Adriamycin-Fe$^{3+}$-Induced Inactivation of Rat Heart Mitochondrial Creatine Kinase: Sensitivity to Lipid Peroxidation

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Adriamycin (ADM)-Fe$^{3+}$ caused inactivation of rat heart mitochondrial creatine kinase (CK) with lipid peroxidation. Superoxide dismutase, catalase and hydroxyl radical scavengers were without effect on the CK inactivation and the lipid peroxidation induced by ADM-Fe$^{3+}$, indicating the lack of involvement of superoxide, hydrogen peroxide or hydroxyl radicals in these reactions. The antioxidant butylated hydroxytoluene strongly inhibited not only lipid peroxidation but also CK inactivation, indicating that mitochondrial CK was inactivated with lipid peroxidation. Reduced glutathione and dithiothreitol (DTT) prevented CK inactivation without inhibiting lipid peroxidation. The CK activity of 5,5′-dithiobis-(nitrobenzoic acid)-treated mitochondria exposed to ADM-Fe$^{3+}$ was partially reversed by addition of DTT, indicating that CK inactivation was due to oxidative damage of sulphydryl groups. In contrast, mitochondrial protein SH groups were not attacked via ADM-Fe$^{3+}$-induced lipid peroxidation. Thus, the SH groups in mitochondrial CK are very susceptible to ADM-Fe$^{3+}$-induced lipid peroxidation.

Keywords  adriamycin; creatine kinase; lipid peroxidation; mitochondria

Adriamycin (ADM) is one of the most potent anticancer drugs in clinical medicine. Unfortunately, its use is limited due to cardiotoxicity. It is believed that ADM-induced cardiotoxicity is mediated via lipid peroxidation and DNA damage in the heart. The cardiotoxicity of ADM is related to the oxidative damage of mitochondria. Some mitochondrial enzymes such as cytochrome oxidase and NADH oxidase are inactivated by ADM and the damage is believed to be due to oxygen radicals generated via the interaction of the ADM semiquinone radical with oxygen.

Mitochondrial creatine kinase (CK) exists on the exterior surface of inner mitochondrial membranes and is involved in energy transport and its activity is related to cardiac function. Therefore, the inactivation in mitochondrial CK may lead to disruption of energy utilization and dysfunction of the heart. Indeed, Bittl et al. have shown that mitochondrial creatine kinase activity was reduced in ischemia-reperfused hearts. Recently, it has been reported that mitochondrial creatine kinase is sensitive to superoxide and hydrogen peroxide generated by the xanthine–xanthine oxidase reaction. However, the relationship between CK inactivation and lipid peroxidation remains to be elucidated.

ADM combines with iron to produce ADM–iron complexes, and ADM-induced lipid peroxidation requires iron. We have previously reported that ADM-Fe$^{3+}$ readily causes lipid peroxidation of erythrocyte membranes. In the present study, we have demonstrated that ADM-Fe$^{3+}$ induces mitochondrial CK inactivation with lipid peroxidation.

MATERIALS AND METHODS

Materials  ADM-HCl was obtained from Kyowa Hakko Co., Ltd., Tokyo, Japan. Superoxide dismutase (SOD, from bovine erythrocytes) and catalase (bovine liver) were from Sigma Chemical Co., St. Louis, MO, U.S.A. Butylated hydroxytoluene (BHT), reduced glutathione (GSH), dithiothreitol (DTT) and 5,5′-dithiobis-(nitrobenzoic acid) (DTNB) were obtained from Wako Pure Chemical Industries Ltd., Osaka, Japan. Thiobarbituric acid (TBA) was from Merck Japan Co. Other reagents were of analytical grade from commercial suppliers. ADM-Fe$^{3+}$ was prepared immediately before use by adding FeCl$_3$ to ADM-HCl (molar ratio of ADM: Fe$^{3+}$ = 2:1). SOD activity was determined by the method of McCord and Fridovich. The specific activities of SOD and catalase are 3000 units/mg protein and 5000 Sigma units/mg protein, respectively.

Preparation of Mitochondria  Mitochondria were prepared from Wistar rats weighing approximately 200–250 g as follows: Rat hearts were minced and homogenized in buffer consisting of 0.25 M sucrose, 1.0 mM EDTA and 10 mM Hepes (pH 7.4). The homogenates were filtered through a double layer of gauze and then centrifuged at 1600 × g. Supernatants were centrifuged at 6000 × g to produce a mitochondrial pellet. To remove sucrose, which interferes with TBA-reactive substance (TBARS) formation, the mitochondrial pellet was washed three times with 10 mM Hepes buffer, pH 7.4, containing 0.15 M NaCl before use.

Determination of CK Activities  Activities of CK were measured at 37°C, using creatine kinase kits (Wako Pure Chemical Industries).

Lipid Peroxidation  The reaction mixture contained heart mitochondria (0.1 mg protein), 0.15 M NaCl and ADM-Fe$^{3+}$ (30 µM as ADM) in 1.0 ml of 10 mM Hepes buffer pH 7.4, unless otherwise stated. The formation of TBARS was determined by the method of Buege and Aust with minor modifications. The reaction was terminated by adding 1.0 ml 30% trichloroacetic acid. After centrifugation for 10 min at 1500 × g the precipitate was discarded. TBARS formation was assayed by measuring the absorption at 535 nm after heating for 30 min at 100°C. Absorbance was expressed as nmol TBARS/mg protein, using ε = 156 mm$^{-1}$ cm$^{-1}$. Protein was measured by the bicinchoninic acid method using bovine serum albumin as
a standard.\(^{29}\)

**Determination of Sulphydryl Group Content** Mitochondrial protein thiol contents were determined with DTNB. Mitochondria (0.1 mg protein/ml) were exposed to ADM-Fe\(^{3+}\) (30 \(\mu\)M as ADM) in 10 mM Hepes buffer, pH 7.4, containing 0.15 M NaCl. After exposure, mitochondrial protein was denatured with 30% trichloroacetic acid (TCA) and centrifuged at 1500 \(\times\) g for 10 min. The precipitate obtained was solubilized with 1% sodium dodecyl sulfate (SDS) and then incubated with 0.1 mM DTNB for 20 min at 37°C. Absorbance at 412 nm was read and the sulphydryl (SH) group content was determined using \(e = 13.6 \text{mm}^{-1} \text{cm}^{-1}\).\(^{30}\)

**RESULTS**

**Inactivation of Mitochondrial CK and Lipid Peroxidation** As shown in Fig. 1, mitochondrial CK was readily inactivated by ADM-Fe\(^{3+}\) in a time-dependent manner. After incubation for 2 h, enzyme inactivation reached approximately 60%. Omission of ADM-Fe\(^{3+}\) resulted in only very slight inactivation of CK. Figure 2 shows that lipid peroxidation, measured by TBARS formation, was induced by ADM-Fe\(^{3+}\) in a time dependent manner. NADH did not affect ADM-Fe\(^{3+}\)-induced mitochondrial CK inactivation and lipid peroxidation, although ADM is reduced by mitochondrial NADH dehydrogenase to produce hydroxyl radicals.\(^{16,17}\) As shown in Fig. 3, ADM-Fe\(^{3+}\) caused inactivation of CK and lipid peroxidation in a concentration-dependent manner.

**Effect of Radical Scavengers** The possible involvement of oxygen radicals in CK inactivation and lipid peroxidation was investigated using various scavengers. As summarized in Table I, the inactivation of CK and lipid peroxidation were not substantially blocked by SOD, catalase or mannitol, indicating a lack of involvement of superoxide, hydrogen peroxide or hydroxyl radicals in the reactions. It is interesting to note that vitamin E inhibits neither ADM-Fe\(^{3+}\)-induced CK inactivation nor lipid peroxidation. In contrast, the antioxidant BHT strongly inhibited both lipid peroxidation and CK inactivation, suggesting that CK may be inactivated via the lipid peroxidation reaction.

**Effect of SH Compounds** CK has SH groups at its active site\(^{31}\) and thiol-containing compounds are good scavengers of reactive oxygen species.\(^{32}\) Therefore, we examined whether loss of mitochondrial protein SH groups was caused by ADM-Fe\(^{3+}\). As shown in Fig. 4, no apparent loss of SH groups occurred during the interaction of ADM-Fe\(^{3+}\) and mitochondria. Thus, CK inactivation seems to be independent of mitochondrial protein SH loss.

**Fig. 1. Inactivation of Mitochondrial CK Induced by ADM-Fe\(^{3+}\)**

Mitochondria (0.1 mg protein/ml) were exposed to ADM-Fe\(^{3+}\) (30 \(\mu\)M as ADM) in 10 mM Hepes buffer, pH 7.4, containing 0.15 M NaCl. After exposure at 37°C, CK activity was measured as described in the Materials and Methods section. (C), ADM-Fe\(^{3+}\) exposed mitochondria; ( ), ADM-Fe\(^{3+}\).

**Fig. 2. Lipid Peroxidation of Mitochondria Induced by ADM-Fe\(^{3+}\)**

Conditions were the same as in Fig. 1. (C), absence of NADH; ( ), presence of NADH.

**Fig. 3. Effect of ADM-Fe\(^{3+}\) Concentration on CK Activity and Lipid Peroxidation**

Conditions were the same as in Fig. 1, except for the variations in ADM-Fe\(^{3+}\) concentration. After exposure for 2 h, CK activity and lipid peroxidation were measured. ( ), CK activity; ( ), TBARS formation.

**Table I. Effect of Oxygen Radical Scavengers on ADM-Fe\(^{3+}\)-Induced CK Inactivation and Lipid Peroxidation**

<table>
<thead>
<tr>
<th>Additions</th>
<th>CK (%)</th>
<th>TBARS (nmol/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. None</td>
<td>100</td>
<td>—</td>
</tr>
<tr>
<td>2. 1 + ADM-Fe(^{3+})</td>
<td>49.2 ± 8.0</td>
<td>21.6 ± 5.1</td>
</tr>
<tr>
<td>3. 2 + SOD (100 (\mu)g/ml)</td>
<td>52.1 ± 10.1</td>
<td>21.7 ± 7.4</td>
</tr>
<tr>
<td>4. 2 + Catalase (100 (\mu)g/ml)</td>
<td>51.1 ± 7.2</td>
<td>17.9 ± 3.7</td>
</tr>
<tr>
<td>5. 2 + Mannitol (10 mM)</td>
<td>55.1 ± 8.0</td>
<td>19.3 ± 3.0</td>
</tr>
<tr>
<td>6. 2 + Vitamine E (10 (\mu)M)</td>
<td>55.3 ± 7.9</td>
<td>18.9 ± 2.6</td>
</tr>
<tr>
<td>7. 2 + BHT (1 (\mu)M)</td>
<td>98.5 ± 3.3</td>
<td>29.0 ± 0.7</td>
</tr>
</tbody>
</table>

The reaction mixture contained mitochondria (0.1 mg protein/ml), ADM-Fe\(^{3+}\) (30 \(\mu\)M as ADM) and 0.15 M NaCl in 10 mM Hepes buffer pH 7.4. Various scavengers were added to the reaction mixture before the start of the reaction. Vitamin E and BHT were solubilized in dimethylsulfoxide and suspended in the reaction mixture. After incubation for 1 h, CK activity and TBARS formation were measured as described in the Materials and Methods section. Each value represents the mean ± S.D. of four experiments.
We next investigated whether SH compounds protect against ADM-Fe^{3+}-induced CK inactivation and lipid peroxidation. As summarized in Table II, GSH and DTT evidently prevented CK inactivation but did not protect against ADM-Fe^{3+}-induced lipid peroxidation. However, another SH compound, captopril, an angiotensin-converting enzyme inhibitor and radical scavenger, did not inhibit CK inactivation or lipid peroxidation. Direct reaction with ADM-Fe^{3+} did not cause inactivation of rabbit muscle or heart CK (data not shown). These findings suggest that the SH groups of CK were attacked by lipid-derived radicals. The enzyme activity of CK treated with DTNB is reversed by addition of DTT. By utilizing this property, we investigated the possibility of oxidative damage of the enzyme SH group. If the SH group at the active site of CK is sequestered by DTNB, thionitrobenzoiate (TNB)-bound CK should be protected from subsequent attack by lipid-derived radicals, and activity should be reversed by treatment with DTT. As summarized in Table III, treatment with DTNB caused about 80% inhibition of mitochondrial CK activity which was reversed, giving up to 50% of control activity by addition of DTT. The CK activity of DTNB-treated mitochondria exposed to ADM-Fe^{3+} was stored to approximately 60% of the control value by addition of DTT. These results suggest that the SH groups of CK may be attacked by lipid-derived radicals, which cause CK inactivation.

Yuan et al. demonstrated that mitochondrial CK inactivation induced by superoxide or hydrogen peroxide can be reversed by DTT, indicating that CK inactivation was due to the formation of disulfide bonds. As summarized in Table IV, GSH, DTT and mercaptoethanol could only partially reverse the activity of ADM-Fe^{3+} treated mitochondrial CK, indicating that the formation of disulfide bonds alone does not cause inactivation of mitochondrial CK. Arsenite reduces sulfenic acid to the sulfhydryl form and does not reduce disulfide. Inactivated mitochondrial CK could not be reversed by arsenite, indicating that the enzyme inactivation induced by ADM-Fe^{3+} was not due to oxidation of sulfhydryls to sulfenic acid.

**DISCUSSION**

The present study demonstrated that rat heart mitochondrial CK was inactivated by ADM-Fe^{3+} with lipid peroxidation. The ADM-Fe^{3+}-induced CK inactivation was due to oxidative modification of SH groups. Robinson et al. observed that chronic administration of ADM causes depression of myocardial CK without TBARS formation. The lack of change in heart levels of TBARS casts doubt on a free radical mechanism as the cause of ADM-induced cardiotoxicity. Since TBARS are water soluble, TBARS produced via ADM-induced lipid peroxidation in vivo may be excreted into the urine. Malondialdehyde, one of the TBARS, did not inhibit
mitochondrial CK activity (data not shown). Conversely, a chain-breaking antioxidant, BHT, strongly inhibited not only lipid peroxidation but also CK inactivation. These findings suggest that lipid-derived radicals, alkoxyl or peroxyl radicals, but not TBARS, contribute to CK inactivation. GSH and DTT protect against CK inhibition but do not inhibit lipid peroxidation. Generally, SH compounds such as GSH or cysteine cannot prevent the peroxidation reaction, indicating that alkoxyl or peroxy radicals, which play an important role in the propagation stage of the peroxidation reaction, exhibit only poor reactivity with these SH compounds. Therefore, it is unlikely that the protective effect of GSH and DTT on CK inactivation was due to scavenging of alkoxyl or peroxy radicals. Captopril, an oxygen radical scavenger, could not inhibit either lipid peroxidation or CK inactivation. It has also been reported that captopril cannot inhibit iron-catalyzed lipid peroxidation, indicating that captopril does not react with alkoxyl and peroxy radicals. However, it is evident that CK inactivation is due to oxidative damage of SH groups because TNB-bound CK enzyme activity was reversed by treatment with DTT, even with lipid peroxidation induced by ADM-Fe\(^{3+}\). Evidently, the SH groups of the enzyme escape attack by alkoxyl or peroxy radicals by binding to DTNB. It is likely that the differences in the effects of different SH compounds on CK protection are due to differences in affinity for the SH groups of CK, i.e., DTT and GSH may easily reach the SH groups of CK to protect it against alkoxyl or peroxy radical attack while captopril cannot.

The SH groups of CK were very susceptible to ADM-Fe\(^{3+}\)-induced lipid peroxidation. However, mitochondrial protein SH groups were not susceptible because no apparent loss of SH groups occurred during ADM-Fe\(^{3+}\)-induced lipid peroxidation. The SH groups of CK exist in a partially ionized state, suggesting that the SH groups of mitochondrial CK may interact with lipid-derived radicals as either hydrogen atom or electron donors as follows:

\[
\begin{align*}
\text{Enz-SH} + R^+ & \rightarrow \text{Enz-S}^+ + RH \quad (1) \\
\text{Enz-S}^- + ROO^- & \rightarrow \text{Enz-S}^+ + ROO^- \quad (2)
\end{align*}
\]

The unusually high susceptibility of CK to peroxidation can be explained by a partial oxidation of its SH groups to disulfide. In contrast, Vile and Winterbourn demonstrated that ADM induces SH loss and inactivation of sarcoplasmic Ca\(^{2+}\)-ATPase with NADPH-dependent microsomal lipid peroxidation. The microsomal SH loss is inhibited by catalase, indicating that H\(_2\)O\(_2\) is involved in the SH loss. Furthermore, BHT inhibits the ADM-induced lipid peroxidation of micromoles but does not inhibit the SH loss and the inactivation of Ca\(^{2+}\)-ATPase. These findings indicate that ADM induces Ca\(^{2+}\)-ATPase inhibition and SH loss independent of lipid peroxidation.

Heart CK serves as an important energy buffer for muscle contraction by quickly transferring creatine phosphoryl groups to ADP to form ATP. The inhibition of this enzyme activity may be one of the mechanisms by which ADM damages cardiac function.

REFERENCES