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EVALUATION OF TISSUE INDICATORS OF OXIDATIVE STRESS IN RATS TREATED CHRONICALLY WITH ADRIAMYCIN

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Abstract—Rats treated chronically with the anticancer agent adriamycin to a cumulative dose of 21 mg/kg, which was sufficient for development of an early stage of cardiomyopathy, were examined for evidence of lipid peroxidation and oxidative stress *in vivo* by several methods. Fluorometric analysis of lipid extracts suggested that fluorescent products of lipid peroxidation reactions were elevated about 3-fold in kidney, 40% in heart, and 10% in liver. However, lipid hydroperoxides and endoperoxides were not found to any significant extent in heart, liver or kidney. By contrast, as previously reported, the serum of adriamycin-treated rats showed substantial levels of lipid peroxide compounds. Measurements of glutathione levels indicated increases of about 50% in kidney and 20% in heart, and a decrease of 20% in liver, on a per gram tissue basis, after adriamycin treatment. Levels of protein-bound mixed disulfides were not altered after adriamycin treatment in heart, liver or kidney. Cardiac glutathione peroxidase activity was increased 30% after chronic adriamycin treatment, whereas glutathione reductase activity was unchanged. The results indicate that the major organs of rats treated chronically with adriamycin exhibit at least some persistent biochemical changes that are consistent with oxidative stress *in vivo*. The different types of lipid peroxidation products found in tissues as compared to serum may reflect, in part, the operation of membrane peroxidation repair processes.

Clinical use of adriamycin as a chemotherapeutic agent is limited by the occurrence of cardiomyopathy associated with chronic cardiotoxicity [1, 2]. Numerous biochemical studies have now demonstrated that adriamycin can stimulate the formation of free radicals through redox cycling interactions with cellular flavoproteins, particularly microsomal NADPH–cytochrome P-450 reductase [3–5] and mitochondrial NADH dehydrogenase [6, 7]. Adriamycin can stimulate free radical-initiated processes such as lipid peroxidation *in vitro* [8–10]. The relevance of such activities *in vivo* in regard to adriamycin toxicity and antitumor activities is less certain, however. Myers *et al.* [11] first demonstrated the formation of malondialdehyde, a breakdown product of lipid peroxides, in hearts of mice treated acutely with a single high dose of adriamycin. This finding suggested that lipid peroxidation may be involved in the cardiotoxicity of adriamycin.

We have studied adriamycin toxicity using chronically treated rats as an animal model for the development of heart muscle disease. We have reported previously that such adriamycin-treated rats exhibit lipid peroxides in the serum, providing a clear indication for the occurrence of lipid peroxidation reactions *in vivo* [12]. Our previous data indicated that the serum lipid peroxides consisted of both hydroperoxides and endoperoxides and were associated mainly with neutral lipid fractions of the serum lipoproteins [12]. In the present paper we report results of systematic studies of the major organs of adriamycin-treated rats evaluated for evidence of the occurrence of lipid peroxidation *in vivo* using methodology similar to that previously employed for detection of the serum lipid peroxides. This was done to investigate the possible origin of the serum lipid peroxides as well as to determine whether an organ-specific pattern of lipid peroxidation occurred in adriamycin-treated rats. We have also measured the sulfhydryl status of these organs as an additional potential indicator of oxidative stress. Previous studies have reported perturbations of glutathione levels following acute adriamycin administration to mice [13] and in cultured cell systems [14, 15], but little information is available in this regard for chronic toxicity models.

MATERIALS AND METHODS

Male, CDF-Fischer rats (Charles River Breeding Laboratories, Raleigh, NC) were treated with 1.5 mg adriamycin hydrochloride (Adria Laboratories, Columbus, OH) per kg body weight once per week subcutaneously for 13 or 14 weeks (cumulative dose: 19.5 to 21.0 mg/kg). control rats received a similar volume of saline. Rats were killed 1–3 weeks after final dosing, following an overnight fast. Organs were rapidly excised, chilled in iced saline, and processed for lipid extraction. For sulfhydryl studies, organs were excised and quick-frozen in liquid nitrogen to minimize GSH auto-oxidation and subsequently processed for analysis on the same day.

For lipid peroxide determinations, aliquots of

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heart tissue were minced and a homogenate was prepared in 130 mM KCl/20 mM KP_i/2 mM EDTA, pH 7.5, using a Polytron tissue disrupter (PT 10, setting number 6.5, 1 min). Butylated hydroxytoluene (BHT*) was added to a final concentration of 0.01%. Duplicate aliquots of homogenate were then extracted by the method of Folch *et al.* [16]. Tissue was homogenized for 2 min at 45° using a Teflon homogenizer during the lipid extraction [17]. The extract was then filtered through Whatman No. 1 paper, partitioned with 0.2 vol. of 0.29% NaCl, and washed with Folch upper phase [16]. Lipid extracts were then divided into three equal portions, evaporated under nitrogen, resuspended, and used for measurements of endoperoxides by thiobarbituric acid reactivity [18], hydroperoxides by an iodometric method [19], and fluorescent lipid peroxidation products [17], respectively, as described in a previous paper [12]. For fluorescence studies, lipid samples were dissolved in 3.0 ml of cyclohexane–ethanol (1 : 1, v/v). Fluorescence intensity was determined from spectra recorded in the uncorrected mode with 10 nm excitation and emission slit widths using a Perkin–Elmer MPF-44B spectrofluorometer. Lipid peroxide analyses of liver and kidney were conducted similarly, except that lipids were extracted directly from minced tissue rather than after preparation of a homogenate. BHT (0.01%) was added to lipid extracts immediately after preparation. Fluorescence intensity was calibrated relative to a solution of quinine sulfate (1.0 μg/ml in 0.01 N H_2SO_4) as 100 units. Each of the various assay methods was conducted with duplicate or triplicate aliquots of each tissue sample.

For analysis of sulfhydryl content, an aliquot of the frozen tissue was allowed to thaw in 2 ml of 1 N $HClO_4$/2mM EDTA, disrupted using the Polytron (1 min at setting number 6.5), and centrifuged (2000 g × 5 min) at 4°. Aliquots of the supernatant fraction were used for total sulfhydryl measurement by colorimetric determination with 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) at pH 8.5 [20]. Absorbance at 412 nm was converted to -SH equivalents using an extinction coefficient of 13.1 $mM^{-1}\,cm^{-1}$ [20]. GSH was measured enzymatically with glutathione-*S*-transferase (No. G-6511 from equine liver, Sigma Chemical Co., St. Louis, MO) catalyzed conjugate formation using 1-chloro-2,4-dinitrobenzene (CDNB) [21]. Formation of the conjugate was monitored by dual wavelength spectroscopy at 340–400 nm using an extinction coefficient of 6.9 $mM^{-1}\,cm^{-1}$ [21].

For determination of protein-bound mixed disulfides [21], the pellet obtained from the acid homogenization was resuspended in 1.0 ml of 0.5 M Tris–HCl/0.5 mM EDTA/4 M urea, adjusted to pH 8.5 with 1 N NaOH, and 0.05 ml octanol (to prevent foaming) and 0.1 ml of freshly-prepared $NaBH_4$ (250 mg/ml) were added. The solution was incubated for 30 min at 37°. Following incubation, 1.0 ml of 2 N $HClO_4$/1 mM EDTA was added, and the mixture was allowed to stand for 10 min on ice to destroy excess BH_4. Subsequently, the mixture was centrifuged and the supernatant fraction filtered through Whatman No. 1 filter paper in a small funnel. Aliquots of the clear filtrate were assayed for total sulfhydryl content by the DTNB method and for GSH by the enzymatic assay with CDNB as described above. In all cases, the samples were neutralized to the assay pH directly in the cuvette at the time of assay to prevent auto-oxidation of GSH [22].

Statistical significance of differences between samples from adriamycin-treated and control rats was evaluated by the unpaired *t*-test. Values of $P < 0.05$ were considered significant.

RESULTS

Effects of chronic adriamycin treatment on pathophysiological characteristics. Previous studies have established that chronic administration of adriamycin to rats produces histopathologic changes typical of adriamycin-induced heart muscle disease [23, 24]. These include a characteristic vacuolization of cardiac myocytes associated with swelling of sarcoplasmic reticulum and mitochondria and disruption of muscle fibers. A total cumulative dose of about 20 mg adriamycin/kg body weight is necessary to induce the early stages of cardiomyopathy in rats [23], and was thus used in the present study so that biochemical changes associated with an early stage of the disease could be identified. Light microscopic examinations confirmed the presence of these lesions in rats treated by the protocol used for the biochemical studies reported in this paper. Multicellular foci of vacuolated myocytes were typically observed. At this dose of adriamycin, rats also exhibited nephrotoxicity characterized by occasional vacuolization of glomeruli and tubular dilation [23, 25]. The liver typically showed a mild fatty degeneration, but neither vacuolization of hepatocytes nor necrosis was observed.

Lipid peroxide analysis of rat organs. The major focus of our investigations has been to determine whether adriamycin treatment is associated with the occurrence of free radical-initiated reactions such as lipid peroxidation *in vivo*. For this purpose we have analyzed tissues of chronically treated rats known to show adriamycin-induced pathology. We have analyzed tissues directly without any subsequent *in vitro* incubation procedures so that the levels of these compounds present *in vivo* could be estimated. In addition, we have employed several different methods for measurement of lipid peroxides and related compounds because lipid peroxidation leads to a wide variety of products.

Lipid hydroperoxides were not detected by a standard iodometric technique in assays of lipid extracts of heart, liver or kidney. In all cases, absorbance values observed with this assay were less than two times the reagent blank values, and were thus considered below the limit for reliable detection by the iodometric assay. Similarly, measurements of thiobarbituric acid reactivity with lipid extracts or with tissue homogenates as an assay for lipid endoperoxides, which degrade to malondialdehyde under

* Abbreviations: BHT, butylated hydroxytoluene; CDNB, 1-chloro-2,4-dinitrobenzene; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); GSH, reduced glutathione; GSSG, oxidized glutathione; and -SH, sulfhydryl.

Table 1. Effect of chronic adriamycin treatment on fluorescent lipid peroxidation products in rat organs*

Organ	Observation wavelengths (λ_{max}) (nm)		Fluorescence intensity (relative units/g wet weight)	
	Excitation	Emission	Control	Adriamycin-treated
Heart	368	440	29.2 ± 10.6 (12)	42.1 ± 15.6† (16)
Liver	368	475	203.0 ± 14.4 (5)	230.6 ± 19.0† (7)
Kidney	338	475	18.3 ± 4.7 (5)	54.2 ± 22.1‡ (7)

* Fluorescence of lipid extracts was measured at the indicated wavelengths as described in Materials and Methods. Intensity values indicate mean ± SD relative to a quinine sulfate (1.0 μg/ml) standard. The number of animals is indicated in parentheses.
† Significantly different from control value at $P < 0.05$.
‡ Significantly different from control value at $P < 0.01$.

the conditions of the assay procedure [26], did not reveal the presence of any endoperoxide-type compounds in tissues of adriamycin-treated rats above the levels observed for control rats. Conjugated diene compounds, detectable by an increase of absorbance at 233 nm in lipid extracts [27], were also not found. However, fluorescence studies of lipid extracts revealed spectra typical of what have been termed fluorescent products of lipid peroxidation [17, 28–30] (Table 1). Previous *in vitro* and *in vivo* studies have demonstrated the formation of a variety of products of peroxidation reactions characterized by fluorescence excitation in the 340–370 nm range and emission in the 420–475 nm range [17, 28–30]. In the case of tissues from adriamycin-treated rats, different organs exhibited somewhat different fluorescence spectra, suggesting the presence of different types and mixtures of compounds. In heart tissue, the level of fluorescence was increased about 40% after adriamycin treatment on a per gram tissue basis. In kidney, a 3-fold increase in fluorescence was found after adriamycin treatment. In liver, only a 10% increase was observed. However, in liver, absolute fluorescence intensities were about 10-fold higher than found in heart or kidney. In addition, the wavelength of maximum emission, 475 nm, suggested that part of the observed fluorescence may have been attributable to retinol [17]. However, UV-irradiation of the lipid extracts in chloroform, expected to cause rapid photodecomposition of retinol [17], did not decrease the observed fluorescence intensity with samples from either control or adriamycin-treated rats.

In agreement with our previous report [12], thiobarbituric acid reactivity of the serum was elevated in adriamycin-treated rats. Values measured with representative groups of rats used for the organ studies reported in this paper were: adriamycin-treated, 24.0 ± 14.8 (N = 22); control, 6.5 ± 2.8 (N = 12) nequiv malondialdehyde per ml serum (significantly different at $P < 0.01$).

Effect of chronic adriamycin treatment on sulfhydryl status of rat organs. Low molecular weight sulfhydryl compounds, particularly glutathione (GSH), are thought to play a major role in protection of cells from oxidative stress through both direct scavenging of free radicals and as a component of the glutathione redox cycle. We measured both total acid-soluble sulfhydryl compounds by a non-enzymatic assay using DTNB, and GSH specifically by a glutathione-*S*-transferase-based assay. In heart, good agreement was found between these two methods, suggesting that GSH is the only major sulfhydryl compound in heart cells. In liver, approximately 90% of total sulfhydryl compounds were accounted for as GSH. In kidney, however, GSH accounted for only 30–40% of total acid soluble -SH groups (Table 2). This apparently reflected the fact that the kidney is the major site of GSH catabolism, where GSH is degraded to amino acids through several intermediate -SH compounds [31, 32].

After chronic adriamycin treatment, liver GSH and total -SH were decreased about 20% on a per gram tissue basis compared to control rats (Table 2). By contrast, in heart, GSH was increased about 20% after long-term adriamycin treatment. In kidney, a 50% increase in the level of GSH was found, while total −SH compounds were increased to a lesser extent. The latter difference did not reach statistical significance, however. The higher proportion of GSH in comparison to total -SH in kidney after adriamycin treatment may reflect some degree of inhibition of GSH catabolism.

Table 2. Effect of chronic adriamycin treatment on acid-soluble sulfhydryl content of rat organs*

Organ	Treatment	Total -SH (μmol/g wet weight)	GSH (μmol/g wet weight)
Heart	Control	1.46 ± 0.19	1.41 ± 0.23
	Adriamycin	1.64 ± 0.30	1.72 ± 0.26†
Liver	Control	6.10 ± 0.88	5.46 ± 0.65
	Adriamycin	4.63 ± 0.97‡	4.35 ± 1.09†
Kidney	Control	2.48 ± 0.55	0.74 ± 0.21
	Adriamycin	2.92 ± 0.71	1.12 ± 0.30†

* Total sulfhydryl compounds were measured chemically with DTNB; GSH was measured enzymatically with GSH-transferase as described in Materials and Methods. Values indicate mean ± SD (N = 8 for control values and N = 10 for adriamycin-treated values).
† Significantly different from control value at $P < 0.05$.
‡ Significantly different from control value at $P < 0.01$.

Table 3. Effect of chronic adriamycin treatment on protein-bound mixed disulfides in rat organs*

Organ	Treatment	Total (μmol/g wet weight)	GSH-specific (μmol/g wet weight)
Heart	Control	0.43 ± 0.13	0.062 ± 0.025
	Adriamycin	0.37 ± 0.07	0.073 ± 0.030
Liver	Control	0.56 ± 0.20	0.042 ± 0.012
	Adriamycin	0.45 ± 0.08	0.049 ± 0.030
Kidney	Control	0.52 ± 0.10	0.033 ± 0.020
	Adriamycin	0.56 ± 0.11	0.060 ± 0.049

* Total protein-bound disulfide groups were determined chemically with DTNB; glutathione bound to proteins as mixed disulfides was measured enzymatically with GSH-transferase as described in Materials and Methods. Data indicate sulfhydryl group equivalents, mean ± SD (N = 8 for control values and N = 10 for adriamycin-treated values).

We also measured the content of protein-bound mixed disulfides in rat tissues, because these have been reported to increase in other models involving oxidative stress [33–35]. Again, we employed both a specific enzymatic assay for GSH bound as mixed disulfides and a chemical procedure for detecting total sulfhydryl compounds bound as mixed disulfides. In all organs, we found that GSH bound to protein as mixed disulfides constituted only a relatively small proportion of total disulfides (Table 3), in agreement with other studies employing a GSH-specific assay method [21, 31]. To verify that the enzymatic assay procedure was adequate for detecting glutathione disulfides after the borohydride reduction procedure, we tested recovery (as GSH) of GSSG added as an internal standard to an aliquot of the samples in several experiments. Recovery of the added internal standard as GSH averaged 88 ± 11% (N = 17), confirming the adequacy of the method.

Chronic adriamycin treatment did not change the amount of total protein-bound mixed disulfides in heart, liver or kidney (Table 3). The amount of GSH bound as mixed disulfides was also not significantly different after adriamycin treatment in all three organs, though a trend toward higher values was seen for kidney. Relatively high variability between different animals was observed in the level of glutathione bound as mixed disulfides. The latter, however, was low in all cases.

To investigate further the effects of adriamycin treatment on glutathione metabolism, we also examined enzymes of the glutathione redox cycle [22]. Glutathione peroxidase activity in homogenates prepared from perfused hearts was elevated about 30% after chronic adriamycin treatment [control, 12.2 ± 1.3 (N = 6); adriamycin-treated, 16.0 ± 2.7 μmol/min/g (N = 9), significantly different at $P < 0.01$]. On the other hand, cardiac glutathione reductase activity was unchanged after chronic adriamycin treatment [control, 1.3 ± 0.3 (N = 5); adriamycin-treated, 1.1 ± 0.1 μmol/min/g (N = 8)].

DISCUSSION

Results of the present study suggest that fluorescent products of lipid peroxidation reactions are present in the kidney and, to a lesser extent, in the heart of rats treated chronically with adriamycin. The data suggest that such compounds may also be present to a small extent in liver, although the high endogenous level of fluorescence found with this tissue makes this less certain. Studies with model lipid systems have shown that fluorescent products of lipid peroxidation represent, at least in part, cross-linked phospholipids, particularly phosphatidylethanolamine dimers [28]. These compounds are thought to arise as end products of free radical reaction sequences which produce malondialdehyde which acts as the cross-linking agent. Fluorescence derives from the formation of Schiff base iminopropene chromophores [28]. Fluorescent lipid peroxidation products appear to be similar to lipofuscin pigments [28]. The absolute amount of these products cannot be readily quantified, however, from the fluorescence data. Nevertheless, the observation that the relative amount of the characteristic fluorescence was increased in lipid extracts obtained from tissues of adriamycin-treated rats in comparison to controls suggests the occurrence of lipid peroxidation reactions *in vivo* in association with adriamycin treatment. Increased amounts of lipofuscin granules have been noted in kidneys of rabbits treated chronically with adriamycin [25]. The variation in fluorescence spectral characteristics (excitation and emission maxima) in different organs suggests that a different mixture of chemical species derived from free radical reactions is present in each organ. This may reflect varied lipid compositions in each organ or a different sequence of reactions leading to the fluorescent products. Previous studies concerned with the formation of fluorescent products during *in vitro* lipid peroxidation have also observed variations in fluorescence spectra with preparations from different tissues [28–30].

The absence of lipid hydroperoxides and endoperoxides from rat organs after adriamycin treatment may reflect the fact that these are generally intermediates in the peroxidation sequence of unsaturated fatty acids [19, 36]. Alternatively, such peroxides, if formed, may be cleaved from membranes by the action of phospholipases. These may subsequently be degraded by glutathione peroxidase, or perhaps exported from the tissue. Recent studies have shown that phospholipase activity is required prior to the action of glutathione peroxidase in metabolism of membrane-associated phospholipid peroxides [37, 38]. In addition, peroxidized phospholipids appear to be preferential substrates for phospholipase A_2 [38]. Increased phospholipase activity has been observed in the rat heart following acute administration of multiple doses of adriamycin over a 4-day period [39].

We have reported previously that rats treated chronically with adriamycin have relatively high amounts of lipid endoperoxides and hydroperoxides in the serum, associated with the serum lipoproteins [12]. Fluorescent products of lipid peroxidation were not observed in the serum, however [12]. Our finding

of lipid peroxides in the serum and lipid peroxidation products in rat organs provides a strong indication that free radical reactions and lipid peroxidation reactions actually occur *in vivo* as a consequence of adriamycin treatment. The different types of lipid peroxide-related compounds found in serum, as compared to tisues may be an indication for the operation of membrane "repair" processes, as previously suggested [12]. Thus, lipid peroxides may be selectively cleaved from membranes and exported to the serum where, as unusual and apparently non-metabolizable species, they might accumulate in storage lipid forms such as the triglycerides of serum lipoproteins. Serum triglycerides are also greatly elevated in adriamycin-treated rats [40]. On the other hand, fluorescent lipid peroxidation products, particularly cross-linked phospholipids, may be non-removable by phospholipase activity and thus tend to be accumulated in the tissue of origin.

The distribution of lipid peroxidation products with respect to the different tissues does not indicate organ specificity of the free radical reactions leading to their formation. It is likely that similar sequences of reactions are responsible for adriamycin toxicity in the various organs. Activation of adriamycin to a free radical form has been demonstrated in subcellular preparations from heart [7], liver [3, 4] and kidney [10].

In regard to total sulfhydryl and glutathione status, the present data indicate a relatively small increase in the heart, a decrease in the liver, and an increase of GSH but not total -SH in kidney after chronic adriamycin treatment. It is not clear, however, which sort of changes are potentially indicative of oxidative stress. Previous studies of glutathione status have also noted increased myocardial GSH in both rabbits [41] and dogs [42] treated chronically with adriamycin. An increase in tissue GSH might be interpreted as an adaptive response to an increased need for antioxidant protection, as has been suggested in some studies [41, 42]. The increased level of cardiac glutathione peroxidase activity observed in this study may likewise reflect an adaptive response to oxidative stress. By contrast, acute administration of high doses of adriamycin has been found to cause a transient decrease in myocardial GSH, an effect attributed to increased flux through the glutathione redox cycle as well as the free radical scavenging activity of GSH [13].

Protein-bound mixed disulfides have been demonstrated in lung tissue following acute paraquat administration to rats [34, 35] and after ozone intoxication [33]. Both paraquat and ozone toxicity are thought to involve free radical reactions [43, 44]. Thus, protein-bound mixed disulfides have been suggested to be a marker for oxidative stress. These may arise as a compensatory mechanism for relief of high GSSG levels through the action of glutathione transferases [45]. In this study, we did not find significantly increased levels of such compounds in tissues of adriamycin-treated rats. This finding, as well as the relatively small changes in acid-soluble sulfhydryl content, suggests that perturbation of the glutathione redox state is not a persistent consequence of adriamycin toxicity during long-term administration of adriamycin.

The finding that only fluorescent lipid peroxidation products, rather than other types of lipid peroxides, were observed in organs, as well as the fact that organ sulfhydryl changes were relatively small in this study, may have been influenced by our choice of sampling intervals after cessation of adriamycin administration. We purposely selected an interval of 1–3 weeks following the final chronic dosing in order to evaluate persistent biochemical changes associated with morphologic alterations rather than the presence of the drug itself. More substantial changes in organ sulfhydryl content or different types of lipid peroxide-related compounds may have been observed at shorter sampling intervals after adriamycin administration when tissue drug levels would be expected to be higher, as have been seen in acute toxicity studies [41, 42]. It is noteworthy, however, that the lipid peroxides found in the serum appear to persist after cessation of chronic adriamycin administration [12].

Overall, the results of this and our previous studies [12, 40] of rats treated chronically with adriamycin support the concept that adriamycin toxicity does involve the generation of free radicals, lipid peroxidation and oxidative stress *in vivo*. However, adriamycin administration induces a range of toxicities manifest by different morphology and impairments of function in a variety of different organs. The numerous biochemical activities of adriamycin [2] may potentially contribute to its toxicity to different degrees in different organs. Thus, free radical reactions and oxidative stress, while involved, are not likely to account for all aspects of adriamycin toxicity.

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