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ORIGINAL ARTICLE

Basic Study

Palmitate induces fat accumulation by activating C/EBPβ-mediated G0S2 expression in HepG2 cells

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Abstract

AIM

To determine the role of G0/G1 switch gene 2 (G0S2) and its transcriptional regulation in palmitate-induced hepatic lipid accumulation.

METHODS

HepG2 cells were treated with palmitate, or palmitate in combination with CCAAT/enhancer binding protein (C/EBP)β siRNA or G0S2 siRNA. The mRNA expression of C/EBPβ, peroxisome proliferator-activated receptor (PPAR)γ and PPARγ target genes (*G0S2*, *GPR81*, *GPR109A* and *Adipoq*) was examined by qPCR. The protein expression of C/EBPβ, PPARγ, and G0S2 was determined by Western blotting. Lipid accumulation was detected with Oil Red O staining and quantified by absorbance value of the extracted Oil Red O dye. Lipolysis was evaluated by measuring the amount of glycerol released into the medium.

RESULTS

Palmitate caused a dose-dependent increase in lipid accumulation and a dose-dependent decrease in lipolysis in HepG2 cells. In addition, palmitate increased

the mRNA expression of C/EBPβ, PPARγ, and PPARγ target genes (*G0S2*, *GPR81*, *GPR109A*, and *Adipoq*) and the protein expression of C/EBPβ, PPARγ, and G0S2 in a dose-dependent manner. Knockdown of C/EBPβ decreased palmitate-induced PPARγ and its target genes (*G0S2*, *GPR81*, *GPR109A*, and *Adipoq*) mRNA expression and palmitate-induced PPARγ and G0S2 protein expression in HepG2 cells. Knockdown of C/EBPβ also attenuated lipid accumulation and augmented lipolysis in palmitate-treated HepG2 cells. G0S2 knockdown attenuated lipid accumulation and augmented lipolysis, while G0S2 knockdown had no effects on the mRNA expression of C/EBPβ, PPARγ, and PPARγ target genes (*GPR81*, *GPR109A* and *Adipoq*) in palmitate-treated HepG2 cells.

CONCLUSION

Palmitate can induce lipid accumulation in HepG2 cells by activating C/EBPβ-mediated G0S2 expression.

Key words: Obesity; Nonalcoholic fatty liver disease; Saturated fatty acid; G0/G1 switch gene 2; CCAAT/enhancer binding protein β; Adipogenesis; Lipolysis; Proliferator-activated receptor γ

Core tip: Obesity-associated nonalcoholic fatty liver disease is characterized by excessive deposition of fat in hepatocytes. The saturated free fatty acid palmitate, the concentration of which is often elevated in obesity, is a major contributor to an increase in intrahepatic triglyceride. G0/G1 switch gene 2 (G0S2) is a critical regulator of hepatic lipid accumulation. However, the role of G0S2 and its transcriptional regulation in palmitate-induced hepatic lipid accumulation is not clear. We found that palmitate can induce lipid accumulation in HepG2 cells by activating C/EBPβ-mediated G0S2 expression.

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INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is characterized by excessive deposition of fat in hepatocytes in the absence of excessive alcohol intake. It is one of the most common emerging liver diseases throughout the world, coinciding with the global obesity epidemic[1]. Elevated plasma free fatty acid (FFA) levels are a common feature of obesity[2] and play an etiological role in the pathogenesis of NAFLD[3]. In particular, the saturated fatty acid palmitate, which makes up 30%-40% of high plasma FFA concentration[4], is a major contributor to an increase in intrahepatic triglyceride[5]. However, the molecular mechanism by which palmitate contributes to the accumulation of excess triglyceride in hepatocytes is not entirely clear.

Several studies of NAFLD have demonstrated that a decreased rate of triglyceride mobilization promotes triglyceride accumulation in the liver[6,7]. The rate-limiting step of intracellular triacylglycerol mobilization is cleavage of the first ester bond in triglycerides, which is catalyzed by adipose triglyceride lipase (ATGL)[8]. In adipocytes, the protein product of G0/G1 switch gene 2 (*G0S2*) is a dominant inhibitor of ATGL[9]. It binds directly to ATGL and attenuates ATGL-mediated lipolysis *via* inhibiting the triglyceride hydrolase activity of ATGL[9-11]. G0S2 is also abundantly expressed in the liver, suggesting that the regulatory function of G0S2 is not limited to adipose tissue[9]. Notably, *G0S2* overexpression in the liver increases the accumulation of triglycerides and promotes fatty liver formation[12,13]. Conversely, loss of *G0S2* in the liver results in a marked decrease in hepatic triacylglycerol levels and protects against high-fat-diet-induced liver steatosis[13]. These findings implicate an important role for G0S2 as a regulator of triglyceride content in the liver and as a contributor to obesity-associated liver steatosis.

G0S2 expression is regulated by a complex transcriptional mechanism that involves proliferator-activated receptor (PPAR)γ. Transactivation, gel shift and chromatin immunoprecipitation assays have identified *G0S2* as a direct target gene of *PPARγ*[14]. The transcription factor CCAAT/enhancer binding protein (C/EBP)β is involved in adipogenesis and is crucial for inducing initial expression of PPARγ during adipogenesis[15,16]. Importantly, C/EBPβ overexpression increases *PPARγ* mRNA level and triglyceride content in the liver, whereas C/EBPβ RNA interference attenuates palmitate-induced PPARγ expression and triglyceride accumulation in hepatocytes[5].

Based on these observations, we propose the following hypothesis: palmitate stimulates C/EBPβ and its downstream target PPARγ and consequent G0S2 expression, and then G0S2 contributes to palmitate-induced fat accumulation in the liver. In this study, using human HepG2 hepatoma cells, a cellular model of hepatic steatosis[17], we examined lipolysis in hepatocytes, hepatocellular triglyceride accumulation, and the expression of C/EBPβ, *PPARγ* and PPARγ-regulated genes (*G0S2*, *GPR81*, *GPR109A* and *Adipoq*) in response to palmitate treatment. In addition, *via* siRNA-mediated gene knockdown experiments, we investigated the relationship between expression of the aforementioned proteins and hepatocyte lipolysis and

lipid accumulation.

MATERIALS AND METHODS

Cell culture

HepG2 cells (China Center for Type Culture Collection, Wuhan, China) were cultured in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA, United States) containing 10% fetal bovine serum (Invitrogen), 100 U/mL penicillin, 100 μg/mL streptomycin, and 1% L-glutamine. Cells were grown at 37 ℃ in an atmosphere of 5% CO_2/95% air in a cell culture flask. The effect of palmitate was examined by addition of this agent to the cells plated in six-well plates at 2 × 10^5 cells per well.

Preparation of palmitate solution

Palmitate (Sigma, St. Louis, MO, United States) stock solution was prepared by coupling palmitate to bovine serum albumin (BSA; Sigma) as previously described[18]. Palmitate was fully dissolved in pure ethanol for a concentration of 195 mmol/L, ensuring that the final concentration of ethanol in the palmitate stock solution did not exceed 1.5% by volume. This palmitate stock solution was then added to a prewarmed BSA solution (10% w/w, 37 ℃) to achieve a final palmitate concentration of 3 mmol/L. The solution was dissolved by incubating at 37 ℃ in a water bath for a further 10 min. The final molar ratio of palmitate to BSA was 2:1. The control vehicle was prepared using a stock of 10% w/w BSA with an equivalent volume of ethanol added to match that contained in the final palmitate stock. The final concentration of ethanol was < 0.2% by volume in all experiments.

Quantitative real-time polymerase chain reaction

Total RNA was isolated from cultured HepG2 cells using TRIzol reagent (Invitrogen), and RNA quality was evaluated *via* electrophoresis. Reverse transcription (RT) was performed using Superscript Ⅱ reverse transcriptase (Invitrogen). The RT conditions for each cDNA amplification were 42 ℃ for 15 min, 85 ℃ for 5 s, and the cDNAs amplified were stored at -20 ℃. Gene expression analysis was performed by quantitative PCR (qPCR) on a StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA, United States) using SYBR Green as the detection dye. Primer sequences used for the detection of genes were designed as follows: *C/EBPβ* forward primer: 5′-CAAGCACAGCGACGAGTACAAGATCC-3′ and reverse primer: 5′- GCTTGAACAAGTTCCGCAGGGTG -3′; *PPARγ* forward primer: 5′-ACCACTCCCACTCC TTTG-3′ and reverse primer: 5′-GCAGGCTCCACTTT GATT-3′; *G0S2* forward primer: 5′-CCTCTTCGGCG TGGTGCT-3′ and reverse prime: 5′-CTGCTGCTT GCCTTTCTCC-3′; *GPR81* forward primer: 5′-CAGA CAGGCTCGGATGAAGAAG-3′ and reverse prime: 5′-TTGTAGAATTTGGGAAAGGAGGG-3′; *GPR109A* forward primer: 5′-TGGACCTGGCGTTC TTTA-3′ and reverse primer: 5′-GCTCGTGCTGCGGTTATT-3′; *Adipoq* forward primer: 5′-AGGAAAGGAGAACCTGGAGAAG-3′ and reverse prime: 5′-ATAGACTGTGATGTGGTAGGC AAA-3′; *β-actin* forward primer: 5′-TGGCACCCAGCA CAATGAA-3′ and reverse primer: 5′-CTAAGTCATAGT CCGCCTAGAA-3′. The expected size of the amplified products was 194 bp (*C/EBPβ*), 169 bp (*PPARγ*), 160 bp (*G0S2*), 240 bp (*GPR81*), 170 bp (*GPR109A*), 204 bp (*Adipoq*) and 186 bp (*β-actin*). *β-Actin* was used as a control housekeeping gene. Cycling conditions were 94 ℃ for 5 s and 60 ℃ for 30 s, followed by 45 cycles. The predicted size of the PCR products was confirmed by 2% agarose gel electrophoresis stained with ethidium bromide. Melting curve analysis was performed for each sample in direct connection to the PCR, to verify the specificity of the amplified PCR product. The results were stated as the fold difference in expression for each target gene compared to that of β-actin as an internal control in the same sample, using the $2^{-\Delta\Delta Ct}$ method. All experiments were carried out in duplicate.

Western blot analysis

To measure the nuclear C/EBPβ protein level, nuclear protein extracts were isolated from HepG2 cells using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Fisher Scientific, Waltham, MA, United States). Meanwhile, HepG2 cells were harvested and lysed with ice-cold RIPA lysis buffer containing protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany) and proteins were extracted from whole-cell lysates. The protein concentration was quantified using Bio-Rad DC Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA, United States). After denaturation by boiling of protein, equal amounts of total protein (40 μg) were loaded and resolved on 10% SDS-PAGE for 2 h at room temperature. The proteins were subsequently transferred to polyvinylidene difluoride membranes (Atto Corporation, Tokyo, Japan). The membranes were blocked with 5% non-fat milk dissolved in Tris-buffered saline/Tween 20 buffer for 2 h and incubated with primary antibodies overnight, and then the secondary antibodies for 1 h. Primary antibodies used were C/EBPβ (1:200; Santa Cruz Biotechnology, Santa Cruz, CA, United States), PPARγ (1:1000; Cell Signaling, NEB, Vienna, Austria) and G0S2 (1:100; Sigma). The β-actin antibody (1:2000;) was used as a loading control. Secondary antibody was goat anti-rabbit IgG-horseradish peroxidase conjugate (1:2000; Bio-Rad Laboratories). The immunoreactive protein bands were visualized using an enhanced chemiluminescence detection system (Amersham Pharmacia Biotech, Piscataway, NJ, United States). The density of the band was quantified using ImageJ software (NIH, Bethesda, MD, United States), and the data were transformed

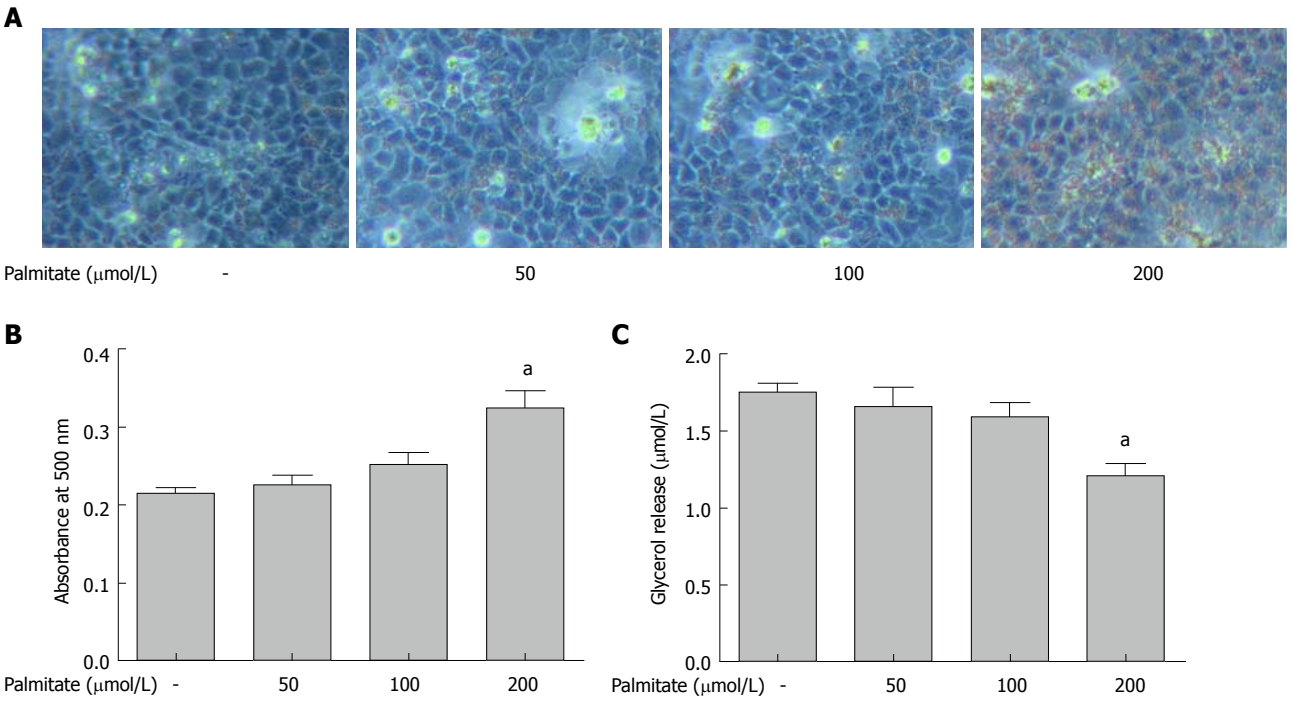


Figure 1 Palmitate-induced lipid accumulation and inhibition of lipolysis in HepG2 cells. A and B: Dose response of lipid accumulation induced by palmitate. HepG2 cells were treated with various concentrations of palmitate for 24 h, and (A) stained with Oil Red O to visualize the intracellular lipid contents (original magnification, × 100); B: Lipid accumulation was quantified by absorbance value of the extracted Oil Red O dye at 500 nm. At least three independent experiments were conducted for each measurement. Data are presented as means ± SE. $^{a}P < 0.05$ *vs* untreated control. C: Dose response of inhibition of lipolysis induced by palmitate. HepG2 cells were treated with various concentrations of palmitate for 24 h, and lipolysis was assessed by glycerol release into the medium. At least three independent experiments were conducted for each measurement. Data are presented as means ± SE. $^{a}P < 0.05$ *vs* untreated control.

and normalized relative to β-actin as the integral optical density ratio. All experiments were performed at least three times and representative data were shown.

siRNA-mediated knockdown

RNA oligonucleotides directed against *C/EBPβ* (sense sequence: GCAATCGGTTTAAACATGGCT) and *G0S2* (sense sequence: GCATCCACCAAAGGAGTTTGG) were purchased from GeneChem Co. Ltd. (Shanghai, China) to silence target proteins. A negative control siRNA was also purchased from GeneChem, which had no matches in the human genome. siRNA transfection to HepG2 cells was conducted using Lipofectamine 2000 (Invitrogen). HepG2 cells were seeded into six-well plates and allowed to culture overnight. The aforementioned siRNAs (2.5 μg) and 5 μL Lipofectamine 2000 Reagent were respectively diluted in 250 μL Opti-MEM Medium (Invitrogen) and incubated separately for 10 min at room temperature. After the 10-min incubation, equal volumes of diluted siRNA and Lipofectamine 2000 Reagent were mixed gently and incubated for 10 min at room temperature to form siRNA–lipid complexes. For transfection, the siRNA–lipid complexes were subsequently combined with HepG2 cells in six-well culture plates at 10^6 cells per well and incubated for 24 h. Knockdown efficiency of the siRNAs was determined by Western blotting. Transfected cells were treated with 200 μmol/L palmitate for 24 h before harvesting.

Oil Red O staining

HepG2 cells were grown on six-well plates, washed three times with phosphate-buffered saline, and fixed with 10% formaldehyde for 30 min at room temperature. The fixed cells were washed with deionized distilled water, dipped in 60% isopropanol for 3 min, stained with 2 mg/mL of Oil Red O staining solution (Sigma) for 60 min, and washed with deionized distilled water three times to remove unbound dye. Cell nuclei were counterstained with hematoxylin for 3 min and washed with deionized distilled water. Images were obtained using an Axiovert 40 CFL microscope (Olympus, Tokyo, Japan). After microscopic examination, the Oil-Red-O-based amount of triglyceride was quantified in each well. After washing and drying completely, 200 μL isopropanol extraction solution was added to each staining well and the mixtures were incubated for 10 min, followed by gentle vibration to release Oil Red O for 10 min at room temperature. The extracted dye was removed by gentle pipetting, and its absorbance was measured at 500 nm by microplate reader (Versamax; Molecular Devices, Sunnyvale, CA, United States). All tests were performed in triplicate.

Lipolysis measurement

Lipolysis was evaluated by measuring the amount of glycerol released into the medium. Aliquots of

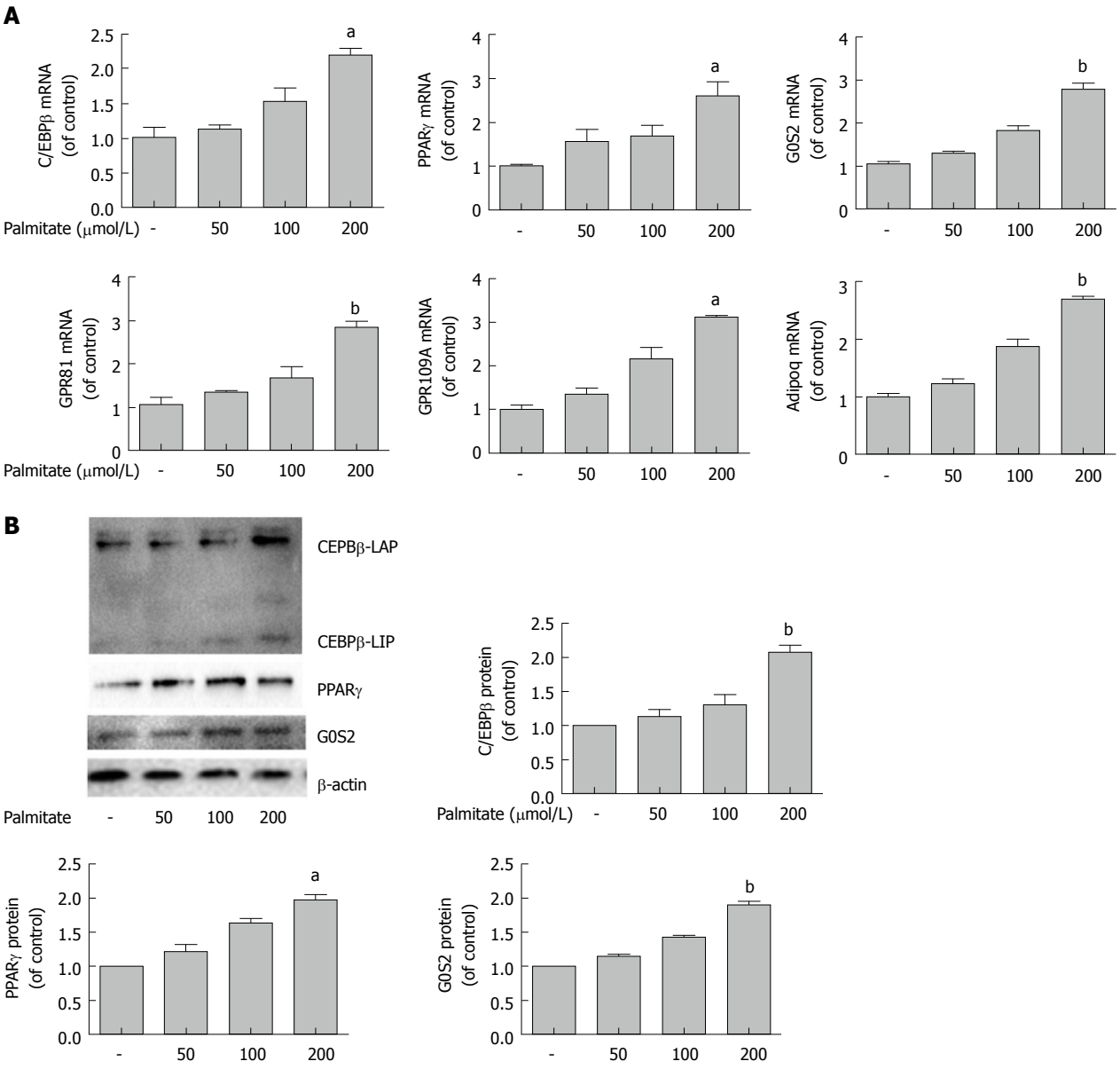


Figure 2 Palmitate-induced expression of C/EBPβ, PPARγ, and PPARγ target genes in HepG2 cells. A: Palmitate increased mRNA expression of C/EBPβ, PPARγ, and PPARγ target genes (*G0S2*, *GPR81*, *GPR109A*, and *Adipoq*) in a dose-dependent manner. mRNA was measured by qPCR. At least three independent experiments were conducted for each measurement. Data are presented as means ± SE. $^{a}P < 0.05$ and $^{b}P < 0.01$ *vs* untreated control. B: Palmitate increased protein expression of C/EBPβ, PPARγ, and G0S2 in a dose-dependent manner. Protein was examined by western blotting. At least three independent experiments were conducted for each measurement. Data are presented as means ± SE. $^{a}P < 0.05$ and $^{b}P < 0.01$ *vs* untreated control.

culture medium were centrifuged to remove debris, and directly subjected to glycerol measurement. The amounts of glycerol released were quantified using a glycerol quantification kit (Biovision Inc., Milpitas, CA, United States). Released glycerol was determined using an autoanalyzer (Cobas-Mira; Roche Diagnostics, Basel, Switzerland) to detect the absorbance at 550 nm. All samples were measured in duplicates.

Statistical analysis

All experimental data were expressed as means ± SE. Statistical differences were evaluated by Student's *t* test or one-way analysis of variance where appropriate using SPSS version 18.0 (SPSS, Chicago, IL, United States). Differences were considered as statistically significant when *P* values were < 0.05.

RESULTS

Palmitate induced lipid accumulation and suppressed lipolysis in HepG2 cells

HepG2 cells were incubated with increasing amounts of palmitate for 24 h, and lipid accumulation was examined by Oil Red O staining. Palmitate caused a dose-dependent increase in lipid accumulation in HepG2 cells (Figure 1A and B). HepG2 cells with palmitate also caused a dose-dependent decrease in lipolysis, demonstrated by reduced glycerol release

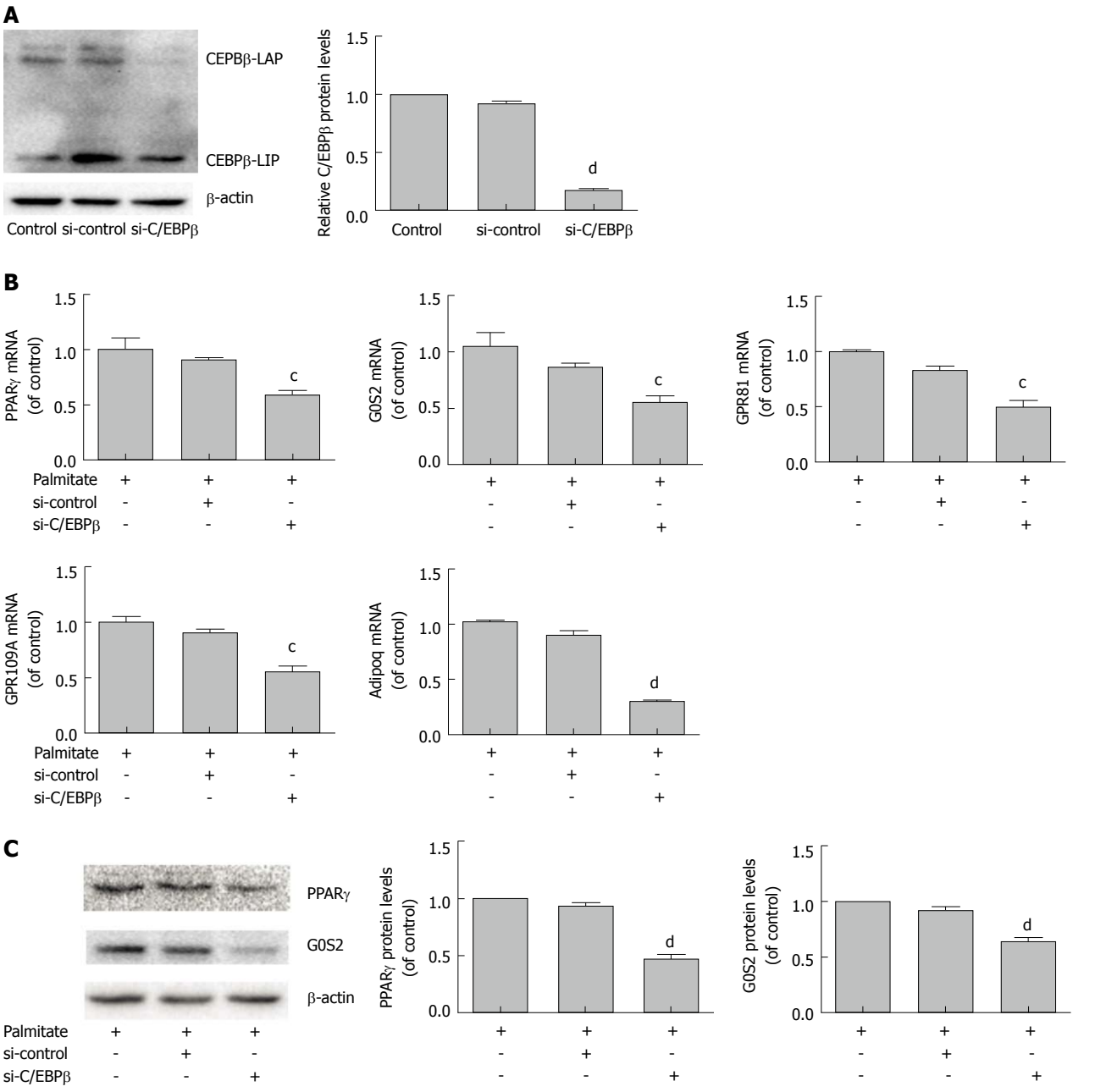


Figure 3 The effects of C/EBPβ knockdown on palmitate-induced *PPARγ* and its target gene expression in HepG2 cells. A: HepG2 cells were transfected with control siRNA or C/EBPβ siRNA, and C/EBPβ expression was measured by Western blotting. At least three independent experiments were conducted. Data are presented as means ± SE. $^{d}P < 0.01$ *vs* control siRNA. B: C/EBPβ knockdown decreased palmitate-induced mRNA expression of *PPARγ* and its target genes (*G0S2*, *GPR81*, *GPR109A*, and *Adipoq*). mRNA was measured by qPCR. At least three independent experiments were conducted for each measurement. Data are presented as means ± SE. $^{c}P < 0.05$ and $^{d}P < 0.01$ *vs* control siRNA. C: C/EBPβ knockdown decreased palmitate-induced protein expression of PPARγ and G0S2. Protein was examined by Western blotting. At least three independent experiments were conducted for each measurement. Data are presented as means ± SE. $^{d}P < 0.01$ *vs* control siRNA.

into the medium (Figure 1C). Palmitate at 200 μmol/L, which represents a high physiological level of circulating palmitate in obesity[2], caused a significant increase in lipid accumulation and a significant decrease in lipolysis. Therefore, this concentration of palmitate was used in all the following siRNA knockdown experiments.

Palmitate induced expression of C/EBPβ, PPARγ, and PPARγ target genes in HepG2 cells

Lipid accumulation is controlled by a few key transcription factors, including C/EBPβ and PPARγ. We assessed the effects of palmitate on the expression of C/EBPβ, PPARγ, and several known PPARγ target genes in HepG2 cells. HepG2 cells were incubated with increasing amounts of palmitate for 24 h, and quantitative PCR analysis revealed that palmitate caused a dose-dependent increase in the mRNA expression of *C/EBPβ*, PPARγ, and PPARγ target genes (*G0S2*, *GPR81*, *GPR109A*, and *Adipoq*) (Figure 2A). Western blotting showed that incubation of HepG2 cells with palmitate also caused a dose-dependent increase in the protein expression of C/EBPβ, PPARγ, and G0S2

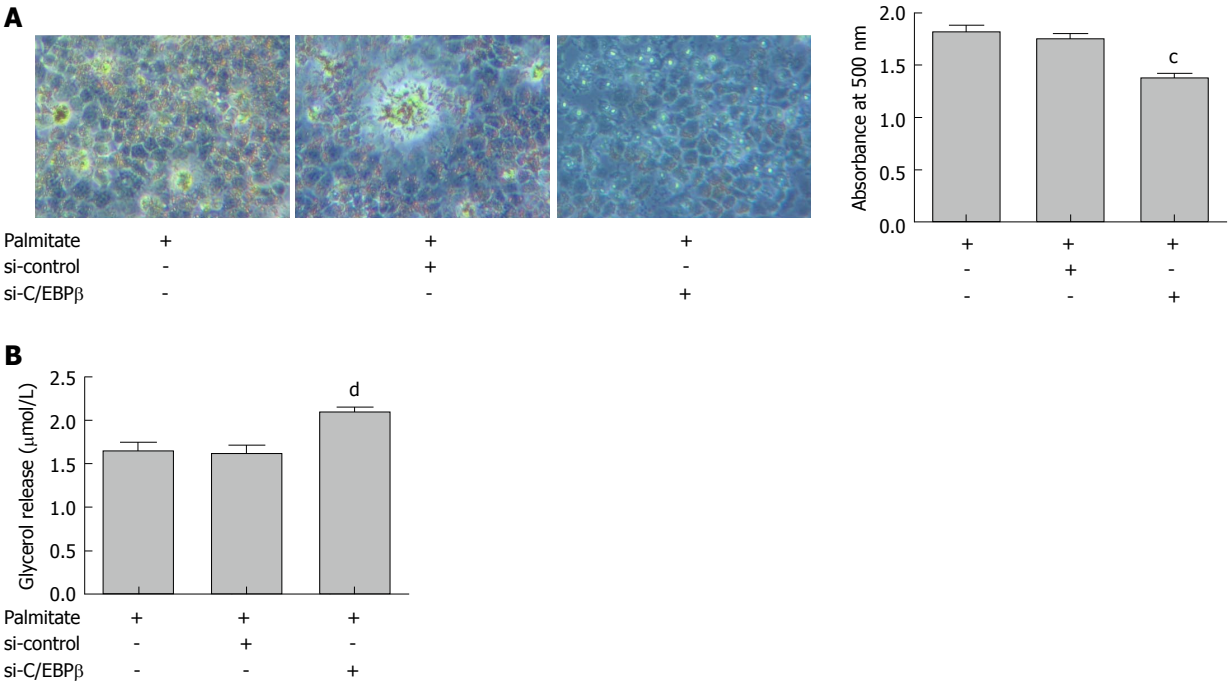


Figure 4 The effects of C/EBPβ knockdown on lipid accumulation and lipolysis in HepG2 cells treated with palmitate. A: C/EBPβ knockdown decreased palmitate-induced lipid accumulation. Lipid accumulation was detected with Oil Red O staining and quantified by absorbance value of the extracted Oil Red O dye at 500 nm. At least three independent experiments were conducted for each measurement. Data are presented as means ± SE. $^{c}P < 0.05$ *vs* control siRNA. B: C/EBPβ knockdown increased lipolysis in HepG2 cells treated with palmitate. Lipolysis was assessed by glycerol release into the medium. At least three independent experiments were conducted. Data are presented as means ± SE. $^{d}P < 0.01$ *vs* control siRNA.

(Figure 2B).

C/EBPβ knockdown reduced palmitate-induced PPARγ and its target gene expression in HepG2 cells

We next examined the role of C/EBPβ in palmitate-induced PPARγ and its target gene expression in HepG2 cells. HepG2 cells were transfected with C/EBPβ siRNA and treated with 200 μmol/L palmitate for 24 h. C/EBPβ siRNA efficiently decreased C/EBPβ protein expression (Figure 3A). qPCR analysis revealed that C/EBPβ knockdown significantly decreased palmitate-induced PPARγ and its target genes (*G0S2*, *GPR81*, *GPR109A*, and *Adipoq*) mRNA expression (Figure 3B). Western blotting showed that C/EBPβ knockdown significantly decreased palmitate-induced PPARγ and G0S2 protein expression (Figure 3C).

C/EBPβ knockdown attenuated lipid accumulation and augmented lipolysis in HepG2 cells treated with palmitate

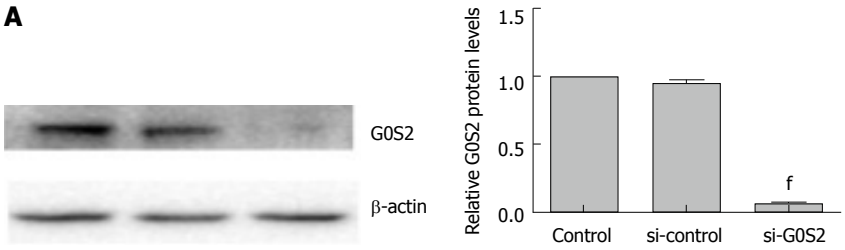
We investigated the effects of C/EBPβ on lipid accumulation and lipolysis in HepG2 cells treated with palmitate. HepG2 cells were transfected with C/EBPβ siRNA and treated with 200 μmol/L palmitate for 24 h. C/EBPβ knockdown significantly attenuated palmitate-induced lipid accumulation in HepG2 cells (Figure 4A). C/EBPβ knockdown significantly augmented lipolysis in HepG2 cells treated with palmitate (Figure 4B).

G0S2 knockdown attenuated lipid accumulation and augmented lipolysis in HepG2 cells treated with palmitate

G0S2 plays an important role in regulating hepatic lipid accumulation and lipolysis. We explored the effects of G0S2 in lipid accumulation and lipolysis in HepG2 cells treated with palmitate. HepG2 cells were transfected with G0S2 siRNA and treated with 200 μmol/L palmitate for 24 h. G0S2 siRNA efficiently decreased G0S2 protein expression (Figure 5A). G0S2 knockdown significantly attenuated palmitate-induced lipid accumulation in HepG2 cells (Figure 5B). G0S2 knockdown significantly augmented lipolysis in HepG2 cells treated with palmitate (Figure 5C). However, G0S2 knockdown had no effects on palmitate-induced mRNA expression of *C/EBPβ*, PPARγ, and other PPARγ target genes (*GPR81*, *GPR109A* and *Adipoq*) (Figure 5D) and palmitate-induced protein expression of C/EBPβ and PPARγ in HepG2 cells (Figure 5E).

DISCUSSION

Obesity is associated with elevation of circulating FFA due to impaired lipid storage capacity in subcutaneous adipose tissue. The increased FFA supply that occurs as a result leads to lipid accumulation in the liver[19,20]. Previous studies showed that the saturated fatty acid palmitate induces lipid accumulation in HepG2 cells[21,22]. In the present study, we also demonstrated that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G0S2
β-actin
Relative G0S2 protein levels
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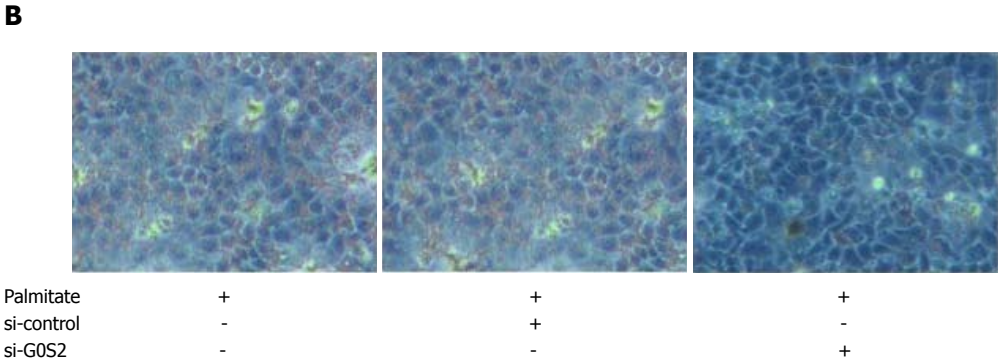
f
Control
si-control
si-G0S2


B
Palmitate + + +
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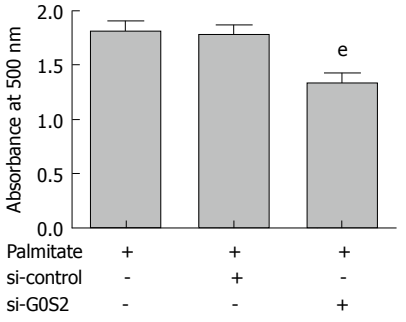
Absorbance at 500 nm
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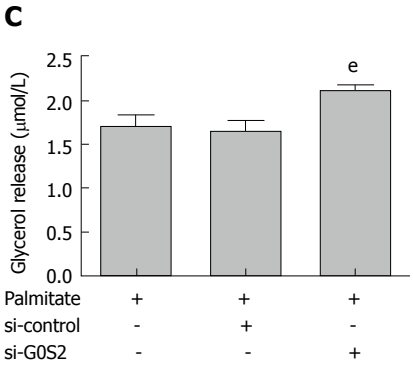
C
Glycerol release (μmol/L)
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2.0
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Palmitate + + +
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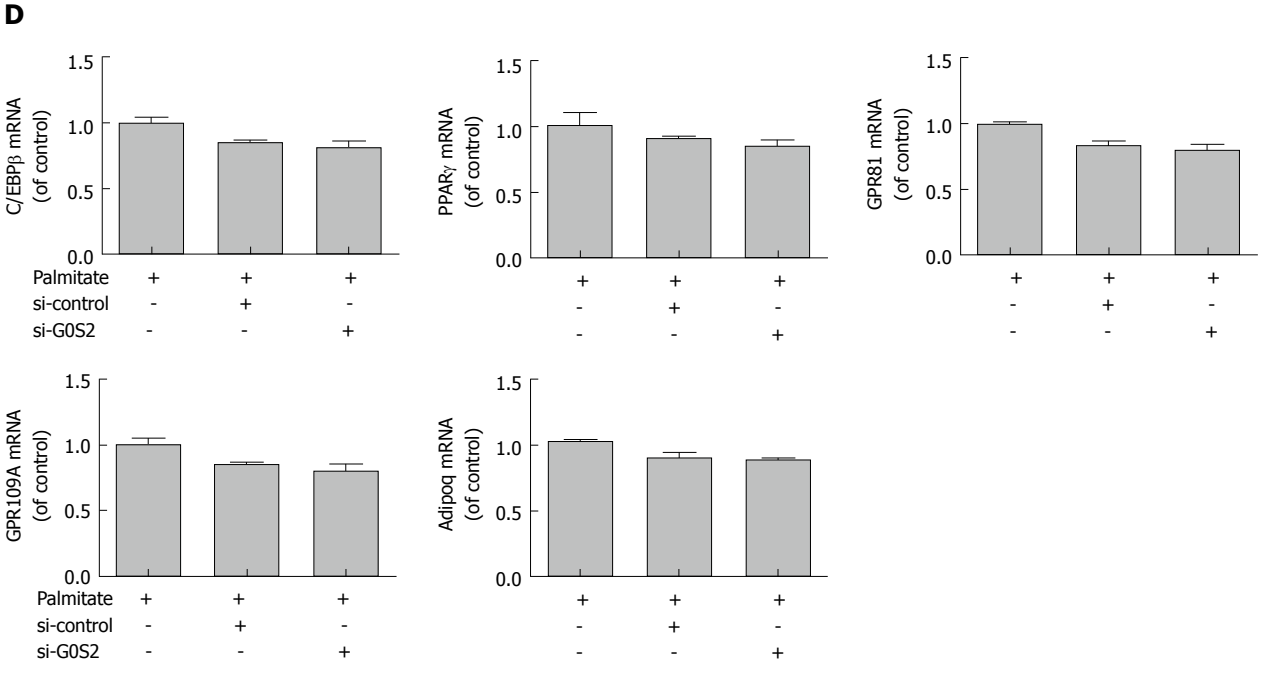
si-G0S2 - - +


D
C/EBPβ mRNA (of control)
PPARγ mRNA (of control)
GPR81 mRNA (of control)
GPR109A mRNA (of control)
Adipoq mRNA (of control)
1.5
1.0
0.5
0.0
Palmitate + + +
si-control - + -
si-G0S2 - - +

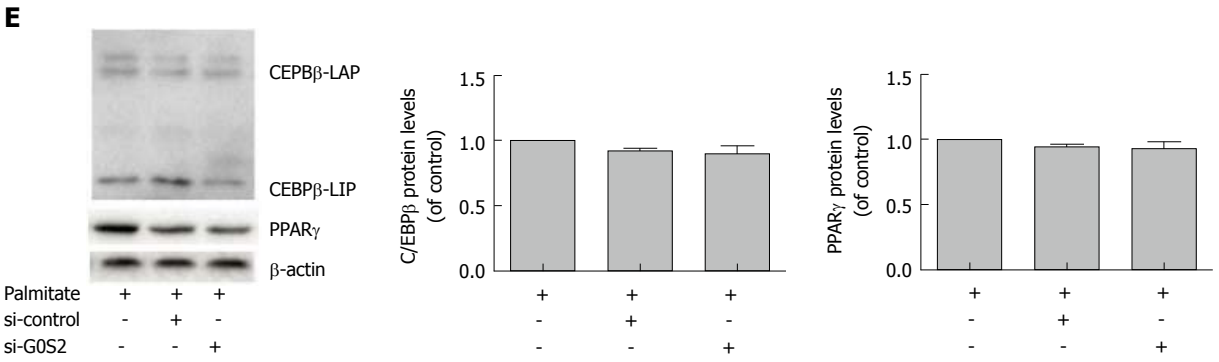


Figure 5 The effects of G0S2 knockdown on *C/EBP*β, *PPAR*γ and PPARγ target genes expression, lipid accumulation, and lipolysis in palmitate-treated HepG2 cells. A: HepG2 cells were transfected with control siRNA or G0S2 siRNA, and G0S2 expression was measured by Western blotting. At least three independent experiments were conducted. Data are presented as means ± SE. $^{f}P < 0.01$ *vs* control siRNA. B: G0S2 knockdown decreased palmitate-induced lipid accumulation. Lipid accumulation was detected with Oil Red O staining and quantified by absorbance value of the extracted Oil Red O dye at 500 nm. At least three independent experiments were conducted for each measurement. Data are presented as means ± SE. $^{e}P < 0.05$ *vs* control siRNA. C: G0S2 knockdown increased lipolysis in HepG2 cells treated with palmitate. Lipolysis was assessed by glycerol release into the medium. At least three independent experiments were conducted. Data are presented as means ± SE. $^{e}P < 0.05$ *vs* control siRNA. D: G0S2 knockdown did not affect palmitate-induced *C/EBP*β, *PPAR*γ, and other PPARγ target genes (*GPR81*, *GPR109A*, and *Adipoq*) mRNA expression. mRNA was measured by qPCR. At least three independent experiments were conducted for each measurement. Data are presented as means ± SE. E: G0S2 knockdown did not affect palmitate-induced PPARγ and G0S2 protein expression. Protein was examined by Western blotting. At least three independent experiments were conducted for each measurement. Data are presented as means ± SE.

palmitate induced lipid accumulation, and moreover, palmitate suppressed lipolysis in hepatocytes.

C/EBPβ is an important basic leucine zipper transcription factor whose mRNA can produce two C/EBPβ isoforms: liver-enriched activating protein (46 kDa) and liver-enriched inhibitory protein (21 kDa)[23]. C/EBPβ is involved in hepatic lipogenesis[5]. In hepatocytes, palmitate upregulates C/EBPβ expression, which in turn induces PPARγ expression and increases hepatic lipid accumulation[5]. Previous studies showed that C/EBPβ directly binds the PPARγ promoter prior to transcriptional activation during the early phase of adipogenesis[24,25]. PPARγ is a master adipogenic transcription factor, and it is necessary for adipogenesis[16]. *G0S2*, *GPR81*, *GPR109A*, and *Adipoq* are known *PPARγ* downstream target genes involved in lipolysis[26,27]. In the present study, we found that palmitate increased the mRNA expression of *C/EBPβ*, *PPARγ* and PPARγ target genes (*G0S2*, *GPR81*, *GPR109A* and *Adipoq*) and the protein expression of C/EBPβ, PPARγ and G0S2 in a dose-dependent manner in hepatocytes. We also found that knockdown of C/EBPβ significantly decreased *PPARγ* and its target genes (*G0S2*, *GPR81*, *GPR109A* and *Adipoq*) mRNA expression and PPARγ and G0S2 protein expression in palmitate-treated HepG2 cells. In addition, gene silencing of C/EBPβ attenuated lipid accumulation and augmented lipolysis in HepG2 cells treated with palmitate. These findings again demonstrate a critical role for C/EBPβ in palmitate-induced hepatic lipid accumulation.

In the above PPARγ target genes, G0S2 acts as an important regulator of triglyceride content in the liver[12,13]. Adipose-tissue-derived fatty acids upregulate fasting G0S2 expression in the liver to induce hepatic triglyceride accumulation[28]. G protein-coupled receptor (GPR)81 functions as a specific receptor for lactate and mediates insulin-induced antilipolytic effects in an autocrine and paracrine manner[27,29]. *GPR109A* is a receptor for the ketone body 3-hydroxybutyric acid and functions as a metabolic sensor that regulates lipolytic activity during starvation to avoid excessive triglyceride degradation[30,31]. Its biological role is related to the ketone body 3-hydroxybutyrate[31]. Adiponectin (Adipoq) is a adipokine that is downregulated in obesity[32]. In the liver, Adipoq can augment the oxidation of fatty acid to alleviate hepatic lipid accumulation[33]. Therefore, G0S2 may play a critical role in C/EBPβ-mediated hepatic lipid accumulation in palmitate-treated HepG2 cells. In this study, we found that knockdown of G0S2 significantly attenuated lipid accumulation and augmented lipolysis in HepG2 cells treated with palmitate. More importantly, inhibition of the G0S2 expression had no effects on mRNA expression of *C/EBPβ*, *PPARγ*, and PPARγ target genes (*GPR81*, *GPR109A* and *Adipoq*) and protein expression of C/EBPβ and PPARγ in palmitate-treated HepG2 cells. Together, these results indicate that palmitate induces lipid accumulation by activating C/EBPβ-mediated expression of G0S2.

In summary, we observed that palmitate can induce lipid accumulation in HepG2 cells by activating C/EBPβ-mediated G0S2 expression. The result provides novel evidence linking G0S2 expression to palmitate-induced hepatic lipogenesis. Considering that liver lipid accumulation is not only a hallmark of NAFLD, but also the first and critical step in the initiation and progression of NAFLD, interfering with G0S2 may represent an effective strategy for the treatment of obesity-related hepatic steatosis.

ARTICLE HIGHLIGHTS

Research background

Obesity-associated nonalcoholic fatty liver disease (NAFLD) is characterized by excessive deposition of fat in hepatocytes. The saturated free fatty acid palmitate, the concentration of which is often elevated in obesity, is a major contributor to an increase in intrahepatic triglyceride. G0/G1 switch gene 2 (G0S2) plays an important role in regulating hepatic lipid metabolism. However, the role of G0S2 and its transcriptional regulation in palmitate-induced hepatic lipid accumulation has remained unclear.

Research motivation

This study was carried out to clarify the molecular mechanism connecting palmitate to obesity-associated NAFLD. As CCAAT/enhancer binding protein beta (C/EBPβ), proliferator-activated receptor gamma (PPARγ) and *G0S2 all* relate to obesity-associated NAFLD, we investigated their roles and interrelationships in palmitate-induced hepatic lipid accumulation. The results lead to important new insights into the molecular mechanism of NAFLD.

Research objectives

The goal of this study was to determine the role of G0S2 and its transcriptional regulation in palmitate-induced hepatic lipid accumulation. The results suggest a previously unknown link between C/EBPβ and G0S2 that contributes to hepatic steatosis.

Research methods

In this study, we examined lipolysis, lipid accumulation, and the expression of C/EBPβ, PPARγ and PPARγ-regulated genes (*G0S2*, *GPR81*, *GPR109A* and *Adipoq*) in response to palmitate treatment in HepG2 cells. Specifically, we investigated the relationships between expression of the aforementioned proteins and hepatocyte lipolysis and lipid accumulation by using siRNA-mediated gene knockdown experiments.

Research results

Palmitate significantly facilitated lipid accumulation and suppressed lipolysis in HepG2 cells. Palmitate also significantly increased the expression of *C/EBPβ*, *PPARγ*, and PPARγ target genes (*G0S2*, *GPR81*, *GPR109A* and *Adipoq*). C/EBPβ knockdown significantly reduced palmitate-induced PPARγ and *G0S2* expression. Moreover, C/EBPβ knockdown attenuated lipid accumulation and augmented lipolysis in palmitate-treated HepG2 cells. Importantly, G0S2 knockdown significantly attenuated lipid accumulation and augmented lipolysis in palmitate-treated HepG2 cells, while G0S2 knockdown had no efects on palmitate-induced expression of *C/EBPβ*, *PPARγ*, and PPARγ target genes (*GPR81*, *GPR109A*, and *Adipoq*).

Research conclusions

Palmitate can induce lipid accumulation in HepG2 cells by activating C/EBPβ-mediated G0S2 expression. The result provides novel evidence linking G0S2 expression to palmitate-induced hepatic lipogenesis. Considering that liver lipid accumulation is not only a hallmark of NAFLD, but also the first and critical step in the initiation and progression of NAFLD, interfering with G0S2 may represent an effective strategy for the treatment of obesity-related hepatic steatosis.

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