

Metabolic Adaptations of Skeletal Muscle to Voluntary Wheel Running Exercise in Hypertensive Heart Failure Rats

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Summary

The Spontaneously Hypertensive Heart Failure (SHHF) rat mimics the human progression of hypertension from hypertrophy to heart failure. However, it is unknown whether SHHF animals can exercise at sufficient levels to observe beneficial biochemical adaptations in skeletal muscle. Thirty-seven female SHHF and Wistar-Furth (WF) rats were randomized to sedentary (SHHFsed and WFsed) and exercise groups (SHHFex and WFex). The exercise groups had access to running wheels from 6-22 months of age. Hindlimb muscles were obtained for metabolic measures that included mitochondrial enzyme function and expression, and glycogen utilization. The SHHFex rats ran a greater distance and duration as compared to the WFex rats ($P < 0.05$), but the WFex rats ran at a faster speed ($P < 0.05$). Skeletal muscle citrate synthase and β -hydroxyacyl-CoA dehydrogenase enzyme activity was not altered in the SHHFex group, but was increased ($P < 0.05$) in the WFex animals. Citrate synthase protein and gene expression were unchanged in SHHFex animals, but were increased in WFex rats ($P < 0.05$). In the WFex animals muscle glycogen was significantly depleted after exercise ($P < 0.05$), but not in the SHHFex group. We conclude that despite robust amounts of aerobic activity, voluntary wheel running exercise was not sufficiently intense to improve the oxidative capacity of skeletal muscle in adult SHHF animals, indicating an inability to

compensate for declining heart function by improving peripheral oxidative adaptations in the skeletal muscle.

Key words

SHHF • Wheel running • Voluntary exercise • Heart failure

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Introduction

Voluntary wheel running (VWR) is an effective exercise model because, given access to running wheels, rodents will run many kilometers per day through a series of short relatively high-intensity bouts (Eikelboom and Mills 1988, Rodnick *et al.* 1989, Swallow *et al.* 1998a, De Bono *et al.* 2006). As an exercise modality, VWR is less stressful because it requires minimal experimental handling and, therefore, does not disrupt the normal diurnal pattern of rodent activity. VWR elicits a variety of physiological modifications including decreases in body mass and body fat (Swallow *et al.* 1999, 2001,

Dumke *et al.* 2001), increases in maximal O₂ consumption, (Lambert and Noakes 1990, Swallow *et al.* 1998b), and metabolic alteration of cardiac and skeletal muscles (Rodnick *et al.* 1989, Ishihara *et al.* 1991, Houle-Leroy *et al.* 2000, Allen *et al.* 2001, Dumke *et al.* 2001, Harrison *et al.* 2002). Furthermore, VWR also reduces risk factors for heart failure (HF), including lowering resting systolic blood pressure as shown in spontaneously hypertensive rats (Overton *et al.* 1986, Kinney LaPier and Rodnick 2001). This is consistent with evidence of low stress exercise in humans inducing beneficial cardiovascular adaptations. As a consequence, low stress exercise is now recommended for HF patients (Aronow 2003, Pina *et al.* 2003, Smart and Marwick 2004).

The Spontaneously Hypertensive Heart Failure (SHHF) rat provides a model that mimics human progression of hypertension from cardiac hypertrophy to heart failure. Animal studies examining the effectiveness of regular exercise training on cardiac remodeling and ventricular function in both the healthy heart and in various models of hypertension and heart failure have used VWR (Allen *et al.* 2001, Natali *et al.* 2002, Konhilas *et al.* 2005, 2006). Most report a beneficial effect of exercise, but one study using a severe transverse aortic constriction model found that exercise accelerated cardiac dysfunction (van Deel *et al.* 2011). Similarly, we have previously reported that 16 months of exercise in SHHF rodents resulted in greater left ventricle (LV) chamber dilation, worsening of LV function, and accelerated signs of heart failure compared to sedentary controls (Schultz *et al.* 2007).

The compensatory effects of endurance exercise on peripheral muscular oxidative adaptations and the progression to heart failure are important in the context of potential treatments for heart failure. However, previous studies have shown that the derangement of skeletal muscle oxidative metabolism associated with heart failure can be prevented, or normalized, with regular endurance exercise and this also alleviated many of the symptoms associated with heart failure (reviewed in Ventura-Clapier *et al.* 2007). As such, we measured markers of skeletal muscle oxidative metabolism after providing running wheels to female SHHF rats from the ages of six months until 22 months. To our knowledge, it is unknown whether SHHF rats can exercise at a sufficient stimulus to observe metabolic adaptations in skeletal muscle, in the presence of progressive heart failure. Thus, the purpose of this study was to determine the potential effectiveness of VWR in compensating for heart failure

through beneficial changes in muscle oxidative capacity.

Methods

Experimental design

Lean female SHHF rats were purchased from Charles River Laboratories (Indianapolis, IN). Age-matched female Wistar-Furth rats (WF; Charles River Laboratories; Wilmington, MA) served as both normotensive and exercise controls. Eleven SHHF and 7 WF female rats were housed individually in clear plastic Nalge™ cages (43"x 27"x 15") with cedar bedding and wire lids. Stainless steel running wheels (Nalgene Activity Wheels for Rodents, Fisher Scientific) were placed inside their cages beginning at six months of age. Teklad Rodent Diet (8604) and water were available *ad libitum*. Wheel-running behavior of the exercise trained SHHF (SHHFex) and WF (WFex) rats was monitored from 6 to 22 months of age. Heart function was also assessed in all rodents and has been reported previously (Schultz *et al.* 2007). Rats were maintained in the same environment including temperature and humidity on a 12:12 dark:light cycle (dark 0600-1800) using an automatic timer. All procedures in this study were approved by the University of South Dakota Animal Care and Use Committee and followed Institutional guidelines for animals.

Wheel running quantification

Running wheels were connected to a Minimitter™ system using a magnetic sensor to record wheel rotations. This system was interfaced with a computer, and recorded using VitalView™ software at one-minute intervals 24 h/day during months 3-9, 12-13, and 15 of the study, which corresponded with ages 9-15, 18-19, and 21 months. The circumference of the running wheel was 1.08 m. Therefore, to obtain the total number of meters run by each animal the number of revolutions of the wheel was multiplied by 1.08. VitalView™ software (Mini Mitter Co., Inc., Bend, OR) was set to store the number of revolutions per minute and to ascertain the total amount of running time and distance.

mRNA expression

Total mRNA was isolated from ~20 mg of rat hindlimb mixed skeletal muscle using TriReagent (Molecular Research Center, Cincinnati, OH), which is based on the guanidine thiocyanate method. Muscle samples were homogenized with a glass homogenizer on

ice in 1 ml of ice-cold TriReagent. RNA was solubilized in RNase-free H₂O and quantitated by measuring the absorbance at 260 nm. Purity was verified by an absorbance (A) 260/280 ratio greater than 1.6 (normally 1.8-2.0). First-strand cDNA was synthesized from 2 µg of total RNA using RETROscript reverse transcriptase (RT, Ambion, Austin, TX). mRNA expression for genes of interest was measured in duplicate with a Stratagene MX3000P real-time PCR machine using SYBR green with ROX as a reference dye (Stratagene, La Jolla, CA). A primer pair was designed against citrate synthase (CS) (NM-130755.1) (forward 5'-CCGTGCTCATGGACTTGGGCCTT-3'; reverse 5'-CCCCTGGCCCAACGTAGATGCTC-3') and β-hydroxyacyl-CoA dehydrogenase (β-HAD) (NM-130826.2) (forward 5'-CCAAACTAAAGCCTGCATTTCATC-3'; reverse 5'-CGCCATCAGTCAGGAAAGAAG-3'). GAPDH (NM-017008.3) was used as the housekeeping gene (Integrated DNA Technologies, Coralville, IA) (Siu *et al.* 2003). The PCR parameters included: initial denaturing at 95 °C for 10 min to activate the DNA polymerase followed by 40 cycles of 30 s at 95 °C, 60 s at an annealing temperature of 55 °C, and 30 s at 72 °C for acquisition of fluorescence signal. The expression levels were calculated by the delta-delta threshold cycle (Ct) method (Livak and Schmittgen 2001).

Protein expression

Citrate synthase and β-HAD protein expression were determined in skeletal muscle samples using Western blot analysis. Frozen muscle was homogenized with a glass homogenizer in freshly prepared ice-cold buffer (150 mM Hepes, 150 mM NaCl, 20 mM beta-glycerophosphate, 10 mM EDTA, 1 % NP-40, 10 % glycerol, 1 mM MgCl₂, 1 mM CaCl₂, 20 mM Na₄P₂O₇, 10 mM NaF, 2 mM sodium orthovanadate, 2 mM PMSF and 5 µl/ml of protease inhibitor cocktail (P8340, Sigma, St. Louis, MO, 10 µl of buffer per mg of muscle). The homogenate was centrifuged for 10 min at 12,000 rpm at 4 °C. Protein concentration was quantified using a commercial BCA protein assay kit (Pierce, Rockford, IL). Aliquots of homogenate were solubilized in lithium dodecyl sulfate (LDS) sample buffer with a final concentration of 1 µg/ul, and 20 µg of protein was separated by 12 % SDS-PAGE. After electrophoresis, the proteins were electro-transferred to a nitrocellulose membrane (Bio-Rad, Hercules, CA). Membranes were blocked in 1 % TBST (10 mM Tris, 100 mM NaCl, and 0.01 % Tween, pH 7.5) containing 5 % nonfat milk

overnight at 4 °C, washed with TBST for 15 min, and incubated with primary antibody (1:5000 dilution) for one hour at room temperature (Diagnostic International, San Antonio, TX). The membranes were washed 5 times for 5 min each wash before incubation with a secondary anti-rabbit IgG antibody (1:10,000; Diagnostic International, San Antonio, TX) for one hour at room temperature. The membranes were washed with TBST again and subjected to enhanced chemiluminescence (Amersham Biosciences, Piscataway, NJ). The membranes were subsequently stripped with 0.5 M NaOH for 15 min at room temperature and reprobed with beta-actin primary antibody (1:1000) for one hour at room temperature (Sigma, St Louis, MO). The membrane was washed several times with TBST before incubation with a secondary anti-mouse IgG antibody (1:10,000; Sigma, St Louis, MO). The results were quantified by scanning densitometry (Kodak, IS 4000R, Rochester, NY).

Mitochondrial enzyme activities

Citrate synthase activity

Spectrophotometric analysis of citrate synthase activity was conducted by the method of Srere (Srere and John 1969) as previously described (Kirwan *et al.* 1990). Muscle was homogenized in potassium phosphate buffer (50 mM KH₂PO₄, 1 mM EDTA, 2 mM MgCl₂) to create a 5 % homogenate. A 5 µl sample of homogenate was added to 815 µl of ddH₂O, 30 µl of 10 mM Acetyl CoA, and 100 µl of 1 mM DNTB. The absorption at 412 nm was followed for 3 min to measure possible acetyl-CoA deacylase activity. The reaction was started by adding 50 µl of 10 mM oxaloacetate. Linear rates were obtained for at least 3 min at 37 °C. Protein extracts of the homogenates were quantified in triplicate using bicinchoninic acid reagent (Pierce, Rockford, IL) and bovine serum albumin standards. The CS activity was then normalized to the total protein content and is reported as nanomoles per milligram protein per minute.

β-hydroxyacyl-CoA dehydrogenase (β-HAD) activity

The muscle tissue (~10 mg) was homogenized in 100 vol/wt of a 100 mM potassium phosphate buffer for measurement of β-HAD activity. Total muscle activity was measured in Tris-HCL buffer (50 mM Tris-HCL, 2 mM EDTA, and 250 µM NADH, pH 7.0) and 0.04 % Triton-X. The reaction was started by addition of 100 µM of acetoacetyl CoA. The activity was assayed spectrophotometrically over 2 min at 37 °C by measuring

the disappearance of NADH at 340 nm (Essen *et al.* 1975).

Skeletal muscle glycogen content

Tissue glycogen was measured in perchloric acid extracts using the amyloglucosidase method (Passonneau and Lauderdale 1974).

Statistical analyses

All data are presented as means \pm SE. Changes in wheel running behavior over time and group differences in muscle gene and protein expression, protein activity, and glycogen were assessed using two-way ANOVA. The Bonferroni *post hoc* test was used to examine statistically significant differences. Student's *t*-tests were used to determine differences in wheel running averages between groups. Results were considered significant when $P < 0.05$.

Results

Body weight did not differ between any of the groups at 22 months of age (SHHFsed=292 \pm 18 g, SHHFex=298 \pm 18 g, WFsed=283 \pm 28 g, WFex=308 \pm 18 g). The SHHFex rats ran an average of 7.5 \pm 0.9 km/day, 339 \pm 46 min/day, and 22.1 \pm 2.6 m/min. In comparison, the WFex rats ran an average of 5.6 \pm 2.0 km/day, 201 \pm 29 min/day, and 31.6 \pm 3.1 m/min. Statistical analysis revealed that SHHFex averaged a greater distance per day ($P < 0.05$) and time per day ($P < 0.05$) than WFex, but the WFex ran at a significantly higher intensity ($P < 0.05$).

Skeletal muscle gene and protein expression

Citrate synthase mRNA levels were similar for the WFsed and SHHFsed groups. Increased physical activity stimulated an increase in citrate synthase gene expression in the WFex animals compared to the control WFsed group ($P < 0.05$) (Fig. 1A). In contrast, the SHHFex group did not show an increase in mRNA levels compared with SHHFsed. Citrate synthase protein expression was also increased in the WFex group compared to the WFsed animals ($P < 0.05$, Fig. 1B). Further, protein expression in the SHHFex group was significantly lower ($P < 0.05$) than the SHHFsed, or the WFex groups.

β -HAD gene expression was higher in the WFex group than either the WFsed or the SHHFex groups ($P < 0.05$). A similar pattern of expression was found for β -HAD protein and the WFex group had increased

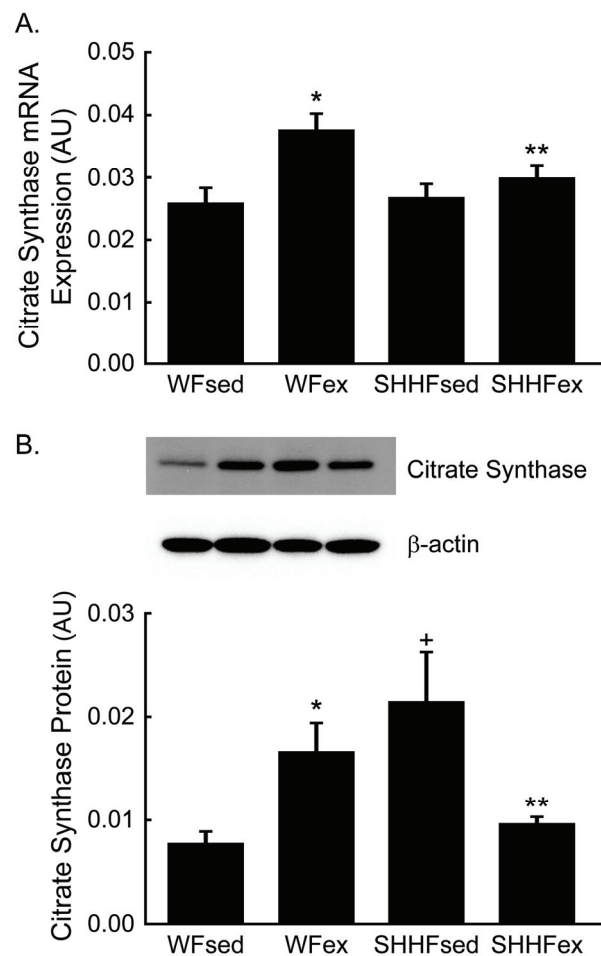


Fig. 1. A. Representative qRT-PCR results for citrate synthase in skeletal muscle from WFsed (n=9), WFex (n=5), SHHFsed (n=9), and SHHFex (n=11) groups. Normalized data are presented as means \pm SE. * $P < 0.05$, WFex group is significantly increased compared to WFsed. ** $P < 0.05$, SHHFex group is significantly lower than WFex. **B.** Citrate synthase protein expression in skeletal muscle of WFsed (n=9), WFex (n=5), SHHFsed (n=11), and SHHFex (n=10) groups. Normalized data are presented as means \pm SE; * $P < 0.05$, WFex significantly increased compared to control WFsed. + $P < 0.05$, SHHFsed group is significantly greater than SHHFex. ** $P < 0.05$, SHHFex group is significantly lower than WFex.

expression compared to the WFsed and SHHFex controls ($P < 0.05$), while there was no difference between the SHHFsed and SHHFex groups (Fig. 2).

Skeletal muscle citrate synthase and β -HAD activity

Citrate synthase enzyme activity was not different in the WFsed group compared with the SHHFsed group. Exercise increased in citrate synthase activity in the WFex animals when compared to the WFsed controls ($P < 0.05$). In contrast, SHHFex rats did not show an increase in citrate synthase activity compared to the SHHFsed control group. Further, citrate synthase activity in SHHFex rats exposed to wheel

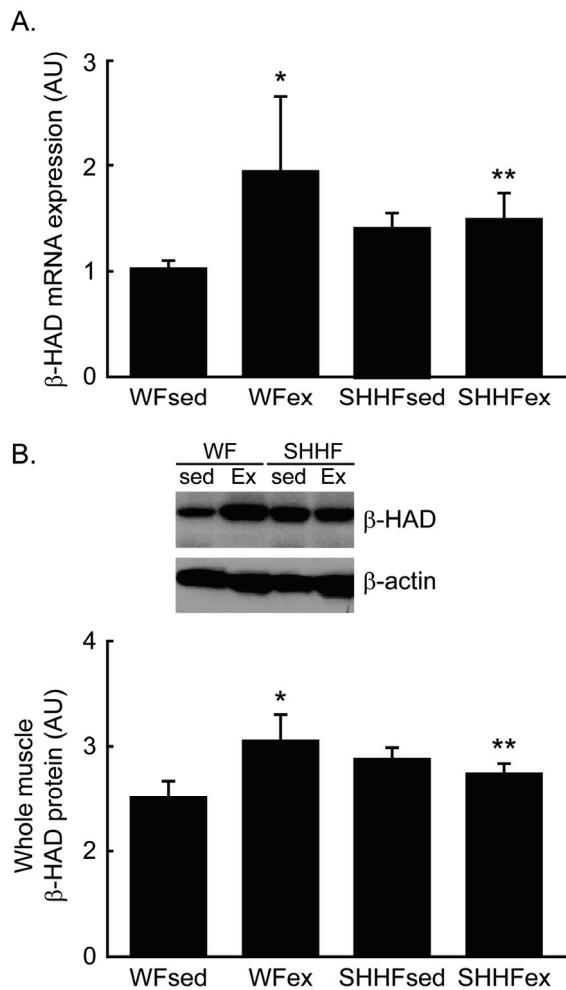


Fig. 2. A. qRT-PCR results for β -HAD in skeletal muscle from WFsed (n=10), WFex (n=5), SHHFsed (n=10), and SHHFex (n=9) groups. Normalized data are presented as means \pm SE. * $P < 0.05$, WFex group is significantly higher than WFsed. ** $P < 0.05$, SHHFex group is significantly lower than WFex. **B.** β -HAD protein expression in skeletal muscle of WFsed (n=10), WFex (n=4), SHHFsed (n=10), and SHHFex (n=9) groups. Normalized data are presented as means \pm SE. * $P < 0.05$, the WFex group was significantly increased compared to WFsed. ** $P < 0.05$, SHHFex group is significantly lower than WFex.

running was lower compared with the WFex group ($P < 0.05$) (Fig. 3A).

Enzyme activity of β -HAD, which is a key enzyme of β -oxidation in skeletal muscle was also used to probe for changes in mitochondrial function. Similar to data for citrate synthase activity, β -HAD activity was greater in the WFex animals compared to WFsed and SHHFex ($P < 0.05$) (Fig. 3B).

Skeletal muscle glycogen content

Muscle glycogen concentrations were measured in samples obtained after the animals had access to the running wheels. Consequently, the active WFex animals had reduced muscle glycogen ($45.0 \pm 7.5 \mu\text{mol/g wwt}$)

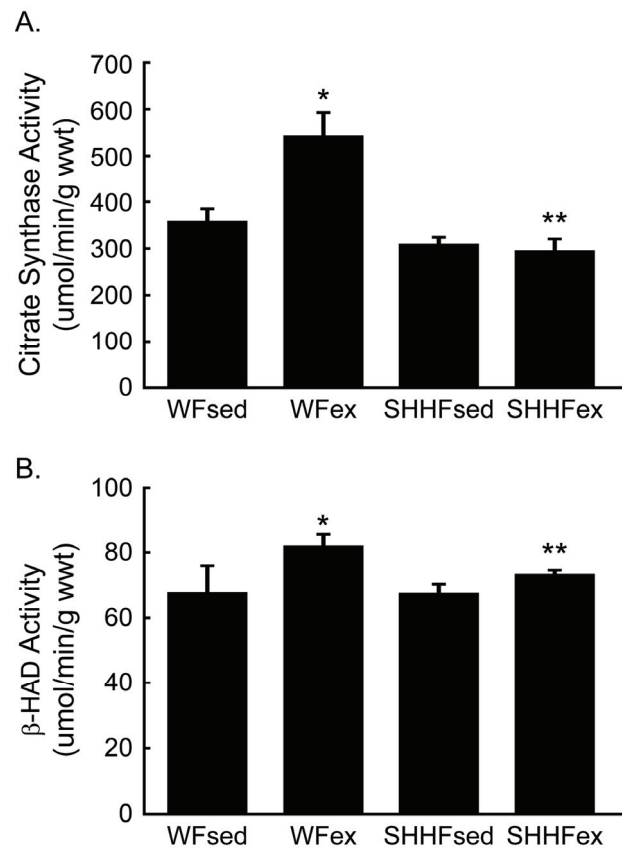


Fig. 3. A. Citrate synthase activity in skeletal muscle of the WFsed (n=8), WFex (n=5), SHHFsed (n=10) and SHHFex (n=8) groups. * $P < 0.05$, the WFex group was significantly increased compared to the WFsed group. ** $P < 0.05$, SHHFex group is significantly lower than WFex. **B.** Enzyme activity of β -HAD in skeletal muscle of WFsed (n=8), WFex (n=5), SHHFsed (n=10) and SHHFex (n=10) groups. Data are presented as means \pm SE. * $P < 0.05$, WFex group was significantly increased compared to the WFsed. ** $P < 0.05$, SHHFex group is significantly lower than WFex.

compared to the sedentary WFsed group ($90.2 \pm 7.9 \mu\text{mol/g wwt}$; $P < 0.05$) (Fig. 4). Glycogen levels in the SHHFsed group ($74.6 \pm 8.3 \mu\text{mol/g wwt}$) were not different from the WFsed group. Importantly, the SHHFex group ($69.1 \pm 3.7 \mu\text{mol/g wwt}$) did not experience any reduction in muscle glycogen compared to SHHFsed despite exposure to the running wheel.

Discussion

This is the first study to examine the effect of VWR on muscle oxidative enzyme adaptations in a rat model of heart failure. Our results indicate that the SHHF rat will run at distances and durations similar to or greater than other species and strains of rodents (Rodnick *et al.* 1989, Jonsdottir *et al.* 1998, Kingwell *et al.* 1998, Allen *et al.* 2001, Kinney LaPier and Rodnick 2001, Burghardt *et al.* 2004, Beatty *et al.* 2005, Nelson *et al.* 2005,

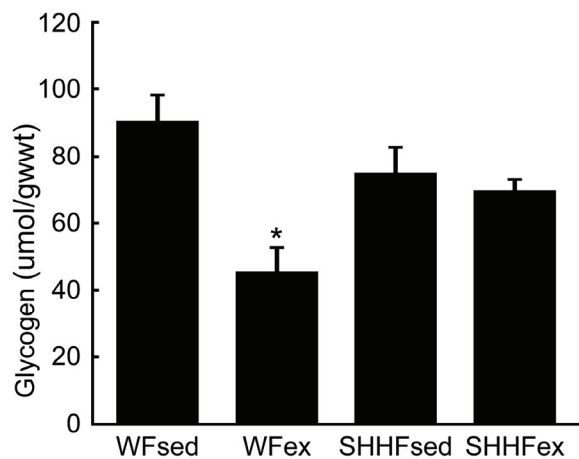


Fig. 4. Glycogen content in WFsed (n=9), WFex (n=5), SHHFsed (n=10), and SHHFex (n=11) groups. * $P < 0.05$, WFex was significantly lower than WFsed.

Konhilas *et al.* 2006). The SHHFex rats in this study tended to maintain their VWR patterns throughout the study, despite the fact that many had already developed or were transitioning to heart failure (previously reported in Schultz *et al.* 2007). However, in contrast to healthy rats this mode of exercise failed to improve peripheral muscular adaptations in SHHF animals.

Previously, it has been shown that heart failure causes exercise intolerance which leads to subsequent declines in muscle oxidative enzyme activity (Pfeifer *et al.* 2001). It is therefore likely that the progression of heart failure in our rats limited their ability to achieve and sustain higher intensity exercise, thus failing to provide an adequate stimulus for oxidative adaptations. Although the SHHFex rats exercised for a longer duration per day on average than the WFex rats, the WFex rats exercised at a higher intensity. This was further evidenced by the reduction of muscle glycogen in the WFex rats, while no such observation was made in SHHFex rats. When considering appropriate stimuli for the increase in abundance and activity of the oxidative enzymes, citrate synthase and β -HAD, short bouts of high-intensity exercise have been shown to be comparable to the effectiveness of the traditional model of training mode of moderate-intensity, long-duration continuous training in rodents (Terada *et al.* 2004, Bexfield *et al.* 2009) and humans alike (den Hoed *et al.* 2008, Green *et al.* 2008). It would appear that the exercise performed by the control rats was of sufficient intensity to cause increased activation of the oxidative enzymes citrate synthase and β -HAD, whereas the lower-intensity exercise of the SHHFex rats was inadequate.

It was somewhat surprising that muscle

oxidative enzyme content and activity was not improved relative to the SHHFsed despite the robust accumulation of physical activity. Several factors, such as gender and mode of exercise may have had an impact on peripheral and central outcomes of the SHHF rats exposed to VWR. Additionally, it is unknown what effect alterations in circulating endothelin-1 and angiotensin II may have had on oxidative adaptations, considering both of these markers have been shown to be elevated in heart failure, and both are inhibitors of PGC-1 α (Garnier *et al.* 2009). However, a recent meta-analysis of exercise in the SHR model (Schluter *et al.* 2010) found that the age at the start of an exercise intervention has a considerable impact on the physiological benefits of the exercise in a disease model. We started our exercise intervention at 6 months of age, which was well into the progression of heart failure as evidenced by increased wall thickness compared to the WF (previously reported in Schultz *et al.* 2007). Relative to most other rodent models of exercise, this is a fairly advanced age. Emter and colleagues (2005) were able to delay the progression of heart failure in SHHF rats, but rodents began exercise at a much younger age and before the onset of pathological changes (Emter *et al.* 2005). Another study employing voluntary wheel running in the hypertrophic cardiomyopathy (HCM) mouse model found prevention and reversal of heart changes in exercised mice, and these benefits were greatest when exercise was initiated before the disease was established (Konhilas *et al.* 2006). Furthermore, these authors reported that animals beginning exercise at 6 months of age failed to prevent the progression of fibrosis and had the greatest increase in cardiac mass. Thus, it appears that in this SHHF rodent model, exercise may only be beneficial before the onset of pathological changes related to heart failure.

In conclusion, this study demonstrates that SHHF rats will engage in VWR at durations and distances greater than other strains and species, but fail to show beneficial oxidative adaptations in skeletal muscle. The likely reason for this is the significantly lower exercise intensity as a result of the progression of heart failure with subsequent exercise intolerance, thereby preventing sufficient stimulus for oxidative muscle adaptations. One major influencing factor appears to have been the advanced age and progression of disease at the initiation of the exercise intervention. We conclude that it is best to begin exercise early to delay the progression of heart failure and to promote beneficial muscular oxidative adaptations.

Conflict of Interest

There is no conflict of interest.

Acknowledgements

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