Adriamycin-Induced Early Changes in Myocardial Antioxidant Enzymes and Their Modulation by Probucol

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Background—The clinical usefulness of adriamycin is restricted by the development of congestive heart failure. It has been suggested that probucol, a strong antioxidant, completely prevents adriamycin-induced cardiomyopathy without interfering with its antitumor properties. The present study investigated the effects of adriamycin and probucol on myocardial antioxidant enzyme activities and immunoreactive protein levels in rats.

Methods and Results—Activities and protein levels of glutathione peroxidase (GSHPx) were significantly decreased from 2 to 24 hours, and those of manganese superoxide dismutase were decreased at 1 and 2 hours after adriamycin treatment. These changes were prevented by probucol. Catalase activity was increased from 2 to 24 hours after adriamycin treatment, but its protein levels were not significantly changed. Copper zinc superoxide dismutase activity and protein level were not changed at any time. Myocardial lipid peroxidation was found to be significantly higher at all time points, and this change was also prevented by probucol. Treatment with probucol alone increased GSHPx activity at 2 weeks, and in these hearts, lipid peroxidation was lower than the control value. Within 24 hours, there was no mortality in any of the groups.

Conclusions—It is suggested that an early and persistent decrease in GSHPx activity and protein may play an important role in the pathogenesis of adriamycin-induced cardiomyopathy, worsening heart failure and mortality. (Circulation. 2000;102:2105-2110.)

Key Words: glutathione peroxidase ■ lipids ■ cardiomyopathy ■ heart failure

Adriamycin (also called doxorubicin) is considered to be one of the most potent drugs against a wide range of tumors. However, a dose-dependent cardiotoxicity limits its clinical potential. Adriamycin has been shown to cause myocardial antioxidant deficit in different animal species, and probucol, a lipid-lowering drug and antioxidant, has been found to modulate these changes. However, the molecular basis and early sequence of the changes in myocardial antioxidant enzyme activities due to adriamycin and probucol remain to be understood.

Because DNA strands and various enzymes can be damaged directly by adriamycin or indirectly by the production of free radicals, it is reasonable to assume that changes in antioxidant enzyme activities could be the result of altered gene expression at the transcriptional or translational level. The possibility of oxidative inactivation of the antioxidant enzymes also cannot be ruled out. Gene expression of different antioxidant enzymes at 3 weeks after the last injection of the drug has been reported. However, there is no information with respect to changes in antioxidant enzyme activities and protein levels at early time points (1 to 24 hours) that could help identify an early defect. Probucol offered partial protection when it was used concurrently with adriamycin treatment. However, complete protection was seen when probucol was started 2 weeks before adriamycin treatment. These findings suggest that the effects of probucol may vary with the duration of exposure. Furthermore, the protective effects of probucol against adriamycin cardiomyopathy may also involve modulation of endogenous antioxidant enzymes at early time points.

We examined the activities and immunoreactive protein levels for different antioxidant enzymes, including glutathione peroxidase (GSHPx), manganese superoxide dismutase (MnSOD), copper zinc superoxide dismutase (CuZnSOD), and catalase (CAT), in the ventricles of rats treated with adriamycin and adriamycin+probucol at 1 to 24 hours after the completion of treatment. Data on lipid peroxidation were also obtained at these early time points. Animals treated with probucol alone for 1 and 2 weeks were examined at 24 hours after the last injection.

Methods

Animal Groups and Treatment
All the studies described in this report conform to the Guidelines of the Canadian Council on Animal Care. Male Sprague-Dawley rats (250±10 g) were obtained from the University of Manitoba breeding...
facility and maintained on a normal rat chow. The animals were randomly divided into 4 groups: control, adriamycin (ADR), adriamycin+probuloc (PROB+ADR), and probuloc (PROB). Animals in the control group (n=4) were treated with normal saline. In the ADR group (n=16), animals were injected intraperitoneally with adriamycin (doxorubicin hydrochloride) for a cumulative dose of 120 mg/kg body wt in 6 injections over 2 weeks as described before. In the PROB+ADR group (n=16), rats were treated with probuloc and adriamycin. These rats were injected with probuloc for 2 weeks before initiation of adriamycin, and for 2 more weeks, probuloc treatment was given on alternate days with adriamycin for a cumulative dose of 120 mg/kg. Animals in these 2 groups (ADR and PROB+ADR) were killed by decapitation at 1, 2, 4, and 24 hours (4 animals at each time point) after the last injection. The PROB group had 2 subgroups with a total of 8 rats that were injected with probuloc (10 mg/kg) 3 times a week for 1 week and 2 weeks with a total dose of 30 or 60 mg/kg body wt, respectively. Animals in the control and PROB groups were killed at 24 hours after the last injection.

Sample Preparation
Animals were killed by decapitation. The hearts were immediately removed; rinsed in saline; trimmed free of the extraneous fat, other connective tissue, and atria; and weighed. Ventricles were quickly cut into very small pieces, and the minced tissue was separated into 2 parts. One part was placed in an ice-cold buffer for analysis of antioxidant enzyme activities and lipid peroxidation. The other part was frozen in liquid nitrogen and kept at −80°C for protein immunoblotting assays.

Measurements of Antioxidant Enzyme Activities and Lipid Peroxidation

GSHPx Activity Assay
GSHPx activity was expressed as nanomoles of reduced NADPH oxidized to NADPH per minute per milligram protein. Cytosolic GSHPx was assayed in a 3-mL cuvette containing 2.4 mL of 75 mmol/L phosphate buffer (pH 7.0). The following solutions were then added: 50 µL of 60 mmol/L reduced glutathione, 100 µL of glutathione reductase (30 U/mL), 50 µL of 120 mmol/L NaN3, 100 µL of 15 mmol/L Na2EDTA, 100 µL of 3.0 mmol/L NADPH, and 100 µL of cytosolic fraction obtained after centrifugation of the heart homogenate at 20,000g for 25 minutes. The reaction was initiated by the addition of 100 µL of 7.5 mmol/L H2O2, and the conversion of NADPH to NADP+ was assayed by measuring the absorbance at 340 nm at 1-minute intervals for 5 minutes.

MnSOD and CuZnSOD Activity Assay
The enzyme activity was assayed by following the inhibition of pyrogallol auto-oxidation. Pyrogallol (24 mmol/L) was prepared in 10 mmol/L HCl and stored at 4°C. CAT 30 µmol/L stock solution was made in an alkaline buffer (pH 9.0). Aliquots of supernatant (150 µg protein) were added to Tris-HCl buffer containing 25 µmol/L pyrogallol and 10 µL CAT stock solutions. The total reaction mixture was made to 3 mL with the same Tris-HCl buffer. Auto-oxidation of pyrogallol was monitored by measuring absorbance at 420 nm 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. MnSOD and CuZnSOD activities were differentiated by measuring the enzyme activity in the presence of 2 mmol/L NaCN. This assay was highly reproducible, and the standard curve was linear up to 250 µg protein, with a correlation coefficient of 0.998. One unit of SOD is defined as the amount that shows 50% inhibition at room temperature and pH 7.8.

CAT Activity Assay
The ventricles were homogenized in 50 mmol/L potassium phosphate buffer (pH 7.4) with a weight-to-volume ratio of 1:10. The homogenate was centrifuged at 40,000g for 30 minutes. Supernatant (50 µL) was added to a cuvette containing 2.95 mL of 19 mmol/L H2O2 solution prepared in potassium phosphate buffer. The disappearance of H2O2 was monitored at 240 nm wavelength at 1-minute intervals for 5 minutes. Specific activity of the enzyme was expressed as µmol/mg protein.

Thiobarbituric Acid–Reactive Substances Assay
Measurement of lipid peroxidation by determination of thiobarbituric acid–reactive substances (TBARS) was performed with a modified TBA method as described previously.

Western Blot Analysis
The tissue samples were thawed in ice-cold Tris/EDTA buffer (100 mmol/L Tris-HCl, 5 mmol/L EDTA, pH 7.4) and homogenized in a Polytron homogenizer with two 10-second pulses and an intervening 10-second rest period. Aprotinin (10 µg/mL), leupeptin (10 µg/mL), pepstatin A (10 µg/mL), and PMSF (20 µmol/L) were included in the buffer to prevent protein degradation. Protein concentrations were determined and used to normalize the protein loading. Protein (20 µg) was loaded for 1-dimensional SDS-PAGE in a discontinuous system using 15% separating gel and 5% stacking gel. The separated proteins were electrophoretically transferred to nitrocellulose membranes with a modified Towbin buffer (20 mmol/L Tris, 150 mmol/L glycine, 2% methanol, 0.02% SDS, pH 8.3) in a cooled Bio-Rad TransBlot unit. After nonspecific protein-binding sites were blocked with 5% nonfat milk in Tris-buffered saline/0.1% Tween-20, the membranes were processed for immunodetection with rabbit anti-human GSHPx antibody (kindly provided by Dr I. Singh, Medical University of South Carolina, Charleston), rabbit anti-MnSOD and CuZnSOD antibodies (kindly provided by Dr L.W. Oberley, University of Iowa, Iowa City), and sheep anti-CAT polyclonal antibody (The Binding Site, Birmingham, UK) as primary antibodies. The bound primary antibodies were detected with anti-rabbit/sheep horseradish peroxidase–conjugated secondary antibody and an ECL Western blotting detection system (Amersham Inc). The photographs generated were quantitatively analyzed for the GSHPx, MnSOD, CuZnSOD, and CAT protein levels with a Bio-Rad GS-670 image densitometer. The molecular weights of the protein bands were determined by reference to the standard molecular weight markers (Bio-Rad).

Protein Determination and Statistical Analysis
Proteins were determined by the methods of Lowry and associates. Data were expressed as the mean±SEM. For a statistical analysis of the data, group means were compared by 1-way ANOVA, and Bonferroni’s test was used to identify differences between groups. Statistical significance was acceptable to a level of P<0.05.

Results

General Observations and Body Weights
Within 24 hours of the last injection, no mortality was seen in any of the control, ADR, PROB, and PROB+ADR groups. Animals in the ADR and ADR+PROB groups did not show any dyspnea or ascites. Animals in the ADR and ADR+PROB groups showed a significant loss in heart and body weights (Table). This reduction in body weight gain is attributed to reduced food intake and inhibition of protein synthesis due to adriamycin. There was a significant loss in the ratio of heart to body weight in the PROB+ADR group. These data are shown in the Table.

GSHPx Enzyme Activity and Proteins
The GSHPx activity was measured at 1, 2, 4, and 24 hours after the last injection of adriamycin; these data are shown in Figure 1. Adriamycin treatment resulted in a significant decrease in GSHPx activity, with values of 98.0%, 81.1%, 77.0%, and 68.2% of the control value at 1, 2, 4, and 24 hours, respectively. The immunoreactive protein levels of
**Effects of Probucol on Adriamycin-Induced Changes in Body Weight, Heart Weight, and Ratio of Heart/Body Weight in Rats**

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of Animals</th>
<th>Body Weight, g</th>
<th>Heart Weight, g</th>
<th>Heart/Body Weight Ratio (×1000)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>4</td>
<td>388.3±17.7</td>
<td>1.05±0.06</td>
<td>2.70±0.03</td>
</tr>
<tr>
<td>ADR</td>
<td>16</td>
<td>289.0±7.6*</td>
<td>0.79±0.05*</td>
<td>2.73±0.10</td>
</tr>
<tr>
<td>PROB + ADR</td>
<td>16</td>
<td>321.0±9.0*</td>
<td>0.80±0.03*</td>
<td>2.49±0.07†</td>
</tr>
</tbody>
</table>

Because there was no difference in the heart and body weights at 1, 2, 4, and 24 hours, these data were pooled and expressed as the mean±SEM of all animals in the control, ADR (adriamycin), and PROB + ADR (probucol + adriamycin) groups.

*Significantly different from the control group (P<0.05).
†Significantly different from the control and ADR groups (P<0.05).

GSHPx were also decreased significantly; the values at 1, 2, 4, and 24 hours after the treatment were 93.0%, 72.0%, 63.8%, and 52.7% of the control values, respectively (Figure 2).

In the PROB + ADR group, the GSHPx activity at 1, 2, 4, and 24 hours was 101.4%, 99.3%, 100.7%, and 95.6% (Figure 1), and protein levels were 91.4%, 114.4%, 95.8%, and 118.9% (Figure 2) of the control values, respectively.

To evaluate the effects of probucol on myocardial antioxidant status in the PROB group, all 4 antioxidant enzymes were measured for activity and protein levels after 1 and 2 weeks of probucol treatment. GSHPx activity was increased significantly at 2 weeks (Figure 3), and this change in enzyme activity was not accompanied by any change in the protein levels (Figure 4).

**SOD Enzyme Activity and Proteins**

In the ADR group, MnSOD activity was decreased significantly at 1 and 2 hours to 73.3% and 80.7% of the control value, respectively, and at 4 and 24 hours, this activity was recovered and the values were 111.3% and 118.0% of the control, respectively (Figure 1). Similarly, the protein levels for MnSOD were also decreased at 1 and 2 hours to 51.1% and 53.7%, respectively. MnSOD protein levels were recovered and the values were 111.3% and 118.0%, respectively, and at 4 and 24 hours, this activity was 101.4%, 99.3%, 100.7%, and 95.6% of the control value, respectively (Figure 2).

In the ADR group, the CAT activity was increased to 117.4%, 136.3%, 134.2%, and 122.4%, respectively (Figure 1). The protein levels of CAT were not changed by probucol treatment alone for 1 and 2 weeks did not have any effect on CAT enzyme activity and protein level (Figures 3 and 4).

**CuZnSOD enzyme activity (Figure 1) and its protein levels (Figure 2) in the ADR and PROB + ADR groups did not show any change at any of the time points. Neither the enzyme activity nor the protein level of CuZnSOD was changed by probucol treatment for 1 and 2 weeks (Figures 3 and 4).**

**CAT Enzyme Activity and Proteins**

In the ADR group, the CAT activity was increased to 117.4%, 153.7%, 161.9%, and 142.7% of the control value at 1, 2, 4, and 24 hours, respectively (Figure 1). In the PROB + ADR group, the CAT activity at 1, 2, 4, and 24 hours was 114.2%, 136.3%, 134.2%, and 122.4%, respectively (Figure 1). The protein levels of CAT were not changed in the ADR and PROB + ADR groups (Figure 2). Furthermore, probucol treatment for 1 and 2 weeks did not have any influence on CAT enzyme activity and protein levels (Figures 3 and 4).

**Lipid Peroxidation (TBARS)**

The extent of lipid peroxidation was measured by assaying TBARS for all groups at different time points. In the ADR group, there was a significant increase in lipid peroxidation, ranging between 30% and 50%, during 1 to 24 hours after treatment compared with the control (Figure 5). In the PROB + ADR group, TBARS were near the control level (Figure 5). Probucol alone caused a small but significant reduction in TBARS in week 2 (Figure 5).

**Discussion**

In biological systems, adriamycin is known to produce free radicals, and antioxidant enzymes play a critical role in the detoxification of these radicals. In a protocol for adriamycin treatment similar to the one used in this study, it was shown that 3 weeks after the last injection, there is a significant reduction in GSHPx activity and increase in oxidative stress. The present study is the first to investigate these changes at earlier time points to determine the chronology of events.

It has been suggested that GSHPx may play an important role in protecting the heart from peroxidative attack. In the present experiments, a decrease in the GSHPx activity, first noticed at 2 hours, continued up to 24 hours. This finding...
may explain the decrease in the GSHPx activity seen at a later time after adriamycin administration. Similar findings have also been reported in rabbits treated with 13.5 mg/kg of adriamycin in 3 weeks. Because a single injection of adriamycin (15 mg/kg) did not have any influence on the activity of GSHPx, it is likely that repeated administration of adriamycin over time is necessary to bring about a decrease in this activity. Furthermore, our data suggest that this decrease in the enzyme activity may be caused by the parallel decrease in the enzyme protein shown by the Western blots in the present study. It has also been shown that the lipid peroxides may inhibit the activity of selected enzymes by oxidation of reduced thiol groups. Malondialdehyde, a byproduct of lipid peroxidation, may also diminish enzyme activity by oxidizing the active site or by forming protein cross-links. In fact, an inverse correlation between GSHPx activity and lipid peroxidation in the hearts of adriamycin-treated rats has been reported.

MnSOD activity was significantly decreased at 1 and 2 hours after the last injection. But this activity returned to the control level at 4 and 24 hours. The protein level of MnSOD was also decreased at 1 to 2 hours and recovered at 4 and 24 hours. This transient change in the MnSOD is also supported by the fact that at longer posttreatment durations, there was no change in the SOD activity. Such a transient decrease in MnSOD may have some role in the early stages of pathogenesis of heart failure. The significant increase in CAT activity seen in this study also appears to be a transient change, because at 3 weeks after treatment, this activity was close to normal. A change in this activity at early time points may represent an early adaptive response to contain oxidative stress; however, this adaptation is not sustained for a longer time. Furthermore, heart has a very low level of CAT activity.
compared with other tissues; thus, adaptive changes in CAT may be inadequate in protecting myocardial cells against oxidative stress.4

Significantly depressed cardiac function at 3 weeks after treatment in this model, as indicated by the elevated left ventricular end-diastolic pressure and decreased left ventricular systolic pressure in the chronic settings, has been reported.5 These changes were accompanied by ascites, dyspnea, and significant mortality, which is as high as 40% to 60% at 3 weeks after the last injection.5–7 In the present study, there was no mortality within 24 hours of the treatment, suggesting that death due to adriamycin treatment begins to occur as heart failure progresses over days and that these events are preceded by decreases in the GSHPx and SOD activities and increases in oxidative stress. A similar course of events has been reported in cancer patients treated with adriamycin who at the time of release were asymptomatic with respect to heart function. In some patients, dyspnea on exertion and increasing fatigue were noted within weeks after release. This was followed by a rapid progression of heart failure and death within weeks.2 Because the decrease in MnSOD activity and protein was only transient, it is likely that during initiation of adriamycin-induced heart failure in rats, both GSHPx and MnSOD may have some role. Progression and worsening of heart failure, however, were accompanied by a significant decrease only in the GSHPx activity and elevated oxidative stress, suggesting a larger role for this enzyme in pathogenesis and progression of heart failure.

Probucol treatment prevented this adriamycin-induced decrease in GSHPx and MnSOD protein contents and enzyme activities. Probucol has been reported to prevent heart failure, ascites, and mortality due to adriamycin in rats.5 A decrease in myocardial oxidative stress with probucol was also noted in the adriamycin-treated animals at the early time points in this study and at later time points in previous reports.6 Probucol treatment alone for 2 weeks also enhanced baseline GSHPx activity without any changes in its protein level, and there was a corresponding decrease in lipid peroxidation. In cultured neonatal rat atrial myocytes, adriamycin suppressed atrial natriuretic peptide (ANP) secretion, steady-state ANP mRNA levels, and ANP gene promoter activity, and probucol reversed the adriamycin-induced inhibition of ANP mRNA accumulation and ANP gene promoter activity.24 The protective effects of probucol against hypercholesterolemic atherosclerosis have been attributed to an increase in the GSHPx activity.25 Probucol reduced lipoperoxide levels in the plasma of atherosclerotic patients by activating the antioxidant enzymes SOD and GSHPx.26,27 Probucol treatment has also been reported to significantly improve the decrease of glomerular MnSOD and GSHPx at both the mRNA and protein levels in rats with subtotal nephrectomy.28 Although the precise mechanism of probucol action is not clear, the data in the present study suggest that the change in GSHPx activity may have been a posttranslational event. Thus, the beneficial effect of probucol may involve some direct antioxidant property of the drug that may also influence antioxidant enzyme gene function.

In conclusion, among different antioxidant enzymes studied, GSHPx and MnSOD showed an early decline in the activity and the change correlated with changes in the enzyme

**Figure 4.** Effects of probucol on GSHPx, MnSOD, CuZnSOD, and CAT protein levels after 1 and 2 weeks of treatment. A, Western blot analysis. Twenty micrograms of protein of each sample were loaded. Blot was probed with GSHPx, MnSOD, CuZnSOD, and CAT antibodies. B, Loading control. C, Densitometric analysis of relative protein levels. Data are mean ± SEM of 4 animals. Samples were processed in triplicate at each time point and represented as percent of control value.
protein levels. Only GSHPx activity remains depressed throughout the posttreatment period, in which heart failure progresses precipitously and is associated with increased mortality in these animals. Furthermore, this change in GSHPx and development of cardiomyopathy and heart failure in these animals require multiple treatments with adriamycin. Probucol treatment, which is most effective in offering optimal protection against adriamycin cardiomyopathy, not only caused a significant increase in the GSHPx activity but also prevented adriamycin-induced decreases in this activity. These changes correlated inversely with oxidative stress. It is known that adriamycin-induced cardiomyopathy in patients is seen only after multiple treatments, and mortality caused by heart failure in patients is a dose-dependent phenomenon. On the basis of these experimental and clinical findings, it is proposed that a decrease in GSHPx activity may be a key defect in the pathogenesis and progression of adriamycin-induced heart failure. It remains to be tested whether analysis of GSHPx in heart biopsies would be an early predictor of impending heart failure.

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