Suppression of Doxorubicin Cardiotoxicity by Overexpression of Catalase in the Heart of Transgenic Mice*

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Doxorubicin is an anthracycline antibiotic and one of the most important anti-cancer agents. It is effective in treatment of acute leukemias and malignant lymphomas as well as a number of solid tumors. However, the clinical use of doxorubicin is limited by its cardiotoxicity (1–3). Studies have suggested that doxorubicin may have at least two mechanisms of action that cause cellular damage. One mechanism involves generation of oxygen free radicals, the damage from which is inhibited by free radical scavengers. This appears to play a major role in the development of cardiomyopathy (4–7). The other mechanism is mediated by the intercalation of the drug to DNA and is unaffected by free radical scavengers. This appears to be the major determinant of doxorubicin cytotoxicity to tumor cells (7).

Catalase is a major enzyme involved in the detoxification of hydrogen peroxide (H₂O₂). This enzyme catalyzes the conversion of two molecules of H₂O₂ to molecular oxygen and two molecules of water. The primary physiological function of this enzyme is to detoxify the H₂O₂ produced as a result of peroxisomal and cytochrome P-450-linked monooxygenase reactions. The importance of catalase in providing protection against extraperoxisomal H₂O₂ is not clear. Studies with isolated hepatocytes, however, have demonstrated that, under conditions of GSH depletion, catalase functions in the metabolism of H₂O₂ produced by the cytochrome P-450-linked monooxygenase system (8). It is thus possible that, under conditions of extreme oxidative stress, which in most cases involves GSH depletion, catalase may become important in providing cytoprotection.

Catalase activities are highest in liver and erythrocytes, relatively high in kidney and adipose tissue, intermediate in lung and pancreas, and very low in heart and brain (9). It has been shown that catalase activity in the heart is about 2% of that in the liver of the human (9), the mouse (10), and the rat (11). Several studies have been undertaken to determine the role of catalase in cardioprotection against oxidative injuries. Supplementation of perfusion medium with catalase significantly reduced ischemia-reperfusion injury to the isolated rat heart by detoxifying H₂O₂ (12). The role of catalase in metabolism of H₂O₂ in the heart tissue was also tested directly by adding H₂O₂ into perfusion medium (13). Accumulated evidence shows that catalase, although present at low levels of activity, functions in detoxification of H₂O₂ in the myocardium.

It is unknown, however, whether the low catalase activity in the heart is responsible for the high sensitivity of this organ to oxidative stress. Current approaches using the isolated heart perfused with catalase have limited application. Exogenously added catalase may not be able to function intracellularly, but may react only with H₂O₂ directly in the perfusion medium. Thus, the relationship between the low constitutive catalase activity and the high sensitivity to oxidative stress in the heart has not been defined. A unique in vivo model is required to overcome these limitations and to precisely determine the role of catalase in protection against oxidative cardiotoxicity.

In the present study, we have developed a transgenic mouse model in which catalase is overexpressed only in the heart. To determine whether catalase elevation provides protection against doxorubicin cardiotoxicity, lipid peroxidation in the heart and serum creatine phosphokinase (CPK) activity were
measured in the doxorubicin-treated transgenic mice and non-transgenic controls. In addition, the atria isolated from the transgenic mice and nontransgenic controls were used to determine the effect of catalase on doxorubicin-induced functional changes. The results demonstrate that overexpression of catalase in the heart provides protection against doxorubicin cardiotoxicity.

MATERIALS AND METHODS

Generation and Identification of Cardiac Catalase Overexpressing Transgenic Mice

FVB mice obtained from the University of North Dakota Biomedical Research Center were used to produce transgenic lines. All animal procedures were approved by the American Association of Accreditation of Laboratory Animal Care certified institutional animal care committee. An 8-kb pair transgene designated MyCat, was constructed for overexpression of catalase in cardiac tissue of transgenic mice (Fig. 1). This transgene contains all of the coding sequences of the rat catalase cDNA (14). Transcription of the transgene is controlled by a fragment of the mouse alpha cardiac myosin heavy chain (MHC) gene previously used by Gullick et al. (15) to produce high level, cardiac-specific expression of cDNA constructs in transgenic mice. A 1730-bp Ncol/HindIII fragment of the rat catalase cDNA (14) which included the catalase start and stop codons was ligated behind a 5.7-kb BamHI/SalI fragment of the MHC gene. The MHC fragment includes the MHC promoter, the first two introns, and the first three noncoding exons of the MHC gene (15). To provide NotI restriction sites for purification of the transgene and to add a polyadenylation signal for the catalase transcript the 7430-bp BamHI/HindIII fragment containing the MHC promoter and catalase coding sequence was ligated in front of a 550-bp NotI/ApaI linker and an additional NotI site was contained in a polylinker upstream of the insulin fragment.

Standard procedures (17) were employed for the development of transgenic mice. The 8-kb MyCat transgene (the Ncol fragment, Fig. 1) was purified (18) on a matrix of diatomaceous earth (Prepaxene, Bio-Rad) and fractionated by electrophoresis on 1.0% agarose gels, and transferred to nylon membranes. The probe corresponding to a 900-base HindIII and BglII fragment of the rat catalase cDNA (14) (Fig. 1) was randomly labeled with [α-32P]dCTP (DuPont NEN) using the Klenow fragment of DNA polymerase I (Life Technologies, Inc.) and purified by chromatography (Sephadex G-50, Sigma) before hybridization. Hybridization and wash procedures were conducted using previously published methods. Autoradiographic images were scanned and analyzed using the MCID system from Imaging Research Inc. (Ontario, Canada).

Catalase—The tissues were homogenized in 1.0% Triton X-100 using a variable speed tissue teaser (Biospec Products, Inc.) at about 20,000 rpm for 30 s on ice. The homogenates were centrifuged at 10,000 rpm at 4 °C for 30 min. The supernatant was diluted with 1.5 volumes of the assay buffer (50 mM KH2PO4, 50 mM NaHPO4, pH 7.0). The enzyme activity was determined by the method described by Aebi (20). Briefly, in a cuvette 2 ml of sample were added. The reaction was initiated by adding 1.0 ml of 30 mM H2O2, and the change in absorbance at 240 nm was monitored at 25 °C for 1.0 min. A portion of the remaining sample was used for protein determination. Specific activity is expressed as μmol H2O2/min/mg of protein. Protein was determined by the method of Smith et al. (21) using bovine serum albumin as the standard.

GSH Peroxidase—Tissue samples were prepared as described above with the exception of the assay buffer (0.1 mM KH2PO4, 1 mM EDTA, pH 7.0). The enzyme activity was determined by the method described by Flohe and Gunzler (22). In 5 ml of assay tubes, 500 μl of buffer containing 2 mM sodium azide, 100 μM of GSH (10 mM), 100 μM of GSH reductase (2.4 units/ml), and 100 μM of sample were incubated for 10 min in 37 °C water bath, then 100 μl of NADPH (1.5 mM) was added, and the reaction mixture was transferred to cuvettes. Absorbance at 340 nm was monitored at 37 °C for 3 min before 100 μl of prewarmed H2O2 (1.5 mM) were added, followed by an additional 5-min monitoring under the same conditions. Enzyme activity was calculated as described previously (22). Specific activity is expressed as μmol H2O2/min/mg of protein.

GSH Reductase—The enzyme activity was determined by the method described by Carberg and Mannervik (23). The tissue preparations were the same as described above for GSH peroxidase. The assay buffer was 0.1 mM KH2PO4 (pH 7.0) containing prewarmed EDTA. In a cuvette, 0.5 ml of buffer, 200 μl of dithiothreitol (20 mM), and 50 μl of NADPH (2 mM) were combined. Following addition of 200 μl of sample, the change in absorbance at 340 nm was monitored at
The enzyme activity was assayed as described by Nebot et al. (24). This method is based on the fact that the rate of alkaline autoxidation of 5,6,6a,11b-tetrahydro-3,9,10-trihydroxy-1,2,12a,13a-tetrahydro-5H-dibenzo[a,d]cycloheptadiene (BHT) is accelerated by SOD activity. This autoxidation yields a chromophore which is detected at 525 nm. A SOD-525 assay kit based on this method was obtained from BioxyTech S.A., France. Briefly, tissue samples were homogenized in 9 volumes of distilled water at 4 C for 5 min at 4°C. A 250-μl aliquot of the supernatant was dialyzed against distilled water at 4°C with a M, 12,000 exclusion membrane for 24 h. The samples were then mixed with a mercaptan scavenger, 1,4-bis(dimethylamino)butane and acrylamide, for 1 min to eliminate major mercaptan interfering substances. Total SOD (Cu,Zn-SOD and Mn-SOD) activity was determined colorimetrically immediately following addition of the chromogenic reagent, BXT-0105. One SOD-525 activity unit is defined as the activity that doubles the BXT-0105 autoxidation background (24). The enzyme activity is expressed as units/g of wet tissue.

GSH and GSH Disulfide—Tissues were homogenized in 10 volumes of 5% (w/v) 5-sulfosalicylic acid at 4°C. The homogenate was centrifuged at 10,000 × g for 15 min, and the supernatant was assayed for GSH by the DTNB-glutathione reductase assay (25). The 1.0 ml reaction mixture contained 190 μl of stock buffer (143 mM sodium phosphate and 6.3 mM Na₂EDTA, pH 7.5), 700 μl of 0.248 mM NADPH stock buffer, 100 μl of 6 mM DTNB, and 10 μl of sample. The assay was initiated by addition of 10 μl of 266 units of glutathione reductase/ml. Standards were assayed in parallel under the same conditions as tissue samples.

Metallothionin (MT)—Total tissue MT concentrations were determined by the cadmium-hemoglobin affinity assay (26). Briefly, tissues were homogenized in 4 volumes of 10 mM Tris-HCl buffer, pH 7.4, at 4°C. Following centrifugation of the homogenate at 10,000 × g for 15 min, 200 μl of supernatant were transferred to microtubes for MT analysis, and 100 μl were transferred to separate microtubes for protein analysis. Samples were then prepared for MT determination as described previously (26).

Determination of Doxorubicin Cardiotoxicity

Transgenic mice and normal controls (7 weeks old, without regard to sex because our preliminary studies showed no difference in cardiac toxic responses to doxorubicin between males and females) were injected intraperitoneally with doxorubicin hydrochloride (Sigma) at 20 mg/kg. Four days after receiving the doxorubicin injection, the experimental animals were anesthetized intraperitoneally with sodium pentobarbital (65 mg/kg, Vet Labs, Lenexa, KS), and cardiovas-cular toxicity was determined as described below.

Lipid Peroxidation—Hearts were rapidly excised, trimmed of connective tissue, and washed free of blood with ice-cold saline. They were then blotted and weighed. Temperature was maintained at 4°C throughout the experiment. Because the classical malondialdehyde (MDA) determination by the thiobarbituric-acid method is influenced by several interferences and its reproducibility is not satisfactory (27), a modified assay was used in the present study (28). This modified method measures the products of lipid peroxidation, MDA, and 4-hydroxynonenal. A commercial kit based on this method was used (LPO-586, BioxyTech S.A., France).

CPK Activity—Serum CPK activity was assayed as described by Oliver (29). A CPK test kit (CK-20, Sigma) based on this method was obtained from Sigma. Blood was collected from the inferior vena cava of the anesthetized animals, and serum was obtained by a serum separator apparatus (Becton Dickinson, Rutherford, N.J.).

Atrial Functional Alteration—Mice were anesthetized with sodium pentobarbital. Atria were separated from ventricles and placed in a 30-ml chamber containing Krebs-Henseleit (KH) buffer of the following composition: NaCl (118 mM), KCl (4.7 mM), NaHCO₃ (3.0 mM), CaCl₂ (3.0 mM), MgSO₄ (1.2 mM), KH₂PO₄ (1.2 mM), glucose (10 mM), Na₂EDTA (0.5 mM), pH 7.4, gassed with 5% CO₂ in O₂. A preload tension of 0.25 g was applied. The atria were allowed to stabilize for 30 min before doxorubicin was added to the incubation buffer to a final concentration of 100 μM. Changes in spontaneous contraction were recorded isometrically by a Grass FT-03 force transducer and a Grass 7 recorder (Grass Instrument Co., Quincy, MA). The effect of doxorubicin on inotropy (contraction force) and chronotropy (heart rate) were determined using isolated left and right atrium, respectively.
Cardiac Catalase and Doxorubicin Toxicity

The highest protection was observed in the hearts expressing this enzyme activity about 200-fold higher than normal. However, the transgenic line overexpressing catalase activity about 200-fold higher than normal demonstrated no protection from doxorubicin.

Doxorubicin toxicity was also revealed by elevated serum CPK in nontransgenic mice (Fig. 7). Catalase overexpression provided essentially the same protection against CPK release as it did for lipid peroxidation. Again, the protection was roughly activity dependent except for the line with 200-fold overexpression, which failed to demonstrate any protection.

Because the loss of protection with 200-fold overexpression of catalase activity was unexpected, we performed preliminary studies with 500-fold catalase overexpressing mice (line 756). The doxorubicin increased MDA levels to 93.1 ± 4.4 nmol/g wet weight in nontransgenic normal mice (Cont, n = 15). Fifteen-fold elevation of catalase activity in the heart slightly decreased the level of doxorubicin-induced lipid peroxides (line 738, n = 8). Sixty (line 742, n = 10) or 100-fold (line 776, n = 10) elevation significantly inhibited doxorubicin-induced lipid peroxidation (p < 0.05). The level of lipid peroxides in these hearts was not significantly different from that in the normal saline-treated hearts. Two hundred-fold elevation did not significantly increase the level of lipid peroxides (line 777, n = 10).

Table I

| Enzyme activities of SOD, GSH peroxidase, and GSH reductase, and concentrations of GSH and MT in the hearts of control and MyCat transgenic mice |
|-----------------|------------------|------------------|------------------|
| **Control**     | **MyCat**        |                  |                  |
| SOD (units/g · wet wt) | 98.6 ± 3.7       | 108.3 ± 13.4     |                  |
| GSH peroxidase (nmol NADPH/min · mg protein) | 29.8 ± 4.4 | 26.2 ± 3.0 |                  |
| GSH reductase (nmol NADPH/min · mg protein) | 14.2 ± 1.7 | 16.0 ± 1.4 |                  |
| GSH (µg/g · wet wt) | 362.2 ± 11.4     | 356.0 ± 10.1     |                  |
| MT (µg/g · wet wt) | 5.1 ± 0.6        | 4.3 ± 0.5        |                  |

Fig. 3. Representative Northern blot analysis of catalase mRNA in the hearts of the 15 different transgenic mouse lines and the controls. The amount of mRNA detected in each line correlates with the level of catalase activity in the heart. Three bands hybridized with the catalase probe in transgenic samples. Only the smallest band corresponds to the expected, full processed transgenic mRNA. The arrow indicates the position of the single control band.

Fig. 4. Comparison of elevated catalase activities in the atria and ventricles in 5 representative transgenic mouse lines. Each value is the average of three determinations (mean ± S.D.). There is no significant difference (p > 0.10) in the catalase activity between the atria and ventricles in any of these transgenic lines.

Fig. 5. Comparison of catalase activities in the liver, kidney, lung, and skeletal muscles from the left rear leg (M1) and the abdominal external oblique muscles (M2) of the five transgenic mouse lines as shown in Fig. 4 (lines 738, 742, 782, 776, 777). The values represent the pooled data from the five lines with three determinations of each line (mean ± S.D., n = 15) for MyCat and from six control animals (Cont).

Fig. 6. Doxorubicin-induced lipid peroxidation in the hearts of normal and transgenic mice. The levels of MDA and 4-hydroxy-alkenals were measured to estimate the extent of lipid peroxidation in the tissue. The concentration of the lipid peroxides in nontransgenic saline-injected mice (Cont, n = 15) was 56.8 nmol/g of wet weight. Controls for each transgenic mouse line (saline injection only) showed no significant difference in the cardiac lipid peroxide level from the nontransgenic controls (data not shown). Doxorubicin significantly (p < 0.05) increased the lipid peroxide level in nontransgenic normal mouse hearts (DOX, n = 15). Fifteen-fold elevation of catalase activity in the heart slightly decreased the level of doxorubicin-induced lipid peroxides (line 738, n = 8). Sixty (line 742, n = 10) or 100-fold (line 776, n = 10) elevation significantly inhibited doxorubicin-induced lipid peroxidation (p < 0.05). The level of lipid peroxides in these hearts was not significantly different from that in the normal saline-treated hearts. Two hundred-fold elevation did not significantly decrease the level of lipid peroxides (line 777, n = 10).
Cardiac Catalase and Doxorubicin Toxicity

**FIG. 7.** Doxorubicin-induced CPK release from the heart. Serum CPK was measured using a CK-20 kit from Sigma. Sera from the same animals treated for determination of lipid peroxidation were collected for the CPK measurement. As shown, doxorubicin significantly increased serum CPK level in the nontransgenic control mice (DOX). Elevation of catalase in the heart to about 60- (line 742) or 100-fold (line 776) higher than normal completely inhibited the increase in the serum CPK level (p < 0.05). However, elevation of cardiac catalase activity to about 200-fold (line 777) higher than normal did not protect against this toxic effect of doxorubicin.

**FIG. 8.** Functional changes induced by doxorubicin in the isolated atria from normal and transgenic mice (line 742). The transgenic atrium contains catalase activity about 60-fold higher than the normal control. The atria were incubated with 300 μM doxorubicin (final concentration) in the perfusion buffer. Changes in spontaneous contraction of the atria were recorded at the times before (0 min) and post doxorubicin treatment (10, 20, and 30 min) as shown. This experiment was repeated five times with atria isolated from different animals. The same result was obtained with the repeated experiments.

**DISCUSSION**

Cardiotoxicity has long been recognized as a complicating factor of cancer chemotherapy with doxorubicin. There are several hypotheses to explain doxorubicin cardiotoxicity. Among these the free radical hypothesis is the most thoroughly investigated. Doxorubicin undergoes one-electron reduction through a metabolic activation caused by NADPH-cytochrome-P-450 reductase, or other flavin-containing enzymes (30). This reduction generates a doxorubicin semiquinone free radical. In the presence of molecular oxygen, the semiquinone rapidly reduces the oxygen to superoxide with regeneration of intact doxorubicin. Superoxide is rapidly converted to hydrogen peroxide spontaneously or by superoxide dismutase. The doxorubicin semiquinone can then react with the hydrogen peroxide to yield a hydroxyl radical (31). These highly reactive oxygen species react with cellular molecules including nucleic acids, protein, and lipids, thereby causing cell damage. Most supportive evidence for this free radical hypothesis has been obtained from in vitro studies including reports that: 1) doxorubicin increases lipid peroxidation and free radical production in the heart tissue (32); 2) free radical scavengers such as N-acetylcysteine (33), vitamin E (34), superoxide dismutase (34), and catalase (7) decrease the severity of doxorubicin-induced oxidative damage; 3) suppression of antioxidant activities enhances doxorubicin toxicity in cultured cardiac cells (35). However, contradictory results have been reported. Some in vivo studies have shown that free radical scavengers failed to prevent cumulative doxorubicin cardiotoxicity (36, 37).

Why have some in vivo studies failed to find antioxidant protection against doxorubicin cardiotoxicity? Under in vivo conditions, three major problems complicate data interpretation: 1) it is impossible to maintain constant plasma antioxidant concentrations and to accurately predict the target tissue concentrations; 2) metabolic activation and inactivation by multiple metabolic organs such as liver and kidney would greatly affect the efficacy of the antioxidants; and 3) high molecular weight antioxidants such as superoxide dismutase and catalase are unlikely transported into intracellular compartments. To overcome the shortcomings of these earlier in vivo studies, we produced a unique transgenic model in the present study. As described under "Results," in this model intracellular catalase activities are maintained at permanently elevated levels specifically in the heart of each transgenic line. Other antioxidant systems were found to be unaffected in the catalase overexpressing heart. Electron microscopic studies (data not shown) demonstrated increased numbers of peroxisomes in transgenic hearts, suggesting a significant amount of elevated catalase is contained in peroxisomes.

The results obtained from this study clearly demonstrate that elevation of catalase activity to an optimum level provides protection against doxorubicin-induced cardiac injury. This suggests that low catalase activity in the heart is a major factor responsible for the high sensitivity of the heart to doxorubicin-induced damage. Because catalase is a major enzyme that metabolizes H_2O_2 in the cell and this enzyme has no activity to react with doxorubicin and its metabolites, the results thus provide direct evidence to support the oxidative injury hypothesis for doxorubicin. Lipid peroxidation is a major cardiotoxic indicator of doxorubicin, particularly under the conditions of acute exposure. It has been found that doxorubicin more selectively induces this oxidative damage to the heart relative to other organs such as the liver in mice (38). We have determined the lipid peroxide levels in the heart induced by doxorubicin as a function of time (days). We found that at the selected dose (20 mg/kg) lipid peroxide levels in the heart reach a peak value on the 4th day after the drug treatment (data not shown). This peak value coincides with high serum CPK activity at the same time, which is in agreement with previous studies (39). Because the elevation in serum CPK results from the myocaidal cell membrane damage due to lipid peroxidation, the increased serum CPK activity has been used as another indicator of...
doxorubicin-induced heart damage (39, 40). The results obtained from this study show that elevated catalase activity protects from doxorubicin-induced lipid peroxidation, which correlates with the protection against CPK release. In both cases, 60- or 100-fold elevation of catalase activity provides maximum protection.

Functionality of the isolated mouse atrium has been increasingly used as a fast screening method for determination of cardiotoxicity induced by doxorubicin (41). Usually, the mouse atrium is incubated with the tested compound and changes in inotropy (contractile force) and chronotropy (heart rate) are recorded as a measurement of cardiotoxicity. It has been shown that doxorubicin depresses the functionality of isolated mouse atria (42–44). Doxorubicin-induced functional changes in the isolated atria were observed in the present study. Both contractile force and heart rate were suppressed. Sixty-fold elevation of catalase activity markedly depressed this effect of doxorubicin on cardiac function. This result demonstrates that doxorubicin-induced cardiac functional alterations most likely result from reactive oxygen species and that the severity of the functional alterations is associated with catalase levels.

In the present study we found that 200-fold elevation of catalase activity in the heart did not provide protection against doxorubicin toxicity and 500-fold elevation may even enhance doxorubicin cardiotoxicity. The same observation has also been reported in vitro. For instance, transfection of L cells with a human catalase cDNA elevated catalase activity by 100-fold. These catalase-enriched cells, however, were more sensitive rather than resistant to the cytotoxicity of paraquat, bleomycin, and doxorubicin than the untransfected cells from which they were derived (45). At least two mechanisms have been proposed for the increased sensitivity of the high catalase containing cells: 1) imbalance between SOD and catalase (46–48) and 2) chelation of heme iron by catalase and release of iron after degradation of catalase. It has been shown that iron-doxorubicin complex is toxic (49). The relevant mechanism for the effect observed in our mice is uncertain at this time.

The transgenic model produced in this study will provide a valuable experimental approach to understanding mechanisms of other cardiac disease conditions. There is, for example, disagreement on whether ischemia-reperfusion-induced heart damage is mediated by oxidative stress and whether low catalase activity in the heart generally makes the heart more sensitive to a variety of oxidative injuries. This experimental model should provide a means to test these related hypotheses.

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Cardiac Catalase and Doxorubicin Toxicity
