早稲田大学審査学位論文
博士（スポーツ科学）

Muscle adaptations to nutritional modulation on rodent models:

High-fat diet regimen and mitochondrial biogenesis

動物モデルでの栄養調節による骨格筋の適応：

高脂肪食とミトコンドリア新生

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早稲田大学大学院　スポーツ科学研究科
李　茜
LI　XI

研究指導教員：　樋口　満　教授

This thesis is based on the following paper.

1. X Li, K Higashida, T Kawamura, M Higuchi.; Alternate-day high-fat diet induces an increase in mitochondrial enzyme activities and protein content in rat skeletal muscle. *Nutrients* 2016, 8, 203.

2. X Li, K Higashida, T Kawamura, M Higuchi.; Time course of decrease in skeletal muscle mitochondrial biogenesis after discontinuing high-fat diet. The paper is being submitted to *Journal of Nutritional Science and Vitaminology*.

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List of Abbreviations

ALT	alternate-day high-fat diet
CaMK Ⅱ	calcium/ calmodulin-dependent protein kinase Ⅱ
CON	control diet
CS	citrate synthase
DHA	docosahexaenoic acid
EPA	eicosapentaenoic acid
FFA	free fatty acid
HFD	high-fat diet
LCAD	long chain acyl-CoA dehydrogenase
mTORC1	mammalian target of rapamycin complex 1
PGC-1α	peroxisome proliferator-activated receptor γ coactivator -1 α
PPARδ	peroxisome proliferator-activated receptor δ
SDH	succinate dehydrogenase
TG	triglyceride
β-HAD	β-hydroxyacyl-CoA dehydrogenase

Chapter 1 Introduction

1-1. Background

Skeletal muscle shows a remarkable capacity to alter its phenotype in response to stimuli. Endurance training induces an increase in skeletal muscle mitochondrial content, resulting in increased fat oxidation and sparing of muscle glycogen during exercise (1). Nutritional modulation is a powerful tool to demonstrate the plasticity of skeletal muscle. Changes in the ratio of ingested macronutrients (carbohydrate, protein, and fat) induce various adaptations in the skeletal muscle. High carbohydrate diet increases the glycogen content of skeletal muscle. The opinion that skeletal muscle glycogen content prior to exercise is related to endurance performance has been widely accepted since the study of Bergstorm *et al.* (2). Therefore, carbohydrate intake before and during exercise has been a topic of extensive discussions. Protein intake increases the availability of amino acids, which stimulates the rate of skeletal muscle synthesis by activating the mammalian target of rapamycin complex 1 (mTORC1) (3). High protein diet increases muscle mass, strength, and function. These skeletal muscle adaptations are beneficial for post exercise recovery. On the other hand, high-fat diet increases skeletal muscle mitochondrial biogenesis (4).

The increase in mitochondrial content results in increased capacity of muscle to generate energy and decrease the utilization of muscle glycogen during exercise. High-fat diet improved exercise performance, both in rodent experiments and human trials, according to a number of studies. After 1 or 5 weeks on high-fat diet, rats ran significantly longer on a rodent treadmill at 35 m/min than the group that ate normal diet (5). The time to exhaustion during moderate-intensity cycling at 50% of peak power

output was significantly longer after high-fat diet in trained cyclists (6). These skeletal muscle adaptations contribute to the improvement of endurance performance.

Compared to the research over almost half a century about carbohydrate and protein diets, there are fewer studies on high-fat diet and its effect on skeletal muscle adaptation. It is important to better understand diet-induced skeletal muscle adaptation, to be able to use nutritional modulation for increasing exercise performance.

1-2. Aims

As described above, high-fat diet increases mitochondrial biogenesis. However, there are some accompanying disadvantages. In order to increase mitochondrial content, long-term high-fat diet consumption is essential. However, long-term high-fat diet consumption leads to body weight gain and accumulation of abdominal fat, which negatively affects exercise performance. It is also necessary to elucidate the time course of the changes in mitochondrial proteins, after withdrawing high-fat diet, for optimal utilization of nutritional regimen to improve exercise performance.

The overall aim of this thesis was to understand at a fundamental level, high-fat diet induced skeletal muscle adaptation, with special focus on the effect of high-fat diet regimen on skeletal muscle mitochondrial biogenesis.

The specific questions that were addressed are as follows:

• Is it necessary to consume high-fat diet every day for increasing skeletal muscle mitochondrial biogenesis?

• How long does skeletal muscle mitochondrial biogenesis remain high after the withdrawal of high-fat diet?

Chapter 2 Alternate-day high-fat diet induces an increase in mitochondrial enzyme activities and protein content in rat skeletal muscle

2-1. Introduction

Endurance exercise training leads to an increase in mitochondrial content in skeletal muscle (1), resulting in increased capacity of muscles to generate ATP. The increase in muscle mitochondrial content also results in a change in substrate utilization: increased fat oxidation and decreased utilization of muscle glycogen (7,8). Since endurance exercise performance is directly related to the muscle glycogen content prior to exercise (2), these biochemical adaptations of skeletal muscle lead to enhanced exercise performance after exercise training.

Skeletal muscle adaptation described above is also caused by high-fat diet consumption. Miller *et al*. demonstrated that a 5-week regimen of high-fat diet elevated mitochondrial enzyme activities in rat skeletal muscle (5). This biochemical adaptation of skeletal muscle has also been reported by other groups in both rodents and human subjects (9-12). Interestingly, in contrast to exercise training (13), the biochemical adaptation to high-fat diet in skeletal muscle occurs slowly, over at least 3–4 weeks (4). Recent studies have proposed possible mechanisms by which a high fat diet induces an increase in mitochondrial biogenesis in skeletal muscle (4,14-16), such as peroxisome proliferator activated receptor (PPAR) δ activation by increased plasma free fatty acid (FFA) and induction of PPARγ coactivator-1α (PGC-1α).

It is well known that consuming high-fat diet leads to accumulation of abdominal fat, insulin resistance, and obesity. Miller *et al*. (5) reported that rats fed with high-fat

diet gained more body weight than those on control diet, despite a significant increase in the activity of mitochondrial enzymes. This might be the reason why endurance athletes do not embrace high-fat diet, even though it has some merit in that there is an increase in mitochondrial enzyme activities and a concomitant decrease in the utilization of glycogen during endurance exercise. A dietary regimen that increases mitochondrial oxidative capacities in skeletal muscle without abdominal fat accumulation and body weight gain will offer many advantages. The objective of the present study was to determine whether the repeated increase in FFA caused by high-fat diet on alternate day results in an increase in the mitochondrial oxidative capacity without accumulation of abdominal fat.

2-2. Methods

2-2-1. Materials

Reagents for SDS-PAGE were obtained from Bio-Rad (Hercules, CA, USA). Monoclonal antibodies against long chain acyl-CoA dehydrogenase and horseradish peroxidase (HRP)-conjugated secondary antibodies were obtained from Sigma (St. Louis, MO, USA) and Cell Signaling Technologies (Danvers, MA, USA), respectively. Enhanced chemiluminescence (ECL) reagent was purchased from Millipore (Temecula, CA, USA). All other chemicals were obtained from Sigma.

2-2-2. Treatment of animals

Four-week old male Wistar rats (70–90 g body weight) were obtained from CLEA Japan (Tokyo, Japan). All rats were housed in rooms lighted from 9:00 a.m. to 9:00 p.m. The temperature in the room was maintained at 22-24 °C. Rats were separated into those receiving control diet (CON: n=6), high-fat diet (HFD: n=6), and high-fat diet on alternate days (ALT: n=6). The high-fat diet was prepared using lard, corn oil, sucrose,

and casein (32%, 18%, 27%, and 23%, respectively, of total calories), and supplemented with minerals (51 g/kg, AIN93G mineral mix: CLEA Japan), vitamins (22 g/kg, AIN93 vitamin mix: CLEA Japan), and methionine (4.4 g/kg: Wako Pure Chemical). The control diet, CE-2 was obtained from CLEA Japan and its calorie composition was 59% carbohydrate, 12% fat, and 29% protein. The energy content of the high-fat diet was 5.1 kcal/g, whereas that of the standard diet was 3.4 kcal/g. The rats were provided with food and water *ad libitum*. Rats in the CON and HFD groups were fed the control diet and the high-fat diet for 4 weeks respectively. Rats in the ALT group were fed control diet or high-fat diet on alternate days. The ALT animals were fed high-fat diet on the day before sacrifice. Food was withdrawn at 9:00 p.m. on the day before muscle dissection. Between 9:00 a.m. and 12:00 a.m. on the next day, rats were anesthetized with an intraperitoneal injection of pentobarbital sodium (50 mg/kg) and blood samples were drawn from the abdominal aorta. After the blood sampling, plantaris muscle and epididymal fat pads were removed. This experimental protocol was approved by the Committee for Animal Experimentation in the Graduate School of Sport Sciences at Waseda University (No. 2014-A096).

2-2-3. Measurement of mitochondrial enzyme activities

For enzyme activity measurements, the plantaris muscles were homogenized in ice-cold buffer containing 175 mM KCl, 10 mM GSH, and 2 mM EDTA, pH 7.4. The homogenates were frozen and thawed three times and mixed thoroughly before enzyme activities were measured. For the β-hydroxyacyl-CoA dehydrogenase (β-HAD) assay, an aliquot of the homogenate was centrifuged at 700 × *g* for 10 min at 4 °C. Citrate synthase (CS), a marker of oxidative enzymes, and β-HAD activities were measured using Srere's (17) and Bass's (18) methods, respectively.

2-2-4. Western blot analysis

A portion of frozen plantaris muscles were homogenized in ice-cold RIPA buffer containing 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.25% deoxycholic acid, 1% NP-40, 1 mM EDTA, and a protease inhibitor cocktail (Cell signaling technologies, Danvers, CA, USA). Protein concentrations were measured using a BCA protein assay kit (Pierce, Rockford, IL, USA) according to the manufacturer's instructions. Samples were diluted in 4 × sample buffer (Invitrogen, Camarillo, CA, USA). Equal amounts of sample protein were subjected to SDS-PAGE (10% resolving gels) and then transferred to PVDF membranes at 200 mA for 90 min. After transfer, the membranes were washed in Tris-buffered saline with 0.1% Tween 20 (TBST; 20 mM Tris base, 137 mM NaCl, pH 7.6). The membranes were then blocked with TBST supplemented with 5% skimmed powdered milk for 1 h at room temperature. After blocking, the membranes were incubated overnight at 4 °C with antibodies specific for long chain acyl-CoA dehydrogenase (LCAD) and PGC-1α at dilutions of 1:2000-5000. The HRP-conjugated secondary antibody (goat anti-rabbit IgG) was used at a concentration of 1:10000. Bands were visualized by ECL and scanned using a chemiluminescence detector (LAS 3000, FUJIFILM). The membranes were stained with Coomassie Brilliant Blue (CBB) to verify and normalize the protein loading (19). Band intensities were quantified using ImageJ (NIH, Bethesda, MD, USA).

2-2-5. Analytical procedure

Concentrations of plasma glucose, FFA, and triglyceride were determined using kits (Glucose C2 Test Wako, NEFA-C Test Wako, Triglyceride E Test Wako, respectively) according to the manufacturer's instructions. Plasma insulin concentration

was measured using enzyme-linked immunospecific assay kit according to the manufacturer's instructions (Mercodia AB, Uppsala, Sweden).

2-2-6. Succinate dehydrogenase (SDH) staining

For histological analysis, plantaris muscles were frozen in isopentane, which had been cooled in liquid nitrogen. Serial cross-sections (5 μm thick) were cut in a cryostat at −20 °C. Sections were stained for succinate dehydrogenase (SDH) activity, complex II of the mitochondrial respiratory chain, as follows. Sections were first allowed to reach room temperature before they were incubated in a solution containing nitro blue tetrazolium (0.5 mg/ml), sodium succinate (50 mM), phosphate buffer (0.12 M potassium dihydrogen phosphate, 0.88 M disodium hydrogen phosphate) for 25 min at 37 °C. Cross-sections were then washed thrice in distilled water, dehydrated in a series of ethanol baths of increasing concentration (1 min each in 70%, 80%, 90%, and 100% ethanol) and then cover-slipped using an aqueous mounting medium.

2-2-7. Statistical analysis

The data were presented as the mean ± standard error of the mean (SEM). Statistical analysis was performed using analysis of variance (ANOVA). The Tukey's test was used for *post-hoc* analysis when the ANOVA test indicated significant differences. When the normality (Shapiro-Wilk test) was not met, variables were analyzed using the Kruskal-Wallis test and the Steel-Dwass *post-hoc* test as needed. $p < 0.05$ indicated statistical significance.

2-3. Results

2-3-1. Body weight, epididymal fat weight, and plasma parameters

Table 2-1 shows the body weight, epididymal fat weight and plasma parameters. High-fat diet for 4 weeks resulted in an increase in epididymal fat weight in the HFD group (CON *vs* HFD, $p < 0.05$). However, at 4 weeks, epididymal fat weight in the ALT group was not significantly different from that in CON group.

Plasma FFA concentration in the HFD and ALT groups was significantly higher than that in the CON group (CON *vs* HFD and ALT, $p < 0.05$). Although the precise mechanism of the decrease was not clear, plasma glucose concentration in the ALT group was significantly lower than that of CON and HFD (ALT *vs* CON and HFD, $p < 0.05$). There was no significant difference in plasma insulin concentration among the three groups (Table 2-1).

Table 2-1. Effects of alternate-day high-fat diet feeding on body weight, epididymal fat mass and plasma parameters in rats.

	CON	HFD	ALT
Initial body weight (g)	87 ± 1	86 ± 5	87 ± 1
Final body weight (g)	298 ± 5	297 ± 9	298 ± 3
Epididymal fat mass (g)	3.1 ± 0.2	5.1 ± 0.3 *	3.8 ± 0.1
Plasma glucose (mg/dL)	96.9 ± 2.6	96.8 ± 6.3	81.1 ± 2.6 #
Plasma FFA (mEq/L)	0.28 ± 0.02	0.44 ± 0.05 *	0.44 ± 0.06 *
Plasma insulin (μg/L)	0.39 ± 0.3	0.42 ± 0.4	0.39 ± 0.3

CON, control group; HFD, high-fat diet group; ALT, alternate-day high-fat diet group. Values are mean ± SEM of 6 animals per group. * indicates significant difference at a level of $p < 0.05$ *vs* CON. # indicates significant difference at a level of $p < 0.05$ *vs* CON and HFD.

2-3-2. Mitochondrial enzymes activities

Citrate synthase activity in the plantaris muscle of the HFD and ALT rats was significantly higher than that in the same muscle of the CON rats (Figure 2-1A) (CON *vs* HFD, $p < 0.01$; CON *vs* ALT, $p < 0.05$). After the 4-week dietary intervention, the β-HAD activity in the HFD and ALT groups was significantly higher than that in the CON group (Figure 2-1B) (CON *vs* HFD and ALT, $p < 0.05$).

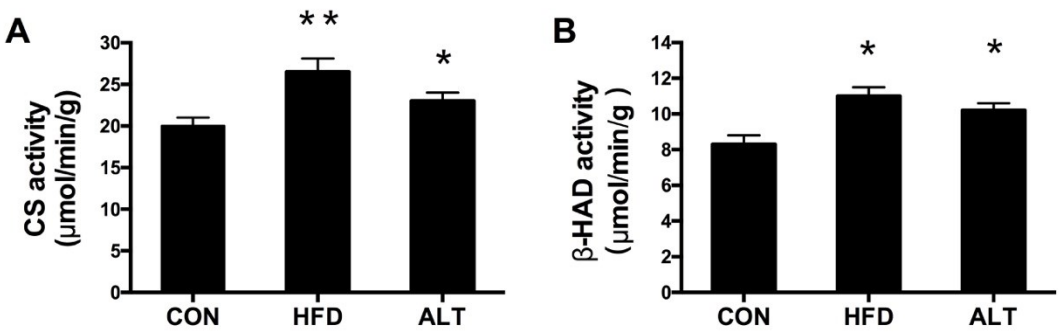

Figure 2-1. Effects of alternate-day high-fat diet feeding on citrate synthase (A), β-HAD (B) enzyme activities in rat skeletal muscle. Values are mean ± SEM of 6 animals per group. * and ** indicate significant differences at levels of $p < 0.05$ and $p < 0.01$ *vs* CON, respectively.

2-3-3. PGC-1α and LCAD protein content

PGC-1α protein content was significantly higher in HFD group than that in the CON group ($p < 0.05$, Figure 2-2A). PGC-1α protein content was also increased by 4-week alternate day high-fat diet ($p < 0.05$, Figure 2-2A). Both HFD and ALT induced a significant increase in LCAD protein content in plantaris muscle (Figure 2-2B) (CON *vs* HFD and ALT, $p < 0.05$).

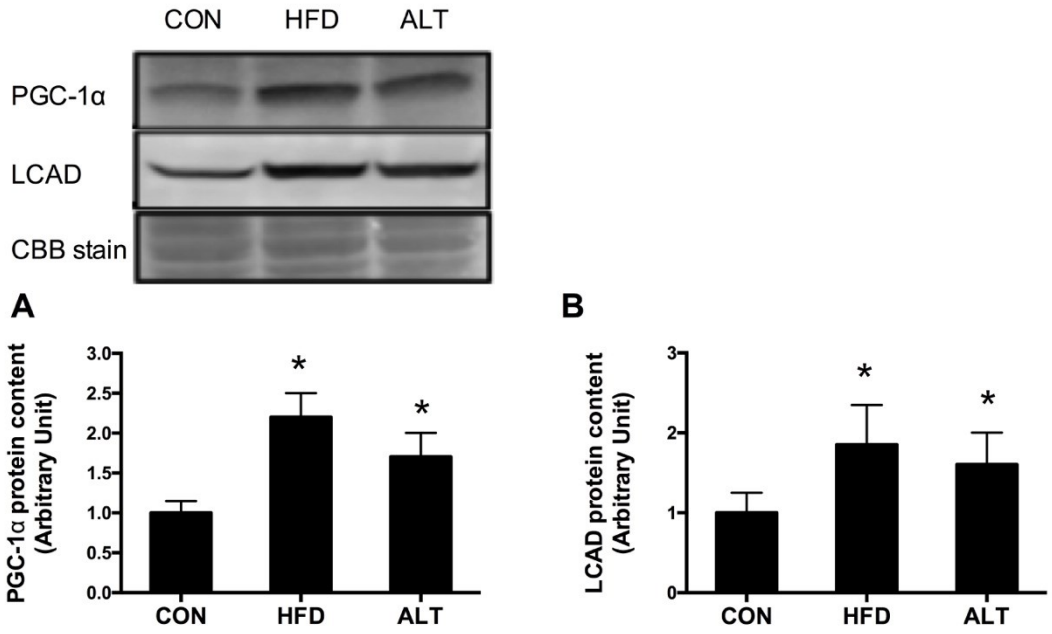

Figure 2-2. Effects of alternate-day high-fat diet feeding on PGC-1α (A) and LCAD (B) protein content in rat skeletal muscle. Values are mean ± SEM of 6 animals per group. * indicates significant difference at a level of $p < 0.05$ *vs* CON.

2-3-4. SDH activity

Next, we assessed the effect of an alternate-day high-fat diet on the oxidative capacity in skeletal muscles using histochemistry. Figure 2-3 shows representative images of succinate dehydrogenase (SDH) staining of the plantaris muscle from CON, HFD, and ALT groups. Succinate dehydrogenase activity staining was increased in HFD and ALT groups compared to the CON group.

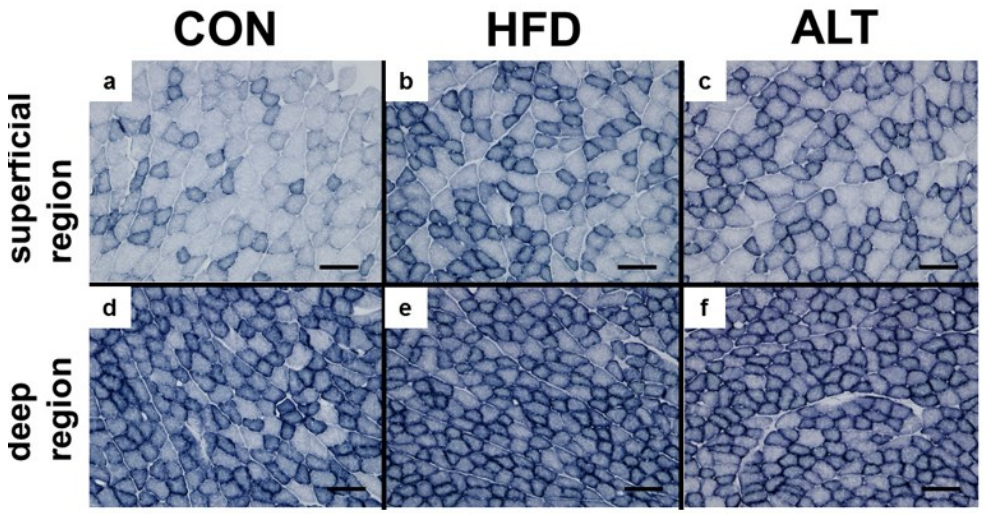

Figure 2-3. Effect of alternate-day high-fat diet feeding on succinate dehydrogenase (SDH) staining in rat plantaris muscle. Representative SDH-stained images are presented. SDH staining of superficial region of plantaris muscle from CON (a), HFD (b) and ALT (c) and deep region from CON (d), HFD (e) and ALT (f). Plantaris muscle of both HFD and ALT showed relatively dark staining for SDH compare to that of CON. Scale bar, 100μm.

2-4. Discussion

It was first reported by Holloszy (1) that endurance exercise training increases mitochondrial enzyme activities in rat skeletal muscle. This finding was confirmed in human skeletal muscles, subsequently by other research groups (20). The most important physiological effect of an increase in mitochondrial content in skeletal muscle is the sparing of muscle glycogen during prolonged, intense exercise. Miller *et al*. (5) reported that rats, which were fed a high-fat diet for 5 weeks ran on a treadmill for a longer duration than those fed a high-carbohydrate diet. The improvement in endurance performance was concomitant with an increase in skeletal muscle citrate synthase (a key enzyme of the tricarboxylic acid cycle), β-HAD enzyme activity (a major index of the β-oxidation) and lower utilization of muscle glycogen. This result suggests that a high-fat diet induces an increase in oxidative capacity and muscle glycogen sparing during submaximal exercise, which thereby prolongs submaximal endurance exercise performance. Consistent with this finding, the present study also showed that a 4-week high-fat diet induces an increase in key mitochondrial enzyme activities in rat skeletal muscle and that an alternate-day high-fat diet induces an increase in mitochondrial enzymes in rat skeletal muscle to a level comparable to that observed after a daily high-fat diet (Figures 2-1A and B). This result suggests that an alternate-day high-fat diet is sufficient to increase mitochondrial biogenesis.

The main disadvantages of a long-term high-fat diet are the huge accumulation of abdominal fat and increased body weight. In the present study, abdominal fat mass in HFD rats was approximately 60% higher than that of CON rats (Table 2-1). Since accumulation of abdominal fat is a strong predicator of insulin resistance (21,22), adopting high-fat diet for long term is contraindicated for endurance athletes. In

addition to being particularly unhealthy, high-fat diet increases body weight, which compromises endurance exercise performance. However, alternate-day high-fat diet induced muscle adaptation, without accompanying body weight gain and accumulation of excessive abdominal fat. Results of the current study suggest that dietary intervention with a high-fat diet can increase mitochondrial oxidative capacities in skeletal muscle without compromising health. However, the duration of alternate-day high-fat diet was only 4 weeks in the present study. If the duration of dietary intervention is prolonged, an increase in body fat might be observed. It is necessary to investigate the time course of changes in abdominal fat accumulation by the alternate-day high-fat diet.

The possible mechanisms involved in high-fat diet induced increase in mitochondrial biogenesis have been studied in the recent times. It has been reported that elevating plasma FFA by high-fat diet results in an increase in PPAR δ activation and mitochondrial biogenesis (4). In this study, plasma concentration of FFA was higher in both HFD and ALT groups than that in the CON group (Table 2-1). Therefore, it is likely that the activation of PPAR δ by raising plasma FFA is similar in both the HFD and ALT groups, resulting in an increase in mitochondrial enzyme activities in skeletal muscle. The transcriptional coactivator, PGC-1α is known to induce mitochondrial biogenesis by activating transcription factors and coordinated expression of a large number of proteins (23). In this study, PGC-1α protein content in HFD was significantly higher than that in CON. The ALT group also exhibited elevated PGC-1α protein content (Figure 2-2A). Although the precise mechanism by which high-fat diet increases PGC-1α protein content is not clear, the finding in this study suggested that repetitive stimulation by high-fat diet on alternate days is sufficient to elevate PGC-1α protein content and induce mitochondrial biogenesis.

Chapter 3 Time course of decrease in skeletal muscle mitochondrial biogenesis after discontinuing high-fat diet

3-1. Introduction

Endurance exercise training induces an increase in the number and size of mitochondria in the skeletal muscles that are recruited during exercise (1). The increase in mitochondria in skeletal muscle minimizes the disruption of homeostasis during exercise, as evidenced by a smaller decrease in muscle glycogen. Since muscle glycogen content prior to exercise is associated with endurance performance (2), these biochemical adaptations in skeletal muscle are responsible for the improvement of exercise capacity after exercise training.

Similar biochemical adaptations are observed in high-fat diet-adapted skeletal muscle. The enzyme activities involved in citrate cycle and β-oxidation were increased after high-fat diet consumption (5). Turner *et al*. demonstrated that feeding high-fat diet for 5 or 20 weeks significantly increased the content of mitochondrial respiratory chain complex proteins (15). These high-fat diet-induced adaptations of skeletal muscle contribute to endurance exercise performance (5).

Although it is generally accepted that the endurance exercise performance is directly related to the muscle glycogen content before starting exercise (24), Miller *et al*. reported that endurance running capacity is enhanced by consuming high-fat diet for 5 weeks, in spite of having lower than normal muscle glycogen stores (5). These results led us to consider that further enhancement of endurance capacity might be possible if high-carbohydrate diet was fed to high-fat diet-adapted animals. However, the time course of changes in the increased mitochondrial oxidative capacity after discontinuing

high-fat diet is not known. Since the high-fat diet-induced increase in plasma FFA concentration seemed to be involved in mitochondrial biogenesis in skeletal muscle (4), switching to high carbohydrate diet might rapidly decrease the elevated mitochondrial proteins. The purpose of the present study was to determine the time course of the reversal of high-fat diet-induced changes in mitochondrial biogenesis after switching to control diet.

3-2. Methods

3-2-1. Animals and experimental procedures

Male C57BL/6J mice (5-week old) were obtained from CLEA Japan (Tokyo, Japan). The mice were housed, four to a standard plastic cage with bedding. Mice had *ad libitum* access to water and a control diet (CON) or high-fat diet (HFD), and were weighed every week. The cages were maintained in an animal house at the temperature of 23 °C with a 12-h light/ dark cycle (lights were off at 9:00 p.m.). After 3 days of acclimatization, mice were randomly divided into control group (CON: n=8) and HFD group (HFD: n=24). HFD group was fed high-fat diet for 4 weeks, and then divided into three subgroups, HFD+0, HFD+3, and HFD+7. Mice in the HFD+0 group were sacrificed immediately at the end of high-fat diet intervention period, whereas HFD+3 and HFD+7 groups were sacrificed on day 3 and 7 after the switch from high-fat diet to control diet, respectively. The high-fat diet was prepared using lard, corn oil, sucrose, and casein (32%, 18%, 27% and 23% of total calories, respectively), supplemented with vitamins (22 g/kg, AIN93 vitamin mix: CLEA Japan), minerals (51 g/kg, AIN93G minerals mix: CLEA Japan) and methionine (4.4 g/kg, Wako Pure Chemical). The control diet CE-2 that contained 59% carbohydrate, 12% fat, and 29% protein in terms of calories, was obtained from CLEA Japan. The energy content of the high-fat diet was

5.1 kcal/g, whereas that of control diet was 3.4 kcal/g. Experiments were carried out in accordance with the guidelines of the Committee for Animal Experimentation in the Graduate School of Sport Sciences at Waseda University (2013-A117).

3-2-2. Tissue preparation

After overnight fasting, animals were anesthetized with sodium pentobarbital (50 mg/kg). Blood was collected by cardiac puncture, allowed to clot at room temperature and centrifuged at 3000 × *g* for 10 min at 4 °C. The supernatant was then transferred to a clean tube and stored at -80 °C for subsequent analysis. Gastrocnemius muscle, plantaris muscle and epididymal adipose tissues were rapidly removed, frozen in liquid nitrogen and stored at -80 °C for subsequent analysis.

3-2-3. Serum biomarkers

Concentrations of plasma triglycerides (TG) and free fatty acids (FFA) were determined using kits (Triglyceride E Test Wako, NEFA-C Test Wako, respectively) according to the manufacturer's instructions.

3-2-4. Western blotting

Frozen gastrocnemius muscle samples (100 mg) were homogenized in ice-cold RIPA buffer containing 50 mM Tris-HCl, PH 7.4, 150 mM NaCl, 0.25% deoxycholic acid, 1% NP-40, 1 mM EDTA, and protease inhibitor cocktail (Cell Signaling Technologies, Danvers, CA, USA). The muscle homogenates were freeze-thawed three times to disrupt mitochondria. Sample protein concentrations were measured by a BCA protein assay kit (Pierce, Rockford, IL, USA). Samples were prepared in Laemmli sample buffer (Wako Pure Chemical, Osaka, Japan). Equal amounts of sample protein were separated on 10% or 12.5% gels by SDS-PAGE and transferred to PVDF membranes. Membranes were blocked in 5% milk powder/TBST for 1 h at room

temperature and then incubated with primary antibodies overnight at 4 °C. Antibodies against long chain acyl-CoA dehydrogenase (LCAD), monoclonal antibodies against complex I subunit, NDUFB8 and ATP synthase subunit alpha (Complex V) were purchased from Sigma (St. Louis, MO, USA), Invitrogen (Carlsbad, CA, USA) and Invitrogen (Carlsbad, CA, USA), respectively. After incubating with HRP-conjugated secondary antibodies (goat anti-rabbit IgG or goat anti-mouse IgG), immobilon western HRP substrate (Millipore, Billerica, MA, USA) was employed to visualize bands. The membranes were stained with Coomassie Brilliant Blue (CBB) to verify and normalize the protein loading (19). The results were analyzed by ImageJ software (NIH, Bethesda, MD, USA).

3-2-5. Mitochondrial DNA analysis

DNA was extracted from 20 mg of plantaris muscle sample using QIAamp DNA mini kit (Qiagen, Hilden, GER) in accordance with manufacturer's instructions. The concentration of extracted DNA was measured at 260 nm using Nanodrop spectrophotometer (ND-1000, Isogen Life Science, De Meern, NE). The extracted DNA was diluted to a final concentration of 10 ng/μl in RNase free water. Mitochondrial DNA encoded cytochrome *b* primers were synthesized prior to the PCR performed by TaKaRa Ex TaqTM (TaKaRa, Japan). The cycle profile included denaturation for 1 min at 94 ℃, annealing for 1 min at 57 ℃, and extension for 1 min at 72 ℃. The primers for cytochrome *b* (forward: ATTCCTTCATGTCGGACGAG; reverse: AGAAGCCCCCTCAAATTCAT) were synthesized by Invitrogen. 18S rRNA was simultaneously measured as an internal standard, using QuantumRNA^{TM} 18S Internal Standards (Ambion, Austin, USA). Equal amounts of PCR products were separated in 2%

agarose gel and then visualized with SYBR Safe (Molecular Probes, Eugene, OR). The transcript intensity is expressed relative to 18S rRNA.

3-2-6. Statistical methods

All statistical analyses were carried out using SPSS statistical software (version 22.0; Chicago, IL, USA). After data were determined to be normally distributed based on a Shapiro-Wilk test for normality, one-way ANOVA Tukey's test was employed to determine the statistical significance of the difference between groups. If the data did not follow normal distribution, the Kruskal-Wallis non-parametric test was performed. All data were presented as mean ± standard error of the means (SEM) and considered as statistically significant at $p < 0.05$.

3-3. Results

3-3-1. Body weight, epididymal fat mass and plasma parameters

Table 3-1 shows the body weight, epididymal fat mass and plasma parameters. High-fat diet for 4 weeks resulted in an increase in body weight (CON *vs* HFD+0, $p < 0.01$). Body weight decreased significantly and reduced to the control value in 3 days after discontinuing high-fat diet feeding. The epididymal fat mass increased significantly by high-fat diet feeding, remained high for 3 days after switching to control diet, and decreased to the control value by the 7th day (CON *vs* HFD+0 and HFD+3, $p < 0.05$ and $p < 0.001$, respectively). Plasma TG concentration was increased by high-fat diet feeding and decreased to the control value in 3 days (CON *vs* HFD+0, $p < 0.01$). High-fat diet increased plasma FFA concentration significantly (CON *vs* HFD+0, $p < 0.05$). FFA concentration decreased rapidly after discontinuing high-fat diet and showed a lower level compared to that of control group (CON *vs* HFD+3 and HFD+7, $p < 0.05$ and $p < 0.01$, respectively).

Table 3-1. Effects of discontinuing high-fat diet on body weight, epididymal fat mass and plasma parameters in mice

	CON	HFD+0	HFD+3	HFD+7
Initial body weight (g)	14.5 ± 0.34	14.9 ± 0.38	14.0 ± 0.18	14.2 ± 0.26
Final body weight (g)	21.4 ± 0.54	24.4 ± 0.37 **	22.7 ± 0.34 §	21.7 ± 0.26 §§§
Epididymal fat mass (g)	0.18 ± 0.01	0.47 ± 0.07 *	0.44 ± 0.02 ***	0.22 ± 0.02 ###
Plasma triglycerides (mg/dL)	50.8 ± 3.38	82.7 ± 8.26 **	57.9 ± 6.21§	45.2 ± 3.72 §§§
Plasma FFA (mEq/L)	0.71 ± 0.05	0.98 ± 0.09*	0.66 ± 0.04 *§§	0.60 ± 0.06 **§§

CON, control group; HFD+0, 4-week high-fat diet group; HFD+3, 3 days after discontinuing 4-week high-fat diet group; HFD+7, 7 days after discontinuing 4-week high-fat diet group. Values are presented as mean ± SEM of 8 animals per group. *, ** and *** represent significant difference at a level of $p < 0.05$, $p < 0.01$ and $p < 0.001$ *vs* CON, respectively. §, §§ and §§§ represent significant difference at a level of $p < 0.05$, $p < 0.01$ and $p < 0.001$*vs* HFD, respectively. ### represent significant difference at a level of $p < 0.001$*vs* HFD+3.

3-3-2. Fatty acid beta-oxidation enzyme protein content

LCAD is an important enzyme involved in fatty acid β-oxidation. High-fat diet for 4 weeks resulted in an increase in LCAD protein content (CON *vs* HFD+0, $p < 0.05$). LCAD protein content remained high for 3 days after discontinuing high-fat diet and decreased to the control level in 7 days (CON *vs* HFD+3, $p < 0.01$).

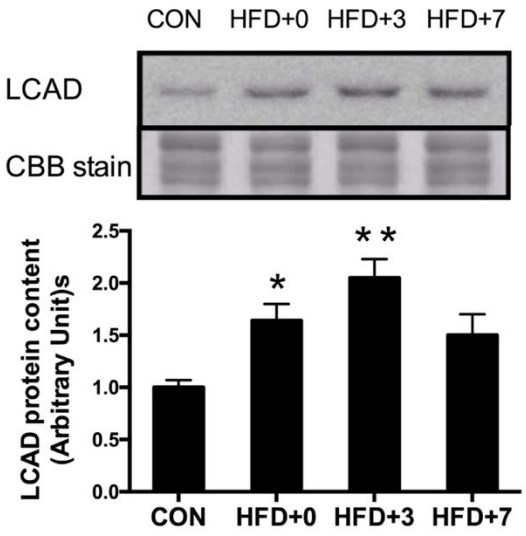

Figure 3-1. Effects of discontinuing high-fat diet on LCAD protein content in mouse skeletal muscle. Values are presented as mean ± SEM of 8 animals per group. * and ** represent significant difference at a level of $p < 0.05$ and $p < 0.01$*vs* CON, respectively.

3-3-3. Electron transport chain protein content

The protein content of one of the electron transport chain protein components, subunit NDUFB8 (Complex I) in the gastrocnemius muscle increased significantly after 4-weeks of high-fat diet and decreased to the control value in 3 days after discontinuing high-fat diet (CON *vs* HFD+0, $p < 0.01$). In addition, the protein content of ATP synthase subunit alpha (Complex V) showed an increase after 4-week high-fat diet and decreased to the control value in 3 days after discontinuing high-fat diet (CON *vs* HFD+0, $p < 0.01$).

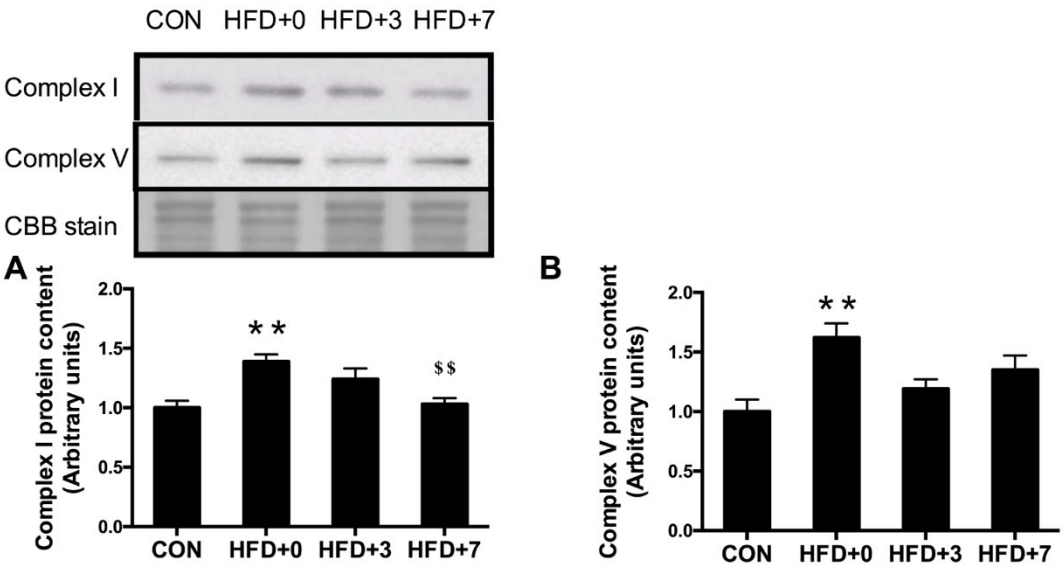

Figure 3-2. Effects of discontinuing high-fat diet on electron transport chain Complex I (A) and Complex V (B) protein content in mouse skeletal muscle. Values are presented as mean ± SEM of 8 animals per group. ** represent significant difference at a level of $p < 0.01$ *vs* CON. §§ represent significant difference at a level of $p < 0.01$*vs* HFD+0.

3-3-4. Mitochondrial DNA copy number

Cytochrome *b* is encoded by mitochondrial DNA and 18s rRNA is encoded by nuclear DNA. Mitochondrial DNA copy number in plantaris muscle was evaluated by determining the ratio of cytochrome *b* DNA to 18s rRNA DNA. As is shown in Figure 3-3, mitochondrial DNA copy number increased after 4 weeks of high-fat diet feeding and stayed high for at least 7 days after discontinuing high-fat diet (CON *vs* HFD+0, HFD+3 and HFD+7, $p < 0.001$, $p < 0.001$, and $p < 0.01$, respectively).

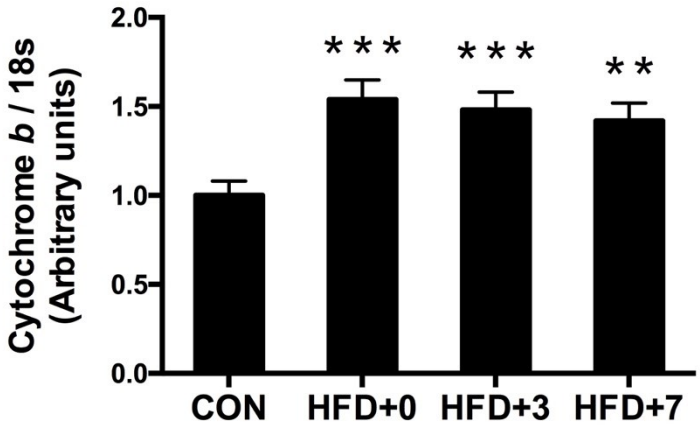

Figure 3-3. Effects of discontinuing high-fat diet on mitochondrial DNA copy number in mice skeletal muscle. Values are presented as mean ± SEM of 8 animals per group. ** and *** represent significant difference at a level of $p < 0.01$and $p < 0.001$ *vs* CON.

3-4. Discussion

Both exercise training and high-fat diet consumption induce an increase in mitochondrial biogenesis in skeletal muscle (1,5). Endurance exercise training-induced increase in muscle mitochondria was earlier thought to be a slow process. However, Wright *et al.* (25) demonstrated that skeletal muscle mitochondrial proteins such as succinate-ubiquinone oxidase and δ-aminolevulinate synthase increase significantly after a 3-hour bout of exercise, suggesting that exercise-induced increase in mitochondrial biogenesis is a rapid process. In contrast, high-fat diet-induced mitochondrial biogenesis occurs slowly. It has been reported that a significant adaptive response of mitochondrial biogenesis to high-fat diet did not occur until after 4 weeks of high-fat diet consumption (4).

Although the higher mitochondrial content in skeletal muscle is beneficial for endurance performance, consuming high-fat diet for 3 to 4 weeks is problematic. Proportionate decrease in carbohydrate intake while on high-fat diet, causes a reduction of muscle glycogen content and may compromise exercise performance. Thus, it is ideal to consume a high-carbohydrate diet after high-fat diet regimen to increase muscle glycogen content, while the mitochondrial content is still elevated. However, the duration for which the increase in high-fat diet-induced mitochondrial biogenesis remains after discontinuing high-fat diet is not known. Therefore, the purpose of this study was to determine the time course of the decline of skeletal muscle mitochondrial biogenesis to basal level after discontinuing high-fat diet. The main findings of the present study were that after feeding high-fat diet for 4 weeks and then discontinuing, electron transport chain complex protein contents returned to control levels (within 3 days) faster than did the enzyme involved in β-oxidation (within 7 days). The increase

in high-fat diet-induced mitochondrial DNA (mtDNA) copy number persisted for at least 1 week after discontinuing high-fat diet. These data showed that the high-fat diet-induced increase in mitochondrial biogenesis is short-lived, and the rate of decrease of each of the mitochondrial components is regulated differently.

After the withdrawal of high-fat diet, mitochondrial respiratory chain Complex I protein content decreased to the baseline value in 3 days, as did that of Complex V (Figure 3-2). It was reported that several components located in the inner membranes of mitochondria in heart and liver have similar half-lives (26). Therefore, the similar rate of decline in the content of these proteins seems to be due to their same location, in the mitochondrial inner membranes. The elevated protein content of LCAD, a β-oxidation enzyme, was maintained until the 3rd day after switching the diet, and returned to basal level by the 7th day. Rabinowitz *et al*. showed that mitochondria are complex organelles composed of at least three major structures: outer and inner membranes, and matrix, where LCAD is located. Components of each of the three major structures are probably synthesized and assembled in different ways (26,27). These differences may explain, in part, the different decline rates of mitochondrial components in the skeletal muscle of animals fed with high-fat diet after high-fat diet withdrawal.

The possible mechanism of high-fat diet-induced increase in muscle mitochondrial biogenesis has been investigated in recent times. High-fat diet consumption activated PPARδ by increasing plasma free fatty acid levels (14). Therefore, we determined the time course of change in plasma FFA concentration following 4 weeks of high-fat diet feeding, to see whether it parallels the changes in mitochondrial components (Table 3-1). After discontinuation of high-fat diet, plasma FFA concentration decreased to the baseline level rapidly and displayed a lower value on the 3rd and 7th days (Table 3-1).

Compared to the time course of change in FFA concentration, LCAD protein content, which is directly regulated by PPARδ, returned to the control level in 1 week after withdrawing high-fat diet. The slower decrease in LCAD protein content compared to that of plasma FFA level indicates that high-fat diet might increase FFA concentration, but other mechanisms may contribute to the maintenance of the increase in LCAD protein content, although we could not demonstrate in this study.

In contrast to the other cellular organelles, mitochondria have their own DNA (mtDNA) that encodes some of the mitochondrial proteins. Consistent with the results of an earlier study, administering high-fat diet for 4 weeks induced a significant increase in mtDNA copy number in skeletal muscle in this study (Figure 3-3). More importantly, after discontinuing the high-fat diet, the mtDNA copy number remained high for at least 1 week. The time course of the change in mtDNA copy number in skeletal muscle indicates that mtDNA has a half-life of 2 weeks, which is consistent with previous studies (28,29).

Since it is reported that the temporary diet change appeared to be tolerated well by subject (30), it is interesting to see how the endurance capacity would increase in humans in response to high-fat diet used to induce muscle mitochondrial biogenesis, and then loading with carbohydrates just prior to a bout of prolonged exercise. Future studies that focus on the time course of muscle adaptation to high-fat diet might help better understanding for the nutritional modulation to improve endurance capacity.

Chapter 4. General discussion

High-fat diet induces skeletal muscle mitochondrial biogenesis and this adaptation is beneficial for endurance exercise performance. However, long term high-fat diet resulted in body weight gain and abdominal fat accumulation. These are disadvantages that need to be addressed, to be able to use high-fat diet for exercise performance enhancement. Alternate-day high-fat diet induced increase in enzyme activities and protein content without increasing the accumulation of abdominal fat. These results indicate that high-fat diet on alternate days is a practical dietary regimen to induce skeletal muscle adaptation. High-fat diet-induced increase in mitochondrial biogenesis occurs slowly, over a minimum of 3-4 weeks. Discontinuing high-fat diet after 4 week results in the reversal of the increases in the skeletal muscle mitochondrial protein content and mitochondrial DNA. The time course of this reversal is different for the two different components. This information is helpful for modulating the dietary regimen using high-fat diet, which induces mitochondrial biogenesis and obviates the need for high energy intake.

In contrast to animal studies, human studies have failed to demonstrate a beneficial effect of a high-fat diet on endurance exercise performance (30,31). The difference in results between animals and human studies may be because of fat composition in diet. The control diet used in most of the animal studies comprised approximately 10% of calories from fat, whereas a typical Japanese and American diet consists of about 25% (32) and 34% fat (33), respectively. It might be difficult to detect a high-fat diet-induced increase in endurance performance in humans, because the fat content in the diet of human is higher than that used in experimental animals. However, because a high-fat

diet, containing 62% calories from fat, induces increase in mitochondrial enzyme in human skeletal muscle (11), human skeletal muscle is capable of adaptation responses to a high-fat diet.

In present thesis, the high-fat diet was prepared using lard and corn oil as the fat source. According to the study of Hancock *et al.* (4), high fat diet that containing flaxseed and olive oil also reported to induce increased skeletal muscle mitochondrial protein content. It implied that elevated plasma FFA induced skeletal muscle mitochondrial biogenesis independent of long chain fatty acid composition. Since it is reported that mice fed a high-fat diet rich in n-3 polyunsaturated fatty acids, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) presented lower body weight than fed high-fat diet rich in vegetable oil (34). It will be interesting in future studies to see the effect of n-3 polyunsaturated fatty acids on high-fat diet induced skeletal muscle adaptation and body weight gain.

The possible mechanism of high-fat diet induces skeletal muscle mitochondrial biogenesis was discussed in recently. Hancock *et al*. demonstrated that increased plasma FFA concentration induced by high-fat diet activates PPARδ, increases PGC-1α protein content, then results in mitochondrial biogenesis (4). FFA is the nature ligand of PPARδ (35). Overexpression of PPARδ in muscle results in an increase in PGC-1α protein and induces an increase in mitochondria (4,36). In the chapter 2 of the present thesis, the increased plasma FFA concentration and muscle PGC-1α protein content after high-fat diet feeding supported the opinions of Hancock *et al*. Moreover, except for high-fat diet feeding, other approach that increases FFA concentration was reported to induce skeletal muscle mitochondrial biogenesis. Increased intracellular FFA levels by

overexpression of muscle lipoprotein lipase was reported to result in the increase of muscle mitochondria (37).

Although agonist activation of PPARδ acts directly on genes of fatty acid β oxidation, such as LCAD and energy uncoupling protein (38,39), there is no evidence for the PPARδ agonist effect on mitochondrial biogenesis and oxidative phosphorylation genes (40). It is possible that activation of PPARδ by increased plasma FFA is not the only possible mechanism of high-fat diet induced skeletal muscle mitochondrial biogenesis, other mechanism may involve in these adaptations. Recently, Jain *et al.* demonstrated that high-fat diet-induced mitochondrial biogenesis is regulated by mitochondrial-derived reactive oxygen species activation of calcium/ calmodulin-dependent protein kinase Ⅱ (CaMK Ⅱ) (41). More details about the mechanism of high-fat diet induced mitochondrial biogenesis need to be discussed in the future.

Chapter 5. Conclusions and Future perspectives

The main findings of this thesis were as follows:

• Administering high-fat diet on alternate days for 4 weeks induces increases in mitochondrial enzyme activities and protein content in rat skeletal muscle without increasing abdominal fat accumulation.

• The time course of the changes following the withdrawal of high-fat diet in skeletal muscle mitochondrial protein content is different from that of the changes in mitochondrial DNA.

In conclusion, the present thesis demonstrated that rational utilization of high-fat diet is a feasible nutritional modulation to induce skeletal muscle adaptations that are beneficial in enhancing endurance exercise performance.

It has been reported recently that administration of ketone ester-based drink alters fuel preference during exercise and improve physical performance in humans (30). Besides the traditional nutritional modulation that includes increased intake of carbohydrate and protein to enhance skeletal muscle adaptation, administering some of the metabolic intermediates as well as increasing FFA by high-fat diet also contributes to the exercise performance. It will be interesting to determine whether manipulating dietary fat or restricting high-fat diet would induce an increase in mitochondrial enzyme activity and protein content in human skeletal muscle and enhance endurance exercise performance.

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