Effects of Different Fatty Acid Chain Lengths on Fatty Acid Oxidation-Related Protein Expression Levels in Rat Skeletal Muscles

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Abstract: Skeletal muscles can adapt to dietary interventions that affect energy metabolism. Dietary intake of medium-chain fatty acids (MCFAs) enhances mitochondrial oxidation of fatty acids (FAO) in type IIa skeletal muscle fibers. However, the effect of MCFAs diet on mitochondrial or cytoplasmic FAO-related protein expression levels in different types of muscle fibers remains unclear. This study aims to examine the effects of a high-fat diet, containing MCFAs, on mitochondrial enzyme activities and heart-type fatty acid-binding protein (H-FABP) levels in different types of skeletal muscle fibers. Five-week-old male Wistar rats were assigned to one of the following three dietary conditions: standard chow (SC, 12% of calories from fat), high-fat MCFAs, or high-fat long-chain fatty acids (LCFAs) diet (60% of calories from fat for both). The animals were provided food and water ad libitum for 4 weeks, following which citrate synthase (CS) activity and H-FABP concentration were analyzed. The epididymal fat pads (EFP) were significantly smaller in the MCFAs group than in the LCFAs group (p < 0.05). MCFAs-fed group displayed an increase in CS activity compared with that observed in SC-fed controls in all types of skeletal muscle fibers (triceps, surface portion of gastrocnemius (gasS), deep portion of gastrocnemius (gasD), and soleus; p < 0.05,). H-FABP concentration was significantly higher in the LCFAs group than in both the SC-fed and MCFAs-fed groups (triceps, gasS, gasD, and soleus; p < 0.05,). However, no significant difference was observed in the H-FABP concentrations between the SC-fed and MCFAs-fed groups. The results of this study showed that the MCFAs diet can increase the expression of the mitochondrial enzyme CS, but not that of H-FABP, in both fast- and slow-twitch muscle fibers, suggesting that H-FABP expression is dependent on the chain length of fatty acids in the cytoplasm of skeletal muscles cells.

Key words: MCFAs diet, H-FABP, mitochondrial enzyme, rat skeletal muscle

1 INTRODUCTION

Lipids and long-chain fatty acids (LCFAs) are energy-rich compounds that play an important role in fatty acid (FA) metabolism. Over the past 20 years, it has been demonstrated that LCFAs act as signaling molecules regulating gene expression and that their target genes encode proteins having a role in FA transport and metabolism1,2. Heart-type fatty acid-binding protein (H-FABP), also known as FABP3, was isolated from a wide range of tissues, including skeletal muscles3. In skeletal muscle cells, H-FABP facilitates the transport of FAs into the mitochondrial β-oxidation system4. The study has demonstrated that polyunsaturated LCFAs that are supplementary to the diet can increase H-FABP protein levels in rat skeletal muscles5. Moreover, exposure to LCFAs can induce the expression of the H-FABP gene in L6 myoblasts6. Physio-

Abbreviations: ANOVA, one-way analysis of variance; CS, citrate synthase; EFP, epididymal fat pad; ELISA, enzyme-linked immunosorbent assay; FA, fatty acids; FAO, fatty acid oxidation; FG, fast-twitch glycolytic; FOG, fast-twitch oxidative glycolytic; gasD, deep portion of gastrocnemius; gasS, surface portion of gastrocnemius; H-FABP, heart-type fatty acid-binding protein; LCFAs, long-chain fatty acid; MCFAs, medium-chain fatty acid; PPAR, peroxisome proliferator-activated receptor; SC, standard chow; SO, slow-twitch oxidative.

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logical experiments showed that an increase in FA uptake and its metabolism results in both elevated H-FABP protein and mRNA levels in skeletal muscles, suggesting that FAs control H-FABP gene expression.

An important adaptation of muscle to endurance exercise is its ability to increase mitochondrial density and oxidative enzyme activity, the latter of which is associated with robust stimulation of mitochondrial biogenesis. Under the conditions of high-fat intake (45%-60% of calories derived from fat), in which LCFAs are available in excess, mitochondrial protein levels and FA oxidation (FAO)-related protein levels increase in both type I and type II skeletal muscle fibers, suggesting that a high-fat diet can enhance the capacity of mitochondrial FAO. In summary, it has been shown that LCFAs can increase the synthesis of FA metabolism-related proteins. However, a diet rich in LCFAs induces widespread insulin resistance in skeletal muscles. Given the close link between lipid accumulation and reduced insulin sensitivity, research on high-fat diet would provide the basis for a primary experimental paradigm to investigate the etiology of insulin resistance in rodents.

Although LCFA-based high-fat diets, which contain saturated FAs, can lead to obesity and insulin resistance, some studies suggested that medium-chain FAs (MCFAs), having a chain of 8–12 carbon atoms, have anti-obesity potential. Compared with LCFAs, MCFAs have several unique nutritional and physiological properties. While LCFAs are first transported into the intestinal lymphatic ducts and then to the systemic circulatory system as chylomicrons, it is well documented that MCFAs are transported by the portal vein system to the liver where they are oxidized. The procedures conformed to the “Fundamental Guidelines for Proper Conduct of Animal Experiment and Related Activities in Academic Research Institutions” (published by the Ministry of Education, Culture, Sports, Science, and Technology, Japan) and were approved by the Ethics Committee for Animal Experimentation of Nara University of Education. Five-week-old male Wistar rats were obtained from Japan SLC (Hamamatsu, Shizuoka, Japan). Animals, with an initial weight of 70–90 g, were ad libitum access to one of the following three diets for 4 weeks: a standard chow diet, MCFA-based high-fat diet, and LCFA-based high-fat diet. Of the total calories obtained from the standard chow diet, 23.5% is derived from proteins, 11.9% is derived from fats, and 64.5% is derived from carbohydrates. Of the total calories obtained from the high-fat diet, 20.0% is derived from proteins (325 g/kg casein), 60.0% is derived from fats (LCFA-fed: 360 g/kg LCFAs oil or MCFA-fed: 324 g/kg MCFAs oil and 36 g/kg LCFAs oil), and 20.0% is derived from carbohydrates (25 g/kg corn starch, 180 g/kg sucrose, and 56 g/kg cellulose). The composition of dietary FAs is presented in Table 1. Both the high-fat diets contained 10 g of a vitamin mix, 35 g of a mineral mix, and 3 g of choline chloride per kg of diet.

2.2 Tissue collection

After 4 weeks of dietary treatment, the rats, fasted overnight, were anesthetized with an intra-peritoneal injection of sodium pentobarbital (50 mg/kg body weight). The deep portion of gastrocnemius (gasD) type Ila fibers (56% fast-twitch glycolytic (FG) fibers, 38% fast-twitch oxidative glycolytic (FOG) fibers, and 4% slow-twitch oxidative (SO) fibers), surface portion of gastrocnemius (gasS) type Ila fibers (58% FG, 37% FOG, and 5% SO fibers), soleus type I fibers (0% FG, 19% FOG, and 80% SO fibers), and triceps type Ila fibers (63% FG, 22% FOG, and 15% SO fibers) were dissected out, clamp-frozen in liquid nitrogen, and stored at −80°C until analysis. The epididymal fat pads (EFP) were collected and weighed.

2.3 Measurements of citrate synthase (CS) activity and H-FABP concentration

Muscle samples were homogenized in a HEPES-EDTA-
sucrose buffer (20 mM HEPES, 1 mM EDTA, and 250 mM sucrose), using a ground-glass homogenizer. These homogenates were frozen and thawed three times and were mixed thoroughly before the measurements of enzymatic activity. For the H-FABP assays, an aliquot of the homogenate was centrifuged at 700 \( \times g \) for 10 min at 4°C. As an index of oxidative enzyme activity, CS activity was measured using Sre re⃞s method\(^\text{21}\)\. H-FABP concentration was measured using an ELISA kit, according to the manufacturer’s instructions (Life Diagnostics, Inc., West Chester, PA, USA).

### 3. Measurements of leptin and adiponectin concentrations

Blood samples were obtained via cardiac puncture. The plasma leptin and adiponectin concentrations were measured using an enzyme-linked immunosorbent assay (ELISA), according to the manufacturer’s instructions (leptin, Quantikine M: R&D system, Minneapolis, MN, USA; adiponectin, Otsuka Pharmaceutical Co., Tokyo, Japan).

### 3.5 Statistical Analysis

The data are expressed as mean \( \pm \) SE. Variables among groups were compared using the one-way analysis of variance (ANOVA). Tukey–Kramer post hoc test was conducted if the ANOVA indicated a significant difference. The level of significance was set at \( p < 0.05 \).

### 3. RESULTS

#### 3.1 Energy intake, body weight, epididymal fat pad (EFP) weight, plasma glucose, leptin, and adiponectin concentrations

Upon completion of the 4-week feeding regimen, body weight was lower in rats fed with MCFA-based high-fat diet (MCFA group) than in rats fed with SC (SC group) and those fed with LCFA-based high-fat diet (LCFA group) (Table 2). As shown in Table 2, although there was no difference in daily energy intake between the SC and MCFA groups, the LCFA group expressed a prominent energy intake. Therefore, the LCFA group showed an increased EFP weight compared with that observed in the SC and MCFA groups (Table 2). The ratio of EFP weight to energy intake did not differ among the three groups (Table 2). Plasma glucose concentrations were significantly higher in the LCFA group than in the SC and MCFA groups (Table 2). Plasma leptin concentrations were significantly higher in

| Table 2 | Body weight, Energy intake, EFP weight, EFP weight/Energy intake, plasma glucose, and adipocytokine concentrations in SC, MCFA and LCFA groups. |
|---------|-----------------------------------------------------------------------------------|
| SC      | MCFA                               | LCFA                               |
| Body weight (g)  | 239 ± 2                           | 216 ± 4*                           | 244 ± 6‡                           |
| Energy intake (kcal/day) | 87 ± 9                           | 77 ± 6                            | 109 ± 8*‡                          |
| EFP weight (g)    | 2.3 ± 0.1                         | 2.1 ± 0.1                         | 2.7 ± 0.2*‡                        |
| EFP weight/Energy intake (g·day/kcal) | 2.6 ± 0.3                         | 2.7 ± 0.2                         | 2.5 ± 0.2                           |
| Plasma glucose (mg/dl) | 202 ± 9                           | 220 ± 25                         | 269 ± 6*‡                          |
| Plasma leptin (ng/dl) | 0.7 ± 0.1                         | 1.4 ± 0.4                         | 2.3 ± 0.4*‡                        |
| Plasma adiponectin (ng/dl) | 3.4 ± 0.1                         | 3.3 ± 0.1                         | 2.0 ± 0.1*‡                        |

Data represent the mean \( \pm \) SE of the values obtained from 5-7 rats. \(^* p < 0.05\) vs. SC group; \(^\# p < 0.05\) vs. MCFA group. EFP, epididymal fat pad; LCFA, long-chain fatty acid; MCFA, medium-chain fatty acid; SC, standard chow.
the LCFA group than in the SC and MCFA groups (Table 2), and plasma adiponectin concentrations were significantly lower in the LCFA group than in the SC and MCFA groups (Table 2).

### 3.2 Mitochondrial enzyme activities

Mitochondria are well known as the major sites of lipid oxidation. Therefore, to examine whether intake of MCFA and LCFA induces a difference in the utilization of fatty acids in skeletal muscles, we measured the activity of the mitochondrial oxidative enzyme CS in the SC, MCFA, and LCFA groups. In soleus and triceps muscles, CS activity was significantly higher in the MCFA and LCFA groups than in the SC group (Fig. 1). In gasS and gasD muscles, CS activity was significantly higher in the MCFA group, but not in the LCFA group, compared to that in the SC group (Fig. 1). In gasS muscle, CS activity of the MCFA group showed a tendency to increase in comparison to that of the LCFA group (p = 0.09).

### 3.3 Fatty acid-binding protein concentrations

H-FABP concentration in the muscles was significantly higher in the LCFA group than in the SC (triceps, gasS, gasD, and soleus) and MCFA (triceps, gasS, gasD, and soleus) groups (Fig. 2). In contrast, there was no significant difference in H-FABP concentrations between the SC and MCFA groups.

### 4 DISCUSSION

To examine the effects of dietary MCFAs on FAO-related protein expression, we measured mitochondrial enzyme activity and H-FABP concentration in rodent skeletal muscles after the 4-week feeding regimen of MCFA-based high-fat diet. The results showed that CS activity increased in both fast- and slow-twitch muscle fibers of MCFA-fed rats, without increased fat accumulation. LCFA-fed rats displayed increased H-FABP concentration in skeletal muscles, while H-FABP concentration did not change in rats fed with the MCFA-based high-fat diet. In view of these results, the intake of MCFAs can increase the expression level of mitochondrial enzyme proteins, but not that of H-FABP, in overall muscle and FAs, depending on their chain length, may induce distinctive FA-binding protein expressions in cytoplasm.

In this study, the MCFA-based diets caused an increase in mitochondrial oxidative enzyme activity in fast- and slow-twitch muscles. An important adaptation of muscle to endurance exercise is its ability to increase mitochondrial density and oxidative enzyme activity (e.g., CS activity) 9, the latter of which is associated with robust stimulation of mitochondrial biogenesis 9. Skeletal muscles that exhibit plasticity can adapt to nutritional interventions. Under the conditions of excess LCFA availability, mitochondrial content and FAO capacity are increased in skeletal muscles 10. Skeletal muscle fibers can be classified into one of the three categories on the basis of their morphological, contractile, and metabolic characteristics. Type I fibers, which are rich in mitochondria and oxidative metabolism enzymes, show low contractile capacity. Type II fibers (including IIa and IIb) are characterized by glycolytic metabolism and fast contraction 22. It has been demonstrated that an LCFA-based high-fat diet feeding for 4 or 5 weeks can increase mitochondrial enzyme activity in type I 10 and type IIb muscle fibers 11. Moreover, MCFA-rich diets also…

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**Fig. 1** Citrate synthase (CS) activity in skeletal muscles of rats fed with the SC (□), MCFA-rich (■), and LCFA-rich (▲) diets. Data represent mean ± SE of the values obtained from 5-7 rats. *p < 0.05 vs. SC group. LCFA, long-chain fatty acid; MCFA, medium-chain fatty acid; SC, standard chow.

**Fig. 2** Heart-type fatty acid-binding protein (H-FABP) concentration in skeletal muscles of rats fed with the SC (□), MCFA-rich (■), and LCFA-rich (▲) diets. Data represent mean ± SE of the values obtained from 5-7 rats. *p < 0.05 vs. SC group; #p < 0.05 vs. MCFA group. LCFA, long-chain fatty acid; MCFA, medium-chain fatty acid; SC, standard chow.
increase mitochondrial protein levels in type IIa fibers of gastrocnemius muscle\textsuperscript{7}. However it remains unresolved whether oxidative enzymes in type I (slow-twitch oxidative) and type IIb (fast-twitch glycolytic) muscle fibers are affected by MCFAs. While another study used an MCFA-diet that was rich in lauric acid (C12) and composed of 50\% LCFA\textsuperscript{17}, we used a diet composed of 90\% MCFAs, in which the percentage of caprylic acid (C8) was 74.3\%. In this study, we measured the activity of the mitochondrial enzyme CS in the soleus type I fibers, gasD type IIa fibers, gasS type IIa fibers, and triceps type IIb fibers after the rats were fed with the MCFA- or LCFA-based diet for 4 weeks. In soleus, gasD, gasS, and triceps, the MCFA-group showed an increase in CS activity compared with that in the SC group. In gasS muscle, moreover, CS activity of the MCFA group expressed a tendency to increase in comparison to that of the LCFA group. This result suggested that the MCFA-based high-fat diet is a more potent inducer of muscle mitochondrial biogenesis than is the LCFA-based high-fat diet. In skeletal muscle, peroxisome proliferator-activated receptor gamma coactivator-1 alpha (PGC-1\textalpha), a master regulator of mitochondrial biogenesis, was increased by LCFA-based high-fat diet feeding\textsuperscript{11}. In addition, plasma FA levels elevated by LCFA-diet feeding were found to induce an increase in muscle mitochondrial biogenesis by activating peroxisome proliferator-activated receptors (PPARs), a family of nuclear transcription factors\textsuperscript{20}. The molecular mechanism underlying MCFA diet-induced up-regulation of muscle mitochondrial biogenesis has not yet been clarified. It seems likely that the MCFA-based diet increases mitochondrial enzyme activity due to activation of PGC-1\textalpha/PPARs and/or an increase in mitochondrial density in skeletal muscles.

To the best of our knowledge, the modulation of H-FABP expression by different lengths of FA chain in skeletal muscles has not been studied previously. We showed that treatment with the LCFA-based high-fat diet could increase H-FABP concentration in both fast- and slow-twitch muscle fibers. Interestingly, H-FABP concentration did not change in the skeletal muscles of rats fed with the MCFA-based high-fat diet. H-FABP might have a buffering action against the damaging accumulation of unbound free FAs in the intracellular environment. It has been demonstrated that changes in circulating hormone levels can affect the FABP concentration of specific animal tissues\textsuperscript{7, 24}. Rats fed with the LCFA-based high-fat diet exhibited elevated levels of H-FABP protein in the liver and adipose tissues\textsuperscript{26}. In addition, exposure to LCFA\textsuperscript{s} was reported to increase expression of the \textit{H-FABP} gene in L6 myoblasts\textsuperscript{6}. Likewise, exposure to LCFA\textsuperscript{s} and 9-cis-retinoic acid can increase the expression of the liver type \textit{FABP} gene in hepatocytes\textsuperscript{20}. Our results showed that the pathway of gene expression induced by MCFAs might be different from that induced by LCFA\textsuperscript{s}. A family of nuclear transcription factors called PPARs was shown to regulate the expression of genes involved in oxidative and FA metabolism upon its activation by FA or other ligands\textsuperscript{11, 12}. LCFA\textsuperscript{s} have a higher binding affinity for PPARs than MCFAs do\textsuperscript{13}. However, it is not clear whether MCFAs specifically affect an oxidative or FA metabolic pathway or influence the activity of other transcription factors. Further studies will be necessary to elucidate the mechanisms underlying H-FABP expression induced by MCFAs and LCFA\textsuperscript{s}.

In agreement with the findings of previous studies\textsuperscript{26, 27}, we found that compared with the LCFA-based high-fat diet, the MCFA-based high-fat diet causes lesser accumulation of visceral fat. The lesser fat accumulation associated with MCFA-based diet is observed due to reduction in daily total energy intake because there is no difference in the ratio of EFP weight to energy intake between LCFA and MCFA groups. Compared with LCFA-based high-fat diet, MCFA-based high-fat diet was also reported to reduce daily food intake\textsuperscript{26}. Obesity is a major public health concern and a major risk factor for insulin resistance. The typical western diet, which is rich in fats and calories is considered the primary cause of both accumulation of visceral fat and development of insulin resistance\textsuperscript{11, 12}. Our above findings suggest that the MCFA-rich diet may prevent LCFA-derived fat accumulation. Moreover, blood glucose levels were lower in rats fed with the MCFA-rich diet than in rats that consumed the LCFA-rich diet. These results suggest that MCFAs accumulate less as body fat compared with LCFA\textsuperscript{s}, which are the main components of general edible oils. In this study, the secretion of adipocytokines could be maintained normally even in rats fed with the MCFA-rich diet. Adipose tissue-derived adipocytokines are well-known as mediators of both metabolic function and metabolic dysfunction\textsuperscript{28, 29}. Adiponectin and leptin are target agents for diabetes research because of their anti-diabetic effects; they are expected to become novel therapeutic tools for treating metabolic disorders\textsuperscript{28, 29}. A decrease in the circulating adiponectin levels was reported to be associated with the development of diabetes and metabolic syndrome\textsuperscript{28}. Leptin also plays a key role in the regulation of energy intake and energy expenditure, and its circulating levels are positively related to adiposity\textsuperscript{29}. Our data show that the plasma adiponectin concentration was significantly higher in the MCFA and SC groups than in the LCFA group. In contrast, the plasma leptin concentration was significantly lower in the MCFA and SC groups than in the LCFA group. Taken together, these results suggest that dietary MCFAs could be instrumental in the prevention of obesity, and, consequently, may attenuate the risk of developing diabetes and metabolic syndrome via alteration in the levels of circulating adipocytokines.
5 CONCLUSIONS

This study shows that the MCFA- and LCFA-based high-fat diets have diverse effects on fat accumulation. We found that the MCFA-based high-fat diet enhances the activity of the mitochondrial oxidative enzyme CS in both fast- and slow-twitch skeletal muscles. This study is the first to demonstrate this effect in skeletal muscles. H-FABP concentration is increased by the intake of LCFA but not MCFA, suggesting that the metabolic fate of FAs is dependent on their chain length in the cytoplasm of skeletal muscle cells.

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