Capric Acid Up-Regulates UCP3 Expression without PDK4 Induction in Mouse C2C12 Myotubes

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Summary Uncoupling protein 3 (UCP3) and pyruvate dehydrogenase kinase 4 (PDK4) in skeletal muscle are key regulators of the glucose and lipid metabolic processes that are involved in insulin resistance. Medium-chain fatty acids (MCFAs) have anti-obesogenic effects in rodents and humans, while long-chain fatty acids (LCFAs) cause increases in body weight and insulin resistance. To clarify the beneficial effects of MCFAs, we examined UCP3 and PDK4 expression in skeletal muscles of mice fed a MCFA- or LCFA-enriched high-fat diet (HFD). Five-week feeding of the LCFA-enriched HFD caused high body weight gain and induced glucose intolerance in mice, compared with those in mice fed the MCFA-enriched HFD. However, the amounts of UCP3 and PDK4 transcripts in the skeletal muscle of mice fed the MCFA- or LCFA-enriched HFD were similar. To further elucidate the specific effects of MCFAs, such as capric acid (C10:0), on lipid metabolism in skeletal muscles, we examined the effects of various FAs on expression of UCP3 and PDK4, in mouse C2C12 myocytes. Although palmitic acid (C16:0) and lauric acid (C12:0) significantly induced expression of both UCP3 and PDK4, capric acid (C10:0) upregulated only UCP3 expression via activation of peroxisome proliferator-activated receptor-δ. Furthermore, palmitic acid (C16:0) disturbed the insulin-induced phosphorylation of Akt, while MCFAs, including lauric (C12:0), capric (C10:0), and caprylic acid (C12:0), did not. These results suggest that capric acid (C10:0) increases the capacity for fatty acid oxidation without inhibiting glycolysis in skeletal muscle.

Key Words medium-chain fatty acids, PDK4, PPARδ, skeletal muscle, UCP3

Diet-induced obesity, which is increasing worldwide, is a major feature of metabolic syndrome and causes the ectopic accumulation of lipid in insulin-sensitive tissues such as skeletal muscle, liver, and adipose tissues (1). Since accumulated intracellular lipids impair the ability to catabolize fatty acids (FAs) and glucose in these insulin-sensitive tissues, the stimulation of FA oxidation in skeletal muscle is important for preventing lipid-induced dysfunctions.

Medium-chain (C8–12) FAs (MCFAs) are FAs that do not make large contributions to the development of obesity (2), because they are absorbed in the small intestine, directly transported to the liver via the portal vein, and immediately consumed by β-oxidation in the liver (3, 4). Therefore, MCFAs increase energy expenditure (2, 5, 6) and diet-induced thermogenesis in the liver (7). Recently, Turner et al. (8) reported that dietary MCFAs directly affected mitochondrial oxidative capacity in skeletal muscle leading to the alteration of peripheral insulin sensitivity, and suggested that MCFAs accumulate in peripheral organs other than the liver and directly regulate their metabolism. However, they mainly examined the effects of dietary lauric acid (C12:0), which possesses similar characteristics to long-chain FAs (LCFAs) (9). There is little evidence that caprylic acid (C8:0) and capric acid (C10:0) directly regulate lipid metabolism in skeletal muscle.
skeletal muscle in vivo.

Uncoupling protein 3 (UCP3) and pyruvate dehydrogenase kinase 4 (PDK4) play important roles respectively in modifying FA metabolism and regulating insulin sensitivity in skeletal muscles (10, 11). UCP3 promotes FA metabolism by increasing the entry of FA into mitochondria (10), while PDK4 is a key enzyme downregulating glycolysis and upregulating lipid oxidation by inhibiting the synthesis of acetyl-CoA from pyruvate (11, 12). Fatty acids regulate expression of UCP3 and PDK4 via activation of peroxisome proliferator-activated receptors (PPARs). However, it remains unclear that MCFAs induce UCP3 and PDK4 expression through PPARs activation in skeletal muscle.

In the present study, we aimed to elucidate the molecular mechanisms for the beneficial effects of MCFAs on lipid and carbohydrate metabolism, especially the expression of UCP3 and PDK4, in the skeletal muscle of mice. We here report that capric acid treatment increased the expression of UCP3, but not PDK4, via PPARδ in C2C12 myotubes. On the basis of our findings, we suggest that MCFAs induce UCP3 and PDK4 expression through PPARs activation in skeletal muscle.

In the present study, we aimed to elucidate the molecular mechanisms for the beneficial effects of MCFAs on lipid and carbohydrate metabolism, especially the expression of UCP3 and PDK4, in the skeletal muscle of mice. We here report that capric acid treatment increased the expression of UCP3, but not PDK4, via PPARδ in C2C12 myotubes. On the basis of our findings, we suggest that capric acid, but not lauric acid, promotes FA oxidation without inhibiting glycolysis in skeletal muscle.

MATERIALS AND METHODS

Animals and diets. C57BL/6j male mice were purchased from Japan SLC, Inc. (Shizuoka, Japan). The mice were housed at 23°C ± 2°C on a 12-h light/dark cycle and allowed free access to laboratory animal diet MF™ (Oriental Yeast Co., Ltd., Tokyo, Japan) and water. Eight-week-old male mice were randomly placed on either a normal diet (ND; 10% of calories from fat, 20% of calories from protein, 70% of calories from carbohydrates; Oriental Yeast) or a HFD enriched with LCFAs (60% of the calories as lard) or with MCFAs without lauric acid [50% of calories from medium-chain triacylglycerol (C8:0 : C10:0 = 3 : 1) and 10% of calories from lard] for 5 wk. The FA composition of these diets is shown in Table 1. After the experimental periods, gastrocnemius muscles were isolated and kept at −80°C until analysis.

Table 1. Fatty acid composition of the diets.

<table>
<thead>
<tr>
<th>Fatty acids</th>
<th>Content (g/100 g diet)</th>
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<tr>
<td></td>
<td>ND</td>
</tr>
<tr>
<td>C8:0</td>
<td>0</td>
</tr>
<tr>
<td>C10:0</td>
<td>0</td>
</tr>
<tr>
<td>C12:0</td>
<td>0.01</td>
</tr>
<tr>
<td>C14:0</td>
<td>0.07</td>
</tr>
<tr>
<td>C16:0</td>
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</tr>
<tr>
<td>C18:0</td>
<td>0.57</td>
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<tr>
<td>C18:1</td>
<td>1.76</td>
</tr>
<tr>
<td>C18:2</td>
<td>0.39</td>
</tr>
<tr>
<td>C18:3</td>
<td>0.02</td>
</tr>
</tbody>
</table>

HFD, high-fat diet; LCFA, long-chain fatty acid; MCFA, medium-chain fatty acid; ND, normal diet.

Table 2. Primer sets used in this study.

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Sequence</th>
<th>Length (bp)</th>
</tr>
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<tbody>
<tr>
<td>UCP3</td>
<td>S 5'-GGAGTGTCACCTGTTACTGACAATC-3'</td>
<td>69</td>
</tr>
<tr>
<td></td>
<td>AS 5'-GACAGAAGCAGGCTTCAAA-3'</td>
<td></td>
</tr>
<tr>
<td>PDK4</td>
<td>S 5'-AAAGGACAGGATGGAAGGATCA-3'</td>
<td>82</td>
</tr>
<tr>
<td></td>
<td>AS 5'-TTCCTCCTGGGTTTGCACAT-3'</td>
<td></td>
</tr>
<tr>
<td>PGC-1α</td>
<td>S 5'-GAGGAAAGGAGACTAAACGGCCA-3'</td>
<td>154</td>
</tr>
<tr>
<td></td>
<td>AS 5'-GCCGATCACAGGAGGCACTTCTT-3'</td>
<td></td>
</tr>
<tr>
<td>PPARδ</td>
<td>S 5'-CCTACAGGTTACCCACTCCAGGAGT-3'</td>
<td>69</td>
</tr>
<tr>
<td></td>
<td>AS 5'-GCCAAATGATTGTGCGCAA-3'</td>
<td></td>
</tr>
<tr>
<td>PPARα</td>
<td>S 5'-GGGGGTCAGGAGGCTTCC-3'</td>
<td>101</td>
</tr>
<tr>
<td></td>
<td>AS 5'-CCTGATGATGAGGCTTCC-3'</td>
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<tr>
<td>GAPDH</td>
<td>S 5'-CCGTGTCTTCTACCCCAATGT-3'</td>
<td>74</td>
</tr>
<tr>
<td></td>
<td>AS 5'-AGTGATCATCATACTTGCGAGGTTTCT-3'</td>
<td></td>
</tr>
</tbody>
</table>

AS, antisense primer; S, sense primer; UCP, uncoupling protein; PDK, pyruvate dehydrogenase kinase; PGC, peroxisome proliferator-activated receptor-gamma co-activator; PPAR, peroxisome proliferator-activated receptor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
Quantitative reverse transcription-polymerase chain reaction (qRT-PCR). qRT-PCR with SYBR Green dye was performed using an ABI 7300 real-time PCR system (Applied Biosystems, Foster City, CA) as described previously (15). The oligonucleotide primers used for PCR are shown in Table 2.

Immunoblotting. Immunoblot analysis was performed as described previously (16). The following antibodies were used: anti-Akt (PharMingen International, Tokyo, Japan) and anti-phospho-S473-Akt antibodies (Cell Signaling Technology, Beverly, MA).

Statistical analysis. All data were statistically evaluated by ANOVA using SPSS version 6.1 (rSPSS Japan, Tokyo, Japan) and are expressed as means ± standard deviation (SD; n = 3–4). Differences between two groups were assessed with Scheffe’s multiple comparison test. Differences were considered significant at p < 0.05.

RESULTS
Effects of a MCF A-enriched HFD on body-weight gain, tissue wet weights and glucose tolerance
We first examined the effects of capric and caprylic acid-enriched HFD on body-weight gain, tissue wet weights and glucose tolerance in mice. Mice fed the LCF A-enriched HFD for 5 wk experienced a body weight gain significantly higher than that of mice fed the ND for the same period (Fig. 1A). In contrast, the weight of mice fed the MCF A-enriched HFD for 5 wk were similar to those of mice fed the ND (Fig. 1A). Although there was no difference in food intake among these diet groups (Fig. 1B), the wet weight of epididymal adipose tissue from mice fed the LCF A-enriched HFD for 5 wk was significantly greater than that of mice fed the MCFA-enriched HFD or ND for the same period (Fig. 1A). In contrast, the weight of mice fed the MCFA-enriched HFD for 5 wk were similar to those of mice fed the ND (Fig. 1A). Although there was no difference in food intake among these diet groups (Fig. 1B), the wet weight of epididymal adipose tissue from mice fed the LCFA-enriched HFD for 5 wk was significantly greater than that of mice fed the MCFA-enriched HFD or ND (Fig. 1C). These HFDs did not affect the wet weight of gastrocnemius muscle, compared with ND (Fig. 1C). Furthermore, we observed significantly impaired glucose tolerance in mice fed the LCFA-enriched HFD for 5 wk (Fig. 1D). Interestingly, the MCFA-enriched HFD improved this glucose intolerance in mice.

Effects of a MCF A-enriched diet on the expression of FA metabolism-associated genes in skeletal muscle
To elucidate the mechanism whereby the MCF A-enriched HFD did not cause obesity or glucose intoler-
ance, we examined the effects of the MCF A-enriched HFD on the expression of FA metabolism-associated genes in skeletal muscles, since skeletal muscle is a major organ for FA oxidation (17). Expression of UCP3 and PDK4 significantly increased in the skeletal muscle of mice fed the LCFA- or MCF A-enriched HFDs, compared with those of mice fed the ND (Fig. 2A). Expression of PDK4 in the skeletal muscle of mice fed the MCF A-enriched HFD was lower than that of mice fed the LCFA-enriched HFD, but this difference was not statistically significant. Since expression of UCP3 and PDK4 is well known to be regulated by PPARα and δ (18), we also examined the
expression of PPARs and PPARγ co-activator-1 (PGC-1α) in the skeletal muscles of mice fed the ND, the LCFA-enriched HFD, or the MCFAs-enriched HFD for 5 wk. There was no significant difference in the expression of PGC-1α, PPARα or δ among these diet groups (Fig. 2B).

Stimulatory effect of capric acid treatment on UCP3 expression, but not on PDK4 induction in C2C12 myotubes

The MCFAs-enriched HFD used in our in vivo experiments contained 10% lard (estimated by calories). We failed to examine effects of diets containing only MCFAs as fat, because these diets induced severe fatty liver in mice (8). To confirm the specific effects of MCFAs, such as capric (C10:0) and caprylic (C8:0) acids, on lipid metabolism in skeletal muscles, we further examined expression of lipid metabolism-associated genes, using C2C12 myocytes treated with various FAs. Since it has been reported that oleic acid (C18:1) up-regulates the expression of UCP3 in C2C12 myotubes (19), we compared the effect of capric or caprylic acid with that of oleic acid. Consistent with the previous report (19), oleic acid induced significant UCP3 expression in C2C12 myotubes (Fig. 3A). Capric and lauric (C12:0) acids significantly increased the amount of UCP3 mRNA, whereas both relative expression levels were lower than that of oleic acid. The former reached the peak value 18 h after the treatment, while the latter induced UCP3 expression in a time-dependent manner. Palmitic (C16:0) and caprylic acids did not change the amount of UCP3 mRNA. Examining a dose-dependent effect of capric acid on UCP3 expression revealed that a concentration of capric acid of over 400 μmol/L was necessary for UCP3 induction (Fig. 3B). Interestingly, caprylic acid hardly induced PDK4 expression in C2C12 myotubes, whereas oleic acid, palmitic acid and lauric acid significantly upregulated the expression of PDK4 (Fig. 3C). Caprylic acid did not affect the expression of PDK4 or UCP3.

Inhibitory effect of PPARδ antagonist on capric acid-induced UCP3 expression

Consistent with our in vivo data (Fig. 2B), no FAs tested in this study changed the expression of PGC-1α, PPARα or δ in C2C12 myotubes (data not shown). These
findings led us to consider that the transcriptional activities of PPARs were more important than their expression, to elucidate the mechanism of MCF A-mediated UCP3 expression. Therefore, we examined the effects of PPARα and δ antagonists on FA-mediated UCP3 and PDK4 expression in C2C12 myotubes. A PPARα antagonist, GW6471, did not change any expression of UCP3 or PDK4, which LCFAs or MCFAs induced in C2C12 myotubes (Fig. 4A). Interestingly, a PPARδ antagonist, GSK0660, significantly suppressed LCFA or MCFA-induced UCP3 and PDK4 expression (Fig. 4B). These findings suggest that capric acid as well as oleic acid stimulates UCP3 expression through PPAR activation in C2C12 myotubes.

**Physiological meaning of capric acid-mediated UCP3 expression without PDK4 induction**

To elucidate the physiological roles of capric acid on metabolism in skeletal muscle, we investigated its effect on insulin signaling in C2C12 myotubes. Our Western blotting for phosphorylated and total Akt showed that palmitic acid significantly disturbed insulin-induced Akt phosphorylation in C2C12 myotubes (Fig. 5). This result was supported by Coll’s report that palmitic acid disturbed the insulin signaling in skeletal muscle cells (20). However, other FAs, including oleic acid, caprylic acid, capric acid, and lauric acid, did not affect the phosphorylation of Akt, indicating that MCFAs are lipids hardly affecting the insulin signaling among saturated FAs.

**DISCUSSION**

MCFAs have been reported to be beneficial dietary oils for human health, compared with LCFAs. Many investigations have indicated that MCFAs stimulate energy expenditure and FA oxidation, and cause the loss of body fat, compared with LCFAs (21, 22). Rapid catabolism of MCFAs in the liver accounts for the decreased accumulation of lipids in peripheral tissues. However, Turner et al. (8) recently reported that besides the liver, skeletal muscle also contributed to such beneficial effects of MCFAs. They showed that a lauric acid-enriched HFD induced the expression of UCP3, a potent enhancer of FA oxidation (10, 22), and did not disturb insulin signaling in skeletal muscles (8). Interestingly, we found that a MCFA-enriched HFD increased UCP3 expression in the skeletal muscle of mice without an increase in body weight. The present study also demonstrated that compared with capric acid and lauric acid, caprylic acid failed to induce UCP3 expression. Based on these findings, we suggest that capric acid exerts more beneficial actions on obesity in the body, compared with other MCFAs.

In general, dietary MCFAs are rapidly transported to the liver after absorption in the small intestine and are converted to ketone bodies (23). However, several lines of investigation reported that MCFA-rich diets increased MCFA concentrations in the blood as well as several target organs including the skeletal muscle of rodents (8, 24, 25). The present study also showed that capric acid directly regulated UCP3 expression in skeletal muscle cells by an in vitro approach. Taken together, it seems to be reasonable that MCFAs are metabolized in skeletal muscle as well as in liver in vivo.

We found that the MCFA- or LCFA-enriched HFD highly induced the expression of UCP3 and PDK4 in our in vivo study. However, PDK4 expression was lower in the skeletal muscle of mice fed the MCFA-enriched HFD than in that of mice fed the LCFA-enriched HFD, although it was not significant. We had to add 10% lard, which mainly contains LCFAs, to the MCFA-enriched diet, because the HFD containing only MCFAs induced...
severe fatty liver in mice (8). Because MCFAs, including lauric acid, capric acid, and caprylic acid, hardly affected PDK4 expression, compared with palmitic acid and oleic acid, in vitro, we suggest that the stimulatory effect of the MCFA-enriched HFD on PDK4 expression was possibly due to the contained LCFA.

PDK4 regulates glucose homeostasis and fuel selection in various tissues through the phosphorylation of pyruvate dehydrogenase complex (PDC) and balances glycolysis and lipid oxidation by inhibiting the synthesis of acetyl-CoA from pyruvate (11, 12, 26, 27). Furthermore, fasting blood glucose levels increased gradually in both PDK4 knockout and wild-type mice fed a high saturated fat diet for 8 mo, but remained significantly lower in the PDK4 knockout mice (28). These findings suggest that suppression of PDK4 expression or activity is one of the most important therapeutic targets to prevent fat-induced insulin resistance. The present study demonstrated that capric acid highly induced fat-induced insulin resistance. The present study demonstrated that capric acid highly induced fat-induced insulin resistance. The present study demonstrated that capric acid highly induced fat-induced insulin resistance. The present study demonstrated that capric acid highly induced fat-induced insulin resistance.

This finding suggests that PPARδ is an important regulator for FA-mediated UCP3 or PDK4 expression in skeletal muscle cells. However, these results also raise another question. What factors caused the distinct expression of UCP3 and PDK4 between C2C12 myotubes treated with oleic acid and capric acid? Why did capric acid induce only UCP3 expression in C2C12 myotubes, although the expression of UCP3 and PDK4 was mediated by PPARδ?

The beneficial effects of MCFAs, especially capric acid, on insulin signaling in vitro may support the anti-diabetic effects of MCFA-enriched diets in vivo. In fact, we also observed glucose intolerance in mice fed the MCFA-enriched HFD, compared with the LCFA-enriched HFD (Fig. 1D). Since Turner et al. (8) suggested that MCFAs accumulate in skeletal muscle in addition to the liver, the beneficial effects of MCFAs on UCP3 and PDK4 expression in skeletal muscle cells may contribute to their improvement of impaired glucose intolerance in vivo, at least in part. However, Montgomery et al. (29) observed an accumulation of ceramide, which inhibits insulin signaling, in the skeletal muscle of mice fed MCFA-rich diets. Furthermore, liver and adipose tissues as well as skeletal muscle are insulin-sensitive tissues. Adiposity increases secretion of insulin resistance-associated adipokines, such as leptin and resistin, and decreases secretion of adiponectin from hypertrophied adipocytes (30). We also found that the size of adipocytes was smaller in the epididymal adipose tissue of mice fed the MCFA-enriched HFD than that of mice fed the LCFA-enriched HFD (data not shown). Based on these findings, we cannot neglect the possibility that MCFAs improved glucose intolerance in vivo via their effects on liver and adipose tissues. Further examination is necessary to elucidate this hypothesis.

Akt phosphorylation by various stimulators, such as insulin and stretching, causes the translocation of glucose transporter 4 from intracellular storage to plasma membranes, resulting in glucose uptake into cells (31, 32). Therefore, we examined the effects of MCFAs on insulin-induced phosphorylation of Akt in C2C12 myotubes (Fig. 5). Palmitic acid treatment decreased the basal and insulin-stimulated phosphorylation of Akt in C2C12 myotubes. In contrast, MCFAs did not affect Akt phosphorylation after insulin treatment, indicating that MCFAs do not disturb insulin signaling in skeletal muscle. Consistent with a previous report (19), oleic acid did not impair insulin signaling in C2C12 myotubes, although it is a LCFA.

In general, expression of UCP3 and PDK4 is regulated by PPARα and δ (17). In the present study, a PPARδ-specific inhibitor, but not a PPARα-specific inhibitor, significantly suppressed the LCFA-induced expression of UCP3 and PDK4, and the MCFA-induced expression of UCP3. This finding suggests that PPARδ is an important regulator for FA-mediated UCP3 or PDK4 expression in skeletal muscle cells. However, these results also raise another question. What factors caused the distinct expression of UCP3 and PDK4 between C2C12 myotubes treated with oleic acid and capric acid? Why did capric acid induce only UCP3 expression in C2C12 myotubes, although the expression of UCP3 and PDK4 was mediated by PPARδ?

Other cofactors, such as PGC-1α, may be involved in this discrepancy. Several PPARδ agonists are available as insulin sensitizers, although detailed mechanisms of the actions remain unclear (33, 34). MCFAs could become more beneficial drugs as insulin sensitizers than PPARδ agonists, if the mechanism for this discrepancy could be elucidated, because PPARδ agonists induce PDK4, which downregulates glycolysis (12). Further examination is necessary to clarify this phenomenon.

Conflicts of interest

No potential conflicts of interest relevant to this article were reported.

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