Original Article

Medium-Chain Triglyceride Activated Brown Adipose Tissue and Induced Reduction of Fat Mass in C57BL/6J Mice Fed High-fat Diet*

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Abstract

Objective To investigate activation of brown adipose tissue (BAT) stimulated by medium-chain triglyceride (MCT).

Methods 30 Male C57BL/6J obese mice induced by fed high fat diet (HFD) were divided into 2 groups, and fed another HFD with 2% MCT or long-chain triglyceride (LCT) respectively for 12 weeks. Body weight, blood biochemical variables, interscapular brown fat tissue (IBAT) mass, expressions of mRNA and protein of beta 3-adrenergic receptors (β3-AR), uncoupling protein-1 (UCP1), hormone sensitive lipase (HSL), protein kinase A (PKA), and adipose triglyceride lipase (ATGL) in IBAT were measured.

Results Significant decrease in body weight and body fat mass was observed in MCT group as compared with LCT group (P<0.05) after 12 weeks. Greater increases in IBAT mass was observed in MCT group than in LCT group (P<0.05). Blood TG, TC, LDL-C in MCT group were decreased significantly, meanwhile blood HDL-C, ratio of HDL-C/LDL-C and norepinephrine were increased markedly. Expressions of mRNA and protein of β3-AR, UCP1, PKA, HSL, ATGL in BAT were greater in MCT group than in LCT group (P<0.05).

Conclusion Our results suggest that MCT stimulated the activation of BAT, possible via norepinephrine pathway, which might partially contribute to reduction of the body fat mass in obese mice fed high fat diet.

Key words: Medium-chain triglyceride; Brown adipose tissue; Uncoupling protein-1; Beta 3-adrenergic receptor

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INTRODUCTION

Adipose tissue is found in mammals in two different forms, white adipose tissue (WAT) and brown adipose tissue (BAT), which perform essentially opposite functions. WAT accumulates surplus energy mainly in the form of triglyceride. BAT dissipates energy directly as heat. BAT is involved in non-shivering as well as diet-induced-thermogenesis[1]. Heat is produced by uncoupling fatty acid oxidation from ATP production by uncoupling protein-1 (UCP1). UCP1 mRNA

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expression and protein levels increase rapidly in response to cold, activation of the sympathetic nervous system and the administration of β-agonists[2]. In rodents, some experiments showed BAT performs anti-obesity function[3]. In those experiments, the surgical denervation or excision of interscapular BAT was followed by an abnormal increase in the amounts of WAT in those animals. In genetically obese mouse models such as the ob/ob and db/db strains, their BATs are dysfunctional[2].

Medium-chain triglyceride (MCT) has been considered to have potential for preventing and treating obesity, although the mechanism was not fully understood. Many investigations, including our previous studies have reported that intake of MCT or medium-chain fatty acids (MCFAs) resulted in less body weight gain and body fat mass in both animals and humans[4-8]. As to mechanism for reduction of body mass by MCT, some studies have reported that MCT, compared with long-chain triglyceride (LCT), could increase energy expenditure and hepatic fatty acid oxidation in rats and humans[7,9-11]. Our previous studies in mice have suggested that MCT irritate the sympathetic nervous system (SNS) to produce more norepinephrine (NE) which would trigger a series of biochemical lipolysis reactions such as the up-regulation of adipose triglyceride lipase and hormone-sensitive lipase signaling in both WAT[12].

Based on the previous experiments, we hypothesized that MCT could induce activation of BAT, which acts as a part of possible mechanisms responsible for the effect of MCT on reduction of body fat.

MATERIALS AND METHODS

Animals

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±1 °C and 40% to 60% humidity with a 12-h light/dark cycle (light on from 8:00-20:00). The experiments were 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-93G diet was purchased from the Academy of Military Medical Sciences for use as a basal diet. The composition of high fat diet and the experimental diets containing 2% MCT or 2% LCT was shown in Tables 1 and 2 respectively. The fatty acid compositions of MCT, LCT, MCT diet and LCT diet measured by gas chromatography were shown in Table 3.

Table 1. Composition of High Fat Diet for C57BL/6J Mice

Ingredients	High Fat Diet
Basal diet (%)	79
Yolk powder (%)	10
Lard (%)	10
Cholesterol (%)	1
Energy (KJ/g)	18.42
Percentage of nutrients	
Protein (%)	18.01
Fat (%)	19.42
Carbohydrate (%)	48.47
Mineral mixture (%)	0.78
Vitamin mixture (%)	0.52
Fiber (%)	1.5
Water (%)	9.5
Others (%)	1.06

Table 2. Composition of MCT and LCT Diet for C57BL/6J Mice

Ingredients	MCT Diet	LCT Diet
High fat diet (%)	90	90
MCT (%)	2	
LCT (%)	2	4
Casein (%)	6	6
Nutritional composition		
Energy (KJ/g)	18.98	18.98
Protein (%)	21.54	21.54
Fat (%)	21.48	21.48
Carbohydrate (%)	43.62	43.62
Mineral mixture (%)	0.78	0.78
Vitamin mixture (%)	0.52	0.52
Fiber (%)	1.5	1.5
Water (%)	9.5	9.5
Others (%)	1.06	1.06

Protocols

One hundred C57BL/6J mice were fed the basal diet and water *ad libitum* for 1 week to stabilize their metabolic condition. Fifteen mice were randomly chosen to feed the basal diet as a non-obesity control. The left mice were fed the high fat diet. After 4 weeks, only those whose body weight gain was over 10% more than that of the non-obesity control mice were used for the experiment. Thus, 30 obese mice were chosen and randomly assigned to two groups and fed the MCT or LCT diet for 12 weeks. The body weight and food intake were monitored twice a week.

Blood, White, and Brown Adipose Tissue Sampling

At the end of the experiment, the mice were deprived of 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 10 mg/kg, and blood samples were collected from the aorta ventralis. The blood samples were centrifuged at 2000 g for 10 min, and sera were collected and stored at -80 °C until analysis. The IBAT, epididymal and perirenal fat pads were excised, rinsed with icy saline and weighed. The IBAT samples were immediately frozen and stored in liquid nitrogen. For histology and immunohistochemistry, a part of IBAT samples were fixed in 4% formaldehyde solution.

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 (No. 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, UK). The concentrations of NE in the serum were measured by a mouse enzyme-linked immunosorbent assay (ELISA) kit (No. E03N0013, Bluegene, China) according to the manufacturer's instructions.

ELISA Analysis

A part of the IBAT sample was homogenized in 10 volumes of a 0.9% NaCl solution. The homogenate was centrifuged at 1200 g for 15 min at 4 °C to create an aqueous layer as the sample for measuring. The levels of hormone sensitive lipase (HSL), cyclic adenosine monophosphate (cAMP), protein kinase A (PKA), and adipose triglyceride lipase (ATGL) in IBAT were determined with their corresponding ELISA kits (No. E03H0281, E03C0027, E03P0574, E03A0893, BlueGene, China). The levels of these parameters were related to the total protein concentration in the tissue, which was measured whit a BCA protein assay kit (No. 23225, Pierce, USA), using bovine serum albumin as a standard.

Table 3. Fatty Acid Composition of MCT, LCT, MCT Diet, and LCT Diet

Fatty Acid	MCT (g/100 g of total fatty acids)	LCT (g/100 g of total fatty acids)	MCT Diet (g/100 g of diet)	LCT Diet (g/100 g of diet)
8:0	75.4	ND[a]	1.5	0
10:0	24.6	ND[a]	0.5	0
14:0	-	ND[a]	0.2	0.2
16:0	-	6.2	4.4	4.5
16:1	-	0.2	0.4	0.4
18:0	-	2.5	2.2	2.3
18:1	-	48.8	7.2	8.2
18:2	-	30.2	3.8	4.4
18:3	-	9.4	0.4	0.5
20:0	-	0.6	0.1	0.1
20:1	-	1.1	0	0
20:4	-	ND[a]	0.1	0.1
22:0	-	0.4	0	0
22:1	-	0.2	0	0
22:6	-	ND[a]	0.1	0.1
24:0	-	0.2	0	0
24:1	-	0.2	0	0
Total	100	100	20.9	20.8

Note. [a]not detectable.

Histology and Immunohistochemistry

After being post-fixed in paraformaldehyde overnight, IBAT samples were embedded in paraffin and cut into 2-μm-thick sections. The sections were either stained with hematoxylin-eosin or processed for immunohistochemistry with polyclonal rabbit anti-UCP1 antibody, or with polyclonal rabbit Anti-beta 3 Adrenergic Receptor (β3-AR) antibody. The dewaxed sections were incubated in 0.3% hydrogen peroxide in methanol to inhibit endogenous peroxidase activity, and then with 10% normal goat serum, polyclonal anti-UCP1 antibody (1:500 diluted, No.ab10983, Abcam, UK), or polyclonal anti-β3-AR antibody (1:100 diluted, No.ab94506, Abcam, UK), goat anti-rabbit IgG (1:400 diluted, Vector, Buringame, CA, USA), and finally with avidin-biotin-peroxidase complex (Vector, Buringame, CA, USA) according to the conventional ABC method. The sections were also counterstained with hematoxylin and examined under a light microscope. The image was captured (microscope from OLYMPUS, Japan, and camera from SONY, Japan) and analyzed using software Image-Pro Plus 6.0. The area of lipid droplet and cell number per 1 mm^2 of brown adipocytes in these samples were counted in three randomly selected fields of each stained specimen. The signal intensity of UCP1 and β3-AR was measured by integrated optical density (IOD).

Western Blot Analysis

A 10 mg amount of frozen IBAT 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. For electrophoresis, sample of 50-100 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 antibody anti-UCP1 (1:1000 diluted, Abcam, UK) or anti-β3-AR (1:1000 diluted, Abcam, UK). 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 IOD, using software Image-Pro Plus 6.0.

Real-time RT-PCR Analysis

The mRNA expression of UCP1 and β3-AR in BAT was measured by quantitative real-time RT-PCR analysis. Total RNA was isolated from the 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: UCP1 NM_009463: forward (5'-3'), GGATGGTGAACCCGACAACT; reverse (5'-3'), GATCTGAAGGCGGACTTTGG. β3-AR NM_ 013462: forward (5'-3'), CCTTCAACCCGGTC ATCTA CTG; reverse (5'-3'), CGCACCTTCATAGCCA TCAAA. β-Actin NM_007393: forward (5'-3'), GAGACCTTC AACACCCCAGC; reverse (5'-3'), ATGTCACGCACGATT TCCC. The β-actin 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 $2^{-\Delta\Delta Ct}[2^{\Delta Ct(actin)-\Delta Ct(target\ gene)}]$.

Statistical Analysis

All data are expressed as the mean values±SD, and the Student's *t*-test was used for comparison between the IBAT excision and sham control or the MCT and LCT group. $P<0.05$ was set to be statistically significant.

RESULTS

Body Weight, Diet Consumption, Body Fat, and Blood Lipid Profiles in C57BL/6J Mice Fed the MCT or LCT Diet

At the end of the 12 weeks experiment, the final body weights, body weight gain, total WAT (the total weights of the epididymal and perirenal fat pad) in the MCT group were significantly lower than those in the LCT group (Table 4). There were no significant differences in the daily average diet and energy intake between the MCT and LCT group. But the food efficiency ratio was significantly higher in the MCT group than in the LCT group (Table 5). Compared with the LCT group, the MCT group had significantly lower levels of blood triglyceride, total cholesterol, LDL-c, and higher levels of HDL-c, HDL-c/LDL-c and NE (Table 6).

Changes in IBAT in C57BL/6J Mice Fed the MCT or LCT Diet

The weight of IBAT, numbers of brown adipose cell per quadratmillimeter were significantly higher in the MCT group than in the LCT group. The area of lipid droplet per brown adipose cell was much lower in the MCT group than in the LCT group (Table 7, Figure 1). The levels of PKA, cAMP, HSL, and ATGL in IBAT were much higher in the MCT group than in the LCT group (Table 8).

Table 4. Body Weight and Body Fat in C57BL/6J Mice Fed the MCT or LCT Diet

Items	MCT (*n*=15)	LCT (*n*=15)
Initial body weight (g)	24.4±0.5	24.4±0.4
Final body weight (g)	29.6±1.0**	30.8±1.0
Body weight gain (g)	5.21±1.25*	6.39±1.00
Total WAT weight (g)	1.30±0.34**	1.81±0.42
Total WAT weight/BW (%)	4.38±1.10**	5.85±1.23

Note. *$^{*}P$<0.05, $^{**}P$<0.01, *vs.* the LCT group.

Table 5. Consumption of Diet in C57BL/6J Mice Fed the MCT and LCT for 12 Weeks

Items	MCT (*n*=15)	LCT (*n*=15)
Food intake (g/d)	3.63±0.56	3.65±0.32
Energy intake (kJ/d)	68.9±10.6	69.2±6.1
Food efficiency ratio (kJ/g BW)	1 113.0±171.7**	909.3±79.7

Note. *$^{**}P$<0.01, *vs*. the LCT group.

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

Items	MCT (*n*=15)	LCT (*n*=15)
TG (mmol/L)	0.711±0.152**	1.343±0.301
TC (mmol/L)	2.66±0.40*	3.19±0.38
HDL-c (mmol/L)	2.49±0.26*	2.03±0.52
LDL-c (mmol/L)	0.302±0.071**	0.684±0.162
HDL-c/LDL-c	8.70±2.17**	3.16±1.08
NE (ng/mL)	7.88±1.98**	4.23±0.99

Note. *$^{*}P$<0.05, $^{**}P$<0.01, *vs.* the LCT group.

Table 7. Changes in IBAT Weight and Numbers of Brown Adipose Cell in C57BL/6J Mice Fed the MCT or LCT Diet for 12 Weeks

Items	MCT (*n*=15)	LCT (*n*=15)
IBAT weight (mg)	154.1±41.2**	90.5±20.3
IBAT weight/BW (‰)	5.21±1.22**	3.01±0.82
Brown adipose cell numbers (×1000/mm^2)	4.81±0.68**	3.37±0.52
Area of lipid droplet (μm^2)	40.1±6.0**	97.5±15.1

Note. *$^{**}P$<0.01, *vs.* the LCT group.

Table 8. Levels of PKA, cAMP, HSL, and ATGL in IBATs from C57BL/6J Mice Fed the MCT or LCT Diet for 12 Weeks

Items	MCT (*n*=15)	LCT (*n*=15)
PKA (ng/mg protein)	4.95±0.84**	3.90±0.51
cAMP (pg/mg protein)	79.9±8.7**	61.5±4.6
HSL (pg/mg protein)	110.4±31.9**	73.9±11.8
ATGL (pmol/mg protein)	650±100**	460±20

Note. *$^{**}P$<0.01, *vs.* the LCT group.

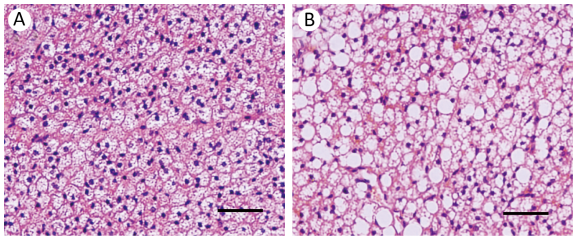

Figure 1. Morphological comparisons in IBATs from C57BL/6J mice fed the MCT or LCT diet for 12 weeks. The bar represents 50 μm as the scale of the image. MCT group (A), LCT group (B).

Levels of Protein and mRNA Expression of UCP1 and β3-AR in IBATs from C57BL/6J Mice Fed the MCT or LCT Diet

Both immunohistochemical detection and western blot analysis showed that the protein expressions of UCP1 and β3-AR were much higher in the MCT group than in the LCT group (Figure 2A-H). Meanwhile the mRNA expressions of UCP1 and β3-AR by real-time RT-PCR analysis were also higher in the MCT group than in the LCT group (Figure 2I).

Immunohistochemical detection of UCP1 (Figure 2A, 2B) and β3-AR (Figure 2D, 2E) in MCT and LCT group, and the IOD analysis for immunohistochemical detection of UCP1 (Figure 2C) and β3-AR (Figure 2F) were shown (n=10). Western blot analysis of UCP1 and β3-AR in IBATs and their gray-scale analysis were also shown in Figure 2G and H (n=4). The Mrna expressions of UCP1 and β3-AR in IBATs by a Real Time RT-PCR were illustrated in Figure 2I (n=5). β-Actin was used as a loading control.

DISCUSSION

It is widely agreed that obesity stems from a prolonged imbalance between the level of energy intake and expenditure, with the resultant surplus being stored as body lipids, predominantly in adipose tissue[13]. Decreased thermogenesis may in itself be causative of obesity; correspondingly, 'induced' thermogenesis counteracts obesity (even without dietary intervention). Thus, activation of thermogenesis is an anti-obesity tool irrespective of whether it is accomplished by artificial uncoupling, exercise, shivering, or recruitment and activation of brown adipose tissue[14-19].

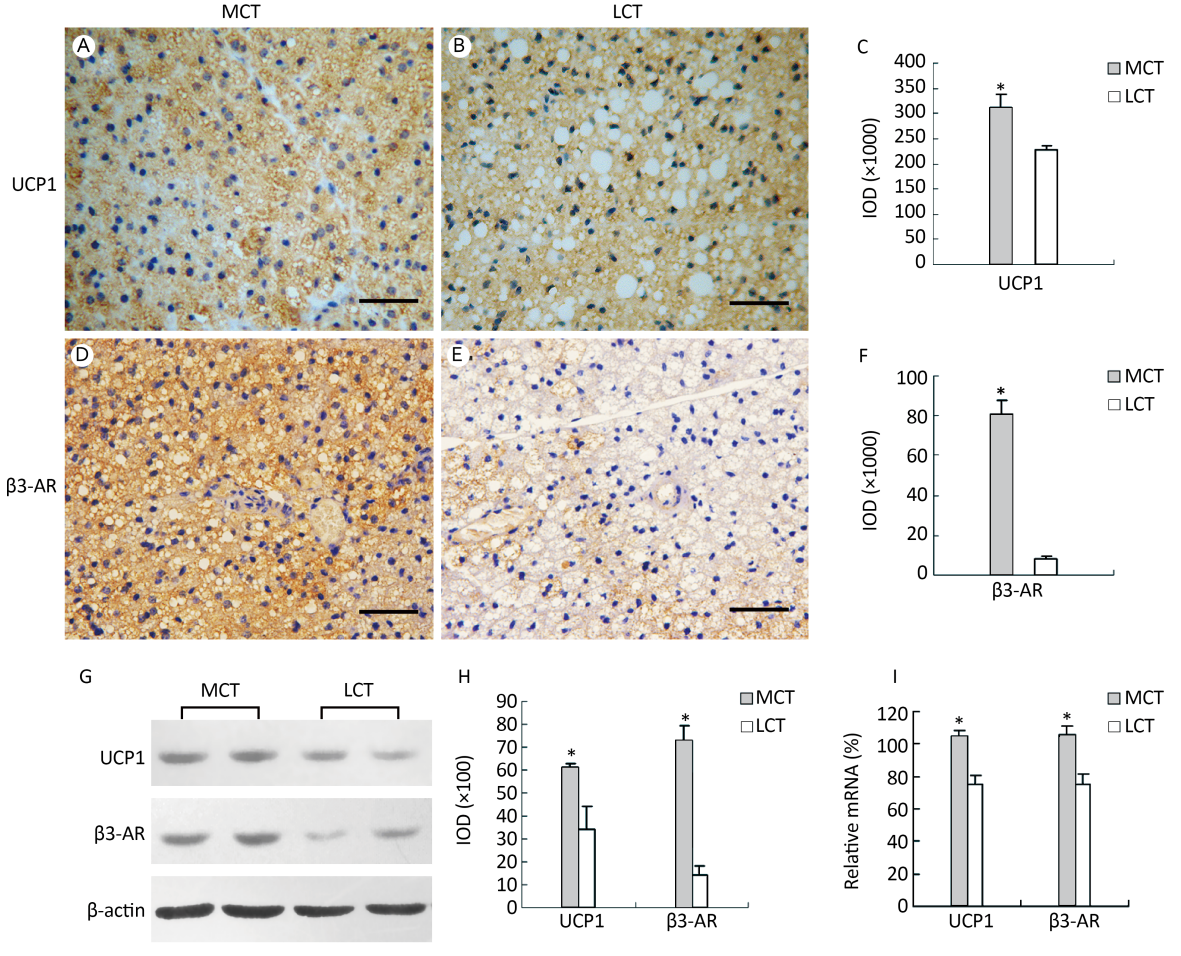

Figure 2. Protein and mRNA expression of UCP1 and β3-AR in IBATs from C57BL/6J mice fed the MCT or LCT diet. The bar represents 50 μm as the scale of the image. *P<0.05, *vs.* the LCT group.

MCT was found to reduce body fat accumulation in the previous clinical trials and animal experiments[8,11,20]. We designed this experiment here to explore a possible effect of MCT on reduction of body weight and body fat mass by activating brown adipose tissue in mice. Our results showed that mice fed MCT had less body weight and body fat mass, and improved blood lipid profiles. These are consistent with our previous study[20]. It was also found in this study that IBAT mass increased markedly in the mice fed MCT (Table 7). BAT depots mainly distribute in interscapular area, intercostal area, periaortic area, perirenal area and subaxile area et al. in mice. IBAT is the major part (about one third) of BAT depots in mice. Multiple independent research teams using combined PET and computed tomography imaging, immunohistochemistry, and gene and protein expression have proven conclusively that adult humans have functional BAT[15]. Therefore, increased BAT mass and its promoted function or activity by MCT might be a potential way to control human obesity.

Uncoupling protein (UCP) is a molecule, which uncouples mitochondrial oxidative phosphorylation by bypassing the electrochemical gradient across the inner membrane from the F1-ATPase and thereby dissipates energy as heat. Among several isoforms of the UCP family so far reported in mammals, UCP1 is the only one whose physiological importance has been firmly established. Present exclusively in BAT, UCP1 is one of the key molecules for adaptive thermogenesis and energy expenditure[21-24]. Our results of increased mRNA and protein expressions of UCP1 in IBAT indicated the enhancement of thermogenic capacity in the mice fed MCT.

The control of BAT activity depends on the adrenergic stimulation of brown adipocytes[25]. BAT depots are densely innervated by efferent branches of SNS. The SNS nerve endings reach individual brown adipocytes and release norepinephrine, which stimulates BAT thermogenic activity and cell differentiation by binding to β3-AR. The latter is a prominent actor in driving the cascade of events necessary for heat production in rodent BAT[25-27]. Norepinephrine induces β3-AR signaling and triggers a series of biochemical reactions related to lipolysis and thermogenesis[28-29]. The lipolysis is regulated in a step-wise pattern by ATGL, HSL and monoacylglycerol lipase (MAGL)[30]. The ATGL initiates lipolysis by cleaving the first fatty acid from triglyceride, and HSL and MAGL respectively act on diacylglycerol and monoacylglycerol, releasing two additional fatty acids and one glycerol molecule[31]. The activation of HSL is due to PKA activation. In brown adipocytes, PKA regulates phosphorylation of the transcription factor of cAMP-response-element-binding protein (CREB). CREB then supposedly activates the expression of genes, including that for UCP1[32]. Some studies have shown that the increase of β3-AR expression, can cause increased UCP1 expression in BAT and heat production[21,28,33-34]. Our team has reported that MCT irritate the SNS to produce more norepinephrine which would trigger a series of biochemical lipolysis reactions in both WAT[12]. The results of the present experiments also showed significant increases in blood norepinephrine and in mRNA and protein expression of UCP1 and β3-AR in IBAT from the mice fed MCT. Taken together, it seems that MCT stimulated norepinephrine, and then triggered the activation of thermogenesis of BAT. Our proposed outline for these processes was illustrated in Figure 3. However, the exact mechanism of increased norepinephrine by MCT is needed to clarify in future.

In conclusion, MCT activated brown adipose tissue, which might be in part responsible for reduction of body fat mass in obese C57BL/6J mice fed high fat diet with MCT.

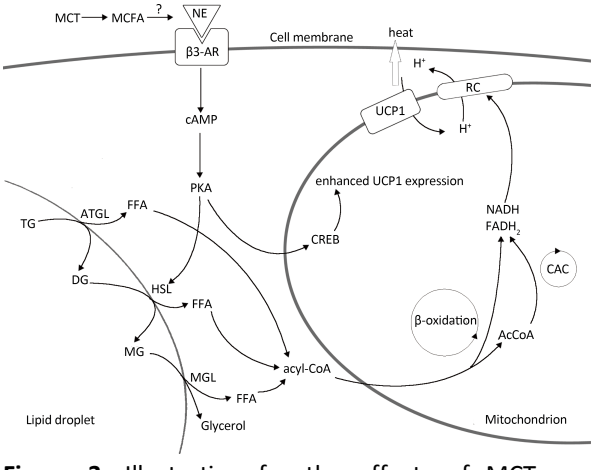

Figure 3. Illustration for the effects of MCT on thermogenesis in BAT. NE, norepinephrine; PKA, protein kinase A; HSL, hormone-sensitive lipase; FFA, free fatty acids; CREB, cAMP-response-element-binding protein; RC, respiratory chain; AcCoA, acetyl CoA; CAC, citric acid cycle; DG, diacylglycerol; MG, monoacylglycerol.

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