

### EFFECTS OF A SINGLE BOUT OF AEROBIC EXERCISE ON SKELETAL MUSCLE PROTEIN TURNOVER IN MICE

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#### ABSTRACT

##### Background and Objective

Aerobic exercise has a strong effect on skeletal muscle metabolism, in both males and females of all ages. However, the effect of a single bout of aerobic exercise on the regulation of protein balance remains unknown. In the present study, we investigated the effect of a single bout of treadmill-based exercise on the levels of various protein synthesis-related eukaryotic proteins (initiation factor 2a, eukaryotic initiation factor 4E, eukaryotic initiation factor 4E-binding protein 1, and eukaryotic elongation factor 2), breakdown-related proteins (microtubule-associated protein 1 light chain 3 alpha (LC3), autophagy-related 7, and muscle RING-finger protein 1 (MuRF1)), and polyubiquitination in 3-month-old male ICR mice.

##### Material and Methods

Twenty-four male mice were randomized into four time-point groups; each group of mice was run on a rodent treadmill for 10 min at 10 m/min at a slope of 5° between 7:00 p.m. and 8:00 p.m. for 2 days during the adaptation period. On the third day, exercise was performed for 50 min at a speed of 12.3 m/min; the control mice did not perform any exercise. Gastrocnemius muscles were collected immediately after the mice were sacrificed by cervical dislocation at 0, 3, 6, or 12 hours post-exercise.

##### Results

Levels of synthesis-related proteins were significantly reduced at 3 and 6 hours into the recovery period, whereas levels of breakdown-related proteins, including that of the autophagy marker LC3, increased immediately after exercise but not during the recovery period. MuRF1 level was determined in the gastrocnemius muscle to identify the factors involved in this increase. We found that increased MuRF1 levels were associated with an increase in polyubiquitination during the recovery period.

## Conclusion

Our results suggest a potential role of optimal time points in muscle protein metabolism during recovery from a single bout of treadmill exercise.

Skeletal muscle proteins are in a constant state of flux, and the balance between protein synthesis and breakdown is influenced by diverse physiological stimuli. Protein synthesis and degradation are both important for the maintenance of muscle tissue, formation of new proteins, and elimination of damaged ones.<sup>1</sup> Generally, increase in skeletal muscle mass with exercise and nutrition due to protein synthesis is greater than the decrease due to protein degradation, resulting in protein accumulation and increased muscle fibers.<sup>2</sup> However, the catabolic pathway is accelerated during exercise to supply energy and nutrients for continued muscle contraction. It is well known that amino acid levels and glucose oxidation increase during endurance exercise, and an increase in energy consumption may be necessary for the induction of autophagy.<sup>3,4</sup> Rose et al. showed that protein synthesis in single-bout-exercised mice decreased 50 min into recovery compared with that in resting skeletal muscles.<sup>5</sup> However, the protein degradation mechanisms relevant to skeletal muscle breakdown during exercise are not well understood.

The magnitude and direction of skeletal muscle protein turnover are influenced by various factors, including mode, intensity, and duration of the exercise performed, as well as training state of the individual. The skeletal muscle protein turnover in response to resistance training is well-established. There is a significant increase in the rates of both mixed muscle fractional synthesis and breakdown after resistance exercise; the magnitude and duration of increased protein synthesis exceeds protein breakdown, resulting in net muscle protein gain.<sup>6</sup> Skeletal muscle protein breakdown occurs through a variety of mechanisms. The most common process associated with protein degradation following exercise is activation of the ubiquitin proteasome pathway. In muscles, the ubiquitin pathway relies on two E3 ubiquitin ligases: muscle RING-finger protein 1 (MuRF1) and muscle atrophy F-box (MAFbx, also known as atrogin-1).<sup>7</sup>

The proteolytic systems as well as the lysosomal and caspase systems generate alternative energy substrates that are used by the cell to maintain internal homeostasis under conditions of energy stress. In addition, autophagy is a major cellular degradation process that sequesters bulk material for luminal breakdown, recycles macromolecules, and provides the cytosol with free fatty and amino acids.<sup>8,9</sup> Autophagy at low basal levels also consumes damaged organelles and protein aggregates, thereby maintaining cellular homeostasis. Furthermore, autophagy may be induced by starvation or cytotoxic events to increase cell survival during metabolic stress. Conversely, activation of the mechanistic target of rapamycin (mTOR) suppresses initiation of autophagy.

Although responses to resistance training are well known, very few studies have examined the changes in skeletal muscle during and after aerobic exercise. Therefore, alterations in protein balance following aerobic exercise are not well-characterized and are poorly understood as. The purpose of this study was to examine the expression of muscle protein synthesis-related and breakdown-related proteins in the gastrocnemius muscle following aerobic exercise.

## METHODS

### *Animals*

ICR male mice (3 months of age) were obtained from Dae Han Bio Link (Eumseong, Korea). Mice housed in standard cages placed in a room at  $22 \pm 2.0^\circ\text{C}$  and  $55 \pm 10\%$  relative humidity under a 12 hour-light/12 hour-dark cycle were fed chow diet and water ad libitum. All animal studies were performed according to procedures approved and guided by the Committee for Experimental Animal Care and Use at the Chungnam National University (CNU-00684).

### *Exercise Protocol*

Twenty-four mice ran on a rodent treadmill for 10 min at 10 m/min at a slope of  $5^\circ$  between 7:00

p.m. and 8:00 p.m. for 2 days during the adaptation period. On the third day, exercise was performed for 50 min at a speed of 12.3 m/min. Gastrocnemius muscles were collected immediately after the mice were sacrificed by cervical dislocation at 0, 3, 6, or 12 hours post-exercise, with six mice in each group. Samples were rinsed with cold saline, weighed, and frozen in liquid nitrogen. Muscle tissues from mice in the 2-day adaptation period were used as controls (con,  $n = 6$ ). Overall, a total of 30 mice was used in these experiments ( $n = 6$  in 5 groups).

### **Western Blot Analysis**

After treadmill exercise, the homogenized gastrocnemius muscle samples were analyzed in a lysis buffer (Invitrogen, Carlsbad, CA, USA) on ice for 30 min and centrifuged at 12,000 rpm for 15 min. Supernatants were collected, and protein concentration was determined using a Bradford assay-based kit. Lysate aliquots were boiled and electrophoresed on 10% sodium dodecyl sulfate-polyacrylamide gels.

The proteins were transferred onto polyvinylidene fluoride (PVDF) membranes, which were incubated with the following primary antibodies from Cell Signaling Technology (Danvers, MA, USA): rabbit anti-phospho Ser 52 and anti-total eukaryotic initiation factor 2a (eIF2a; both at 1 : 1,000 dilution), rabbit anti-phosphorylated and anti-total eukaryotic initiation factor 4E (eIF4E; both at 1 : 800), rabbit anti-phosphorylated and anti-total eukaryotic elongation factor 2 (eEF2E; 1 : 800), rabbit anti-phosphorylated and anti-total eukaryotic initiation factor 4E-binding protein 1 (4EBP1; 1 : 800), rabbit anti-microtubule-associated protein 1B light chain 3 (LC3B; 1 : 1,000), rabbit anti-MuRF1 (1 : 2,000), and mouse IgG1 anti-ubiquitin (1 : 1,000). Then, the membranes were incubated with horseradish peroxidase-linked secondary anti-mouse or anti-rabbit antibodies (Alpha Innotech, Santa Clara, CA, USA).

Finally, protein bands were detected using an enhanced chemiluminescence detection kit (Millipore, Billerica, MA, USA). Integrated optical density of the protein bands was calculated using the ImageJ software (National Institutes of Health, Maryland, USA), and values were normalized to those of the housekeeping  $\alpha$ -tubulin gene or the non-phosphorylated protein. The

acquired images were digitized and analyzed using AlphaEase FC software (Alpha Innotech).

### **Statistical Analysis**

Data are expressed as means and standard deviations. Significant differences between time-point groups were assessed by one-way repeated analysis of variance (ANOVA) using SPSS version 18.0 (SPSS, Chicago, IL, USA). Statistical significance was accepted for  $p < 0.05$ .

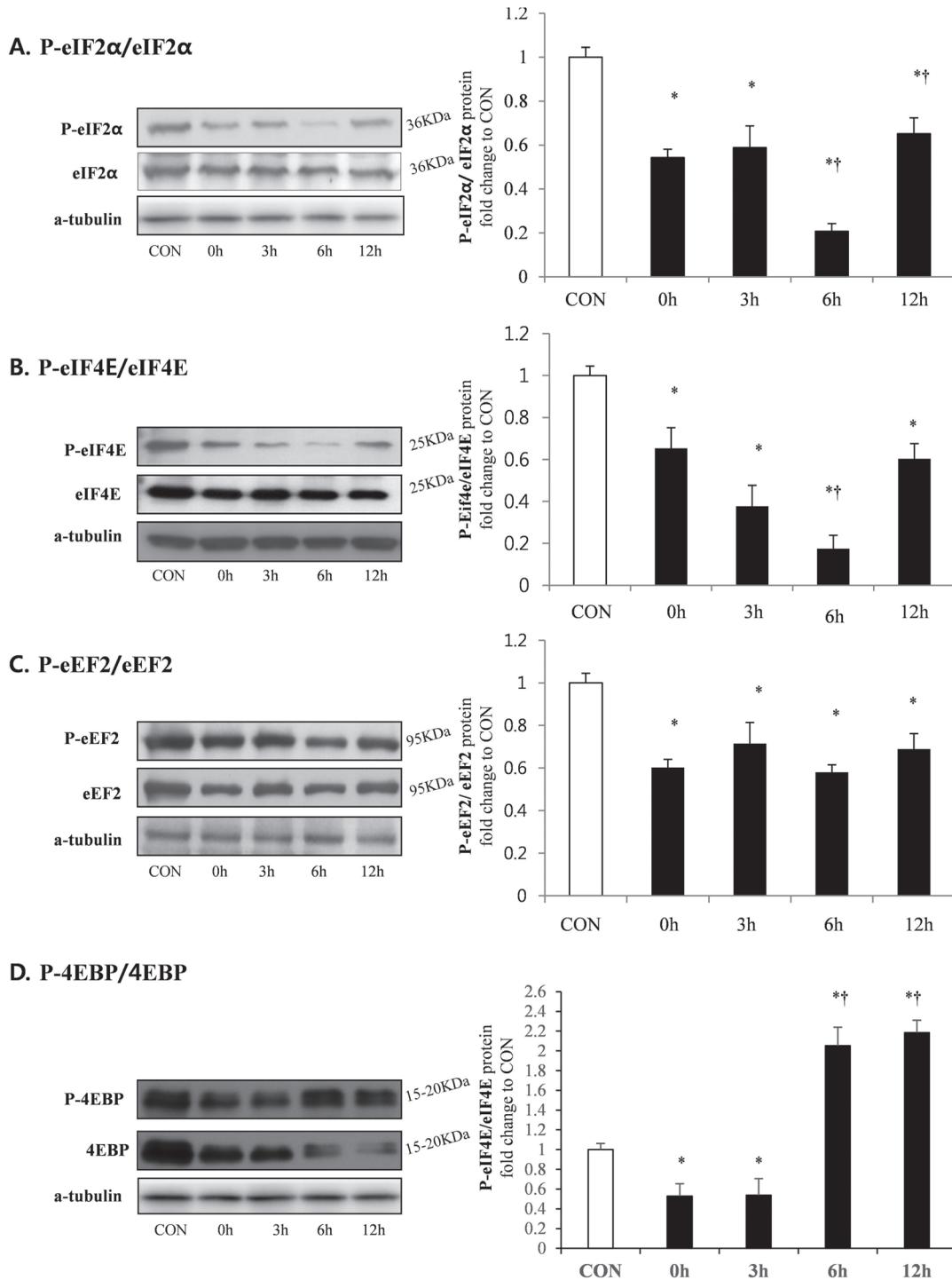
## **RESULTS**

To determine whether the muscle protein balance changes with time, we analyzed muscle proteins, such as synthesis-related and breakdown-related proteins, after a single bout of treadmill exercise in mice. First, we analyzed eIF2 $\alpha$  induction and observed that phosphorylation of eIF2 $\alpha$  was attenuated at all time points after exercise compared with that in the control group, with the lowest level at 6 h ( $p < 0.05$ ; Figure 1A). Phosphorylation of eIF4E was also significantly attenuated during the recovery period after exercise compared with that in the control group, with the lowest level at 6 h ( $p < 0.05$ ; Figure 1B). Phosphorylation of eEF2 and 4EBP was significantly attenuated at 3 and 6 h after exercise compared with that in the control group; however, a consistent trend was not observed during the recovery period (Figure 1C and D).

Phosphorylation of protein synthesis markers returned to basal levels at 12 h after recovery. However, LC3-II levels increased immediately after exercise, but not after 3, 6, and 12 h of recovery (Figure 2A). Autophagy-related 7 (ATG7) and p62 protein expression decreased significantly at all time points after exercise compared with that in the control group ( $p < 0.05$ ; Figure 2B and C). MuRF1 expression was significantly attenuated at 0 and 3 h compared with that of the control group; however, it significantly increased at 6 and 12 h compared with that of the basal level and the 0 h time point ( $p < 0.05$ ; Figure 2D).

Polyubiquitin level decreased immediately after exercise. However, the rate of decline decreased progressively with the recovery time (0 h vs. 3, 6, and 12 h; Figure 2E). DNA fragmentation, a marker of apoptosis, was not observed at any time point (Figure 3).

**FIG. 1** Expression of muscle protein synthesis markers.



Expression and phosphorylation of synthesis-related proteins (A) eIF2α, (B) eIF4E, (C) eEF2, and (D) 4EBP in the gastrocnemius muscle after exercise; \* indicates a significant difference compared with the control group; † indicates a significant difference compared with the 0 h time point (both  $p < 0.05$ ).

## DISCUSSION

It is known that ubiquitin-mediated degradation is involved in programmed cell death, as evidenced by dramatic increases in polyubiquitin message or protein observed in specific examples of programmed cell death (such as apoptosis).<sup>10–12</sup> Although increased apoptosis can be induced by exercising to exhaustion, we did not observe any DNA fragmentation following exercise in this study, suggesting that the exercise program did not activate cell death signaling. Our results suggest that a single bout of aerobic exercise induces an imbalance in muscle protein synthesis and breakdown during the non-apoptotic recovery period.

During translation, ribosomes build proteins based on the information encoded by mRNAs. The first step involves the GTP-dependent and eIF2a-catalyzed recruitment of the initiator methionyl-tRNA to the 40S ribosomal subunit, which is required for translation initiation.<sup>5</sup> This can be blocked by the competitive binding of phosphorylated eIF2a to eIF2B,<sup>13</sup> as phosphorylation of eIF2a occurs in response to cellular stress.<sup>5,8</sup> This may act as a regulatory mechanism that attenuates skeletal muscle protein synthesis during exercise. Dephosphorylated 4EBP binds to eIF4E and prevents initiation complex formation, thus representing a major regulator of translation initiation.<sup>5</sup> Global translation is reduced in response to most, if not all, types of cellular stress. This preserves cellular energy stores, up to 50% of which can be consumed by translation depending on the organism. Decreased translation also prevents the synthesis of unwanted proteins. Interestingly, stress-induced attenuation of global translation is often accompanied by selective translation of proteins required for cell survival under stress.<sup>10,14</sup>

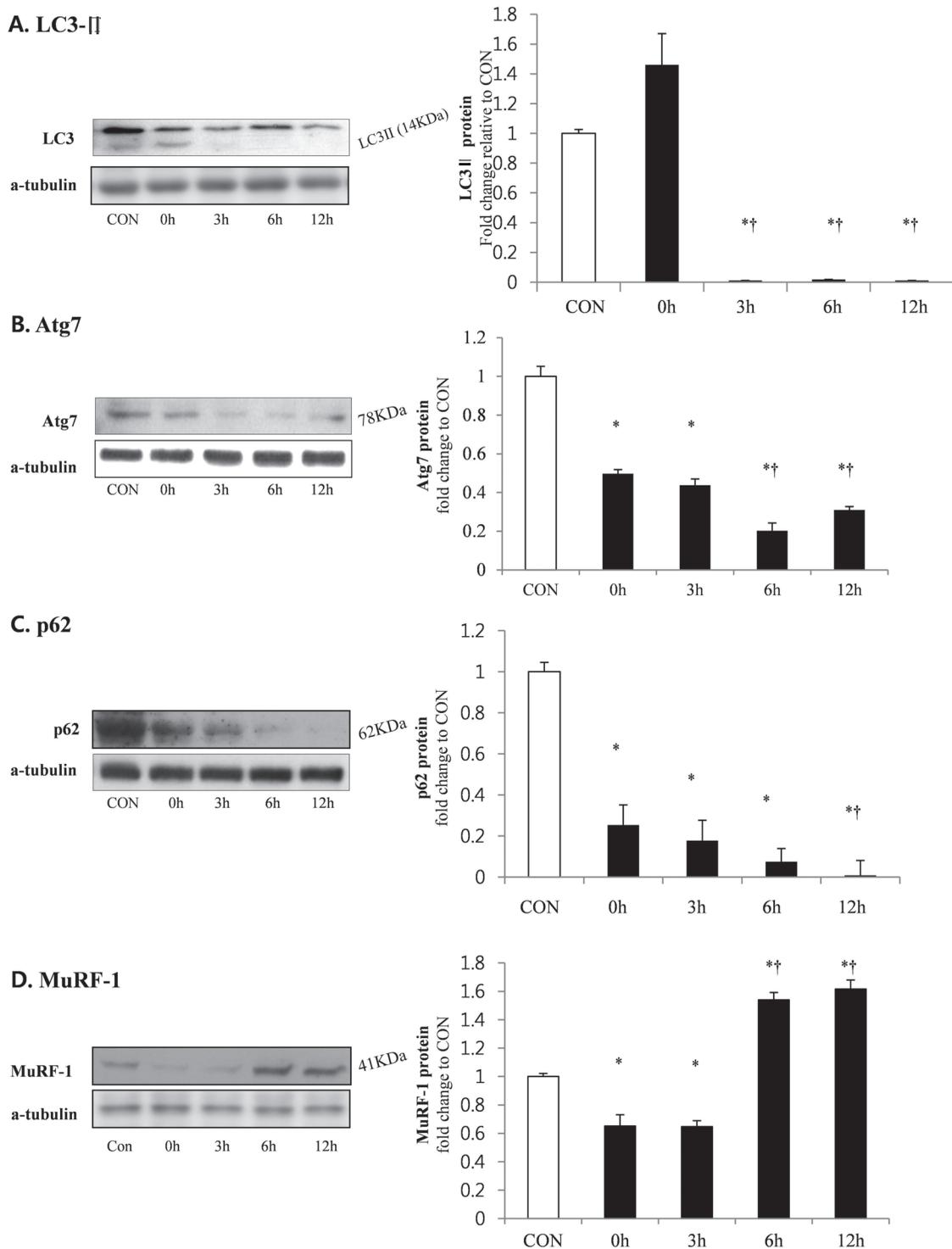
In this study, eIF2a and eIF4E expression decreased significantly compared with that of the controls at 3 and 6 h after a single bout of aerobic exercise. This is consistent with the results of previous studies by the Richter laboratory that demonstrated that skeletal muscle protein synthesis decreases during exercise.<sup>14,15</sup> Inhibition of protein synthesis during exercise is mediated at least in part by a decrease in mRNA translation initiation, and each extension involves changes in 4EBP1 and eEF2 phosphorylation.

Conversely, Williamson et al. demonstrated polyribosome disaggregation during murine skeletal muscle contractions, which can also attenuate translation initiation.<sup>17,18</sup> In particular, dephosphorylated 4EBP1 binds to eIF4E and inhibits initiation by preventing its association with eIF4G, which is observed in skeletal muscle during exercise.<sup>19,20</sup> Rose et al. reported that the combined use of Ca<sup>2+</sup> release agents and ATPase inhibitors caused 60–70% suppression of fast-twitch skeletal muscle protein synthesis during contraction, which was evenly distributed between Ca<sup>2+</sup>- and energy turnover-related mechanisms.<sup>14,15</sup> An eEF2 kinase inhibitor completely attenuated the increase in eEF2 phosphorylation and partially attenuated (30–40%) the suppression of protein synthesis during contractions. The 3–5-fold increase in skeletal muscle eEF2 phosphorylation during contractions in situ was rapid and sustained, and was restricted to working muscles. Furthermore, suppression of protein synthesis correlated more closely with changes in eEF2 than 4EBP1 phosphorylation. In the present study, 4EBP1 was dephosphorylated at 3 and 6 hours after aerobic exercise. This may occur due to the suppression of skeletal muscle protein synthesis during the recovery period, which is consistent with previously published studies on running.<sup>16,21</sup>

Williamson et al. suggested that a single bout of resistance exercise can increase mTOR signaling, which is associated with an increase in global protein synthesis.<sup>18</sup> In addition, electrical stimulation at high frequency can increase protein synthesis.<sup>22</sup> The fundamental differences in signal transduction pathway activation and regulation of mRNA translation may lie in the intensity, frequency, and duration of different types of exercise.

Conversely, the muscle protein breakdown response after exercise increases the breakdown of skeletal muscle protein.<sup>23</sup> Proteins related to degradation after exercise include MuRF1, polyubiquitin, and components of the autophagy system, including LC3-II, the levels of which immediately increased during exercise and decreased during the recovery period in this study. The autophagy-related proteins involved included ATG7 and p62. E1 ligases, such as ATG7, are important during elongation, including in the lipidation of LC3B-I to form LC3B-II. The level of p62, a

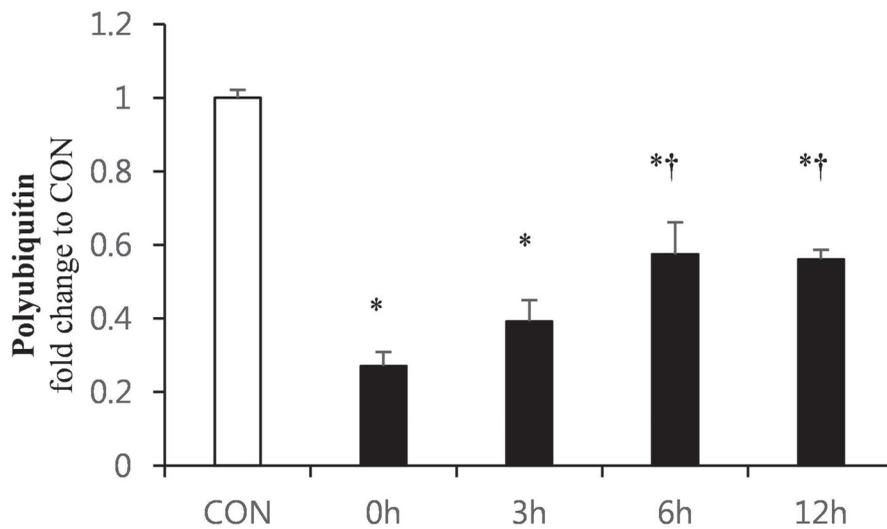
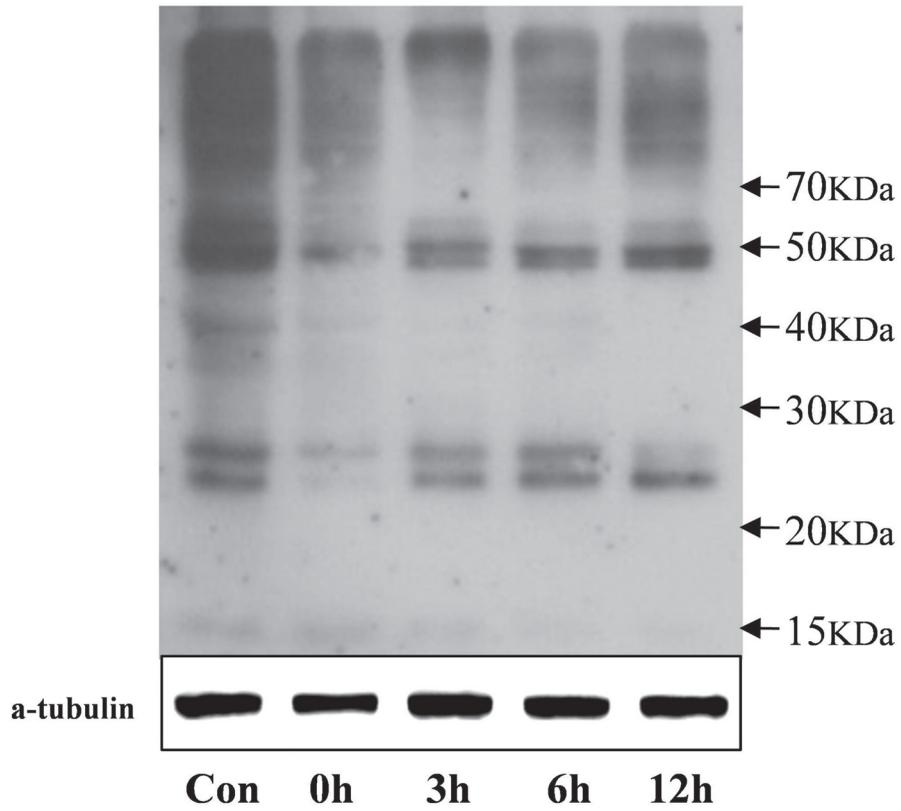
**FIG. 2a** Expression of muscle protein breakdown markers.



Expression of breakdown-related proteins (A) LC3-II, (B) ATG7, (C) p62, (D) MuRF1, and (E) polyubiquitin in the gastrocnemius muscle after exercise; \* indicates a significant difference compared with the control group; † indicates a significant difference compared with the 0 h time point (both  $p < 0.05$ ).

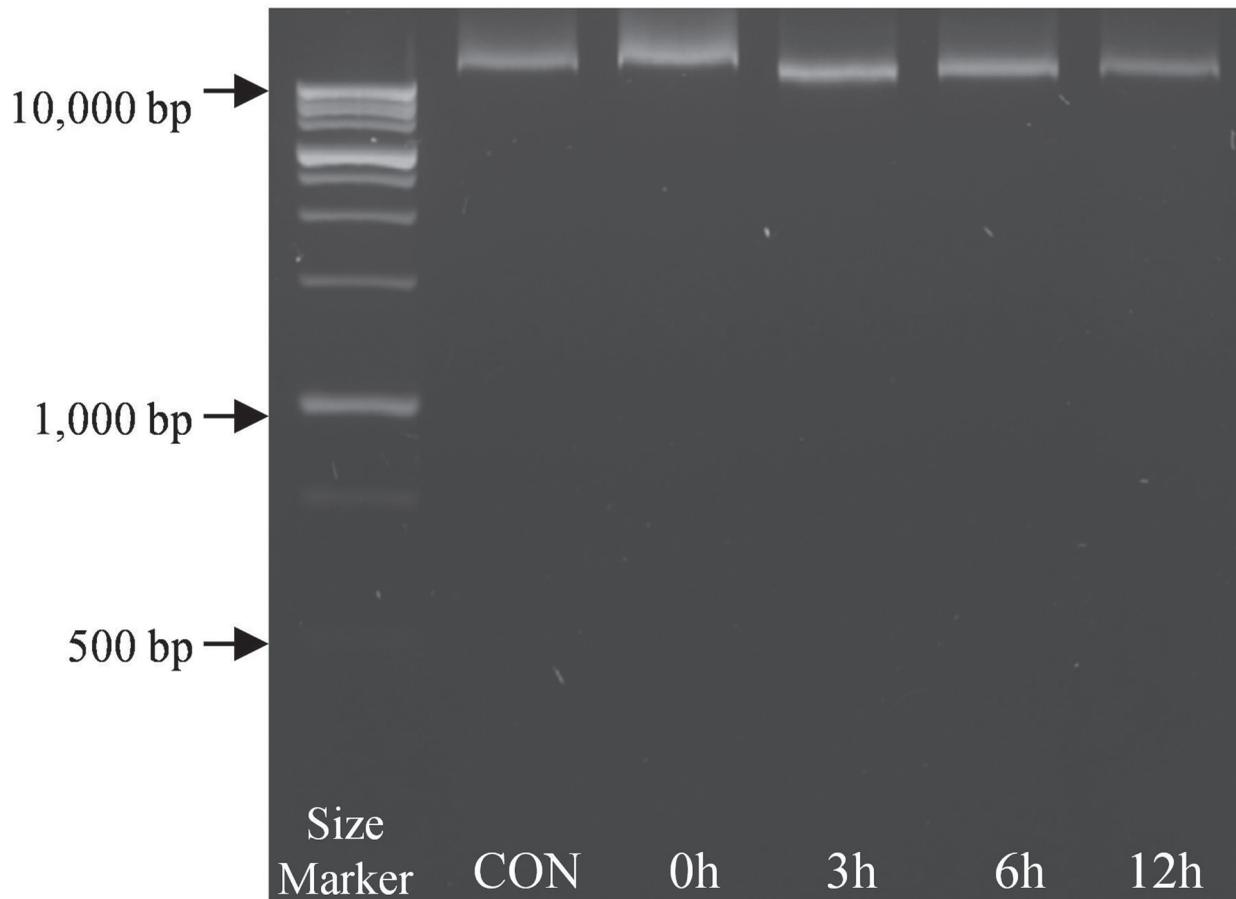
**FIG. 2b** Expression of muscle protein breakdown markers.

**E. Polyubiquitin**



Expression of breakdown-related proteins (A) LC3-II, (B) ATG7, (C) p62, (D) MuRF1, and (E) polyubiquitin in the gastrocnemius muscle after exercise; \* indicates a significant difference compared with the control group; † indicates a significant difference compared with the 0 h time point (both  $p < 0.05$ ).

**FIG 3.** DNA fragmentation after exercise.



CON = control.

protein constitutively degraded during autophagy,<sup>24,25</sup> decreased significantly at all time points after exercise compared with that of the control group.

Notably, MuRF1 expression and polyubiquitin levels were highest at 6 and 12 h, when the production of synthesis-associated proteins had been attenuated for over 6 h. A few recent studies have investigated attenuated protein synthesis response after resistance exercise. Fry et al. reported that markers of protein breakdown were elevated and autophagy was reduced in both younger and older adults after an acute bout of resistance exercise.<sup>20</sup> Indeed, the abatement of skeletal muscle protein synthesis during contractions should occur through rapid mechanisms such as alterations in peptide chain translation initiation or elongation, independently of the amount and distribution of

ribosomes and translatable mRNA.<sup>5</sup> The distribution of ribosomes between polysome aggregates and disaggregated monosomes should be investigated to verify this hypothesis.<sup>26</sup> Selective inhibition of initiation versus elongation would enable the distinction between polysome disaggregation and accumulation of monosomes *in vivo*.<sup>27</sup>

### CONCLUSION

Our results suggest a potential role of optimal time points in muscle protein metabolism during recovery from a single bout of treadmill exercise.

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### CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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