



Molecular Adaptations to Aerobic Exercise Training in Skeletal Muscle of Older Women

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Background. We have recently shown that 12 weeks of progressive aerobic exercise training improves whole-muscle size and function in older women. The purpose of this investigation was to evaluate molecular markers that may be associated with muscle hypertrophy after aerobic training in aging skeletal muscle.

Methods. Muscle biopsies were obtained before and after 12 weeks of aerobic exercise training on a cycle ergometer in nine older women (70 ± 2 years) to determine basal levels of messenger RNA and protein content of select myogenic, proteolytic, and mitochondrial factors.

Results. The training program increased ($p < .05$) aerobic capacity $30 \pm 9\%$, whole-muscle cross-sectional area $11 \pm 2\%$, and whole-muscle force production $29 \pm 8\%$. Basal messenger RNA levels of FOXO3A, myostatin, HSP70, and MRF4 were lower ($p < .05$) after aerobic training. FOXO3A, FOXO3A phosphorylation, and HSP70 protein content were unaltered after training. Mitochondrial protein COX IV was elevated ($p < .05$) $33 \pm 7\%$ after aerobic training, whereas PGC-1 α protein content was $20 \pm 5\%$ lower ($p < .05$).

Conclusions. These data suggest that reductions in FOXO3A and myostatin messenger RNA are potentially associated with exercise-induced muscle hypertrophy. Additionally, it appears that mitochondrial biogenesis can occur with aerobic training in older women independent of increased PGC-1 α protein. Aerobic exercise training alters molecular factors related to the regulation of skeletal muscle, which supports the beneficial role of aerobic training for improving muscle health in older women.

Key Words: FOXO3A—myostatin—PGC-1 α —aging—hypertrophy.

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AGING is associated with significant reductions in muscle mass termed sarcopenia (1). Sarcopenia is clinically relevant as it is associated with elevated risk of cardiac, pulmonary, and metabolic disease processes. Due to these health concerns, the importance of developing interventions that reverse the negative consequences associated with aging is essential.

Aerobic exercise is commonly recommended for older adults due to the cardiovascular and metabolic health benefits (2). Despite the prevalence of aerobic exercise, there has been limited scientific inquiry regarding the effects of aerobic exercise on muscle size. We have recently shown that 12 weeks of progressive aerobic training dramatically increases muscle size in addition to improvements in muscle function and aerobic capacity in older women (3). Although these whole-body adaptations have been established, the underlying molecular mechanisms remain to be elucidated.

The ubiquitin–proteosomal pathway is responsible for the majority of protein degradation within skeletal muscle, and our laboratory has recently shown that older women exhibit greater basal proteolytic gene expression (FOXO3A,

MuRF-1, and myostatin) compared with young women (4,5). Additionally, although the influence of aging on mixed skeletal muscle protein breakdown is equivocal (6,7), our laboratory has reported that myofibrillar proteolysis is elevated in older men (8). Due to the elevated myofibrillar proteolysis and related genetic markers in aging skeletal muscle, it is possible that a reduction in these proteolytic mechanisms partially contributes to the improvements in muscle health in response to exercise training.

Thus, the global purpose of this investigation was to examine a cohort of molecular factors within aging skeletal muscle that may be associated with the recently observed enhancements in muscle size and aerobic capacity after an aerobic exercise training program (3). Interestingly, markers associated with muscle oxidative capacity (ie, PGC-1 α) and muscle protein preservation (ie, HSP70) have been recently linked to skeletal muscle health in animal models. Mechanistically, these factors appear to decrease protein breakdown through the ubiquitin–proteosomal pathway by inhibiting FOXO signaling (9–11). Although these relationships have not been examined in human skeletal muscle,

they represent potential mechanisms for the muscle hypertrophy induced by aerobic training. Therefore, we hypothesized that resting levels of HSP70, PGC-1 α , and TFAM (mitochondrial transcription factor A) would be elevated in skeletal muscle of older women after aerobic training and that these muscle oxidative factors will be associated with reductions in proteolytic markers (FOXO3A, MuRF-1, and atrogin-1).

METHODS

Participants

Nine older women (70 ± 2 years) volunteered to participate in this investigation. All participants were cleared to participate by the study physician following a thorough physical examination. Volunteers were excluded based on criteria that have been previously described (3). Written informed consent, approved by the institutional review boards of Ball State University and Ball Memorial Hospital, was obtained from each participant prior to study participation. Data from a subset ($n = 7$) of these participants regarding whole-muscle and single myofiber contractile physiology have been reported previously (3).

Experimental Design and Methodology

Each participant completed the experimental protocol over a period of approximately 15 weeks consisting of several visits to the laboratory for baseline measurements of aerobic capacity, body composition using dual energy x-ray absorptiometry, whole-muscle cross-sectional area, a muscle biopsy, and 42 exercise training sessions (3). All measurements were repeated after the 12-week training protocol.

Aerobic Exercise Training Protocol

Participants performed 12 weeks of aerobic training on a cycle ergometer (Stairmaster Stratus 3300 CE, Kirkland, WA) as previously described in detail (3). A total of 42 exercise sessions were performed. Duration (20–45 minutes), intensity (60%–80% heart rate reserve), and frequency (three or four sessions per week) of exercise were progressively increased throughout the 12 weeks. The last 5 weeks consisted of four 45-minute sessions at 80% intensity per week.

Aerobic Capacity

Participants performed a physician-supervised graded exercise test for the assessment of VO_2max before and after the 12-week aerobic training intervention. The test was performed on an electronically braked cycle ergometer (SensorMedics Ergometrics 800, Yorba Linda, CA) beginning at a very low workload (~ 10 W). After a brief warm-up at the initial workload, the workload was progressively increased 10 W every 1-minute stage until exhaustion with a total test

time of 10–12 minutes. During the test, participant's heart rate, blood pressure, rating of perceived exertion, and electrocardiogram were monitored, and ventilation and expired air samples were measured by a metabolic cart (TrueOne 2400 Metabolic System; ParvoMedics, Inc., Sandy, UT) for the determination of VO_2 .

Whole-Muscle Function

Peak power and peak isometric force of the knee extensor muscle group was assessed before and after the 12-week aerobic training intervention using an inertial ergometer (Inertial Technology, Stockholm, Sweden) connected to a strain gauge load cell and potentiometer interfaced with a personal computer (Gateway E-4200, Irvine, CA). Following multiple orientation sessions with the knee extensor device, participants performed three identical sessions separated by at least 2 days. All tests were bilateral. Prior to any testing, participants performed a 10-minute warm-up on a stationary bicycle at a self-selected intensity followed by small loads on the resistance apparatus. Peak isometric force was assessed at a fixed knee joint angle of 120° . For peak power and peak force, participants completed three submaximal repetitions, followed by three maximal attempts with three minutes rest between sets. The concentric power output was recorded throughout the full range of motion.

Whole-Muscle Size

Before and after the 12-week training intervention, proton magnetic resonance images of the leg were measured using a General Electric Signa 1.5 Tesla imaging system (Milwaukee, WI) at standard settings (TR/TE = 2000/9 milliseconds) as we have previously described (3,12). Images were analyzed in a blinded fashion by the same investigator via NIH Image software (version 1.60). Muscle cross-sectional area was taken as the average of each slice from the first distal image containing the medial rectus femoris and the last proximal image not containing the gluteal muscle. The cross-sectional area (square centimeters) of the whole quadriceps femoris was taken as the sum of the averaged slices for the vastii (vastus lateralis, vastus medialis, and vastus intermedius) and rectus femoris.

Skeletal Muscle Biopsy

Before and after the 12-week aerobic training intervention, a muscle biopsy was obtained from the vastus lateralis of each participant. The posttraining biopsy sample occurred ~ 48 hours after the last exercise session. Tissue was obtained following local anesthetic (Lidocaine HCl 1%) using a 5-mm Bergstrom needle with suction. One piece (~ 15 mg) was placed in RNAlater and stored at -20°C until RNA extraction, whereas the other pieces were immediately frozen and stored in liquid nitrogen.

Gene Expression

Total RNA extraction and RNA quality check.—Total RNA was extracted in TRI reagent (Molecular Research Center, Cincinnati, OH). The quality and integrity (RNA Integrity Number of 8.4 ± 0.07) of extracted RNA (158.1 ± 17.17 ng/ μ L) was evaluated using an RNA 6000 Nano Lab-Chip kit on Agilent 2100 Bioanalyzer (Wilmington, DE).

Reverse transcription and real-time polymerase chain reaction.—Oligo(dT) primed first-strand cDNA was synthesized (150ng total RNA) using SuperScript II reverse transcription (Invitrogen, Carlsbad, CA). Quantification of messenger RNA (mRNA) transcription (in duplicate) was performed in a 72-well Rotor-Gene 3,000 Centrifugal Real-Time Cycler (Corbett Research, Mortlake, New South Wales, Australia). Housekeeping gene GAPDH was used as a reference gene (5). The validation of GAPDH was performed to ensure that its expression was unaffected by the experimental treatment as we have previously described (13,14). All primers used in this study were mRNA specific (on different exons and/or over an intron) and were designed for SYBR Green chemistry using Vector NTI Advance 9 software (Invitrogen). Primers for MyoD, myogenin, muscle regulatory factor 4 (MRF4), atrogin-1, muscle RING-finger protein-1 (MuRF-1), forkheadbox (FOXO)3A, peroxisome proliferator-activated receptor-gamma coactivator 1- α (PGC-1 α), and mitochondrial transcription factor (TFAM) have been reported previously by our laboratory (13–15). The primer sequence for heat-shock protein 70 (HSP70, NM_005345.5) is as follows: forward primer, 5'-CCACGGACAAGAGCACCGGCAA-3', and reverse primer, 3'-CGCCTCCTGCACCATGCGCTC-5'. A melting curve analysis was generated validating that only one product was present. The details regarding reverse transcription and polymerase chain reaction parameters have been reported previously (5,14).

Relative quantitative polymerase chain reaction data analysis.—Relative gene expression analysis comparing the expression of a gene of interest in relation to a reference gene is based on the distinct cycle (Ct) differences. The $2^{-\Delta\text{CT}}$ method (16) was used in this study as described in detail previously (4,5).

Western Blotting

Muscle samples (~20 mg) were homogenized using an electrically powered glass pestle in 40 volumes of cold RIPA buffer (Pierce, Rockford, IL) with Halt Protease Inhibitor Cocktail, Halt Phosphatase Inhibitor Cocktail, and 0.5 M EDTA (Pierce). Proteins (20 μ g) were separated with a 4%–20% gradient gel (Pierce) using sodium dodecyl sulfate–polyacrylamide gel electrophoresis for 90 minutes at 100 V (Mini Protean 3 system; Bio-Rad Laboratories, Hercules, CA) and then transferred to polyvinylidene

Table 1. Body Composition Before (PRE) and After (POST) 12 Weeks of Aerobic Exercise Training

	PRE	POST
Weight (kg)	67.2 \pm 4.1	67.5 \pm 3.9
Body fat (%)	41.4 \pm 2.7	40.4 \pm 2.9*
Fat-free mass (kg)	39.0 \pm 1.1	39.5 \pm 1.0*
Lean body mass (kg)	36.7 \pm 1.0	37.2 \pm 1.0*

Notes: All values are presented in mean \pm SE. * $p < .05$ vs PRE.

fluoride membrane (Immobilon-P; Millipore, Bedford, MA) for 2 hours at 200 mA at 4°C. The membrane was blocked with 5% milk for 60 minutes and then incubated with a human primary antibody (PGC-1 α #2178S, HSP70 #4872, FOXO3A #9467, phospho-FOXO3A Serine 253 #9466, or COX IV #4844; Cell Signaling, Danvers, MA) at 4°C overnight. Blots were identified by incubation with horseradish peroxidase–conjugated secondary antibody (#7074, Cell Signaling) and then exposed to an enhanced chemiluminescent substrate (Pierce ECL Western Blotting Substrate or Amersham ECL Plus Western Blotting Detection System, GE, Milwaukee, WI). Digital images were captured using a chemiluminescent imaging system (FluorChem SP; Alpha Innotech, San Leandro, CA). Phosphorylation of FOXO3A was determined by normalizing the expression of the phosphorylated protein to total protein content. Sizes of the immunodetected proteins were confirmed by molecular weight markers (see Blue & Magic Marker; Invitrogen). Equal protein loading was established by results from a bicinchoninic acid protein assay and confirmed by Ponceau S staining. Also, a control sample was included on each blot to serve as an internal standard to verify uniform transfer and protein detection. Both pre- and posttime points for each participant were analyzed on the same blot to control for interassay variability.

Statistics

Pre- and posttraining values for all variables were analyzed using a paired two-tailed Student's *t* test. Significance was accepted as $p < .05$. All values are presented as mean \pm SE.

RESULTS

Body Composition

All participants maintained body weight ($p > .05$) throughout the training program. Although body weight was unchanged, percent body fat decreased ($p < .05$) while fat-free mass and lean body mass increased ($p < .05$) after training (Table 1).

Aerobic Capacity

Absolute VO₂max increased ($p < .05$) $30 \pm 9\%$ from 1.07 ± 0.05 to 1.38 ± 0.10 L/min. Additionally, maximum workload

Table 2. Whole-Body Adaptations to 12 Weeks of Aerobic Training in Older Women

	PRE	POST
Graded exercise test		
VO ₂ max (L/min)	1.07 ± 0.05	1.38 ± 0.10*
Maximum workload (W)	87 ± 9	122 ± 10*
Whole-muscle size		
Cross-sectional area (cm ²)	40 ± 3	44 ± 3*
Whole-muscle function		
Knee extensor isometric force (Nm)	247 ± 34	301 ± 35*
Knee extensor peak power (W)	273 ± 49	328 ± 50*

Notes: All values are presented in mean ± SE. **p* < .05 vs PRE.

during the graded cycle test increased (*p* < .05) 43% (87 ± 9 to 122 ± 10 W) after aerobic training (Table 2).

Whole-Muscle Size and Function

Cross-sectional area of the quadriceps femoris increased 11 ± 2% (*p* < .05) in response to 12 weeks of aerobic training (Table 2). Functional capacity of the knee extensor muscles, measured as isometric force and peak power during knee extension, improved (*p* < .05) 28 ± 8% and 25 ± 4%, respectively.

Basal mRNA Expression

For the proteolytic genes, FOXO3A was reduced 24 ± 9% (*p* < .05), whereas atrogin-1 and MuRF-1 were unaltered by aerobic training. Myostatin mRNA expression was decreased (*p* < .05) 49 ± 17% after the aerobic training program (Figure 1). Additionally, HSP70 mRNA was 23 ± 8% lower (*p* < .05), whereas PGC-1α and TFAM mRNA were not altered after the training intervention (Figure 2). Resting levels of myogenic gene MRF4 was decreased (*p* < .05) 22 ± 8%, and myogenin trended to decrease (*p* = .09) 11 ± 6% after training (Figure 3).

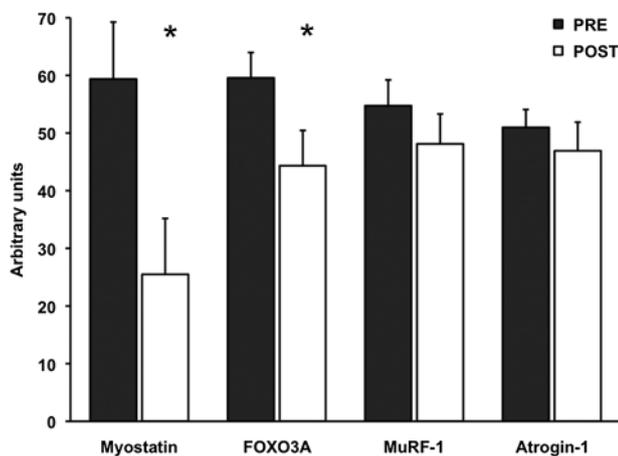


Figure 1. Basal proteolytic mRNA expression before (PRE) and after (POST) 12 weeks of progressive aerobic exercise training. Data are presented in mean ± SE. **p* < .05 compared to PRE.

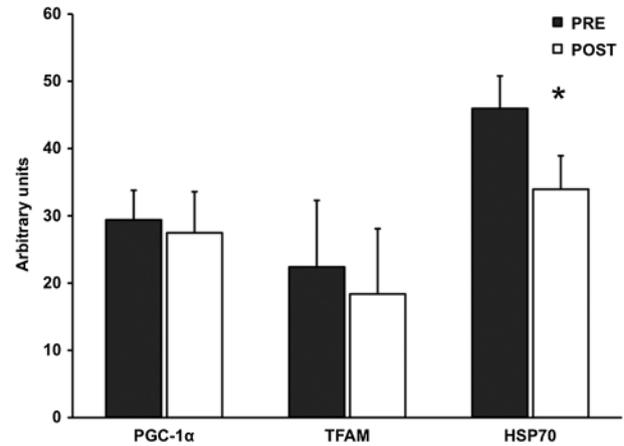


Figure 2. Basal levels of PGC-1α, TFAM, and HSP70 mRNA before (PRE) and after (POST) 12 weeks of progressive aerobic training. Data are presented in mean ± SE. **p* < .05 compared to PRE.

Protein Expression

COX IV protein content increased (*p* < .05) 33 ± 7%, whereas basal levels of PGC-1α protein decreased (*p* < .05) 20 ± 5% after the aerobic training intervention. There were no changes in the protein content of HSP70, FOXO3A, or FOXO3A phosphorylation after 12 weeks of progressive aerobic training in older women (Figures 4 and 5).

DISCUSSION

The purpose of this investigation was to elucidate the cellular factors within skeletal muscle that are associated with improvements of skeletal muscle size, function, and aerobic capacity after an aerobic training intervention in older women. The main findings from this investigation were that aerobic training: (a) reduced basal FOXO3A and myostatin mRNA expression, although FOXO3A total protein content and phosphorylation status remained unchanged; (b)

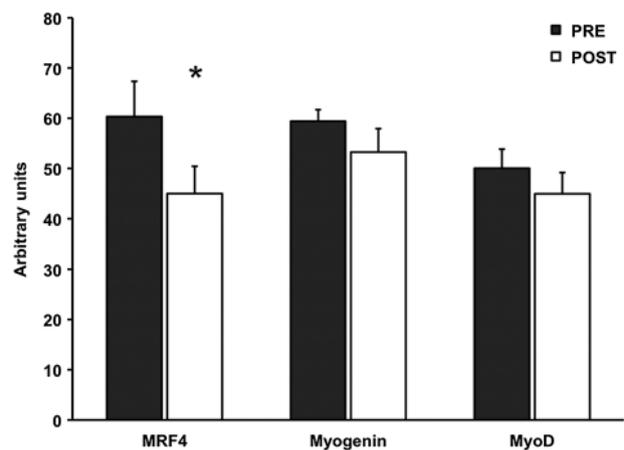


Figure 3. Myogenic mRNA expression before (PRE) and after (POST) 12 weeks of progressive aerobic exercise training. Data are presented in mean ± SE. **p* < .05 compared to PRE.

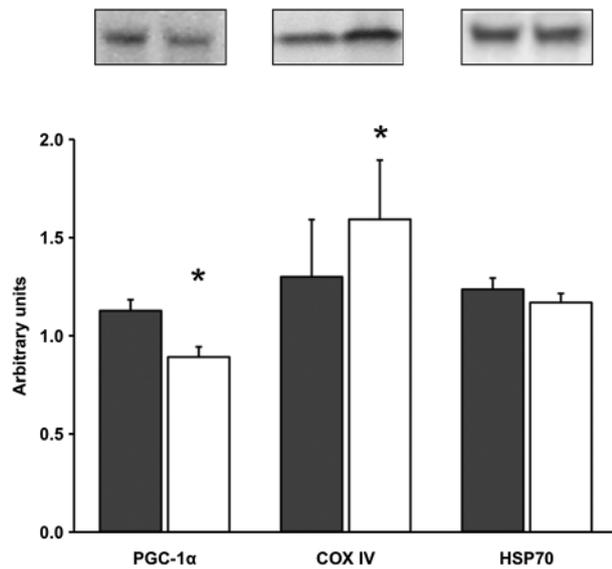


Figure 4. Basal levels of PGC-1 α , COX IV, and HSP70 protein content before (PRE) and after (POST) 12 weeks of progressive aerobic training. A representative Western blot is shown for each respective protein. Data are presented in mean \pm SE. * p < .05 compared to PRE.

reduced basal HSP70 mRNA without a change in HSP70 protein content; and (c) increased mitochondrial protein (COX IV) but reduced PGC-1 α protein content. These findings suggest that downregulation of these proteolytic mechanisms may be partially responsible for the training-induced muscle hypertrophy in older women. Furthermore, these data indicate that improvements in whole-body oxidative capacity and muscle mitochondrial content can be enhanced with aerobic training independent of increases in PGC-1 α .

Molecular adaptations to aerobic exercise training in older participants are limited in the literature. FOXO3A and

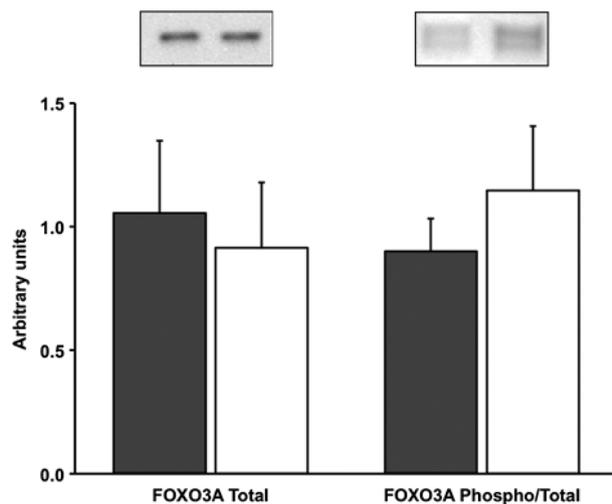


Figure 5. Basal levels of FOXO3A total protein content and FOXO3A phosphorylation normalized to total FOXO3A protein before (PRE) and after (POST) 12 weeks of progressive aerobic exercise training. A representative Western blot is shown for each respective protein. Data are presented in mean \pm SE.

myostatin represent attractive targets because they have been implicated in sarcopenia (4,5,17,18). As hypothesized, we observed a decrease in FOXO3A mRNA after training. We also observed a reduction in myostatin mRNA expression, a known potent inhibitor of muscle growth (19). Data from our laboratory suggest that at rest untrained older women display greater expression of these catabolic genes (FOXO3A and myostatin) and exhibit lower muscle mass than their younger counterparts (4,5). Furthermore, it has been purported that elevated levels of myostatin are associated with skeletal muscle atrophy in aging (18) and chronic obstructive pulmonary disease participants (20), whereas a reduction in myostatin mRNA is associated with muscle hypertrophy following resistance training (21). Although the relationship between FOXO3A and myostatin in sarcopenia (4,5,18) and exercise-induced muscle hypertrophy (21) is not clear, their roles in skeletal muscle mass regulation likely target different aspects of protein balance. A decline of FOXO3A mRNA suggests a reduction in muscle protein degradation that is elevated in aging skeletal muscle (8), whereas a reduction of myostatin may indicate an elevation in protein synthesis (22,23). Therefore, it is possible the reduction of myostatin (ie, increase protein synthesis) and FOXO3A (ie, decrease protein breakdown) may act synergistically to create a favorable environment for the muscle hypertrophy induced by aerobic exercise in older women.

Despite reductions in FOXO3A mRNA, we reported no change in FOXO3A total protein content and phosphorylation status. Generally, alterations in mRNA expression are assumed to represent a new demand placed on the cell for the corresponding protein. However, this assumption is not often verified in human skeletal muscle after acute or chronic exercise. To our knowledge, this is the first study to report decreases in the basal levels of FOXO3A mRNA without changes in related protein content after an exercise training program. Urso et al. (24) have also presented discrepancies between mRNA expression and protein content when investigating skeletal muscle in individuals with spinal cord injury. The reason for this disparity between mRNA and protein levels is not readily apparent. We measured total and phospho-FOXO3A in the whole cell lysate, which does not delineate the specific location (ie, cytosol or nucleus) of FOXO3A within the myofiber. Our laboratory has recently shown that resistance training alters the cellular location of FOXO3A (25), which influences its proteolytic activity (26,27). Fujita et al. (28) have shown that aerobic exercise acutely restores the muscle's anabolic response to insulin through AKT signaling, which may also alter FOXO3A signaling and reduce proteolytic gene expression in the fed state following each exercise session. It should be noted that the posttraining muscle sample was obtained at least 48 hours after the last exercise session in the fasted state to avoid any transient changes of FOXO3A mRNA or signaling from an acute exercise bout as we have previously shown (14,15). For this reason, we are confident

that our measure represents a new steady-state level within the muscle.

PGC-1 α has also been implicated in reducing muscle protein breakdown by attenuating FOXO3A signaling (11). Therefore, we measured PGC-1 α mRNA and protein expression due to its potential role in reducing atrophy through FOXO as well as being a regulator of mitochondrial biogenesis. Interestingly, we observed a 20% reduction in PGC-1 α protein, although COX IV was elevated $33 \pm 7\%$ after training in our older women, suggesting the training program resulted in mitochondrial biogenesis (29). Acute and chronic aerobic exercise has been shown to increase PGC-1 α gene and protein levels in mouse (30,31) and human (32–34) models. PGC-1 α is widely believed to be essential for controlling exercise-induced mitochondrial biogenesis (35). However, there are limited data related to PGC-1 α protein in older participants. A cross-sectional investigation recently reported that PGC-1 α content was higher in skeletal muscle of older aerobically trained than sedentary participants (36). Consequently, we hypothesized that aerobic training would increase PGC-1 α in our older women. Our findings may differ from the previous study due to the dissimilarity in study design and participant training history. However, the most unique difference was our stimulus simultaneously induced muscle hypertrophy and increased aerobic capacity. All the above-mentioned differences between investigations could create divergent environments for adaptations to occur.

Although our observed decrease in PGC-1 α protein was unexpected, recent research has revealed that PGC-1 α protein is not required to initiate adaptations of mitochondrial proteins (37,38). The decrease in PGC-1 α protein is uncharacteristic of aerobic exercise in young participants but potentially relates to the effects of the observed muscle hypertrophy, or the elevation of PGC-1 α protein expression is not mandatory to induce mitochondrial biogenesis (37). Most importantly, on a global aspect, our participants were able to increase their aerobic capacity by $30 \pm 9\%$ without increased PGC-1 α mRNA or protein expression.

We also examined HSP70 mRNA and protein content due to its possible role in the preservation of skeletal muscle during atrophic stimuli through FOXO signaling (10,39). Our data do not support this connection in skeletal muscle of older women as we observed a decrease in HSP70 mRNA, although the related protein content of HSP70 was not affected after 12 weeks of aerobic training. HSP70 is responsive to acute aerobic exercise (40); however, it has been reported that aerobic training status may not influence HSP70 protein levels (41). HSP70 is one essential component in remodeling skeletal muscle by repairing and protecting proteins from future insult (42). Thus, a reduction in HSP70 mRNA may reflect a new steady-state level of reduced basal stress in skeletal muscle of older women after aerobic training.

With regard to the myogenic genes, MRF4 decreased and myogenin trended to decrease after aerobic training. Interestingly, basal levels of MyoD, MRF4, and myogenin are elevated in older participants, presumably to offset the increased basal stress that induces muscle loss seen in sarcopenic muscle (5,43). The decrease in myogenic mRNA (MRF4 and myogenin) in our study could potentially be linked to diminished proteolytic machinery. Thus, there may be less need for MRF4 and myogenin to offset elevated proteolysis regulated by catabolic markers (FOXO3A and myostatin).

The aim of this investigation was to examine basal levels of mRNA expression and related protein content of factors associated with whole-muscle adaptations after aerobic exercise training in older women. Consequently, there are inherent limitations when describing skeletal muscle adaptations before and after a 12-week exercise intervention from a relatively small number of older participants ($n = 9$). Although we based our posttraining biopsy time point on previous investigations (14,25), we acknowledge that acquiring muscle samples 48 hours after training may not have captured alterations in mRNA transcript levels, protein content, or posttranslational modifications that were present at other time points. These limitations reiterate why investigations attempting to connect molecular and whole-muscle adaptations are scarce within the aging literature and warrant additional research.

In conclusion, the current data suggest that aerobic training alters basal levels of growth-related mRNA and proteins within skeletal muscle of older women. More specifically, the decreased mRNA of FOXO3A and myostatin are two factors potentially associated with the exercise-induced muscle hypertrophy. Additionally, these data provide evidence that the enhancement of mitochondrial markers following aerobic training can occur despite decreased PGC-1 α protein in older women. These molecular adaptations following aerobic exercise training support the beneficial adaptations seen in the myocellular and whole-muscle environments (3). Therefore, an aerobic training intervention provides an exercise prescription that dynamically combats the debilitating effects of sarcopenia in sedentary older individuals at the molecular, cellular, and whole-muscle levels.

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