Effect of prior chronic aerobic exercise on overload-induced skeletal muscle hypertrophy in mice

Short running title: Aerobic exercise and muscle hypertrophy

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Summary

This study aimed to examine how regular aerobic training can affect the muscle hypertrophy induced by overloading. Male C57BL/6J mice were randomly divided into three groups: rest group, low-intensity aerobic exercise group, and high-intensity aerobic exercise group. Mice in the exercise groups were assigned to run at a speed of 10 m/min (low-intensity) or 25 m/min (high-intensity) for 30 min/day, five days/week, for four weeks. Then, the right hind leg gastrocnemius muscles were surgically removed to overload the plantaris and soleus muscles, while the left hind leg was subjected to a sham-operation. Both the plantaris and soleus muscles grew larger in the overloaded legs than those in the sham-operated legs. Muscle growth increased in the plantaris muscles in the low-intensity exercise group compared to that in the rest or high-intensity exercise groups at one and two weeks after overloading. This enhancement was not observed in the soleus muscles. Consistently, we observed changes in the expression of proteins involved in anabolic intracellular signaling, including Akt, mechanistic target of rapamycin (mTOR), and p70S6K, in the plantaris muscles. Our data showed for the first time that chronic low-intensity aerobic exercise precipitates overload-induced muscle growth.

Keywords aerobic exercise・skeletal muscle・hypertrophy・mTOR protein

Introduction

Skeletal muscle is a critical organ for maintaining physical strength and metabolic function. The importance of maintaining muscle mass and function has gathered the attention of scientists in aging society. Sarcopenia, the age-related loss of muscle mass and strength, which is accompanied by accumulation of muscle fat, is the main cause of frailty among the elderly (Marcell 2003, Xue 2011), which has been recognized as the main medical issue in aging societies.

Physical exercise, together with nutrition, is the main intervention used for preventing and treating muscle loss (Dickinson *et al.* 2013). Exercise training can cause the molecular and metabolic remodeling of skeletal muscles (Egan *et al.* 2013). Although the effectiveness of high-intensity resistance exercise on the increase of muscle mass and strength has been established by many studies (Peterson *et al.* 2014), such exercise is difficult to sustain and may be risky for the elderly or people with chronic diseases. The benefits of aerobic training have been linked mostly to the resultant increases in endurance capacity and insulin sensitivity (Jiang *et al.* 2010). However, aerobic training has also been shown to alter protein metabolism and induce muscle hypertrophy (Fujita *et al.* 2007, Harber *et al.* 2010, Harber *et al.* 2009, Harber *et al.* 2009, Harber *et al.* 2012, Konopka *et al.* 2014, Short *et al.* 2004).

In the present study, we investigated the effect of chronic aerobic exercise on overload-induced skeletal muscle hypertrophy. We aimed to examine how regular aerobic training, which has multiple effects such as increasing insulin sensitivity (Yuan *et al.* 2013, Cho *et al.* 2014) and suppressing chronic inflammation

69 (Jung *et al.* 2013, Kwon *et al.* 2014), can alter the muscle growth induced by overloading. We examined

70 whether insulin-AKT-mechanistic target of rapamycin (mTOR)-p70S6K signaling pathway, one of the

71 major pathways responsible for muscle protein synthesis, and muscle RING finger 1 (MuRF1) and

72 Forkhead box O1 (FoxO1) expression, which regulates muscle protein breakdown, are affected during

73 overloading by the prior aerobic exercise. Furthermore, we tested whether the intensity of aerobic

74 exercise changes the effect of the prior aerobic training.

Materials and methods

Animals

All experimental procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals of Nagoya University. C57BL/6J male mice (8 weeks of age) were obtained from Chubu Kagakushizai Co. Ltd. (Nagoya, Japan). Mice were housed individually and fed with standard chow (Oriental Yeast Co., Ltd., Tokyo, Japan) and water ad libitum. After a week of acclimation, the mice were randomly divided into three groups: the rest group, the low-intensity exercise group, and the high-intensity exercise group. The mice were maintained in a 12:12 h reversal light-dark environment at 23°C.

Overload-induced muscle hypertrophy

Overload-induced muscle hypertrophy is the model used to examine molecular and cellular mechanisms that regulate muscle growth (Spangenburg 2009). The sequence of the overloading study procedure is shown in Fig. 1. Mice were anesthetized during operation with sodium pentobarbital (50 mg/kg, intraperitoneally). Overload-induced muscle hypertrophy was induced in the right hind legs by surgical excision of gastrocnemius muscles from the Achilles' tendon to the belly of the muscle as described previously (Makanae *et al.* 2013, Serrano *et al.* 2008). This operation induces the compensatory growth of the soleus and plantaris muscles. An incision through the skin was made, and the Achilles tendon was exposed in the left hind legs (sham-operated), which were used as controls. After one week or two weeks

of overloading, muscles and epididymal fat were dissected under anesthesia, and mice were sacrificed. The wet weight of muscles was measured, and then, the muscles were frozen with liquid nitrogen and stored at -80°C until analysis.

Exercise protocol

Mice were assigned to treadmill running exercise (SEEDS Inc., Nagoya, Japan) with 0º inclination. Mice in the low-intensity exercise group were assigned to run at a speed of 10 m/min. Mice in the high-intensity exercise group were assigned to run at 10 m/min initially with an increment of 2 (or 1 on the eighth training day) m/min each training day to 25 m/min. Accordingly, mice in the high-intensity exercise group were assigned to run at 25 m/min for 30 min per day on the eighth training day and the following training days. When mice in the high-intensity exercise group could not continue running with the increased pace, the treadmill speed was decreased so that the mice could continue running. Mice in the exercise groups ran 30 min per day, five days a week, for four weeks until the day prior to the operation. The total exercise volume was 300 m/day and 750 m/day in the low-intensity group and the high-intensity group, respectively. Mice were not assigned to the exercise during overloading. To understand the effect of low-intensity or high-intensity aerobic exercise (Fig. 2, Table 1, Fig. 5d), muscles of mice in the exercise groups were dissected 24 hours after the final exercise. The wet weight of muscles was measured, and then, the muscles were frozen with liquid nitrogen and stored at -80°C until analysis.

Western blotting

111 Western blotting was performed as described previously (Li *et al.* 2008). Briefly, 10 μg of protein
112 extracts from muscle were separated by SDS-PAGE at 20 mA. The proteins were transferred to
113 polyvinylidene difluoride (PVDF) membranes (EMD Millipore Corporation, Billerica, MA, USA) by
114 semi-dry transfer at 25V for 60 mins. After blocking membranes with 5% nonfat milk for one hour at
115 room temperature, membranes were incubated overnight with a 1:1000 dilution of the primary antibody at
116 4°C. The blots were then rinsed in PBS with 0.05% Tween 20 and incubated with a 1:1000 dilution of the
117 appropriate horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature.
118 Immunoreactive bands were detected using an ECL detection system (GE Healthcare UK Limited,
119 Buckinghamshire, UK). Images of each membrane were taken on film and analyzed using Image-J
120 software (National Institutes of Health, Bethesda, MD, USA). The individual rest/overload data points
121 were divided by the group mean, thus the mean of the normalized rest/overload group is 1 with variability.
122 The density of the protein band of the rest/sham-operated, low-intensity exercise/overload and
123 sham-operated, and high-intensity exercise/overload and sham-operated groups was expressed as the fold
124 change of the density of the rest/overload values.
125 Primary antibodies against phospho-Akt (Ser473), phospho-mTOR (Ser2448), total mTOR,
126 phospho-p70S6K (Ser371), total p70S6K, total FoxO1, and total AMPK were obtained from Cell
127 Signaling Technology (Beverly, MA, USA). Primary antibodies against total Akt1/2/3 and total MuRF1
128 were obtained from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Horseradish peroxidase

129 (HRP)-conjugated goat anti-rabbit (Bio-Rad, Laboratories Inc., Hercules, CA, USA) and anti-mouse

130 (KPL, Gaithersburg, MD, USA) IgG antibodies were used as secondary antibodies.

131 *Statistical analysis*

132 All data were expressed as mean ± S.D. The multiple group comparisons were made by one-way analysis

133 of variance (ANOVA) followed by Tukey's test. A two-way ANOVA analysis was initially performed for

134 Fig 3, 4, and 5, but the interaction was found between the variables (the type of exercise and

135 overloading/sham-operated). One-way ANOVA analysis was performed among the 6 groups

136 (rest/overload or sham-operated, low-intensity exercise/overload or sham-operated, and high-intensity

137 exercise/overload or sham-operated) followed by Tukey's test. Significance was accepted at $P < 0.05$. All

138 analyses were performed using GraphPad Prism 6.0 (GraphPad Software Inc., La Jolla, CA, USA).

Results

Effect of low-intensity or high-intensity aerobic exercise on leg muscles

The effect of 4-week treadmill exercise on muscle weight and consequent protein anabolic signaling was examined (Fig. 2, Table 1). There were no significant differences in body weight, lower leg muscle weight, or epididymal fat weight among the groups. Total food intake was significantly higher in the high-intensity exercise group than in the rest group. The analysis of Akt-mTOR-p70S6K signaling showed increased Akt phosphorylation only in the low-intensity exercise group. mTOR and p70S6K phosphorylation and Akt, mTOR, and p70S6K protein levels were not different among the rest, low-intensity, and high-intensity exercise groups.

Effect of prior aerobic exercise on overload-induced muscle growth

The effect of prior 4-week treadmill exercise on the growth of overloaded muscles for one week or two weeks was examined. To evaluate the time course of muscle growth, we measured muscle weights at one week and two weeks of overloading. Overloaded muscles were significantly heavier than muscles from sham-operated leg muscles in all groups for both the soleus and plantaris muscles (Fig. 3). In addition, the plantaris muscles from the overloaded legs of mice in the low-intensity exercise group but not in the high-intensity exercise group was heavier than those of mice in the rest group at both one week and two weeks of overloading (Fig. 3a). Soleus muscle weight in the overloaded leg was significantly lower in the high-intensity exercise group than in the rest group at two weeks (Fig. 3b). Table 2 shows the changes in

157 body weight, overloaded leg muscle weight, and epididymal fat weight after one week or two weeks of
158 overloading. Body weight was not significantly different among groups. Total food intake was not
159 different among groups. Epididymal fat weight was significantly lower in the low-intensity exercise and
160 high-intensity exercise groups than in the rest group.
161 *Effect of prior aerobic exercise on overload-induced anabolic signaling in muscles*
162 To evaluate the time course of the signaling related to muscle protein synthesis, we examined the muscles
163 dissected at one week and two weeks of overloading. Phosphorylation and protein expression of Akt,
164 mTOR, and p70S6K were examined (Fig. 4). Phosphorylation and expression of Akt were increased in
165 the overloaded legs compared to those in the sham-operated legs. Phosphorylation of Akt in the
166 overloaded legs was significantly higher in the low-intensity exercise and high-intensity exercise groups
167 than in the rest group after one week, but was lower after two weeks of overloading in high-intensity
168 exercise group than in the rest group (Fib. 4a). Expression of Akt was significantly higher in the
169 overloaded legs in the high-intensity exercise group than in the rest group after one week, but was lower
170 after two weeks in the low-intensity exercise and high-intensity exercise groups than in the rest group
171 (Fig 4b).
172 Phosphorylation of mTOR was increased in overloaded legs compared to that in sham-operated legs.
173 mTOR phosphorylation in the overloaded legs in the low-intensity exercise group but not in the
174 high-intensity exercise group was significantly higher than that in the rest group after one week and two

175 weeks of overloading (Fig. 4c). After one week of overloading, mTOR protein expression was increased
176 in the overloaded legs compared to that in the sham-operated legs, with the exception of the low-intensity
177 exercise group; mTOR protein expression in the overloaded legs in the low and high-intensity exercise
178 groups was lower than that in the rest group. After two weeks of overloading, mTOR protein expression
179 in the overloaded legs was increased compared to that in the sham-operated legs, and this expression in
180 the overloaded legs in the low-intensity exercise group was increased compared to that in the rest group
181 (Fig. 4d).
182 Phosphorylation and expression of p70S6K were increased in the overloaded legs compared to those in
183 the sham-operated legs. Phosphorylation of p70S6K in the overloaded legs of the low-intensity exercise
184 group was significantly higher than that in the rest group after one week and was significantly higher in
185 the low- and high-intensity exercise groups than that in the rest group after two weeks (Fig. 4e). Protein
186 expression of p70S6K was higher in the low-intensity exercise group than in the rest group after one week
187 and two weeks (Fig. 4f).
188 *Effect of prior aerobic exercise on the protein expression of FoxO1 and MuRF-1*
189 Protein expression of FoxO1 was decreased in overloaded legs compared to that in sham-operated legs,
190 and was lower in the overloaded legs in the low- or high-intensity exercise groups than in the rest group
191 after two weeks (Fig. 5a). Furthermore, expression of MuRF1 was lower in the overloaded legs compared
192 to that in the sham-operated legs, and was lower in the overloaded legs in the low-intensity exercise and

193 the high-intensity exercise groups than in the rest group after two weeks (Fig. 5b).

194 *Effect of prior aerobic exercise on the expression of AMPK*

195 Expression of AMP-activated kinase (AMPK) in the overloaded legs was lower than that in the

196 sham-operated legs, and was lower in the overloaded legs in the low-intensity exercise group than in the

197 rest group (Fig. 5c). AMPK expression was increased after four weeks of both the low-intensity and the

198 high-intensity aerobic training (Fig. 5d).

Discussion

In this study, we showed that prior chronic aerobic training enhanced mechanical load-induced muscle hypertrophy. This effect was observed for low-intensity, but not high-intensity, aerobic training. To our knowledge, this study showed for the first time that chronic aerobic training can affect muscle growth induced by resistance stimuli. We believe that our data indicates the benefits of regular aerobic exercise. It is noteworthy that our results support the benefits of low-intensity exercise rather than high-intensity exercise.

The effect of aerobic training on skeletal muscle hypertrophy has yet to be established. Konopka et al. showed that aerobic exercise is effective for preventing age-related muscle loss through various mechanisms (Konopka *et al.* 2014) including increased muscle protein synthesis (Harber *et al.* 2010, Harber *et al.* 2009, Harber *et al.* 2009, Short *et al.* 2004). Aerobic exercise has been shown to restore anabolic insulin signaling and increase protein synthesis in older adults (Fujita *et al.* 2007). In the present study, aerobic training itself did not affect muscle mass or mTOR-p70S6K signaling. AMPK is a serine/threonine kinase that is activated by intense exercise in an intensity-dependent manner (Egan *et al.* 2013). Continuous AMPK activation can decrease insulin resistance (Ruderman *et al.* 2013) and suppress chronic inflammation, which may enhance muscle growth.

Muscle mass is regulated by the balance between muscle protein synthesis and breakdown (Schiaffino *et al.* 2013). One of the reasons for decreased muscle mass in aging individuals is the anabolic resistance

217 that occurs with age (Burd *et al.* 2013, Durham *et al.* 2010). mTOR has been shown to function as a
218 signaling node that leads to muscle protein synthesis (Kennedy *et al.* 2016). Resistance exercise increases
219 protein synthesis via three distinct signaling pathways initiated by insulin/insulin-like growth factor 1
220 (IGF1), mechanical loading, or amino acids (Kim *et al.* 2008). Activation of Akt precedes the activation
221 of mTOR in the insulin/IGF1 pathway, but mechanical loading or amino acids can activate mTOR in an
222 Akt-independent manner (Kim *et al.* 2008). The mTOR protein kinase is also found in two complexes:
223 mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). The dynamic and complex changes in
224 mTOR protein expression that occurred during our overloading experiment might be due to the
225 interaction between diverse upstream signaling pathways and the involvement of the two distinct mTOR
226 complexes, but we could not explain how this response of total-mTOR occurred. In the present study,
227 however, the overall data on signaling is consistent with the increase of overload-induced muscle growth
228 that occurred in the low-intensity exercise group.
229 The mTORC1-p70S6K pathway is required for protein synthesis in skeletal muscle. We found that
230 prior high-intensity exercise did not increase overloading-induced muscle growth. This is not consistent
231 with the increase of p70S6K phosphorylation observed after two weeks of overloading, and might be due
232 to the delayed activation of p70S6K phosphorylation in the high-intensity exercise group compared with
233 the activation of p70S6K in the low-intensity group. This difference in the profile of p70S6K may reflect
234 the difference in AMPK activation between the low-intensity group and high-intensity group, which

235 suppress the mTOR-p70S6K pathway (Bolster *et al.* 2002). Finally, we examined the muscle protein
236 degradation pathway. In catabolic conditions, activation of the degradation pathway contributes to muscle
237 loss. FOXO transcription factors control the ubiquitin-proteasome system, and MuRF1 levels increase
238 when the degradation pathway is activated (Sandri 2010). Expression of MuRF1, muscle-specific atrophy
239 related ubiquitin ligase, was not different between the low-intensity and high-intensity exercise groups.
240 The level of MuRF1 and FOXO1 expression seems to reflect the activation pattern of AKT in our study.
241 AKT activation inhibits protein degradation by suppressing FOXO activity, which decreases MuRF1
242 expression (Sandri 2013). Our data show the degradation pathway related to MuRF1 is not activated, and
243 does not explain our results showing the differential effect on muscle growth between the low-intensity
244 and high-intensity exercises.
245 In the present study, the hypertrophic effect of the prior low-intensity aerobic exercise was only
246 observed in the plantaris muscle, a primarily type II muscle, but not in the soleus, a primarily type I
247 muscle. Studies have shown that type II muscles are more sensitive to the effects of various physiological
248 and pathological conditions than type I muscles (Holecek *et al.*, 2017, Muthny *et al.* 2008, Koopman *et al.*
249 2006). The observed lack of effect of prior aerobic training on muscle growth or signaling in the soleus
250 muscle (data not shown) may be due to the difference in susceptibility to overloading between type I and
251 type II muscles, but it may also suggest that the mechanism underlying muscle growth (e.g., signaling
252 pathway) differs between the plantaris and soleus muscles. Thomson et al. reported that AMPK activation

253 diminished hypertrophy in aged fast-twitch (type II) skeletal muscle but not in slow-twitch (type I) soleus
254 muscle (Thomson *et al.* 2005). A previous study showed that the signaling proteins regulating
255 hypertrophy may act differently between soleus and plantaris muscles (Gordon *et al.* 2001).
256 Recently, both aerobic training and resistant training are recommended for maintaining health (Garber
257 *et al.* 2011). The optimal exercise mode, amount, and intensity for maintaining skeletal muscle have not
258 been established. Although the present study suggests the benefit of low intensity aerobic training to
259 muscle, we cannot address the combined effect of aerobic and resistance training on muscle mass because
260 each exercise was introduced separately. Concurrent training, defined as simultaneous incorporation of
261 endurance and resistance exercises, has been suggested to attenuate gains in muscle mass, strength, and
262 power with resistant exercise alone (Fyfe *et al.* 2014), Lundberg et al. significantly showed that an acute
263 aerobic exercise bout performed 6 h before power training enhanced the anabolic signaling compared
264 with power training by itself (Lundberg *et al.* 2012). It should also be addressed that the total exercise
265 volume of mice in the low-intensity exercise group and the high-intensity exercise group was different in
266 the present study. Further experiments with matched total volume of exercise between low-intensity and
267 high-intensity groups are warranted.

268 **Conclusion**

269 Our study showed that chronic low intensity aerobic training enhanced muscle growth, indicating that
270 mild aerobic exercise play a role in maintaining muscle mass.

Conflict of Interest

There is no conflict of interest.

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Figure Legends and Tables

Figure 1. Sequence of the study procedure for functional overloading.

Figure 2. Effect of aerobic exercise on the PI3K-Akt-mTOR pathway in the plantaris muscles. Phosphorylation and protein expression of Akt, mTOR, and p70S6K in the plantaris muscles after 4 weeks of aerobic exercise were analyzed by western blotting. Representative immunoblots are shown in the top panels. REST, rest group (n=6); LOW, low-intensity exercise group (n=6); HIGH, high-intensity exercise group (n=6). The comparison of data used ANOVA followed by Tukey's test. Data are expressed as mean ± SD. The density of the protein band of the low-intensity and high-intensity exercise groups was expressed as the fold change of the density of the mean of the rest group values. Statistical difference vs. REST group ($^{*}p<0.05$).

Figure 3. Effect of prior 4 weeks of aerobic exercise on muscle weight after 1 or 2 weeks of overloading. Weight of the plantaris muscle (a) and soleus muscle (b) of functionally overloaded legs (OL) or sham-operated legs (S) was measured after 1 or 2 weeks of overloading. REST, rest group (1 week: n=8; 2 weeks: n=6); LOW, low-intensity exercise group (1 week: n=8; 2 weeks: n=7); HIGH, high-intensity exercise group (1 week: n=6; 2 weeks: n=8). A significant difference was observed between OL and S legs after 1 or 2 weeks of overloading (***p<0.001, ****p<0.0001). The statistical analysis on the differences between 1 week and 2 weeks overloading were not made. The comparison of data from overloaded vs. sham operated leg used paired T-test. The comparison of data from different treatment groups used ANOVA followed by Tukey's test. Data are expressed as mean ± SD. Statistical difference versus REST OL legs ($^{\&\&\&}p < 0.001$, $^{\&\&\&\&}p<0.0001$).

Figure 4. Effect of prior 4 weeks of aerobic exercise on PI3K-Akt-mTOR pathway in the plantaris muscles, after overloading. Phosphorylation and protein expression of Akt, mTOR, and p70S6K in the plantaris muscles after 1 or 2 weeks of overloading were analyzed by western blotting. Representative immunoblots are shown at the top of the figures. REST, rest group (1 week: n=8; 2 weeks: n=7); LOW, low-intensity exercise group (1 week: n=8; 2 weeks: n=7); HIGH, high-intensity exercise group (1 week: n=6; 2 weeks: n=8); OL, overloaded legs; S, sham-operated legs; 1 W, Overload of 1 week; 2 W, Overload of 2 weeks. The statistical analysis on the differences between 1 week and 2 weeks overloading were not made. The density of the protein band of the low-intensity and high-intensity exercise groups was expressed as the fold change of the density of the mean of rest group (overloaded legs) values. Data are expressed as mean ± SD. The comparison of data from overloaded vs. sham operated leg used paired T-test. The comparison of data from different treatment groups used ANOVA followed by Tukey's test. A significant difference was observed between OL and S legs (*$p < 0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$). Significant differences vs. REST OL legs ($^{\&}p < 0.05$, $^{\&\&}p<0.01$, $^{\&\&\&}p<0.001$).

Figure 5. Effect of prior 4 weeks of aerobic exercise on FoxO1, MuRF1, and AMPK expression in the plantaris muscles, after overloading. Protein expression of FoxO1 (a), MuRF1 (b), and AMPK (c) after 1 or 2 weeks of overloading were analyzed by western blotting. Representative immunoblots are shown at the top of the figures. REST, rest group (1 week: n=8; 2 weeks: n=7); LOW, low-intensity exercise group (1 week: n=8; 2 weeks: n=7); HIGH, high-intensity exercise group (1 week: n=6; 2 weeks: n=8); OL, overloaded legs; S, sham-operated legs; 1 W, Overload of 1 week; 2 W, Overload of 2 weeks. The statistical analysis on the differences between 1 week and 2 weeks overloading were not made. The density of the protein band of the low-intensity and high-intensity exercise groups was expressed as the fold change of the density of the mean of rest group (overloaded legs) values. Protein expression of AMPK (d) after 4 weeks of aerobic training in the plantaris muscles were analyzed by western blotting.

425 REST, rest group (n=6); LOW, low-intensity exercise group (n=6); HIGH, high-intensity exercise group
426 (n=6); Data are expressed as mean ± SD. The comparison of data from overloaded vs. sham operated leg
427 used paired T-test. The comparison of data from different treatment groups used ANOVA followed by
428 Tukey's test. The density of the protein band of the low-intensity and high-intensity exercise groups was
429 expressed as the fold change of the density of the mean of the rest group values. Significant differences
430 were observed between OL and S legs (*$p < 0.05$, **$p<0.01$, ***$p<0.001$), and vs. REST OL legs ($^{\&}p <$
431 0.05, $^{\&\&}p<0.01$, $^{\&\&\&}p<0.001$). Significant difference vs. REST group ($^{\$}p<0.05$) in (d).
432

Table 1. Body weight, weight of muscles, and epididymal fat weight after 4 weeks of aerobic exercise

	REST (n=6)	LOW(n=6)	HIGH(n=6)
Body weight (g)	24.4 ± 1.3	24.0 ± 1.2	23.2 ± 0.1
Weight of muscle (mg)			
Gastrocnemius	140 ± 10	137 ± 9	135 ± 11
Plantaris	23.8 ± 2.3	22.4 ± 1.9	22.0 ± 2.0
Soleus	11.6 ± 1.5	10.4 ± 1.0	10.3 ± 1.4
Tibialis anterior	49.0 ± 4.1	46.8 ± 3.1	44.5 ± 3.6
Extensor digitorum	11.5 ± 0.8	10.9 ± 0.8	11.1 ± 0.7
Epididymal fat weight (mg)	349 ± 71	356 ± 73	316 ± 81
Total food intake (g/day)	3.25 ± 0.19	3.31 ± 0.32	3.48 ± 0.23*

REST: rest group; LOW: low-intensity exercise group; HIGH: high-intensity exercise group.

Data are expressed as mean ± SD.

Statistical difference vs. the REST group (*$p<0.05$)

Table 2. Body weight, weight of muscles, and epididymal fat weight after 1 week or 2 weeks of overloading

	1 week of overloading			2 weeks of overloading		
	REST (n=8)	LOW (n=8)	HIGH (n=6)	REST (n=6)	LOW (n=7)	HIGH (n=8)
Body weight (g)	25.4 ± 1.7	25.8 ± 0.7	25.5 ± 1.6	25.7 ± 1.4	25.7 ± 1.8	24.7 ± 1.5
Weight of muscle[#] (mg)						
Tibialis anterior	46.2 ± 2.6	49.6 ± 5.5	46.2 ± 3.9	45.7 ± 1.9	48.6 ± 2.4	47.5 ± 2.6
Extensor digitorum longus	11.6 ± 0.8	11.3 ± 0.5	12.1 ± 1.3	11.6 ± 0.5	12.4 ± 0.7	11.6 ± 0.7
Epididymal fat weight (mg)	354 ± 27	221 ± 53****	216 ± 63***	413 ± 11	291 ± 45 *	281 ± 56 *
Total food intake (g/day)	3.64 ± 0.12	3.72 ± 0.15	3.73 ± 0.10	3.60 ± 0.05	3.65 ± 0.11	3.64 ± 0.10

REST: rest group; LOW: low-intensity exercise group; HIGH: high-intensity exercise group.
Data are expressed as mean ± SD. [#] Muscles of overloaded legs.
Statistical difference vs. the REST group in each group. (*p<0.05,***p<0.001,****p<0.0001).

Figure 1. Sequence of the study procedure for functional overloading.

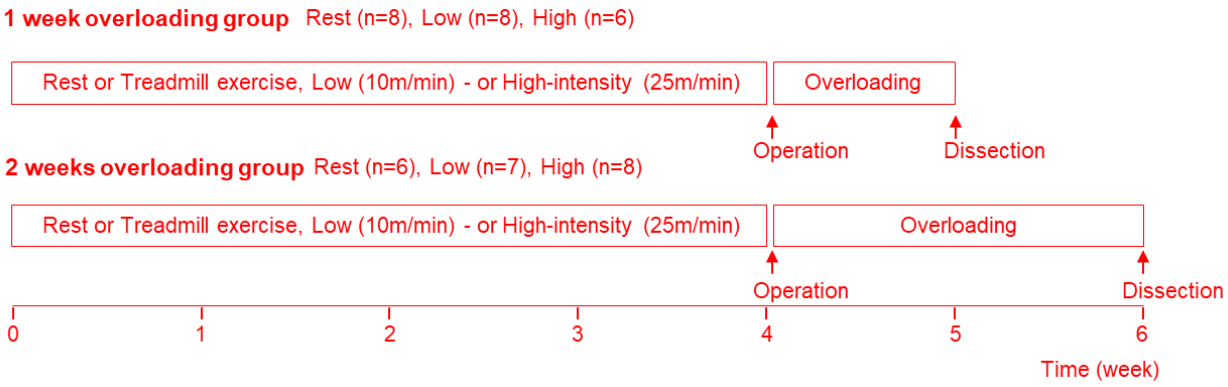

Figure 2. Effect of aerobic exercise on the PI3K-Akt-mTOR pathway in the plantaris muscles.

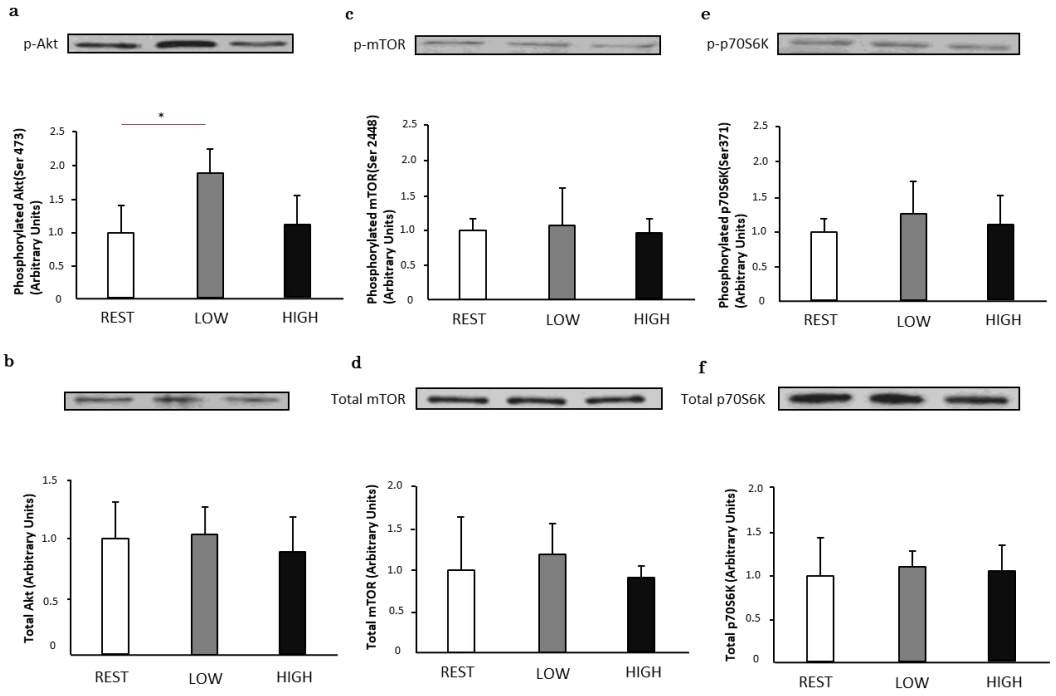

Figure 3. Effect of prior 4 weeks of aerobic exercise on muscle weight after 1 or 2 weeks of overloading.

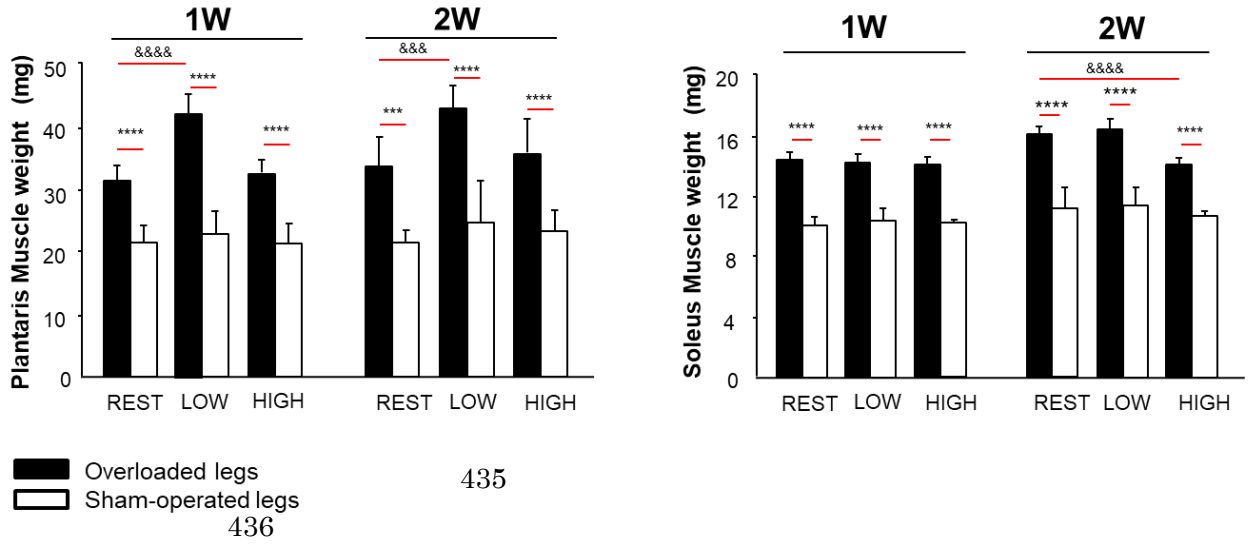

Figure 4. Effect of prior 4 weeks of aerobic exercise on PI3K-Akt-mTOR pathway in the plantaris muscles, after overloading.

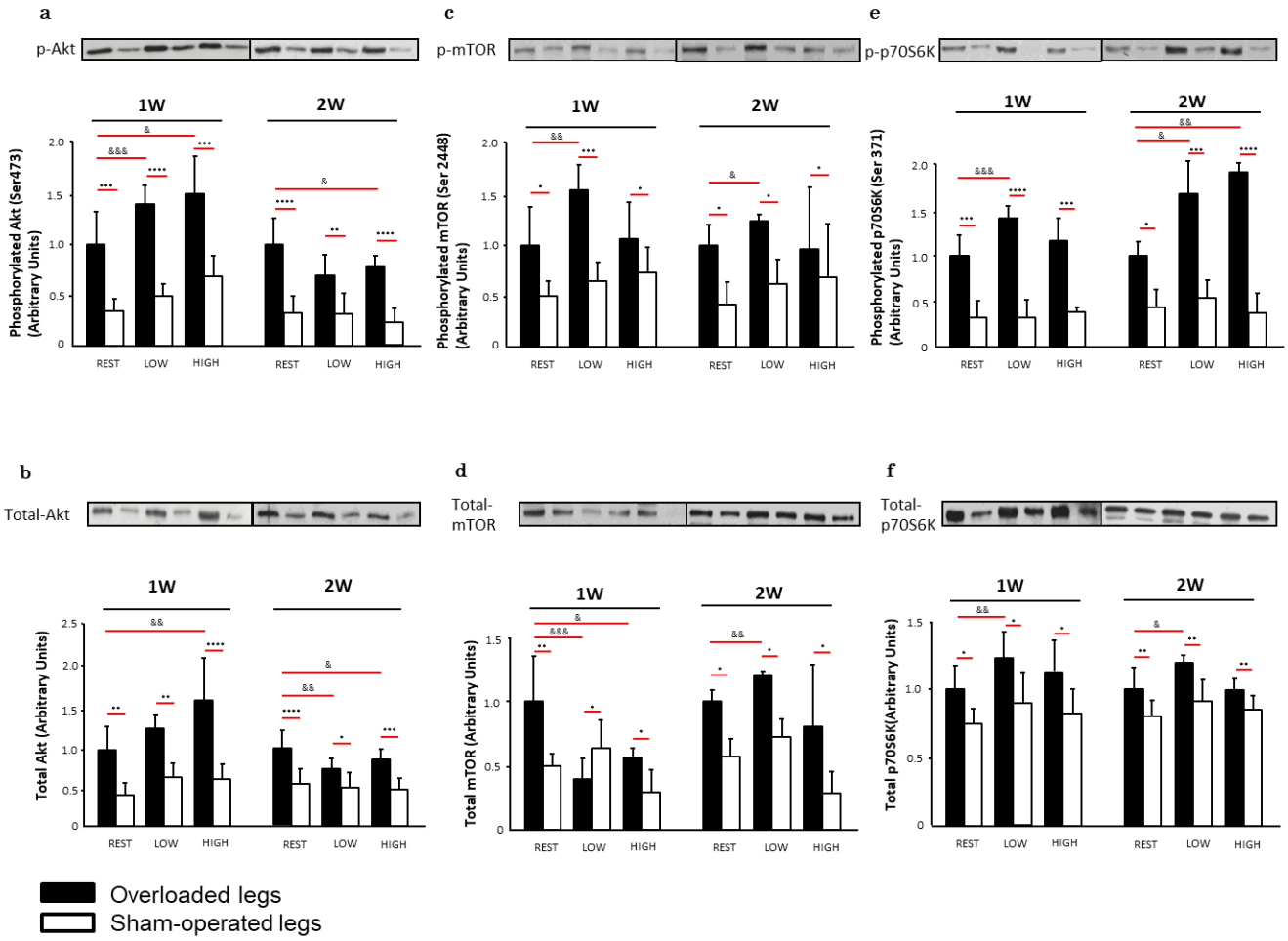

Figure 5. Effect of prior 4 weeks of aerobic exercise on FoxO1, MuRF1, and AMPK expression in the plantaris muscles, after overloading.