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Doxorubicin-Induced Thiol-Dependent Alteration of Cardiac Mitochondrial Permeability Transition and Respiration

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Abstract—Doxorubicin (DOX) is a highly effective treatment for several forms of cancer. However, clinical experience shows that DOX induces a cumulative and dose-dependent cardiomyopathy that has been ascribed to redox-cycling of the drug on the mitochondrial respiratory chain generating free radicals and oxidative stress in the process. Mitochondrial dysfunction including induction of the mitochondrial permeability transition (MPT) and inhibition of mitochondrial respiration have been implicated as major determinants in the pathogenesis of DOX cardiotoxicity. The present work was aimed at investigating whether the inhibition of mitochondrial respiration occurs secondarily to MPT induction in heart mitochondria isolated from DOX-treated rats and whether one or both consequences of DOX treatment are related with oxidation of protein thiol residues. DOX-induced oxidative stress was associated with the accumulation of products of lipid peroxidation and the depletion of α-tocopherol in cardiac mitochondrial membranes. No changes in mitochondrial coenzyme Q_9 and Q_{10} concentrations were detected in hearts of DOX-treated rats. Cardiac mitochondria from DOX-treated rats were more susceptible to diamide-dependent induction of the MPT. Although DOX treatment did not affect state 4 respiration, state 3 respiration was decreased in heart mitochondria isolated from DOX-treated rats, which was reversed in part by adding either cyclosporin A or dithiothreitol, but not Trolox. The results suggest that in DOX-treated rats, (i) induction of the MPT is at least in part responsible for decreased mitochondrial respiration, (ii) heart mitochondria are more susceptible to diamide induced-MPT, (iii) thiol-dependent alteration of mitochondrial respiration is partially reversible *ex vivo* with dithiothreitol. Collectively, these data are consistent with the thesis that thiol-dependent alteration of MPT and respiration is an important factor in DOX-induced mitochondrial dysfunction.

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Key words: doxorubicin, heart, mitochondria, oxidative stress

The anthracycline quinone doxorubicin (DOX; adriamycin) is prescribed for the treatment of several human tumors and leukemias. Despite its recognized effectiveness against such neoplasias, the clinical success of DOX is limited due to its dose-dependent and cumulative cardiotoxicity [1]. DOX generates oxygen free radicals as a consequence of undergoing redox-cycling on complex I of the mitochondrial electron transport chain [2, 3], with the resulting oxidative stress being implicated as a primary cause of doxorubicin-induced cardiac toxicity [4]. Mitochondrial oxidative damage and calcium overload, both of which are associated with DOX toxicity [5-8], are potent inducers of the mitochondrial permeability transition (MPT) [9]. Consequently, the pro-oxidant nature of DOX has been implicated as a primary cause for its toxicity. In fact, several authors have demonstrated that DOX and its metabolites decrease the mitochondrial calcium loading capacity that determines MPT pore opening, both *in vitro* [10, 11] and *in vivo* [5-8]. The evidence clearly suggests that, by inducing the MPT, DOX interferes with mitochondrial calcium regulation, which may be one of the principal features in the pathogenesis of DOX-induced cardiomyopathy. Furthermore, enhanced induction of the MPT in heart mitochondria may also be responsible for the decreased respiratory rates observed in heart mitochondria isolated from DOX-treated animals [12].

The oxidation of specific thiol residues in mitochondrial proteins has been described as a critical regulator of

Abbreviations: DOX) doxorubicin; SAL) saline; MPT) mitochondrial permeability transition; ANT) adenine nucleotide translocator; DTT) dithiothreitol; BSA) bovine serum albumin; TBARS) thiobarbituric acid-reactive substances.

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MPT induction [13]. The protein containing critical thiol residues has not been definitively identified, but was suggested to be the adenine nucleotide translocator (ANT) [14]. The correlation between mitochondrial glutathione and thiol redox status, induction of the MPT, and inhibition of mitochondrial respiration suggests possible causal relationships amongst these otherwise independent events. However, it has yet to be demonstrated that oxidation of thiol groups caused by DOX-induced oxidative stress [15] is causally related to rendering mitochondria more susceptible to induction of the permeability transition. Also, it remains to be determined if the inhibition of mitochondrial respiration is a direct effect of DOX-induced thiol oxidation or if it is secondary to induction of the MPT.

The present investigation was thus designed to assess whether increased MPT induction caused by *in vivo* DOX treatment is responsible for a secondary inhibition of state 3 respiration. We also investigated whether changed thiol status impacts in regulating both altered MPT and respiration. The hypothesis was that cyclosporin A could revert the inhibition of respiration observed in DOX-treated animals. Also, heart mitochondria from DOX-treated samples were suspected to be more susceptible to the MPT induced by diamide, and that dithiothreitol (DTT), as a thiol reducing agent, is able to restore normal mitochondrial respiration.

MATERIALS AND METHODS

Chemicals. Ultra-pure sucrose was obtained from Schwarz/Mann Biotech (USA). DOX was purchased from Pharmacia & Upjohn Co. (USA). Cyclosporin A was a gift from Sandoz Pharmaceuticals (USA). All other chemicals were of the highest grade available from Sigma Chemical Co. (USA).

Animals and treatment protocol. Male Sprague–Dawley rats (Harlan Labs, USA) weighing 200-300 g were maintained in AAALAC (Association for Assessment and Accreditation of Animal Laboratory Care)-accredited, climate-controlled facilities and allowed free access to food (Purina Chow) and water. All studies were carried out in accordance with the *Guide for the Care and Use of Laboratory Animals*. Rats were randomly divided in two experimental groups: saline (SAL)-treated and DOX-treated. Rats received seven weekly subcutaneous (s.c.) injections of either DOX (2 mg/kg) or an equivalent volume of SAL solution (1 ml/kg). DOX dosage was established on the basis of previous studies that showed cardiac mitochondrial damage and histopathology [6, 7]. The animals were killed by decapitation one week after the last injection and the hearts immediately excised to cold buffer for mitochondrial isolation. Body and heart weights were recorded on the day of the experiments. At that time, DOX-treated rats had a lower heart (1.17 ± 0.09 versus 1.54 ± 0.06 g, $p < 0.05$, $n = 10$) and body (287.3 ± 5.6 versus 386.6 ± 6.6 g, $p < 0.05$, $n = 10$) weight.

Isolation of cardiac mitochondria. Cardiac mitochondria were prepared by differential centrifugation. Briefly, the hearts were finely minced in an ice-cold isolation medium containing 250 mM sucrose, 1 mM EGTA, 10 mM HEPES-KOH (pH 7.4), and 0.1% defatted bovine serum albumin (BSA). The minced tissue was suspended in 40 ml of the isolation medium containing 0.5 mg protease type VIII (Sigma, P5390) per g of tissue and homogenized with a tightly fitted homogenizer (Teflon/glass). The suspension was incubated for 1 min (4°C) and then re-homogenized. The homogenate was then centrifuged at 9500*g* for 10 min. The supernatant was decanted and the pellet was gently re-suspended to its original volume. The suspension was centrifuged at 900*g* for 10 min, and the resulting supernatant was centrifuged at 9000*g* for 10 min. This pellet was resuspended using a brush and repelleted twice at 9000*g* for 10 min. EGTA and defatted BSA were omitted from the final washing medium. Mitochondrial protein content was determined by the Bradford method calibrated with BSA [16]. The yield of mitochondrial protein (mg protein/g heart tissue) was not different between treatment groups (5.5 ± 0.5 for SAL and 5.2 ± 0.2 for DOX group, $n = 8$).

Oxygen consumption. Oxygen consumption by isolated heart mitochondria was monitored polarographically with a Clark oxygen electrode connected to a suitable recorder. Reactions were carried out at 25°C in 1 ml of media containing 100 mM KCl, 50 mM sucrose, 10 mM Tris-Mops, 10 μM EGTA, and 2.5 mM KH_2PO_4 (pH 7.4). Mitochondria were suspended at a concentration of 0.25 mg/ml in the respiratory medium. State 4 respiration was measured in the presence of 5 mM glutamate/malate and ADP (210 nmol) was added to induce state 3 respiration. The ADP/O was calculated according to standard procedures [17]. Where indicated, Trolox (100 μM), DTT (1 mM), and cyclosporin A (0.5 μM) were added 3 min before mitochondrial energization.

Mitochondrial swelling. Mitochondrial volume changes were estimated by monitoring changes in light scattering measured at 540 nm. The assays were performed in 1.5 ml of non-ionic media composed of 200 mM sucrose, 10 mM Tris-Mops, 10 μM EGTA, 5 mM KH_2PO_4 (pH 7.4, 25°C), and 2 μM rotenone to which was added 0.5 mg of mitochondrial protein. The incubation medium is different from the one used for respiratory experiments in order to provide better osmotic stability for mitochondria. Succinate (4 mM) was added 2 min after calcium addition (200 nmol $CaCl_2$/mg protein). Diamide concentrations ranging from 0.665 to 13.33 μM were added 2 min after calcium addition. Initial rates of mitochondrial swelling were measured for at least 3 min after the onset of the decrease in absorbance. The time elapsed between diamide addition and the beginning of the initial decrease in the suspension absorbance was also recorded.

Extraction and quantification of CoQ_9, CoQ_{10}, and vitamin E. Aliquots of mitochondria containing 1 mg of protein/ml were extracted according to the method described by Takada et al. [18]. The extract was evaporated to dryness under a stream of N_2 and resuspended in absolute ethanol. CoQ content was determined by reverse-phase HPLC (Spherisorb RP18, S5ODS2 column). Samples were eluted with methanol–heptane (10 : 2 v/v) at a flow rate of 2 ml/min. Detection was performed by an ultraviolet (UV) detector at 269 nm. Vitamin E was extracted and quantified by following the method described by Takayanagi et al. [19]. The extract was evaporated to dryness under a stream of N_2 and resuspended in *n*-hexane. Vitamin E content was determined by reverse-phase HPLC (4.6 × 200 mm; Spherisorb S10w column). Samples were eluted with *n*-hexane supplemented with 0.9% methanol at a flow rate of 1.5 ml/min. Detection was performed by a UV detector at 287 nm.

Thiobarbituric acid-reactive substances (TBARS) determination. TBARS content in mitochondrial samples was measured by HPLC according to Wong et al. [20]. Liquid chromatography was performed using Gilson (USA) HPLC apparatus with a reverse phase column (RP18, S5 ODS_2). The samples were eluted from the column at a flow rate of 1 ml/min and detection was performed at 532 nm. The TBARS content in the samples was calculated from the standard curve prepared using the thiobarbituric–malonyldialdehyde complex and expressed in nmol/mg protein.

Statistical analysis. The results are presented as mean ± SEM of at least five independent experiments. Statistical comparison between selected experimental groups was performed by using Student's paired *t* test or paired ANOVA, followed by Bonferroni's post-test. A $p < 0.05$ was considered statistically significant.

RESULTS

In our model for *in vivo* DOX-induced mitochondrionopathy, oxidative stress to mitochondrial samples was confirmed on the basis of drug-related changes in thiobarbituric acid-reactive substances (TBARS) and vitamin E and coenzyme Q content as quantified by high-performance liquid chromatography. Despite no changes in the mitochondrial content of coenzymes Q_9 and Q_{10}, DOX treatment resulted in a 35% increase in cardiac TBARS and a 25% decrease in vitamin E content (table).

Diamide, which is known to promote thiol cross-linking [21], was used to titrate the rate of mitochondrial swelling in the presence of calcium in order to assess treatment-related alterations in the thiol-dependent regulation of the MPT complex, as described previously [13, 21]. The rational behind this protocol is that oxidation of thiol groups in critical elements of the MPT pore complex would be manifested as a susceptibility to diamide. Mitochondrial swelling in both saline and DOX-treated animals demonstrated biphasic kinetics (Fig. 1a). Swelling curves in the presence of 13 μM diamide showed that mitochondria from DOX-treated animals displayed a shorter lag phase when compared with the corresponding control (9.8 ± 0.5 versus 12.6 ± 0.9 min, $p < 0.05$, $n = 4$). Furthermore, the rate of swelling with increasing concentrations of diamide was significantly greater in cardiac mitochondria isolated from DOX-treated animals compared to cardiac mitochondria isolated from saline-treated control rats (Fig. 1b). In particular, for the highest diamide concentration (13 μM), swelling rates were 0.0197 ± 0.0018 optical density unit per min for the DOX group compared to 0.0123 ± 0.0023 optical density unit per min for samples from saline treated rats ($p < 0.05$, $n = 4$) (Fig. 1b).

Levels of coenzyme Q_9, coenzyme Q_{10}, vitamin E, and malonyldialdehyde in heart mitochondria isolated from saline- and DOX-injected animals (values are means ± SEM of seven different mitochondrial samples; * $p < 0.05$ versus saline control)

Experimental group	Control (saline)	DOX
Coenzyme Q_9 (pmol/mg protein)	8573.3 ± 298.3	8827.8 ± 588.0
Coenzyme Q_{10} (pmol/mg protein)	333.0 ± 16.5	335.6 ± 37.7
TBARS (nmol/mg protein)	1.61 ± 0.22	2.19 ± 0.18*
Vitamin E (nmol/mg protein)	0.55 ± 0.06	0.41 ± 0.04*

Treatment of rats *in vivo* with DOX resulted in a statistically significant inhibition of state 3 respiration for cardiac mitochondria (Fig. 2b), whereas there was no treatment-related effect on state 4 respiration (Fig. 2a). Although not shown, there was no treatment-related difference in the phosphorylation efficiency of cardiac mitochondria isolated from DOX compared to saline treated rats (ADP/O ratio was 2.7 ± 0.1 versus 2.8 ± 0.2, respectively, $n = 7$), which is in accordance with previously published results [12]. Glutamate/malate was the substrate used in the experimental protocol as previous works showed a stronger inhibition of respiration by DOX when using this substrate [12]. The DOX-induced inhibition of cardiac mitochondrial state 3 respiration was partially reversed by both DTT and cyclosporin A. DTT by itself, however, exhibited a direct inhibition of state 3 respiration in cardiac mitochondria from saline-treated rats.

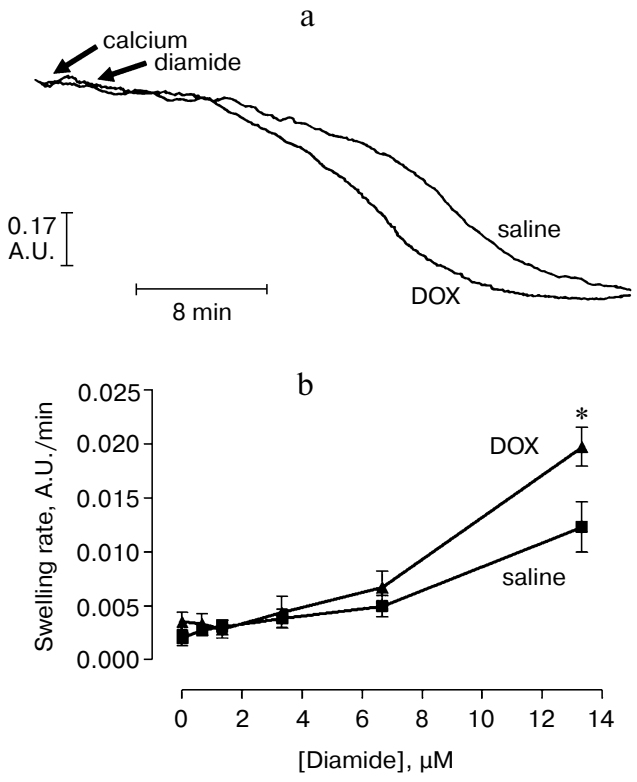

Fig. 1. a) Typical recording of mitochondrial swelling induced by calcium and diamide (13 μM) in heart mitochondria from DOX- or saline-treated rats. Mitochondrial swelling was followed by the variation of absorbance at 540 nm. The assays were performed in 1.5 ml of a non-ionic media composed of 200 mM sucrose, 10 mM Tris-Mops, 10 μM EGTA, 5 mM KH_2PO_4 (pH 7.4, 25°C), and 2 μM rotenone to which was added 0.5 mg of mitochondrial protein. Mitochondria were energized with 4 mM succinate. Diamide concentrations ranging from 0.665 to 13.33 μM were added 2 min after calcium (200 nmol $CaCl_2$/mg protein) addition. The recording is typical of four different preparations. b) The initial rate of mitochondrial swelling induced by calcium in the presence of diamide in heart mitochondria from saline- and DOX-treated rats. The rates expressed in the graph were recorded in the initial phase of the absorbance decrease as described in "Materials and Methods". The values are means ± SEM, * $p < 0.05$ versus saline, $n = 4$.

DISCUSSION

The widely described effect of DOX to generate oxygen free radicals and cause generalized oxidative stress was evidenced by the loss of vitamin E and accumulation of products of lipid peroxidation (TBARS) in cardiac tissue from drug-treated rats. The results are consistent with previous reports that DOX causes oxidative stress in heart mitochondria [15, 22], although no changes in the levels of coenzyme Q_9 and Q_{10} were observed. The hypothesis of the present investigation was that the increased cardiac MPT observed in DOX-treated rats is at least in part responsible for the inhibition of respiratory function in cardiac mitochondria. Also, our secondary hypothesis was that DOX-induced oxidation of thiol residues in mitochondrial proteins [15, 22] is responsible not only for altering the MPT sensitivity to calcium and thiol-targeted agents, but also for the effects of *in vivo* DOX treatment on mitochondrial respiration. Diamide and DTT, two thiol specific agents, were used *ex vivo* to demonstrate that thiol redox status is changed in cardiac mitochondria from DOX-treated rats, which may be partially responsible for the altered mitochondrial bioenergetics and opening of the MPT pore.

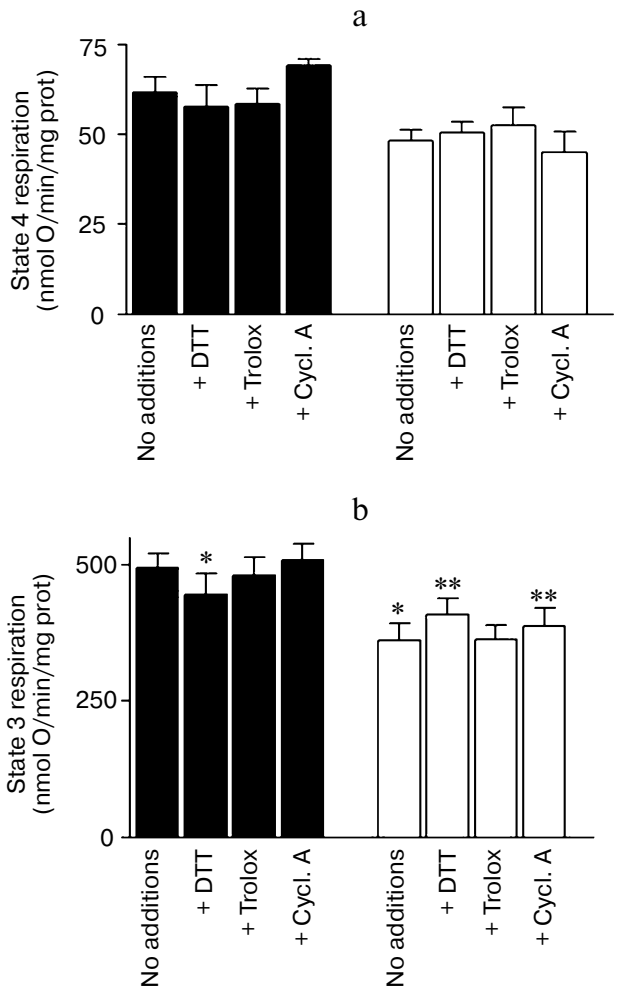

Fig. 2. Mitochondrial oxygen consumption during state 4 (a) or state 3 (b) respiration in the presence of DTT (1 mM), Trolox (100 μM), and cyclosporin A (Cycl.A, 0.5 μM). Reactions were carried out at 25°C in 1 ml of media containing 100 mM KCl, 50 mM sucrose, 10 mM Tris-Mops, 10 μM EGTA, and 2.5 mM KH_2PO_4 (pH 7.4). Mitochondria were suspended at a concentration of 0.25 mg/ml in the respiratory medium. State 4 respiration was measured in the presence of 5 mM glutamate/malate, and ADP (210 nmol) was added to induce state 3 respiration. Where indicated, DTT (1 mM), Trolox (100 μM), and cyclosporin A (0.5 μM) were added 3 min before mitochondrial energization. Cardiac mitochondria were isolated from saline- (filled bars) or DOX-treated (open bars) rats. The values are means ± SEM, $n = 6$; * $p < 0.05$ versus saline without additions; ** $p < 0.05$ versus DOX without additions.

In fact, the results indicate that heart mitochondria isolated from DOX-treated rats were more susceptible to *ex vivo* diamide-induced swelling. Diamide, a bifunctional thiol cross linking agent [21], increases the probability for pore opening due to oxidation of protein thiol sites that are in equilibrium with the mitochondrial pool of pyridine nucleotides and reduced glutathione [13]. Although the specific regulatory protein has not been conclusively identified, it has been suggested that the adenine nucleotide translocator (ANT) is a major target for thiol-dependent induction of the MPT and is most likely the protein containing the redox sensor of the pore [23, 24]. However, there remains some controversy regarding ANT as a primary component of the pore complex [25]. The shorter lag-phase preceding diamide-induced swelling in heart mitochondria from DOX-treated animals suggests a greater degree of oxidized proteins involved in inducing the MPT pore. Initial swelling rates were also higher for heart mitochondria from DOX-treated animals. The results suggest increased amounts of oxidized thiol residues in proteins of the MPT pore complex in heart mitochondria from DOX-treated rats, which may be a relevant factor for the enhanced calcium-induced MPT detected in those animals. As mentioned above, the ANT is a primary target for thiol-dependent regulation of the MPT pore. In fact, it has previously been demonstrated that decreased functional ANT protein numbers exist in hearts from DOX-treated animals [7]. The decrease in mitochondrial antioxidants (such as vitamin E) may also be relevant because it could contribute to the lack of protection of critical protein thiol groups in the pore complex against oxygen free radicals derived from DOX redox cycling.

State 4 respiration in DOX-treated animals was not significantly different from saline control, which is consistent with previously published results [6]. The higher ionic strength of the buffer used in these experiments may explain slight differences with previous reports [12]. Mitochondrial state 3 respiration was significantly depressed in DOX-treated animals, which again confirms earlier reports [12]. The novelty in this study was that DTT and cyclosporin A, when added *ex vivo*, were independently able to partially reverse the inhibitory effect of *in vivo* DOX treatment on state 3 respiration, indicating that the inhibition of mitochondrial respiration by DOX is in part secondary to oxidation of thiol groups and induction of the MPT, rather than exclusively being a direct effect of the drug itself. In contrast to DTT, Trolox did not restore mitochondrial respiration, which can be explained by a different mechanism of action (unrelated to specific targeting of thiol groups).

An alternate interpretation of our data is that the oxidation of thiol groups occurs secondarily, rather than causally, to MPT induction, which would explain the effect of DTT on mitochondrial respiration of DOX-treated animals. Evidence supporting this proposal is the result obtained by Zhou et al. [7]. The authors demonstrated that unlike cyclosporin A, DTT is not able to reverse the decrease in mitochondrial calcium loading capacity in cardiac mitochondria isolated from DOX-treated rats. Nevertheless, the lack of inhibition of the MPT by DTT could also indicate a more permanent alteration of protein thiol residues *in vivo* that cannot be reversed by adding thiol reducing agents *ex vivo*. The data obtained with diamide titration suggests that oxidation of thiol residues occurs *in vivo* during DOX-treatment and is relevant for the decreased calcium loading capacity [7, 12].

The fact that both DTT and cyclosporin A, the specific MPT inhibitor [26], were able to partially restore respiration in cardiac mitochondria isolated from DOX-treated animals suggests that (i) the inhibition of mitochondrial respiration is secondary to MPT induction and (ii) DOX reversibly affects one or more proteins associated with oxidative phosphorylation that is also modulated by its thiol oxidation state. This later proposal is supported by the fact that DTT inhibits state 3 respiration in heart mitochondria from saline-treated rats (Fig. 2b), as opposed to the partial improvement of respiration observed in the DOX-treated group. One protein involved in mitochondrial energy metabolism and whose thiol status determines its activity is mitochondrial complex I [27], the activity of which has been shown to be decreased in DOX-treated rats [12]. The mechanism relating decreased mitochondrial respiration and MPT alterations in DOX-treated rats is so far unknown. There are nevertheless two likely possibilities. The first is that cyclosporin A may restore the activity of mitochondria that were previously non-functional due to pore opening. Another hypothesis is that the binding of cyclosporin A to cyclophilin D [28] may cause the disaggregation of preformed pores, thus allowing a crucial component to again become involved in the oxidative phosphorylation process.

In conclusion, the results of the current investigation provide the first evidence that *in vivo* oxidation of critical thiol residues in mitochondrial proteins following *in vivo* DOX treatment affects both the MPT pore and oxidative phosphorylation machinery. MPT induction would then be in part responsible for the observed decrease in mitochondrial respiration. A particular consequence of DOX toxicity is that mitochondrial proteins involved in both oxidative phosphorylation and in the MPT pore complex may be significantly more susceptible to external stimuli that would lead to an increase of intracellular oxidative stress. The results also establish a cause–effect relation between enhanced MPT activation and decreased mitochondrial respiration, which is indicated by the partial restoration of mitochondrial respiration by cyclosporin A, a potent and specific inhibitor of the MPT. Accordingly, the MPT may play a so far unknown role in modulating the adverse bioenergetic and functional consequences associated with DOX treatment.

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