ORIGINAL ARTICLE

Autophagic response to a single bout of moderate exercise in murine skeletal muscle

Yong An Kim · Young Sang Kim · Wook Song

Abstract The effect of a single bout of exercise on autopahgy in murine gastrocnemius muscle was investigated. Autophagy is a process for the degradation system of cytoplasmic components, which may help maintain intracellular quality control of cell survival and turnover under normal conditions. The present study investigated the changes of autophagy-related proteins including microtubule-associated protein 1b light chain 3 (LC3), Beclin-1, Atg7 (autophagy-related gene 7), conjugation form of Atg12 to Atg5, lysosome-associated membrane protein (LAMP2a), and muscle-specific RING finger protein-1 (MURF-1) protein level in gastrocnemius muscle after a single bout of treadmill exercise. Mice exercised on a treadmill for 50 min at a speed of 12.3 m/min with a slope of 5°. The animals were sacrificed by cervical dislocation 0, 3, 6, or 12 h after exercise, and muscle samples were collected immediately. Western blot analysis demonstrated that the autophagy marker LC3-II was significantly decreased during the recovery period (3, 6, and 12 h) whereas there was no decrease immediately after exercise (0 h). To identify factors related to this decrease, autophagosome component proteins were examined in murine gastrocnemius muscle. A decrease in Beclin-1, Atg7, and LAMP2a during recovery period was concomitant with the decreased level of LC3-II. Additionally, MuRF-1 expression was significantly increased after a single bout of exercise. This study is the first to demonstrate autophasic related protein expression after a single bout of treadmill exercise and our results suggest that a single bout of treadmill exercise attenuates the autophagic response in murine skeletal muscle.

Keywords Autophagy · Single bout of moderate exercise · Skeletal muscle

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Introduction

Skeletal muscles, which comprise about 40% of body weight and are abundant in protein, can serve as a major amino acid source during energy-depleting stress. Muscle is influenced by the balance of protein synthesis and protein degradation. Importantly, the proteolytic systems including the lysosomal system, caspase system, and the proteasome can produce alternative energy substrates that are used by the cell to maintain internal homeostasis in conditions of energy

stress. Autophagy is a ubiquitous catabolic process that accompanies the bulk degradation of cytoplasmic components through a lysosomal pathway. Autophagy functions in catabolism condition and the cellular processes of the degradation of macromolecules and organelles, and is activated under nutritional stresses of skeletal muscle, such as metabolic stress [4]. These autophagic functions are required for cellular survival and for the clearance of damaged proteins and altered organelles [6, 16]. Under normal conditions, the intracellular amino acid pool can be maintained by the proteasome, which continuously degrades cytoplasmic proteins. In contrast, this pool is largely maintained by autophagy during starvation [17]. The primary role of this basal autophagy is very important for intracellular quality control through the constitutive turnover of cytoplasmic components [10].

The initial step in autophagy is the formation of autophagosomes [4]. This formation begins with the engulfment of damaged cytoplasmic material and sequestration of the material in double-membrane vesicles. This regulatory process involves the association of Atg13 from a protein complex containing Atg1 kinase and Atg17. When mTOR is inhibited, re-association of dephosphorylated Atg13 with Atg1 stimulates its catalytic activity and induces autophagy. Vesicle nucleation depends on the formation of a multi-protein complex involving Beclin-1. Beclin-1 forms a complex with the class III phosphatidyl–inositol 3 kinase (PI3-kinase) and vacuolar protein sorting 34 (Vps34) [2]. Subsequent vesicle elongation involves the covalent conjugation of Atg12 to Atg5, with the help of the E1-like enzyme Atg7 and the E2-like enzyme Atg10. This pathway also participates in the conjugation of phosphatidylethanolamine (PE) to microtubule-associated protein 1b light chain 3(LC3) by the sequential action of the conversion of the soluble form of LC3 (LC3-I) to the autophagic vesicle-associated form (LC3-II) that is required for membrane expansion. LC3-II is used as a marker of autophagy because its lipidation and specific recruitment to autophagosomes provides a shift from diffuse to punctate staining of the protein and increases its electrophoretic mobility on gels compared with LC3-I [1, 4, 5]. Autophagosomes undergo maturation by fusion with lysosomes to create autolysosomes, and LAMP2a is likely to play a role in this process as a lysosomal membrane protein [5]. Fusion of the autophagosome with a lysosome provides hydrolases [4].

Recent evidence has indicated that the growth-promoting pathways and autophagy are coordinately regulated. Atg7 is an ubiquitin E1-like enzyme homolog that is required for conjugation of PE to LC3-I, and conjugation of Atg12 to Atg5 (hereafter referred to as Atg12 + Atg5). Impaired autophagy of Atg7 null muscles is characterized by muscle atrophy, weakness, and features of myofiber degeneration. Thus, autophagy was implicated as being essential for myofiber maintenance and for the clearance of damaged proteins and altered organelles [7]. In addition, muscle-specific RING finger protein-1 (MURF-1) is also shown to be involved in the regulation of myofibril replacement and turnover [8]. However, MURF-1 expression with the autophagy regulation in response to exercise is largely unknown.

Catabolic pathways are accelerated during exercise to supply the energy and substrates to muscle for continuation of contractions. It is well established that the rates of amino acid oxidation and glucose are increased during endurance exercise and increased energy consumption is likely required for autophagy induction. However, it is currently unknown whether or not autophagy is changed with a single bout of exercise in skeletal muscle. The present study addressed this issue by examining the expression of light chain 3 (LC3), autophagy-related genes, lysosome-associated membrane protein 2a (LAMP2a), and MURF-1 in mice gastrocnemius muscle after a single bout of treadmill exercise.

Materials and methods

Animals

Male ICR mice 3 months of age were obtained from Daehan Biolink (Eumseong, Korea). Mice were provided with chow and water ad libitum. All animal procedures were approved and guided by the Committee for Experimental Animal Care and Use at Chungnam National University.

Exercise protocol

Running exercise was performed on a rodent treadmill. Mice were forced to run on a treadmill for 10 min at a speed of 10 m/min at a slope of 5° once a day for 2 days to adapt them to running exercise before the start of the main experiments. The running exercise was carried out between 7:00 p.m. and 8:00 p.m. On day 3,

the mice were exercised on a treadmill for 50 min at a speed of 12.3 m/min with a slope of 5°. The animals were sacrificed by cervical dislocation 0 ($n=6$), 3 ($n=6$), 6 ($n=6$), or 12 h ($n=6$) after exercise, and muscle samples were collected immediately. The removed gastrocnemius skeletal muscle was rinsed in ice-cold saline, weighed, and quickly frozen in liquid nitrogen. As a contro ($n=6$)l, muscle was collected from a group of mice after the 2-day adaptation phase.

Western blot analysis

Frozen gastrocnemius muscle was powdered using a mortar and pestle, and then homogenized in tissue extraction reagent I (Invitrogen, Carlsbad, CA). The homogenized sample was centrifuged at $12,000 \times g$ for 10 min, and the protein content in the extracted supernatants was measured using the Bradford method. The supernatants of each sample were adjusted to 2 μg/μl with lysis buffer (tissue extraction reagent I), and then 80 μl of each sample was mixed 20 μl of a 5× sodium dodecyl sulfate (SDS) sample buffer. The mixture was boiled for 5 min and quickly transferred into ice. The protein samples separated by 40% SDS–polyacrylamide gel electrophoresis and the resolved proteins were blotted onto a polyvinylidene fluoride membrane (Schleicher and Schuell, Keene, NH), followed by blocking overnight in 10 mM Tris, pH 7.0, 150 mM NaCl (TBS) containing 5% skim milk at 4°C. After gentle shaking of the blocking solution for 1 h at room temperature, each membrane was washed three times in TBS+0.05% Tween-20 (TBST) and incubated 24 h at 4°C with the particular dilutions of primary antibody. After washes in TBST, each membrane was incubated for 1 h at room temperature in TBST containing a

1:20,000 dilution of horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG. Following this, the membranes were washed as above and developed in chemiluminescent HRP substrates (Millipore, Billerica, MA). Rabbit anti-Beclin-1 (Atg6; Santa Cruz Biotechnology, Santa Cruz, CA; 1:1,500 dilution), rabbit anti-LC3B (microtubule-associated protein 1B light chain 3, Atg8; Cell Signaling, Danvers, MA; 1:1,000 dilution), rabbit anti-Atg7 (Cell Signaling; 1:1,000 dilution), rabbit anti-Atg12 + Atg5 (Cell Signaling; 1:800 dilution), rabbit anti-LAMP2a (Abcam, Cambridge, UK; 1:500 dilution), and rabbit anti-MURF-1 (Cell Signaling; 1:2,000 dilution). The acquired images were digitized and analyzed using AlphaEase FC software (Alpha Innotech, Santa Clara, CA).

Statistical analysis

All data are reported as the mean ± SEM. Statistical analysis was performed using SPSS 12.0 software (SPSS, Chicago, IL). A one-way repeated ANOVA was employed to detect differences among control (CON) and test animals at 0, 3, 6, and 12 h. For all tests the significance level was set at $p < 0.05$.

Results

LC3-II was significantly decreased at all time points after exercise compared to the control group ($p < 0.05$), with the lowest level evident at 3 h. The decrease was not significant at 0 h but was at 3, 6, and 12 h compared to control ($p < 0.05$; Fig. 1). The level of Beclin-1 protein was also significantly decreased immediately after exercise, and also at 3, 6, and 12 h compared to the control
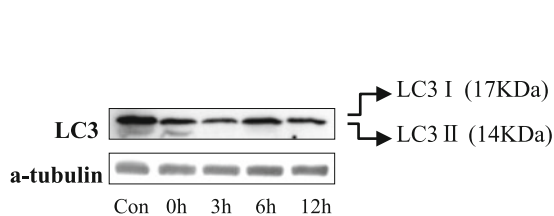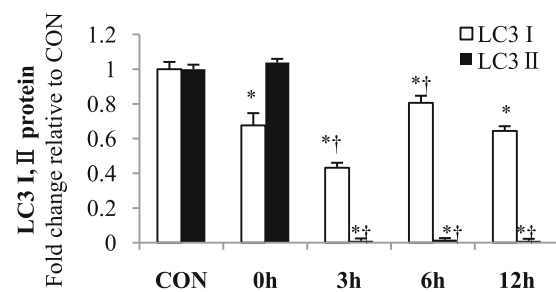

Fig. 1 LC3 protein expression in gastrocnemius muscle after exercise. LC3 protein levels were expressed as a fold change relative to CON at 0, 3, 6, and 12 h following treadmill exercise.

* indicates significantly different from CON ($p < 0.05$); † significantly different from 0 h level ($p < 0.05$)

group ($p<0.05$). However, the level of reduction progressively lessened with recovery time (0 h versus 3, 6, and 12 h; $p<0.05$; Fig. 2a). Atg7 protein was significantly decreased at all time points after exercise compared to the control group ($p<0.05$), and was lowest at 6-h post-exercise (Fig. 2b). Atg12 covalent conjugation form with Atg5 expression differed significantly from that of the control group at all time points ($p<0.05$). However, there was not a consistent trend in the expression level during the recovery period (Fig. 2c). The expression of LAMP2a was similar at 0 h compared to the control group. However, at 3, 6, and 12 h of recovery after exercise, the level

of LAMP2a expression was significantly decreased compared to the control group ($p<0.05$; Fig. 3). MuRF-1 protein was significantly decreased at 0 and 3 h compared to the control group. But, the levels at 6 and 12 h were significantly increased compared to the control group and at 0 h ($p<0.05$; Fig. 4).

Discussion

The major finding of our study is that the autophagy marker LC3-II was significantly decreased after a single
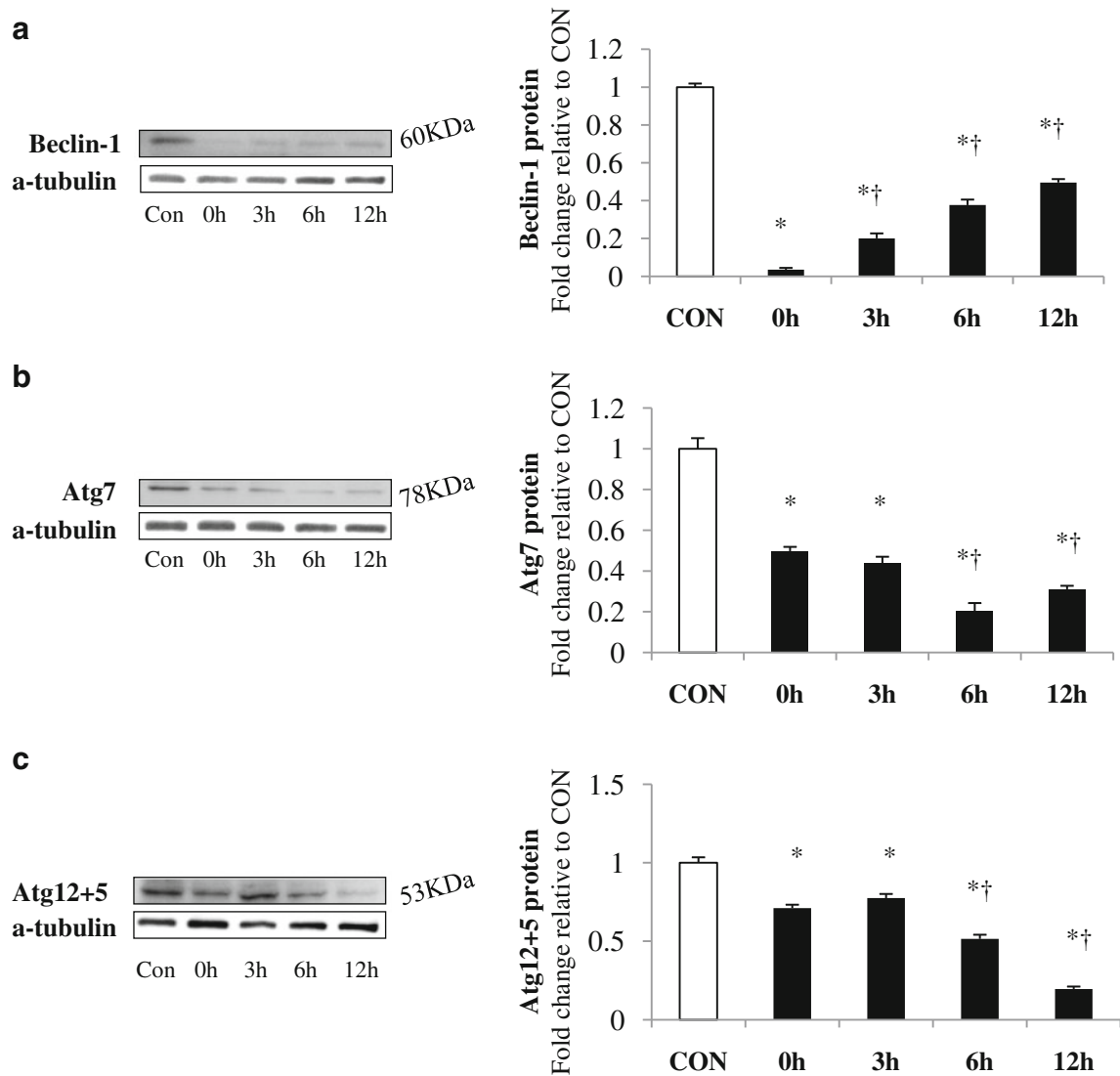

Fig. 2 Autophagy-related protein expression in gastrocnemius muscle after exercise. **a** Bechin-1, **b** Atg 7, **c** Atg 12 conjugate Atg 5. Protein levels were expressed as a fold change relative to CON at 0, 3, 6, and 12 h following treadmill exercise. * indicates significantly different from CON ($p<0.05$); † significantly different from 0 h level ($p<0.05$)
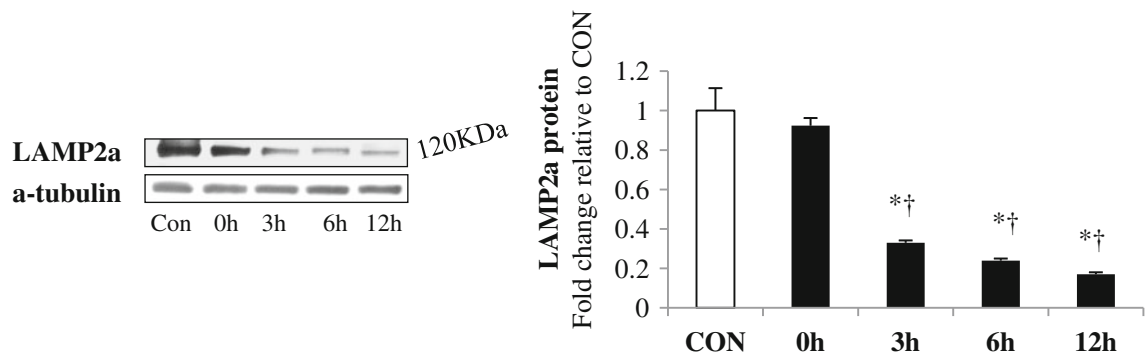
Fig. 3 LAMP2a protein expression in gastrocnemius muscle after exercise. LAMP2a protein levels were expressed as a fold change relative to CON at 0, 3, 6, and 12 h following treadmill exercise. * indicates significantly different from CON ($p<0.05$); † significantly different from 0 h level ($p<0.05$)

bout of treadmill exercise during the recovery period, and expressions of autophagosome component proteins including Beclin-1, Atg7, and LAMP2a were concomitantly decreased with the level of LC3-II. The current study is the first to demonstrate autophasic related protein expression after a single bout of exercise. The results suggest that a single bout of treadmill exercise attenuates autophagic response in murine skeletal muscle.

Autophagy in skeletal muscle is peculiar when compared to other important metabolic tissues such as the liver and pancreas. During fasting most tissues show a transient activation of autophagy that only lasts a few hours. But, autophagy is not required for glycogen transport to lysosomes in skeletal muscle [12]. In contrast, skeletal muscle shows a persistent generation of autophagosomes that continues for days [11, 16]. Therefore, the maintenance of autophagy is important for skeletal muscle homeostasis.

In the present study, LC3-II protein expression, as a major marker of autophagy, decreased significantly compared to control during the recovery from exercise after a single bout of treadmill (50 min running) exercise. In addition, LC3-I protein expression also markedly decreased even immediately after (0 h) a single bout of exercise. It is well established that the expression of LC3-II is strongly correlated with the number of autophagosomes, and increased LC3-II levels can be associated with either enhanced autophagosome synthesis or reduced autophagosome turnover, perhaps due to delayed trafficking to the lysosomes, reduced fusion between compartments, or impaired lysosomal proteolytic activity [4]. The autophagosome turnover reduced by increased LC3-II accompanies accumulated LC3-I upon the impairment of autophagic flux [1]. Since, in our results, decreased LC3-I expression accompanied the decreased level of LC3-II (Fig. 1), it is speculated that autophagic flux is likely not impaired after a single bout of exercise.
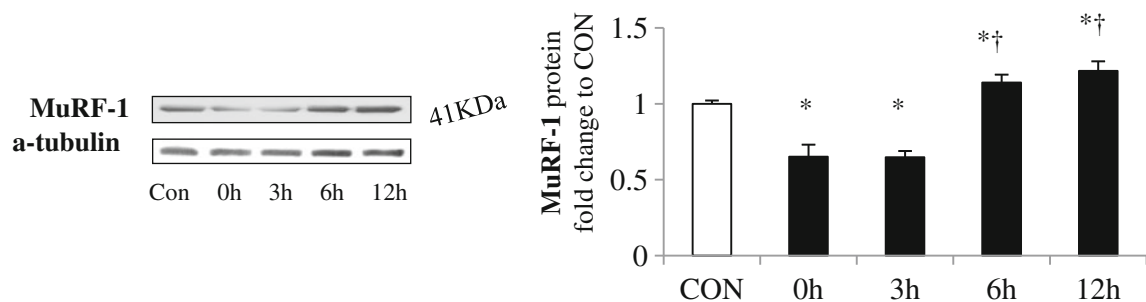

Fig. 4 MuRF-1, muscle-specific ubiquitin-related protein expression in gastrocnemius muscle after exercise. MuRF-1 protein levels were expressed as a fold change relative to CON at 0, 3, 6, and 12 h following treadmill exercise. * indicates significantly different from CON ($p<0.05$); † significantly different from 0 h level ($p<0.05$)

Subsequently, in order to determine potential mechanisms by which LC3-II was decreased after an acute bout of exercise, the protein expressions of autophagosome-related proteins including Beclin-1, Atg7, and Atg12+Atg 5 were measured. As hypothesized, levels of Beclin-1, Atg7, and Atg12 + Atg5 were significantly decreased immediately after a single bout of exercise and even during recovery period (3–12 h; Fig. 2a–c).

Beclin-1 is involved in an early phase of autophagy formation [2, 18]. Consistent with this context, the level of protein was dramatically decreased immediately after exercise (0 h) and the magnitude of decrease became less with recovery period and was not fully recovered, even at 12 h (Fig. 2a).

While Beclin-1 is involved in autophasic vesicle formation, Atg7 plays a role in autophasic vesicle elongation. Consistent with the expression of Beclin-1, we found that Atg7 protein expression was decreased after a single bout of exercise until 12 h of recovery. In addition, since Atg7 is an ubiquitin E1-like enzyme homolog that is required for conjugation of Atg12 to Atg5, the trend of decrease of conjugation form Atg12 + Atg5 is concomitant with the decrease of Atg7 (Fig. 2b and c).

Since LAMP2a, as a receptor protein in the lysosomal membrane [5], is involved in autophagosome maturation where lysosomes are fused with autophagosome resulting in lysis of the inner membrane and breakdown of the inner components of autophagosome, the protein level of LAMP2a was determined subsequent to the levels of autophagosome-related proteins. Consistent with the expression of autophagosome-related proteins, LAMP2a expression significantly decreased at 3, 6, and 12 h of recovery after a single bout of exercise (Fig. 3). However, no difference was found in the expression of LAMP2a at 0 h compared to control group, which contrasted with the other autophagosome-related protein expression.

Recently, muscle-specific Atg7$^{-/-}$ mice (blocking of autophagy) has revealed that the muscle-specific and atrophy-related ubiquitin-ligases atrogin-1/MAFbx and MuRF1 are basally overexpressed suggesting compensation of autophagy by induction of other degradation mechanism [7]. In order to determine whether MuRF1 expression changed with an acute bout of exercise, we measured MuRF-1 expression. Interestingly, MuRF-1 protein expression was significantly increased after a single bout of exercise, contrary to the results of LC3-II and autophagosome-related proteins (Fig. 4). Our results also suggest that there

should be a compensation of autophagy by induction of another protein degradation system regulated by MuRF-1. However, further study applying exercise of long duration or high intensity will be required to elucidate the mechanism of autophagy regulation with exercise, since the exercise protocol in this study should not have been long or intense enough to induce proteolysis.

Growing evidence suggests that autophagy can be induced during deprivation and starvation status where turnover system is adopted to reuse degraded cellular components in order to replenish insufficient energy source [10]. However, since an acute bout of exercise applied in our study was the condition where energy consumption was relatively temporal and energy stored in the cell would not be used up, it is considered that marker of autophagy was not induced.

Autophagy is known to be attenuated with aging because autophagy plays a pivotal role in cellular quality control and maintenance of homeostasis [3, 9, 13–15, 19–21]. A recent study showed that mild caloric restriction can increase the expression of Beclin-1, Atg 7, and LC3-II, and that the additive effect of life-long voluntary wheel running when combined with caloric restriction (8%) was not detected in 24-month-old rats compared to 6-month-old rats [20]. However, limitation of the study was that exercise only group was not included, making it difficult to interpret the effect of exercise separated from caloric restriction.

Conclusion

In summary, in the present study, a single bout of moderate treadmill exercise induced the attenuation of autophagy and expression of autophagosome-related proteins in murine gastrconemius skeletal muscle. Further study will be required to elucidate how acute and long-term exercise regulates the autophagy process in skeletal muscle.

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