Biochemical and Biophysical Research Communications 456 (2015) 519–526

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

A treadmill exercise reactivates the signaling of the mammalian target of rapamycin (mTor) in the skeletal muscles of starved mice

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ARTICLE INFO

Article history:
Received 25 November 2014
Available online 6 December 2014

Keywords:
Autophagy
Exercise
Treadmill
Skeletal muscle
mTor
GFP-LC3

ABSTRACT

It has been well established that a starvation-induced decrease in insulin/IGF-I and serum amino acids effectively suppresses the mammalian target of rapamycin (mTor) signaling to induce autophagy, which is a major degradative cellular pathway in skeletal muscles. In this study, we investigated the systematic effects of exercise on the mTor signaling of skeletal muscles. Wild type C57BL/6J mice were starved for 24 h under synchronous autophagy induction conditions. Under these conditions, endogenous LC3-II increased, while both S6-kinse and S6 ribosomal protein were dephosphorylated in the skeletal muscles, which indicated mTor inactivation. Using GFP-LC3 transgenic mice, it was also confirmed that fluorescent GFP-LC3 dots in the skeletal muscles increased, including soleus, plantaris, and gastrocnemius, which clearly showed autophagosomal induction. These starved mice were then subjected to a single bout of running on a treadmill (12 m/min, 2 h, with a lean of 10 degrees). Surprisingly, biochemical analyses revealed that the exercise elicited a decrease in the LC3-II/LC3-I ratio as well as an inversion from the dephosphorylated state to the rephosphorylated state of S6-kinase and ribosomal S6 in these skeletal muscles. Consistently, the GFP-LC3 dots of the skeletal muscles were diminished immediately after the exercise. These results indicated that exercise suppressed starvation-induced autophagy through a reactivation of mTor signaling in the skeletal muscles of these starved mice.

1. Introduction

Mammalian skeletal muscles constitute the largest protein pool in the body. This pool is maintained by a continuous flow of protein synthesis and degradation, and is regulated by diverse conditions as well as external stimuli. Under nutrient-endowed conditions, skeletal muscles grow or become hypertrophied in response to mechanical stimuli such as exercise. The mTor-dependent mechanism by which exercise stimuli evoke protein synthesis in the use of nutrients, including amino acids and carbohydrates, has been well characterized [1,2]. Under nutrient austerity, skeletal muscles can serve as a major source of amino acids for the body [3]. In extreme cases where muscular movement is mechanically or functionally restrained, the degradation of myofibrils is markedly induced, which results in disuse muscle atrophy [4].

It is well known that the protein degradation of skeletal muscles is conducted mainly by the ubiquitin–proteasome system. Muscle proteasome constitutively participates in the turnover of myofibrillar and soluble sarcoplasmic proteins and two muscle-specific E3 ubiquitin ligases, Atrogin-1 and MuRF1, make a major contribution to this degradation [5–8]. Proteasomal degradation of skeletal muscles also plays a major role in disuse muscle atrophy in the forms of denervation-induced atrophy and ventilator-induced diaphragmatic dysfunction [9–11]. Another important catabolic pathway of skeletal muscles is autophagy. Autophagy is an intracellular catabolic pathway that is involved in the turnover

Abbreviations: mTor, mammalian target of rapamycin; GFP, green fluorescent protein; LC3, microtubule-associated protein IA/IB light chain 3; IGF-1, insulin-like growth factor 1.

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http://dx.doi.org/10.1016/j.bbrc.2014.11.118
0006-291X/

of cell constituents via lysosomes [12,13]. Constitutive or basal autophagy participates in the clearance of denatured or injured organelles and proteins, playing an essential role in the quality control of the cytoplasm. Skeletal muscle-specific autophagy (Atg7)-deficiency is known to cause muscle atrophy [14]. Accumulation of myofibrillar aggregates and inactivated mitochondria under autophagy deficiency is thought to impair normal muscle function, which eventually leads to elevated oxidative stress and muscle cell death. In addition to the role of maintaining cell homeostasis, induced muscle autophagy under fasting conditions most likely helps supply amino acids to other peripheral tissues.

Recently, muscle autophagy has attracted growing attention as it relates to exercise. In humans, ultra-endurance running is known to induce a coordinated expression of proteasome subunit b, MuRF1, and Atrogin-1 as well as autophagy marker LC3-II [15]. The induction of the ubiquitin proteasome system and autophagy markers is consistently regulated by mTor inactivation, AMPK phosphorylation, and FoxO3 dephosphorylation. Meanwhile, beneficial effects have been seen with the exercise-stimulated autophagy of skeletal muscles as the muscles adapted to metabolic changes during exercise in mice [16,17]. He et al. showed that acute exercise-induced autophagy is strictly dependent on the disruption of the BCL2-beclin-1 complex [16]. Grumati et al. revealed that exercise-stimulated autophagy contributes to maintaining muscle homeostasis, whereas physical training exacerbated a pathological phenotype in autophagy-defective collagen VI null mice [17]. More recently, Lira et al. reported that exercise increased the expression of autophagy-related genes to a greater extent in glycolytic fast-twitch muscles than in oxidative slow-twitch muscles [18]. To better understand the relationship between exercise and autophagy, it is important to investigate the muscle-type responses that are induced by exercise. In this study, we conducted a systematic analysis on how exercise influences the autophagic response and the autophagy-related signaling pathway in control wild type mice as well as GFP-LC3-transgenic mice.

2. Materials and methods

2.1. Animals

C57BL/6J mice were purchased from Charles River Laboratories (Wilmington, MA, USA) and GFP-LC3-transgenic mice were obtained from RIKEN Bioresource Center (Tsukuba, Ibaragi, Japan). All mice were maintained in an environmentally controlled room (lights on 8:00–20:00) and fed a pelleted laboratory diet and tap water *ad libitum*, unless otherwise stated. For synchronous autophagy induction, mice that had been previously fasted for 24 h were fed a diet for 2 h (20:00–22:00) in the dark to suppress autophagic activity to a minimal level, as described previously [19]. The diet was then withdrawn and all mice were again starved for 24 h. These re-starved mice were divided into two groups (group 1 and group 2). Group 1 was used as the control (untrained). Group 2 was subjected to running on a motor-driven treadmill. They were exercised on a treadmill at 12 m/min for 2 h, with a lean of 10 degrees.

All animal experiments involving animal care and sample preparation were approved by the Animal Experimental Committee of Juntendo University Graduate School of Medicine (Permit Number: 260189) and performed in compliance with the regulations and guidelines for the care and use of laboratory animals of Juntendo University Graduate School of Medicine.

2.2. Analytical methods

The concentrations of insulin, glucagon and IGF-1 in plasma were determined using an ELISA kit (AKRIN-011, Shibayagi Co. Ltd., Shibukawa, Gunma, Japan), a glucagon enzyme-immunoassay kit (YK090, Yanaihara Institute Inc., Fujinomiya, Shizuoka, Japan), and a Milliplex MAP Rat/Mouse IGF-1 kit (#RMIGF187K, EMD Millipore Corporation, Billerica, MA, USA), respectively. The concentrations of glucose, non-esterified fatty acids, and triacylglycerol in plasma were determined using an automatic biochemical analyzer, JCA-BM8000 (JEOL Ltd., Akishima, Tokyo, Japan), with a hexokinase-coupled spectrophotometric assay kit (#326023042 and #326023059, Sino-Test Corporation, Kanda, Tokyo, Japan), an acyl-CoA synthetase/acyl-CoA oxidase-coupled colorimetric assay kit (G-HE99, Eiken Chemical Co., Ltd., Taito, Tokyo, Japan), and a lipoprotein lipase-coupled colorimetric assay kit (#412-37494 and #418-37594, Wako Pure Chemical Industries, Osaka, Japan), respectively.

2.3. Measurement of GFP-LC3 dots in the skeletal muscles

GFP-LC3 dots in the frozen sections of fast-twitch muscles (gastrocnemius and plantaris) and slow-twitch muscle (soleus) of GFP-LC3-transgenic mice were analyzed using a confocal fluorescence microscope (Olympus FV1000; Olympus, Japan).

2.4. Immunoblotting

Mouse skeletal muscles were homogenized in nine volumes of ice-cold RIPA buffer supplemented with protease inhibitors (Complete Mini, Roche Diagnostics, Indianapolis, IN, USA) and phosphatase inhibitors (PhosSTOP, Roche Diagnostics, Indianapolis, IN, USA). Homogenates were centrifuged at 500×*g* and 4 °C for 10 min. The resultant supernatant (extract) was subjected to SDS–polyacrylamide gel electrophoresis (SDS–PAGE) [20]. Separated polypeptides were analyzed by immunoblotting analyses according to the published procedure [21], with the exception that SuperSignal West Pico Chemiluminescent Substrate (#34080) or SuperSignal West Dura Extended Duration Substrate (#34075) (Thermo Fisher Scientific, Inc., Waltham, MA, U.S.A.) was used as the substrate for the horseradish peroxidase-conjugated secondary antibodies. The antibodies against Atg7 and LC3 were prepared as described previously [22]. The antibodies against S6 ribosomal protein (#2317), phospho-S6 ribosomal protein (Ser235/236, #2211), p70 S6 kinase (#2708), phospho-p70 S6 kinase (Thr 389, #9205), phospho-p70 S6 kinase (Thr421/Ser424, #9204), Akt (#9272), and phospho-Akt (Ser473, #9271) were purchased from Cell Signaling Technology (Danvers, MA, USA). The antibody against actin (clone C4, MAB1501) was obtained from Millipore Corporation (Billerica, MA, USA). Quantitative densitometry was performed using NIH imaging system. Each value is reported as the mean ± SD of data.

2.5. Determination of free plasma and tissue amino acids

Plasma samples were mixed with 50% TCA to give a final concentration of 3.3%. After centrifugation at 10,000×*g* and 4 °C for 20 min, the free amino acids in the supernatants were analyzed using a L8500 amino acid analyzer (Hitachi, Tokyo, Japan). For determination of free amino acid concentration in tissue, liver and muscle, the samples were homogenized with five volumes of 10% TCA and centrifuged at 10,000×*g* and 4 °C for 20 min. The supernatants (0.5 ml) were mixed with an equal volume of 0.02 N HCl and analyzed using a L8500 amino acid analyzer.

2.6. Statistical analysis

All data are reported as the means ± SD. The statistical differences between the two groups were evaluated using a two-tailed Student's *t* test. A *p* value of less than 0.05 was considered statistically significant.

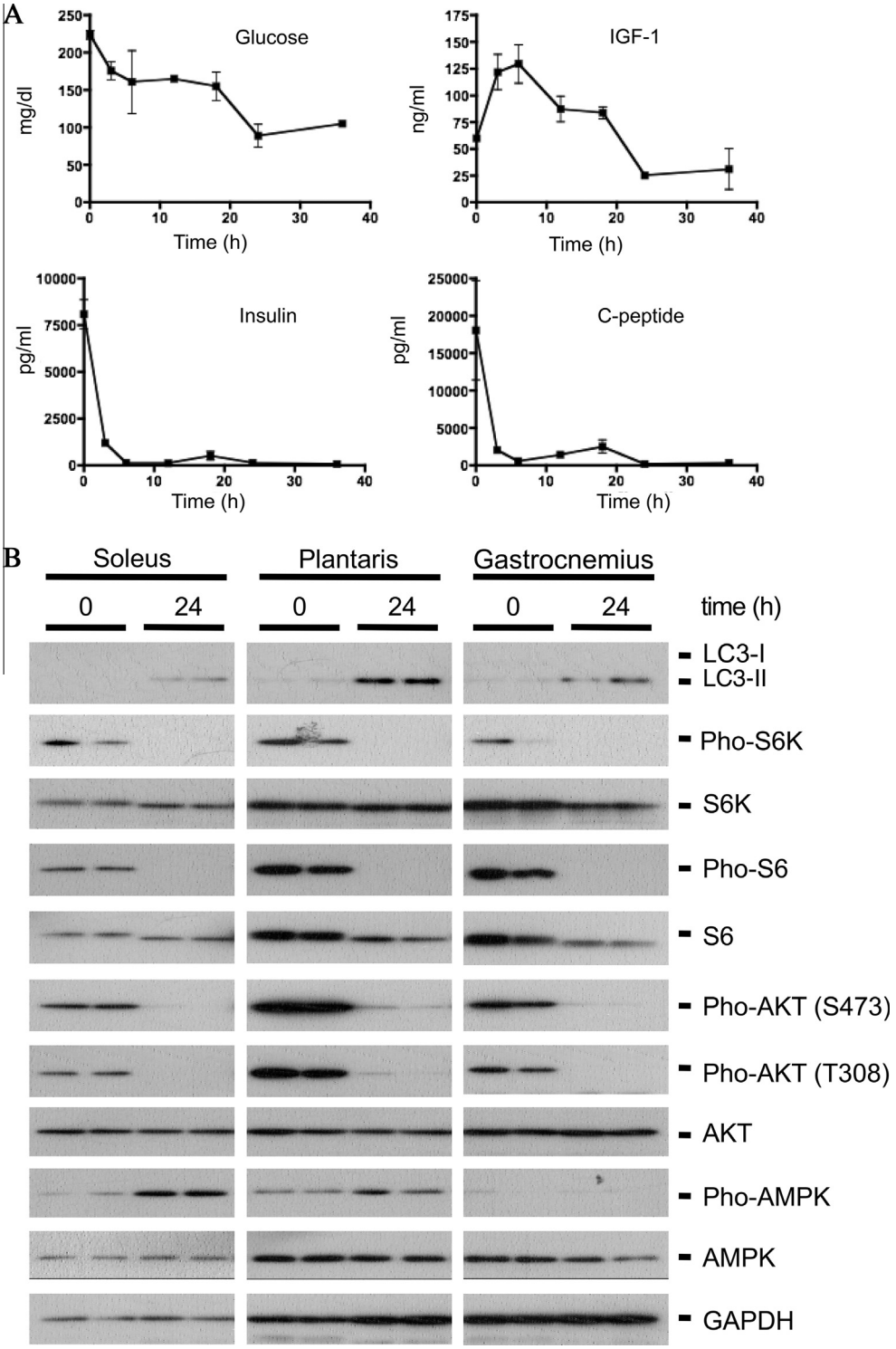

Fig. 1. (A) Changes in plasma glucose, IGF-1, insulin, and C-peptide in wild-type C57BL/6J mice during synchronous starvation. At the times indicated after the initiation of starvation, plasma samples were taken from mice, and the concentrations of insulin, IGF-1, insulin, and C-peptide were determined. Each value is presented as the mean ± SD of data from at least four mice. (B) Immunoblotting analysis of muscle extracts (10 μg protein per lane) prepared from muscle tissues of wild-type C57BL/6J mice before (0) and after (24 h) starvation. Endogenous LC3 in the extracts recognized by an anti-LC3 antibody is shown in the top row. LC3-I; soluble form of LC3, LC3-II; membrane bound phosphatidylethanolamine-conjugated form of LC3. Phosphorylated states of p70 S6 kinase (S6K), S6 ribosomal protein (S6), AKT, and AMP-activated protein kinase (AMPK) are shown in the lower rows. Two representative data of four mice of each experimental group are shown. (C) Quantitative densitometry of the immunoblotting data for LC3-II, phosphorylated p70 S6 kinase, and phosphorylated S6 ribosomal protein shown in (B). $n = 4$, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

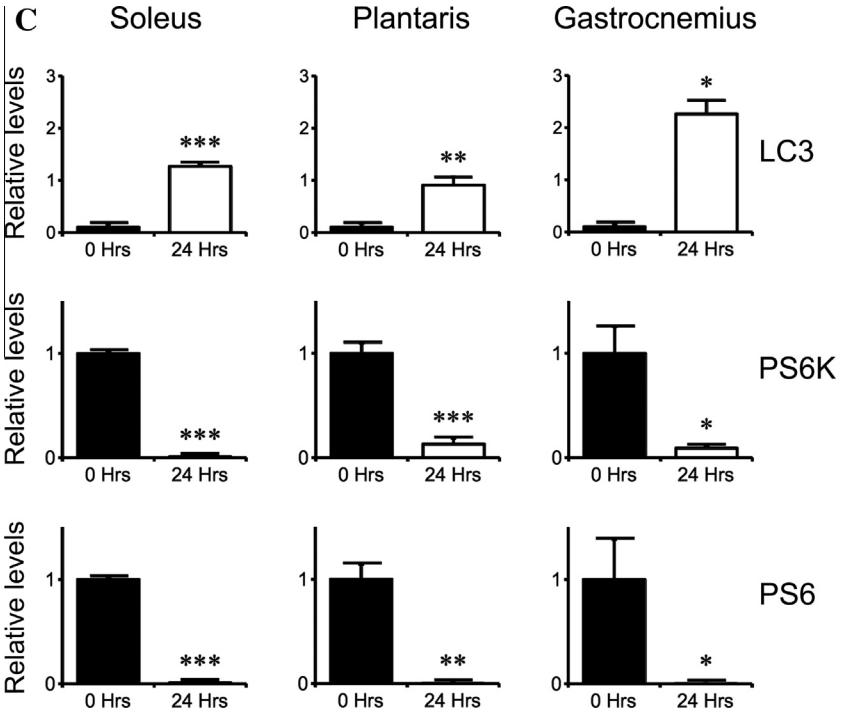

Fig. 1 (*continued*)

3. Results

3.1. Starvation-induced mTor inactivation and autophagosome formation of skeletal muscles

To elucidate the effect of exercise on the autophagy of skeletal muscles, two experimental approaches were conducted. First, endogenous LC3 as well as autophagy-specific mTor signaling molecules, including S6-kinase and S6 ribosomal subunits, in the skeletal muscles of wild-type C57BL/6J mice were examined by immunoblotting analyses. Second, GFP-LC3 dots in the skeletal muscles, including soleus, plantaris, and gastrocnemius, of GFP-LC3 transgenic mice were determined. As clearly shown by Mizushima et al. [23], an increase in the fluorescent GFP dots corresponds to an increase in autophagosomes/autolysosomes. We first attempted to determine how treadmill running influences autophagosomes/autolysosomes in the skeletal muscles of GFP-LC3-transgenic mice fed *ad libitum*. Unexpectedly, however, the fluctuation of GFP dot numbers as well as biochemical markers (S6-kinase and S6) among individual mouse muscles before and after exercise was large and no consistent results could be obtained. We therefore switched our approaches to starved mice. For this purpose, we investigated the autophagic responses of the skeletal muscles of mice placed under a synchronous starvation induction regimen [19], as described in the Section 2. This protocol enabled the synchronous induction of autophagy in livers as well as in the skeletal muscles of all mice, as reported previously [19]. Consistent with the previous results [19], blood glucose concentration exhibited an initial drop during the first 3 h of starvation, but remained stable between 6 and 18 h of starvation (Fig. 1A). Then, the concentration decreased further during 18–24 h to reach its lowest level (Fig. 1A). Compared with blood glucose, plasma insulin and C-peptide concentrations showed a more rapid decrease to reach the lowest levels and maintained these basal levels for a further 20 h (Fig. 1A). Insulin-like growth factor 1 (IGF-1) showed a different response to nutrient starvation. During the beginning of starvation, IGF-1 levels increased steeply to the highest level, then decreased gradually, and finally reached a basal level after 24 h of starvation (Fig. 1A).

We first performed immunoblotting analyses of the endogenous LC3 and the phosphorylation state of S6-kinase, the S6 ribosomal subunits, and the Akt in control wild type mice before (0 h) and after 24 h of starvation (Fig. 1B and C). It is well known that insulin/IGF-1-dependent activation of class I PI3-kinase also activates mTor (mTORC1 complex). Activated mTORC1 in turn induces the phosphorylation of Akt, S6-kinase, and ribosomal S6 while simultaneously suppressing autophagy [24,25]. Nutrient deprivation shuts off the insulin/PI3-kinase signaling resulting in the dephosphorylation of Akt and downstream effectors of mTor. As shown in Fig. 1B, the LC3-II in skeletal muscles increased markedly (top row), indicating full autophagy induction at 24 h. Ribosomal S6, S6-kinase, and Akt of all skeletal muscles examined were phosphorylated when starvation began, but these became almost completely dephosphorylated after 24 h of starvation. These data are consistent with previous data that showed a maximal increase in the endogenous LC3-II in the skeletal muscles of wild type mice at 24 h [19]. In accordance with these data, fluorescent GFP-LC3 dots in GFP-LC3 transgenic mice were scarcely detected in the beginning of starvation (0 h) in all muscles investigated. After 24 h of starvation, the number of GFP-LC3 dots markedly increased (Fig. 2).

3.2. Effect of a single bout of exercise on autophagy and on the mTor signaling pathway

We next examined the mechanical stimuli caused by a single bout of exercise and the effect on autophagic activity in the skeletal muscles of starved mice. We imposed a single bout of treadmill exercise (12 m/min for 2 h with a lean of 10 degrees) on control wild type mice and GFP-LC3 transgenic mice that had been starved for 24 h to see whether or not the exercise would enhance autophagic responses. Surprisingly, endogenous LC3-II was diminished, whereas LC3-I was increased in control wild type mice immediately after the exercise (Fig. 3A). In addition, the exercise elicited

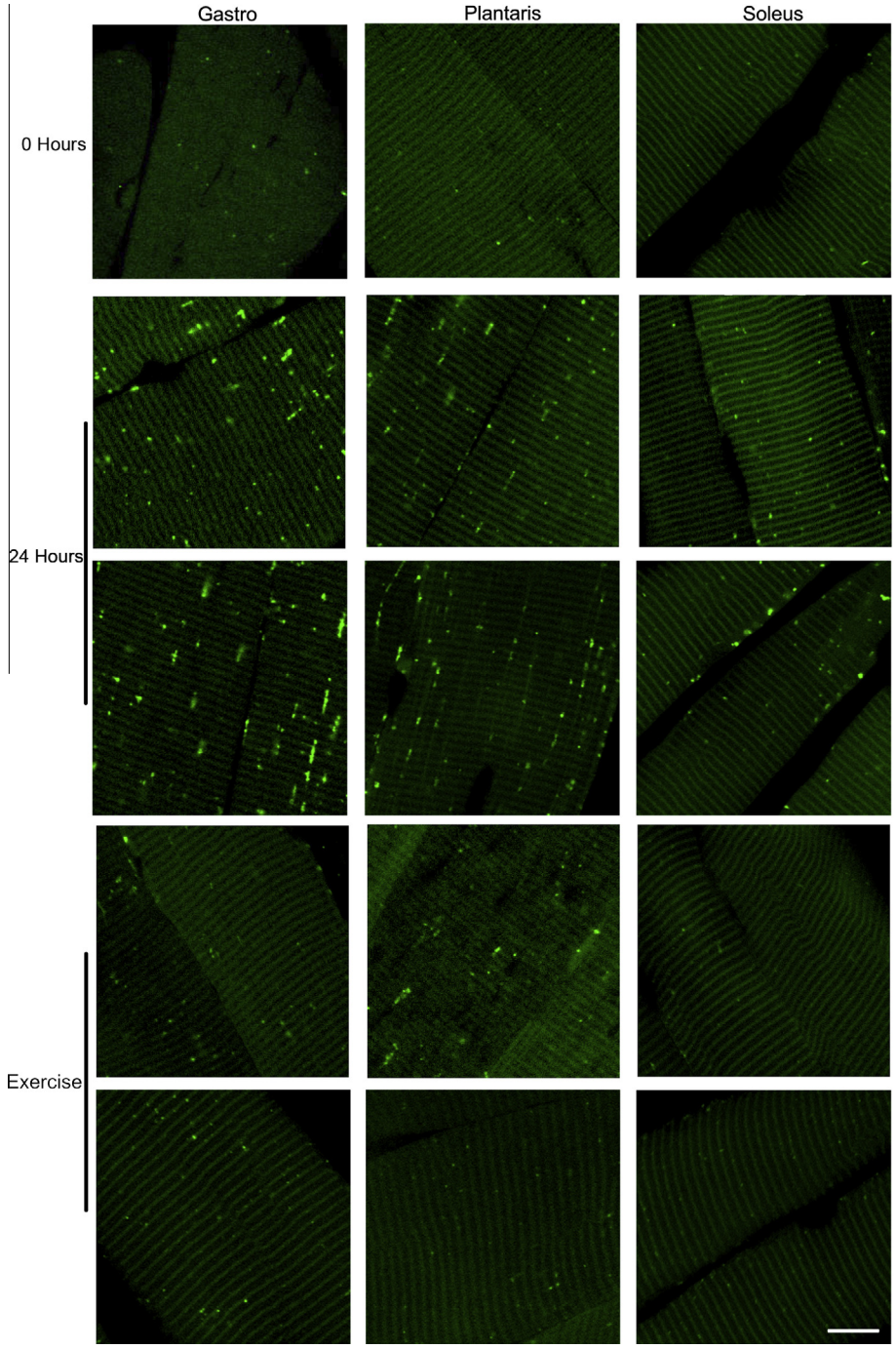

Fig. 2. GFP-LC3 puncta in skeletal muscles (soleus, plantaris, and gastrocnemius) of non-starved GFP-LC3 transgenic mice (0 Hours), 24 h-starved GFP-LC3 transgenic mice without exercise (24 Hours), and 24 h-starved GFP-LC3 transgenic mice immediately after treadmill exercise at 12 m/min for 120 min (Exercise). Representative images of GFP-LC3 puncta in gastrocnemius, plantaris, and soleus muscles are shown. Cryosections were analyzed by fluorescence microscopy as described in Section 2.

a significant transition from the dephosphorylated to the phosphorylated state of S6-kinase, ribosomal S6, and Akt (Fig. 3A and B), indicating mTor reactivation. These changes were most prominent in the soleus and plantaris muscles.

Along with the data of immunoblotting, the exercise elicited a decrease in the GFP-LC3 dots in the skeletal muscles that had been detected in starved mice of GFP-LC3 transgenic mice (Fig. 2). The bright fluorescent GFP dots were markedly decreased immediately after the exercise, and smooth green fluorescence, probably attributable to unconjugated GFP-LC3, remained in the sarcoplasm as well as along the sarcolemma. The decrease in the GFP-LC3 dots was more evident in soleus muscles than in plantaris or gastrocnemius muscles, too (Fig. 2).

As mTor reactivation appeared to play a key role in exercise-induced autophagy suppression, we further explored whether either extrinsic or intrinsic factors were involved as exercise-induced stimuli. However, as shown in Fig. 4A, neither insulin, nor IGF-1, nor glucagon, exhibited any significant change either before or after the exercise. There also were no significant changes either in plasma fatty acids or triacylglycerol. Only plasma glucose levels showed a significant reduction after exercise (Fig. 4A). Because amino acids also play key roles in mTor activation, we next

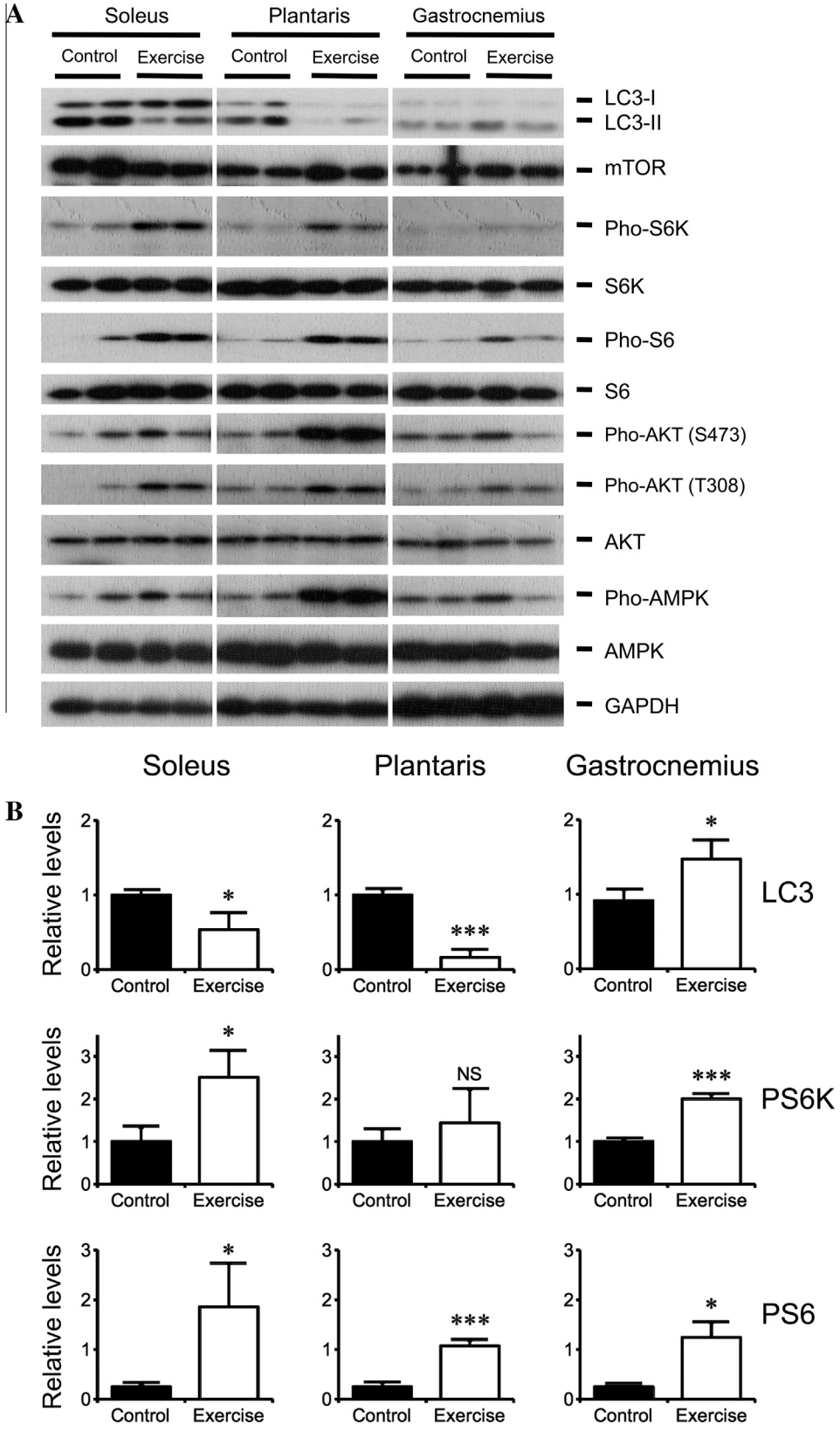

Fig. 3. (A) Immunoblotting analysis of muscles extracts (10 μg protein per lane) prepared from muscle tissues of wild-type C57BL/6J mice with (Exercise) or without (Control) exercise. Endogenous LC3-I (soluble form) and LC3-II (phosphatidylethanolamine-conjugated form) are shown in the top row. Phosphorylated states of p70 S6 kinase (S6K), S6 ribosomal protein (S6), AKT, and AMP-activated protein kinase (AMPK) are shown in the lower rows. Two representative data of four mice of each experimental group are shown. (B) Quantitative densitometry of immunoblotting data for phosphorylated p70 S6 kinase, phosphorylated S6 ribosomal protein, and LC3-II shown in (A). $n = 4$, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, NS; not significant.

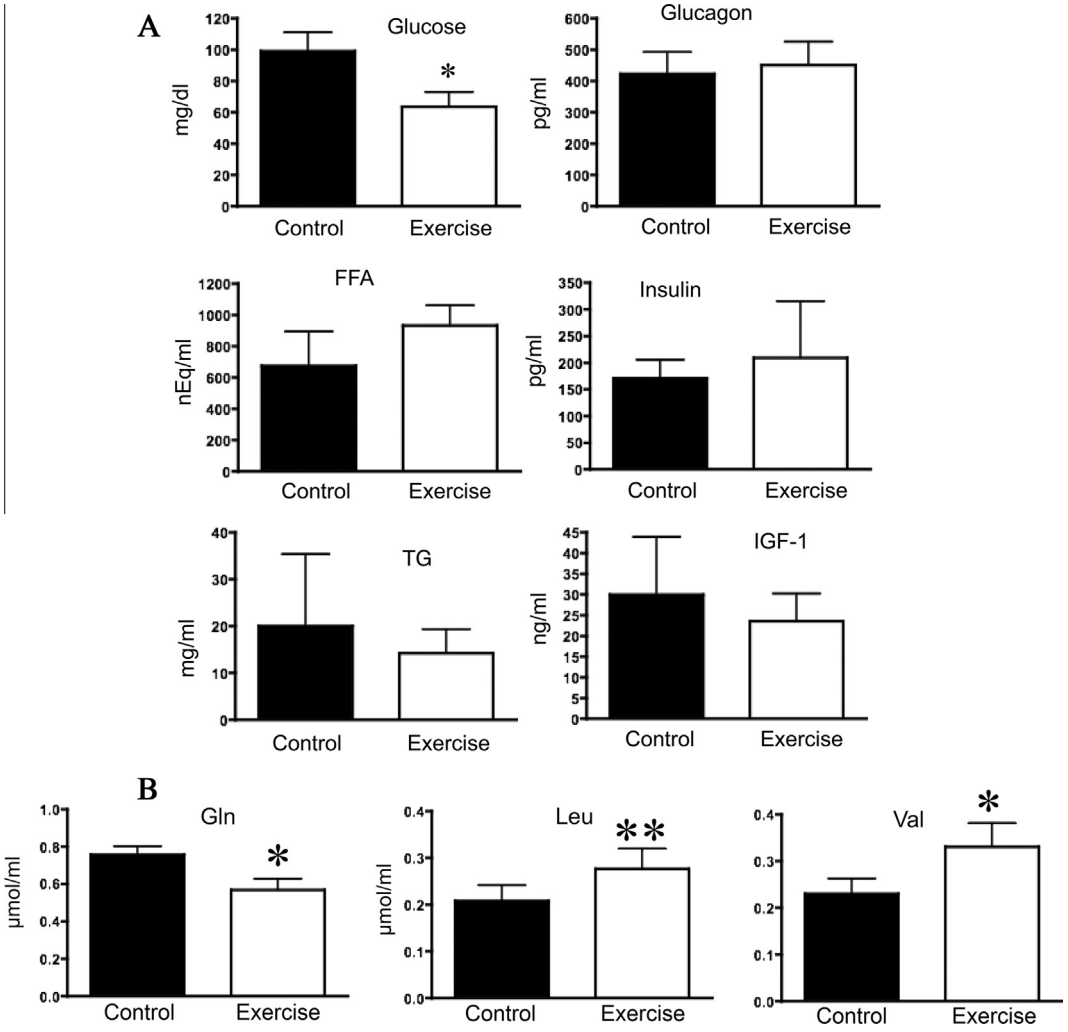

Fig. 4. (A) Plasma levels of glucose, free fatty acid (FFA), triglyceride (TG), glucagon, insulin, and IGF-1 of 24 h-starved mice at rest and those after exercise at 12 m/min for 120 min, with a 10 degree lean. Each value is presented as the mean ± SD of data from at least four mice. $^{*}p < 0.05$ (B) Effects of treadmill running exercise on amino acid concentrations of plasma. Plasma samples were collected from 24 h-starved mice that were subjected to exercise (Exercise) and those at rest (Control). Free amino acids, except for cysteine and tryptophan, were determined using an amino acid analyzer. The concentrations of three amino acids (Gln, Leu, Val) that have exhibited significant changes after the exercise are expressed as μmol/ml. Each value is reported as the mean ± SD of data from at least four mice. $^{*}p < 0.05$, $^{**}p < 0.01$.

examined whether the exercise influenced plasma amino acids. The concentrations of 20 plasma free amino acids were determined. As shown in Fig. 4B, the concentrations of valine and leucine significantly increased, whereas glutamine concentration was significantly decreased (Fig. 4B). None of other plasma free amino acids exhibited any significant increases or decreases (data not shown).

4. Discussion

As with liver, skeletal muscles respond well to nutrient starvation for autophagy induction [23], and the inactivation of mTor (TORC1 complex) is thought to play a principal role due to reduced insulin/IGF-1 signaling [7,14,24,25]. Recently, more attention has been focused on the mechanistic relationship between exercise and skeletal muscle autophagy. Some studies have shown that exercise stimulates autophagy, which promotes metabolic adaptation and ameliorates muscle function [16–18]. Jamart et al. showed that low-intensity exercise increased the expression of autophagy-related gene products, including LC3, GABARAP, and Atg12, as well as dephosphorylation of S6-kinase in the gastrocnemius muscles (fast-twitch muscle) in fasted mice [26]. On the other hand, Eliasson et al. reported that maximal eccentric contraction promoted p70 S6 kinase phosphorylation in humans who were without a nutrient supply [27]. In view of these differences under different experimental conditions, we decided to use a treadmill (12 m/min, 2 h, with a lean of 10 degrees) to systematically examine the effect of moderate single-bout exercise on autophagy in various types of skeletal muscles. For this purpose, however, we had to change our experimental settings to the skeletal muscles of mice synchronously starved for 24 h, under which the conditions of autophagy were preliminarily activated under mTor inactivation before beginning the exercise (Fig. 2).

To our surprise, the exercise effectively suppressed muscle autophagy through reactivation of mTor-dependent signaling molecules, such as Akt, S6-kinase, and ribosomal S6 (Fig. 3A and B). GFP-LC3 dots of skeletal muscles of GFP-LC3 transgenic mice markedly decreased after the exercise (Fig. 2), while the increased LC3-II levels in the soleus and plantaris muscles of the control wild type mice were also suppressed after exercise (Fig. 3A and B). A significant simultaneous re-phosphorylation of S6-kinase and ribosomal S6 was noted in these muscles, particularly in the soleus and plantaris muscles. Thus, our results with these starved mice were in

sharp contrast to those previously reported concerning fed mice [17,18]. The implication of autophagy suppression immediately after exercise may be attributed to the maintenance or protection of muscle mass to maintain the functional integrity of the muscles during exercise under a fasted state.

The exercise-evoked autophagy suppression with reactivation of mTor signaling seemed to be caused neither by changes in extracellular insulin nor in plasma amino acids. Insulin and IGF-1 concentrations changed only marginally both before and after exercise under starvation conditions (Fig. 4A). Out of 20 plasma amino acids leucine and valine exhibited small but significant increases, whereas glutamine significantly decreased after the exercise. Plasma leucine (~2 mM) is known to stimulate mTor signaling of rat skeletal muscles [28]. The increase in leucine (~0.1 mM, Fig. 4B) was not sufficient for mTor activation [28]. These results are reminiscent of an earlier observation whereby a rapamycin-sensitive, but growth factor-independent, mechanism was observed operating during mechanical stimuli-induced muscle hypertrophy [29]. Thus, it is more likely that changes or factor(s) inside the muscle cells other than those of an extracellular milieu function in autophagy suppression and mTor reactivation. Another consideration was that the exercise-evoked suppression of autophagy and the mTor reactivation was more evident in soleus muscles, which are slow-twitch muscles. Thus, this effect of exercise seems to be dependent on muscle type. To clarify a cause-effect relationship between exercise under starvation and autophagy suppression, further investigation is necessary.

Conflicts of interest

The authors declare that they have no conflicts of interest.

Acknowledgments

This work was supported in part by a Grant-in-aid for Scientific Research (C) (16590253 to JE), Grants-in-aid for Scientific Research (B) (19390079 to EK and 14380308 to TU), a Grant-in-aid for Scientific Research on Priority Areas (18076005 to MK, TU), a Grant-in-aid for "High-Tech Research Center" Projects for Private Universities, a matching fund subsidy (to EK) from the Ministry of Education, Culture, Sports, Science and Technology of Japan, a Grant-in-Aid for the Third-Term Comprehensive 10-Year Strategy for Cancer Control from the Ministry of Health, Labor, and Welfare of Japan (16271401 to TU), and a Research Grant from the Takeda Science Foundation of Japan (TU). The authors thank Dr. Norihiro Tada, Research Support Center, Juntendo University Graduate School of Medicine for technical assistance and James L. McDonald for language editing.

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