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Effect of electrical stimulation-induced resistance exercise on mitochondrial fission and fusion proteins in rat skeletal muscle

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Running Head: Resistance exercise and mitochondrial dynamics

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ABSTRACT

It is well known that resistance exercise increases muscle protein synthesis and muscle strength. However, little is known about the effect of resistance exercise on mitochondrial dynamics, which is coupled with mitochondrial function. In skeletal muscle, mitochondria exist as dynamic networks that are continuously remodeling through fusion and fission. The purpose of this study was to investigate the effect of acute and chronic resistance exercise, which induces muscle hypertrophy, on the expression of proteins related to mitochondrial dynamics in rat skeletal muscle. Resistance exercise consisted of maximum isometric contraction, which was induced by percutaneous electrical stimulation of the gastrocnemius muscle. Our results revealed no change in mitochondrial fission (Fis1 and Drp1) or fusion (Opa1, Mfn1, and Mfn2) regulatory protein levels over the 24-h period following acute resistance exercise. Phosphorylation of Drp1 at Ser616 was increased immediately after exercise (P<0.01). Four weeks of resistance training (three times/week) increased Mfn1 (P<0.01), Mfn2 (P<0.05), and Opa1 (P<0.01) protein levels without altering mitochondrial oxidative phosphorylation proteins. These observations suggest that resistance exercise has little effect on mitochondrial biogenesis, but alters the expression of proteins involved in mitochondrial fusion and fission, which may contribute to mitochondrial quality control and improved mitochondrial function.

Key Words: skeletal muscle; resistance exercise; mitochondrial dynamics; Fis1; Drp1; Opa1; Mfn

INTRODUCTION

It has been well recognized that resistance exercise increases muscle protein synthesis and muscle strength, and thus can delay age-associated declines in muscle mass and function. However, the impact of resistance exercise on energy metabolism in skeletal muscle remains relatively unknown. Previous studies have shown that resistance exercise largely does not alter the maximal activity of mitochondrial enzymes such as citrate synthase and succinate dehydrogenase, which are considered to reflect mitochondrial volume (Tesch et al. 1990; Green et al. 1999b). More recent studies have found mitochondrial respiration to increase after resistance training in human skeletal muscle (Pesta et al. 2011; Salvadego et al. 2013; Porter et al. 2014). Therefore, resistance exercise training may increase mitochondrial function without changes in mitochondrial mass.

In skeletal muscle, mitochondria exist as dynamic networks that are continuously remodeling through fusion and fission. These processes, termed mitochondrial dynamics, are important for maintenance of functional mitochondria. Mitochondrial fusion is regulated by mitofusin proteins (Mfn1 and Mfn2) and by optic atrophy 1 (Opa1) in the outer and inner mitochondrial membrane, respectively (Meeusen et al. 2006; Chen et al. 2010). Opa1 undergoes proteolytic processing, which has been shown to be essential in fusion activity (Duvezin-Caubet et al. 2006; Wang et al. 2014). Mitochondrial fission is mediated by dynamin related protein 1 (Drp1), a cytosolic protein that translocates to the outer surface of the mitochondria when activated, in combination with fission protein 1 (Fis1) (Romanello et al. 2010). Drp1 is activated by the phosphorylation of serine 616 (Taguchi et al. 2007). Disorders of mitochondrial dynamics can lead to reduced mitochondrial respiratory capacity (Bach et al. 2003; Chen et al. 2005; Parone et al. 2008), suggesting that balanced mitochondrial fisson and fusion events are critical to the maintenance of mitochondrial function.

Dysfunction of skeletal muscle mitochondria in type 2 diabetes or chronic muscle disuse has been reported to be associated with a decrease in the abundance of mitochondrial fusion proteins or with an increase in the activation status of the mitochondrial fission protein Drp1 (Bach et al. 2003; Iqbal et al. 2013; Picard et al. 2015). In contrast, a recent study demonstrated that an endurance exercise-type chronic muscle contractile activity increased mitochondrial fusion protein levels (Iqbal et al. 2013). Moreover, exercise training decreases the activation status of Drp1 in insulin-resistant human skeletal muscle (Fealy et al. 2014). Therefore, the increase in skeletal muscle oxidative capacity with exercise training may not only attribute to mitochondrial biogenesis, but also to changes in mitochondrial dynamics. However, the effects of resistance exercise on mitochondrial dynamics proteins have not yet been examined. The purpose of this study was to examine the abundance and activation status of the mitochondrial fission (Fis1 and Drp1) and fusion (Opa1 and Mfn2) regulatory proteins following acute and chronic resistance exercise in rat skeletal muscle.

MATERIALS AND METHODS

Animals

Thirty-five male Sprague-Dawley rats (male, aged 10 weeks) were obtained from CLEA Japan (Tokyo, Japan). All animals were housed individually in an environment maintained at 22-24□ with a 12-h light-dark cycle and were allowed food and water ad libitum. Rats were randomly assigned to one of seven groups: sedentary (SED), acute resistance exercise (RE), acute resistance exercise followed by 1 h of recovery (RE+1h), as well as 3 h (RE+3h), 6 h (RE+6h), and 24 h (RE+24h), and four weeks of resistance exercise training (RT). This study was approved by the Ethics Committee for Animal Experiments at Ritsumeikan University.

Experimental protocol

Under isoflurane anesthesia, the hair was shaved off the right lower leg of each rat, and then the rats were positioned with their right foot on a footplate (the ankle joint angle was positioned at

90°) in the prone posture. The triceps surae muscle was stimulated percutaneously with with electrodes (Vitrode V, Ag/AgCl; Nihon Kohden, Tokyo, Japan), which were cut into 10×5-mm sections and connected to an electric stimulator and isolator (SS-104J; Nihon Kohden, Japan). The right gastrocnemius muscle was isometrically exercised as previously described (Ogasawara et al. 2013; Ogasawara et al. 2014). For all exercise sessions, the gastrocnemius muscle was trained by stimulating ten 3-s contractions, with a 7-s interval between contractions, per set for 5 sets, with 3-min rest intervals. The voltage (~30 V) and stimulation frequency (100 Hz) were adjusted to produce maximal isometric tension. Animals were euthanized and the target tissues were removed at a basal state, and at immediately, 1 h, 3 h, 6 h, 24 h after the completion of exercise. Rats in the RT group were trained every other day for 4 weeks (12 exercise sessions in total) using the same electrical stimulation protocol described above, and the left gastrocnemius muscle served as an internal control for the examination of training adaptations. Animals were euthanized and the target tissues were removed 48 h after the last exercise session. The tissues were rapidly frozen in liquid nitrogen and stored at -80□ until further analysis.

Western blotting

Whole gastrocnemius muscle samples were homogenized using a Polytron homogenizer in homogenization buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 1 mM EDTA and 0.1% SDS) supplemented with protease and phosphatase inhibitor cocktail (Thermo Fisher Scientific, Waltham, MA). The homogenates were centrifuged at 10,000 g for 10 min at 4°C. The supernatant was removed, and the protein concentration of each sample was quantified using a Protein Assay Rapid Kit (Wako, Japan). Equal amounts of protein (5–15 μg, depending on the protein of interest) were loaded onto 10–12% SDS-PAGE gels and separated by electrophoresis. Proteins were transferred to polyvinylidene difluoride membranes, and Western blotting was carried out using the primary antibodies: Total OXPHOS Rodent WB Antibody Cocktail [NDUFB8 (NADH dehydrogenase (ubiquinone) 1 beta

subcomplex 8), SDHB (succinate dehydrogenase complex subunit B), UQCRC2 (ubiquinol-cytochrome c reductase core protein II), ATP5A (ATP synthase, H+ transporting, mitochondrial F1 complex, alpha subunit 1); ab110413], LDH (lactate dehydrogenase; ab134187), FAT/CD36 (fatty acid translocase; ab137320), Fis1 (ab96764), Drp1 (ab56788), Mfn2 (ab124773), and Parkin (ab77924) from Abcam (Cambridge, MA); COX IV (cytochrome c oxidase subunit 4; #4850), SOD2 (Superoxide dismutase 2; #13141), and Phospho-Drp1 (Ser616, #3455) from Cell Signaling Technology (Danvers, MA); Opa1 (#612606) from BD transduction laboratories (Tokyo, Japan); Mfn1 (sc-50330) and PFK (phosphofructokinase; sc-31712) from Santa Cruz Biotechnology (Santa Cruz, CA); GLUT4 (#07-1404) from Millipore (Billerica, MA). Antibodies against MCT (monocarboxylate transporter)1 and MCT4 were raised in rabbits against the C-terminal region of the respective MCT (Qiagen, Japan) and have been used in previous studies (Enoki et al. 2006; Kitaoka et al. 2014). Membranes were then incubated with the appropriate secondary antibody (A106PU or A102PT; American Qualex, San Clemente, CA or sc-2020; Santa Cruz Biotechnology) and visualized by enhanced chemiluminescence detection reagent (Thermo Fisher Scientific). Blots were scanned and quantified using ChemiDoc XRS (Bio-Rad Laboratories, Hercules, CA) and Quantity One (version 4.5.2, Bio-Rad). Ponceau staining was used to verify consistent loading.

Statistical analysis

Data were expressed as mean ± SEM. Multi-group comparisons in acute study were performed by one-way analysis of variance (ANOVA), followed by the Dunnett post hoc test. Paired t-tests were used to test for differences between groups in chronic study (GraphPad Prism 5.0, La Jolla, CA). Values of $P < 0.05$ were considered significant.

RESULTS

Effect of acute resistance exercise on mitochondrial dynamics proteins

To examine the adaptations of mitochondrial fusion and fission proteins to resistance exercise,

we first investigated the time course of protein expression after acute exercise. We did not identify any changes in the level of mitochondrial fission protein Fis1. Phosphorylation of Drp1 at Ser616 was increased immediately after exercise (+137%, $P<0.01$) and 1 hour after exercise (+75%, $P<0.05$), without changes in total Drp1 protein abundance. There was no change in the level of mitochondrial fusion proteins Mfn1, Mfn2, or total Opa1 over the 24-h period following acute resistance exercise. As it has been previously reported, knockdown of Opa1-induced mitochondrial fragmentation is recovered by the expression of the long isoform of Opa1 (L-Opa1) but not the short isoform of Opa1 (S-Opa1) (Ishihara et al. 2006), which indicates that L-Opa1 is the active isoform. We quantified these isoforms separately, but did not find any changes in either isoform of Opa1 or the ratio of L-Opa1 to S-Opa1 (Figure 1).

Effect of chronic resistance training on mitochondrial dynamics proteins

Next, we examined the effect of chronic resistance exercise on the levels of mitochondrial dynamics proteins. Four weeks of resistance training (12 exercise sessions) increased Mfn1 (+24%, $P<0.01$) and Mfn2 (+14%, $P<0.05$) protein levels. Both S- and L-isoforms of Opa1 and total Opa1 levels were also increased (+20%, $P<0.01$). There were no changes in the levels of Fis1, total Drp1, or Drp1 phosphorylation at Ser616 (Figure 2).

Effect of chronic resistance training on energy metabolism proteins

Mitochondrial oxidative phosphorylation (OXPHOS) protein levels were not altered by 4 weeks of resistance training, except for UQCRC (complex □), which slightly increased (+8%, $P<0.05$). Levels of superoxide dismutase 2 (SOD2), a mitochondrial specific antioxidant enzyme, increased after resistance training (+11%, $P<0.05$). We did not observe any changes in Parkin, a protein involved in mitophagy (Figure 3A), coinciding with a recent human study (Ogborn et al. 2015). Glycolytic enzyme protein levels (phosphofructokinase and lactate dehydrogenase) were not altered by resistance training (Figure 3B). Levels of glucose, lactate, and fatty acid transporter proteins did not change after resistance training, except for MCT4, which markedly increased (+24%, $P<0.05$) (Figure 3C).

DISCUSSION

To the best of our knowledge, changes in the abundance and activation status of mitochondrial fusion and fission regulatory proteins by resistance exercise have not been investigated in skeletal muscle. In this study, we used the rat electrical stimulation model and have shown that 1) acute resistance exercise increased the activation status of Drp1 and 2) chronic resistance training increased mitochondrial fusion protein Mfn1, Mfn2, and Opa1 levels without altering mitochondrial mass in rat skeletal muscle. In our previous studies, we demonstrated that this exercise protocol induces mammalian target of rapamycin (mTOR) signaling activation, associated with increased muscle protein synthesis, and that long-term training induces significant increases in muscle size and strength (Ogasawara et al. 2013; Ogasawara et al. 2014). To further validate our rat isometric training model, we examined proteins involved in glycolysis, OXPHOS, and metabolite transport. We observed no changes in most of these proteins related to energy metabolism, which is consistent with previous human studies that reported no changes in either glycolytic or oxidative enzyme activities after resistance training (Tesch et al. 1990; Green et al. 1999a). This adaptation is clearly different from endurance training or high-intensity interval training, which are known to upregulate metabolite transport proteins for each specific substrate uptake, accompanied by mitochondrial biogenesis (Burgomaster et al. 2008; Perry et al. 2008; Hoshino et al. 2013). In this study, we observed a significant increase in MCT4, a protein that facilities lactate release from muscle cells. This observation is consistent with a previous human study (Juel et al. 2004). Importantly, expression of MCT1, but not MCT4, increased after 1 and 3 weeks of chronic muscle stimulation (10 Hz, 24 h/day), an endurance exercise model in rats (Bonen et al. 2000). Thus, in this study we utilized high-frequency muscle contractions as the model of resistance exercise in rats. Our findings suggest that acute and chronic resistance exercise alters the activation status and abundance of mitochondrial dynamics proteins without altering mitochondrial volume.

209

210 Previous studies have demonstrated that acute exercise may potentially promote the
211 mitochondrial fission process. Treadmill running for 150 min reported to increase Fis1 protein
212 level and decrease Mfn1 protein in rat skeletal muscle (Ding et al. 2010). Another study has
213 shown that 90 min of treadmill running increased phosphorylation of Drp1 at Ser616 without
214 altering expression of Mfn1 and Mfn2 in skeletal muscle of mice (Jamart et al. 2013). In this
215 study, we found that the activation status of Drp1 was increased immediately after acute
216 resistance exercise. Recent studies have shown that Drp1 phosphorylation at Ser616 is regulated
217 by extracellular signal-regulated protein kinase (ERK)1/2 (Kashatus et al. 2015; Serasinghe et al.
218 2015). Interestingly, this pathway is well known to be activated by resistance exercise (Karlsson
219 et al. 2004; Moore et al. 2011). In addition, skeletal muscle contraction-induced reactive oxygen
220 species (ROS) production can induce mitochondrial fragmentation (Fan et al. 2010). Acute
221 exercise-activated fission process might be important in removing damaged mitochondria, since
222 autophagy is reported to be required for training adaptation in skeletal muscle (Lira et al. 2013).
223 In addition, fission may be conceivably required to remodel the mitochondrial network.
224 However, at present, very little information is available regarding changes in mitochondrial
225 morphology followed by acute exercise. One study has demonstrated that 3-h voluntary exercise
226 remodels mitochondrial membrane interactions, without changes in Mfn2 and Opa1 in mouse
227 skeletal muscle (Picard et al. 2013). Further studies are needed to understand the acute
228 exercise-induced mitochondrial remodeling.

229

230 In contrast to acute exercise, chronic exercise training may induce the mitochondrial fusion
231 process. A recent study demonstrated that 7 days of chronic muscle contractile activity (10 Hz, 3
232 h/day), an endurance exercise model in rats, increased protein levels of Mfn2 and Opa1, while
233 Drp1 was decreased and Fis1 was not affected (Iqbal et al. 2013). In a human study, 2 weeks of
234 high-intensity interval training was shown to increase protein levels of Fis1, Drp1, and Mfn1,

but not Mfn2 (Perry et al. 2010). Other studies in humans (Cartoni et al. 2005) and rats (Ding et al. 2010) have also reported an increase in Mfn1 and Mfn2 transcript levels. However, transcript and protein levels of mitochondrial dynamics proteins may not directly translate to altered fission/fusion, given that these proteins are regulated by post-translational modifications and proteolytic processing (Duvezin-Caubet et al. 2006; Ishihara et al. 2006; Taguchi et al. 2007). Therefore, in the current study, we evaluated the phosphorylation of Drp1 at Ser616 and L-Opa1, which represented the activation status of these proteins, in addition to their total protein abundances. We found that 4 weeks of resistance training increased L-Opa1 protein level, while phosphorylation of Drp1 at Ser616 was not altered in rat skeletal muscle. Protein levels of total Opa1, Mfn1, and Mfn2 also increased, whereas total Drp1 and Fis1 levels were unchanged. Our results suggest that chronic resistance training activates the mitochondrial fusion process, which possibly leads to an expanded mitochondrial reticular network along with muscle hypertrophy, although the absence of actual mitochondrial morphology data via electron microscopy is the main limitation of this study. Nevertheless, previous studies which demonstrated that repression of Mfn2 reduces respiratory capacity and mitochondrial membrane potential in muscle cells (Bach et al. 2003), or loss of Mfn1 and Mfn2 causes severe mitochondrial dysfunction and muscle atrophy with high levels of mtDNA mutations (Chen et al. 2010), support the importance of increased mitochondrial fusion proteins in response to resistance training.

It was generally assumed that resistance training has little effect on skeletal muscle mitochondria, since maximal activity of oxidative enzymes is not altered. In the current study, we demonstrated that acute and chronic resistance exercise altered the activation status and total protein abundance of proteins involved in mitochondrial fusion and fission, which may contribute to improved mitochondrial and subsequently muscle function.

Conflict of interest statement: There is no conflict of interest.

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Figure Legends.

Figure 1. **Mitochondrial dynamics protein expression after acute resistance exercise in rat skeletal muscle.** (A) Mitochondrial fission proteins. (B) Mitochondrial fusion proteins. Data are presented as mean ± S.E.M. n = 5 in each group. **Significantly different from sedentary subjects (P<0.01) and *Significantly different from sedentary subjects (P<0.05).

Figure 2. **Mitochondrial dynamics protein expression after 4 weeks of resistance training in rat skeletal muscle.** Data are presented as mean ± S.E.M. n = 5 in each group. **Significantly different from control subjects (P<0.01) and *Significantly different from control subjects (P<0.05).

Figure 3. **Energy metabolism protein expression after 4 weeks of resistance training in rat skeletal muscle.** (A) Mitochondrial proteins. (B) Glycolytic enzymes. (C) Metabolite transporters. Data are presented as mean ± S.E.M. n = 5 in each group. *Significantly different from control subjects (P<0.05).