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THIOL OXIDATION AND INHIBITION OF Ca-ATPase BY ADRIAMYCIN IN RABBIT HEART MICROSOMES

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Abstract—Incubation of rabbit heart microsomes with Adriamycin® and NADPH resulted in the oxidation of approximately 25% of protein thiols and 66% inhibition of Ca-ATPase activity. Thiol oxidation and Ca-ATPase inactivation were iron-dependent and could be catalysed by ferritin. Removal of contaminating catalase revealed that both processes required H_2O_2 which could be supplied by O_2 under aerobic conditions. However, O_2^- was not involved. Butylated hydroxytoluene (BHT), α-tocopherol and β-carotene inhibited lipid peroxidation of microsomes, but did not inhibit thiol oxidation or the inactivation of Ca-ATPase. Likewise, the hydroxyl radical scavengers benzoate, formate and mannitol were not inhibitory. Glutathione (GSH), however, prevented inactivation of Ca-ATPase. It is concluded that Adriamycin®-enhanced redox reactions involving iron and H_2O_2 are responsible for oxidizing microsomal thiol groups and inhibition of Ca-ATPase. Disruption of Ca transport within the myocyte by this process could contribute to the cardiotoxicity of Adriamycin.®

Ca pumps on the endoplasmic reticulum and in mitochondria of the cell maintain cytostolic Ca concentrations at levels much lower than in the extracellular space. Disruption of this distribution results in loss of function and cell death [1–3]. Myocytes are particularly susceptible since the contraction/relaxation cycle is dependent on Ca. The cardiotoxicity of Adriamycin® may be related to the chemical reduction of Adriamycin® [4] and subsequent reactions of free radical species with cardiac cell components such as membrane lipids [5, 6] or enzymes [7, 8]. There is considerable evidence that Adriamycin® disrupts the Ca milieu of cardiac microsomes [7, 9, 10], mitochondria [11, 12] and isolated atria [2].

Ca-ATPases maintain Ca levels within the myocyte [13]. Reduced thiol groups in the enzyme are essential for Ca-ATPase activity, and oxidant stress of muscle and liver microsomes has been shown to cause thiol oxidation and Ca-ATPase inhibition [7, 14, 15]. Adriamycin® can undergo redox cycling as a result of reduction by either NADH dehydrogenase or NADPH-cytochrome P-450 reductase in microsomes [4, 16], and inhibition by Adriamycin® of cardiac microsomal Ca-ATPase via the NADH dehydrogenase enzyme has been described [7]. From results with a variety of microsomal oxidizing systems, species such as O_2^-, H_2O_2, OH· and Fe(IV) [7, 17] have been proposed as initiators of thiol oxidation and Ca-ATPase inactivation. However the mechanism is unclear. Contamination of microsomes with superoxide dismutase, catalase and ferritin [18] may be one reason for this.

Lipid oxidation products have been proposed as inactivators of Ca-ATPase in heart microsomes [17], though the evidence is equivocal [14]. A number of redox active agents, including Adriamycin®, can induce peroxidation of heart microsomal lipids [6, 19, 20]. Recently we have shown that peroxidation promoted by Adriamycin® and the microsomal NADPH-cytochrome P-450 reductase system is maximal at low pO_2 and requires Fe^{3+} or ferritin [6], and can be inhibited by α-tocopherol or β-carotene [21], but not by the H_2O_2 scavenger catalase [6].

This study investigates thiol status and Ca-ATPase activity of purified rabbit heart microsomes after incubation with Adriamycin® and NADPH. The pO_2, and hence the extent of lipid peroxidation, has been varied, and the effects of inhibiting lipid peroxidation with α-tocopherol, BHT and β-carotene have been investigated. A requirement for Fe^{3+} or ferritin has been demonstrated, and the involvement of H_2O_2, O_2^- and OH· examined.

MATERIALS AND METHODS

Chemicals. All biochemicals were from the Sigma Chemical Co. (St Louis, MO). Ferritin was used as supplied and contained 1.5 nmol iron/mg protein determined by the method of Hoy *et al.* [22]. Procedures were carried out in phosphate buffer pH 7.4, except Ca uptake which was performed in 25 mM Hepes buffer pH 7.4 containing 125 mM KCl, 2 mM K_2HPO_4 and 4 mM $MgCl_2$ [15]. Both buffers were treated with Chelex resin (Biorad Laboratories, Richmond, CA) to decrease iron contamination. The Hepes buffer was treated before addition of Mg.

Microsome preparation. Heart microsomes were prepared in 10 mM phosphate buffer pH 7.4 [23]. Protein concentration was determined according to Lowry *et al.* [24]. Microsomes were further purified from catalase, superoxide dismutase and ferritin present in the preparation by passage down a Sepharose CL-2B column [18]. This decreased the catalase content 10-fold and the superoxide dismutase content 5-fold. Preparations were standardized such that 0.5 mg microsomal protein/mL, with 100 μM NADPH, reduced cytochrome *c* at a

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rate of 3 μM/min. All experiments were performed with purified microsomes except where indicated. α-Tocopherol, β-carotene and BHT were incorporated into the microsomal membrane by gently homogenizing 5 mL of the 105,000 *g* microsomal pellet with each antioxidant added in 10 μL chloroform, (final concentration of antioxidant was 200 nmol/mg protein) using a Potter–Elvejheim homogenizer as in Ref. 21.

Incubation of microsomes with Adriamycin® Microsomes (0.5 mg protein/mL) were incubated with Adriamycin® (30 μM), NADPH (100 μM) and other additives where indicated, for 30 min at 22° with continuous rotation. Incubations of microsomes at low pO_2 were performed in 10-mL rubber-stoppered glass tubes. The contents were bubbled with O_2-free N_2 and the requisite volume of N_2 was replaced with air, using a gas-tight syringe.

Thiol oxidation. The thiol content of the microsomes, before and after treatment with Adriamycin®, was determined by adjusting the pH of the suspension to 8.0 with 0.5 M Tris buffer pH 8.5 then adding 0.1% sodium dodecyl sulphate (SDS) and 100 μM dithiobisnitrobenzoic acid (DTNB). A_{412} of each solution was measured against an appropriate blank containing all the reagents except DTNB, and loss of thiols calculated from ΔA_{412} between treated and untreated microsomes.

To determine if thiol groups were reversibly oxidized to disulphides the proportion of thiols regenerated with dithiothreitol (DTT) was determined. Microsomes (0.5 mg/ml) were incubated for 30 min with Adriamycin® (30 μM), $FeSO_4$ (2.0 μM), H_2O_2 (100 μM) and NADPH (100 μM) in phosphate buffer at 22°. A control incubation was performed without NADPH. Thiol content of 1-mL samples from each incubation was determined by adding SDS and DTNB. The remainder of each microsomal preparation was treated for 4 hr with DTT (3 mM), dialysed against deionized water until no DTT was detectable in the dialysate, then assayed with DTNB in the presence of SDS.

Ca-ATPase activity. Following preincubation with or without Adriamycin® and additives, microsomes (0.5 mg/ml) were incubated with ATP (100 μM), Ca(20 μM) and arsenazo III (30 μM) in Hepes buffer, and the changes in A_{654} were monitored continuously. Rates of Ca uptake were calculated from the disappearance of the Ca–arsenazo III complex using $\varepsilon_{654} = 21{,}400\ M^{-1}\ cm^{-1}$.

RESULTS

Microsomal thiol loss

Incubation of microsomes with NADPH and 2.0 μM $FeSO_4$ resulted in the loss of approximately 10% of total thiol groups, most of which occurred in the first 10 min (Fig. 1). Addition of 30 or 60 μM Adriamycin® increased the loss to 30%. No thiol loss occurred in the absence of NADPH. Eighty per cent of thiols lost were regenerated with DTT indicating that most were oxidized to disulphides (data not shown). When microsomes were assayed with DTNB in the absence of SDS only 60% of the thiols measured in the presence of SDS were detectable,

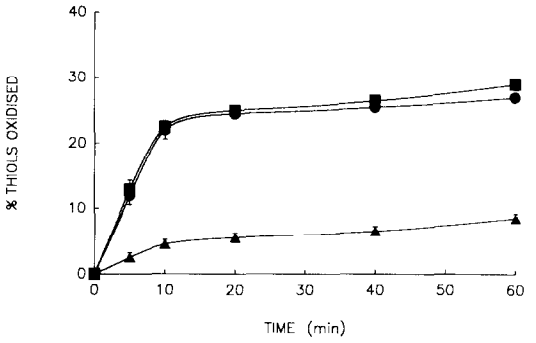

Fig. 1. Time course of thiol oxidation in the presence of (▲) 0 μM, (■) 30 μM and (●) 60 μM Adriamycin®. Microsomes were incubated with NADPH (100 μM) and $FeSO_4$ (2.0 μM) in phosphate buffer in air. Each point represents the mean and range of two sets of duplicates. Total thiol groups = 59 μmol/mg protein.

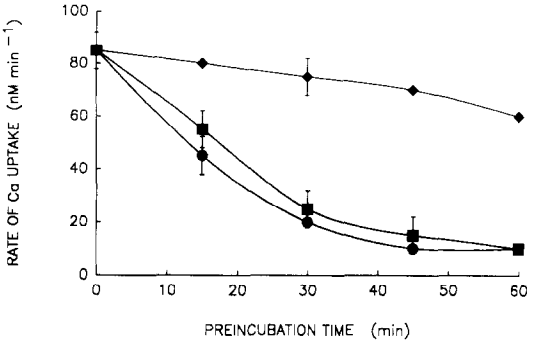

Fig. 2. Time course of microsomal Ca-ATPase inactivation in the presence of (◆) 0 μM, (■) 30 μM and (●) 60 μM Adriamycin®. Solutions also contained NADPH, $FeSO_4$ (2 μM), Ca (20 μM), ATP (100 μM) and arsenazo III (30 μM). Each point represents the mean and range of two sets of duplicates.

and 88% of these were lost on treatment with Adriamycin®, NADPH and $FeSO_4$. The absolute thiol loss (18 μmol/mg protein) remained the same as when SDS was present, indicating that only thiols near the surface of the microsomes were oxidized by Adriamycin® treatment.

Inactivation of Ca-ATPase

Microsomal Ca-ATPase activity was measured as the rate of Ca uptake from a medium containing Ca–arsenazo III complex. In agreement with the findings of Refs. 14 and 15 microsomes took up Ca, but only in the presence of ATP. As shown in Fig. 2, preincubation with NADPH and $FeSO_4$ caused only a slight time-dependent loss in Ca-ATPase activity. There was a much greater loss in the presence of Adriamycin®, with only 33% of the original activity remaining after 30 min.

Iron or ferritin dependence

Thiol oxidation and Ca-ATPase inactivation were dependent on iron. Fe^{3+}-ADP and ferritin iron were

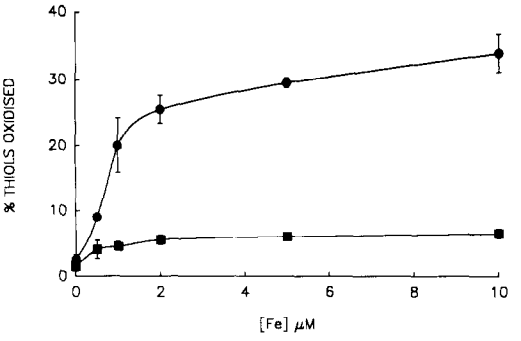

Fig. 3. Dependence of thiol oxidation on iron concentration. Microsomes were incubated for 30 min with NADPH, Adriamycin® and $FeSO_4$ at the final concentrations shown; (■) with and (●) without desferrioxamine (100 μM). Each point represents the mean and range of two sets of duplicates.

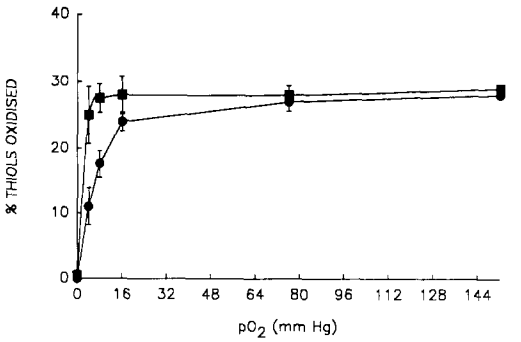

Fig. 4. Dependence of thiol oxidation on pO_2. Solutions (1 mL) contained microsomes, NADPH, Adriamycin® (30 μM) and (■) ferritin (50 μg) or (●) $FeSO_4$ (2.0 μM). pO_2 was adjusted as described in Materials and Methods. Each point is the mean and range of two sets of duplicates.

Table 1. Effect of $FeSO_4$ and ferritin on microsomal thiol oxidation and Ca uptake

Addition	% Thiols oxidized	% Ca uptake
No NADPH	0	100
NADPH	4	100
$FeSO_4$ (2.0 μM)	26	23
$FeSO_4$ (2.0 μM) + ADP (1 mM)	24	25
$FeSO_4$ (2.0 μM) + EDTA (100 μM)	8	75
Ferritin (50 μg/mL)	24	33
Ferritin (100 μg/mL)	28	11
Ferritin (50 μg/mL) + desferrioxamine (100 μM)	2	88

Solutions containing microsomes (0.5 mg/mL), NADPH (100 μM) except where indicated, Adriamycin® (30 μM) and additions as above, were incubated in air. Thiol oxidation was measured with DTNB and Ca uptake with ATP (100 μM), Ca (20 μM) and arsenazo III (30 μM) added after a 30 min preincubation. Each value is the mean of two sets of duplicates which differed by no more than 10%.

good catalysts, Fe^{3+}-EDTA was not (Fig. 3 and Table 1). Ferritin catalysed thiol oxidation and Ca-ATPase inactivation was almost completely prevented by desferrioxamine, demonstrating the need to release the iron from ferritin.

Involvement of O_2

O_2 was required for the oxidation of thiol groups (Fig. 4). Oxidation was maximal with a $pO_2 \geq 16$ mm Hg, when the reaction was catalysed by $FeSO_4$, and at a lower pO_2 for the ferritin-dependent reaction. Ca-ATPase was not inactivated under N_2.

Involvement of H_2O_2 *and* O_2^-

Thiol loss in microsomes incubated with ferritin, NADPH and Adriamycin® was inhibited by catalase, but was unaffected by SOD (Table 2, 1st column). Consistent with the involvement of H_2O_2 was an increase in thiol loss with increasing concentration of H_2O_2 added (Fig. 5). The reducing system was required in addition to H_2O_2 since no inactivation occurred with ferritin and H_2O_2 alone (Fig. 5). The O_2 requirement was abolished when H_2O_2 was added to the microsomes (Fig. 6).

With added H_2O_2, thiol loss promoted by $FeSO_4$ was independent of pO_2, whereas with ferritin it was maximal under anaerobic conditions, This can be explained by the need for iron to be released from ferritin to be active (see Table 1) and the greater efficiency of release by microsomally-reduced Adriamycin® in the absence of O_2 [25].

Catalase prevented inactivation of Ca-ATPase by Adriamycin®, NADPH and ferritin and inactivation was increased by adding H_2O_2 (Table 2). SOD was without effect. The experiments in Table 2 were carried out with microsomes that had been purified by passage through Sepharose. Unless the purification was performed, contamination by catalase obscured the H_2O_2 requirement for thiol oxidation and Ca-ATPase inactivation. There was less oxidation and inactivation than occurred with purified microsomes and there was no inhibition by catalase.

Involvement of $OH^{\cdot}$

In the presence of 20 mM benzoate, formate or mannitol (which would be sufficient to scavenge any hydroxyl radical produced) thiol loss (26 ± 2%) was the same as with no scavenger present. Inhibition of

Table 2. Effect of SOD and catalase on thiol oxidation and Ca uptake by heart microsomes

Addition	% Thiols oxidized	% Ca uptake
—	0	100
NADPH	26	34
NADPH + catalase (20 μg)	5	85
NADPH + SOD (30 μg)	24	32
NADPH + H_2O_2 (150 μM)	ND	16

Solutions (1 mL) contained microsomes, Adriamycin®, ferritin (50 μg), NADPH and additions as shown. Thiol oxidation was measured with DTNB and Ca uptake was measured after a 30 min preincubation with Ca, arsenazo III and ATP. Each value is the mean of two sets of duplicates which differed by no more than 10%.

ND, not done.

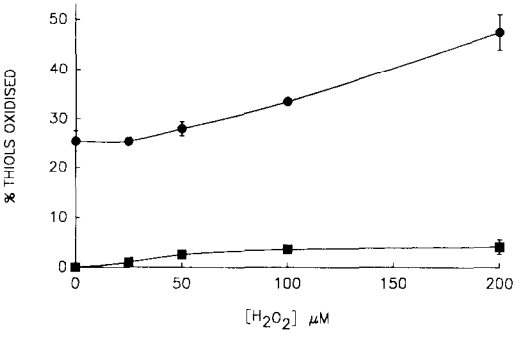

Fig. 5. Dependence of thiol oxidation on H_2O_2 concentration. Microsomes were incubated with Adriamycin® (30 μM), ferritin (50 μg/mL) and either no NADPH (■) or NADPH (100 μM) (●) in air. Each point represents the mean and range of two sets of duplicates.

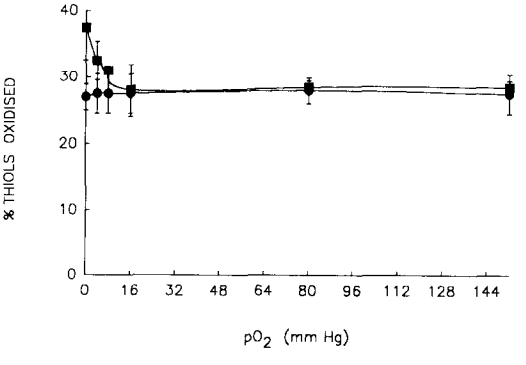

Fig. 6. Thiol oxidation dependence on pO_2 in the presence of H_2O_2. Solutions contained microsomes, NADPH, Adriamycin® (30 μM), H_2O_2 (150 μM) and either (■) ferritin (50 μg/mL) or (●) $FeSO_4$ (2.0 μM). pO_2 was adjusted as described in Materials and Methods. Each point represents the mean and range of two sets of duplicates.

Ca uptake (67%) was also unaltered by any of the scavengers.

Involvement of lipid peroxidation

Microsomes incubated with Adriamycin® NADPH and $FeSO_4$ or ferritin undergo lipid peroxidation. This is maximal at pO_2 values of 4–8 mm Hg, where peroxidation is 2–3-fold higher than in air [6]. No corresponding O_2 dependence was seen for thiol loss (Fig. 4). Lipid peroxidation is inhibited by butylated hydroxytoluene (BHT), α-tocopherol and β-carotene (Table 3). However, these lipid antioxidants gave no corresponding inhibition of thiol loss nor prevented the inactivation of Ca-ATPase.

Prevention of Ca-ATPase inactivation by reduced glutathione

Only 12% of the activity of Ca-ATPase was lost when reduced glutathione (GSH) (150 μM) was present during preincubation of microsomes with ferritin, NADPH and Adriamycin®. Without GSH Ca-ATPase activity was inhibited by 68% (Table 3). Protection by GSH is likely to have been the result of interception of the oxidizing species, since Ca-ATPase activity was not restored by incubating the treated microsomes with the same concentration of GSH for 30 min before monitoring Ca uptake.

DISCUSSION

We have shown that NADPH-dependent reduction of Adriamycin® by rabbit heart microsomes results in oxidation of thiols and inactivation of Ca-ATPase. Both processes required Fe^{3+}, H_2O_2, but not $O_2^{\cdot-}$. Ferritin was a good source of Fe^{3+}. Inhibition of lipid peroxidation did not alter thiol oxidation or Ca-ATPase inactivation and hydroxyl radical scavengers were without effect. A major proportion of heart microsomal thiol groups are associated with Ca-ATPase [26] and maintenance of reduced thiols is essential for its activity. Therefore it is not surprising that Adriamycin®-enhanced oxidation of thiols results in Ca-ATPase inactivation.

Adriamycin® is reduced by microsomal NADPH-cytochrome P-450 reductase [4]. Reduction was a requirement for thiol oxidation and inactivation of Ca-ATPase. A lack of reducing equivalents is likely to have been the reason why no inhibition of cardiac microsomal Ca-ATPase by Adriamycin® was observed in a previous study [26]. Harris and Doroshow [7], using a NADH/NADH-dehydrogenase

Table 3. Effect of antioxidants on microsomal thiol oxidation and Ca uptake

Addition	% Thiols oxidized	% Ca uptake	% Inhibition of lipid peroxidation
—	27	32	0
BHT (25 μM)	27	33	90
α-Tocopherol (50 μM)	28	33	70
β-Carotene (50 μM)	28	32	60

Solutions contained microsomes, NADPH, ferritin (50 μg/mL), Adriamycin® and antioxidants as shown above. Antioxidants were incorporated into the microsomal pellet by homogenization as described in Materials and Methods. Thiol oxidation was measured with DTNB; Ca uptake with arsenazo III, Ca and ATP. Values are means of two sets of duplicates which differed by no more than 10%.

system in microsomes, have also measured inactivation of Ca-ATPase in the presence of Adriamycin® and observed a requirement for H_2O_2. Thiol loss and inactivation of Ca-ATPase have been demonstrated in microsomes exposed to a variety of oxidant stresses [7, 15, 17] but there is no consensus as to the roles of H_2O_2, O_2^- and iron. Standard preparations of microsomes have been shown to contain catalase, superoxide dismutase and ferritin [18], and different levels of contamination could be a reason for this ambiguity. It was only when contaminant catalase was removed from our microsomal preparation that we saw inhibition by catalase and a requirement for H_2O_2.

Microsomal reduction of Adriamycin® to the semiquinone enables subsequent reduction of Fe^{3+} complexes and efficient release of iron from ferritin [25, 27]. We have shown that this iron can mediate peroxidation of microsomal lipids [6]. The present results show that ferritin can also provide iron for Adriamycin®-dependent oxidation of thiols and inactivation of Ca-ATPase. We did not see inactivation unless we added Fe^{3+} or ferritin to our microsomes, presumably because their Fe^{3+} content was very low. When microsomes contain residual ferritin, iron can be released by Adriamycin® [28] and should be able to participate in the reactions seen here. Ferritin content of normal heart is approximately 30–60 μg ferritin/g [29]. Therefore ferritin is a possible source of Fe^{3+} to catalyse thiol oxidation and Ca-ATPase inactivation *in vivo*. Low molecular weight Fe^{3+}-chelates would not be necessary.

Inhibition of cardiac enzymes containing thiol groups has been proposed as a mechanism for Adriamycin® cardiotoxicity [7, 8, 12] and a direct link between thiol groups and the activity of Ca-ATPase has been established in a number of microsomal systems under oxidative stress [9, 14, 15]. In intact cells, GSH levels are decreased in conjunction with a loss of protein thiols [3, 30, 31], and other studies [32, 33] have found that GSH can protect against inactivation of Ca-ATPase by *t*-butyl hydroperoxide and A23187. Intracellular GSH levels in the rat heart are approximately 1.2 mmol/g heart [34] and we found that concentrations of GSH much less than this prevented Adriamycin®-dependent inactivation of Ca-ATPase. The mechanism appeared to involve protection rather than repair.

The reaction mechanism for the oxidative inactivation of microsomal Ca-ATPase is unknown. It has been proposed that it occurs secondary to lipid peroxidation [17]. Previously we have shown that Fe^{3+}-ADP is a good catalyst of Adriamycin®-promoted microsomal lipid peroxidation, whereas Fe^{3+}-EDTA and ferrioxamine are not [6]. The abilities of Fe^{3+}-chelates to promote thiol oxidation and Ca-ATPase inactivation are similar to their abilities to promote lipid peroxidation and Olafsdottier *et al.* [33] have shown that in oxidant stressed intact hepatocytes, α-tocopherol can protect against protein thiol loss. However our finding that inhibition of peroxidation by lipid antioxidants did not affect the inactivation of Ca-ATPase or the oxidation of thiols indicates that lipid peroxidation products are not responsible in the Adriamycin®/microsomal system investigated here. Our results agree with those of Scherer and Deamer [14] who concluded that thiol oxidation and Ca-ATPase inactivation are independent of lipid oxidation in a lobster abdominal muscle microsomal system exposed to several different oxidant stresses.

The requirement for Fe^{3+}, H_2O_2 and a reductant suggests the mechanism of inactivation could involve a Fenton reaction, producing $OH^{\cdot}$, or possibly an Fe(IV) species, depending on the nature of the Fe^{2+}-complex:

$$Fe^{2+}\text{-}(chelate) + H_2O_2 \rightarrow Fe^{3+}\text{-}(chelate) + OH^{\cdot} + OH^-.$$

However, the lack of inhibition by high concentrations of benzoate, formate or mannitol rules out the involvement of freely diffusible $OH^{\cdot}$ and probably also Fe(IV). Kukreja *et al.* [17] also found that $OH^{\cdot}$ scavengers did not prevent Ca-ATPase inactivation in microsomes oxidized by dihydroxyfumarate and Fe^{3+}-ADP. The lack of inhibition by the scavengers could indicate a site-localized reaction that is inaccessible to scavengers. However, this work demonstrates that thiol oxidation occurred at sites accessible to DTNB without disrupting the membranes with SDS, and was inhibited by GSH. A plausible mechanism could involve a concerted reaction between iron, H_2O_2 and a thiol, perhaps involving an iron–thiol complex, in which the iron mediates oxidation of the thiol by H_2O_2, without the release of the oxidizing intermediate.

As a thiol-rich protein, Ca-ATPase is particularly vulnerable to oxidation and inactivation. As shown here Adriamycin®, in conjunction with iron from ferritin, can readily oxidize thiol groups of Ca-

ATPase, diminishing Ca uptake by cardiac microsomes. Disruption of this enzyme may be related to the cardiotoxicity of Adriamycin®.

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