Beneficial Effects of Long-Term Use of the Antioxidant Probucol in Heart Failure in the Rat

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Background—Congestive heart failure (CHF) is a disease that is characterized by progressive left ventricular (LV) dysfunction and dilatation. Oxidative stress is thought to contribute to the progression of CHF, and antioxidants have been shown to have beneficial effects when started early after myocardial infarction (MI). In this study, we tested whether the powerful antioxidant probucol would attenuate progression of CHF once it was established after MI in the rat.

Methods and Results—Ligation of a coronary artery was used to create an MI in rats (n=266). Survivors were then randomized 20 days after MI to either probucol 61 mg · kg⁻¹ · d⁻¹ or vehicle and followed up for a total of 100 days after MI. Studies of cardiac hemodynamics, LV remodeling, cardiac apoptosis and morphology, systemic neurohumoral activation, oxidative stress, and renal function were then evaluated. Probucol improved LV function (LV maximum rate of pressure rise from 3103 to 4250 mm Hg/s, \( P<0.05 \), and LV end-diastolic pressure decrease from 28 to 24 mm Hg, \( P<0.05 \)), reduced pulmonary weights, prevented right ventricular systolic hypertension, and preserved renal function compared with vehicle. Probucol also prevented LV dilatation, prevented wall thinning (1.70 versus 1.42 mm, \( P<0.05 \)), reduced cardiac fibrosis and cardiac apoptosis, attenuated increased myocardial cell cross-sectional area, and increased scar thickness.

Conclusions—In chronic CHF, probucol exerts multiple beneficial morphological effects that result in better LV remodeling and function, reduced neurohumoral activation, and preserved renal function. (Circulation. 2002;105:2549-2555.)

Key Words: myocardial infarction ■ probucol ■ heart failure ■ remodeling ■ apoptosis

Large myocardial infarction (MI) is a major cause of chronic heart failure (CHF). CHF is a progressive disease characterized by prolonged excessive neurohumoral activation and progressive ventricular dilatation.¹ Despite current aggressive medical therapy with neurohumoral inhibitors, morbidity and mortality of patients with ischemic CHF remain unacceptably high. Evidence from both animal and human studies suggests that increased free radical formation and oxidative stress are associated with a poor prognosis and may play an important role in the pathogenesis and progression of CHF.² A number of studies suggest that antioxidants may be beneficial after MI and in Adriamycin-induced cardiomyopathy.³ Palace et al³ and Takeshita et al⁵ demonstrated that the antioxidants vitamin E and dimethylthiourea (DMTU), respectively, helped preserve ventricular function when started before or early after MI. These observations provide evidence for an adverse role of oxidative stress after large MI and suggest that administration of exogenous antioxidants may be an important component in the management of chronic CHF.

In the present study, we tested the hypothesis that the antioxidant probucol would improve left ventricular (LV) function and LV remodeling in the rat model of chronic CHF. Probucol is a lipid-soluble, cholesterol-lowering drug with potent antioxidant properties that has been shown to protect myocardial function and endogenous antioxidant reserve in both early and late Adriamycin-induced cardiomyopathy.³,⁶

Methods

Animal Care

Male Wistar rats weighing 200 to 250 g were obtained from Charles River Breeding Laboratories (Saint-Constant, Quebec, Canada). All care was in accordance with the Canadian Council for Animal Care and the Animal Care Committee guidelines of the Montreal Heart Institute.
MI Operative Procedure
MI was induced in rats by ligation of the left anterior coronary artery as described previously. Animals surviving 20 days after MI were randomized to 2 treatment groups. Animals were classified into sham, small- to medium-MI, or large-MI groups at the end of the study according to previously described criteria based on a relationship between MI circumference, scar to body weight (BW) ratio, and LV end-diastolic pressure. Rats with a large MI had an LV scar >45% of the LV circumference or a scar to BW ratio >0.2 g/kg, and those with a small to medium MI had an LV scar <45% or scar to BW ratio <0.2 g/kg. Rats that died after randomization but before hemodynamic monitoring also had morphological assessment for classification of MI size.

Pharmacological Interventions
Rats surviving 20 days after MI were randomly assigned to receive either vehicle (soybean oil) or probucol (61 mg·kg⁻¹·d⁻¹). All rats were then treated for 80 days by daily gavage (0.5 mL).

Renal Function at 100 Days After Infarction
A subgroup of 28 rats had 0.5 mL of blood drawn from the tail to measure serum creatinine and sodium by chromatography. Rats were then placed in metabolic cages for 24-hour urine collection for measurement of urinary creatinine and sodium

\[ \frac{U_{\text{creatinine}} \times \text{urine flow rate}}{P_{\text{creatinine}}} \]

where \( U_{\text{creatinine}} \) and \( P_{\text{creatinine}} \) represent, respectively, urine and plasma concentrations of creatinine (\( \mu \)mol/L).

Cardiac Hemodynamic Studies
After a total of 100 days had passed after MI, rats were anesthetized with 1% halothane mixed with 100% \( \text{O}_2 \), and LV and right ventricular hemodynamics were recorded as described previously.

Plasma Neurohumoral Measurements
Approximately 10 mL of plasma was drawn from each rat for measurement of plasma atrial natriuretic peptide and plasma norepinephrine as described previously.

Circulating Oxidative Stress by Gas Chromatography–Mass Spectrometry
Circulating oxidative stress was assessed by measurement of plasma malondialdehydes by gas chromatography mass spectrometry by a method previously described by Luo et al.

Passive Pressure-Volume Relationship
After cardiac hemodynamic measurements were completed, 21 rats had 3 passive pressure-volume curves obtained within 10 minutes, and an average of these 3 curves was used as the final value, as described previously.

Cardiac Remodeling
Once the pressure-volume curve was completed, the LV was filled with saline solution to a pressure of 15 mm Hg, sealed, and fixed in its distended form in 10% formalin phosphate buffer for 24 hours. Two cross sections were obtained at 1-mm intervals midway between the base and the apex of the LV for measurement of epicardial and endocardial circumferences by planimetry, as described previously. Average scar thickness was obtained directly by planimetry.

LV fibrosis was assessed by determination of collagen density (picrosirius red) on tissues obtained from the 2 cross sections as described previously. Quantification was performed in cardiac regions away from the scar and away from collagen-rich vascular areas (5 in epicardium, 3 in endocardium, and 2 in septa). To explore the characteristics of the scar that led to increased thickness in probucol-treated MI rats, slides were stained with hematoxylin and eosin, and stains for connective tissue with hematoxylin, phloxine, and safranine were performed on mid-LV cross sections of 8 probucol-treated and 8 vehicle-treated large-MI rats. All histological sections were analyzed in a blinded manner, and cell counts were performed at a magnification of ×20 at 5 predetermined points in the endocardial and epicardial portions of the scar: 2 junctional areas of the infarct, the center of the scar, and midway between the junctional area and the center of the scar.

Cardiomyocyte Cross-Sectional Area Measurement
Myocyte cross-sectional areas were evaluated on histological sections as described previously. For each mid-LV histological section, 3 epicardial and 3 endocardial areas that displayed cross sections of cardiomyocytes were selected. In each selected area, the areas of 20 to 30 cross-sectioned cardiomyocytes were calculated with Northern Eclipse version 6.0 software (Empix Imaging Inc).

Detection of Apoptosis
In Situ End Labeling
Sections 5 µm thick from the LV mid cavity were stained with the CardioTACS in situ apoptosis detection kit according to the manufacturer’s instructions (R&D Systems). For each heart, the number of ISEL-positive cells was scored per total number of cells per high-power field. A total of 17 high-power fields were scored in the peri-infarct area for each rat.

DNA Laddering
The methodology used was similar to that reported by Hu and Van Eldik.

Statistical Analyses
All values are expressed as mean±SEM. Results were analyzed by 2-tailed Student’s \( t \) test for unpaired data and by ANOVA for multiple comparisons, followed by a 2-sided Dunnett test when appropriate. Statistical significance was assumed at \( P<0.05 \). Kaplan-Meier survival curves over the follow-up period were constructed and analyzed by the generalized Savage (Mantel-Cox) test.

Results
Survival
The 116 rats that survived 20 days after MI were randomly allocated to either soybean oil (vehicle; \( n=61 \)) or probucol (\( n=55 \)). The survival rate of sham-operated rats was excellent. The mortality rate of rats with a medium MI was similar in both treatment groups (15.4% in control group and 13.3% in the probucol-treated group). The mortality rate of rats with a large MI treated with vehicle (12 deaths [35.3%]) was not statistically different from the probucol group (5 deaths [16.1%]; \( P=0.13 \)). The numbers of rats that survived the full 100 days were as follows: sham vehicle, \( n=14 \); sham probucol, \( n=7 \); medium-MI vehicle, \( n=13 \); medium-MI probucol, \( n=17 \); large-MI vehicle, \( n=34 \); and large-MI probucol, \( n=31 \).

Hemodynamic Measurements
In sham-operated rats, probucol treatment had no effect on any of the hemodynamic variables measured (Table 1). In rats with medium MI, probucol also had little effect, except for an increase in heart rate compared with control (vehicle) medium MI. Control rats with large MI had a decrease in heart rate, LV systolic pressure, maximal rate of pressure rise and decline (LV \( +/- \) dP/dt), and mean aortic pressure compared with their sham counterparts. These were accompanied by an increase in both right ventricular systolic and end-diastolic pressure.
pressures and LV end-diastolic pressure. Rats with a large MI treated with probucol had less of a decrease in heart rate, mean aortic pressure, and LV systolic pressure and had an improvement in LV $+\overline{d}P/dt$, LV end-diastolic pressure, right ventricular systolic pressure, and right ventricular end-diastolic pressure compared with vehicle rats with a large MI.

Renal Function at 100 Days After Infarction
In sham-operated rats, few differences in renal function occurred. Control (vehicle) rats with large MI had a decrease in BW (537±14 versus 613±25 g; P<0.05), which was not found in the probucol large-MI group (599±15 g). In control large-MI rats, serum creatinine increased (54±3 versus 44±2 $\mu$mol/L for sham control; P<0.05) and creatinine clearance decreased (2.2±0.1 versus 3.1±0.2 mL/min for sham control; P<0.05), which suggests chronic renal dysfunction. Treatment with probucol attenuated the increase in serum creatinine (49±2 $\mu$mol/L) and decrease in creatinine clearance (2.7±0.1 mL/min; P<0.05 versus control large MI). Serum sodium did not decrease in either large-MI group.

Plasma Neurohumoral Levels at 100 Days After Infarction
In the 2 sham-operated groups, plasma norepinephrine (307±77 versus 324±114 pg/mL) and atrial natriuretic peptide (48±15 versus 50±14 pg/mL) levels for control (vehicle) and probucol, respectively, were similar. Compared with sham, there was a significant increase in plasma norepinephrine (938±203 pg/mL, P<0.05) in the large-MI control group, but because of a large standard deviation, the increase in atrial natriuretic peptide (92±42 pg/mL) did not reach statistical significance. Probucol treatment in rats with large MI resulted in a significant decrease in norepinephrine (450±77 pg/mL; P<0.05), but there was no effect on atrial natriuretic peptide (92±60 pg/mL) compared with the control large-MI group.

Circulatory Oxidative Stress
There was no difference in circulating malondialdehydes in the 2 sham groups (410±48 pg/mL for vehicle versus 346±48 pg/mL for probucol; P=NS). In the vehicle large-MI group, malondialdehydes were increased (552±39 pg/mL; P=0.04 versus sham vehicle), and this increase was inhibited by probucol treatment (430±40 pg/mL; P=0.05 versus vehicle large MI).

Passive Pressure-Volume Relationship
The passive pressure-volume relationships of the 2 sham-operated groups were similar (Figure 1). A large MI caused a rightward shift of this relationship in both groups compared with the 2 sham-operated groups, which indicates ventricular dilatation after large MI. Consistent with less ventricular dilatation after large MI. Consistent with less ventricular

### Table 1. Hemodynamic Monitoring at 100 Days Postinfarction

<table>
<thead>
<tr>
<th></th>
<th>HR, bpm</th>
<th>RVSP, mm Hg</th>
<th>RVEDP, mm Hg</th>
<th>LVSP, mm Hg</th>
<th>LVEDP, mm Hg</th>
<th>LV $+\overline{d}P/dt$, mm Hg/s</th>
<th>LV $-\overline{d}P/dt$, mm Hg/s</th>
<th>MAP, mm Hg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle</td>
<td>414±11</td>
<td>27.5±1.4</td>
<td>1.8±0.6</td>
<td>130.5±4.4</td>
<td>8.2±1.2</td>
<td>6211±370</td>
<td>−6025±388</td>
<td>113.1±4.1</td>
</tr>
<tr>
<td>Probucol</td>
<td>404±39</td>
<td>27.3±1.2</td>
<td>0.9±0.4</td>
<td>121.6±10.6</td>
<td>6.5±2.3</td>
<td>5571±582</td>
<td>−4850±484</td>
<td>103.5±9.4</td>
</tr>
<tr>
<td>Large MI</td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle</td>
<td>356±11</td>
<td>47.9±1.8*</td>
<td>9.8±1.19*</td>
<td>90.3±3.0*</td>
<td>27.5±1.6*</td>
<td>3103±171*</td>
<td>−2458±153*</td>
<td>80.9±3.1*</td>
</tr>
<tr>
<td>Probucol</td>
<td>393±10†</td>
<td>39.6±2.8†</td>
<td>5.7±1.3†</td>
<td>110.3±3.7†</td>
<td>24.3±2.4†</td>
<td>4250±290†</td>
<td>−3369±314†</td>
<td>98.6±3.7†</td>
</tr>
</tbody>
</table>

*HR indicates heart rate; RVSP, right ventricular systolic pressure; RVEDP, right ventricular end-diastolic pressure; LVSP, LV systolic pressure; LVEDP, LV end-diastolic pressure; and MAP, mean arterial pressure.

*P<0.05 vs sham; †P<0.05 vs vehicle.
dilatation and wall thinning in mid-LV cross sections of probucol-treated hearts with a large MI, the pressure-volume relationship in this group was shifted leftward compared with the untreated large-MI group, which indicates less ventricular dilatation \( (P<0.05) \).

**Ventricular Remodeling and Morphology**

Probucol treatment had little effect on cardiac morphology in the sham-operated rats (Table 2). Only the control large-MI group had a decrease in BW. Both large-MI groups had an increase in right ventricular weight/BW ratio and atrial weight/BW ratio compared with sham-operated rats. This increase was less in the probucol-treated rats, which suggests that there was less evidence of lung congestion, a finding supported by an increase in wet to dry lung weight ratio in vehicle large-MI compared with probucol large-MI rats. As expected, scar weight and scar weight/BW ratios were similar in both large-MI groups. No difference was found in LV weight/BW ratio between large-MI groups.

No difference was found in mid-LV cross-section characteristics between the 2 sham-operated groups (Table 2; Figure 2). As expected, both large-MI groups had similar MI size. In control (vehicle) rats with a large MI, LV epicardial and endocardial circumferences were significantly increased compared with their sham counterparts. There was also an important decrease in LV wall thickness, which suggests that progressive ventricular dilatation was not accompanied by concentric hypertrophy after large MI. Probucol treatment resulted in significantly thicker LV walls than in the control large-MI group. This was accompanied by a decrease in epicardial and endocardial circumferences. This reflected a tendency toward a more appropriate balance between dilatation and ventricular hypertrophy, which would be beneficial in heart failure from both mechanical and bioenergetic points of view.

Cardiac fibrosis was not modified by probucol in the sham groups. The control (vehicle) large-MI group had a significant increase in cardiac fibrosis, an increase that was only partially attenuated by the use of probucol.

<table>
<thead>
<tr>
<th>TABLE 2. Morphology Parameters of Heart at 100 Days Postinfarction</th>
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<tbody>
<tr>
<td><strong>Sham</strong> (Vehicle)</td>
</tr>
<tr>
<td>Body weight, g</td>
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<tr>
<td>Scar weight, g</td>
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<tr>
<td>SW/BW, g/kg</td>
</tr>
<tr>
<td>Infarct size, %</td>
</tr>
<tr>
<td>LVW/BW, g/kg</td>
</tr>
<tr>
<td>RVW/BW, g/kg</td>
</tr>
<tr>
<td>AW/BW, g/kg</td>
</tr>
<tr>
<td>Lung wet/dry weight</td>
</tr>
<tr>
<td>Mean myocardial wall thickness, mm</td>
</tr>
<tr>
<td>Endocardial circumference, mm</td>
</tr>
<tr>
<td>Epicardial circumference, mm</td>
</tr>
<tr>
<td>Cardiac fibrosis‡</td>
</tr>
<tr>
<td>Endocardium myocyte cross-sectional area, ( \mu m^2 )</td>
</tr>
<tr>
<td>Epicardium myocyte cross-sectional area, ( \mu m^2 )</td>
</tr>
</tbody>
</table>

SW indicates scar weight; LVW, LV weight; RVW, right ventricular weight; and AW, atria weight.

*\( P<0.05 \) vs sham; †\( P<0.05 \) vs vehicle.

‡Collagen density is expressed as ratio of area occupied by collagen on total area.

\( n=14 \) to 21

Figure 2. Representative LV midventricular cross sections of sham rat (A) and rats with large MI treated with vehicle (B) or probucol (C) and LV collagen images (original magnification \( \times100 \)) of sham rat (D) and rats with large MI treated with vehicle (E) or probucol (F).
Cardiomyocyte Cross-Sectional Area
No difference was found in individual cardiomyocyte cross-sectional area (Figure 3) between the 2 sham groups (sham endocardium vehicle 230±33 μmol/L, probucol 209±33 μmol/L; epicardium vehicle 199±46 μmol/L, probucol 196±33 μmol/L). In the control large-MI group, LV cardiomyocyte cross section was increased compared with control sham, but this increase reached statistical significance only in the epicardial layer (large-MI vehicle endocardium 248±19 μmol/L, epicardium 264±34 μmol/L; P<0.05 versus sham). Probucol prevented this increase in cardiomyocyte cross-sectional area in the large-MI group (endocardium 216±23 μmol/L, epicardium 238±19 μmol/L; P<0.05 versus vehicle large MI).

Assessment of Cardiac Apoptosis
There was little evidence of apoptosis in the sham-operated rat hearts as evaluated by either ISEL-positive cell staining (TUNEL staining, Figure 4) or DNA agarose gel electrophoresis (DNA laddering, Figure 5). Rat hearts from control (vehicle) rats with a large MI had evidence of significant apoptosis by both techniques. Probucol-treated hearts with a large MI had a significant reduction of apoptosis by both techniques.

Discussion
This study demonstrates that the use of probucol, once chronic CHF is established after MI, results in improved hemodynamics and renal function, as well as reduced pulmonary congestion and neurohumoral activation. These beneficial effects of probucol appear to be the result of improved ventricular remodeling, which includes less ventricular dilatation, cardiac fibrosis, ventricular wall thinning, cardiac apoptosis, and reactive hypertrophy of remaining cardiomyocytes and an increase in scar thickness. These beneficial effects of probucol may have been related to its antioxidant properties, as reflected by normalization of circulating malondialdehydes, an index of oxidative stress. Taken together, these findings suggest that the antioxidant probucol has beneficial effects when given chronically once post-MI CHF is established.

This is the first study to evaluate the effects of an antioxidant in chronic CHF and thus the first to show beneficial effects in this setting. Probucol prevented the rise in circulating malondialdehydes that was found in control rats with large MI. In a study of Adriamycin-treated rats and in a previous early post-MI study, a similar dose of probucol was shown to be both an effective antioxidant and cardioprotective. Congestive heart failure has been associated with an increase in free radical formation and subsequent oxidative stress. When endogenous antioxidant reserve is exhausted, oxidative stress damages the myocytes and extracellular matrix through lipid peroxidation and oxidative modification of cellular proteins and DNA, and together this can lead to progressive heart failure. Indeed, a decline of antioxidant reserve coincides with the occurrence of heart failure in the post-MI setting. Moreover, free radicals stimulate cytokine expression and apoptotic cell death in failing myocardium. Thus, in the present study, probucol may have exerted its beneficial effects via its antioxidant effects.

The most remarkable findings of the present study are the beneficial effects of probucol on LV remodeling. In previous studies, the antioxidant DMTU resulted in extremely beneficial ventricular remodeling and preservation of LV function when started early (6 hours) after MI in the mouse; probucol started early (<24 hours) after MI in the rat did not result in beneficial LV remodeling, yet it had impressive benefits for LV function and survival. In the present study, probucol was started 20 days after MI, a time when CHF is well established, significant adverse ventricular remodeling has already occurred, and further ventricular remodeling does not involve the scar but only slowly progressive remodeling of the remaining viable heart. In this setting, the long-term use of probucol resulted in a decrease in LV dilatation and wall thinning, a decrease in reactive cardiomyocyte hypertrophy and cardiac fibrosis, and an increase in scar thickness. The difference in LV remodeling between this and our previous study may have resulted from a longer follow-up period in the present study (80 versus 27 days of treatment), a period of time that may have permitted the full expression of the changes that accompany LV remodeling in CHF and the effects of probucol on this process.

In the control large-MI hearts, there was an increase in cardiac apoptosis, cardiac fibrosis, and reactive hypertrophy of the remaining cardiomyocytes. This, perhaps combined with cardiomyocyte cell slippage, resulted in marked LV dilatation and reactive hypertrophy of the remaining cardiomyocytes that was inadequate to maintain normal LV wall thickness despite maintenance of LV weight. The result was LV dysfunction and most likely an increase in wall stress,
unfavorable cardiac bioenergetics that served as further stimuli for hypertrophy and cell slippage.

We identified 3 potential mechanisms by which probucol may have exerted beneficial effects on ventricular remodeling, thereby reducing LV dysfunction. First, probucol reduced cardiac apoptosis, a finding consistent with previous work with antioxidants. Cardiac apoptosis is known to occur after MI, particularly in the peri-MI region, and this is thought to contribute to progressive deterioration of LV function after MI. Second, probucol decreased cardiac fibrosis and hypertrophy, thereby helping to preserve LV function. Third, by its antifibrotic and antiapoptotic properties, it is possible that probucol prevented extracellular matrix disruption and cell-to-cell slippage. Indeed, the antioxidant DMTU has been shown to reduce activation of metalloproteinase-2, which along with other metalloproteinases is thought to contribute to cardiomyocyte slippage and thus ventricular dilatation. Reduced activation of metalloproteinase inhibitors combined with local fibroblast activation in the scar could account for the increase in scar thickness in the probucol group.

The long-term use of probucol helped preserve LV function. This was accompanied by reduced neurohumoral activation, reduced pulmonary congestion and right ventricular hypertrophy, improved renal function, and prevention of weight loss. Preservation of LV function with probucol was likely the result of the convergence of a number of mechanisms, the most important being the beneficial effect of probucol on ventricular remodeling. By reducing LV dilatation, LV wall thinning, and cardiac fibrosis, and perhaps by increasing scar thickness, probucol would be expected to reduce LV wall stress and to improve cardiac efficiency and bioenergetics. A second mechanism by which probucol may have improved LV function is by reducing the direct negative effects on heart function.
inotropic effects of oxygen free radicals associated with CHF and neurohumoral activation.

All of these beneficial effects would be expected to result in improved survival. Although the improvement in survival in the present study was not significant (84% versus 65%, P = 0.1), the study was not powered for a survival benefit, and the lack of statistical significance may have resulted from a type II error. We thus conclude that once chronic congestive heart failure is established, probucol markedly improves LV remodeling, which results in improved LV and renal function and reduced neurohumoral activation.

References


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