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ARTICLE

Acetic Acid Upregulates the Expression of Genes for Fatty Acid Oxidation Enzymes in Liver To Suppress Body Fat Accumulation

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We investigated the effect of acetic acid (AcOH) on the prevention of obesity in high-fat-fed mice. The mice were intragastrically administrated with water or 0.3 or 1.5% AcOH for 6 weeks. AcOH administration inhibited the accumulation of body fat and hepatic lipids without changing food consumption or skeletal muscle weight. Significant increases were observed in the expressions of genes for peroxisome-proliferator-activated receptor α (PPARα) and for fatty-acid-oxidation- and thermogenesis-related proteins: acetyl-CoA oxidase (ACO), carnitine palmitoyl transferase-1 (CPT-1), and uncoupling protein-2 (UCP-2), in the liver of the AcOH-treatment groups. PPARα, ACO, CPT-1, and UCP-2 gene expressions were increased *in vitro* by acetate addition to HepG2 cells. However, the effects were not observed in cells depleted of α2 5′-AMP-activated protein kinase (AMPK) by siRNA. In conclusion, AcOH suppresses accumulation of body fat and liver lipids by upregulation of genes for PPARα and fatty-acid-oxidation-related proteins by α2 AMPK mediation in the liver.

KEYWORDS: Acetic acid; acetate; 5′-AMP-activated protein kinase; peroxisome-proliferator-activated receptor α; body weight; body fat; fatty acid oxidation

INTRODUCTION

Obesity, a pathophysiological status of excessive body fat accumulation, is caused by an imbalance between energy intake and expenditure, leading to conditions such as impaired glucose tolerance, hypertension, and hyperlipidemia; it also increases the risk of developing atherosclerosis (*1*, *2*). Prevention and improvement of obesity are important issues in today's modern society, and an effective countermeasure is urgently required.

Vinegar has not only been consumed as seasoning throughout the world but has also been used as a folk medicine since ancient times (*3*, *4*). Scientific evidence for the beneficial effects of vinegar was scarce until this decade. We investigated the health effects of acetic acid (AcOH) as a main component of vinegar (*5*) and showed that AcOH effects hyperglycemia (*6*), dyslipidemia (*7*), and hypertension (*8*). In addition, Yamashita et al. recently reported that AcOH suppresses body fat accumulation (*9*). The mechanism was explained as follows: AcOH is absorbed immediately after oral administration and metabolized to acetyl-CoA with the production of AMP in the liver (*10*). This results in elevation of the AMP/ATP ratio and subsequent phosphorylation of 5′-AMP-activated protein kinase (AMPK) (*6*, *11*, *12*). Consequently, genes involved in glucose metabolism or lipogenesis, downstream of AMPK, are downregulated, resulting in suppression of body fat accumulation (*9*). In support of this mechanism, we reported that AcOH reduces the expressions of genes for lipogenesis-related proteins [acetyl-CoA carboxylase (ACC), fatty acid synthase (FAS), sterol regulatory element binding protein (SREBP-1), and ATP-citrate lyase (ATP-CL)] through activation of AMPK (*7*). In addition to the inhibitory effect of AcOH on lipogenesis, AcOH feeding induces transient enhancement of fatty acid use in the liver through inhibition of glucose use (*13*). AcOH feeding might induce activation of fatty acid oxidation; however, very little is known about the effect of AcOH on fatty acid oxidation.

AMPK is a heterotrimer consisting of a catalytic α subunit (α1 or α2) and regulatory β (β1 or β2) and γ (γ1, γ2, or γ3) subunits (*14*, *15*). Recently, it was reported that α2β2γ1 AMPK translocates to the nucleus, where Thr172 of the α2 subunit is phosphorylated and subsequently induces peroxisome proliferator-activated receptor α (PPARα) gene expression (*16*). PPARα regulates mRNA expression of fatty acid oxidation enzymes, such as acyl-CoA oxidase (ACO) (*17*) and carnitine palmitoyl transferase-1 (CPT-1) (*18*), and thermogenic protein, uncoupling protein-2 (UCP-2) (*19*). The data also suggest AcOH enhances fatty acid oxidation by upregulating the expression of genes for PPARα and its downstream enzymes, through α2 AMPK subunit phosphorylation.

In this study, we investigated the effect of AcOH administration on body fat accumulation and the involvement of fatty acid oxidation in mice fed a high-fat diet. Further investigation using hepatic HepG2 cells was performed to verify the detailed mechanism.

MATERIALS AND METHODS

Experiments with Diet-Induced Obese Mice. Six-week-old male C57BL/6J mice were obtained from CLEA Japan (Tokyo, Japan). Mice

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Table 1. Composition of the Experimental Diet[a] (g/kg)

	high-fat diet
casein (80 mesh)	240.9
L-cystine	3.6
corn starch	27.1
maltodextrin 10	150.6
sucrose	177.7
cellulose (BW200)	60.2
soybean oil	30.1
lard	240.9
salt mix S10026	12.0
dicalcium phosphate	15.7
calcium carbonate	6.6
potassium citrate (1 H_2O)	19.9
vitamin mix V10001	12.0
choline bitartrate	2.4
energy (kJ/g)	20.5
protein (% of energy)	20.0
carbohydrate (% of energy)	30.1
fat (% of energy)	49.9

[a] Dietary components were prepared by Research Diets.

were individually housed in an animal laboratory controlled at a temperature between 22 and 24 °C and humidity of 45−65% with a 12 h light/dark cycle (7:00−19:00/19:00−7:00). The animals had free access to water and a basic diet for 7 days. On the first day of the experimental period, the animals were divided into three groups, each with a similar mean body weight and a similar mean food intake. The mice were cared for in accordance with the Guidelines for Animal Experimentation at the 34th Annual Meeting of the Japanese Association for Laboratory Animal Science, held May 22, 1987. From the first day of the experimental period, each group was given the high-fat diet (50% of total energy) for the 42 day experimental period (**Table 1**). Mice were treated with 1.5% AcOH, high-dose group; 0.3% AcOH, low-dose group; or water (0% AcOH), control group at 10 mL/kg body weight via a stomach tube. Body weight and food intake were monitored every other day. On the final day of the experiments, food was withdrawn at 7:00 and mice were treated with water or AcOH, as described above, at 12:00. After 2 h, whole blood was collected from the inferior vena cava under anesthesia with diethyl ether. The serum was separated by a standard centrifugation method and stored at −80 °C until needed for measurement of triacylglycerol (TG) and total cholesterol (T-chol). Liver samples were immediately immersed in RNA later (Ambion, Austin, TX) and kept at −80 °C until total RNA was prepared. Epididymal, mesenteric, and retroperitoneal white adipose tissue (WAT) mass was measured.

Serum Lipid Concentration and Liver Lipid Content Analysis. Liver T-chol and TG were extracted using solvents (chloroform/methanol, 2:1) (*20*). Extracts were evaporated until dry, and lipids were dissolved in a minimal volume of isopropanol for TG measurement or isopropanol with 10% TritonX-100 for T-chol measurement. The concentration of T-chol (276-64909; Wako Chemicals, Osaka, Japan) and TG (274-69807; Wako Chemicals, Osaka, Japan) in the serum and liver were determined by enzymatic colorimetric methods.

RNA Extraction and Reverse Transcriptase Polymerase Chain Reaction (RT-PCR). Total RNA was prepared using TRIzol (Invitrogen, Carlsbad, CA) and an RNA Easy kit (QIAGEN, Hilden, Germany), according to the instructions of the manufacturer. The expression of mRNAs for PPARα, ACO, CPT-1, and UCP-2 were measured by PCR using the TaqMan method (*21*) and the ABI prism 7000 sequence detection system (Perkin-Elmer Biosystems, Foster City, CA). The primer sets for target genes in mice are PPARα [Ppara; Mm00440939_m1], ACO [Acox1; Mm00443579_m1], CPT-1 [Cpt1a; Mm00550438_m1], UCP-2 [Ucp2; Mm00627599_m1], SREBP-1 [Srebf1; Mm00550338_m1], FAS [Fasn; Mm01253292_m1], ACC [Acaca; Mm01304289_m1], and β-actin [Actb; Mm00607939_s1] and, in humans, are PPARα [PPARA; Hs00231882_m1], ACO [ACOX1; Hs00244515_m1], CPT-1 [CPT1A; Hs00157079_m1], UCP-2 [UCP2; Hs00163349_m1], AMPK α2 [PRKAA2; Hs00178903_m1], and β-actin [ACTB; Hs99999903_m1]. The relative amounts of these mRNAs were normalized to the amount of β-actin.

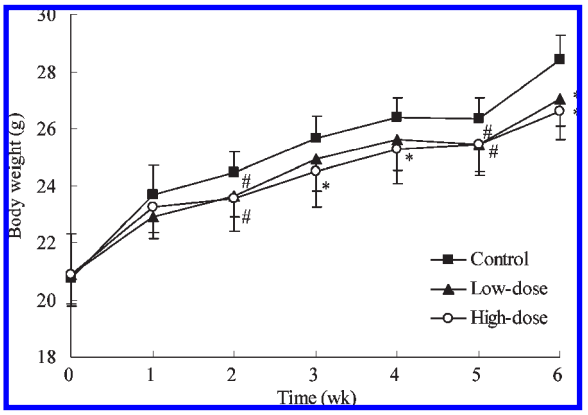

Figure 1. Effect of the acetic acid on body weight during the 6 week experimental period. Values are means ± SD; $n = 9-10$. (*) Significantly different ($p < 0.05$, Dunnett's multiple-comparison test) versus the control. (#) Nearly significantly different ($p < 0.1$, Dunnett's multiple-comparison test) versus the control.

HepG2 Cell Experiments. Human hepatoblastoma cell line HepG2 was purchased from American Type Culture Collection (HB-8065), and the cells were cultured in a 96-well plate in Dulbecco's modified Eagle's medium−high glucose (D5796; Sigma, St. Louis, MO), 10% fetal bovine serum (5796; Sigma, St. Louis, MO), and MEM non-essential amino acid solution (M7145; Sigma, St. Louis, MO). Cells were grown at 37 °C in a humidified atmosphere of 95% air/5% CO_2. A total of 4×10^3 cells per 96-well plate were transfected with a negative-control number 1 siRNA (4611, Ambion, Austin, TX) or validated siRNAs targeting human α2 AMPK (4390824, Ambion, Austin, TX) using siPORT NeoFx (M4510, Ambion, Austin, TX) following the instructions of the manufacturer. After 24 h, the knockdown efficiency of α2 AMPK was measured by RT-PCR. The effects of 1−3 h treatment with AcOH (0−500 μmol/L; 0 μmol/L, the control) were assessed using sodium acetate (acetate) in the form of neutralized AcOH to avoid changes in pH. Before treatment with acetate, cells were starved of serum overnight. PPARα, SREBP-1, ACO, CPT-1, UCP-2, ACC, and FAS mRNA levels were analyzed by RT-PCR.

Statistical Analyses. All experimental data were analyzed using SPSS for Windows (version 11.5 J; SPSS, Inc., Chicago, IL). Statistical evaluation of the results was performed by Dunnett's multiple-comparison test. The Dunnett procedure compares the means as measured for the treatment groups to the control mean. Differences are considered significant at $p < 0.05$. Each value is presented as the mean ± standard deviation (SD).

RESULTS

Body Weight Gain and Food Intake. There were no significant differences in body weight on the initial day of the experimental period (**Figure 1**) and total energy intake throughout the experimental period among the three groups (**Table 2**). Body weight gain in the control group was significantly higher when compared to the low-dose group at 6 weeks and the high-dose group at 3, 4, and 6 weeks of the experimental period. Both the low- and high-dose groups tended to have lower body weight than the control group at 2 and 5 weeks of the experimental period ($p < 0.1$). At the end of the experiment, the incremental ratios of body weight gain during the experimental period were 37.3% (from 20.8 ± 1.5 to 28.4 ± 0.8) in the control group, 29.5% (from 20.9 ± 1.1 to 27.0 ± 0.9) in the low-dose group, and 27.7% (from 20.9 ± 1.0 to 26.6 ± 1.0) in the high-dose group.

Fat, Liver, and Muscle Weights. As shown in **Table 2**, both the high- and low-dose groups had significantly lower weights of total WAT, mesenteric WAT, perirenal and retroperitoneal WAT, and liver when compared to the control group. The epididymal WAT in the low-dose group tended to be lower when compared to the control group ($p < 0.1$), but there was no significant difference between the high-dose group and the control group.

Table 2. Energy Intake, Tissue Weight, Serum Lipid Concentration, and Liver Lipid Contents[a]

		control	low dose	high dose
total energy intake (kJ/mouse)		2755.1 ± 340.2	2755.0 ± 423.8	2637.9 ± 446.2
tissue weight (g)	liver	1.08 ± 0.09	0.93 ± 0.06[b]	0.92 ± 0.06[b]
	total WAT	1.77 ± 0.19	1.39 ± 0.15[b]	1.37 ± 0.29[b]
	mesenteric WAT	0.50 ± 0.04	0.39 ± 0.07[b]	0.40 ± 0.08[b]
	perirenal and retroperitoneal WAT	0.38 ± 0.14	0.26 ± 0.06[b]	0.24 ± 0.08[b]
	epididymal WAT	0.89 ± 0.17	0.74 ± 0.10[c]	0.74 ± 0.18
	gastrocnemius muscle	0.18 ± 0.03	0.17 ± 0.01	0.17 ± 0.01
tissue weight/body weight ratio (%)	liver	3.80 ± 0.30	3.46 ± 0.22[b]	3.47 ± 0.23[b]
	total WAT	6.24 ± 0.65	5.16 ± 0.47[b]	5.12 ± 0.92[b]
	mesenteric WAT	1.77 ± 0.14	1.45 ± 0.27[b]	1.49 ± 0.28[b]
	perirenal and retroperitoneal WAT	1.33 ± 0.50	0.96 ± 0.20[c]	0.88 ± 0.29[b]
	epididymal WAT	3.14 ± 0.60	2.74 ± 0.33	2.75 ± 0.58
	gastrocnemius muscle	0.62 ± 0.10	0.63 ± 0.04	0.62 ± 0.04
serum lipid (mg/L)	TG	629 ± 216	625 ± 248	535 ± 118
	T-chol	1632 ± 189	1507 ± 117	1377 ± 157[b]
liver lipid (mg/total tissue)	TG	113.8 ± 12.0	96.9 ± 8.6[b]	94.8 ± 12.3[b]
	T-chol	3.79 ± 0.42	3.29 ± 0.29[b]	3.26 ± 0.32[b]

[a] Values are means ± SD; $n = 9-10$. [b] Significantly different ($p < 0.05$, Dunnett's multiple-comparison test) versus the control. [c] Nearly significantly different ($p < 0.1$, Dunnett's multiple-comparison test) versus the control.

Table 3. mRNA Levels in Mice Liver[a]

gene name	control[b]	low dose	high dose
PPARα	1.00 ± 0.08	1.15 ± 0.09[c]	1.16 ± 0.13[c]
ACO	1.00 ± 0.23	1.78 ± 0.78[c]	1.60 ± 0.46[c]
CPT-1	1.00 ± 0.14	1.42 ± 0.25[c]	1.28 ± 0.19[c]
UCP-2	1.00 ± 0.15	1.13 ± 0.17	1.23 ± 0.27[c]
SREBP-1	1.00 ± 0.23	1.04 ± 0.21	0.96 ± 0.15
FAS	1.00 ± 0.68	0.73 ± 0.13	0.79 ± 0.15
ACC	1.00 ± 0.38	1.03 ± 0.35	1.03 ± 0.30

[a] Values are means ± SD; $n = 9-10$. [b] Control refers to cells without the addition of acetate. [c] Significantly different ($p < 0.05$, Dunnett's multiple-comparison test) versus the control.

Tissue weight/body weight ratios of liver, total WAT, and mesenteric WAT in both the high- and low-dose groups were significantly lower when compared to the control group. No significant difference was observed in gastrocnemius muscle weight among the three groups.

Serum Lipid Concentration and Liver Lipid Contents. Serum T-chol was significantly lower in the high-dose group when compared to the control group, but no significant difference was observed in serum TG concentrations among the three groups. For the liver lipid contents, T-chol and TG in both the high- and low-dose groups were significantly lower when compared to the control group (**Table 2**).

Gene Expression in the Liver. As shown in **Table 3**, both the high- and low-dose groups had significantly greater levels of mRNA for PPARα when compared to the control group. In addition, the expression of genes for ACO and CPT-1 in both the high- and low-dose groups and for UCP-2 in the high-dose group were significantly higher when compared to the control group. No significant differences were observed in the mRNA expression levels of SREBP-1, FAS, and ACC among the three groups.

Gene Expression in HepG2 Cells. We examined whether acetate induced PPARα and its down stream genes via the α2 AMPK pathway using HepG2 cells and the RNAi method (**Figure 2**). Inhibition of α2 AMPK gene expression was verified by RT-PCR (approximately 20% mRNA remaining, data not shown). There was a significant increase in mRNA for PPARα 3 h after the addition of 100−500 μmol/L acetate in the negative-control siRNA-transfected HepG2 cells, but no significant differences were observed at ∼2 h (data not shown). Furthermore, a significant increase was observed in mRNA for ACO, CPT-1, and UCP-2 under the same conditions. Dose-dependent changes were particularly obvious for ACO and CPT-1. No significant differences were observed in the expression of genes for PPARα, ACO, CPT-1, and UCP-2 in the α2 AMPK siRNA-transfected HepG2 cells.

DISCUSSION

We examined the effect of AcOH on body fat accumulation and its mechanism, mainly focusing on fat oxidation. The body weight of high-fat-fed mice was significantly lower in both the high- and low-dose groups when compared to the control group. Moreover, lower fat pad weight in mesenteric tissue and perinephric and retroperitoneal tissues were observed in both the acetate treatment groups. Therefore, it was confirmed that AcOH is effective in decreasing fat pad weight. No apparent dose dependency in body weight and WAT weight was observed, suggesting that the effect reached a plateau at the low dose. There was no significant difference in skeletal muscle weight among the three groups. Therefore, it is suggested that AcOH suppresses body fat accumulation without lowering skeletal muscle weight.

The effect of AcOH on body fat accumulation was previously reported by Yamashita et al. (*9*). Although they advocated the mechanism of suppressing body fat accumulation by downregulating lipogenesis, significantly lower energy intake was also observed in AcOH treatment groups in their study. Therefore, AcOH may have another effect, reducing energy intake, leading to the loss of body fat. In contrast, no difference in energy intake or downregulation of lipogenesis was observed in this study. Additionally, although it has been reported that the composition of gut flora affects obesity (*22*), no significant differences were observed in the cecal contents of the three groups of mice used in this study in the pattern of microbial diversity of *Bifidobacterium*, *Lactobacillales*, *Bacteroides*, *Prevotella*, and *Clostridium* (data not shown). Therefore, the main mechanism by which AcOH intake suppressed body fat accumulation in this study is suggested to be upregulation of fatty acid oxidation, as described below.

We analyzed the expression of genes for SREBP-1 and PPARα, which regulate gene expression of lipogenetic enzymes and fatty acid oxidation, respectively (*23*, *24*), in the liver, where almost all acetate in the portal circulation is metabolized. No changes were observed in the genes for SREBP-1 or lipogenic enzymes, such as FAS and ACC. However, Yamashita et al.

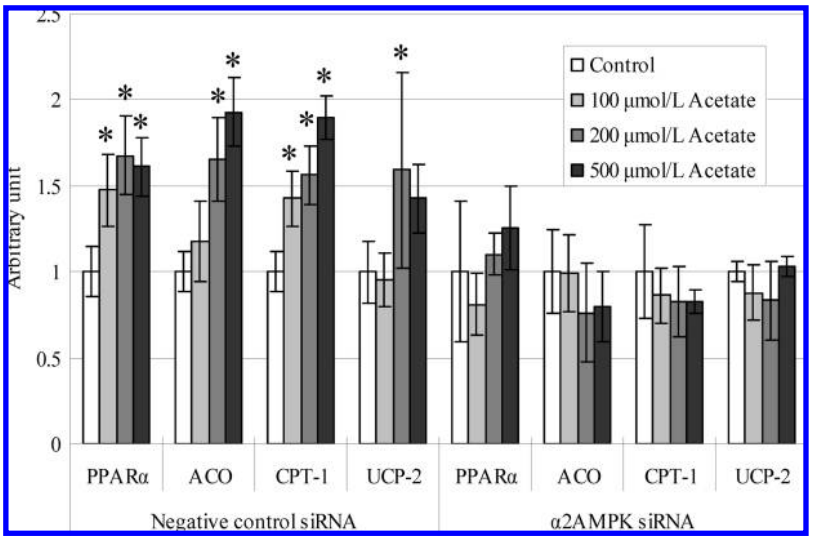

Figure 2. Effect of acetate on mRNA levels of PPARα, ACO, CPT-1, and UCP-2 in HepG2 cells transfected with negative-control siRNA or α2 AMPK siRNA. Values are means ± SD; $n = 6$. (∗) Significantly different ($p < 0.05$, Dunnett's multiple-comparison test) versus the control.

reported that AcOH administration to Otsuka Long-Evans Tokushima fatty (OLETF) rats, fed a normal laboratory diet, reduced the expression of lipogenic genes, such as FAS and ACC (*9*). This discrepancy may be explained by two possibilities: first, Yamashita et al. used OLETF rats, while we used C57BL/6J mice. Second, Yamashita et al. fed their rats a normal fat diet, while we fed our mice a high-fat diet. High-fat diet loading is reported to suppress gene expression of lipogenic enzymes, such as FAS, and rarely causes elevation of blood TG in C57BL/6J mice (*25*). Further investigation is required to ascertain whether inhibition of lipogenesis plays a role in suppression of body fat accumulation by AcOH.

PPARα gene expression in both the high- and low-dose groups was significantly upregulated. Gene expression of molecules regulated by PPARα (ACO, a rate-controlling enzyme involved in fatty acid β oxidation; CPT-1, regulating acyl-CoA inflow and β oxidation in the mitochondrial outer membrane; and UCP-2, involved in thermogenesis) were also elevated. The results of this study suggest that AcOH suppresses body fat accumulation by increasing fatty oxidation and thermogenesis in the liver through PPARα.

AMPK is a key enzyme related to energy adjustment in the cells. Recently, Ravnskjae et al. reported that AMPK promotes transcription of PPARα (*26*). In addition, Suzuki et al. reported that, to increase the transcription of PPARα, the catalytic subunit α2 of AMPK needed to be phosphorylated and form a complex with regulatory subunit β2γ1 (*16*). We validated gene expression of each subunit in this study: α2, β2, and γ1 for AMPK in the liver of C57BL/6J (data not shown), and suggest that AcOH leads to PPARα gene expression through phosphorylation of AMPK consisting of α2, β2, and γ1 subunits.

Ingested AcOH or vinegar is immediately absorbed into the bloodstream, and the blood acetate concentration can reach up to several hundred micromoles per liter (*9*, *27*). Consistent with this, it was observed that PPARα transcription is increased by the addition of 100−500 μmol/L acetate to HepG2 cells. In addition, the expression of genes for ACO, CPT-1, and UCP-2 was also elevated. Although initiation of their transcriptional activation took 3 h from the time of acetate addition, it has been reported that a comparable time duration is necessary for PPARα transcription by leptin (*16*). Therefore, a 3 h time lag may occur between phosphorylation of α2 AMPK and translocation to the nucleus to induce PPARα gene transcription.

PPARα gene expression was increased by acetate in negative-control siRNA-transfected HepG2 cells. Furthermore, the expression of genes for ACO, CPT-1, and UCP-2, regulated by PAPRα, was also increased by acetate. However, an increase in the expression of these genes was not observed in α2 AMPK siRNA-transfected HepG2 cells. These results indicate that AcOH can enhance the expression of genes for PPARα, fatty-acid-oxidation-related enzymes, and UCP-2, through α2 AMPK in liver, that is, enhancing fatty oxidation and therefore suppressing body fat accumulation.

In conclusion, the results of this study reveal that AcOH upregulates the expression of genes for fatty acid oxidation enzymes and thermogenic protein, such as ACO, CPT-1, and UCP-2, through a α2 AMPK/PPARα-mediated pathway and suppresses accumulation of body fat and liver lipids. We intend to perform further clinical studies to confirm fat pad reduction and energy consumption enhancement by vinegar intake. Moreover, we will investigate the effect of AcOH on fatty oxidative activation in other organs, particularly skeletal muscles.

ABBREVIATIONS USED

ACC, acetyl-CoA carboxylase; ACO, acyl-CoA oxidase; AcOH, acetic acid; AMPK, 5′-AMP-activated protein kinase; ATP-CL, ATP citrate lyase; CPT-1, carnitine palmitoyl transferase-1; FAS, fatty acid synthase; PPARα, peroxisome-proliferator-activated receptor α; SREBP-1, sterol regulatory element binding protein-1; T-chol, total cholesterol; TG, triacylglycerol; UCP-2, uncoupling protein-2; WAT, white adipose tissue.

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