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Original Article

Comparative changes in antioxidant enzymes and oxidative stress in cardiac, fast twitch and slow twitch skeletal muscles following endurance exercise training

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Abstract: The aim of this study was to evaluate exercise-induced transcriptional and protein responses of heart, soleus (slow oxidative), and plantaris (fast glycolytic) muscle in response to ten days of endurance exercise training. Four-month old female Sprague-Dawley rats were assigned to either a sedentary (SED) or endurance exercise-training (EXE) group (n=8 per group). The heart, plantaris, and soleus were excised and used for biochemical analyses. Our results show that heart and plantaris from EXE animals had higher protein levels of superoxide dismutase 2 (SOD2) compared to SED animals ($P<0.05$). Also, the protein levels of catalase were higher in plantaris of EXE animals compared to SED animals ($P<0.05$). No significant differences existed for 4 hydroxynonenal (4HNE) conjugated proteins (index of oxidative damage) in the three tissues between SED and EXE animals. mRNA levels of peroxisome proliferator-activated receptor gamma coactivator 1 alpha (PGC-1α) were higher in plantaris of EXE animals compared to SED animals ($P<0.05$), and mRNA levels of estrogen-related receptor alpha (ERRα) were lower in the heart of EXE animals compared to SED animals. In conclusion, heart and plantaris are responsive to ten days of treadmill training, while greater exercise intensities or durations may be needed to elicit alterations in soleus.

Keywords: Muscle, antioxidants, exercise

Introduction

Physical activity results in profound physiological adaptations in the body, which enhance functional capacity and improve overall health [1]. While physical inactivity is indicated in the causation of many chronic conditions (i.e. type 2 diabetes, osteoporosis, congestive heart failure etc.), increased physical activity can successfully mitigate many diseases [2, 3]. Indeed, both cardiac and skeletal muscles exhibit remarkable alterations to exercise and display exceptional plasticity to changes in activity level. Specifically, exercise provides a signaling cascade that results in a multiplicative response in transcriptional and translational outcomes, mediated largely by the effects of increased muscle contraction. Importantly, an acute bout of exercise results in transient changes in gene transcription, while repeated exercise induces changes in protein expression and muscle function. Unfortunately, many disease conditions preclude exercise training and cessation of exercise results in the rapid reversal of training adaptations. Therefore, it is important to understand how exercise training alters gene transcription to determine key proteins responsible for the cytoprotective effects of exercise.

In this regard, endurance training increases intramuscular mitochondrial content [4] and endogenous antioxidants [5] through various transcriptional pathways. These adaptations are necessary to protect the body from damaging events that occur to cell structures during exercise, and enable the muscles to perform at higher metabolic demands. However, the physiological adaptations to short-term endurance exercise training have not been well described across the various muscle types. Therefore, these experiments determined the differential

alterations in gene expression of mitochondrial function related mRNAs and antioxidant proteins between heart, plantaris, and soleus muscles of rats undergoing ten days of endurance exercise training on the treadmill. Differences were detected in antioxidant protein levels and transcriptional factors [e.g. peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) and estrogen related receptor alpha (ERRα), etc.] in cardiac and plantaris muscles but not soleus following ten days of treadmill training.

Materials and methods

Animals

Adult female Sprague Dawley rats (4 months old) were assigned to a sedentary (SED) or endurance exercise-training (EXE) group (n=8 per group). Female rats were chosen for this study because typically female rats run better than male rats and we did not want to force the rats to run and cause additional stress that may add variability to the study. The animals were housed on a 12-h: 12-h light-dark cycle (20-22°C) and provided food and water ad libitum throughout the experiment. The University of Florida Institutional Animal Care and Use Committee approved the use of animals in this experiment.

Experimental design

The EXE animals were familiarized to treadmill running for five consecutive days (10, 20, 30, 40, and 50 min of exercise/day, respectively). Following two days of rest, EXE animals were trained on the treadmill for ten days at a speed of 30 m/min at 0° incline (estimated work rate of 70% maximum oxygen consumption [6]) for 60 min per day. SED and EXE animals were acutely anesthetized with 60 mg/kg sodium pentobarbital by an intraperitoneal injection 24 hours after the final training bout and the heart, plantaris, and soleus muscles were immediately harvested and flash frozen in liquid nitrogen and stored at -80°C until analysis.

RT-PCR for skeletal muscle mRNA expression

RNA was isolated from heart, plantaris, and soleus using the Ribozol method (Amresco, Solon, OH) according to the manufacturer's instructions. Concentration and purity of the extracted RNA were measured spectrophotometrically at 260 and at 280 nm using the NanoDrop Lite Spectrophotometer (Thermo Fisher Scientific). Following isolation, 1 μg of RNA was reverse transcribed into cDNA using a cDNA synthesis kit (Quanta, Gaithersburg, MD) per manufacturer's recommendations. Real-time PCR was performed using SYBR green chemistry (Quanta) with gene-specific primers: PGC-1α (Peroxisome proliferator-activated receptor gamma coactivator 1-alpha): forward primer 5'-ATGTGTCGCCTTCTTGCTCT-3', reverse primer 5'-ATCTACTGCCTGGGGACCTT-3'; TFAM (mitochondrial transcription factor A): forward primer 5'-ATCAAGACTGTGCGTGCATC-3', reverse primer 5'-AAAGCCCGGAAGGTTCTTAG-3'; Pyruvate dehydrogenase kinase, isozyme 4 (PDK4): forward primer 5'-AAAGTGGGTCTACGGCAGTG-3', reverse primer 5'-TGCGGAAACAAGAGTCCACA-3'; Estrogen-related receptor alpha (ERRα): forward primer 5'-ACCTCCTCTCCAGAGCAGAG-3', reverse primer 5'-ATGCAATGAGGAGAGGAGCG-3'; Beta glucuronidase (Gusb): forward primer 5'-CCAGAGCGAGTATGGAGCAG-3', reverse primer 5'-CCTCACTGAACATGCGAGGT-3'. Notably, Gusb expression was not different between the two groups and was used as the reference gene. Relative quantification of gene expression was performed using the $2\Delta\Delta CT$ method whereby ΔCT [CT (reference gene)-CT (gene of interest)].

Western blot analysis

Heart, plantaris, and 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, St Louis, MO) and centrifuged at 1500× g for 10 minutes at 4°C. The resulting supernatant (cytosolic) fraction was collected and protein content was assessed by the method of Bradford (Sigma). Equal amounts of protein were separated by polyacrylamide gel electrophoresis via 12% polyacrylamide gels containing 0.1% sodium dodecyl sulfate for ~2 hours at 120 V (C.B.S. Scientific Company, San Diego, CA). After electrophoresis, the proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Amresco) via the C.B.S. Scientific Company system for 2 hours at 200 mA. Membranes were blocked for 1 hour at room temperature in PBS solution containing 0.05% Tween and 5% non-fat milk. Membranes were then incubated for 1 hour with primary antibodies directed against the proteins of interest. The primary antibodies us-

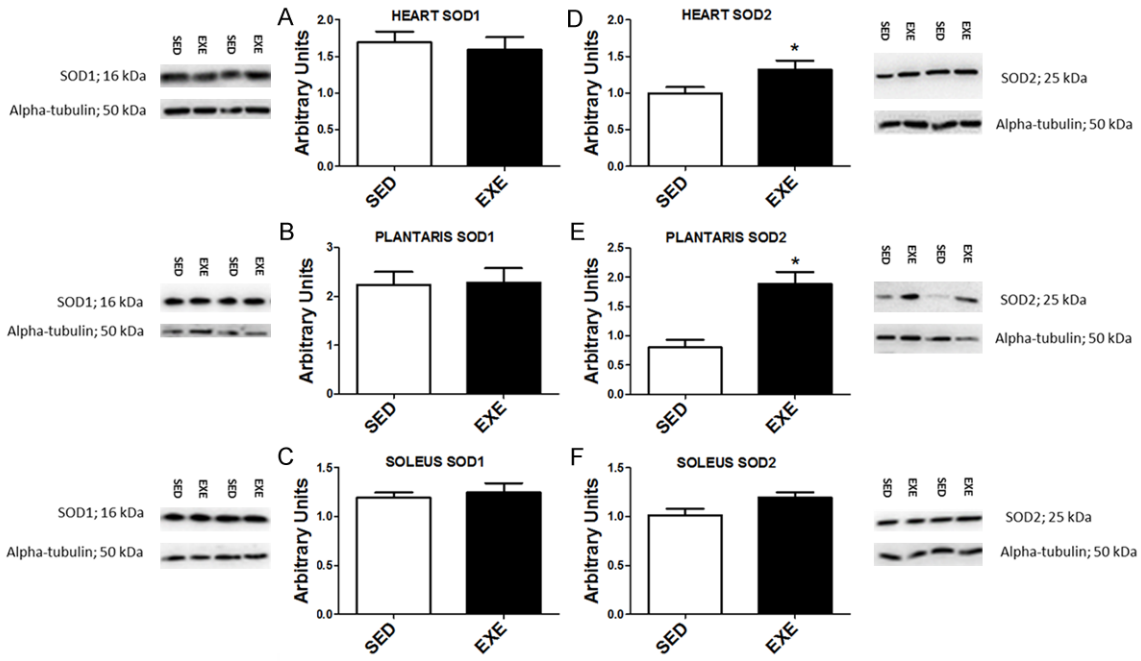

Figure 1. Protein levels of SOD1 (A-C) and SOD2 (D-F) in heart, plantaris, and soleus in sedentary (SED) and exercise (EXE) animals. Representative Western blots are shown (n=6-8 per group). *P<0.05.

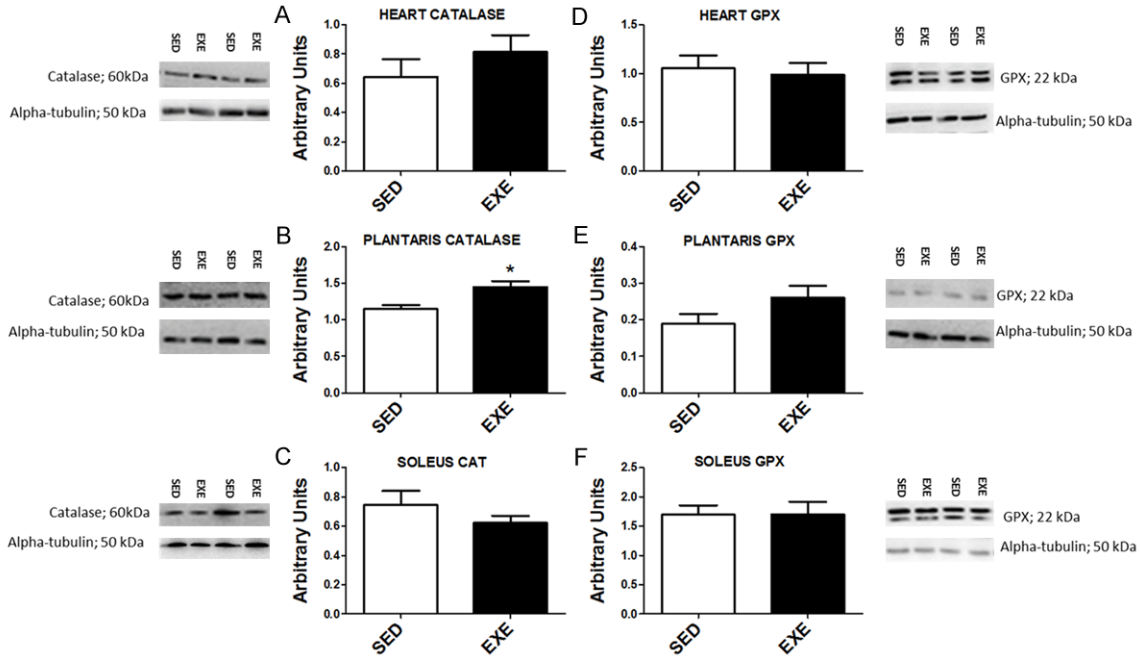

Figure 2. Protein levels of catalase (A-C) and GPX (D-F) in heart, plantaris, and soleus in sedentary (SED) and exercise (EXE) animals. Representative Western blots are shown (n=6-8 per group). *P<0.05.

ed were: superoxide dismutase 2 (SOD2; #GTX-116093; GeneTex, Irvine, CA), superoxide dismutase 1 (SOD1; GeneTex, #GTX100554), glutathione peroxidase (GPX; GeneTex, #GTX11-6040) catalase (GeneTex, #GTX110704), and 4-hydroxynonenal conjugated proteins (4-HNE, #ab46545, Abcam, Cambridge, MA). Alpha-tubulin (#12G10, Developmental Studies Hy-

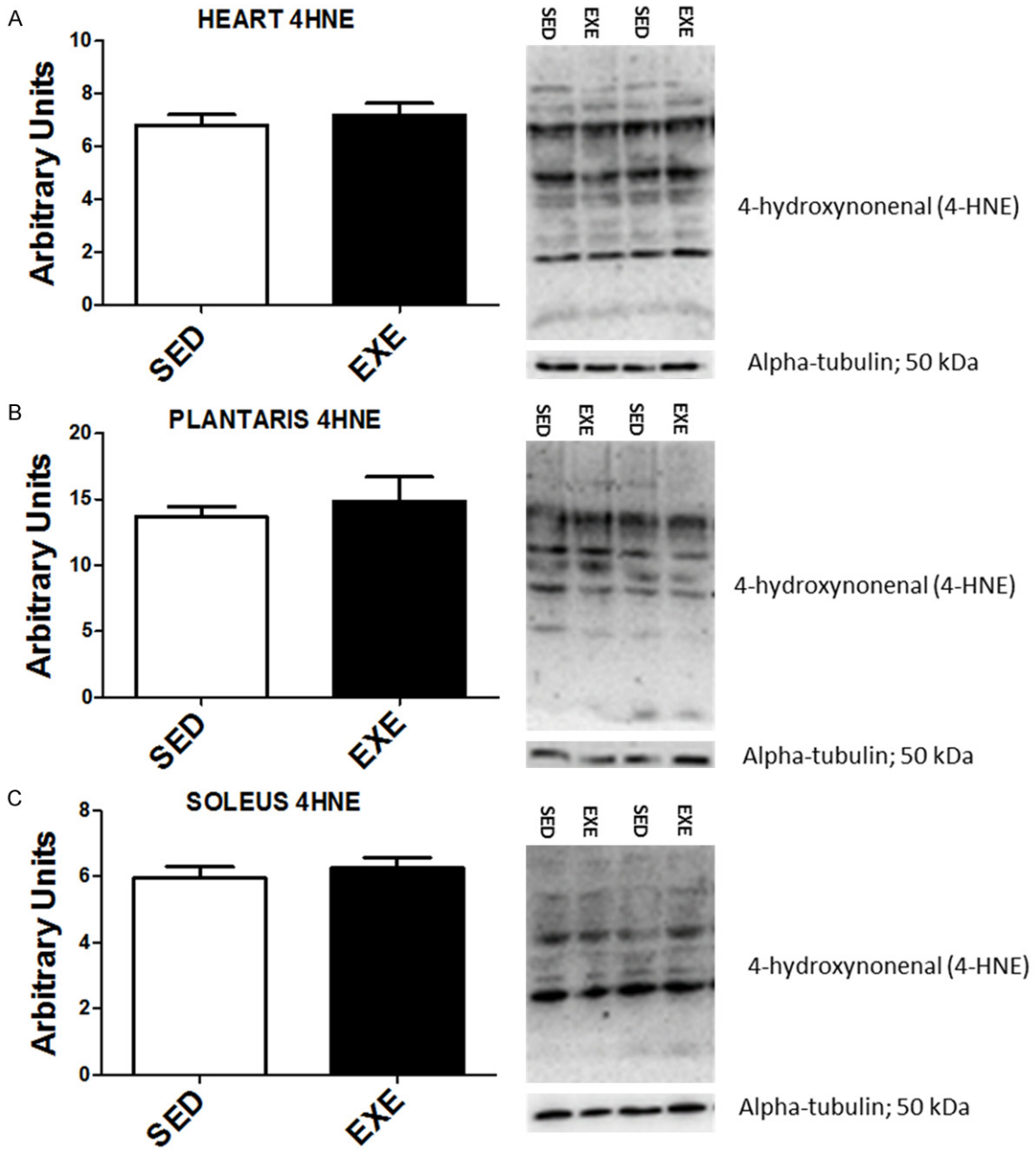

Figure 3. Levels of lipid peroxidation in heart, plantaris, and soleus (A-C) in sedentary (SED) and exercise (EXE) animals were compared by using 4-hydroxynonenal (4-HNE). Representative Western blots are shown (n=7-8 per group).

bridoma Bank, Iowa City, IA) was used as the normalizing control. Following incubation with primary antibodies, membranes were washed extensively with PBS-Tween and then incubated with secondary antibodies. Membranes were then developed using an enhanced chemiluminescent reagent (Amersham, Pittsburgh, PA), and band densitometry was performed through the use of a UVP Imager and associated densitometry software (UVP, LLC, Upland, CA).

Statistical analysis

Dependent variable comparisons between groups were made by independent t-tests with the significance set at $P<0.05$. Data are presented as means ± standard error (SE).

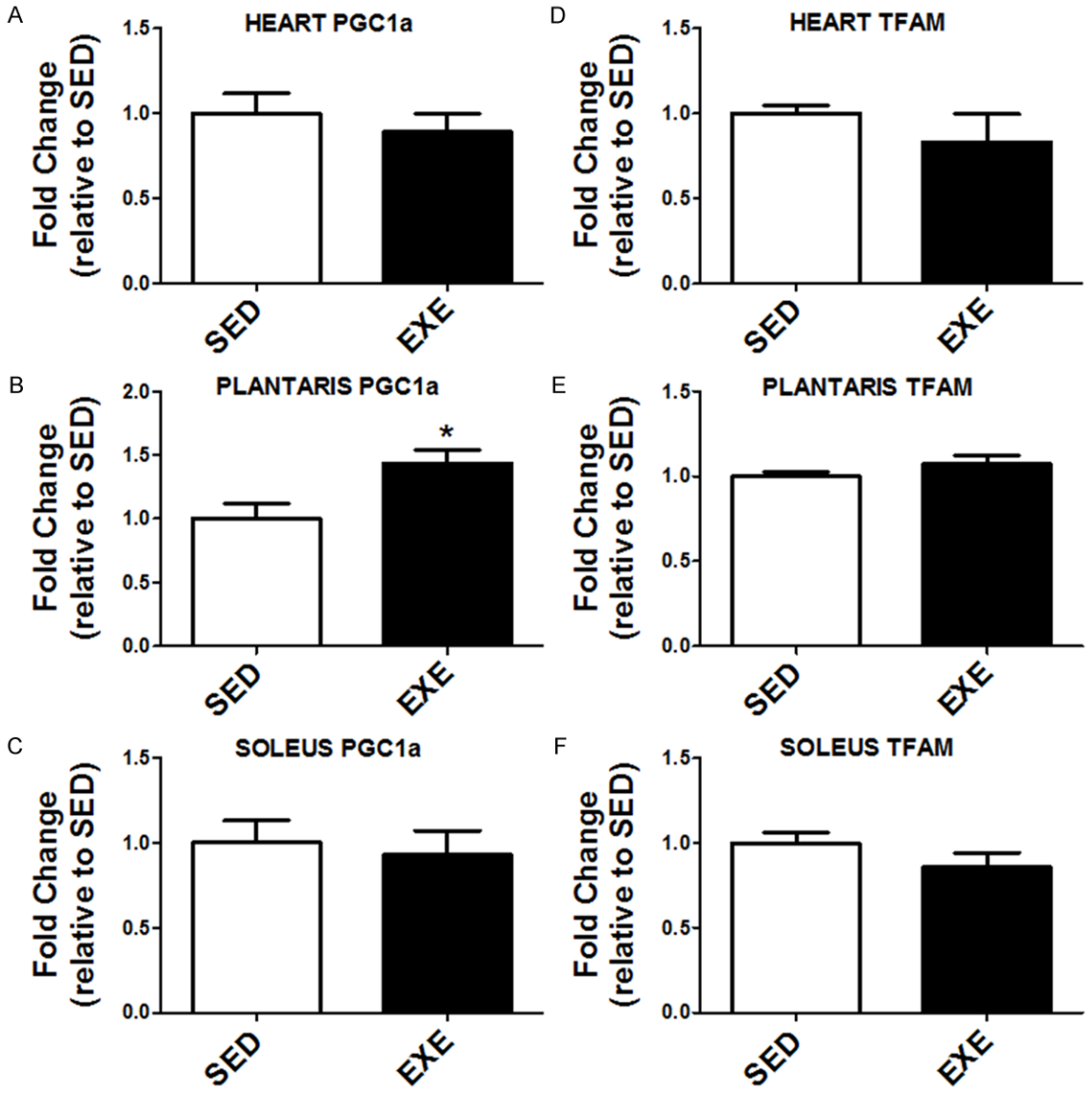

Figure 4. mRNA levels of PGC-1α (A-C) and TFAM (D-F) in heart, plantaris, and soleus in sedentary (SED) and exercise (EXE) animals. (n=7-8 per group). *P<0.05.

Results

Effects of endurance exercise training on muscle antioxidant capacity

Exercise training results in a dose-response effect on the levels of endogenous antioxidant enzymes in both cardiac and skeletal muscle [7-9]. Specifically, this adaptive response is affected by both the duration and intensity of the exercise protocol. In this regard, our data demonstrate that ten days of treadmill training in rats results in a significant increase in the protein levels of SOD2 in the heart and plantaris of EXE animals compared to SED animals (P<0.05), however there were no significant differences for the soleus (**Figure 1**; n=6-8). In addition, the plantaris of EXE animals had higher catalase protein levels compared to SED animals (P<0.05), but no significant differences were detected in the heart and soleus (**Figure 2**; n=6-8). Finally, no significant differences existed for SOD1 and GPX protein levels in heart, plantaris, and soleus between SED and EXE animals (**Figures 1** and **2**). Please note that in the case of heart and soleus our antibody for GPX detected two bands around the predicted molecular target of 22 kDa. In these two cases

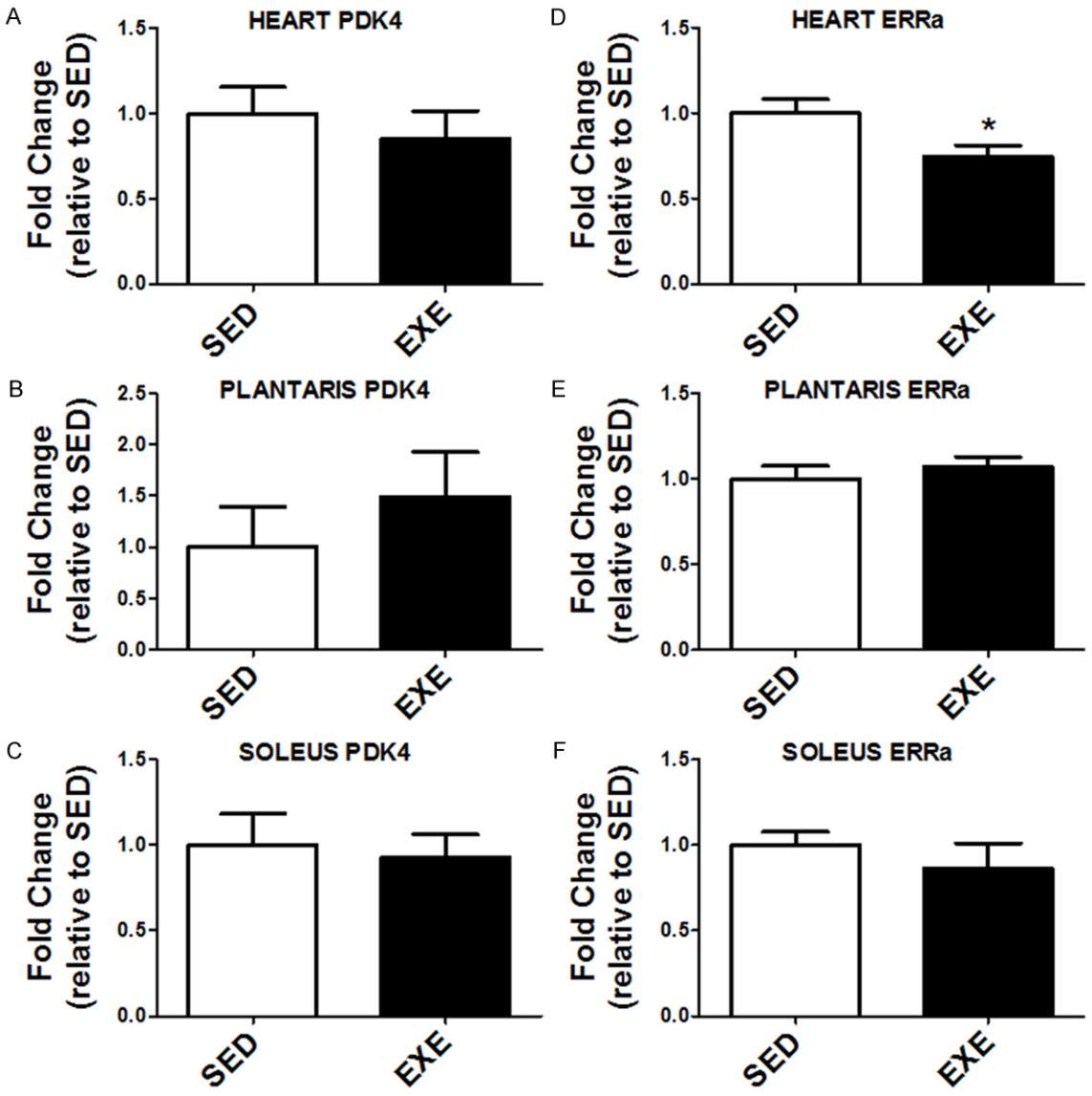

Figure 5. mRNA levels of PDK4 (A-C) and ERRα (D-F) in heart, plantaris, and soleus in sedentary (SED) and exercise (EXE) animals. (n=6-8 per group).

both bands were analyze since it has been previously reported that the existence of a double band represents a proteolytically processed form of GPX [10].

In addition to the effects of exercise on antioxidant capacity, evidence also indicates that exercise can induce reactive oxygen species (ROS) production [5, 11, 12]. Therefore, we measured the protein expression of 4HNE, a marker of oxidative damage, in heart, plantaris, and soleus muscle. The whole lane of 4HNE detected proteins were analyzed and no significant differences were detected in the levels of 4HNE between SED and EXE animals (**Figure 3**; n=7-8).

Effects of endurance exercise training on markers of mitochondrial biogenesis

PGC-1α serves as an upstream transcriptional mediator for many important processes of mitochondrial function, including metabolic regulation and oxidative metabolism in skeletal muscle [13, 14]. Previous studies have observed alterations in skeletal muscle gene expression involved in mitochondrial function in response to a single bout of endurance training

[15], however to our knowledge no studies have observed a differential responses between cardiac, plantaris, and soleus in response to ten days of treadmill training. In this regard, our data demonstrates that PGC-1α mRNA levels were higher ($P<0.05$) in plantaris of EXE animals compared to SED animals, but no significant differences were detected in the heart or soleus (**Figure 4**; n=7-8).

PGC-1α is also a known co-activator of ERRα. The co-activation of ERRα by PGC-1α has been indicated as an essential mechanism to induce transcription of genes important in energy production [16, 17]. Specifically, ERRα encodes for proteins related to fatty acid oxidation and lactate metabolism via lactate dehydrogenase B [18]. Furthermore, abolishing ERRα expression significantly decreases the ability of PGC-1α to mediate mitochondrial biogenesis [19]. In these experiments, ERRα mRNA levels were lower in the heart of EXE animals compared to SED animals, but no significant differences were detected in the plantaris and soleus (**Figure 5**; n=6-8).

Finally, PGC-1α is also associated with the regulation of the downstream transcription factors TFAM and PDK4, which regulate mitochondrial DNA and metabolism, respectively. No significant differences were found for TFAM or PDK4 mRNA levels between SED and EXE animals in heart, plantaris, or soleus (**Figures 4** and **5**).

Discussion

Understanding the differing adaptive responses to exercise across distinct muscle types can provide useful informational cues on the inherent biochemical properties of these tissues, and can serve to enhance the benefit of prescribing exercise as a preventative or rehabilitative therapeutic for chronic disease. In this study, we observed differential transcriptional and protein responses of cardiac and skeletal muscles in response to ten days of endurance treadmill training in rats. Our results show that, in comparison to a sedentary control group, ten days of treadmill training resulted in higher antioxidant protein levels in heart and plantaris muscles, but not soleus. Transcriptional alterations of PGC-1α and key downstream markers of metabolism and mitochondrial biogenesis were also observed in cardiac and plantaris muscles. Specific details of these findings are discussed in the following paragraphs.

Antioxidant response to exercise training

Reactive oxygen species (ROS) occur naturally in the body through processes of energy production and metabolism, however an imbalance between ROS production and a counteracting antioxidant system can result in oxidative damage [5, 7]. More specifically, ROS can result in the breakage of intact DNA strands, DNA base modification, protein oxidation, and lipid oxidation each having the potential to cause catastrophic cellular malfunctioning [20]. However due to the threat of oxidative damage, cellular mechanisms exist that enable ROS to be recognized as a signal for facilitating beneficial adaptations in the affected tissue. In this regard, acute conditions of elevated ROS production, such as exercise [12], can confer a protective status by signaling for increases in endogenous antioxidant enzymes. In this regard, our results indicate that ten days of treadmill training results in increased protein levels of SOD2 in both plantaris and cardiac muscle, but not soleus. SOD1 and GPX protein levels were unchanged in all tissue types between sedentary and exercise groups. Our data agree with findings of Hollander et al. [21] that training can induce increases in SOD2 protein expression and that higher intensities of training are likely required for increasing SOD1. However, Hollander et al. observed training effects after ten weeks of treadmill running at a similar intensity (27 m/min, 12% grade), while our current study demonstrates that only ten days of treadmill training is sufficient to significantly increase antioxidant protein expression in plantaris and cardiac tissue. It is likely that the variation in antioxidant adaptations between plantaris, heart, and soleus muscles are due to initial levels of inherent antioxidant protein content pre-training or rather the relative degree of oxidative stress induced by exercise. Indeed, the fast-glycolytic muscle fiber phenotype in plantaris may experience higher rates of oxidative stress and compensate by increasing antioxidant proteins to a greater extent than the more oxidative-fiber types of soleus and cardiac muscle.

Mitochondrial response to exercise training

Acute bouts of exercise have been shown to increase PGC-1α mRNA expression [22, 23], yet the effect of training on PGC-1α mRNA expression is not fully understood. One study

observing PGC-1α related training adaptations (i.e. fiber type shift) in plantaris muscle showed no changes in PGC-1α mRNA expression 24-hours post cessation of a four week voluntary wheel running program [15]. Our results indicated that higher levels of PGC-1α mRNA were present in plantaris muscle in EXE animals, however no statistically significant differences were detected in cardiac and soleus muscles. Several potential mechanisms exist to explain the lack of exercise-induced effects on PGC-1α signaling. First, we speculate that cardiac and soleus muscles did not experience similar changes in PGC-1α due to their pre-existing oxidative-slow twitch fiber type orientation and/or insufficient exercise stimulus (e.g., intensity or duration). In addition, it is well described that ROS production is a requirement for contraction-induced expression of PGC-1α [5, 24], however our data demonstrates no significant differences in 4HNE between groups, as well as no changes in several transcriptional factors downstream of PGC-1α. Finally, there is also evidence that mitochondrial adaptations may be initiated prior to measurable changes in PGC-1α expression [25]. Therefore, changes to PGC-1α expression may not be required for exercise-induced mitochondrial adaptations.

In conclusion, with increasing evidence linking exercise and health, it is important to observe and highlight the important physiological benefits of exercise that remain absent in a sedentary lifestyle. Our data demonstrate that ten days of treadmill training results in differential tissue response between plantaris, soleus, and heart antioxidant protein levels and mitochondrial gene expression. Ten days of endurance exercise training resulted in increased antioxidant protein expression in heart and plantaris muscles, providing the potential to combat greater cellular levels of ROS. Plantaris mRNA PGC-1α levels increased in EXE animals in comparison to their sedentary counterparts. Finally, our results indicate that plantaris and cardiac muscles are responsive to ten days of treadmill training, and that greater exercise intensity or duration is likely required to illicit alterations in soleus muscle.

Disclosure of conflict of interest

None.

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