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MODULATION OF GLUTATHIONE AND GLUTATHIONE DEPENDENT ANTIOXIDANT ENZYMES IN MOUSE HEART FOLLOWING DOXORUBICIN THERAPY[1]

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The toxicity of the antineoplastic agent doxorubicin (DOX) has been shown to be moderated by the antioxidant enzyme glutathione peroxidase. It has been reported that acute doses of DOX can cause an inhibition of glutathione peroxidase in cardiac tissue, that may render this tissue especially susceptible to further prooxidant damage. In this study, multiple DOX treatments at a therapeutic dose were assessed for their effect on the antioxidant enzyme status of cardiac and kidney tissue. DOX was administered *i.p.* (5 mg/kg) once a week for two weeks to male balb/c mice. The activities of the antioxidant enzymes superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPOX) and glutathione reductase (GR) were measured 1, 2 and 7 days following the second DOX treatment in both heart and kidney. Levels of reduced glutathione (GSH) were also measured in cardiac tissue at these same times. Cardiac levels of GPOX and GR showed a time-dependent decrease in activity, with 10% and 12% inhibition for GPOX and GR, respectively, at 7 days post second treatment. Cardiac levels of GSH also showed a significant decrease, approximately 15%, at 7 days post second treatment. Cardiac levels of SOD and CAT as well as kidney levels of all four antioxidant enzymes were not affected by DOX treatment. These data suggest that DOX given in a therapeutic regimen, at a therapeutic dose, can cause decreases in cardiac levels of GPOX, GR and GSH that could render the heart especially susceptible to further oxidative challenge.

KEY WORDS: Doxorubicin, antioxidant enzymes, glutathione.

Abbreviations used: BUN; blood urea nitrogen, CAT; catalase, CK; creatine phosphokinase, CPK-MB; cardiac specific isoenzyme of creatine phosphokinase, DOX; doxorubicin, GPOX; glutathione peroxidase, GR; glutathione reductase, GSH; reduced glutathione, SOD; superoxide dismutase.

INTRODUCTION

Doxorubicin (DOX) is a widely used chemotherapeutic agent useful in the treatment of a variety of human neoplastic diseases[1]. The clinical usefulness of this antineoplastic antibiotic is hindered by the dose-dependent cardiotoxicity of DOX, which is often dose-limiting[2]. DOX is thought to cause cardiotoxicity through a

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mechanism involving the generation of reactive oxygen species following enzymatic bioreduction[3-6]. DOX has been shown to interact with NADH:oxidoreductase[7,8], an integral membrane protein of the mitochondrial electron transport chain, and NADPH:cytochrome c reductase[9,10], giving rise to a DOX intermediate capable of generating oxygen radicals. Oxidative damage induced by DOX in cardiac tissue is thought to include lipid peroxidation[3,5] as well as the inactivation of vital transport proteins[11-13].

Cellular defenses against oxidative stress include the antioxidant enzymes superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPOX) and glutathione reductase (GR). These enzymes function to detoxify reactive oxygen intermediates in the case of SOD (O_2^-) and CAT and GPOX (H_2O_2), or to regenerate reduced glutathione (GSH) in the case of GR. GPOX also functions to detoxify lipid hydroperoxides generated by the interaction of reactive oxygen with cellular lipids. Increases in cellular GPOX activity by scrape loading purified GPOX into human MCF-7 mammary carcinoma cells[14], and treatment of MCF-7 and Ehrlich tumor cells with the seleno-organic antioxidant PZ-51[15-16], which has GPOX-like activity[17], have been shown to decrease sensitivity to DOX induced toxicity. Depletion of GSH with buthionine sulfoximine, an inhibitor of GSH *de novo* synthesis, has been shown to increase the sensitivity of DOX-resistant MCF-7 cells to DOX induced toxicity[18], suggesting that GSH mediated mechanisms may play a role in cellular defenses against DOX induced damage.

The effect of DOX on the activity of GPOX in cardiac tissue has been extensively studied. DOX given *i.v.* (1.5 mg/kg) 3 times a week for 3 weeks to rabbits caused a 40% decrease in GPOX activity in cardiac tissue, but caused a 20% increase in GPOX activity in liver, and no effect in kidney[19]. Studies using mice given a single DOX treatment (10–15 mg/kg) *i.p.* caused a significant decrease (20%–50%) in cardiac, but not liver, GPOX activity between 24 and 72 hours following DOX administration[20]. Other studies using rats given DOX (15 mg/kg) *i.v.*[21], and rabbits treated with DOX (1.1, 5 and 10 mg/kg) *i.v.*[22] have shown no effect on cardiac GPOX activity. Cardiac levels of GSH have also been shown to be decreased[23] or increased[22] in response to acute DOX treatment. The discrepancies between these studies brings in to question the role of GPOX inhibition and GSH depletion in DOX induced oxidative damage in cardiac tissue.

In the studies presented in this paper, therapeutic doses of DOX (5 mg/kg), given once a week for two weeks, were assessed for their effects on the antioxidant enzyme status in heart and kidney up to one week following the second treatment. GSH levels were also measured in cardiac tissue in this same time frame. The results suggest that two enzymes involved in cellular GSH metabolism (GPOX and GR) have decreased activities in cardiac tissue following DOX treatment, but that their activities are unaffected by DOX in kidney. Kidney tissue was selected as a control tissue as other studies have shown no perturbations in glutathione metabolism in this tissue following DOX treatment[19]. These data suggest that DOX therapy may render cardiac tissue especially susceptible to further oxidative insult.

MATERIALS AND METHODS

Chemicals

Catalase (from bovine liver, C10), doxorubicin (D4305), diethylenetriaminepentaacetic acid (D1133), reduced glutathione (G4251), oxidized glutathione (G4626),

glutathione reductase (G4751, type III from bakers yeast), nitro blue tetrazolium (N6876), superoxide dismutase (S2515, from bovine erythrocytes) and the diagnostic kits for the determination of total-CK (47-50), CPK-MB isozyme (715-AM) and blood urea nitrogen (535-A) were purchased from Sigma Chemical Co. (St. Louis, MO.). All other reagents were of analytical grade.

Experimental Animals

Animals used for these studies were 10–12 week old, male Balb/c mice given food and water *ad libitum*. Control and DOX treated animals were treated once a week for two weeks with either DOX (5 mg/kg) or carrier solvent (sterilized water). This lower, non-lethal, multiple drug dose schedule more closely resembles DOX administration in the clinical setting[24-25]. All injections were given *intra peritoneally*, according to a schedule B injection protocol (0.05 ml/20 g). Treated and control animals were sacrificed via cervical dislocation 1, 2 and 7 days post 2nd injection, and all assays were done immediately on the fresh tissue.

Enzyme Assays

Heart and kidney tissue was excised and washed with ice-cold 50 mM potassium phosphate buffer (pH 7.8) to remove any excess blood. The tissue was then homogenized in this same buffer and centrifuged at $600 \times g$ for 10 minutes. The supernatant was then collected and stored on ice until used.

SOD activity was measured by the method described by McCord and Fridovich[26] as modified by Oberley and Spitz[27]. Briefly, a superoxide generating system consisting of xanthine and xanthine oxidase was used along with nitroblue tetrazolium (NBT) to monitor superoxide formation by following the colorimetric absorbance change at 560 nm. A standard curve for SOD activity was generated using known amounts of SOD (from bovine erythrocytes) and monitoring the decrease in absorbance change at 560 nm due to enzymatic breakdown of superoxide by SOD. From this standard curve, heart and kidney levels of SOD activity were calculated based upon the samples' ability to decrease the absorbance change at 560 nm and extrapolating to the corresponding SOD activity on the standard curve. Reaction mixtures consisted of the following final concentrations: 50 mM potassium phosphate (pH 7.8), 1 mM diethylen etriaminepentaacetic acid, 1 unit of catalase, 56 μM NBT, 10 mM xanthine, and xanthine oxidase. All assays were run at 25°C, and SOD activity is expressed in units/mg protein.

CAT activity was measured by monitoring the breakdown of H_2O_2 at 240 nm as previously described[28]. Reaction mixtures consisted of the following: 50 mM potassium phosphate (pH 7.0), 1 mM EDTA and H_2O_2 to give an absorbance between 0.52 and 0.55 at 240 nm. One unit of catalase activity was defined as that amount that decomposes 1 μmol H_2O_2/min/mg protein at 25°C.

GR activity was determined by the method of Racker[29]. Reaction mixtures consisted of 0.15 ml of 1 M potassium phosphate (pH 7.6), 0.3 ml 1 mM NADPH, 1.65 ml water, 0.3 ml 1% BSA, and 0.3 ml of a 2% solution of glutathione disulfide. Reaction was monitored at 340 nm to measure NADPH disappearance and one unit of GR activity is expressed as 1 nmol NADPH consumed/min/mg protein at 25°C.

GPOX activity was determined by the method of Strauss *et al*[30]. Reaction mixture consisted of 2.59 ml potassium phosphate (pH 7.0) containing 1 mM EDTA, 0.01 ml 1.25 M sodium, azide, 0.1 ml 150 mM reduced glutathione and 5 μl

glutathione reductase. To this solution 0.1 ml of 2.2 mM H_2O_2 was added and the reaction monitored at 340 nm until a linear rate was established followed by the addition of the sample. The difference between the two rates corresponds to the GPOX activity. One unit of GPOX activity is defined as 1 nmol NADPH consumed/min/mg protein at 25°C.

Determination of GSH Levels in Heart

Hearts were excised, washed with ice-cold 50 mM potassium phosphate buffer (pH 7.4) to remove any excess blood, and as quickly as possible homogenized in 10% (v/v) perchloric acid containing 1 mM bathophenanthrolinedisulfonic acid. This acid extract was then spun at *15,000* × *g* for 15 minutes, and the supernatant derivitized and analyzed by HPLC as previously described[31]. HPLC analysis was carried out using a Beckman 338 binary gradient system (Beckman Instruments, Palo Alto, CA) fitted with a 3-Aminopropyl-Spherisorb column (Custom LC, Inc., Houston, TX).

Determination of CPK-MB Isoenzyme and BUN Levels in Serum

Collected blood was centrifuged for 15 mins. in a refrigerated microcentrifuge, and the serum removed. Total-CK was determined spectrophotometrically (Diagnostic kit 47–50, Sigma Chemical Co.). The CPK-MB isozyme was measured following separation of the isozymes electrophoretically on agarose gels[32], followed by active enzyme staining (Diagnostic kit 715-AM, Sigma Chemical Co.), and the bands were quantitated using a Beckman DU-64 spectrophotometer equipped with a gel scanning apparatus (Beckman Instruments). CPK-MB activity was then calculated by taking the fraction of CPK-MB isozyme in serum multiplied by the total-CK activity. Serum BUN levels were measured using a colorimetric determination (Diagnostic kit 535-A, Sigma Chemical Co.) without deproteinization of the serum.

Lipid Peroxidation Studies

Hearts and kidneys were excised and washed with 50 mM potassium phosphate buffer (pH 7.8) to remove any excess blood, weighed, and subsequently homogenized in the wash buffer. The homogenate was assayed for lipid peroxidation by the thiobarbituric acid test[33].

Protein Determination

Protein was determined using the BCA Protein Assay Reagent (Pierce, Rockford, IL), and bovine serum albumin (BSA) as a standard.

Statistical Analysis

The student's t test was used to assess the differences between control and DOX treated groups. Significance was attributed to $P < 0.05$.

RESULTS

The effect of two weeks DOX treatment on the activity of the antioxidant enzymes GPOX, GR, CAT and SOD was measured in heart and kidney tissue and the results are shown in tables 1 and 2. Cardiac GPOX activity was decreased 2 and 7 days post 2nd DOX treatment, with the activity showing about a 7% decrease at 2 days and 10% at 7 days. The activity of GR in cardiac tissue was also decreased at 2 and 7 days post 2nd DOX treatment, showing 5% and 12% decreases respectively. The activities of SOD and CAT in heart were not decreased by DOX treatment. Kidney levels of GPOX, GR, SOD and CAT were unchanged by DOX treatment.

Cardiac levels of GSH were measured at 1, 2 and 7 days post 2nd DOX treatment and the results are shown in table 3. Cardiac GSH levels decreased approximately 10% (marginally significant) at 24 hours post treatment, but returned to control levels at 2 days post treatment. At 7 days post 2nd DOX treatment, cardiac GSH levels were significantly decreased ($P < .05$) from the control levels, to about 85% of control levels.

TABLE 1
Effect of 2 weeks DOX (5 mg/kg) treatment (once a week for 2 weeks) on antioxidant enzyme activity in Balb/C mouse cardiac tissue[a]

Day following 2nd DOX treatment	glutathione peroxidase[b]	glutathione reductase[c]	catalase[d]	superoxide dismutase[e]
control	100 ± 2.6	100 ± 1.2	100 ± 2.2	100 ± 5.6
1 day	98.4 ± 2.2	97.3 ± 2.5	106.7 ± 4.2	113.0 ± 7.8
2 days	92.8 ± 3.0	94.7 ± 1.5[∓]	97.0 ± 3.3	104.8 ± 4.2
7 days	90.2 ± 2.1[∓]	88.0 ± 2.3[∓]	105.6 ± 4.4	102.4 ± 6.6

[a]Values represent the mean ± SE of at least 6 independent determinations and are represented as percent of control value.
[b]control activity was 59.1 ± 1.5 nmols NADPH oxidized min^{-1} mg $protein^{-1}$.
[c]control activity was 20.8 ± 0.3 nmols NADPH oxidized min^{-1} mg $protein^{-1}$.
[d]control activity was 14.1 ± 0.3 μmols H_2O_2 decomposed min^{-1} mg $protein^{-1}$.
[e]control activity was 9.2 ± 0.5 SOD units mg $protein^{-1}$.
[∓]significantly different ($P < 0.05$) from control value.

TABLE 2
Effect of 2 weeks DOX (5 mg/kg) treatment (once a week for 2 weeks) on antioxidant enzyme activity in Balb/C mouse kidney tissue[a]

Days following 2nd DOX treatment	glutathione peroxidase[b]	glutathione reductase[c]	catalase[d]	superoxide dismutase[e]
control	100 ± 2.2	100 ± 2.7	100 ● 3.3	100 ± 5.6
1 day	100.7 ± 4.0	100.1 ± 3.4	91.7 ± 3.5	107.2 ± 3.5
2 days	96.4 ± 3.8	99.1 ± 1.7	92.8 ± 3.0	91.5 ± 4.3
7 days	102.3 ± 3.7	97.9 ± 2.5	110.3 ± 5.2	103.4 ± 5.5

[a]Values represent the mean ± SE of at least 6 independent determinations and are represented as percent of control value.
[b]control activity was 459 ± 10 nmols NADPH oxidized min^{-1} mg $protein^{-1}$.
[c]control activity was 89.8 ± 2.4 nmols NADPH oxidized min^{-1} mg $protein^{-1}$.
[d]control activity was 153 ± 5 μmols H_2O_2 decomposed min^{-1} mg $protein^{-1}$.
[e]control activity was 13.0 ± 0.8 SOD units mg $protein^{-1}$.

Lipid peroxidation in heart and kidney measured 7 days post 2nd DOX treatment was also assessed by measuring thiobarbituric acid reactive substances (TBARS), and the results are shown in table 4. Cardiac lipid peroxidation was increased significantly to 13.62 ± 1.13 nmols malondialdehyde equivalents formed/mg tissue from the control level of 8.52 ± 0.77. Kidney TBARS were not significantly affected by DOX treatment. Serum levels of CPK-MB, the cardiac specific isozyme of creatine phosphokinase, were elevated at 7 days, and serum blood urea nitrogen (BUN) levels were unaffected, suggesting that there was damage to cardiac tissue and no apparent damage to kidney (table 5).

TABLE 3
Effect of 2 weeks DOX (5 mg/kg) treatment (once a week for 2 weeks) on reduced glutathione (GSH) levels in Balb/C mouse cardiac tissue[a]

Days following 2nd DOX treatment	GSH levels[b] (% of control)
control	100 ± 3.2
1 day	89.6 ± 3.8∓∓
2 days	100.7 ± 4.4
7 days	85.1 ± 3.5∓

[a]Values represent the mean ± SE of at least 6 independent determinations.
[b]Control GSH levels were 647 ± 29 nmols GSH/mg tissue.
∓Significantly different ($P < 0.05$) from control value.
∓∓Marginal significant difference ($P < 0.08$) from control value.

TABLE 4
Lipid peroxidation in Balb/C mouse heart and kidney following 2 weeks DOX treatment (5 mg/kg) as measured by TBARS 7 days after last treatment[a]

Treatment Group	Heart[b]	Kidney
Control	8.52 ± 0.77	15.42 ± 0.87
DOX treated	13.62 ± 1.13	17.87 ± 0.96

[a]Values are expressed as nmols malondialdehyde equivalents formed/mg tissue ± SE of at least seven independent determinations.
[b]DOX treated group was significantly increased ($P < .05$) from control group.

TABLE 5
Measurement of heart and kidney damage in Balb/C mice induced by 2 weeks DOX treatment (5 mg/kg) 7 days following last treatment[a]

Treatment group	Heart[b]	Kidney[c]
Control	0	17.96 ± 4.78
DOX treated	0.44 ± 0.14	18.78 ± 5.36

[a]Values are expressed as the mean ± SE of at least 6 independent determinations.
[b]Heart damage was assessed as the amount of the cardiac specific isozyme of creatine phosphokinase (CPK-MB) in serum as described in the materials and methods. Values are expressed as units CPK-MB activity per ml. None of the 6 animals tested in the control group showed any measurable amount of CPK-MB isoenzyme in serum. The value of the DOX group is the mean value for the 4 of 8 animals in that group that showed any measurable CPK-MB isozyme in serum.
[c]Kidney damaged was assessed by measuring blood urea nitrogen levels in serum as described in the materials and methods.

DISCUSSION

The generation of reactive oxygen species by DOX following bioreductive activation has been proposed to play a major role in the cardiotoxic effects of this chemotherapeutic agent[3-6]. The abundance of mitochondria in cardiac myocytes, as well as the ability of DOX to redox cycle with mitochondrial proteins[7,8], makes cardiac tissue a preferential site for DOX-induced oxygen radical generation. Cardiac levels of GPOX and CAT activity were found to be approximately 10% of that found in kidney (tables 1 and 2) and liver (data not shown) in balb/c mice, and GR and SOD were also significantly lower in heart by approximately 75% and 50% respectively. This lower antioxidant enzyme activity observed in cardiac tissue could lead to an increased susceptibility to oxidative stress in heart as opposed to kidney or liver.

The role of glutathione-dependent processes in DOX-induced cardiotoxicity has been previously studied. Conflicting results from various studies however have not allowed for a clear understanding of this role. Decreases in cardiac GPOX activity following DOX treatment have been shown in rabbits[19] and mice[20] but other studies using rats[21] and rabbits[22] have shown no effect on cardiac GPOX activity. Likewise, GSH levels in rabbit cardiac tissue were shown in one study to increase following acute DOX treatment[22], while another study demonstrated an acute depletion of GSH in mouse cardiac tissue following acute DOX treatment[23]. Some of the discrepancies noted in the results of these various studies may be attributed to species differences, assay techniques, time frames of study and DOX dosages. Our studies used a highly sensitive and specific HPLC analysis for GSH levels and a non-toxic, therapeutic dosage of DOX. Our treatment regimen followed a therapeutic protocol for which we have previously described drug-induced cardiotoxicity[34]. This non-lethal, multiple dose schedule, with a break in between drug administrations, more closely approximates DOX administration in man than the single high dose bolus administration used in several other studies[24,25]. Our assay time points were chosen to reflect acute (1 and 2 day) changes in GSH and enzyme activities as well as to reflect the tissue status at the time point for a subsequent drug administration (7 day).

In this study, cardiac GPOX and GR were both significantly decreased 7 days after the second DOX treatment. While 1 day post treatment activities were not significantly lower, a steady decrease in activity with time was observed. No significant effects or patterns were observed for the other antioxidant activities tested in cardiac tissue, SOD or CAT. Kidney GPOX, GR, SOD and CAT showed no significant changes or patterns of change either. These observations are consistent with DOX's well documented cardiotoxicity and apparent lack of renal toxicity as well as our observation in table 5 of no increase in serum BUN but a significant serum CPK-MB isozyme level in DOX treated animals vs. controls.

The mechanism by which DOX causes a decrease in GPOX activity has been previously investigated and does not appear to involve a direct interaction between DOX and the enzyme[19,20], but may involve the oxidation of the selenocysteine at the active site to a diselenide, which cannot be subsequently reduced. It has also been proposed that DOX may cause a depletion in free selenium in cardiac tissue[19], which would cause a decrease in GPOX activity due to the selenium dependence of its enzymatic function. Previous studies in our laboratory have shown that pretreatment with PZ-51, a seleno-organic antioxidant with GPOX-like activity[17], can significantly reduce DOX-induced heart and liver lipid peroxidation

as well as serum CK activity without significantly increasing GPOX levels[35]. This suggests that even minor changes in cardiac levels of GPOX may influence the development of oxidative damage.

The mechanism for the DOX-induced decrease in GR activity may be very similar in mechanism in that it may involve the oxidation of important thiols in the active site of GR to an oxidation state incapable of being biologically reduced back to an active state. Inhibition of Ca-ATPase activity in heart microsome preparations by DOX has been previously shown to involve this type of modification of important thiol groups[12,13].

The decrease in cardiac GSH levels at 7 days post 2nd DOX treatment is in contrast with other studies that have shown either an acute decrease in cardiac GSH levels, that rebound to control levels by 1 day post treatment[23], or an increase in cardiac GSH in response to either acute or chronic DOX treatment[22]. It seems unlikely that GSH levels would show significant depletion due to the decrease in GR activity (about 12%), as previous reports have shown that inhibition of GR by 30%–40% with BCNU did not lead to a significant decrease in cardiac or hepatic GSH levels[36]. This lack of GSH depletion following GR inhibition by BCNU led the authors to conclude that there was excess GR activity within these tissues. Excess oxidative stress, inhibition of glutathione synthesis or an increase in lipid peroxidation byproducts requiring conjugation and detoxification by glutathione-S-transferases are all possible explanations for this observed decrease in GSH. While these studies do not elucidate a mechanism for DOX-induced GSH depletion, it clearly demonstrates that DOX treatment results in a decrease of cardiac GSH levels.

The importance of cardiac thiol status in the cardiotoxicity of DOX has been shown through the protective effects of N-acetylcysteine and cysteamine, two thiol donors, against DOX induced cardiotoxicity[23,25]. DOX given in a single i.p. dose (15 mg/kg) to mice was shown to decrease cardiac GSH levels by approximately 25% at 12 hrs. post treatment, while pretreatment with cysteamine (50 mg/kg) completely eliminated this DOX-induced decrease. In these same studies, pretreatment with N-acetylcysteine or cysteamine prior to DOX treatment, eliminated the appearance of cardiac damage as measured by electron microscopic examination of the tissue. Pretreatment with cysteamine caused an approximate 16% increase in the cardiac thiol pool, suggesting that even small changes in the cardiac free thiol pool may be important in the manifestation of DOX-induced cardiotoxicity[23]. N-acetylcysteine pretreatment prior to DOX-treatment has also been shown to significantly decrease the lethality of DOX to CDF_1 mice without affecting cardiac DOX concentrations or metabolism[25].

This study shows a perturbation of glutathione metabolism within cardiac tissue, but not kidney tissue, following DOX treatment. The generation of oxygen radicals in cardiac tissue by DOX has been well documented[3–8,37], and much work has gone into understanding the role of glutathione dependent processes in moderating DOX toxicity in both normal and neoplastic tissue. The tissue specific effects of DOX on decreasing GPOX and GR activity as well as GSH levels in cardiac, but not kidney tissue, are consistent with the increases in lipid peroxidation and damage seen in heart but not kidney. This suggests that DOX renders cardiac tissue especially susceptible to oxidative insult due to lowering of the cellular defenses against reactive oxygen intermediates. This may be important in understanding DOX's cumulative toxicity and DOX's enhancement of mitomycin C's oxidative toxicity[34] in that the subsequent oxidative toxicity, produced by repeated drug administration,

may be exacerbated by the prior tissue specific depletion of cardiac glutathione-dependent protective enzymes.

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