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ADRIAMYCIN-INDUCED LIPID PEROXIDATION IN MITOCHONDRIA AND MICROSOMES*

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Abstract—The effect of the anti-neoplastic agent adriamycin on the peroxidation of lipids from rat liver and heart mitochondria and rat liver microsomes was investigated. The extent of total lipid peroxidation was determined by assaying for malondialdehyde (MDA), while the degradation of unsaturated fatty acids was monitored using gas chromatography. For liver mitochondria and microsomes, the formation of MDA was dependent on the concentrations of adriamycin, Fe^{3+}, and protein, as well as time. In the presence of 50 μM adriamycin and saturating amounts of NADH, 1.5 ± 0.2 nmol MDA/mg protein/60 min was produced with liver mitochondria. Upon addition of 25 μM Fe^{3+}, the amount of MDA generated was increased to 6.5 ± 0.1 nmol/mg protein/60 min. Liver microsomes produced amounts which were approximately 2-fold higher under all conditions. No MDA formation could be detected in rat heart mitochondria. The addition of 50 μM chlorpromazine completely inhibited peroxidation, whereas 0.5 to 1.0 mM *p*-bromophenacyl bromide blocked MDA formation by 50%. Analysis of fatty acids by gas chromatography showed that there was about a 50% decrease in arachidonic and docosahexaenoic acids in liver mitochondria and microsomes, but no change in the fatty acid content of heart mitochondria when incubated with both 50 μM adriamycin and 25 μM Fe^{3+} for 1 hr. These results suggest that (1) therapeutic concentrations of adriamycin enhance the peroxidation of lipids in liver mitochondria and microsomes through an enzymatic mechanism, especially in the presence of Fe^{3+}; and (2) toxicity of this drug may be related to the degradation of membrane lipids.

Adriamycin, a cytotoxic antibiotic in the anthracycline family, is a widely employed and very effective antineoplastic agent. The quinone moiety of this drug has been shown to be capable of a one-electron reduction to a semiquinone free radical by NADH dehydrogenase in mitochondria [1]. The semiquinone radical can react with molecular oxygen to form a superoxide anion and, subsequently, hydrogen peroxide and a hydroxyl radical [2]. All these highly reactive compounds participate in the peroxidation of membrane lipids, resulting in membrane degradation and protein aggregation and, hence, could be responsible for the known toxicity of the drug.

In an effort to provide evidence for this hypothesis, we analyzed the extent of lipid peroxidation in rat liver and heart mitochondria, as well as in rat liver microsomes. These *in vitro* experiments were performed under identical conditions, such that the lipid peroxidation in all three systems could be directly compared. Both the detection of malondialdehyde (MDA) formation, as well as the loss of total fatty acids as analyzed by gas chromatography, were used to determine the extent of peroxidation.

MATERIALS

Chemicals. Adriamycin/HCl (with lactose), chlorpromazine, *p*-bromophenacyl bromide, heptadecanoic acid and the fatty acid methyl ester standards, palmitic, stearic, oleic, linoleic, linolenic, arachidonic, and docosahexaenoic, were purchased from the Sigma Chemical Co. (St. Louis, MO). All other reagents were of the highest purity available.

Adriamycin/HCl was dissolved in 15 mM NaCl, 10 mM Na_2HPO_4, pH 6.2, to a final concentration of 3.4 mM and stored at 4°. Fe^{3+} was added to the incubations in the form of $FeCl_3$; Fe^{2+} was added in the form of $FeCl_2 \cdot 4H_2O$; ADP/Fe^{3+} and ADP/Fe^{2+} complexes were obtained by combining the appropiate concentrations of the two species in distilled/deionized H_2O and stirring overnight at 4°. Stock solutions (up to 50 mM) of chloropromazine and *p*-bromophenacyl bromide were prepared in distilled/deionized H_2O and absolute ethanol respectively.

Mitochondrial and microsomal preparations. Male Sprague–Dawley rats (200–250 g) were fed with Purina Rat Chow and water *ad lib.* until the time of sacrifice. Liver mitochondria from one rat were prepared according to the procedure of Schneider and Hogeboom [3], with the addition of 1 mM EDTA to the isolation medium. The crude mitochondrial pellet was washed twice in 140 mM KCl, 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes), pH 7.4, to remove sucrose and EDTA (which interfere with the MDA assay for lipid peroxidation) and were suspended in this medium to approximately 35 mg/ml. The post-mitochondrial supernatant fraction was further centrifuged in a Beckman ultracentrifuge at 105,000 *g* for 30 min to obtain a microsomal fraction. The microsomal pellet was taken up in 140 mM KCl, 10 mM Hepes, pH 7.4,

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washed twice by centrifugation as above, and finally suspended in the same buffer at approximately 20 mg/ml. Aliquots (1 ml) were frozen (−80°) and used for subsequent experiments. The purity of the mitochondrial fraction from this procedure was determined by assaying for the microsomal marker enzyme, 5′-nucleotidase [4]. The mitochondrial preparation was contaminated less than 20% with microsomes.

Heart mitochondria were obtained according to the procedure of Fuller *et al.* [5] in the absence of bovine serum albumin. Analogous to liver mitochondria, the mitochondrial pellet was washed twice with 140 mM KCl, 10 mM Hepes, pH 7.4, and suspended in this medium at a concentration of approximately 20 mg/ml (a total of 50–60 mg protein was obtained from four hearts).

Protein was determined by the biuret method, using bovine serum albumin as the standard [6].

Measurement of MDA formation. Rat liver and heart mitochondria and rat liver microsomes (1 mg/ml) in a total volume of 1.75 ml were incubated at 37° in the dark under an atmosphere of oxygen for 0–120 min in oxygen-saturated 140 mM KCl, 10 mM Hepes, pH 7.4, 2.5 mM NADH or 2.5 mM NADPH, 0–75 μM adriamycin, and 0–25 μM Fe^{3+}. All incubations were initiated by the addition of protein. The incubations were quenched at the appropriate time by the addition of 0.75 ml of 2 M trichloroacetic acid (TCA)/1.7 N HCl. After pelleting the protein, 0.5 ml of the supernatant fraction was removed and mixed with 2 ml of 0.7% thiobarbituric acid (TBA) solution. The mixture was heated at 95° for 20 min. After cooling, the absorbance of the sample was determined at 533 nm in a Bausch & Lomb Spectronic 210 UV/VIS spectrophotometer. From the extinction coefficient of the MDA–TBA adduct ($1.53 \times 10^5\ M^{-1}\ cm^{-1}$ [7]), the amount of MDA equivalents produced was calculated. In some experiments, the incubations were quenched with 2 M TCA/1.7 N HCl containing 0.01% butylated hydroxytoluene (BHT). The addition of BHT had no effect on the amount of MDA produced.

Several experiments using liver mitochondria included the phospholipase A_2 inhibitors, chlorpromazine (0–100 μM) or *p*-bromophenacyl bromide (0–1 mM). The above incubation procedure was used in the case of chlorpromazine; however, *p*-bromophenacyl bromide was preincubated with the protein at 37° for 30 min, followed by the addition of adriamycin, Fe^{3+} and NADH. The incubation continued for an additional 60 min, and the extent of MDA formation was measured as stated above.

Analysis of fatty acid content. Rat liver and heart mitochondria and rat liver microsomes were incubated for 60 min with 50 μM adriamycin and 25 μM Fe^{3+} as described in the preceding section. (Some experiments were also performed in the presence of 25 μM chlorpromazine.) All incubations were initiated by the addition of protein. The peroxidation reaction was terminated by transferring 1 ml of the incubation mixture to 5 ml of cold $CHCl_3$/MeOH (2:1) containing 0.05% BHT. The exogenous fatty acid, heptadecanoic acid (100 nmol), was added as an internal standard. The two phases were stirred vigorously for 2 hr at room temperature or overnight at 4°. The samples were filtered through glass wool, and the final volume was adjusted to 8 ml by the addition of $CHCl_3$/MeOH (2:1)–0.05% BHT. Water (1.6 ml) was then added and the samples were vortexed for 2 min. After a low speed centrifugation to cleanly separate the two phases, the upper layer was removed. The above washing procedure was repeated twice using 2.4 ml of Folch upper phase ($CHCl_3$/MeOH/H_2O, 3:48:47) instead of H_2O. The $CHCl_3$ layer was transferred to vials and the solvent evaporated under N_2 to complete dryness. For total fatty acid analysis by gas chromatography, the acids were methylated by treatment with borontrifluoride in methanol (0.5 ml MeOH, 0.2 ml 50% BF_3/MeOH, 100°, 15 min). The methyl esters were extracted into hexane by the addition of 1.7 ml H_2O and 1.4 ml hexane and were vortexed for 1 min. The phases were separated completely by a brief centrifugation, and the H_2O/MeOH layer was removed. The hexane was evaporated under N_2 and the residue was suspended in 50 μl hexane. For free fatty acid analysis, a portion of the $CHCl_3$ layer was methylated with ethereal diazomethane for 10 min at room temperature. The etheral diazomethane was evaporated to dryness followed by the addition of 600 mg of silicic acid. The methyl esters of the free fatty acids were then extracted twice with 3.5 ml hexane/ether (20:1, v/v). The solvent was evaporated to dryness, and 25 μl of hexane was added to the residue. The fatty acid methyl esters, both free and total, were analyzed on an SP2330 capillary column (Supelco, Bellefonte, PA) in a Hewlett–Packard 5890 gas chromatograph [split injector system; column head pressure, 25 psi; temperature program: 170° (1 min), 6°/min, 230° (1 min); flame ionization detector]. Nelson Analytical Software was used for collection and analysis of data. Identification of the individual fatty acids was based on the retention times of the fatty acid methyl ester standards. The results for total fatty acids were calculated with respect to the endogenous fatty acid, stearic acid (18:0), which did not undergo peroxidation. For free fatty acid analysis, the results were calculated as percentages of heptadecanoic acid (17:0), because the amount of all endogenous free fatty acids varied under the different conditions employed.

RESULTS

Characterization of adriamycin-induced MDA formation in rat liver mitochondria. Initial investigations were undertaken to define the conditions of maximal lipid peroxidation under our *in vitro* experimental system. The extent of peroxidation was quantified by measuring the amount of MDA produced. MDA formation was found to be dependent on the concentrations of adriamycin, Fe^{3+}, and protein, as well as time. The amount of MDA produced in the presence of adriamycin alone was quite small, with a maximum at 25 μM adriamycin (Fig. 1, bottom curve). The reaction was linear for 90 min (Fig. 2, bottom curve). The addition of Fe^{3+} produced a significant increase in adriamycin-induced MDA formation, approaching a plateau at 5 μM $FeCl_3$ (Fig. 3). With 25 μM Fe^{3+}, this increased production of MDA exhibited the same dependence on adriamycin

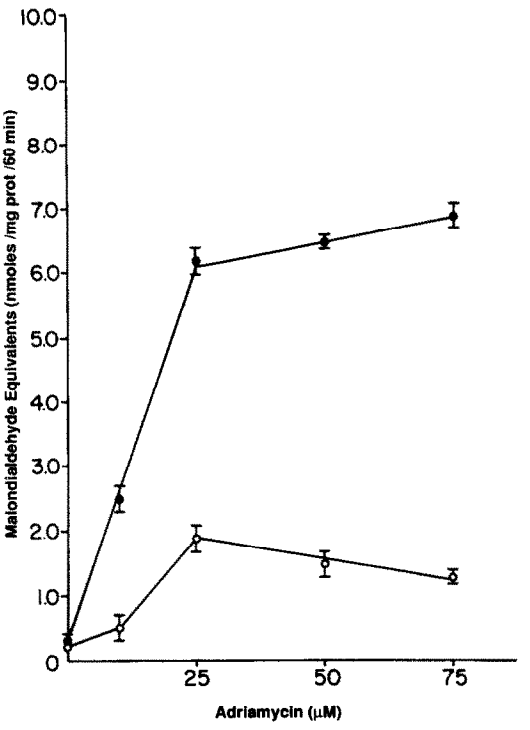

Fig. 1. Dependence of lipid peroxidation from rat liver mitochondria on adriamycin concentration. Malondialdehyde formation was measured either with (●) or without (○) 25 μM Fe^{3+} as described in Materials and Methods. Incubation time was 60 min. Values are means ± SEM, N = 3–4 experiments.

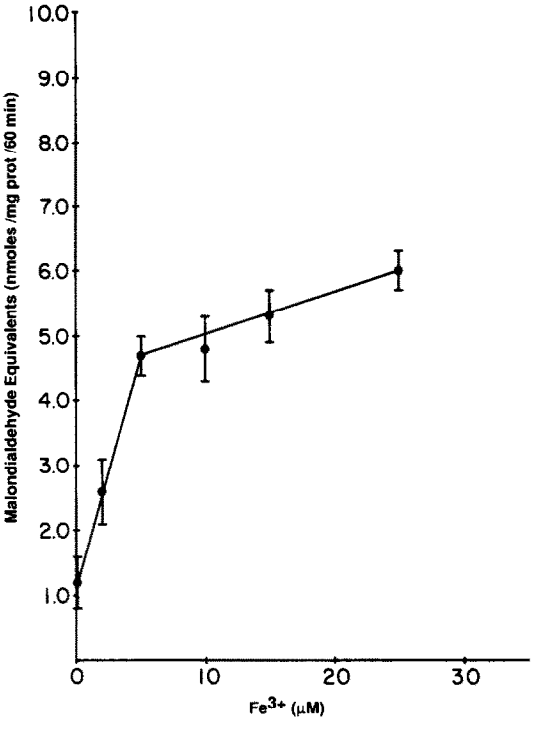

Fig. 3. Effect of Fe^{3+} on adriamycin-induced peroxidation of membrane lipids from rat liver mitochondria. Adriamycin concentration was 50 μM. Other experimental details are provided under Materials and Methods. Values are means ± SEM, N = 3–4 experiments.

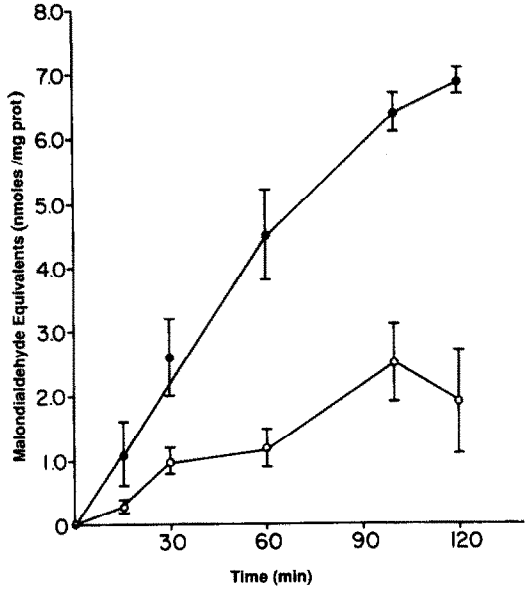

Fig. 2. Dependence of the peroxidation of membrane lipids from rat liver mitochondria on time. Malondialdehyde formation was measured either with 50 μM adriamycin (○) or with 50 μM adriamycin plus 25 μM Fe^{3+} (●) as described in Materials and Methods. Values are means ± SEM, N = 3–4 experiments.

concentration (Fig. 1, top curve) and time (Fig. 2, top curve) as that in the absence of Fe^{3+}. Using the optimal conditions of 50 μM adriamycin, 25 μM Fe^{3+}, 2.5 mM NADH, and an incubation time of 60 min, MDA formation was linear up to a protein concentration of 2 mg/ml. Raising the concentration of NADH to 5.0 mM did not increase any further the amount of MDA formed. In some experiments, 2.5 mM NADH was replaced by 2.5 mM NAD^+. Under such a condition, MDA production was approximately 60% less than that seen with NADH.

To determine whether lipid peroxidation exhibited a requirement for a specific form of iron, the rates of MDA production were measured using various means of iron administration (Table 1). It was found that the absolute rates, both with and without adriamycin, were dependent on the form of iron added to the incubation. In the absence of adriamycin, Fe^{2+}, both alone and with ADP, yielded larger amounts of MDA produced than the corresponding forms of Fe^{3+}. Moreover, complexes of either reduced or oxidized iron with ADP were more effective than their non-complexed counterparts. With adriamycin, the rates of MDA formation increased substantially in all cases. However, the net amounts, i.e. the differences due to the presence of the drug, did not vary greatly.

Addition of either EDTA (10 μM) or deferoxamine (0.5 μM) to incubations containing 50 μM adriamycin and 25 μM Fe^{3+} completely inhibited the formation of MDA.

Table 1. Effect of various forms of iron on the peroxidation of membrane lipids from rat liver mitochondria incubated with and without adriamycin

Iron administered	Malondialdehyde produced (nmol × mg protein^{-1} × 60 min^{-1})	
	0 Adriamycin	50 μM Adriamycin
Fe^{3+}	1.1 ± 0.1	4.2 ± 0.3*
Fe^{2+}	4.4 ± 0.4	7.4 ± 0.3†
Fe^{3+}/Fe^{2+}	1.9 ± 0.3	6.0 ± 0.1*
ADP/Fe^{3+} (33:1) complexed	5.1 ± 0.4	7.6 ± 1.1
ADP/Fe^{3+} (33:1) non-complexed	1.4 ± 0.2	6.8 ± 0.5*
ADP/Fe^{3+} (17:1) complexed	4.8 ± 0.2	8.9 ± 1.1†
ADP/Fe^{3+} (17:1) non-complexed	1.2 ± 0.1	5.3 ± 0.9†
ADP/Fe^{2+} (33:1) complexed	5.6 ± 0.4	8.5 ± 1.4
ADP/Fe^{2+} (33:1) non-complexed	4.9 ± 0.4	8.7 ± 1.4†

Experimental conditions are described in detail in Materials and Methods. Total iron was 25 μM. Results are presented as means ± SEM for four experiments.

*† Significantly different from 0 adriamycin (paired Student's *t*-test): *$P < 0.005$, and †$P < 0.05$.

Effect of adriamycin on the fatty acid content of rat liver mitochondria. The peroxidation of rat liver mitochondrial lipids was analyzed by evaluating the fatty acid profiles in the presence and absence of adriamycin and/or Fe^{3+}. The major fatty acids of rat liver mitochondrial lipids were palmitic (16:0), stearic (18:0), oleic (18:1), linoleic (18:2), arachidonic (20:4), and docosahexaenoic (22:6). Table 2 shows that there was no detectable loss in total fatty acids after a 1-hr incubation in the absence of both adriamycin and Fe^{3+}, with 25 μM Fe^{3+} alone, or with 50 μM adriamycin alone. However, there was approximately a 50% loss in arachidonic and docosahexaenoic acids when both 50 μM adriamycin and 25 μM Fe^{3+} were present. When the non-complexed form of ADP/Fe^3 (33:1) was used in place of 25 μM Fe^{3+}, the same result was obtained (data not shown).

To determine whether phospholipid-bound or free fatty acids were more or equally susceptible to peroxidation, two phospholipase A_2 inhibitors, chlorpromazine and *p*-bromophenacyl bromide, were used. Chlorpromazine completely prevented MDA formation, with a half-maximal concentration of 25 μM. *p*-Bromophenacyl bromide also inhibited MDA production, but much higher concentrations were necessary, with a half-maximal value around 0.5 mM (data not shown). Analyses of free and total fatty acids of mitochondria treated with 50 μM adriamycin, 25 μM Fe^{3+}, and 25 μM chlorpromazine showed no loss in any of the detectable total fatty acids, and only a slight increase in the free fatty acid content over control samples (Table 3).

Lipid peroxidation of heart mitochondria in the presence of adriamycin. Once conditions were established for the liver mitochondrial system, analogous experiments were performed on heart mitochondria. Under a variety of conditions, adriamycin (0–100 μM), Fe^{3+} (0–25 μM), protein (1 mg/ml and 1.5 mg/ml), and time (30–60 min), net MDA production could not be detected. An analysis of total fatty acids in the presence of 50 μM adriamycin and 25 μM Fe^{3+} showed no change in the fatty acid profiles after 60 min of incubation. When expressed as a percentage of stearic acid (18:0), the amount of arachidonic acid was 90% of that at time zero in the absence of the drug and Fe^{3+} and 91% in their

Table 2. GC analysis of the fatty acid content in membranes from rat liver mitochondria incubated with and without adriamycin and/or Fe^{3+}

		Fatty acid (relative content)				
	Conditions:					
	Adriamycin (μM)	0	0	0	50	50
Fatty acid	Fe^{3+} (μM)	0	0	25	0	25
type	Time (min)	0	60	60	60	60
16:0		79 ± 7	87 ± 9	86 ± 12	84 ± 3	95 ± 9
18:1		24 ± 4	23 ± 2	23 ± 3	23 ± 3	25 ± 4
18:2		93 ± 8	91 ± 10	94 ± 12	92 ± 10	87 ± 10
20:4		104 ± 11	99 ± 5	100 ± 10	99 ± 14	52 ± 3*
22:6		50 ± 4	45 ± 4	47 ± 5	45 ± 7	21 ± 2*

Experimental conditions and details of procedures are described in Materials and Methods. The results for stearic acid (18:0) were normalized and all other fatty acids are expressed as percentages of this value ± SEM for three experiments.

* Significance of the effect of adriamycin was calculated with respect to the samples incubated for 60 min with 25 μM Fe^{3+}: $P < 0.02$ (paired Student's *t*-test).

Table 3. Effect of chlorpromazine on the content of free and total fatty acids in rat liver mitochondria incubated with adriamycin and Fe^{3+}

Fatty acid type	Fatty acid (relative content)					
	0 time		No Chlorpromazine		+25 μM Chlorpromazine	
	Free	Total	Free	Total	Free	Total
16:0	12	152	9	118	13	115
18:0	19	188	14	157	18	187
18:1	4	54	3	44	6	48
18:2	4	148	2	119	4	152
20:4	1	178	2	102	4	162
22:6	2	129	4	61	4	108

The concentration of adriamycin was 50 μM and of Fe^{3+}, 25 μM. Incubation time was 60 min. All other details are described in Materials and Methods. Results were calculated as percentages of the externally added standard heptadecanoic acid (17:0) and represent means of two experiments.

presence. The respective values for docosahexaenoic acid were 83 and 81% respectively.

Peroxidation of liver microsomal lipids by adriamycin. The dependence of MDA formation on adriamycin, Fe^{3+}, and protein concentrations was also investigated with rat liver microsomes. The incubations were performed in the presence of saturating amounts of NADH (2.5 mM) for 60 min. The amount of MDA formed increased in a hyperbolic manner with the rise in adriamycin concentration, both with and without Fe^{3+}. The rates were linear up to 10 μM drug and reached a plateau at approximately 75 μM. (Similar results were obtained when 2.5 mM NADPH was used; however, the absolute amounts were 2- to 3-fold higher than those seen with NADH.) The dependence on iron and protein with microsomes was essentially the same as with mitochondria, although in all situations MDA generation was twice higher.

Analysis of total fatty acids of microsomal lipids from three different preparations showed consistent losses of arachidonic and docosahexaenoic acids. Two experiments, performed in the spring, gave similar results with a 31% loss in arachidonic acid and a 40% loss in docosahexaenoic acid. A third experiment, performed in winter, exhibited much greater losses: 50% for 20:4 and 67% for 22:6. These values correlated well with the MDA formation, with the first two experiments yielding 4–7 nmol/mg protein/60 min and 15 nmol/mg protein/60 min for the third experiment.

DISCUSSION

The object of this study was to investigate the importance of lipid peroxidation in the toxicity of adriamycin. Our studies focused primarily on mitochondria for several reasons: (i) ultrastructure studies of human [8] and rabbit [9, 10] hearts with adriamycin-induced cardiomyopathy show mitochondrial swelling and degeneration; (ii) cytofluorescence microscopy techniques reveal a specific localization of adriamycin in rat heart mitochondria [11]; (iii) adriamycin appears to have a high affinity for the negatively-charged phospholipid, cardiolipin, which is located exclusively in the inner membrane of mitochondria [12–17]. This interaction may be responsible for the inhibition of certain respiratory chain enzymes, especially in the cytochrome bc_1 complex and the cytochrome oxidase region [18]; (iv) adriamycin is capable of a one-electron reduction to the semiquione free radical by mitochondrial NADH dehydrogenase [1]; (v) the free radical species, $O_2^{\cdot}$ and $OH^{\cdot}$, have been detected in mitochondria in the presence of adriamycin [19, 20].

The *in vitro* experimental conditions to induce lipid peroxidation by adriamycin had already been established for mitochondria and microsomes from the liver [7, 21, 22]. However, since the extent of lipid peroxidation may be dependent on the species (the content of unsaturated fatty acids varies from species to species) [23], the strain, and the age of the animal, as well as the diet consumed (which influences the fatty acid content), it was important to find *in vitro* conditions of maximal lipid peroxidation for our animal model, male Sprague–Dawley rats fed on a diet sufficient in α-tocopherol. In fact, this rationale was supported by our observations that the amount of lipid peroxides was greatly dependent on the time of year in which the experiments were performed. Membrane preparations obtained in fall and winter resulted in more active peroxidation, under a given set of conditions, than preparations from spring and summer.

The extent of lipid peroxidation was determined by two methods: the spectrophotometric analysis of the amount of MDA using thiobarbituric acid and the analysis of the fatty acid content of mitochondrial membranes in the presence and absence of adriamycin with gas chromatography. MDA, which is cleaved from peroxidized fatty acids, is thought to be derived from polyunsaturated fatty acids containing three or more double bonds [24]. Several investigators have calculated that 1 mol of MDA is generated for every 14–21 mol of unsaturated fatty acids consumed [25–27]. With liver microsomes and an NADPH-supported lipid peroxidation system, Jordan and Schenkman [23] showed experimentally a linear relationship between MDA production and arachidonic acid loss. No other polyunsaturated fatty acid exhibited such a relationship.

Our results show that the amount of MDA produced in the presence of adriamycin alone was very small, but could be stimulated markedly (3- to 5-fold, Fig. 3) by low (μM) levels of iron. It is possible that, under the former conditions, the presence of trace amounts of iron in KCl/Hepes buffer (which had not been treated with iron-chelating agents) was responsible for lipid peroxidation. Extrapolation of the linear portion of the curve in Fig. 3 indicates that the concentration of contaminating iron could not have been greater than 2 μM, in agreement with the studies of Mimnaugh *et al.* [7]. It would appear, therefore, that iron is necessary for the manifestation of the peroxidative capability of adriamycin.

The role of iron in adriamycin-induced lipid peroxidation can be appreciated when one considers a probable chain of events. The anthracycline can be

reduced enzymatically to the semiquinone radical by NADH dehydrogenase [1], which in turn can react with molecular oxygen to generate superoxide [2]. Superoxide dismutase may convert superoxide to hydrogen peroxide, which then can cycle into the superoxide-driven Fenton reaction. It is here that both Fe^{2+} and Fe^{3+} participate: Fe^{2+} donates an electron to hydrogen peroxide to form hydroxyl radicals and Fe^{3+} (either formed from this reaction or supplied externally from an exogenous source) accepts electrons from superoxide to generate Fe^{2+}. In addition, there may be a direct electron transfer between adriamycin and Fe^{3+}, whereby the drug donates an electron to Fe^{3+}. The Fe^{2+} thus generated can then reduce oxygen to hydrogen peroxide, which enters the Fenton reaction. Such a transfer has been shown for the adriamycin/Fe^{3+} complex [28], but this may also occur when both are free in solution. It follows from the above that the semiquinone radical of adriamycin can be formed enzymatically (via mitochondrial NADH dehydrogenase or microsomal NADPH dehydrogenase) or non-enzymatically. A 60% decrease in MDA formation with NAD^+ as compared to NADH suggests that in the presence of mitochondria the predominant pathway of adriamycin-enhanced lipid peroxidation is the result of the enzymatic formation of the semiquinone radical.

The extent of peroxidation was also determined by observing the loss in total unsaturated fatty acids from rat liver mitochondria in the presence of adriamycin and iron. Both arachidonic and docosahexaenoic acids were involved in peroxidation and the extent of their degradation correlated well with the amount of MDA produced. Rat liver microsomes exhibited similar losses in arachidonic and docosahexaenoic acids, with a 2-fold greater production of MDA. Considering the fact that approximately 50% of 20:4 and 22:6 are degraded within 60 min, the effect of adriamycin and Fe^{3+} may produce dramatic changes in membrane structure and function. It is also interesting to note that such effects occur at adriamycin concentrations of 10–50 μM, just slightly higher than the therapeutic plasma concentrations of this drug [29]. Since other subcellular organelles, as well as the plasma membrane, contain arachidonic and docosahexaenoic acids, it is possible that overall membrane integrity may be compromised upon administration of this drug.

One of the questions posed in this work was whether or not membrane-bound or free unsaturated fatty acids were equally susceptible to peroxidation. Thus, to block the release of fatty acids, chlorpromazine and *p*-bromophenacyl bromide, inhibitors of phospholipase A_2, were used. Both chlorpromazine and *p*-bromophenacyl bromide inhibited MDA formation, and the former prevented losses in arachidonic and docosahexaenoic acids. Although these results could suggest that phospholipase A_2 is important in adriamycin/Fe^{3+}-induced lipid peroxidation, one must be aware of the non-specificity of these inhibitors. *p*-Bromophenacyl bromide, the more specific of the two, alkylates histidine residues at the active site of various enzymes. It has been shown to inhibit completely phospholipase A_2 purified from porcine pancreas [30] and rat liver mitochondria [31]. However, *p*-bromophenacyl bromide has also been found to block irreversibly yeast alcohol dehydrogenase, bovine pancreatic α-chymotrypsin, and human platelet phosphoinositol-specific phospholipase C [32]. Chlorpromazine is thought to act at the substrate–enzyme interface, thereby inhibiting this enzyme [33]. But this drug is also capable of a one-electron transfer to Fe^{3+} to form a cation free radical [34]. It is this species which can act as an antioxidant and thus terminate a free radical cascade. It is quite possible that, in our experiments, chlorpromazine may be acting not only as an inhibitor of phospholipase A_2, but also as a free radical scavenger. If so, chlorpromazine or similar drugs may prove useful therapeutically when administered along with adriamycin to alleviate the membrane damage that might occur as a result of lipid peroxidation. In support of this, it has been observed that pretreatment of rats with chlorpromazine before induction of ischemia and subsequent reperfusion prevents Ca^{2+} overload and degradation of phospholipids [35].

In spite of the paucity of information on adriamycin-induced lipid peroxidation in heart mitochondria, it is widely hypothesized that such a process is responsible for the known cardiotoxicity of the drug. It came, therefore, as a surprise that neither MDA production nor changes in fatty acids could be detected in the heart organelle under conditions in which hepatic mitochondria showed such evidence of peroxidation. The reasons for this are difficult to explain, in view of the reports that the heart has depressed levels of superoxide dismutase, catalase, and glutathione peroxidase [36–38], mechanisms whereby the cell detoxifies oxygen free radicals. One possible explanation is that different tissues may contain varying amounts of antioxidants, such as α-tocopherol, which imbed themselves into the lipid bilayer and are readily accessible to quench peroxidation. Consistent with this is the observation that heart microsomes from rat and rabbit have more α-tocopherol than liver microsomes from these species [39]. Moreover, Mimnaugh *et al.* [40] could only detect adriamycin-stimulated MDA formation with heart microsomes from rats maintained on diet deficient in α-tocopherol. However, some MDA production could be detected in heart mitochondria using similar conditions from rabbits [41]. If there is any relevance of these results on experimental animals to the situation in humans, our work suggests that varying the levels of antioxidant in patients receiving adriamycin treatment could influence the severity of the toxic side-effects of this drug.

Note added in proof: While this manuscript was submitted for publication, a report by Solaini *et al.* [42] showed that endogenous coenzyme Q is beef heart mitochondria can effectively protect the organelles from membrane lipid oxidative damage induced by an adriamycin–iron complex.

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