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Thiol Oxidation Induced by Oxidative Action of Adriamycin

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To clarify the mechanism of the cardiotoxic action of adriamycin (ADM), the participation of free radicals from ADM in cardiotoxicity was investigated through the protective action of glutathione (GSH) or by using electron spin resonance (ESR). Oxidation of ADM by horseradish peroxidase and H_2O_2 (HRP–H_2O_2) was blocked by GSH concentration dependently. Inactivation of creatine kinase (CK) induced during interaction of ADM with HRP–H_2O_2 was also protected by GSH. Other anthracycline antitumor drugs that have a *p*-hydroquinone structure in the B ring also inactivated CK, and GSH inhibited the inactivation of CK. These results suggest that ADM was activated through oxidation of the *p*-hydroquinone in the B ring by HRP–H_2O_2. Although ESR signals of the oxidative ADM B ring semiquinone were not detected, glutathionyl radicals were formed during the interaction of ADM with HRP–H_2O_2 in the presence of GSH. ADM may be oxidized to the ADM B ring semiquinone and then reacts with the SH group. However, ESR signals of ADM C ring semiquinone, which was reductively formed by xanthine oxidase (XO) and hypoxanthine (HX) under anaerobic conditions, were not diminished by GSH, but they completely disappeared with ferric ion. These results indicate that oxidative ADM B ring semiquinones oxidized the SH group in CK, but reductive ADM C ring semiquinone radicals may participate in the oxidation of lipids or DNA and not of the SH group.

Keywords: Adriamycin; Anthracycline; Glutathione; Horseradish peroxidase; Semiquinone; Quinone

INTRODUCTION

Among anthracycline antitumor drugs, adriamycin (ADM) especially is toxic to cardiac tissue.[1–3] The clinical use of ADM has been limited because it causes cumulative and dose-dependent cardiotoxicity.[4,5] Although several modes of action have been proposed, the exact molecular mechanisms of the biological activities of anthracycline drugs are not well understood. ADM is reduced to a semiquinone radical by microsomal nicotinamide-adenine dinucleotide phosphate (NADPH)-P450 reductase and mitochondrial NADH dehydrogenase.[6–9] In the presence of oxygen, the reductive semiquinone radical species produces superoxide and hydroxyl radicals, which cause peroxidation of unsaturated membrane lipids.[6–9] These events lead to the disturbance of membrane structure and dysfunction of mitochondria in cardiac cells.

We previously showed[10] that creatine kinase (CK) and succinate dehydrogenase, which are distributed abundantly in the mitochondria of cardiac cells and are associated with the physiological role of ATP generation in conjunction with the contractile and transport system,[11] are strongly inactivated by anthracycline drugs, including ADM oxidized by horseradish peroxidase (HRP) in the presence of H_2O_2 (HRP–H_2O_2). Reduced glutathione (GSH) levels in tissues decrease with the administration of ADM.[12,13] ADM with HRP–H_2O_2 decreases sulfhydryl (SH) groups in rabbit muscle CK.[14] Thiol compounds, including GSH, reduce cardiotoxicity induced by ADM.[15,16] These studies suggest that cardiac toxicity by anthracycline drugs is due to oxidation of SH groups in tissue. However, whether reductive free radicals or oxidative activation of ADM has a crucial role in cardiac toxicity remains unclear. In this study, we show that ADM activated oxidatively, but not reductive semiquinone radicals from ADM, reacts with GSH to produce glutathionyl radicals and that GSH strongly blocks inactivation of CK.

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MATERIALS AND METHODS

Materials

ADM was obtained from Kyowa Hakko Co. Ltd., Tokyo, Japan; HRP and GSH were obtained from Wako Pure Chemical Industries, Co. Ltd., Osaka, Japan; epirubicin and idarubicin were obtained from Pharmacia Japan; pirarubicin was obtained from Meijiseika Co. Ltd.; aclarubicin was obtained from Yamanouchi Pharmaceutical Co. Ltd.; daunorubicin and CK (rabbit muscle) were obtained from Sigma Chemical Co., St Louis, MO, USA. 5,5-dimethyl-1-pyrroline-1-oxide (DMPO; ultra pure grade) was obtained from Mitsui Toatu Co. Ltd. Xanthine oxidase (XO; butter milk) was obtained from biozyme Laboratories Ltd., Gwent, UK. Other chemicals were highly analytical grade products obtained from commercial suppliers.

Measurement of Enzyme Activities

CK activity was measured at 30°C by using a creatine kinase kit from Wako Pure Chemical Industries. The CK kit consisted of 22 mM creatine phosphate, 0.89 mM ADP, 1 mM $MgCl_2$, hexokinase (1.1 U/ml), 0.72 mM $NADP^+$ and glucose-6-phosphate dehydrogenase (1.1 U/ml) in 80 mM *Tris* buffer at pH 6.8. CK produces ATP from creatine phosphate and ADP, hexokinase produces glucose-6-phosphate from ATP and glucose, and glucose-6-phosphate dehydrogenase produces NADPH from glucose-6-phosphate and $NADP^+$. The activity of CK was measured by the amount of NADPH formed at 340 nm. The activity of HRP was measured by using the method of Das and Banerjee.[17] The reaction mixture contained 0.27 mM H_2O_2, 1.7 mM KI and HRP in 50 mM acetate buffer at pH 5.0. After incubating for 5 min at 37°C, the absorbance was measured at 375 nm. Protein was measured by using the bicinchoninic acid method.[18]

Measurement of the SH Group

The number of SH groups was measured by using 5,5′-dithiobis-(2-aminobenzoic acid) (DTNB).[19] The reaction mixture contained CK (0.5 mg/ml), 10 μM of ADM, 1.2 μM of HRP and 100 μM of H_2O_2 in 50 mM Hepes buffer at pH 7.4. The reaction was stopped by adding trichloroacetic acid (10.0%) at various time points and then the sample was centrifuged for 10 min at 3,000 rpm. The precipitate was dissolved in 1% sodium dodecylsulfate and then 1.0 mM DTNB was added. After the mixture was incubated for 30 min, the absorbance at 412 nm was measured.

Electron Spin Resonance (ESR) Measurement

ESR signals of glutathionyl radicals were detected in 50 mM Hepes buffer at pH 7.4 containing ADM (0.5 mM), 100 μM H_2O_2, 0.75 μM HRP, 5.0 mM GSH and 100 mM DMPO. The ESR signal of the ADM C ring semiquinone radical was measured in 50 mM Hepes containing hypoxanthine (HX; 0.1 mM), XO (0.05 U/ml), ADM (1.0 mM), 5.0 mM glucose and 20 U/ml of glucose oxidase. For anaerobic experiments, the reaction mixture was bubbled with nitrogen gas for 10 min. The ESR settings of these radicals were: microwave power, 10 mM; modulation frequency, 100 kHz; modulation field, 0.1 G; receiver gain, 2,000 and time constant, 0.3 s.

RESULTS

Inhibition of ADM Oxidation by GSH

We previously showed[10] that diphenolic hydroxyl groups of the ADM B ring are rapidly oxidized with decolorization by HRP–H_2O_2. Figure 1 shows the structure of ADM. Figure 2 shows the inhibitory effect of GSH on the decolorization of ADM. The red color of ADM was almost lost during the incubation with HRP–H_2O_2 for about 5 min. When GSH was added to the reaction mixture, the decolorization was delayed, the periods of which depended on the concentrations of GSH. At 50, 100 and 150 μM of GSH, the lag times were about 50, 100 and 150 s, respectively. Evidently, GSH reacted with ADM activated by HRP–H_2O_2 and decolorization of ADM have been caused after oxidation of GSH in the reaction system.

Oxidation of SH Groups in CK

Previously, we indicated[14] that ADM activated by HRP–H_2O_2 inactivates SH enzymes, including CK and succinate dehydrogenase, suggesting that SH enzymes are very sensible to ADM. Figure 3 shows

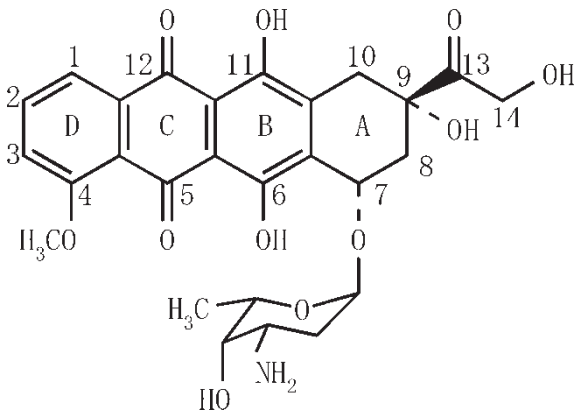

FIGURE 1 Structure of ADM.

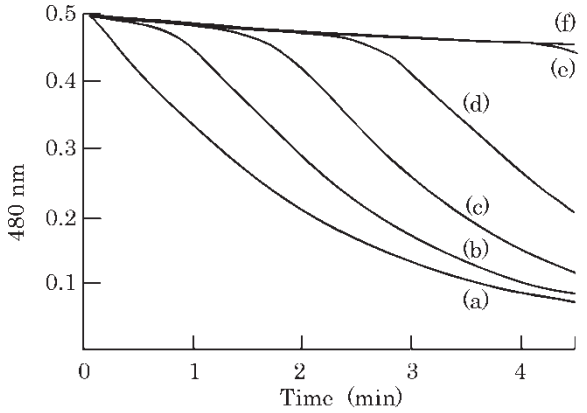

FIGURE 2 Inhibition of red color loss of ADM by GSH. The reaction mixture contained 50 μM ADM, 0.75 μM HRP and 100 μM H_2O_2 in 50 mM Hepes buffer pH 7.4. Various concentrations of GSH were added to the reaction mixture. Oxidation of ADM was continuously monitored by measuring the absorbance at 480 nm. The results of one of the three experiments that varied less than 10% are shown. (a) 0; (b) 50; (c) 100; (d) 150; (e) 200 and (f) 500 μM GSH, respectively.

a relationship between loss of SH groups and inactivation of CK. When CK was incubated with ADM in the presence of HRP–H_2O_2, the activity of CK rapidly decreased with loss of SH groups. After incubation for 10 min, about 60% of the enzyme activity and 40% of the SH groups in the CK were lost. After incubation for 30 min, about 90% of the enzyme activity and 50% of SH groups in CK were lost. The enzyme activity and the SH groups were not lost when CK was incubated with ADM in the absence of HRP–H_2O_2 (data not shown).

Figure 4 shows that the inactivation of CK induced by ADM with HRP–H_2O_2 was blocked by GSH. Protection by GSH of the enzyme activity depended

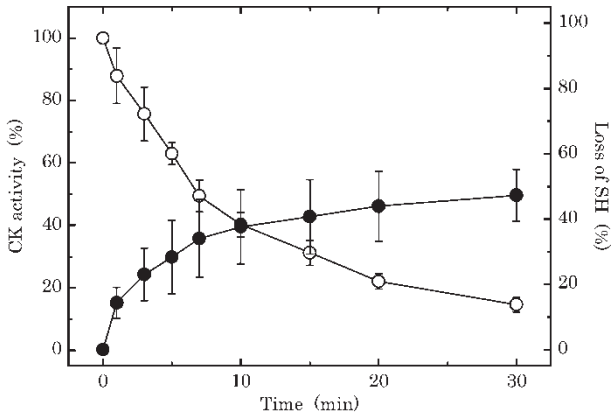

FIGURE 3 Inactivation and SH loss of CK induced by ADM with HRP–H_2O_2. CK (0.5 mg/ml) was incubated with 10 μM of ADM in the presence of HRP (1.2 μM) and 100 μM of H_2O_2. After the incubation at 37°C, SH and enzyme activity were measured as described in "Materials and methods" section. CK activity and SH groups are expressed as percentages of the control that corresponds to 3.8 U/ml enzyme activity and 30.2 nmol/mg protein of SH groups at zero time, respectively. Each point represents the mean ± SD of five experiments. (○), enzyme activity and (•), SH groups.

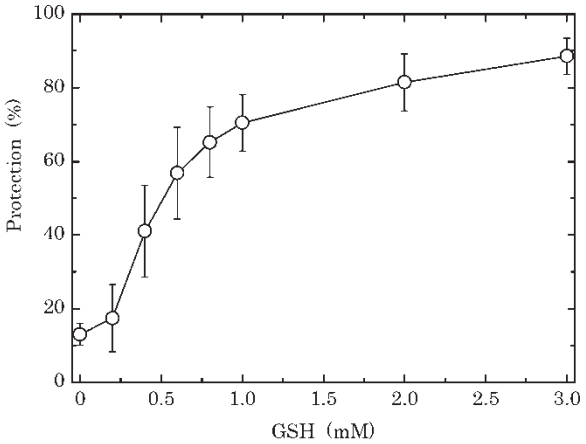

FIGURE 4 Inhibition of ADM-induced CK inactivation by GSH. Conditions were the same as described in Fig. 2 except for adding 0.1 mg/ml of CK and various concentrations of GSH. After incubation for 30 min, the activity of the enzyme was measured. Each point represents the mean ± SD of 5 experiments.

on the concentrations. Blocking of the inactivation of CK was 90% at 3.0 mM GSH.

Formation of Glutathionyl Radicals

Table I shows that inactivation of CK induced by other anthracycline antitumor drugs, including epirubicin, pirarubicin, daunorubicin and aclarubicin with HRP–H_2O_2, were also inhibited by GSH. Except for aclarubicin, anthracycline drugs used in this study have a *p*-hydroquinone structure in the B ring. Hydroquinone is easily oxidized by HRP–H_2O_2.[20] Two phenolic hydroxyl groups of the B ring in ADM should be oxidized through generation of semiquinone radicals of the B ring to quinone or epoxide. We tried to detect ESR signals of the ADM B ring semiquinone radical, but were unsuccessful. The disappearance of the ADM red color and loss of CK activity induced by HRP–H_2O_2 were blocked by GSH, indicating interaction of ADM B ring semiquinone radicals with GSH. If GSH acts as a scavenger of ADM B ring semiquinone, GSH by itself should convert to a glutathionyl radical, which

TABLE I Inhibition of anthracycline-induced CK inactivation by GSH

	CK activity (%)	
Drugs	−GSH	+GSH
Adriamycin	13.3 ± 1.5	79.6 ± 0.6
Epirubicin	9.2 ± 0.3	56.3 ± 3.0
Daunorubicin	30.9 ± 7.2	98.4 ± 1.9
Pirarubicin	44.7 ± 7.8	98.1 ± 1.7
Idarubicin	13.4 ± 1.4	99.8 ± 0.7
Aclarubicin	65.8 ± 5.4	98.9 ± 1.7

Conditions were the same as described in Fig. 3 except for adding anthracycline drugs with or without GSH (2.0 mM). After incubation for 30 min, the enzyme activities were measured. Each value represents the mean ± SD of five experiments.

has been detected by using ESR together with DMPO as a spin trap agent.[21] We used this technique to investigate the formation of ADM B ring semiquinone radicals.

Figure 5 shows ESR signals ($a_N = 15.0$ G, $a_H = 16.3$ G) that indicate the formation of glutathionyl radicals during the interaction of ADM with HRP–H_2O_2 in the presence of GSH and DMPO. ESR signals were consistent with those of the DMPO-glutathionyl radical adduct. The formation of glutathionyl radicals was extremely accelerated during the interaction of ADM with HRP–H_2O_2 (Fig. 5a). Weak signals were detected in the absence of ADM (Fig. 5e) and H_2O_2 (Fig. 5c). No signals were detected in the absence of GSH (Fig. 5b) and HRP (Fig. 5d). These results suggest that semiquinone radicals of the oxidative ADM B ring are produced and cause loss of SH groups in CK during the interaction with HRP–H_2O_2.

Formation of Reductive ADM C Ring Semiquinone Radicals

Mitochondrial NADH dehydrogenase, microsomal P450 reductase and XO can reduce ADM to the ADM C ring semiquinone radical that immediately reacts with oxygen to form the superoxide.[6–9,22] Figure 6 shows ESR signals of the ADM C ring semiquinone radical produced by XO with HX (XO–HX).

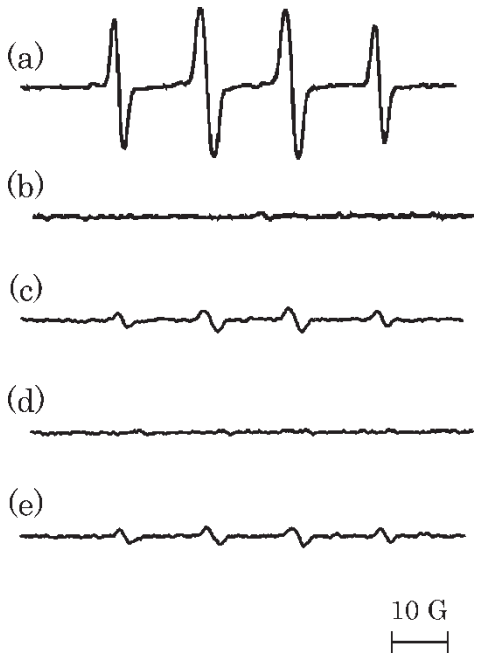

FIGURE 5 ESR signals indicating the formation of glutathionyl radical during interaction of ADM with HRP–H_2O_2 in the presence of GSH. The reaction mixture contained 0.5 mM ADM, 0.75 μM HRP, 100 μM H_2O_2, 5 mM GSH and 50 mM DMPO in 50 mM Hepes buffer pH 7.4. ESR signals were recorded as described in the "Materials and methods" section. a, complete reaction mixture; b, without GSH; c, without H_2O_2; d, without HRP and e, without ADM.

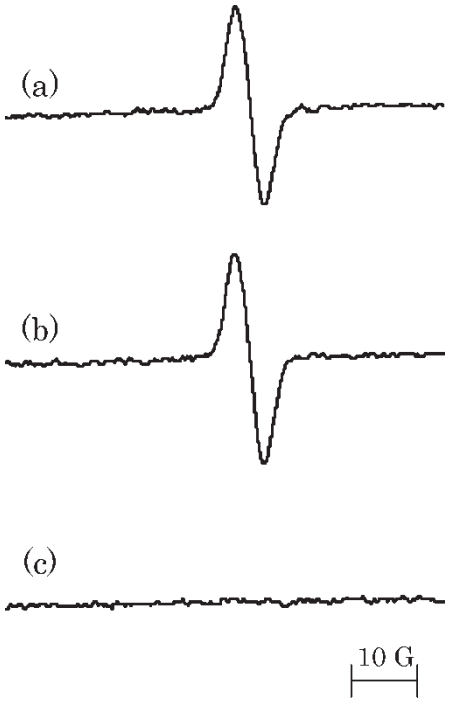

FIGURE 6 ESR spectrum of ADM C ring semiquinone radicals formed from interaction of ADM with XO–HX. The reaction mixture contained 1.0 mM ADM, 0.1 mM HX, 0.05 U/ml of XO, 5 mM glucose and 20 U/ml of glucose oxidase in 0.1 M *tris*–HCl buffer pH 7.4. a, complete reaction mixture; b, a with GSH (5 mM) and c, a with Fe^{3+} (50 μM).

When ADM was reduced by XO–HX under anaerobic conditions, ESR signals with *g* value 2.0038 were detected. These ESR signals were consistent with those of the ADM C ring semiquinone as previously reported.[22] Of interest, GSH had no effect on the ADM C ring semiquinone radical. In contrast, adding ferric ion completely diminished the ESR signals of the ADM C ring semiquinone radical, indicating that ferric ion is readily reduced by the ADM C ring semiquinone radical.

DISCUSSION

This study showed that GSH blocks oxidation of ADM to protect CK from attack by ADM activated by HRP–H_2O_2. Figure 7 shows the redox of ADM. When GSH was in the reaction system that included ADM and HRP–H_2O_2, ADM decolorized after lag times. The periods of the lag time depended on the concentrations of GSH, indicating that oxidation of ADM proceeds after GSH is consumed in the reaction system. Administration of ADM severely decreases GSH in tissues.[12] We previously showed[10] that mitochondrial CK and succinate dehydrogenase, which are typical SH enzymes, are strongly inactivated by anthracycline antitumor drugs activated by HRP–H_2O_2. Administration of SH agents attenuates the cardiotoxicity by

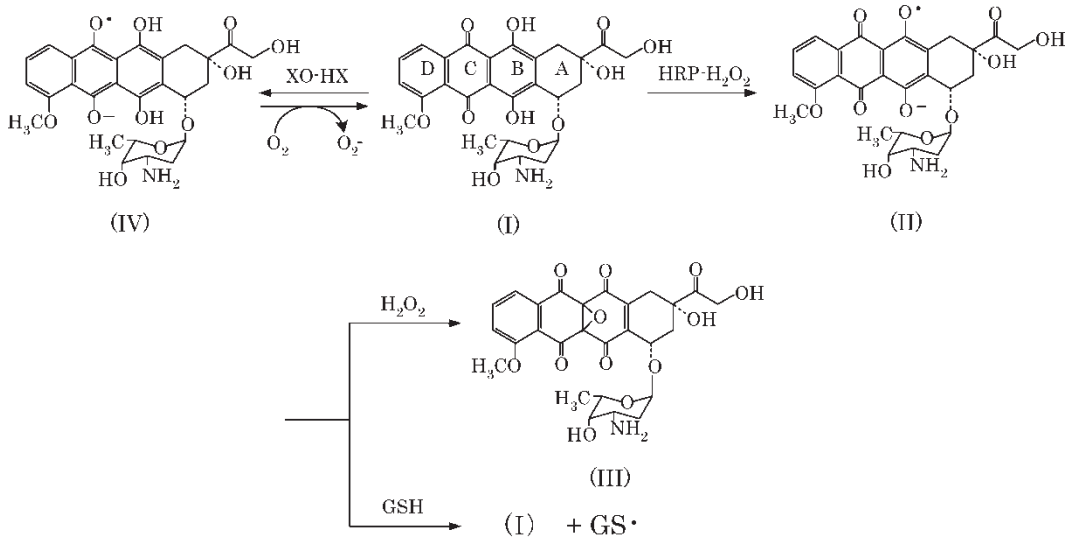

FIGURE 7 Redox of ADM.

ADM.[13,14] These findings indicate that the decrease in SH groups contribute to cardiotoxicity induced by anthracycline anticancer drugs.

Peroxidase oxidizes various compounds by an one-electron transfer to form free radicals.[23–27] We failed to detect directly oxidative ADM B ring semiquinone radicals during interaction of ADM with HRP–H_2O_2. In the presence of GSH, however, we observed formation of ESR signals of the DMPO-glutathionyl radical adduct during interaction of ADM with HRP–H_2O_2. Evidently glutathionyl radicals were produced through interaction of GSH with ADM, oxidatively activated by HRP. The ADM B ring semiquinone seems to be too unstable to be detected by ESR. The red colored solution of ADM was rapidly decolorized by HRP–H_2O_2, suggesting that ADM B ring semiquinone may be further oxidized to epoxide of ADM that did not produce ESR signals (Fig. 7).

ADM is reduced to an ADM C ring semiquinone by various biological systems.[6–9,22] The redox cycling of the reductive ADM C ring semiquinone radical forms the superoxide, H_2O_2 and hydroxyl radicals and lipid peroxidation is caused by these reactive oxygen species themselves or through reduction of iron by these reactive oxygen species.[28–33] The ADM C ring semiquinone radicals formed by ADM with XO–HX were not affected by GSH but was strongly diminished by ferric ion. SH enzymes, including CK and alcohol dehydrogenase, were little inhibited by ADM activated with XO–HX under anaerobic conditions (data not shown). These results indicate that the B ring, but not the C ring ADM semiquinone participated in oxidation of the SH group.

Cardiotoxic action of anthracycline drugs seems to relate to the oxidation of hydroquinone structure. Except for aclarubicin, anthracycline drugs used in this study were steadily metabolized and the red color of the drugs disappeared during interaction with HRP–H_2O_2. Aclarubicin, which has a phenolic hydroxyl group in the B ring, weakly inhibited CK compared with other anthracycline drugs in this study.[34] The yellow color of aclarubicin was constant during the interaction with HRP–H_2O_2.

Ca^{2+}-ATPase of cardiac microsomes is inhibited by ADM independent of lipid peroxidation, and GSH strongly prevents inactivation of Ca^{2+}-ATPase;[35] reductive ADM semiquinone radicals may participate in the inactivation of Ca^{2+}-ATPase.[35] We previously showed[30] that GSH protects mitochondrial CK, but does not inhibit lipid peroxidation induced by ADM-Fe^{3+}. In this case, the SH groups in CK might be oxidized by lipid peroxides produced from the reaction system, not by the ADM C ring semiquinone, because strong antioxidant butylated hydroxytoluene inhibits both lipid peroxidation and inactivation of mitochondrial CK. Ferric ion completely diminished the ESR signals of ADM C ring semiquinone produced from XO–HX. However, ferrous ion did not affect the ESR signal of ADM C ring semiquinone (data not shown). These findings suggest that ADM C ring semiquinone contributes to lipid peroxidation through reduction of ferric ion. In contrast, ADM B ring semiquinone should participate in oxidation of SH groups in proteins to contribute to cardiotoxicity. ADM may cause cardiotoxicity through formation of reductive and oxidative ADM semiquinone radicals.

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