Probucol Promotes Endogenous Antioxidants and Provides Protection Against Adriamycin-Induced Cardiomyopathy in Rats

N. Siveski-Iliskovic, MD; N. Kaul, PhD; P.K. Singal, PhD

Background The potential usefulness of adriamycin (ADR) is restricted because of its cardiotoxic side effects. Since free radicals and lipid peroxidation are suggested to be involved in ADR cardiomyopathy, we examined the beneficial effects of probucol, a lipid-lowering drug with strong antioxidant properties.

Methods and Results ADR was administered to rats in six equal intraperitoneal injections over a period of 2 weeks (cumulative dose of 15 mg/kg). After a 3-week posttreatment period, cardiomyopathy and congestive heart failure were characterized by ascites, congested liver, depressed cardiac function, elevated left ventricular end-diastolic pressure, and myocardial cell damage. Myocardial glutathione peroxidase (GSHPx) activity was decreased, and lipid peroxidation was increased. Probucol (cumulative dose, 60 mg/kg IP) was administered in six equal injections over a 2-week period on days alternating with ADR treatment. Probucol significantly attenuated the myocardial effects of ADR, improved left ventricular function, and lowered mortality as well as the amount of ascites. Treatment with probucol was also accompanied by an increase in myocardial GSHPx and superoxide dismutase activities, with a concomitant decrease in lipid peroxidation.

Conclusions These data provide evidence that ADR cardiomyopathy is associated with an antioxidant deficit. Improved cardiac function resulting from treatment with probucol may be related to the maintenance of the antioxidant status of the heart. The study suggests potential usefulness of antioxidant (probucol) therapy in ADR cardiomyopathy. (Circulation. 1994;90:2829-2835.)

Key Words • heart failure, congestive • glutathione peroxidase • superoxide dismutase • lipids

Adriamycin, also called doxorubicin, is an effective antitumor drug used against a variety of carcinomas. However, the potential usefulness of adriamycin is restricted because of its cardiotoxic side effects and the development of congestive heart failure that is refractory to all known therapeutic procedures.1,2 Adriamycin-induced myocardial dysfunction has been suggested to involve inhibition of nucleic acid as well as protein synthesis,3,4 release of vasoactive amines,5 changes in adrenergic functions,6 abnormalities in the mitochondria,7 lysosomal alterations,8 alterations in sarcoplasmic Ca2+ transport,9,10 membrane-bound enzymes,11 imbalance of myocardial electrolytes,12 free radical formation,13,14 and lipid peroxidation.2,8,15 Although a close examination of this list indicates that adriamycin-induced injury may be multifactorial and complex,16 one mechanism common to most of these suggestions is the increased oxidative stress.2 Because of the presence of semiquinone in the tetracyclic aglycone molecule of adriamycin, the drug is reported to increase the oxygen radical activity13,14 as well as peroxidation of polysaturated fatty acids within the membrane phase.15 This may also explain adriamycin-induced defects in membrane function caused by this drug.2,9,17

An acute study involving a single injection of adriamycin (15 mg/kg) in mice showed that a single pretreatment with 85 IU of vitamin E provided protection with respect to cardiac cell damage that was also accompanied by a reduction in lipid peroxidation.15 In the present study, we examined the beneficial effects of repeated treatment with probucol, a lipid-lowering drug with strong antioxidant property, in a chronic model of adriamycin-induced congestive heart failure in rats. In addition, hemodynamic function, myocardial ultrastructure, lipid peroxidation, and various antioxidant enzyme activities were also studied.

Methods

Animal Model

Male Sprague-Dawley rats, body weight 250±10 g, were maintained on a normal rat chow diet. Rats were divided into four groups: CONT (control), ADR (adriamycin treated), PROB (probucol treated), and PROB+ADR (probucol+adriamycin treated). Adriamycin (doxorubicin hydrochloride) was administered intraperitoneally in six equal injections (each containing 2.5 mg/kg adriamycin) to animals in the ADR and PROB+ADR groups over a period of 2 weeks for a total cumulative dose of 15 mg/kg body weight. Probucol (cumulative dose, 60 mg/kg body weight) was also administered intraperitoneally to PROB and PROB+ADR groups in six equal injections (each treatment containing 10 mg/kg) over a period of 2 weeks, alternating with adriamycin injections. CONT animals were injected with the vehicle alone (lactose, 75 mg/kg in saline) in the same regimen as ADR. Treated as well as CONT animals were observed for up to 3 weeks after the last injection for their body weight, general appearance, behavior, and mortality. At the end of the 3-week
posttreatment period, animals were hemodynamically assessed. Hearts were used for the study of myocardial antioxidants, lipid peroxidation, and ultrastructure.

**Hemodynamic Studies**

Animals were anesthetized with sodium pentobarbital (50 mg/kg IP). A miniature pressure transducer (Millar MicroTip) was inserted into the left ventricle via the right carotid artery. Left ventricular systolic (LVSP), left ventricular end-diastolic (LVEDP), aortic systolic (ASP), and aortic diastolic (ADP) pressures were recorded on a Beckman Dynograph.

**Bioassays**

**Catalase Assay**

Ventricles (1 g) were homogenized in 10 mL 0.05 potassium phosphate buffer (pH 7.4) and centrifuged at 40,000g for 30 minutes. Supernatant, 50 μL, was added to the cuvette containing 2.95 mL of 19 mmol/L H2O2 solution prepared in potassium phosphate buffer.18 The color was read at 240 nm on a Zeiss spectrophotometer every minute for 5 minutes. Commercially available catalase was used as a standard. Specific activity of the enzyme was expressed as units per milligram tissue protein.

**Glutathione Peroxidase (GSHPx) Assay**

GSHPx activity was expressed as nanomoles reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidized to nicotinamide adenine dinucleotide phosphate (NADP) per minute per milligram protein, with a molar extinction coefficient for NADPH at 340 nm of 6.22×10³.19 Cytosolic GSHPx was assayed in a 3-mL cuvette containing 2.0 mL of 75 mmol/L phosphate buffer, pH 7.0. The following solutions were then added: 50 μL of 60 mmol/L glutathione, 100 μL glutathione reductase solution (30 U/mL), 50 μL of 0.12 mol/L Na2S, 0.10 of 15 mmol/L Na2EDTA, 100 μL of 3.0 mol/L NADPH, and 100 μL of cytosolic fraction obtained after centrifugation at 20,000g for 25 minutes. Water was added to make a total volume of 2.9 mL. The reaction was started by the addition of 100 μL of 7.5 mmol/L H2O2, and the conversion of NADPH to NADP was monitored by a continuous recording of the change of absorbance at 340 nm in 1-minute intervals for 5 minutes. Enzyme activity of GSHPx was expressed in terms of milligrams of protein.

**Superoxide Dismutase Assay**

Supernatant (2000g for 20 minutes) was assayed for superoxide dismutase (SOD) activity by following the inhibition of pyrogallol auto-oxidation.20 Pyrogallol (24 mmol/L) was prepared in 10 mmol/L HCl and kept at 4°C before use. Catalase (30 μmol/L stock solution) was prepared in an alkaline buffer (pH 9.0). Aliquots of supernatant (150 μg protein) were added to Tris HCl buffer containing 25 μL pyrogallol and 10 μL catalase. The final volume of 3 mL was made up of the same buffer. Changes in absorbance at 420 nm were recorded at 1-minute intervals for 5 minutes. SOD activity was determined from a standard curve of percentage inhibition of pyrogallol auto-oxidation with a known SOD activity. This assay was highly reproducible, and the standard curve was linear up to 250 μg protein with a correlation coefficient of 0.998. Data are expressed as SOD units per milligram protein compared with the standard.

**Malondialdehyde Assay**

Measurement of lipid peroxidation by determination of myocardial malondialdehyde (MDA) content was performed by a modified thiobarbituric acid (TBA) method.21 Hearts were quickly excised and washed in buffered 0.9% KCl (pH 7.4). After the atria, extraneous fat, and connective tissue were removed, the ventricles were homogenized in the same buffer (10% wt/vol). The homogenate was incubated for 1 hour at 37°C in a water bath. A 2-mL aliquot was withdrawn from the incubation mixture and pipetted into an 8-mL Pyrex tube. One milliliter of 40% trichloroacetic acid (TCA) and 1 mL of 0.2% TBA were promptly added. To minimize peroxidation during the subsequent assay procedure, 2% butylated hydroxytoluene was added to the TBA reagent mixture.22 Tube contents were vortexed briefly, boiled for 15 minutes, and cooled in a bucket of ice for 5 minutes. Two milliliters of 70% TCA was then added to all tubes, and contents were again vortexed briefly. The tubes were allowed to stand for 20 minutes. This was followed by a centrifugation of the tubes for 20 minutes at 3500 rpm. The color was read at 532 nm on a Zeiss spectrophotometer and compared with a known MDA standard.

**Ultrastructural Studies**

For ultrastructural studies, three to five hearts in each group were processed as described.9,10 Hearts were washed in cold 0.1 mol/L sodium phosphate buffer (pH 7.4). Tissue samples 6 to 11 mm in size were taken from four different areas of the subendocardium as well as the subepicardium of the free left ventricular wall between the midregion and apex of the heart. The tissue pieces were immersed for 15 minutes in 0.1 mol/L phosphate buffer (pH 7.4) containing 3% glutaraldehyde. This briefly fixed tissue was further cut into cubes smaller than 1 mm. Aldehyde fixation was continued for a total duration of 2 hours. The tissues were washed for 1 hour in the above phosphate buffer containing 0.05 mol/L sucrose. Postfixation was done in 2% OsO4 for 1.5 hours, after which the tissue pieces were dehydrated in graded alcohol series. Tissue embedding was done in epoxy. Ultrathin sections were placed on Formvar-coated grids and stained with uranyl acetate and lead citrate. Electron micrographs of the subendocardial and subepicardial regions from the four groups were compared to establish ultrastructural differences.

**Proteins and Statistical Analysis**

Proteins were determined by the method of Lowry and associates.23 Data were expressed as the mean±SEM. For a statistical analysis of the data, group means were compared by one-way ANOVA, and Bonferroni's test was used to identify differences between groups. Statistical significance was acceptable to a level of P<.05.

**Results**

**General Observations and Mortality**

The general appearance of all groups of animals was recorded during the time course of the study. After completion of adriamycin treatment, the animals’ fur became scruffy and developed a light yellow tinge, and there was red exudate around the eyes in both ADR and PROB+ADR groups, although more extensively in the ADR group. Animals in the ADR group also appeared to be sicker, weaker, and lethargic compared with the PROB+ADR group. The most predominant feature in the ADR group animals was the development of a grossly enlarged abdomen and ascites. This condition became apparent within a week after the completion of treatment with adriamycin. When they were killed, all ADR group animals had a significant amount of peritoneal fluid (Table 1). In addition, the liver was enlarged and congested in all ADR group animals. In the PROB+ADR group animals, the amount of peritoneal fluid was about one fifth that seen in animals in the ADR group (Table 1). During the posttreatment period, the mortality rate was significantly higher in the ADR group than in the PROB+ADR group (Table 1). There were no deaths in the CONT and PROB groups.
Data on heart weight and heart weight/body weight ratio are also given in Table 1. Despite the ascites, the body weight gain in the ADR group was significantly less. Treatment with adriamycin resulted in a significant decrease in heart weight and ratio of heart to body weight in the ADR group. In the PROB+ADR group, ratio of heart to body weight was not significantly different from the CONT and PROB groups. Heart weight in the PROB+ADR group was significantly higher than in the ADR group but was still lower than in the CONT and PROB groups.

**Hemodynamic Studies**

ASP, ADP, LVSP, and LVEDP were recorded in all groups; these data are shown in Table 2. There were significant changes in cardiac performance in the ADR group. LVEDP in both ADR and PROB+ADR groups was higher than values in the CONT and PROB groups; however, this value was relatively more increased in the ADR than the PROB+ADR group. LVSP was significantly decreased in the ADR group alone, whereas in the PROB+ADR group it was no different from the CONT and PROB groups. There was no difference between CONT and treated groups with respect to ADP. ASP values were significantly lower in the ADR group compared with all other groups.

**Morphological Studies**

Electron microscopic analysis of left ventricular free wall was conducted on heart tissue excised from all four groups of rats. The morphological appearance of different subcellular structures, including mitochondria, sarcoplasmic reticulum, sarcomeres, myofibrils, and intercalated disks, in hearts from control and probucol groups were typical of normal cells. Morphological changes due to adriamycin treatment alone included disruption of several subcellular elements including loss of myofibrils, swelling of mitochondria, vacuolization of the cytoplasm, formation of lysosomal bodies, and dilation of the sarcotubular system (Fig 1A). Mitochondrial injury in addition to the swelling of these organelles was also accompanied by disarrangement and disruption of cristae (Fig 1B). Some of the electron-dense bodies showed lamellar inclusions (Fig 1B). These structural changes are typical for adriamycin cardiomyopathy.2,12,15 The ultrastructure of hearts from the PROB+ADR group showed regular myofibrillar structure, maintained sarcotubular reticulum, and preserved mitochondria (Fig 2A). At higher magnification, intramitochondrial details were quite normal, but some intracellular edema was noticeable around the mitochondria (Fig 2B).

**Antioxidant Enzymes and Lipid Peroxidation**

Different antioxidant enzyme activities were examined in all groups; these data are shown in Table 3. GSHPx activity in the ADR group was reduced by about 32% compared with the CONT group. In the PROB+ADR group, GSHPx activity was near control levels. Total SOD activity in the PROB and PROB+ADR groups was significantly higher than in the CONT and ADR groups. Catalase activity did not change in any group. The amount of lipid peroxidation was determined by evaluating myocardial MDA content; these data are also shown in Table 3. MDA levels were almost the same in the CONT, PROB, and PROB+ADR groups, whereas in the ADR group alone the MDA content was significantly higher.

### Table 1. Effects of Probucol on Adriamycin-Induced Changes in Heart Weight, Body Weight, Mortality Rate, and Ascites

<table>
<thead>
<tr>
<th>Animal Group</th>
<th>Heart Weight, g</th>
<th>Heart Weight/Body Weight Ratio×100</th>
<th>Mortality, %</th>
<th>Ascites, mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>CONT</td>
<td>1.25±0.05</td>
<td>2.84±0.12</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>ADR</td>
<td>0.75±0.03*</td>
<td>2.38±0.08*</td>
<td>30</td>
<td>92.2±13.2*</td>
</tr>
<tr>
<td>PROB</td>
<td>1.18±0.04</td>
<td>2.78±0.13</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>PROB+ADR</td>
<td>0.92±0.04†</td>
<td>2.73±0.14</td>
<td>10</td>
<td>19.9±6.4†</td>
</tr>
</tbody>
</table>

CONT indicates control; ADR, adriamycin; and PROB, probucol. Data are mean±SEM of six to eight animals in all studies. Mortality data are mean±SEM of 50 animals each in the ADR and PROB+ADR groups and 25 animals each in the CONT and PROB groups.

* and †P<.05 compared with all other groups.

### Table 2. Effects of Probucol on Adriamycin-Induced Pressure Changes

<table>
<thead>
<tr>
<th>Animal Group</th>
<th>ASP</th>
<th>ADP</th>
<th>LVSP</th>
<th>LVEDP</th>
</tr>
</thead>
<tbody>
<tr>
<td>CONT</td>
<td>102.1±1.8</td>
<td>66.9±4.4</td>
<td>126.0±11.3</td>
<td>5.9±3.0</td>
</tr>
<tr>
<td>ADR</td>
<td>84.2±3.2*</td>
<td>57.8±9.4</td>
<td>89.5±6.7*</td>
<td>33.7±8.6*</td>
</tr>
<tr>
<td>PROB</td>
<td>91.0±9.8</td>
<td>62.6±13.5</td>
<td>113.9±6.5</td>
<td>12.4±6</td>
</tr>
<tr>
<td>PROB+ADR</td>
<td>110.5±8.2</td>
<td>72.1±6.3</td>
<td>123.3±7.2</td>
<td>20.1±5.3†</td>
</tr>
</tbody>
</table>

ASP indicates aortic systolic pressure; ADP, aortic diastolic pressure; LVSP, left ventricular systolic pressure; LVEDP, left ventricular end-diastolic pressure; CONT, control; ADR, adriamycin; and PROB, probucol. Values are mm Hg, mean±SEM of six to eight experiments.

*and †P<.05 significantly different from CONT and ADR groups, respectively.

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Fig 1. Photomicrographs showing myocardial cell damage in rats exposed to adriamycin. A, Swelling of mitochondria (M) as well as sarcoplasmic reticulum (arrow). Vacuolization (*) and dense bodies (double arrow) are also apparent. B, At a higher magnification, loss of cristae from the mitochondria and some membrane cisternae in the dense bodies are seen. Bar=1 μm.
Fig 2. Photomicrographs showing portion of a myocardial cell from probucol- and Adriamycin-treated rat. A, Mitochondria (M), myofibrils (MF), sarcoplasmic reticulum (arrows), and other cellular details are better maintained. B, At higher magnification, mitochondrial (M) cristae arrangement and sarcomeres (S) are quite normal. However, some perimitochondrial edema (*) is still apparent. Bar=1 μm.
TABLE 3. Effects of Probucol on Adriamycin-Induced Changes in Antioxidant Enzyme Activities and Lipid Peroxidation

<table>
<thead>
<tr>
<th>Animal Group</th>
<th>GSHPx, nmol/mg protein</th>
<th>SOD, U/mg protein</th>
<th>Catalase, U/mg protein</th>
<th>MDA, nmol/g heart</th>
</tr>
</thead>
<tbody>
<tr>
<td>CONT</td>
<td>59.9±5.7</td>
<td>34.7±4.4</td>
<td>31.3±3.8</td>
<td>49.1±3.2</td>
</tr>
<tr>
<td>ADR</td>
<td>40.7±5.1†</td>
<td>41.0±2.7</td>
<td>32.7±2.0</td>
<td>82.1±3.1*</td>
</tr>
<tr>
<td>PROB</td>
<td>52.4±2.9</td>
<td>46.2±6.5†</td>
<td>36.7±3.2</td>
<td>54.3±3.2</td>
</tr>
<tr>
<td>PROB+ADR</td>
<td>54.6±5.3</td>
<td>64.1±4.2†</td>
<td>30.0±2.4</td>
<td>58.2±7.1</td>
</tr>
</tbody>
</table>

GSHPx indicates glutathione peroxidase; SOD, superoxide dismutase; MDA, malondialdehyde; CONT, control; ADR, adriamycin; and PROB, probucol. Data are mean±SEM from six to eight experiments.

*P<.05, †P<.02 different from all other groups. $P<.05$ different from CONT and ADR groups.

Discussion

Repeated administration of adriamycin beyond a certain dose has been shown to cause cardiomyopathic changes in patients as well as in a variety of animal species. The rat model is considered to be a good, reproducible, and cost-effective system for testing beneficial effects of different drugs. Both development of cardiomyopathy and congestive heart failure in rats in the present study were established by the myocardial cell damage, depressed systolic pressures, increase in LVEDP, ascites, and congestive changes in the liver. These failing hearts in vivo as well as in isolated myocardial preparations ex vivo have been shown to respond poorly to inotropic interventions. The present study demonstrates for the first time that a simultaneous treatment with probucol mitigates adriamycin-induced cardiomyopathic changes as well as congestive heart failure, as indicated by the improved cardiac structure and function and a reduced mortality in the PROB+ADR group.

Probucol in the plasma is transported predominantly by low-density, very-low-density, and high-density lipoproteins. Oral administration of probucol at 1 g/d increases its level in the blood as well as the adipose tissue. However, there seems to be no absolute correlation between the plasma levels of probucol and the extent of cholesterol lowering. Although it is difficult to draw any parallel between the dosage used by us in rats (6×10 mg/kg IP) and therapeutic dosage (2×500 mg/d for 3 to 6 months), the probucol treatment protocol used in our study was well tolerated.

Probucol treatment in heterozygous familial hypercholesterolemia caused the regression of xanthomas, which did not correlate with the level of cholesterol reduction. Cholestyramine, another cholesterol-lowering drug, and probucol both sharply lowered the serum cholesterol levels in nonhuman primates, but only probucol caused regression of atherosclerotic lesions. These observations clearly suggest that beneficial effects of probucol may be independent of cholesterol lowering. Because of the two phenolic groups in its molecular structure, probucol has been reported to be a strong antioxidant, and it appears that the protection offered by probucol in this study may involve antioxidant mechanisms. In this regard, adriamycin has been shown to promote the production of free radicals; these toxic species are known to cause myocardial dysfunction. Data on lipid peroxidation are also in concert with this suggestion, inasmuch as probucol caused a significant attenuation in the adriamycin-induced increase in MDA levels. The beneficial effect of probucol against restenosis after percutaneous transluminal coronary angioplasty has also been suggested to be due to its antioxidant properties.

In addition to its cholesterol-lowering and antioxidant properties, probucol may also have an effect on endogenous antioxidant enzyme activities. Probucol not only prevented adriamycin-induced decreases in GSHPx activity but also increased SOD activity. It is important to note that in the ADR group, there was a small although statistically not significant increase in the SOD activity. Probucol alone caused a 35% increase in SOD; however, in the PROB+ADR group, there may have been some synergistic effect, since the increase in SOD activity was about 88%. Thus, probucol clearly improves "endogenous antioxidant reserve," and the latter has been suggested to improve myocardial structure and function. The mechanisms for an adriamycin-induced decrease in GSHPx and probucol-induced increase in antioxidants (GSHPx and SOD) are not clear. This study, however, clearly demonstrates that probucol may be providing protection by acting as an antioxidant as well as by promoting endogenous antioxidants.

In conclusion, it can be said that adriamycin cardiomyopathy is associated with an antioxidant deficit and that probucol treatment improves the antioxidant status of the heart. Improved cardiac function due to treatment with probucol may be related to the maintenance of the antioxidant status of the heart. Adriamycin, because of its histophlic nature, is cleared from the plasma and appears in the tissues within minutes. Since treatments with PROB and adriamycin were 24 hours apart, it is unlikely that PROB influenced adriamycin absorption. Nevertheless, future studies are required to resolve two important questions: (1) What are the molecular mechanisms for these antioxidant changes; and (2) does this antioxidant protection also influence absorption, body distribution, and thus antitumor properties of the drug? It would also be of interest to examine these beneficial effects of PROB in a larger animal model.

Acknowledgments

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