Effects of Dietary Fat Restriction on Endurance Training-induced Metabolic Adaptations in Rat Skeletal Muscle

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Abstract: Endurance exercise training enhances muscle fat oxidation while concomitantly reducing carbohydrate (glycogen) utilization during exercise, thereby delaying the onset of fatigue. This study examined the effects of dietary fat restriction on endurance training-induced metabolic adaptations in rat skeletal muscle. Male Sprague-Dawley rats were placed on either a control diet (CON: 19.2% protein, 21.6% fat, and 59.2% carbohydrate as a percentage of total energy) or a fat-restricted diet (FR: 21.5% protein, 2.4% fat, and 76.1% carbohydrate as a percentage of total energy) for 4 wks. Half the rats in each dietary group performed daily 6-h swimming exercise (two 3-h sessions separated by 45 min of rest) on 5 days each wk. Endurance training significantly increased the expression of β-hydroxyacyl CoA dehydrogenase (βHAD), a key enzyme of fat oxidation, and pyruvate dehydrogenase kinase 4 (PDK4), an inhibitory regulator of glycolytic flux, in the skeletal muscle of rats fed the CON diet. However, such endurance training-induced increases in muscle βHAD and PDK4 were partially suppressed by the FR diet, suggesting that a FR diet may diminish the endurance training-induced enhancement of fat oxidation and reduction in glycogen utilization during exercise. We then assessed the muscle glycogen utilization rate during an acute bout of swimming exercise in the trained rats fed either the CON or the FR diet and consequently found that rats fed the FR diet had a significantly higher muscle glycogen utilization rate during exercise compared with rats fed the CON diet. In conclusion, dietary fat restriction may attenuate the endurance training-induced metabolic adaptations in skeletal muscle.

Key words: low-fat, endurance training, glycogen, rat, skeletal muscle

1 Introduction

Endogenous carbohydrate stores are limited and thus almost completely emptied within only a few hours of continuous submaximal (70%–80% maximal oxygen uptake) exercise. The depletion of carbohydrate reserves is associated with onset of fatigue and impairment of exercise capacity3. Meanwhile, body fat deposits constitute another, much larger, source of fuel for exercise. It is well known that long-term endurance exercise training increases the expressions of mitochondrial enzymes, especially fatty acid oxidation (FAO) enzymes, in skeletal muscle3. Muscle pyruvate dehydrogenase kinase 4 (PDK4), which inactivates the pyruvate dehydrogenase complex and inhibits glycolytic flux, is also increased by endurance training3. These adaptations lead to enhanced muscle FAO and diminished glycogen utilization during acute bouts of exercise. This phenomenon is called the "glycogen sparing effect," and it plays a key role in delaying the onset of fatigue during acute endurance exercise at a given intensity3.

To be successful in endurance sport events such as the marathon, race walking, and road cycling, it is advantageous not only to optimize the training-induced adaptations as mentioned above but also to reduce body weight and body fat mass. Some endurance athletes therefore tend to restrict their dietary intake of fat, which has higher energy density compared with carbohydrates and proteins5. However, such dietary fat restriction is likely to attenuate the muscle enzyme adaptations induced by endurance exercise training.
ance exercise training. This is because peroxisome proliferator-activated receptor (PPAR)β, which is suggested to be involved in the endurance training-induced increase in mitochondrial enzymes and PDK4, is also activated by free fatty acid (FFA). It is therefore hypothesized that dietary fat restriction, which is associated with a lower blood FFA concentration, attenuates muscle enzyme adaptations in response to endurance training. This hypothesis is further supported by the previous finding that the partial silencing of PPARβ expression by shRNA resulted in marked attenuation of the exercise training-induced mitochondrial adaptations in rat skeletal muscle. Moreover, another study reported that pharmacologically lowering serum FFA resulted in a 30% decrease in the mRNA content of mitochondrial proteins in human skeletal muscle. Based on these findings, it is more plausible that a substantial reduction in dietary fat intake has negative effects on endurance training-induced muscle enzyme adaptations, particularly the increase in mitochondrial FAO enzymes, and that the muscle glycogen sparing effect during acute exercise is blunted.

The purpose of this study was therefore to determine whether long-term intake of a fat-restricted diet (2.4% of total energies) blunts endurance training-induced metabolic enzyme adaptations in rat skeletal muscle. If it does, we then evaluated muscle and liver glycogen utilizations during an acute bout of exercise in trained rats fed a fat-restricted or a control diet.

2 Experimental Procedures

2.1 Experimental animals

Four-week-old male Sprague-Dawley rats (CLEA Japan, Tokyo) with body weights of 70–90 g were kept in individual cages. The environment was maintained at 22 ± 1°C with 50 ± 5% humidity and illumination from 09:00 to 21:00. All animals were treated in accordance with national guidelines for the care and use of laboratory animals (Notification of the Prime Minister’s Office of Japan). The Animal Experimental Committee of the University of Tokyo approved this experimental protocol (approval no. 29–10).

2.2 Experiment 1

In Experiment 1, we evaluated the effects of dietary fat restriction on endurance training-induced metabolic enzyme adaptations in rat skeletal muscle. During an acclimation period of 5 days, the rats were allowed free access to water and a control diet (19.2% protein, 21.6% fat, and 59.2% carbohydrate as a percentage of total energy, 4.17 kcal/g), which was based on the AIN-93G formula with the modification that the macronutrient composition was comparable to that in the diet consumed by typical endurance athletes. All rats were acclimated to the swimming exercise for 10 min a day for 2 days before being divided into two dietary groups, matched for body weight and food efficiency: a group continued on the control diet (CON; n = 14) and a group fed a fat-restricted diet (FR; n = 15: 21.5% protein, 2.4% fat, and 76.1% carbohydrate as a percentage of total energy, 3.72 kcal/g), in which 90% of the fat in the CON diet was replaced with carbohydrates. The compositions of the diets are presented in Table 1.

Table 1 Composition of the experimental diets.

| Ingredients            | Experimental diet (g/kg diet) |
|------------------------|-----------------------------|
|                        | CON  | FR   |
| Cornstarch             | 499.48 | 589.48 |
| Casein                 | 200.00 | 200.00 |
| Sucrose                | 100.00 | 100.00 |
| Soybean oil            | 100.00 | 10.00 |
| Cellulose fiber        | 50.00  | 50.00  |
| Mineral mix (AIN-93G)  | 35.00  | 35.00  |
| Vitamin mix (AIN-93)   | 10.00  | 10.00  |
| L-Cystine              | 3.00   | 3.00   |
| Choline bitartrate     | 2.50   | 2.50   |
| tert-Butylhydroquinone | 0.02   | 0.02   |

CON, control diet; FR, fat-restricted diet.
because they have been shown to be mainly recruited during swimming exercise in rats. This is evidenced by the occurrence of glycogen depletion in response to a bout of swimming exercise\textsuperscript{14} and adaptive increases in glucose transporter (GLUT) 4 and mitochondrial enzymes\textsuperscript{12, 13}. Biochemical analyses in the muscle and plasma samples were performed as described below.

2.3 Experiment 2

In Experiment 2, we assessed the muscle and liver glycogen utilizations during an acute bout of exercise in trained rats fed either a control diet or a fat-restricted diet. After a 6-day acclimation period, rats were fed either the CON diet (n = 16) or the FR diet (n = 16) for 4 wks. All rats performed the 4-wk swimming exercise training as described in Experiment 1. At 18 h after the last training session, the rats in each dietary group were further divided into two groups for the evaluation of the glycogen and triacylglycerol utilizations during an acute bout of exercise. One group was subjected to a 1-h period of swimming exercise with a weight equal to 5% of their body weight tied to their body. Four rats swam simultaneously in a barrel filled to a depth of 45 cm and with an average surface area of 400 cm\textsuperscript{2}/rat. Immediately after the acute bout of swimming exercise, the rats were sacrificed under anesthesia with isoflurane. The epitrochlearis and triceps muscles and the liver were removed, rapidly frozen, and stored at −80°C until analysis. The other group was sacrificed without performing an acute bout of swimming exercise to serve as a baseline control. The glycogen and triacylglycerol utilizations in the muscles and liver during the acute bout of exercise were estimated by subtracting the concentrations of each of these substrates in each rat that had exercised from the corresponding mean values in the baseline control rats in each dietary group.

2.4 Analytical procedures

2.4.1 Sample homogenization

The epitrochlearis muscles were homogenized in an ice-cold radio-immunoprecipitation assay lysis buffer (Merck Millipore, Billerica, MA) containing 50 mmol/L Tris-HCl pH 7.4, 150 mmol/L NaCl, 0.25% deoxycholic acid, 1% NP-40, and 1 mmol/L ethylenediaminetetraacetic acid (EDTA) with protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO). The homogenates were subjected to three freezing-thawing cycles to disrupt intracellular organelles before being rotated end-over-end at 4°C for 90 min to solubilize the protein. The homogenized samples were centrifuged at 700 × g for 5 min at 4°C, after which the supernatants were collected.

2.4.2 Western blot analysis

The protein concentration of the supernatants was determined using a BCA protein assay kit (Thermo Fisher Scientific, Waltham, MA). Samples were prepared in Laemmli buffer consisting of 277.8 mM Tris-HCl, 44.4% (w/v) glycerol, 4.4% (w/v) lithium dodecyl sulfate, and 0.02% (w/v) bromphenol blue, at a pH of 6.8 (Bio-Rad Laboratories, Hercules, CA) with dithiobisreitol (Bio-Rad). The mixture was heated at 95°C for 5 min in a heating block. The sample for GLUT4 measurement was prepared without using dithiobisreitol or heating. Equal amounts of sample protein were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (7.5%–10% resolving gels) and then transferred to polyvinylidene difluoride membranes (Merck Millipore) at 200 mA for 90 min. Following this transfer, the membranes were blocked with 5% (w/v) skim milk in Tris-buffered saline (TBS; 20 mmol/L Tris base, 137 mmol/L NaCl, pH 7.6) containing 0.1% Tween 20 (TBS-T) for 1 h at room temperature. The membranes were incubated overnight with the following primary antibodies at concentrations of 1:500–5,000 at 4°C: anti-β-hydroxyacyl CoA dehydrogenase (anti-βHAD), 1:500; Proteintech, Rosemont, IL), anti-PDK4 (1:1,000; Protein-tech), anti-GLUT4 (1:1,000; from the laboratory of Dr. John O. Holloszy, Washington University, St. Louis, MO), and anti-Δ-aminolevulinic acid synthetase (ALAS, 1:5,000; from the laboratory of Dr. John O. Holloszy). The membranes were then incubated at room temperature for 1 h with secondary antibodies (anti-mouse IgG or goat anti-rabbit IgG; Jackson ImmunoResearch, West Grove, PA) at dilution of 1:5,000 in TBS-T containing 1% skim milk. Bands visualization was performed using an enhanced chemiluminescence prime reagent (GE Healthcare, Chicago, IL) and quantified by Image Studio Digits (Ver. 5.2; LI-COR Biosciences, Lincoln, NE). The membranes were stained with Ponceau (Sigma-Aldrich) to verify equal protein loading across lanes\textsuperscript{15}. The intensities of immunobands were normalized to the total protein determined by quantifying all Ponceau red-stained bands in the relevant sample lane.

2.4.3 Citrate synthase activity measurement

The triceps muscles were homogenized in 175 mM KCl, 10 mM glutathione, and 2 mM EDTA, pH 7.4. After the homogenates were subjected to three freezing-thawing cycles, citrate synthase activity was measured using Sreer’s method\textsuperscript{16}.

2.4.4 Glycogen measurement

The epitrochlearis muscles and liver were homogenized in 0.3 mol/L perchloric acid. After acid hydrolysis, the glycogen concentration was measured using the enzymatic method described by Lowry and Passonneau\textsuperscript{17}.

2.4.5 Triacylglycerol measurement

The triceps muscle samples were homogenized in 0.9% (w/v) NaCl and extracted with chloroform-methanol (2:1 v/v) as described by Folch et al.\textsuperscript{18}, separating the chloroform and methanol-water phases, and then further processed using the method of Denton and Randle\textsuperscript{19} with modifications by Frayn and Maycock\textsuperscript{20}. The triacylglycerol concentration was then measured using a Triglyceride E-test.
Tokyo and intra-abdominal fat weight were observed for final body weight, body weight gain, and after the acute bout of swimming exercise. In Experiment 2, we thus performed Student’s t-test to evaluate the directional hypotheses. All statistical analyses were performed using BellCurve for Excel software (Social Survey Research Information, Tokyo). Statistical significance was defined as \( p < 0.05 \).

3 Results
3.1 Experiment 1
3.1.1 Final body weight, body weight gain, total energy intake, food efficiency, and intra-abdominal fat weight

No significant interactions between exercise training and diet were observed for final body weight, body weight gain, and intra-abdominal fat weight (Table 2). Main effects of exercise training on these parameters were observed and resulted in significantly lower final body weight, body weight gain, and intra-abdominal fat weight in the EX group compared with the SED group. Moreover, the FR group had significantly lower final body weight, body weight gain, and intra-abdominal fat weight compared with the CON group.

There was no significant interaction between exercise training and diet for total energy intake or food efficiency (Table 2). The total energy intake and food efficiency were significantly lower in the EX group than the SED group. A significant main effect of diet on food efficiency but not on total energy intake was observed and resulted in significantly lower food efficiency in the FR group compared with the CON group.

3.1.2 CS activity and ALAS protein expression in skeletal muscle

To clarify the effects of the FR diet on endurance exercise training-induced mitochondrial adaptations in skeletal muscle, we measured the CS activity and protein content of ALAS, which are frequently used as markers of mitochondrial adaptation to endurance exercise training\(^{21, 22}\). There was no significant interaction between exercise training and diet for either CS activity in triceps or ALAS protein content in epitrochlearis muscle (Figs. 1A and B). The CS activity and ALAS protein content in the muscle tissues were found to be significantly higher in the EX group than in the SED group. However, no significant main effect of diet on these enzymes was observed.

3.1.3 \( \beta \)HAD protein expression in epitrochlearis muscle

\( \beta \)HAD is a key enzyme in fatty acid \( \beta \)-oxidation, and the activity of this enzyme in muscle has been shown to be significantly correlated with the FAO rate during exercise\(^{23}\). In this study, we therefore measured the \( \beta \)HAD protein content as a marker of FAO capacity in skeletal muscle. No significant interaction between exercise training and diet was observed for \( \beta \)HAD protein content in the epitrochlearis muscle.

Table 2 Total energy intake, body weight, body weight gain, food efficiency, and intra-abdominal fat weight in rats.

|                          | CON-SED | FR-SED | CON-EX | FR-EX | \( p \)-values*        |
|--------------------------|---------|--------|--------|-------|------------------------|
| Total energy intake (kcal) | 2988 ± 84  | 2863 ± 39  | 2524 ± 56  | 2531 ± 54  | \(<0.001\) n.s. n.s. |
| Initial body weight (g)   | 140 ± 3   | 140 ± 2   | 140 ± 2   | 140 ± 2   | n.s. n.s. n.s.         |
| Final body weight (g)     | 425 ± 12  | 395 ± 6   | 312 ± 8   | 296 ± 6   | \(<0.001\) \(<0.001\) n.s. |
| Body weight gain (g)      | 285 ± 10  | 255 ± 4   | 172 ± 7   | 156 ± 5   | \(<0.001\) \(<0.01\) n.s. |
| Food efficiency (\( \Delta \)g/100 kcal) | 9.5 ± 0.1 | 8.9 ± 0.1 | 6.8 ± 0.2 | 6.2 ± 0.1 | \(<0.001\) \(<0.001\) n.s. |
| Intra-abdominal fat weight (g) | 20.2 ± 2.0 | 16.4 ± 0.8 | 8.3 ± 0.9 | 5.9 ± 0.3 | \(<0.001\) \(<0.05\) n.s. |

Values are means ± SEM; \( n=7 \) for all groups except FR-EX \( (n=8) \). *The main effects of swimming exercise training (exercise), diet, and their interaction (exercise × diet) were analyzed by two-way ANOVA. n.s., not significant.
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The muscle βHAD protein content was significantly higher in the EX group than the SED group. On the other hand, a main effect of diet on βHAD protein expression was also observed, with the FR group having significantly lower βHAD protein expression compared with the CON group.

3.1.4 PDK4 protein expression in epitrochlearis muscle

Previous studies have shown that endurance exercise training decreases glycolytic flux and glucose oxidation in skeletal muscle during exercise via upregulation of the PDK4 content, which phosphorylates and inactivates the pyruvate dehydrogenase complex \(^3\). Therefore, we evaluated the PDK4 protein content in skeletal muscle, and the results showed that the EX group had significantly higher muscle PDK4 protein content compared with the SED group (Fig. 3). On the other hand, groups fed the FR diet had significantly lower PDK4 protein content compared with the groups fed the CON diet. The interaction between exercise training and diet for the PDK4 protein content in epitrochlearis muscle was not significant.

3.1.5 GLUT4 protein expression in epitrochlearis muscle

Endurance exercise training is the most potent stimulus in terms of enhancing the muscle GLUT4 content, which is highly correlated with glucose transport activity and glycogen concentration in skeletal muscle \(^26\). In our experiments, no significant interaction between exercise training and diet was observed for the GLUT4 protein content in the epitrochlearis muscle (Fig. 4). The EX group had significantly higher muscle GLUT4 protein content compared with the SED group. Moreover, a significant main effect of

Fig. 1 Effects of endurance exercise training and dietary fat restriction on CS activity in the rat triceps muscle (A) and on the protein expression of ALAS in the rat epitrochlearis muscle (B). Values are means ± SEM; n = 7 for all groups except FR-EX (n = 8). The main effects of swimming exercise training (exercise), diet, and their interaction (exercise × diet) were analyzed by two-way ANOVA. SED, sedentary group; EX, exercise training group; CON, control diet group; FR, fat-restricted diet group.

Fig. 2 Effects of endurance exercise training and dietary fat restriction on the protein expression of βHAD in the rat epitrochlearis muscle. Values are means ± SEM; n = 7 for all groups except FR-EX (n = 8). The main effects of swimming exercise training (exercise), diet, and their interaction (exercise × diet) were analyzed by two-way ANOVA. SED, sedentary group; EX, exercise training group; CON, control diet group; FR, fat-restricted diet group.
diet on GLUT4 protein content in the epitrochlearis muscle was also observed and resulted in significantly higher GLUT4 protein expression in the FR group compared with the CON group. The interactions between exercise training and diet for the plasma glucose, FFA, triacylglycerol, and insulin concentrations were not statistically significant (Table 3). The plasma glucose, triacylglycerol, and insulin concentrations but not the FFA concentration were significantly lower in the EX group than the CON group. On the other hand, a significant main effect of diet was observed for the plasma FFA concentration, with the FR group having a significantly lower plasma FFA concentration than the CON group.

3.2 Experiment 2

3.2.1 Energy substrate concentrations in the muscle and liver before and immediately after an acute bout of 1-h swimming exercise, and their utilizations during exercise

No significant interaction between time and diet for gly-
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4 Discussion

Endurance athletes such as marathon runners, race walkers, and road cyclists strive to reduce their body weight and body fat mass by restricting their daily intake of fat, which has high energy density. In the present investigation, although the FR and CON groups had similar total energy intakes, the FR group showed significantly lower body weight and total intra-abdominal fat mass (Table 2). As a result, the food efficiency of the FR group was significantly lower than that of the CON group, which means that the mechanism by which dietary fat restriction prevents weight gain may be an increase in energy expenditure. A previous study demonstrated that the intake of a high-carbohydrate diet leads to an increase in the conversion of carbohydrates to fat (27), which is an energetically costly process, converting 25% of the energy content of carbohydrates into heat (28). Therefore, we speculate that the rats fed the FR diet, in which carbohydrates accounted for 78% of the total energy, had markedly increased fat synthesis from carbohydrates, thereby increasing their energy expenditure.

The main metabolic consequence of the adaptation of skeletal muscle to long-term endurance exercise training is the enhancement of fat oxidation and the concomitant reduction in carbohydrate utilization during exercise. This “glycogen sparing effect” in skeletal muscle may be attributed to the endurance training-induced increase in muscle mitochondria, particularly FAO enzymes (3). Consistent with previous findings, endurance swimming exercise training for 4 wks induced significant increases in CS activity and in the expressions of ALAS and HAD in skeletal muscle (Figs. 1 and 2). It is thus plausible that the muscle glycogen sparing effect occurred during exercise in the EX groups. On the other hand, although no effect of diet was observed for CS activity and ALAS expression in skeletal muscle, the endurance training-induced increase in muscle βHAD, a key enzyme in fatty acid β-oxidation, was partially suppressed by feeding rats the FR diet (Fig. 2). The fact that the dietary fat restriction suppressed βHAD expression but not CS activity or ALAS expression may have been due to the distinct regulatory mechanisms of their expressions. While the exercise-induced expression of CS and ALAS is coordinately regulated by factors such as nuclear respiratory factor (NRF)-1 and -2 and PPARγ coactivator-1α (PGC-1α) (30), βHAD expression is also regulated by the nuclear receptor, PPARβ, which is activated by fatty acids, as well as NRFs and PGC-1α (29). A previous study demonstrated that increasing serum FFA in rats by feeding them a high-fat diet resulted in the upregulation of PPARβ activity and subsequent increases in FAO enzyme expressions in skeletal muscle (29). In the present investigation, the FR group had significantly lower plasma FFA levels compared with the CON group (Table 3), which may have resulted in the diminished activation of PPARβ and the attenuation of the endurance training-induced increase in βHAD expression but not in CS activity or ALAS expression in skeletal muscle. Because the activity of βHAD has been shown to be significantly correlated with the FAO rate during exercise (26), it is plausible that dietary fat restriction negated the endurance training-induced enhancement of the muscle FAO capacity.

Previous studies have shown that endurance training induces an increase in the expression of PDK4, an inhibitory regulator of glycolytic flux, in skeletal muscle, which may also contribute to the endurance training-induced muscle glycogen sparing effect during exercise (8). Whereas our results also showed that endurance training resulted in a significant increase in muscle PDK4 expression (Fig. 3), this increase was partially suppressed by feeding of the FR diet as well. This may also be attributed to the diminished activation of PPARβ by lower plasma FFA levels in the FR group, because PPARβ is also involved in the regulation of PDK4 as well as βHAD expression in skeletal muscle (7). Our findings that FR diet feeding suppressed the endurance training-induced increases in the expression of βHAD and PDK4 in skeletal muscle led us to hypothesize that dietary fat restriction may negate the endurance training-induced muscle glycogen sparing effect during exercise.

To assess this hypothesis, the glycogen and triacylglycerol utilizations during an acute 1-h bout of swimming ex-
Fig. 5  Glycogen concentrations in the epitrochlearis muscle (A) and liver (C) and triacylglycerol concentration in the triceps muscle (E) before and immediately after an acute 1-h bout of swimming exercise, and their utilizations during exercise (B, D, and F). Values are means ± SEM; n = 8 for all groups. The main effects of time, diet, and their interaction were analyzed by two-way ANOVA (A, C, and E). Student’s one-tailed t-test was used to evaluate the directional hypotheses that glycogen utilization is higher and triacylglycerol utilization is lower in the FR group than the CON group during the acute bout of exercise (B, D, and F). CON, control diet group; FR, fat-restricted diet group.
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In conclusion, the present investigation demonstrated that dietary fat restriction may attenuate the endurance training-induced enhancement of the expressions of muscle FAO enzyme and PDK4, thereby negating the glycogen sparing effect during exercise.

5 Conclusion

In conclusion, the present investigation demonstrated that dietary fat restriction may attenuate the endurance training-induced enhancement of the expressions of muscle FAO enzyme and PDK4, thereby negating the glycogen sparing effect during exercise.

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