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Inactivation of Creatine Kinase by Adriamycin® during Interaction with Horseradish Peroxidase

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ABSTRACT. Oxidative damage of creatine kinase (CK) induced by Adriamycin® (ADM) with peroxidase was investigated using horseradish peroxidase (HRP). ADM oxidatively inactivated CK during its interaction with HRP in the presence of H_2O_2 (HRP–H_2O_2). The red color of ADM was lost during oxidation by HRP–H_2O_2. Adding catalase stopped the color change of ADM induced by HRP–H_2O_2, indicating that ADM was oxidized by HRP complex I or II. CK was inactivated readily, even when it was added to the reaction mixture containing colorless ADM. Some sulfhydryl groups of CK, which have an important role in its enzyme activity, were very sensitive to ADM activated by HRP–H_2O_2, suggesting that inactivation of CK is due to oxidation of SH groups at the active center. Presumably, oxidative ADM quinone is involved dominantly in the inactivation of CK. Among the anthracycline drugs tested in this study, only ADM and epirubicin caused inactivation of CK and alcohol dehydrogenase and loss of the red color during oxidation by HRP–H_2O_2. BIOCHEM PHARMACOL **60**;1: 95–99, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. Adriamycin; anthracycline drugs; creatine kinase; horseradish peroxidase; quinone

Anthracycline antibiotics, which include ADM,† are widely used to treat various solid and hematologic malignancies [1, 2], but their clinical use has been limited because they cause cumulative and dose-dependent cardiotoxicity [3]. The cytotoxic action of ADM has been explained by intercalation into DNA, which results in inhibition of replication, transcription, and ultimately translation [4], and generation of free radicals [4–6]. Intercalation dominantly accounts for the antitumor action of the drug, and free radicals that are produced by reductive activation of the drug by cytochrome P450 reductase account for the related cardiotoxicity. The mechanism of cytotoxicity mediated by free radicals involves one-electron reduction of the C ring of the quinone moiety of these drugs to a semiquinone radical. This semiquinone radical reacts with molecular oxygen to initiate a cascade of reactions resulting in the generation of a variety of reactive oxygen-derived species, such as superoxide, H_2O_2, and hydroxyl radicals. These species have been implicated in the damage of cellular proteins, nucleic acids, and cell membrane components.

Kolodziejczyk *et al.* [7] showed that the quinone-modified anthracycline drug 5-iminodaunorubicin is readily activated oxidatively by HRP. In addition, Inchiosa and Smith [8] showed that ibuprofen, a nonsteroidal anti-inflammatory drug, ameliorates the toxicity of ADM, and proposed that the drug inhibits neutrophil infiltration. Neutrophils contain a large amount of myeloperoxidase. These findings suggest that peroxidases are involved in ADM metabolism. However, the potential for oxidative activation of ADM by peroxidase is not recognized in the metabolism of ADM. However, CK is distributed abundantly in the heart and is associated with the physiological role of ATP generation in conjunction with the contractile or transport system [9]. In this study, we investigated the enzymatic oxidative activation of ADM using HRP–H_2O_2, and we showed that CK is inactivated readily by oxidatively activated ADM.

MATERIALS AND METHODS

Chemicals

ADM was obtained from the Kyowa Hakko Co. Ltd., and HRP from Wako Pure Chemical Industries, Co. Ltd. Epirubicin (4-epi-adriamycin) and idarubicin were purchased from Pharmacia Japan; pirarubicin was obtained from Meijiseika Co. Ltd.; and daunorubicin, CK (rabbit muscle), catalase (beef liver), and superoxide dismutase (bovine erythrocyte) were purchased from the Sigma Chemical Co. ADH (yeast) was obtained from Oriental Yeast Co. Ltd. Other chemicals were high analytical grade products obtained from commercial suppliers.

Determination of Enzyme Activities

CK activity was measured at 30° using a Wako Pure Chemical Industries CK kit. One unit of CK transfers 1.0

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† *Abbreviations*: ADH, alcohol dehydrogenase; ADM, Adriamycin®; CK, creatine kinase; DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid); HRP, horseradish peroxidase; HRP–H_2O_2, horseradish peroxidase in the presence of H_2O_2; and SH, sulfhydryl.

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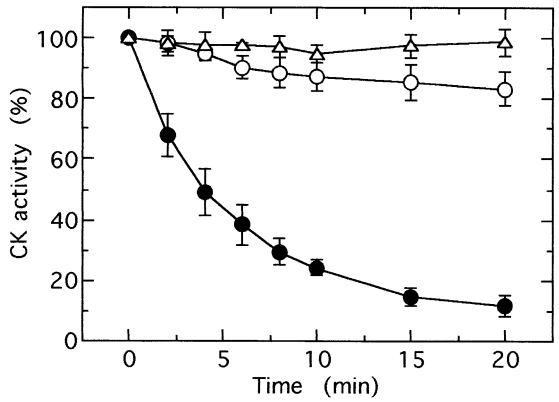

FIG. 1. Inactivation of rabbit muscle CK induced by ADM with HRP–H_2O_2. The reaction mixture contained CK (1.23 μM), HRP (1.25 μM), and H_2O_2 (200 μM) in 10 mM HEPES buffer containing 0.15 M NaCl at pH 7.4. The reaction was started by adding ADM (10.0 μM). After incubating at 37° for 20 min, an aliquot of the reaction mixture was removed, and then the activity of CK was determined as described in Materials and Methods. CK activity is expressed as a percentage of the control, which corresponds to 3.8 U/mL of the enzyme activity at zero time. Each point represents the mean ± SD of five experiments. Key: (○) ADM not added; (●) ADM added; and (Δ) ADM without HRP–H_2O_2 added.

μmol of phosphate from phosphocreatine to ADP per minute at 30°. The activity of peroxidase was determined as follows. The reaction mixture contained 0.27 mM hydrogen peroxide, 1.7 mM KI, and peroxidase in 10 mM acetate buffer at pH 5.0. After incubating for 5 min at 37°, the absorbance was measured at 375 nm. The ADH activity was determined by the method of Bonnichsen and Brink [10]. The protein was measured by the bicinchoninic acid assay using BSA as a standard [11].

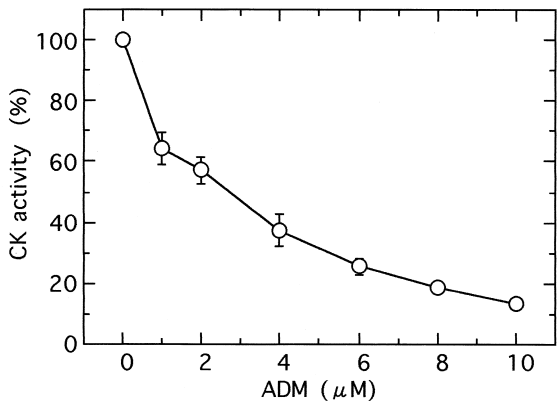

FIG. 2. Effect of ADM concentration on the inactivation of CK. Conditions were the same as described in Fig. 1, except for the concentrations of ADM. After incubating for 15 min, the activity of CK was measured. CK activity is expressed as a percentage of the control, which corresponds to 3.0 U/mL of enzyme activity. Each point represents the mean ± SD of five experiments.

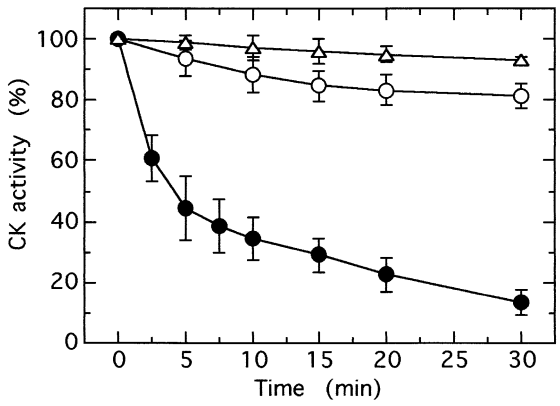

FIG. 3. Inhibition of CK activity of heart homogenate induced by ADM with HRP–H_2O_2. Heart homogenate (0.5 mg protein/mL) was suspended in 10 mM HEPES buffer containing 0.15 M NaCl at pH 7.4. Other conditions were the same as described for Fig. 1. CK activity is expressed as a percentage of the control that corresponds to 1.8 U/mL of enzyme activity. Each point represents the mean ± SD of five experiments. Key: (○) ADM not added; (●) ADM added; and (△) ADM without HRP–H_2O_2 added.

Preparation of Homogenate

Heart homogenates were prepared from male Wistar strain rats, weighing about 200 g, as follows: hearts were minced and homogenized in 10 mM HEPES buffer containing 0.15 M NaCl at pH 7.4. The homogenate (10%) was dialyzed against the same buffer overnight at 4°. The specific activity of CK in homogenate was 72 U/mg protein.

Determination of SH Group

The SH groups were measured using DTNB. The reduction of DTNB was calculated using the value of $\epsilon_{412} = 1.36 \times 10^4\ M^{-1}\ cm^{-1}$ [12].

RESULTS

Inactivation of CK

Figure 1 shows that ADM inactivated rabbit muscle CK

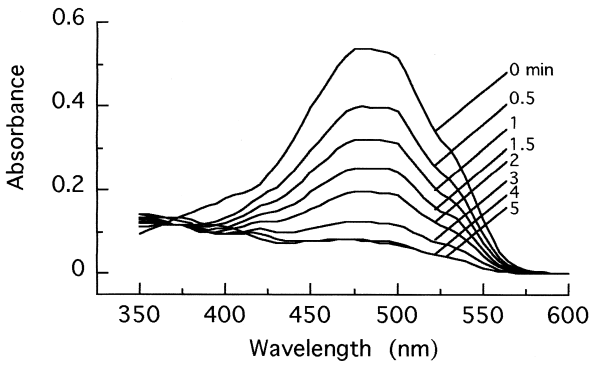

FIG. 4. Spectral change of ADM induced by HRP–H_2O_2. Conditions were the same as described for Fig. 1, except for the concentration of ADM (50.0 μM). Numbers in the figure refer to the incubation time (min). After incubating, the spectrum of ADM was recorded.

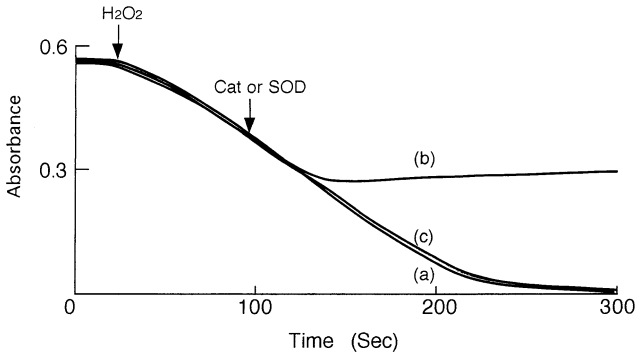

FIG. 5. Effect of catalase and superoxide dismutase on oxidation of ADM. The reaction mixture contained HRP (1.25 μM) in 10 mM HEPES buffer containing 0.15 M NaCl at pH 7.4. The reaction was started by adding H_2O_2 (200 μM, curve a). Catalase (Cat) (0.02 μM, curve b) or superoxide dismutase (SOD) (0.3 μM, curve c) was added to the reaction mixture at the arrow.

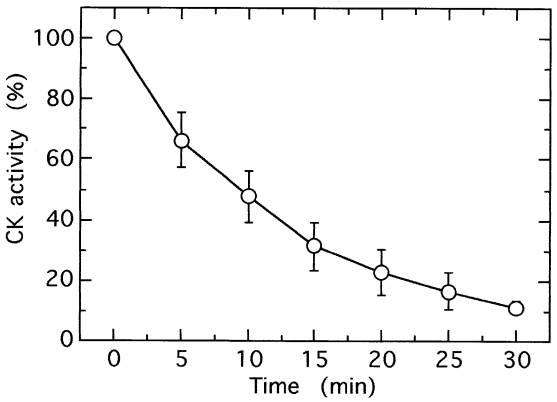

FIG. 6. CK inactivation induced by a colorless compound produced from ADM. Conditions were the same as described for Fig. 1, except for CK. After incubating for 5 min, catalase (0.02 μM) was added to the reaction mixture, and then CK (1.23 μM) was added and the mixture was incubated further. CK activity is expressed as a percentage of the control, which corresponds to 3.8 U/mL of enzyme activity. Each point represents the mean ± SD of five experiments.

during interaction with HRP–H_2O_2. After incubating for 20 min, about 90% of the enzyme activity was lost. In the absence of ADM, CK activity was decreased very slightly by HRP–H_2O_2. ADM without HRP–H_2O_2 had no effect on the enzyme activity. Figure 2 shows that the inactivation of CK depended on the concentration of ADM up to 10 μM. The IC_{50} of ADM was about 3.0 μM. Figure 3 shows that CK activity in heart homogenate was also almost lost oxidatively by ADM with HRP–H_2O_2. In the absence of ADM, the CK activity in the homogenate decreased about 20%. However, when ADM was incubated with homogenate in the absence of HRP–H_2O_2, CK activity in the homogenate was not affected. These results indicated that CK activity was lost during the interaction of ADM with HRP–H_2O_2.

Spectral Change of ADM

The oxidation of ADM by HRP–H_2O_2 was accompanied by a marked change from red color to the colorless state. Figure 4 shows the spectral change in ADM during the interaction with HRP–H_2O_2. The absorption around 480 nm decreased rapidly, and after 4 min a complete loss of the red color of ADM occurred. Under anaerobic conditions, CK inactivation and color disappearance also occurred, indicating no involvement of oxygen in the inactivation of CK and conversion of ADM to the colorless compound (data not shown). These results indicate that CK was inactivated by ADM that had been activated oxidatively by HRP–H_2O_2. Figure 5 shows that adding catalase stopped the decrease in absorbance at 480 nm. In contrast, superoxide dismutase did not affect the color change. These results indicated that catalase decomposed H_2O_2 to interfere with the formation of HRP complex I or II and subsequently blocked oxidation of ADM. Peroxidases oxidize various organic compounds by a one-electron transfer [13–16], suggesting that an oxidative ADM semiquinone radical forms during the interaction with HRP–H_2O_2. However, we failed to detect ESR signals of oxidative ADM semiquinone radicals. Presumably, oxidative ADM semiquinone is too unstable or low in amount to give detectable ESR signals. Of interest, CK was also inactivated when it was added to the reaction system containing colorless ADM (Fig. 6). These results suggested that not only oxidative semiquinone, but also ADM quinone is involved in the CK inactivation.

Oxidation of SH Groups

CK is a typical SH enzyme. Therefore, we tested whether SH groups of CK and BSA also were oxidized by ADM

TABLE 1. Diminution of protein SH groups by ADM–peroxidase

	BSA		CK	
Additions	SH (nmol/mg/protein)	% of Loss	SH (nmol/mg protein)	% of Loss
None	4.62 ± 1.20		30.15 ± 5.09	
HRP–H_2O_2	2.52 ± 1.12	45.7	19.75 ± 1.48	35.6
AD with HRP–H_2O_2	2.57 ± 1.03	44.4	14.85 ± 0.35	50.7

The reaction mixture contained CK (12.3 μM) or BSA (36.2 μM) in 10 mM HEPES buffer containing 0.15 M NaCl at pH 7.4. HRP (1.25 M) and hydrogen peroxide (200 μM) were added to the reaction mixture with or without ADM (50 μM). After incubating for 30 min, the SH groups of the proteins were measured. Each value represents the mean ± SD of five experiments.

TABLE 2. Inactivation of SH enzymes induced by various anthracycline drugs with HRP–H_2O_2

Additions	CK	ADH
	Enzyme activity (%)	
Adriamycin	13.5 ± 2.4	6.6 ± 1.4
Epirubicin	7.8 ± 1.5	3.9 ± 0.9
Idarubicin	71.2 ± 6.3	64.9 ± 5.6
Pirarubicin	70.9 ± 8.4	65.6 ± 4.0
Daunorubicin	71.5 ± 6.6	52.9 ± 6.4

Conditions were as described for Fig. 1. Anthracycline drugs (10.0 μM) were added to the reaction mixture containing CK (1.23 μM) or ADH (6.76 μM). After incubating for 15 min, the activity of the enzymes was measured. CK activity is expressed as the percentage of the control that corresponds to 3.0 U/mL of the enzyme activity. ADH activity is expressed as the percentage of the control that forms 0.12 μmol NADH/mL/min. Each value represents the mean ± SD of five experiments.

activated by HRP–H_2O_2; Table 1 summarizes the results. About 50 and 45% of the SH groups in CK and BSA, respectively, were lost by exposure to ADM with HRP–H_2O_2. However, even when CK and BSA were incubated with HRP–H_2O_2 in the absence of ADM, about 35 and 45% of the SH groups in CK and BSA, respectively, were lost. Furthermore, adding H_2O_2 at 200 μM also caused oxidation of SH groups in CK and BSA to the same extent (data not shown). Evidently, all oxidation of SH groups in BSA was due to H_2O_2. About 35% of the SH groups in CK were also oxidized directly by H_2O_2. However, about 15% of the SH groups, which have an important role in the enzyme activity, were oxidized by ADM activated by HRP–H_2O_2. SH groups at the active center of CK may be very sensitive to oxidative attack by ADM.

CK Inactivation Induced by Other Anthracycline Drugs

We examined whether anthracycline antibiotic drugs, including ADM, daunorubicin, epirubicin, idarubicin, and pirarubicin, cause inactivation of SH enzymes, such as CK and ADH, during interaction with HRP–H_2O_2. Table 2 summarizes our findings that only ADM and epirubicin caused inactivation of SH enzymes by about 90%. The red color of epirubicin was also lost by the interaction with HRP–H_2O_2 (data not shown). However, inhibition of SH enzymes by daunorubicin, idarubicin, and pirarubicin was only about 30–50%. The color of these anthracycline drugs was not changed by HRP–H_2O_2 (data not shown). The color disappearance of the anthracycline drugs was closely related with inactivation of SH enzymes. Lactic acid dehydrogenase, which is a non-SH enzyme, was not inactivated by the anthracycline drugs tested here during the interaction with HRP–H_2O_2 (data not shown).

DISCUSSION

This study showed that CK is inactivated by ADM that has been activated oxidatively by HRP–H_2O_2. ADM undergoes oxidation readily and loses its red color during interaction with HRP–H_2O_2. The decrease in absorbance around 480 nm is closely related to inactivation of CK. A frequently proposed mechanism of quinone toxicity involves the reduction of quinones by various flavoproteins to form semiquinones, which, in turn, reduce oxygen to superoxide and subsequently form other partially reduced oxygen species [4–6, 17, 18]. However, superoxide and hydroxyl radical did not participate in the cause of CK inactivation induced by ADM with HRP–H_2O_2 because superoxide dismutase and mannitol, which are superoxide and hy-

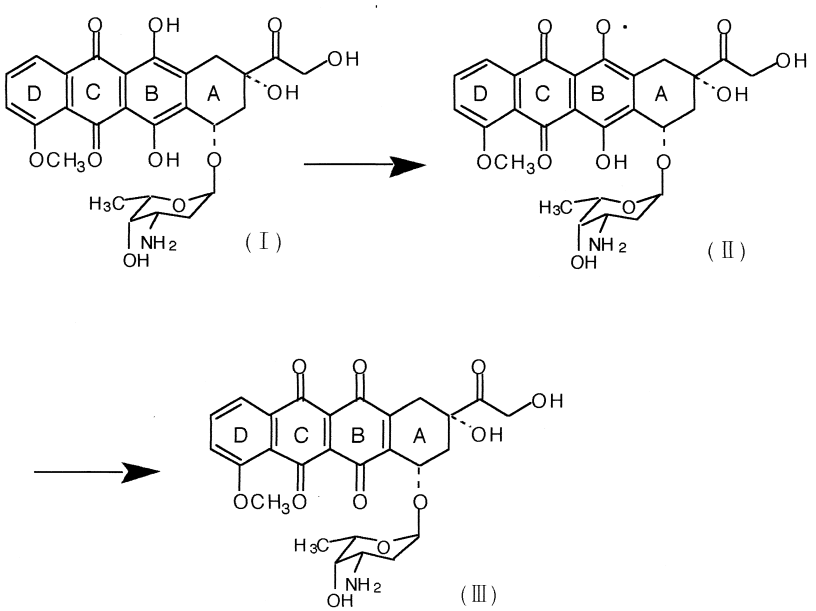

FIG. 7. Scheme showing oxidation of ADM.

droxyl radical scavengers, respectively, gave no protection against CK inactivation (data not shown).

The oxidation of ADM may be analogous to enzymic oxidation of hydroquinone [19, 20]. If ADM is oxidized through a one-electron transfer, it should become an oxidative semiquinone radical (II in Fig. 7). In this study, however, it seems unlikely that oxidative semiquinone of ADM dominantly caused CK inactivation because stable colorless compounds produced from ADM also inactivated CK. Oxidation of hydroquinone by HRP–H_2O_2 produces stable *p*-benzoquinone, which is a strong oxidizing agent and reacts with SH groups [19, 20], suggesting that the colorless compound is ADM quinone (III in Fig. 7). Indeed, SH groups markedly decreased in CK, indicating that ADM quinone oxidatively attacked cysteine residues of CK. *p*-Benzoquinone can be quite toxic in its own right without causing a free radical-based mechanism of toxicity [19, 20]. ADM quinone may be produced through further oxidation of oxidative semiquinone by HRP complex I or II or by interaction with two molecules of semiquinone.

Among the anthracycline drugs we tested, only ADM and epirubicin caused inactivation of CK and loss of red color. Although daunorubicin, idarubicin, and pirarubicin have a hydroquinone structure in the B ring, they did not cause CK inactivation and loss of red color. Evidently, these anthracycline drugs were unsuitable substrates for HRP.

Mammalian tissues contain microsomal enzymes and prostaglandin synthase systems that have hydroperoxidase activity [21, 22]. Involvement of ADM in an alternative oxidative process, quite different from that already established for reductive metabolites, may be important to understand its biological properties. CK is distributed abundantly in the heart and serves as an important energy buffer for muscle concentration by quickly transferring creatine phosphoryl groups to ADP to form ATP [9]. Inhibiting this enzyme activity may cause critical damage to the heart.

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