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Effects of different modes and intensities of exercise on longevity proteins in middle-aged mouse skeletal muscle

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ABSTRACT

Physical exercise represents one of the most effective approaches to anti-aging. The goal of this study was to verify the effects of different modes and intensities of exercise on longevity proteins in the skeletal muscle in midlife. Middle-aged mice were trained in aerobic or resistance exercise for 8 weeks, and the changes in sirtuin 1 (SIRT1), adenosine monophosphate-activated kinase (AMPK), and mammalian target of rapamycin (mTOR) pathways in the skeletal muscle were evaluated by western blotting. Long-term exercise had no effects on skeletal muscle SIRT1 abundance, whereas high-intensity aerobic exercise increased AMPK phosphorylation and peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α). Low-intensity resistance exercise facilitated Akt/mTOR/p70 ribosomal protein kinase S6 (p70S6K) signaling but did not induce muscle hypertrophy. Conversely,

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high-intensity resistance exercise stimulated muscle hypertrophy without phosphorylation of mTOR signaling-related proteins. These results suggest the importance of setting exercise modes and intensities for anti-aging in midlife.

KEYWORDS

exercise, skeletal muscle, SIRT1, AMPK, mTOR

INTRODUCTION

Aging is a serious problem, and one in five people in the world will be over 60 by 2050 [1]. Exercise is a potent anti-aging approach in the growing aging population. It has been well known that regular exercise can attenuate the major hallmarks of aging by treating and preventing age-related diseases including diabetes, cancer, cerebrovascular and cardiovascular diseases, dementia, and sarcopenia [2]. Notably, moderate exercise and physical activity in midlife are associated with healthy aging and longevity decades later [3], highlighting the importance of exercise in midlife for anti-aging.

Substantial efforts have been devoted to understanding the cellular and molecular mechanisms of aging to develop strategies targeting aging. Sirtuin 1 (SIRT1), adenosine monophosphate-activated kinase (AMPK), and mammalian target of rapamycin (mTOR) pathways are regarded as the central pathways for the aging process [4]. SIRT1 and AMPK are evolutionarily conserved fuel-sensing molecules that have similar functions in energy balance, cellular survival, and lifespan expansion, which mutually regulate each other [5]. SIRT1 and AMPK phosphorylation at Thr172 are induced by a decrease in a cellular energy state and can enhance mitochondrial biogenesis and function via peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α) [5]. mTOR is involved in cellular growth, differentiation, autophagy, and lifespan by regulating the phosphorylation levels of other protein kinases [6]. Phosphorylation of mTOR at Ser2448 can phosphorylate multiple substrates such as p70 ribosomal protein S6 kinase (p70S6K) and eukaryotic initiation factor 4E binding protein 1 (4E-BP1), leading to increased protein synthesis [6]. In addition, the phosphorylation state of mTOR can be regulated not only by Akt phosphorylated at Ser473 but also by the SIRT1/AMPK pathway [4], which extensively communicate with each other to control various aging-related biological functions. Considered together, elucidating exercise-induced changes in these longevity pathways is of central importance for anti-aging. Many studies in the literature have documented that exercise can affect the longevity pathways in humans [7, 8] and rodents [9]. Acute or chronic aerobic exercise activates the SIRT1 and AMPK pathways [7, 9], whereas the Akt/mTOR signaling pathway is stimulated by resistance exercise [8]. Although the systemic responses to exercise differ by exercise modes and intensities [10], how these differences affect the longevity pathway remains unclear. Understanding exercise modes and intensities to optimize longevity pathway responses is necessary for developing exercise strategies for anti-aging in midlife and beyond.

Skeletal muscle is a power source for locomotion and also functions as an endocrine organ that influences systemic metabolic conditions. The longevity proteins (SIRT1, AMPK, and mTOR) play important roles in modulating age-related skeletal muscle degeneration, leading to chronic diseases and increased mortality [11]. Therefore, regulating the longevity proteins in middle-aged muscle is crucial not only for the skeletal muscle but also for systemic anti-aging. Hence, the goal of this study was to verify the effects of different modes and intensities of exercise on the longevity proteins in the skeletal muscle in midlife. To reach our goal, we examined the changes in the SIRT1, AMPK, and mTOR pathways in the skeletal muscle after long-term aerobic or resistance exercise in middle-aged mice.

MATERIALS AND METHODS

Experimental design

Twenty male C57BL/6J retired breeder mice (9-month-old, Japan SLC Inc., Shizuoka, Japan) were used in this study, which corresponds to the late 30s to early 40s of human age [12]. Male mice were selected to avoid the effects of sex hormones on the skeletal muscle [13]. The mice were randomly divided into 3 groups: control (no exercise), aerobic exercise, and resistance exercise groups. The animals in each exercise group were assigned 2 subgroups, corresponding to the exercise intensity: 12 and 18 m min^{-1} for the aerobic exercise group; 30 and 100% of the body weight (BW) for the resistance exercise group ($n = 4$ per group). The mice were trained once a day for 8 weeks.

All experimental procedures were approved by our institutional animal care and use committee and according to the Kobe University Experimental Regulations (approval number: P180602). The mice were housed in polycarbonate cages with bedding and were maintained under artificial conditions at a constant temperature of 22 ± 1 °C with a constant humidity of 55 ± 5% and a 12-h light-dark cycle. They were allowed free access to standard food and water 24 h a day. All animals were fasted overnight prior to sacrifice and euthanized by exsanguination under general anesthesia and analgesia at the end of the experimental period. Skeletal muscle samples were collected 48 h after the last exercise session.

Exercise protocol

The mice in the aerobic exercise group were trained on a treadmill (MK-680, Muromachi Kikai Co., Ltd., Tokyo, Japan) for 60 min day^{-1}, 7 days $week^{-1}$, for 8 weeks. The treadmill was set at a speed of 12 or 18 m min^{-1} and 0° incline. For the resistance exercise group, the mice were trained 7 days $week^{-1}$ to climb a custom ladder (110 cm in length, 80° incline, 1 cm grid steps) for 8 weeks. A weight equivalent to 30 or 100% of the body weight was attached to the mouse tail. The daily training protocol consisted of 4 sets of 3 repetitions with a 1–2 min rest.

Western blotting

The gastrocnemius muscle was selected for protein quantification because of its diverse fiber composition and pronounced responsiveness to physical exercise [14, 15]. The muscles were homogenized in cold lysis buffer containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1%

Triton X-100, 1 mM EDTA, and 5 mM EGTA with protease inhibitor cocktail (PIC-2, ITSI-Biosciences, PA, USA) and Halt™ phosphatase inhibitor cocktail (Thermo Fisher Scientific, MA, USA). The homogenates were centrifuged at 12,000 g for 15 min at 4 ℃, and the supernatants were collected. Total protein concentration was determined using a BCA protein assay kit (Takara Bio, Shiga, Japan). Protein extracts were mixed with an equal volume of 2X Laemmli buffer and equal amounts (20 μg) of protein were loaded onto Any kD™ Mini-PROTEAN TGX™ precast gels (Bio-Rad, CA, USA). The same batch of 20 samples was run into two gels within the same electrophoresis chamber and then transferred simultaneously to PVDF membranes (Trans-Blot® Turbo™ Transfer Pack, Bio-Rad) using the Trans-Blot Turbo Blotting System. The membranes were then blocked with 5% skim milk in Tris-buffered saline containing 0.1% Tween-20 (TBS-T) for SIRT1, AMPKα, mTOR, PGC-1α, and GAPDH or 2% bovine serum albumin in TBS-T for phospho-AMPK, -mTOR, -Akt, and -p70S6K for 60 min at room temperature. The membranes were incubated overnight at 4℃ with the specific primary antibodies. Antibodies against SIRT1 (#07-131, 1:1000) and PGC-1α (#ab54481, 1:3000) were obtained from Merck Millipore (MA, USA) and Abcam (Tokyo, Japan), respectively. Anti-AMPKα (#2532, 1:2000), phospho-Thr172-AMPK (#2535, 1:1000), mTOR (#2972, 1:1000), phospho-Ser2448-mTOR (#2971, 1:1000), phospho-Ser473-Akt (#9271, 1:500), phospho-Thr389-p70S6K (#9205, 1:1000), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH; #2118, 1:10000) were provided by Cell Signaling Technology (MA, USA). The membranes were washed and incubated for 60 min at room temperature with the secondary antibody (#A16110, Thermo Fisher Scientific). The membranes were then visualized by chemiluminescence using ImmunoStar LD (#290-69904, FUJIFILM Wako Pure Chemical, Tokyo, Japan) and imaged using the OptimaShot CL-420α system (FUJIFILM Wako Pure Chemical). To ensure the reliable quantitative comparison between samples on different gels, the simultaneous transfer, incubation with antibodies, and chemiluminescence imaging were executed for the multiple samples according to the established protocol [16]. The band intensities were quantified using ImageJ software (National Institutes of Health, MD, USA). All western blotting experiments were repeated 3 times.

Statistical analysis

Statistical analyses were conducted with EZR (Saitama Medical Center, Jichi Medical University, Saitama, Japan), which is a graphical user interface for R. The results were compared among all groups with the one-way ANOVA test followed by the Tukey HSD test. All values are presented here as the mean ± standard error. *P* values less than 0.05 were considered significant. A post hoc power analysis for the one-way ANOVA test by the G*Power 3 program [17] was used to confirm that sufficient numbers of animals were used.

RESULTS

Skeletal muscle mass

Body weights were statistically comparable between the groups after 8 weeks of exercise (data not shown). The ratio of the gastrocnemius wet weight to body weight tended to increase in the

100% BW group compared to the control group after 8 weeks of resistance exercise ($P = 0.08$, Power = 0.80) (Fig. 1A). The tibialis anterior and soleus muscle masses were not different among groups (Fig. 1B and C).

Changes in longevity protein levels in skeletal muscle

The abundance of longevity proteins was determined by western blotting of the gastrocnemius (Fig. 2A). Total SIRT1 and AMPK protein abundance did not change significantly after 8 weeks of exercise (Fig. 2B and C). The phosphorylated AMPK levels significantly increased in the 18 m min^{-1} group compared to the control and 30% BW groups ($P = 0.04$, Power = 0.80) (Fig. 2D). The phosphorylation levels of the 12 m min^{-1} group were also higher than that of the control group, but the differences were not significant ($P = 0.10$, Power = 0.80). Aerobic exercise at 18 m min^{-1} tended to increase the PGC-1α protein abundance compared with the control group ($P = 0.10$, Power = 0.81) (Fig. 2E).

After 8 weeks of resistance exercise, the phosphorylated Akt levels tended to be higher in the 30% BW group than in the control and 12 m min^{-1} groups ($P = 0.09$ and 0.08, respectively; both Power = 0.80) (Fig. 2F and G). Total mTOR protein abundance was comparable between groups (Fig. 2H). The phosphorylation of mTOR increased after resistance exercise at 30% body weight compared to the control group ($P = 0.04$, Power = 0.80) (Fig. 2I), and its phosphorylation level tended to be higher than the 100% BW group ($P = 0.10$, Power = 0.80). Resistance exercise at 30% BW increased the p70S6K phosphorylation level when compared to the control group, although this was not statistically significant ($P = 0.07$, Power = 0.80) (Fig. 2J).

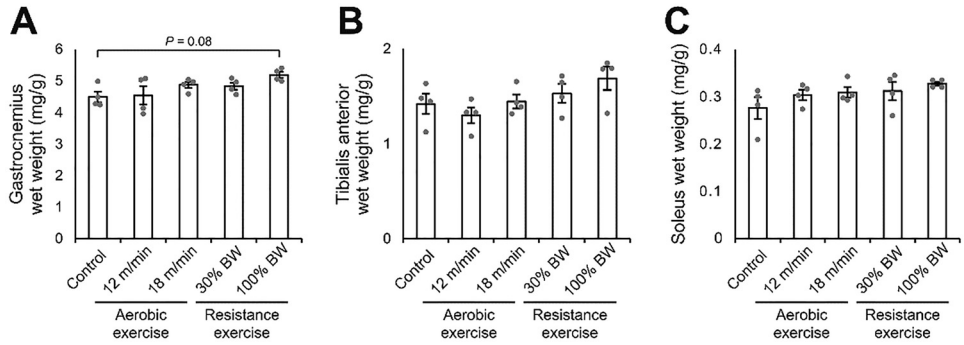

Fig. 1. The graphs show the ratio of (A) the gastrocnemius, (B) tibialis anterior, and (C) soleus muscle wet weight to body weight after 8 weeks of exercise. $n = 4$ mice per group. Data are presented as mean ± standard error. Dot plots represent individual data

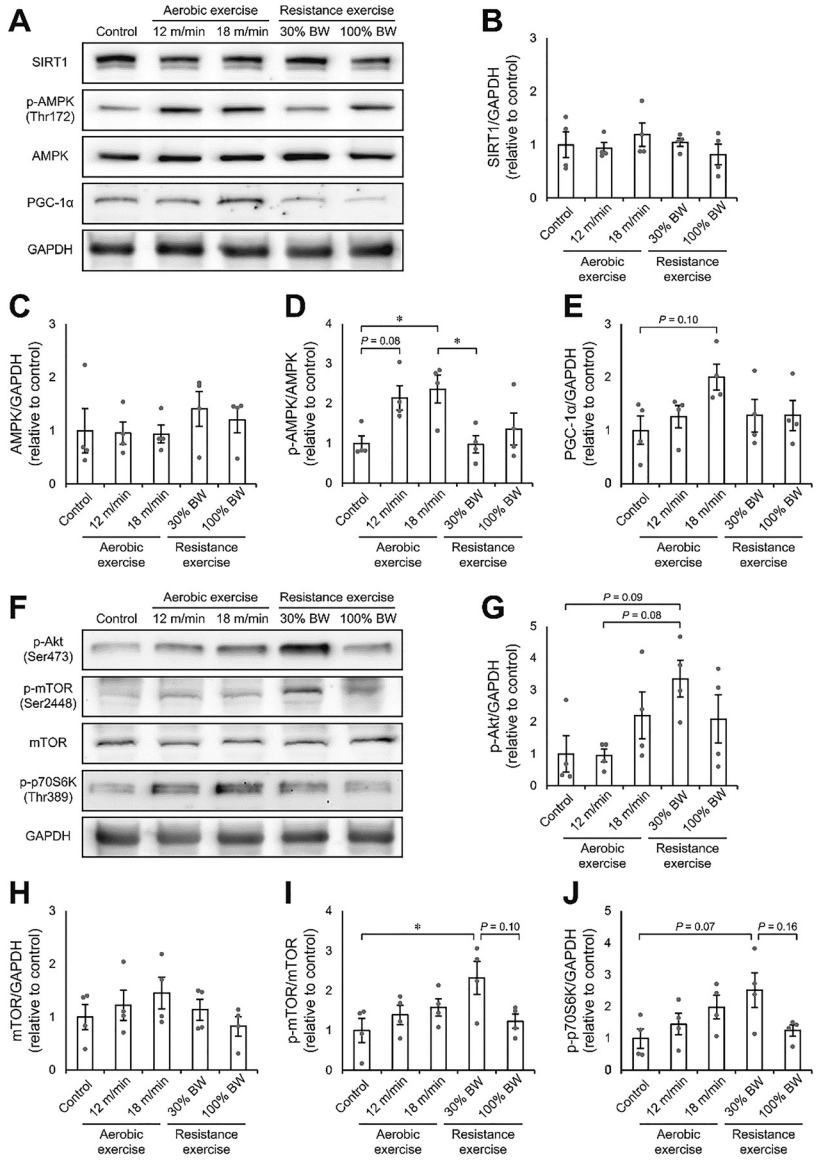

Fig. 2. (A) Representative blots of SIRT1 and AMPK pathway-related proteins in the gastrocnemius after 8 weeks of exercise are shown. The graphs show the band intensities of (B) SIRT1, (C) AMPK, (D) phosphorylated AMPK (Thr172), and (E) PGC-1α. (F) Representative blots of mTOR pathway-related proteins in the gastrocnemius are shown. The graphs show the band intensities of (G) phosphorylated Akt (Ser473), (H) mTOR, (I) phosphorylated mTOR (Ser2448), and (J) phosphorylated p70S6K (Thr389). The band intensities were normalized to the intensity of GAPDH. $n = 4$ mice per group. Data are presented relative to respective controls as mean ± standard error. Dot plots represent individual data. The asterisk (*) indicates $P < 0.05$

DISCUSSION

This study investigated the effects of aerobic or resistance exercise with different intensities on the longevity pathways in middle-aged muscle. Our results showed that long-term exercise did not affect SIRT1 protein abundance, and AMPK phosphorylation was induced by high-intensity aerobic exercise, but not by resistance exercise. We also found that low-intensity resistance exercise stimulated the Akt/mTOR pathway but did not induce skeletal muscle hypertrophy. Conversely, high-intensity resistance exercise stimulated muscle hypertrophy without increasing Akt, mTOR, and p70S6K phosphorylation in middle-aged muscle.

SIRT1 is linked to attenuating cellular senescence and extending lifespan [18]. Long-term aerobic exercise increased SIRT1 protein abundance in the skeletal muscle [9]. However, our results showed no effects of long-term exercise on SIRT1 in the skeletal muscle, which corresponded well with a previous report [19]. Aerobic exercise can enhance systemic antioxidant capacity, whereas SIRT1 protein abundance does not correlate with exercise-stimulated muscle oxidative capacity [19]; and therefore the role of SIRT1 in the muscle remains debatable. Additionally, an increased SIRT1 level after exercise was seen in young rats (3-month-old), but not aged rats (18-month-old) [20]. Middle-aged mice may similarly be less responsive to exercise-induced SIRT1 activation due to aging. Given that SIRT1 is highly sensitive to cellular nutritional status [18], exercise strategies with nutritional supplementation may be required to activate SIRT1 in midlife and beyond.

AMPK is a crucial factor for skeletal muscle metabolism and aging regulation [21]. Consistent with a previous report [7], high-intensity aerobic exercise increased the level of phosphorylated AMPK Thr172 in the skeletal muscle. AMPK phosphorylation at Thr172 is normally induced by high-intensity exercise at more than 60% VO_2 max [7]. In our exercise protocol, treadmill speeds at 12 and 18 m min^{-1} were predicted below and above 60% VO_2 max intensities, respectively, which confirmed previous findings of intensity-dependent increase in phosphorylated AMPK. AMPK phosphorylation at Thr172 transduces exercise-induced signals to PGC-1α, the master regulator of mitochondrial biogenesis, through post-translational modifications [21]. Likewise, in our study, the PGC-1α protein abundance increased only after high-intensity aerobic exercise, suggesting the beneficial effects of this type of exercise on the oxidative phosphorylation in muscle. We also found that the level of phosphorylated AMPK induced by high-intensity aerobic exercise was higher than that induced by low-intensity resistance exercise. This result supports the findings that aerobic-like muscle contractions activate AMPK, but not resistance-like contractions [22]. In contrast to our results, some researchers observed that AMPK phosphorylation levels were comparable between aerobic and resistance exercise [23]; that is, responses of different exercise modalities to AMPK are still controversial. Our results clearly show that high-intensity aerobic exercise increases AMPK phosphorylation and PGC-1α in skeletal muscle, whereas resistance exercise does not, suggesting that this particular type of exercise can improve energy metabolism in midlife and beyond.

The Akt/mTOR signaling pathway has been implicated in various cellular processes that modulate skeletal muscle hypertrophy, aging, and even lifespan [6]. Our low-intensity resistance exercise increased the phosphorylation of Akt Ser473 and mTOR Ser2448, in accordance with a previous report of resistance exercise with 35% of body weight loading [24]. Phosphorylation of Akt Ser473 and mTOR Ser2448 is required for their signaling function, resulting in the phosphorylation of downstream effector proteins such as p70S6K at Thr389 [6]. Similarly, p70S6K

phosphorylation was increased only by low-intensity resistance exercise in this study. Although increased phosphorylation of p70S6K is well known to induce skeletal muscle hypertrophy by promoting mRNA translation and thereby increasing protein synthesis [6], muscle hypertrophy was not observed after low-intensity resistance exercise in this study. Excessive phosphorylation of mTOR signaling-related proteins, including p70S6K at Thr389, is observed in aged skeletal muscle [25] and contributes to various age-related diseases [6]. Moreover, rapamycin, an mTOR inhibitor, extends the lifespan [6]. Therefore, the exercise-induced increase in phosphorylation of mTOR-related proteins in middle-aged muscle may not necessarily result in superior outcomes in terms of muscle mass and lifespan. Unlike low-intensity resistance exercise, high-intensity resistance exercise promoted skeletal muscle hypertrophy without increasing Akt/mTOR/p70S6K phosphorylation, similarly to previous reports in aged rats [26]. Supporting these findings, a recent report demonstrated that mTOR signaling is unnecessary for protein synthesis [27]. Other pathways may be involved in skeletal muscle hypertrophy in midlife and beyond (e.g., the mitogen-activated protein kinase [MAPK] or proteolysis pathway) [26, 28]. Taken together, our results suggest that high-intensity resistance exercise is beneficial for the maintenance and increase of skeletal muscle mass via mTOR-independent mechanisms in midlife.

A potential limitation of this study is that it only focused on exercise effects in midlife. Our results suggest optimal exercise modes and intensities for longevity protein responses in midlife, but it remains unclear whether these responses in midlife lead to healthy aging and longevity. On the other hand, there is evidence that exercise habits and physical activity in midlife are linked to successful aging and longevity decades later [3]. Our findings in middle-aged mice may shed light on the molecular mechanisms underlying this evidence. Determining the effectiveness of exercise-induced changes in the longevity proteins on healthy aging and longevity requires a future longitudinal study from midlife to older adulthood.

Our results demonstrate that high-intensity aerobic exercise increases AMPK phosphorylation and PGC-1α in the skeletal muscle and may contribute to enhancing systemic energy metabolism in midlife. We also show that high-intensity resistance exercise can induce skeletal muscle hypertrophy without enhancing the Akt/mTOR/p70S6K signaling. Our findings highlight the importance of exercise in midlife for anti-aging, especially in setting exercise modes and intensities.

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