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Palmitate induces insulin resistance without significant intracellular triglyceride accumulation in HepG2 cells

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Abstract

Previous studies showed that increased release of free fatty acids from adipocytes leads to insulin resistance and triglyceride (TG) accumulation in the liver, which may progress into hepatic steatohepatitis. We and other investigators have previously reported that palmitate induces endoplasmic reticulum stress-mediated toxicity in several tissues. This work investigated whether palmitate could induce insulin resistance and steatosis in HepG2 cells. We treated cells with either saturated fatty acid (palmitate) or unsaturated fatty acid (oleate), and observed that palmitate significantly activated c-jun N-terminal kinase and inactivated protein kinase B. Both 4-phenylbutyric acid and glycerol significantly activated protein kinase B, confirming the involvement of endoplasmic reticulum stress in palmitate-mediated insulin resistance. Oleate, but not palmitate, significantly induced intracellular TG deposition and activated sterol regulatory element binding protein–1. Instead, diacylglycerol level and protein kinase C ε activity were significantly increased by palmitate, suggesting the possible role of diacylglycerol in palmitate-mediated lipotoxicity. Therefore, the present study clearly showed that palmitate impairs insulin resistance, but does not induce significant TG accumulation in HepG2 cells.

1. Introduction

Obesity can be considered a chronic low-grade inflammatory state with adipose tissue secreting multiple cytokines, hormones, and free fatty acids (FFAs) [1]. Obesity is also the most significant single risk factor for the development of nonalcoholic fatty liver [2], which may be due to an increased uptake of FFAs by hepatocytes [3]. The rate of hepatic FFA uptake is unregulated and therefore proportional to plasma FFA concentration. Elevated levels of cytokines and FFAs are thought to contribute to the induction of insulin resistance and diabetes by modifying glucose and lipid metabolism, as well as inflammatory cascades in skeletal muscle, liver, and adipose tissues [4,5]. In a variety of experimental systems, saturated and unsaturated fatty acids differ significantly in their contributions to lipotoxicity. This selectivity has been attributed to the generation of specific lipid species or signaling molecules in response to saturated but not unsaturated FFAs [6].

Although several studies strongly suggest that hepatic lipid accumulation causes hepatic insulin resistance, the molecular mechanisms responsible for this relationship remain uncertain. Fat in the liver in the form of steatosis has been suggested a source for potential mediators of insulin resistance and may play a potentially important role by leading to subacute hepatic "inflammation" via cytokine production [7]. Recent studies demonstrated the association between an increase in tissue diacylglycerol (DAG) content and insulin resistance in the liver and muscles [8-11]. Protection of fat-induced hepatic insulin resistance was correlated with reduced fasting concentrations of DAG, implicating the role of DAG as a potentially important mediator of fat-induced hepatic insulin resistance [12]. Diacylglycerol is a potent activator of protein kinase C ε (PKCε), a novel protein kinase C isotype that enhances Ser/Thr phosphorylation of the insulin receptor and inhibits its tyrosine kinase activity [13].

Activation of the unfolded protein response has been suggested to contribute significantly to palmitate-induced lipotoxicity in various types of cells [14-17]. Several studies have suggested that the endoplasmic reticulum (ER) is the site to monitor and translate cellular stress into inflammatory

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signaling and responses [18] and that ER stress is involved in the induction of insulin resistance and diabetes [19,20]. The ER stress pathway was activated in the liver and adipose tissue of obese mice, leading to increased c-jun N-terminal kinase (JNK) activation, which in turn suppresses insulin action by inhibiting insulin receptor substrate–1 and its downstream signaling pathways [19]. Mammalian cells express several membrane-bound transcription factors that are activated by regulated intramembrane proteolysis. Sterol depletion activates the sterol regulatory element-binding proteins (SREBPs); and ER stress activates the activating transcription factor 6 (ATF6), resulting in the translocation of these transcription factors into the nucleus [21]. Several studies showed that induction of ER stress could activate SREBP and triglyceride (TG) accumulation. In the intragastric ethanol feeding model, hepatic TG accumulation is mainly determined by the action of SREBP-1c [22,23], suggesting a causal link between ER stress and SREBP activation. In addition, homocysteine-induced ER stress has been shown to induce hepatic SREBP-1 messenger RNA (mRNA) expression of mice [24]. In the present study, we therefore investigated whether ER stress could be the underlying mechanism involved in FFA-mediated steatosis in HepG2 cells.

2. Methods and materials

2.1. HepG2 cell culture and treatment

HepG2 human hepatocarcinoma cell line was obtained from the American Type Culture Collection (ATCC, Manassas, VA) and cultured in Dulbecco modified Eagle medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin at 37°C/5% CO_2. HepG2 cells have been shown to exhibit many differentiated functions of human parenchymal cells, including expression of secretory proteins [25]. The cells were treated with FFA after overnight FBS deprivation. The FFA solutions were prepared as described [14] and was freshly diluted in Dulbecco modified Eagle medium without FBS to a final concentration of 1 mmol/L FFA/1% bovine serum albumin (BSA) or 0.5 mmol/L FFA/0.5% BSA before treatment. Selected samples were treated with inhibitors of ceramide biosynthesis pathway (50 μmol/L fumonisin B_1 or 1 mmol/L cycloserine) just before adding palmitate. Similar concentration of the inhibitor has been shown to inhibit palmitate-induced de novo ceramide accumulation in liver and other cells [26-28].

2.2. Oil red O staining

To detect intracellular lipid accumulation, the cells were seeded onto 4-well chamber slides (Nunc, Rochester, NY) that were coated with poly-L-lysine (Sigma, St Louis, MO). After treatment, the cells were washed twice with ice-cold phosphate-buffered saline (PBS) and then fixed with 7.5% paraformaldehyde for 15 minutes. The cells were stained with freshly diluted 0.3% Oil red O solution for 1 hour. The stain was then removed, and the cells were washed twice with water and then examined in light microscopy.

2.3. Fluorometric determination of fat content in intact cells by Nile red assay

The lipid content in cultured cells was determined fluorometrically using Nile red (Sigma), a vital lipophilic dye used to label fat accumulation in the cytosol. After treatment, the cells were stained with Nile red as described previously [29]. Briefly, the cells were washed with Hank buffered salt solution (HBSS) (Sigma); and background fluorescence was determined (535-nm excitation, 580-nm emission) using a Victor3 plate reader (Perkin-Elmer, Waltham, MA). After 4-hour incubation at room temperature in the dark, Nile red was removed; and the cells were washed with HBSS. After further incubation for 16 hours in HBSS, fluorescence was again determined as described above. Background fluorescence was subtracted to obtain bound Nile red fluorescence, which was adjusted to cell protein content. Cell protein content was determined using Bio-Rad protein assay reagent (Bio-Rad, Hercules, CA).

2.4. Cell extract preparation and immunoblotting

After treatment with FFAs, the cells were rinsed with PBS and lysed in ice-cold lysis buffer containing 50 mmol/L Hepes-KOH, pH 7.5, 150 mmol/L NaCl, 1 mmol/L NaF, 10% glycerol, 1 mmol/L EDTA, 2.5 mmol/L EGTA, 10 mmol/L β-lycerophosphate, 0.1 mmol/L Na_3VO_4, 1 mmol/L dithiothreitol, 0.1% Tween-20, and 0.2 mmol/L phenylmethylsulfonyl fluoride. For the measurement of SREBP-1 activation, the treated cells were rinsed in PBS; and nuclear protein extracts were prepared using NE-PER nuclear and cytoplasmic extraction reagents (Pierce, Rockford, IL) according to the manufacturer's protocol. Equal amounts of protein were loaded into the lanes of a sodium dodecyl sulfate polyacrylamide gel electrophoresis gel, separated, and blotted onto a polyvinylidene difluoride membrane (Millipore, Billerica, MA). After the membranes had been blocked with either 5% nonfat milk or BSA, they were probed with a specific antibody (anti-JNK [Cell Signaling, Danvers, MA], anti–p-JNK [Cell Signaling], anti-Akt [Cell Signaling], anti–p-Akt [Cell Signaling], anti–SREBP-1 [Santa Cruz Biotechnology, Santa Cruz, CA], anti–p-PKCε [Santa Cruz], or anti–β-actin [Sigma]) and then incubated with horseradish-peroxidase–linked secondary antibody for chemiluminescent detection. The band intensities were quantified with Quantity One software (Bio-Rad).

2.5. Lipid extraction and separation by thin-layer chromatography

Lipids were extracted in chloroform-methanol (2:1, vol/vol) according to the method of Folch et al [30]. Diacylglycerol content was assayed as described previously

[31]. Briefly, the total lipid extract was separated using heptane–isopropyl ether–acetic acid (60:40:3, vol/vol/vol). Plates were then dipped in a solution of 10% cupric sulfate (wt/vol) in 8% phosphoric acid (vol/vol) for 10 seconds, thoroughly dried under a stream of hot air, and immediately heated at 200°C for 2 minutes. The spot intensities were quantified with Quantity One software.

2.6. Statistical analysis

The data were analyzed using SAS software (version 9.1; SAS, Cary, NC). For all experiments, 1-way analysis of variance followed by Duncan multiple range test was used to assess statistical significance. Data were expressed as means ± SEM, and differences were considered statistically significant at $P < .05$.

3. Results

3.1. Effect of FFAs on insulin resistance in HepG2 cells

We treated HepG2 cells with either 1 mmol/L palmitate or oleate for 6 hours to investigate the effect of elevated FFA levels on hepatic insulin resistance. In the previous study, the relative cell viability was significantly decreased in a dose-dependent manner in the cells treated with various concentrations of palmitate (0-0.5 mmol/L) for 12 hours [17]. The venous blood concentration of FFAs is known to vary widely (~0.25-3.0 mmol/L) and is chronically higher in individuals with obesity and/or diabetes [32]. We examined whether palmitate activates JNK in HepG2 cells. The levels of phosphorylated forms of JNK were found to be increased by palmitate, although levels of total JNK proteins were unchanged (Fig. 1A). To investigate the effect of palmitate on the insulin signaling pathway, we measured the activation of Akt, a key transducer in the insulin signaling pathway, using a Ser 473 phosphate-specific Akt antibody (Fig. 1B). The Akt phosphorylation was found to be dramatically decreased after 6 hours of palmitate treatment. To further evaluate whether ceramide synthesis represents a mechanism whereby palmitate impairs insulin resistance, cells were incubated with palmitate in the presence of an inhibitor of ceramide synthesis (50 μmol/L fumonisin B_1 or 1 mmol/L cycloserine). Insulin signaling was not significantly affected by ceramide inhibitors as shown in phosphorylation status of Akt (Fig. 1C).

We also treated HepG2 cells with 5 mmol/L 4-phenylbutyric acid (PBA) and 0.5 mol/L glycerol to assess whether chemical chaperones attenuate palmitate-induced insulin resistance. The JNK activity was significantly reduced exclusively by glycerol (46.8% ± 18.8% of control). Phosphorylation of Akt was significantly increased by 239.7% ± 18.9% and 181.3% ± 21.1% relative to the palmitate-treated group in PBA- and glycerol-treated cells, respectively (Fig. 2).

3.2. Effect of FFAs on lipid metabolism in HepG2 cells

Intracellular lipid accumulation was analyzed by microscopy after staining of cells with Oil red O. We clearly observed visible lipid droplets in cells incubated with oleate. In contrast, barely visible lipid droplets with light red cytoplasmic staining were observed after incubation with palmitate. Staining of cells with Oil red O (Fig. 3A) was consistent with the quantitative data obtained using the Nile red assay (Fig. 3B). Nile red assay also showed that oleate at concentrations of more than 0.1 mmol/L significantly increased TG accumulation. To investigate the role of chemical chaperones in the regulation of TG accumulation, we incubated cells with either PBA or glycerol in the presence of palmitate. Both glycerol and PBA had no effect on TG accumulation when cells were cotreated with palmitate (data not shown).

We also measured the effect of fatty acids on activation of SREBP-1. Immunoblot assay was used to measure and compare the levels of hepatic membrane-bound precursor

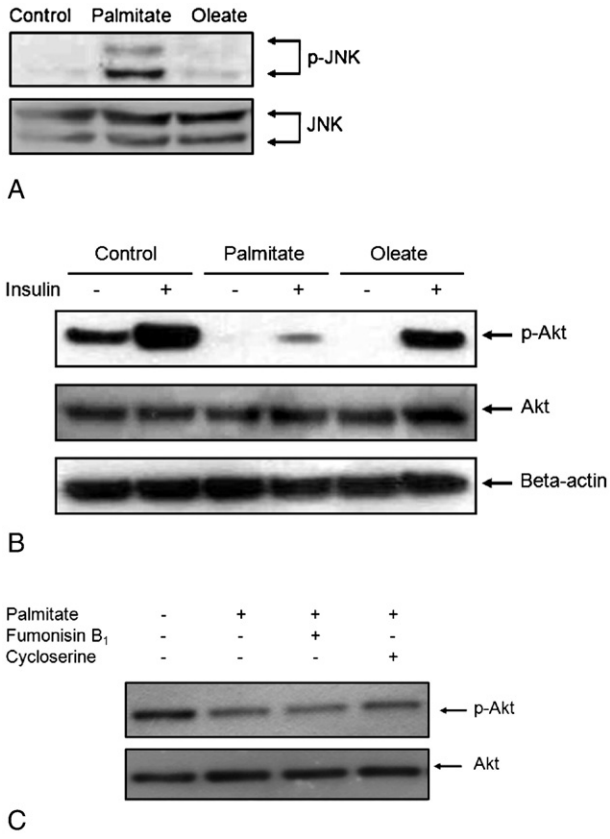

Fig. 1. Palmitate induces insulin resistance in HepG2 cells. A, Protein expressions of p-JNK and JNK were measured by immunoblotting. Cells were treated with 1% BSA, 1 mmol/L palmitate/1% BSA, or 1 mmol/L oleate/1% BSA in media without serum for 6 hours. Results are representative of 3 independent experiments. B, Protein expressions of p-Akt and Akt were measured by immunoblotting. Cells were treated with FFAs in media without FBS for 6 hours and activated with 50 nmol/L insulin for 20 minutes before harvest. To confirm the equal loading of the lysates, the membrane was reprobed with anti–β-actin antibody. Results are representative of 3 independent experiments. C, Protein expressions of p-Akt and Akt were measured by immunoblotting. Cells were treated with 50 μmol/L fumonisin B_1 or 1 mmol/L cycloserine for 6 hours in the presence of palmitate and activated with 50 nmol/L insulin for 20 minutes before harvest. Results are representative of 3 independent experiments.

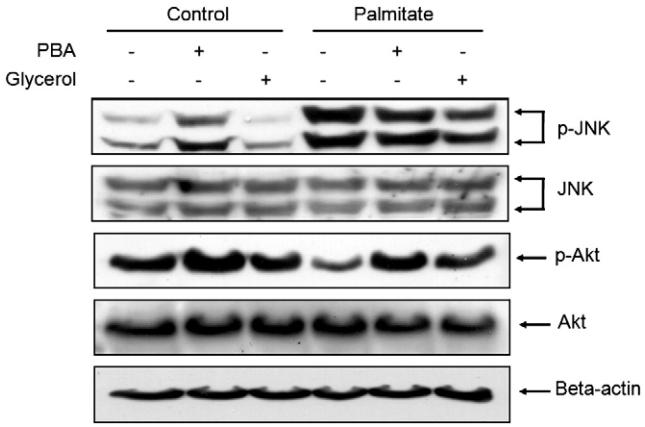

Fig. 2. Chemical chaperones attenuate palmitate-induced insulin resistance in HepG2 cells. Protein expressions of p-JNK, JNK, p-Akt, and Akt were measured by immunoblotting. Cells were treated with 1% BSA or 1 mmol/L palmitate/1% BSA for 6 hours, with or without pretreatment with 5 mmol/L PBA or 0.5 mol/L glycerol for 2 hours. To confirm the equal loading of the lysates, the membrane was reprobed with anti–β-actin antibody. Results are representative of 3 independent experiments.

and mature nuclear forms of SREBP-1 (Fig. 3C). When the cells were treated with oleate, but not with palmitate, a significant increase in the ratio of mature to precursor form of SREBP-1 was observed.

To investigate the involvement of DAG accumulation in the palmitate-induced lipotoxicity in HepG2 cells, DAG was separated by thin-layer chromatography. After separation, DAG spots were quantitatively assessed. Palmitate significantly increased DAG content in HepG2 cells compared with that in control cells; however, oleate did not increase DAG content (Fig. 4A). We also observed increased levels of TG accumulation in cells treated with oleate. To confirm the involvement of DAG in the palmitate-induced lipotoxicity in HepG2 cells, the expression of p-PKCε was investigated by immunoblotting. Protein kinase C ε was significantly activated by palmitate; however, we could not observe any significant change in cells treated with oleate compared with control (Fig. 4B).

4. Discussion

Here we showed that palmitate has the capacity to induce ER stress-mediated insulin resistance, but not a significant increase in TG accumulation, in HepG2 cells. We previously reported that palmitate induces ER stress-mediated lipotoxicity in HepG2 cells, which was alleviated by treatment with chemical chaperone [17]. Although lipotoxicity induced by a single species of fatty acid in transformed cells may be considered too simple a model to explain the metabolic changes associated with obesity [33], the results presented here can be used to explain the molecular mechanism involved in the individual FFA-mediated insulin resistance and steatosis, which are known to be induced in the liver of high-fat diet–mediated and gene-related obesity models.

The present study shows that chemical chaperone inhibited the activation of JNK and the insulin resistance induced by palmitate, thereby confirming the role of ER stress in the insulin resistance pathway. Ozcan et al [34] showed that chemical chaperones, such as PBA and the taurine-conjugated derivative of ursodeoxycholic acid, reduce ER stress in Fao liver cells treated with tunicamycin. These authors also showed that chemical chaperones restore glucose homeostasis in a mouse model of type 2 diabetes mellitus.

In the present study, palmitate did not lead to a significantly elevated accumulation of TG regardless of insulin resistance in HepG2 cells. Instead, we observed a significant increase in intracellular DAG in cells treated with palmitate. Significantly increased hepatic lipid accumulation was observed in triolein (36% fat calories)-fed animals when compared with tristearin-fed animals [35]. Triglyceride synthesis requires endogenously synthesized monounsaturated fatty acids as critical substrates in studies using stearoyl–coenzyme A desaturase −/− mice [36,37]. In vitro overexpression of stearoyl–coenzyme A desaturase 1 resulted in an increase in TG esterification and protected myotubes from fatty acid–induced insulin resistance by reducing ceramide and DAG accumulation [38]. Diacylglycerol acyltransferase, which catalyzes the conversion of DAG to TG, showed maximal activity with diolein and minimal saturable activity with dipalmitin [39]. Therefore, palmitate could accumulate as DAG, whereas oleate was readily used to synthesize TG, resulting in marked differences in lipid accumulation between cells treated with either palmitate or oleate [8,40,41]. In addition, intracellular TG accumulation observed in oleate-treated cells may exert a protective role against insulin resistance and apoptosis. Previous study using CHO cells showed that oleate supplementation leads to TG accumulation and is well tolerated, whereas excess palmitate is poorly incorporated into TG and causes apoptosis [6]. In both muscle cells [42] and pancreatic islet cells [43], intracellular TG has been suggested to exert a protective role against apoptosis by preventing an intracellular increase in toxic metabolites from palmitate.

We observed increases in SREBP-1 activation and TG accumulation by oleate. Consistently, recent study showed that increased TG accumulation by oleate was associated with increased gene expressions of SREBP-1 and peroxisome proliferator-activated receptor γ in cultured hepatocytes [40]. Several studies have investigated the effect of various fatty acids on SREBP-1 mRNA expression and SREBP-1 protein activation; but there is inconsistency in findings, and the involved mechanism has not been clearly investigated [44-46]. Although various ER stress inducers have been shown to activate SREBP-1 in hepatocytes [47] and islet β-cells [48], the magnitude of SREBP-1 activation was not correlated with extent of ER stress determined by X-box binding protein–1 mRNA splicing. Activation of ATF6 did not require SREBP cleavage activating protein, an essential protein for SREBP activation [49]. Interestingly, X-

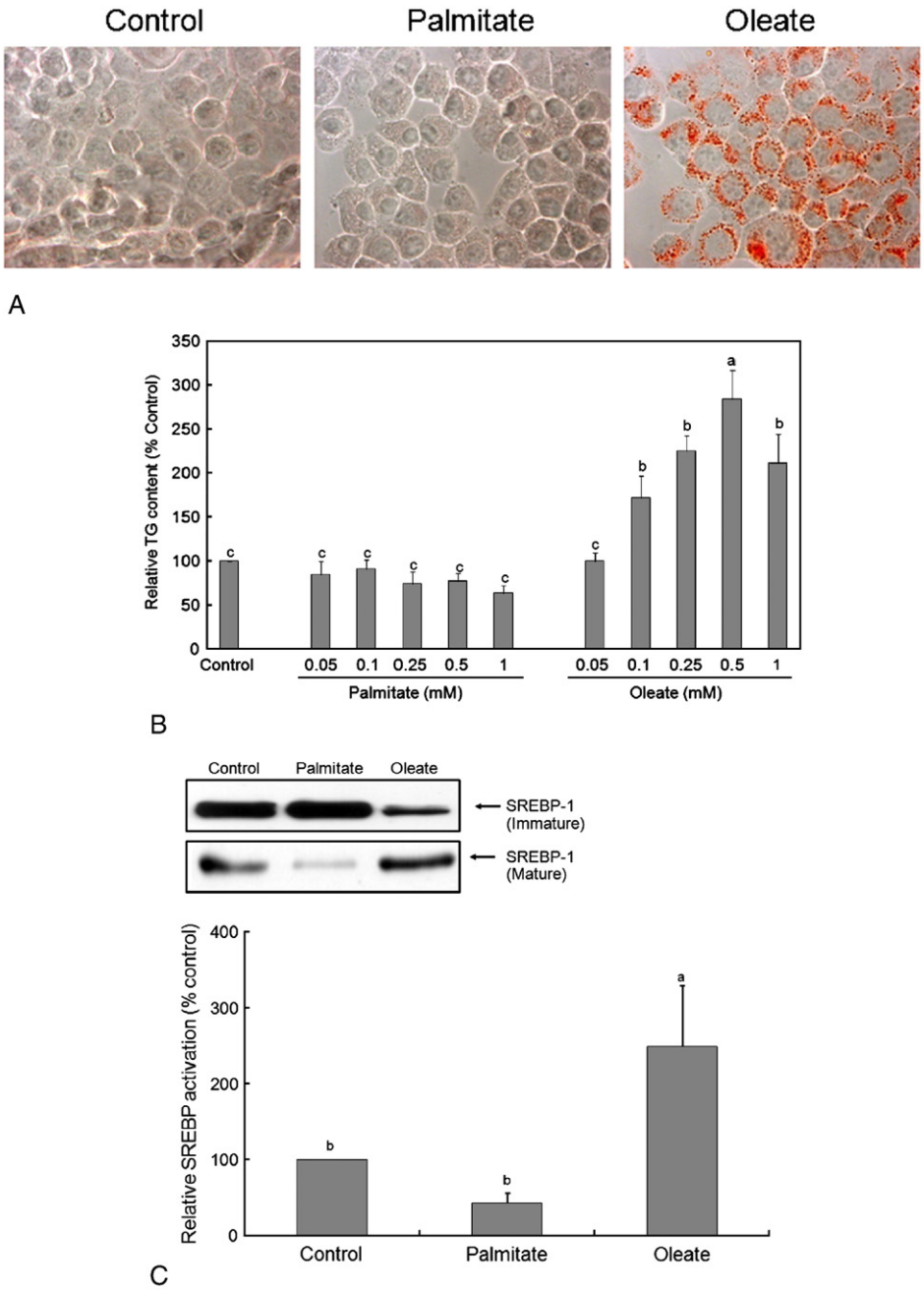

Fig. 3. Oleate induces intracellular lipid accumulation in HepG2 cells. A, Cells were treated with 0.5% BSA (control), 0.5 mmol/L palmitate/0.5% BSA (palmitate), or 0.5 mmol/L oleate/0.5% BSA (oleate). After 6 hours, the cells were stained with Oil red O to measure intracellular lipid accumulation and examined by light microscopy. Results are representative of 3 independent experiments. B, Cells were treated with various concentrations of FFAs for 6 hours and stained with Nile red. Fluorescence was detected to measure intracellular lipid accumulation. Each bar represents mean ± SEM (n = 3), and bars with different superscripts are significantly different at $P < .05$. C, Oleate activates SREBP-1 in HepG2 cells. Cells were treated with FFAs for 6 hours. Representative SREBP-1 immunoblots are shown (n = 3). Immature and mature denote the precursor and cleaved nuclear forms of SREBP-1, respectively. Each bar represents mean + SEM (n = 3), and bars with different superscripts are significantly different at $P < .05$.

box binding protein−1 regulates lipogenesis regardless of ER stress in the liver [50], suggesting that SREBP-1 activation may not be accompanied by ER stress.

Ceramide, a bioactive sphingolipid derived from palmitate, has been shown to activate atypical PKC [11,51], resulting in lipid-induced insulin resistance [9,52]. The inhibition of ceramide synthesis ameliorates obesity-induced insulin resistance in skeletal muscles and liver [52] and myoblasts [53] without changes in intracellular level of DAG. Watson et al [11] observed an increased DAG synthesis from palmitate and activation of novel PKC of rat muscle cells with sustained reduction in the capacity to synthesize ceramide. In

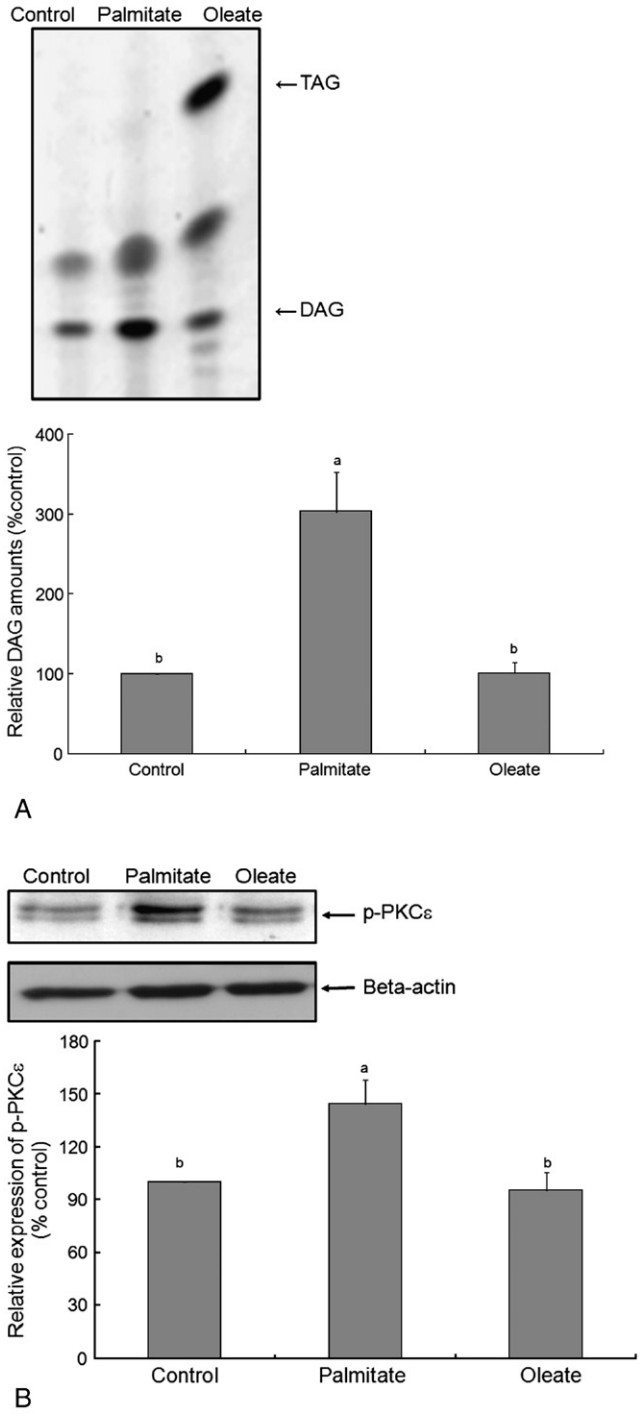

Fig. 4. Palmitate increased DAG content and PKCε activity in HepG2 cells. A, Cells were treated with FFAs for 24 hours. Representative thin-layer chromatogram of DAG is shown (n = 3). B, Protein expressions of p-PKCε and β-actin were measured by immunoblotting. Cells were treated with FFAs for 6 hours. Results are representative of 3 independent experiments. Each bar represents mean + SEM (n = 3), and bars with different superscripts are significantly different at $P < .05$.

addition to the direct phosphorylation of serine residues in insulin receptor, an activated novel PKC causes insulin resistance by increasing oxidative stress and activating IκB kinase and/or the nuclear factor–κB pathway [54,55]. In the present study, de novo synthesis of ceramide was not necessary for palmitate-induced insulin resistance in HepG2 cells. Protein kinase C ε was significantly increased in cells treated with palmitate, suggesting the important role of DAG in inducing insulin resistance. In the muscle cells, the alleviation of palmitate-induced insulin insensitivity is accomplished by cotreatment with oleate, which did not modify the fatty acid composition in microsomal phospholipids but rather redirected palmitate metabolism from DAG to TG [8]. Suppression of diacylglycerol acyltransferase–2 with antisense oligonucleotides improved high-fat diet–induced hepatic steatosis and insulin resistance by lowering hepatic DAG content and PKCε activity via reducing SREBP-1c–mediated lipogenesis and increasing β-oxidation of fatty acids [56]. The relative contribution of palmitate metabolites and PKC isoforms to the induction of hepatic insulin resistance remains to be clarified.

In summary, our data suggest that individual FFA has different contribution to hepatic pathophysiologic progress in obesity. We also demonstrated that chemical chaperones inhibited the ER stress-mediated insulin resistance induced by palmitate, suggesting that these chaperones represent promising therapeutic agents for insulin resistance.

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