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Modulation of mitochondrial dynamics in skeletal muscle during endurance training: early activation of fission and late induction of fusion protein expression

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Abstract

The mitochondria are highly plastic organelles. Exercise training alters mitochondrial dynamics (mitochondrial fission and fusion) in skeletal muscles and improves overall mitochondrial function by altering mitochondrial morphology and forming new networks. However, changes in mitochondrial dynamics over time during exercise have not previously been observed. In the present study, we examined the changes in mitochondrial fission and fusion protein expression in rats over four weeks of endurance swimming training. The expression of the GTPase protein dynamin-related protein 1 (Drp1), a mitochondrial fission protein, increased during the early phase of the training period. In contrast, the expression of optic atrophy type 1 (OPA1), a mitochondrial fusion protein, was increased in the late phase of the training period. These data suggest that mitochondrial fission was increased in the early phase, and mitochondrial fusion was initiated partially in the late phase, of the training period. In conclusion, mitochondrial dynamics may be modulated depending on the phase of muscular adaptation to exercise training. This modulation contributes to enhanced mitochondrial function in skeletal muscle.

Keywords: endurance training; mitochondrial fusion and fission; protein expression; skeletal muscle

論文タイトル: ミトコンドリアの分裂と融合は持久力的トレーニング期間中の異なるタイミングで促進される

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ランニングタイトル

筋組織内のミトコンドリアダイナミクスについて

抄録

ミトコンドリアは非常に可塑性の高い細胞小器官である。運動トレーニングは骨格筋のミトコンドリアダイナミクス（ミトコンドリアの分裂と融合）に影響を及ぼす。その結果として、ミトコンドリア形態は変化し、新しいネットワークが形成されることにより、ミトコンドリア機能は改善する。しかしながら、運動トレーニング期間中のミトコンドリア動態の経時的変化はこれまで検討されていなかった。本研究では、ラットに 4 週間の持久的水泳トレーニングを行わせ、ミトコンドリアの分裂および融合タンパク質の発現量の変化を調べた。ミトコンドリア分裂タンパク質である GTPase protein dynamin-related protein 1（Drp1）の発現は、トレーニング期間の初期に増加した。一方、ミトコンドリア融合タンパク質である optic atrophy type 1（OPA1）の発現は、トレーニング後期に増加した。これらの結果から、ミトコンドリアの分裂はトレーニング期間の初期に、ミトコンドリアの融合は後期に部分的に生じていることが示唆された。結論として、ミトコンドリアダイナミクスは運動トレーニングに対する適応の段階に応じて調節される可能性が考えられた。また、この変化は骨格筋におけるミトコンドリア機能の増強に寄与していると考えられる。

Introduction

Mitochondria are intracellular organelles that produce adenosine triphosphate (ATP) through oxidative phosphorylation and are essential for endurance exercise performance in skeletal muscles. Mitochondria exhibit high plasticity in response to physiological and pathological stimuli [1-2]. These stimuli influence the content (biogenesis), dynamics (fusion/fission), and degradation (mitophagy) of mitochondria, ultimately affecting their metabolic functions and oxidative capacity [1]. While mitochondrial biogenesis in skeletal muscle has been well studied in response to endurance training [3-4], mitochondrial fusion and fission responses have not been fully elucidated.

The mitochondrial dynamics of fusion and fission have emerged as important processes for the maintenance of functional mitochondria [5-6]. Mitochondrial fission utilizes dynamin-like protein 1 (Drp1) and membrane-bound adaptor proteins, including fission 1 (Fis 1) to cleave the outer mitochondrial protein membranes [7-8]. In contrast, mitochondrial fusion utilizes the GTPase transmembrane proteins, mitofusion 1 (Mfn1) and mitofusion 2 (Mfn2), located on the outer mitochondrial membrane, to fuse the outer membrane of the mitochondria [9]. The optic protein atrophy 1 (OPA1) protein is located in the inner mitochondrial membrane and is responsible for the fusion of the inner mitochondrial membrane [7].

Previous studies on mitochondrial dynamics in response to exercise have typically focused on changes in the expression of mitochondrial dynamics-related proteins and mRNAs in three contexts: acutely exercised muscle, fully adapted muscle to endurance training, and knockout muscle of mitochondrial dynamics-related proteins [10-12]. Indeed, Ding et al. [11] observed that continuous low-intensity exercise rapidly increased (within 30 min) Fis1 mRNA levels, followed by increases in Mfn1 (3 h post) and Mfn2 (12 h post) mRNA levels. Additionally, Arribat et al.[10] reported that 16wks of continuous moderate-intensity training in elderly subjects did not increase Mfn1 or Mfn2 content but increased OPA1 content and

mitophagy effectors. Moreover, in mice with a muscle-specific heterozygous deficiency of dynamin-related protein 1 (Drp1), a mitochondrial fission protein, a reduction in Drp1 signaling was associated with decreased muscle endurance and running performance, suggesting that Drp1 signaling plays a role in adaptations to endurance exercise training [12]. However, changes in mitochondrial dynamics over time during exercise training were not evident. Therefore, we aimed to examine the changes in mitochondrial dynamics-related proteins over time during four weeks of endurance swimming training.

Materials and Methods

Ethical approval

All the experimental procedures were conducted in accordance with the Guide for the Care and Use of Laboratory Animals of the Physiological Society of Japan. This study was approved by the Ethics Committee on Animal Experimentation at Kanazawa University (Protocol # AP-132797).

Animal care and exercise endurance training program

Male Wistar rats were obtained from the Japan SLC Corporation. All rats were acclimated to their new environment for 7 days. Experimental animals were housed in an air-conditioned room under laboratory environmental conditions (12:12 light/dark cycle, room temperature: 23 ± 2 ˚C, humidity: 55 ± 5%). A standard diet (MF oriental yeast) and water were provided ad libitum.

Eight-week-old Wistar rats (body weight: 194–227 g) were randomly divided into control (Con) and training (Tr) groups. The Tr groups were further divided into four groups according to the training period from 1 to 4 wks (9 wks , 10 wks, 11 wks, and 12 wks). Age-matched Con groups corresponding to the Tr groups were prepared as follows: 9 wks, 10 wks, 11 wks, and 12 wks. The rats in the 1 wk training group swam for 2 h in four 30-min bouts separated by 5 min of rest, during which a weight equaling 2% of the rats' body weight was tied to the rats. The training protocol for the other swimming groups was as follows: on the first and second days, the rats swam for 1 h in two 30-min bouts separated by 5 min of rest. On the third and fourth days, the rats swam for 1.5 h in three 30-min bouts, separated by 5 min of rest. On and after the fifth day, the rats swam for 2 h in four 30-min bouts separated by 5 min of rest. Except for the first bout of swimming training until the sixth day, a weight equal to 2% of their body weight was tied to the rats. The rats performed the swimming protocol six days/wk. The

water temperature was maintained at approximately 35 ˚C. The tank was square-shaped: 48 cm deep, 80 cm long, and 60 cm wide. It had an average surface area of at least 400 cm^2/rat. In addition, we monitored the rats to prevent climbing, diving, and bobbing during swimming training. Cases in which these behaviors were observed were dealt with immediately.

Preparation of tissue and sample homogenization

All surgical procedures were performed under anesthesia with an intraperitoneal (*i.p.*) injection of medetomidine (0.4 mg/kg), midazolam (2 mg/kg), or butorphanol (2.5 mg/kg). Twenty-four h after the training period, the gastrocnemius muscles of the hind limbs were removed and washed with ice-cold saline. After removing the connective tissue, fat, and nerve, the muscles were weighed, clamp-frozen in liquid nitrogen, and then stored at -80 ˚C until subsequent analyses.

The deep portion of the gastrocnemius muscle was homogenized and fractionated according to a modified method described in previous studies [13-14]. Briefly, the tissue was homogenized in 17 volumes of ice-cold Solution A (250 mM sucrose, 5 mM NaN_3, 2 mM EGTA, 20 mM HEPES-Na, pH 7.4) with the strokes of a Teflon pestle in a Potter-Elvehjem glass tissue homogenizer at 1,000 rpm. The homogenate was centrifuged at 600 × g for 10 min at 4 ˚C to remove nuclei and debris. Part of the supernatant was stored as a whole fraction solution. The remaining supernatant was centrifuged at 10,000 *g* for 30 min at 4 ˚C to precipitate crude mitochondrial pellets. The crude mitochondrial pellet was washed twice with Solution A re-suspended in Solution A. The mitochondrial pellet was re-pelleted by centrifugation at 10,000 *g* for 30 min at 4 ˚C. This pellet was washed in Solution C (1 mM EDTA, 10 mM Tris, pH 7.4) and was resuspended in 200 µl of Solution C containing 1% NP-40 and centrifuged at 1,100 *g* for 20 min at room temperature; the supernatant was used for immunoblots as a mitochondrial fraction. The protein concentration in the resuspension was determined using the

Bradford method with a protein assay kit (Bio-Rad Laboratories). All samples were adjusted to a constant protein concentration of 2.0 μg/μl with Solution A or C, and then equal amounts of 2X SDS sample buffer (125 mM Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.002% bromophenol blue) were added. The final concentration of the sample was adjusted to 1.0 μg/μl.

Western blotting and citrate synthase activity in skeletal muscle

Western blot analysis was performed according to a modified version of the method described by Yamada et al.[14]. Samples with equivalent protein contents were resolved by 15% SDS polyacrylamide gel electrophoresis, and the separated proteins were electrophoretically transferred onto PVDF membranes (Clear Blot Membrane-P plus, ATTO, Japan) using a semi-dry system (WSE-4045, HorizeBLOT 4M, ATTO, Japan). Membranes were then incubated in blocking buffer followed by incubation with anti-Mfn1 (H-65, Santa Cruz Biotechnology, USA), anti-Fis1 (ALX-210-1037, Enzo Life Sciences, USA), anti-VDAC (ab15895, Abcam, USA), anti-OPA1 (612606, BD Biosciences, USA), anti-Drp1 (611112, BD Biosciences, USA), or anti-GAPDH (ab8245, Abcam, USA) antibodies at 4 ˚C overnight. After exposure to the secondary antibody for 1 h, the membranes were treated with ECL prime reagents (GE Healthcare, NJ, USA), and chemiluminescent signals were visualized using MicroChemi (Berthold Technologies, Baden Württemberg, Germany). Signal intensities were quantified using ImageJ imaging software (NIH, USA).

Citrate synthase (CS) activity, a mitochondrial enzyme and marker of muscle oxidative potential, was measured in whole muscle homogenates using the spectrophotometric method described by Srere [15].

Measurement of mitochondrial respiration

Mitochondria respiration was measured using an oxygen electrode via a modified method reported in a previous study[16]. Briefly, soleus muscle was minced using stainless steel scissors in the isolation medium and homogenized with Potter–Elvehjem tissue grinder in the isolation medium at pH 7.2 at 4°C (225 mM mannitol, 100 mM KCl, 20 mM Sucrose, 20 mM Hepes-2Na, 1 mM EDTA (ethylenediaminetetraacetic acid), 0.2% BSA (bovine serum albumin), 0.25 mg/mL proteinase, bacterial and 5 mM L-carnitine). The homogenized mixture was centrifuged at 700 g for 10 min at 4°C to remove nuclei and debris, and the supernatant was further centrifuged at 10,000 g for 10 min at 4°C to collect the isolated mitochondria. The mitochondrial pellet was washed once in the isolation medium and resuspended in a ratio of 0.4 μm per mg initial muscle weight with resuspension buffer at pH 7.2 at 4°C (215 mM mannitol, 75 mM Sucrose, 20 mM Hepes-2Na, 1 mM EDTA, 0.2% BSA and 5 mM L-carnitine). Mitochondrial respiration was monitored using a Clark-type oxygen electrode (Model-5300A, YSI Japan, Japan) at 37 °C in a thermostatically controlled chamber by adding 3 mL of reaction buffer (pH 7.2) at 37°C (225 mM mannitol, 75 mM Sucrose, 20 mM Hepes-2Na, 10 mM KCl, 10 mM K_2HPO_4, 1 mM $MgCl_2$ $6H_2O$, 1 mM EDTA, 0.1% BSA and 5 mM L-carnitine) to it. After applying the substrate of Pyruvate (5 mM) and L-malate (2 mM), 60 μL of isolated mitochondria under resuspension was added. After confirming that state 4 remained stable, ADP at the final concentration of 200 μM was added to the medium to initiate state 3.

Statistical Analysis

All data are expressed as mean ± SD. Statistical differences were examined using a two-way unpaired analysis of variance (ANOVA) (group × age). A simple main-effect test was applied if the interaction or main effect showed a significant difference. Pearson's correlation coefficient was calculated when the relationship between two variables was evaluated. The level of significance was set at $p << 0.05$.

Results

Figure 1 shows the changes in CS activity and mitochondrial respiration rates for states 3 and 4 during endurance swimming training. CS activity differed significantly between groups ($F(1, 40) = 108.85$, $p < 0.05$), ages ($F(3, 40) = 27.05$, $p < 0.05$), and with their interaction ($F(3, 40) = 2.88$, $p < 0.05$). Comparing groups of the same age using simple main effects, CS activity was significantly higher in the Tr group than in the Con group at all time points ($p < 0.05$). The simple main effects revealed significant differences between the ages (Con: 9 wks < 11-12 wks and 10-11 wks < 12 wks, Tr: 9 wks < 11-12 wks and 10-11 wks < 12wks, $p < 0.05$). The state 4 mitochondrial respiration rate differed significantly between groups ($F(1, 34) = 7.67$, $p < 0.05$), ages ($F(3, 34) = 12.06$, $p < 0.05$), and with their interaction ($F(3, 34) = 3.91$, $p < 0.05$). Comparing groups of the same age using simple main effects, the state 4 rate was significantly higher in the Tr group than in the Con group at 11 and 12 wks ($p < 0.05$). The simple main effects revealed significant differences in the state 4 rate between the ages (Tr: 9 wks < 11-12 wks and 10 wks < 11-12 wks, $p < 0.05$). The state 3 respiration rate differed significantly between the ages ($F(1, 34) = 8.96$, $p < 0.05$) and with the interaction between age and group ($F(3, 34) = 3.31$, $p < 0.05$), but not between groups ($F(1, 40) = 2.64$, n.s.). Comparing groups of the same age using simple main effects, the state 3 rate was significantly higher in the training group than in the control group at 11 and 12 wks ($p < 0.05$), and differed significantly with age (Con: 9 wks < 11-12 wks, Tr: Tr: 9 wks < 11-12 wks and 10 wks < 11-12 wks, $p < 0.05$). *Please insert Figure 1 after this paragraph*

Figure 2 shows the changes in the expression of mitochondrial fission proteins (Drp1 and Fis1) over time. Drp1 expression in the whole fraction showed a statistical difference in the interaction factor ($F(3, 40) = 3.23$, $p < 0.05$), but not in the group factor ($F(1, 40) = 0.19$, n.s.) or age factor ($F(3, 40) = 1.23$, n.s.). The simple main effects showed that Drp1 expression was significantly higher in the Tr group than in the Con group only at 9wks of age ($p < 0.05$), and

159 significant differences were observed in age (Con: 9-10 wks < 12wks, Tr:10wks < 9wks, $p <$
160 0.05). Fis1 expression in the mitochondrial fraction did not show a significant change in the
161 group factor ($F (1, 40) = 0.04$, n.s.), age ($F (3, 40) = 0.42$, n.s.), or interaction ($F (3, 40) = 1.40$,
162 n.s.). The relationship between CS activity and Drp1 expression levels showed significant
163 positive correlations in Con group ($r = 0.53$, $p < 0.05$, $n = 24$), and significant negative
164 correlations in Tr group (Figure 3; $r = -0.49$, $p < 0.05$, $n = 24$). *Please insert Figure 2 & 3
165 after this paragraph*

166 Figure 4 shows the changes in the expression of mitochondrial fusion proteins (OPA1
167 and Mfn1) in the whole fraction over time. OPA1 expression showed a statistically significant
168 difference in the group factor ($F (1, 40) = 14.27$, $p < 0.05$) and age factor ($F (3, 40) = 4.82$, $p <$
169 0.05), but not in the interaction factor ($F (3, 40) = 0.15$, n.s.). The simple main effects showed
170 that OPA1 expression was significantly higher in the Tr group than in the Con group only at
171 12wks of age ($p < 0.05$), and there were significant differences in the age factor (Con:10wks <
172 11wks, Tr:10 wks < 11-12 wks, $p < 0.05$). Mfn1 expression showed a statistical difference in
173 the age factor ($F (3, 40) = 3.97$, $p < 0.05$), but not in the group factor ($F (1, 40) = 3.70$, n.s.) or
174 interaction factor ($F (3, 40) = 1.18$, n.s.). The simple main effects showed significant differences
175 by age (Con: 10 wks < 11 wks, $P < 0.05$). *Please insert Figure 4 after this paragraph*

Discussion

Mitochondria continuously change shape in response to fission and fusion events, which ultimately enables them to maintain proper function by forming a mitochondrial network[17]. Mitochondrial dynamics are highly dependent on the nutrient availability and energy demand of the cell [18]. Therefore, the mitochondrial morphology changes in response to exercise [19-21]. In this study, we examined changes in the expression levels of mitochondrial fusion/fission proteins in rats during four weeks of endurance swimming training. Drp1, a mitochondrial fission protein, increased in the early phase of the training period, whereas OPA1, a mitochondrial fusion protein, increased in the late phase. After the third week of the training period, swimming training increased both mitochondria volume and mitochondrial respiratory function in skeletal muscle.

The increase in Drp1 expression found in this study suggests that mitochondrial fission is promoted during the early phase of an endurance swimming training program. Mitochondrial fission leads to an improved mitochondrial network as it separates dysfunctional mitochondrial sections, thereby improving overall function [22]. These fission events occur at an early stage in response to continuous physiological stimuli to improve mitochondrial quality, and this adaptation precedes the enlargement of the mitochondrial network [17]. The general research consensus is that mitochondrial fission is regulated by Drp1 [23]. Drp1 is recruited to the mitochondrial outer membrane in response to changes in the intracellular environment, such as decreased cellular energy, high cytosolic ADP levels, and acute hypoxia [24-26]. Acute exercise is likely to create such a cellular environment, especially during the early phases of swimming training. In theory, the phosphorylation status of Drp1 should be measured to determine the degree of mitochondrial fission. We could not measure phosphorylated Drp1 in skeletal muscles because Drp^{ser616} levels returned to the basal phosphorylation status within a few hours of exercise stimuli [12]. Nevertheless, based on the finding that the Ser616:Ser637

phosphorylation status of Drp1 correlates with Drp1 abundance on the outer mitochondrial membrane [12,27], an increase in Drp1 expression in the whole fraction would indicate the promotion of mitochondrial fission if the degree of Drp1 activation and Drp1 abundance on the outer mitochondrial membrane relative to Drp1 content in the whole fraction remains constant. In contrast, one of the receptor factors, Fis1, showed no change in expression throughout the training period, suggesting that the Fis1 protein expression level may not be as important for exercise-induced mitochondrial fission, as reported in recent studies [28].

OPA1 expression increased during the late phase of endurance swimming training in this study. Mitochondrial fusion is essential for the maintenance of mitochondrial and cellular functions as it controls mtDNA replication, mitochondrial nucleoid distribution, and OXPHOS capacity [29-30]. Mitochondrial fusion is independently regulated at the outer and inner mitochondrial membranes [17]. Outer mitochondrial membrane fusion is regulated by the activities of Mfn1 and Mfn2, whereas fusion of the inner mitochondrial membrane fusion is coordinated by the activity of OPA1 [31-32]. Our results showed an increase in expression of only OPA1 and no change in Mfn1 expression, but we did not measure the expression level of Mfn2. Moreover, in the previous study of Arribat et al. [10] Mfn1 expression was not altered in trained muscle, whereas OPA1 and Mfn2 expression were elevated. These results suggest that mitochondrial fusion may be partially promoted during the late phase of the training period.

OPA1 plays a role in cristae remodeling and is considered an integral regulator of OXPHOS [33]. Cristae membranes are highly dynamic and continuously undergo morphological changes mediated by OPA1, an i-AAA protease, ATP-dependent zinc metalloprotease 1 (YME1L), metalloendopeptidase (OMA1), the mitochondrial contact site and cristae organizing system (MICOS), and sorting and assembly machinery complex 50 (Sam50) [34]. Patten et al. [35] showed that OPA1 dynamically regulates cristae shape in healthy cells and that OPA1-dependent modulation of cristae structure is necessary for cellular adaptation to energy

substrate availability. Cristae are invaginations of the inner mitochondrial membrane, where the essential machinery for mitochondrial respiration, the ETC, is located[17]. Therefore, oxidative phosphorylation activity is highly dependent on the cristae shape and ETC organization[36]. In summary, the elevated OPA1 expression may have induced changes in the inner mitochondrial membrane cristae in the late phase of the training period. Future studies will need to examine the effects of extended training periods on mitochondrial fusion.

In the training group, whole-fraction Drp1 expression was negatively correlated with CS activity, an indicator of mitochondrial volume, whereas in the control group, CS activity and Drp1 expression were positively correlated (Figure 3). As Drp1 expression is also an indicator of mitochondrial dynamics [12,27], these findings suggest that when mitochondria volume is increased by exercise training, the intracellular environment is more likely to be conducive to mitochondrial fusion. Although the functional interaction between CS activity levels and Drp1 expression remains unclear, exercise training is likely to alter the relationship between mitochondrial dynamics and mitochondrial biogenesis in skeletal muscle.

In this study, mitochondrial proteins and respiratory function were shown to increase and accelerate with age. Because CS activity in the soleus muscle was shown to increase from day 28 to 140 after birth in the previous study [37] and the breeding environment during the training period was similar between groups in the present study, the age-related changes in respiratory function and CS activity in the control group were considered to be due to development.

Our study reveals that the mitochondrial fission was promoted in the early phase of the training period, and the mitochondrial fusion was partially induced in the late phase of the training period. Such mitochondrial dynamics may properly manage mitochondrial functionality in response to changes in the intracellular environment caused by exercise training.

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Conflict of Interest

There is no conflict of interest for this study.

Author Contribution

H.T., H.B., T.Y., and K.M. designed the study; H.B. and T.Y. conducted the experiments and analyzed the data; H.T. and H.B. wrote the manuscript; and K.M., R.K., T.S., and Y.N. edited the paper and helped with the experiments.

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Figure Legends

Figure 1. Change in CS activity, and mitochondrial respiration rates for states 3 and 4 over time from 9 to 12 wks old during endurance swimming training in the training and control groups.

(A) A two-way ANOVA for CS activity revealed a statistical difference in the group factor ($F(1, 40) = 108.85$, $p < 0.05$), age ($F(3, 40) = 27.05$, $p < 0.05$), and interaction ($F(3, 40) = 2.88$, $p < 0.05$). The simple main effects showed that CS activity was significantly higher in the Tr group than in the Con group at all time points ($p < 0.05$), and there were significant differences in age ($p < 0.05$). (B) A two-way ANOVA for the state 4 of respiration rate showed a statistically significant difference in the group factor ($F(1, 34) = 7.67$, $p < 0.05$), age ($F(3, 34) = 12.06$, $p < 0.05$), and interaction ($F(3, 34) = 3.91$, $p < 0.05$). The simple main effects showed that state 4 was significantly higher in the Tr group than in the Con group at 11 and 12 wks ($p < 0.05$), and and there were significant differences in age ($p < 0.05$). (C) A two-way ANOVA for the state 3 of respiration rate showed a statistically significant difference in the age factor ($F(1, 34) = 8.96$, $p < 0.05$) and interaction ($F(3, 34) = 3.31$, $p < 0.05$), but not in the group factor ($F(1, 40) = 2.64$, n.s.). The simple main effects showed that state 4 was significantly higher in the Tr group than in the Con group at 11 and 12 wks ($p < 0.05$), and and there were significant differences in age ($p < 0.05$). Values are represented as mean ± SD ($n = 6$ for CS activity in each group, $n = 4$-6 for mitochondrial respiration rate in each group). Abbreviations: Con, control; Tr, training; CS, citrate synthase. *: $p < 0.05$ vs. age-matched Con group, a: $p < 0.05$ vs. 9 wk-Con group, b: $p < 0.05$ vs. 9 wk-Tr group, c: $p < 0.05$ vs. 10 wks-Con group, d: $p < 0.05$ vs. 10 wks-Tr group, e: $p < 0.05$ vs. 11 wks-Con group.

Figure 2. Change in Drp1 and Fis1 over time from 9 to 12 wks old during endurance swimming training in the training and control groups.

(A) Two-way ANOVA for Drp1 expression in the whole fraction revealed a statistical difference in the interaction factor ($F(3, 40) = 3.23$, $p < 0.05$), but not in the group factor ($F(1, 40) = 0.19$, n.s.) and the age factor ($F(3, 40) = 1.23$, n.s.). The simple main effects showed that the Drp1 expression was significantly higher in Tr than in Con groups only in 9 wks old ($p < 0.05$), and there were significant differences in the age factor ($p < 0.05$). (B) The Fis1 expression in the mitochondrial fraction did not show significant change in the group factor ($F(1, 40) = 0.04$, n.s.), the age factor ($F(3, 40) = 0.42$, n.s.), and the interaction ($F(3, 40) = 1.40$, n.s.). Values are represented as mean ± SD (n = 6 in each group). Abbreviations: Con, control group; Tr, training group; Drp1, dynamin-like protein 1; Fis1, fission 1. *: $p < 0.05$ vs. age-matched Con group, a: $p < 0.05$ vs. 9 wks-Con group, b: $p < 0.05$ vs. 9 wks-Tr group, c: $p < 0.05$ vs. 10 wks-Con group.

Figure 3. Relationship between CS activity and Drp1 expression level in control and training groups.

The relationship between CS activity and Drp1 expression levels showed significant positive correlations in Con group ($r = 0.53$, $p < 0.05$, $n = 24$), and significant negative correlations in Tr group ($r = -0.57$, $p < 0.05$, $n = 24$). Values are represented as mean ± SD (n = 6 in each group). Abbreviations: Con, control group; Tr, training group; Drp1, dynamin-like protein 1; CS, citrate synthase.

Figure 4. Change in Opa1 and Mfn1 over time from 9 to 12 wks of age during endurance swimming training in the Tr and Con groups.

(A) Two-way ANOVA for the Opa1 expression level in the whole fraction revealed a statistical difference in the group factor ($F(1, 40) = 14.27$, $p < 0.05$) and the age factor ($F(3, 40) = 4.82$, $p < 0.05$), but not in the interaction factor ($F(3, 40) = 0.15$, n.s.). The simple main

435 effects showed that the Opa1 expression was significantly higher in Tr than in Con groups
436 only in 12 wk-old rats ($p < 0.05$), and there were significant differences in the age factor ($p <$
437 0.05). (B) The Mfn1 expression in the whole fraction showed a statistical difference in the age
438 factor ($F (3, 40) = 3.97$, $p < 0.05$), but not in the group factor ($F (1, 40) = 3.70$, n.s.) or the
439 interaction factor ($F (3, 40) = 1.18$, n.s.). The simple main effects showed the significant
440 differences in the age factor ($p < 0.05$). Values are represented as mean ± SD ($n = 6$ in each
441 group). Abbreviations: Con, control group; Tr, training group; Opa1, optic protein atrophy 1;
442 Mfn1, mitofusion 1. *: $p < 0.05$ vs. age-matched Con group, c: $p < 0.05$ vs. 10 wks-Con
443 groups, d: $p < 0.05$ vs. 10 wks-Tr group.

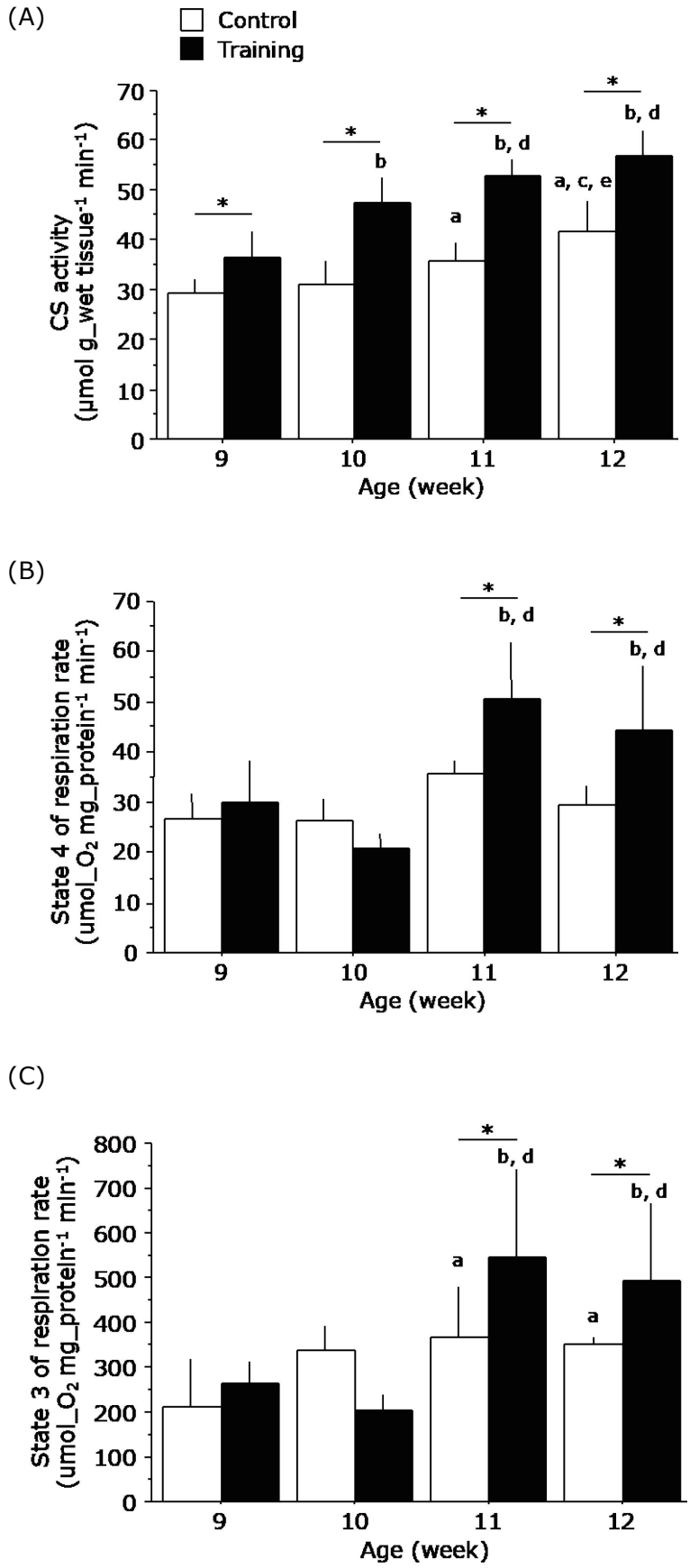

(A)
Control
Training
CS activity (µmol g_wet tissue-1 min-1)
70
60
50
40
30
20
10
0
*
*
*
*
b
a
b, d
b, d
a, c, e
9
10
11
12
Age (week)
(B)
State 4 of respiration rate (umol_O2 mg_protein-1 min-1)
70
60
50
40
30
20
10
0
*
*
b, d
b, d
9
10
11
12
Age (week)
(C)
State 3 of respiration rate (umol_O2 mg_protein-1 min-1)
800
700
600
500
400
300
200
100
0
*
*
a
a
b, d
b, d
9
10
11
12
Age (week)

Figure 1.

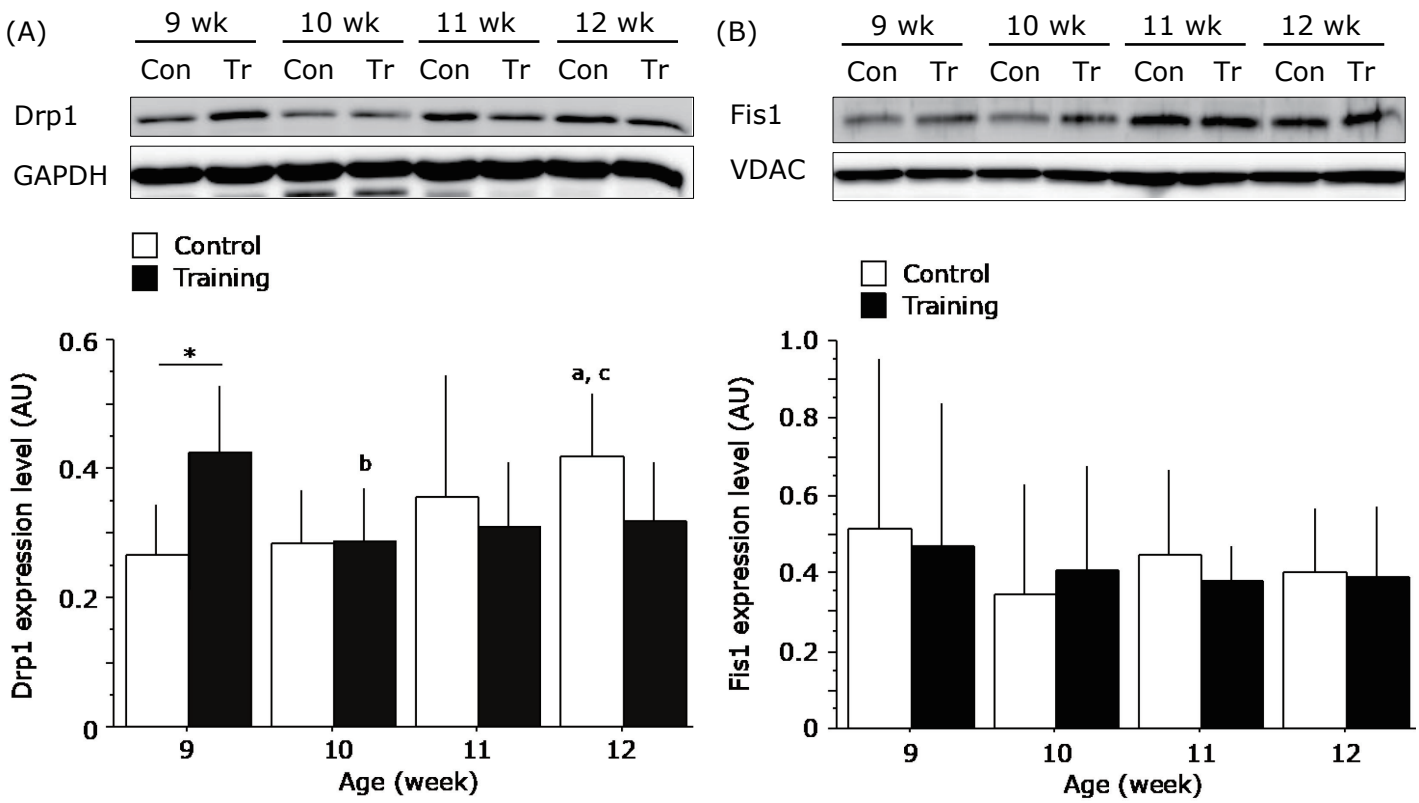
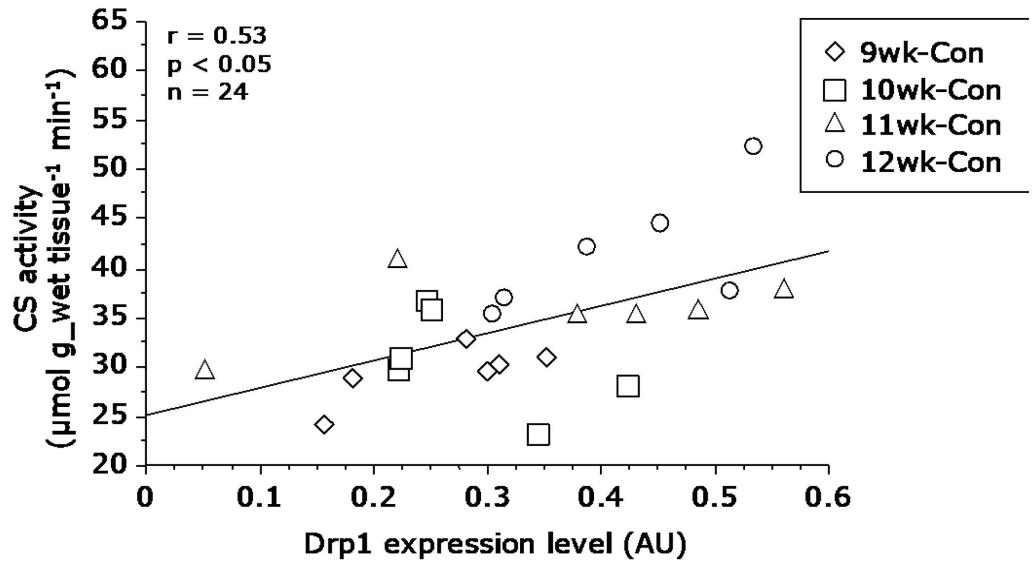

(A)
9 wk
10 wk
11 wk
12 wk
Con Tr Con Tr Con Tr Con Tr
Drp1
GAPDH
(B)
9 wk
10 wk
11 wk
12 wk
Con Tr Con Tr Con Tr Con Tr
Fis1
VDAC
Control
Training
Drp1 expression level (AU)
0.6
0.4
0.2
0
*
b
a, c
9
10
11
12
Age (week)
Control
Training
Fis1 expression level (AU)
1.0
0.8
0.6
0.4
0.2
0
9
10
11
12
Age (week)

Figure 2.

Con group



Tr group

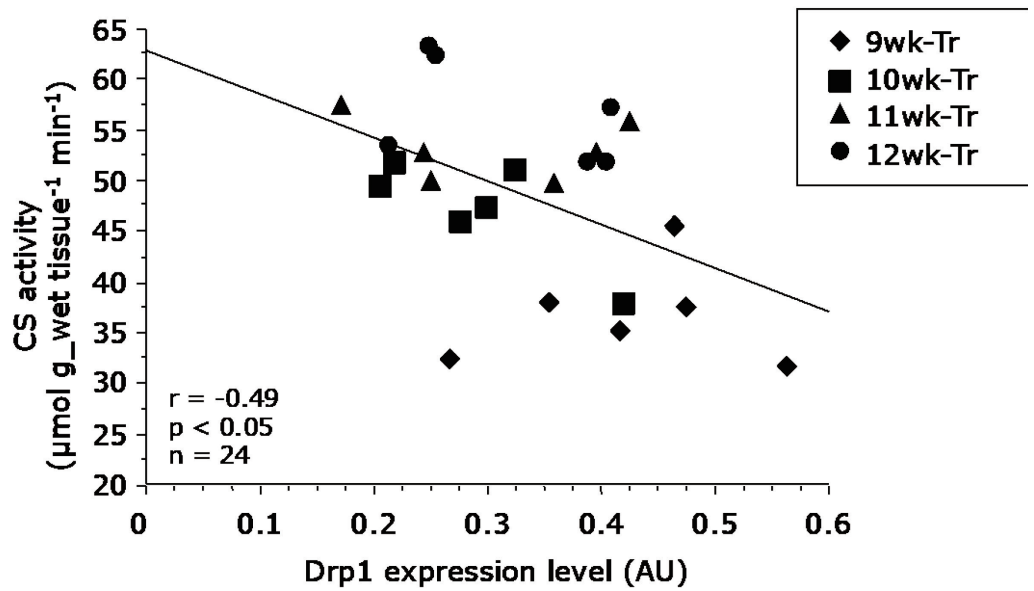
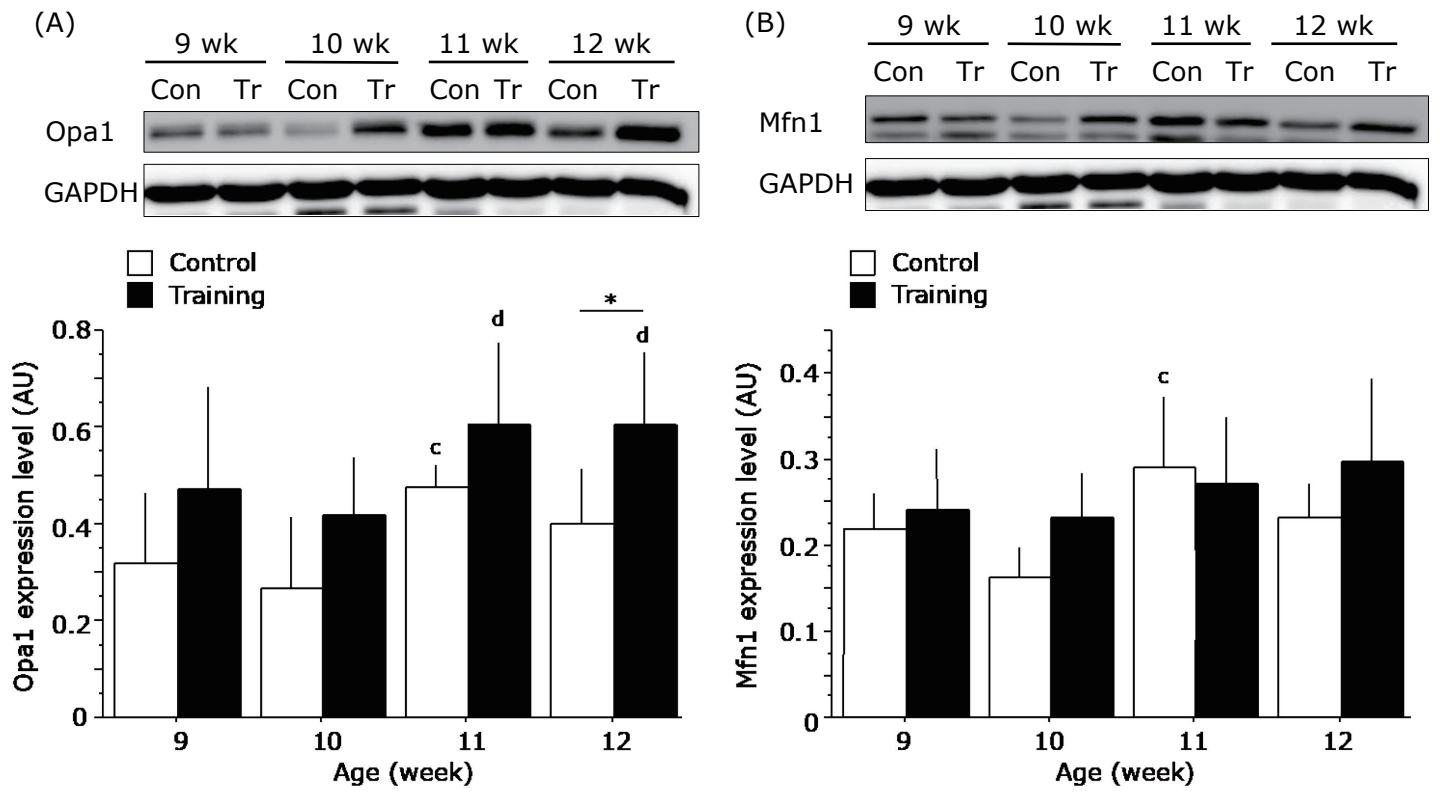


Figure 3.


(A)
9 wk
10 wk
11 wk
12 wk
Con Tr Con Tr Con Tr Con Tr
Opa1
GAPDH
(B)
9 wk
10 wk
11 wk
12 wk
Con Tr Con Tr Con Tr Con Tr
Mfn1
GAPDH

Control
Training
Opa1 expression level (AU)
0
0.2
0.4
0.6
0.8
c
d
*
d
9
10
11
12
Age (week)

Control
Training
Mfn1 expression level (AU)
0
0.1
0.2
0.3
0.4
c
9
10
11
12
Age (week)

Figure 4.