Biochemical and Biophysical Research Communications 394 (2010) 136–140

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

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

Fasting-related autophagic response in slow- and fast-twitch skeletal muscle

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ARTICLE INFO

Article history:
Received 18 February 2010
Available online 23 February 2010

Keywords:
Autophagy
Skeletal muscle
LC3
p62
mTOR

ABSTRACT

This study investigated regulation of autophagy in slow-twitch soleus and fast-twitch plantaris muscles in fasting-related atrophy. Male Fischer-344 rats were subjected to fasting for 1, 2, or 3 days. Greater weight loss was observed in plantaris muscle than in soleus muscle in response to fasting. Western blot analysis demonstrated that LC3-II, a marker protein for macroautophagy, was expressed at a notably higher level in plantaris than in soleus muscle, and that the expression level was fasting duration-dependent. To identify factors related to LC3-II enhancement, autophagy-related signals were examined in both types of muscle. Phosphorylated mTOR was reduced in plantaris but not in soleus muscle. FOXO3a and ER stress signals were unchanged in both muscle types during fasting. These findings suggest that preferential atrophy of fast-twitch muscle is associated with induction of autophagy during fasting and that differences in autophagy regulation are attributable to differential signal regulation in soleus and plantaris muscle.

1. Introduction

Nutrient starvation in living cells leads to depletion of glycogen and amino acids, which are essential for life. In response, cellular proteins are proteolyzed and used as a nutrient source under starvation conditions. One of the proteolytic responses to starvation is the induction of autophagy. Lysosome-mediated macroautophagy is a major pathway for bulk protein degradation during starvation [1]. Macroautophagy may be especially important in skeletal muscle. Muscle can serve as a major amino acid source during starvation because it has abundant protein and comprises about 40% of the body's weight [2].

Skeletal muscle atrophy due to proteolysis is observed in immobilization, denervation, and aging, and leads to declining muscle function [3]. Fasting is also known to induce muscle atrophy. Interestingly, fast-twitch muscle is more sensitive to starvation than slow-twitch muscle, and more severe muscle atrophy is observed in fast-twitch muscle with fasting [4,5]. Although it is unknown why fast-twitch muscle is preferentially degraded during starvation, bulk degradation by macroautophagy may be involved in muscle type-specific atrophy during fasting. Mizushima et al. (2004) have reported that expression of LC3-II, an autophagosomal membrane protein that is a phosphatidylethanolamine-conjugated form of LC3-I, is greater in fast-twitch extensor digitorum longus muscle than in slow-twitch soleus muscle after 24 h of fasting [6].

It is unclear how autophagy is regulated in skeletal muscle during starvation. We hypothesized that it is regulated differently in slow- and fast-twitch muscle. Although macroautophagy is mediated through several pathways, it is known that Akt/mTOR and Akt/FOXO3a signaling are major regulators [7–9]. In addition, recent studies have reported that activation of ER stress signals is also related to induction of macroautophagy [10,11]. However, regulation of autophagy-related signals in starved skeletal muscle has not been characterized. Thus, we examined the expression levels of LC3-II, a marker for macroautophagy induction [12], and the co-regulator p62 in slow-twitch soleus and fast-twitch plantaris muscles after 1, 2, or 3 days of fasting. In addition, expression of proteins in the Akt/mTOR, Akt/FOXO3a, and ER stress pathways was examined to elucidate the regulation of autophagy in skeletal muscles during fasting.

2. Methods

2.1. Animal care and fasting

All procedures were performed in accordance with the guidelines of the Committee on Animal Care and Use at Waseda University and the Guiding Principles for the Care and Use of Animals in the Field of Physical Sciences established by the Physiological Society of Japan. Five-week-old male Fischer-344 rats were assigned to 4 groups: sedentary control (Cont; $n = 6$), 1 day fasting (F-1d;

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doi:10.1016/j.bbrc.2010.02.130

$n = 6$), 2 days fasting (F-2d; $n = 6$), or 3 days fasting (F-3d; $n = 6$). Rats were housed in a temperature-controlled (22 ± 2 °C) room with a 12:12-h light/dark cycle. Control rats were provided rat chow and water *ad libitum*. Fasting rats were provided only water *ad libitum* for 1, 2, or 3 days. All rats were anesthetized with sodium pentobarbital (50 mg/kg i.p.) and soleus and plantaris muscles were removed after each fasting period.

2.2. Homogenization

Muscle samples were homogenized in 15 volumes of homogenizing buffer (1% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate in Tris-buffered saline (TBS)). Total protein per muscle mass was measured using the Bradford method [13]. Homogenized sample was centrifuged at 10,000*g* for 20 min. The extracted supernatant protein content was measured at a final protein concentration of 2 μg/μL and then boiled in sample buffer for 2 min.

2.3. Western blotting

Sample buffer containing protein extract from each muscle was separated on an 8% (for large molecular weight proteins) or 15% (for small molecular weight proteins) acrylamide gel and a 4% stacking wide-gel using standard electrophoretic methods [14]. A total of 50 μg protein was applied to each lane. The gels were transferred to polyvinylidene difluoride membranes using semi-dry blotting. After transfer, blots were blocked with 5% non-fat milk in TBS containing 0.05% Tween 20 (TBS-T) for 1 h at room temperature. The blots were incubated overnight at 4 °C with the primary antibodies diluted 1:100 to 1:2000 in TBS-T containing 5% bovine serum albumin (BSA). Primary antibodies used in this study were: anti-LC3 (M115-3; MBL, Nagoya, Japan), anti-p62/SQSTM1 (PM045; MBL), anti-mTOR (#2972; Cell Signaling, Beverly, MA), anti-Phospho-mTOR (#2971; Cell Signaling), anti-FOXO3a (3673-100; BioVision, Mountain View, CA), anti-Phospho-FOXO3a (#9464, Cell Signaling), anti-eIF-2α (#9722; Cell Signaling), anti-Phospho-eIF2α (#9721, Cell Signaling), anti-CHOP/GADD153 (sc-7351; Santa Cruz Biotechnology, Santa Cruz, CA), and anti-KDEL (reacted with GRP78, SPA-827; Assay Designs, Ann Arbor, MI). The membranes were then exposed to horseradish peroxidase-conjugated goat anti-mouse IgG (074-1806; KPL, Gaithersburg, MD) or goat anti-rabbit IgG (074-1506; KPL) in TBS-T containing 5% BSA for 1 h at room temperature. The membranes were then processed using enhanced chemiluminescence (ECL; RPN2106; GE Healthcare, Buckinghamshire, England). ECL signals on the immunoblots were detected and measured using a Fuji LAS3000 luminescent imaging system (Las-3000; FUJI FILM, Tokyo, Japan) with MultiGauge software (Ver.3.0; FUJI FILM).

All data are presented as means ± SEM. Significant differences among groups were determined using one-way ANOVA and Tukey–Kramer post hoc tests. All differences were determined to be significant at the $P < 0.05$ level.

3. Results

3.1. Characteristics of soleus and plantaris muscles during fasting

Body weight declined in a fasting duration-dependent manner, and F-3d rats were 31% lighter than control rats (Table 1). Compared with control muscle, absolute soleus muscle weight was not changed in the F-1d or F-2d groups, but it decreased significantly by 10% in the F-3d group. In contrast, notable plantaris muscle loss was observed with fasting. Weight loss was 11% in F-1d, 19% in F-2d, and 23% in F-3d rats relative to control rats. Total protein content was not changed by fasting in either muscle type.

3.2. Fasting-related changes in the autophagic marker LC3-II and the adaptor protein p62

Patterns of LC3-I to LC3-II conversion during fasting and LC3-II levels in soleus and plantaris muscle are shown in Fig. 1A. Although LC3-II was barely detectable under control conditions in plantaris muscle, fasting drastically increased the protein in a duration-dependent manner. The greatest enhancement was observed in the F-3d group. Increased expression of LC3-II in soleus muscle occurred only in the F-3d group, and the magnitude of increase during fasting was lower than in plantaris muscle. p62 expression also increased in response to fasting (Fig. 1B). In plantaris muscle, p62 was higher in F-3d rats than in F-1d rats, and the peak value was 5.1-fold higher in the F-2d group than in the control group. The enhancement of p62 was lesser in soleus muscle than in plantaris muscle, and p62 expression levels were increased 1.7-fold only in the F-2d rats.

3.3. Regulation of Akt, mTOR, and FOXO3a during fasting

Total and phosphorylated Akt were downregulated in both muscles in response to fasting (Fig. 2A). Total Akt declined in plantaris muscle, and expression relative to control was 33.5% in F-1d, 43.6% in F-2d, and 36.0% in F-3d rats. Phosphorylated Akt also decreased to 21.8% of the control value in F-1d rats and to 12.4% of controls in F-3d rats. Downregulation of Akt was also observed in soleus muscle after fasting, and expression decreased to 55.0% of control values for total Akt and 7.4% of control for phosphorylated Akt in the F-3d group.

Expression of mTOR and FOXO3a, downstream targets of Akt, is shown in Fig. 2B and C. Total and phosphorylated mTOR in soleus muscle were not changed by fasting. In contrast, in plantaris muscle, phosphorylated mTOR declined to 53.6% of control levels in F-1d rats and to 34.9% of controls in F-3d rats, while total mTOR protein was not changed by fasting (Fig. 2B). Finally, FOXO3a levels were unchanged during fasting; levels of both total and phosphorylated FOXO3a were not different than in the control group in either type of muscle (Fig. 2C).

Table 1
Morphological changes in experimental animals.

	Body weight (g)		Soleus			Plantaris		
	Pre	Post	Absolute wt. (mg)	Muscle/body wt.	Total protein (mg/g wet wt.)	Absolute wt. (mg)	Muscle/body wt.	Total protein (mg/g wet wt.)
Cont	137 ± 1	142 ± 2	51 ± 1	0.372 ± 0.005	135 ± 6	133 ± 3	0.971 ± 0.016	130 ± 3
F-1d	131 ± 2	112 ± 2*	49 ± 1	0.434 ± 0.010*	140 ± 5	119 ± 2*	1.058 ± 0.013*	146 ± 6
F-2d	136 ± 3	104 ± 2*,#	47 ± 1	0.447 ± 0.010*	146 ± 8	108 ± 2*,#	1.036 ± 0.016*	141 ± 4
F-3d	136 ± 2	98 ± 1*,#	46 ± 1*	0.474 ± 0.009*,#	145 ± 8	103 ± 1*,#	1.054 ± 0.014*	145 ± 7

Values are means ± SEM.
* $P < 0.05$ vs. Cont.
# $P < 0.05$ vs. F-1d.

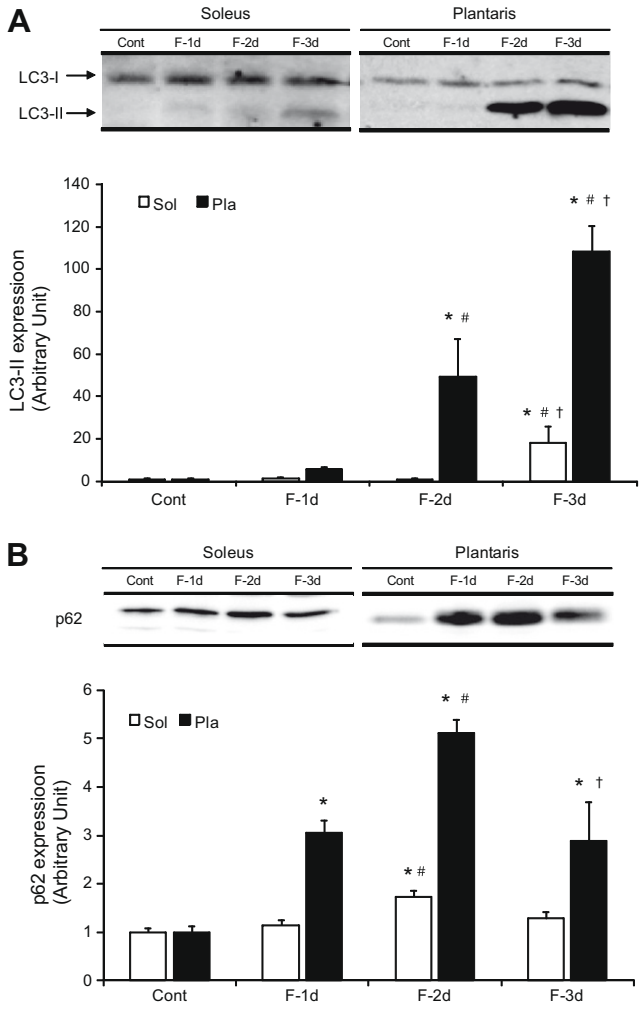

Fig. 1. Fasting-induced LC3 conversion and p62 expression in soleus and plantaris muscles. Protein expression of LC3-II (A) converted by glycosylation and p62 (B) was measured by western blotting after 1, 2, and 3 days of fasting. Cont, control; F, fasting; d, day. Values are means ± SEM. $^{*}P < 0.05$ compared with control; $^{\#}P < 0.05$ compared with F-1d; $^{\dagger}P < 0.05$ compared with F-2d in soleus (open bar) and plantaris (filled bar) muscles.

3.4. Expression of ER stress signaling proteins during fasting

Expression of ER stress signaling proteins is shown in Fig. 3. Fasting had no effect on total or phosphorylated eIF2α protein expression in either soleus or plantaris muscle (Fig. 3A). CHOP/GADD153 protein expression was also not different in the fasting and control groups (Fig. 3B). However, GRP78 content in plantaris muscle decreased to 43.7% of the control level in F-2d rats and to 44.6% of controls in F-3d rats, while no change was observed in fasted soleus muscle. Thus, we saw no evidence of activation of ER stress signals by fasting (Fig. 3C).

4. Discussion

Skeletal muscle contains a large amount of protein that can serve as an energy source [2], and atrophy is induced under fasting conditions [2,4]. Interestingly, the degree of fasting-related atrophy is greater in fast-twitch muscle than in slow-twitch muscle [4]. However, the reason for muscle type specificity in protein degradation with fasting is unknown. Expression of LC3-II is highly

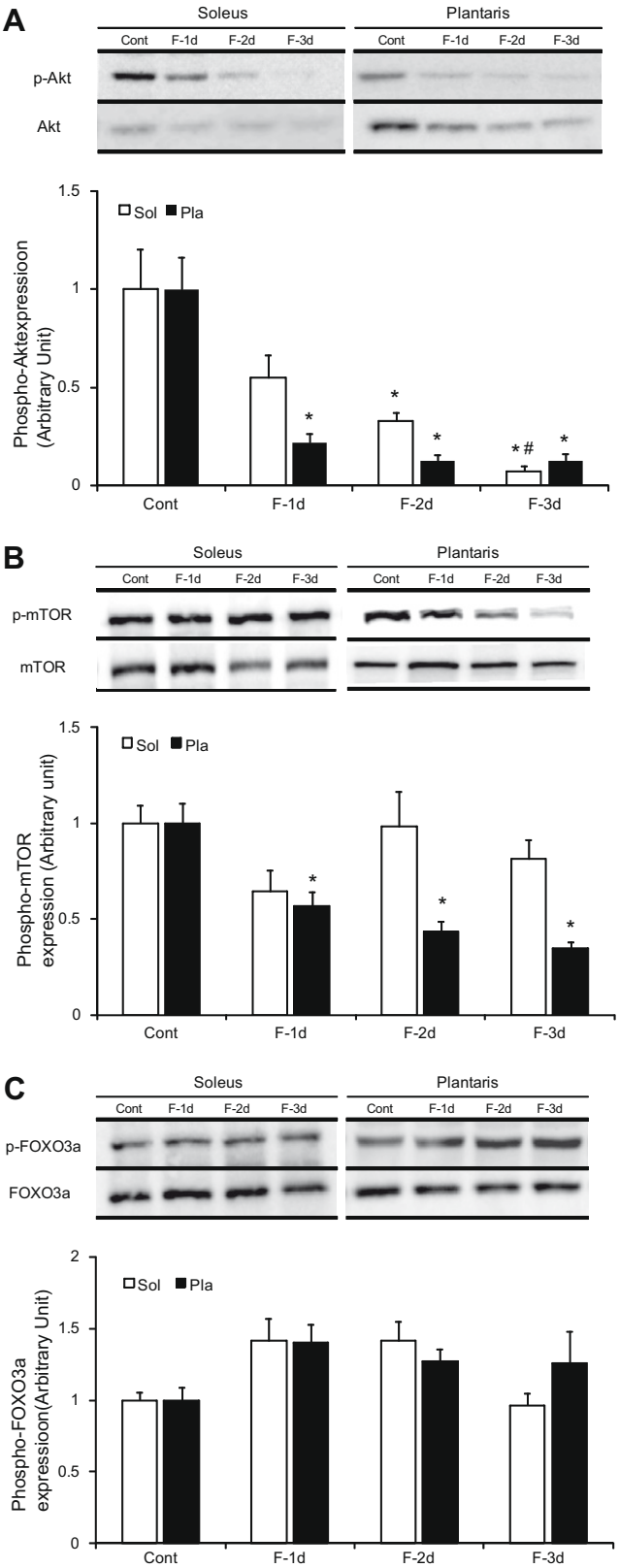

Fig. 2. Changes in phosphorylation of Akt, mTOR, and FOXO3a after fasting in soleus and plantaris muscles. Protein expression of phosphorylated-Akt (A), -mTOR (B) and -FOXO3a (C) were measured by western blotting after 1, 2, and 3 days of fasting. Cont, control; F, fasting; d, day. Values are means ± SEM. $^{*}P < 0.05$ compared with control; $^{\#}P < 0.05$ compared with F-1d in soleus (open bar) and plantaris (filled bar) muscles.

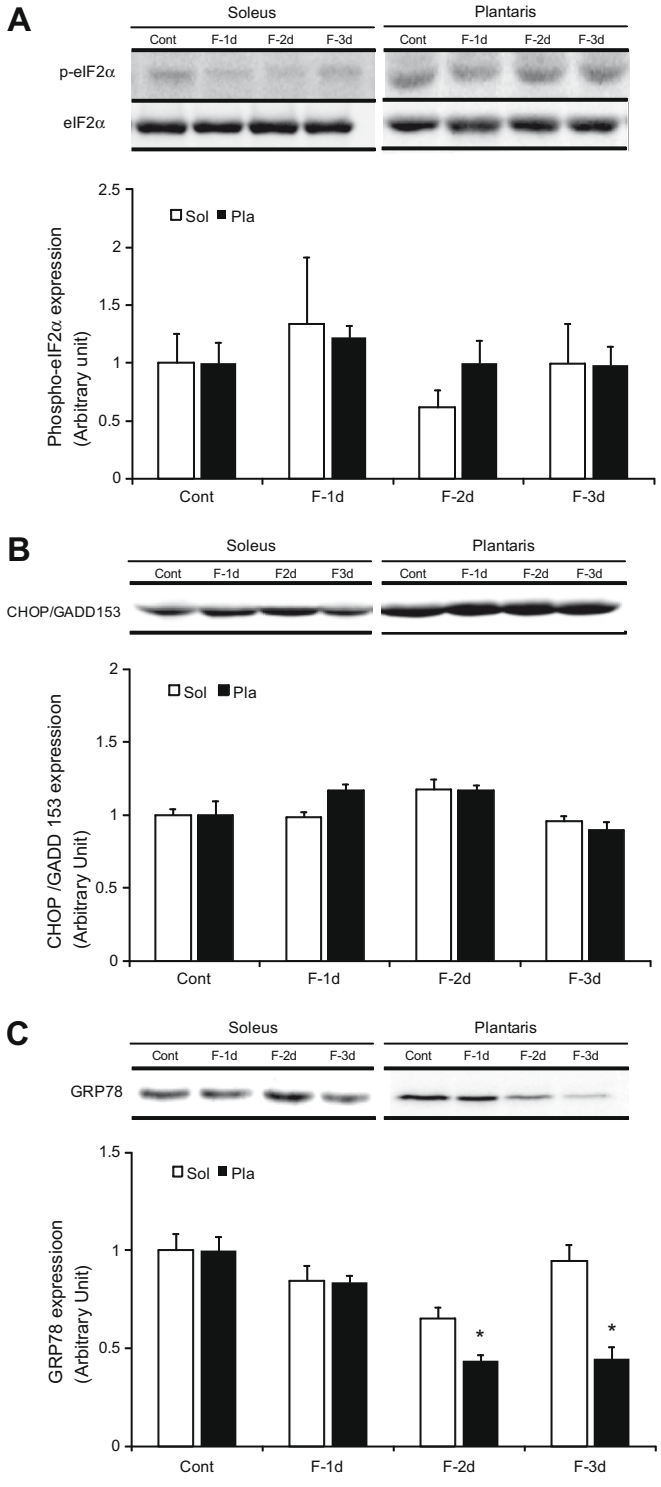

Fig. 3. Expression of ER stress signaling proteins after fasting in soleus and plantaris muscles. Expression of phosphorylated-eIF2α (A), CHOP/GADD153 (B), and GRP78 (C) protein was measured by western blotting after 1, 2, and 3 days of fasting. Cont, control; F, fasting; d, day. Values are means ± SEM. $^{*}P < 0.05$ compared with control in soleus (open bar) and plantaris (filled bar) muscles.

correlated with the number of autophagosomes [12]. In the present study, LC3-II expression was shown to be enhanced in a fasting duration-dependent manner in skeletal muscle, and the increase was notably greater in fast-twitch plantaris muscle than in slow-twitch soleus muscle. Similar results were observed for p62, an adaptor protein for LC3. This enhancement of autophagy-related proteins is consistent with the pattern of muscle weight loss rate during fasting. These findings suggest that induction of muscle type-specific autophagy during fasting is partially associated with greater weight loss in fast-twitch muscle than in slow-twitch muscle.

To identify factors involved in the preferential activation of autophagy in fast-twitch muscle, we examined expression of mTOR and FOXO3a, which are involved in Akt signaling, and proteins involved in ER stress signaling. A differential mTOR signaling response was observed in soleus and plantaris, with mTOR phosphorylation decreased by fasting only in fast-twitch muscle. There was no evidence of fasting-induced activation of FOXO3a or ER stress signals in either muscle type. These findings suggest that decreased mTOR phosphorylation plays a role in the enhancement of autophagy in fast-twitch muscle, because inactivation of mTOR is known to induce autophagy [15]. However, it is not clear how mTOR phosphorylation is regulated in skeletal muscle during fasting.

Akt has been shown to negatively regulate induction of autophagy through activation of mTOR [15] and inactivation of FOXO3a [7] by phosphorylation of these proteins. However, both total and phophorylated Akt decreased in both types of muscle, phosphorylated mTOR was decreased only in plantaris muscle, and phosphorylated FOXO3a was unchanged in both muscle types. These data indicate that Akt does not play a central role in regulation of autophagy and that skeletal muscle mTOR phosphorylation is mediated by an Akt-independent pathway during fasting.

Differences in frequency of mechanical loading between soleus and plantaris muscle may contribute to Akt-independent mTOR regulation. Slow-twitch muscle has a higher contraction frequency than fast-twitch muscle because it is used for constitutive activity such as weight bearing [16,17]. Hornberger et al. reported that constitutive mechanical loading stimulates phosphorylation of mTOR through activation of phospholipase D [18]. In addition, they showed that mechanical stimulation suppressed inactivation of mTOR by rapamycin in skeletal muscle. Thus, sustained phosphorylation of mTOR may result from mechanical loading with constant muscle contraction in soleus muscle. However, it is unclear whether regulation of mTOR in fast-twitch muscle actually contributes to the induction of autophagy, because little is known about autophagy regulation in skeletal muscle under fasting conditions. In addition, rapamycin-induced inhibition of mTOR does not cause notable autophagy in skeletal muscle [19]. Therefore, the role of mTOR in skeletal muscle autophagy remains unclear and further study is needed, although the differential regulation of mTOR during fasting shown in this study provides an important clue as to the mechanism of autophagy induction in fasted skeletal muscle.

FOXO3a is known to be one of the central regulators of autophagy, activating LC3 in skeletal muscle [7,19]. Mammucari et al. reported that activation of FOXO3a induces muscle fiber atrophy in vivo via lysosomal proteolysis by macroautophagy [19]. However, total and phosphorylated FOXO3a levels were not changed in fasted atrophying muscle in this study. Therefore, FOXO3a may not play a central role in fasting-related autophagy in skeletal muscle. In contrast, activation of ER stress signaling, a response to denatured protein in the ER lumen, is known to induce autophagy [10]. ER stress activation had not been examined in fasted skeletal muscle prior to this work. This study demonstrates that ER stress signaling is not activated by fasting and is not associated with induction of autophagy in fasted skeletal muscle.

In conclusion, this study shows that autophagy is preferentially induced in fast-twitch muscle in a fasting duration-dependent manner, and that downregulation of mTOR phosphorylation in fasting is fast-twitch muscle-specific. These findings led us to the hypothesis that differential regulation of autophagy in skeletal

muscle is related to differences in contraction frequencies between soleus and plantaris muscles. To examine this hypothesis, it will be necessary to determine whether fasting-induced autophagy is suppressed by high-frequency mechanical stimulation.

4.1. Perspective

Little is known about the induction of starvation-related muscle atrophy, although weight reduction through extremely low calorie feeding or fasting is often observed in athletes competing in weight category sports such as boxing and wrestling. Therefore, our investigation of the induction of fasting-related autophagy may provide important information for understanding mechanisms of muscle atrophy and contribute to our understanding of sports medicine and health.

Acknowledgments

This work was supported by Grants to Tomonori Ogata from Grant-in-Aid for Young Scientists (B) (#20700531) and a Grant-in-Aid for the Academic Frontier Project (Waseda University: 2005-2010) of the Ministry of Education, Culture, Sports, Science and Technology, Japan.

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