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STIMULATION BY ADRIAMYCIN OF RAT HEART AND LIVER MICROSOMAL NADPH-DEPENDENT LIPID PEROXIDATION

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Abstract—Rat liver and heart microsomes catalyze the transfer of single electrons from NADPH to adriamycin forming semiquinone radicals which, in turn, activate molecular oxygen. This process stimulated lipid peroxidation 5- to 7-fold as measured by malonaldehyde formation. Adriamycin-augmented lipid peroxidation was linear with time to 60 min, optimal at 1.0 mg of microsomal protein/ml and pH 7.5, and was proportional to the adriamycin concentration up to 100 μM. An NADPH-generating system was superior to NADPH, and an oxygen atmosphere tripled the rate of peroxidation as compared to air. Nitrogen abolished adriamycin-stimulated peroxidation. Superoxide dismutase, reduced glutathione, α-tocopherol, EDTA, dioxopiperazinylpropane (ICRF-187), and dimethylurea were effective inhibitors of lipid peroxidation. This suggests that superoxide anion and possibly hydroxyl radical may be formed by the oxidation of the adriamycin semiquinone radical and thus stimulate the peroxidation of microsomal unsaturated fatty acids. Although adriamycin failed to stimulate lipid peroxidation in heart microsomes from control animals, peroxidation was dramatically increased when adriamycin was added to cardiac microsomes from α-tocopherol-deficient rats. Lipid peroxidation in α-tocopherol-deficient liver microsomes was four times greater than in control microsomes with the NADPH-generating system, and adriamycin did not further increase that high rate of peroxidation; however, when NADPH was used as the source of electrons in place of the NADPH-generating system, adriamycin stimulated peroxidation more than 2-fold. These results suggest that microsomal lipid peroxidation may play a role in the cytotoxicity and cardiotoxicity of adriamycin.

There is accumulating evidence that demonstrates that the antineoplastic drug, adriamycin, facilitates the generation of oxygen radicals by microsomes. Handa and Sato first reported that adriamycin stimulated the initiation of sulfite oxidation [1] and promoted NADPH oxidation by hepatic microsomes [2]. They postulated that a transitory adriamycin semiquinone free radical was formed by an enzymatic single-electron reduction of adriamycin, and that this species could function as an electron carrier [2]. Their suggestion was subsequently supported by Bachur *et al.* [3, 4], who showed that adriamycin increases the rate of reduction of oxygen to superoxide anion by microsomes and NADPH by a cyclic redox reaction involving a semiquinone free radical intermediate. Not only adriamycin, but similar quinone-containing anticancer drugs interact with microsomes and function as free radical carriers capable of augmenting oxygen reduction [5]. Superoxide anions (O_2^-) and/or secondary reactive oxygen species such as hydrogen peroxide (H_2O_2), hydroxyl radicals ($\cdot OH$), and electronically excited singlet oxygen ($^1O_2$) are believed to initiate and propagate the peroxidation of unsaturated membrane lipids [6–10]. In a preliminary communication, it was shown that adriamycin can stimulate NADPH-dependent microsomal lipid peroxidation, apparently by generating reactive oxygen [11].

Recently, lipid peroxidation *in vivo* has been implicated as a possible biochemical mechanism of adriamycin cardiotoxicity [12], and the observation that α-tocopherol pretreatment produced a time-dependent protection from the lethal effects of adriamycin [13, 14] is consistent with the view that lipid peroxidation is stimulated by oxygen radicals produced by the reoxidation of reduced anthracycline semiquinone. In this connection, we have investigated in detail the effects of adriamycin *in vitro* on liver and heart microsomal NADPH-dependent lipid peroxidation.

MATERIALS AND METHODS

Chemicals. Adriamycin hydrochloride and dioxopiperazinylpropane (ICRF-187) were obtained from the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute, National Institutes of Health, Bethesda, MD. Glucose-6-phosphate, glucose-6-phosphate dehydrogenase, NADPH, NADP, NADH, superoxide dismutase (2900 units/mg protein), catalase (10,000 units/mg protein), D-α-tocopherol (820 units/ml), glutathione, mannitol, 1,3-dimethylurea, and bovine serum albumin (fraction V) were obtained from the Sigma Chemical Co., St. Louis, MO. All other chemicals were of the highest purity available.

Animals and microsome preparation. Male

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Sprague–Dawley rats (Taconic Farms, Germantown, NY), weighing 200–300 g, were fed Purina Rat Chow and water *ad lib*. For the α-tocopherol deficiency experiments, a separate group of weanling rats (23-days-old) were fed an α-tocopherol-deficient diet (ICN, Cleveland, OH) for up to 8 weeks. The tocopherol contents of the deficient and the control diets were < 0.05 μg/g and 110 μg/g of diet, respectively. Animals were killed by cervical dislocation, the hearts and livers were removed, homogenized in 150 mM KCl–50 mM Tris–HCl buffer, pH 7.4 (0–4°), in a Potter-type glass and teflon homogenizer, and microsomes were isolated by differential centrifugation as described previously [15].

Assay for lipid peroxidation. Liver and heart microsomes (1.0 mg protein/ml) were incubated in the absence or presence of adriamycin (100 μM) in KCl–Tris–HCl buffer with an NADPH-generating system [NADP (1.9 mM), glucose-6-phosphate (20 mM), glucose-6-phosphate dehydrogenase (1.1 I.U./ml), and magnesium chloride (8.6 mM)] in a 1.75-ml volume. Reactions were conducted under an oxygen atmosphere in Dubnoff metabolic shakers for 60 min. Modifications of this basic incubation mixture are noted in the tables and figures. Reactions were stopped by adding 0.75 ml of cold 2.0 M trichloroacetic acid–1.7 N HCl, and the samples were centrifuged to pellet denatured proteins. Aliquots (0.5 ml) of the clear supernatant fractions were then added to 2.0 ml of 1.0% (w/v) 2-thiobarbituric acid, heated to 95° and cooled, and the absorption at 533 nm was measured spectrophotometrically [16]. Zero-time blanks containing all the components of the incubation mixture were included to compensate for any interference by adriamycin. Because adriamycin forms a complex with ferrous and ferric iron [17], exogenous iron salts were not added to the microsomes. In addition, preliminary experiments revealed that, when 20 mm ferrous sulfate was added to the incubation mixtures, the rate of lipid peroxidation was increased more than 15-fold over control rates in the absence of exogenous iron, and that adriamycin did not further stimulate that high rate of lipid peroxidation. Microsomal lipid peroxidation is expressed as nmoles of malonaldehyde equivalents per mg protein per 60 min. Standard malonaldehyde was prepared by the acid hydrolysis of tetraethoxypropane (K & K Laboratories, Plainview, NY), and the molar extinction coefficient was found to be $1.53 \times 10^5\ M^{-1}\ cm^{-1}$ at 533 nm.

Inhibitors. The following substances were added directly to incubation mixtures in a wide range of concentrations: superoxide dismutase, catalase, reduced glutathione, ICRF-187, ethylenediaminetetraacetic acid (EDTA), diethylenetriaminepentaacetic acid (DTPA), 1,3-dimethylurea, mannitol and diazabicyclooctane. Boiled superoxide dismutase, boiled catalase, and bovine serum albumin (3.3 mg/ml, equivalent to the protein concentration of 10^{-4} M superoxide dismutase) were added as controls. α-Tocopherol in an ethanol solution was homogenized with microsomes prior to diluting them to the appropriate protein concentration. Ethanol alone was added to a separate aliquot of microsomes to account for any effects of the solvent since ethanol is a radical scavenger.

Other assays. The tocopherol content of the diets and tissues was determined by high pressure liquid chromatography as described by Tangney *et al.* [18] following saponification and extraction into hexane [19]. Protein was measured by the method of Lowry *et al.* [20]. The thin-layer chromatographic method of Bachur *et al.* [21] was used to determine whether adriamycin was metabolized or degraded during the lipid peroxidation reactions. Data were analyzed statistically by Student's *t*-test [22].

RESULTS

Conditions for assay for microsomal lipid peroxidation. An earlier study provided preliminary evidence that adriamycin moderately stimulated (65 per cent) microsomal lipid peroxidation in the presence of NADPH [11]; however, it is difficult to assess whether the conditions for measuring the stimulation of lipid peroxidation by adriamycin were optimal. By contrast, in this report, adriamycin caused a dramatic 3- to 5-fold increase in the extent of lipid peroxidation, depending upon the conditions of assay (Fig. 1). The rate of peroxidation was roughly linear with time to at least 60 min in the absence and presence of adriamycin. Under the conditions described herein, microsomal lipid peroxidation was optimal at 1.0 mg protein/ml; the pH optimum was 7.5 in KCl–Tris–HCl buffer, and the stimulation of lipid peroxidation was proportional to the adriamycin concentration up to 100 μM (Fig. 1). Chromatographic studies revealed that adriamycin was unchanged chemically when incubations were conducted under an oxygen atmosphere.

Cofactor requirements. The stimulation of hepatic microsomal lipid peroxidation by adriamycin was studied using NADPH, an NADPH-generating system, or NADH. Table 1 shows that, although NADPH supported peroxidation in microsomes, the NADPH-generating system was unequivocally superior. This generating system was capable of cycling NADP to produce an equivalent of 20 mM NADPH. Doubling the concentration of the generating system did not further increase the reaction rates. As expected, NADH was relatively ineffective even at 5.0 mM. Note that NADPH (5 mM) was nearly ten times more effective in promoting lipid peroxidation in α-tocopherol-deficient microsomes than in control microsomes, but even under these conditions the generating system was still more effective (Table 1). In liver microsomes from α-tocopherol-deficient animals, however, when the NADPH-generating system was used, adriamycin failed to stimulated peroxidation, perhaps because the endogenous unsaturated lipid substrates were exhausted at 90–100 nmoles malonaldehyde/mg of microsomal protein. Kornbrust and Mavis [23] reported an upper limit to malonaldehyde formation in microsomes of 75–100 nmoles/mg protein and showed that the total depletion of microsomal arachidonic and docosohexaenoic acids corresponded to maximal malonaldehyde formation. The NADPH-generating system was used exclusively in subsequent experiments.

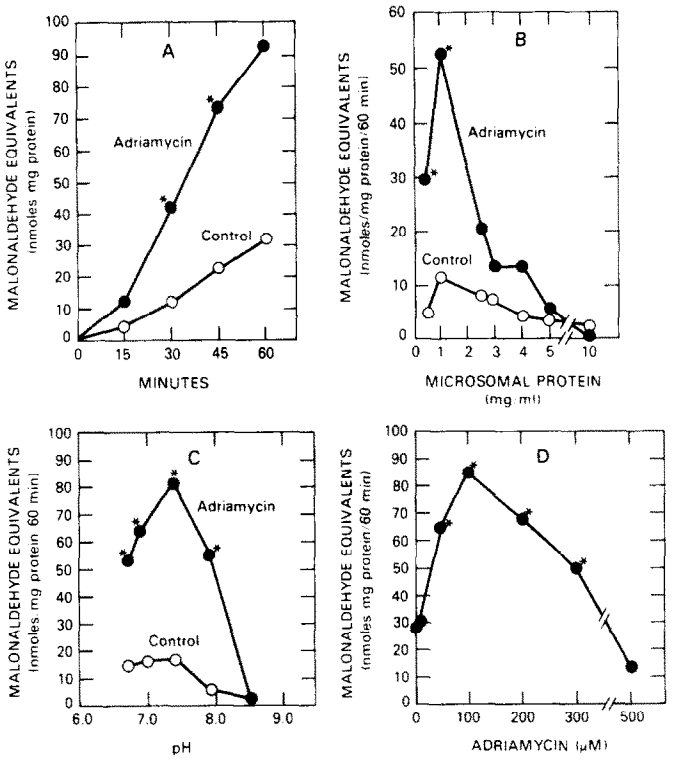

Fig. 1. Effects of varying the time of incubation (A), the microsomal protein concentration (B), the pH (C) and the adriamycin concentration (D) on lipid peroxidation by control and adriamycin-stimulated liver microsomes. The basic incubation mixture was as described in Materials and Methods. Lipid peroxidation was measured by the 2-thiobarbituric acid method. Values significantly different from controls are shown by an asterisk ($P < 0.05$, $N = 3$–6).

Table 1. Cofactor requirements for adriamycin-stimulated hepatic microsomal lipid peroxidation*

	Malonaldehyde equivalents†			
	Control diet		α-Tocopherol-deficient diet‡	
Cofactor (concn)	Control	Adriamycin (100 μm)	Control	Adriamycin (100 μm)
NADPH (1 mM)	3.19 ± 1.62	2.55 ± 1.64	14.4 ± 2.1	35.2 ± 6.9§
NADPH (5 mM)	4.96 ± 3.26	32.7 ± 19.9§	43.8 ± 11.9	81.3 ± 7.1§
NADPH-generating system‖	23.1 ± 11.5	81.9 ± 15.6§	93.1 ± 5.8	97.8 ± 1.3
NADPH-generating system × 2	15.9 ± 5.8	80.6 ± 14.6§		
NADH (1 mM)	3.32 ± 1.78	3.72 ± 3.71		
NADH (5 mM)	1.48 ± 1.35	0.14 ± 0.13		

* Microsomes (1 mg/ml) were incubated with the appropriate cofactor in KCl–Tris–HCl buffer (pH 7.4) at 37°, under oxygen for 60 min. Lipid peroxidation was measured by the 2-thiobarbituric acid method.

† Values are expressed as nmoles malonaldehyde produced per mg protein per 60 min (mean ± S.D.; N = 6–12).

‡ α-Tocopherol-deficient animals were fed a deficient diet for 8 weeks.

§ Significantly greater than values without adriamycin, $P < 0.001$.

‖ The NADPH-generating system was: NADP (1.9 mM), glucose-6-phosphate (20 mM), glucose-6-phosphate dehydrogenase (1.1 I.U./ml) and magnesium chloride (8.6 mM).

Effect of atmosphere. The relationship between oxygen consumption and lipid peroxide formation in liver microsomes has been well documented [24, 25]. The molar ratio of oxygen reduction to malonaldehyde formation has been reported to be between 15 and 20 under a wide range of experimental conditions [26]. To demonstrate that oxygen was essential to adriamycin-stimulated lipid peroxidation, reactions were carried out under atmospheres of air, oxygen or nitrogen. Compared against air, an oxygen atmosphere nearly doubled the rate of lipid peroxidation in the absence of adriamycin and more than tripled the rate in the presence of adriamycin (Table 2). Nitrogen markedly reduced or abolished microsomal lipid peroxidation. It should be noted that adriamycin is rapidly biotransformed by microsomes to its aglycone under anaerobic conditions [27], and considerable amounts of aglycone are formed during incubations under an air atmosphere after dissolved oxygen is consumed (unpublished observation).

Effects of inhibitors. It has been suggested that, following the NADPH-cytochrome P-450 reductase catalyzed single-electron transfer from NADPH to adriamycin, the semiquinone radical thus formed may shuttle electrons to molecular oxygen to form superoxide anion [1, 28]. Furthermore, superoxide can, in turn, produce other forms of reactive oxygen such as hydroxyl radical and singlet oxygen. In order to determine whether these reactive-oxygen species participate in adriamycin-stimulated lipid peroxidation, the effects of a number of inhibitors were studied. The antioxidant α-tocopherol (10^{-5} M) inhibited lipid peroxidation more than 90 per cent in control microsomes and 99 per cent in the presence of adriamycin (Table 3). Lower concentrations of α-tocopherol were less effective. Superoxide dismutase inhibited both control and adriamycin-stimulated peroxidation by more than 90 per cent (Table 3). The effect of superoxide dismutase was abolished by boiling the enzyme; indeed, boiled superoxide dismutase stimulated peroxidation slightly (20 per cent). An equivalent amount of unboiled bovine serum albumin had no effect. The fact that superoxide dismutase, albeit at a rather high concentration (4×10^{-5} M), inhibited adriamycin-stimulated lipid peroxidation strongly implicates a role for superoxide anion in these reactions.

Table 2. Oxygen requirement during microsomal lipid peroxidation*

	Malonaldehyde equivalents‡	
Atmosphere†	Control	Adriamycin (100 μM)
Air	15.4 ± 9.5	24.7 ± 10.6§
Oxygen	26.8 ± 14.1‖	87.7 ± 5.1 §,‖
Nitrogen	3.22 ± 1.55‖	0.00 ± 0.00§,‖

Microsomes from rat livers (1.0 mg protein/ml) were incubated in KCl–Tris–HCl buffer (pH 7.4) with an NADPH-generating system under an air, oxygen or nitrogen atmosphere for 60 min. Lipid peroxidation was measured by the 2-thiobarbituric acid method.

† Gases were delivered to covered incubators at rates of 5.0 l/min.

‡ Values are expressed as nmoles malonaldehyde produced per mg protein per min (mean ± S.D.; N = 3–10).

§ Significantly different from values without adriamycin, $P < 0.05$.

‖ Significantly different from values for air, $P < 0.05$.

EDTA and DTPA, both chelators of divalent cations, were nearly 100 per cent effective as inhibitors of adriamycin-stimulated peroxidation and, most likely, inhibited by sequestering catalytic amounts of free ferrous iron necessary for the promotion of lipid peroxidation in microsomes. When rat liver microsomes were isolated from homogenates that contained 1.0 mM EDTA, and then resuspended in EDTA-free buffer, NADPH (2.5 mM)-supported lipid peroxidation was 11.6 ± 1.23 and 43.5 ± 3.35 nmoles malonaldehyde·mg protein)$^{-1}$·60 min^{-1} in the absence and presence of adriamycin. These results suggest that only very small amounts of iron are required or that NADPH or the buffer was contaminated with trace amounts of iron salts. ICRF-187, an anticancer drug that has chelation properties and has been shown to reduce the lethal toxicity of daunorubicin in hamsters [29], was also an effective inhibitor of both liver and heart microsomal lipid peroxidation.

Thiol compounds have been shown to strongly inhibit NADPH-induced peroxidation [26]. Reduced glutathione added to incubation mixtures quite effectively decreased lipid peroxidation, although increasing the glutathione concentration above 1.0 mM did not markedly increase the inhibition. Lower concentrations of glutathione were less effective. Both catalase and boiled catalase (10^{-8} to 10^{-5} M) added to reaction mixtures produced a marked inhibition of peroxidation. Inasmuch as boiled catalase was as effective as the active enzyme, no substantive conclusions can be drawn concerning the role of the enzyme or hydrogen peroxide in these reactions. It should be noted, however, that these data do not negate participation of hydrogen peroxide in adriamycin-stimulated lipid peroxidation. Mannitol, a hydroxyl radical scavenger, and diazabicyclooctane, the singlet oxygen quencher, were ineffective inhibitors at concentrations as high as 10^{-2} M. However, 1,3-dimethylurea, another hydroxyl radical scavenger [30], inhibited lipid peroxidation by 90 per cent, which suggests that hydroxyl radical may participate in these reactions. 1,3-Dimethylurea at either 1.0 or 10.0 mM had no effect on rat liver microsomal NADPH-cytochrome *c* reductase or aminopyrine *N*-demethylase activities and, therefore did not block the flow of electrons from NADPH through the flavoprotein. UDP-glucuronyltransferase, a microsomal enzyme especially sensitive to detergent effects *in vitro* [31], was similarly unaltered by dimethylurea, indicating that dimethylurea did not inhibit microsomal lipid peroxidation by a detergent-like effect which might have disrupted the microsomal membrane or solubilized NADPH-cytochrome P-450 reductase (data not presented).

Effects of α-tocopherol deficiency. Although adriamycin did not significantly stimulate lipid peroxidation in microsomes prepared from control rat hearts, it dramatically stimulated lipid peroxidation 5-fold in cardiac microsomes from α-tocopherol-

Table 3. Effects of various inhibitors on control and adriamycin-stimulated microsomal lipid peroxidation*

Inhibitor† (conc)	Percent inhibition‡ Control	Adriamycin (100 μM)
α-Tocopherol (10^{-5} M)§	91 ± 3	99 ± 1
Superoxide dismutase		
(2×10^{-5} M)	8 ± 5	1 ± 1
(4×10^{-5} M)	81 ± 8	42 ± 22
(6×10^{-5} M)	94 ± 3	96 ± 2
Boiled superoxide dismutase (10^{-4} M)	0 ± 0	0 ± 0
Reduced glutathione (10^{-3} M)	79 ± 17	93 ± 5
Catalase (10^{-5} M)	97 ± 2	99 ± 1
Boiled catalase (10^{-5} M)	99 ± 1	100 ± 0
Bovine serum albumin (3.3 mg/ml)‖	0 ± 0	0 ± 0
Dioxopiperazinylpropane (ICRF-187) (5×10^{-4} M)	97 ± 2	98 ± 2
Ethylenediamine tetraacetic acid (10^{-5} M)	98 ± 1	98 ± 2
Diethylenetriamine pentaacetic acid (5×10^{-6} M)	95 ± 3	99 ± 1
Dimethylurea (10^{-2} M)	88 ± 6	91 ± 8
Mannitol (10^{-2} M)	0 ± 0	0 ± 0
Diazabicyclooctane (10^{-2} M)	16 ± 6	7 ± 10

* Control lipid peroxidation was 28.6 ± 16.8 and 88.6 ± 15.9 nmoles malonaldehyde per mg protein per 60 min for incubations in the absence and presence of adriamycin respectively.

† Inhibitors were added directly 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 = 3. No statistical analysis was performed.

§ α-Tocopherol was added to microsomes in an ethanol solution and homogenized prior to incubation. Ethanol alone inhibited lipid peroxidation only 11 and 21 per cent.

‖ The concentration of bovine serum albumin (3.3 mg/ml) was equivalent to the protein concentration produced at 10^{-4} M superoxide dismutase.

deficient animals (Table 4). Lipid peroxidation was also greater in liver microsomes from α-tocopherol-deficient rats compared to controls. Adriamycin doubled peroxidation when NADPH was used but, in contrast, when the NADPH-generating system was substituted for NADPH, the adriamycin stimulation was abolished because of the higher, perhaps maximum, endogenous activities in the deficient microsomes. There was little difference in cardiac or hepatic microsomal lipid peroxidation at 4, 6 and 8 weeks of α-tocopherol deficiency (Table 4). Bieri has shown that both heart and liver concentrations of α-tocopherol are depleted rapidly in weanling rats fed an α-tocopherol-deficient diet, with steep declines during the first 2 weeks, followed by slower rates of change thereafter [32]. There was little further change in the α-tocopherol content of heart or liver after 4-weeks deficiency.

Next we investigated the effects of inhibitors of lipid peroxidation in rat liver and heart microsomes from α-tocopherol-deficient rats. In general, reduced glutathione, EDTA, ICRF-187 and superoxide dismutase inhibited adriamycin-stimulated lipid peroxidation in heart and liver microsomes from both control and α-tocopherol-deficient animals (Table 5); however, the degree of inhibition was consistently less in microsomes from α-tocopherol-deficient animals. For example, glutathione (1 mM) inhibited peroxidation 44 and 97 per cent respectively, in control heart and liver microsomes but, by contrast, 5 mM glutathione inhibited only 11 and 77 per cent in the α-tocopherol-deficient group. Superoxide dismutase, likewise, was less effective in inhibiting lipid peroxidation in microsomes from α-tocopherol-deficient animals. It appears that the concentration of α-tocopherol is decidely important in determining the effects of adriamycin on NADPH-supported lipid peroxidation in both heart and liver microsomes.

DISCUSSION

The interaction of adriamycin with microsomes and NADPH generates adriamycin semiquinone radicals that may then transfer electrons to molecular oxygen, forming superoxide anion, and thereby stimulate microsomal lipid peroxidation. As demonstrated in this manuscript, the magnitude of adriamycin-augmented lipid peroxidation is dependent upon the concentrations of adriamycin and microsomal protein, the presence of sufficient reducing equivalents (NADPH-generating system), and the degree of oxygen saturation.

Table 4. Effect of α-tocopherol deficiency on the stimulation by adriamycin of heart and liver microsomal lipid peroxidation*

		Malonaldehyde equivalents†			
		Control diet		α-Tocopherol-deficient diet‡	
Organ	Time (weeks)	Control	Adriamycin (100 μM)	Control	Adriamycin (100μM)
Heart	4	0.41 ± 0.24	1.67 ± 1.54	7.65 ± 1.34	33.5 ± 1.2§
	6	1.49 ± 0.63	1.67 ± 0.94	6.30 ± 0.55	31.9 ± 2.9§
	8	0.34 ± 0.31	2.15 ± 1.97	3.94 ± 0.34	29.6 ± 1.1§
Liver	4	28.6 ± 16.8	88.6 ± 15.9§	102 ± 21.2	97.2 ± 20.4
	6	31.4 ± 10.2	88.9 ± 3.6 §	83.9 ± 13.7	82.9 ± 11.7
	8	26.9 ± 19.7	80.3 ± 16.2§	92.5 ± 5.0	100 ± 6.6

* Lipid peroxidation was measured in heart and liver microsomes by the 2-thiobarbituric acid method.

† Values are expressed as nmoles malonaldehyde produced per mg protein per 60 min (mean ± S.D.; N = 3–6).

‡ Animals were fed either a control (110 μg α-tocopherol/g diet) or an α-tocopherol-deficient diet (< 0.5 μg α-tocopheral/g diet) for 4, 6 and 8 weeks.

§ Significantly different from values without adriamycin, $P < 0.05$.

Cardiac and hepatic microsomal lipids are presumably peroxidized by the same mechanism. Lipid peroxidation in both preparations required NADPH and oxygen, was maximally stimulated at 100 μM adriamycin, and was effectively inhibited by glutathione, superoxide dismutase and EDTA. Thus, it is reasonable to expect that *in vivo*, cardiac sarcoplasmic reticulum could activate adriamycin enzymatically to a semiquinone radical metabolite that could then accentuate reactive oxygen generation, cause lipid peroxidation and, subsequently, sarcoplasmic reticular membrane damage. Alternatively, electrons could be passed directly from the semiquinone to microsomal lipids, forming lipid radicals that could then undergo peroxidation by oxygen addition. As shown in Fig. 2, there are at least two possible mechanisms whereby adriamycin can stimulate peroxidation of membrane unsaturated lipids. Furthermore, products originating from the peroxidation of microsomal lipids have been demonstrated to bind covalently to microsomal proteins [33], and it is tempting to speculate that these substances could also cause damage to biomolecules other than phospholipids.

Whether superoxide anion itself or secondary reactive oxygen products are responsible for initi-

Table 5. Differences in the inhibition of adriamycin-stimulated lipid peroxidation in heart and liver microsomes from control and α-tocopherol-deficient rats*

	Percent inhibition			
	Control		α-Tocopherol deficient‡	
Inhibitor† (conc)	Heart	Liver	Heart	Liver
Glutathione (1 mM)	44 ± 11	97 ± 1	11 ± 2	55 ± 11
Glutathione (5 mM)	51 ± 4	98 ± 1	11 ± 1	77 ± 4
EDTA (10^{-5} M)	47 ± 2	95 ± 5	27 ± 6	40 ± 5
EDTA (10^{-4} M)	59 ± 5	98 ± 1	87 ± 1	94 ± 1
ICRF-187 (5×10^{-4} M)	100 ± 0	99 ± 1	84 ± 0	71 ± 23
Superoxide dismutase (10^{-4} M)	0 ± 0	85 ± 9	19 ± 3	32 ± 10

* Lipid peroxidation for control hearts and livers was 2.04 ± 0.50 and 92.4 ± 3.4 nmoles malonaldehyde per mg protein per 60 min, respectively, and for α-tocopherol-deficient hearts and livers 35.0 ± 3.6 and 95.6 ± 2.3 nmoles malonaldehyde per mg protein per 60 min respectively (N = 3–6). These values were set at 100 per cent.

† Inhibitors were added directly to the incubation mixtures.

‡ Weanling rats were fed an α-tocopherol-deficient diet for 6 weeks.

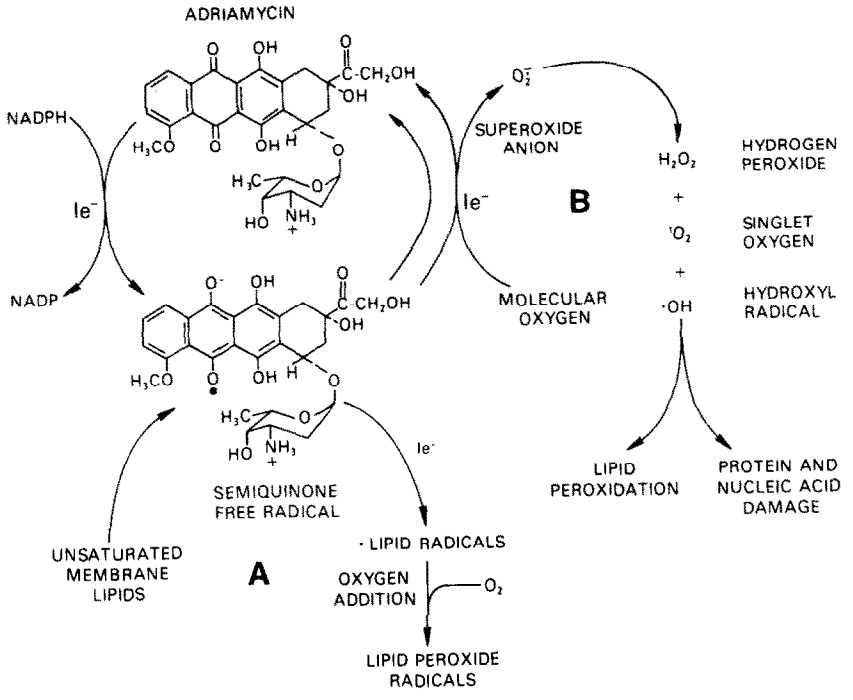

Fig. 2. Possible mechanism of action of adriamycin-stimulated microsomal lipid peroxidation. Following the formation of adriamycin semiquinone radicals by NADPH-cytochrome P-450 reductase, single electrons may be passed directly to unsaturated membrane lipids (A) forming lipid free radicals, which then peroxidate by oxygen addition. Alternatively, electrons may transfer to molecular oxygen (B) forming superoxide anions and other reactive oxygen species that initiate and promote lipid peroxidation and possibly damage other macromolecules *in vivo*.

ating and propagating lipid peroxidation has not been established conclusively. Goodman and Hochstein [11] reported that superoxide dismutase partially inhibited daunomycin-augmented sulfite oxidation. In the present study 4×10^{-5} M superoxide dismutase effectively inhibited adriamycin-stimulated microsomal lipid peroxidation, strongly suggesting participation of superoxide anion. This concentration of superoxide dismutase is approximately twice the endogenous rat liver cytosolic concentration [34]. Although superoxide is a likely intermediate leading to peroxidation of membranous unsaturated fatty acids, singlet oxygen and hydroxyl radical have also been implicated as powerful initiators of lipid peroxidation [9, 10]. As shown in this report, 1,3-dimethylurea, a hydroxyl radical scavenger, inhibited microsomal lipid peroxidation 90 per cent. This finding makes it likely that more than one reactive oxygen species participates in adriamycin-augmented lipid peroxidation, just as superoxide, hydrogen peroxide, singlet oxygen and hydroxyl radical may all participate in paraquat [35, 36] or oxygen [37] promoted lipid peroxidation.

Bachur *et al.* [3] showed that α-tocopherol prevented adriamycin-promoted NADPH-dependent oxygen consumption by liver microsomes. Similarly, we found that α-tocopherol added to control microsomes effectively prevented adriamycin-stimulated lipid peroxidation, presumably by preferential oxidation of α-tocopherol which spared microsomal lipids [38, 39]. It appears that control rat heart microsomes are well protected against peroxidative damage by endogenous α-tocopherol, whereas α-tocopherol-deficient heart microsomes are peroxidized extensively in the presence of adriamycin. Only after the endogenous microsomal α-tocopherol is depleted can the redox cycling of adriamycin accelerate the peroxidation of microsomal fatty acyl groups. Thus, the α-tocopherol content of microsomes may represent the "front-line defense" against lipid peroxidation, as suggested by Bieri and Anderson [40]. The observation that α-tocopherol partially protects against the lethal toxicity of adriamycin in mice [14] and rats [41] supports this contention. It should be mentioned, however, that the importance of α-tocopherol as a defense mechanism may vary from tissue to tissue.

Cells have several crucial defenses against reactive oxygen toxicity in addition to α-tocopherol. Catalase, superoxide dismutase and glutathione peroxidase enzymatically dispose of hydrogen peroxide, superoxide anions and lipid hydroperoxides respectively. Glutathione can diminish lipid peroxidation through oxidation of its thiol group, thus protecting unsaturated fatty acids. These defenses, however, are confined to the cell cytosol and would appear to be of secondary importance to α-tocopherol in checking adriamycin-stimulated reactive oxygen-mediated damage to membranes. The cytosolic defenses are undoubtedly important in preventing

reactive oxygen-mediated damage to cellular target molecules other than lipids and, interestingly, it appears that the heart is deficient in some of these cytosolic defenses [42, 43].

It seems clear to us that the powerful stimulation of microsomal lipid peroxidation is but only one consequence of adriamycin-augmented reactive oxygen production. *In vivo*, not only endoplasmic reticulum lipids but other target molecules such as membrane and cytosolic enzymes and nucleic acids may be susceptible to free radical and reactive oxygen-mediated damage. Although a site of adriamycin-accentuated reactive oxygen generation is the endoplasmic reticulum, it is possible that a shower of reactive oxygen or, alternatively, cytotoxic lipoperoxide products could diffuse to and damage organelles beyond the vicinity of the endoplasmic reticulum. It is well known that adriamycin treatment causes morphologic changes in cardiac mitochondria and nuclei [44], and it is possible that reactive oxygen plays a role in those pathologic changes. Although the mechanisms are not understood completely, adriamycin interacts with vital cellular macromolecules and abrogates essential biochemical functions. For example, adriamycin has been shown to cause DNA strand scission [45], to interfere with mitochondrial respiration [46], and most recently it has been reported that adriamycin temporarily reduces cardiac cytosolic glutathione peroxidase activity [43]. Some or all of these deleterious effects of adriamycin may be mediated by adriamycin radicals and/or reactive oxygen. Elucidation of the mechanism(s) of damage to all intercellular target molecules is integral to the full understanding of adriamycin cardiotoxicity.

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