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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.

Keywords

Heart; Anthracycline; Apoptosis

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

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(Doroshov 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-II β -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 Hoppel, 2010; Weinstein et al., 1986). Specifically, in response to H₂O₂, 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 colorimetric 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 (Adhietty et al., 2005). Using previously described techniques (Adhietty 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 μ M CaCl_2 and 75 μ M 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 AmplexTM 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).

2.2.5. Western blot analysis—The abundance of SS and IMF mitochondrial proteins was determined via western blot as previously described (Kavazis et al., 2009a; Smuder et al., 2012). Specifically, SS and IMF mitochondrial proteins were probed for: cytochrome c, AIF, SOD1, SOD2 (Santa Cruz Biotechnology, Dallas, TX), OPA1, DRP1 (BD, Franklin Lakes, NJ), total OXPHOS, GPX1, catalase, p62, Parkin, PINK1 (Abcam, Cambridge, MA), Tfam (Calbiochem, Darmstadt, Germany), Nrf1 (Rockland Immunochemicals, Limerick, PA), pUB (Boston Biochem, Cambridge, MA), Fis1, HSP70, HSP10 (Enzo Life Sciences, Farmingdale, NY), Mfn2 and LON (Sigma, St. Louis, MO). VDAC (Santa Cruz Biotechnology) was used as a mitochondrial marker to control for protein loading and transfer differences.

2.3. Data analyses

Data are presented as mean $\pm$ SEM. Comparisons between groups for DOX concentration was made by a one-way analysis of variance (ANOVA). All other measurements were evaluated by a two-way ANOVA. When appropriate, Tukey HSD (honestly significant different) tests were performed post-hoc. Significance was established at $p < 0.05$.

3. Results

3.1. Mitochondrial accumulation of DOX

Compared to other cellular compartments, DOX has a high affinity for mitochondrial localization (Jung and Reszka, 2001; Wallace, 2003). Our results verify these findings, as the

mitochondrial concentration of DOX was significantly greater than the cytosolic concentration (Fig. 1). In addition, our results also demonstrate that there is a significant difference in DOX accumulation between SS and IMF mitochondria. Specifically, two days after DOX injection SS mitochondria contain greater concentrations of DOX compared to IMF mitochondria.

3.2. Differential cardiac mitochondrial function between SS and IMF subpopulations after DOX treatment

Cardiac and mitochondrial function are both compromised 48 h after acute administration of DOX (Min et al., 2015). To determine if DOX treatment differentially alters the function of SS and IMF mitochondria, we assessed several biomarkers of mitochondrial function. First, we measured the rates of oxidative phosphorylation using complex I substrates (pyruvate/malate). Our results demonstrate no significant differences in state 3 or state 4 respiration in either mitochondrial subpopulation as a result of DOX. However, state 4 respiration was significantly reduced in the IMF compared to the SS mitochondria (Fig. 2A–B). Moreover, DOX treatment resulted in a significant decrease in mitochondrial coupling in the IMF mitochondria compared to CON, as demonstrated by the diminished respiratory control ratio (RCR) (Fig. 2C). Finally, the mitochondrial concentration of proteins required for electron transport chain (complex I: NDUF8, complex II: SDHB, complex III: UQCRC2, complex IV: MTCO1, and complex V: ATP5A) and tricarboxylic acid (TCA) cycle function (citrate synthase) were measured in SS and IMF mitochondria with and without DOX treatment. Our results demonstrate that the mitochondrial expression of citrate synthase is reduced in both SS and IMF cardiac mitochondria treated with DOX compared to CON animals (Fig. 2D). In regards to mitochondrial respiratory chain content, no difference existed between CON and DOX animals for any complex. However, complex I and complex V protein expression was significantly greater in IMF mitochondria compared to SS mitochondria (Fig. 2E).

Increasing evidence supports the postulate that DOX-induced apoptosis is a primary mechanism of DOX-induced cardiomyopathy (reviewed in (Zhang et al., 2009)). Specifically, increased mitochondrial ROS production leads to oxidative damage and calcium overload in the mitochondria, triggering mitochondrial outer membrane permeabilization. Therefore, we measured the rate of H₂O₂ release and mtPTP opening characteristics by assessing the maximal rate of pore opening (V_{max}) and the time to reach V_{max} during exposure to both calcium and ROS. In both SS and IMF mitochondria, DOX resulted in a significant increase in ROS emission (Fig. 3A). In addition, IMF mitochondria appear to produce more ROS than SS mitochondria independent of the treatment. Our results also show that V_{max} is increased in both SS and IMF mitochondria as a result of DOX. However, time to V_{max} was only significantly reduced in the IMF subfraction (Fig. 3B–C).

3.3. DOX reduces the mitochondrial content of pro-apoptotic proteins

DOX-induced apoptosis may occur as a result of activation of the intrinsic apoptosis pathway. Activation of this pathway is mediated by the release of cytochrome *c* and AIF from the mitochondria, and measurement of their mitochondrial protein levels can be used as an index of mitochondrial damage. Our results reveal that mitochondrial cytochrome *c* content is greater in IMF mitochondria compared to SS, and that DOX treatment results in a

significant reduction in IMF mitochondrial cytochrome *c* compared to CON animals (Fig. 4A). In addition, IMF mitochondria contain less AIF than SS mitochondria, and DOX treatment reduces the mitochondrial content of AIF in both SS and IMF mitochondria (Fig. 4B).

3.4. DOX treatment alters mitochondrial antioxidant enzymes in the heart

Mitochondrial antioxidant enzyme capacity was measured to determine if alterations to the mitochondria's endogenous oxidant buffering system were responsible for the DOX-induced increase in mitochondrial ROS emission. Compared to control animals DOX resulted in a significant increase in GPX1 expression in the SS mitochondria (Fig. 5A), while the protein expression of SOD1, SOD2 and catalase were not affected by DOX administration in either mitochondrial subfraction (Fig. 5B–D). However, our results show that independent of treatment, IMF mitochondria contain significantly lower catalase protein levels than SS mitochondria.

3.5. DOX-induced alteration of mitochondrial fission and fusion proteins

Mitochondria exist in a network that constantly changes to adapt to the environment by altering the rate of fission and fusion to create new mitochondria and remove damaged ones (Iqbal and Hood, 2015). In this regard, our data demonstrate that DOX alters mitochondrial dynamics as the protein expression of the fusion protein optic atrophy 1 (OPA1) was reduced in IMF mitochondria treated with DOX (Fig. 6A). In addition, our data demonstrate that protein levels of both OPA1 and the fission protein Fission 1 (Fis1) are distinctly less in the IMF mitochondria compared to the SS mitochondrial subfraction (Fig. 6A and C). No differences existed in the protein expression of the fusion protein mitofusin 2 (Mfn2) or the fission protein dynamin-related protein 1 (DRP1) (Fig. 6B and D).

3.6. DOX-induced alterations to markers of mitophagy

Several mitochondrial proteins are required to regulate mitophagy in cardiac muscle. Specifically, PINK1 recruits Parkin to the mitochondria, where it promotes ubiquitination of outer mitochondrial membrane proteins that are then recognized by p62 (Narendra and Youle, 2011; Sandri, 2013). Our data demonstrates that DOX appears to increase mitophagy in IMF mitochondria, as p62 expression was significantly reduced and PINK1 expression was significantly elevated in IMF mitochondria treated with DOX compared to CON (Fig. 7A–B). No differences existed in the protein expression of Parkin (Fig. 7C).

3.7. DOX-induced alterations to markers of mitochondrial biogenesis

Published reports show that DOX can disrupt cardiac mitochondrial biogenesis to promote apoptosis (Wallace, 2003; Zhou et al., 2001). TFAM and Nrf1 are key proteins in activating mitochondrial gene transcription and replication. Our data demonstrates that DOX does not induce any alterations to TFAM protein expression. However, Nrf1 expression is reduced in IMF mitochondria of animals treated with DOX (Fig. 8A–B).

3.8. Doxorubicin and protein quality control

Accumulation of damaged proteins within the mitochondria is a constant threat as a result of increased oxidative stress. The mitochondria contain multiple mechanisms to protect against protein misfolding and aggregation, including mitophagy, heat shock proteins and Lon protease (Lon). Therefore, we assessed the protein expression of markers of each of these pathways. Our data demonstrate that DOX results in the accumulation of polyubiquitinated (pUB) mitochondrial proteins in SS mitochondria compared to CON. In addition, our data also demonstrate that independent of treatment, SS mitochondria contain greater amounts of pUB compared to IMF mitochondria (Fig. 9A). In addition, IMF mitochondria contain a significantly greater amount of HSP70 compared to SS mitochondria (Fig. 9B). Finally, no differences exist between treatment groups of HSP10 or Lon (Fig. 9C–D).

4. Discussion

Anthracycline compounds are major culprits in chemotherapy-induced cardiomyopathy, which is the chief limiting factor in delivering optimal chemotherapy to cancer patients. Specifically, mitochondria have been identified as both the target and origin of major pathogenic pathways, which lead to the progression of DOX-induced cardiac injury (Zhang et al., 2009). Cardiac muscle contains two distinct subpopulations of mitochondria that possess differential functional, compositional and biochemical properties. While differences have been demonstrated between these two subpopulations in their reaction to many cellular stresses, to date it is unknown if cardiac SS and IMF mitochondria differ in their contribution to DOX-induced cardiac dysfunction. Therefore, the primary goal of this study was to investigate the potential spatial influence of SS and IMF mitochondria to DOX treatment. A brief overview of our results follows.

4.1. Cellular compartment affects mitochondria DOX accumulation

Cardiac muscle mitochondrial subpopulations are located in specialized cellular compartments and are named according to their location within the muscle fiber. SS mitochondria are located immediately beneath the sarcolemmal membrane and are situated nearest to the capillaries and are thought to contribute to the energy requirement for chemical transport in and out of the cell. IMF mitochondria are located between the myofibrils and are thought to provide energy for contraction. It has been hypothesized that the heterogeneity between the mitochondrial subpopulations may be related to differential rates of protein import, which is dependent on their concentration of proteins required for influx into the mitochondria (Takahashi and Hood, 1996). Indeed, divergent rates of protein import in SS and IMF mitochondria may be dependent on specific differences in the concentration of various components necessary for import. In this regard, our data demonstrate that SS mitochondria contain a higher concentration of DOX compared to IMF mitochondria. While the exact mechanism for this disparity is unknown, it is possible that the variance can be accounted for by differences in mitochondrial phospholipid content. Specifically, the phospholipid cardiolipin has been demonstrated to play an important role in the binding of proteins destined for the mitochondria, and it is well established that DOX accumulates in the mitochondria due to its high affinity to cardiolipin (Jung and Reszka, 2001; Wallace, 2003). In addition, it has also been reported that SS mitochondria contain

greater amounts of cardiolipin compared to the IMF mitochondria (Takahashi and Hood, 1996). Therefore, SS mitochondria may accumulate greater amounts of DOX as a result of enhanced cardiolipin-mediated DOX import.

4.2. DOX increases mitochondrial dysfunction and oxidative stress in both SS and IMF mitochondria

While the precise mechanisms for DOX-induced cardiotoxicity have not been fully elucidated, several reports indicate a key role of free radical-induced oxidative stress (Iarussi et al., 2001; Neilan et al., 2007; Wallace, 2003; Zhang et al., 2009). Specifically, it is well known that during its metabolism DOX generates ROS (Iarussi et al., 2001; Neilan et al., 2007; Wallace, 2003; Zhang et al., 2009). Indeed, endothelial nitric oxide synthase can convert DOX to an unstable semiquinone intermediate that favors ROS generation and both ROS and DOX can lead to mitochondrial damage and further production of ROS via respiratory chain failure (Neilan et al., 2007).

Previously, studies looking at the cardiac mitochondria population as a whole have demonstrated that DOX does indeed cause increased mitochondrial ROS production and reduced mitochondrial coupling (Kavazis et al., 2010). While our data confirm these findings, they also demonstrate important differences between the mitochondrial subpopulations. Specifically, we show unique differences in both the rate of ROS emission and the RCR between SS and IMF mitochondria, which highlights the divergent functions of these subfractions, whereby the SS mitochondria typically have lower oxidative capacity and produce lower levels of ROS compared to the IMF. As a result, the IMF mitochondria are highly specialized toward energy production for contractile activity and the associated calcium handling, whereas SS mitochondria are thought to provide energy for membrane related events (Powers et al., 2012; Romanello and Sandri, 2010; Walsh et al., 2006).

4.3. SS and IMF mitochondria differ in their susceptibility to DOX-induced apoptosis and proteolytic activity

Apoptosis is a highly conserved process that can be initiated in a cell by either an extrinsic or intrinsic pathway. The intrinsic pathway is mediated by mitochondria, as they contain AIF and cytochrome *c*, which can activate cell death upon release into the cytosol (Adhihetty et al., 2005; Zhang et al., 2009). Release of these proteins from the mitochondria is dependent upon the formation of a specialized mtPTP. DOX administration has been shown to increase cardiac apoptosis as a result of increased mitochondrial ROS production. ROS can induce pore opening by directly interacting with components of the mtPTP or indirectly by activating redox-sensitive transcription factors involved in the expression of apoptotic genes (Adhihetty et al., 2005; Zhang et al., 2009). In this regard, both mitochondrial subfractions display alterations to mtPTP. Specifically, the IMF subfraction had reduced mitochondrial cytochrome *c* and AIF content after DOX treatment, and also demonstrated a greater rate of pore opening. Conversely, the DOX induced apoptotic events in the SS mitochondria were not as pronounced. The SS mitochondria showed no difference in cytochrome *c* content and no difference in the time to reach V_{max} . Similar findings were demonstrated in IMF and SS mitochondria isolated from quadriceps muscle and treated with H_2O_2 (Adhihetty et al., 2005). Indeed, it was demonstrated that differences in mtPTP composition between the two

subpopulations results in a differential responses to ROS, resulting in a greater release of cytochrome *c* and AIF from the IMF mitochondria (Adhihetty et al., 2005). A potential explanation for the differences between the SS and IMF mitochondria may be due to the expression of endogenous mitochondrial antioxidants. Specifically, activation of apoptosis by ROS has been demonstrated to be inhibited by altered expression of mitochondrial antioxidant proteins (i.e. SOD1/2 and GPX1) (Adhihetty et al., 2005). In this regard, our data demonstrate a SS mitochondria-specific increase in GPX1 protein expression after DOX injection, which may serve as a protective mechanism to combat DOX-induced ROS production and may account for the difference in apoptotic markers between SS and IMF mitochondria, whereby IMF mitochondria appear to be more susceptible to apoptosis after DOX exposure.

Finally, the intrinsic pathway of apoptosis can also be activated by DOX through the downregulation of cardiac mitochondrial biogenesis (Piantadosi et al., 2008; Suliman et al., 2007; Zhang et al., 2009). This correlates with our findings as Nrf1 protein expression was reduced in IMF mitochondria in DOX treated animals. Prevention of the DOX-induced reduction in mitochondrial biogenesis may prove to be an important therapeutic strategy as evidence suggests that activation of mitochondrial biogenesis can prevent cardiomyopathy by protecting mitochondrial DNA and reducing apoptosis (Piantadosi et al., 2008; Suliman et al., 2007).

4.4. DOX treatment results in subpopulation-specific differences in the expression of markers of mitochondrial dynamics and mitophagy

While the role of apoptosis has been well defined in DOX-induced cardiotoxicity, recently it has been suggested that mitochondrial function and turnover may also be regulated by both changes to mitochondrial dynamics and mitophagy. Mitochondria are constantly being remodeled via the process of fission and fusion. Recent evidence reveals that alterations to the cellular expression of key fission and fusion proteins is linked to increased apoptosis (Romanello et al., 2010; Youle and Karbowski, 2005). Specifically, it was demonstrated that the loss of OPA1 in cells can cause disorganization of the mitochondrial inner membrane, release of cytochrome *c* and apoptosis (Olichon et al., 2003). In regards to treatment with DOX, previous studies have demonstrated reductions in the fusion proteins Mfn1/2 and OPA1 (Dirks-Naylor et al., 2014). Indeed, our results also show a decrease in OPA1 concentration after DOX administration that is specific to the SS mitochondria. Therefore, this decrease in protein expression could contribute to DOX induced apoptosis.

Autophagy is a process by which damaged and unwanted cytosolic proteins and organelles can be degraded by lysosomal proteases, and recently autophagy has been demonstrated to play a role in DOX-induced cardiomyopathy (Dirks-Naylor, 2013; Smuder et al., 2013b). Specific elimination of mitochondria by autophagy has been termed mitophagy, and the proteins PINK1 and Parkin play an important role in the selective recognition and elimination of dysfunctional mitochondria by mitophagy (Narendra and Youle, 2011). In addition to PINK1 and Parkin, p62 is also a marker of mitophagy. p62 is required to bind ubiquitinated proteins to the autophagosome via its interaction with LC3, and degradation of p62 is a marker of increased mitophagy (Narendra and Youle, 2011). Our results suggest that

IMF mitochondria are susceptible to increased mitophagy as this subpopulation demonstrated an increase in PINK1 and a reduction in p62 in DOX treated hearts. In addition, SS cardiac mitochondria treated with DOX had a greater concentration of pUB proteins than control animals. However, this change could be due to either increased rates of mitophagy or proteolytic processing by the ubiquitin proteasome system.

5. Conclusions

In summary, DOX administration results in acute cardiac dysfunction in the days following treatment as a result of both apoptosis and mitophagy, which occurs in part as a result of increased mitochondrial ROS production. While current research emphasizes the important contribution of mitochondrial dysfunction to DOX-induced myopathy, to date, the extent to which SS and IMF mitochondria are individually affected remained unknown. In this regard, the regulation of mitochondrial quality control is vital to maintaining optimally functioning cells, and our results demonstrate that spatial differences exist in the response of mitochondria to DOX exposure with each mitochondrial subpopulation eliciting independent phenotypic changes.

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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 $\pm$ SEM. § Significantly different versus Cytosolic and IMF ($p < 0.05$). ^ Significantly different versus Cytosolic ($p < 0.05$).

Fig. 2. Mitochondrial respiration and protein content from isolated SS and IMF mitochondria. A) state 3 respiration; B) state 4 respiration; C) respiratory control ratio; D) citrate synthase protein expression and; E) mitochondrial respiratory chain protein content (complex I (CI), complex II (CII), complex III (CIII), complex IV(CIV) and complex V (CV)) in isolated SS and IMF mitochondria from CON and DOX treated animals. Pyruvate/malate was used as a complex I substrate to measure mitochondrial respiration. Representative western blot

images are shown with the graph. Values are mean $\pm$ SEM. * Significantly different versus control (CON) ($p < 0.05$). # Significantly different versus SS mitochondria ($p < 0.05$).

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Fig. 3. Mitochondrial ROS emission and pore opening. A) mitochondrial hydrogen peroxide emission; B) maximal rate of pore opening (V_{max}) and; C) time to reach V_{max} during exposure to both calcium and reactive oxygen species in isolated SS and IMF mitochondria from CON and DOX treated animals. Values are mean $\pm$ SEM. * Significantly different versus control (CON) ($p < 0.05$). # Significantly different versus SS mitochondria ($p < 0.05$).

Fig. 4. Mitochondrial pro-apoptotic factors. A) Cytochrome c and B) AIF protein expression in isolated SS and IMF mitochondria from CON and DOX treated animals. Representative western blot images are shown to the right of the graph. Values are mean $\pm$ SEM. * Significantly different versus control (CON) ($p < 0.05$). # Significantly different versus SS mitochondria ($p < 0.05$).

Fig. 5. Markers of mitochondrial antioxidant capacity. A) GPX1; B) SOD1; C) SOD2; and D) catalase protein expression in isolated SS and IMF mitochondria from CON and DOX treated animals. Representative western blot images are shown below the graph. Values are mean $\pm$ SEM. * Significantly different versus control (CON) ($p < 0.05$). # Significantly different versus SS mitochondria ($p < 0.05$).

Fig. 6. Markers of mitochondrial dynamics. Mitochondrial fusion protein expression: A) OPA1 and B) Mfn2; and mitochondrial fission protein expression: C) Fis1 and D) DRP1 in isolated SS and IMF mitochondria from CON and DOX treated animals. Representative western blot images are shown below the graph. Values are mean $\pm$ SEM. * Significantly different versus control (CON) ($p < 0.05$). # Significantly different versus SS mitochondria ($p < 0.05$).

Fig. 7. Markers of mitophagy. A) p62; B) PINK1 and; C) Parkin protein expression in isolated SS and IMF mitochondria from CON and DOX treated animals. Representative western blot images are shown to the right of the graph. Values are mean $\pm$ SEM. * Significantly different versus control (CON) ($p < 0.05$).

Fig. 8.

Markers of mitochondrial biogenesis. A) TFAM and B) Nrf1 protein expression in isolated SS and IMF mitochondria from CON and DOX treated animals. Representative western blot images are shown to the right of the graph. Values are mean $\pm$ SEM. * Significantly different versus control (CON) ($p < 0.05$).

Fig. 9.

Markers of protein quality control. A) pUB; B) HSP70; C) HSP10; and D) Lon protein expression in isolated SS and IMF mitochondria from CON and DOX treated animals. Representative western blot images are shown below the graph. Values are mean $\pm$ SEM. * Significantly different versus control (CON) ($p < 0.05$). # Significantly different versus SS mitochondria ($p < 0.05$).