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t10,c12 Conjugated Linoleic Acid Upregulates Hepatic *De Novo* Lipogenesis and Triglyceride Synthesis *via* mTOR Pathway Activation

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In mice, supplementation of *t10,c12* conjugated linoleic acid (CLA) increases liver mass and hepatic steatosis *via* increasing uptake of fatty acids released from adipose tissues. However, the effects of *t10,c12* CLA on hepatic lipid synthesis and the associated mechanisms are largely unknown. Thus, we tested the hypothesis that gut microbiota-producing *t10,c12* CLA would induce *de novo* lipogenesis and triglyceride (TG) synthesis in HepG2 cells, promoting lipid accumulation. It was found that treatment with *t10,c12* CLA (100 μM) for 72 h increased neutral lipid accumulation *via* enhanced incorporation of acetate, palmitate, oleate, and 2-deoxyglucose into TG. Furthermore, treatment with *t10,c12* CLA led to increased mRNA expression and protein levels of lipogenic genes including SREBP1, ACC1, FASN, ELOVL6, GPAT1, and DGAT1, presenting potential mechanisms by which CLA may increase lipid deposition. Most strikingly, *t10,c12* CLA treatment for 3 h increased phosphorylation of mTOR, S6K, and S6. Taken together, gut microbiota-producing *t10,c12* CLA activates hepatic *de novo* lipogenesis and TG synthesis through activation of the mTOR/SREBP1 pathway, with consequent lipid accumulation in HepG2 cells.

Keywords: Conjugated linoleic acid, hepatic steatosis, *de novo* lipogenesis, triglyceride synthesis, nutrient sensing mTOR pathway

Introduction

Conjugated linoleic acids (CLA) are a group of geometric and positional isomers of linoleic acid (C18:2) and characteristically contain conjugated double bonds [27, 28]. CLA is synthesized *via* biohydrogenation and isomerization of linoleic acid by rumen bacteria [21] and delta-9 dehydrogenation of *trans*-11 vaccenic acid in mammalian tissues [9, 26]. The predominant CLA isomer in nature is *cis-9,trans-11* CLA (*c9,t11* CLA, >80%), and is chiefly found in meat and dairy products, whereas *trans-10,cis-12* CLA (*t10,c12* CLA) is only present in minor amounts in food.

Numerous studies have demonstrated that the biochemical and physiological properties of CLA include reduction of body fat and modulation of lipid metabolism [2, 3, 6, 13], modulation of immune function [20, 32], antioxidative activity [33], and growth promotion [34]. It is well established that CLA has the ability to reduce adipogenesis in adipose tissues both *in vitro* and *in vivo*. Indeed, CLA prevented lipid accumulation through reduction of peroxisome proliferator-activated receptor gamma (PPARγ) in rodent [5] and bovine preadipocytes [31]. Dietary supplementation with 0.5% CLA for 5 weeks resulted in decreased adipose tissue cellularity in Sprague-Drawley rats [1]. Similarly, *t10,c12* CLA supplementation was associated with decreased expression of C/EBPa in 3T3-L1 adipocytes [19], followed

by attenuation of adipocyte differentiation, proliferation, and lipogenesis in adipose tissue [16].

Interestingly, certain populations of gut microbiota are involved in health-promoting properties *via* CLA [30], but their role is still debated. Recently, it was reported that CLA-producing bacteria could mediate the metabolism in mice supplemented with both high-fat diet feeding and prebiotic substrates [12]. Specifically, among various CLA isomers associated with the presence of gut microbiota, *t10,c12* CLA could be responsible for anti-obesity [25] and anti-colon cancer activities [22].

However, gut microbiota-producing *t10,c12* CLA exerts contrary effects on hepatic lipid metabolism, with CLA supplementation promoting increased risk factors for steatosis and enhanced fat mass in the liver of mice [8, 14, 18, 24, 36]. Evidence providing a biochemical basis for the effect of CLA isomers on hepatic lipogenesis has recently emerged. Supplementation with CLA led to increased triacylglycerol and lipogenic enzyme mRNA levels in the liver of mice [18]. Hepatic lipogenesis increased progressively, leading to hepatic steatosis, upon CLA supplementation in mice [14]. Furthermore, feeding mice a diet supplemented with 0.3% *t10,c12* CLA for 6 weeks resulted in increased liver mass, while adipose tissue mass decreased markedly [24]. Hepatic steatosis is induced by multiple factors, including increased fatty acid influx, diminished β-oxidation, impaired ApoB containing particle secretion, and increased lipid synthesis. However, the mechanisms that underlie CLA-induced hepatic *do novo* lipogenesis and lipid synthesis are largely unknown. The present study was designed to examine the hypothesis that CLA augments hepatic lipid accumulation *via* activation of *de novo* lipogenesis and lipid synthesis in human hepatoma HepG2 cells. We demonstrate a novel role for the nutrient sensing mTOR pathway in the stimulatory function of gut microbiota-producing CLA in hepatocytes.

Materials and Methods

Materials and Reagents

Antibodies for ACC1 (3662s, 1:1,000 dilution; Cell Signaling), FASN (3189s, 1:1,000 dilution; Cell Signaling), SCD1 (2794s, 1:1,000 dilution; Cell Signaling), ELOVL6 (PA5-13455, 1:500 dilution; Thermo Scientific), DGAT1 (3845-100, 1:500 dilution; Bio Vision), GPAT1 (GTX85034, 1:1,000 dilution; GeneTex), AKT-pT308 (4058s, 1:500 dilution; Cell Signaling), AKT (9272s, 1:500 dilution; Cell Signaling), mTOR-pS2448 (2971s, 1:1,000 dilution; Cell Signaling), mTOR (2972s, 1:1,000 dilution; Cell Signaling), S6K-pT389 (9205s, 1:400 dilution; Cell Signaling), S6K (2708s, 1:400 dilution; Cell Signaling), S6-pS240/244 (5364s, 1:1,500 dilution; Cell Signaling), S6 (2217s, 1:1,500 dilution; Cell Signaling), and GAPDH (3683s, 1:1,500 dilution; Cell Signaling) were purchased from Cell Signaling Technology. Linoleic acid (free fatty acid dissolved in ethanol, #90150) and *t10,c12* CLA (free fatty acid dissolved in ethanol, #90145) were purchased from Cayman Chemical.

Cell Culture

HepG2 cells, a human hepatoma cell line, were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% heat-inactivated fetal bovine serum and 1× penicillin-streptomycin at 37°C in a humidified O_2/CO_2 (19:1) atmosphere. For treatment, HepG2 cells were supplemented with the indicated concentrations of *t10,c12* CLA dissolved in ethanol, or linoleic acid for the indicated time periods.

Nile Red Staining

HepG2 cells were cultured on collagen-coated glass coverslips and treated with *t10,c12* CLA for 72 h. Cells were fixed with 4% paraformaldehyde and washed three times with PBS, followed by staining with 0.1 μg/ml Nile-red (MP Biochemicals) in the dark for 30 min at ambient temperature. Specimens were examined using a Nikon Ti-E Eclipse inverted microscope equipped with Perfect Focus, with excitation and emission filters at 561 nm. Images were acquired using consistent laser output, gain, and offset settings for three independent experiments, and 20 cells were randomly selected and analyzed from each coverslip. Total fluorescence density was quantified using ImageJ.

Cellular Triglyceride and Cholesterol Ester Analysis

Following a 72 h treatment with linoleic acid (100 μM) or *t10,c12* CLA (100 μM), cells were washed with PBS and homogenized in 5% NP-40 in water. Cells were slowly heated to 95°C for 5 min and then were cooled down to ambient temperature; this was repeated to solubilize triglyceride. After centrifugation at 14,000 rpm for 2 min, the supernatant was collected for TG and cholesterol ester (CE) determination using enzymatic kits (Wako). Protein concentrations were measured using the Bio-Rad protein DC assay kit with bovine serum albumin as a standard. The TG and CE contents were normalized to protein concentration.

Incorporation of ^{14}C-Acetate, ^{14}C-Palmitate, ^{14}C-Oleate, and ^{3}H-2-Deoxyglucose into Triglycerides

HepG2 cells were treated with either linoleic acid (100 μM) or *t10,c12* CLA (100 μM) for 24 h, followed by insulin stimulation (100 nM) for 4 h. Insulin-stimulated HepG2 cells were incubated with 25.0 nmol of [1,2-^{14}C]-acetate (specific activity = 54.3 mCi/mmol; Perkin Elmer), 12.5 nmol [1-^{14}C]-palmitate (specific activity = 60.0 mCi/mmol; Perkin Elmer), 12.5 nmol [1-^{14}C]-oleate (specific activity = 54.4 mCi/mmol; Perkin Elmer), and 4.0 nmol [1,2-^{3}H]-2-deoxyglucose (specific activity = 50 Ci/mmol; Perkin Elmer) for 2 h at 37°C in a humidified O_2/CO_2 (19:1) atmosphere. Cells were washed three times with PBS prior to harvest using Cellstripper

(Cellgro). Total cellular lipid was prepared according to the Bligh and Dyer method [4]. The lower phase containing neutral lipids was collected and dried under nitrogen gas. Concentrated neutral lipids were then dissolved in chloroform:methanol (2:1 (v/v)) and lipid fractions were separated by thin layer chromatography (TLC, Silica 60 F_{254}; Merck) with organic mobile solvents, hexane/diethyl ether/acetic acid (70:30:1 (v/v)). Lipid dots were visualized under UV light and subsequently scraped off for radioactivity determination using a Beckman LS6500 scintillation counter.

Quantitative RT-PCR

HepG2 cells were treated with either linoleic acid (100 μM) or *t10,c12* CLA (100 μM) for 72 h. Total RNA was isolated using an RNeasy Plus Mini Kit (Qiagen Sciences) and complementary DNA (cDNA) was synthesized from 5 μg of total RNA primed with random hexamer using Superscripts II reverse transcriptase. Real-time PCR amplification was performed using iQ SYBR Green Supermix (Bio-Rad) in an Eppendorf Master cycler RealPlex2 after adjusting the threshold cycle (Ct). Reactions were performed in quadruple with an 18S internal control. Relative mRNA levels were expressed as fold increase compared with the control. Primer sequences were as shown in Table 1.

Immunoblotting

Phosphorylation levels of mTOR pathway proteins were determined by western blotting. HepG2 cells were treated with *t10,c12* CLA (50 or 100 μM) or insulin (100 nM) for 3 h. After treatment, whole tissue lysates were prepared using cell lysis buffer (Cell Signaling, #9803) supplemented with a protease and phosphatase inhibitor cocktail. Protein concentration was determined using the Bio-Rad protein assay reagent, and extracted protein was solubilized in Laemmli sample loading buffer. A Bio-Rad mini-gel system was employed to perform SDS-polyacrylamide gel electrophoresis, and a Bio-Rad electroblot system was used to transfer proteins to a PVDF membrane. Transferred proteins were immunoblotted using target primary antibodies and appropriate HRP-conjugated secondary antibodies. Enhanced chemiluminescence reagents were applied to develop the blots, which were then quantified using Bio-Rad Image Lab software.

Statistical Analysis

All data represent the results from independent quadruples. Data are expressed as means ± SEM. Comparisons between two groups were performed using the Student's t-test. For multiple comparisons, the LSD test in conjunction with ANOVA was carried out. A probability value of $P < 0.05$ was considered as statistically significant for all experiments.

Results and Discussion

HepG2 Cells Treated with Gut Microbiota-Producing *t10,c12* CLA Exhibit Increased Accumulation of Cellular TG

After digestion and absorption of lipid in the gut, the liver has a major role in maintaining lipid homeostasis. When the supply of glucose exceeds its demand, the excess is converted to fatty acids, which primarily undergo esterification with glycerol to produce TG and consequently function as energy stores. In the present study, to investigate the effect of gut microbiota-producing *t10,c12* CLA on hepatic lipid accumulation, HepG2 cells were treated with linoleic acid

Table 1. Human primer sequences for qRT-PCR.

Target gene	Reverse	Forward	Accession No.
SREBP1	Forward	5′-ACTTCTGGAGGCATCGCAAGCA-3′	NM_001005291
	Reverse	5′-AGGTTCCAGAGGAGGCTACAAG-3′	
ACC1	Forward	5′-TTCACTCCACCTTGTCAGCGGA-3′	NM_000664
	Reverse	5′-GTCAGAGAAGCAGCCCATCACT-3′	
FASN	Forward	5′-TTCTACGGCTCCACGCTCTTCC-3′	NM_004104
	Reverse	5′-GAAGAGTCTTCGTCAGCCAGGA-3′	
SCD1	Forward	5′-CCTGGTTTCACTTGGAGCTGTG-3′	NM_005063
	Reverse	5′-TGTGGTGAAGTTGATGTGCCAGC-3′	
ELOVL6	Forward	5′-CCATCCAATGGATGCAGGAAAAC-3′	NM_001130721
	Reverse	5′-CCAGAGCACTAATGGCTTCCTC-3′	
GPAT1	Forward	5′-TTGTGGCTTGCCTGCTCCTCTA-3′	NM_001244949
	Reverse	5′-AATCACGAGCCAGGACTTCCTC-3′	
DGAT1	Forward	5′-GCTACAGGTCATCTCAGTGCTC-3′	NM_001253891
	Reverse	5′-GTGAAGTAGAGCACAGCGATGAG-3′	

SREBP1, sterol regulatory element-binding protein 1; ACC1, acetyl-CoA carboxylase 1; FASN, fatty acid synthase; SCD1, stearoyl CoA desaturase 1; ELOVL6, elongation of very long chain fatty acids protein 6; GPAT1, glycerol-3-phosphate acyltransferase 1; DGAT1, diacylglycerol O-acyltransferase 1.

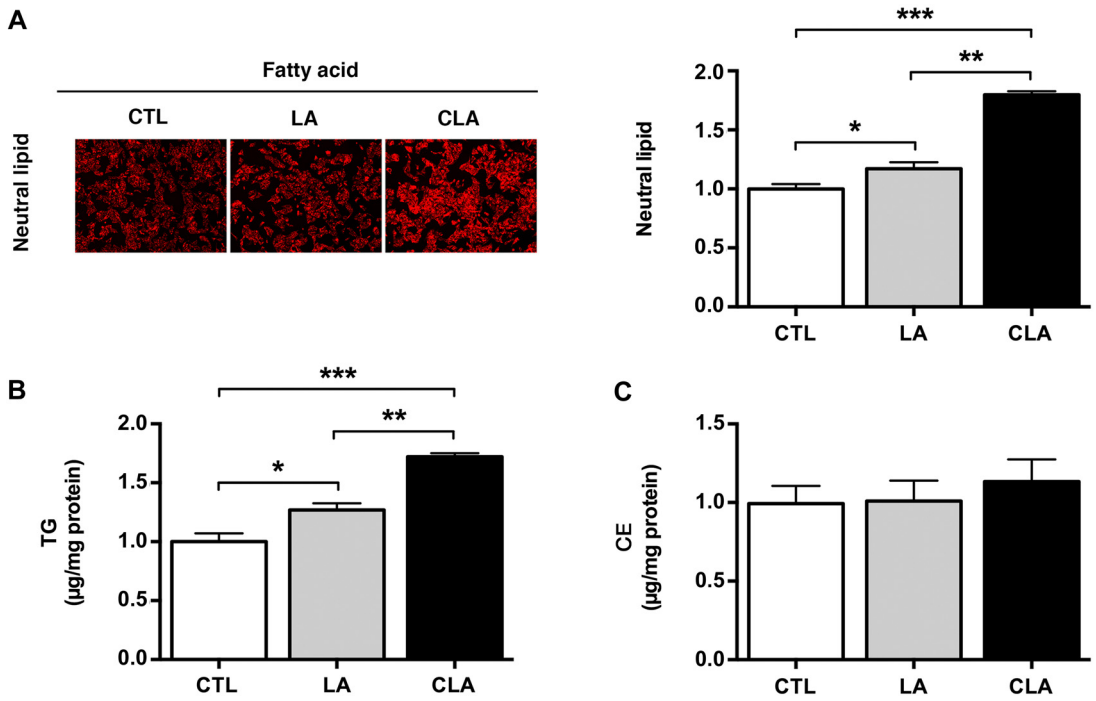

Fig. 1. (**A**) Neutral lipid content of HepG2 cells by Nile-red staining and its quantification by ImageJ and (**B**) triglyceride (TG) and (**C**) cholesterol ester (CE) contents measured by enzymatic assays in HepG2 cells.

Means ± SEM of two independent experiments are shown. LA, linoleic acid; CLA, *t10,c12* conjugated linoleic acid. **P* < 0.05; ***P* < 0.01; ****P* < 0.001.

(100 μM) or *t10,c12* CLA (100 μM) for 72 h. Neutral lipid storage was examined by Nile-red staining, and was significantly greater in CLA treated HepG2 cells compared with untreated and linoleic acid-treated cells (Fig. 1A). Accordingly, the cellular content of TG, but not CE, was also considerably higher in CLA-treated HepG2 cells (Figs. 1B and 1C). Likewise, *in vivo* studies have shown that *t10,c12* CLA supplementation in mice resulted in increased lipid deposition, followed by hepatic steatosis [8, 11, 29]. Our findings are consistent with these previous data and, furthermore, reveal that gut microbiota-producing *t10,c12* CLA increases TG accumulation, but not CE, in hepatic cells.

Gut Microbiota-Producing *t10,c12* CLA Enhances Lipid Synthesis Through Increased Incorporation of Fatty Acids and Glucose into TG

The causes of hepatic steatosis are multifactorial and include increased fatty acid uptake, impaired fatty acid oxidation and secretion of apoB-containing lipoproteins, and lipid synthesis. In particular, steatotic conditions are associated with increased lipid synthesis; thus, the current study investigated the effects of gut microbiota-producing *t10,c12* CLA on lipid synthesis. To this end, HepG2 cells treated with 100 μM linoleic acid or 100 μM CLA were incubated with radioactive substrates including ^{14}C-acetate, ^{14}C-palmitate, ^{14}C-oleate, and ^{3}H-2-deoxyglucose. TG was isolated by TLC and the ^{14}C incorporations were measured using a scintillation counter. Treatment with 100 μM *t10,c12* CLA resulted in dramatically increased incorporation of acetate, palmitate, oleate, and 2-deoxyglucose (72%, 43%, 37%, and 80%, respectively) into TG when compared with treatment with linoleic acid (Figs. 2A–2D). These results indicate that the observed increase in cellular lipid content upon gut microbiota-producing *t10,c12* CLA treatment may be associated with enhanced TG synthesis in HepG2 cells.

CLA-Induced *De Novo* Fatty Acid and TG Syntheses in HepG2 Cells Is Associated with Increased mRNA and Protein Levels of Lipogenic Genes

To study the fundamental mechanisms underpinning increased hepatic lipid synthesis, mRNA and protein levels of key lipogenic enzymes and transcription factors were determined after 24 h of *t10,c12* CLA treatment (100 μM). The mRNA levels of acetyl-CoA carboxylase 1 (ACC1), fatty acid synthase (FASN), elongation of very long chain fatty acids 6 (ELOVL6), and glycerol-3-phosphate acyltransferase 1 (GPAT1) were significantly increased in CLA-treated

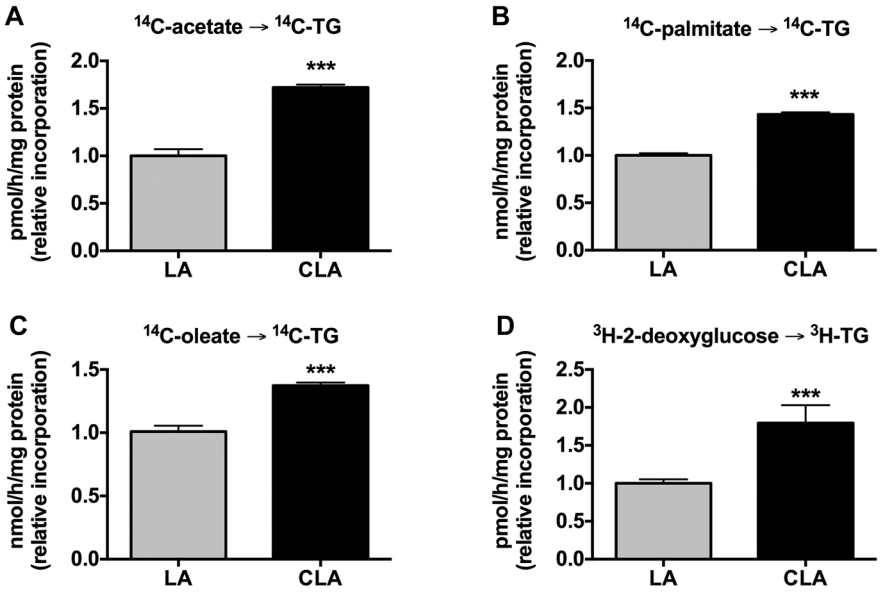

Fig. 2. Incorporation of (**A**) ^{14}C-acetate, (**B**) ^{14}C-palmitate, (**C**) ^{14}C-oleate, and (**D**) ^{3}H-2-deoxyglucose into triglyceride (TG) in HepG2 cells.

Means ± SEM of three independent experiments are shown. LA, linoleic acid; CLA, *t10,c12* conjugated linoleic acid. ***$P < 0.001$.

HepG2 cells compared with linoleic acid-treated cells (Fig. 3). Accordingly, protein levels of lipogenic enzymes, including ACC1, FASN, ELOVL6, GPAT1, and diacylglycerol acyltransferase 1 (DGAT1), were significantly increased following CLA treatment. ACC1 is the initial key and rate-limiting enzyme in *de novo* fatty acid synthesis. Several studies concluded that ACC1 plays a pivotal role in development of hepatic steatosis related to CLA supplementation [18, 35]. The second central enzyme in this pathway is FASN, which utilizes cytosolic malonyl-CoA produced by ACC1 for *de novo* fatty acid synthesis and produces 16-carbon palmitic acid (C16:0). Thus, by increasing the expressions of ACC1 and FASN, *t10,c12* CLA accelerates hepatic *de novo* lipogenesis in HepG2 cells.

Newly synthesized palmitic acid is further desaturated by stearoyl-CoA desaturase 1 (SCD1) and/or elongated by ELOVL6. Palmitic acid and stearic acid (C18:0) can be desaturated to palmitoleic acid (C16:1) and oleic acid

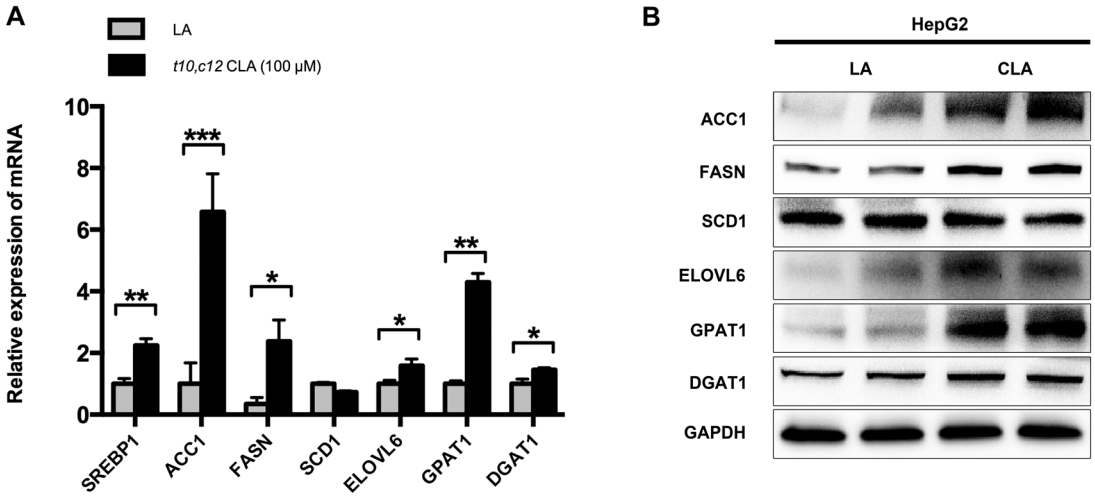

Fig. 3. (**A**) mRNA expression and (**B**) protein levels of *de novo* lipogenesis and triglyceride synthesis.

Means ± SEM of three independent experiments are shown. LA, linoleic acid; CLA, conjugated linoleic acid. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

(C18:1) by SCD1. Palmitic acid and palmitoleic acid (C16:1 n7) are converted to stearic acid (C18:0) and vaccenic acid (C18:1 n7) by ELOVL6. It has been previously shown that CLA treatment increases the concentration of palmitic acid and stearic acid [15]. In the current study, we observed increased mRNA and protein expression levels of ELOVL6, but not of SCD1, in response to gut microbiota-producing *t10,c12* CLA treatment, supporting that saturated fatty acids are increased by CLA supplementation.

Multiple steps and several enzymes are involved in the formation of TG in the liver, including GPAT1, 1-acylglycerol-3-phosphate acyltransferase (AGPAT), LIPIN, and DGAT1. Our results showed that mRNA and protein levels of GPAT1 and DGAT1 were significantly induced by *t10,c12* CLA, which supports higher TG accumulation and TG incorporation of various fatty acids and glucose. SREBP1 is a key regulator of these lipogenic enzymes, and expression levels of this transcription factor were also significantly greater following treatment with 100 μM CLA, compared with linoleic acid treatment, in HepG2 cells. Thus, the increased lipid content and TG incorporation observed upon gut microbiota-producing *t10,c12* CLA treatment in HepG2 cells can be explained by augmented expression levels of lipogenic enzymes and their transcriptional regulator SREBP1.

CLA Triggers Nutrient Sensing Pathway of mTOR/S6K/S6 to Activate SREBP1 in HepG2 Cells

Lipid homeostasis is largely influenced by environmental factors, particularly the digestion and absorption of nutrients in the gut. The nutrient sensing kinase mTOR is the core component of the multi-protein complex mTORC1, which regulates lipid homeostasis by phosphorylating several ribosomal proteins, including S6K and S6 [23]. It has recently been reported that mTORC1 directly stimulates SREBP1, and thus induces the expression of various lipogenic enzymes [10, 17, 37-39]. In this current study, strikingly, phosphorylation of mTOR, S6K, and S6 were all significantly greater in gut microbiota-producing *t10,c12* CLA-treated HepG2 cells compared with the control (Fig. 4). Chung *et al.* [7] demonstrated that *t10,c12* CLA activated mTOR and its downstream pathway, followed by activation of lipolysis in human adipocytes. The effects of CLA were found to be AKT-independent in our study, highlighting the need for future clarification of the mechanisms of AKT-independent activation of mTOR. In brief, the current findings suggest that *t10,c12* CLA stimulates mTOR/S6K/S6 activities, with consequential increased expression of SREBP1 and activation of lipogenic enzymes in HepG2 cells.

In summary, we successfully confirmed the hypothesis that CLA increases hepatic steatosis *via de novo* lipogenesis and TG synthesis through the nutrient sensing pathway. We observed a dramatic increase in hepatic lipid accumulation, cellular TG content, and TG incorporation of fatty acids and glucose in gut microbiota-producing *t10,c12* CLA-treated HepG2 cells. Through careful dissection of the lipogenic pathways, we showed enhanced expression of SREBP1, which is a key transcription factor in lipid

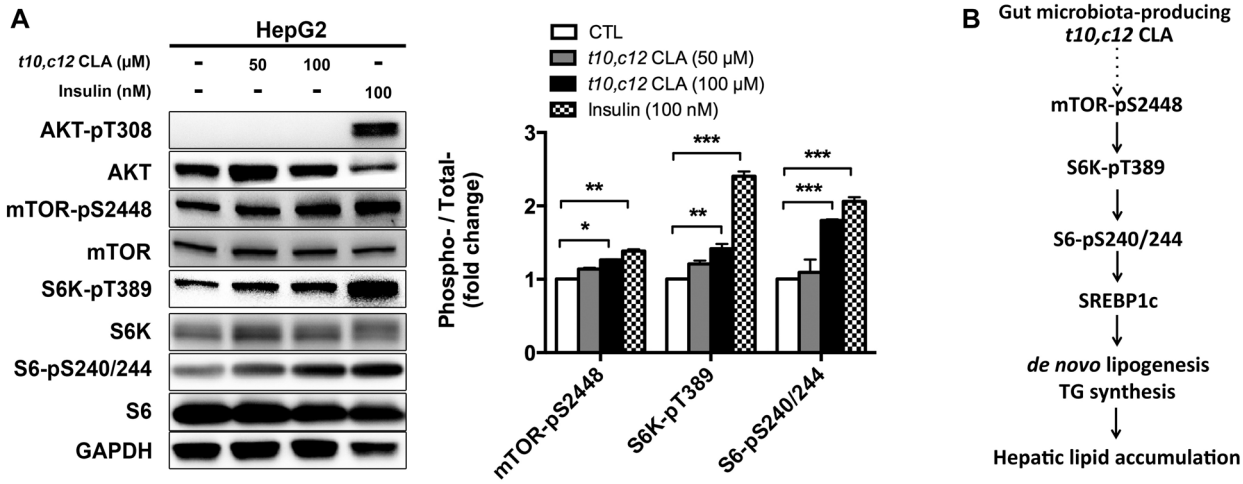

Fig. 4. Analysis of the mTOR pathway and its activation by *t10,c12* CLA.

(**A**) Western blot analysis of mTOR pathways in HepG2 cells. The ratios of phosphorylated to total proteins as measured by densitometry are shown. Data represent three independent experiments. (**B**) The schematic of gut microbiota-producing *t10,c12* CLA activation of mTOR pathways, SREBP1, *de novo* lipogenesis, lipid synthesis, and hepatic lipid deposition. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

synthesis pathways. The most notable finding of the present study was that CLA induced the activation of the nutrient sensing mTOR/S6K/S6 pathway in HepG2 cells. Taken together, our study underscores the critical role of gut microbiota-associated CLA in hepatic steatosis on the liver following digestion/absorption in the gut and identifies the mTOR/SREBP1 pathway as a major contributor to this pathology.

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