Effect of a Single Bout of Exercise on Autophagy Regulation in Skeletal Muscle of High-Fat High-Sucrose Diet-Fed Mice

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Background: Autophagy maintains metabolic homeostasis of muscles, and its impairment may cause muscle dysfunction. Exercise can improve muscle dysfunction induced by long-term high-fat diet. This study aimed to explore the association of autophagy with impaired muscle dysfunction in obese conditions and investigate its relationship with exercise-induced muscle function improvement.

Methods: Male C57BL/6 mice (n=24) were randomly assigned to four groups: low-fat diet+plain water feeding sedentary (CON) group, low-fat diet+plain water feeding exercise (CON+EX) group, high-fat high-sucrose (HFHS) diet-fed sedentary group, and HFHS diet-fed exercise (HFHS+EX) group, and subjected to a single bout of exhaustive exercise.

Results: HFHS diet resulted in shorter hanging time, reduced grip force, and lower exhaustion time and distance, and decreased lean mass per body weight. Moreover, in the soleus, which is chosen as a representative red (oxidative) muscle, LC3II/LC3I ratio, P62, and Bnip3 levels were altered following the HFHS diet, and were negatively correlated with muscle performance parameters; exercise significantly decreased the LC3II/LC3 ratio while P62 increased with HFHS diet. Autophagy-related protein changes were not found in the white (glycolytic) gastrocnemius.

Conclusion: The study revealed that 20-week HFHS diet causes a significant increase in body weight and fat mass, along with a decrease in muscle function. Autophagy-related LC3 and P62 protein expression was negatively correlated with muscle function, and they were reduced when a single bout of exercise stimulated the soleus of obese mice. However, no change of autophagy-related proteins was seen in the gastrocnemius.

Key words: Acute exercise, High-fat diet, Sucrose, Autophagy, Muscle, Obesity

INTRODUCTION

Higher muscle mass is linked with better physical function, representing a better quality of life.¹ An unhealthy diet (especially those which have a high content in fats, free sugars and salt) is a high-risk factor for causing metabolic diseases such as metabolic syndrome, obesity, diabetes, and even increased mortality.²,³ Long-term high-fat diet contributes to the catabolism of muscle tissue, declined muscle fiber area, mitochondrial dysfunction, and affects exercise capacity and muscle growth, possibly leading to muscle fiber atrophy at an age preceding prevalent sarcopenia.⁴,⁵ Exercise has proven to be an effective intervention to reverse...
metabolic syndrome. Growing evidence show that regular exercise could reduce abdominal fat deposits, change the body composition, recover insulin resistance, and improve muscle function. Although mechanisms underlying some of these effects of exercise are well documented, that underlying muscle function improvement in obesity remains to be clarified. Studies have indicated that beneficial metabolic effects of exercise are unrelated to activated autophagy through AMP-activated protein kinase (AMPK) and sestrin interaction. Autophagy has been documented to mediate chronic exercise-induced metabolic benefits and exercise-induced adaptations, such as improved glucose and lipid homeostasis, as well as enhanced endurance performance in multiple metabolically relevant organs (i.e., skeletal muscle).

Autophagy, which means “self-devouring,” is the natural, regulated, destructive mechanism of the cell that disassembles unnecessary or dysfunctional components. It serves as a dynamic recycling system for cell regrowth and homeostasis; its damage or abnormal activation could contribute to disease pathogenesis, with clear evidence that autophagy regulation strongly affects skeletal muscle plasticity. Autophagy has been suggested to contribute to the prevention of accumulation of dysfunctional mitochondria during muscle contraction, and for maintaining exercise-dependent glucose homeostasis. Autophagy impairment could be induced by high-fat or high-sugar consumption. Mice lacking autophagy-related proteins failed to show any improvement in skeletal muscle adaptation and exercise performance, induced by exercise.

However, little is known on how nutrition might interact with acute exercise to modulate autophagy in obesity. Moreover, an acute bout of exercise is proven to activate autophagy through post-translational modifications, and more chronically by the enlistment of a transcriptional program. Furthermore, a single bout of endurance exercise can increase the autophagy-related and -regulated gene expression and even lead to a prolonged adaptive response. Therefore, to verify the mechanism concerning the changes of muscle functions brought by exercise in obese condition, and the relation between the mechanism and autophagy, this study applied a single bout of exercise that can cause changes in autophagy to stimulate the long-term high-fat high-sucrose (HFHS)-fed obesity mice, and observed the changes of autophagy-related proteins in different fibers of obesity model, and the relationship between changed autophagy proteins with the stimulation of exercise and the muscle function. This study hypothesized that the effect of exercise on muscle function in obesity is related to autophagy.

### METHODS

#### Animals and diets

Male C57BL/6 mice (n = 24; Japan SLC Inc., Haruno & Oh-hara Production Facility) were purchased from Central Lab. Animal Inc. (Seoul, Korea), at 8 weeks of age. After 1 week of acclimatization, all mice were individually caged and switched to either a 60% fat (D12492; Research Diets Inc., New Brunswick, NJ, USA) diet with 30% sucrose (SS016; Sigma-Aldrich, St. Louis, MO, USA) liquid feed, or a normal diet and normal water feed, for 20 weeks, to induce obesity; they were randomly assigned to HFHS feeding groups (HFHS [sedentary], HFHS+EX [exercise]) and low-fat diet+plain water feeding groups (CON [sedentary], CON+EX [exercise]). All groups were allowed to eat ad libitum, and food intake and body weight were recorded twice a week over the duration of the study. After 20-week diet intervention, all groups took the muscle function tests which consisted of whole-limb grip strength test and hanging time test. And one single bout of exhaustion exercise was performed in CON+EX and HFHS+EX groups 72 hours after the end of muscle function tests. The gastrocnemius (GAS) and soleus (SOL) muscles were examined to investigate the autophagic flux and mitophagy-related protein expression using Western blot analysis. The animals were housed under reverse light-dark cycle (lights on at 7:00 AM and off at 7:00 PM) at 21°C–23°C. All animal procedures were approved by the Institute of Animal Care and Use Committee, Seoul National University (No. SNU-170818-1-2) (Fig. 1).

#### Whole-limb grip strength test

The maximum strength of whole-limb grip in mice was measured in grams by a grip strength meter (Bioseb, Vitrolles, France). The mouse was placed on a tension grid while restrained manually by the scruff of the neck and base of the tail. After visual confirmation of firm gripping, the mouse was gently pulled back, until it released its grip from the grid. Each mouse was allowed three trials, and the greatest force was used for analysis. To adjust for potential
effects of body weight on test performance, results from the grip strength test were normalized to body weight.22

Hanging test
Balance, coordination, and muscle condition were assessed by testing the hanging time of each mouse, using a hanging device (made in-house). Mice were allowed to grasp the grid with all four paws; the grid was 40 cm above soft bedding to prevent any injury upon falling. The timer was started when the hanging grid (with the mouse) was inverted. The test session ended if the mouse could hang for a duration of 600 seconds. Mice that fell off the grid once, were allowed up to two more trials. The maximum hanging time (i.e., the longest of the trials) was used for future analysis. To adjust for potential effects of body weight on test performance, results obtained from hanging tests were multiplied by body weight.22

Treadmill exhaustion exercise
At the end of the diet treatment, mice were acclimatized to and trained for 2 days on a 12° uphill Exer-3/6 open treadmill (Columbus Instruments, Columbus, OH, USA) for 5 minutes at 8 m/min and/or followed by another 5 minutes at 10 m/min. On day 3, the mice were allowed to start at a speed of 10 m/min for 40 minutes, after which the treadmill speed was increased at a rate of 1 m/min every 10 minutes for a total of 30 minutes, and subsequently increased at the rate of 1 m/min every 5 minutes, until the mice were exhausted. Exhaustion was defined as the point at which the mice spent more than 5 seconds on hands without attempting to resume running. Total running time was recorded and the total distance run was calculated for each mouse.14

Body composition
Total body composition was determined immediately before sacrifice, using the Minispec Contrast Agent Analyzer (Bruker Optik, Ettlingen, Germany).
Western blot analyses

Mouse tissue extracts were prepared by homogenizing tissues in lysis buffer containing 50 mM Tris (pH 7.9), 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 1% Triton X-100, proteinase inhibitor cocktail (Roche Applied Sciences, Mannheim, Germany), and Halt phosphatase inhibitor cocktail (Roche Applied Sciences), and then subjected to Western blot analysis with anti-LC3 (1:500, Sigma-Aldrich), anti-P62 (1:500; Cell Signaling Technology, Danvers, MA, USA), anti-Bnip3 (1:500, Cell Signaling Technology), anti-AMPK (1:500, Cell Signaling Technology), anti-p-AMPK (1:500, Cell Signaling Technology), anti-Beclin1 (1:500, Cell Signaling Technology), and anti-GAPDH (1:3,000, Cell Signaling Technology) antibodies. Activation of AMPK was expressed as the ratio of phosphorylated AMPK (pAMPK) to total AMPK (tAMPK).

Statistical analysis

Statistical analyses were performed using IBM SPSS version 23.0 (IBM Corp., Armonk, NY, USA) and GraphPad Prism 7 (GraphPad Software Inc., La Jolla, CA, USA), and data were presented as mean ± standard deviation. The t-test was used to estimate the body weight, grip strength, hanging time, and speed and distance of exhaustion, for comparison between CON and HFHS groups, before or postintervention. Two-way analysis of variance was used for comparison of the magnitude of changes between sedentary and exercise groups in mice with two different diets. Values with $P < 0.05$ were considered statistically significant. Association of autophagy-related protein level in SOL and GAS with muscle function was calculated with Pearson’s correlation coefficient.

RESULTS

Chronic HFHS diet induced body weight, body composition, and tissue mass changes

As shown in Fig. 2A-C, HFHS-fed mice had significantly higher body weight ($P < 0.05$) and average energy intake ($P < 0.05$) compared to CON-fed mice, at all time-points, with more fat mass ($P < 0.01$), lean mass ($P < 0.05$), and free body fluid ($P < 0.01$) than CON (Fig. 2D). However, contrary to the fat mass per body weight induced by HFHS diet, lean mass per body weight in HFHS groups were significantly decreased (Fig. 2E). In Fig. 2F, wet weight of liver, inguinal white adipose tissue, and interscapular brown adipose tissue were all found to be significantly ($P < 0.01$) higher in HFHS than in CON, whereas muscle (SOL, GAS, quadriceps [QUAD], tibialis anterior [TA] muscle) mass had no difference between the two groups.

Chronic HFHS diet induced exercise performance changes

After the 20-week diet regime, hanging time was significantly ($P < 0.01$) increased in CON-fed mice compared to baseline (Fig. 2G). The grip force of mice significantly decreased in both CON and HFHS groups ($P < 0.01$) (Fig. 2H). At 20 weeks, both hanging time and grip strength of HFHS mice were significantly lower than those of CON mice ($P < 0.01$). Twenty-week HFHS feeding regime induced significant reduction in both exercise exhaustion time ($P < 0.05$) and exhaustion distance ($P < 0.05$) (Fig. 2I).

A bout of exhaustive exercise induced autophagy- and mitophagy-related protein expression changes in SOL

Western blot analysis showed that, after a 20-week HFHS diet, there was no significant increase in pAMPK/tAMPK ratio compared to that in the control group (Fig. 3B). However, the expression levels of autophagy-related proteins LC3I ($P < 0.01$), LC3II ($P < 0.05$), and LC3II/LC3I ratio ($P < 0.05$) in HFHS group were all significantly increased compared to those in the CON group (Fig. 3D). Interestingly, P62 expression level was also increased ($P < 0.01$) (Fig. 3F). This phenomenon also occurred in the mitochondria, accompanied by an increase in the expression of BCL2/adenovirus E1B 19-kDa interacting protein 3 (Bnip3, $P < 0.01$) (Fig. 3E) while the expression of Beclin1 remained unchanged.

In response to a single acute exercise stimulation, the pAMPK/tAMPK ratio increased significantly ($P < 0.01$) in CON group, though not in HFHS group (Fig. 3B). The expression levels of LC3III, LC3II/LC3I ratio, and P62 were not affected by exercise in CON group, except for the increased LC3I expression level (Fig. 3D, F). On the contrary, the expression levels of LC3II, LC3II/LC3I ratio, and P62 decreased, except for that of LC3I ($P < 0.05$) in HFHS group (Fig. 3D, F). Upon exercising, Bnip3 expression was significantly increased in CON mice ($P < 0.05$), but not in
Figure 2. Chronic high-fat high-sucrose (HFHS) diet induced body weight, body composition, and tissue mass, and muscle function changes. HFHS diet-fed mice had significantly higher body weight (A), average energy intake (B, C), with more fat mass, lean mass, free body fluid (D), while a decreased lean mass per body weight (E), and more wet weight of liver, inguinal white adipose tissue (iWAT), and interscapular brown adipose tissue (iBAT) (F). After 20-week diet regime, hanging time was increased in control diet-fed mice (G), and the grip force of mice decreased in both two diet-fed groups (H) compared to baseline. At 20 weeks, 20-week HFHS feeding regime induced significant reduction in hanging time, grip strength, and both exercise exhaustion time and exhaustion distance (G-I). Values are presented as mean ± standard deviation (A-C, G, H: n = 12; D-F: n = 6). *P < 0.05, †P < 0.01 vs. CON groups (A-C) or CON groups at 0 week (G, H) or CON (D-F); ‡P < 0.01 vs. CON groups at 20 weeks; §P < 0.05 vs. CON+EX (I). The t-test was used. CON, low-fat diet+plain water feeding group; HFHS, HFHS diet-fed sedentary group; CON+EX, CON exercise group; HFHS+EX, HFHS diet-fed exercise group; SOL, soleus; GAS, gastrocnemius; TA, tibialis anterior; QUAD, quadriceps; eWAT, epididymal white adipose tissue.
HFHS mice (Fig. 3E). Besides, the expression level of Beclin1 was not affected by either diet or exercise intervention (Fig. 3C).

A bout of exhaustive exercise induced autophagy- and mitophagy-related protein expression changes in GAS

Immunoblotting of GAS in all groups is shown in Fig. 4A. The 20-week HFHS diet was accompanied by a significant increase ($P<0.05$) in the levels of pAMPK/tAMPK ratio compared to the CON diet (Fig. 4B). Autophagy-related proteins LC3I, LC3II, LC3II/LC3I ratio, and P62 expression, and mitophagy-related protein Bnip3, did not change following the intervention (Fig. 4C-F). After a single bout of exhaustive exercise stimulation, the level of pAMPK/tAMPK ratio increased significantly in CON mice, but not in HFHS mice (Fig. 4B). Unlike the changes seen in SOL, a single exercise did not cause any change in autophagy pathway proteins in GAS in both the groups.

Pearson’s correlation between muscle performance parameters and molecular expression level of autophagy in SOL

Correlations between muscle performance parameters and molecular expression level of autophagy in SOL, after 20 weeks of treatment, are shown in Table 1. The expression of mitophagy-related protein Bnip3 was negatively correlated with three muscle performance parameters, including grip strength ($P<0.01$), exhaustion time, and distance ($P<0.05$). Hanging time also had a moderately negative relationship with the expression of P62 in the autophagy pathway ($P<0.05$), and LC3II/LC3I ratio was negatively correlated with grip strength ($P<0.05$) and exhaustion distance ($P<0.05$) (Supplementary Fig. 1).
DISCUSSION

Effect of HFHS diet on general parameters and muscle function

In this study, 20 weeks of HFHS feeding induced a significant increase in body weight, adipose tissue, liver mass, fat mass, as well as lean mass. However, long-term HFHS diet did not cause the same growth of skeletal muscle tissues in hind limbs, such as GAS and SOL, but has led to a decreased lean mass per body weight. The 20-week HFHS feeding sufficiently impaired functional performance, with reduced submaximal muscle grip strength, lower hanging time, and decreased distance and time of exhaustion. These may be consistent with previous results that showed that prolonged HFHS diet leads to skeletal muscle atrophy with a decrease in myofibrillar proteins, and phenotypically characterized by loss of muscle mass and strength.\textsuperscript{23-25} Growing evidence support the role of fatty acids and their derived lipid intermediates in the regulation of skeletal muscle mass and function. Furthermore, continuous glucose (15\%) intake was suggested to contribute to intramyocellular lipid accumulation and lead to the induction of autophagy.\textsuperscript{19} Inhibition/alteration of autophagy is proven to contribute to myofiber degeneration and weakness in muscle-specific Atg7 knockout mice generation, thereby indicating that autophagy is required to maintain muscle mass.\textsuperscript{26} While an essential role of autophagy in the maintenance of lipo-homeostasis has been revealed in recent years, whether muscle dysfunction is the result of impaired autophagy regulation in obesity still remains speculative.

HFHS diet affects the autophagy pathway

In SOL, we observed HFHS feeding to increase both LC3II/
LC3I ratio as well as P62 expression (Fig. 3), which means that the long-term unhealthy diet promoted the formation of autophagosomes, but blocked their degradation. Intramyocellular lipid content and ectopic fat storage, increased by excess lipid in skeletal muscle, had been proven to result in skeletal muscle dysfunction.27 Thus, the increased LC3II/LC3I ratio with HFHS diet may be an attempt to mitigate the associated metabolic result. However, the accumulation of P62 indicated a decrease of the combination of autophagosomes and lysosomes, thus leading to a decrease of ubiquitinilated protein degradation or blockage of lipid droplet breakdown, thereby influencing the homeostasis of muscle cells.28,29 The Pearson’s correlation coefficients, obtained in this study, showed a negative correlation between autophagy responses and muscle function parameters, under the regime of HFHS diet (Table 1). Together, it indicated that the dynamics of autophagic flux (LC3 and P62) determined the muscle function of mice feeding on HFHS diet. However, our article lacks the determination of intracellular fat content.

As mentioned earlier, high-fat and high-sugar feeding induced the disequilibrium of autophagy, and the recovery of this disequilibrium seems to be the key to avoid loss of muscle function caused by diet. The improvement effects of muscle function of long-term exercise have been well known. Moreover, an acute bout of exercise is proven to activate autophagy.13,20 In order to prove whether this autophagy disequilibrium caused by diet is interfered by exercise and restore muscle function, this study used a single exercise to stimulate different metabolic type muscle. Under exhaustive exercise, P62 expression decreased, in accord with the decrease of LC3II/LC3I ratio in HFHS+EX mice, which implied that acute endurance exercise accelerates autophagosome degradation while not facilitating LC3I generation (Fig. 5). For normal diet-fed mice, exercise increased LC3I expression. Therefore, the effect of long-term exercise on LC3II generation in obese mice was worth exploring. For the unchanged LC3II/LC3I ratio in CON+EX mice, a debatable reduction, or no change of LC3-II level, was reported after less strenuous endurance exercise.28,30 Unlike P62 and LC3II conversion, upon exercise stimulation, the expression of Bnip3 had no response in HFHS-fed mice. It is, however, contrary to the study that reported autophagy to be important in preventing mitochondrial damage during damaged muscle contraction.31 Consistent with the previous study that showed non-lipid substrates to be necessary for the activation of autophagy with exercise, in this study, Bnip3 was seen to change only by exercise in CON mice, thereby confirming that a low-lipid substrate is essential for the activation of autophagy with exercise.32 In this study, HFHS did not influence the expression of Beclin-1, same as in the previous report, indicating that more definitive studies would be required to determine the role of Beclin-1 complex in the regulation of autophagy pathways.33 Autophagy pathway had no significant change owing to the

### Table 1. The association between muscle performance parameters and autophagy

| Variable                  | Grip strength | Hanging time | Exhaustion time | Exhaustion distance |
|---------------------------|--------------|--------------|-----------------|--------------------|
| LC3II/LC3I ratio          | r* = -0.653† | r* = -0.468† | r* = -0.543     | r* = -0.606†       |
| P62                      | 0.021        | 0.146        | 0.068           | 0.037              |

*The association between muscle performance parameters and molecular biological expression level of autophagy in soleus of control diet-fed mice and high-fat high-sucrose diet-fed mice, calculated with Pearson’s correlation coefficient; †P < 0.05 (n = 6).
HFHS diet and exhaustive exercise in GAS. Similarly, our previous study found that the response of autophagy-related proteins to acute exercise had no change of LC3II in the GAS of mice immediately sacrificed after exercise. For the reasons why a single bout of exercise did not improve the autophagy-related proteins in GAS like SOL is possibly the response time to exercise due to a basal autophagy level and oxidative capacity in different muscle fiber. Furthermore, several studies have indicated different levels of basal autophagy among the different muscle types to reflect the specific physiological demands, suggesting that autophagy maybe a protective mechanism to maintain complete muscle structure and function. When facing a metabolic challenge, muscle integrity of glycolytic muscle is rapidly altered within just 3 days, whereas SOL muscle structural integrity is essentially conserved over 28-week exposure to HFHS diet. Moreover, contrary to aerobic muscle SOL, glycolytic muscle was proven to cause an early mitochondrial dysfunction of the fructose-fed insulin resistant rat. Thus, unlike oxidative SOL muscle, glycolytic muscle appears to lack a protective mechanism to preserve muscle integrity in animals exposed to HFHS diet. Due to the inherently low oxidative capacity of glycolytic muscle compared to that of aerobic muscle, an endurance exercise would protect the intramuscular fat increase or tissue fibrosis that appeared in the glycolytic muscle of an HFHS-fed animal through an improved oxidative capacity.

As a nutrient and energy sensor that maintains energy homeostasis, AMPK is a critical molecule in the initiation of autophagy. In the current study, the different responses of pAMPK/tAMPK ratio and LC3II and P62 expression in both GAS and SOL, following diet and exercise, suggested that AMPK activation during exercise may not be sufficient to regulate macroautophagy in muscles. However, AMPK phosphorylation of Ulk1 has been proven to be required for targeting mitochondria to lysosomes in exercise-induced mitophagy, which is consistent with the responses of pAMPK/tAMPK ratio and Bnip3 expression to exercise in SOL of both CON and HFHS mice, in our study.

There were several limitations of the current study. First, since LC3II conversion after exercise is debatable, lack of group settings at different times of sacrifices is a limitation in this study. Second, the detection of intramyocellular lipid content is insufficient. Third, since autophagy is a dynamic system that is difficult to observe, the control group settings with an injection of chloroquine that inhibits autophagic flux would help in the observation of autophagic flux in future studies.

In conclusion, our results suggested that impaired autophagy may contribute to muscle dysfunction in obese conditions, and the damage is muscle-fiber dependent. To combat muscle dysfunction in obesity, maintenance of autophagic flux is essential. The mechanism of autophagosomes and impaired organelles or excessive lipid accumulation in obese individuals, and the effect of long-term exercise on dysfunctional autophagy would require further studies. Most importantly, these data demonstrated that HFHS diet plays a role in muscle dysfunction through autophagy regulation, and that a single bout of exercise could alter the key proteins of autophagy pathway, which were negatively correlated with muscle performance parameters.

**CONFLICTS OF INTEREST**

The authors declare no conflict of interest.

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**AUTHOR CONTRIBUTIONS**

Study concept and design: DZ, HYM, LT, and WS; acquisition of data: DZ, JHL, SEK, and HES; analysis and interpretation of data: DZ and WS; drafting of the manuscript: DZ; critical revision of the manuscript: DZ, HYM, LT, and WS; statistical analysis: DZ and YZ; obtained funding: DMS, JKS, and WS; administrative, technical, or material support: HYM, DMS, JKS, and WS; and study supervision: WS.
SUPPLEMENTARY MATERIALS

Supplementary Figure 1. The association between muscle performance parameters and autophagy. It can be found via https://doi.org/10.7570/jomes.2019.28.3.175.

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