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ENHANCEMENT OF REACTIVE OXYGEN-DEPENDENT MITOCHONDRIAL MEMBRANE LIPID PEROXIDATION BY THE ANTICANCER DRUG ADRIAMYCIN*

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Abstract—Mitochondrial degeneration is a consistently prominent morphological alteration associated with adriamycin toxicity which may be the consequence of adriamycin-enhanced peroxidative damage to unsaturated mitochondrial membrane lipids. Using isolated rat liver mitochondria as an *in vitro* model system to study the effects of the anticancer drug adriamycin on lipid peroxidation, we found that NADH-dependent mitochondrial peroxidation—measured by the 2-thiobarbituric acid method—was stimulated by adriamycin as much as 4-fold. Marker enzyme analysis indicated that the mitochondria were substantially free of contaminating microsomes (<5%). Lipid peroxidation in mitochondria incubated in KCl–Tris–HCl buffer (pH 7.4) under an oxygen atmosphere was optimal at 1–2 mg of mitochondrial protein/ml and with NADH at 2.5 mM. Malonaldehyde production was linear with time to beyond 60 min, and the maximum enhancement of peroxidation was observed with adriamycin at 50–100 μM. Interestingly, in contrast to its stimulatory effect on NADH-supported mitochondrial peroxidation, adriamycin markedly diminished ascorbate-promoted lipid peroxidation in mitochondria. Superoxide dismutase, catalase, 1,3-dimethylurea, reduced glutathione, α-tocopherol and EDTA added to incubation mixtures inhibited endogenous and adriamycin-augmented NADH-dependent peroxidation of mitochondrial lipids, indicating that multiple species of reactive oxygen (superoxide anion radical, hydrogen peroxide and hydroxyl radical) and possibly trace amounts of endogenous ferric iron participated in the peroxidation reactions. In submitochondrial particles freed of endogenous defenses against oxyradicals, lipid peroxidation was increased 7-fold by adriamycin. These observations suggest that some of the effects of adriamycin on mitochondrial morphology and biochemical function may be mediated by adriamycin-enhanced reactive oxygen-dependent mitochondrial lipid peroxidation.

While the mechanism of antineoplastic activity of the anthracycline antitumor drug adriamycin (doxorubicin) is generally considered to result from the interaction of adriamycin with DNA [1], the biochemical mechanism of adriamycin toxicity, especially its cardiotoxicity, remains essentially unknown. Numerous investigators have hypothesized that the interactions of adriamycin with cellular macromolecules contribute to the pathogenesis of the chronic, life-threatening cardiomyopathy elicited by adriamycin, yet no single hypothesis has emerged which adequately explains the multiple biochemical and morphological alterations which are associated with adriamycin toxicity. Currently, the most acceptable proposal involves the formation of the adriamycin semiquinone free radical intermediate catalyzed by flavin-containing oxidoreductase enzymes [2], the most active of which is the membrane-bound enzyme, NADPH-cytochrome P-450 reductase [3]. Under anoxic conditions, the adriamycin semiquinone radical can rearrange to a species which can alkylate both proteins [4] and nucleic acids [5]. Alternatively, under normoxic conditions, the semiquinone rapidly reacts with oxygen to generate superoxide anion free radical [6]. The redox cycling of adriamycin also results in the production of hydrogen peroxide [7] and hydroxyl radical [8]. These partially reduced reactive oxygen species, especially the latter, are extremely cytotoxic, since they can react with and damage enzymes [9], nucleic acids [10] and membrane lipids [11]. Abundant recent evidence *in vivo* [12–14] and *in vitro* [15–18] supports the contention that oxyradical-mediated membrane lipid peroxidation is enhanced in the presence of adriamycin, and these results further suggest that uncontrolled lipid peroxidation may play a major role in adriamycin toxicity.

The most prominent and consistent ultrastructural alterations observed in adriamycin-damaged tissues are vacuolization, resulting from dilation and disintegration of the sarcoplasmic reticulum, and extensive mitochondrial degeneration ultimately leading to dissolution of the cristae [19]. Concomitant with or preceding these pathological lesions in mitochondria, adriamycin causes disturbances in oxidative phosphorylation [20]. It is especially interesting that adriamycin stimulates superoxide and hydrogen peroxide formation in mitochondria [21]

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and in submitochondrial particles [22], and this accentuation of oxyradical production appears to depend upon mitochondrial enzyme activity which catalyzes the activation of adriamycin to the semiquinone radical [23].

Since mitochondria are important subcellular sites of adriamycin toxicity, and since mitochondria contain electron-transport enzymes which can activate adriamycin and stimulate the production of toxic oxyradicals, we hypothesized that oxyradical-mediated membrane lipid peroxidation may play a toxicologically important role in adriamycin-caused mitochondrial degeneration and dysfunction. Therefore, we have investigated the effects of adriamycin *in vitro* on NADH-dependent mitochondrial membrane lipid peroxidation and, further, by the use of specific reactive oxygen scavengers, determined the forms of reactive oxygen which are involved in adriamycin-enhanced peroxidation of mitochondrial membrane lipids.

MATERIALS AND METHODS

Chemicals and drugs. NADH, bovine serum albumin, reduced glutathione, D-α-tocopherol (820 units/ml), superoxide dismutase (2,600 units/mg protein), thymol-free catalase (10,000 units/mg protein), cytochrome *c*, EDTA, DETPAC* 2-thiobarbituric acid and ascorbic acid were purchased from the Sigma Chemical Co. (St. Louis, MO); 1,3-dimethylurea was obtained from the Aldrich Chemical Co. (Milwaukee, WI); ultrapure Tris was from Bethesda Research Laboratories (Gaithersburg, MD); potassium chloride with very low iron content (<0.5 ppm) was from the Fisher Scientific Co. (Fair Lawn, NJ); and ultra-pure sucrose was purchased from the J. T. Baker Chemical Co. (Phillipsburg, NJ). All other chemicals used in this study were of the highest purity available. Adriamycin hydrochloride (NSC 123127) and ICRF-187 (NSC 169780) were provided by the Drug Development Branch, Division of Cancer Treatment, National Cancer Institute, NIH (Bethesda, MD). Adriamycin was dissolved in oxygenated (bubbled with 100% oxygen for at least 10 min) 150 mM KCl–50 mM Tris–HCl buffer, pH 7.4 (KCl–Tris) just before use and was protected against light exposure with aluminium foil. The water was first deionized and then distilled in glass.

Animals and mitochondria preparation. Adult, male Sprague–Dawley rats (Taconic Farms, Germantown, NY), weighing 200–300 g, were fed Purina Rodent Chow and water *ad lib.* for at least 2 weeks after delivery to the laboratory. Animals were killed by cervical fracture and the livers were removed, rinsed in KCl–Tris buffer and gently homogenized (10%, w/v) on ice in 0.25 M sucrose:25 mM Tris:1 mM EDTA:1% (w/v) bovine serum albumin isolation solution. The EDTA diminished any microsomal–mitochondrial association which is facilitated by divalent cations [24], and chelated any free iron released from the tissue during homogenation; bovine serum albumin was included to stabilize mitochondria structure and function [25]. Usually two livers were combined to make one sample. Mitochondria were isolated by differential centrifugation essentially by the method of Schneider and Hogeboom [26] with modifications as described by Estabrook and Pullman [27]. Following the second sedimentation of the mitochondria in the isolation solution, the mitochondrial pellets were gently resuspended and repelleted by centrifugation (11,500 *g* × 6 min) three additional times in KCl–Tris buffer to remove the bovine serum albumin (BSA) which would contribute to an erroneous measurement of the protein content of the mitochondria and to remove the sucrose and EDTA which interfere with the assay for lipid peroxidation. For certain experiments, submitochondrial particles were prepared by sonicating the isolated mitochondria and sedimenting the particles by ultracentrifugation, essentially as described by Pfeifer and McCay [28].

Assay for lipid peroxidation. Mitochondria or submitochondrial particles (1.0 mg protein/ml) were incubated in the dark at 37° for 60 min with adriamycin (100 μM) and NADH (2.5 mM) in oxygenated KCl–Tris (1.75 ml final volume) under a 100% oxygen atmosphere, unless otherwise noted. Incubations were conducted under oxygen to prevent the enzymatic reductive deglycosidation of adriamycin [29]. Zero-time blanks, containing all the components of the incubation mixtures, were always included to compensate for any interference by adriamycin or other additions to the reaction mixtures in the measurement of peroxidation.

It is emphasized that the mitochondria were isolated and washed in an EDTA-containing medium which minimized contamination by adventitious metal ions; moreover, neither iron salts nor iron chelates were added to the reaction mixtures. Although some investigators have included exogenous iron (up to 100 μM) in their studies of the effects of adriamycin on lipid peroxidation [30–33], we have chosen *not* to include exogenous iron. This is because adriamycin is not administered to cancer patients as an iron complex, and because it is virtually impossible to interpret peroxidation data obtained from incubations which include preformed iron–adriamycin complexes, since the iron may dissociate from the anthracycline drug during incubations and function as "free" iron, producing spurious results. Sufficient trace-amounts of iron (2–5 μM) which are required to support lipid peroxidation [34] were apparently present in the KCl–Tris buffer or, alternatively, were intimately associated with the mitochondria. Recently, Tangeras [35] reported that the non-heme iron content of rat liver mitochondria was 2.7 ± 0.6 nmoles/mg protein, but it is not known how much of this iron can participate in lipid peroxidation reactions.

Lipid peroxidation was terminated by adding 0.75 ml of cold 2 M trichloroacetic acid:1.7 N HCl, and the precipitated proteins were removed by centrifugation. Following the addition of 2 ml of 1% (w/v) 2-thiobarbituric acid to 0.5-ml aliquots of the resulting supernatant fractions, they were heated at 95° for 15 min, cooled to room temperature, and the

* Abbreviations: DETPAC, diethylene-triamine pentaacetic acid; and ICRF-187, (±)-1,2-bis(3,5-dioxopiperazin-1-yl)-propane.

malonaldehydethiobarbiturate adduct was quantitated spectro-photometrically at 533 nm as described by Bernheim *et al.* [36]. Negligible lipid peroxidation occurred when mitochondria were inactivated by boiling (0.34 ± 0.30 nmol malonaldehyde/mg protein/60 min) or when the source of reducing equivalents, NADH, was omitted (0.46 ± 0.11 nmole malonaldehyde/mg protein/60 min). This reaction mixture for adriamycin-enhanced lipid peroxidation *in vitro* has been extensively characterized previously for microsomal lipid peroxidation [16]. Results are expressed as nmoles of malonaldehyde equivalents per mg of mitochondrial protein per 60 min. Standard malonaldehyde was prepared by the acid hydrolysis of tetraethoxypropane (K & K Laboratories, Plainview, NY), and the molar extinction coefficient of the 2-thiobarbiturate-malonaldehyde adduct was found to be $1.53 \times 10^5\ M^{-1}\ cm^{-1}$ at a peak absorption of 533 nm. This value was used in all calculations.

Reactive oxygen scavengers and chelating agents. To determine whether activated oxygen species might participate in adriamycin-enhanced mitochondrial lipid peroxidation, various scavengers of reactive oxygen were added to the incubation mixtures in a range of concentrations. These included: the enzymes, superoxide dismutase and catalase; the hydroxyl radical scavenger, 1,3-dimethylurea; the antioxidants and free radical scavengers, α-tocopherol and ascorbic acid; and the nucleophilic thiol, reduced glutathione. In addition, EDTA, a potent chelator of transition-metal cations, and ICRF-187, an anticancer drug with chelation properties, were added to investigate any involvement of metal ions in mitochondrial membrane peroxidation. Heat-inactivated superoxide dismutase and intact bovine serum albumin were added to incubations to rule out any inhibition attributable to nonenzymic effects of superoxide dismutase.

Other assays. Mitochondrial protein was measured by the method of Lowry *et al.* [37], with bovine serum albumin as the standard. The activity of NADPH-cytochrome P-450 reductase was determined by the method of Williams and Kamin [38] with cytochrome *c* as the substrate, cytochrome P-450 content was determined by its carbon monoxide-difference spectrum as described by Omura and Sato [39], aminopyrine *N*-demethylase activity was quantitated by formaldehyde production [40] and glucose-6-phosphatase activity was assayed by the method of Baginski *et al.* [41]. All enzyme assays except the measurement of cytochrome P-450 were conducted at 37°, and in each case the products of the enzymatic reactions were measured. These enzymes were used to estimate the microsomal contamination of the mitochondrial preparations. The activities of the mitochondrial marker enzymes monoamine oxidase [42] and succinate-cytochrome *c* reductase [43] were also determined. All assays were performed in triplicate. Mitochondrial respiration was measured by monitoring oxygen consumption using a Clark electrode in the presence of glutamate and ADP exactly as described by Sordahl *et al.* [44]. NADH oxidation was followed spectrophotometrically at 340 nm.

Statistics. Data were analyzed by Student's *t*-test [45], and differences between mean values at $P < 0.05$ were considered to be significant.

RESULTS

Assessment of mitochondrial purity. To rule out the possibility that the isolated mitochondria fraction might be contaminated with microsomes, the activities of several microsomal and mitochondrial marker enzymes were determined. As shown in Table 1, NADPH-cytochrome *c* (P-450) reductase activity, cytochrome P-450 content, and aminopyrine *N*-demethylase activity in mitochondria ranged from undetectable to less than 8% of the corresponding values measure in microsomes, indicating minimal contamination of the mitochondrial preparation by microsomes. Conversely, succinate-dependent cytochrome *c* reductase and monoamine oxidase activities in the mitochondrial fractions were 10- and 6-fold greater than those values found in microsomes, and corresponded to previously published values [46]. Mitochondrial glucose-6-phosphatase activity was 17% of the activity measured in microsomes.

It was found that mitochondria isolated and washed as described above were capable of oxidizing

Table 1. Marker enzyme activities in isolated mitochondrial and microsomal fractions*

Enzyme activity	Mitochondria	Microsomes
NADPH-cytochrome *c* reductase†	24.2 ± 2.1	309 ± 22
Succinate-cytochrome *c* reductase†	410 ± 42	4.2 ± 1.6
Monoamine oxidase†	12.0 ± 2.1	2.0 ± 1.0
Aminopyrine *N*-demethylase†	0.16 ± 0.04	8.71 ± 0.86
Cytochrome P-450‡	Undetectable	0.69 ± 0.05
Glucose-6-phosphatase†	54 ± 9	327 ± 36

* Mitochondria or microsomes were incubated with the appropriate substrates and cofactors, and enzyme activities were measured at 37° as described in Materials and Methods.

† Values are expressed as nmoles of product formed per mg of protein per min (mean ± S.D., N = 4–8).

‡ Expressed as nmoles of reduced, carbon monoxide-bound cytochrome P-450 per mg of microsomal protein by difference spectrum.

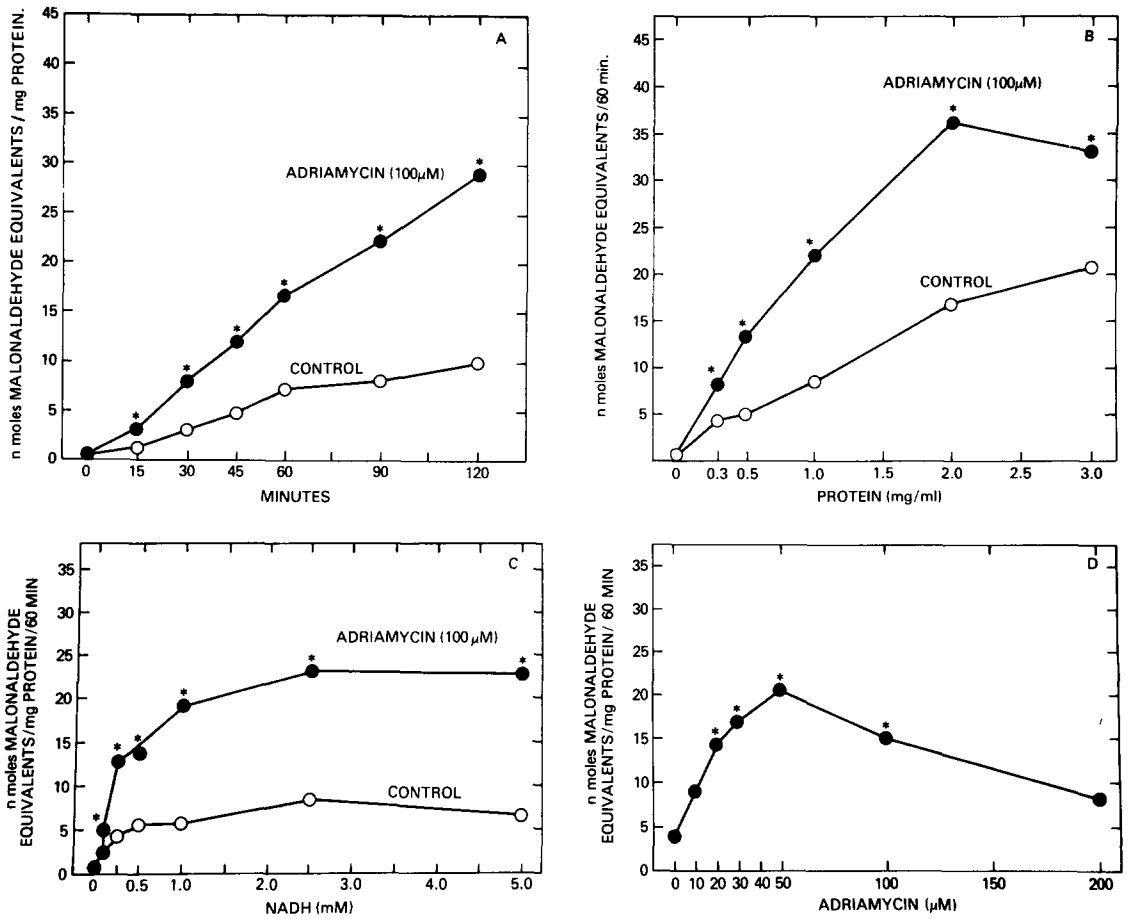

Fig. 1. Effects of varying the time of incubation (A), the mitochondrial protein concentration (B), and the NADH concentration (C) on mitochondrial lipid peroxidation in the absence (○) and presence (●) of adriamycin at 100 μM. Panel D shows the concentration-dependent stimulation of NADH-dependent mitochondrial peroxidation by adriamycin. Mitochondria were incubated in KCl–Tris–HCl buffer (pH 7.4) at 37° under an oxygen atmosphere at the indicated conditions. Lipid peroxidation was measured by the 2-thiobarbituric acid method, and the data are expressed as nmoles of malonaldehyde equivalents per mg of mitochondrial protein per 60 min (except for the time course) for the mean of four samples. Values significantly different from control values are shown by an asterisk ($P < 0.05$).

NADH (12 ± 1 nmoles of NADH oxidized/mg mitochondrial protein/min) and catalyzing NADH-dependent oxygen consumption (7.1 ± 0.8 nmoles/mg protein/min). These results suggest that the mitochondria had been made at least partially permeable to NADH during the isolation and purification procedures. The ADP:O ratio was 3.4 ± 0.3 and the respiratory control index (state 3/state 4) with glutamate as substrate was 2.6 ± 0.3. However, it should be noted that it was not crucial to the lipid peroxidation experiments that the mitochondria be tightly coupled; the primary considerations were that the mitochondrial enzyme(s) capable of interacting with adriamycin and activating it to the semiquinone free radical retained activity, and that the isolated mitochondria were reasonably free of microsomal contamination.

NADH-dependent mitochondrial lipid peroxidation. The effects of adriamycin on the rate and extent of lipid peroxidation in isolated mitochondria are shown in Fig. 1. NADH-dependent mitochondrial membrane peroxidation was enhanced as much as 4-fold by adriamycin. Mitochondrial peroxidation was optimal in KCl–Tris buffer, pH 7.4, at 1–2 mg of mitochondrial protein/ml, and NADH was saturating at 2.5 mM. It was found that NADPH could substitute for NADH in supporting endogenous and adriamycin-stimulated peroxidation of mitochondrial lipids; however, NADH was used exclusively throughout this study. NADPH-supported mitochondrial lipid peroxidation has been reported previously by a number of investigators [47, 48]. Peroxidation was roughly linear with time to at least 90 min and was maximally stimulated by adriamycin at 50–100 μM. Higher concentrations of the drug resulted in less peroxidation, as has been observed previously for NADPH-dependent lipid peroxidation with microsomes [16]. The extent of mitochondrial lipid peroxidation observed in the presence of adriamycin at 120 min (35 nmoles of malonaldehyde equivalents/mg protein) represents nearly complete peroxidation of unsaturated mitochondrial membrane lipids [47]. Complete peroxidation refers to the maximum amount of malonaldehyde which

Table 2. Comparison of the effects of adriamycin on NADH-dependent lipid peroxidation in intact mitochondria or submitochondrial particles*

Enzyme source	Malonaldehyde equivalents (nmoles/mg protein/60 min)	
	Endogenous	Adriamycin (100 μM)
Intact mitochondria	3.3 ± 0.4	17.2 ± 1.3†
Sonicated mitochondria	2.1 ± 0.5	21.0 ± 0.7†
Submitochondrial particles	9.5 ± 0.6	73.5 ± 7.4†

* Mitochondria or submitochondrial particles were incubated at 37° in KCl–Tris-HCl buffer (pH 7.4) with NADH (2.5 mM) under a 100% oxygen atmosphere for 60 min. Lipid peroxidation was measured by the 2-thiobarbituric acid method. Values are expressed as mean ± S.D., N = 6.

† Significantly different from values without adriamycin ($P < 0.05$).

was produced under optimum condition at extended incubation times. Kornbrust and Mavis [34] have shown that the maximum thiobarbituric acid-reacting material generated during membrane lipid peroxidation correlated with the maximum depletion of polyunsaturated fatty acids.

Within the mitochondrion there are multiple internal defenses against oxyradicals and lipid peroxidation including superoxide dismutase [49], reduced glutathione [50] and glutathione peroxidase [51]. Since many of these defenses can be removed by simply disrupting mitochondria by ultrasound and sedimenting the resulting membranous particles by ultracentrifugation [52], we investigated the effects of adriamycin on lipid peroxidation in submitochondrial particles. As shown in Table 2, sonicating the mitochondria had little effect on either endogenous or adriamycin-stimulated mitochondrial lipid peroxidation. Similarly, freeze-thawing of the mitochondria had no significant effect on either endogenous or adriamycin-enhanced peroxidation (data not presented). In contrast, NADH-supported specific lipid peroxidation in submitochondrial particles was markedly higher than in intact or sonicated-mitochondria (Table 2), most likely because endogenous defenses against oxyradicals were removed. Adriamycin increased NADH-dependent membrane lipid peroxidation in submitochondrial particles more than 7-fold.

Inhibition of adriamycin-stimulated mitochondrial lipid peroxidation by oxyradical scavengers. The concentration-dependent inhibition of mitochondrial lipid peroxidation by superoxide dismutase and catalase is shown in Fig. 2, panels A and B. Bovine copper-zinc-superoxide dismutase decreased both endogenous and adriamycin-enhanced mitochon-

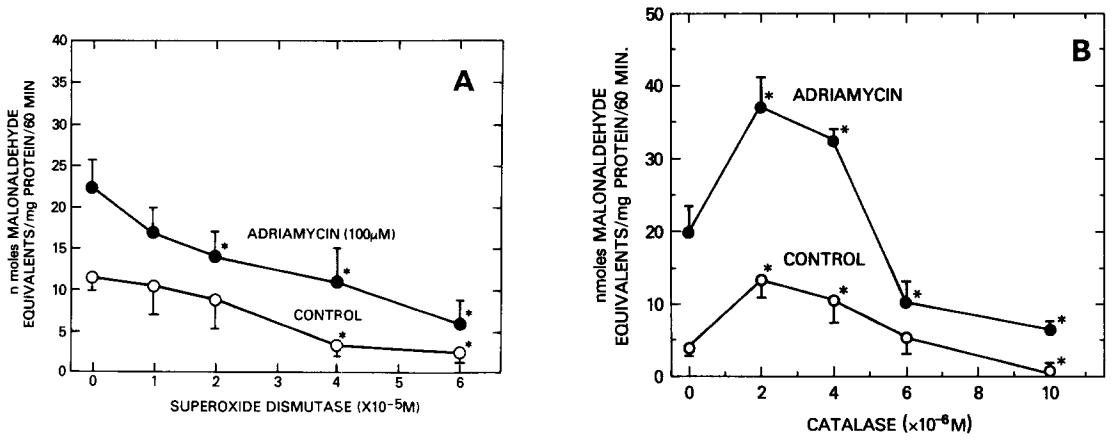

Fig. 2. Concentration-dependent effects of superoxide dismutase (A) and catalase (B) on endogenous (○) and adriamycin-enhanced (●) mitochondrial lipid peroxidation. The scavenging enzymes were added to incubation mixtures prior to adding adriamycin and NADH. Neither bovine serum albumin nor heat-inactivated (95° for 1 min) superoxide dismutase was inhibitory, although boiled (10 min) superoxide dismutase inhibited mitochondrial lipid peroxidation, possibly by the release of copper and zinc which are potent peroxidation-inhibiting metals. Values are expressed as nmoles malonaldehyde equivalents per mg protein per 60 min (means ± S.D., N = 4). Values that are statistically different from those measured in the absence of the scavenging enzymes are denoted by an asterisk ($P < 0.05$).

Table 3. Inhibition of NADH-dependent mitochondrial lipid peroxidation by reactive oxygen scavengers and chelating agents*

Additions	Malonaldehyde equivalents† (nmoles/mg protein/60 min)	
	Endogenous	Adriamycin (100 μM)
None	4.6 ± 0.6	20.7 ± 1.6
Reduced glutathione (5 mM)	0.9 ± 0.1‡	4.5 ± 0.9‡
α-Tocopherol§ (10^{-5} M)	1.9 ± 0.5‡	1.8 ± 1.1‡
1,3-Dimethylurea (20 mM)	1.8 ± 0.3‡	2.1 ± 0.4‡
EDTA (10^{-5} M)	3.3 ± 0.3‡	4.2 ± 0.4‡
DETPAC (10^{-5} M)	0.1 ± 0.1‡	1.3 ± 1.3‡
ICRF-187 (5×10^{-4} M)	0.2 ± 0.2‡	2.2 ± 0.7‡

* Inhibitors were added to incubation mixtures in a range of concentrations. The lowest concentration that provided effective inhibition is noted in parentheses.

† Values are means ± S.D. (N = 4–8).

‡ Significantly different from values without added chemicals ($P < 0.05$).

§ α-Tocopherol, dissolved in ethanol, was added to the mitochondria in the appropriate concentrations, and the mixture was homogenized, using a Teflon and glass tissue grinder, prior to adding the mitochondria to the incubation mixtures.

drial peroxidation by more than 75%, indicating a role for superoxide anions in NADH-dependent mitochondrial lipid peroxidation. The effects of catalase were less clear in that small amounts of the enzyme potentiated both endogenous and adriamycin-accentuated lipid peroxidation, while a higher concentration of catalase (10^{-5} M) significantly diminished mitochondrial peroxidation (Fig. 2). The anomalous stimulatory effect of catalase has been reported previously for microsomal lipid peroxidation [53].

Other scavengers of reactive forms of oxygen, such as reduced glutathione, α-tocopherol, and the hydroxyl radical scavenger 1,3-dimethylurea, potently inhibited both endogenous and adriamycin-augmented peroxidation of mitochondrial phospholipids (Table 3). In addition, cation-chelating agents [EDTA, DETPAC and dioxopiperazinylpropane (ICRF-187)] were also effective in preventing adriamycin-enhanced peroxidation in mitochondria, suggesting an important role for metal ions, most likely iron.

These collective results which show that adriamycin-enhanced mitochondrial NADH-dependent membrane lipid peroxidation was inhibited by various specific and nonspecific oxyradical scavengers strongly indicate that mitochondrial enzyme-activated adriamycin free radical stimulates a complex oxyradical cascade which includes superoxide, hydrogen peroxide and hydroxyl radical. The result of this increased mitochondrial oxyradical generation is greatly increased peroxidation of mitochondrial unsaturated lipids.

Effect of adriamycin on ascorbate-promoted mitochondrial lipid peroxidation. Ascorbic acid generally functions as an antioxidant; however, at low concentrations (0.5 mM) ascorbic acid has a prooxidant effect on lipid peroxidation. This effect is generally considered to result from the ability of ascorbic acid to reduce trace amounts of ferric iron to ferrous iron which autoxidizes to generate superoxide or, alternatively, the iron may react with hydrogen peroxide to form hydroxyl radical [54]. Therefore, we examined the effects of adriamycin on NADH-independent, ascorbate-promoted mitochondrial lipid

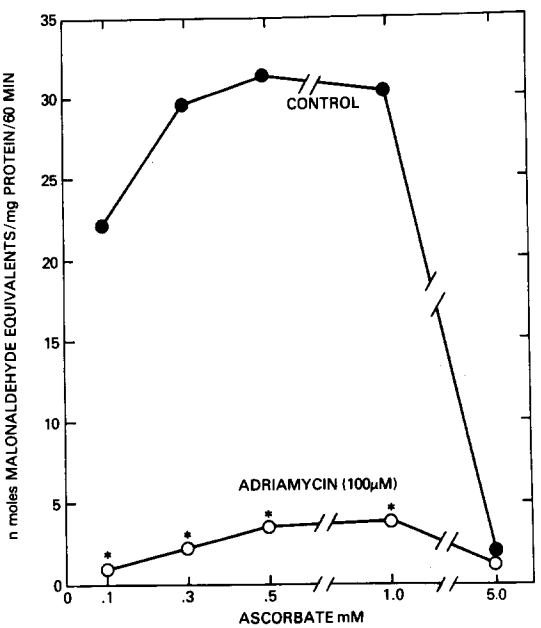

Fig. 3. Inhibition by adriamycin of nonenzymatic, NADH-independent, ascorbate-promoted lipid peroxidation in mitochondria. Lipid peroxidation was measured by the 2-thiobarbituric acid method and is expressed as nmoles of malonaldehyde equivalents per mg protein per 60 min (mean for four samples). Note the biphasic effect of ascorbic acid on mitochondrial lipid peroxidation and the potent blocking of ascorbate-dependent peroxidation by adriamycin. Peroxidation in the presence of adriamycin at 200 μM with ascorbate at 0.5 mM was 2.2 ± 0.9 nmoles of malonaldehyde equivalents per mg per 60 min. Significantly inhibited values are shown by an asterisk ($P < 0.05$).

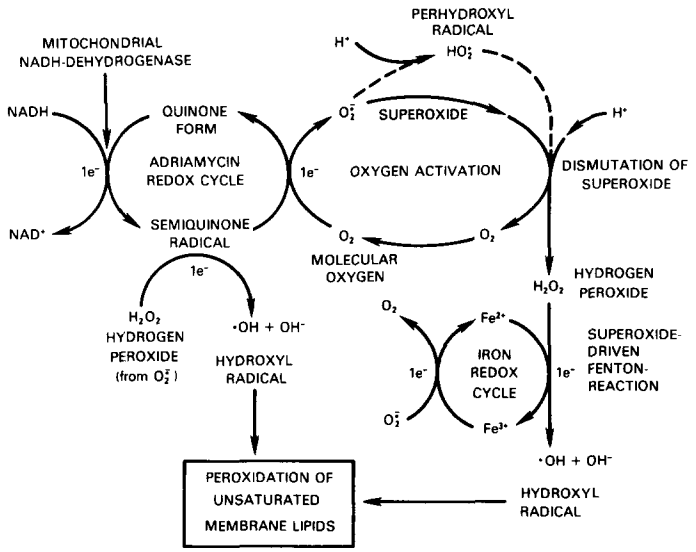

Fig. 4. Schematic representation which shows the activation of adriamycin by mitochondrial NADH-dehydrogenase to establish the drug semiquinone-quinone redox cycle in the presence of molecular oxygen. Also shown is the reductive activation of oxygen to the superoxide anion free radical and the cascade of reactions and interactions which generate secondary reactive oxygen species, which eventually results in greatly-enhanced peroxidation of unsaturated mitochondrial membrane phospholipids.

peroxidation. The concentration-dependent effects of ascorbic acid on mitochondrial peroxidation are shown in Fig. 3. As little as 0.1 mM ascorbic acid caused extensive peroxidation of mitochondrial lipids, while ascorbic acid at 5 mM caused an opposite effect in that it failed to promote peroxidation. Interestingly, adriamycin (100 μM) markedly diminished the non-enzymatic ascorbic acid-dependent peroxidation in mitochondria. Adriamycin at 200 μM nearly abolished ascorbate-promoted mitochondrial lipid peroxidation. The inhibitory effect of adriamycin may result from the removal of trace amounts of iron ions that are required for ascorbate-dependent peroxidation [55] since adriamycin tenaciously binds iron in a 3:1 adriamycin–iron complex [56]. The inhibitory effect of adriamycin on the nonenzymatic, ascorbate-promoted peroxidation of microsomal membrane lipids has been reported previously [57].

DISCUSSION

In this investigation we examined the enhancement by adriamycin of NADH-dependent reactive oxygen-mediated membrane lipid peroxidation in isolated mitochondria. This process depends upon the enzymatic reduction of the drug to the semiquinone free radical [2] which redox cycles to generate a cascade of several reactive oxygen species. We have reported previously that adriamycin and many other anthracyclines greatly stimulate hepatic and cardiac microsomal lipid peroxidation as a consequence of adriamycin-enhanced superoxide ($O_2^{\cdot}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($^{\cdot}OH$) generation [17, 57]. Since adriamycin also increases superoxide, hydrogen peroxide and hydroxyl radical production in the presence of NADH and isolated mitochondria [21], submitochondrial particles [22] or partially purified NADH-dehydrogenase [23, 58], it was reasonable to speculate that adriamycin could also enhance lipid peroxidation in mitochondria as a result of greatly-enhanced oxyradical output. Indeed, most recently, Kharasch and Novak [18] reported that adriamycin increased NAD(P)H-dependent malonaldehyde production in hepatic microsomes, cardiac sarcosomes and cardiac mitochondria isolated from rabbits. The present study extends those observations and demonstrates that the mechanism of adriamycin enhancement of mitochondrial peroxidation involves several forms of reactive oxygen. Figure 4 is a schematic representation of some of the features of the *in vitro* stimulation of mitochondrial membrane lipid peroxidation by adriamycin which are consistent with the results we and other investigators have obtained. The mitochondrial flavoprotein, NADH-dehydrogenase, is the enzyme which most likely reduces adriamycin to the semiquinone free radical [22, 23, 58] and catalyzes the redox cycling of the anthracycline in the presence of oxygen to generate prodigious amounts of superoxide anion radical [22, 58]. A portion of the superoxide, even at physiological pH, is converted to the perhydroxyl radical (dissociation constant = 4.8) [59] which rapidly reacts with additional superoxide to generate hydrogen peroxide and oxygen as products (rate constant = $10^8\,M^{-1}\,sec^{-1}$) [60]. Some direct dismutation of superoxide to hydrogen peroxide probably also occurs, although at physiological pH the rate is slow [59]. The inhibition of adriamycin-stimulated mitochondrial lipid peroxidation by superoxide dismutase and catalase implicates the involvement of *both* superoxide and hydrogen peroxide. The hydrogen peroxide can react with either ferrous iron [61] or adriamycin semiquinone radical (at decreased oxygen concentrations) [52] to generate the extremely

reactive hydroxyl radical. The participation of hydroxyl radical in the peroxidation of mitochondrial lipids is indicated by the fact that the hydroxyl radical scavenger 1,3-dimethylurea [63] prevented malonaldehyde formation. Finally, because EDTA and DETPAC also inhibited lipid peroxidation, the involvement of trace amounts of endogenous iron must be considered. We believe that the primary function of iron ions in this system is to catalyze the formation of hydroxyl radical by the superoxide-driven Fenton reaction [61]. Thus, the enhancing effect of adriamycin on mitochondrial NADH-supported lipid peroxidation appears to be mediated by a complex, interdependent oxyradical cascade involving superoxide anion, hydrogen peroxide and hydroxyl radical. The latter species is probably the predominant lipid-attacking oxyradical by virtue of its extreme reactivity [64].

Mitochondria fortunately contain multiple and elaborate defenses against oxyradicals and lipid peroxidation including: superoxide dismutase [49], glutathione peroxidase and glutathione reductase [51], reduced glutathione [50], catalase [52] and the intramembrane antioxidant α-tocopherol [53]. Although most of these defenses are found in the mitochondrial interior, they can be removed by ultrasonic disruption of the mitochondria followed by sedimentation of the mitochondrial fragments by ultracentrifugation [52]. NADH-dependent lipid peroxidation in submitochondrial particles washed free of soluble enzymatic and biochemical defenses was increased 3- to 4-fold compared to values measured in intact mitochondria. And in the presence of adriamycin, peroxidation of submitochondrial membranes, freed of matrix protein, yielded an impressive 74 ± 7 nmoles of malonaldehyde equivalents per mg protein. Although the specific peroxidation of submitochondrial particles was greater than that measured in whole mitochondria, the considerable peroxidation of intact mitochondria membrane lipids, especially in the presence of adriamycin, revealed that membrane lipid peroxidation can occur in intact mitochondria despite the presence of endogenous defenses against oxygen radicals. This suggests that the greatly increased production of oxyradicals originating from adriamycin semiquinone autoxidation inevitably overwhelms the mitochondrial enzymatic defenses against oxyradicals and eventually lipid peroxidation can progress unchecked.

The biochemical and enzymatic components of the inner mitochondrial membrane are extremely sensitive to damage resulting from the peroxidation of the membrane phospholipids [65]. For example, lipid peroxidation in mitochondria decreases mitochondrial membrane fluidity, increases the negative surface charge distribution, and alters membrane ionic permeability including proton permeability which uncouples bioenergetic reactions, oxidative phosphorylation and active calcium accumulation [66]. Lipid peroxidation also induces mitochondrial swelling and inactivates membrane-bound enzymes possibly by oxidizing sensitive SH-groups [67]. Recently, it has been shown that NADPH-dependent lipid peroxidation in bovine heart submitochondrial particles diminished ubiquinone reduction by NADH-dehydrogenase and decreased electron transfer at several sites of the respiratory chain [68]. It is therefore apparent that lipid peroxidation can be extremely damaging to both mitochondrial structure and mitochondrial function.

Anthracyclines cause multiple deleterious biochemical alterations in mitochondria, and one of the most significant effects is perturbation of the mitochondrial electron transport chain which causes impaired respiratory control [69]. Other adriamycin-caused effects are inhibition of the activities of the coenzyme Q_{10}-requiring enzymes, succinooxidase and NADH oxidase [70], and diminution of mitochondrial isocitrate dehydrogenase activity [71]. Similarly, Demant and Jensen [72] found that adriamycin progressively inactivated NADH-oxidase, cytochrome oxidase and NADH-cytochrome *c* reductase activities in submitochondrial particles, concomitant with the disappearance of polyunsaturated fatty acids. Recently, it was demonstrated that adriamycin causes uncoupling of energy production from energy utilization which may have resulted from adriamycin-mediated inhibition of the binding of creatine phosphokinase to the inner mitochondrial membrane [73], and Burns and Dow [74] have reported that anthracycline-induced membrane damage is associated with the development of irreversible work failure by the heart. Finally, several investigators [75–77] have reported that adriamycin causes abnormal calcium translocation in mitochondria which could contribute extensively to mitochondrial damage.

Prominent morphological alterations in mitochondria are produced by adriamycin and these include swelling and degeneration followed by progressive membrane damage which eventually leads to dissolution of the cristae which disintegrate to membrane myelin figures [19, 78]. Mitochondrial damage may be so extensive that only mitochondrial remnants in the form of fine filamentous membranes are found [79], and this condition results in a paucity of mitochondria within severely damaged cells. It, therefore, appears likely that, in addition to adriamycin-caused damage to mitochondrial biochemical functions, the disruption of mitochondrial membrane structural integrity may also result from the stimulation of mitochondrial lipid peroxidation by adriamycin.

Some of the deleterious effects of lipid peroxidation in mitochondria may result directly from the disintegration of the mitochondrial membrane ultrastructure; alternatively, an indirect mode of damage which should be considered involves toxic carbonyl-containing products which are formed during lipid peroxidation. These aldehyde compounds have been shown to be cytotoxic to isolated cells [80], to inhibit protein synthesis in reticulocyte lysates, possibly by reacting with -SH containing enzymes [81], and to covalently bind to microsomal proteins [82]. It is possible that the inactivation of mitochondrial enzymes as a result of mitochondrial lipid peroxidation may be due to alkylation by reactive aldehydes derived from peroxidized phospholipids. Some of these products have been isolated and identified as 4-hydroxy-alkenals, the most reactive of which is 4-hydroxynonenal [80], and these reactive,

lipid-derived aldehydes have been shown to impair the activities of membrane-bound microsomal glucose-6-phosphatase and cytochrome P-450 [83], although their effects on mitochondrial enzymes have not yet been determined. In view of the fact that products originating from the peroxidative breakdown of membrane unsaturated fatty acids are extremely reactive and even cytotoxic, their contribution to mitochondrial damage is unlikely to be trivial.

In summary, it appears that adriamycin interacts with mitochondrial NADH-dehydrogenase to facilitate the production of the adriamycin semiquinone free radical intermediate which in the presence of oxygen redox cycles to generate superoxide anion, hydrogen peroxide and hydroxyl radical. These reactive oxygen species greatly enhance NADH-dependent peroxidation of mitochondrial membrane unsaturated phospholipids in spite of the presence of endogenous biological defenses against oxyradicals. Whether there is a correlation between the ability of adriamycin to enhance mitochondrial membrane lipid peroxidation *in vitro*, and the various damaging biochemical and morphological alterations caused by adriamycin treatment *in vivo*, remains to be determined. However, the results presented in this paper strongly support the contention that oxyradical-mediated membrane lipid peroxidation plays a toxicologically important role in adriamycin-caused mitochondrial degeneration and dysfunction.

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