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INTERACTION OF ADRIAMYCIN AGLYCONES WITH ISOLATED MITOCHONDRIA

EFFECT OF SELENIUM DEFICIENCY

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Abstract—Adriamycin (AdM) aglycones have dramatic effects on isolated heart mitochondria, oxidizing pyridine nucleotides, modifying sulfhydryl groups, and triggering a permeability transition of the inner membrane that results in free passage of solutes smaller than 1500 Da. In this investigation, the role of glutathione (GSH) peroxidase in these actions of the aglycones was evaluated, by comparing mitochondria from selenium-deficient and selenium-supplemented rats, with the following results. Selenium deficiency was without effect on the permeability transition of heart mitochondria, followed via Ca^{2+} release and triggered by AdM aglycone or by *t*-butyl hydroperoxide (TBH) or H_2O_2, both of which are authentic substrates of the peroxidase. The permeability transition of liver mitochondria was delayed by selenium deficiency regardless of the triggering agent; however, substantial triggering by the aglycone and TBH persisted in mitochondria from selenium-deficient animals. Selenium deficiency inhibited thiol modification elicited by AdM aglycone and H_2O_2 in heart mitochondria and by the aglycone, TBH, and possibly H_2O_2 in liver mitochondria. It would thus appear that AdM aglycone, TBH, and H_2O_2 can induce the permeability transition of isolated heart mitochondria via a process (or processes) distinct from the catalytic activity of the peroxidase. Furthermore, even in liver, where involvement of the peroxidase is observed, mechanisms other than the GSH cycle can contribute to transition induction by the aglycone and by TBH. Finally, mitochondrial -SH group modification by the aglycones appeared not to be causally linked to induction of the permeability transition. This laboratory has suggested that the effects of aglycone metabolites of AdM on mitochondria mediate the cardiotoxicity that limits use of the parent drug. The data presented in this paper argue against the involvement of GSH preoxidase in that process. They are in agreement with *in vivo* studies, which have generally failed to find evidence for amelioration of AdM cardiotoxicity in selenium-deficient animals.

Adriamycin (AdM†; doxorubicin) is one of the most potent antineoplastic agents known, but its clinical use is limited by cumulative and irreversible cardiotoxicity [see, for example, Ref. 1]. The biochemical mechanisms underlying this important toxicity have yet to be unequivocally identified [for reviews, see Refs. 2 and 3], although a substantial body of evidence suggests involvement of redox reactions of the quinone moiety of the molecule [4].

This laboratory has reported that aglycone derivatives of AdM, at concentrations that would occur intracellularly during therapeutic use of the drug, profoundly alter the function of isolated rat heart mitochondria. Mitochondrial pyridine nucleotides [NAD(P)H] are oxidized [5], mitochondrial sulfhydryl (-SH) groups are modified [6], and a permeability transition is induced in the inner mitochondrial membrane (i.m.) permitting passage of solutes smaller than 1500 Da [7]. The last of these effects would perhaps be the most serious since it would disrupt both cellular ATP synthesis and homeostatic control of cytosolic Ca^{2+}. We have hypothesized that the effects of AdM aglycones on mitochondria are responsible for the cardiotoxicity of the parent drug.

The biochemical mechanisms by which AdM aglycones alter mitochondrial function have yet to be elucidated. However, we have presented evidence for electron transfer in intact mitochondria from NAD(P)H to the aglycones and thence to O_2, giving rise to the superoxide ($O_2^{\cdot-}$) radical [5]. This is in good agreement with results obtained by exposing disrupted mitochondrial systems to AdM [8, 9].

Scheme 1 presents alternative biochemical pathways by which reduction of AdM aglycone could lead to modification of mitochondrial -SH groups. The four pathways shown are: (1) direct modification of thiols by the aglycone free radical (aglycone$^{\cdot}$), (2) aglycone-mediated $O_2^{\cdot-}$ generation followed sequentially by production of H_2O_2 via superoxide dismutase (SOD) and thiol modification mediated by glutathione (GSH) peroxidase, (3) aglycone-mediated production of both $O_2^{\cdot-}$ and H_2O_2 as outlined in (2) with subsequent Fe^{3+}-dependent

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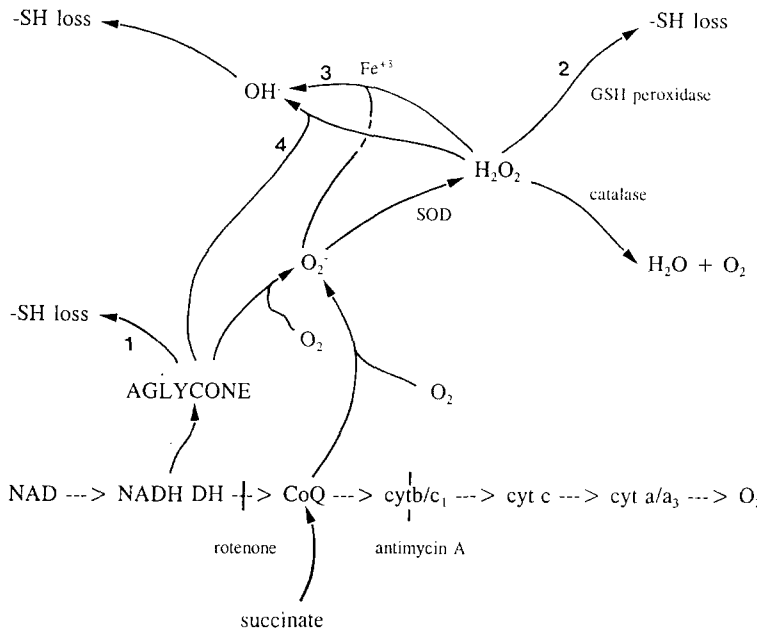
† Abbreviations: AdM, Adriamycin; BSA, bovine serum albumin; DMSO, dimethyl sulfoxide; DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid); F-CCP, carbonyl cyanide *p*-trifluoromethoxy-phenylhydrazone; GSH, glutathione; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethane-sulfonic acid; i.m., inner mitochondrial membrane; MOPS, 3-(*N*-morpholino)propanesulfonic acid; -SH, sulfhydryl; SOD, superoxide dismutase; and TBH, *t*-butyl hydroperoxide.



Scheme 1. Biochemical pathways leading to modification of mitochondrial -SH groups. Arrows indicate the transfer of electrons. Catalysts and cofactors are shown in smaller print adjacent to the arrows. Line segments perpendicular to the electron transport arrows indicate the sites of action of inhibitors.

interaction of those two species (the Haber–Weis reaction) to produce the hydroxyl radical (OH·), which would then attack adjacent -SH groups, and (4) reduction of H_2O_2 by aglycone· in a potentially Fe^{3+}-independent fashion [10], again with attack by the OH· on mitochondrial thiols.

Several observations suggest that H_2O_2 may play a key role in the interaction of AdM aglycones with mitochondria. The cytotoxicity of daunorubicin, a related anthracycline, is inversely related to the ability of cells to metabolize H_2O_2 [11]. Furthermore, although washed submitochondrial particles produce O_2^- [8], the high SOD/catalase ratio in cardiac ti sue [12] suggests that H_2O_2 may accumulate in more intact systems. Finally, we have observed that aglycone-induced -SH loss is catalase sensitive [13].

In the experiments described in this paper, selenium deficiency was used to decrease mitochondrial glutathione peroxidase activity in order to evaluate the importance of the second of the pathways outlined above, namely, aglycone-induced H_2O_2 production followed by peroxidase-mediated thiol oxidation, in aglycone-induced alterations in mitochondrial function. Specifically, we have examined the effect of selenium deficiency on the aglycone-induced permeability transition and on -SH modification mediated by the aglycone in isolated rat heart mitochondria. H_2O_2 and *t*-butyl hydroperoxide (TBH) were included in these studies as authentic substrates of GSH peroxidase as were liver mitochondria since the role of GSH peroxidase in the TBH-induced permeability transition of these organelles has been established [14].

METHODS

Male Sprague–Dawley rats (150–200 g) were maintained on a selenium-deficient diet, obtained from ICN Biochemicals, Cleveland, OH, for 6–12 weeks. The diet consisted of 30% Torula yeast, 59% sucrose, 5% tocopherol-stripped lard, 5% salt HMW (Hubbel, Mendel and Wakeman), 100 IU vitamin E, and 1% ICN vitamin fortification mixture without vitamin E. Control rats were fed the same diet supplemented with 1.2 ppm sodium selenite.

Heart [7] and liver [15] mitochondria were isolated as previously described. Assays of mitochondrial function were carried out in a standard resin (Chelex-100)-treated buffer containing 100 mM sucrose, 50 mM KCl, and 20 mM 3-(*N*-morpholino)propanesulfonic acid (MOPS)-KOH (pH 7.2), supplemented with 0.8 μM rotenone. For experiments using heart mitochondria, the protein concentration was 0.2 mg/mL and KH_2PO_4 was present at 1.7 mM; for liver mitochondria, the protein concentration was increased to 0.4 mg/mL and inorganic phosphate was reduced to 0.2 mM. Temperature was maintained at 30°. Mitochondrial Ca^{2+} fluxes were measured with a Ca^{2+}-selective electrode [7]. Sulfhydryl groups were quantified via reaction with 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) [6]. Protein was determined according to the method of Lowry *et al.* [16] using bovine serum albumin (BSA) as standard. No difference in mitochondrial protein yield between selenium-deficient and control tissues was observed.

Mitochondrial extracts were prepared by adding mitochondria (50 μL) to 250 μL of 50 mM *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (HEPES)-KOH, pH 7.2. Triton X-100 was added to give a final concentration of 1%. After a 10-min incubation on ice, samples were centrifuged at 15,000 *g* for 15 min [17].

GSH peroxidase activity in the extracts was

Table 1. Effect of selenium deficiency on the activity of mitochondrial GSH peroxidase

Tissue	Substrate	Control (nmol·min^{-1}·mg^{-1})	Se$^-$ (nmol·min^{-1}·mg^{-1})	Se$^-$/Control (%)
Heart	H_2O_2	265 ± 32.0 (3)	22.6 ± 23.7* (3)	8.5
	TBH	295 ± 43.3 (3)	22.6 ± 23.5* (3)	7.6
Liver	H_2O_2	428.3 (2)	0.55 (2)	0.13
	TBH	482.1 (2)	0.90 (2)	0.19

Activity was determined for each substrate using 5-, 10- and 20-μL aliquots of mitochondrial extract and the results were averaged. The control and Se$^-$ values in the heart tissue are given as means ± SD for the number of mitochondrial preparations indicated in parentheses.

* The range of the values obtained was large: 4.3 to 24.1 nmol·min^{-1}·mg^{-1} for H_2O_2, and 3.2 to 24.3 nmol·min^{-1}·mg^{-1} for TBH.

assayed at 30° according to Sies and Moss [17] as modified by Lotscher and co-workers [14] in a reagent consisting of 50 mM HEPES-KOH (pH 7.2), 1 mM EDTA, 1 mM NaN_3, 1 mM GSH, 50 μM NADPH and 0.2 U/mL GSH reductase, with 60 μM H_2O_2 or TBH as substrate. In this coupled enzyme assay, substrate reduction is linked to the stoichiometric oxidation of NADPH which can be followed spectrophotometrically at 340 nm. Activities were corrected for the rate of NADPH oxidation obtained in the presence of substrate prior to the addition of mitochondrial extract.

GSH reductase was purchased from Boehringer-Mannheim, Indianapolis, IN, and Chelex-100 (200 mesh) from Bio-Rad, Richmond, CA. Biochemicals were from the Sigma Chemical Co., St. Louis, MO. All other reagents were of the highest quality available. Adriamycin was the gift of Adria Laboratories, Columbus, OH. The 7-hydroxy aglycone derivative of AdM was prepared by cleavage in dilute acid [18] and dissolved in dimethyl sulfoxide (DMSO). This aglycone was used in all studies rather than the 7-deoxy form that is generated in biological systems. The latter is considerably more difficult to produce chemically. The two aglycones have been demonstrated previously to be similarly active in altering mitochondrial function [5–7].

RESULTS

The efficacy of the selenium-deficient diet in lowering mitochondrial GSH peroxidase levels was determined directly. GSH peroxidase activity was reduced to less than 10% of control values in heart mitochondria and to less than 1% of controls in liver mitochondria (Table 1). The factors responsible for this tissue-specific difference in efficacy are unclear. Control peroxidation rates measured in liver mitochondria were substantially greater than those reported previously by Lotscher and co-workers [14]. This may reflect, in part, the higher temperature used in the assays reported here. The fractional reduction in peroxidase activity elicited by selenium deprivation was at least as great as in the earlier experiments.

Peroxidation rates measured with H_2O_2 and with TBH as substrate were essentially the same for control heart mitochondria. The rates observed with control liver mitochondria were somewhat (*ca.* 1.6-fold) higher, but, again, there was no significant difference in the rates measured with the two substrates. For mitochondria from each tissue, selenium deficiency decreased the rate of peroxidation, with similar decreases being observed for both H_2O_2 and TBH.

Organic hydroperoxides are metabolized by both selenium-dependent and -independent GSH peroxidases whereas H_2O_2 is a substrate only of the former [19]. The lack of enhancement of TBH peroxidation relative to H_2O_2 in extracts from selenium-deficient animals argues against the presence of a selenium-independent GSH peroxidase in either mitochondrial preparation. This is in keeping with the report of Lawrence and Burk [20] that the selenium-independent form of the enzyme is cytosolic.

The mitochondrial permeability transition was monitored via triggered Ca^{2+} release. Because the ability of mitochondrial preparations to take up and retain Ca^{2+} is somewhat variable, total uptake capacity was determined [7] and a 25% load was used in measurements of the permeability transition.

The effects of selenium deficiency on the transition in representative preparations of heart and liver mitochondria are shown in Figs. 1 and 2, respectively. When four preparations of heart mitochondria from selenium-deficient animals were compared with three control preparations, no differences in the ability of AdM aglycone, H_2O_2, or TBH to trigger Ca^{2+} release could be discerned. In contrast, when liver mitochondria were examined, selenium deficiency delayed the Ca^{2+} release triggered by each of these agents. The permeability transition induced by H_2O_2 was essentially eliminated, whereas both AdM aglycone and TBH retained substantial triggering ability.

In preceding publications [5, 6], this laboratory has suggested that induction of the mitochondrial permeability transition by AdM aglycone is linked to modification of mitochondrial -SH groups by the drug. The effect of exposure to H_2O_2 or TBH, both of which trigger the transition, on mitochondrial -SH groups was therefore determined and compared with the effect of AdM aglycone (Table 2). In heart mitochondria, all three agents significantly reduced

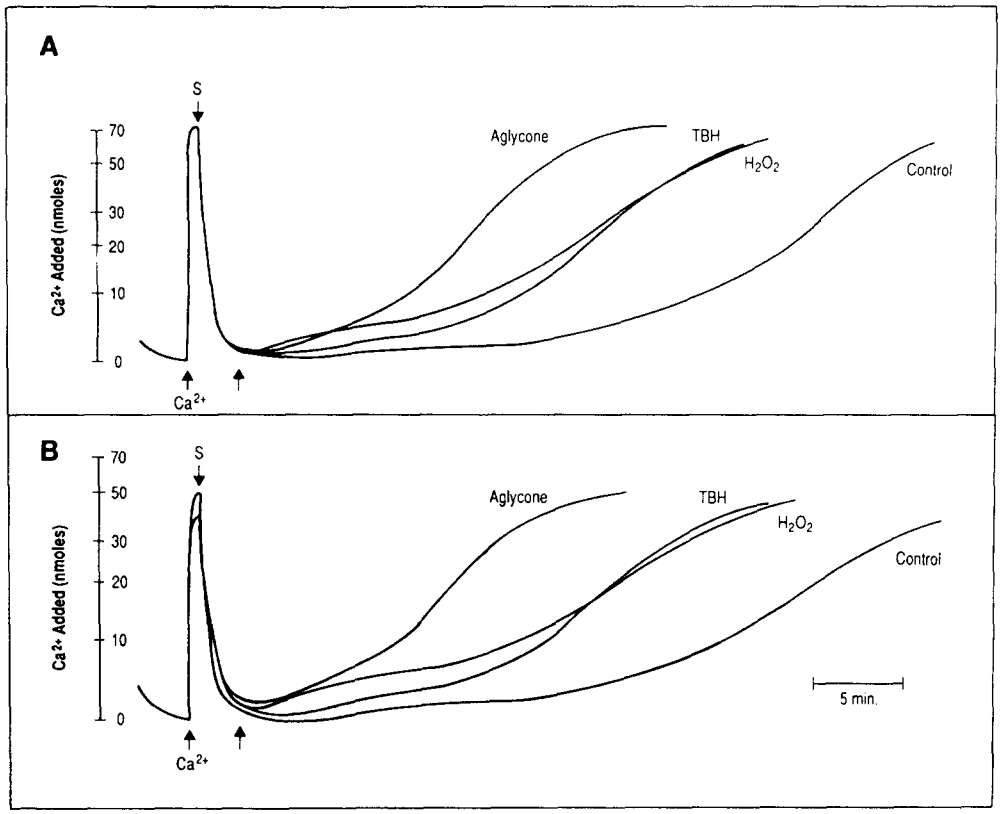


Fig. 1. Effect of selenium deficiency on Ca^{2+} retention by isolated rat heart mitochondria. Mitochondria were incubated in standard buffer supplemented with rotenone for 3 min. At the times indicated by the arrows, Ca^{2+}, succinate (5 mM) and triggering agent were added. Triggering agent concentrations were: AdM aglycone (17.6 μM), TBH (3 mM), and H_2O_2 (30 μM). (A) Mitochondria from control (selenium-supplemented) rats; 155 nmol Ca^{2+}/mg protein was added to give 25% loading. (B) Mitochondria from selenium-deficient rats; the Ca^{2+} addition was 127.9 nmol/mg. In each case, the DMSO control run was superimposed on the trace obtained in the presence of H_2O_2.

the number of thiol groups detected during a 30-min reaction with DTNB.

None of the agents tested significantly decreased -SH group detection in liver mitochondria. In the case of H_2O_2, this appears to reflect relative inactivity of the agent. Presumably this is due to the high levels of catalase present in liver [12]. (Were AdM aglycone acting solely through H_2O_2, its activity should be similarly low in the liver system.) For both AdM aglycone and TBH, the data suggest thiol modification but the number of animals available was too small to provide statistical confirmation.

The effect of selenium deficiency on -SH group modification by AdM aglycone, H_2O_2, and TBH was determined. In contrast to data obtained from animals fed a selenium-supplemented diet, in selenium-deficient animals, only TBH produced a marked change in the heart mitochondrial -SH group number compared with the controls. Furthermore, in heart mitochondria, the number of thiol groups detectable with DTNB during a 30-min incubation in the presence of AdM aglycone or H_2O_2 was increased by selenium deficiency, although the increase was not statistically significant in either case. In liver mitochondria, the effects of AdM aglycone and TBH appeared to be decreased by selenium deficiency (Table 2). Again, because absolute numbers of detectable -SH groups were highly variable and sample size was small, these apparent differences were not statistically significant. When the data were re-expressed in terms of the percentage of thiol groups remaining after treatment with the various transition triggering agents (Table 3), the effects of selenium deficiency were more apparent, i.e. selenium deficiency decreased the ability of all agents tested to modify mitochondrial -SH groups with two exceptions. The effects of TBH on thiol groups of heart mitochondria and possibly of H_2O_2 on liver mitochondrial thiols were not altered.

Lenartowicz [21] has presented evidence recently for reduction of DTNB by thioredoxin reductase (TR) in rat liver mitochondria. Were that reaction occurring in the experiments summarized in this paper, reductase-mediated DTNB reduction would contribute to the parameter reported as -SH number. Furthermore, competition for electrons from TR by O_2^- generated via electron flow from AdM aglycone would decrease DTNB reduction and could account for apparent inhibition of sulfhydryl detection by

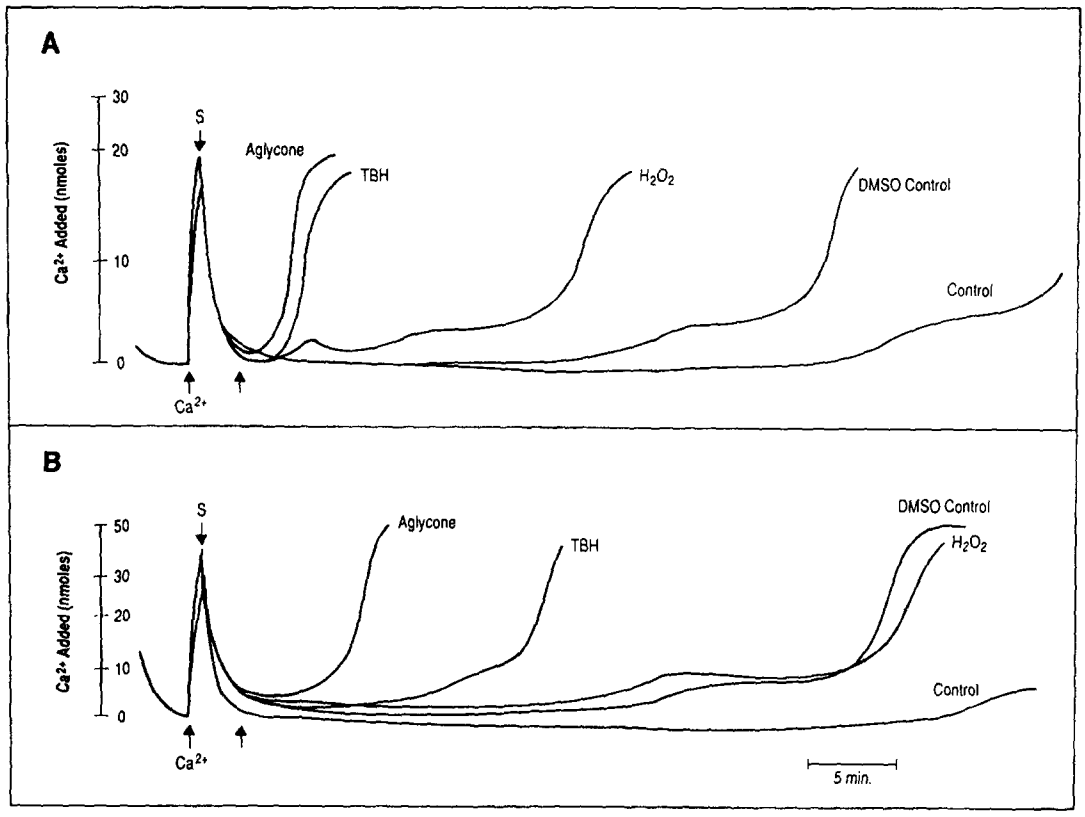


Fig. 2. Effect of selenium deficiency on Ca^{2+} retention by isolated rat liver mitochondria. (A) Mitochondria from control rats; the Ca^{2+} addition was 73.6 nmol/mg. (B) Mitochondria from selenium-deficient rats; 71 nmol Ca^{2+}/mg was added. Other details were as for Fig. 1.

Table 2. Effect of selenium deficiency on modification of mitochondrial -SH groups

Tissue	Agent*	Control -SH groups† (nmol·mg^{-1})	Se^- -SH groups †‡ (nmol·mg^{-1})
Heart	DMSO control	36.2 ± 5.3§ (4)	37.0 ± 11.7 (3)
	AdM aglycone	14.3 ± 2.4‖ (4)	24.0 ± 7.4 (3)
	Control	54.3 ± 3.7 (3)	52.7 (2)
	H_2O_2	23.5 ± 3.3¶ (3)	42.7 (2)
	TBH	7.0 ± 3.4¶ (3)	8.8 (2)
Liver	DMSO control	26.5 (2)	35.9 (2)
	AdM aglycone	12.2 (2)	26.8 (2)
	Control	38.8 (2)	34.4 (2)
	H_2O_2	35.2 (2)	43.6 (2)
	TBH	0.6 (2)	13.5 (2)

* Sulfhydryl group modifications were induced by AdM aglycone (17.6 μM), H_2O_2 (30 μM), and TBH (3 mM).

† The number of -SH groups capable of reacting with DTNB in 30 min was determined. The values obtained for DMSO control and aglycone-treated and for control and either H_2O_2- or TBH-treated preparations were compared (Student's *t*-test).

‡ The values obtained for control and selenium-deficient (Se^-) preparations were compared using Student's *t*-test. No significant differences were obtained.

§ Mean ± SD for the number of mitochondrial preparations indicated in parentheses.

‖ Treatment induced significant -SH loss compared to DMSO control, $P < 0.01$.

¶ Treatment induced significant -SH loss compared to control, $P < 0.001$.

Table 3. Normalized effect of selenium deficiency on -SH group modification

Tissue	Agent	Percent -SH groups remaining (control)	Percent -SH groups remaining (Se^-)
Heart	AdM aglycone	40.0 ± 8.3* (4)	65.6 ± 9.1† (3)
	H_2O_2	43.3 ± 5.8 (3)	81.2 (2)
	TBH	13.1 ± 6.9 (3)	16.6 (2)
Liver	AdM aglycone	45.3 (2)	74.1 (2)
	H_2O_2	91.4 (2)	100.0 (2)
	TBH	1.1 (2)	39.1 (2)

* Mean ± SD for the number of mitochondrial preparations indicated in parentheses.
† Data obtained for the selenium-deficient preparations were significantly different from the control, $P < 0.05$ (Student's t-test).

the aglycone. Several observations indicate that TR-mediated DTNB reduction did not contribute in any significant way to the measurements reported here. First, the uncoupler F-CCP (carbonyl cyanide p-trifluoromethoxy-phenylhydrazone) has never been observed to decrease -SH detection (unpublished observation). F-CCP inhibits TR-mediated DTNB reduction by decreasing the supply of NADPH, which is the electron donor for the reaction [20]. Second, the experiments reported here used succinate and rotenone to drive electron transport; only minimal rates of DTNB reduction mediated by TR are supported by this substrate combination [20]. Third, the K_m for DTNB of the TR reaction is 1.4 mM [20]; DTNB was used in these experiments at 100 μM.

DISCUSSION

In agreement with the previous report of Lotscher and co-workers [14], selenium deficiency was found to decrease the abilities of TBH and H_2O_2 to trigger the i.m. permeability transition of isolated liver mitochondria. The transition induced by AdM aglycone was also slowed. However, both the aglycone and TBH retained considerable triggering capacity in the relative absence of GSH peroxidase (Fig. 2).

In contrast to the results obtained with liver mitochondria, selenium deficiency was without effect on the permeability transition of heart mitochondria whether the triggering agent was AdM aglycone, TBH, or H_2O_2 (Fig. 1). A similar difference in the interaction of AdM aglycones with mitochondria from heart and liver was suggested by our earlier observation that butylated hydroxytoluene is able to slow the aglycone-induced permeability transition in liver but not in heart [15].

Differences in the effect of selenium deficiency on mitochondria from heart and liver cannot be attributed to the presence of a selenium-independent GSH peroxidase in the heart mitochondria; no evidence for such an enzyme was found either here (Table 1) or in earlier studies [20]. It could be argued that the insensitivity of the permeability transition of heart mitochondria to the GSH peroxidase status of the organelle reflects the greater residual enzyme activity of the selenium-deficient heart preparation. Two observations argue against this conclusion. First, similar levels of GSH peroxidase activity persisted in liver mitochondria in an earlier study, yet the permeability transition was eliminated [14]. Second, although the permeability transition of heart mitochondria was unaltered by selenium deficiency, -SH modification by both AdM aglycone and H_2O_2 was overcome, i.e. the reduction in enzyme activity was substantial enough to alter the interaction of each of these agents with the mitochondria.

We propose that AdM aglycone and TBH and H_2O_2 can induce the permeability transition of isolated heart mitochondria via a process (or processes) unrelated to the catalytic activity of GSH peroxidase, even though TBH and H_2O_2 are substrates for the heart mitochondrial enzyme. Furthermore, we suggest that, even in liver mitochondria, where GSH peroxidase does appear to play a role in transition induction, induction of the transition by AdM aglycone and TBH can include a substantial contribution from mechanisms other than GSH peroxidase-mediated oxidation of NAD(P)H. It is impossible, however, to draw firm conclusions based on these data as to the importance of different reactions to transition induction. It may be that the reactions outlined in Scheme 1 represent flexibly interconnected pathways and that their relative contributions to induction of the permeability transition vary with tissue and conditions.

This laboratory has suggested that modification of mitochondrial -SH groups by AdM aglycones leads to induction of the mitochondrial permeability transition [5, 6]. In light of the data collected in Tables 2 and 3, this hypothesis must be re-examined. In heart mitochondria, selenium deficiency decreased the ability of both AdM aglycone and H_2O_2 to modify sulfhydryls (suggesting that GSH peroxidase is central to this effect) without altering the ability of these agents to elicit the i.m. permeability transition. The possibility must therefore be considered that electron transport events facilitated by the aglycones lead to both -SH modification and Ca^{2+} release but that there is no causal link between these two sequelae. In liver mitochondria, the situation may be quite different. For the agents tested, both induction of the transition and

modification of thiol groups are to some extent dependent on GSH peroxidase.

The findings summarized here should also be interpreted in light of studies of the effect of selenium deficiency on the cardiotoxicity of AdM *in vivo*. Were aglycone effects on mitochondria responsible for the cardiotoxicity associated with AdM administration, and were GSH peroxidase required for the actions of the aglycones, then selenium deficiency would protect an organism from AdM cardiotoxicity. Several studies have examined the effect of selenium deficiency on rats exposed chronically to AdM. In four studies, cardiac GSH peroxidase levels were reduced to less than 10% of control values. In one, AdM-induced loss of heart function and increase in malondialdehyde production were enhanced, while mitochondrial function was disrupted [22], i.e. the effects of the anthracycline were accentuated. Two studies failed to find evidence of increased AdM-induced cardiomyopathy in selenium-deficient animals [23, 24]. The fourth investigation examined AdM-induced lipid peroxidation and discerned no effect of selenium deficiency [25]. An additional study utilizing a diet with reduced selenium content again failed to document any significant effects on AdM-induced decreases in heart function or increases in indicators of lipid peroxidation [26]. In both the mouse [12] and the rat [25], selenium deficiency was found to increase mortality due to AdM exposure, but in the rat model this effect was not associated with alterations in redox metabolism or lipid peroxidation. Thus, in agreement with the results outlined in this paper, no data are available to support the participation of GSH peroxidase in expression of the cardiotoxic effects of AdM.

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