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REACTIONS OF ADRIAMYCIN WITH MICROSOMAL IRON AND LIPIDS

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Iron plays a central role in oxidative injury, reportedly because it catalyzes superoxide- and hydrogen peroxide-dependent reactions yielding a powerful oxidant such as the hydroxyl radical. Iron is also thought to mediate the cardiotoxic and antitumour effects of adriamycin and related compounds. NADPH-supplemented microsomes reduce adriamycin to a semiquinone radical, which in turn re-oxidizes in the presence of oxygen to form superoxide and hence hydrogen peroxide. During this redox cycling membrane-bound nonheme iron undergoes superoxide dismutase- and catalase-insensitive reductive release. Membrane iron mobilization triggers lipid peroxidation, which is markedly enhanced by simultaneous addition of superoxide dismutase and catalase. The results indicate that : i) lipid peroxidation is mediated by the release of iron, yet the two reactions are governed by different mechanisms; and ii) oxygen radicals are not involved in or may actually inhibit adriamycin-induced lipid peroxidation. Microsomal iron delocalization and lipid peroxidation might represent oxyradical-independent mechanisms of adriamycin toxicity.

KEY WORDS: Microsomes, ferric nonheme iron, adriamycin semiquinone, ferric reduction, ferrous release.

INTRODUCTION

Microsomes isolated by the usual methods of differential centrifugation are contaminated by ferritin, which can be readily and very effectively removed by means of affinity chromatography[1] or Sepharose CL-2B chromatography.[2,3] Recent studies in this laboratory[3] and earlier reports from other laboratories[1,2] have shown that chromatographed ferritin-free microsomes still contain significant amounts of ferric nonheme iron, which is tentatively referred to as nonheme-nonferritin. Any attempt to characterize structure, binding sites and other basic features of this iron species has been so far unsuccessful. From a biological viewpoint, it would appear that the microsomal pool of nonheme-nonferritin iron is utilized to form the heme iron group of inducible cytochrome P-450 isozymes. This conclusion stems from the repeated finding that induction of cytochrome P-450 by phenobarbital treatment is paralleled by the depletion of nonheme-nonferritin iron[1,3,4] and that such nonheme iron → to heme iron conversion is blocked by the simultaneous administration of inhibitors of heme synthesis.[4]

The microsomal flavoenzyme NADPH-cytochrome P-450 reductase catalyzes one-electron reduction of the cardiotoxic anticancer drug adriamycin (ADR)[3] to a semiquinone radical ($ADR^{\cdot -}$), which in turn regenerates the parent compound by

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Abbreviations: ADR, adriamycin; $O_2^{\cdot -}$ superoxide; H_2O_2, hydrogen peroxide; ·OH, hydroxyl radical; SOD, superoxide dismutase; p-HMB, p-hydroxymercuribenzoate; MDA, malondialdehyde.

virtue of its reoxidation in the presence of oxygen to form $O_2^{\cdot-}$ and its dismutation product H_2O_2.[5,6] Subsequently, a Fenton's reaction of H_2O_2 with Fe^{2+} may eventually threaten cell integrity via liberation of a powerful oxidant such as $\cdot OH$.[7]

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH^- + \cdot OH \quad (1)$$

The involvement of $\cdot OH$ has been implicated in a number of pathological processes, varying from lipid peroxidation to protein degradation and DNA double strand breakage.[8]

Keeping in mind that both $ADR^{\cdot-}$ and $O_2^{\cdot-}$ are Fe^{3+} reductants,[9] and that Fe^{3+} to Fe^{2+} reduction facilitates mobilization of iron from iron-binding proteins[10] and membranes,[2] it was of interest to establish whether microsomes may couple the redox cycling of ADR with the reductive release of nonheme-nonferritin iron and $\cdot OH$-mediated lipid peroxidation.

MATERIALS AND METHODS

Chemicals

NADPH, type VI horse heart cytochrome c, bathophenanthroline disulfonate, rat liver ferritin, Sepharose CL-2B, p-HMB and thiobarbituric acid were purchased from Sigma Chemical Co. (St. Louis, MO). Sephadex G-25 was a product of Pharmacia Fine Chemicals (Uppsala, Sweden). Chelex 100 ion exchange resin was obtained from Bio Rad (Richmond, CA) and was used to remove contaminating metals from all solutions and reagents. All experiments were carried out in oxygen-saturated 50 mM NaCl, carefully adjusted to pH 7.0 just prior to use. This was done to avoid artifactual ligand-catalyzed Fe^{2+} oxidation that occurs in most common laboratory buffers.[11] Although unbuffered, the pH of incubations did not vary from 7.0 throughout the experiment time.

Enzymes

Bovine erythrocyte SOD (EC. 1.15.1.1.) and thymol-free bovine liver catalase (EC. 1.11.1.6) were from Sigma. The enzymes were first incubated with 10 mM EDTA on ice for 1 h and then chromatographed over a Sephadex G-25 column, previously equilibrated with 0.3 M NaCl, pH 7.0. Following this treatment, that was intended to remove loosely associated iron, the activity of SOD and catalase was determined according to McCord and Fridovich,[12] and Beers and Sizer,[13] respectively.

Microsome preparation

Microsomes were isolated from the liver of male Wistar rats (150–180 grams) according to the procedure described by Pederson and Aust,[14] with minor modifications.[2] The 105,000 xg pellets were washed once in 50 mM NaCl-0.2% nicotinamide, suspended in 0.02 M Tris-HCl/0.15 M KCl, pH 7.4 and applied to a Sepharose CL-2B column (2.5 × 25 cm), previously equilibrated with the same buffer. Chromatographed microsomes were washed twice in 50 mM NaCl-0.2% nicotinamide and suspended in 50 mM NaCl-20% glycerol, pH 7.0. Proteins were determined as described by Layne.[15]

Assays

NADPH-cytochrome P-450 reductase was measured by reduction of unmodified cytochrome c,[16] whereas superoxide formation was measured as SOD-inhibitable reduction of acetylated cytochrome c.[17] NADPH oxidation was measured as decrease in absorbance at 340 nm.[17] Fe^{2+} release was studied spectrophotometrically by taking advantage of the absorptivity of bathophenanthroline-Fe^{2+} complex at 530–560 nm.[18] Lipid peroxidation was assayed as MDA formation by means of the thiobarbituric acid test.[19] Other assay details are given in legends to tables. Nonheme iron was determined according to Brumby and Massey.[20] Ferritin was determined by means of an ELISA relying on the cooxidation of o-phenylendiamine by H_2O_2 and antiferritin IgG-conjugated horseradish peroxidase (Ferrizyme, Abbott Diagnostic Division); purified rat liver ferritin was used as internal standard.

RESULTS

As shown in Table I, Sepharose CL-2B chromatography substantially modifies the composition of microsomal membranes. For example, chromatography removes

TABLE I
Effects of Sepharose CL-2B chromatography on microsomal membranes.

	Catalase	Cytochrome P-450 reductase	Ferritin	Nonheme iron
	Units/mg prot.		μg/mg prot.	nmoles/mg prot.
Microsomes	36.2	0.21	5.7	17.2
Chromatographed microsomes	3.1	0.38	0.0	5.8

Note. Enzymes, ferritin and nonheme iron were assayed as described under Materials and Methods.

TABLE II
Adriamycin-induced microsomal oxidation of NADPH, formation of O_2^- and release of Fe^{2+}.

Addition	NADPH Oxidation[a)]	O_2^- formation[b)]	Fe^{2+} release[c)]
	nmoles mg prot.$^{-1}$ min.$^{-1}$		
NADPH	17.8	6.4	0.05
NADPH, ADR	91.4	54.1	0.44

Note. [a]Incubations (1 ml final volume) contained chromatographed microsomes (0.05 mg prot./ml) in 50 mM NaCl, pH 7.0, 37°C. Reactions were started by the addition of NADPH (0.25 mM) and the decrease in absorbance at 340 nm was monitored continuously.

[b]Incubations (1 ml final volume) contained chromatographed microsomes (0.1 mg prot./ml) and acetylated cytochrome c (0.1 mM) in 50 mM NaCl, pH 7.0, 37°C. Reactions were started by the addition of NADPH (0.5 mM) and the increase in absorbance at 550 nm was monitored continuously. Values are given as differences between rates obtained before and after addition of SOD (200 U/ml).

[c]Incubations (1 ml final volume) contained chromatographed microsomes (0.5 mg prot./ml) and bathophenanthroline (0.5 mM) in 50 mM NaCl, pH 7.0, 37°C. Reactions were started by the addition of NADPH (1 mM) and the increase in absorbance at 530–560 nm was monitored vs reagent blanks lacking bathophenanthroline.

In (a–c) the concentration of ADR was held constant at 0.25 mM.

TABLE III
The effects of SOD, catalase and p-HMB on ADR-induced release of Fe^{2+}.

System	(nmoles Fe^{2+} mg prot.$^{-1}$ min^{-1})
Complete	0.44
+ SOD	0.44
+ catalase	0.45
+ SOD	
+ catalase	0.49
+ p-HMB	0.01

Note. Incubations prepared as described in Legend to Table II, point (c). Where indicated, SOD (200 U/ml), catalase (400 U/ml) and p-HMB (0.25 mM) were included in the reaction mixtures.

TABLE IV
NADPH and ADR-induced microsomal lipid peroxidation : The effects of SOD, catalase and p-HMB.

Addition	nmoles MDA mg prot.$^{-1}$ min.$^{-1}$
NADPH	0.02
NADPH, ADR	0.21
+ SOD	0.14
+ catalase	0.48
+ catalase	
+ SOD	0.45
+ catalase	
+ SOD	
+ p-HMB	0.00

Note. Incubations (2.5 ml final volume) contained chromatographed microsomes (0.6 mg prot./ml) in 50 mM NaCl, pH 7.0, 37°C. Reactions were started by addition of NADPH (1 mM) and aliquots (0.5 ml) were taken at regular times for MDA assay. Where indicated. SOD (200 U/ml), catalase (400 U/ml) and p-HMB (0.25 mM) were included in the reaction mixtures.

loosely associated proteins or enzymes, such as ferritin or catalase, thereby increasing specific activity of integral membrane constituents, e.g. NADPH-cytochrome P-450 reductase. It should be noted, however, that a complete chromatographic removal of ferritin is not paralleled by a complete disappearance of nonheme iron (see also Table I). This indicates that microsomes contain an "endogenous" pool of nonheme-nonferritin iron.[1-4]

The addition of ADR to a suspension of chromatographed microsomes stimulates the oxidation of NADPH and the formation of $O_2^{\cdot-}$ (Table II). This effect is indicative of a sustained flux of reducing equivalents from NADPH to molecular oxygen, via the NADPH-cytochrome P-450 reductase and the continuous reduction-reoxidation of ADR. During this redox cycling, nonheme-nonferritin iron is reductively released, as evidenced by mobilization of bathophenanthroline-chelatable Fe^{2+} (see also Table II). The reductive release of iron is blocked by addition of p-HMB, an inhibitor of the reductase,[21] but not by separate or simultaneous addition of SOD and catalase (Table III).

In the presence of ADR, i.e. under conditions that favour iron release, the addition of NADPH causes lipid peroxidation of chromatographed microsomes (Table IV). The reaction is partially inhibited by SOD and greatly enhanced by either catalase or

the combination of catalase with SOD; no lipid peroxidation can be observed in the presence of p-HMB (see also Table IV).

DISCUSSION

Adriamycin has long been recognized as a redox cycling compound, in that it may shuttle electron from donor(s) to acceptor(s) by virtue of its facile reduction-reoxidation. In the presence of NADPH-supplemented microsomes, ADR will first pick up one electron from NADPH-cytochrome P-450 reductase and will subsequently return this electron to molecular oxygen, thereby accelerating oxidation of NADPH and formation of $O_2^{\cdot-}$ (cf. Table II). This redox cycling liberates intermediates that reduce membrane-bound iron and initiate lipid peroxidation, as evidenced by mobilization of Fe^{2+} and formation of MDA (cf. Table II and Table IV). Lipid peroxidation is notoriously contingent on iron-catalyzed reactions[8,22,23] hence, the potential for ADR to couple the release of iron with the formation of MDA is not surprising. However, while Fe^{2+} release is SOD- and catalase-insensitive, lipid peroxidation can either be inhibited or stimulated by SOD and catalase (cf. Table III and Table IV). Therefore, mechanism(s) of ADR-induced membrane iron reduction are different from mechanism(s) of ADR-induced, Fe^{2+}-catalyzed lipid peroxidation.

Lack of inhibition of iron release by SOD and/or catalase would imply that : i) membrane iron is reduced by $ADR^{\cdot-}$, with negligible contribution by the $O_2^{\cdot-}$ which stems from its reoxidation; ii) $O_2^{\cdot-}$ does participate in the reductive release of iron, yet the reaction occurs at membrane sites which cannot be entered by SOD; iii) H_2O_2 does not interfere with the mechanisms of iron release. On the other hand, stimulation of lipid peroxidation by catalase implies that H_2O_2 inhibits formation and/or reactivity of ultimate oxidant(s). Consistently, lipid peroxidation is inhibited by SOD, which accelerates dismutation of $O_2^{\cdot-}$, and such inhibition can be easily reverted to stimulation by adding catalase for decomposing H_2O_2. Viewed from another point of consideration, the data rule out the intermediacy of $\cdot OH$ in ADR-induced lipid peroxidation. In fact, lipid peroxidation is maximal when catalase scavenges the H_2O_2 required for Fenton's reaction, and is minimal when SOD facilitates Fenton's reaction by accumulating H_2O_2 from $O_2^{\cdot-}$. Overall, it is rather likely that ADR-induced lipid peroxidation is mediated by iron-oxygen complex(es), as observed by other investigators under different experimental conditions.[24–26] It should also be emphasized that lipid peroxidation is a two step reaction,[22,27,28] in which "initiation" (i.e. formation of lipid hydroperoxides from polyunsaturated fatty acids) is followed by "propagation" (i.e. ferricytochrome P-450- or Fe^{2+}-catalyzed cleavage of lipid hydroperoxides to highly reactive lipid alkoxyl radicals). Therefore, the addition of catalase would shift the released Fe^{2+} from reaction with H_2O_2 to reaction with lipid hydroperoxides, ultimately favouring propagation and formation of MDA.

Irrespective of their precise mechanism(s), both iron release and lipid peroxidation can be prevented by inhibiting the reductase with p-HMB (cf. Table III and Table IV). This indicates that electron transport and $ADR^{\cdot-}$ formation remain absolute prerequisites for the generation of iron reductant(s) and lipid oxidant(s).

Tumour and myocardial cells are characterized by low content of SOD and catalase,[29,30] and this has been invoked as a reason for their unique sensitivity to an $O_2^{\cdot-}$ and H_2O_2– generating drug like ADR.[30] The present study provides evidence that the combination of SOD with catalase not only fails to prevent microsomal iron mobilization but also paradoxically exaggerates subsequent iron-catalyzed lipid peroxi-

dation. Thus, the regulation of membrane iron content and mobilization may overshadow the availability of SOD and catalase as a factor of ADR-sensitivity. Work is in progress to validate this hypothesis.

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