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

Ryo Nakamura, MD; Kensuke Egashira, MD; Youji Machida, MD; Shunji Hayashidani, MD; Motohiro Takeya, MD; Hideo Utsumi, MD; Hiroyuki Tsutsui, MD; Akira Takeshita, MD

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
ylthiourea 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 AB) was placed in the apical area of the right ventricle. Ventricular pacing was then performed at 240 bpm continuously for 4 weeks. Before and 7, 14, and 21 days after ventricular pacing began, dogs were randomly assigned to receive an intraperitoneal injection of PBS (HF$^+$ PBS group) or 100 mg/kg probucol (HF$^+$ probucol group). PBS or probucol was injected after the echocardiographic study was completed. Probucol scavenges superoxide\textsuperscript{20} and inhibits oxidative modification of low-density lipoprotein.\textsuperscript{21} This dose of probucol was chosen because intraperitoneal injection of probucol at doses ranging from 12.5 to 120 mg/kg every 3 to 7 days prevents doxorubicin-induced oxidative stress and myocardial dysfunction in rats and dogs.\textsuperscript{22,23} Intraperitoneal injection was selected because probucol is poorly absorbed from the gastrointestinal tract, with only 2% to 8% of the dose reaching the circulation. To determine whether probucol reached the myocardial tissues, we measured the probucol concentration in the myocardial tissues using high-performance liquid chromatography.\textsuperscript{24} The probucol concentration in myocardial tissues after 28 days of pacing (7 days after the 4th injection of probucol) was 21.9±2.0 μg/g wet tissue. Control dogs were treated in the same manner as HF dogs without pacing.

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).\textsuperscript{3} The LV ejection fraction (percent) was then calculated.\textsuperscript{3} 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{3} Dogs were then killed by an overdose of pentobarbital, and the hearts were excised for histopathological and biochemical analysis.

### TABLE 1. Hemodynamic Parameters in Anesthetized Dogs After 4 Weeks

<table>
<thead>
<tr>
<th>Group</th>
<th>Control</th>
<th>HF$^+$ PBS</th>
<th>HF$^+$ Probucol</th>
</tr>
</thead>
<tbody>
<tr>
<td>(n=6)</td>
<td>(n=6)</td>
<td>(n=7)</td>
<td></td>
</tr>
<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>LV dP/dt, mm Hg/s</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>

LV dP/dt 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$_2$ and H$_2$O$_2$) was measured by the electron spin resonance (ESR) method.\textsuperscript{3,25}

Northern Blot Analysis

Immediately after the hemodynamic measurements, the hearts were removed, snap-frozen in liquid nitrogen, and stored at −80°C. Total RNA was extracted from each sample by the acid guanidinium thiocyanate–phenol-chloroform method. Northern blot hybridization was performed as described previously.\textsuperscript{26} The cDNA probes used included dog MCP-1 (a gift of Dr Leonardo Mendoza, Baylor College of Medicine, Waco, Tex). Relative amounts of MCP-1 were normalized against the amount of GAPDH mRNA.

Immunohistochemistry

Immunohistochemistry was performed with frozen OCT compound–embedded sections and antibodies against monocyte (AM-3K), 27 T lymphocyte (CD3, Dako), MCP-1 (Catalog No. 43279, Genzyme), or nonimmune IgG. The slides 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.\textsuperscript{17} 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.\textsuperscript{29}

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$^+$ probucol groups. Mean aortic pressure was significantly decreased in the HF$^+$ PBS and HF$^+$ probucol 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, 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).

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+PBS 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. 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.

**Acknowledgments**

This study was supported by Grants-in-Aid for Scientific Research (11470164, 11158216, 11557056) from the Ministry of Education, Science, and Culture, Tokyo, Japan; by the Ryouichi Naito Foundation for Medical Research, Osaka, Japan; and by a Research Grant from Takeda Medical Research Foundation and Takeda Science Foundation, Osaka, Japan.

**References**


**TABLE 3. Plasma ANP, Angiotensin II, Renin Activity, and LV Tissue ACE Activity**

<table>
<thead>
<tr>
<th></th>
<th>Control (n=6)</th>
<th>HF+PBS (n=6)</th>
<th>HF+Probucol (n=7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma ANP, pg/mL</td>
<td>47.2±8</td>
<td>340.4±45</td>
<td>239.1±19†</td>
</tr>
<tr>
<td>Plasma angiotensin II, pg/mL</td>
<td>43.6±6</td>
<td>194.5±58</td>
<td>56.2±24†</td>
</tr>
<tr>
<td>Plasma renin activity, ng·mL⁻¹·h⁻¹</td>
<td>1.7±0.6</td>
<td>7.4±1.9*</td>
<td>2.1±0.7†</td>
</tr>
<tr>
<td>LV tissue ACE activity, nmol·mg wet wt⁻¹·h⁻¹</td>
<td>0.25±0.01</td>
<td>0.36±0.03*</td>
<td>0.27±0.04†</td>
</tr>
</tbody>
</table>

ANP indicates atrial natriuretic peptide. Plasma ANP, angiotensin II, and renin activity were measured from blood samples taken while animals were conscious. Data are expressed as mean±SEM.

*P<0.01 vs control; †P<0.05 vs HF; ‡P<0.01 vs HF.


Probucol Attenuates Left Ventricular Dysfunction and Remodeling in Tachycardia-Induced Heart Failure: Roles of Oxidative Stress and Inflammation
Ryo Nakamura, Kensuke Egashira, Youji Machida, Shunji Hayashidani, Motohiro Takeya, Hideo Utsumi, Hiroyuki Tsutsui and Akira Takeshita

Circulation. 2002;106:362-367; originally published online June 24, 2002;
doi: 10.1161/01.CIR.0000021430.04195.51
Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2002 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/106/3/362

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation is online at:
http://circ.ahajournals.org//subscriptions/