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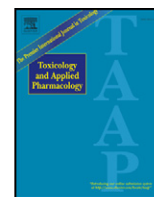

Early transcriptional changes in cardiac mitochondria during chronic doxorubicin exposure and mitigation by dexrazoxane in mice

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ABSTRACT

Identification of early biomarkers of cardiotoxicity could help initiate means to ameliorate the cardiotoxic actions of clinically useful drugs such as doxorubicin (DOX). Since DOX has been shown to target mitochondria, transcriptional levels of mitochondria-related genes were evaluated to identify early candidate biomarkers in hearts of male $B6C3F_1$ mice given a weekly intravenous dose of 3 mg/kg DOX or saline (SAL) for 2, 3, 4, 6, or 8 weeks (6, 9, 12, 18, or 24 mg/kg cumulative DOX doses, respectively). Also, a group of mice was pretreated (intraperitoneally) with the cardio-protectant, dexrazoxane (DXZ; 60 mg/kg) 30 min before each weekly dose of DOX or SAL. At necropsy a week after the last dose, increased plasma concentrations of cardiac troponin T (cTnT) were detected at 18 and 24 mg/kg cumulative DOX doses, whereas myocardial alterations were observed only at the 24 mg/kg dose. Of 1019 genes interrogated, 185, 109, 140, 184, and 451 genes were differentially expressed at 6, 9, 12, 18, and 24 mg/kg cumulative DOX doses, respectively, compared to concurrent SAL-treated controls. Of these, expression of 61 genes associated with energy metabolism and apoptosis was significantly altered before and after occurrence of myocardial injury, suggesting these as early genomics markers of cardiotoxicity. Much of these DOX-induced transcriptional changes were attenuated by pretreatment of mice with DXZ. Also, DXZ treatment significantly reduced plasma cTnT concentration and completely ameliorated cardiac alterations induced by 24 mg/kg cumulative DOX. This information on early transcriptional changes during DOX treatment may be useful in designing cardioprotective strategies targeting mitochondria.

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1. Introduction

Cardiomyopathy is a serious side effect associated with clinical use of the potent *anti*-cancer drug, Doxorubicin (DOX) that can occur during or after the completion of the treatment (Wouters et al., 2005; Lipshultz and Adams, 2010). Increased oxidative stress through generation of reactive oxygen species (ROS) is a widely accepted mechanism in the development of cardiotoxicity (Minotti et al., 2004; Tokarska-Schlattner et al., 2006). It has been consistently demonstrated that cardiac mitochondria are a prime target of DOX (Zhou et al., 2001; Wallace, 2007; Berthiaume and Wallace, 2007) and a major source of ROS production (Davies and Doroshow, 1986). Various ROS sources include, mitochondrial NADH dehydrogenase, at which, the quinone moiety in the DOX molecule undergoes one-electron reduction to a semiquinone radical that reduces molecular oxygen to superoxide anion and hydrogen peroxide (Minotti et al., 1999) and the interference with the electron transport within the mitochondrial respiratory chain by DOX and its lipophilic aglycone metabolites (Davies and Doroshow, 1986; Licata et al., 2000; Minotti et al., 2004).

Cellular iron is another important component in ROS production during DOX exposure. DOX interacts with iron to form a DOX-iron complex, which can redox cycle between the Fe^{2+} and Fe^{3+} oxidation states in the presence of oxygen, generating toxic radicals (Minotti et al., 2004; Mordente et al., 2012). It has been shown that both DOX and DOX-iron complex have a strong affinity for the cardiolipin that is abundantly

Abbreviations: cTnT, cardiac troponin T; DOX, doxorubicin; DXZ, dexrazoxane; FDR, false discovery rate; GO, Gene Ontology; ROS, reactive oxygen species; SAL, saline; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling.

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present in the inner mitochondrial membrane (Goormaghtigh et al., 1980; Hasinoff and Davey, 1988). Cardiolipin is an integral part of many membrane-bound proteins, including the respiratory chain proteins. An association of DOX or its iron complex with cardiolipin, thus can disrupt the cardiolipin–protein interface, resulting in altered protein activities and increased ROS formation (Goormaghtigh et al., 1990; Schlame et al., 2000). A role of iron accumulation within mitochondria in the development of DOX cardiotoxicity has been illustrated by Ichikawa et al. (2014) that reported higher levels of iron within heart mitochondria from patients with DOX-induced cardiomyopathy than hearts from patients with other types of cardiomyopathy. Altogether, there is strong evidence for a substantial involvement of mitochondria in DOX-induced cardiotoxicity. Moreover, the selective toxicity of DOX in the heart can be attributed to high mitochondrial density (40–50%) in cardiomyocytes (Marin-Garcia et al., 2001; Torres and Simic, 2012) compared to mitochondrial density of approximately 8–35% of the cell volume in the liver, skeletal muscle, lungs, and kidney (Else and Hulbert, 1985).

Considering a major role of ROS in DOX cardiotoxicity, various antioxidants have been tested to prevent DOX-induced oxidative damage in animal models and humans (Speyer and Wasserheit, 1998; Quiles et al., 2002; Santos et al., 2002; Wouters et al., 2005). However, only a few of these interventions have shown encouraging outcomes under certain conditions. Currently, dexrazoxane (DXZ) is the only FDA-approved drug that has been proven effective in reducing the prevalence of DOX cardiotoxicity in animal models and humans (Imondi et al., 1996; Herman and Ferrans, 1998; Herman et al., 2000; Lipshultz et al., 2004; Lipshultz and Adams, 2010). The hydrolyzed metabolite of DXZ is a strong iron-chelator that binds to free iron and also can remove iron from the DOX-iron complex (Hasinoff, 1989; Buss and Hasinoff, 1993). One of the proposed mechanisms of cardioprotection by DXZ is through prevention of site-specific iron-mediated oxidative damage to mitochondria induced by DOX and its iron complex (Hasinoff et al., 2003; Hasinoff and Herman, 2007).

Extensive efforts have been invested in different model systems to understand the mechanisms underlying the pathogenesis of DOX cardiomyopathy (Yi et al., 2006; Berthiaume and Wallace, 2007; Thompson et al., 2010; Todorova et al., 2012; Holmgren et al., 2015; Zhao et al., 2015). However, the knowledge of early molecular changes within cardiac mitochondria that may progress to cardiomyopathy is still lacking. Such information can serve as predictive biomarkers of cardiotoxicity and may help in protecting heart from toxic DOX effects. Clinical approaches, both invasive and non-invasive, that are currently used in predicting cardiotoxicity in cancer patients on DOX therapy are with technical complications, sub-optimal or insensitive (Shan et al., 1996; Gharib and Burnett, 2002). Also, cardiac-specific markers, troponins, are detected in blood only after cardiac tissue damage has occurred. Thus, it is imperative to identify early predictive molecular markers of cardiotoxicity. In the present study, therefore, transcriptional changes for genes related to mitochondria were examined in hearts from male $B6C3F_1$ mice during progression to cardiotoxicity using a clinically relevant DOX regimen. In addition, effect of DXZ on DOX-induced transcriptional changes was evaluated in these mice. Many cardiac genes associated with various mitochondrial pathways, including energy metabolism and apoptosis, were differentially expressed in DOX-treated mice before and after the occurrence of myocardial injury at 18 mg/kg cumulative DOX dose, suggesting their potential as early genomics markers of cardiotoxicity. Pretreatment with DXZ significantly mitigated many of these DOX-induced transcriptional changes as well as reduced plasma cardiac troponin T (cTnT) concentration and showed a complete recovery from cardiac pathology. These findings provide important insights into early transcriptional changes in cardiac mitochondria that may lead to overt cardiotoxicity during chronic DOX treatment and enable identification of candidate early genomics markers of cardiotoxicity in mouse heart.

2. Materials and methods

2.1. Animal husbandry

Male $B6C3F_1$ mice from the breeding colony at the National Center for Toxicological Research (NCTR) were raised in a pathogen-free environment at the NCTR and treated according to the Institutional Animal Care and Use Committee guidelines. Mice were housed individually in standard polycarbonate cages with hardwood chip bedding and were maintained at 23 °C with a relative humidity of 50%. The animals were conditioned to a 12/12-hour light/dark cycle and had free access to NIH-41 IR diet (LabDiet, Richmond, IN) and water.

2.2. Experimental design

Animal treatments began at 8 weeks of age. Mice on the study were randomly assigned to different treatment groups: (1) *Doxorubicin treatment groups (DOX)*: mice were given a weekly dose of 3 mg/kg body weight DOX (Chempacific, Baltimore, MD) by intravenous (i.v.) injection via tail vein for 2, 3, 4, 6, and 8 weeks, resulting in cumulative DOX doses of 6, 9, 12, 18, and 24 mg/kg, respectively. These cumulative doses correspond to human equivalent doses (HED) of 17.8, 26.6, 35.5, 53.3, and 71 mg/m^2 body surface area, respectively. Each DOX treatment group consisted of 12 mice. (2) *Saline treatment groups (SAL)*: mice were administered once a week an equivalent volume (2 ul/g body weight) of sterile 0.9% saline (i.v.) (Sigma-Aldrich, St. Louis, MO) for 2, 3, 4, 6, and 8 weeks. Each SAL treatment group consisted of 10 mice. (3) *Dexrazoxane (DXZ) plus DOX treatment groups (DXZ ± DOX)*: mice were injected with a weekly dose of 60 mg/kg DXZ (177.5 mg/m^2) intraperitoneally (i.p.) approximately thirty minutes before i.v. administration of DOX for 2, 3, 4, 6, and 8 weeks, resulting in cumulative DXZ doses of 120, 180, 240, 360, and 480 mg/kg. These cumulative doses correspond to HED of 355.0, 532.4, 709.9, 1064.9, and 1419.8 mg/m^2. Each DXZ + DOX treatment group consisted of 12 mice. (4) *Dexrazoxane plus saline treatment groups (DXZ ± SAL)*: mice were administered every week 60 mg/kg DXZ (i.p.) approximately thirty minutes before i.v. injection of sterile 0.9% saline for 2, 3, 4, 6, and 8 weeks. Each DXZ + SAL treatment group consisted of 10 mice. All animals were observed daily for abnormal signs during the course of the study.

2.3. Collection of tissues

Animals were euthanized a week after the last dose to mimic the clinical situation, where DOX cardiotoxicity has been reported to occur one year to decades after cessation of the treatment (Lipshultz et al., 1991). Also, delayed cardiotoxicity weeks after DOX exposure has been shown in laboratory animals (Herman et al., 1998; Lebrecht et al., 2007). Mice were anesthetized by inhalation of 0.5–2.0% isoflurane mixed with oxygen at each sacrificial time point. Blood was collected from each mouse by *retro*-orbital puncture into EDTA-coated Microtainer® tubes and centrifuged at 1000 × g at 4 °C to separate plasma for measurement of cTnT concentration. Following *retro*-orbital bleeding, mice were euthanized by exsanguination and the hearts were immediately excised and separated from the pericardium. A transverse section of the heart was collected midpoint between the base and apex and fixed in 4% paraformaldehyde for examination by light microscopy. Approximately 1 mm piece of left ventricle of heart was fixed in 4% glutaraldehyde for examination of mitochondrial morphology by transmission electron microscopy. The remaining heart tissue was immediately frozen in liquid nitrogen for storage at −80 °C for genomics.

2.4. Investigations

2.4.1. Measurement of plasma cTnT concentration. This was performed using fourth-generation immunoassay (Elecsys® Troponin T STAT; Roche Diagnostics, Indianapolis, IN) on the Elecsys 2010 instrument

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