Effect of Randomly Interesterified Triacylglycerols Containing Medium- and Long-Chain Fatty Acids on Energy Expenditure and Hepatic Fatty Acid Metabolism in Rats

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In our previous studies, medium- and long-chain triacylglycerols (MLCT), randomly interesterified triacylglycerols containing medium-chain and long-chain fatty acids in the same glycerol molecule, significantly reduced body fat accumulation in humans and rats. To clarify mechanism(s) for this effect of MLCT, we measured energy expenditure and hepatic fatty acid metabolism in rats by comparison with long-chain triacylglycerols (LCT) or medium-chain triacylglycerols (MCT). MLCT, compared with LCT, showed significantly lower body fat accumulation, higher 24-h energy expenditure and acyl-CoA dehydrogenase activity measured using octanoyl-CoA as a substrate, and similar lipogenic activity. MCT, compared with LCT, showed significantly higher energy expenditure, but fat accumulation was comparable. Additionally, MCT exhibited significantly higher lipogenic activity than the other oils. These data suggest that enhancement of energy expenditure and medium-chain fatty acids (MCFA) oxidation without activating de novo lipogenesis are responsible at least for the lower body fat accumulation in rats fed MLCT. The activation of hepatic lipogenesis by excessive intake of MCFA might counteract their preventive effects on body fat accumulation.

Key words: medium-chain fatty acid; body fat accumulation; energy expenditure; fatty acid oxidation; lipogenesis

Medium-chain fatty acids (MCFA, composed of chains of 8 to 10 carbon atoms) are found in edible oils such as coconut oil. Compared with long-chain fatty acids (LCFA), MCFA have several unique physiological and biological characteristics, as reported elsewhere over the past half-century.1–3 Medium-chain triacylglycerols (MCT), composed exclusively of MCFA, are metabolized differently from long-chain triacylglycerols (LCT), composed exclusively of LCFA. MCFA are absorbed via the portal system and are transported to the liver directly, whereas LCT are absorbed via the intestinal lymphatic ducts and transported as chylomicrons through the thoracic duct to reach systemic circulation. MCFA and hence MCT are easily oxidized because their intramitochondrial transport does not require carnitine palmitoyltransferase (CPT), a rate-limiting enzyme of mitochondrial β-oxidation.4 MCT are absorbed and metabolized as rapidly as glucose and have more than twice the caloric density of protein and carbohydrate. They are utilized to prevent obesity and several lifestyle-related diseases.4 However, it is difficult to substitute MCT for LCT in the diet for long-term therapy, largely because their lower smoke point and higher tendency to bubble make MCT difficult to use as a cooking oil.

Recently, to overcome this disadvantage, we developed a new cooking oil containing triacylglycerols composed of medium- and long-chain fatty acids (MLCT), which are randomly interesterified triacylglycerols containing MCFA and LCFA in the same glycerol molecule.5 MLCT contain about 13% of MCFA, in contrast to MCT, which contain MCFA exclusively. MLCT can replace LCT in the daily diet, and in human and rat studies this replacement has been shown to cause significant lower body fat accumulation.6–10 Previous studies have suggested possible mechanisms for the effect of MLCT on body fat accumulation, including enhancement of diet-induced energy expenditure in humans6 and activation of hepatic fatty acid oxidation in rats.10,11 However, although MLCT contain only one-eighth the amount of MCFA found in MCT, the reasons MLCT cause significant lower body fat accumulation than LCT are still unclear. Furthermore, the relationship...
between changes in energy expenditure and fatty acid metabolism with respect to the effect of MLCT on body fat accumulation is unknown.

In the present study, we examined the relevance of energy expenditure and fatty acid metabolism to known mechanism(s) responsible for body fat reduction by MLCT. We measured 24-h energy expenditure in Sprague-Dawley rats after 4 weeks of feeding diets containing MLCT, LCT, or MCT, and then measured several parameters of fatty acid metabolism in the liver.

Materials and Methods

Materials. MCT (consisting of octanoate and decanolate), MLCT (consisting of MCFA and LCFA), and LCT (rapeseed oil) were from Nisshin OilliO (Tokyo). Their fatty acid compositions as measured by gas chromatography are shown in Table 1. Acetyl-CoA, malonyl-CoA, octanoyl-CoA, and palmitoyl-CoA were purchased from Sigma-Aldrich Japan (Tokyo).

Animals and diets. All rats were treated in accordance with the guidelines established by the Japanese Society of Nutrition and Food Science (Law No. 105 and Notification No. 6 of the Japanese Government). Male Sprague-Dawley rats (6 weeks old) were obtained from Japan SLC (Hamamatsu, Shizuoka, Japan). The rats were housed individually in a room with controlled temperature (20° to 24°C), humidity (40% to 60%), and lighting (lights on from 0800 to 2000), and given a commercial standard diet (PicoLab Rodent Diet 20 5053, PMI Feeds, St. Louis, MO) and tap water ad libitum for 3 d. Fifteen rats were randomly divided into three groups. Each group of rat was allowed free access to an experimental diet and water for 31 d. All diets were formulated according to the AIN-93G diet,12 which was modified to contain sucrose at 40 g/kg of diet and pregelatinized instead of dextrinized cornstarch. The composition of the experimental diets is shown in Table 2. At the end of the 4-week feeding period, the rats were measured for 24-h energy expenditure. After 3 d, the rats were weighed and killed by decapitation under anesthesia with diethyl ether without fasting. Blood was collected in centrifuge tubes (VenojectII; Terumo, Tokyo). Serum was separated by centrifugation at 600 × g for 15 min and stored at −70°C until analyzed. The liver was removed, weighed, and used for RNA analysis. The remaining portion of the liver was frozen using liquid N2 and stored at −70°C until assayed. Carcass fat weight was measured using Soxhlet extraction apparatus, and expressed as a percentage of body weight. Carcass protein was determined by the Kjeldahl method.

Energy expenditure. Rats were placed in a plastic chamber and allowed test-diet and water ad libitum. Oxygen consumption and carbon dioxide production were measured for 26 h on a metabolism measuring system for small animals (MK-5000R; Muromachi Kikai, Tokyo). Oxygen consumption and carbon dioxide production were measured every 3 min, but the data from 0 to 2 h after placing the animals in a chamber were excluded to avoid variation due to restlessness in the new environment. Total energy expenditure was calculated according to the method reported previously.13

Preparation of liver fraction and enzyme activity measurements. Fractions measuring fatty acid metabolism-related enzyme activities in rat liver were prepared as described previously,13 except that about 6 g of each liver was homogenized. Carnitine palmitoyltransferase (CPT), acyl-CoA dehydrogenase (ACAD), acyl-CoA oxidase (ACO), fatty acid synthase (FAS), ATP-citrate lyase (ACL), and glucose-6-phosphate dehydrogenase (G6PDH) were measured with a spectrophotometer.14–19 ACAD activity was measured using octanoyl-CoA or palmitoyl-CoA as a substrate. Activities were expressed as nanomoles per min per milligram protein. Protein concentration was determined with a BCA protein assay kit (Pierce, France) using bovine serum albumin as a standard.
RNA extraction and RT-PCR analysis. Total RNA was isolated from the liver using an RNeasy Mini Kit (Qiagen, Tokyo) according to the manufacturer’s instructions. To remove genomic DNA, DNase I digestion was performed with an RNase-Free DNase Set (Qiagen, Tokyo). Reverse transcriptional reaction was carried out using a 1st Strand cDNA Synthesis Kit for RT-PCR (AMV) (Roche Diagnostics, Tokyo). Real-time quantitative PCR was performed using a fluorescence temperature cycler (LightCycler; Roche Diagnostics). To confirm the exactitude of RNA extraction and RT-PCR, all samples were subjected to PCR amplification using a LightCycler-FastStart DNA Master SYBR Green I kit (Roche Diagnostics). The primers used in this study were designed on-line with Primer 3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3 www.cgi) and purchased from Sigma Genosys Japan (Hokkaido, Japan). The primer sequences used were as follows: long-chain acyl-CoA dehydrogenase (LCAD) (sense, 5'-CGCCCGATGTTCTCATTCT-3'; antisense, 5'-GGCTTTTCCTCCATTCCTGGT-3'), medium-chain acyl-CoA dehydrogenase (MCAD) (sense, 5'-CGCCCCAGACTACGATAAAA-3'; antisense, 5'-CAAGACCAACCACAACCTCCTCC-3'), malic enzyme (ME) (sense, 5'-CGGCACAGAAAATGAGGAGTT-3'; antisense, 5'-CCTCTTGTGCGCTTTCCGAT-3'), peroxisome proliferator activated receptor-alpha (PPARα) (sense, 5'-TGAAAAACAGGAGGCAGG-3'; antisense, 5'-AAGGAGGACACGATCGTGAA-3'), sterol regulatory element binding protein-1 (SREBP-1) (sense, 5'-CAAAAAACGCTCTCCAGAGA-3'; antisense, 5'-AGTCCCATCCACAGAAGAA-3'), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (sense, 5'-GTCGGTGTTGGAAGCCCTCCTAG-3'; antisense, 5'-GTGTTGGAAACGCAGGTCTAGA-3'). These were expressed as arbitrary units. The data were normalized to the expression levels of the constitutive housekeeping gene. These were calculated using an internal standard curve based on a 1st Strand cDNA Synthesis Kit for RT-PCR. PCR-grade water instead of first strand DNA was used as a negative control. Melting curve analysis was used to determine the specific PCR products. We used an internal standard curve based on serial dilutions of first-strand DNA from the liver of rats fed a commercial standard diet. The level of transcripts for the constitutive housekeeping gene product GAPDH was measured in each sample, and the data were normalized to the expression levels of this housekeeping gene. These were expressed as arbitrary units.

Analytical measurement. Serum triacylglycerol, cholesterol, and non-esterified free fatty acid were measured with a 7170 automated system (Hitachi, Tokyo) by an enzymatic method. Serum acetocetic and 3-hydroxybutyric acids were measured with a JCA-BM12 automated system (JEOL, Tokyo). Liver lipids were extracted with an ice-cold mixture of chloroform and methanol (2:1, v/v), and triacylglycerol was analyzed using Triglyceride E-test Wako (Wako Pure Chemicals, Osaka, Japan).

Statistical analysis. Results are expressed as means ± SEM. The significance of the changes was evaluated by analysis of variance, followed by Tukey’s HSD test for multiple comparisons. Significant differences between groups were identified using SPSS for Windows (version 10.0J; SPSS Japan, Tokyo). Results were considered significant at P < 0.05.

Results

After 4 weeks of consumption of the test diets, no significant differences were observed among the groups in final body weight, body weight gain, or food intake, and hence, food efficiency (Table 3). Daily average intake of MCFA was calculated to be 4.1 g/d/kg body weight in the MCT group and 0.7 g/d/kg body weight in the MLCT group. As shown in Table 4, epidydimal and mesenteric adipose tissue weights were significantly lower in the MLCT group than in the LCT group. On the other hand, although there was a tendency for adipose tissue weights to be lower in the MCT group than in the LCT group, the difference was not significant. No changes were observed in liver weight or carcass protein contents among the groups. The hepatic triacylglycerol contents of MCT-fed rats were significantly higher than those of LCT-fed rats. The hepatic triacylglycerol contents of MLCT-fed rats showed a tendency to be higher than those of LCT-fed rats, but the difference was not significant.

As shown in Table 5, MCT-fed rats had significantly lower serum triacylglycerol concentrations than did LCT-fed rats. The triacylglycerol concentrations of MLCT-fed rats showed a tendency to be lower than those of LCT-fed rats, but the difference was not significant. Even though MLCT contain MCFA, total ketone body concentrations in MLCT-fed rats were significantly lower than those in MCT-fed rats.

To evaluate energy expenditure in rats fed the experimental diets, we measured energy expenditure over 24 h in individual animals after 4 weeks on each

<table>
<thead>
<tr>
<th>Dietary group</th>
<th>LCT</th>
<th>MCT</th>
<th>MLCT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial body weight (g)</td>
<td>233 ± 3</td>
<td>230 ± 3</td>
<td>230 ± 3</td>
</tr>
<tr>
<td>Final body weight (g)</td>
<td>412 ± 9</td>
<td>403 ± 12</td>
<td>401 ± 9</td>
</tr>
<tr>
<td>Body weight gain (g)</td>
<td>180 ± 6</td>
<td>173 ± 12</td>
<td>172 ± 10</td>
</tr>
<tr>
<td>Food intake (g)</td>
<td>718 ± 12</td>
<td>725 ± 38</td>
<td>696 ± 17</td>
</tr>
<tr>
<td>Food efficiency (%)</td>
<td>25.0 ± 0.7</td>
<td>23.8 ± 0.7</td>
<td>24.6 ± 0.9</td>
</tr>
</tbody>
</table>

1 All values are expressed as means ± SEM.
2 Body weight gain (g)/food intake (g) × 100 (%).
diet, and calculated the area under the curve of energy expenditure. As shown in Fig. 1, both MLCT- and MCT-fed rats showed significantly higher expenditure values than LCT-fed rats.

We used spectrophotometric methods to evaluate enzyme activity related to hepatic fatty acid metabolism. With regard to β-oxidation activity, the activity of ACAD measured using octanoyl-CoA as a substrate was
significantly higher in the MLCT group than in the LCT group. In contrast, the activity was comparable when palmitoyl-CoA was used as a substrate (Table 6). No differences were observed in the activities of other enzymes in the β-oxidation pathway. On the other hand, the activities of the lipogenic enzymes measured were all markedly higher in the MCT group than in the others.

To examine the underlying mechanism(s) responsible for changes in fatty acid metabolism after feeding MLCT, we measured mRNA expression levels by real-time PCR (LightCycler). The data for each sample were normalized to the expression level of the housekeeping gene GAPDH and expressed as arbitrary units. The expression of this housekeeping gene showed no significant differences in the respective parameters among the groups (data not shown). As shown in Fig. 2, among fatty acid oxidation genes, the mRNA level of LCAD was significantly higher in the MCT and MLCT groups than in the LCT group. No differences were observed in the mRNA levels of MCAD or PPARα among the groups. The mRNA levels for the hepatic de novo lipogenic enzymes ACC and ME showed no differences between the MLCT and LCT groups. On the other hand, in the MCT group the hepatic de novo lipogenic enzymes measured were significantly up-regulated compared with the other groups. SREBP-1, the nuclear transcription factor for lipogenesis, was also up-regulated.

Discussion

The purpose of this study was to examine the relevance of energy expenditure and hepatic fatty acid metabolism to the body fat-lowering effect of MLCT. We observed a significantly lower body fat accumulation in MLCT than in LCT, while MCT showed an intermediate effect. Also, we observed a significant enhancement of energy expenditure in MLCT and MCT over that in LCT. With respect to fatty acid metabolism, MLCT but not MCT significantly activated ACAD as measured with an octanoyl-CoA substrate compared with LCT. In contrast, the activities of all the lipogenic enzymes measured were significantly enhanced in MCT compared with the other oils. Although no enhanced activities of fatty acid oxidation-related enzymes were observed in MCT-fed rats compared with LCT-fed rats, a significant elevation of serum ketone bodies was seen (Tables 5 and 6). These results suggest an enhancement in MCFA oxidation in the livers of MCT-fed rats. On the other hand, ACAD activity measured with an octanoyl-CoA substrate was significantly enhanced in MLCT-fed rats.

Table 6. Activities of Hepatic Key Enzymes in Fatty Acid Metabolic Pathways in Rats Fed LCT, MCT, or MLCT for 4 Weeks

<table>
<thead>
<tr>
<th>Enzymes</th>
<th>Dietary group</th>
<th>LCT</th>
<th>MCT</th>
<th>MLCT</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(nmol/min/mg protein)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ACAD</td>
<td></td>
<td>14.3 ± 1.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>19.7 ± 1.4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>21.6 ± 1.7&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>C8:0</td>
<td></td>
<td>15.8 ± 0.8</td>
<td>15.1 ± 0.7</td>
<td>14.6 ± 0.7</td>
</tr>
<tr>
<td>C16:0</td>
<td></td>
<td>0.49 ± 0.11</td>
<td>0.32 ± 0.05</td>
<td>0.46 ± 0.11</td>
</tr>
<tr>
<td>ACO</td>
<td></td>
<td>0.90 ± 0.09</td>
<td>0.63 ± 0.04</td>
<td>0.74 ± 0.08</td>
</tr>
<tr>
<td>FAS</td>
<td></td>
<td>6.72 ± 0.87&lt;sup&gt;b&lt;/sup&gt;</td>
<td>7.94 ± 0.64&lt;sup&gt;b&lt;/sup&gt;</td>
<td>7.41 ± 0.23&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>ACL</td>
<td></td>
<td>14.1 ± 1.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>23.2 ± 0.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>16.7 ± 1.1&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>G6PDH</td>
<td></td>
<td>26.0 ± 3.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>48.2 ± 2.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>26.3 ± 2.8&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> All values are expressed as means ± SEM. Data were analyzed by ANOVA, followed by Tukey’s HSD test for multiple comparisons. Values with different superscript letters indicate significant differences, P < 0.05.

<sup>b</sup> ACAD activity was measured using octanoyl-CoA (C8:0) or palmitoyl-CoA (C16:0) as a substrate.
rats compared with LCT-fed rats (Table 6). The results thus suggest that MCFA are subjected to fatty acid oxidation more than LCFA in the livers of MLCT-fed rats. The mRNA expression levels of MCAD and PPARα showed no differences among the groups (Fig. 2). LCFA, especially polyunsaturated fatty acids, are known to serve as ligands for PPARα, whereas MCFA are poor activators and PPARα is known to regulate the mRNA expression level of MCAD. 21) These observations suggest that after intake of MCT or MLCT, the unresponsiveness of the mRNA expression level of MCAD might be the result of unresponsiveness of the PPARα gene. LCAD is the first-step enzyme in mitochondrial β-oxidation of C6–C18 fatty acids, and its mRNA expression level is known to be under the regulation of PPARα. 21) In the present study, the mRNA expression level of LCAD was significantly higher both in MLCT- and MCT-fed rats than in LCT-fed rats, although ACAD activity measured using palmitoyl-CoA as a substrate showed no difference among the groups (Fig. 2, Table 6). The reason for this discrepancy is presently unclear.

MLCT is not a physically mixed oil made of MCFA and LCFA but rather randomly interesterified triacylglycerols in the same glycerol molecule. We cannot completely rule out the possibility that the body fat-lowering effect of MLCT reflects their structural characteristics. Some studies have indicated that intestinal absorption after intake of the test oil varies depending on whether the oil was structured. 23,24) However, in our previous study, a physical mixture of LCT and MCT, which contained the same amount of MCFA found in MLCT, showed a significantly lower body fat accumulation than LCT, and that the adipose tissue weight of rats fed MLCT and the physical mixture of LCT and MCT were not significantly different. 8) We suppose that the effect of MLCT on body fat accumulation might not be determined by the structure of MCFA and LCFA within triacylglycerol but rather by the amount of MCFA ingested.

Many studies have reported effects of MCT on body fat accumulation, 25–28) suggesting that daily intake of MCT can reduce body fat accumulation and that it might be effective for preventing obesity and lifestyle-related diseases. However, in the present study, although MCT-fed rats exhibited a tendency for adipose tissue weight to be lower than that of LCT-fed rats, the difference was not significant (Table 3). The body fat accumulation of MLCT-fed rats was less than that of LCT-fed rats, even though the amount of MCFA consumed in the MLCT-fed rats was less than that in the MCT-fed rats (Table 3). The experimental diets used in previous studies were generally composed of high MCT (45 to 60% of total energy) and low carbohydrate (10 to 40% of total energy). On the other hand, the diet used in the present study was based on AIN-93G, which is composed of low MCT (11% of total energy) and high carbohydrate (64% of total energy) in the MCT group. The vitamins and minerals were also different from our AIN-93G standard diets. Dietary composition has been shown to influence body weight, body fat, and whole-body energetics. 29) It is thus likely that one cause of the discrepancies is the difference in the compositions of the experimental diets.

Metabolic processes such as lipogenesis, gluconeogenesis, and ketogenesis are known to influence the calculation of rates of carbohydrate and fat oxidation from measurements of oxygen consumption and carbon dioxide production. 30) Hill et al. 31) observed in humans an increase in energy expenditure and fasting total serum triglycerides and a decrease in total lipid oxidation on day 6 as compared with day 1 of an MCT overfeeding week. They reported that the measured energy expenditure on day 1 might be due to increased formation and oxidation of ketone bodies, and that the much larger energy expenditure on day 6 than on day 1 most likely reflects increased de novo fatty acid synthesis. Hence they concluded that enhanced energy expenditure after intake of MCT was probably due to enhanced lipogenesis in the liver. Accordingly, the enhancement of energy expenditure observed in MCT-fed rats in the present study might be due to enhanced de novo lipogenesis and ketogenesis in the liver. On the other hand, unlike MCT, MLCT showed significant enhancement of energy expenditure without enhancing de novo lipogenesis and ketogenesis, suggesting the possibility that enhancement of fatty acid oxidation in other tissues besides the liver might well be involved. Furthermore, no significant difference in body fat accumulation was observed in MCT-fed rats, in spite of apparent enhancement of energy expenditure as compared with LCT-fed rats (Table 3, Fig. 1). St-Onge et al. 32) reported that long-term consumption of MCT significantly enhanced energy expenditure and fat oxidation as compared with LCT, but resulted in only a small, insignificant difference in body composition in overweight women. The body fat-lowering effect of MCFA is thus difficult to explain by an enhancement of energy expenditure alone.

Daval et al. 33) in a study using chickens, clarified the contribution of enhanced de novo lipogenesis to body fat accumulation. Hwang et al. 34) reported that fat deposition after MCT feeding is accomplished not by incorporation of dietary fatty acids but by de novo fatty acid synthesis. Other reports have indicated the ability of MCT to enhance de novo lipogenesis in the liver. 35–38) It is thought that the magnitude of the enhancement in lipogenesis by MCFA might be one reason for the inconsistency in demonstrating a body fat-lowering effect of MCT. Its effect appears to depend on experimental conditions and the characteristics of subjects. In the present study, liver triacylglycerol contents were significantly higher in MCT-fed rats than in rats fed other oils (Table 4). Excess acetyl-CoA produced by β-oxidation of MCFA was used in the synthesis of LCFA without being oxidized in liver mitochondria. 39) MCT-fed rats showed significantly higher hepatic de


**References**


23) Tso, P., Lee, T., and Demichele, S. J., Lymphatic


