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Effects of doxorubicin on cardiac muscle subsarcolemmal and intermyofibrillar mitochondria

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ABSTRACT

Doxorubicin (DOX) is a highly effective chemotherapeutic used in the treatment of a broad spectrum of malignancies. However, clinical use of DOX is highly limited by cumulative and irreversible cardiomyopathy that occurs following DOX treatment. The pathogenesis of DOX-induced cardiac muscle dysfunction is complex. However, it has been proposed that the etiology of this myopathy is related to mitochondrial dysfunction, as a result of the dose-dependent increase in the mitochondrial accumulation of DOX. In this regard, cardiac muscle possesses two morphologically distinct populations of mitochondria. Subsarcolemmal (SS) mitochondria are localized just below the sarcolemma, whereas intermyofibrillar (IMF) mitochondria are found between myofibrils. Mitochondria in both regions exhibit subtle differences in biochemical properties, giving rise to differences in respiration, lipid composition, enzyme activities and protein synthesis rates. Based on the heterogeneity of SS and IMF mitochondria, we hypothesized that acute DOX administration would have distinct effects on each cardiac mitochondrial subfraction. Therefore, we isolated SS and IMF mitochondria from the hearts of female Sprague-Dawley rats 48 h after administration of DOX. Our results demonstrate that while SS mitochondria appear to accumulate greater amounts of DOX, IMF mitochondria demonstrate a greater apoptotic and autophagic response to DOX exposure. Thus, the divergent protein composition and function of the SS and IMF cardiac mitochondria result in differential responses to DOX, with IMF mitochondria appearing more susceptible to damage after DOX treatment.

1. Introduction

Doxorubicin (DOX) is an anthracycline antibiotic, used in the treatment of a broad spectrum of human cancers (Lu, 2005). Despite the efficacy of this anticancer drug, the clinical use of DOX is limited due to deleterious dose-related effects on cardiac muscle function (Doroshow et al., 1985). Specifically, DOX treatment can induce the development of cardiomyopathy and cardiac dysfunction leading to congestive heart failure and death (Jeyaseelan et al., 1997; Kavazis et al., 2010; Singal et al., 2000; Zhang et al., 2009). In this regard, mitochondria have repeatedly been implicated as the main target of DOX-induced toxicity as a result of the dose-dependent increase in the mitochondrial accumulation of DOX.

In muscle, DOX accumulates in the mitochondria due to its high affinity to cardiolipin, a phospholipid that is uniquely expressed on the inner mitochondrial membrane (Jung and Reszka, 2001; Wallace, 2003). The complex that DOX forms with cardiolipin places it in close proximity to the electron transport chain, and redox cycling of DOX is mediated through its interaction with NADH dehydrogenase (complex I) of the mitochondrial electron transport chain (Ascensao et al., 2006; Ascensao et al., 2005a; Ascensao et al., 2005b; Chicco et al., 2006a; Chicco et al., 2005, 2006b; Childs et al., 2002; Green and Leeuwenburgh, 2002; Jang et al., 2004). The quinone moiety of DOX can undergo a one-electron conversion to a semiquinone by several cellular oxidoreductases. One-electron oxidation of the DOX-semiquinone radical to the DOX-quinone form leads to the generation of the highly reactive superoxide. In addition, DOX administration also results in a topoisomerase-IIβ-dependent reduction in antioxidant enzyme gene transcription, increased mitochondrial ROS production and reduced expression of genes necessary for mitochondrial biogenesis (Vejpongsa and Yeh, 2014; Zhang et al., 2012). Therefore, mitochondrial dysfunction plays a critical role in DOX-induced cardiotoxicity due to its direct ability to generate ROS.

In this regard, cardiac muscle possesses two distinct populations of mitochondria. Subsarcolemmal (SS) mitochondria are located just below the sarcolemmal membrane, while intermyofibrillar (IMF) mitochondria are found between the myofibrils. Importantly, these mitochondrial subpopulations possess many divergent inherited properties and differ in their response to stress (Palmer et al., 1977; Rosca and

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Hoppel, 2010; Weinstein et al., 1986). Specifically, in response to H_2O_2, it was demonstrated that IMF mitochondria exhibit greater release of cytochrome *c* and apoptosis-inducing factor (AIF), compared to SS mitochondria (Adhihetty et al., 2005). This ROS challenge also resulted in a greater rate of mitochondria permeability transition (mtPTP) pore opening in the IMF compared to the SS (Adhihetty et al., 2005). In contrast, in response to both denervation and aging, SS mitochondria appear to be more affected than IMF mitochondria (Adhihetty et al., 2007; Chabi et al., 2008). These heterogeneous responses displayed by the mitochondrial subpopulations emphasize the importance of determining the spatial influence on mitochondrial function during pathological conditions. Therefore, the goal of this study was to determine if differences exist in the susceptibility of cardiac SS and IMF mitochondria to DOX treatment.

2. Materials and methods

2.1. Experimental design

Adult six-month old female Sprague-Dawley (SD) rats were used in these experiments. The Animal Care and Use Committee of the University of Florida approved these experiments. Animals were maintained on a 12:12 h reverse light-dark cycle and provided rat chow ad libitum throughout the experimental period. Rats were randomly assigned to one of two treatment groups (n = 10/group): 1) Acute saline administration (CON) and 2) Acute doxorubicin administration (DOX).

2.1.1. DOX administration

Animals in the DOX group received doxorubicin hydrochloride (20 mg/kg body weight IP) 48 h prior to sacrifice. Equal volumes of saline were administered to the CON group. The dose of DOX administered is a human clinical dose of this drug that has been pharmacologically scaled for use in rats (Childs et al., 2002; Kang et al., 1997; Yen et al., 1996), and has been demonstrated to cause cardiac muscle toxicity (Kavazis et al., 2010; Smuder et al., 2011, 2013a). At the completion of the experimental period, animals were acutely anesthetized with sodium pentobarbital (60 mg/kg IP). After reaching a surgical plane of anesthesia, the heart was removed and subsarcolemmal (SS) and intermyofibrillar (IMF) mitochondria were isolated.

2.1.2. Mitochondrial isolation

Differential centrifugation was used to fractionate SS and IMF mitochondria as described previously (Cogswell et al., 1993) with minor modifications (Kavazis et al., 2009b; Kavazis et al., 2008; Kavazis et al., 2009c). Briefly, cardiac tissue was finely minced on ice and then homogenized with a Polytron tissue processor (VirTis, Gardiner, NY) for 7 s at 50% power at 4 °C. The homogenate was centrifuged at 500 *g* for 10 min at 4 °C, with the resulting supernatant containing SS mitochondria. The pellet, containing IMF mitochondria, was resuspended in isolation buffer and homogenized with the same Polytron tissue processor for 5 s. After 10 min of incubation in isolation buffer containing trypsin (5 mg/g muscle weight) at 4 °C, an equal volume of isolation buffer was added to attenuate the activity of trypsin. The homogenate was centrifuged at 500 *g* for 10 min, leaving IMF in the supernatant. Both SS and IMF mitochondria were pelleted with centrifugations at 3000 *g* for 10 min at 4 °C. In addition, 1 mL of supernatant from the SS isolation was saved to analyze the cytosolic concentration of DOX. The pellets of SS and IMF were washed twice and resuspended using a Dounce homogenizer in resuspension buffer at 4 °C (220 mM of mannitol, 70 mM of sucrose, 2 mM of Tris base, and 20 mM of HEPES, pH 7.4). Mitochondrial integrity after this isolation procedure has been previously described by our laboratory (Kavazis et al., 2009b; Kavazis et al., 2008; Kavazis et al., 2009c).

2.2. Biochemical analyses

2.2.1. Mitochondrial DOX accumulation

The relative concentration of DOX was measured in cardiac SS and IMF mitochondria and in the cytosolic fraction using a commercially available Rat Adriamycin ELISA kit according to manufacturer's instructions (MyBioSource, San Diego, CA). Briefly, this is a competitive colormetric immunoassay technique utilizing a monoclonal anti-Adriamycin and an Adriamycin-HRP conjugate. Intensity of the color of the samples was measured spectrophotometrically at 450 nm in a plate reader and the Adriamycin concentration was calculated compared to a standard curve which relates the intensity of the sample's optical density to the concentration of the standards.

2.2.2. Mitochondrial respiration

Mitochondrial oxygen consumption was measured using previously described techniques (Kavazis et al., 2009c). Maximal ADP-stimulated respiration (state 3) was obtained using complex I substrates (i.e., 2 mM pyruvate and 2 mM malate) in the presence of 0.25 mM ADP and state 4 respiration was recorded following the complete phosphorylation of ADP. Thereafter, the respiratory control ratio (RCR) was calculated as the quotient of state 3 and state 4 respiration.

2.2.3. Mitochondrial permeability transition pore (mtPTP) assessment

mtPTP opening is facilitated by increased concentrations of calcium and/or oxidative stress, which leads to mitochondrial swelling, outer membrane rupture, and release of proapoptotic factors (Adhihetty et al., 2005). Using previously described techniques (Adhihetty et al., 2005; Kavazis et al., 2008), the assessment of mtPTP opening was accomplished by monitoring the decrease in light scattering associated with mitochondrial swelling at 540 nm. Isolated cardiac mitochondria were treated with 400 μM $CaCl_2$ and 75 μM tert-butyl hydroperoxide. Subsequently, the decrease in absorbance was monitored through a spectrophotometer for 30 min. The major dependent variable measures were maximal rate of pore opening (V_{max}) and time to reach V_{max}.

2.2.4. Mitochondrial ROS production

Cardiac mitochondrial ROS production was determined using Amplex™ Red (Molecular Probes, Eugene, OR). The assay was performed at 37 °C in 96-well plates using succinate as the substrate as previously described (Kavazis et al., 2009c).

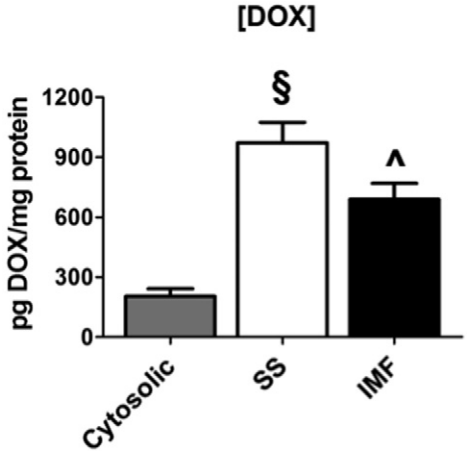

Fig. 1. Mitochondrial DOX accumulation. Cardiac DOX concentration from cardiac cytosolic fraction (non-mitochondrial fraction) (Cytosolic), subsarcolemmal (SS) and intermyofibrillar (IMF) mitochondrial subfractions from animals treated with DOX. Values are mean ± SEM. § Significantly different versus Cytosolic and IMF ($p < 0.05$). ^ Significantly different versus Cytosolic ($p < 0.05$).