Effects of short-term endurance exercise training on acute doxorubicin-induced FoxO transcription in cardiac and skeletal muscle

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Submitted 4 March 2014; accepted in final form 17 June 2014

Kavazis AN, Smuder AJ, Powers SK. Effects of short-term endurance exercise training on acute doxorubicin-induced FoxO transcription in cardiac and skeletal muscle. J Appl Physiol 117: 223–230, 2014. First published June 19, 2014; doi:10.1152/japplphysiol.00210.2014.—Doxorubicin (DOX) is a potent antitumor agent used in cancer treatment. Unfortunately, DOX can induce myopathy in both cardiac and skeletal muscle, which limits its clinical use. Importantly, exercise training has been shown to protect against DOX-mediated cardiac and skeletal muscle myopathy. However, the mechanisms responsible for this exercise-induced muscle protection remain elusive. These experiments tested the hypothesis that short-term exercise training protects against acute DOX-induced muscle toxicity, in part, due to decreased forkhead-box O (FoxO) transcription of atrophy genes. Rats (n = 6 per group) were assigned to sedentary or endurance exercise-trained groups and paired with either placebo or DOX treatment. Gene expression and protein abundance were measured in both cardiac and skeletal muscles to determine the impact of DOX and exercise on FoxO gene targets. Our data demonstrate that DOX administration amplified FoxO1 and FoxO3 mRNA expression and increased transcription of FoxO target genes [i.e., atrogin-1/muscle atrophy F-box (MaFbx), muscle ring finger-1 (MuRF-1), and BCL2/adenovirus E1B 19 kDa protein-interacting protein 3 (BNIP3)] in heart and soleus muscles. Importantly, exercise training protected against DOX-induced increases of FoxO1 and MuRF-1 in cardiac muscle and also prevented the rise of FoxO3, MuRF-1, and BNIP3 in soleus muscle. Furthermore, our results indicate that exercise increased peroxisome proliferator-activated receptor-gamma coactivator-1 alpha (PGC-1α) in both the heart and soleus muscles. This is important because increased PGC-1α expression is known to suppress FoxO activity resulting in reduced expression of FoxO target genes. Together, these results are consistent with the hypothesis that exercise training protects against DOX-induced myopathy in both heart (FoxO1 and MuRF-1) and skeletal muscles (FoxO3, MuRF-1, and BNIP3).

Several mechanisms have been proposed to explain DOX-induced myotoxicity. However, the primary mechanism responsible for DOX-induced muscle myopathy is predicted to be increased production of reactive oxygen species (ROS) due to aberrant electron cycling in the mitochondrial electron transport chain (47). Several studies have demonstrated that physiological doses of DOX induce a significant elevation of mitochondrial ROS production in cardiac myocytes (6, 25, 41) and skeletal muscle fibers (19, 22). In this regard, it has been reported that DOX stimulates ROS generation by mitochondrial NADH dehydrogenase leading to the generation of a free radical cascade with potent oxidizing potential (12, 15).

Increased muscular ROS production has been reported to play an important role during both beneficial and pathological muscle adaptations (38). Whereas low levels of muscle ROS production are involved in cell signaling and muscle adaptation to exercise training, high levels of ROS production can contribute to muscle myopathy (38). In this case, elevated levels of ROS production could activate the forkhead-box O (FoxO) signaling pathway (14). This amplified FoxO signaling is important because increased FoxO nuclear translocation results in amplified transcription of FoxO target genes [e.g., atrogin-1 (also called muscle atrophy F-box; MaFbx), muscle ring finger-1 (MuRF-1), and BCL2/adenovirus E1B 19 kDa protein-interacting protein 3 (BNIP3)] involved in muscle catabolism (7, 11, 23). Regulation of FoxO transcriptional activity is complex, but evidence indicates that increased peroxisome proliferator-activated receptor-gamma coactivator-1 alpha (PGC-1α) expression can protect myocytes by inhibiting FoxO transcriptional activity (44).

In general, the transcriptional coactivator PGC-1α targets promoters by interacting directly with numerous transcription factors including FoxO. Furthermore, PGC-1α functions as a major regulator of mitochondrial biogenesis in cardiac and skeletal muscles (2, 30, 55). PGC-1α expression in the heart and skeletal muscle can be induced by a variety of stimuli including endurance exercise training (4, 5). In this regard, previous work has shown that endurance exercise training is an effective intervention for protecting both heart and skeletal muscle from DOX-induced damage (3, 9, 25, 54). Nonetheless, the specific mechanisms responsible for exercise-induced protection against DOX-induced cardiac and skeletal muscle atrophy remain unknown.

Therefore, these experiments tested the hypothesis that short-term exercise training protects against acute DOX-induced muscle toxicity, in part, due to decreased FoxO transcription of atrophy genes. Our data support this hypothesis; and also suggest that the exercise-induced upregulation of PGC-1α in cardiac and skeletal muscle is a possible upstream mechanism that could be respon-
sible for the observation that exercise training suppresses DOX-induced FoxO gene expression.

METHODS

Experimental design. Adult 6-mo-old male Sprague-Dawley rats were used in these experiments. 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 (n = 6 per group): 1) sedentary control–placebo treated (SED PLA); 2) short-term exercise training–placebo treated (EXE PLA); 3) sedentary–treated with doxorubicin (SED DOX); and 4) short-term exercise training–treated with doxorubicin (EXE DOX). The University of Florida Institutional Animal Care and Use Committee approved these experiments.

Exercise training. Animals assigned to exercise groups were habituated to running by increasing durations of treadmill exercise on 5 consecutive days (10, 20, 30, 40, and 50 min/day on days 1–5, respectively). Following 2 days of rest, animals then performed 5 consecutive days of treadmill exercise for 60 min/day at 30 m/min, 0% grade. This intensity has previously been demonstrated to be ~70% of the animals’ VO2 max (28).

Doxorubicin administration. The EXE DOX animals received DOX hydrochloride (20 mg/kg body wt ip) immediately after the final exercise bout and animals were killed 24 h later. The SED DOX animals received DOX hydrochloride (20 mg/kg body wt ip) 24 h prior to death. Saline was used as both the vehicle and the placebo treatment. These doses of DOX are comparable to human clinical trials. Significant main effects for drug were observed for heart FOXO3 mRNA (B) where #DOX increased (P < 0.05) these levels regardless of activity level. Significant main effects for drug were observed for soleus FOXO3 mRNA (D) where #DOX increased (P < 0.05) these levels only in the SED animals. Data are mean ± SD and fold difference is expressed relative to SED PLA. DOX, doxorubicin; EXE, short-term exercise training; FoxO, forkhead-box O; PLA, placebo; SED, sedentary.

Fig. 1. FoxO1 and FoxO3 mRNA levels in heart and soleus. A significant interaction effect was present for heart FoxO1 mRNA (A) where *SED DOX was higher (P < 0.05) compared with EXE DOX. Significant main effects for drug were observed for heart FOXO3 mRNA (B) and soleus FoxO1 mRNA (C) where #DOX increased (P < 0.05) these levels regardless of activity level. Significant main effects for drug were observed for soleus FoxO3 mRNA (D) where #DOX increased (P < 0.05) these levels only in the SED animals. Data are mean ± SD and fold difference is expressed relative to SED PLA. DOX, doxorubicin; EXE, short-term exercise training; FoxO, forkhead-box O; PLA, placebo; SED, sedentary.
protease inhibitor cocktail (Sigma) and centrifuged at 1,500 g for 10 min at 4°C. The resulting supernatant was collected and protein content was assessed by the Bradford method (Sigma). Proteins 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 for 90 min at 65 V. Nonspecific sites were blocked for 2 h at room temperature in phosphate-buffered saline (PBS) 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. The protein abundance of AMP-activated protein kinase (AMPK) and phosphorylated AMPK (pAMPK) (Cell Signaling), mitofusin-2 (Mfn2; Sigma), and PGC-1α (Calbiochem) were determined in both heart and skeletal muscle samples. In addition, membranes were stripped and reprobed for α-tubulin (Santa Cruz Biotechnology), which served as a loading control to normalize protein loading and transfer. Following incubation with primary antibodies, membranes were washed extensively with PBS-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).

**RESULTS**

DOX results in increased transcription of select FoxO target genes. DOX increases the activity of several proteolytic systems in both cardiac and skeletal muscles (27, 52–54). Specifically, activation of both the ubiquitin-proteasome pathway and

**Fig. 2.** Atrogin-1/MaFbx, MuRF-1, and BNIP3 mRNA levels in heart and soleus. Significant main effects for drug were observed for heart atrogin-1/MaFbx mRNA (A) where #DOX increased (P < 0.05) mRNA expression regardless of activity level. A significant interaction effect was present for heart MuRF-1 mRNA (B) and soleus MuRF-1 mRNA (E) where @SED DOX was higher (P < 0.05) compared with both SED PLA and EXE DOX. A significant interaction effect was present for heart BNIP3 mRNA (C) and soleus atrogin-1/MaFbx mRNA (D) where @DOX increased (P < 0.05) the expression of these genes regardless of activity level. Significant main effects for drug and activity were observed for soleus BNIP3 mRNA (F) where SED DOX was higher (P < 0.05) than #SED PLA and †EXE DOX. Data are mean ± SD and fold difference is expressed relative to SED PLA. MaFbx, muscle atrophy F-box (also called atrogin-1); MuRF-1, muscle ring finger-1; BNIP3, BCL2/adenovirus E1B 19 kDa protein-interacting protein 3.
the autophagy/lysosomal system play significant roles in DOX-induced myotoxicity (40, 52). In this regard, activation of the FoxO family of transcription factors has been attributed to the activation of these important proteolytic pathways in cardiac and skeletal muscle through increased transcription of specific atrophy-related genes (i.e., atrogin-1/MaFbx, MuRF-1, and BNIP3) (7, 8, 23, 33, 35). Therefore, we investigated how the expression of these genes is affected in the heart and soleus muscles following acute DOX administration in sedentary and short-term exercise-trained animals. Our data demonstrate that short-term exercise training attenuated DOX-induced increases in cardiac FoxO1 mRNA and soleus FoxO3 mRNA (Fig. 1). However, cardiac FoxO3 mRNA and soleus FoxO1 mRNA remained elevated in exercise-trained animals treated with DOX (Fig. 1).

Furthermore, our data show that atrogin-1/MaFbx, MuRF-1, and BNIP3 mRNA increased in heart and soleus muscles in sedentary animals following DOX treatment (Fig. 2). Interestingly, exercise training attenuated the DOX-induced increase in MuRF-1 mRNA in both heart and soleus muscles, but...
atrogin-1/MaFbx mRNA remained elevated in heart and soleus muscles of exercise-trained animals treated with DOX (Fig. 2). Also, the DOX-induced BNIP3 mRNA increase was attenuated in the soleus, but not in the heart of exercise-trained animals (Fig. 2).

**Short-term exercise training induces the upregulation of PGC-1α and pAMPK/AMPK.** It is established that repetitive muscle contractions (i.e., endurance exercise training) elevates the AMP:ATP ratio in working muscles, which activates the AMPK pathway (13). Increased AMPK activity can induce transcriptional activation of PGC-1α (13). Indeed, our exercise training protocol increased PGC-1α mRNA and protein expression in cardiac and soleus muscles (Fig. 3). Additionally, our results indicate that compared with sedentary animals treated with DOX, exercise-trained animals that were treated with DOX possessed higher protein levels of PGC-1α in both the heart and soleus muscles (Fig. 3).

Mfn2 is a target of PGC-1α that has been shown to augment mitochondrial fusion (57). Specifically, Mfn2 facilitates the merging of adjacent mitochondrial membranes leading to mitochondrial fusion (36). Our data indicate that Mfn2 was increased in the heart and soleus muscles of exercise-trained animals (Fig. 4). Importantly, treatment of animals with DOX did not change the protein levels of Mfn2 in cardiac or soleus muscles in either sedentary or exercise trained animals (Fig. 4).

**Myostatin gene expression is altered in heart and soleus.** Myostatin is a potent negative regulator of muscle mass and can increase atrogin-1/MaFbx expression via FoxO activation in an IGF1-PI3K-AKT-dependent manner (34). Our data revealed that DOX administration increased myostatin mRNA in the heart of sedentary animals, but not in exercise-trained animals (Fig. 5). In the soleus muscle, sedentary animals treated with DOX contained higher myostatin mRNA compared with exercise trained animals treated with DOX (Fig. 5).

**DISCUSSION**

**Overview of principal findings.** These experiments provide new and important information regarding the potential mechanisms responsible for exercise-induced protection against DOX-induced cardiac and skeletal muscle wasting. Our findings show that short-term endurance exercise training prevents acute DOX-induced increases in the expression of MuRF-1 mRNA in both heart and soleus muscles. Our data also reveal that exercise training increases the expression of PGC-1α in both heart and soleus muscles. This is potentially important...
because increased PGC-1α can play an important role in mediating exercise-induced protection against cardiac and skeletal muscle wasting because this transcriptional coactivator can prevent muscle atrophy by suppressing FoxO action and gene transcription (44). Figure 6 summarizes the potential pathways of DOX-induced myopathy and how exercise training can protect against this myopathy. A detailed discussion of these findings follows.

**DOX activates the FoxO pathway of proteolysis.** The FoxO family of transcription factors contributes to the regulation of muscle mass through the regulation of muscle atrophy-related genes (i.e., MuRF-1, atrogin-1/MaFbx, and BNIP3). Indeed, muscle-specific overexpression of FoxO1, FoxO3, or both is sufficient to cause muscle atrophy in vivo (45, 50). Therefore we investigated the effects of DOX administration on the activation of FoxO transcription.

During conditions of muscle wasting, FoxO is dephosphorylated and translocated to the nucleus, which leads to the induction of atrophy genes including atrogin-1/MaFbx and MuRF1. Atrogin-1/MaFbx and MuRF-1 are two muscle-specific E3 ligases that play an instrumental role in targeting proteins for breakdown by the proteasome (7, 23, 39). In regard to skeletal muscle atrophy, studies involving several experimental models of muscle wasting reveal that prevention of FoxO-specific transcription of atrogin-1/MaFbx and MuRF-1 can impede muscle atrophy (29, 43). Specifically, our data reveal that short-term endurance exercise training prevents DOX-induced increases in the expression of MuRF-1 mRNA in both heart and soleus muscles. In contrast, our results indicate that exercise training does not protect heart or skeletal muscle against DOX-induced increases in atrogin-1/MaFbx mRNA. This finding raises an interesting question as to why these two muscle atrophy genes respond differently to exercise training. The mechanisms to explain this finding are unclear and remain an interesting area for future work.

In addition to contributing to proteasome-specific muscle atrophy, FoxO activation can also increase the transcription of numerous proteins important for autophagy (42). In this regard, we have previously shown that biomarkers of autophagy are increased in both cardiac and skeletal muscle following DOX administration (53). Thus we measured the expression of BNIP3, which is a FoxO target gene (31). Specifically, BNIP3 is a proapoptotic BH3-only protein primarily located in the mitochondria that promotes mitochondrial dysfunction/apoptosis and is a potent inducer of autophagy (24). In reference to autophagy, the autophagosome initiation protein Beclin-1 forms a complex with Bcl-2 in cells during homeostatic conditions. During stressful conditions in the cell, Bcl-2 releases Beclin-1, which then binds to BNIP3 to promote autophagy. Therefore, increased transcription of BNIP3 can promote both apoptosis and autophagy in cardiac and skeletal muscle fibers. Our data establish that DOX administration increased the expression of BNIP3 in both cardiac and skeletal muscles, which is consistent with previous evidence indicating DOX induces autophagy and apoptosis in both cardiac and skeletal muscles (27, 52, 54). Also, note that short-term exercise training significantly decreased the DOX-induced increase of BNIP3 mRNA in soleus. In contrast, short-term exercise training did not attenuate the DOX-induced increase of cardiac BNIP3 mRNA.

Our results show that DOX treatment increases FoxO1 and FoxO3 transcription in cardiac and skeletal muscle. Importantly, short-term exercise training significantly reduced the DOX-induced increase in FoxO1 mRNA in the heart and FoxO3 mRNA in soleus. This observation reveals that heart and soleus muscles were differentially affected by exercise training followed by DOX treatment and future experiments should investigate the mechanisms responsible for this differential response. It appears feasible that these exercise-induced decreases in DOX-mediated FoxO mRNA and the expression of FoxO target genes could result from exercise-induced increases in PGC-1α. Indeed, transgenic overexpression of PGC-1α in skeletal muscle results in a reduction of atrogin-1/MaFbx and MuRF1 expression in denervated muscles due to the reduced capacity of FoxO3 to bind to the promoter of these genes (44). Therefore, exercise-induced increases in PGC-1α could contribute to exercise-induced protection against FoxO catabolic signaling.

**Exercise increases mitochondrial biogenesis and expression of mitochondrial fusion proteins in both cardiac and skeletal muscle.** The transcriptional coactivator PGC-1α is a functional activator of the peroxisome proliferator-activated receptor-γ (PPAR-γ) and plays a critical role in numerous aspects of metabolism. Reports indicate that exercise-induced adaptations

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**Fig. 6. Potential mechanisms of exercise-induced protection from DOX-induced myopathy.** DOX can increase expression of FoxO1, or FoxO3, or both leading to increased transcription of target proteolytic genes (e.g., atrogin-1/MaFbx, MuRF-1, BNIP3) and myostatin resulting in myopathy. Exercise training upregulates PGC-1α, which can inhibit FoxO1/FoxO3 DNA binding and can lead to increased Mn2 and improved mitochondria function that can protect from DOX-induced myopathy. In our experiments, exercise training prior to DOX treatment protected against DOX-induced decreases of FoxO1, MuRF-1, and myostatin in cardiac muscle and FoxO3, MuRF-1, BNIP3, and myostatin in soleus muscle, but did not protect against atrogin-1/MaFbx in either heart or soleus muscles. See text for additional details.
to PGC-1α expression are responsible for stimulating mitochondrial biogenesis in muscle cells (2, 30, 55). Importantly, our data support the observation that exercise can stimulate increases in PGC-1α expression in both cardiac and skeletal muscles of exercise-trained animals. This is potentially important because we and others have demonstrated that DOX administration results in mitochondrial damage in both cardiac and skeletal muscles (3, 19, 27). Specifically, DOX-induced mitochondrial damage leads to inhibition of the mitochondrial electron transport system and increased ROS emission. As a result, the increased emission of ROS can lead to further inhibition of the electron transport system and redox modifications of cell macromolecules (e.g., proteins, lipids, DNA) with detrimental downstream effects on whole organ function (19). Importantly, increased ROS can deplete vital redox buffering systems, resulting in an inability of the antioxidant buffering system to maintain a proper cellular redox environment.

In addition to promoting mitochondrial biogenesis, PGC-1α is also involved in the control of mitochondrial fission and fusion. In this regard, the fusion proteins Mfn1 and Mfn2 promote mitochondrial elongation and fusion in cultured cells (37). Moreover, Mfn2 expression is driven by a PGC-1α coactivation with estrogen-related receptor α (ERRα), which is also essential for the expression of genes involved in mitochondrial biogenesis and select antioxidant systems (49). Independent of its role in mitochondrial fusion, Mfn2 may also play a role in the regulation of oxidative phosphorylation (37). Our data indicate that Mfn2 expression is increased following exercise training in both cardiac and skeletal muscles, and it is feasible that increased levels of Mfn2 in cardiac and skeletal muscle contribute to the exercise-induced protection against DOX-mediated myopathy. Nonetheless, whether or not increased Mfn2 is required to achieve exercise-induced protection against DOX-induced myopathy is unclear and warrants additional study.

Cardiac and skeletal muscle myostatin expression following DOX administration. In addition to its role in proteasome and autophagy activation, FoxO signaling has also been reported to regulate myostatin expression and signaling (1). This is important because myostatin is part of the transforming growth factor-β (TGF-β) superfamily of secreted growth factors and is a negative regulator of muscle growth (16). Myostatin signaling can promote the expression of atrogin-1/MalFbx-1 and induce muscle atrophy (46). Therefore, we investigated the effect of DOX treatment and exercise on both cardiac and skeletal muscle levels of myostatin mRNA. DOX treatment increased cardiac myostatin mRNA levels in sedentary animals. In the soleus muscle, short-term exercise training prevented the DOX-induced increases in myostatin mRNA. These findings suggest that the myostatin signaling pathway could contribute to DOX-induced myotoxicity.

Summary and conclusions. DOX is a potent anticancer drug but its clinical usefulness is limited because of cardiotoxicity that occurs due to several interrelated factors including increased radical production, calcium overload, mitochondrial damage, and protease activation. In addition to cardiotoxicity, DOX administration results in skeletal muscle myopathy. Endurance exercise training prior to DOX administration can protect both cardiac and skeletal muscles against DOX-induced toxicity. Importantly, our findings suggest that the beneficial effects of short-term exercise training may be due to the downregulation of FoxO transcriptional activity (FoxO1 in cardiac muscle; FoxO3 in soleus) and the repression of FoxO-target genes (MuRF-1 in cardiac muscle; MuRF-1 and BNIP3 in soleus), possibly as a result of increased PGC-1α expression. Collectively, these new findings provide the basis for future translational studies to develop therapeutic countermeasures to retard DOX-induced myotoxicity.

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

AUTHOR CONTRIBUTIONS

REFERENCES


