Cumulative and Irreversible Cardiac Mitochondrial Dysfunction Induced by Doxorubicin

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ABSTRACT

Interference with mitochondrial calcium regulation is proposed to be a primary causative event in the mechanism of doxorubicin-induced cardiotoxicity. We previously reported disruption of mitochondrial calcium homeostasis after chronic doxorubicin administration (Solen et al. Toxicol. Appl. Pharmacol., 129: 214–222, 1994). The present study was designed to characterize the dose-dependent and cumulative interference with mitochondrial calcium regulation and to assess the reversibility of this functional lesion. Sprague Dawley rats were treated with 2 mg/kg/week doxorubicin s.c. for 4–8 weeks. With succinate as substrate, cardiac mitochondria isolated from rats after 4 weeks of treatment with doxorubicin expressed a lower calcium loading capacity compared with control. This suppression of calcium loading capacity increased with successive doses to 8 weeks of treatment (P < 0.05) and persisted for 5 weeks after the last doxorubicin injection, and was corroborated by dose-dependent and irreversible histopathological changes. Preincubation of mitochondria with tamoxifen, DTT, or monobromobimane did not reverse the diminished calcium loading capacity caused by doxorubicin. In contrast, incubation with cyclosporin A abolished any discernible difference in mitochondrial calcium loading capacity between doxorubicin-treated and saline-treated rats. The decrease in cardiac mitochondrial calcium loading capacity was not attributable to bioenergetic changes in the electron transport chain, because the mitochondrial coupling efficiency was not altered by doxorubicin treatment. However, the ADP/ATP translocase content was significantly lower in mitochondria from rats that received 8 weeks of doxorubicin treatment. These data indicate that doxorubicin treatment in vivo causes a dose-dependent and irreversible decrease in mitochondrial calcium loading capacity. Suppression of adenine nucleotide translocase content may be a key factor altering the calcium-dependent regulation of the mitochondrial permeability transition pore, which may account for the cumulative and irreversible loss of myocardial function in patients receiving doxorubicin chemotherapy.

INTRODUCTION

DOX, an anthracycline drug widely used for the treatment of various cancers, causes a cumulative dose-dependent cardiac toxicity that is characterized by an irreversible dilated cardiomyopathy and congestive heart failure (1, 2). Although several mechanisms have been suggested to explain this cardiotoxicity, the exact mechanism and its metabolic consequences remain unclear. Biochemical and physiological data favor the hypothesis that DOX causes the formation of free radicals that stimulate lipid peroxidation and alter cellular membrane integrity (3–7). Accordingly, a number of efforts have been made to use exogenously supplemented antioxidants to protect the heart from DOX-induced damage (8, 9). However, the effectiveness is varied and limited. Alternatively, transgenic mice that overexpress methallothionein are resistant to DOX-induced morphological changes in the myocardium and creatine kinase release from the heart (10). Yet, the protection of methallothionein on DOX cardiotoxicity is still controversial (11). Additional studies are needed to delineate the exact mechanism of DOX-induced oxidative damage, particularly to explore the clinical potential of antioxidants in protecting against DOX cardiotoxicity.

Induction of the MPT by oxidants has been proposed to play an important role in chemical-induced tissue injury and cell killing both in vitro and in vivo (12–17). This leads to the implication of oxidative alteration of mitochondrial calcium transport as the mechanism of toxicity. DOX and its aglycone metabolite alter mitochondrial calcium retention and diminish the capacity of isolated mitochondria to accumulate calcium in vitro (18–20) as well as in vivo (21, 22). This DOX-induced interference with mitochondrial calcium regulation is a consequence of selective activation of the CsA-sensitive calcium release channel (20, 23). A relationship has been suggested between the induction of mitochondrial calcium cycling and DOX cardiotoxicity. Ruthenium red, an inhibitor of mitochondrial calcium uptake, and CsA protect cardiac myocytes from DOX-induced cell killing (22, 24). This is also supported by the observation of in vivo prevention of DOX cardiotoxicity by CsA or FK506 (25).

We recently demonstrated that activation of the selective CsA-sensitive calcium channel of cardiac mitochondria by DOX in vitro (23) is also manifested in cardiac mitochondria isolated from rats after chronic in vivo treatment with DOX (21). Furthermore, we have characterized the cumulative dose-dependent interference with mitochondrial calcium transport by DOX and found that this is manifested as an increased sensitivity to calcium-induced injury in cardiac myocytes isolated from rats exposed in vivo (22). These data suggest that interference with cardiac mitochondrial calcium homeostasis may be a critical factor underlying the DOX-induced cardiotoxicity and may be responsible for the clinical manifestations of cardiomyopathy. Because the DOX-induced cardiomyopathy is irreversible, we question whether the disruption of mitochondrial calcium regulation can be restored after discontinuation of DOX administration. In this study, we assessed the dose-dependence and reversibility of the decreased mitochondrial calcium loading capacity and explored the potential mechanisms underlying this important pathogenic process.

MATERIALS AND METHODS

Chemicals. D-mannitol was purchased from Aldrich Chemical Co. (Milwaukee, WI) and ultra-pure sucrose from Schwarz/Mann Biotech (Cleveland, OH). DOX was purchased from Pharmacia & Upjohn Co. (Kalamazoo, MI). CsA was a generous gift from Sandoz Pharmaceuticals (East Hanover, NJ). CsATR was a gift from Dr. A. Starkov (Moscow State University). All of the other chemicals were of the highest grade available from Sigma Chemical Co. (St. Louis, MO).

Animals. Male Sprague Dawley rats (Harlan Labs, Madison, WI) were maintained in AAALAC-accredited, climate-controlled facilities and allowed free access to food (Purina Chow) and water. Rats received 4–8 weekly s.c. injections of either DOX (2 mg/kg) or an equivalent volume of saline (1 ml/kg). The animals were killed by decapitation 1, 3, or 5 weeks after the last injection and the heart immediately excised to cold buffer.

Isolation of Cardiac Mitochondria. Cardiac mitochondria were isolated by differential centrifugation after homogenization and proteolytic digestion as
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Mitochondrial calcium loading capacity is a measure of the total amount of Ca$^{2+}$ that mitochondria are capable of accumulating from the incubation medium before undergoing the MPT and releasing it back to the medium. This process is a measure of calcium-induced calcium release, which may be synonymous with induction of the Ca$^{2+}$-dependent MPT. It was estimated by using a calcium-selective electrode made of commercial calcium-ionophore membrane from Fluke Chemical Corp. (Milwaukee, WI) and an AgCl reference electrode. The calcium electrode was calibrated by sequential additions of CaCl$_2$ solution: 200 μM, 1 μM, 40 μM, 80 μM, and 100 μM (total 260 μM). The calcium loading capacity is expressed as nmoles of Ca$^{2+}$ per mg of mitochondrial protein.

Light and Electron Microscopy. Small blocks of cardiac muscle from the mid-portions of the lateral wall of the left ventricle were fixed in 2.5% glutaraldehyde-2% formaldehyde in 0.1 M phosphate buffer (pH 7.4), postfixed in 1% OsO$_4$ in the same buffer, dehydrated in graded acetone solutions, and embedded in epon-araldite. One-μm thick sections for light microscopy were stained at 55°C with 0.25% toluidine blue in 1% sodium borate after treatment with 0.1 N HCl (28) and 1% ruthenium red (29). Thin sections for electron microscopy were stained with lead citrate and uranyl acetate.

Cytchrome Content. Mitochondrial cytochrome content was determined spectrophotometrically as described previously (30, 31). Two mg of isolated heart mitochondria were dissolved in 2 ml of 2% Triton X-100 in 0.1 M phosphate buffer (pH 7.0). The reaction was started with the addition of 5 mM succinate. Absorbance at 560 nm was recorded for 4 min before mitochondria.

Mitochondrial Calcium Loading Capacity. Mitochondrial calcium loading capacity was estimated using an ion-selective electrode to measure the distribution of the tetraphenylphosphonium ion (TPP$^+$) according to previously established methods (27). The reference electrode was AgCl. Mitochondria (0.25 mg) were suspended in 1 ml of medium containing 200 mM sucrose, 10 mM Tris-MOPS (pH 7.2), 10 mM KH$_2$PO$_4$, 1 μM rotenone, and 1 μg/ml oligomycin. Once a steady-state membrane potential was established, sequential additions of Ca$^{2+}$ were made to calibrate the Ca$^{2+}$ electrode (20 μM, 20 μM, 40 μM, 80 μM, and 100 μM, total 260 μM). The reaction was started by adding 5 mM succinate. Absorbance at 560 nm was recorded for 4 min for 4 min before adding succinate to start the reaction. Where indicated, CsA (1 μM) was added before mitochondria.

Three control rats from time periods 6–1 week and 8–1 week after the last of 6 and 8 weekly injections, respectively. 8 + 3w and 8 + 5w represent mitochondria isolated 3 or 5 weeks after the last of 8 weekly injections. Data are expressed as the mean ± SE of three separate mitochondrial preparations. *, a statistically significant difference compared with saline control (P < 0.05).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>n$^a$</th>
<th>Score$^b$</th>
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<tr>
<td>Control$^c$</td>
<td>10</td>
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<tr>
<td>DOX 6 + 1w</td>
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<td>DOX 8 + 1w</td>
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<td>DOX 8 + 3w</td>
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<td>DOX 8 + 5w</td>
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$^a$ There were originally 10 rats in each treatment group; some rats died during DOX treatment.

$^b$ Means of values obtained from blinded examination of coded sections (by R. L. L. and M. K. F.) using the 0–3 scoring method of Billingham et al. (57) and Bristow et al. (58). There was a close correspondence of scores between the two scorers.

$^c$ Three control rats from time periods 6 + 1w and 8 + 5w each; and two control rats each from 8 + 1w and 8 + 3w treatment groups. 6 + 1w and 8 + 1w, heart tissue isolated from rats 1 week after the last of 6 and 8 weekly injections, respectively. 8 + 3w and 8 + 5w, heart tissue from rats 3 and 5 weeks, respectively, after the last of 8 weekly treatments.
wavelengths were then measured: 550–535 (cytochrome c), 554–540 (cytochrome c1), 563–577 (cytochrome b), and 605–630 nm (cytochrome a). The calculation of cytochrome content was based on the millimolar extinction coefficient for the respective cytochromes as reported by Williams (30).

Mitochondrial Respiration. Mitochondria were incubated at a concentration of 0.25 mg/ml in 1 ml of 200 mM sucrose-10 mM Tris-MOPS (pH 7.2)-10 mM KH2PO4 supplemented with 5 mM glucose and 20 units of hexokinase. The incubations were performed at 30°C in a closed and magnetically stirred reaction chamber equipped with a Clark-type electrode. The reactions were started by adding 5 mM glutamate plus malate. State 3 respiration was initiated by adding 50 μM ADP. After static state 3 respiration was obtained (about 2 min), cATR was then added. A titration curve was obtained by stepwise addition of cATR to respiring isolated mitochondria. Plots of O2 consumption versus cATR appeared biphasic, with an increasing inhibitory segment followed by a steady respiration indicating that state 3 respiration was completely inhibited. The amount of cATR corresponding to complete inhibition of state 3 respiration was used to estimate ANT content assuming a 1:1 binding stoichiometry, which was expressed as cATR content per mg mitochondrial protein.

Statistical Data Analysis. All of the data were expressed as the mean ± SE of three to five independent experiments. The data were analyzed using one-way ANOVA, and differences among individual means were compared using the Bonferroni/Dunn test. A probability of P < 0.05 was used as the criterion for statistical significance.

RESULTS

Mitochondrial Calcium Loading Capacity. Mitochondria possess a finite capacity for accumulating calcium before undergoing the calcium-dependent MPT. In this study we define mitochondrial calcium loading capacity as the total amount of Ca2+ that mitochondria are able to accumulate before they start releasing it back to the incubation medium. This is an important parameter to characterize mitochondrial integrity and indicates the resistance of mitochondria to induction of the Ca2+-dependent MPT. As shown in Fig. 1, the Ca2+ electrode was calibrated by sequential additions of CaCl2 solution. Succinate-supported mitochondria started to take up Ca2+ to a point (downward deflection in the trace), then released Ca2+ back into the medium (upward deflection, Fig. 1, c). This Ca2+-induced mitochondrial Ca2+ release indicated the opening of the MPT pore, which was
confirmed by adding CsA (Fig. 1, trace d), a specific inhibitor of the mitochondrial pore (32), to block the Ca\(^{2+}\) release. In the presence of CsA, mitochondria accumulated and retained all of the added Ca\(^{2+}\) (Fig. 1, d). Associated with this Ca\(^{2+}\)-induced mitochondrial Ca\(^{2+}\) release was a depolarization of the \(\Delta \Psi\) (Fig. 1, a, upward deflection), which was prevented by CsA (Fig. 1, b). Induction of the MPT by loading with Ca\(^{2+}\) was further evidenced by the fact that adding CsA prevented the calcium-induced mitochondrial swelling (Fig. 2). It should be noted that the prevention of calcium-induced mitochondrial swelling by CsA was not complete, as it was for \(\Delta \Psi\). This is because of different experimental conditions. Unlike the \(\Delta \Psi\) experiment conducted in an open chamber, mitochondrial swelling was performed in a small cuvette in which oxygen diffusion was limited. Under these conditions, \(\Delta \Psi\) consumption by Ca\(^{2+}\)-uncoupled mitochondria leads to relative hypoxia, which causes mitochondrial swelling independent of CsA-sensitive MPT (33).

Irreversible Mitochondrial Dysfunction. Mitochondria isolated from rat hearts receiving 4 weeks of treatment with DOX expressed a slightly lower calcium loading capacity compared with control (Fig. 3). The decrease in mitochondrial calcium loading capacity was most evident after 8 weeks of treatment (\(P < 0.05\)). The diminished capacity to accumulate Ca\(^{2+}\) did not reverse over 3–5 weeks after discontinuation of DOX administration, which is consistent with the clinically observed irreversible cardiomyopathy.

Histopathological Examination. When examined by light and electron microscopy, cardiac myocytes of saline-injected rats had normal morphology (Table 1; Figs. 4A and 5A). Incipient pathology occurred in most cardiac myocytes of rats receiving 6 weeks of DOX injections (Figs. 4B and 5B). The most common ultrastructural change was a slight enlargement of the T-tubules that was often accompanied by distension of the sarcoplasmic reticulum (Fig. 4B). After 8 weeks of treatment with DOX, frank ultrastructural pathology was apparent, including cytoplasmic vacuolation, mitochondrial and myofibrillar damage and loss, and cellular edema. Ultrastructural injury was not repaired at 3 and 5 weeks after cessation of DOX treatment and became even more extensive (Fig. 4, C and D), which was correlated with the persistent decrease in mitochondrial calcium loading capacity. Worsening pathology with length of DOX administration was also evident at the light microscopic level (Fig. 5, B, C, and D), with increasing duration of treatment causing increased degrees of cytoplasmic vacuolization and frank degeneration.

Effect of DOX Treatment on the Bioenergetics of Mitochondria. It is well established that the MPT is strongly influenced by the transmembrane potential (34). Previous studies demonstrated that DOX suppresses the expression of COXII of COX (35), which is a pivotal enzyme in the mitochondrial electron transport chain. Therefore, the mitochondrial content of cytochromes a, b, c, and c1 was determined as an indication of the efficiency of the electron transport chain. However, no differences were found in the content of any cytochrome between cardiac mitochondria from DOX-treated and saline-treated animals (Fig. 6). There is still a possibility that DOX treatment may depress the activity of COX by means other than modulating gene expression. Instead of estimating the activity of COX, mitochondrial coupling efficiency was compared by titrating \(\Delta \Psi\) with FCCP. Mitochondrial coupling efficiency is indicative of respiratory chain efficiency and may directly influence mitochondrial calcium loading capacity. As illustrated in Fig. 7, DOX treatment did not significantly alter the relationship between FCCP concentration and \(\Delta \Psi\), which indicated that alteration of mitochondrial bioenergetics is not a critical factor causing the decrease in mitochondrial calcium loading capacity by DOX.

Effect of CsA and Antioxidants on Mitochondrial Calcium Loading Capacity. Considering the hypothesis of the oxidative alteration of mitochondria by DOX treatment, it might be possible to reverse the decreased mitochondrial calcium loading capacity by adding selected antioxidants. Previous studies have reported that TAM, DTT, and mBrB inhibit the induction of the MPT by Ca\(^{2+}\) \textit{in vitro} (20, 33, 36, 37). Cardiac mitochondria from DOX-treated rats preincubated with TAM, however, exhibited only a marginal increase in calcium loading capacity (\(P > 0.05\)), whereas mBrB (data not shown) and DTT did not show any protection compared with control (Fig. 8). Interestingly, in the presence of CsA, mitochondria from DOX-treated animals displayed a >2-fold increase in calcium loading capacity, which was not different from saline control in the presence of CsA. This indicates that the reversible oxidative alteration of mitochondrial proteins is not a major factor responsible for the decrease in mitochondrial calcium loading capacity caused by DOX.

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vivo treatment with DOX. cATR reacts with ANT in a 1:1 stoichiometry (39, 40). Accordingly, the ANT content in mitochondria can be estimated by titrating the active-state respiration with cATR as illustrated in Fig. 9A. It was found that ANT content was significantly diminished in cardiac mitochondria from DOX-treated animals compared with control (Fig. 9B). The ANT contents in treated and control animals were 1885 ± 686 and 1585 ± 31 (pmol cATR/mg mitochondrial protein), respectively. This 15% decrease in ANT content correlates with the decreased mitochondrial calcium loading capacity. Because ADP/ATP translocase is a principal component of the MPT pore complex (41, 42), it is not surprising that the decrease in translocase content correlates with the increased sensitivity of mitochondria to calcium-induced pore opening.

**DISCUSSION**

Induction of the MPT is implicated in the mechanism of cell injury caused by many chemicals (12–17), including the pathogenesis of DOX-induced cardiotoxicity (21, 23, 24). It has been proposed that DOX-induced free radical generation causes oxidative alteration of the MPT pore, which contributes to the development of DOX-induced cardiotoxicity. We recently reported that both cardiac mitochondria
Treatment with DOX suppresses COX gene expression (51). However, in the present study no difference was found in mitochondrial content of any of the cytochromes examined. Although the activity of COX was not estimated, the mitochondrial coupling efficiency was found to be similar between treated and control animals. Therefore, inhibition of mitochondrial bioenergetics does not appear to be responsible for the decrease in mitochondrial calcium loading capacity. TAM, DTT and mBrB, three antioxidants known to prevent induction of the MPT in vitro (20, 33, 36, 37) did not reverse the sensitivity of cardiac mitochondria from DOX-treated rats to induction of the calcium-dependent permeability transition. This suggests that oxidative alteration of mitochondrial proteins is not a critical factor responsible for the decreased calcium loading capacity.

A number of studies have demonstrated that ADP/ATP translocase is involved in the MPT, although the exact mechanism is unclear (42, 52, 53). Mitochondria from rat heart and liver vary in their ANT content (40, 54), which is proportional to their ability to accumulate calcium. In the present study, the content of this translocase was found to be less in cardiac mitochondria from DOX-treated rats compared with control. Moreover, the extent of decrease in this translocase content correlated with the decreased mitochondrial calcium loading capacity. Therefore, it may be that the decrease in ADP/ATP translocase content is responsible for the diminished mitochondrial calcium loading capacity after DOX treatment. Recently, Jeyaseelan et al. (38) reported that ADP/ATP translocase gene was down-regulated in cardiac myocytes exposed to DOX in culture. This suggests that the decrease in ANT content might be attributable to the altered regulation of gene expression caused by DOX. Considering the fact that the change in mitochondrial calcium loading capacity was irreversible, modulation in ANT gene regulation might be persistent as well. Interestingly, we recently found that mitochondrial DNA adducts persist for over 5 weeks after discontinuation of chronic DOX administration (55, 56). This dose-dependent and irreversible accumulation of DNA adducts correlates well with the cumulative and persistent histopathology and decrease in mitochondrial function.

In conclusion, we demonstrate that mitochondria from DOX-treated rats expressed a dose-dependent and irreversible decrease in calcium loading capacity that correlates with the accumulation of DNA adducts, histopathology, and clinically observed cardiomyopathy. We attribute the decrease in mitochondrial calcium loading capacity, in part, to the diminished ANT content and suggest that this altered regulation of mitochondrial calcium homeostasis may be a critical factor involved in the pathogenic pathway of the cumulative and irreversible cardiomyopathy associated with long-term DOX cancer chemotherapy.

REFERENCES

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