



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

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

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12 Running Head: Resistance exercise and mitochondrial dynamics

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27 **ABSTRACT**

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

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

46 Mfn

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**53 INTRODUCTION**

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

64

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

78

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

92

## 93 **MATERIALS AND METHODS**

### 94 **Animals**

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

### 102 **Experimental protocol**

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

105 90°) in the prone posture. The triceps surae muscle was stimulated percutaneously with with  
106 electrodes (Vitrode V, Ag/AgCl; Nihon Kohden, Tokyo, Japan), which were cut into 10×5-mm  
107 sections and connected to an electric stimulator and isolator (SS-104J; Nihon Kohden, Japan).  
108 The right gastrocnemius muscle was isometrically exercised as previously described  
109 (Ogasawara et al. 2013; Ogasawara et al. 2014). For all exercise sessions, the gastrocnemius  
110 muscle was trained by stimulating ten 3-s contractions, with a 7-s interval between contractions,  
111 per set for 5 sets, with 3-min rest intervals. The voltage (~30 V) and stimulation frequency (100  
112 Hz) were adjusted to produce maximal isometric tension. Animals were euthanized and the  
113 target tissues were removed at a basal state, and at immediately, 1 h, 3 h, 6 h, 24 h after the  
114 completion of exercise. Rats in the RT group were trained every other day for 4 weeks (12  
115 exercise sessions in total) using the same electrical stimulation protocol described above, and  
116 the left gastrocnemius muscle served as an internal control for the examination of training  
117 adaptations. Animals were euthanized and the target tissues were removed 48 h after the last  
118 exercise session. The tissues were rapidly frozen in liquid nitrogen and stored at -80°C until  
119 further analysis.

#### 120 **Western blotting**

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

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

#### 148 **Statistical analysis**

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

153

## 154 **RESULTS**

### 155 **Effect of acute resistance exercise on mitochondrial dynamics proteins**

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

157 we first investigated the time course of protein expression after acute exercise. We did not  
158 identify any changes in the level of mitochondrial fission protein Fis1. Phosphorylation of Drp1  
159 at Ser616 was increased immediately after exercise (+137%,  $P<0.01$ ) and 1 hour after exercise  
160 (+75%,  $P<0.05$ ), without changes in total Drp1 protein abundance. There was no change in the  
161 level of mitochondrial fusion proteins Mfn1, Mfn2, or total Opa1 over the 24-h period following  
162 acute resistance exercise. As it has been previously reported, knockdown of Opa1-induced  
163 mitochondrial fragmentation is recovered by the expression of the long isoform of Opa1  
164 (L-Opa1) but not the short isoform of Opa1 (S-Opa1) (Ishihara et al. 2006), which indicates that  
165 L-Opa1 is the active isoform. We quantified these isoforms separately, but did not find any  
166 changes in either isoform of Opa1 or the ratio of L-Opa1 to S-Opa1 (Figure 1).

#### 167 **Effect of chronic resistance training on mitochondrial dynamics proteins**

168 Next, we examined the effect of chronic resistance exercise on the levels of mitochondrial  
169 dynamics proteins. Four weeks of resistance training (12 exercise sessions) increased Mfn1  
170 (+24%,  $P<0.01$ ) and Mfn2 (+14%,  $P<0.05$ ) protein levels. Both S- and L-isoforms of Opa1 and  
171 total Opa1 levels were also increased (+20%,  $P<0.01$ ). There were no changes in the levels of  
172 Fis1, total Drp1, or Drp1 phosphorylation at Ser616 (Figure 2).

#### 173 **Effect of chronic resistance training on energy metabolism proteins**

174 Mitochondrial oxidative phosphorylation (OXPHOS) protein levels were not altered by 4 weeks  
175 of resistance training, except for UQCRC (complex  $\square$ ), which slightly increased (+8%,  $P<0.05$ ).  
176 Levels of superoxide dismutase 2 (SOD2), a mitochondrial specific antioxidant enzyme,  
177 increased after resistance training (+11%,  $P<0.05$ ). We did not observe any changes in Parkin, a  
178 protein involved in mitophagy (Figure 3A), coinciding with a recent human study (Ogborn et al.  
179 2015). Glycolytic enzyme protein levels (phosphofructokinase and lactate dehydrogenase) were  
180 not altered by resistance training (Figure 3B). Levels of glucose, lactate, and fatty acid  
181 transporter proteins did not change after resistance training, except for MCT4, which markedly  
182 increased (+24%,  $P<0.05$ ) (Figure 3C).



183

184 **DISCUSSION**

185 To the best of our knowledge, changes in the abundance and activation status of mitochondrial  
186 fusion and fission regulatory proteins by resistance exercise have not been investigated in  
187 skeletal muscle. In this study, we used the rat electrical stimulation model and have shown that  
188 1) acute resistance exercise increased the activation status of Drp1 and 2) chronic resistance  
189 training increased mitochondrial fusion protein Mfn1, Mfn2, and Opa1 levels without altering  
190 mitochondrial mass in rat skeletal muscle. In our previous studies, we demonstrated that this  
191 exercise protocol induces mammalian target of rapamycin (mTOR) signaling activation,  
192 associated with increased muscle protein synthesis, and that long-term training induces  
193 significant increases in muscle size and strength (Ogasawara et al. 2013; Ogasawara et al. 2014).  
194 To further validate our rat isometric training model, we examined proteins involved in  
195 glycolysis, OXPHOS, and metabolite transport. We observed no changes in most of these  
196 proteins related to energy metabolism, which is consistent with previous human studies that  
197 reported no changes in either glycolytic or oxidative enzyme activities after resistance training  
198 (Tesch et al. 1990; Green et al. 1999a). This adaptation is clearly different from endurance  
199 training or high-intensity interval training, which are known to upregulate metabolite transport  
200 proteins for each specific substrate uptake, accompanied by mitochondrial biogenesis  
201 (Burgomaster et al. 2008; Perry et al. 2008; Hoshino et al. 2013). In this study, we observed a  
202 significant increase in MCT4, a protein that facilitates lactate release from muscle cells. This  
203 observation is consistent with a previous human study (Juel et al. 2004). Importantly, expression  
204 of MCT1, but not MCT4, increased after 1 and 3 weeks of chronic muscle stimulation (10 Hz,  
205 24 h/day), an endurance exercise model in rats (Bonen et al. 2000). Thus, in this study we  
206 utilized high-frequency muscle contractions as the model of resistance exercise in rats. Our  
207 findings suggest that acute and chronic resistance exercise alters the activation status and  
208 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,

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

253

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

259

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

261

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391 **Figure Legends.**

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

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

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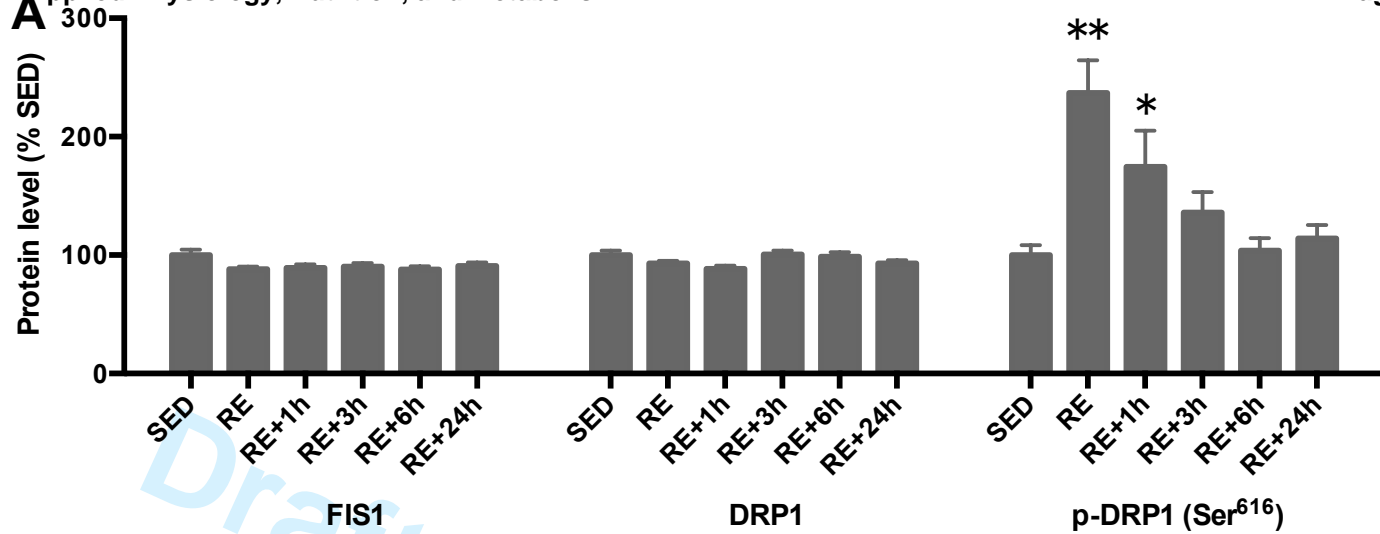
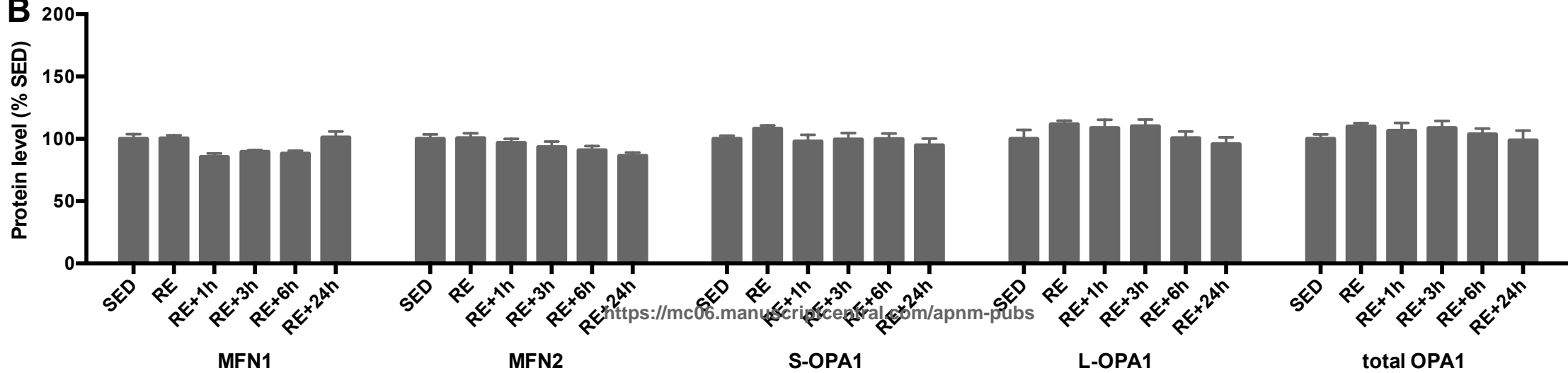
404 **Figure 3. Energy metabolism protein expression after 4 weeks of resistance training in rat**  
405 **skeletal muscle.** (A) Mitochondrial proteins. (B) Glycolytic enzymes. (C) Metabolite  
406 transporters. Data are presented as mean  $\pm$  S.E.M.  $n = 5$  in each group. \*Significantly different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

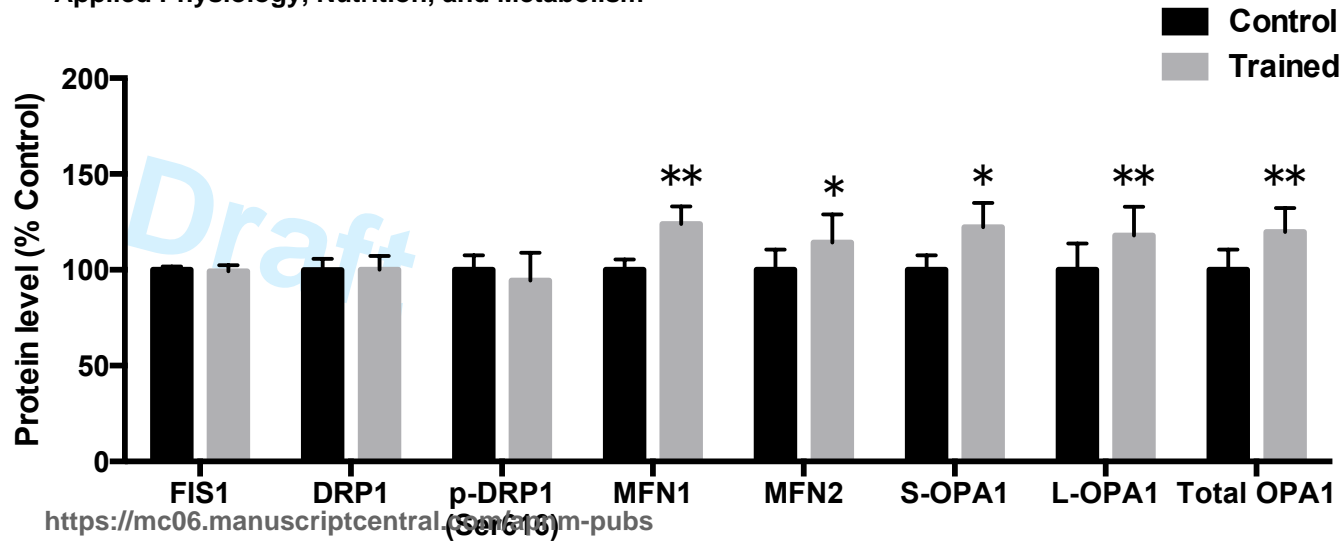
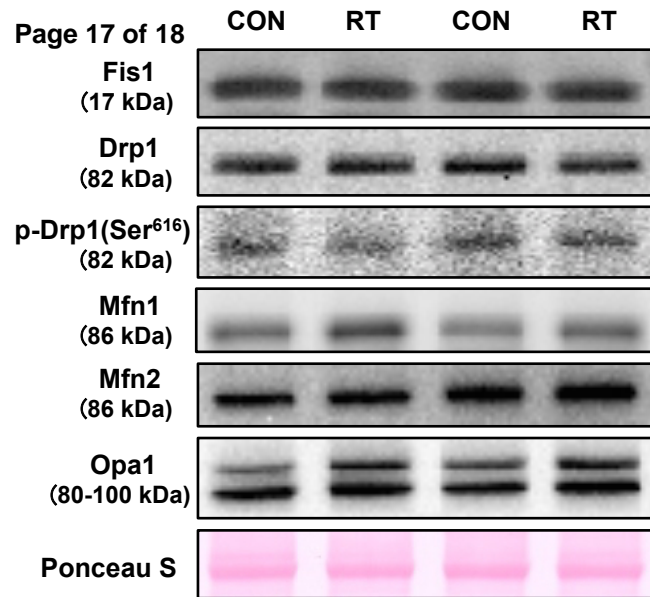
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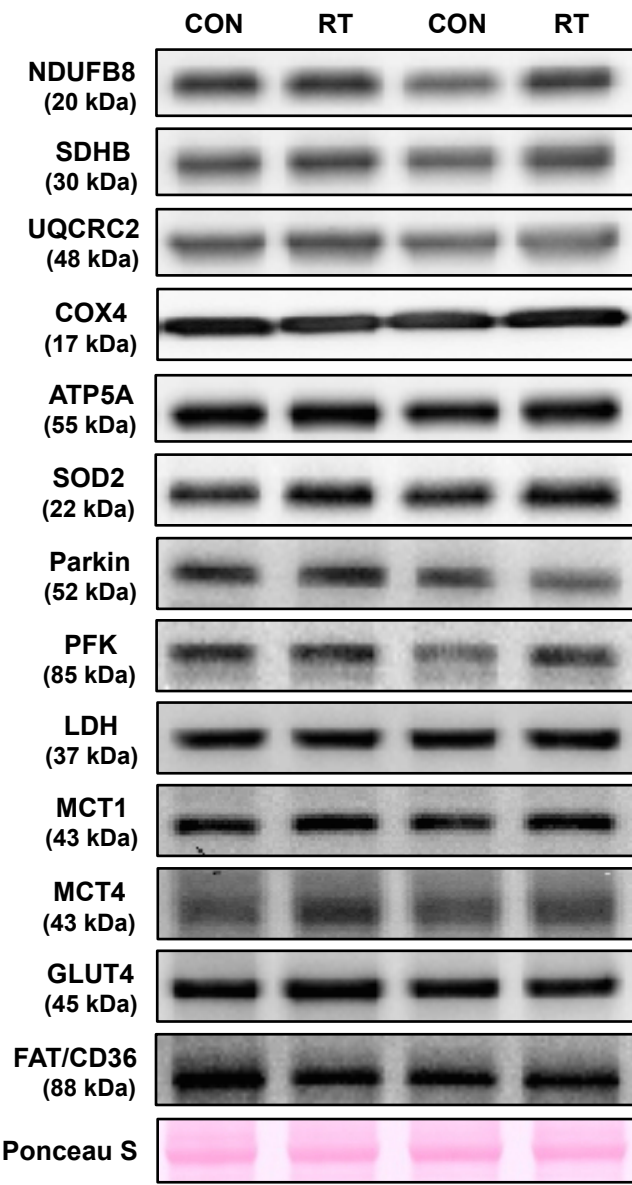
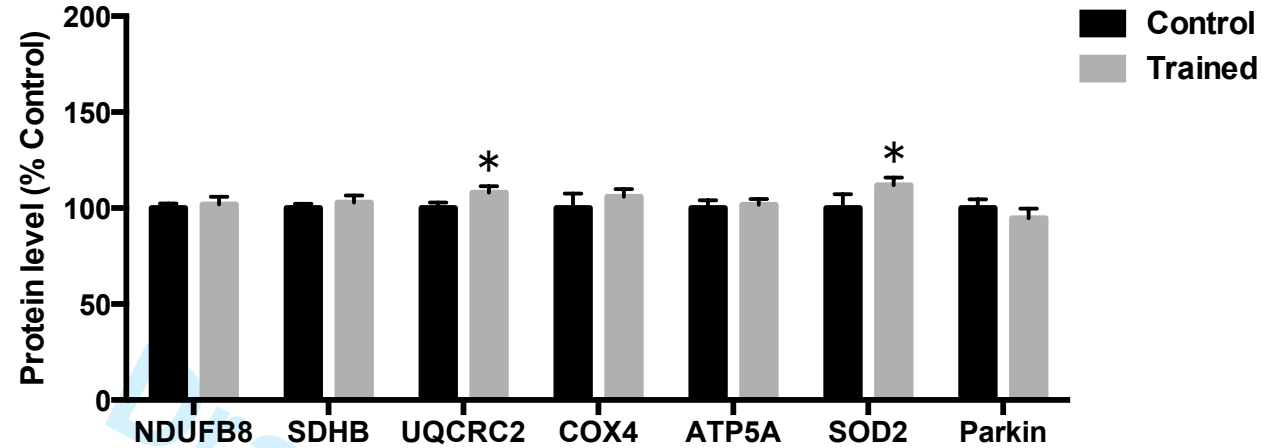
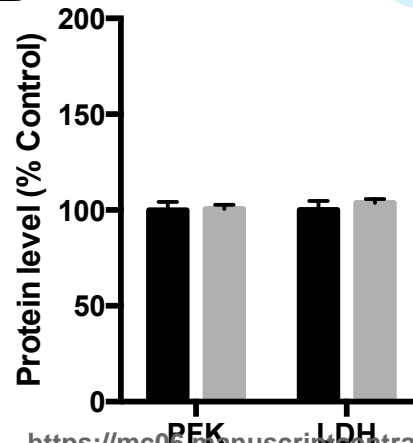
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**A****B**



**A****B****C**