Doxorubicin-induced persistent oxidative stress to cardiac myocytes

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Abstract

We recently reported a cardioselective and cumulative oxidation of cardiac mitochondrial DNA (mtDNA) following subchronic administration of doxorubicin to rats. The mtDNA adducts persist for up to 5 weeks after cessation of doxorubicin treatment. Since the evidence suggests that this persistence of mtDNA adducts cannot be attributed to a lack of repair and replication, we investigated whether it might reflect a long-lasting stimulation of free radical-mediated adduct formation. Male Sprague–Dawley rats received weekly s.c. injections of either doxorubicin (2 mg/kg) or an equivalent volume of saline. Cardiac myocytes isolated from rats following 6 weekly injections of doxorubicin expressed a much higher rate of reactive oxygen species (ROS) formation compared to saline controls. This higher rate of ROS formation persisted for 5 weeks following the last injection. Associated with this was a persistent depression of GSH in heart tissue, while protein-thiol content was not markedly altered. These data suggest that the accumulation and persistence of oxidized mtDNA may be due, not to the stability of the adducts, but to some as yet undefined toxic lesion that causes long-lasting stimulation of ROS generation by doxorubicin. This persistent generation of ROS may contribute to the cumulative and irreversible cardiotoxicity observed clinically with the drug. © 2001 Elsevier Science Ireland Ltd. All rights reserved.

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1. Introduction

Long-term administration of doxorubicin (DOX), a potent antineoplastic agent for treating various human tumors, causes a cumulative dose-dependent cardiomyopathy. The mechanism of this DOX-induced cardiotoxicity is attributed to free radical generation, stimulation of lipid peroxidation, and subsequent alteration of cellular membrane integrity. This hypothesis is supported by the reported cytoprotective effect of antioxidants against DOX toxicity (Kimura et al., 2000; Wang and Kang, 1999; Wu and Kang, 1998). An increasing number of studies demonstrate that

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DOX causes a dose-dependent disruption of cardiac mitochondrial structure and bioenergetic function (Nicolay and De Kruijff, 1987; Papadopoulos et al., 1999; Praet and Ruyschaert, 1993; Solem et al., 1994, 1996), and implicate mitochondrial dysfunction in the pathogenesis of DOX-induced cardiomyopathy. We hypothesize that DOX-induced cardiotoxicity is mediated through altered mitochondrial function caused by DOX-induced free radical generation.

We recently demonstrated that DOX causes the preferential accumulation of 8-hydroxydeoxyguanosine (8OHdG) in cardiac mitochondrial DNA (mtDNA) (Palmeira et al., 1997). Moreover, this cardioselective oxidation of mitochondrial DNA is cumulative with repeated dosing and persists for 5 weeks after cessation of DOX treatment (Serrano et al., 1999). The accumulation and persistence of 8OHdG caused by DOX is in accord with the irreversible cardiomyopathy observed clinically. Since expression of the mitochondrial genome is essential to the integrity of the respiratory chain, oxidation of mtDNA may interfere with the expression of critical mitochondrial electron transport proteins, which might underlie the pathogenesis of DOX cardiomyopathy.

The formation of 8OHdG adducts is attributed to reactive oxygen species. Indeed, a strong correlation has been characterized between the rate of generation of hydroxyl free radicals in submitochondrial particles and the occurrence of 8OHdG adducts to the mitochondrial genome (Giulivi et al., 1995). Although a number of initial studies reported deficiencies in mtDNA repair mechanisms that might explain the persistence of mtDNA adducts (LeDoux et al., 1992; Miyaki et al., 1977; Niranjan et al., 1981; Stairs et al., 1983), recent evidence demonstrates that mitochondria are capable of repairing oxidative damage to mtDNA (Salazar and Van Houten 1997; Yakes and Van Houten, 1997). Moreover, mtDNA adducts can be eliminated by mitochondrial turnover. Thus, the persistence of mtDNA adducts is not likely attributable to the stability of the adducts in vivo. Therefore, we questioned whether the persistence of 8OHdG adducts might reflect prolonged stimulation of ROS generation and oxidative stress that continues long after termination of drug treatment.

2. Materials and methods

2.1. Materials

Doxorubicin was purchased from Pharmacia & Upjohn Co. (Kalamazoo, MI). 5-(and 6)-chloromethyl-2',7'-dichlorodihydro-fluorescein diacetate (CM-H$_2$DCFDA) was purchased from Molecular Probes (Eugene, OR). All other chemicals were of analytical grade and obtained from common commercial sources.

2.2. Animals

Male Sprague–Dawley rats (Harlan Labs, Madison, WI) weighing 175 ± 5 g were divided randomly between treatment groups and maintained in AAALAC-accredited, climate-controlled facilities and allowed free access to food (Purina Chow) and water. Rats received 6 weekly s.c. injections of either doxorubicin (2 mg/kg) or an equivalent volume of saline (1 ml/kg). The animals were killed by decapitation 1 or 5 weeks after the last injection. The hearts were excised immediately to isolate cardiac myocytes for determination of ROS generation or frozen in liquid nitrogen and stored at –80°C until assayed for GSH and protein-thiol.

2.3. Determination of reactive oxygen species (ROS)

ROS were determined fluorometrically using a Labsystems type 374 plate-reader fluorometer. Cardiac myocytes were isolated as previously described (Solem et al., 1996). Isolated cardiac myocytes were suspended in Rosewell Park Memorial Institute (RPMI) medium at a density of 5 × 10$^3$ live cells/ml. Cells were loaded with 50 μM CM-H$_2$DCFDA (prepared in DMSO) for 15 min at 37°C. Cell suspension (200 μl; 10$^5$ cells) were loaded into a 96-well plate. The formation of the oxidized derivative of CM-H$_2$DCF, was monitored fluorimetrically at an excitation wavelength
485 nm and an emission wavelength 538 nm. The fluorescence was monitored for 30 min to calculate the rate of ROS formation. The results were expressed as fluorescence intensity increase per 10^5 cells per 10 min.

2.4. Analysis of GSH and protein-thiol

Determination of GSH was performed by a modified fluorometric method (Hissin and Hilf, 1976). Rat heart tissue (250 mg) was homogenized on ice using a Polytron homogenizer. The solution used for homogenization consisted of 3.75 ml of phosphate–EDTA buffer (0.1 M sodium phosphate, 5 mM EDTA, pH 8.0) and 1 ml of 25% HPO_3. The total homogenate was centrifuged at 4°C at 100000 × g for 30 min. The supernatant was removed for the assay of GSH, and the pellet was used for determining protein-thiol content. For the GSH assay, 10 μl of the 100000 × g supernatant was added to 1.89 ml of phosphate–EDTA buffer, and 100 μl of 1 mg/ml o-phthalaldehyde (OPT) solution (prepared in methanol). After thorough mixing and incubation at room temperature for 15 min, 250 μl of the solution was loaded into a 96-well plate. Fluorescence at 405 nm was measured with the excitation at 320 nm. Quantification of GSH was obtained by employing commercial GSH standards. The results are expressed as μmole GSH per gram wet heart weight.

Determination of protein-thiol was performed by a modification of the method of Riddles et al. (1979). Briefly, the 100000 × g pellet was resuspended in 2 ml phosphate buffer. To 0.5 ml of this mixture, 4.5 ml phosphate buffer and 50 μl of 10 mM 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) was added. After thorough mixing and incubation at room temperature in the dark for 15 min, the solution was transferred to a cuvette. Absorbance was measured at 412 nm using a Beckman DU 7400 spectrophotometer. The results are expressed as μmole 2-nitro-5-thiobenzoic acid (TNB) per gram wet heart weight using a coefficient of 13600 M⁻¹ cm⁻¹.

2.5. Statistical analysis

All data are expressed as the mean ± SE for two–five separate animals. Differences between DOX and saline treatment were analyzed using the Student t test. A probability of P < 0.05 served as the criterion for statistical significance.

3. Results

The initial weights for saline and DOX groups were 176 ± 4 and 175 ± 2 g, respectively. As shown in Table 1, 6 weeks of DOX treatment retarded rat body weight gain by about 10% compared to controls. This was associated with a 15% decrease in ventricular heart weight as compared to saline-treated rats but there was no difference in the heart weight to body weight ratio between DOX-treated and saline-treated rats. Moreover, the decrease in both body weight and heart weight of DOX-treated rats persisted for a 5 week period after cessation of DOX treatment: DOX rats maintained body weight, while control rats continued to gain weight (Table 1).

The yield and viability of cardiac myocytes were not different between 6 week DOX and saline-treated rats. However, both cell yield and cell viability decreased markedly after 5 weeks recovery following the sixth injection (cell yields

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Rat body and heart weight (g)*</th>
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<tr>
<td></td>
<td>Saline</td>
</tr>
<tr>
<td>6+1b</td>
<td></td>
</tr>
<tr>
<td>Body weight (Bw)</td>
<td>387 ± 29</td>
</tr>
<tr>
<td>Heart weight (Hw)</td>
<td>1.66 ± 0.12</td>
</tr>
<tr>
<td>Ratio (Hw/Bw) × 100</td>
<td>0.43 ± 0.01</td>
</tr>
<tr>
<td>6+5w</td>
<td></td>
</tr>
<tr>
<td>Body weight (Bw)</td>
<td>420 ± 2</td>
</tr>
<tr>
<td>Heart weight (Hw)</td>
<td>1.57 ± 0.05</td>
</tr>
<tr>
<td>Ratio (Hw/Bw) × 100</td>
<td>0.37 ± 0.02</td>
</tr>
</tbody>
</table>

*a Data represent the mean ± SE for four animals.

b 6+1w and 6+5w represent samples from rats killed 1 or 5 weeks, respectively, following the last of 6 weekly injections of DOX or saline.

c Significant difference compared to saline control at P < 0.05.
Fig. 1. DOX-induced ROS formation in cardiac myocytes. Rats received 6 weekly injections of DOX (2 mg/kg) or saline. 6 + 1w and 6 + 5w represent rats killed 1 or 5 weeks, respectively, after the last injection. Reactive oxygen species (ROS) were estimated fluorometrically using the probe CM-H$_2$DCFDA, and expressed as fluorescent intensity increase per 10$^5$ cells per 10 min. Data represent the mean ± SE of measurements from three 6 + 1w rats and two 6 + 5w rats, respectively. *, significant difference compared to saline control at $P < 0.05$.

Fig. 2. GSH content in heart tissue harvested from DOX/saline treated rats. Rats received 6 weekly injections of DOX (2 mg/kg) or saline. 6 + 1w and 6 + 5w are defined in Fig. 1. Determination of GSH was performed by a modified fluorometric method using OPT. Values are expressed as μmole GSH per gram wet heart weight. Data represent the mean ± SE of measurements from five separate rats. *, significant difference compared to saline control at $P < 0.05$.

4. Discussion

In agreement with our previous findings (Solem et al., 1994, 1996), both body weight and heart weight were significantly less in 6-week DOX-treated rats compared to controls. Moreover, in the present study we found that the decrease in both body weight and heart weight persisted for a 5-week period after cessation of DOX treatment. This suggests an irreversible, and possibly progressive pathogenic process.

In correlation with the effect on body weight gain, we found a persistent stimulation of ROS formation. This persistent ROS formation is of particular importance in elucidating the mechanism of DOX-induced cardiotoxicity, as it correlates well with our recent finding of the persistence of oxidized mtDNA adducts. The accumulation of mtDNA 8OhdG adducts has been ascribed to ROS generation (Giulivi et al., 1995). Despite initial reports of deficiencies in mtDNA repair enzymes, recent studies demonstrate that oxidative DNA damage can be repaired in mito-
residual doxorubicin is undetectable 7 days following the last injection (Solem et al., 1994, 1996). Therefore, we propose the alternate hypothesis that this persistent ROS generation occurs as a consequence of damage to the mitochondria caused by DOX. Indeed, it is well established that inhibition of the mitochondrial electron transport chain in vitro by antimycin A, results in a significant increase in the mitochondrial free radical production (Boveris and Chance, 1973; Turrens and Boveris, 1980; Turrens et al., 1982, 1985). In a recent study, Esposito et al. (1999) demonstrated that inhibition of oxidative phosphorylation by knocking out the adenine nucleotide translocator gene also results in increased ROS production and consequent damage to the mtDNA. Accordingly, it is feasible that repetitive DOX dosing causes some type of permanent impairment damage that leads to the persistent stimulation of ROS generation. For example, this could be a manifestation of the biogenesis of poorly coupled mitochondria (Esposito et al., 1999; Jeyaseelan et al., 1997; Papadopoulou et al., 1999) or the conversion of xanthine dehydrogenase to xanthine oxidase (Gustafson et al., 1991).

Because of its proximity to oxidative phosphorylation in the mitochondrial inner membrane, mtDNA is highly susceptible to oxidation by ROS and the oxidized DNA may directly contribute to mitochondrial genomic mutations. Unlike nuclear DNA, however, mtDNA encodes rRNAs, tRNAs and mitochondrial proteins with no nonsense sequences, except for a small region involved in the regulation of replication and translation of mtDNA. Thus, it is highly probable that any mutation in the mtDNA will result in a phenotypic response. This hypothesis is supported by a number of studies which show that damage to mtDNA causes a decrease in the levels of mitochondrial transcripts (Andreu et al., 1998; Calleja et al., 1993). Indeed, it has been demonstrated that DOX suppresses the expression of several mitochondrial genes, including genes encoding cytochrome c oxidase (Papadopoulou and Tsiftsoglou, 1996; Papadopoulou et al., 1999) and the adenine nucleotide translocator (Jeyaseelan et al., 1997). All these effects may lead to substantial alterations in the synthesis of mitochondrial

Fig. 3. Protein-SH content in heart tissue harvested from DOX/saline treated rats. Rats received 6 weekly injections of DOX (2 mg/kg) or saline. 6+1w and 6+5w are defined in Fig. 1. The protein-SH was determined spectrophotometrically at 412 nm by using 5,5’dithiobis(2-nitrobenzoic acid). Values are expressed as μmole TNB per gram wet heart tissue. Data represent the mean ± SE of measurements from five separate rats.
proteins. Thus, following the last DOX dosing, it is possible that the newly synthesized dysfunctional mitochondria may continue to generate high levels of ROS, which in turn provide a continuous source of oxidation of mtDNA.

The overall antioxidant defense system is also an important factor determining the fate of DOX-induced ROS. It has been suggested that the occurrence of oxidative injury reflects stimulated generation of ROS that exceeds the capacity of antioxidant defense systems. GSH is the most important antioxidant against ROS. In agreement with previous reports that DOX treatment decreases GSH (Gustafson et al., 1993; Olson et al., 1980; Yin et al., 1998), we found that GSH is diminished in heart tissue from 6-week DOX-treated rats (c.a., 20% less than control) and remained suppressed 5 weeks after discontinuation of DOX treatment (Fig. 2). This persistently lower GSH is consistent with the persistent ROS formation. However, there was no difference in protein-thiol content of heart tissue between DOX and saline-treated rats. This might be due to the effect of GSH, which though depleted, may still provide sufficient reduction of heart tissue protein-thiols against ROS-induced oxidation. In addition, there might be compensatory actions of other antioxidant systems in response to this increased oxidative stress in the cells. For example, it has been reported that DOX treatment increases metallothionein content and catalase activity in heart tissues (Yin et al., 1998).

In summary, we demonstrate a higher rate of ROS formation in cardiac myocytes isolated from rats following 6 weekly injections of DOX, and that this enhanced ROS generation persists for up to 5 weeks after the last injection. This correlates well with our previous finding of the cumulative and persistent oxidation of cardiac mtDNA. Associated with the sustained high rate of ROS formation is a persistent depression of GSH in heart tissue. The enhanced generation of ROS may directly damage mitochondria or alter mitochondrial genomic expression, thus altering the synthesis of proteins associated with the mitochondrial electron transport chain. All of these events might lead to a catastrophic bioenergetic dysfunction of mitochondria in a feed-forward cascade, eventually accounting for the cumulative and progressive pathogenesis of DOX-induced cardiomyopathy.

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References


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