Note

Effects of Dietary Medium-Chain Triacylglycerol on mRNA Level of Gluconeogenic Enzymes in Malnourished Rats

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Summary We have reported previously that dietary medium-chain triacylglycerol (MCT) improved serum albumin concentration and protein balance in malnourished rats. To clarify the mechanisms for this effect of MCT, hepatic messenger RNA levels of gluconeogenic enzymes, pyruvate dehydrogenase (PDH) and alanine aminotransferase (ALT) were measured in rats fed low-protein diets containing either MCT or isocaloric long-chain triacylglycerol (LCT) for 2 wk. The serum albumin concentration in rats fed the MCT diet was significantly higher compared with those fed the LCT diet. Serum free fatty acids and ketone body fraction were higher in rats fed MCT compared with those fed the LCT diet. The hepatic mRNA level of PDH was significantly lower in rats fed MCT than those fed LCT. But, there was no significant difference between the two groups in mRNA of gluconeogenic enzymes or ALT. These results suggest that ketone bodies, which are an alternative energy source and might spare blood glucose, increase by MCT feeding, and the reason for the PEM (protein-energy malnutrition)-improving effect of MCT is not caused by suppression of gluconeogenesis.

Key Words rat, medium-chain triacylglycerol, gluconeogenesis, protein-energy malnutrition

Protein-energy malnutrition (PEM) is the general term for the disease resulting from a lack of protein and energy. Many elderly people, especially those residing at nursing homes or hospitals, tend to suffer from PEM (1). PEM is regarded as questionable by raising the other disease merger rates and the mortality rates after its morbidity (2, 3). Earlier, we produced an animal model having similar symptoms as clinical PEM, such as hypoalbuminemia and growth suppression, by feeding a low-protein diet to rats for 4 wk (4). It was shown that in this PEM rat model, hypoalbuminemia improved and protein balance became high by feeding medium-chain triacylglycerol (MCT) in substitution for long-chain triacylglycerol (LCT) as the dietary oil. It is a factor of PEM that the consumption of meals relatively decreases with elderly people. When caloric or carbohydrate intake runs short and blood sugar level decreases, muscle protein is disassembled into amino acids (5). Among these amino acids, glucogenic amino acids are carried to the liver and gluconeogenesis is promoted to compensate for the lack of blood sugar (6). Ketone bodies are synthesized in the liver at the same time from

free fatty acids (7). The ketone bodies are also utilized as alternative energy sources of the blood glucoses by the peripheral (8). We thought that the improvement of the protein balance with MCT intake occurred through suppression of the gluconeogenesis which mainly started from the body protein degradation. Therefore, we investigated the expression of some genes of gluconeogenic enzymes in MCT-fed rats.

Materials and Methods

Materials. Rapeseed oil, a long-chain triacylglycerol, was used as the control oil. MCT was purchased from Nisshin Oillio, Ltd. (Tokyo, Japan) and used as the test oil. Rapeseed oil contained 4.6% palmitic acid, 2.1% stearic acid, 58.7% oleic acid, 20.7% linoleic acid, 9.9% linolenic acid and 4.0% other fatty acids. MCT contained 74.4% caprylic acid and 25.6% capric acid.

Animals and diets. All animals were treated in accordance with the guidelines for the care and use of laboratory animals (Notification of the Prime Minister's Office in Japan). The experimental plan was approved by the Laboratory Animal Care Committee of the Research Laboratory, Nisshin-Oillio Group. Specific pathogen-free male Wistar rats (Japan SLC, Inc., Hamamatsu, Japan) were individually housed in stainless-steel wire cages and allowed free access to sterilized water. The temperature of the animal room was $22 \pm 2^\circ\text{C}$, with a humidity of $50 \pm 10\%$, and illumination from 08:00 to 20:00 h. The animals were also allowed free access to a standard

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Abbreviations: ALT, alanine aminotransferase; G6P, glucose-6-phosphatase; LCT, long-chain triacylglycerol; MCT, medium-chain triacylglycerol; PC, pyruvate carboxylase; PCR, polymerase chain reaction; PDH, pyruvate dehydrogenase; PEM, protein-energy malnutrition; PEPCK, phosphoenolpyruvate carboxykinase.

Table 1. Composition of the experimental diets.

Ingredients	LCT diet	MCT diet
	(g/kg)	
Beta-corn starch	547.5	543.5
Lactic casein	50.0	50.0
Alfa-corn starch	132.0	132.0
Sucrose	100.0	100.0
Rapeseed oil ¹	70.0	10.0
MCT ¹	0.0	64.0
Cellulose fiber	50.0	50.0
Mineral mix (AIN-93G)	35.0	35.0
Vitamine mix (AIN-93)	10.0	10.0
L-Cystine	3.0	3.0
Choline bitertrate	2.5	2.5

LCT, long-chain triacylglycerol; MCT, medium-chain triacylglycerol.

¹ Contains 0.02% (w/w) *tert*-butylhydroquinone.

commercial diet (Labo MR Stock, Nosan Corporation, Yokohama, Japan) before the experiment. Blood of 3-wk-old rats were drawn from the tail and these rats were separated into 2 groups of 7 animals each, having a similar mean body weight and mean serum albumin concentration. They were allowed free access to the semi-purified experimental diet (Table 1) for 2 wk. At day 7, blood of rats was drawn from the tail vein. At the end of the feeding period, the rats were killed by bleeding. All these blood drawings were performed from 13:00 h after 4 h of food deprivation. Serum was separated by centrifugation at 600 × *g* for 15 min and stored at −20°C until analysis. The liver was removed and was frozen using liquid N₂ and stored at −70°C until RNA assay.

Analysis of the serum parameters. Serum concentration of total protein was measured by Biuret's method and albumin was measured by the BCG method (A/G B-Test Wako; Wako Pure Chemical Industries, Ltd., Osaka, Japan). Transferrin was estimated by the TIA method (SRL, Tokyo, Japan). Ketone bodies and free fatty acids were estimated enzymatically (SRL).

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). Reverse transcriptional reaction was carried out using a dNTP mix, oligo dT (12–18) primer, and SuperScript III (Invitrogen, Tokyo) according to the manufacturer's instructions. Real-time quantitative PCR was performed with a fluorescence temperature cyler (LightCycler; Roche Diagnostics, Tokyo). To confirm the precision of RNA extraction and RT-PCR, all samples were subjected to PCR amplification with a QuantiTect SYBR Green PCR Kit (Qiagen). The primer sequences used were as follows: alanine aminotransferase (ALT) (forward, 5'-CAGCCTGGGGTCTTACAGTG-3'; reverse, 5'-G CTCGCTCCAGTAGTCAGGT-3'), pyruvate dehydrogenase (PDH) (forward, 5'-ATAAACCCCA-

Table 2. Serum proteins in rats fed experimental diets.¹

		LCT diet	MCT diet
Albumin (g/dL)	day 0	3.27±0.07	3.25±0.10
	day 7	2.73±0.06	2.87±0.05
	day 14	2.54±0.04	2.77±0.05**
Total protein (g/dL)	day 0	4.89±0.14	4.76±0.15
	day 7	3.94±0.05	3.95±0.07
	day 14	3.47±0.04	3.72±0.04**
Transferrin (mg/dL)		119±1	126±2*

LCT, long-chain triacylglycerol; MCT, medium-chain triacylglycerol.

¹ Values are means±SE. Significantly different from LCT group: **p*<0.05, ***p*<0.01.

CGGACCATCT-3'; reverse, 5'-TAGCACAGCCTCTCTTCGT-3'), glucose-6-phosphatase (G6P) (forward, 5'-CAGCCTCCAGAGGTACTGTCTC-3'; reverse, 5'-ACCATCTCTTGGCCTTCTCC-3'), phosphoenolpyruvate carboxykinase (PEPCK) (forward, 5'-CATTCCTGGATGAAGTTTG-3'; reverse, 5'-ATGGCGTTCCGATTTGTCT-3'), pyruvate carboxylase (PC) (forward, 5'-TGCTAATGGACACAACCTTCC-3'; reverse, 5'-TCCTCCCCAGTTCTCTATGCT-3'), beta actin (forward, 5'-CGTTGACATCCGTAAAGACC-3'; reverse, 5'-TGTGTGGATTGGTGGCTCTA-3'). PCR-grade water instead of first-strand DNA was used as a negative control. A melting curve analysis was generated at the end of the cycling program and was used to determine the specific PCR products. We used an internal standard curve based on a serial dilution of each PCR product for each target gene. The data was normalized to the expression level of the beta-actin gene. The results are expressed in arbitrary units.

Statistical analysis. All results were expressed as the mean value±standard error (SE). Statistical significance of the difference among values was conducted by two-tailed unpaired *t*-test. The program used was Microsoft Excel software (version 2000 for Windows, Microsoft Corp., USA).

Results

Growth, body composition and body fat content

There were no differences in food intake or energy intake between the LCT (9.75±0.02 g/d and 38.5±0.1 kcal/d) and MCT groups (9.77±0.02 g/d and 38.4±0.1 kcal/d).

The body weight gain of the MCT group (1.43±0.04 g/d) was significantly lower than that of the LCT group (1.65±0.05 g/d).

There were no differences in epididymal or perirenal fat weight between the LCT (0.715±0.057 g and 0.693±0.063 g) and MCT groups (0.646±0.034 g and 0.546±0.030 g). But, mesenteric and total abdominal fat weights were lower in the MCT group (0.99±0.03 g and 2.18±0.09 g) than in the LCT group (1.22±0.07 g and 2.63±0.18 g).

Serum analysis

There were no significant differences in serum total protein or albumin concentration between the LCT and

Table 3. Serum free fatty acids and ketone body fraction in rats fed experimental diets.¹

	LCT diet	MCT diet
Free fatty acids ($\mu\text{Eq/L}$)	601 $\pm$ 23	721 $\pm$ 26**
Acetoacetic acid ($\mu\text{mol/L}$)	80.3 $\pm$ 15.2	141.4 $\pm$ 26.7
β -hydroxybutyric acid ($\mu\text{mol/L}$)	959 $\pm$ 140	1,528 $\pm$ 203*
Total ketone bodies ($\mu\text{mol/L}$)	1,039 $\pm$ 155	1,670 $\pm$ 228*

LCT, long-chain triacylglycerol; MCT, medium-chain triacylglycerol.

¹ Values are means $\pm$ SE. Significantly different from LCT group: * p <0.05, ** p <0.01.

Fig. 1. Messenger RNA levels in rats fed experimental diets. Rats were fed the LCT diet (white boxes) or MCT diet (black boxes) for 2 wk. Messenger RNA levels of alanine aminotransferase (ALT), pyruvate dehydrogenase (PDH), glucose-6-phosphatase (G6P), phosphoenolpyruvate carboxykinase (PEPCK), and pyruvate carboxylase (PC) were measured by real-time PCR. Values were normalized to the levels of the β -actin gene and expressed as arbitrary units. All values are expressed as means $\pm$ SE. Significantly different from LCT group: * p <0.05.

MCT groups on day 0 or 7 (Table 2). However, they were significantly higher in the MCT group than in the LCT group on day 14. Serum transferrin concentration was also higher in the MCT group on day 14.

Serum free fatty acids and ketone body fraction were 1.2 to 1.8 times higher in the MCT group (Table 3).

Messenger RNA expression levels

No difference was observed in the expression of the beta-actin gene (data not shown). The mRNA levels were normalized to the level of beta-actin and expressed as arbitrary units. The level of PDH mRNA was significantly lower in the MCT group than in the LCT group (Fig. 1). On the other hand, there were no differences in the levels of ALT, G6P, PEPCK or PC mRNA between the two groups.

Discussion

In this study, we assumed that the improvement of the protein balance by MCT ingestion occurred by suppression of several enzymes which regulate gluconeogenesis, and examined their gene expression. We have reported previously that a 4-wk dietary intake of MCT

improved serum albumin concentration (4). This improvement had already begun in week 2. Thus, we examined gene expression at 2 wk in this study. In this study, blood drawings were performed after 4 h of food deprivation. Influence of the dietary lipids would not be completely eliminated under the conditions. But, mobilization of free fatty acid from the adipose tissue might increase and the difference in free fatty acid derived from the difference in test oils becomes relatively small after further fasting. Therefore, we set the fasting the conditions mentioned above. Consequently, expression of the PDH which converted pyruvic acid into acetyl CoA was suppressed. However, no change was observed in the expression of ALT, PC, PEPCK or G6P. When intake of protein decreases and runs short, the living body increases the blood amino acid pool through muscle protein breakdown, and tends to maintain its turnover. Muscle protein is also disassembled into amino acids when the blood glucose level decreases because of energy deficiency (5). Among these amino acids, gluconeogenic amino acids such as alanine are carried to the liver, and gluconeogenesis increases. Then, the generated glucose is discharged to the blood and maintains blood glucoses (6). Furthermore, ketone bodies are synthesized in the liver from free fatty acids mobilized from the periphery (7). These ketone bodies are discharged into blood and are also utilized in the periphery as alternative energy sources of blood glucoses (8).

Shinohara et al. showed that serum ketone body fractions in rats fed a diet containing 5% MCT for 4 wk was higher than that of an LCT-fed group (9). Tsuji et al. fed healthy volunteers 10 g/d of MCT as an ingredient of their breakfast for 12 wk (10). As a result, after the eighth week, the serum total ketone bodies were significantly higher than in the LCT-fed group. Claeysens et al. reported that plasma ketone body was higher in rats fed a low-protein diet than in control rats (11). So, serum ketone body might be higher in rats of the LCT group compared to rats fed the normal diet. In this study, serum acetoacetic acid, β -hydroxybutyrate and total ketone body in the MCT group were even higher than in the LCT group. Utilization of the ketone body in the periphery is promoted so that the serum ketone body level is high (12). Therefore, the increase in the ketone body utilization of the periphery in the MCT group is also speculated in this study. Increasing utilization of the ketone body as an alternative to glucose might spare the blood glucose.

Hepatic gluconeogenesis starts with mobilization of alanine from muscle and is converted into pyruvate by ALT (13). Subsequently, pyruvate is converted into glucose by rate-limiting enzymes of gluconeogenesis such as PC, PEPCK and G6P. At the time of starvation, these gluconeogenic enzyme activities go up, and gluconeogenesis increases (14). In previous studies, a low-protein diet increased PEPCK activity (15) in rat liver. Meanwhile, PDH was not affected by dietary protein content (16). So, gluconeogenesis might be stimulated but the TCA cycle might remain unchanged in the liver of rats of the LCT group compared to rats fed the normal

diet.

In this study, there was no difference in the mRNA expression of ALT or gluconeogenic enzyme between the 2 groups. Thus, it was supposed that the possibility that dietary MCT restrains alanine utilization by suppressing gluconeogenesis and improves protein balance is low. On the other hand, the RNA expression of PDH was significantly suppressed by MCT intakes. While pyruvate is a starter substance of gluconeogenesis, it is also a starter substance of the TCA cycle. There is a possibility that even if gluconeogenesis does not change, utilization of pyruvate would be suppressed by inhibition of PDH. This suppression of PDH may be the feedback inhibition by acetyl-CoA. The ingested MCT is digested and absorbed immediately, and it is more than 90% metabolized after arriving at the liver (17). A large quantity of acetyl CoA is generated by this metabolizing process. It is thought that this acetyl CoA suppresses PDH.

Serum ketone bodies increased with MCT ingestion in this study. Many studies have reported similar observations (18). However, Noguchi et al. reported that serum ketone bodies of the MCT-fed rats were significantly low adversely for the LCT-fed rats (19). They considered that longer fasting of 18 h might be a cause of the opposite result. Another reason for the low serum ketone body in their study might be because ketone body usage at the peripherals increases during longer feeding periods and it exceeds the ketogenesis of the liver with long-term MCT intake, but this remains unknown. Further examination is expected by measurement of the ketone body metabolizing enzyme activity in the periphery.

In conclusion, the ketone bodies, which are alternative energy source, increase when fed MCT. It is assumed that this increasing ketone body spares blood glucose. In addition, key enzymes of gluconeogenesis were not altered by MCT feeding, suggesting that the reason for the PEM-improving effect of MCT is not suppression of gluconeogenesis.

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