Exercise protects against doxorubicin-induced markers of autophagy signaling in skeletal muscle

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Submitted 8 April 2011; accepted in final form 20 July 2011

DOXORUBICIN (DOX) IS A HIGHLY effective antitumor agent, widely used in the treatment of solid tumors and hematological malignancies (6, 10, 12). However, DOX has been shown to induce deleterious effects in several tissues and organs, including skeletal and cardiac muscle (12, 49, 51). In this regard, numerous studies have attempted to identify the molecular mechanisms responsible for DOX toxicity in muscle (13–15). Nevertheless, a complete understanding of the mechanisms responsible for DOX-mediated muscle toxicity remains elusive.

A potential mechanism to account for DOX-mediated cellular toxicity is its ability to generate ROS (41, 46). Specifically, ring B of DOX’s four-ring anthracylene structure is an unsubstituted quinone that can readily form a redox cycle with appropriate electron donors and promote superoxide radical production at complex I within the electron transport chain within the mitochondria (5, 29). Importantly, our laboratory has shown that DOX administration results in increased mitochondrial ROS production and mitochondrial dysfunction (20). It follows that DOX’s generation of ROS by the mitochondria could lead to cellular oxidative modification, as well as the activation of several proteolytic pathways. Specifically, ROS can induce damage to calcium-handling proteins, increasing intracellular calcium, which can trigger autophagy by the activation of calmodulin-dependent kinase kinase and AMP-activated protein kinase (17, 53). In addition, changes in the rate of mitochondrial fission can also increase mitochondrial susceptibility to degradation via autophagy (37, 38).

Macroautophagy (hereafter referred to as autophagy) is a highly regulated dynamic process involving long-lived cytosolic proteins and organelle degradation through engulfment into double-membraned vesicles called autophagosomes. The autophagosomes then fuse with lysosomes to degrade their contents. Autophagy plays important roles in cell growth and development, organelle biogenesis and turnover, and controlling the precise balance between protein synthesis and degradation (53). Nonetheless, evolving evidence suggests that autophagy serves as a double-edge sword: on one hand, it functions by removing protein aggregates and damaged organelles as a prosurvival pathway maintaining energy homeostasis, while, on the other hand, intense enhancement of autophagy can lead to cell death (53). Indeed, autophagy can also cause damage to organelles, and increased degradation of mitochondria and/or endoplasmic reticulum could release compounds into the cytoplasm (e.g., calcium, cytochrome c) that can induce cell death (30, 31, 53). Currently, the effects of DOX administration on autophagic signaling in skeletal muscle remain unknown. Therefore, to investigate the role of DOX administration on autophagic signaling in skeletal muscle, we tested the hypothesis that DOX administration results in increased markers of autophagy. Our results support our hypothesis, as DOX administration did increase numerous markers of autophagy in skeletal muscle. These novel findings reveal that autophagy may play an important role in contributing to skeletal muscle toxicity resulting from DOX administration.

In regard to protecting skeletal muscles against the damaging effects of DOX, previous work has shown that exercise training is an effective intervention for protecting both heart and skeletal muscle from DOX-induced oxidative damage and activation of select proteases (e.g., calpain, caspase-3) (19, 20, 43). The mechanism responsible for exercise-induced protection against DOX-induced oxidative stress is unknown, but could be associated with the exercise-induced increase in muscle antioxidant capacity (24, 32, 36, 43, 50). Given that oxidative stress in cells can upregulate autophagy (2, 25), it is feasible that one mechanism by which exercise training pro-
tects skeletal muscle against DOX-induced toxicity is by the prevention of DOX-induced oxidative stress and the subsequent protection against the oxidative stress-mediated increases in the expression of autophagy genes. Therefore, our experiments also tested a second hypothesis, that exercise training before DOX administration would protect against DOX-induced increases in autophagic signaling in skeletal muscle. Our results reveal that exercise training before DOX administration was successful in preventing the DOX-induced increase in specific markers of autophagy in skeletal muscle.

METHODS

Experimental Design

Adult 6-mo-old male Sprague-Dawley rats were used in these experiments. The Animal Care and Use Committee of the University of Florida approved these experiments, and animals were housed at the University of Florida Animal Care facility. Animals were maintained on a 12:12-h reverse light-dark cycle and provided food and water ad libitum throughout the experimental period. Rats were randomly assigned to one of four experimental groups: 1) sedentary control (SED, n = 7); 2) exercise trained, no drug treatment (EXTR, n = 6); 3) sedentary, treated with DOX (SEDDOX, n = 5); and 4) exercise trained, treated with DOX (EXDOX, n = 6).

Animals assigned to exercise groups were habituated to running by increasing durations of treadmill exercise for 5 days (10, 20, 30, 40, 50 min/day on days 1–5). After 2 days of rest, animals then performed 5 consecutive days of treadmill exercise for 60 min/day at 30 m/min, 0% grade. This work rate represents an estimated 70% of maximum oxygen consumption (23). The EXDOX animals received DOX hydrochloride (20 mg/kg body wt ip) immediately after the final exercise bout, and animals were killed 24 h later. The SEDDOX animals received DOX hydrochloride (20 mg/kg body wt ip) 24 h before death. Saline was used as both the vehicle and the placebo treatment. These doses of DOX are human clinical doses of this drug that are pharmacologically scaled for use in rats (7, 18, 52).

At the conclusion of the experimental period, animals in each group were acutely anesthetized with pentobarbital sodium (60 mg/kg ip). After reaching a surgical plane of anesthesia, soleus muscle was removed and immediately frozen in liquid nitrogen and stored at −80°C for subsequent analyses. The animals were subsequently euthanized by removal of the heart. The soleus muscle was used as the representative skeletal muscle because the effects of DOX have been shown to be more pronounced in the soleus compared with other hindlimb skeletal muscles (11). Specifically, DOX-induced skeletal muscle impairments occur in both fast- and slow-twitch muscle fibers; however, slow-twitch muscle fibers appear to be predominantly affected, possibly due to defective intracellular calcium handling (11). Therefore, the current experiments have exclusively focused on the slow-twitch soleus muscle.

Histological Analyses

Hematoxylin and eosin staining. Sections from frozen diaphragm samples were cut at 10-μm using a cryostate (Shandon, Pittsburgh, PA) and stained for hematoxylin and eosin for analysis of soleus muscle damage.

Apoptosis. To determine the level of myonuclear apoptosis in the soleus muscle, we employed the terminal deoxynucleotidyl transferase nick-end labeling (TUNEL) method using a histochemical fluorescent detection kit (Roche Applied Scientific, Indianapolis, IN), as previously described by our group (26, 35).

Biochemical Analyses

Western blot analysis. Soleus muscles were homogenized 1:10 (wt/vol) in 5 mM Tris (pH 7.5) and 5 mM EDTA (pH 8.0) with a protease inhibitor cocktail (Sigma) and centrifuged at 1,500 g for 10 min at 4°C. The resulting supernatant (cytosolic) was collected, and protein content was assessed by the method of Bradford (Sigma). Proteins (40 μg) were then separated by polyacrylamide gel electrophoresis via 4–20% gradient polyacrylamide gels containing 0.1% sodium dodecyl sulfate for ~1 h at 200 V. After electrophoresis, the proteins were transferred to nitrocellulose membranes via the criterion system for 90 min at 65 V. Nonspecific sites were blocked for 2 h at room temperature in phosphate-buffered saline solution containing 0.05% Tween and 5% nonfat milk. Membranes were then incubated overnight at 4°C with primary antibodies directed against the proteins of interest. Beclin-1 (Cell Signaling) was probed as a measurement of the induction of autophagy. Atg7 (Cell Signaling), Atg4 (Biosensis), Atg12, Atg12-Atg5 (Cell Signaling), and LC3 (Cell Signaling) were measured as markers of autophagosome formation. Finally, the protein level of the lysosomal protease cathepsin L (Santa Cruz) was also measured. In addition, membranes were stripped and reprobed for α-tubulin (Santa Cruz), which served as a loading control to normalize equal protein loading and transfer. Following incubation with primary antibodies, membranes were washed extensively with phosphate-buffered saline-Tween and then incubated with secondary antibodies (Amersham Biosciences). After washing, a chemiluminescent system was used to detect labeled proteins (GE Healthcare). Membranes were developed using autoradiography film, and images of the film were captured and analyzed using the 440CF Kodak Imaging System (Kodak, New Haven, CT).

RNA isolation and cDNA synthesis. Total RNA was isolated from muscle tissue with TRIzol Reagent (Life Technologies, Carlsbad, CA), according to the manufacturer’s instructions. Total RNA and RNA content (μg/mg muscle) were evaluated by spectrophotometry. Total RNA (5 μg) was then reverse transcribed with the Superscript III First-Strand Synthesis System for RT-PCR (Life Technologies), using oligo(dT)20 primers and the protocol outlined by the manufacturer.

Real-time polymerase chain reaction. One microliter of cDNA was added to a 25-μl PCR reaction for real-time PCR using Taqman chemistry and the ABI Prism 7000 Sequence Detection system (ABI, Foster City, CA). Relative quantification of gene expression was performed using the comparative computed tomography method (ABI, User Bulletin no. 2). This method uses a single-calibrator sample for comparison of every unknown sample’s gene expression. ∆ΔCT [ΔCT(calibrator) − ΔCT(sample)] was then calculated for each sample, and relative quantification was calculated as 2ΔΔCT. β-Glucuronidase, a lysosomal glycoside hydrolase, was chosen as the reference gene for soleus muscle samples based on previous work showing unchanged expression with our experimental manipulations (8, 9, 44).

Fivefold dilution curves were assayed on selected samples to confirm the validity of this quantification method for each gene. Beclin-1, Atg4, Atg7, Atg12, LC3, cathepsin B, cathepsin D, and cathepsin L mRNA transcripts were assayed using predesigned rat primer and probe sequences commercially available from Applied Biosystems (Assays-on-Demand).

Data Analyses

Data are presented as means ± SE. Comparisons between groups for each dependent variable were made by a one-way ANOVA, and, when appropriate, Tukey honestly significantly different tests were performed post hoc. Significance was established at P < 0.05.

RESULTS

All animals in the exercise groups completed the exercise protocol without incident, with no noticeable differences in exercise performance and without apparent complications.
DOX Administration Increases Muscle Damage

Hematoxylin and eosin staining was performed histologically to visualize the muscle fiber damage that occurs in response to DOX treatment. Our histological slides reveal that the SEDDOX animals exhibited damaged myofiber ultrastructure in the soleus muscle compared with all other groups (Fig. 1). Importantly, exercise training before DOX administration prevented this damage to the muscle. In addition, TUNEL staining was used as a biomarker of myonuclear apoptosis. Our results reveal that, compared with all other groups, the soleus muscles from the SEDDOX animals had a significantly higher number of TUNEL-positive nuclei, which is indicative of a higher rate of myonuclear apoptosis (Fig. 2).

DOX Administration Increases Markers of Autophagosome Initiation

Activation of the autophagic signaling pathway begins with the formation of the autophagosome, which involves a series of steps. The induction step of autophagy involves formation of a small isolation membrane (phagophore) to which necessary proteins will be recruited to form the mature autophagosome. This process is regulated by a system of autophagy proteins (Atg proteins). Beclin-1 is part of a phosphoinositide 3-kinase complex and seems to play an important role during the initial steps of autophagosome formation by mediating the localization of other Atg proteins to the isolation membrane (16, 21). Beclin-1 mRNA levels in soleus muscle were elevated \( \text{(}\text{P} < 0.05) \) in the SEDDOX group compared with all other groups (Fig. 3A). Beclin-1 protein levels were also higher in the soleus of the SEDDOX group compared with the SED group \( (P < 0.05) \) (Fig. 3B). Importantly, note that exercise training protected the soleus muscle against DOX-induced expression of Beclin-1.

DOX Administration Increases Markers of Autophagosome Formation

Synthesis of the autophagosome requires the interaction of many Atgs. Specifically, Atg12, Atg7, Atg4, and LC3 all play important roles in elongation and formation of the autophagosome. Our results show that Atg12 mRNA expression in the soleus muscle was significantly increased \( (P < 0.05) \) in the SEDDOX group compared with all other groups (Fig. 4A). In addition, there was a significant increase \( (P < 0.05) \) in soleus Atg12 protein levels in the SEDDOX group compared with SED and EXTR. Also, the levels of the Atg12-Atg5 complex were significantly increased \( (P < 0.05) \) in soleus muscle of the SEDDOX group compared with SED and EXTR groups, but exercise training before DOX treatment did not attenuate this increase (Fig. 4, B and C). Soleus mRNA expression and protein expression of Atg4 did not differ between experimental groups (Fig. 5). Similarly, soleus muscle Atg7 mRNA levels did not differ between experimental groups; however, muscle protein levels of Atg7 were significantly increased \( (P < 0.05) \) in the SEDDOX animals compared with SEDs (Fig. 6). Finally, LC3 mRNA expression in the soleus muscle was increased \( (P < 0.05) \) in the SEDDOX group compared with all other groups. In addition, the ratio of LC3II to LC3I (LC3II/LC3I) was also measured as a marker of LC3 cleavage, which

**Fig. 1.** Representative hematoxylin and eosin staining of soleus muscle: 1, sedentary control (SED); 2, exercise trained, no treatment (EXTR); 3, sedentary, treated with doxorubicin (SEDDOX); and 4, exercise trained, treated with doxorubicin (EXDOX).
is an indicator of LC3 activation, and, therefore, the LC3II/LC3I is often regarded as a biomarker of increased autophagy in cells (47). In the SEDDOX group, the LC3II/LC3I was increased ($P < 0.05$) in the soleus compared with all other groups (Fig. 7).

**DOX Administration Increases Lysosomal Proteases**

Cathepsins B, D, and L are all ubiquitously expressed lysosomal proteases that are charged with the removal of both organelles and nonmyofibril cytosolic protein aggregates. In the soleus, there was no change in the mRNA expression of either cathepsin B or D; however, there was a significant increase ($P < 0.05$) in cathepsin L mRNA expression in the SEDDOX group compared with all other groups. Furthermore, compared with all other groups, there was a significant increase ($P < 0.05$) in the protein levels of cathepsin L in the soleus muscle of the SEDDOX group, and this DOX-induced increase in cathepsin L protein levels was attenuated in the group of animals performing endurance exercise before DOX treatment; however, the EXDOX group still remained significantly greater than the SED group (Fig. 8).

**DISCUSSION**

**Overview of Principal Findings**

These experiments provide new and important information regarding the effects of DOX administration on skeletal muscle. First, our findings support the hypothesis that DOX treatment increases the expression of numerous markers of autophagy in locomotor skeletal muscle. Furthermore, our results reveal that exercise training before DOX treatment can attenuate DOX-induced increases in autophagic signaling in skeletal muscle. A detailed discussion of these findings follows.

**DOX Administration Promotes Skeletal Muscle Damage**

Muscle weakness and fatigue are common symptoms in patients receiving DOX treatment (45). Consistent with
these reports in humans, our data indicate that DOX administration resulted in injury to the rat soleus muscle, as indicated by both the damage to muscle structure (Fig. 1) and the increased number of TUNEL-positive nuclei in muscle fibers (Fig. 2). Collectively, these histological findings indicate that systemic delivery of DOX results in alterations of normal muscle structure. Furthermore, the observation that a higher number of TUNEL-positive nuclei

Fig. 3. Beclin-1 in soleus muscle was analyzed as a marker of the initiation of autophagosome formation. Representative Western blots are shown at top. Values are mean fold differences (mRNA) and mean percent change (protein) from SED ± SE. A: soleus Beclin-1 mRNA expression. §SEDDOX significantly different vs. all groups. B: soleus Beclin-1 protein expression. *SEDDOX significantly different vs. SED.

Fig. 4. Atg12 in soleus muscle was analyzed as a marker of the elongation and formation of the autophagosome. Representative Western blots are shown in the middle. Values are mean fold differences (mRNA) and mean percentage change (protein) from SED ± SE. A: soleus Atg12 mRNA expression. §SEDDOX significantly different vs. all groups. B: soleus Atg12 protein expression. §SEDDOX significantly different vs. SED and EXTR. C: soleus Atg12-Atg5 protein expression. §SEDDOX significantly different vs. SED and EXTR. ||EXDOX significantly different vs. SED and EXTR.
exist in muscle fibers of DOX-treated animals suggests that DOX promotes myonuclear apoptosis, leading to a loss of nuclei from the soleus muscle.

**DOX Administration Increases Markers of Autophagy**

Autophagy is a highly regulated lysosomal pathway for the degradation of nonmyofibril cytosolic proteins and organelles (4, 39). Although basal autophagy is important for maintaining cell survival by recycling old and damaged organelles and cytosolic proteins, high levels of autophagy can induce pathological results, such as apoptosis-mediated cell death and cellular atrophy (16, 39, 53). The induction of autophagy occurs by activation of the Atg1 complex, which is followed by a cascade of reactions resulting in autophagosome formation. For example, the mammalian homologue of yeast Atg6, Beclin-1, associates with numerous Atg regulatory proteins and is required for induction of autophagy (i.e., formation of the pre-autophagosome structure) (3, 4). Our results indicate that DOX administration does indeed cause an increase in Beclin-1 mRNA and protein abundance in skeletal muscle.

Furthermore, the interaction of other key Atg proteins is required for autophagosome formation to occur. Specifically, Atg12 and Atg5 form a complex that requires Atg7 and Atg10 to covalently conjugate Atg12 to Atg5 (16, 40). The Atg12-Atg5 complex interacts noncovalently with Atg16, and this complex then initiates the elongation of the membrane by recruiting LC3 after it has been cleaved by the cysteine protease Atg4 (1, 16, 22, 27, 28). Therefore, we measured Atg12, Atg7, Atg4, and LC3 as markers of autophagosome formation. Our findings reveal that Atg12, the Atg12-Atg5 complex, Atg7, and LC3 are all significantly

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**Fig. 5.** Atg4 in soleus muscle was analyzed as a marker of the elongation and formation of the autophagosome. Representative Western blots are shown at top. Values are mean fold differences (mRNA) and mean percentage change (protein) from SED ± SE. A: soleus Atg4 mRNA expression. B: soleus Atg4 protein expression.

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**Fig. 6.** Atg7 in soleus muscle was analyzed as a marker of the elongation and formation of the autophagosome. Representative Western blots are shown at top. Values are mean fold differences (mRNA) and mean percentage change (protein) from SED ± SE. A: soleus Atg7 mRNA expression. B: soleus Atg7 protein expression. *SED/DOX significantly different vs. SED.
increased following DOX administration compared with the SED group. Therefore, our data indicate that DOX administration increases numerous markers of autophagosome formation in skeletal muscle that could promote increased protein breakdown by the lysosomal system of proteases.

DOX Administration Increases Expression of Lysosomal Proteases

During elongation of the autophagosome, the membrane surrounds proteins and organelles in the cytosol to be seques-

Fig. 7. LC3 in soleus muscle was analyzed as a marker of the elongation and formation of the autophagosome. Representative Western blots are shown at top. Values are mean fold differences (mRNA) and mean percentage change (protein) from SED ± SE. A: soleus LC3 mRNA expression. §SED/DOX significantly different vs. all groups. B: soleus LC3 protein expression. §SED/DOX significantly different vs. all groups.

Fig. 8. Cathepsins B, D, and L were analyzed as markers of increased degradation by the lysosomal proteolytic system. Representative Western blots are shown in the middle. Values are mean fold differences (mRNA) and mean percentage change (protein) from SED ± SE. A: soleus cathepsin B mRNA expression. B: soleus cathepsin D mRNA expression. C: soleus cathepsin L mRNA expression. §SED/DOX significantly different vs. all groups. *EX/DOX significantly different vs. SED and EXTR. D: soleus cathepsin L protein expression. §SED/DOX significantly different vs. all groups. *EX/DOX significantly different vs. SED.
tered. The mature autophagosome can fuse with the lysosome to form the autolysosome where the contents of the autophago-
some are degraded (16, 22, 28). In this regard, numerous lysosomal proteases exist (e.g., cathepsins B, D, and L), and each plays a vital role in Atg-related protein breakdown (4, 39). Specifically, the cathepsins B, D, and L function as ubiquitous lysosomal proteases and are expressed in many tissues (4, 48). During conditions of muscle damage, higher levels of expres-
sion of cathepsins are often observed in tissues presenting high rates of protein turnover. The present data indicate that there is an increase in cathepsin L expression (i.e., elevated mRNA and protein abundance) in the soleus muscle following DOX admin-
istration. In contrast, DOX administration did not increase muscle mRNA levels of cathepsin B and D. Among all lysosomal proteases that are implicated in proteolysis, cathepsin L is recognized as a general marker of muscle protein break-
down, and our data confirm that cathepsin L mRNA and protein is overexpressed in the DOX-induced pathological condition (3, 4, 48). Therefore, collectively, our results are consistent with the concept that the autophagic/lysosomal sys-
tem is upregulated in skeletal muscle following DOX admin-
istration.

Exercise Protects Skeletal Muscle Against DOX-induced Expression of Autophagy Genes

It is established that endurance exercise increases the ex-
pression of several key antioxidant enzymes (e.g., superoxide
dismutase, glutathione peroxidase, etc.) in skeletal muscles, and that these muscles are protected against subsequent oxidative stress (24, 32, 36, 43, 50). Specifically, prior work from our group has shown that exercise training can protect skeletal muscle against DOX-induced oxidative damage, as well as reduce activation of the proteases calpain and caspase-3 in both cardiac and skeletal muscle (20, 33, 34, 42, 43). In addition to the calpain and caspase-3 proteolytic pathways, the autophagic/
lysosomal proteolytic system has been shown to increase protein breakdown and, therefore, promote muscle atrophy (20, 39). Therefore, we determined whether exercise training can also shield against DOX-induced increases in the expression of autophagy genes. Our data reveal that exercise training before DOX administration protects skeletal muscle from increased muscle damage, as well as increased autophagic signaling. Specifically, when animals were exercised before administration of DOX, the exercise intervention protected the soleus muscle against DOX-induced expression of numerous au-
tophagy-related genes and also prevented the DOX-induced increase in the LCII/LCI.

Conclusions

In summary, our study provides the first evidence that DOX administration is associated with an increase in markers of autophagy in skeletal muscle. In addition, we also showed that exercise training before DOX administration can attenuate many of the DOX-induced changes to autophagy markers in skeletal muscle. Collectively, we interpret these findings as an indication that exercise training can protect skeletal muscles against DOX-induced increases in autophagy. The present work provides the experimental rationale for future studies to determine whether direct inhibition of autophagy before DOX administration can protect skeletal muscle against DOX-induced toxicity.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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