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Increased Norepinephrine by Medium-Chain Triglyceride Attributable to Lipolysis in White and Brown Adipose Tissue of C57BL/6J Mice

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A further investigation of the lipolysis induced by medium-chain triglyceride (MCT) was conducted on C57BL/6J mice fed with a diet containing 2% MCT or 2% long-chain triglyceride (LCT). Blood norepinephrine, body fat and blood lipid variables, and the protein or mRNA expression of the genes relevant to lipolysis were measured and analyzed in the white and brown adipose tissue (WAT, BAT). Decreased body fat and improved blood lipid profiles attributable to MCT were confirmed. A higher level of blood norepinephrine was observed with the MCT diet. The adipose triglyceride lipase (ATGL) activity and its mRNA expression, the expression of protein and mRNA of the beta 3 adrenergic receptor (β3-AR) in both WAT and BAT, and the hormone-sensitive lipase (HSL) activity and its mRNA expression in BAT were significantly increased in the mice with MCT feeding. The lipolysis induced by MCT might be partially mediated by increasing norepinephrine, thereafter signaling the up-regulation of β3-AR, ATGL, and HSL in WAT and BAT.

Key words: medium-chain fatty acids; norepinephrine; beta 3 adrenergic receptor; hormone-sensitive lipase; adipose triglyceride lipase

A poor dietary composition, particularly involving a high intake of dietary fats, is considered to be an important causative factor of obesity, so control of both the amount and type of dietary fat may help to prevent overweight and obesity.[1)] Dietary fats are mainly long-chain triglycerides (LCT) with long-chain fatty acids (LCFA). However, medium-chain triglycerides (MCT) are composed of medium-chain fatty acids (MCFA) with 8 and/or 10 carbon atoms. MCT was introduced into clinical nutrition in the 1950s for the dietary treatment of malabsorption syndromes because of its rapid absorption without bile and pancreatic juice.[2)] Many investigations in the past few years have found that the intake of MCFA or MCT produced less body weight gain and body fat mass in both animals and humans.[3–6)] The exact mechanism, for this however has not been fully identified. Some studies have reported that the fatty acid metabolism altered by dietary MCT in the liver and white adipose tissue (WAT) could be responsible for the reduced adiposity.[7,8)] Our previous study has shown that lipolysis due to the increased level and activity of hormone-sensitive lipase (HSL), which was induced by the activation of cyclic adenosine monophosphate (cAMP)-dependent protein kinase A (PKA) in WAT, was partially attributed to less fat accumulation in C57BL/6J mice fed with MCT.[9)] HSL was considered to be the major enzyme responsible for the hydrolysis of stored triglycerides in adipose tissue. This notion was challenged by the finding that triglycerides in the adipose tissue of HSL-deficient mice were still hydrolyzed and the mice were leaner than normal mice.[10)] Zimmermann *et al.* have reported that adipose triglyceride lipase (ATGL) catalyzed the initial step in the triglyceride hydrolysis.[11)] A cascade of biochemical reactions is involved during the hydrolysis of stored triglyceride and the oxidation of fatty acids. Active sympathetic hormones such as norepinephrine and epinephrine stimulate both the alpha- and beta-adrenergic systems which induce the stimulation of adenylate cyclase and protein kinase. Thereafter, increased ATGL and HSL promote triglyceride hydrolysis inside adipose tissue. The beta 3 adrenergic receptor (β3-AR) has been shown to be the most important for lipid mobilization in adipose tissue as only this receptor type stimulated lipolysis.[12)] We therefore hypothesized that MCFA would possess the potential to produce more norepinephrine sympathetic nerve hormone to induce triglyceride hydrolysis in adipose tissues. We tried to verify this hypothesis in the present study with C57BL/6J mice fed an MCT or LCT diet.

Materials and Methods

Animals. 80 male C57BL/6J mice (4–5 weeks old) were purchased from the Institute of Laboratory Animal Science, Chinese Academy of Medical Sciences (SCXK license no. JING2009-0007). The mice were kept in a room at $22 \pm 1\,^{\circ}C$ and 40% to 60% humidity with a 12-h light/dark cycle (light on from 8:00–20:00). The experiments were

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Abbreviations: ATGL, adipose triglyceride lipase; β3-AR, beta 3 adrenergic receptor; BAT, brown adipose tissue; cAMP, cyclic adenosine monophosphate; FFA, free fatty acids; HDL-C, high-density lipoprotein cholesterol; HSL, hormone-sensitive lipase; LCFA, long-chain fatty acids; LCT, long-chain triglyceride; LDL-C, low-density lipoprotein cholesterol; MAGL, monoacylglycerol lipase; MCFA, medium-chain fatty acids; MCT, medium-chain triglyceride; PKA, protein kinase A; T3, thyroid hormone (3,5,3′-triiodothyronine); TC, total cholesterol; TG, triacylglycerol (triglyceride); WAT, white adipose tissue

Table 1. Composition of Diets for C57BL/6J Mice

Ingredient	MCT diet	LCT diet	Control diet
Basal diet (%)	54	54	100
MCT (%)	2		
LCT(%)		2	
Casein (%)	6	6	
Soybean oil (%)	18	18	
Yolk powder (%)	10	10	
Lard (%)	10	10	
Energy (KJ/g)	18.98	18.98	14.22
Percentage of nutrients			
Protein (%)	21.54	21.54	21.50
Fat (%)	21.48	21.48	4.1
Carbohydrate (%)	43.62	43.62	54.33
Mineral mixture (%)	0.78	0.78	1.97
Vitamin mixture (%)	0.52	0.52	0.8
Fiber (%)	1.5	1.5	2.3
Water (%)	9.5	9.5	9.6
Others (%)	1.06	1.06	5.4

conducted in accordance with the guidelines for the Animal Care and Use Committee of the Chinese PLA General Hospital.

Diets. A standard commercial diet based on the AIN-96G diet[13] was purchased from the Academy of Military Medical Sciences for use as the basal diet. The mice had free access to the basal diet and water *ad libitum* for 1 week to stabilize their metabolic condition. 15 mice were randomly selected and fed the basal diet as a non-obesity control. The other mice were fed the high-fat diet based on AIN-96G. After 4 weeks, only those whose body weight gain was over 20% more than that of the non-obesity control mice were chosen for the experiment. The obese mice were randomly assigned to two groups according to their fasting body weight and were fed two different experimental diets containing 2% MCT or 2% LCT (Table 1) for 12 weeks. MCT (consisting of octanoate and decanolate) and LCT (canola oil) were presented by Nisshin OilliO (Tokyo, Japan). Their fatty acid compositions measured by gas chromatography are shown in Table 2. The body weight and food intake were monitored twice a week.

Blood, white and brown adipose tissue sampling. After 12 weeks of feeding, the mice were deprived of the diet, but not water, for fasting overnight (at least 12 h). In the morning after fasting, the mice were anesthetized by an intramuscular injection of xylazine hydrochloride at a dose of 1 mg/kg, and blood samples were collected from the aorta ventralis. Several technicians worked together to complete the blood and tissue sampling as rapidly as possible. The blood samples were centrifuged at 2,000 *g* for 10 min, and sera were collected and stored at −80 °C until needed for analysis. The mesenteric, epididymal and perirenal fat pads, and interscapular BAT were excised, rinsed with iced-cooled saline and weighed. A part of the epididymal fat pad and interscapular BAT samples was immediately frozen in liquid nitrogen and stored at −80 °C.

Measurement of the blood biochemical variables. The serum levels of total cholesterol (TC) and triglyceride (TG) were determined by enzymatic colorimetric methods with commercial kits (nos. 290-63701 and 294-65801, Wako, Japan), and high-density lipoprotein cholesterol (HDL-C) and low-density lipoprotein cholesterol (LDL-C) were determined by sediment methods with commercial kits (no. ab65390, Abcam, USA). The concentrations of free fatty acids (FFA), glycerol, norepinephrine, and thyroid hormone (3,5,3′-triiodothyronine, T3) in the serum were measured by a mouse enzyme-linked immunosorbent assay (ELISA) kit (Bluegene, China) according to the manufacturer's instructions.

ELISA analysis. A part of the epididymal adipose tissue or BAT sample was homogenized in 10 volumes of a 0.9% NaCl solution. The homogenate was centrifuged at 1,200 *g* for 15 min at 4 °C to create a floating fat layer as the sample for measuring. The levels of HSL, cAMP and PKA in BAT, and the level of ATGL in WAT and BAT were determined with their corresponding ELISA kits (HSL kit from GBD, USA, the other kits from BlueGene, China). The levels of these parameters were related to the total protein concentration in the tissue, which was measured with a BCA protein assay kit (no. 23225, Pierce, USA), using bovine serum albumin as a standard.

Table 2. Fatty Acid Composition of LCT and MCT (g/100 g of total fatty acids)

Fatty acid	LCT	MCT
C8:0	ND[a]	75.4
C10:0	ND[a]	24.6
C16:0	6.2	
C16:1	0.2	
C18:0	2.5	
C18:1	48.8	
C18:2	30.2	
C18:3	9.4	
C20:0	0.6	
C20:1	1.1	
C22:0	0.4	
C22:1	0.2	
C24:0	0.2	
C24:1	0.2	
Total	100	

[a]Not detectable.

Real-time RT-PCR analysis. The mRNA expression of HSL in BAT, and of ATGL and β3-AR in WAT and BAT was measured by quantitative real-time RT-PCR analysis. Total RNA was isolated from the epididymal white adipose tissue and BAT by using the Trizol reagent (Invitrogen, USA). cDNAs were synthesized from 3 μg of RNA by using M-MLV reverse transcriptase (Invitrogen, USA). After the cDNA synthesis, quantitative real-time PCR was performed in 25 μL of Bioeasy SYBR Green PCR Master Mix (Sun Biomedical Technology Co., China), using a fluorometric thermal cycler (Line-Gene fluorometric PCR detection system, BoRi Technology, China). Each reaction mixture was incubated for initial denaturation at 95 °C for 2 min, followed by 45 cycles of 95 °C for 20 s, 59 °C for 25 s and 72 °C for 30 s. Primers were designed by using a type of Primer Express 3.0 software and based on the mRNA sequences from a database. The sequences of the primers used were as follow: HSL NM_001039507: forward (5′-3′), CAGAAGGCACTAGGCGTGATG; reverse (5′-3′), GGGCTTGCGTCCACTTAGTTC. ATGL NM_001163689: forward (5′-3′), GGTGCCAACATTATTGAGGT; reverse (5′-3′), GTCACTCTCGCCTGAGAATTG. β3-AR NM_013462: forward (5′-3′), CCTTCAACCCGGTCATCTACTG; reverse (5′-3′), CGCACCTTCATAGCCATCAAA. β-Actin NM_007393: forward (5′-3′), GAGACCTTCAACACCCCAGC; reverse (5′-3′), ATGTCACGCACGATTTCCC) was used as a control to normalize the gene expression. The ΔCt method was used to assess the relative quantification. The ΔCt value for each sample was determined by calculating the difference between the Ct value of the target gene and the Ct value of the β-actin reference gene. The normalized target gene expression level in the sample was calculated by using the formula $2^{-\Delta\Delta Ct}(2^{\Delta Ct(actin)-\Delta Ct(target\ gene)})$.

Western blot analysis. A 10 mg amount of frozen WAT or BAT tissue was added to 200 μL of a protein lysate solution, and homogenized with a glass grinder on ice. The homogenate was centrifuged at 16,000 *g* for 15 min at 4 °C to create a supernatant for subsequent measurement. Protein of the supernatant was measured by using the BCA kit, before the electrophoretic sample of 50 μg–100 μg of total protein was mixed with a 5-fold loading buffer; the mixture was boiled for 5 min and then immediately cooled in an ice box. The sample was size-fractionated on 10% SDS–PAGE gel and then electrophoretically transferred to a nitrocellulose membrane, using 0.48 mol/L Tris base, 0.39 mol/L glycine, and 20% methanol (pH 8.0) as the transfer buffer. The membrane was then incubated overnight at 4 °C with the Tris buffer and a 0.5% blocking solution containing the primary anti-β3-AR antibody (Sigma, USA). The diluted HRP membrane labeled with the closure of the secondary antibody (Santa

Cruz, USA) at a dilution ratio of 1:3000, and the secondary antibody-diluted membrane were incubated for 2–3 h. They were next processed further by using a chemiluminescence Western blotting kit (Sun Biomedical Technology Co., Beijing, China) according to the manufacturer's protocol. The signal intensity was measured by densitometry, using LabWorks Image software (Sun Biomedical Technology Co., Beijing, China).

Statistical analysis. All data are expressed as the mean ± SD, and the *t*-test was used between the MCT and LCT groups. An analysis of covariance (ANCOVA) was performed to compare differences in the body weight and diet consumption by using SPSS software version 17.0. $p < 0.05$ was set to be statistically significant.

Results

Body weight, diet consumption and adipose tissue pad weight

At the end of the first 4 weeks, the average body weight in the non-obese control group (n = 15) was 21.28 ± 0.96 g. There were 30 mice fed on the high-fat diet, whose body weight of 24.39 ± 0.46 g was over 20% higher than that of the mice in the non-obese control group. This weight increase indicated that these animals were an appropriate model for high-fat diet-induced obesity.

No significant differences in the daily average diet and energy intake, nor in the food efficiency ratio between the MCT and LCT group were apparent (Table 3). The body weights of the MCT group were significantly lower than those of the LCT group from the 8th week onwards. The weights of the mesenteric, epididymal, perirenal fat pad, and total WAT (the sum of the mesenteric, epididymal and perirenal fat pad weights) were much lower in the MCT group than in the LCT group (Table 4).

Serum biochemical variables

Compared with the LCT group, the MCT group had significantly lower levels of blood triglyceride, total cholesterol, LDL-C and FFA, and higher levels of HDL-C, HDL-C/LDL-C and norepinephrine. However, no significant differences were found in the levels of blood glycerol and T3 between the MCT and LCT groups (Table 5).

Levels of cAMP, PKA, HSL, ATGL, mRNA and protein expression of β3-AR in WAT and BAT

The level of ATGL in WAT and the levels of cAMP, PKA, HSL and ATGL in BAT were significantly higher in the MCT group than in the LCT group (Table 6). The mRNA expression of HSL in BAT and of ATGL and β3-AR in both WAT and BAT in the MCT group was significantly higher than that in the LCT group (Fig. 1). The Western blot analysis also showed that the protein expression of β3-AR was 53.6% higher in WAT and 30.9% higher in BAT for the MCT group than the figures for the LCT group (Fig. 2).

Table 3. Consumption of the Diet by C57BL/6J Mice Fed with MCT and LCT for 12 Weeks

Average diet intake	MCT (n = 15)	LCT (n = 15)
Week 1 (g/d)	4.13 ± 0.51	4.19 ± 0.64
Week 4 (g/d)	4.98 ± 0.56	5.01 ± 0.46
Week 8 (g/d)	4.36 ± 0.41	4.65 ± 0.57
Week 12 (g/d)	4.67 ± 0.52	4.88 ± 0.61
Total 12 weeks (g/d)	4.54 ± 0.52	4.68 ± 0.57
Total 12 weeks (KJ/d)	86.4 ± 10.2	89.7 ± 8.28
Food efficiency ratio[a]	1440 ± 88.2	1281 ± 93.1

[a]Energy intake (KJ/d)/body weight gain (g/d)

Table 4. Changes in Body Weight, and in White and Brown Fat Mass at the End of 12 Weeks of Study in C57BL/6J Mice Fed with the MCT or LCT Diet

	MCT (n = 15)	LCT (n = 15)
BW at week 0 (g)	24.4 ± 0.5	24.4 ± 0.4
BW at week 4 (g)	27.7 ± 0.8	28.4 ± 1.0
BW at week 8 (g)	29.0 ± 0.8#	30.1 ± 0.9
BW at week 12 (g)	29.6 ± 1.0#	30.8 ± 1.0
Mesenteric fat pad (g)	0.47 ± 0.2	0.51 ± 0.2
Epididymal fat pad (g)	0.65 ± 0.2#	1.02 ± 0.3
Perirenal fat pad (g)	0.18 ± 0.1*	0.28 ± 0.1
Total WAT (g)	1.30 ± 0.3*	1.81 ± 0.4

*$p < 0.01$, #$p < 0.001$ compared to the LCT group.

Table 5. Levels of Blood Lipids and Other Blood Variables in C57BL/6J Mice Fed with the MCT or LCT Diet for 12 Weeks

	MCT (n = 15)	LCT (n = 15)
TG (mmol/L)	0.84 ± 0.4#	1.31 ± 0.3
TC (mmol/L)	2.72 ± 0.4*	3.13 ± 0.4
HDL-C (mmol/L)	2.49 ± 0.2**	2.09 ± 0.5
LDL-C (mmol/L)	0.35 ± 0.2*	0.62 ± 0.2
HDL-C/LDL-C	8.00 ± 2.6#	3.83 ± 1.9
FFA (mmol/L)	93.1 ± 15.7#	113.1 ± 11.5
Glycerol (μg/mL)	186 ± 45.1	230 ± 62.6
Norepinephrine (ng/mL)	6.82 ± 2.2*	4.65 ± 1.5
T3 (ng/mL)	1.12 ± 0.04	1.10 ± 0.1

*$p < 0.05$, **$p < 0.01$, #$p < 0.001$ compared to the LCT group.

Table 6. Levels of ATGL in WAT, and of cAMP, PKA, HSL and ATGL in BAT of C57BL/6J Mice Fed with the MCT and LCT Diets for 12 Weeks

	MCT (n = 15)	LCT (n = 15)
ATGL in WAT (nmol/mg of protein)	2.01 ± 0.8*	1.30 ± 0.5
cAMP in BAT (pg/mg of protein)	71.1 ± 16.3*	51.4 ± 8.6
PKA in BAT (ng/mg of protein)	5.08 ± 1.2*	4.09 ± 0.8
HSL in BAT (ng/mg of protein)	101.9 ± 33.4*	75.3 ± 13.3
ATGL in BAT (nmol/mg protein)	0.61 ± 0.1#	0.45 ± 0.04

*$p < 0.05$, #$p < 0.001$ compared to the LCT group.

Discussion

Some studies have proposed that the lipid metabolism of mice was no different from that of humans to a certain extent.[14–16] Some mice did not become obese in spite of being fed with a high-fat diet. We therefore used obese mice induced by a high-fat diet whose body weight gain was over 20% more than that of mice fed with a normal diet. This was carried out according to the report of Tschop and Heiman.[15]

In good agreement with previous reports,[6,9,17] we found that the MCT diet induced less deposition in the visceral fat pads than the LCT diet did in C57BL/6J mice. Furthermore, the results of the present experiment

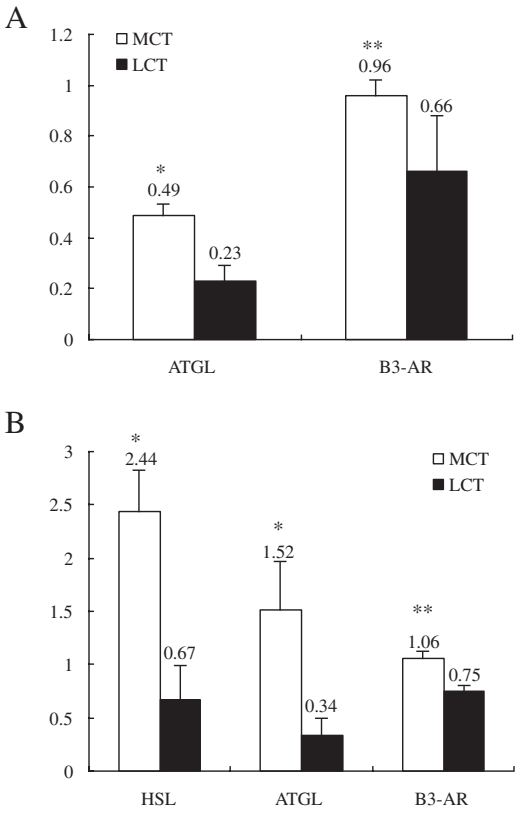

Fig. 1. mRNA Expression of ATGL and β3-AR in WAT (A) and of HSL, ATGL and β3-AR in BAT (B) of C57BL/6J Mice Fed with the MCT or LCT Diet by a Real Time RT-PCR Analysis.
Results are expressed as the mean, and SD is shown as error bars. $^{*}p < 0.05$, $^{**}p < 0.01$ compared to the LCT group, n = 15.

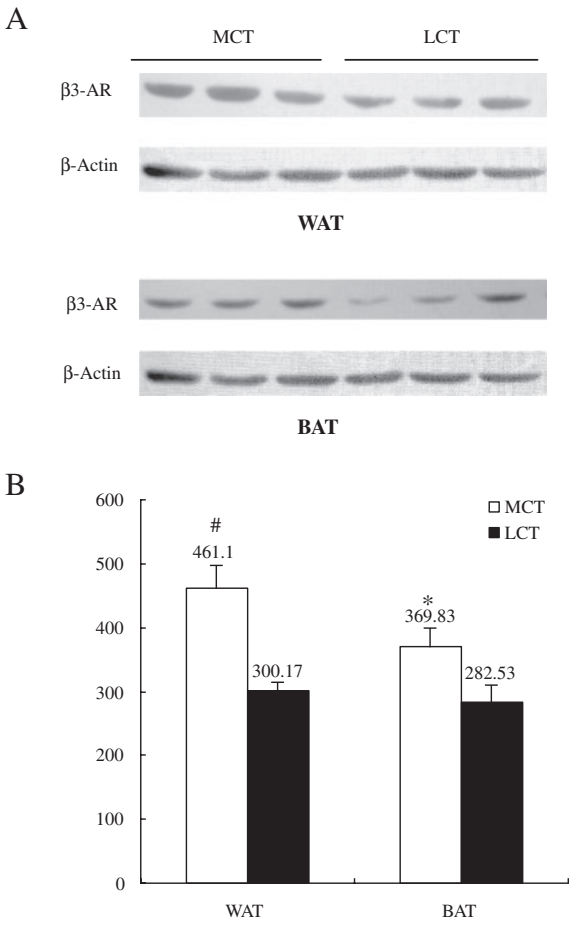

Fig. 2. Protein Expression of β3-AR in WAT and BAT from C57BL/6J Mice Fed with the MCT or LCT Diet by a Western Blot Analysis.
β-Actin was used as a loading control. The blots are representative of n = 3 for each group (A). The results of a gray-scale analysis are also shown (B), and SD is shown as error bars. $^{*}p < 0.01$, $^{\#}p < 0.001$ compared to the LCT group.

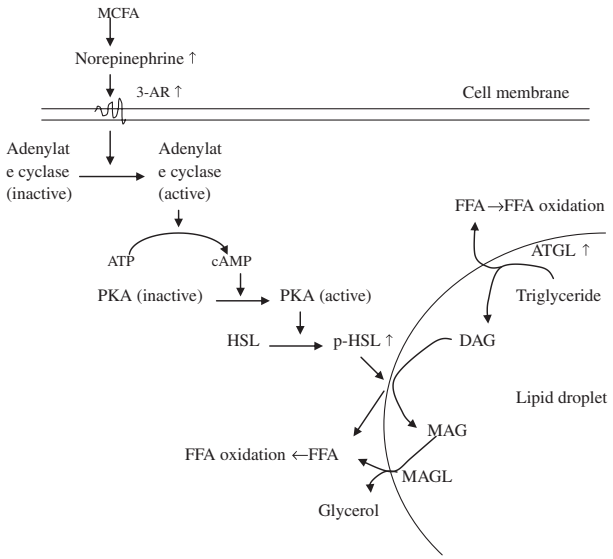

Fig. 3. Outline of the Effects of MCFA on Triglyceride Mobilization in Adipocytes.
Arrows (↑) represent an increasing level and up-regulation of activity, or of protein or mRNA expression. The MCFA diet stimulated the effect of norepinephrine on β3-AR signaling and increased the level of cAMP and PKA activation. The phosphorylation of HSL was subsequently triggered. MCFA diet also up-regulated the protein and mRNA expression of ATGL, resuting in the triglycerides of lipid droplets inside white and brown adipocytes being mobilized. DAG, diacylglycerol; MAG, monoacylglycerol.

were also consistent with those of the previous clinical trials and animal experiments[4,9,18] in that the MCT diet reduced the concentrations of triglyceride, total cholesterol, LDL-C and FFA, and increased the levels of HDL-C and the ratio of HDL-C/LDL-C.

Obesity means the excessive accumulation of body fat.[19] On the contrary, catabolism of fat represents the mobilization of fat or lipolysis. Mobilization of stored fat is mediated by lipolytic enzymes which degrade adipose triglycerides and release FFAs into the circulation.[20] Body fat exits in the form of triglyceride in WAT. WAT and BAT are similar with regard to a number of highly specialized biochemical functions such as the synthesis and storage of triglycerides (lipogenesis) and the release of FFA (lipolysis). The main explanations for the effect of MCT on less body fat accumulation have previously been based on results that MCT increased the energy expenditure and accelerated fatty acid oxidation in the liver or WAT of rats.[7,8] Our previous study has found a reduction in size and an increase in number of adipocytes.[9] Since the size of adipocytes is determined by intracellular lipids,[21] the reduction in size resulted from the increased mobilization of intracellular triglyceride. According to the results of the previous[9] and present experiments, triglyceride catabolism or mobilization in adipose tissue was substantially stimulated by a diet containing MCT.

HSL was once thought to be the major enzyme responsible for the lipolytic breakdown of cellular fat stores.[22] However, HSL-deficient mice were lean, and they efficiently mobilized FFAs from stored triglyceride,[23] suggesting that other triglyceride hydrolases played an important role in the fat mobilization. Some recent investigations almost simultaneously reported the discovery of a new enzyme which was named adipose triglyceride lipase (ATGL) in accordance with its physiological activity.[11,24,25] They reported that ATGL specifically hydrolysed triglyceride and was predominantly expressed in adipose tissue, and to a lesser extent in cardiac muscle, skeletal muscle, testis tissue and other tissues. ATGL mRNA expression was regulated by fasting/feeding[24] as well as hormones and cytokines,[26,27] and the inhibition of ATGL *in vitro*[11,28] markedly decreased triglyceride catabolism, implying that the enzyme could play an important role in lipolysis. It was therefore considered that lipolysis in the adipose tissue of humans and rodents would be regulated in a step-wise pattern by ATGL, HSL and monoacylglycerol lipase (MAGL).[29] The current explanation is that ATGL initiates lipolysis by cleaving the first fatty acid from triglyceride, and then that HSL and MAGL respectively act on diacylglycerol and monoacylglycerol, releasing two additional fatty acids and one glycerol molecule.[30] The up-regulation of both protein and mRNA of ATGL and HSL would therefore have promoted triglyceride breakdown,[31] and induced a reduction in the body fat mass. This notion is supported by our previous[9] and present results, indicating that the protein levels and mRNA expression of ATGL and HSL were all increased in both WAT and BAT of C57BL/6J mice fed with the MCT diet. Additionally, the phosphorylation of HSL, which is required for cAMP-dependent PKA-mediated lipolysis, was essential.[32] Our previous study found increased levels of cAMP and PKA in the blood and WAT of C57BL/6J mice fed with the MCT diet,[9] and the present study has shown higher levels of cAMP and PKA in BAT of C57BL/6J mice fed with the MCT diet, both experiments being accompanied by less accumulation of fat.

Body fat storage and mobilization is mainly regulated by the adrenergic system. Norepinephrine induces β3-AR signaling and triggers a series of biochemical reactions related to lipolysis.[33] It has always been believed that β3-AR agonists could stimulate the sympathetic nerve and lead to lipolysis in white fat tissue and the consequent liberation of fatty acids into the blood.[34,35] Chronic treatment of obese rodents with β3-AR agonists has led to a decrease in body weight due

to the reduction of fat tissue.[36] The β3-AR agonist can increase intracellular cAMP and phosphorylation of PKA in brown fat cells, so that triglycerides break down into free fatty acids.[37] Miyoshi *et al.* have reported that β3-AR stimulation activated PKA and promoted phosphorylated HSL.[38] The results of the present experiment show a significant increase in blood norepinephrine and in the mRNA and protein expression of β3-AR in both WAT and BAT of the MCT group. Taken together, it seems that MCT stimulated the sympathetic nerve, and triggered a cascade of biochemical reactions for fat mobilization or lipolysis and the oxidation of fatty acids. Among these, the stimulation of norepinephrine secretion and β3-AR expression might be the key mechanism for reducing the fat-mass effectiveness of MCT. Our proposed outline for these processes is illustrated in Fig. 3.

Based on the results of the previous and present experiments,[9] we conclude that MCT could irritate the sympathetic nervous system to produce more norepinephrine which would trigger a series of biochemical lipolysis reactions such as the up-regulation of β3-AR, ATGL and HSL signaling in WAT and BAT.

Acknowledgments

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