeks of pacing, the 3 groups of dogs underwent echocardiographic examinations while conscious and in sinus rhythm, after a 15-minute stabilization period. The internal LV dimensions and the wall thickness of the LV were measured with an ultrasonograph (SSH-160A, Toshiba Medical Inc.). The LV ejection fraction (percent) was then calculated.\textsuperscript{1}

After 4 weeks of ventricular pacing, venous blood was sampled from the antecubital vein of conscious dogs for measurement of hormonal factors. The animals were then anesthetized with pentobarbital and ventilated with a respirator. After thoracotomy, aortic pressure, heart rate, LV pressure, and the positive first derivative of LV pressure (LV dP/dt) were recorded with a polygraph (RM-6000, Nihon-Kohden) while animals were in sinus rhythm.\textsuperscript{2} Dogs were then killed by an overdose of pentobarbital, and the hearts were excised for histopathological and biochemical analysis.

### Hemodynamic Parameters in Anesthetized Dogs After 4 Weeks

<table>
<thead>
<tr>
<th></th>
<th>Control (n=6)</th>
<th>HF+PBS (n=6)</th>
<th>HF+Probul (n=7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart rate, bpm</td>
<td>135±8</td>
<td>137±5</td>
<td>127±6</td>
</tr>
<tr>
<td>Mean aortic pressure, mm Hg</td>
<td>104±4</td>
<td>77±3*</td>
<td>80±3*</td>
</tr>
<tr>
<td>LVEDP, mm Hg</td>
<td>8±1</td>
<td>26±2*</td>
<td>21±2†</td>
</tr>
<tr>
<td>LV dP/dt, mm Hg/s</td>
<td>2371±162</td>
<td>956±37*</td>
<td>1390±76‡</td>
</tr>
</tbody>
</table>

LVEDP indicates LV end-diastolic pressure. Data are mean±SEM. *P<0.01 vs control; †P<0.05 vs HF+PBS; ‡P<0.01 vs HF+PBS.

### Measurement of Myocardial Oxidative Stress

As we described previously, myocardial oxidative stress (OH originating from O\textsubscript{2} and H\textsubscript{2}O\textsubscript{2}) was measured by the electron spin resonance (ESR) method.\textsuperscript{23,24}

### Immunohistochemistry

Immunohistochemistry was performed with frozen OCT compound-embedded sections and antibodies against monocyte (CD14, Dako), MCP-1 (Catalog No. 3279, Enzyme), or nonimmune IgG. The sections were washed and incubated with biotinylated, affinity-purified goat anti-mouse IgG as the secondary antibody. After avidin-biotin amplification, the slides were incubated with 3,3'-diaminobenzidine and counterstained with hematoxylin.

### Myocardial MMP Activity and Tissue ACE Activity

MMP activity in the myocardial tissue was measured with gelatin zymography according to methods described previously. Briefly, the samples extracted from LV myocardium were loaded directly onto electrophoretic gels (SDS-PAGE) containing 1 mg/mL of gelatin under nonreducing conditions. Myocardial tissues of the LV free wall were isolated, and the ACE activity was measured by fluorometric assay as previously described.

### Neurohormonal Factors

Plasma atrial natriuretic peptide, plasma renin activity, and angiotensin II levels were determined with commercially available kits.

### Statistical Analysis

Data are expressed as mean±SEM. Differences between 3 experiments were determined by 2-way ANOVA and Bonferroni’s multiple comparison test. A value of P≤0.05 was considered statistically significant.

### Results

#### Hemodynamics and Echocardiography

Hemodynamic parameters after 4 weeks of pacing are summarized in Table 1. Heart rate was similar in the control, HF+PBS, and HF+probuloc groups. Mean aortic pressure was significantly decreased in the HF+PBS and HF+probuloc groups compared with control groups. LV end-diastolic pressure was significantly elevated and LV...
dP/dt was decreased in the HF/PBS group and attenuated in the HF/PROBUCOL group.

Serial echocardiographic examination revealed progressive LV dilatation and contractile impairment in the HF/PBS group (Figure 1A). Table 2 summarizes the data for echocardiographic measurements after 4 weeks for each group. These changes were attenuated in the HF/probucol group. LV end-diastolic diameter increased and LV ejection fraction decreased in a time-dependent manner in the HF/PBS group (Figure 1, B and C). Probucol treatment significantly inhibited the increase in LV end-diastolic diameter and the decrease in LV ejection fraction after 2, 3, and 4 weeks of pacing.

Myocardial Oxidative Stress

ESR spectroscopy signals of 4-hydroxy-2,2,6,6-tetramethylpiperidine-N-oxyl (hydroxy-TEMPO) declined more rapidly in HF dogs than in controls, and there was a linear relation in the semilogarithmic plot of peak signal intensity versus time. As we reported previously,25 the decay rate of ESR signals of hydroxy-TEMPO did not differ between the control and HF/PBS group after 2 weeks of pacing. At 4 weeks, the decay rate was significantly greater in the HF/PBS group than in controls (Figure 2). The increased decay rate was attenuated by probucol treatment (Figure 2).

AM-3K–Positive Monocyte Infiltration and MCP-1 Expression

After 4 weeks, MCP-1 mRNA expression in the myocardial tissues was not detected in the control group but was markedly increased in the HF/PBS group. The increased expression of MCP-1 mRNA was significantly suppressed by probucol treatment (Figure 3, A and B). There was intense staining of immunoreactive MCP-1 in the endothelial layers (possibly endothelial cells) and infiltrated monocytes (Figure 3C). AM-3K–positive monocytes diffusely infiltrated the interstitial space of the myocardium in HF/PBS dogs (Figure 4A). The number of immunopositive cells per section was significantly greater in HF/PBS dogs than in controls. The increases in AM-3K positive cells were markedly reduced in the HF/probucol group (Figure 4B). The number of SG8H6-positive neutrophils and CD3-positive T lymphocytes did not differ among the 3 groups.

Neurohumoral Parameters and Tissue ACE Activity

After 4 weeks, plasma renin activity, angiotensin II, and atrial natriuretic peptide concentrations were significantly increased in the HF/PBS group. Probucol treatment markedly
Figure 3. Effect of antioxidant treatment with probucol on MCP-1 mRNA levels. A, Representative expression of cardiac MCP-1 and GAPDH mRNA in control, HF + PBS, and HF + probucol groups. B, Densitometric analysis of data shown in A. Expression of MCP-1 mRNA in each sample is normalized relative to GAPDH mRNA expression in same sample. n = 6 in each group. *P < 0.01 vs control group. #P < 0.05 vs HF + probucol group. C, Representative micrographs of myocardial tissues stained immunohistochemically with anti-MCP-1 antibody in control, HF + PBS, and HF + probucol groups. Arrows denote infiltrated monocytes.

Figure 4. Inflammatory changes and MMP activity in LV tissues in control, HF + PBS, and HF + probucol groups. A, Immunohistochemical micrography of LV myocardial sections. AM-3K-positive monocytes diffusely infiltrated myocardium in HF + PBS group. Magnification ×200. B, Number of AM-3K−, SG8H6−, and CD3-positive cells per section. Cells were counted by a single observer who was blinded to treatment protocols. Each section (5 per heart) immunostained with an antibody against AM-3K, SG8H6, and CD3 was scanned at 200× magnification with a light microscope. Number of positive cells in each section was determined, and average number of positive cells per section was calculated for each animal. Values are mean ± SEM. n = 6 in each group. *P < 0.01 vs control group. #P < 0.05 vs HF group. C, Summary data for densitometric analysis of MMP activity in HF + PBS (n = 5) and HF + probucol (n = 5) groups. Data are expressed as ratio to control values run concurrently on same gel. Values are mean ± SEM. *P < 0.01 vs control group. #P < 0.01 vs HF + PBS group.
suppressed these increases (Table 3). Probucol treatment also reduced increased LV tissue ACE activity (Table 3).

Myocardial MMP Activity
After 4 weeks, gelatin zymography of the LV myocardial tissues revealed that MMP activity was significantly increased in the HF+PBS group and suppressed in the HF+probucol group compared with the HF+PBS group (Figure 4C).

Discussion
The major new finding of the present study is that treatment with probucol attenuated myocardial oxidative stress and inflammatory processes (monocyte infiltration, MCP-1 expression, and MMP activation), as well as LV dysfunction and remodeling, in tachycardia-induced HF. These data suggest that oxidative stress and inflammation have an important role in the progression of HF.

Our group has recently provided direct evidence of increased myocardial oxidative stress in dogs with tachycardia-induced HF using the ESR method and immuno-histochemical detection of lipid peroxidation. Here, we report suppression of myocardial oxidative stress by probucol, indicating that probucol prevented increased myocardial oxidative stress in this dog model of HF.

Oxidative stress activates redox-sensitive transcription factors (NF-κB and activating protein-1), which in turn lead to the transcription of many inflammation-related genes. Such redox-sensitive transcription pathways are activated in HF. We demonstrated inflammatory cells in the failing myocardium that were exclusively AM-3K–positive monocytes, but not neutrophils or T lymphocytes. An important finding of the present study is that treatment with probucol not only attenuated LV dysfunction and remodeling but also inhibited myocardial inflammation (monocyte infiltration and MCP-1 expression). MCP-1 is now recognized to be a potent chemokine for monocyte infiltration and activation. Therefore, our present data support the hypothesis that the beneficial cardioprotective effects of probucol are a result of its antiinflammatory effects through attenuated expression of MCP-1. Further studies are needed to determine whether the cardioprotective effects of probucol are a result of its primary antiinflammatory action or its secondary antioxidant action.

Consistent with previous reports, systemic and local renin-angiotensin systems were activated in our model of pacing-induced HF. Angiotensin II induces oxidative stress; activates redox-sensitive transcription, resulting in MCP-1 gene expression; and activates MMP. Another important finding is that treatment with probucol prevented increases in plasma angiotensin II levels, renin activity, and LV tissue ACE activity, suggesting that the beneficial effects of probucol can be explained by the reduction in local and circulating angiotensin II. Further studies are needed to elucidate the mechanism by which probucol prevents activation of the renin-angiotensin system.

It is generally accepted that progressive myocyte cell loss (necrotic and apoptotic cell death), reactive hypertrophy of remaining myocytes, and extracellular matrix changes (matrix degradation caused by MMP activation) contribute to progressive LV dysfunction and remodeling in HF. Recent data suggest that all of these pathological changes occur in tachycardia-induced HF. MMP activation contributes to the degradation of the extracellular matrix, which in turn leads to side-to-side slippage of myocytes and thus accounts for the LV remodeling (dilatation) in HF. Spinale et al demonstrated that inhibition of MMP activity in pacing-induced HF limited LV dilatation and dysfunction. In the present study, treatment with probucol attenuated myocardial MMP activation in failing myocardium. The present data therefore extend our previous findings and those of others and suggest that myocardial inflammatory processes activate myocardial MMPs, which in turn leads to the development of LV dysfunction and remodeling in HF. Further studies are needed to determine whether lesional monocytes are responsible for increased MMP activity in the failing myocardium.

In summary, treatment with probucol attenuated LV dysfunction and remodeling in tachycardia-induced HF. The beneficial effect of probucol is a result of not only its inhibition of myocardial oxidative stress but also inhibition of inflammatory processes (monocyte infiltration and activation by MCP-1). The present study suggests that inflammatory processes, which cause abnormal interactions between failing myocardium and activated monocytes, are involved in the progression of HF.

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References
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