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Multi-Tissue Examination of Exercise or Metformin on the Consequences of Doxorubicin Treatment

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A dissertation submitted to the faculty of
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Doctor of Philosophy

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ABSTRACT

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Doxorubicin (DOX is an effective chemotherapeutic treatment with lasting deleterious side effects in heart and skeletal muscle. As an increased percentage of patients live many years past their cancer treatments, addressing the long-term side effects of chemotherapy treatment becomes critical. In an attempt to prevent heart and skeletal muscle damage caused by DOX, two co-treatments, exercise (EX or metformin (MET were studied for their effectiveness in maintaining muscle function, mitochondrial respiration and iron regulation. DOX is known to bind with iron, contributing to oxidative damage resulting in cardiac and skeletal muscle toxicity. However, the degree to which the toxic side effects are due to iron dysregulation is poorly understood. To address this gap in understanding, the changes in proteins involved with iron regulation following DOX treatment with or without EX or MET was examined in liver, heart, and skeletal muscle. To study the effects of EX or MET on DOX muscle toxicity and the effect of DOX on iron regulation, C2C12 myotube cell culture and a mouse model were used. Results from this research suggest that the some of the toxic effects of DOX treatment can be reduced with EX or MET treatments. EX is effective at preventing an impairment in muscle relaxation, promoting positive iron regulation changes in the liver and blunting DOX-induced changes in iron regulation in muscle. MET partially prevents loss of mitochondrial respiration and promotes positive changes in iron regulation in the liver. Additionally, study of DOX on iron regulation in liver, heart, and skeletal muscle suggests that DOX promotes iron dysregulation. However, the cellular response is protective against excessive iron dysregulation and increased oxidative stress. This cellular response is at least partially dependent on NF-κB activation.

Keywords: doxorubicin, exercise, metformin, muscle function, mitochondrial respiration, iron regulation, oxidative stress, NF-κB, skeletal muscle, cardiac muscle, liver

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CHAPTER 1: Introduction

Cancer is the second leading cause of death in the United States, with nearly 600,000 new cases diagnosed in 2015. As cancer screenings and treatments have improved, the rate of death due to cancer continues to drop with a 13% decrease from 2004-2013 ("Doxorubicin Hydrochloride," 2017). The long-term ill effects of chemotherapy treatment become more apparent as an increased percentage of patients survive many years past their cancer diagnosis and treatment. One chemotherapy agent, Doxorubicin (DOX), is known to have toxic effects in a variety of off-target tissues (Martins et al., 2012; Mohamed, Karam, & Amer, 2011; Shivakumar, Rani, Reddy, & Anjaneyulu, 2012). Clinically known as Adriamycin, DOX is widely used and effective against a variety of solid tumors and leukemias("Doxorubicin Hydrochloride," 2017). DOX's chemotherapeutic mechanism of action occurs through inhibiting replication by intercalating into DNA to inhibit topoisomerase II and DNA polymerase (Momparler, Karon, Siegel, & Avila, 1976). A cumulative clinical DOX dosage is set based on its side effect of causing cardiomyopathy, which occurs independent of its chemotherapeutic action (Gilliam & St Clair, 2011; Schwartz, Winters-Stone, & Gallucci, 2007).

Even within accepted clinical dosage, DOX is known to cause the degeneration of heart (Shivakumar et al., 2012), skeletal muscle (Smuder, Kavazis, Min, & Powers, 2011a), kidney (Shivakumar et al., 2012), liver (Shivakumar et al., 2012), testis (Shivakumar et al., 2012), and brain (Mohamed et al., 2011). The toxic effects of DOX on cardiac and skeletal muscle will be discussed here in detail due to their importance for long-term patient health and quality of life. Figure 1.1 summarizes the effects DOX is known to have in cardiac and skeletal muscle. To better understand the pathway by which DOX causes toxicity, the effects of DOX on muscle function, mitochondrial function, oxidative stress, and iron regulation will be discussed.

Additionally, exercise and metformin will be discussed as potential treatments to combat toxicity from DOX treatment.

DOX Causes Clinical Degeneration of Cardiac and Skeletal Muscle

Patients commonly experience cardiac and skeletal muscle degeneration as a side effect of chemotherapy treatment. Of course, maintaining cardiac function is of critical importance for patient survival. Therefore, total DOX dosage is limited based on preventing cardiomyopathy. Despite this limit, some patients still exhibit signs of impaired heart function, which can manifest acutely or several years after cessation of treatment (Mitry & Edwards, 2016; Schlitt et al., 2014). Skeletal muscle dysfunction leading to severe fatigue is a side effect experienced by nearly 90% of chemotherapy patients (Gilliam & St Clair, 2011; Luthy et al., 2011). These patients find completing simple daily activities to be exhaustive. This imposes limitations on their everyday lives, has a profound effect on their quality of life, and extends several years past the cessation of treatment (Elbl et al., 2006; Gilliam & St Clair, 2011; Villani et al., 2009). Additional research is required to understand the pathway by which DOX induces muscle toxicity and to identify potential counter treatments.

DOX Causes Heart and Skeletal Muscle Dysfunction

Animal studies have been used to better understand the mechanism by which DOX induces toxicity in heart and skeletal muscle. During time-course studies, DOX causes continual depression in cardiac and skeletal muscle function (Hayward et al., 2013; Jensen, Lien, Hydock, Schneider, & Hayward, 2013). Mice given DOX treatment have reduced in-vivo cardiac function with decreased mitral and aortic blood flow velocities (Hydock et al., 2012; Jensen et al., 2013). Ex-vivo studies reveal depressed left ventricular function through a reduction in developed

pressure, maximal rate of pressure development, and maximal rate of pressure decline (Chicco, Hydock, Schneider, & Hayward, 2006; Hayward et al., 2013; Hydock et al., 2012; Jensen et al., 2013). DOX treatment causes a reduction in skeletal muscle function (Bredahl & Hydock, 2017; Bredahl, Pfannenstiel, Quinn, Hayward, & Hydock, 2016; Ertunc, Sara, Korkusuz, & Onur, 2009; Hayward et al., 2013; Hydock, Lien, Jensen, Schneider, & Hayward, 2011; van Norren et al., 2009). Muscle function has been analyzed using an ex-vivo method, which measures the force of a slice of muscle, typically the extensor digitorum (EDL) or soleus (SOL). DOX has a greater effect on the SOL, which contains a high proportion of highly oxidative type 1 muscle fibers compared to other muscles like the EDL which is composed of primarily fast-twitch glycolytic muscle fibers (Bredahl & Hydock, 2017; Bredahl et al., 2016; Ertunc et al., 2009; Hayward et al., 2013). However, the effect of DOX in an in vivo model has not been evaluated.

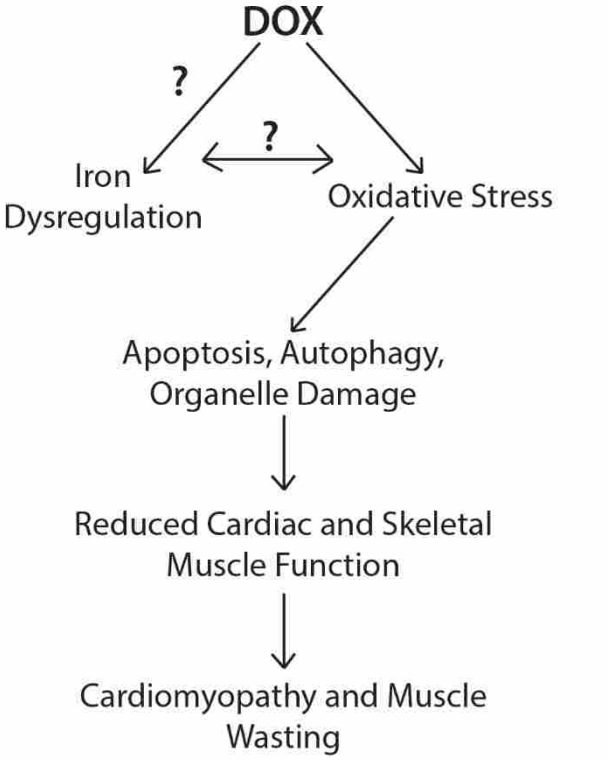

Figure 1.1: The Known Effects of DOX on Cardiac and Skeletal Muscle. The nature of the interactions between DOX, iron regulation, and oxidative stress are not well understood.

Exercise has been studied as a potential co-treatment to prevent muscle function loss due to DOX (Bredahl et al., 2016). Bredahl et al reported that muscle function in rats that exercised for 10 weeks prior to receiving DOX treatment had partially preserved SOL muscle function, but

EDL muscle function was not restored. It's not currently known if short-term exercise treatment is sufficient to prevent muscle function loss due to DOX.

DOX Causes Mitochondrial Damage in Cardiac and Skeletal Muscle

To better understand the mechanism of cardiomyopathy and muscle dysfunction associated with DOX treatment, mitochondrial function has been studied. DOX causes a reduction in electron transport chain function and disrupted mitochondrial calcium handling. Together, this causes disrupted mitochondrial function which contributes to the toxic side effects of DOX. Studies show reduced mitochondrial respiration in response to DOX in heart and skeletal muscle using a variety of techniques (Asensio-Lopez et al., 2014; Davies & Doroshow, 1986; Gilliam et al., 2013; Kavazis, Smuder, Min, Tümer, & Powers, 2010; Marques-Aleixo et al., 2015; Montaigne et al., 2010; Mouli et al., 2015; Yen, Oberley, Gairola, Szweda, & St Clair, 1999). When using intact heart or skeletal muscle mitochondria, DOX causes a decrease in maximum respiration (state 3) and ADP/O coupling efficiency(Gilliam et al., 2013; Marques-Aleixo et al., 2015; Yen et al., 1999). The effects of DOX on mitochondrial respiration appear to have distinct acute and long-term effects. Gilliam et. al (Gilliam et al., 2013) showed in a time course study that respiration decreased 2 hours after DOX administration and was restored to normal after 24 hours. At 72 hours, mitochondrial function had deteriorated to the lowest observed respiration levels. These results indicate that there are short and long-term effects of DOX on mitochondria with a 'rebound' intermediate period, an effect that has been observed elsewhere in the literature (Kavazis et al., 2010). The mechanisms behind the short and long-term effects are still largely unknown. Acutely, DOX inhibits complex I of the electron transport chain, which causes redox cycling and leads to increased reactive oxygen species (Berthiaume & Wallace, 2007; Davies & Doroshow, 1986; Doroshow & Davies, 1986; Gilliam et al., 2013;

Montaigne et al., 2010). There are two proposed mechanisms for long-term electron transport dysfunction. The first is that DOX-induced mitochondrial calcium signaling causes long-term depressed respiratory capacity (Wallace, 2007). This occurs through decreasing mitochondrial calcium reuptake(Ascensão et al., 2011; Marques-Aleixo et al., 2015) and reducing mitochondrial calcium retention(Gilliam et al., 2013). Second, DOX can cause mitochondrial DNA damage, resulting in irreversible mitochondrial disruption(Berthiaume & Wallace, 2007; Lebrecht, Setzer, Ketelsen, Haberstroh, & Walker, 2003). Treatments that prevent the mitochondrial dysregulation associated with DOX may decrease the overall toxicity of DOX in cardiac and skeletal muscle.

DOX Causes Oxidative Stress, Cell Death, and Inflammation in Cardiac and Skeletal Muscle

Oxidative stress is generally considered to be central to the cardio-toxic effects of DOX. Despite this, measurements of DOX-induced oxidative stress are not always consistent across studies. This suggests that the elevated oxidative stress from DOX treatment is complex and still poorly understood. Additionally, while increased oxidative stress following DOX treatment has been well studied in cardiac tissue, only a few studies have examined this in skeletal muscle. In cardiac tissue and cells, measurements of oxidative species such as hydrogen peroxide (Akolkar et al., 2017; Kavazis et al., 2010; Ludke, Akolkar, Ayyappan, Sharma, & Singal, 2017), nitric oxide (Akolkar et al., 2017), protein oxidation (Akolkar et al., 2017; Granados-Principal et al., 2014; Smuder et al., 2011a), reduced glutathione (Doroshow, Locker, Baldinger, & Myers, 1979; Khafaga & El-Sayed, 2018), and lipid peroxidation (Benzer, Kandemir, Ozkaraca, Kucukler, & Caglayan, 2018; Kavazis et al., 2010; Khafaga & El-Sayed, 2018; Shaker, Abboud, Assad, & Hadi, 2018) follow a general pattern of being elevated 1-2 days post DOX treatment followed by a restoration to normal at 3-5 days (Chicco et al., 2006; Deng et al., 2015), although

there are exceptions to this trend (Benzer et al., 2018). In contrast, expression of antioxidant enzymes such as superoxide dismutases and glutathione peroxidase in response to DOX do not exhibit a clear pattern of change. For example, superoxide dismutase activity is reported to be increased (Ascensão et al., 2011; Kavazis et al., 2010; Smuder et al., 2011a) decreased (Khafaga & El-Sayed, 2018; Zhang et al., 2017), or not changed (Chicco et al., 2006; Deng et al., 2015; Dirks-Naylor, Tran, Yang, Mabolo, & Kouzi, 2013) with no clear pattern regarding dosage or time since treatment. Glutathione peroxidase exhibits similar variability, with reports of increased (Chicco et al., 2006; Kavazis et al., 2010) or decreased (Anghel et al., 2017; Benzer et al., 2018; Khafaga & El-Sayed, 2018; Zhang et al., 2017) levels after DOX treatment. Overall, measurements of antioxidant enzyme activity are not consistent across studies. Oxidative species measurements, especially lipid peroxidation and glutathione, appear to follow a more consistent pattern.

DOX uniformly increases markers of apoptosis (Ascensão et al., 2011; Benzer et al., 2018; Chicco et al., 2006; Kavazis et al., 2010; Ludke et al., 2017; Shaker et al., 2018; Zhang et al., 2017), autophagy (Kavazis et al., 2010; Smuder et al., 2011a; Smuder, Kavazis, Min, & Powers, 2013) and inflammation (Akolkar et al., 2017; Benzer et al., 2018; Shaker et al., 2018; Zhang et al., 2017) in cardiac muscle. Of particular interest is the role of NF-κB activity in response to DOX treatment. NF-κB is activated in response to inflammation or oxidative stress signals and can result in pro-inflammatory and pro-apoptotic responses or anti-inflammatory and anti-apoptotic responses by the cell (Lawrence, 2009; Lawrence & Fong, 2010). The mechanisms behind this dichotomy are complex and are dependent upon cell type, inflammatory cytokines present, and involvement of specific NF-κB subunits. Each of these factors play a role in determining if anti- or pro- inflammatory pathways are activated (Lawrence, 2009; Lawrence

& Fong, 2010). While the mechanism is not completely understood, the pro- and anti- apoptotic effects of NF-κB activity with response to DOX have been studied. In cancer cell lines, DOX activates NF-κB causing anti-apoptoic signaling (Arlt et al., 2001; Cho et al., 2008; Ho, Dickson, & Barker, 2005; Seubwai et al., 2016). However, in cardiac cells, NF-κB causes pro-apoptotic signaling (Benzer et al., 2018; Wang et al., 2002; Zhang et al., 2017). The results of these studies suggest that the effects of DOX on NF-κB function are dependent upon cell type. Further research is required to determine the effects of DOX-induced NF-κB function in other cell types, including skeletal muscle.

DOX Interacts Directly with Iron

In addition to the effects of DOX on muscle function, cardiomyopathy, and oxidative stress, DOX can directly bind to iron. Furthermore, this interaction is known to increase free radical formation and is a potential mechanism by which DOX exerts its toxic effects (Eliot, Gianni, & Myers, 1984; Muindi, Sinha, Gianni, & Myers, 1984). DOX can bind to iron in its ferrous (Fe^{2+}) or ferric (Fe^{3+}) forms, which allows redox reactions to occur with oxygen through the fenton and haber-weiss reactions (shown on the next page). The iron chelator Deferoxamine reduces iron availability and prevents this DOX-iron interaction. Deferoxamine is the only clinically approved DOX co-treatment available to reduce cardiomyopathy (Wiseman & Spencer, 1998).

Fenton and Haber-Weiss Reactions

$Fe^{3+} + O_2^{\bullet -} \rightarrow Fe^{2+} + O_2$

$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH^{\bullet}$

Net Reaction: $O_2^{\bullet -} + H_2O_2 \rightarrow OH^- + OH^{\bullet} + O_2$

The role that DOX plays in changing proteins that regulate iron is complex and has only been analyzed in cardiac tissue. Studies report conflicting results on the effects of DOX on key proteins related to iron regulation (Corna, Galy, Hentze, & Cairo, 2006; Xu, Persson, & Richardson, 2005; Xu, Sutak, & Richardson, 2008) and potential mechanisms by which this change occurs (Bernuzzi, Recalcati, Alberghini, & Cairo, 2009; Corna et al., 2006; Ghigo, Li, & Hirsch, 2016; Muindi et al., 1984). Additionally, studies have almost exclusively focused on the effects of DOX on iron regulation in the heart and ignored other tissues of interest. Study of iron regulation in skeletal muscle is of interest because the iron needs of muscle are critically important for high energy turnover for respiration function.

In addition to cardiac and skeletal muscle, the effects of DOX on liver iron regulation need to be studied. The liver is central to systemic iron regulation and any changes in liver iron will have systemic ramifications. Currently, the effects of DOX on liver iron regulation are not known

The Major Iron Regulatory Pathway

To understand the effects and ramifications of DOX on iron regulation, a brief overview of the major iron regulatory pathway will be outlined (figure 1.2). Iron plays an essential role in virtually all organisms from bacteria to humans. In addition to binding oxygen in heme, iron is critical for DNA synthesis and electron transfer in the electron transport chain (Oliveira, Rocha, & Fernandes, 2014; von Drygalski & Adamson, 2013). While necessary for survival, iron has a high potential for creating reactive oxygen species and causing oxidative stress by reacting with oxygen. For this reason, iron absorption, transport and storage are highly regulated. Iron is absorbed into enterocytes through divalent metal transporter 1 (DMT1) and heme carrier protein 1 and is transported in the blood bound to transferrin. Most iron uptake occurs at the bone

marrow for erythropoiesis and the majority of the remaining iron is transported to liver or muscle. Iron is transported and stored in the cell in a manner that will facilitate low oxidative stress but keep iron available for use (figure 1.3). Once at the cell, transferrin binds transferrin receptor (TfR) and is endocytosed. A proton pump lowers the pH of the endosome, causing iron to release from transferrin. Iron is then pumped into the cytosol via DMT1. The iron enters a small labile iron pool that is loosely chelated, but available for cell needs. However, due to the highly oxidative nature of iron, the majority is transported to other organelles or stored in

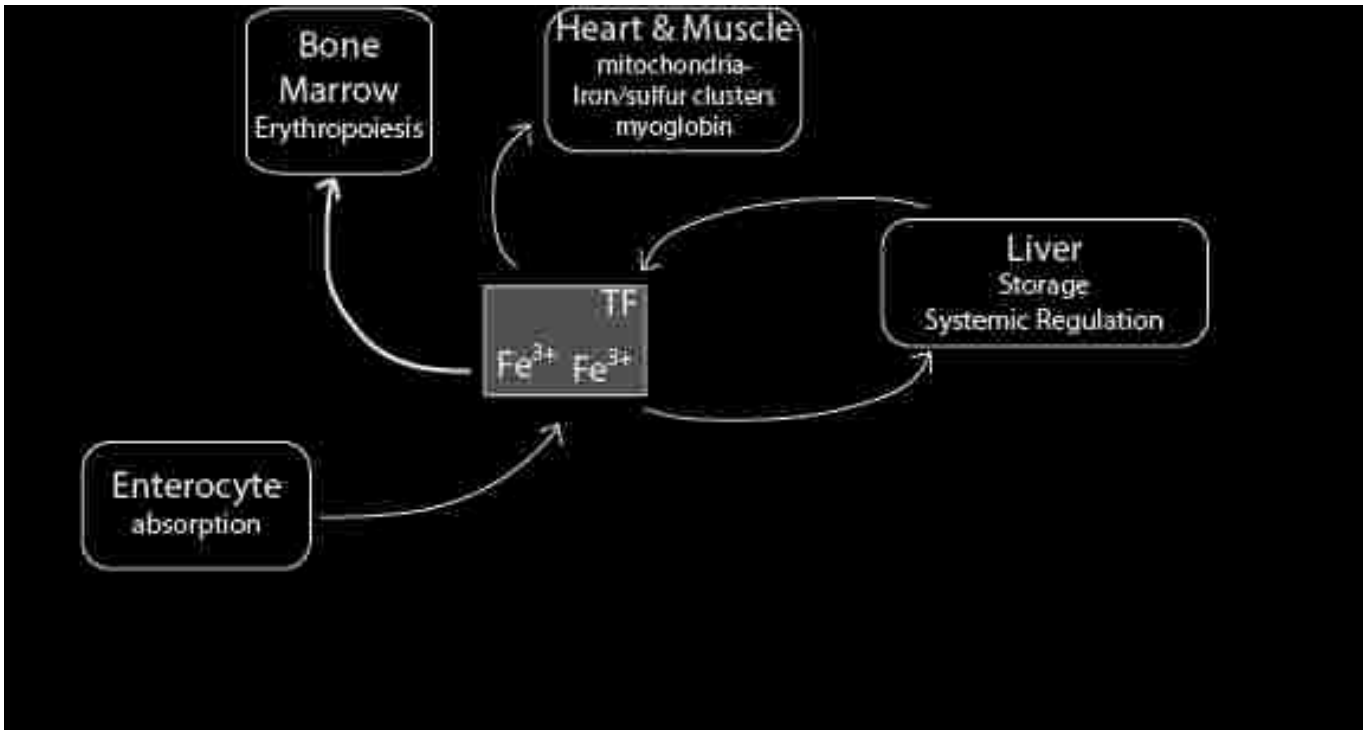

Figure 1.2: Overview of Systemic Iron Transport. TF- transferrin. Iron is absorbed through enterocytes and deposited onto TF. TF circulates through the blood stream and delivers iron to tissues throughout the body.

ferritin. Ferritin is the major storage protein for iron and is composed of 24 subunits including heavy (FHC) and light chains (FLC). FHC is a known antioxidant that oxidizes Fe^{2+} to Fe^{3+}, allowing it to enter the protein. FLC catalyzes the formation of iron phosphate crystals that allows iron to accumulate within the protein (Watt, 2011). Iron is exported from the cell by

ferroportin where it can then bind to transferrin for transport through the blood (Oliveira et al., 2014; von Drygalski & Adamson, 2013).

The Role of Iron Regulation with DOX

Given the iron chelator Deferoxamine reduces cardiomyopathy with DOX treatment, understanding the DOX-iron interaction is critically important. Currently, the literature has focused almost entirely on this interaction in cardiac tissue and there have been no reports in skeletal muscle or liver. Additionally, most DOX related studies limit their analysis of iron regulation to FHC. While FHC plays a major role in managing cellular iron regulation, TfR is also important for iron regulation/iron import and should also be evaluated.

In an attempt to clarify the relationship between DOX and iron in cardiac tissue, three studies have used iron overload to test whether or not more iron availability exacerbated the effects of DOX treatment (Corna, Santambrogio, Minotti, & Cairo, 2004; Guenancia et al., 2015; Panjrath et al., 2007). Interestingly, these studies report conflicting

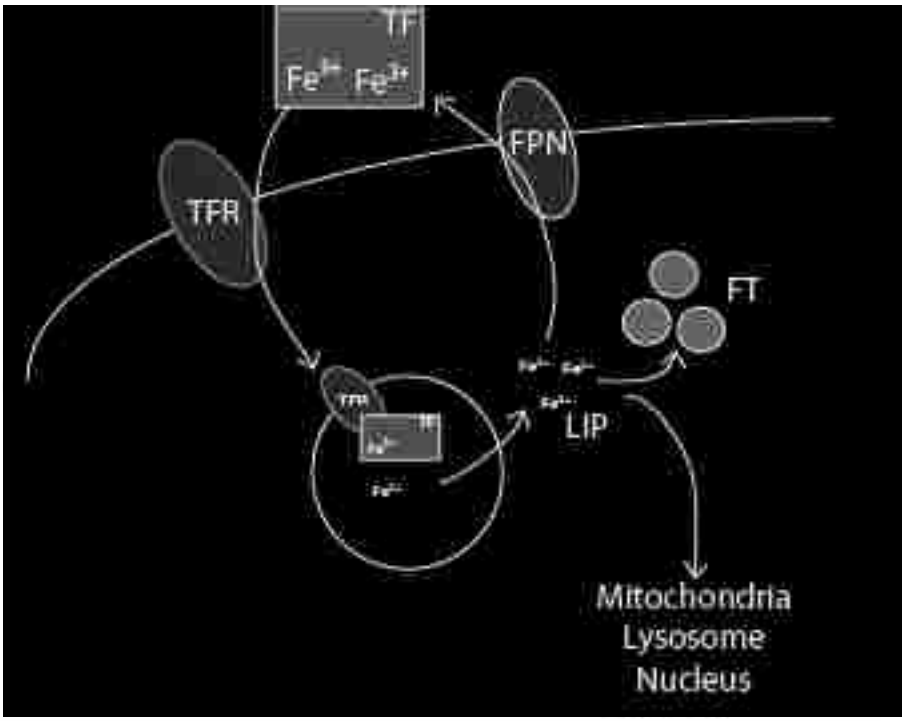

Figure 1.3: Overview of Cellular Iron Regulation. TF- transferrin. TFR- transferrin receptor. FPN- ferroportin. LIP-labile iron pool. FT- ferritin complex including heavy and light chains.

results. Corna et al reported that pre-treatment with DOX protected cardiomyocytes against oxidative stress induced by high iron treatment (Corna et al., 2004). Guenancia et al found that iron overload did not exacerbate decreased cardiac function with DOX in mice (Guenancia et al., 2015). The reason behind this phenomenon was not investigated and no data were shown in proteins involved in managing cellular iron like FHC or TfR. In direct contradiction to the previous studies, a Panjrath et al. (Panjrath et al., 2007) found iron overload increased cardiac toxicity and apoptosis with DOX. These results illustrate the complex nature of iron regulation and DOX treatment and the need for a more thorough analysis of proteins involved with iron regulation.

To further understand the effect of DOX on iron regulation in cardiac tissue, FHC and TfR have been measured. FHC protein levels are uniformly increased in response to DOX (Bernuzzi et al., 2009; Corna et al., 2006; Corna et al., 2004; Kwok & Richardson, 2003). The effects of DOX on TfR may be cell-type dependent. TfR levels are decreased in cardiac tissue (Corna et al., 2006) and fibroblast cells (Xu et al., 2008), but are increased in endothelial cells (Kotamraju, Chitambar, Kalivendi, Joseph, & Kalyanaraman, 2002). With data limited primarily to cardiac tissue, a multi-tissue analysis of iron regulation is needed to clarify the role of DOX on the major iron regulation pathway.

The mechanism behind DOX inducing changes in FHC, TfR, and other proteins involved in iron regulation is not understood. In light of the lack of a thorough, multi-tissue analysis of iron regulation, this is not surprising. Oxidative stress dependent pathways have been proposed as one mechanism and iron regulatory proteins as a second mechanism. Initially, it was believed DOX-induced changes to iron regulation were directly through increased oxidative stress (Eliot et al., 1984; Muindi et al., 1984). Indeed, many cell culture studies (Gilliam et al., 2012;

Kalivendi, Kotamraju, Zhao, Joseph, & Kalyanaraman, 2001; Kotamraju et al., 2002) have shown that antioxidants ameliorate the effects of DOX treatment. However, antioxidants are only sometimes effective in animal models and are ineffective in clinical models (Afsar, Razak, Batoo, & Khan, 2017; Akolkar et al., 2017; Ghigo et al., 2016; van Dalen, Caron, Dickinson, & Kremer, 2011). Additionally, Bernuzzi et al., using very low DOX concentrations, revealed that DOX can increase FHC protein levels without inducing apoptosis or reporting an increase in measures of oxidative stress (Bernuzzi et al., 2009). This suggests that a DOX-induced increase in FHC levels is at least partially independent of oxidative stress. The DOX-induced increase in FHC expression may be mediated through activating NF-κB. When NF-κB is activated by inflammation and oxidative stress, it translocates to the nucleus. NF-κB activity typically causes anti-apoptotic effects, although in some models it can be pro-apoptotic. DOX has been shown to mediate both anti-apoptotic (Arlt et al., 2001; Ho et al., 2005) and a pro-apoptotic (Wang et al., 2002) effects of NF-κB, depending on the experimental setup. There is an NF-κB binding site on the promotor for FHC gene transcription (Bresgen & Eckl, 2015; Kwak, Larochelle, Beaumont, Torti, & Torti, 1995; Pham et al., 2004), suggesting that DOX may exert its effects on iron regulation in an NF-κB dependent manner.

The second mechanism by which DOX may facilitate iron regulation is through specific iron regulatory proteins (IRPs). IRPs bind to mRNA of transcripts of several proteins involved in iron regulation, promoting mRNA stabilization or degradation, depending on the location of binding (Bresgen & Eckl, 2015). IRPs are activated under conditions of low iron availability and induce decreased FHC protein levels and increased TfR protein levels. Under high cellular iron, IRPs are not active, allowing for high FHC levels and low TfR levels. The role of DOX on IRP regulation is conflicting. Two studies report that IRPs are required for DOX-induced changes in

FHC (Ghigo et al., 2016; Xu et al., 2005) while a third study shows that DOX acts independent of IRPs (Corna et al., 2006). The reason for this conflict is not clear. Given the major cellular changes DOX treatment induces, it seems plausible that multiple mechanisms are at play. FHC and other iron regulation proteins may be regulated in response to DOX through oxidative stress, IRPs, or other mechanisms.

Using Exercise to Decrease DOX Toxicity

Treating or preventing the long-term side effects of DOX is of critical importance to prevent heart damage and increase quality of life. Exercise (EX) training has positive effects on maintaining heart and skeletal muscle function with DOX treatment. The obvious caveat here is that chemotherapy patients typically experience a range of ill side effects including weakness, tiredness, nausea, and vomiting that would prevent them from maintaining a rigorous EX program (Gilliam & St Clair, 2011; Luthy et al., 2011). However, studies done in breast cancer patients receiving chemotherapy, including DOX, have shown that completing moderate EX is possible (Schwartz, 2000; Schwartz et al., 2007). These patients successfully completed daily exercise routines, experienced reduced fatigue (Schwartz, 2000) and maintained bone mineral density (Schwartz et al., 2007).

Previous animal studies have typically completed EX treatment before DOX to avoid complications of exercising during chemotherapy. Studies on EX treatments prior to receiving DOX have lasted anywhere from 1 day to 10 weeks (Ascensão et al., 2011; Jensen et al., 2013; Kavazis et al., 2010; Kavazis, Smuder, & Powers, 2014; Smuder et al., 2011a; Smuder, Kavazis, Min, & Powers, 2011b; Smuder et al., 2013). Additionally, a few studies have continued EX regimens after receiving DOX (Chicco et al., 2006; Dickinson et al., 2017; Huang et al., 2017; Hydock et al., 2012). EX treatment with DOX partially preserves cardiac function (Hydock et

al., 2012; Jensen et al., 2013; Kouzi & Uddin, 2016) and decreases oxidative stress, autophagy, and apoptotic markers in cardiac tissue (Chicco et al., 2006; Kavazis et al., 2010; Smuder et al., 2013). Similarly, in skeletal muscle, EX with DOX results in maintenance of partial muscle function (Bredahl et al., 2016) and fiber size (Dickinson et al., 2017). Additionally, markers of autophagy, proteolysis and oxidative stress are decreased (Kavazis et al., 2014; Smuder et al., 2011a, 2011b). EX treatment has been shown to not interfere with, or decrease the efficacy, of chemotherapy treatment (Sturgeon et al., 2014). This evidence suggests that EX treatment can safely attenuate the toxic side effects of DOX. The effect EX has on iron regulation with DOX treatment has not been studied.

Using Metformin to Decrease DOX Toxicity

Metformin (MET) has recently become a drug of interest for its potential to decrease DOX toxicity in the heart. MET is a commonly used anti-diabetic drug that has a variety of mechanisms of action; some of which are still under investigation. MET increases cardiac cell survival after DOX treatment through activating AMPK, which activates NF-κB, causing increased FHC expression (Asensio-López, Lax, Pascual-Figal, Valdés, & Sánchez-Más, 2011; Asensio-López et al., 2013; Asensio-Lopez et al., 2014). Follow up studies have shown that MET treatment preserves cardiac function, decreases markers of heart failure, autophagy, and oxidative stress in cardiac tissue (Argun et al., 2016; Kelleni, Amin, & Abdelrahman, 2015; Sheta, Elsakkar, Hamza, & Solaiman, 2016). Additionally, MET has been shown to have a variety of beneficial effects on aiding cancer treatment. Clinically, MET use is associated with lower rates of some types of cancer, despite people with type 2 diabetes having an increased risk for developing cancer (Li, Yeung, Hassan, Konopleva, & Abbruzzese, 2009; Viollet et al., 2012; Yue, Yang, Dipaola, & Tan, 2014). MET has also been shown to decrease proliferation of cancer

cells (Demir, Koehler, Schneider, Schweiger, & Klocker, 2014; Lee et al., 2014) and to increase the efficacy of DOX treatment in cancer cells (El-Ashmawy, Khedr, El-Bahrawy, & Abo Mansour, 2017; Wu et al., 2016). With its potential to increase the effectiveness of DOX treatment and protect against its toxic side effects, MET is an excellent co-treatment candidate. The effects of MET on skeletal muscle in the DOX model have not yet been tested and the mechanism behind how MET protects against the toxic effects of DOX treatment is still not well understood.

In conclusion, as the rate of cancer survival increases, the need to address the severe side effects associated with chemotherapy also increases. Specifically, multi-tissue analysis of the effect of DOX on iron regulation has not been investigated. Identifying co-treatments that will combat the toxic side effects of DOX is of critical importance. Exercise and metformin treatments have both been shown to be beneficial at preserving cardiac function. However, their effectiveness in skeletal muscle and on iron regulation are still under investigation. Decreasing DOX toxicity in muscle will improve patient outcomes and long-term quality of life.

Study Design

The purposes of this research were to (1) characterize how iron regulation changes in response to DOX in liver, heart, and skeletal muscle and (2) analyze the effectiveness of EX or MET on preventing DOX toxicity in skeletal muscle.

Experimental Set Up

Skeletal muscle cell culture and an animal model were employed to study DOX, EX, and MET treatments. In cell culture, differentiated C2C12 myotubes were used. To analyze the acute effects of DOX and MET treatments on myotubes, 0.5 μM DOX and/or 0.5 mM MET were incubated in differentiation media for 16 hours.

In the animal model, the effects of DOX were studied after three days of treatment, as shown in figure 1.4. Mice were assigned to one of six groups: CON, DOX, EX, DOX+EX, MET, or DOX+MET. DOX mice received an intraperitoneal injection of 15 mg/kg DOX and were euthanized 3 days later. After acclimating to the treadmill, EX mice ran at a moderate pace of ~70% max for 60 minutes for 5 days. DOX+MET mice received DOX treatment after completion of the last bout of running. MET mice received daily 500 mg/kg oral MET beginning 2 days before DOX treatment and extending until euthanized.

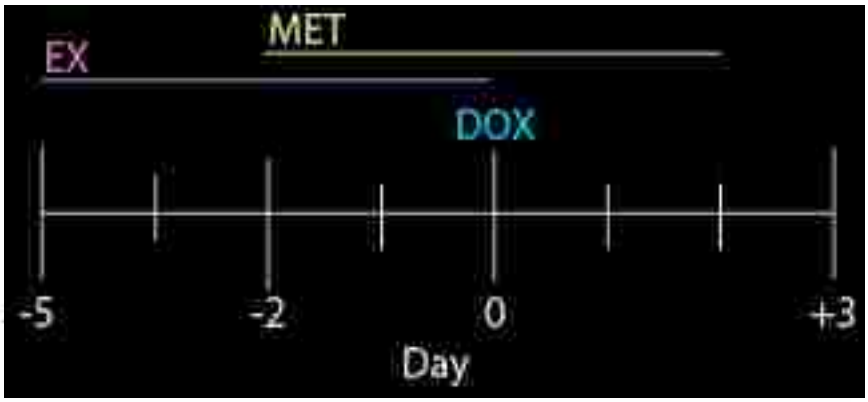

Figure 1.4: Timeline of Mice Receiving Exercise Training, Metformin, and/or DOX Treatments.

Specific Aims

- Determine if DOX induced skeletal muscle dysfunction can be prevented with EX or MET treatments using a novel in-situ model of assessing muscle function.

- Determine if MET treatment can prevent the mitochondrial dysfunction associated with DOX treatment in C2C12 mouse skeletal muscle myotubes.

- Characterize the effect DOX has on the major iron regulatory pathway in cardiac muscle, skeletal muscle, and liver. Determine if EX or MET exert their effects against DOX through modulating changes in iron regulation.

- Determine if the changes in iron regulation from DOX and/or MET treatment are due to NF-κB activity in C2C12 mouse skeletal muscle myotubes.

CHAPTER 2: Exercise, But Not Metformin Prevents Loss of Muscle Function Due to Doxorubicin in Mice Using an in-situ Method

Introduction

The chemotherapeutic Doxorubicin (DOX) is used clinically to treat a variety of solid tumor and leukemia cancers by intercalating into DNA and inhibiting replication (Minotti, Menna, Salvatorelli, Cairo, & Gianni, 2004). A cumulative clinical DOX dosage is set based on DOX's side effect of causing cardiomyopathy (Gilliam & St Clair, 2011; Schwartz et al., 2007). Even within accepted clinical dosage, patients receiving DOX experience severe fatigue, muscle loss, and some heart damage. These side effects are due to the accumulation of DOX in cardiac, skeletal, and smooth muscle and remain in effect long term (Elbl et al., 2006; Gilliam & St Clair, 2011; Villani et al., 2009). Research into the mechanism of action of DOX on muscle is ongoing with most studies focusing on cardiac muscle at both the molecular (Kavazis et al., 2010; Kouzi & Uddin, 2016; Smuder et al., 2013) and functional (Chicco et al., 2006; Ertunc et al., 2009; Hayward et al., 2013; Hayward, Lien, Jensen, Hydock, & Schneider, 2012; Jensen et al., 2013; Kouzi & Uddin, 2016) levels. Additional study of DOX on skeletal muscle is warranted due to the importance of skeletal muscle in metabolism, mobility in carrying out daily activities, and quality of life. Treatments that combat the side effects of DOX without altering its chemotherapeutic potency will make DOX a more usable drug and increase patients' long-term quality of life.

Skeletal muscle function is central to carrying out basic everyday activities and its dysfunction is a contributor to severe fatigue. Fatigue is experienced by nearly 90% of chemotherapy patients (Gilliam & St Clair, 2011; Luthy et al., 2011). Patients find completing simple daily activities to be exhaustive, which imposes limitations on their everyday lives and has a profound effect on quality of life. Research into the mechanism behind these side effects is

ongoing and studies have shown DOX causes increased oxidative stress and activation of proteolytic pathways in skeletal muscle (Dickinson et al., 2017; Smuder et al., 2011a, 2011b). Additionally, a decrease in muscle force production has been measured in an ex vivo model (Bredahl & Hydock, 2017; Bredahl et al., 2016; Ertunc et al., 2009; Gilliam & St Clair, 2011; Hayward et al., 2013; Hydock et al., 2011; van Norren et al., 2009). In this study, we further analyzed muscle function of the gastrocnemius-plantaris-soleus (GPS) complex using an in-situ model.

Recent studies have shown exercise treatment (EX) with DOX to limit skeletal muscle proteolysis (Kavazis et al., 2014; Smuder et al., 2011a, 2011b), maintain mitochondrial respiration (Gilliam et al., 2013; Marques-Aleixo et al., 2015) and preserve muscle function (Bredahl et al., 2016). Clinically, moderate exercise in breast cancer patients is beneficial to reduce fatigue (Schwartz, 2000) and maintain bone mineral density (Schwartz et al., 2007). The effects of EX on preserving muscle function is still not fully understood and muscle function analysis has never been conducted using an in-situ method. Acute moderate exercise completed before DOX treatment was used in this study due to the difficulties associated with exercise after receiving chemotherapy.

The anti-diabetic drug metformin (MET) was also used as a potential treatment to limit the toxicity of DOX in skeletal muscle. Emerging literature suggests that MET may be beneficial at decreasing the toxic side effects of DOX in cardiac cells (Asensio-López et al., 2011; Asensio-López et al., 2013; Asensio-Lopez et al., 2014; Kobashigawa, Xu, Padbury, Tseng, & Yano, 2014) and in cardiac tissue of animal models (Argun et al., 2016; Kelleni et al., 2015; Sheta et al., 2016). MET is also under investigation as a potential co-treatment to enhance the effectiveness of chemotherapeutic treatments (El-Ashmawy et al., 2017). The effects of MET in

skeletal muscle remain unknown. Here we used a daily high dose of MET beginning 2 days before DOX treatment and continuing until euthanasia.

The purpose of this study is to determine if EX or MET treatment can prevent skeletal muscle dysfunction due to DOX treatment. The negative effects of DOX treatment on skeletal muscle function were analyzed with or without EX or MET treatments in the GPS complex using an in-situ method. Additionally, the cause for decreased muscle function was investigated by measuring mitochondrial function via high resolution respirometry.

Methods

Animal Care

All animal care and experimental procedures were approved by the Institutional Animal Care and Use Committee of Brigham Young University. 5-week old C57BL/6 mice were fed standard chow (Harlan Teklad 8064) and water ad libitum and housed in a temperature controlled environment (21-22°C) with a 12 hour light/dark cycle.

Mice were randomly assigned to 4 groups: control (CON), doxorubicin (DOX), doxorubicin + exercise (DOX+EX), or doxorubicin + metformin (DOX+MET). Mice with DOX treatments received a single 15 mg/kg intraperitoneal injection, which has been previously shown to be sufficient to cause muscle dysfunction in a rodent model (Bredahl et al., 2016; Hayward et al., 2013), and were sacrificed 3 days after injection. Mice with exercise treatments were acclimated to the treadmill on days 1-3 (5-10 m/min for 10 min/day), performed a maximum running test on day 4, and rested on days 5-6. On days 7-11, mice performed 60 minutes of running at about 70% max speed. DOX treatments were given on the final day of exercise, after completion of the exercise bout. Mice with MET treatments were given 5 daily

doses at 500 mg/kg body weight via oral gavage beginning two days before DOX treatment. Control mice received appropriate saline treatments to mimic MET and DOX treatments.

In-situ Muscle Function

To assess muscle function In-situ, contractile function and fatigue of the gastrocnemius-plantaris-soleus (GPS) complex measurements were made as described previously (Hancock, Janssen, & Terjung, 2005; Hardman, Hall, Cabrera, Hancock, & Thomson, 2014). Mice were anesthetized with 2.5-3.5% vaporized isofluorane in supplemental oxygen. The calf complex was isolated and the Achilles tendon excised with a small portion of the heel. The Achilles tendon was then attached to the lever arm and held in place during the contraction bout. Tetanic contractions of the GPS were elicited by direct stimulation of the sciatic nerve (2-4 V) for 6 minutes using a Grass S88X stimulator (0.5 Hz train frequency, 100 ms train duration, 150 Hz pulse frequency). Results were analyzed using ASI 610A Dynamic Muscle Control V5.500 software. Data were analyzed for peak force, and half relaxation times.

Mitochondrial Respiration

A sample of gastrocnemius (~6 mg) fibers were dissected then permeabilized with saponin (20 μg/mL) for 30 minutes. After washing, the fibers were placed in the Oxygraph-2K respirometer (OROBOROS INSTRUMENTS, Innsbruck, Austria) chambers with 2 mL of MiR05 respiration buffer (110 mM sucrose, 60 mM potassium lactobionate, 3 mM magnesium chloride, 20 mM taurine, 10 mM potassium phosphate, 0.5 mM EGTA, 20 mM HEPES, and 1 g/L BSA, pH=7.4). After adding oxygen to ~450 nmol/mL the chamber was sealed and allowed to equilibrate. Glutamate (2 mM), malate (10 mM) and ADP (2.5 mM) were added to stimulate Complex I respiration (CI). Succinate (10 mM) was added next to stimulate full oxidative

phosphorylation capacity (CI+CII). Maximum uncoupled respiration was measured using FCCP (0.5 μM steps). Complex I was then inhibited with rotenone (0.5 μM). Cytochrome C (10 μM) was added to ensure mitochondrial membrane integrity, and any data with an increase in respiration more than 10% were discarded. Finally, all respiration was inhibited at Complex III with antimycin A (2.5 μM) and resulting residual oxygen consumption was subtracted from the data set. Experiments were performed at 25°C and results confirmed at 37°C.

Hydrogen Peroxide Production

Hydrogen peroxide production was measured simultaneously with respiration measurements as described previously (Tueller, Harley, & Hancock, 2017). Briefly, horse radish peroxide (1 Unit/mL) and Amplex® Ultrared (10 μM) were added to the chamber. Three 0.1 μM hydrogen peroxide steps were added for a calibration curve. Fluoresce readings were taken with 563 nm excitation and 587 nm emission wavelengths.

Statistics

Comparisons between CON, DOX, and DOX+EX or CON, DOX, and DOX+MET were analyzed using one-way ANOVA and a Tukey-Kramer post-hoc. All significant values were tested with an alpha level of 0.05. Results are displayed in two groups for clarity.

Results

DOX Treatment Caused Severe Body Weight Loss at 3 Days

A single bolus of DOX was administered to 6-week old mice which were euthanized 3 days later. Consistent with previous studies, DOX treatment caused a significant reduction in body weight (Table 2.1). All groups had similar starting weight and groups that received DOX

lost a significant amount of weight ($p \leq 0.05$). This weight loss was partially blunted in the DOX+EX group ($p \leq 0.05$) and DOX+MET group ($p = 0.0506$) indicating that DOX+EX and DOX+MET treatments had some beneficial effect in preventing weight loss due to DOX. There was no change in average calf weight (gastrocnemius-soleus-plantaris complex) between groups (Table 2.2). The calf weight expressed as % of total body weight was higher in the DOX and DOX+EX groups ($p \leq 0.05$).

DOX Treatment Caused a Minor Change in Force Production of GPS Complex

Force production of GPS complex over a 6-minute fatigue protocol is shown in figure 2.1. DOX treatment caused a slight decrease in force production, which trended toward significance ($0.08 \geq p \leq 0.04$) at time points 0, 160, 180 and 360 seconds in the EX analysis (figure 2.1A). DOX+EX resulted in restoration of force production back to CON levels. Histogram representation of force produced are shown at time 0 seconds (figure 2.1B) and time 360 seconds (figure 2.1C). In the MET mice (figure 2.1D), DOX+MET trended towards lower force production ($0.09 \geq p \leq 0.06$) at time points 180, 200, 240, 260 and 360 seconds. Histogram representation of force produced are shown at time 0 seconds (figure 2.1E) and time 360 seconds (figure 2.1F).

DOX Treatment Impaired Half Relaxation Rate

DOX increased the time to half relaxation following a contraction, indicating a slower muscle recovery (figure 2.2). DOX slowed half relaxation time by an average of 18% over CON. In the EX mice, this difference reached significance ($p \leq 0.05$) at time points 60 and 100-360 seconds (figure 2.2A). The impairment in half relaxation time was prevented in the DOX+EX group, which was different from DOX at time points 60-360 ($p \leq 0.05$) (figure 2.2A). Histogram

representation of half relaxation time shown at time 120 seconds (figure 2.2B). In the MET mice, DOX was different from CON at time points 80-360 seconds ($p \leq 0.05$) (figure 2.2C). The half relaxation rate was impaired in the DOX+MET group versus CON at time points 0-360 seconds ($p \leq 0.05$). Histogram representation of half relaxation time shown at time 120 seconds (figure 2.2D).

DOX Treatment Does Not Impair Mitochondrial Respiration

High resolution respirometry was used to investigate if the loss in muscle function due to DOX treatment was because of a disruption in the functionality of the electron transport chain. There was no difference in respiration rates or hydrogen peroxide production among any groups (figure 2.3). These results suggest that maximal electron transport chain capacity is not responsible for impairment of half relaxation time.

Discussion

The purpose of this study was to determine if exercise or metformin treatment can prevent skeletal muscle dysfunction due to DOX treatment. Here we report that DOX causes a small decrease in GPS complex force and a significant impairment of half relaxation time. These changes were effectively prevented with exercise, but not metformin treatment. Additionally, mitochondrial respiration was not different between groups, suggesting that changes in muscle function were not due to a decrease in respiration capacity.

While a small decrease in force production was observed due to DOX treatment, the effect was not as large as expected. This is the first study to use an in-situ method to test muscle function and some differences are expected when comparing results from ex-vivo and in-situ models. The in-situ method is a more physiologically relevant method because the muscle is left

intact with continual blood circulation for the duration of the experiment. While skeletal muscle proteolysis has been shown to occur as early as 24 hours after DOX administration (Kavazis et al., 2014; Smuder et al., 2011a), skeletal muscle dysfunction is still progressing at day 5 (Hayward et al., 2013). At the 3-day time point used in this study, skeletal muscle dysfunction may not have been fully developed.

DOX treatment also has a greater effect on the slow, highly oxidative type I fibers than the fast, glycolytic type II fibers. Most other muscle function studies with DOX treatment have been done in a rat model (Bredahl & Hydock, 2017; Bredahl et al., 2016; Ertunc et al., 2009; Hayward et al., 2013; Hydock et al., 2011). During these studies, the ex-vivo function of the soleus (SOL) and extensor digitorum longus (EDL) muscles were analyzed. The SOL in rats is composed of ~80% type I fibers and ~20% type II fibers and the EDL is has ~5% type I fibers and 95% type II fibers (Punkt, Naupert, & Asmussen, 2004). DOX has a more profound effect on SOL, than the EDL (Bredahl & Hydock, 2017; Bredahl et al., 2016; Ertunc et al., 2009; Hayward et al., 2013). Here we show data from the GPS complex of mice, which is composed primarily of the gastrocnemius muscle (GAS). The GAS is ~6% type I fibers and 94% type II fibers-primarily type IIb fibers which are the most glycolytic. The GAS is more similar to the EDL of the rat, which has a less pronounced effect from DOX treatment (Augusto, Padovani, & Campos, 2004). Additionally, the study that examined the effects of chronic EX with DOX treatment reported that EX partially preserved muscle function in the SOL but not the EDL (Bredahl et al., 2016). Future studies should focus on the effects of DOX on whole muscles with mixed fiber types using the more physiologically relevant in-situ method.

The data from this study show that DOX impaired the half relaxation rate, an effect that was prevented in the DOX+EX group but not the DOX+MET group. The half relaxation rate is

the time required for the muscle to relax by 50% following the completion of an electrical stimulus. As the muscle fatigues, the time to half relaxation increases. Because the muscle was given time to recover between contractions, the impaired relaxation rate did not have a large effect on the force production of the muscle. Previous work done by Hancock et al. (Hancock, Brault, Wiseman, Terjung, & Meyer, 2005; Hancock, Janssen, et al., 2005) suggests that the half relaxation rate is tied directly to the energy state of the muscle. An impairment of half relaxation rate suggests a transient lack of energy availability in the cell resulting in slower actin-myosin release and a transient decrease in the rate of calcium sequestering in the sarcoplasmic reticulum. To better understand the mechanism behind how the half relaxation rate was impaired, mitochondrial respiration was measured as an indicator of energy production capacity.

In this study, no change was found in mitochondrial respiratory capacity, which is different from previous reports (Gilliam et al., 2013; Marques-Aleixo et al., 2015). Gilliam et al. (Gilliam et al., 2013) used the same technique to measure respiration and found a DOX-induced decrease at 2 hours, restoration at 24 hours and depression again at 72 hours in a rat model. These data suggest that there is an acute effect of DOX on mitochondrial function, followed by a recovery period and an eventual loss of function. In the present study done in mice, there was no change in mitochondrial respiration at the 3-day time point, which may suggest that mice have a longer, more profound recovery effect than what is found in rats. It would therefore be expected that an acute treatment or longer treatment would result in mitochondrial depression in a mouse model. These data also suggest that the impairment of half relaxation rate is not due to mitochondrial dysfunction, indicating that mitochondrial dysfunction is not required for the development of muscle dysfunction.

Table 2.1: Average Mouse Body Weight Before and After DOX Treatment and Percent Weight Change. Results are shown as a mean ± SEM. DOX main effect on final BW $p<0.05$. Values with different letters are significantly different $p<0.05$. Percent body weight change DOX+MET vs DOX $p=0.0506$.

Body Weight	CON	DOX	DOX+EX	DOX+MET
Starting BW (g)	19.8±0.3	20.0±0.3	20.3±0.3	19.7±0.4
Final BW (g)	20.2±0.3^{a}	16.7±0.3^{b}	17.7±0.4^{b}	17.0±0.3^{b}
Change BW (%)	2.0±0.7^{a}	-19.8±1.3^{b}	-15.2±1.9^{c}	-15.6±1.5

Table 2.2: Average Weights of Gastrocnemius-Soleus-Plantaris Complex. Average calf weights (mg) ± SEM. Calf weights as percent of total body weight (BW). Values with different letters are significantly different $p<0.05$.

Group	CON	DOX	DOX+EX	DOX+MET
Calf weight (mg)	129.9±4.3	121.4±4.5	113.7±3.2	125.7±3.2
% of BW	0.62±0.02^{a}	0.71±0.01^{b}	0.65±0.02^{b}	0.69±0.02^{a}

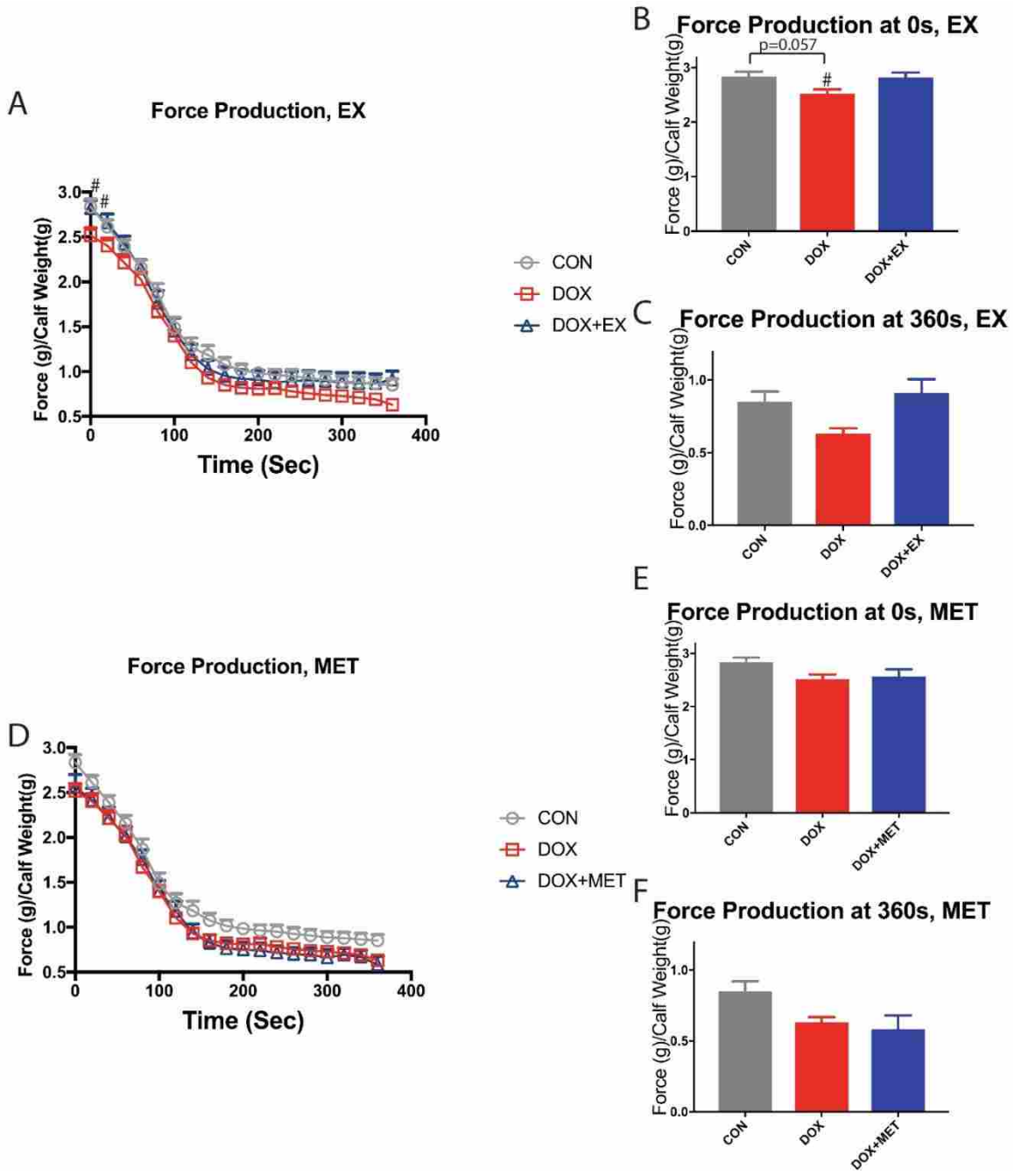

Figure 2.1: Force Production of GPS Complex Over 6 Minute Fatigue Protocol. (A) Force produced over 6 minute protocol of CON, DOX, and DOX+EX groups. (B) Histogram representation of force at time point 0 seconds of CON, DOX, and DOX+EX groups. (C) Histogram representation of force at time point 360 seconds of CON, DOX, and DOX+EX groups. (D) Force produced over 6 minute protocol of CON, DOX, and DOX+MET groups. (E) Histogram representation of force at time point 0 seconds of CON, DOX, and DOX+MET groups. (F) Histogram representation of force at time point 360 seconds of CON, DOX, and DOX+MET groups. N=6-8 ± SEM. # $p<0.05$ DOX vs DOX+EX

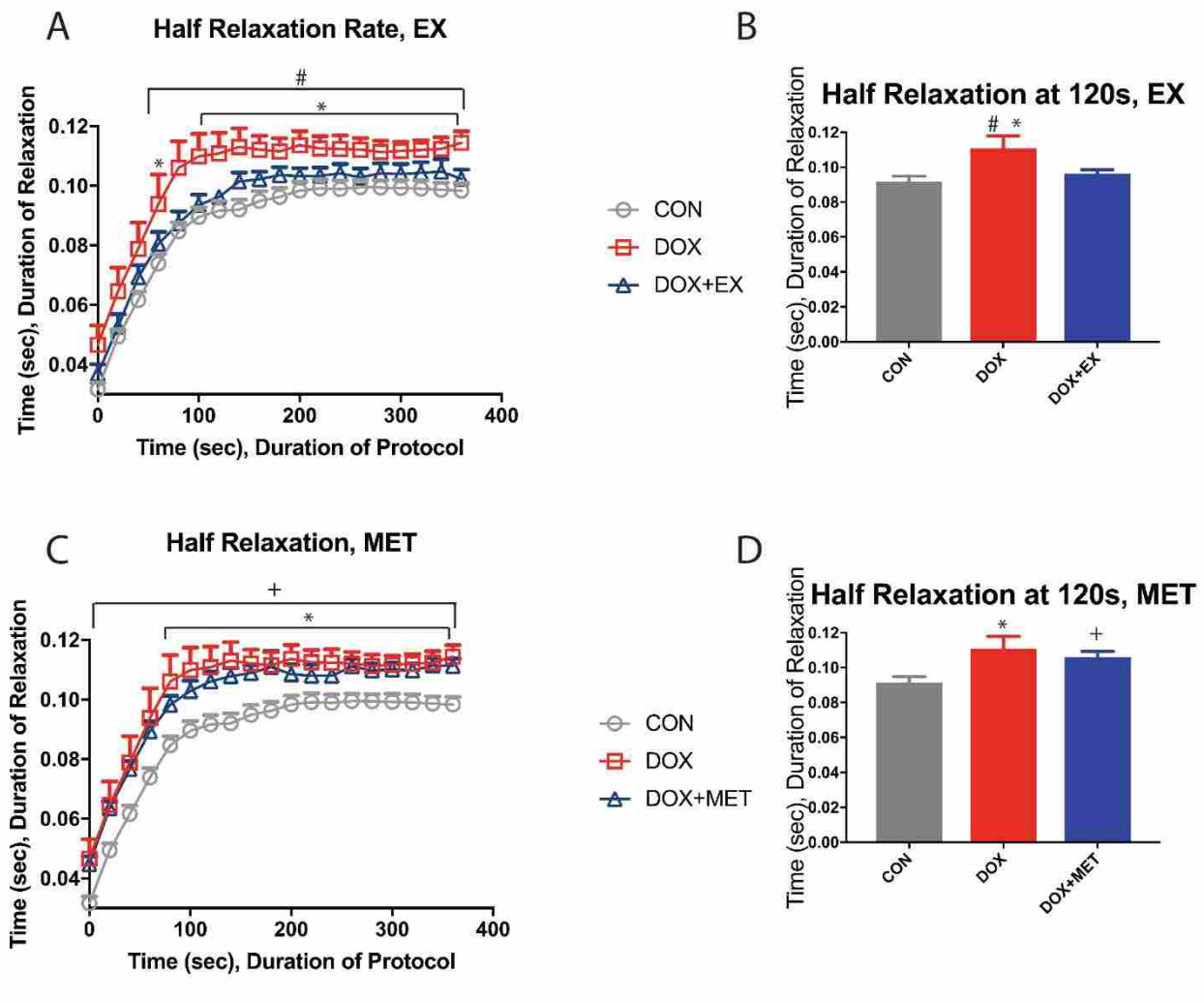

Figure 2.2: Half Relaxation Rate of GPS Complex over 6 Minute Fatigue Protocol. (A) Half relaxation rate over 6 minute protocol of CON, DOX, and DOX+EX groups. (B) Histogram representation of force at time point 120 seconds of CON, DOX, and DOX+EX groups. (C) Half relaxation rate over 6 minute protocol of CON, DOX, and DOX+MET groups. (D) Histogram representation of force at time point 120 seconds of CON, DOX, and DOX+MET groups. N=6-8 ± SEM. * $p<0.05$ CON vs DOX, # $p<0.05$ DOX vs DOX+EX, + $p<0.05$ CON vs DOX+MET.

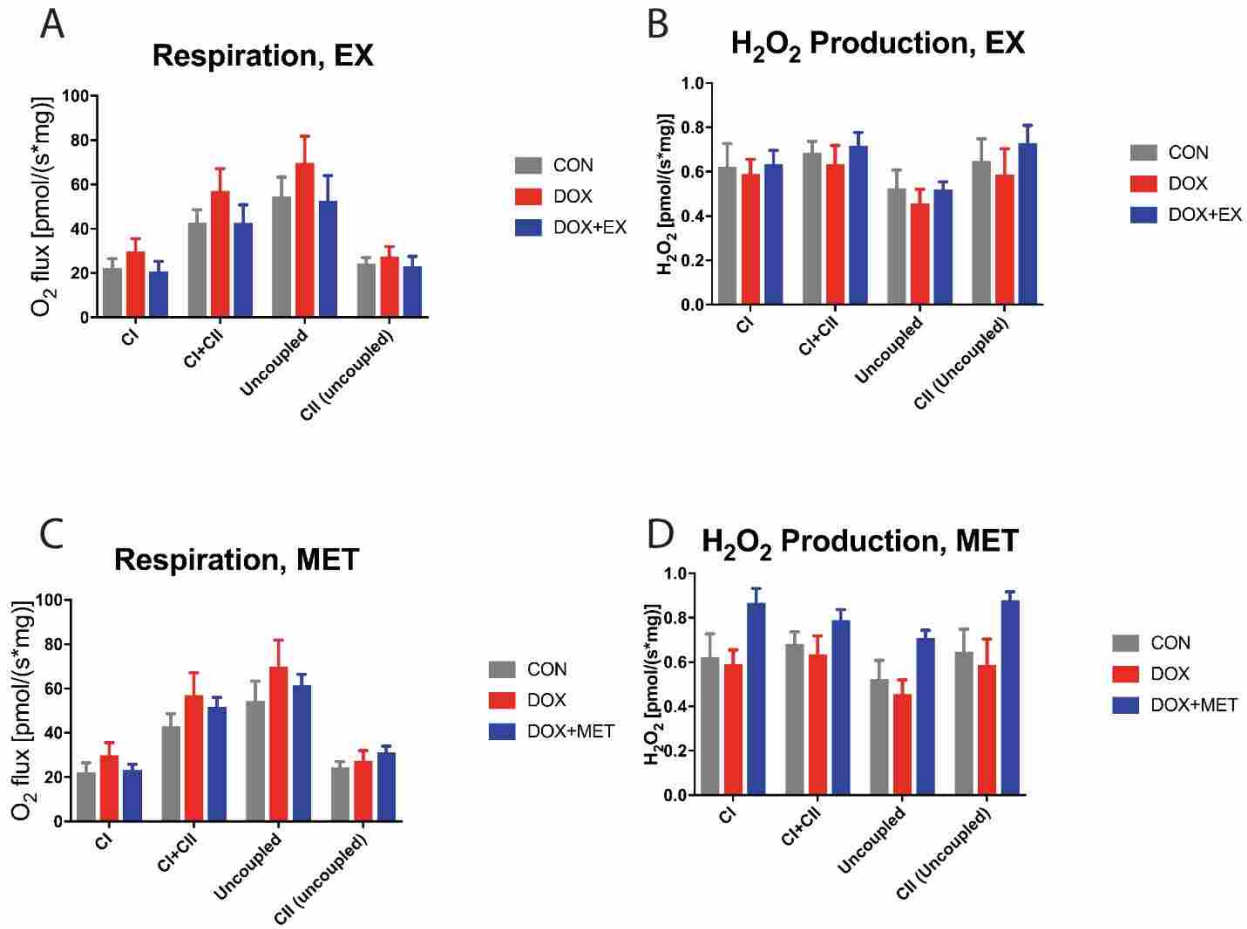

Figure 2.3: High Resolution Respirometry of Red Gastrocnemius. (A) Respiration rate of CON, DOX, and DOX+EX groups, n=6-9 ± SEM. (B) Hydrogen peroxide production of CON, DOX, and DOX+EX groups, n=4-6 ± SEM. (C) Respiration rate of CON, DOX, and DOX+MET groups n=6 ± SEM. (D) Hydrogen peroxide production of CON, DOX, and DOX+MET groups, n=3-5 ± SEM.

CHAPTER 3: Metformin Prevents Doxorubicin-Induced Inhibition of Complex II of the Electron Transport Chain

Introduction

The widely used chemotherapeutic drug doxorubicin (DOX) is known to have severe side effects in muscle (Mitry & Edwards, 2016; Shivakumar et al., 2012). As a chemotherapy agent, DOX exerts its effects by inhibiting DNA replication and is effective in a wide variety of solid tumors and leukemia cancers ("Doxorubicin Hydrochloride," 2017). A total dosage cap is in place clinically to limit the cardiotoxic effects of DOX (Schwartz et al., 2007). Even within acceptable dosage limits, patients experience severe muscular side effects including impaired heart function (Mitry & Edwards, 2016; Schlitt et al., 2014; Shivakumar et al., 2012) and severe fatigue (Gilliam & St Clair, 2011; Shivakumar et al., 2012; Smuder et al., 2011a) from DOX treatment. These side effects can last several years past the cessation of treatment and have a profound effect on patient quality of life (Gilliam & St Clair, 2011; Schlitt et al., 2014). To improve long-term patient outcomes, co-treatments that limit the side effects of DOX without inhibiting its chemotherapeutic action need to be investigated.

One potential DOX co-treatment is the commonly used anti-diabetic drug metformin (MET). This drug is associated with preserved cardiac function (Sheta et al., 2016) and enhanced chemotherapeutic effectiveness with DOX (Wu et al., 2016). MET co-treatment with DOX decreases cardiac oxidative stress (Kelleni et al., 2015), decreases autophagy (Kelleni et al., 2015), increases cardiomyocyte cell survival (Asensio-López et al., 2011), and preserves cardiac function (Argun et al., 2016; Sheta et al., 2016). Additionally, long-term clinical MET use is associated with lower rates of some types of cancer despite the increased cancer risk of patients with diabetes (Li et al., 2009; Yue et al., 2014). MET has also been shown to decrease proliferation of cancer cells (Demir et al., 2014; Lee et al., 2014) and increase efficacy of DOX

treatment on cancer cells (El-Ashmawy et al., 2017; Wu et al., 2016). The potential for MET to preserve muscle viability and increase the chemotherapeutic efficacy of DOX makes it an attractive candidate to study.

One mechanism by which DOX causes muscle toxicity is through disrupting mitochondrial function. DOX decreases maximal mitochondrial respiration (state 3) in both cardiac (Marques-Aleixo et al., 2015; Yen et al., 1999) and skeletal muscle (Gilliam et al., 2013; Kavazis et al., 2010). Additionally, DOX is a known inhibitor of complex I in the electron transport chain (ETC) (Berthiaume & Wallace, 2007; Davies & Doroshow, 1986; Doroshow & Davies, 1986). MET is also a known inhibitor of complex I of the ETC (Andrzejewski, Gravel, Pollak, & St-Pierre, 2014; Luengo, Sullivan, & Heiden, 2014). While MET co-treatment with DOX is beneficial to preserve cardiac function, the effects of MET and DOX treatment on mitochondrial function have not been studied. The purpose of this study was to determine the effect of DOX and MET combined treatments on mitochondrial respiration in mouse skeletal muscle cells.

Methods

Cell Culture

Mouse skeletal muscle C2C12 myoblasts were grown to confluence on growth media (DMEM/HG, GE Lifesciences; 10% fetal bovine serum, Fisher Scientific; and 1% antibiotic antimyotic, Sigma) and differentiated to myotubes for three days on differentiation media (DMEM/HG, GE Lifesciences; 10% horse serum, Sigma; and 1% antibiotic antimyotic, Sigma). Cells were treated with 0.5 μM DOX and/or 0.5mM MET for 16 hours.

Cell Viability (MTT) Assay

Differentiated cells were incubated with 0.5 mg/mL Thiazolyl Blue Tetrazolium Bromide (MTT) in differentiation media for 45 minutes. Cells were rinsed with PBS and incubated with solubilizing solution (4 mM HCl, 0.1% nondel in isopropanol) until MTT was dissolved. Absorbance was read at 570 nm. Results shown here are also shown in chapter 5.

Mitochondrial Respiration

High resolution respirometry was performed in real-time using the Oroboros Oxygraph-2k (OROBOROS INSTRUMENTS, Innsbruck, Austria). Confluent cells from a single well of a 6 well plate (8.87 cm^2) were harvested using trypsin, centrifuged at 100 rcf for 5 minutes and resuspended in Mir05 buffer (0.5 mM EGTA, 3mM $MgCl_2$, 60 mM lactobionic acid, 20 mM taurine, 10 mM KH_2PO_4, 20 mM Hepes, 110 mM sucrose, 1g/L BSA, pH=7.1). Suspended cells were placed into the 2 mL chamber, media was oxygenated to 425-475 µM O_2, and sealed. Cells were permeabilized using 4 µM digitonin for 15 minutes in the presence of 2 mM glutamate and 10 mM malate. The rate of oxygen consumption was allowed to stabilize between substrates and data was taken based on the average rate of oxygen consumption over at least 3 minutes. The electron transport chain was stimulated by adding the following substrates sequentially: 2.5 mM ADP (complex I), 10 µM cytochrome C (control), 10 mM succinate (complex I + complex II), 1 µM FCCP (uncoupler), 0.5 µM rotenone (complex I inhibitor), and 2.5 µM antimycin A (complex III inhibitor). Residual oxygen consumption after the addition of antimycin A was subtracted from the entire run. Any prep that resulted in cytochrome C increasing respiration by more than 10% indicates mitochondrial disruption and was discarded.

Statistics

Statistical significance was determined using 2-way ANOVA and Tukey-Kramer post-hoc test with an alpha level of 0.05 using JMP software.

Results

0.5 μM DOX Does Not Decrease Cell Viability at 16 Hours

Mouse skeletal muscle C2C12 myotubes were treated with 0.5 μM DOX and/or 0.5 mM MET for 16 hours. Cell viability was assessed using MTT. At the low concentration of 0.5 μM DOX, there was no change in cell viability compared to CON. Interestingly, there was a main effect of MET to decrease cell viability ($p<0.05$, figure 3.1), most of which can be explained by the DOX+MET combination treatment. However, compared to the CON group, DOX+MET was not different but, compared to DOX treatment, DOX+MET did trend towards significance ($p=0.068$, figure 3.1).

High Concentration of DOX Causes Loss of Mitochondrial Integrity

Initially, 5 μM DOX was tested for respiration capacity. 5-10 μM DOX is commonly used in other studies and is within the range of peak plasma concentrations reached during a chemotherapy infusion (Gianni et al., 1997). However, this level of DOX caused severe loss in mitochondrial integrity, as shown by a cytochrome C response (figure 3.2). Cytochrome C is routinely added as a control. Under proper preparation conditions, Cytochrome C cannot enter the mitochondria, and should cause no change in respiration. Respiration measurement in high DOX-treated cells always resulted in a cytochrome C response. In order to avoid severe disruption of the mitochondria and ensure valid experimental procedures, the concentration of DOX used was decreased 10-fold to 0.5 μM.

Mitochondrial Respiration is Reduced with DOX and/or MET Treatments

Real-time mitochondrial respiration was measured using high resolution respirometry. A representative trace of each group is shown in figure 3.3. LEAK respiration was measured with glutamate and malate. Complex I (CI) respiration was next stimulated by adding ADP. Cytochrome C was added to ensure mitochondrial integrity- as seen by a small increase in oxygen flux that was not sustained. Succinate was next added to stimulate Complex I + Complex II (CI+CII), or state 3 respiration. The electron transport chain was then uncoupled from ATP synthase through the addition of FCCP (state 3, uncoupled). Next, CI was inhibited through addition of rotenone. Finally, antimycin A was added to inhibit complex III. Residual respiration was subtracted off the entire run.

Results of each stage of respiration were quantified in figure 3.4. MET inhibited CI respiration ($p<0.05$, figure 3.4A). DOX treated cells had somewhat decreased CI respiration, which was not significantly different from CON or MET treatments. CI+CII supported respiration, also known as state 3, was analyzed next (figure 3.4B). There was a main effect of DOX ($p<0.05$) and a main effect of MET ($p<0.05$) to reduce CI+CII respiration. Combination treatment of DOX+MET resulted in similar respiration to DOX or MET treatments alone. Uncoupled CI+CII respiration was reduced with MET, DOX, and DOX+MET treatments compared to CON ($p<0.05$, figure 3.4C). CI inhibited respiration via rotenone was lower with DOX ($p<0.05$, figure 3.4D) and trended towards a reduction with MET ($p=0.066$) and DOX+MET ($p=0.051$).

CII Respiration is Reduced with DOX Treatment

CII respiration was estimated by subtracting CI+CII respiration from CI respiration. While uncoupled CI respiration is measured after the addition of rotenone, the pattern of

respiration seen with rotenone inhibition is different than expected. This observation may be due to rotenone not being a completely specific inhibitor to CI. When analyzing calculated CII respiration, DOX had reduced respiration compared to all other groups ($p<0.05$, figure 3.5). DOX+MET treatment restored respiration back to CON levels. This result suggests that DOX reduces maximal respiration at CII, but this can be prevented with MET co-treatment.

Changes in Mitochondrial Respiration are More Visible After Accounting for Cell Viability

Initial analysis of mitochondrial respiration was done by controlling for cell density when seeding the plate and observing 100% confluency before differentiation. However, initial results did not take into account changes in cell viability due to treatments. After the treatment, cell viability was different between groups. Most noticeably there was a reduction in the DOX+MET group. To account for this difference, an estimation of respiration was made by dividing respiration by average cell viability. The results of this approximation are shown in figures 6-7. The resulting pattern is the same as seen previously, but the effects are more pronounced.

Discussion

DOX (Davies & Doroshow, 1986) and MET(Luengo et al., 2014) are both known to inhibit the electron transport chain, specifically at CI. We show that at a low dose of DOX (0.5 μM) cell viability is not damaged and CI respiration is not inhibited. However, CI+CII supported and uncoupled respiration is reduced with DOX treatment, an effect that appears to be primarily due to inhibition at CII. As expected, MET treatment inhibited CI respiration, which resulted in lower CI+CII supported and uncoupled respiration. The combination treatment of MET+DOX resulted in lower CI, CI+CII, and uncoupled respiration. However, using DOX and MET

together did not result in additive inhibition of the ETC. Additionally, use of MET with DOX restored CII respiration back to levels seen in the control group.

Previous studies have shown DOX to disrupt mitochondrial function and decrease state 3 respiration in cardiac (Marques-Aleixo et al., 2015; Yen et al., 1999) and skeletal muscle (Gilliam et al., 2013). DOX is a well-known ETC inhibitor through binding to CI (Davies & Doroshow, 1986), an effect that results in lower CI supported and CI+CII supported respiration in heart (Yen et al., 1999) and skeletal muscle (Gilliam et al., 2013) from rats. Additionally, some studies also report that CII supported respiration is decreased (Gilliam et al., 2013; Yen et al., 1999) with DOX treatment. Here we report that with 0.5 μM DOX for 16 hours, CI respiration is not significantly inhibited, but CII respiration is inhibited. This resulted in a total decrease in CI+CII supported respiration.

To our knowledge, this is the first study to examine the effects of DOX+MET treatments on mitochondrial respiration. While maximum respiration was similar between DOX+MET, DOX and MET treatments, the complex inhibited differed depending on treatment. Interestingly, MET treatment was able to prevent CII inhibition by DOX. Further research is required to understand the mechanism by which MET maintains CII supported respiration with DOX treatment.

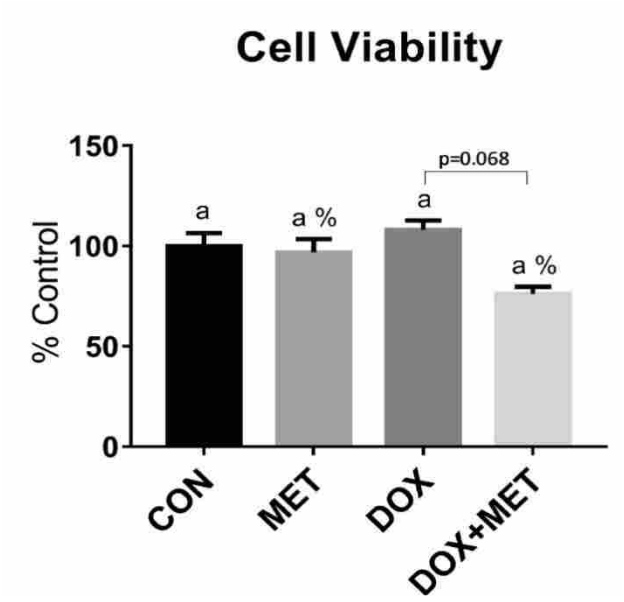

Figure 3.1: Myotube Cell Viability Measured Using MTT. 2-way ANOVA: main effect of MET % $p<0.05$. Post-hoc analysis: bars with different letters are significantly different $p<0.05$.

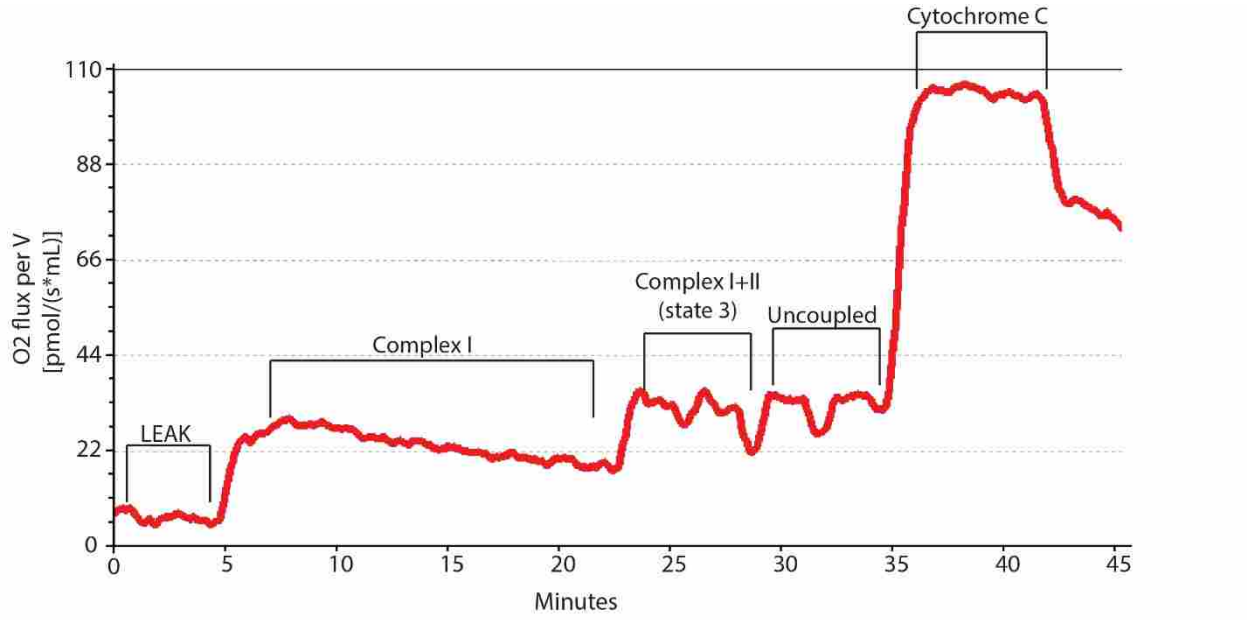

Figure 3.2: Representative Trace of Respiration at High DOX. 5 µM DOX causes cytochrome C response at 16 hours.

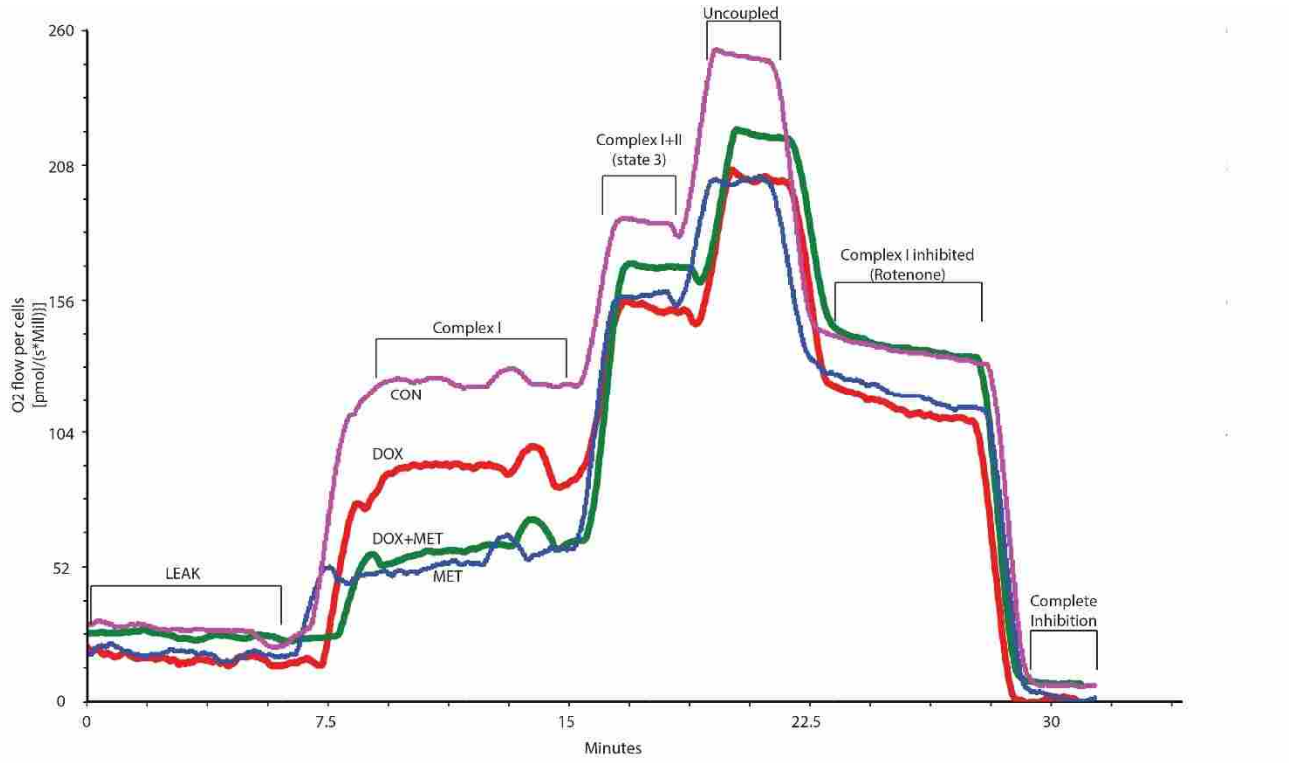

Figure 3.3: Representative Trace of Respiration with 0.5 µM DOX and/or 0.5 mM MET. CON in purple, DOX in red, MET in blue, and DOX+MET in green. LEAK respiration measured after addition of glutamate and malate. Complex I supported respiration measured after addition of ADP. Complex I+II measured after addition of succinate. Uncoupled respiration measured after addition of FCCP. Complex I inhibition measured after addition of rotenone. Complete inhibition measured after addition of antimycin A.

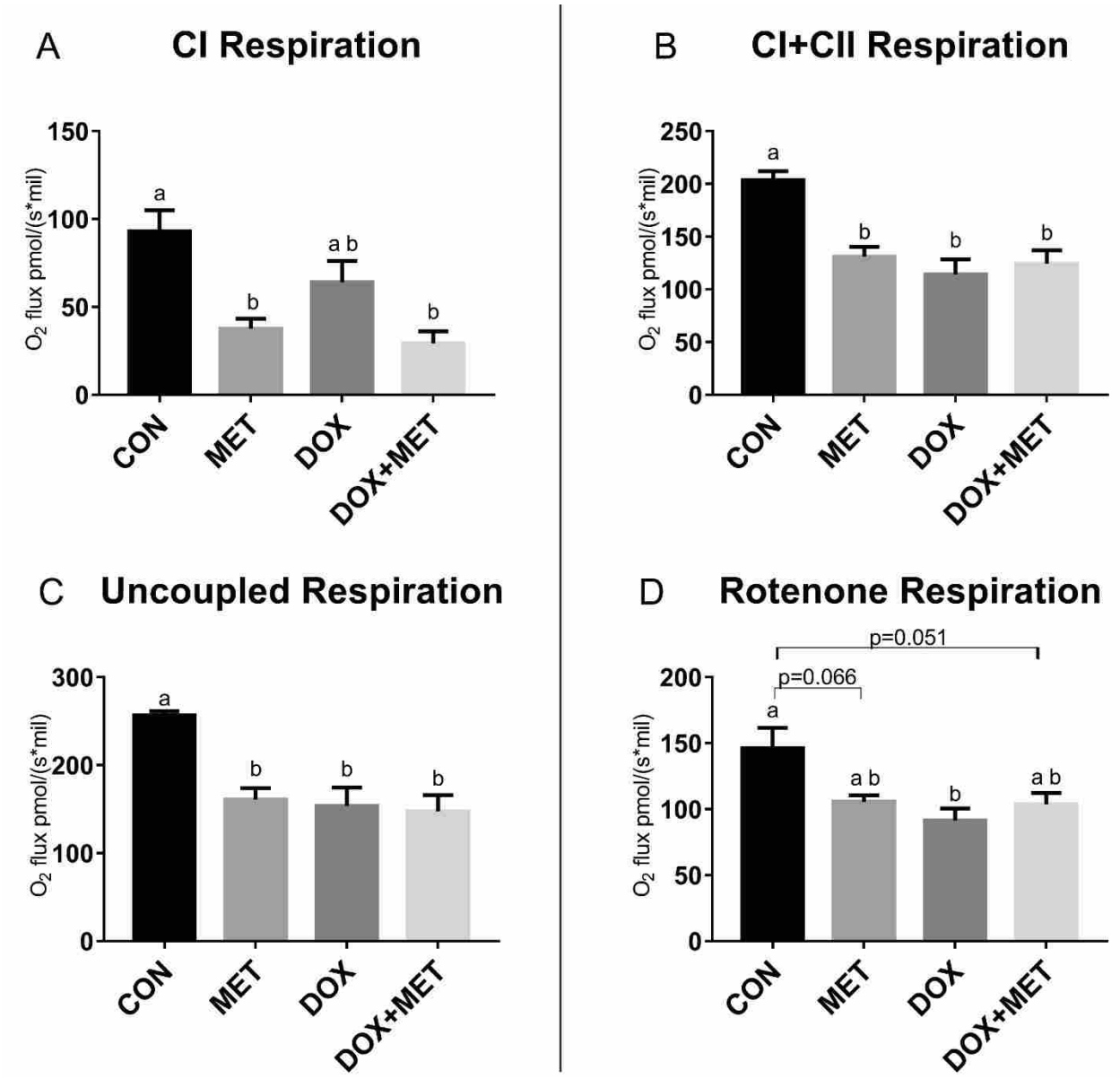

Figure 3.4: Mitochondrial Respiration of Myotubes. N=4 ± SEM. (A) Respiration of CI, measured with glutamate, malate, and ADP. (B) Respiration of CI+CII measured after addition of CI substrates and succinate (state 3 respiration). (C) Uncoupled state 3 respiration measured after addition of FCCP. (D) CI inhibited respiration measured after addition of rotenone. Post hoc analysis: bars with different letters are significantly different $p<0.05$.

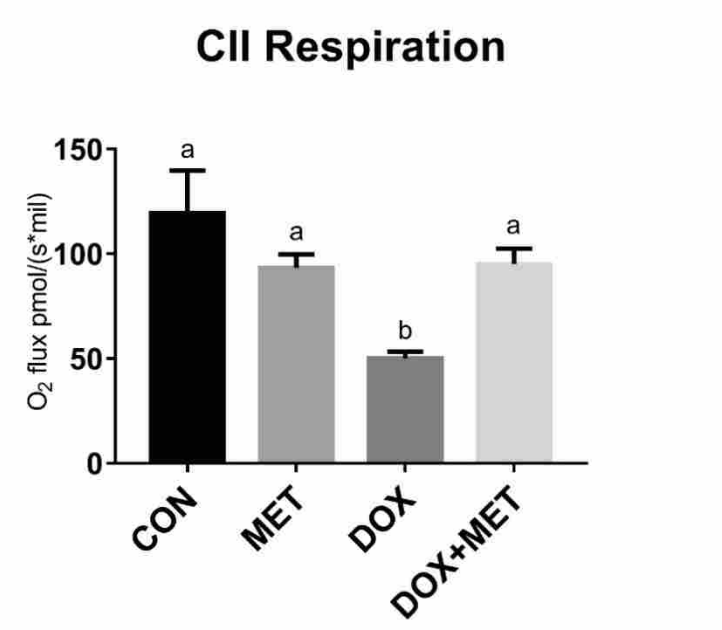

Figure 3.5: CII Estimated Respiration. Respiration estimated by subtracting state 3 respiration – CI respiration. Post hoc analysis: bars with different letters are significantly different $p<0.05$.

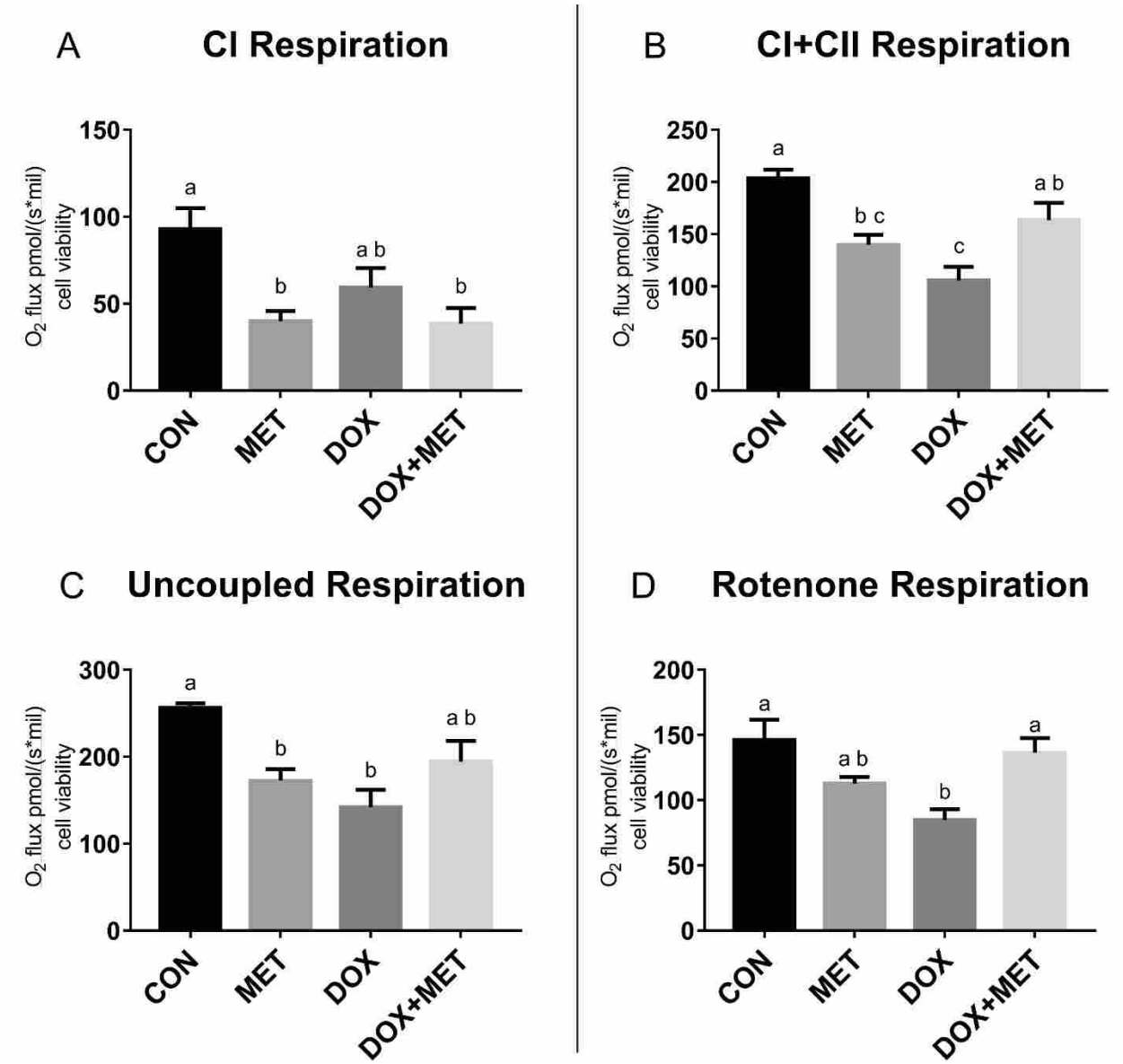

Figure 3.6: Mitochondrial Respiration of Myotubes Divided by Cell Viability. N=4 ± SEM. (A) Respiration of CI, measured with glutamate, malate, and ADP. (B) Respiration of CI+CII measured after addition of CI substrates and succinate (state 3 respiration). (C) Uncoupled state 3 respiration measured after addition of FCCP. (D) CI inhibited respiration measured after addition of rotenone. Post hoc analysis: bars with different letters are significantly different $p<0.05$.

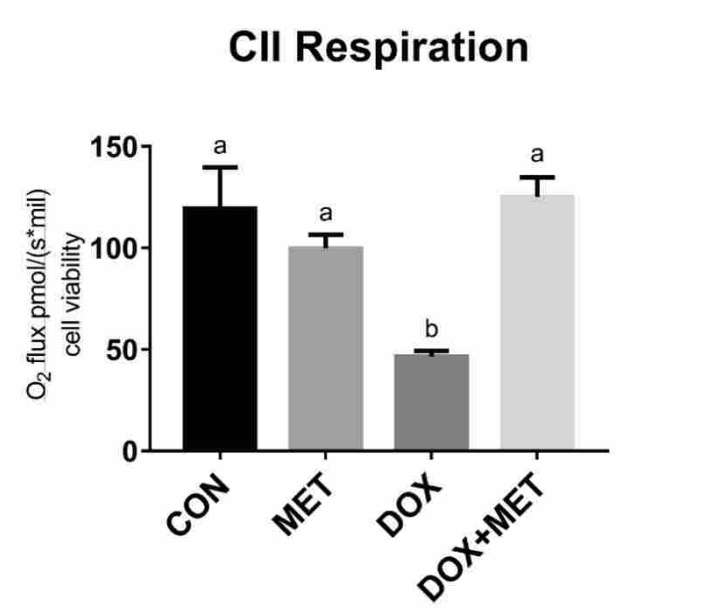

Figure 3.7: CII Estimated Respiration Corrected for Cell Viability. Respiration estimated by subtracting state 3 respiration – CI respiration. Post hoc analysis: bars with different letters are significantly different $p<0.05$.

CHAPTER 4: Multi-Tissue Analysis of Exercise or Metformin on Doxorubicin-Induced Iron Dysregulation

Introduction

Doxorubicin (DOX) is a common chemotherapeutic used to treat a variety of solid tumor and leukemia cancers by intercalating DNA and inhibiting replication (Minotti et al., 2004; Momparler et al., 1976). DOX is also known to cause toxic side effects in organs independent of its chemotherapeutic properties. The most commonly studied DOX-related side effect is cardiomyopathy (Hayward et al., 2013), albeit not exclusively affecting this organ, but negatively affecting many other organs including liver, brain, lung, kidney, and skeletal muscle (Martins et al., 2012; Mohamed et al., 2011; Shivakumar et al., 2012; Smuder et al., 2011a). The ramifications of these side effects are severe and persist several years past the cessation of treatments (Elbl et al., 2006; Villani et al., 2009). Continued research into mechanisms and possible treatments of the side effects of DOX is therefore warranted.

DOX is able to bind directly with iron (Eliot et al., 1984; Muindi et al., 1984), cause iron dysregulation (Corna et al., 2006; Xu et al., 2008), mitochondrial disruption (Gilliam et al., 2013; Ichikawa et al., 2014), and oxidative stress (Asensio-López et al., 2013; Eliot et al., 1984). While DOX has long been known to bind with iron in-vitro and contribute to oxidative stress, the effects of DOX on iron regulation across multiple tissues in an animal model has not been studied. To understand the effects of DOX on iron regulation, a more comprehensive study of the major cellular pathways of iron is required. Briefly, the major cellular iron trafficking pathway occurs when iron enters the cell via the transferrin receptor. After endocytosis, iron is released into the cytosol where it is available for transport into organelles or storage in ferritin. The only known iron export protein is through ferroportin (von Drygalski & Adamson, 2013).

The relationship between DOX and iron regulation is complex and still not fully understood but some work demonstrates the ability of DOX to perturb iron-related pathways. Studies indicate that DOX causes an increase in ferritin heavy chain expression in cardiac cells and tissue (Bernuzzi et al., 2009; Corna et al., 2006; Corna et al., 2004; Kwok & Richardson, 2003). Transferrin receptor expression is decreased in cardiac tissue (Corna et al., 2006) and fibroblast cells (Xu et al., 2008), but increased in endothelial cells in response to DOX treatment (Kwok & Richardson, 2003) suggesting tissue specific responses to DOX treatment. Some studies find that adding antioxidants ameliorates the negative effects of DOX (Kalivendi et al., 2001; Kotamraju et al., 2002), while others find no benefit of antioxidant supplementation (van Dalen et al., 2011). Additionally, DOX-induced iron regulation can change without the presence of high oxidative stress (Bernuzzi et al., 2009). This suggests that the negative effects of DOX are likely a consequence of iron dysregulation but are only partially dependent on oxidative stress. Data are also conflicting on whether or not high iron diets have any effect on cardiac function with DOX treatment (Corna et al., 2004; Guenancia et al., 2015; Panjrath et al., 2007). Measurements of iron regulation have been incomplete, with each study typically only reporting on a single iron protein. Thorough, multi-tissue iron analyses in an animal model has not been completed. In this study, we report on the effects of DOX on iron regulation in liver, skeletal muscle, and cardiac muscle. It is crucial to understand liver iron regulation due to the central role that it plays in systemic iron regulation. Thorough analysis of iron regulation is also needed in heart and skeletal muscle because of their high iron needs and high toxicity with DOX treatment.

In an effort to ameliorate the negative side effects of DOX, a variety of co-treatments were tested. Currently, the only approved DOX co-treatment is the iron chelator Deferoxamine (DFO) (Wiseman & Spencer, 1998). While antioxidants were initially good candidates as

potential treatments, a recent study has indicated that antioxidants are not effective in humans (van Dalen et al., 2011). Two other treatments that are of interest and beneficial in animal models are exercise (EX) and metformin (MET). EX with DOX has been well studied in animals and shown to partially prevent heart failure (Hydock et al., 2012; Jensen et al., 2013; Kouzi & Uddin, 2016), maintain muscle function (Bredahl et al., 2016), and limit proteolysis (Smuder et al., 2011a) and autophagy (Smuder et al., 2011a). Two studies done in women with breast cancer receiving chemotherapy including DOX have shown EX to be beneficial in reducing fatigue (Schwartz, 2000) and maintaining bone mineral density (Schwartz et al., 2007). While there was high individual variability in fatigue level and patterns, women participating in aerobic exercise reported fewer days of severe fatigue and an overall decrease in average fatigue levels (Schwartz, 2000). The anti-diabetic drug MET has been shown in an animal model to have cardio-protective effects from DOX (Argun et al., 2016; Kelleni et al., 2015; Sheta et al., 2016). In cardiac cells, MET ameliorates the negative side effects of DOX through increasing ferritin heavy chain expression (Asensio-López et al., 2011; Asensio-López et al., 2013; Asensio-Lopez et al., 2014). The effects of EX or MET treatment on iron regulation in an animal model are unknown.

The purpose of this study is to characterize the changes to the major iron regulation by measuring total iron, transferrin receptor (TfR), and ferritin (FHC and FLC) due to DOX treatment in liver, heart, and skeletal muscle. Second, we determined if EX or MET treatments minimized iron dysregulation that occurs with DOX.

Methods

Animals

All animal procedures were approved by Brigham Young University's Institutional Animal Care and Use Committee and carried out in compliance with the Animal Welfare Act. Five-week old male C57BL/6 mice were housed at 22°C on a 12 hour light/dark cycle and fed standard chow (Harlan Teklad 8064) and water ad libitum. Animals were randomly assigned to one of six groups: Control (CON), doxorubicin (DOX), exercise (EX), doxorubicin + exercise (DOX+EX), metformin (MET), or metformin + doxorubicin (MET+DOX). DOX, DOX+EX and DOX+MET mice received one DOX intraperitoneal injection of 15 mg/kg. EX and DOX+EX mice were acclimated to the treadmill for 3 days (5-10 m/min for 10 min/day). On day 4, mice were tested for maximum speed by running at 12 m/min for 2 minutes and 15 m/min for 3 minutes, then increasing speed by 1 m/min every minute until exhaustion. After 2 days of rest, mice were run at 70% max speed for 60 minutes each morning. DOX+EX mice received a dose of DOX one hour after the last bout of exercise. MET and DOX+MET mice received daily oral doses of metformin at a concentration of 500 mg/kg beginning 2 days before DOX treatment and continuing until euthanasia. CON mice received corresponding oral and injection saline treatments. All mice were euthanized 3 days after DOX/saline treatment.

Western Blotting

Samples from muscle, liver, and heart were homogenized to have a final concentration of 5% tissue using glass-on-glass grinding in ice-cold homogenization buffer and protein content was measured using a standard BSA protein assay (Thermo Fisher Scientific). All samples were processed and prepared in tandem to ensure equal protein concentrations in final western blotting

samples. Samples were resolved through SDS-PAGE and blotted onto nitrocellulose membrane. The membrane was stained with Ponceau S and analyzed to ensure even protein loading and transfer across lanes. After blocking, primary antibody was applied against ferritin heavy chain (Cell Signaling Technologies, 3998S), ferritin light chain (LSBio, B9977) and transferrin receptor (Abcam, ab84036) at 4°C. Appropriate secondary antibody conjugated with horse radish peroxidase was applied, after which chemiluminescent reagents were applied and detected using autoradiography film. Resulting bands were analyzed for densitometry using image-J and Microsoft Excel software. Each blot was normalized against itself before comparing between membranes.

mRNA Analysis

Real Time PCR analysis of ferroportin was performed due to a lack of a reliable commercially available antibody. Samples were homogenized, and RNA was prepared according to directions in the GenElute^{TM} Mammalian Total RNA Miniprep Kit (Sigma, RTN350). RT-PCR was carried out according to the protocol found in RT^2 SYBR Green system (Qiagen, 330514) using PPR466085A (Qiagen) for ferroportin and PPR06557B (Qiagen) for Gapdh using the QuantStudio 6 Flex system (Applied Biosystems).

Thiobarbituric Acid Reactive Substances (TBARS) Assay

Frozen samples were homogenized to 10% tissue concentration in RIPA buffer with 5 mM butylated hydroxytoluene and cleared through centrifugation (1000 rcf) then mixed with 0.67% thiobarbituric acid (TBA) and 6N HCl and boiled for 30 minutes. After reaching room temperature, samples were aliquoted in triplicate on a microplate and read at an absorbance of

532 nm (Mihara & Uchiyama, 1978). Results were normalized against protein concentration and compared to control endpoints.

Glutathione Assay

Liver and muscle samples were sonicated and snap frozen in 5% perchloric acid, 0.2M boric acid, and 10μ γ-glutamylglutamate. After thawing, samples were prepared as dansylated derivatives as described previously (Harris et al., 2015). Reduced (GSH) and oxidized (GSSG) glutathione were resolved and quantified using reverse-phase HPLC analysis (Waters 2695 Alliance Separations Module) with a Supelcosil LC-NH_2 column (Sigma). The GSH redox potential (E_h) was calculated through the Nernst equation $-264 + 30LOG(\frac{\mu M\ GssG}{\mu M\ GSH^2})$ as described previously, at 37°C and pH 7.4 (Harris et al., 2015).\

Total Iron

Total Iron measurements were made using inductively coupled plasma mass spectrometry (ICP-MS). Samples were prepared in water with 0.1-0.3% homogenate and 3-5% nitric acid. Samples were then aerosolized into the ICP-MS (Agilent Technologies 7800). All three isotopes of iron (54, 56, and 57) had very similar results. Isotope 56 levels were normalized to protein content and used for analyses.

Statistics

2-way ANOVA and Tukey-Kramer post-hoc tests were performed on two data groups: CON, DOX, EX, DOX+EX and CON, DOX, MET, DOX+MET. All data were analyzed with for significance at an alpha level of $p<0.05$ using JMP software.

Results

DOX Treatment Caused Severe Body Weight Loss at 3 Days

Six-week old mice were given a single bolus of 15 mg/kg DOX and euthanized 3 days later. DOX treatment caused significant weight loss ($p<0.05$, table 4.1), similar to results from previous studies. All groups had similar starting weight except for EX group, due to variation in body weights of young mice. This difference was not observed 3 days later at time of euthanasia. DOX, DOX+EX, and DOX+MET groups had significantly lower final body weights ($p<0.05$). The percentage of weight loss was partially blunted in DOX+EX ($p<0.05$) and DOX+MET ($p=0.064$) versus DOX suggesting some beneficial effects of EX or MET treatment with DOX. Next, analysis of iron regulation is reported in the liver, heart, and skeletal muscle. Results of EX and MET treatments are found in separate paragraphs and figures to allow for better organization and ease of understanding. After discussing iron regulation, an analysis of glutathione and TBARs levels will be analyzed as measurements of oxidative stress.

DOX Treatment Increased Ferritin Content in the Liver

Hepatic iron regulation is critical for systemic iron distribution. While DOX is known to modulate iron regulation proteins in the heart, little is known concerning DOX effects on iron regulation in liver. Due to the central role the liver plays in systemic iron regulation, TfR, both ferritin subunits (heavy and light) and Fpn are reported here. The ferritin complex functions to sequester iron within the cell. There was a main effect of DOX to increase FHC ($p<0.05$, figure 4.1A) and FLC ($p<0.05$, figure 4.1B). In addition, there was a DOX+EX interaction that led to an increase in both FHC ($p<0.05$) and FLC ($p<0.05$). Transferrin receptor (TfR) binds to transferrin and brings iron into the cell. While there were main effects of DOX ($p<0.05$, figure

4.1C) and EX ($p<0.05$) on TfR, this is explained primarily by the marked reduction in TfR levels with combined treatment of DOX+EX ($p<0.05$, figure 4.1C). There was no change in Fpn mRNA expression (figure 4.1D). DOX treatment also caused a main effect of increased total iron content ($p<0.05$, figure 4.1E). These results show that DOX treatment increases total iron content and storage capacity in the liver. Exercise prior to DOX treatment resulted in a reduction in iron transport capacity through TfR. Interestingly, this did not result in lower total iron levels at three days.

Animals treated with MET had similar changes in liver iron regulation as seen with EX treatment (figure 4.2). There was a main effect of DOX that increased FHC levels ($p<0.01$, figure 4.2A) and FLC levels ($p<0.05$, figure 4.2B). Furthermore, there was a main effect of DOX that resulted in a reduction of TfR content ($p<0.01$, figure 4.2C) which is primarily explained by the low levels of TfR observed with the combination treatment of DOX+MET ($p<0.01$, figure 4.2C). There was no change in Fpn mRNA expression (figure 4.2D). Total iron content was increased with DOX compared to CON ($p<0.05$, figure 4.2E), but DOX+MET treatment restored levels back to CON. In summary, DOX treatment increased total iron and iron storage capacity in the liver. The combination of EX or MET treatment with DOX resulted in a marked reduction in TfR expression, but this only resulted in a decrease in total iron levels with MET treatment.

DOX Treatment Increased FHC Content and Decreased TfR Content in the Heart

Decreased heart function is a major side effect of DOX, an effect that is at least in part due to DOX interacting with iron. In EX treatment groups, DOX had a main effect of increased FHC expression ($p<0.05$, figure 4.3A) and decreased TfR expression ($p<0.05$, figure 4.3B). Total iron content in the heart was not statistically different between groups (figure 4.3C). These

results indicate that DOX increases iron storage capacity and decreases iron import capacity with little effect of EX treatment.

The effects of iron regulation in the heart of MET animals is almost identical to observations with EX animals (figure 4.4). DOX had a main effect of increased FHC expression ($p<0.05$, figure 4.4A). A main effect of DOX on decreasing TfR expression was also observed ($p<0.05$, figure 4.4B). DOX+MET treatment slightly blunted the decrease in TfR and was not different from CON or DOX groups (figure 4.4B). There was a main effect of DOX to increase total iron content ($p<0.05$, figure 4.4C), but there was no statistical difference between individual groups (figure 4.4C). The EX or MET treatments had very minor effects on iron regulation in the heart with DOX. This suggests that the previously reported benefits of EX or MET in cardiac tissue treated with DOX are likely independent of iron regulation.

DOX Treatment Increased FHC Content and Decreased TfR Content in the Skeletal Muscle

Skeletal muscle wasting and fatigue due to DOX are of major concern for patient quality of life and long-term health. In animals treated with exercise, there was a main effect of DOX to increase FHC expression ($p<0.05$, figure 4.5A) in muscle. This was partially blunted in the DOX+EX group, which was not significantly different from CON or DOX (figure 4.5A). Furthermore, there was a main effect of DOX that lead to a reduction in TfR expression ($p<0.05$, figure 4.5B). Additionally, the combination treatment of DOX+EX resulted in intermediate TfR expression that was not different from CON or DOX (figure 4.5B). Total iron content was not different between groups (figure 4.5C). EX treatment in addition to DOX partially blunted the effects of DOX on iron regulation in muscle.

In animals treated with MET, DOX had a main effect on increasing FHC expression in the muscle ($p<0.05$, figure 4.6A). DOX and MET treatments both had main effects on decreasing

TfR expression ($p<0.05$, figure 4.6B). Additionally, there was no difference in the reduced level of TfR expression between DOX, MET, or DOX+MET groups (figure 4.6B). There was no change in total iron between any groups (figure 4.6C). In muscle, MET treatment in combination with DOX had no effect on changes in iron regulation due to DOX.

DOX Treatment Caused No Change in Glutathione Redox Potential

Glutathione redox potential (E_h) was calculated using the Nernst equation. E_h is measured to analyze the reducing potential and oxidative state of the cell. Three days following treatment with DOX, with or without EX resulted in no differences in GSH E_h (figure 4.7A). Interestingly, in the muscle, there was a trend for DOX to induce a more reduced state ($p=0.063$ vs CON, $p=0.055$ vs DOX+EX, figure 4.7B).

Similar to EX treatment, there were no differences in liver GSH E_h with MET treatment (figure 4.8A). In the muscle, there was a main effect of DOX to have a more reduced GSH E_h ($p<0.05$, figure 4.8B). Additional post-hoc analysis revealed a trend for DOX to have a more negative E_h compared to CON ($p=0.067$, figure 4.8B). These results suggest that by the three-day timepoint, DOX induced oxidative stress is tempered, with a trend towards increased reducing potential in muscle.

DOX Treatment Increased Lipid Peroxidation in Liver

Malondialdehyde reactive lipid peroxidation (TBARS) was measured in liver, heart, and muscle as an indicator of overall oxidative stress. In the liver, DOX had a main effect of increased TBARS ($p<0.05$, figure 4.9A). There was no change in TBARS in the heart (figure 4.9B) or muscle (figure 4.9C).

In MET treated animals, TBARS in the liver was increased in DOX, MET, and DOX+MET groups compared to CON ($p<0.05$, figure 4.10A). There was no difference in TBARS levels in the heart (figure 4.10B) or muscle (figure 4.10C). DOX caused a moderate increase in lipid peroxidation in the liver, yet no changes were observed in the heart or muscle. Taken together with the glutathione results, this is suggestive that DOX causes only moderate oxidative stress at the three-day timepoint.

Discussion

The purpose of this study was twofold: first to characterize the changes in iron regulation in response to DOX treatment in heart, liver, and skeletal muscle. Second, to determine if the changes in iron regulation following DOX treatment were improved with either exercise or metformin.

To our knowledge, this is the first multi-tissue analysis of iron following treatment with DOX. DOX is capable of binding ferrous (Fe^{2+}) ore ferric (Fe^{3+}) iron, causing free radical formation through the fenton and Haber Weiss reactions. Increasing iron storage capacity would therefore be beneficial to reduce DOX interactions with iron and oxidative stress. Here we show that DOX induced positive changes in iron regulation by increasing iron storage capacity through ferritin in the liver, heart, and skeletal muscle. FHC expression has antioxidant capacities (Bresgen & Eckl, 2015) and is expressed under conditions of high oxidative stress (Bresgen & Eckl, 2015; Watt, 2011). We report that increased FHC protein content persists past the period of high oxidative stress caused by DOX. Additionally, iron transport capacity was reduced through decreased TfR levels in cardiac and skeletal muscle. However, there was still a small increase in total iron in liver and cardiac muscle. While these changes promote iron sequestering and reduced oxidative stress, it is clearly not sufficient to protect against the toxic effects of DOX.

Studies in cardiac tissues or cells have shown that DOX increases ferritin expression (Bernuzzi et al., 2009; Corna et al., 2006; Corna et al., 2004; Kwok & Richardson, 2003). Our results in the heart confirm these studies. Two studies in cardiac tissue (Corna et al., 2006) and fibroblast cells (Xu et al., 2008) show decreased TfR expression and one study observed increased TfR expression in endothelial cells (Kotamraju et al., 2002). We observed decreased TfR expression in cardiac and muscle tissue and no change in expression in the liver. Taken together, this suggests that the effects of DOX on TfR are tissue dependent. Additionally, ours was the first study to measure total iron content in the heart after DOX treatment. We saw a small increase in iron, which is not unexpected given that iron storage is stable and slow to change. While proteins involved in iron regulation were changed in a manner that would limit an increase in cellular iron, it was not sufficient to completely prevent an increase in total iron levels at 3 days.

The iron chelator DFO is currently the only approved co-treatment with DOX. Since DFO exerts its effects through limiting iron availability, other potential co-treatments should be investigated for their effects on iron regulation. Exercise and metformin treatments have both previously been found to be beneficial co-treatments with DOX (Argun et al., 2016; Kavazis et al., 2010; Kelleni et al., 2015; Sheta et al., 2016; Smuder et al., 2011b), however their effects on iron regulation have not been studied. Interestingly, while very different in nature, the overall effects of exercise or metformin treatment on iron regulation were similar. Exercise or metformin treatments in combination with DOX blunted weight loss compared to DOX alone, indicating an overall beneficial effect. Exercise or metformin co-treatment did not reverse or temper the increase in FHC or FLC expression that was observed with DOX treatment. Both treatments caused a reduction in TfR expression in the liver, which would be expected to limit cellular iron

uptake and cause a reduction in total iron levels. However, the exercise plus DOX treatment had iron content similar to that of DOX alone and higher levels than control. In contrast, co-treatment of metformin and DOX resulted in restoration of total iron levels to control. This suggests that the reduction in TfR expression was sufficient to prevent an increase in total iron with metformin treatment, but not with exercise treatment.

Exercise before or during DOX treatment partially preserves cardiac function through preserving left ventricular function in-vivo (Jensen et al., 2013) and ex-vivo(Chicco et al., 2006; Hydock et al., 2012; Jensen et al., 2013). Exercise also decreases autophagy (Smuder et al., 2013), and apoptosis (Chicco et al., 2006) due to DOX. Exercise had no effect on the changes in iron regulation observed in response to DOX. This strongly suggests that the previously reported positive effects of exercise on cardiac function may be independent of iron dysregulation caused by DOX.

Metformin exerts its benefits by preserving cardiac function (Argun et al., 2016), decreasing apoptosis (Argun et al., 2016; Kelleni et al., 2015; Sheta et al., 2016), and decreasing oxidative stress factors (Kelleni et al., 2015; Sheta et al., 2016) due to DOX. A series of studies in cardiac cells showed that metformin treatment before DOX induced FHC expression, preserving cell viability and reducing oxidative stress (Asensio-López et al., 2011; Asensio-López et al., 2013; Asensio-Lopez et al., 2014). We did not confirm this pathway in a mouse model. Instead, there was no effect of metformin on FHC expression and DOX increased FHC in the heart. Future work would determine if the iron chelator DFO with exercise or metformin treatments would have additive benefits of preventing cardiac dysfunction due to DOX.

Fewer studies have been done on the effects of DOX and exercise on skeletal muscle but reports so far have been positive. Exercise treatment protects muscle against DOX induced

oxidative stress (Smuder et al., 2011b), autophagy (Smuder et al., 2011a), and proteolysis (Smuder et al., 2011b). Additionally, ex-vivo analysis of skeletal muscle function is preserved in animals that exercised before receiving DOX treatment (Bredahl et al., 2016). In this study, we show that exercise treatment tempered changes in iron regulation due to DOX.

The effects of metformin co-treatment with DOX in skeletal muscle have not been studied. The classic mechanism of action of metformin on skeletal muscle is to increase AMP-activated protein kinase (AMPK) activity, which increases insulin sensitivity (Musi et al., 2002). Metformin was previously shown to increase FHC expression and limit DOX-induced cardiomyocyte apoptosis in an AMPK dependent manner (Asensio-López et al., 2011; Asensio-López et al., 2013). In our study, metformin with DOX did not modulate iron regulation in heart or skeletal muscle. Despite using a high dose of metformin, we did not observe evidence of metformin increasing FHC expression, suggesting that the AMPK induced FHC expression pathway was not present in our animal model.

A single high level of DOX dose resulted in nearly 20% body weight loss in three days. Despite this deleterious effect, changes in oxidative stress were relatively mild, with only a small increase in TBARS in liver and a trend towards higher GSH E_h in muscle. The oxidative response due to DOX is complex, with studies regularly reporting opposite effects of the same measurement. This heterogeneity appears to be due, at least in part, to the time point chosen after DOX administration. DOX induced lipid peroxidation in heart is initially increased at 1-2 days (Benzer et al., 2018; Kavazis et al., 2010; Khafaga & El-Sayed, 2018; Shaker et al., 2018), decreased at 3 days (Deng et al., 2015), and restored to normal after 5 days (Chicco et al., 2006). Our results show no change in lipid peroxidation at 3 days, which largely agrees with this pattern. GSH levels appear to follow a similar pattern with a depletion of reduced GSH observed

at 1-2 days (Benzer et al., 2018; Khafaga & El-Sayed, 2018) and restoration to control at 3 days (Deng et al., 2015). Our results of GSH E_H potential follow this pattern. These results suggest that DOX initially causes high levels of oxidative stress followed by a restoration to control levels.

One study used low dose DOX exposure to cardiomyocytes to determine if changes in iron regulation occurred without inducing oxidative stress. They reported that DOX exposure did not increase reactive oxygen species formation or the oxidative markers aldose reductase or catalase. However, FHC expression was increased, indicating that DOX can induce changes to iron regulation without increased oxidative stress (Bernuzzi et al., 2009). We report multi-tissue changes in iron regulation at a time point without severe increases in oxidative stress markers. Taken together, this suggests that DOX induced modulation of iron regulation is at least partially independent of oxidative stress factors at this time point.

In conclusion, DOX positively modifies iron regulation in liver, heart, and skeletal muscle to decrease DOX and iron interactions. The addition of exercise or metformin treatments further modulated iron regulation in the liver or skeletal muscle. These effects are not dependent upon depleted GSH and persist in the absence of high levels of oxidative stress. Future studies examining short and long-term time points could further characterize the effects of DOX on iron regulation and its association with oxidative stress. Additionally, combining treatments of DFO plus EX or MET would determine if two co-treatments has additive benefits against DOX treatment.

Table 4.1: Mean Body Weight ± SEM of EX Animals. DOX main effect #p<0.05. Values with different letters are significantly different p<0.05.

	CON	DOX	EX	DOX+EX
Starting BW (g)	19.8±0.3	20.0±0.3	17.9±0.2	20.3±0.3
Final BW (g)	20.2±0.3^{a}	16.7±0.3^{b}	19.5±0.3^{a}	17.7±0.4^{b}
Change BW (%)	2.0±0.7^{a}	-19.8±1.3$^{\#}$	8.0±0.7^{a}	-15.2±1.9$^{\#}$

Table 4.2: Mean Body Weight ± SEM of MET Animals. DOX main effect #p<0.05. Values with different letters are significantly different p<0.05. Percent BW change DOX vs DOX+MET p=0.064.

	CON	DOX	MET	DOX+MET
Starting BW (g)	19.8±0.3	20.0±0.3	19.6±0.4	19.7±0.3
Final BW (g)	20.2±0.3^{a}	16.7±0.3^{b}	20.0±0.4^{a}	17.0±0.3^{b}
Change BW (%)	2.0±0.7^{a}	-19.8±1.3$^{b\#}$	2.0±0.9^{a}	-15.6±1.5$^{b\#}$

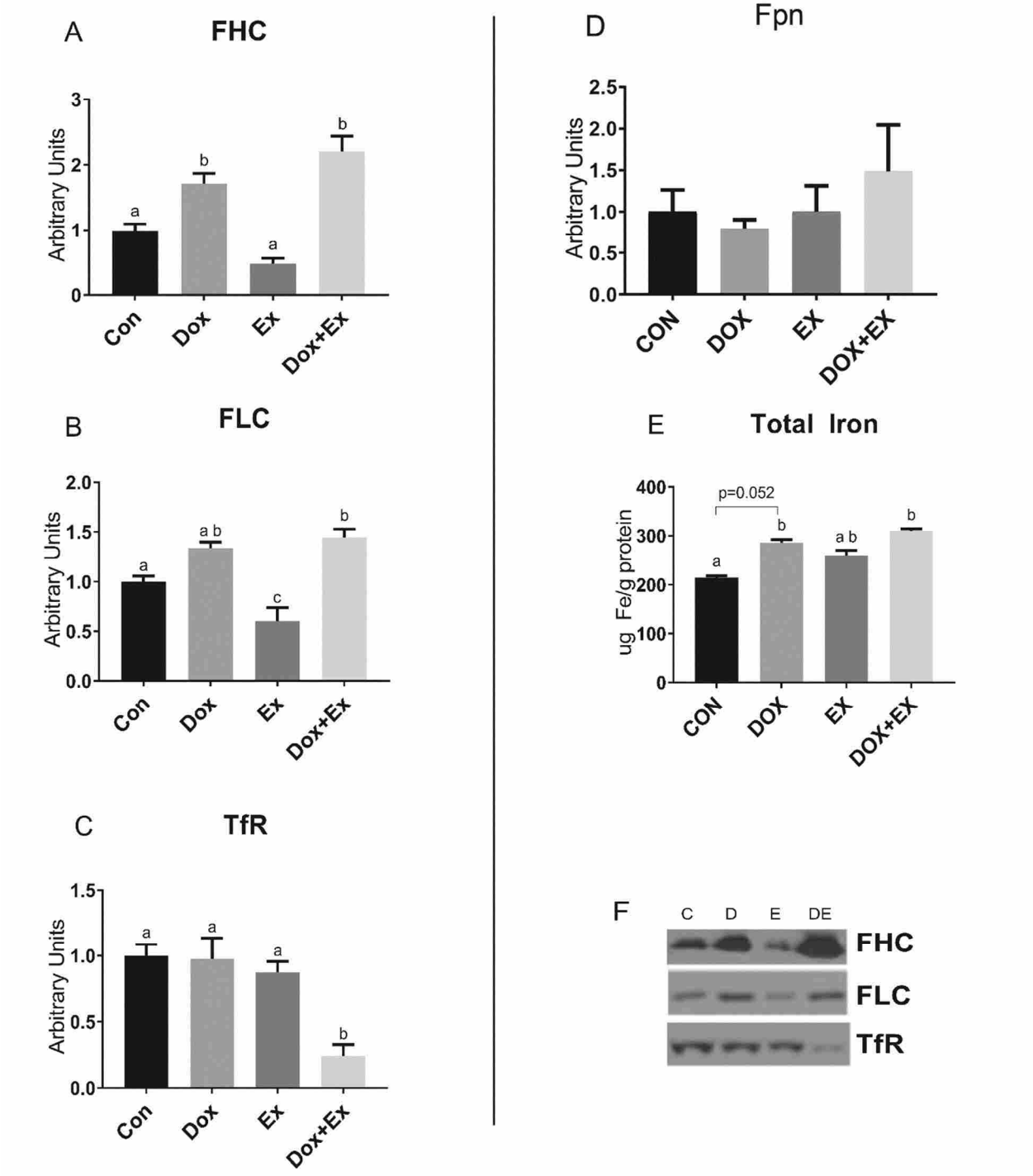

Figure 4.1: DOX Increases Ferritin Protein Content and Total Iron in the Liver, EX Treatment. (A) FHC protein content, n=7-9 ±SEM. Main effect of DOX $p<0.05$. (B) FLC protein content, n=5-7 ±SEM. Main effect of DOX $p<0.05$. (C) TfR protein content, n=5-8 ±SEM. (D) Fpn mRNA, n=5-6 ±SEM. (E) Total iron, n=5-9 ±SEM. Main effect of DOX $p<0.05$. (F) Representative blots. All samples were derived and processed at the same time. Post hoc analysis: bars with different letters are significantly different $p<0.05$.

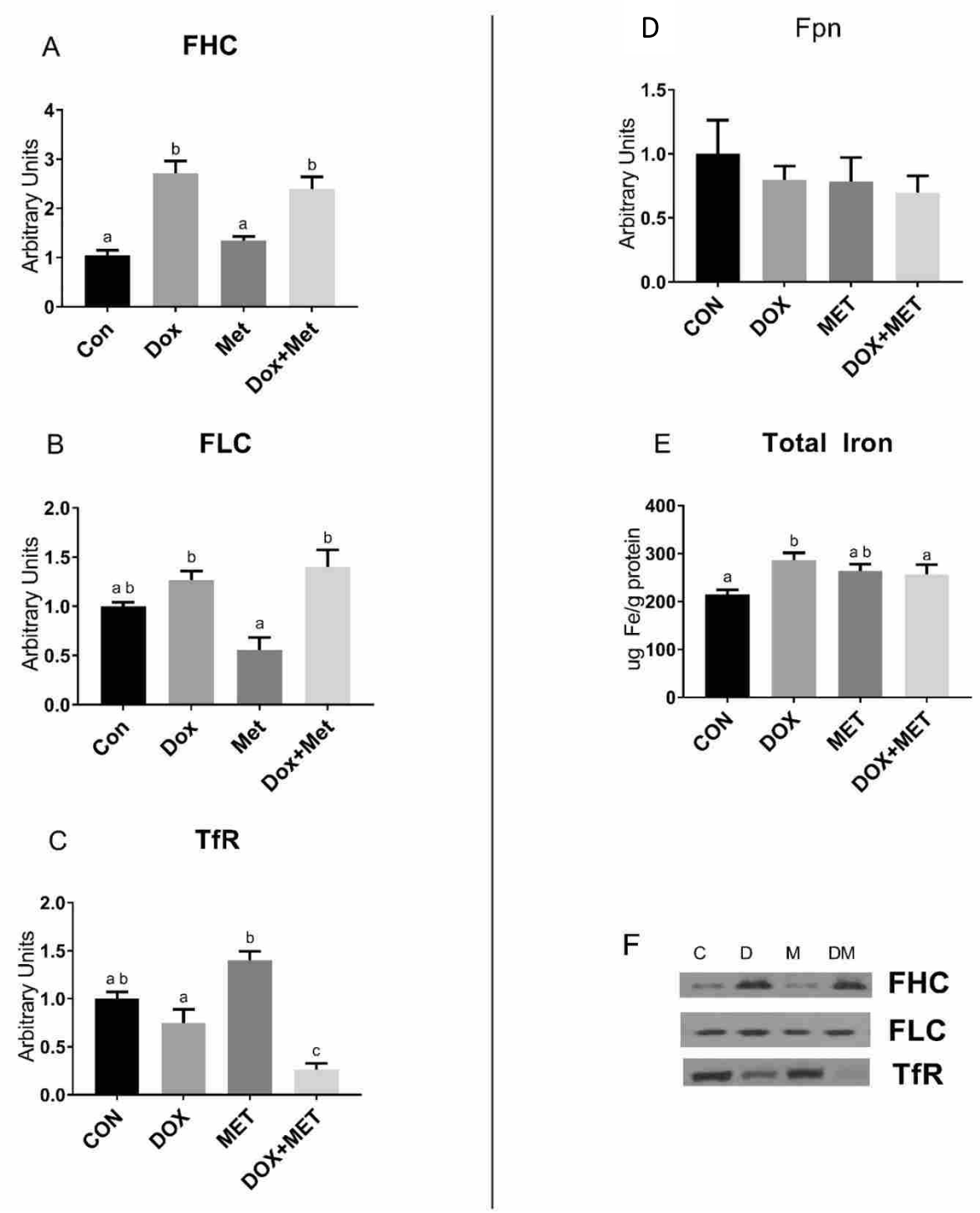

Figure 4.2: DOX Increases Ferritin Protein Content and Total Iron in the Liver, MET Treatment. (A) FHC protein content, n=6-9 ±SEM. Main effect of DOX $p<0.05$. (B) FLC protein content, n=5-7 ±SEM. Main effect of DOX $p<0.05$. (C) TfR protein content, n=5-10 ±SEM. (D) Fpn mRNA, n=5-6 ±SEM. (E) Total iron, n=6 ±SEM. (F) Representative blots. All samples were derived and processed at the same time. Post hoc analysis: bars with different letters are significantly different $p<0.05$.

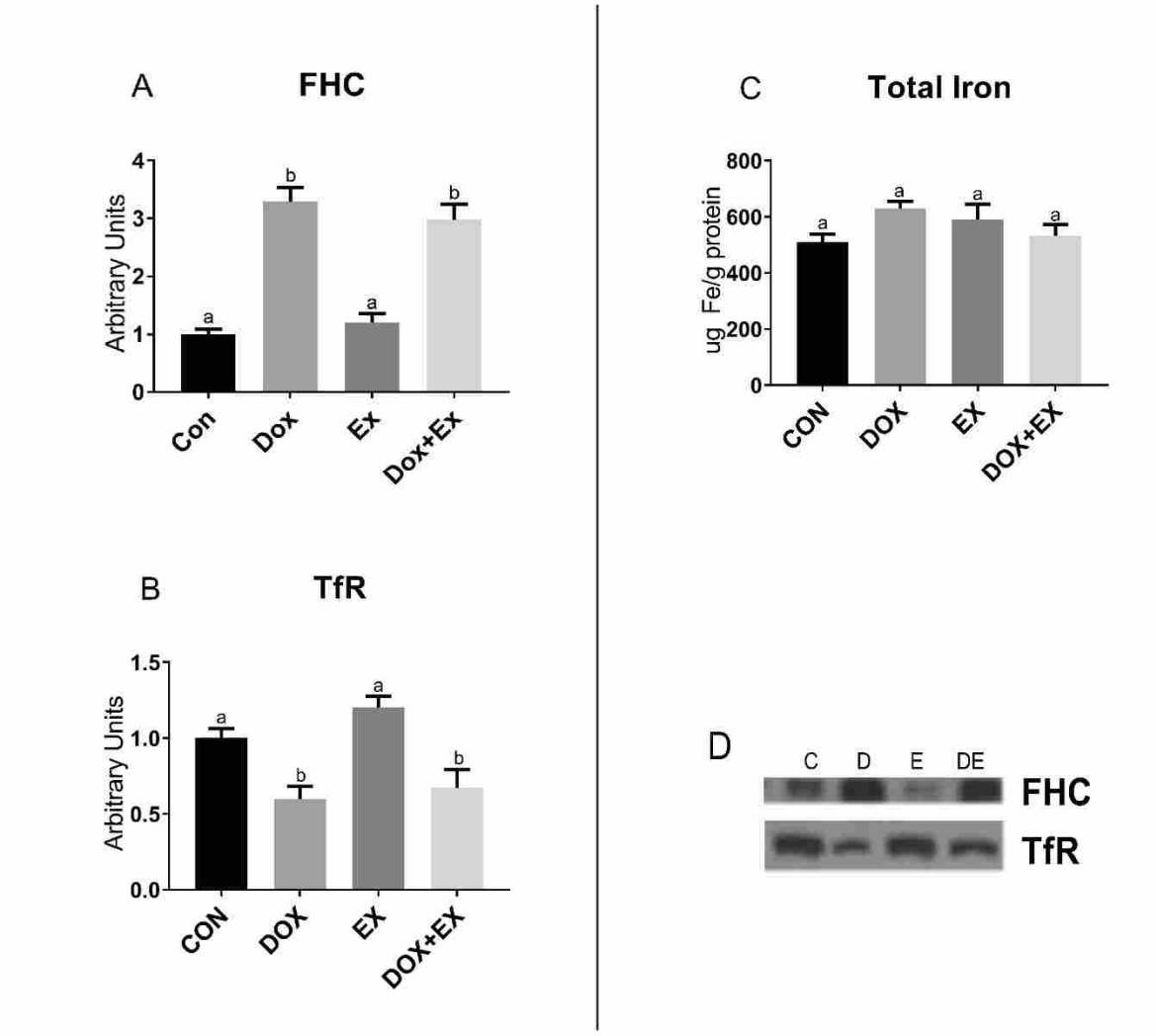

Figure 4.3: DOX Increases FGC and Decreases TfR Protein Content in the Heart, EX Treatment. (A) FHC protein content, n=6-7 ±SEM. (B) TfR Protein Content, n=6 ±SEM. (C) Total iron, n=6-7 ±SEM. (D) Representative blots. All samples were derived and processed at the same time. Post hoc analysis: bars with different letters are significantly different $p<0.05$.

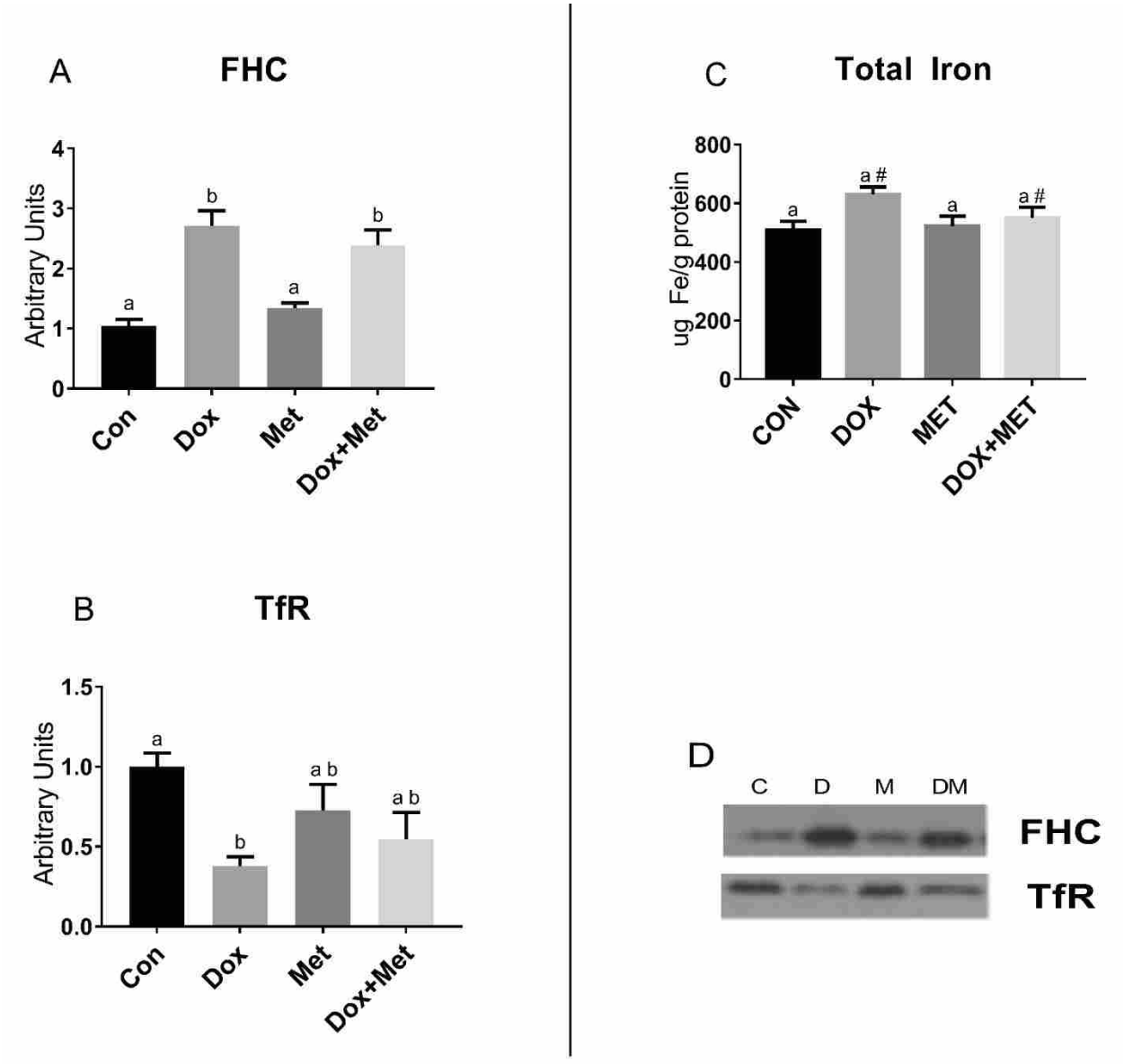

Figure 4.4: DOX Increases FHC and Decreases TfR Protein Content in the Heart, MET Treatment. (A) FHC protein content, n=5 ±SEM. Main effect of DOX $p<0.05$. (B) TfR protein content, n=5 ±SEM. Main effect of DOX $p<0.05$. (C) Total iron, n=6-7 ±SEM. Main effect of DOX # $p<0.05$. (D) Representative blots. All samples are derived and processed at the same time. Post hoc analysis: bars with different letters are significantly different $p<0.05$.

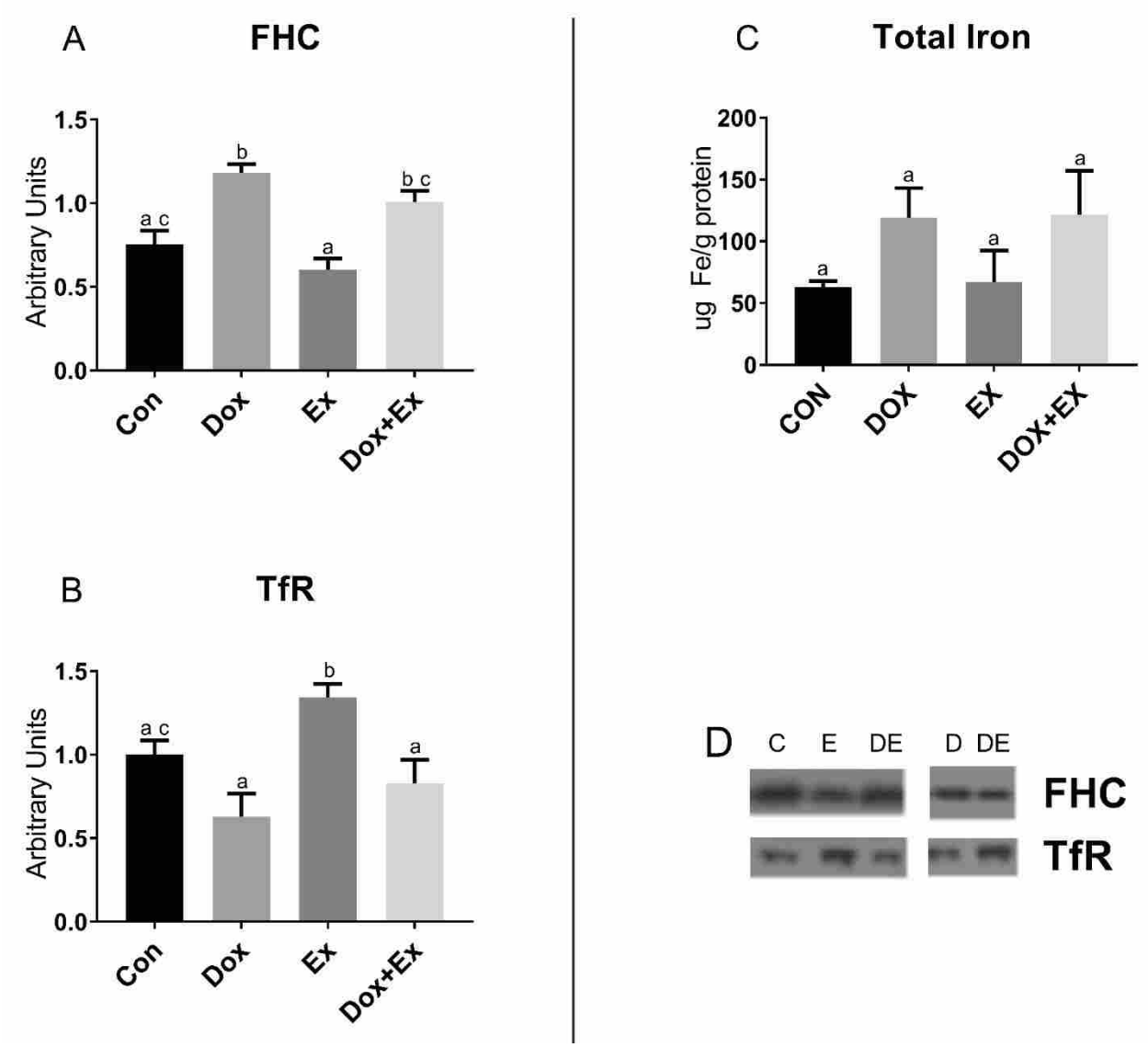

Figure 4.5: DOX Increases FHC and Decreases TfR Protein Content in the Muscle, EX Treatment. (A) FHC protein content, n=5 ±SEM. Main effect of DOX $p<0.05$. (B) TfR protein content, n=5-6 ±SEM. Main effect of DOX $p<0.05$. (C) Total iron, n=5-6 ±SEM. (D) Representative blots. All samples were derived and processed at the same time. Blots are split to allow for better resolution between groups. Post hoc analysis: bars with different letters are significantly different $p<0.05$.

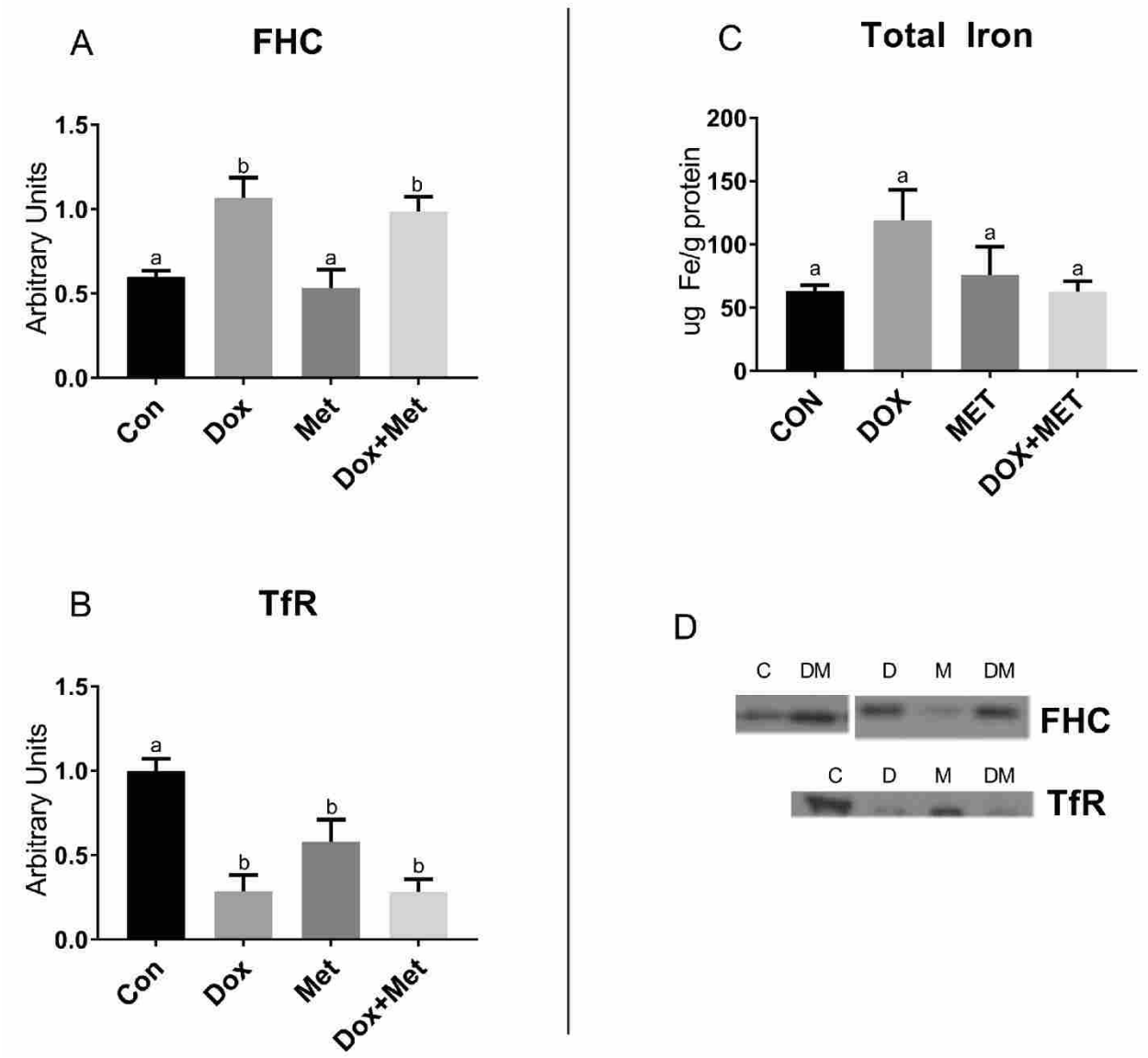

Figure 4.6: DOX Increases FHC and Decreases TfR Protein Content in the Muscle, MET Treatment. (A) FHC protein content, n=5-6 ±SEM. (B) TfR protein content, n=5-6 ±SEM. Main effect of DOX $p<0.05$. (C) Total iron, n=5-6 ±SEM. (D) Representative blots. Blot is split to allow for better resolution between groups. All samples were derived and processed at the same time. Post hoc analysis: bars with different letters are significantly different $p<0.05$.

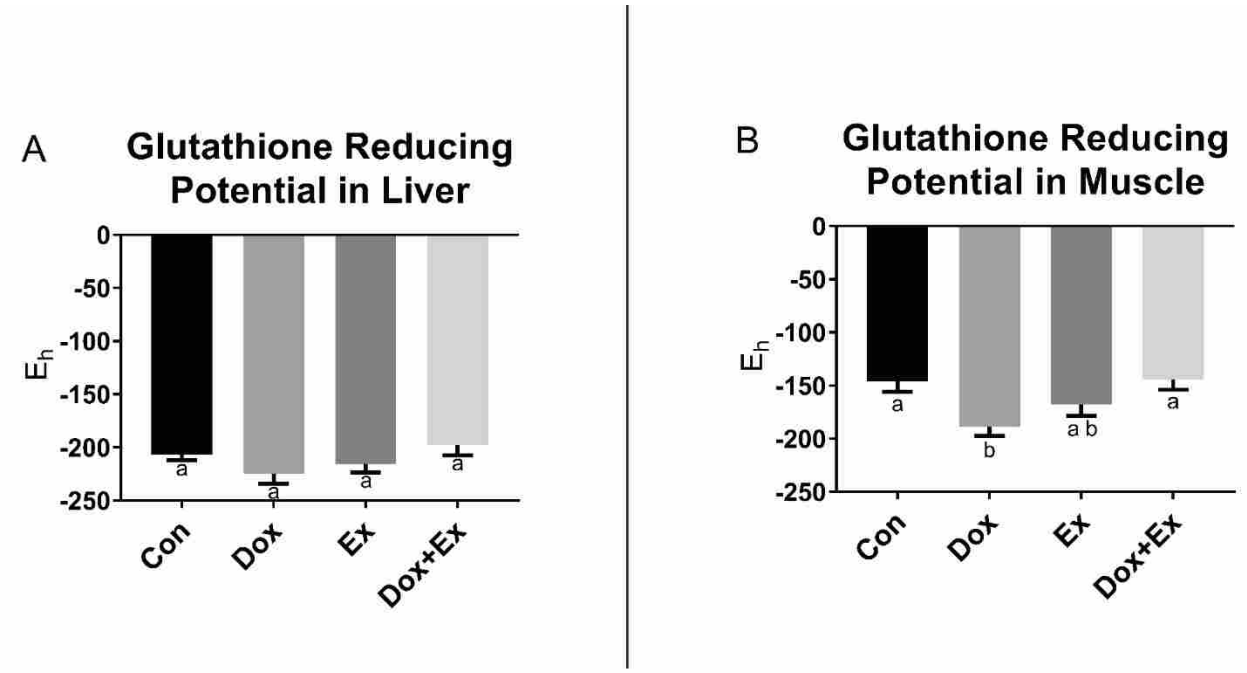

Figure 4.7: GSH Redox Potential with EX Treatment. Calculated using the Nernst equation. (A) Reducing potential in the liver, n=5-6 ±SEM. DOX+EX interaction p=0.062. (B) Reducing potential in the muscle, n=5-6 ±SEM. DOX+EX interaction p<0.05. Post hoc analysis: bars with different letters are significantly different p<0.05.

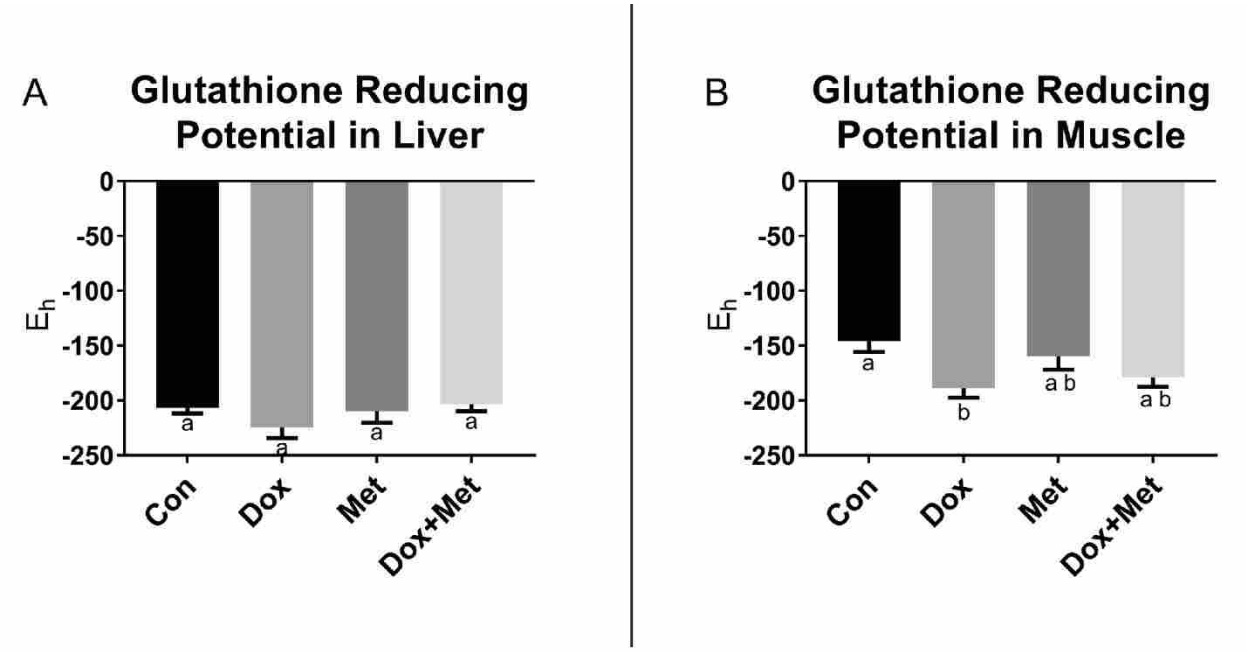

Figure 4.8: GSH Redox Potential with MET Treatment. Calculated using the Nernst equation. (A) Reducing potential in the liver, n=5-6 ±SEM. (B) Reducing potential in the muscle, n=5-6 ±SEM. Main effect of DOX p<0.05. Post hoc analysis: bars with different letters are significantly different p<0.05.

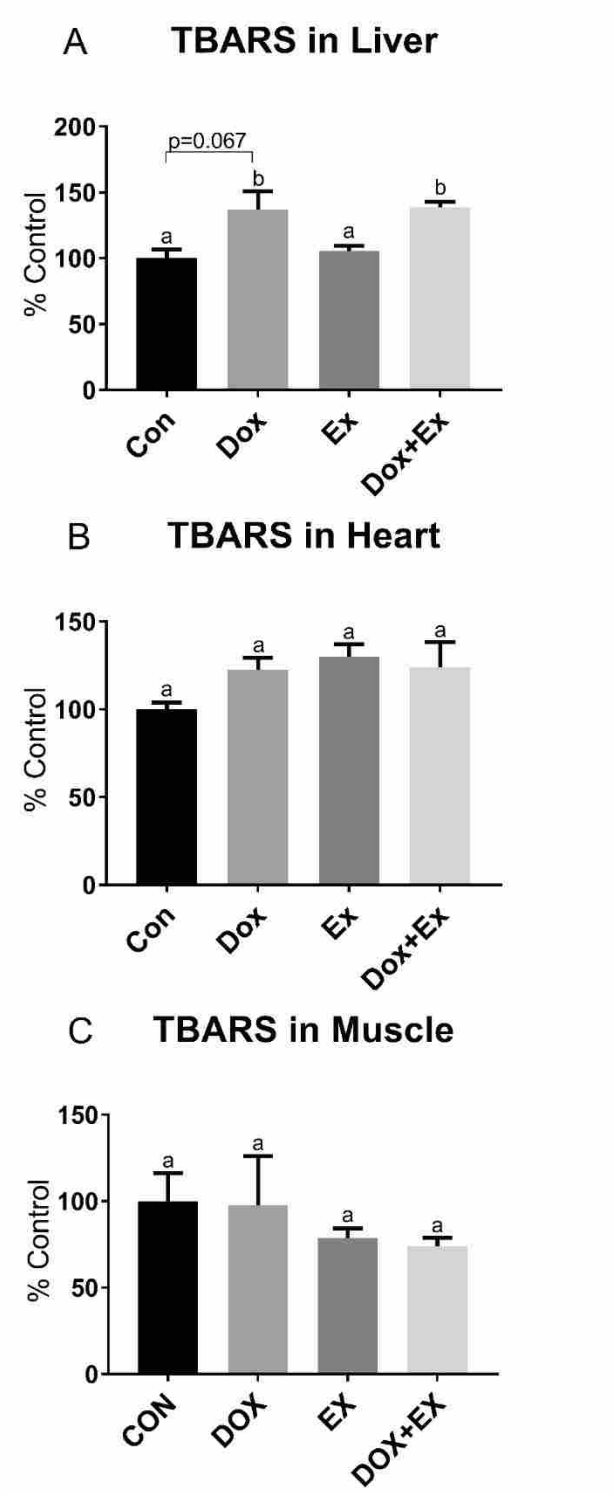

Figure 4.9: TBARS in the Liver, Heart, and Muscle, EX Treatment. (A) Liver, n=5-7 ±SEM. DOX main effect $p<0.05$. Post hoc analysis: bars with different letters are significantly different $p<0.05$.

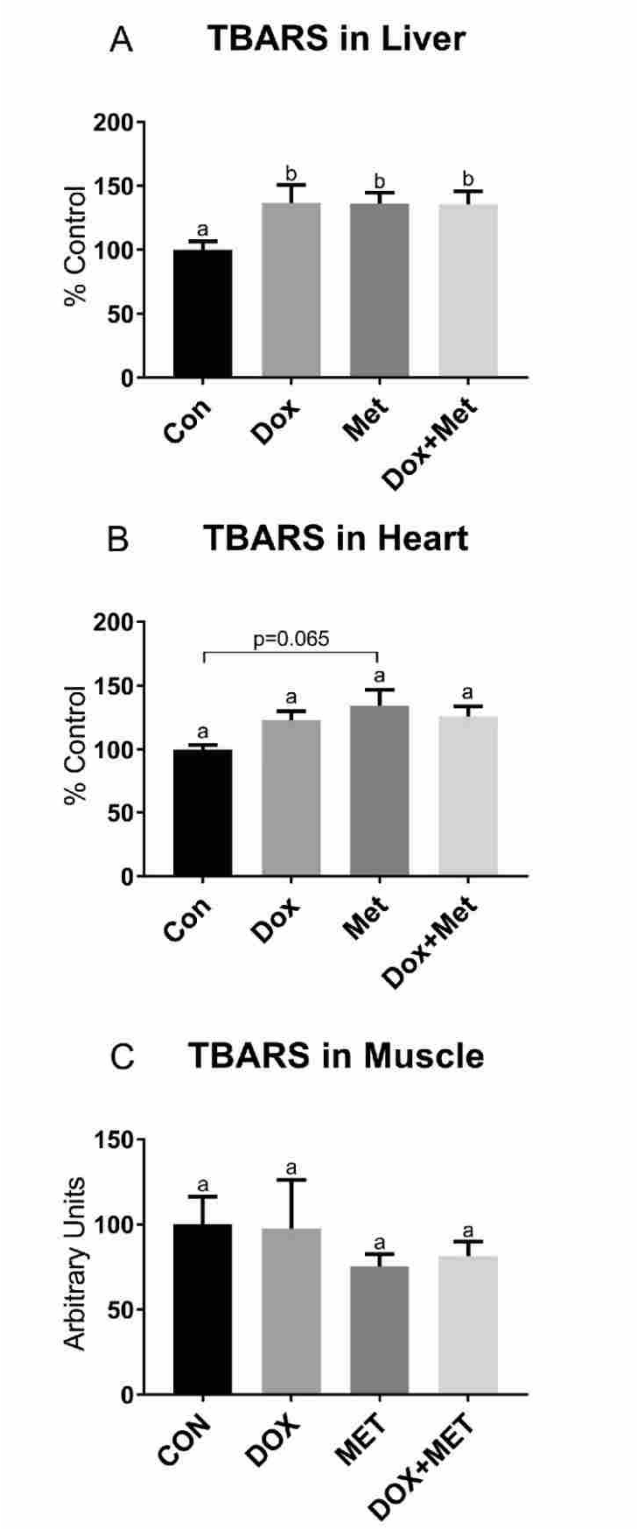

Figure 4.10: TBARS in the Liver, Heart, and Muscle, MET Treatment. (A) Liver, n=5-7 ±SEM. Post hoc analysis: bars with different letters are significantly different $p<0.05$.

CHAPTER 5: Doxorubicin Induces NF-κB Activity to Preserve Cell Viability and Modulate Iron Regulation in Skeletal Muscle Cells

Introduction

Doxorubicin (DOX) is a chemotherapy drug used to treat a variety of solid tumor and leukemia cancers. While an effective chemotherapeutic, the cardio-toxicity caused by DOX limits its clinical total dosage (Schwartz, 2000). In addition to cardiotoxicity, DOX causes similar side effects in skeletal muscle (Gilliam et al., 2013; Smuder et al., 2011a). In both cardiac and skeletal muscles, DOX causes oxidative stress (Benzer et al., 2018; Ludke et al., 2017; Smuder et al., 2011b), autophagy (Smuder et al., 2011a, 2013), decreased mitochondrial function (Gilliam et al., 2013; Kavazis et al., 2010), and decreased heart (Chicco et al., 2006; Hayward et al., 2013) and skeletal muscle function (Hydock et al., 2011). DOX also interacts directly with iron (Eliot et al., 1984; Muindi et al., 1984) and causes cardiac changes in iron regulation through increased levels of the iron storage protein ferritin and decreased levels of the iron transport protein transferrin receptor (Corna et al., 2006). Multiple mechanisms have been investigated to determine how DOX induces changes in iron regulation. Here, we describe the role NF-κB activity plays in responding to DOX treatment in skeletal muscle cells.

NF-κB is activated by a variety of inflammation and oxidative stress factors to induce changes in gene expression. Typically, NF-κB promotes anti-apoptotic (Arlt et al., 2001; Ho et al., 2005) changes in gene expression, but in some models, it increases pro-apoptotic factors (Wang et al., 2002; Zhang et al., 2017). NF-κB plays differing roles in response to DOX treatment depending on cell type. In cancer cell lines, DOX increases NF-κB activity to elicit anti-apoptotic signaling (Arlt et al., 2001; Seubwai et al., 2016). Additionally, inhibiting NF-κB signaling increases the toxicity of DOX in these cancer cell models. In contrast, DOX-induced NF-κB activity causes pro-apoptotic signaling in cardiac tissue or cells (Benzer et al., 2018;

Wang et al., 2002; Zhang et al., 2017). Inhibiting NF-κB activity in cardiac cells exposed to DOX resulted in a decrease in apoptosis (Wang et al., 2002). However, the effects of DOX on NF-κB activity has not been well studied in skeletal muscle. Given the similar responses of cardiac and skeletal muscle to DOX treatment, skeletal muscle NF-κB activity is expected to be similar to cardiac muscle.

In addition to determining the mechanism by which DOX elicits toxic side effects in the muscle, potential co-treatments that limit these side effects need to be investigated. Metformin (MET) has been suggested as one such treatment. Recent studies have shown that MET blunts DOX-induced autophagy, oxidative stress, and cardiac loss of function (Argun et al., 2016; Kelleni et al., 2015; Sheta et al., 2016). Additionally, MET has been shown to decrease cancer risk (Li et al., 2009; Viollet et al., 2012; Yue et al., 2014) and increase the efficacy of DOX as a chemotherapeutic (El-Ashmawy et al., 2017; Wu et al., 2016). Study of the role that MET plays in iron regulation with DOX treatment is incomplete. Furthermore, the role MET plays with DOX treatment in skeletal muscle is not well understood.

A series of papers by Lopez et al. show that MET treatment prevents DOX-induced apoptosis in cardiomyocytes (Asensio-López et al., 2011; Asensio-López et al., 2013; Asensio-Lopez et al., 2014). Using siRNA methods, it was shown that MET acts through NF-κB to increase the ferritin heavy chain protein levels and maintain cell viability. This finding is interesting given other research showing the pro-apoptotic effects of NF-κB with DOX treatment in cardiac tissue. Given the contradictory evidence for the role of NF-κB in cardiomyocytes with DOX and/or MET treatment, we tested the effects of MET and DOX treatment with and without NF-κB activity.

A model with NF-κB activity specifically and permanently inhibited has not been tested in skeletal muscle with DOX treatment. We therefore tested the hypothesis that NF-κB has pro-apoptotic effects in response to DOX in skeletal muscle cell culture. We also investigated whether MET exerted its effects with DOX treatment in an NF-κB dependent manner. To test our hypothesis, we used a mouse skeletal muscle C2C12 cell line with an inactive form of IκBα that inhibits NF-κB activity (designated IκBαSR). The NF-κB /IκBα complex must dissociate to activate and translocate NF-κB to the nucleus. The expressed IκBα can no longer be phosphorylated, which prevents dissociation of the NF-κB /IκBα complex causing potent and specific inhibition of NF-κB (Guttridge, Albanese, Reuther, Pestell, & Baldwin, 1999).

The primary purpose of this study is to determine if DOX exerts its effects on cell viability and iron regulation in an NF-κB dependent manner and to determine if NF-κB exerts anti-apoptotic or pro-apoptotic effects in this model. The secondary purpose of this study is to determine if MET causes beneficial adaptations to cell viability and iron regulation with DOX treatment in an NF-κB dependent manner.

Methods

Cell Culture

Mouse skeletal muscle C2C12 myoblasts were grown to confluence on growth media (DMEM/HG, GE Lifesciences; 10% fetal bovine serum, Fisher Scientific; and 1% antibiotic antimyotic, Sigma) and differentiated to myotubes for three days on differentiation media (DMEM/HG, GE Lifesciences; 10% horse serum, Sigma; and 1% antibiotic antimyotic, Sigma). IκBαSR knockout C2C12 cells were a generous gift from Denis Guttridge (Ohio State University Medical Center). IκBαSR cells contain a mutant plasmid of IκBα that can no longer be

phosphorylated, which results in specific inhibition of NFkB as shown previously (Guttridge et al., 1999). IκBαSR myoblasts were grown to confluence on growth media (DMEM/HG, GE Lifesciences; 10% fetal bovine serum, Fisher Scientific; and 10% Geneticin, Life Technologies) and differentiated to myotubes for three days on differentiation media (DMEM/HG, GE Lifesciences; 2% horse serum, Sigma; and 10% Geneticin, Life Technologies). Cells were treated with 0.5 μM DOX and/or 0.4mM MET for 16 hours.

Cell Viability (MTT) Assay

Differentiated cells were incubated with 0.5 mg/mL Thiazolyl Blue Tetrazolium Bromide (MTT) in differentiation media for 45 minutes. Cells were rinsed with PBS and incubated with solubilizing solution (4 mM HCl, 0.1% nondel in isopropanol) until MTT was dissolved. Absorbance was read at 570 nm. Results shown here are also shown in chapter 3.

Western Blotting

Cells were harvested by scraping in lysis buffer and sonicating. Protein concentration was determined using a BSA protein assay kit (23225, Thermo Scientific). Samples were made to an equal protein concentration, resolved through SDS-PAGE and blotted onto nitrocellulose membrane. The membrane was stained with Ponceau S and analyzed to ensure even protein loading and transfer across lanes. After blocking, primary antibody was applied against ferritin heavy chain (cell signaling, 3998S) and transferrin receptor (abcam, ab84036) at 4°C. Appropriate secondary was then applied (LI-COR, 926-32213) and images were taken using the Li-COR Odyssey CLx. The bands were then analyzed using Image Studio Light v5.2.

Statistics

Statistical significance was determined using 2-way Anova and Tukey-Kramer post-hoc test with an alpha level of 0.05 using JMP software.

Results

Maintaining Cell Viability Under DOX Treatment Requires NF-κB Activity

Mouse skeletal muscle C2C12 cells containing an inactive form of IκBα were used to inhibit NF-κB activity (Guttridge et al., 1999). Standard C2C12 cells (SC2C12) and inactivated IκBα cells (IκBαSR) were grown to confluency then differentiated into myotubes for 3 days. Myotubes were treated with 0.5 μM DOX and/or 0.5 mM MET for 16 hours.

Cell viability was assessed using the MTT assay (figure 5.1). A low concentration of 0.5 μM DOX was used and did not cause a reduction is cell viability in SC2C12 cells (figure 5.1A). Surprisingly, there was a main effect of MET to reduce cell viability ($p<0.05$, figure 5.1A) in SC2C12 cells. Most of this effect can be attributed to the combination treatment of DOX+MET, although significance was not reached between any individual groups. In IκBαSR cells, viability was reduced with DOX treatment ($p<0.05$, figure 5.1B) and there was no effect of MET treatment. These results suggest that DOX activates NF-κB to induce anti-apoptotic changes and maintain cell viability.

DOX-Induced FHC Protein Content is Blunted Without NF-κB Activity

Ferritin heavy chain (FHC) is an iron storage protein with an NF-κB gene binding site(Bresgen & Eckl, 2015). There was a main effect of DOX to induced FHC expression in SC2C12 ($p<0.05$, figure 5.2A). Post-hoc analysis confirmed increased FHC expression in DOX and DOX+MET groups versus CON ($p<0.05$, figure 5.2A). In IκBαSR cells, there was a main

effect of DOX to increase FHC expression ($p<0.05$, figure 5.2B). Post-hoc analysis revealed that DOX and DOX+MET groups were not different from CON (figure 5.2B). There was no effect of MET treatment on FHC expression in either cell type. These results suggest that without NF-κB activity, the DOX-induced increase in FHC expression is blunted.

DOX-Induced Reduction of TfR Protein Content is Restored Without NF-κB Activity

The major cellular iron transporter, transferrin receptor (TfR), was measured next. In SC2C12 cells, there was a main effect of DOX to reduce TfR expression ($p<0.05$, figure 5.3A). This pattern was not observed in IκBαSR cells, which had no differences in TfR expression between groups (figure 5.3B).

Discussion

We demonstrate that NF-κB activation is required to maintain cell viability with low concentration DOX treatment. We also show that the DOX-induced increase in FHC and reduction of TfR is at least partially due to NF-κB activation. Given that the FHC gene has a known binding site for NF-κB(Bresgen & Eckl, 2015; Pham et al., 2004), this result is not surprising. Additionally, MET treatment does not affect changes in cell viability or iron regulation with or without NF-κB activity.

Previous reports in cardiac tissue and cell lines have suggested that NF-κB functions as a pro-inflammatory factor and increases apoptosis in response to DOX(Benzer et al., 2018; Wang et al., 2002; Zhang et al., 2017). Two studies used indirect means of inhibiting NF-κB activity through adding tannic acid(Zhang et al., 2017) or curcumin(Benzer et al., 2018). In both studies, NF-κB activity, pro-apoptotic, and pro-inflammatory factors were reduced with treatment. The authors suggested that the reduction in apoptosis and inflammation was through reduced NF-κB

activity but did not test this hypothesis directly. A third study by Wang et al(Wang et al., 2002) used multiple means to reduce NF-κB activity in cardiac cell culture including a similar IκB mutant vector. In this study, inhibited NF-κB activity resulted in a reduction of apoptosis after DOX treatment. Interestingly, this study used the same DOX concentration as was used here (5 μM), but for 4 hours instead of the 16 hours used here. Additionally, Wang et al used a cardiac cell line while a skeletal muscle cell line was used here. These differences in experimental set up may account for some differences in results. Additional study is needed to better understand the differing roles NF-κB activity may play in a time and/or cell line dependent manner.

The mechanisms by which DOX induces increased FHC expression are still under debate. The roles of oxidative stress, NF-κB, and iron regulatory proteins (IRPs) have all been investigated. DOX can bind to iron directly in its ferrous (Fe^{2+}) or ferric (Fe^{3+}) form, which allows free radical formation by the fenton and Heiber-Weiss reactions(Eliot et al., 1984; Muindi et al., 1984). These reactions, in addition to the known effect for DOX to increase many oxidative stress measurements, resulted in the theory that DOX increases FHC expression as a response to a general increase in oxidative stress(Eliot et al., 1984; Muindi et al., 1984). However, a study that used low concentrations of DOX (0.25 μM) showed FHC levels were increased without inducing oxidative stress(Bernuzzi et al., 2009). This suggests that the effects of DOX on iron regulation can be independent of oxidative stress. In this study, low concentrations of DOX (0.5 μM) did not decrease cell viability but did induce FHC expression. However, this effect was dependent upon NF-κB activity, which is activated in response to oxidative stress. It may be that low levels of oxidative stress are sufficient to induce NF-κB activity to increase FHC protein levels.

A series of studies by Lopez et. al. (Asensio-López et al., 2011; Asensio-López et al., 2013; Asensio-Lopez et al., 2014) suggested that MET treatment improves cell viability through

NF-κB activation and upregulation of FHC. These studies used siRNA to knock down NF-κB activity, which resulted in blunted FHC expression. Additionally, the effectiveness of MET in preserving cell viability was shown to operate through the NF-κB/FHC pathway. Our results do not confirm this pathway. We show that MET did not improve cell viability or upregulate FHC expression with or without NF-κB. Our model of inhibition through a mutant IκBα allows for permanent, specific, and potent inactivation of NF-κB activity. Use of siRNA, as done in the Lopez et al studies, is a temporary and much less effective way to reduce NF-κB activity.

Another proposed mechanism for the DOX-induced increase in FHC levels is through iron regulatory proteins (IRP). Increased IRP activity occurs under conditions of low iron resulting in decreased FHC and increased TfR protein levels. Current literature is split on whether IRP activity is an important factor in DOX-induced increased FHC and decreased TfR. Two studies show DOX-induced changes in FHC protein content is dependent upon IRP activity(Ghigo et al., 2016; Xu et al., 2005) while a third clearly shows that DOX can induce increased FHC and decreased TfR independent of IRP(Corna et al., 2006). This study did not examine IRP activity, but did find that FHC response was blunted, but not eliminated without NF-κB activity. Since DOX is known to cause a wide range of cellular changes, it is entirely possible that FHC and TfR regulation occur through multiple mechanisms of action including IRPs and NF-κB. We therefore suggest that NF-κB activity is partially, but not completely responsible for DOX-induced changes in iron regulation.

In conclusion, NF-κB promotes anti-apoptotic effects in response to DOX treatment in skeletal muscle cells. Additionally, NF-κB activity is at least partially responsible for DOX-induced increased FHC and decreased TfR in skeletal muscle cells. MET treatment failed to induce positive changes with DOX treatment with or without NF-κB activity. Additional study is

required to better understand the mechanisms by which DOX elicits its toxic effects in cardiac and skeletal muscle.

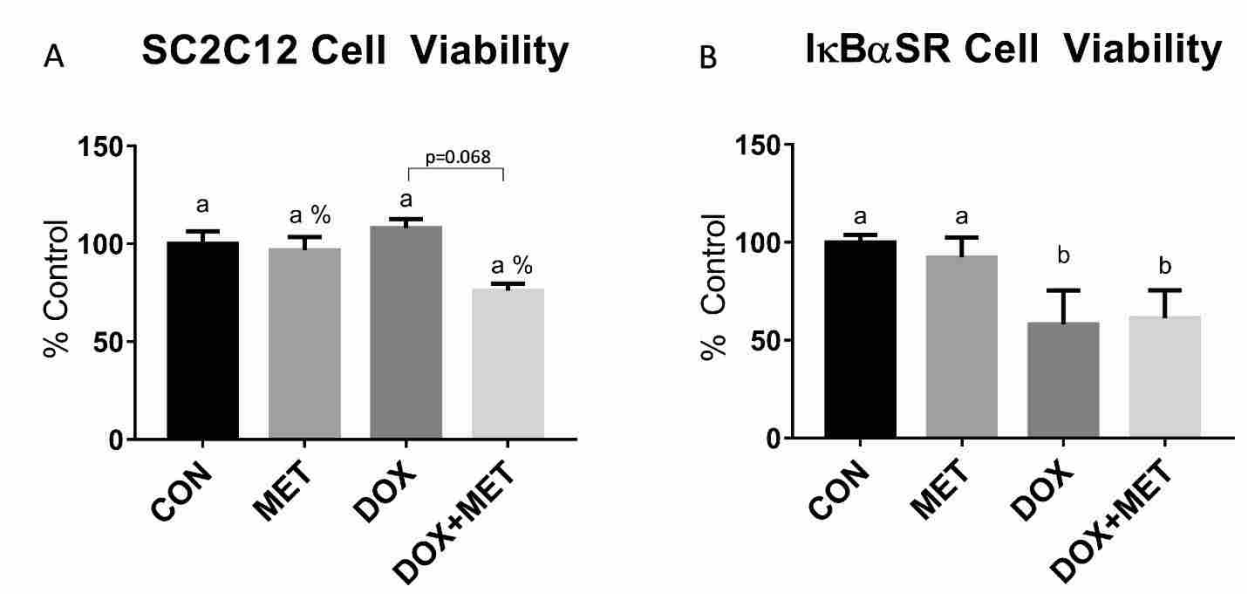

Figure 5.1: Cell Viability Assessed Using MTT Assay. Cells treated with 0.5 μM DOX and/or 0.5 mM mET for 16 hours. (A) Viability of SC2C12 cells, n=3-5 ±SEM. (B) Viability in IκBαSR cells, n=3-5 ±SEM. MET main effect % $p<0.05$. Bars with different letters are significantly different $p<0.05$.

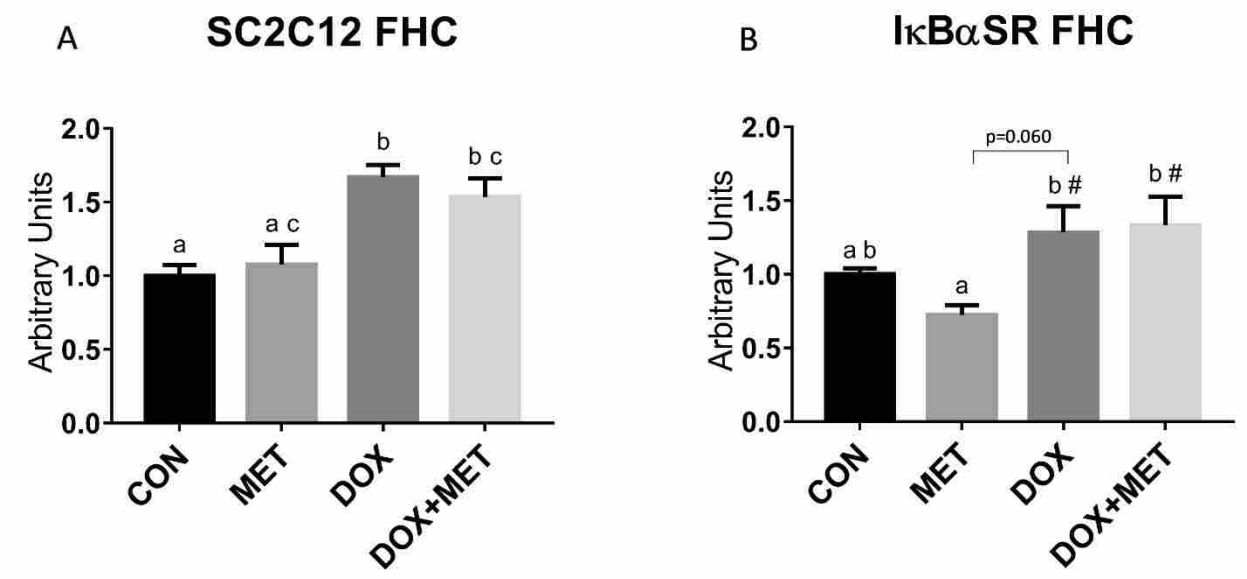

Figure 5.2: FHC Protein Content. Cells treated with 0.5 µM DOX and/or 0.5 mM MET for 16 hours. (A) FHC protein content of SC2C12 cells, n=3-5 ±SEM. (B) FHC protein content of IκBαSR cells, n=4-5 ±SEM. DOX main effect # $p<0.05$. Bars with different letters are significantly different $p<0.05$.

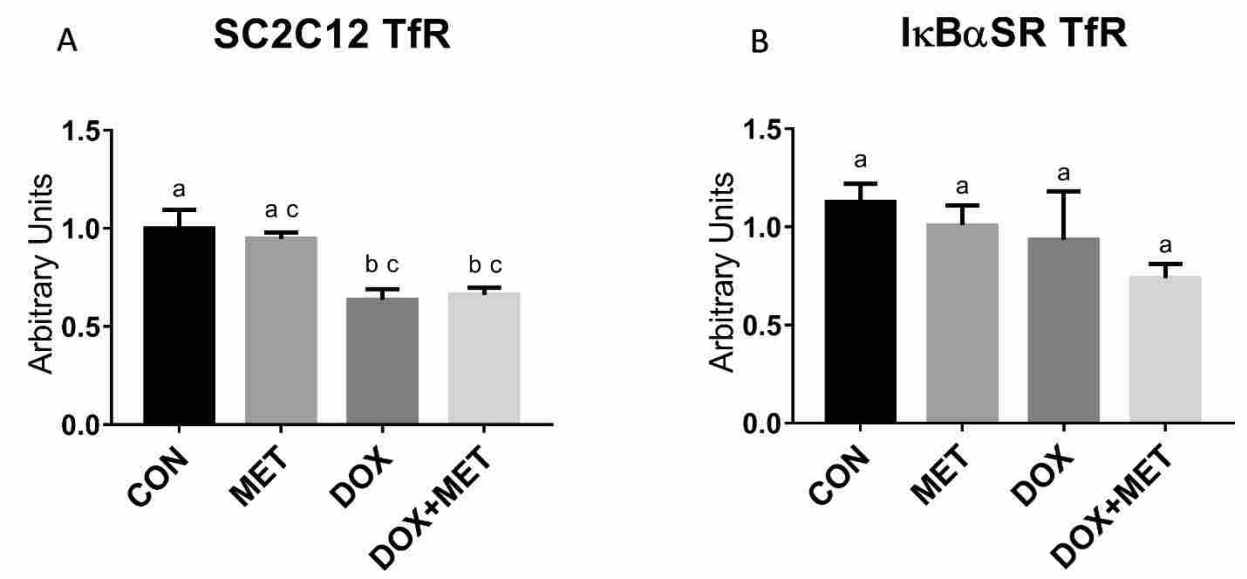

Figure 5.3: TfR Protein Content. Cells treated with with 0.5 µM DOX and/or 0.5 mM MET for 16 hours. (A) TfR protein content of SC2C12 cells, n=3 ±SEM. (B) TfR protein content of IκBαSR cells, n=4-5 ±SEM. Bars with different letters are significantly different $p<0.05$.

CHAPTER 6: Conclusion

The purposes of this research were four-fold. The first purpose was to investigate if DOX-induced muscle dysfunction can be prevented with EX or MET treatments. Second, was to determine if MET could prevent the mitochondrial dysfunction caused by DOX treatment. The third purpose was to characterize the effect of DOX on the major iron regulatory pathway in three tissues of interest and determine if EX or MET exert their effects against DOX in through modulating iron regulation. Last, the fourth purpose was to determine if NF-κB plays a role in DOX-induced changes to iron regulation.

For the first specific aim we measured in-situ muscle function after DOX treatment with or without EX or MET co-treatments. We showed that DOX impaired skeletal muscle half relaxation time in the gastrocnemius-soleus-plantaris complex. By using an in-situ method, we had the advantage of a more physiologically relevant model to analyze whole muscle function with continued blood circulation. We demonstrate for the first time that in-situ total force production by the muscle was only marginally decreased with DOX treatment. However, the half relaxation time after a muscle contraction was significantly impaired with DOX treatment. Additionally, we found that EX prevented half relaxation time impairment, but MET did not. In this animal model, mitochondrial function was not significantly affected by any of our treatments. While surprising, this result indicates that decreased muscle function due to DOX can occur without inhibiting mitochondrial function.

In the second specific aim, we measured the mitochondrial function of mouse C2C12 myotubes with DOX and/or MET treatment. We report that DOX reduced respiration at complex I and complex II of the electron transport chain in cell culture. While this phenomenon has been demonstrated before, the effect of MET co-treatment with DOX has not been previously studied.

We show that while MET inhibited complex I respiration, the effects of DOX and MET treatment were not additive. Additionally, MET co-treatment with DOX was able to prevent the decrease in complex II respiration from DOX. However, maximum respiration was lower than control and not different between MET, DOX, or DOX plus MET groups.

Mitochondrial function results were not consistent between animal and cell culture models. While DOX is known to decrease mitochondrial respiration, a recovery period in respiration has been observed before in animals; an effect that was also seen in this study. In the cell culture experiments, acute (16 hrs) DOX treatment resulted in reduced respiration. The finding that MET could prevent a DOX-induced decrease in complex II respiration in cell culture is novel and should be followed up with animal studies at additional time points.

For the third specific aim, we analyzed the changes in the major iron regulatory pathway due to DOX and/or EX or MET treatments in liver, skeletal muscle, and heart. While FHC and TfR had been analyzed in heart previous to this study, the effects of DOX on iron regulation in skeletal muscle and liver were unknown. Additionally, DOX with EX or MET treatment had not been studied with regards to iron regulation. We report that DOX induced general changes that seem to be protective against free radical damage, although these changes are clearly not adequate to prevent oxidative stress and damage to muscle. DOX increased ferritin levels in liver, heart, and skeletal muscle and decreased TfR levels in the heart and skeletal muscle. EX promoted protective effects in the liver by decreasing TfR content and partially blunted the DOX-induced changes in skeletal muscle. MET also decreased TfR content but had no effect in the heart or skeletal muscle.

Lastly for specific aim four, we determined if DOX modulates iron regulation through an NF-κB dependent pathway. To accomplish this task, we used mouse C2C12 myotubes without

NF-κB activity. The effect of NF-κB activity with DOX treatment on off-target tissues is not well understood generally and has not previously been studied in skeletal muscle. We report that DOX increased FHC levels and decreased TfR levels in myotubes. Without NF-κB activity, these changes were blunted, suggesting that NF-κB activity is at least partially required for DOX-induced changes in iron regulation. Additionally, at the low concentration of DOX used, cell viability was not damaged when NF-κB activity was present. However, without NF-κB activity, cell viability was reduced with DOX. This suggests that NF-κB activity is promoting anti-apoptotic effects in the cell. While determining the mechanism by which DOX exerts its effects on iron regulation was not central to the purpose of this study, we report evidence that NF-κB is at least partially responsible for the increased FHC and decreased TfR levels observed with DOX treatment.

DOX-induced changes in FHC and TfR were consistent between the animal and cell culture models. In both cases, DOX caused an increase in skeletal muscle FHC protein content and a decrease in TfR protein content. These results in skeletal muscle are novel and similar to previous reports in cardiac muscle. Additionally, the results of MET with DOX treatment in skeletal muscle have not been previously reported. We show that MET plus DOX has no effect on myotube cell viability, FHC, or TfR. This result is different from previous findings in cardiomyocytes. Additional study is required to better understand the role MET may play in decreasing DOX toxicity.

In conclusion, this body of work significantly contributes to our understanding of the effect of EX and MET treatments on the consequences of DOX treatment. For the first time, EX treatment with DOX was found to prevent impairment of half relaxation rate using an in-situ model of muscle analysis. EX treatment also modulated iron regulation in response to DOX

treatment in the liver and skeletal muscle. These results add to the body of literature suggesting that moderate EX treatment with DOX can limit DOX toxicity in cardiac and skeletal muscle. We report for the first time on the effects of MET and DOX treatment outside of cardiac tissue. MET treatment did not prevent muscle function loss due to DOX but was able to partially preserve mitochondrial function. MET treatment modulated iron regulation in response to DOX in the liver but not the heart or skeletal muscle. A novel multi-tissue analysis of DOX on iron regulation shows that DOX increases ferritin in liver, heart, and skeletal muscle, decreases TfR in heart and skeletal muscle, and increases total iron in liver and heart. In skeletal muscle, these effects are partially due to NF-κB activity. This thorough analysis clarifies the role of DOX on systemic iron regulation.

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CURRICULUM VITAE

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EDUCATION

Brigham Young University- Doctoral Degree 2012-2018

Physiology and Developmental Biology

Utah State University- Bachelor of Science 2008-2012

Biochemistry, Cum Laude

EXPERIENCE

Graduate Research Assistant
Brigham Young University, Hancock Lab 2012-2018

- Independently designed dissertation project by developing and maintaining an understanding of the body of published scientific literature.
- Identified scientific evidence needed to support side effects of chemotherapeutic treatments.
- 5 years' experience as lab manager leading a team of 8-10 undergraduate students.

Teaching Assistant
Brigham young University

- Nutrition and Metabolism in Sports and Exercise
- Advanced Physiology
- Advanced Physiology Lab

Undergraduate Research Assistant
Utah State University, Johnson Lab

- Worked on a team to isolate proteins involved with mRNA degradation.

SCHOLARSHIPS AND AWARDS

Research Assistant Fellowship, Brigham Young University 2012-2018

Teaching Assistant Fellowship, Brigham Young University 2012-2018

Full Tuition Scholarship Award, Brigham Young University 2012-2017

Excellent Undergraduate Researcher Award, Utah State University 2012

Poster Award, Hansen Biochemistry Retreat 2011

PUBLICATIONS

- Nutrition and Metabolism in Sports and Exercise Student Manual
    - Currently used in place of course textbook at Brigham Young University

MANUSCRIPTS IN PREPARATION OR UNDER REVIEW

- [Under Review] Mackay A.D., Munk, D.J., Marchant E.D., Hansen J., Watt R.K., Hancock C.R. Multi-Tissue Analysis of Exercise or Metformin on Doxorubicin-Induced Iron Dysregulation. Journal of Physiology-Endocrinology and Metabolism.

- [In Preparation] Mackay A.D., Louw M.G., Hancock C.R. Exercise, but not Metformin Prevents Loss of Muscle Function Due to Doxorubicin in Mice Using an in-situ Method. Journal of Applied Phyiology.

- [In Preparation] Mackay A.D., Hintze K., Marchant E.D., Anderson J., Munk D.J., Hancock C.R. Doxorubicin Induced NF-kappaB Activity to Preserve Cell Viability and Modulate Iron Regulation in Skeletal Muscle Cells. Journal of Physiology and Biochemistry.

ABSTRACTS

- Mackay A.D., Louw M.G, Hancock C.R., Exercise, but not Metformin Prevents Loss of Muscle Function Due to Doxorubicin in Mice Using an in-situ Method. Experimental Biology. 2018.

VOLUNTEER WORK

- Americorps STEM Outreach Volunteer 2016
    - Developed STEM curriculum and lead in-class activities for students k-8 grades.