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Conjugated linoleic acid supplementation caused reduction of perilipin1 and aberrant lipolysis in epididymal adipose tissue ☆

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ABSTRACT

Perilipin1, a coat protein of lipid droplet, plays a key role in adipocyte lipolysis and fat formation of adipose tissues. However, it is not clear how the expression of perilipin1 is affected in the decreased white adipose tissues (WAT) of mice treated with dietary supplement of conjugated linoleic acids (CLA). Here we obtained lipodystrophic mice by dietary administration of CLA which exhibited reduced epididymal (EPI) WAT, aberrant adipocytes and decreased expression of leptin in this tissue. We found both transcription and translation of perilipin1 was suppressed significantly in EPI WAT of CLA-treated mice compared to that of control mice. The gene expression of negative regulator tumor necrosis factor α (TNFα) and the positive regulator Peroxisome Proliferator-Activated Receptor-γ (PPARγ) of perilipin1 was up-regulated and down-regulated, respectively. In cultured 3T3-L1 cells the promoter activity of perilipin1 was dramatically inhibited in the presence of CLA. Using ex vivo experiment we found that the basal lipolysis was elevated but the hormone-stimulated lipolysis blunted in adipose explants of CLA-treated mice compared to that of control mice, suggesting that the reduction of perilipin1 in white adipose tissues may at least in part contribute to CLA-mediated alternation of lipolysis of WAT.

1. Introduction

Conjugated linoleic acids (CLA), a group of positional and geometric isomers of fatty acid, are reported to benefit human health by biological processes including anti-obesity, anti-carcinogenesis, anti-inflammation and anti-atherogenesis [1,2]. Natural CLA are produced by the biohydrogenation in rumen of ruminant animals in which the main isomer is cis-9, trans-11 (c9t11) [3], while commercial CLA supplements are usually synthetic isomeric mixtures of c9t11 and trans-10, cis-12 (t10c12) in equal amounts. c9t11- and t10c12-isomer are implicated to be responsible for anti-tumorigenesis [4,5] and for the regulation of energy metabolism [6,7], respectively. Dietary administration of either the mixture of c9t11 and t10c12 or t10c12 alone is efficient to mediate the adipose-lowering effects in mice [8]. However, numerous investigations demonstrate that CLA cause mouse lipodystrophy with metabolic disorders [9–11], making it necessary to seek insight into the CLA-mediated fat loss in order for the safe utilization of this nutrient in humans.

Lipid droplet formation, a crucial factor for the determination of fat mass, depends on the participation of lipid droplet coat proteins [12] such as Perilipin–Adipophilin–Tip47 (PAT) family including perilipin1 (Perilipin A), Perilipin2 (Adipophilin), Perilipin3 (Tip47), Perilipin4 (OXPAT/MLDP) and Perilipin5 (S3–12). Perilipin1 is most abundant on the surface of large adipocyte lipid droplets and plays an important role in adipocyte lipolysis [13]. The expression of perilipin1 is regulated positively by PPARγ and negatively by TNFα [14,15].

It is still elusive how perilipin1 is regulated in adipose tissues in response to the CLA treatment. In primary human adipocytes the treatment of t10c12 at a dose of 30 μM for 7 days [16] or 9 days [17] suppresses perilipin1 expression. A time course experiment [18] conducted in cultured SV human adipocytes shows that protein signals of perilipin1 in response to t10c12 at the same dose above increases at the first 12 h, decreases at day 4 and vanishes at day 8. However, studies in cultured 3T3-L1 cells [19] demonstrate that the addition of t10c12 at a dose of 75.4 μM for 8 days makes perilipin1 expression increase more than 6-fold. The contrary results in vitro studies, reflecting the different effects of CLA on perilipin1 expression in different circumstances, also exist

Abbreviations: CLA, conjugated linoleic acids; c9t11, cis-9, trans-11; EPI, epididymal; HSL, hormone-sensitive lipase; LA, linoleic acids; PPARγ, Peroxisome Proliferator-Activated Receptor-γ; t10c12, trans-10, cis-12; TNFα, tumor necrosis factor α; TG, triglyceride; WAT, white adipose tissue.

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in investigations in mice. Ippagunta et al. [20] reports that 0.5% of CLA supplement in diets containing soy oil or coconut oil for 10 days facilitates the expression of perilipin1 in EPI fat pad of lean mice, whereas House et al. found that addition of 1% of dietary CLA for 14 days inhibits perilipin1 expression in EPI adipose tissue of M16 polygenic obese line of mice [21].

In this study lipodystrophic mice were established by dietary CLA supplement at a dose of 1.5% for 13 days. We investigated the gene expression of perilipin1 and its regulators as well as the lipolysis of EPI adipose tissues which depends on the participation of perilipin1.

2. Materials and methods

2.1. Animal welfare and diet-treated mice

All of the experiments involving animals were reviewed and approved by the Institutional Animal Care and Use Committee of Henan Province. Male Kunming mice at 5 weeks old were purchased from Henan Laboratory Animals Science Center (HLAC) (Zhengzhou, China), housed at 22 °C with 50% of humidity on a 12/12-h light/dark cycle and allowed free access to water and diet. These mice were fed a normal chow diet (HLAC) for 1 week for acclimation, and then randomized into two groups ($n = 20$) with one group receiving a diet supplemented with LA (1.5% w/w) and another receiving a diet with CLA (1.5% w/w) for 13 days. The CLA used were a mixture of equal amount of c9t11 and t10c12, which reduces adipose tissue weight to the same extent as t10c12 isomer does alone [8]. Table 1 showed the components of the supplements. After 13 days feeding, euthanasia of mice was performed by anesthesia using intraperitoneal injection of pelltobarbitalum natricum (80 mg/kg).

2.2. Plasmid construction

A DNA fragment of perilipin1 promoter from −946 to +104 was amplified by PCR using mouse genomic DNA as the template with forward primer GTGGCATCTCAGGCTAAGT and reverse primer ACACTCACTGCAGCAGAC in TaKaRa LA Taq® Hot Start Version system. The PCR product was cloned into pMD®19-T Simple Vector(TAKARA), and from which a Sal1-Kpn1 fragment was harvested and inserted into between Xho1–Kpn1 sites of multicloning site of pGL3-basic to generate a new construct pGL3–plin1.

2.3. Reagents and antibodies

All chemical reagents were from Sigma Chemical Co., LA and CLA were purchased from Zhongshan Unicare Natural Medicine Co., Ltd. Adrenaline hydrochloride was from Shanghai Harvest Pharmaceutical Co., Ltd. TRIzol reagent and DMEM 11995 were from Invitrogen. Reverse transcriptase and Dnase I were from Promega, Glycerol GPO-POD assay kit was from Applygen Technologies Inc. Protease inhibitor Cocktail was from Roche. Antibodies against β-actin and perilipin1 were from Santa Cruz Biotechnology Inc. ECL Plus™ Western Blotting Detection Reagents was from GE Healthcare Bio-Sciences Corp. Higene transfection reagent was from Beijing Applygene Company. Luciferase reporter assay system was from Promega.

Table 1
Fatty acid profile of the conjugated linoleic acids supplements[a].

Fatty acid	LA	CLA
C16:0	3.58	5.24
C18:0	1.26	2.15
C18:1	11.21	11.65
Linolenic acid	2.35	ND
Linoleic acid (LA)	80.13	0.75
cis-9,trans-11 CLA	ND	37.15
trans-10,cis-12 CLA	ND	36.96
Other CLA isomers	ND	5.57
Other fatty acids	1.47	ND

ND: not detected.
[a] The percentage of compositions by weight.

2.4. Morphometry

At the terminal day of LA or CLA feeding mice were sacrificed. Sections (5 μm) of EPI adipose tissues were fixed in 10% phosphate-buffered Formalin, stained with hematoxylin/eosin Y and visualized histologically using a Leica DMIL light microscope with a 20× objective. The digital images were captured with Leica EC3 camera. Total number of adipocytes in a section was counted and the cross-sectional areas of the cells were calculated. We regarded the cell shape as nearly round and determined the cross-sectional area of adipocyte by the formula πr^2 (r = radius) in μm^2.

2.5. Real time PCR analysis of gene expression

Quantitative PCR analysis was performed using SYBR Premix Ex Taq™ PCR Master Mix in Mastercycler® ep realplex PCR detection system (Eppendorf). The EPI fat was homogenized in TRIzol reagent, and total RNA was isolated according to the manufacturer's specifications (Invitrogen). In each sample the concentration of RNA was measured using NanoDrop™ 2000 (Thermo) followed by the assessment of RNA quality in electrophoresis of denatured agarose gel. One microgram of RNA samples was treated with Dnase I and reverse transcribed using random hexamer primers with reverse transcription reagents. The gene transcription was quantified using 25 ng of each cDNA sample, normalized by internal control gapdh, and assayed in triplicates. Table 2 showed the sequences of the primers for real time PCR.

2.6. Western blotting

The EPI WAT were homogenized in 1× RIPA buffer (50 mM Tris–Cl pH 7.4, 150 mM NaCl, 1% NP40, 0.25% Na-deoxycholate, 1 mM PMSF, 1 mM sodium orthovanadate and 1× Roche complete mini protease inhibitor cocktail). Lysate was incubated on ice for 20 min with gentle rocking and centrifuged at 14,000*g* for 10 min at 4 °C. Equivalent amounts of supernatant samples were resolved by 10% SDS–PAGE, transferred to a nitrocellulose membrane and immunoblotted subsequently using antibodies against Perilipin1 and β-actin in 5% milk TBST (50 mM Tris–Cl, pH 7.4, 150 mM NaCl, 0.1% Tween 20). The membrane was stained by ECL plus and allowed to expose X-ray film.

2.7. Preparation of fatty acids solution

Purified CLA (c10t12) or LA (Sigma) was mixed with 100% ethanol, dried in nitrogen atmosphere, and then dissolved in 0.1 M NaOH for a fatty acid–NaOH stock solution of 100 mM. This fatty acid stock solution was added into the serum-free media to give a final concentration of 100 μM for working medium.

2.8. Cell culture and luciferase assay

3T3-L1 cells were cultured in DMEM containing 10% fetal bovine serum in a 5% CO_2 37 °C incubator and transfected with relevant DNA constructs for 16 h. Subsequently the medium of transfected cells were replaced with the fresh working medium for 24 h. Luciferase activity of the cells was measured on a lumino-

Table 2
Primer sequences used for gene expression analysis by real-time PCR.

Target	Forward primer	Reverse primer	GeneBank Accession No.
GAPDH	TTGCTGTTGAAGTCGCAGGAG	TGTGTCCGTCGTGGATCTGA	NM_008084.2
PPARγ	CATAAAGTCCTTCCCGCTGA	GAAACTGGCACCCTTGAAAA	NM_001127330.1
TNF-α	GCCACCACGCTCTTCTG	GGTGTGGGTGAGGAGCA	NM_013693.2
LEPTIN	GACACCAAAACCCTCAT	CAGAGTCTGGTCCATCT	NM_008493.3
PLIN1	GGGACCTGTGAGTGCTTCC	GTATTGAAGAGCCGGGATCTTTT	NM_175640.2
PLIN2	GTGGAAAGGACCAAGTCTGTG	GACTCCAGCCGTTCATAGTTG	NM_007408.3
PLIN3	ACATCCACCGCTCCTGTGAC	GGCTGGACAGACTGCAGGAA	NM_025836.3
PLIN4	TCCTGCTCTGAGGGACCCTT	TCTTGCCTTTGGATTTGGGG	NM_020568.3
PLIN5	TGTCCAGTGCTTACAACTCGG	CAGGGCACAGGTAGTCACAC	NM_025874.3

meter (Thermo Scientific Fluoroskan Ascent, FL) according to the instruction of luciferase reporter assay system.

2.9. Lipolysis

The lipolysis assay was conducted according to the previous report [20]. The conditioned media were collected and stored at −80 °C for analysis. Glycerol concentrations of the media were measured using Glycerol GPO-POD assay kit following the manufacturer's instructions.

2.10. Statistical analysis

The data are presented as the means ± SEM. Comparisons of data were made using two-tailed Student's *t* test for independent data. The significance level was set at $p < 0.05$.

3. Results

3.1. Body weight and food intake

We supplemented 1.5% CLA (CLA group) or 1.5% LA (LA group) to the diet of male Kunming mice at 6 weeks old for 13 days. During the 12 day period we found no changes of body weight between the two groups (Fig. 1A) and there was a trend that CLA group ate more than LA group from day 2 to day 6 but the amount of food consumption was only significantly different at day 3 and day 11 (Fig. 1B) between groups.

3.2. CLA reduced WAT

Dietary CLA supplement at a dose of 1.5% diminished fat so fast that we were not able to find the obvious EPI WAT at day 14 of the treatment (data not shown). Photos at day 13 showed the gross reduction (Fig. 2A, upper panel, arrows) and the histological alternation (Fig. 2A, lower panel) of EPI WAT, in which the size of adipocytes was relatively uniform in LA group but divergent in CLA group. Wet weight of WAT normalized by body weight in CLA group reduced 53% compared to that in LA group and is significantly different between the two groups (Fig. 2B). Under microscope with objective of 20 magnifications, the adipocyte area was measured and the percentage of cells with different area was calculated. As Fig. 2C illustrated, the distribution pattern of adipocytes changed dramatically in which the peak of cell abundance left shifted from the medium size in LA mice to larger size in CLA mice, and the cells larger than 10,000 μm^2 only appeared in CLA mice. The mRNA level of leptin, which is a marker of adipose tissues, was remarkably decreased in WAT (Fig. 2D), demonstrating the fat loss of CLA mice.

3.3. The gene expression of perilipin1 and its regulators

In EPI WAT of CLA mice the mRNA level of perilipin1 significantly reduced 71% compared to that in LA mice (Fig. 3A); parallel

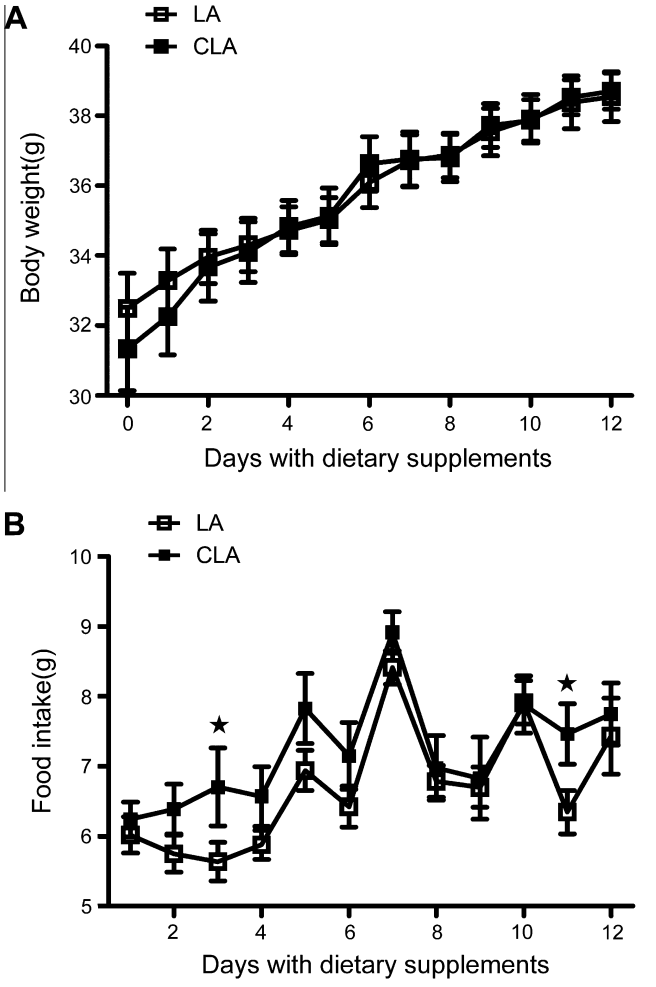

Fig. 1. Body weight and food intake. Male Kunming mice of 6 weeks old were fed the diets with 1.5% CLA or LA supplement for 13 days. Body weights (A) and the food intake (B) were measured daily for 12 days. Data are means ± SEM of 12 mice per group. *$P < 0.05$.

with this observation, Western blotting showed that the protein perilipin1 remarkably decreased (Fig. 3B). The expression of some other members of PAT family was also detected, with the significant rising of perilipin2 and the decline of perilipin4 (Fig. 3A). The mRNA levels of TNFα, the negative regulator of perilipin, increased 3.29-fold, while that of PPARγ, the positive regulator, lost 81% (Fig. 3C). In combination, these data suggested that CLA not only inhibited the expression of perilipin1 but also affected its regulators.

3.4. CLA inhibited the activity of perilipin1 promoter

Since we demonstrated dietary CLA decreased the expression of perilipin1 gene in mouse EPI WAT, it is reasonable that CLA

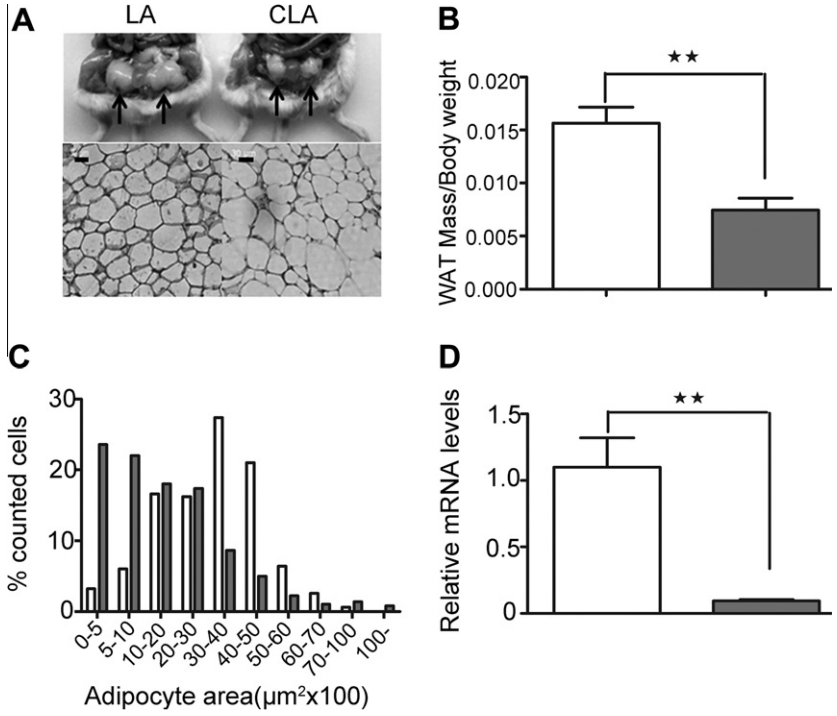

Fig. 2. Alternation of WAT. At day 13 of the supplement of dietary CLA or LA, mice were sacrificed for terminal experiments. (A) Photograph showing the gross differences of the EPI adipose tissues (WAT) (arrows, upper panel) between LA or CLA mice. Sections (5 μm thick) of EPI adipose tissues were stained with hematoxylin/eosin and illustrating the histological alternations (lower panel). Scale bar represents 30 μm. (B) Mass of the WAT corrected by their body weights. In the tissue sections, adipocyte area was measured, and the percentage of adipocytes with different area was calculated (C) under the objective of 20 magnification. (D) The transcription level of leptin gene in WAT was assessed by real-time PCR. Open or filled box represented the data from LA or CLA mice, respectively. (B and D) Data are means ± SEM of 6 mice per group. $^{**}P < 0.01$.

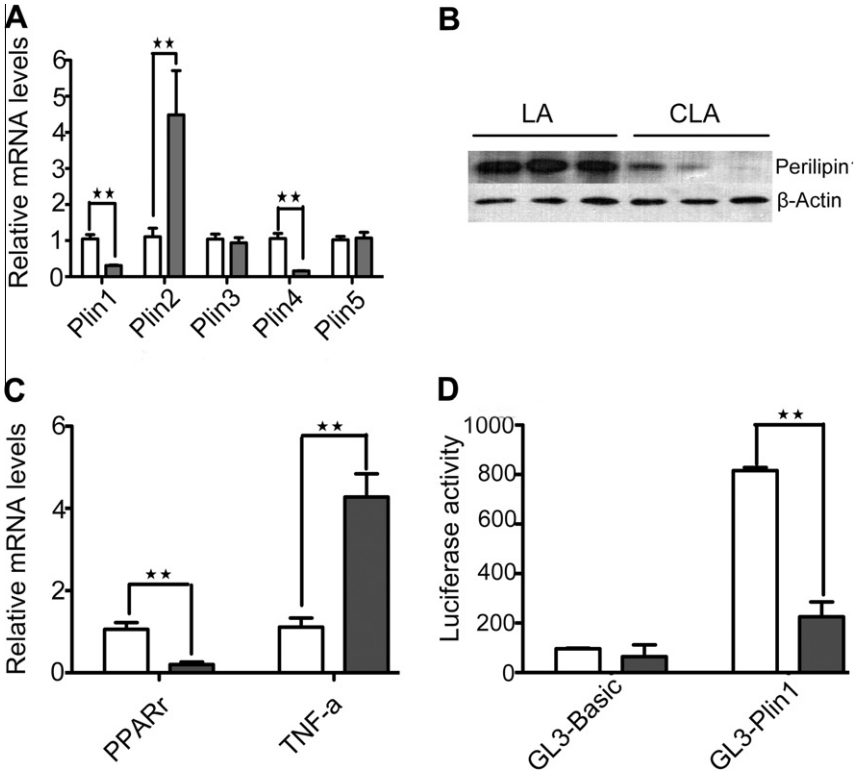

Fig. 3. Gene expression of perilipin1 and its regulators. With real-time PCR analyses, the mRNA levels of PAT (A) including perilipin1 (Plin1) as well as the cytokines TNFα and PPARγ (C) were quantified in 1 μg of total RNA of WAT from LA (open box) or CLA (filled box) mice. Data normalized by internal control gapdh are shown as means ± SEM of 6 mice per group. $^{**}P < 0.01$. (B) Protein signals of perilipin1 were detected from the lysate of epididymal adipose tissues of LA or CLA mice by immuno-blotting against perilipin1 and β-actin. (D) 3T3-L1 cells were transfected with pGL3-plin1 or promoter-less pGL3-basic as negative control, 16 h later the cells were incubated in fresh working medium containing 100 μM of LA (open box) or CLA (filled box) for 24 h and harvested for the assessment of luciferase activity on a luminometer.

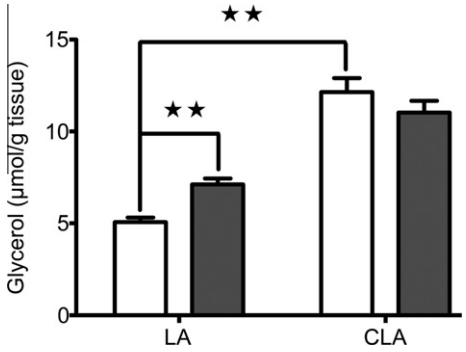

Fig. 4. Lipolysis assay in ex vivo. EPI adipose tissues was dissected and incubated at 37 °C in the serum-free culture medium in the absence (open box) or presence (filled box) of 0.01 mg/ml adrenaline hydrochloride for 3 h. Data are presented as means ± SEM of 10 mice per group. $^{**}P < 0.01$.

suppresses the promoter activity of perilipin1 gene in adipocytes. To prove this assumption, we transiently transfected 3T3-L1 cells with a luciferase reporter construct containing the proximal promoter of the mouse perilipin1 gene. The result of luciferase assay showed that the addition of t10c12 in the culture medium dramatically inhibited the promoter activity in cells compared to that of LA (Fig. 3D).

3.5. CLA caused the aberrant lipolysis of adipose tissues

Given that perilipin1 plays the crucial roles in lipolysis, we predicted that the reduction of perilipin1 would affect the rate of this biological activity, which is one of the determinants of fat mass. The EPI WAT of mice fed with LA or CLA supplement for 13 days were dissected and incubated in serum-free medium for the assessment of basal and hormone-stimulated lipolysis. The released glycerol in the media from a piece of fat in both presence and absence of epinephrine (Fig. 4) increased significantly in CLA group compared to that in LA group. Epinephrine significantly promoted the release of glycerol in LA group, but failed to do so in CLA group. The combined data above indicated that the WAT of CLA mice had an increased basal lipolysis but lost the ability to respond to epinephrine treatment.

4. Discussion

CLA-induced lipodystrophy is observed in many experiments in mice, providing an animal model for the pathophysiological study of adipose tissue. Here we obtained this model by feeding male mice with 1.5% of CLA supplement for 13 days in which fat loss and adipocyte aberrance were accompanied by insulin resistance and hepatomegaly (data not shown). Because of the importance of perilipin1 in the lipid droplet formation the expression of this gene were detected in EPI WAT of the CLA treated mice. We found both transcription and translation of perilipin1 was suppressed significantly in EPI WAT of CLA-treated mice compared to that of control mice. It was difficult to reconcile our result with that reported by Ippagunta et al. [20] because of several differences of experiment conditions. First, 1.5% of CLA supplement was used in this study compared to their 0.5%. The increased dose of CLA could result in different responses in mice. Second, unlike their experiment, neither soy oil nor coconut oil was added to the base diet. Previous reports address PPARγ facilitates the perilipin1 expression by binding the PPAR response elements (PPREs) located in the promoter [22] whereas TNFα down-regulates perilipin1 expression [23,24]. In the present study CLA-mediated suppression of perilipin1 was concomitant to the down-regulation of PPARγ and up-regulation of TNFα. It was conceivable that the drop of perilipin1 was attributed to the network regulation of regulators.

The increase of basal lipolysis parallel to the reduction of the perilipin1 under CLA treatment in this study agreed with the previous reports that in perilipin1 null mice the lipolysis of adipose tissue was promoted [13] and supported the point that perilipin1 could inhibit the basal lipolysis by forming a protective barrier on lipid droplets [12]. The down-regulation of perilipin1 and the up-regulation of perilipin2 would lead to the displacement of perilipin1 by perilipin2 on the surface of lipid droplets as Soonkyu Chung indicates [18]. Given the report that perilipin2-coated lipid droplets in adipocytes from perilipin1 null mouse embryonic fibroblasts fail to support the docking of HSL after the stimulation of β-adrenergic receptors [25], the compromised hormone-stimulated lipolysis of EPI WAT of CLA mice in this study may result from the interchange of perilipin1 and perilipin2 on the surface of lipid droplets, which blocked HSL to get access to its substrates in the cells in response to hormone stimulation. The rising of TNFα in adipose tissues of CLA-treated mice may also activate inflammation pathways by which TNFα promote lipolysis in adipocytes [23].

We also demonstrated in a luciferase reporter system that the addition of CLA inhibited the expression of perilipin1 gene. Previous promoter studies indicate that perilipin1 is regulated by PPARγ through Peroxisome Proliferator Response Element (PPRE) located around −2000 [22] and by estrogen receptor-related receptor α(ERRα) via the ERRα-response element [26] in the promoter. Since the promoter(−946 to +104) used here lacked the PPRE element we supposed that CLA-mediated perilipin suppression would be independent of direct action of PPARγ in the WAT of mice. Whether the down-regulation of perilipin1 was attributed to the alternation of ERRα in CLA mice needs to be exploited in the future.

Conflict of interest

None declared.

Acknowledgments

G.Q.Y. designed the research; C.D.M., H.J.L., B.Z. and X.M.Z conducted the research; L.Q.H. provided the technical support, G.Y.Y. analyzed the data; G.Q.Y. wrote the paper and G.Q.Y. had primary responsibility for the final content; and all authors read and approved the final manuscript. We thank Shaofang Lu for care of animals and secretarial assistance.

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