Fenofibrate and PBA prevent fatty acid-induced loss of adiponectin receptor and pAMPK in human hepatoma cells and in hepatitis C virus-induced steatosis

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Abstract **Adiponectin receptors play a key role in steatosis and inflammation; however, very little is known about regulation of adiponectin receptors in liver. Here, we examined the effects of palmitate loading, endoplasmic reticulum (ER) stress, and the hypolipidemic agent fenofibrate on adiponectin receptor R2 (AdipoR2) levels and AMP-activated protein kinase (AMPK) in human hepatoma Huh7 cells and in Huh.8 cells, a model of hepatitis C-induced steatosis. Palmitate treatment reduced AdipoR2 protein and basal AMPK phosphorylation in Huh7 cells. Fenofibrate treatment preserved AdipoR2 and phosphorylated AMPK (pAMPK) levels in palmitate-treated cells accompanied by reduced triglyceride (TG) accumulation and less activation of ER stress markers CCAAT/enhancer binding (C/EBPβ) and eukaryotic translation initiation factor 2 α. ER stress agents thapsigargin and tunicamycin suppressed AdipoR2 and pAMPK levels in Huh7 cells, while fenofibrate and the chemical chaperone 4-phenylbutyrate (PBA) prevented these changes. AdipoR2 levels were lower in Huh.8 cells and fenofibrate treatment increased AdipoR2 while reducing activation of c-Jun N-terminal kinase and C/EBPβ expression without changing TG levels. Taken together, these results suggest that fatty acids and ER stress reduce AdipoR2 protein and pAMPK levels, while fenofibrate and PBA might be important therapeutic agents to correct lipid- and ER stress-mediated loss of AdipoR2 and pAMPK associated with nonalcoholic steatohepatitis.**—Rahman, S. M., I. Qadri, R. C. Janssen, and J. E. Friedman. **Fenofibrate and PBA prevent fatty acid-induced loss of adiponectin receptor and pAMPK in human hepatoma cells and in hepatitis C virus-induced steatosis.** ***J. Lipid Res.*** **2009.** 50: **2193–2202.**

Supplementary key words insulin resistance • inflammation • signal transduction • diabetes • obesity • palmitate

Hepatic steatosis is a hallmark of nonalcoholic fatty liver disease; however, it is a frequent histological finding in other metabolic liver diseases, including chronic hepatitis C (1, 2). Nonalcoholic fatty liver disease encompasses a wide range of liver injury, from simple steatosis to nonalcoholic steatohepatitis (NASH) or even cirrhosis or fibrosis (3, 4). The pathogenesis of NASH begins with increased synthesis and/or decreased oxidation of triglycerides (TG) in the hepatocyte, thereby leading to increased fat accumulation. Because steatosis may render the liver more vulnerable to inflammation and fibrosis (5), there is increasing interest in the role of steatosis-induced liver injury. Excess TG accumulation activates a number of cellular stress signaling and inflammatory pathways, such as c-Jun N-terminal kinase (JNK) and nuclear factor κB, that may eventually lead to apoptosis, giving rise to the phenomenon termed lipotoxicity (6, 7).

NASH is associated with a significant decrease in plasma adiponectin levels, which correlates with the severity of liver histology (8). The metabolic effects of adiponectin are mediated by two putative receptors: adiponectin receptor R1 (AdipoR1) and adiponectin receptor R2 (AdipoR2). The former is primarily expressed in muscle, while the latter is expressed in liver (9). At the cellular/molecular level, AdipoR2 mRNA expression is decreased in most (10–13) but not all (14, 15) liver samples from

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Abbreviations: AdipoR1, adiponectin receptor R1; AdipoR2, adiponectin receptor R2; AMPK, AMP-activated protein kinase; C/EBP, CCAAT/enhancer binding protein; CHOP, CCAAT/enhancer binding protein homologous protein; eIF2α, eukaryotic translation initiation factor 2 α; ER, endoplasmic reticulum; HCV, hepatitis C virus; JNK, c-Jun N-terminal kinase; NASH, nonalcoholic steatohepatitis; pAMPK, phosphorylated AMP-activated protein kinase; PBA, 4-phenylbutyrate; pJNK, phosphorylated c-Jun N-terminal kinase; PPARα, peroxisome proliferator-activated receptor α; TG, triglyceride; TPG, thapsigargin; TUN, tunicamycin.

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NASH patients as compared with control patients, with no difference in AdipoR1 mRNA expression (10). Yamauchi et al. (16) have shown that adenovirus-mediated overexpression of either AdipoR1 or AdipoR2 in liver reversed steatosis-linked insulin resistance in diabetes. This was recently confirmed by Tomita et al. (17) in a methionine-deficient and choline-deficient mouse model of NASH. Conversely, simultaneous disruption of these two receptors resulted in glucose intolerance and insulin resistance. AdipoRl is reportedly linked with AMP-activated protein kinase (AMPK) activation and AdipoR2 with peroxisome proliferator-activated receptor α (PPARα) (16). In addition, a hepatoprotective role of adiponectin is now emerging in which adiponectin acts as an antiinflammatory hormone participating in the repair process of liver injury (18, 19). Thus, this raises the possibility that the loss of hepatic AdipoR2 receptor in NASH patients may play an important role in worsening many of the metabolic, enzymatic, and gene regulatory changes that characterize human NASH patients (8, 20). However, very little information is currently available about the interaction between steatosis and loss of adiponectin receptor expression in hepatic cells.

Emerging evidence suggests that the ability to counterregulate inflammatory responses plays an important role in metabolic control through regulation of endoplasmic reticulum (ER) stress (21, 22). Metabolic disorders including obesity and alcohol ingestion can cause protein misfolding or overloading in the ER, triggering a stress cascade with pathological consequences including inflammation and cell death (23, 24). When mammalian cells are subjected to protein overload in the ER, an immediate response is activation of the pancreatic eukaryotic translation initiation factor α kinase (eIF2α) to inhibit protein biosynthesis through phosphorylation of eIF2α (21). If the overload of unfolded or misfolded proteins in the ER is not resolved, prolonged activation leads to programmed cell death requiring increased CCAAT/enhancer binding protein (C/EBP) homologous protein (CHOP/GADD153), a transcription factor that potentiates apoptosis (25). The transcription factor C/EBPβ is also activated in the liver through the actions of cytokines, hormones, nutrients, and ER stress (26, 27) and has been suggested to play an important role in proinflammatory and apoptotic pathways (28).

The main aim of the present study was to investigate the effects of lipid loading and the therapeutic effects of the PPARα agonist fenofibrate on expression of AdipoR2 and levels of phosphorylated AMPK (pAMPK) in Huh7 human hepatoma cells and in the Huh.8 cell line, which stably expresses a hepatitis C virus (HCV) subgenome replicon (29). These cells display many features of HCV-induced fatty liver disease including increased intracellular lipid accumulation and activation of JNK by a reactive oxygen species-ER stress pathway (30, 31). Fenofibrate is widely used as a hypolipidemic drug that activates PPARα and thereby regulates the expression of a number of genes critical for lipid and lipoprotein metabolism (32–34), leading to lipid catabolism in the liver and reduction in total body fat as well as circulating plasma lipids (35). We also investigated whether ER stress played a causal role in the loss of AdipoR2 and pAMPK in response to lipid overload. We found that ER stress inducers thapsigargin (TPG) and tunicamycin (TUN) caused a loss of AdipoR2 and pAMPK in Huh7 cells, while treatment with the chemical chaperone 4-phenylbutyrate (PBA), an agent used to reduce ER stress, restored palmitate- and TPG-mediated reduction of AdipoR2 expression and AMPK phosphorylation in both the parental Huh7 cell line and in Huh.8 derived cells. Our results demonstrate that ER stress is associated with decreased AdipoR2 and pAMPK levels and suggest that

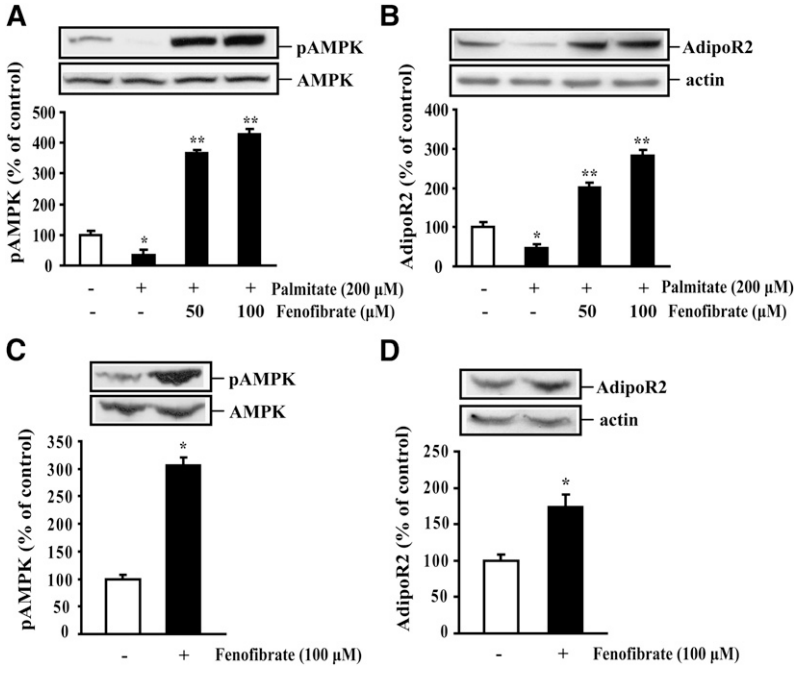

Fig. 1. Fenofibrate treatment prevented palmitate-induced loss of AMPK phosphorylation and AdipoR2 protein expression in Huh7 cells. Immunoblots and densitometric values for pAMPK (A and C) and AdipoR2 (B and D). Representative blots are shown. A and B: Huh7 cells were grown in high glucose medium (25 mM), serum-starved overnight, and treated without (control, with 0.1% ethanol and 0.5% albumin), with 200 μM palmitate or with 200 μM palmitate plus 50 or 100 μM fenofibrate for 24 h. C and D: Huh7 cells were grown in high glucose medium (25 mM), serum-starved overnight, and treated without (control, with 0.1% ethanol) or with 100 μM fenofibrate for 24 h. A–D: Cytosolic (for pAMPK) and membrane fractions (for AdipoR2) were prepared as described in Materials and Methods. Values are means ± SE of two independent experiments run with three replicates and expressed as % change over control (setting control to 100) after normalization to AMPK for pAMPK and actin for AdipoR2. Statistical significance was estimated using a nonpaired Student's *t*-test. $*P < 0.05$ versus control, $**P < 0.05$ versus palmitate-treated cells.

fenofibrate and PBA might be important therapeutic agents to prevent metabolic abnormalities by reducing ER stress associated with fatty liver disease.

MATERIALS AND METHODS

Cell culture and treatment

The cell lines used in this study, Huh7 and Huh.8, were provided by Dr. Charles Rice (Rockefeller University, New York, NY). The Huh.8 cell line was derived from the parental Huh7 cells and contains an HCV-derived expression vector stably integrated into the Huh7 background. The expression vector includes the HCV proteins NS2, NS3, NS4A, NS4B, NS5A, and NS5B linked to the antibiotic selection marker G418 (29). The Huh7 parental cell lines were cultured in DMEM containing high glucose (25 mM) supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, and 10% fetal bovine serum (all from Sigma-Aldrich, St. Louis, MO). Huh.8 cells were maintained in complete DMEM (25 mM glucose) supplemented with 10% heat-inactivated fetal bovine serum, nonessential amino acids, and 1 mg/ml G418 (Gemini Bio-Products, West Sacramento, CA). Cells were maintained at 37°C for two to three passages in a humidified environment containing 5% CO_2. For experimental treatments, Huh7 and Huh.8 cells were serum-starved overnight and treated with different concentrations of palmitate, fenofibrate (Sigma), TUN (Sigma), or PBA (Calbiochem) as indicated in the appropriate figure legends.

Palmitate preparation

The conjugation of palmitate and albumin was prepared to deliver a final concentration of 200 μM palmitate in the tissue culture medium. One mol of palmitic acid was conjugated to 3 mol of albumin (both from Sigma). A 200 mM palmitic acid solution was prepared by dissolving 0.256 g palmitic acid in 5 ml of 100% ethanol and separately, a 27% (wt/v) albumin solution was prepared in PBS. From there, the conjugate (palmitate:albumin) was prepared by adding 4.5 ml of 27% albumin to 250 μl of 200 mM palmitate solution. The final volume was brought to 5 ml with PBS after adjusting to pH 7.4. The 10 mM palmitate stock was added directly to the culture without further dilution. The appropriate controls for experiments using palmitate contained 0.1% ethanol and 0.5% albumin.

Preparation of cytosolic, membrane, and nuclear fractions

Cytosolic and nuclear extracts were prepared as previously reported (28). For analysis of AdipoR2 protein levels, membrane fractions were prepared by centrifuging the cytosolic extract in a

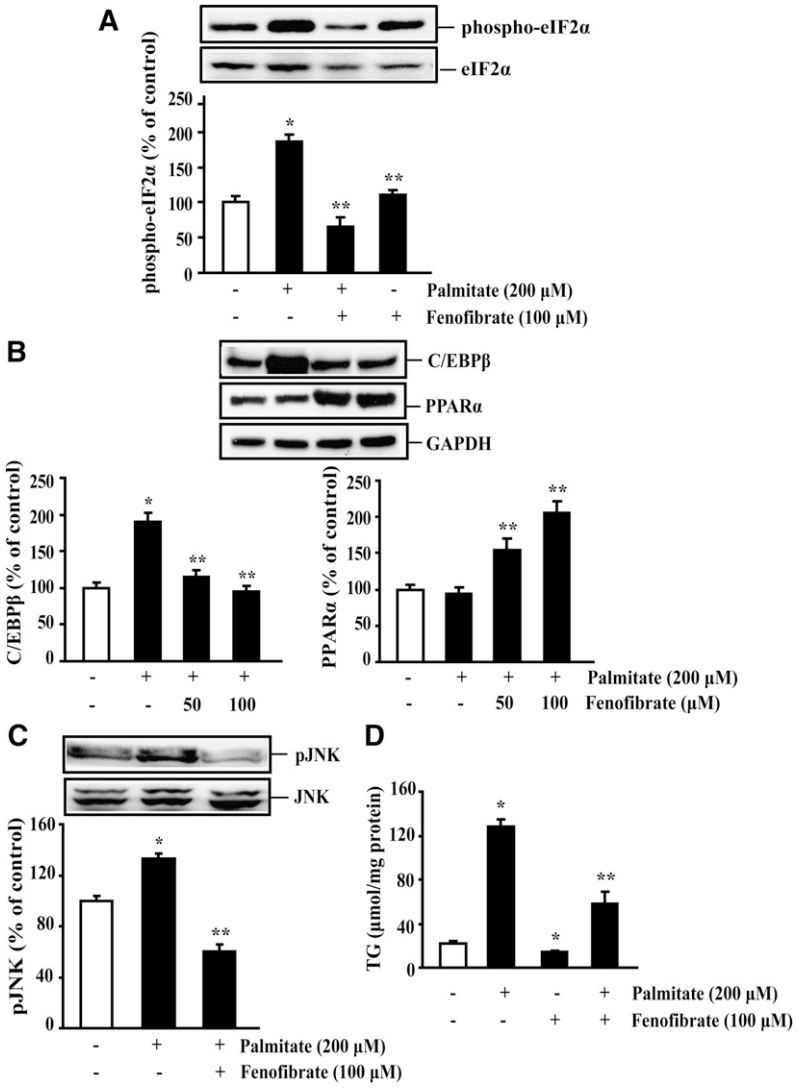

Fig. 2. Fenofibrate reduced stress and inflammatory proteins but increased PPARα protein expression and reduced pJNK activation and TG accumulation in palmitate-treated Huh7 cells. Immunoblots and densitometric values for phospho-eIF2α (A), C/EBPβ and PPARα (B), and pJNK (C). Representative blots are shown. A: Huh7 cells were grown in high glucose medium, serum-starved overnight, and treated without (control), with 200 μM palmitate, 200 μM palmitate plus 100 μM fenofibrate, or 100 μM fenofibrate for 24 h. B: Huh7 cells were treated without (control), with 200 μM palmitate, or with 200 μM palmitate plus 50 or 100 μM fenofibrate for 24 h. C: Huh7 cells were grown in high glucose medium, serum-starved overnight, and treated without (control), with 200 μM palmitate, or with 200 μM palmitate plus 100 μM fenofibrate for 24 h. Values are means ± SE of two independent experiments run with three replicates and expressed as % change over control (setting control to 100) after normalization to eIF2α for phospho-eIF2α, GAPDH for C/EBPβ and PPARα, and JNK for pJNK. D: Huh7 cells were grown in high glucose medium, serum-starved overnight, and treated without (control), with 200 μM palmitate, with 100 μM fenofibrate, or with 200 μM palmitate plus 100 μM fenofibrate for 24 h. A–D: All experiments, including controls, had 0.1% ethanol and 0.5% albumin final concentrations. Statistical significance was estimated using a nonpaired Student's *t*-test. $^*P < 0.05$ versus control, $^{**}P < 0.05$ versus palmitate-treated cells.

Beckman ultracentrifuge at 60,000 rpm for 2 h. The pellets were resuspended in 50 μl of hypotonic buffer (10 mM HEPES, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol, 1 mM PMSF, 2 μg/ml each of aprotinin and leupeptin, and 0.5 mg/ml benzamidine). For phospho-protein assays, cells were prepared and quantitated as described previously (28).

Immunoblot analysis

Lysates were subjected to SDS-PAGE and Western blot analysis as previously described (28). Primary antibodies used in this study were C/EBPβ, CHOP, PPARα, actin, and GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA), phosphorylated JNK (pJNK), pAMPK (Thr172), AMPK, phospho-eIF2α, and eIF2α (Cell Signaling Technology, Danvers, MA), JNK (R and D Systems, Minneapolis, MN), and AdipoR2 (Phoenix Pharmaceuticals, Burlingame, CA).

Extraction of lipid and measurement of TG content

Lipids were extracted from cells using the procedure of Bligh and Dyer (36). Quantitative enzymatic measurements of TG content were performed in triplicate using TG determination reagents (Sigma).

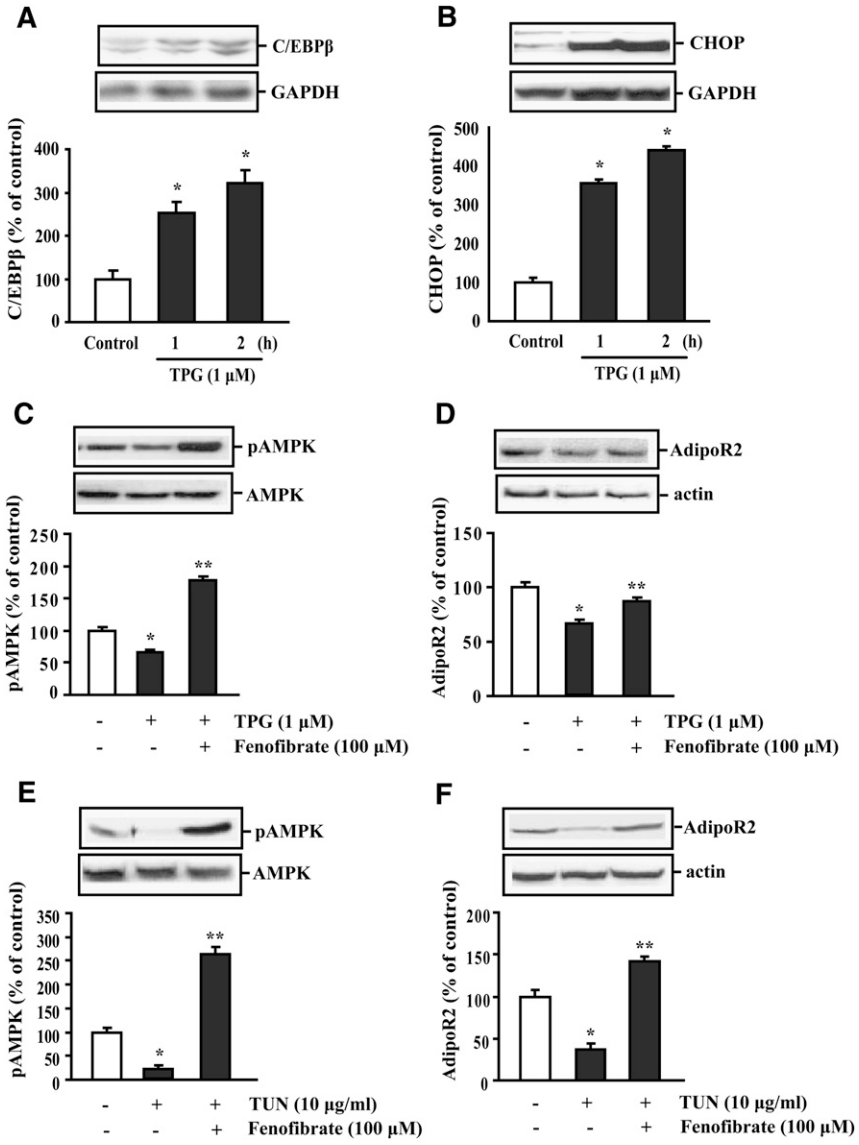

Fig. 3. ER stress-inducer TPG increased C/EBPβ and CHOP protein expression and fenofibrate prevented TPG- and TUN-mediated reduction of AMPK phosphorylation and AdipoR2 expression in Huh7 cells. Immunoblots and densitometric values for C/EBPβ (A), CHOP (B), pAMPK (C and E), and AdipoR2 (D and F). Representative blots are shown. A and B: Huh7 cells were grown in high glucose medium, serum-starved overnight, and treated without (control, with 0.05% DMSO) or with 1 μM TPG for 1 and 2 h. C–F: Huh7 cells were grown in high glucose medium, serum-starved overnight, treated without (control) or pretreated with 1 μM TPG for 2 h (C and D) or 10 μg/ml TUN for 6 h (E and F) and incubated with 100 μM fenofibrate for 24 h (all experiments had 0.1% ethanol and 0.05% DMSO final concentrations). Values are means ± SE of two independent experiments run with three replicates and expressed as % change over control (setting control to 100) after normalization to GAPDH for C/EBPβ and CHOP, AMPK for pAMPK, and actin for AdipoR2. Statistical significance was estimated using a nonpaired Student's *t*-test. $^{*}P < 0.05$ versus control, $^{**}P < 0.05$ versus TPG- or TUN- treated cells.

RESULTS

Fenofibrate treatment prevented palmitate-induced loss of AMPK phosphorylation and AdipoR2 expression in Huh7 cells

We checked the effects of palmitate and palmitate in combination with fenofibrate on pAMPK and AdipoR2 levels in Huh7 human hepatoma cells. Huh7 cells were grown in complete DMEM (25 mM glucose) treated with either 200 μM palmitate alone or in combination with two concentrations of fenofibrate (50 and 100 μM), a known hypolipidemic agent. Palmitate significantly suppressed both pAMPK and AdipoR2 levels and coincubation with fenofibrate prevented palmitate-induced suppression of pAMPK and AdipoR2 expression in Huh7 cells (**Fig. 1, A** and **B**). In addition, we found that fenofibrate treatment alone (100 μM) for 24 h increased pAMPK levels and AdipoR2 protein expression in Huh7 cells (Fig. 1, C and D).

Fenofibrate reduced palmitate-mediated induction of ER stress and inflammatory proteins

The ER stress response has recently been recognized in the pathogenesis of liver disease, including apoptosis, fat accumulation, and inflammation (37). To determine whether the positive effects of fenofibrate had any effect on reducing ER stress in palmitate-treated Huh7 cells, we monitored the expression of C/EBPβ and phospho-eIF2α, two key proteins induced by two different arms of the ER stress pathway (38, 39). Palmitate treatment (200 μM) significantly increased phospho-eIF2α levels while coincubation with fenofibrate (100 μM) markedly reduced phospho-eIF2α expression (**Fig. 2A**), consistent with a reduction in ER stress. Palmitate alone induced a marked 100% increase in nuclear C/EBPβ, an inducer of fatty liver and ER stress in hepatic cells (28, 40), while addition of fenofibrate significantly decreased C/EBPβ protein expression and increased PPARα levels in palmitate-treated Huh7 cells (Fig. 2B).

Fenofibrate blocked TG accumulation and pJNK activation in palmitate-treated Huh7 cells

Palmitate treatment has been shown to induce sustained JNK activation and insulin resistance in mouse hepatocytes (41). To determine whether fenofibrate treatment could prevent JNK activation in Huh7 cells, cells were treated with 200 μM palmitate or 200 μM palmitate plus 100 μM fenofibrate for 24 h. pJNK was significantly induced in palmitate-treated Huh7 cells, while fenofibrate reduced palmitate-induced pJNK by >50% (Fig. 2C). Huh7 cells incubated with palmitate showed a 6-fold increase in TG concentration, while fenofibrate alone or coincubation with palmitate significantly lowered TG levels compared with levels in control or palmitate-treated Huh7 cells, respectively (Fig. 2D). These results demonstrate that fenofibrate treatment prevented TG accumulation, reduced

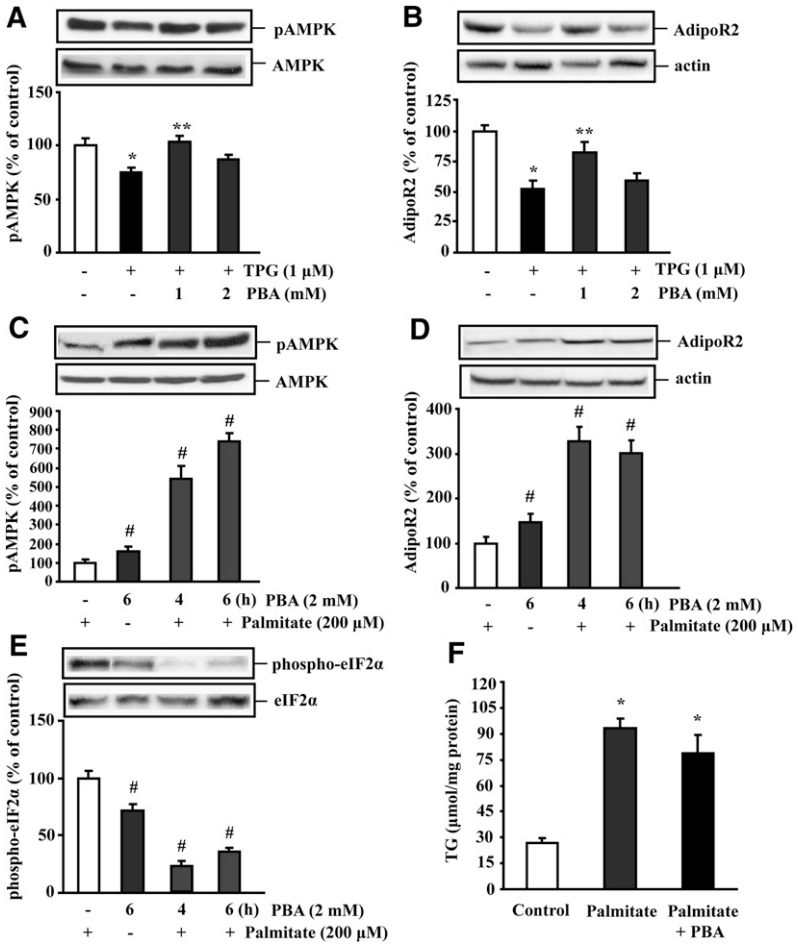

Fig. 4. PBA treatment attenuated TPG- and palmitate-mediated reduction of pAMPK and AdipoR2 expression and decreased phospho-eIF2α expression in Huh7 cells. Immunoblots and densitometric values for pAMPK (A and C), AdipoR2 (B and D), and phospho-eIF2α (E). A and B: Huh7 cells were grown in high glucose medium, serum-starved overnight, and treated without (control, with 0.05% DMSO), with 1 μM TPG, or with 1 μM TPG plus 1 mM or 2 mM PBA for 24 h. C–E: Huh7 cells were grown in high glucose medium, serum-starved overnight, and treated without (control), with either 200 μM palmitate for 24 h or cells were pretreated with 2 mM PBA for 4 or 6 h and then treated with 200 μM palmitate for 24 h (all experiments had 0.1% ethanol, 0.5% albumin, and 0.05% DMSO final concentrations). Values are means ± SE of two independent experiments run with three replicates and expressed as % change over control (setting control to 100) after normalization to AMPK for pAMPK, actin for AdipoR2, and eIF2α for phospho-eIF2α. F: TG levels in Huh7 cells. Cells were grown in high glucose medium, serum-starved overnight, and treated without (control), with 200 μM palmitate for 24 h, or pretreated with 2 mM PBA for 6 h and then with 200 μM palmitate for 24 h (all experiments had 0.1% ethanol, 0.5% albumin, and 0.05% DMSO final concentrations). Statistical significance was estimated using a nonpaired Student's *t*-test. $^{*}P < 0.05$ versus control, $^{**}P < 0.05$ versus TPG-treated cells, $^{\#}P < 0.05$ versus palmitate-treated cells.

pJNK activation, and reduced phospho-eIF2α and C/EBPβ protein levels, suggesting the ability to lower ER stress levels.

TPG increased the expression of C/EBPβ and CHOP proteins while fenofibrate restored TPG- and TUN-mediated reduction of AMPK phosphorylation and AdipoR2 expression in Huh7 cells

Having established an effect of fenofibrate on lowering markers of ER stress and restoration of AdipoR2, we then went on to ask whether treatment with two ER stress-inducing agents, TPG and TUN, might be causative for reduced AdipoR2 and pAMPK levels in Huh7 cells and to test the efficacy of fenofibrate. Huh7 cells treated with 1 μM TPG increased C/EBPβ and CHOP protein levels (**Fig. 3, A** and **B**), indicative of ER stress. Huh7 cells were then pretreated with 1 μM TPG and incubated with or without 100 μM fenofibrate for 24 h. Both AMPK phosphorylation and AdipoR2 expression were significantly lower in Huh7 cells pretreated with TPG; however, fenofibrate treatment significantly increased AMPK phosphorylation and AdipoR2 expression (Fig. 3, C and D). Similar results were found when Huh7 cells were pretreated with 10 μg/ml TUN for 6 h and then incubated with or without 100 μM fenofibrate for 24 h (Fig. 3, E and F).

PBA prevented TPG- and palmitate-mediated reduction of AMPK phosphorylation and AdipoR2 expression

Treatment of obese and diabetic *ob/ob* mice with PBA or tauroursodeoxycholate, both chemical chaperones, has been shown to restore glucose tolerance and insulin sensitivity and resolve fatty liver in parallel with marked inhibition of ER stress markers pancreatic eIF2α kinase, inositol-requiring enzyme-1 alpha, and JNK activation (22, 42). To determine whether PBA can restore AMPK phosphorylation and AdipoR2 expression in TPG- and palmitate-loaded Huh7 cells, we coincubated Huh7 cells with the chemical chaperone PBA. PBA prevented TPG- and palmitate-mediated reduction of AMPK phosphorylation and AdipoR2 expression (**Fig. 4, A–D**). PBA also reduced palmitate-mediated induction of phospho-eIF2α expression (Fig. 4E), suggesting a reduction in ER stress. However, PBA did not reduce palmitate-mediated induction of TG levels in Huh7 cells (Fig. 4F). This result provides evidence that ER stress, rather than lowering TG, might contribute to reduced AMPK phosphorylation and AdipoR2 expression and that suppression of ER stress can restore AMPK phosphorylation and AdipoR2 expression.

Fenofibrate treatment restored AMPK phosphorylation and AdipoR2 expression and reduced pJNK activation in HCV-induced Huh.8 cells

Hepatitis C is well known to induce insulin resistance and steatosis (43, 44) and is associated with loss of AdipoR2 mRNA expression in human liver in vivo (11). Furthermore, it is well recognized that HCV invades the ER and induces ER stress with profound effects on normal ER functions (45). In the Huh7-derived cell line Huh.8 engineered with HCV, AdipoR2 expression was significantly lower compared with Huh7 cells (**Fig. 5A**). Interestingly, AdipoR2 levels were further reduced by addition of TPG in Huh.8 cells (Fig. 5A), suggesting that the initial decrease in AdipoR2 in Huh.8 compared with Huh7 cells might have additional causes. We next treated Huh.8 cells with increasing fenofibrate concentrations to investigate the effects on pAMPK and AdipoR2 levels. Addition of fenofibrate, especially at concentrations of 75 and 100 μM, increased (50–800%) both pAMPK and AdipoR2 expres-

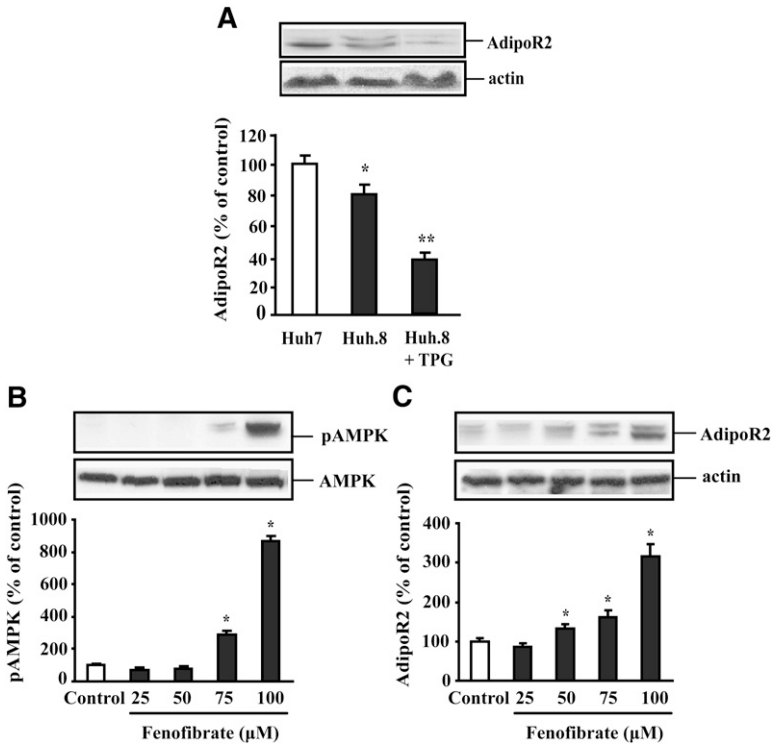

Fig. 5. Fenofibrate treatment restored pAMPK and AdipoR2 expression in Huh.8 cells. Immunoblots and densitometric values for AdipoR2 in Huh7 and Huh.8 cells (A) and pAMPK (B) and AdipoR2 (C) in Huh.8 cells. A: Huh7 and Huh.8 cells were grown in high glucose medium, serum-starved overnight, and treated without TPG (controls, with 0.05% DMSO) or Huh.8 cells were treated with 1 μM TPG for 12 h. $*P < 0.05$ versus Huh7 control, $**P < 0.05$ versus Huh.8 control. B and C: Huh.8 cells were grown in high glucose medium, serum-starved overnight, and treated without (control, with 0.1% ethanol) or with fenofibrate (25, 50, 75, or 100 μM) for 24 h. Values are means ± SE of two independent experiments run with three replicates and expressed as % change over control (setting control to 100) after normalization to AMPK for pAMPK and actin for AdipoR2. Statistical significance was estimated using a nonpaired Student's *t*-test. $*P < 0.05$ versus control.

sion (Fig. 5, B and C), while fenofibrate at 100 μM for 24 h significantly reduced C/EBPβ and pJNK activation (**Fig. 6, A** and **B**). Huh.8 cells contain a 2-fold increase in levels of TG storage in the absence of palmitate treatment compared with the parental Huh7 cell line (Fig. 2D vs. Fig. 6C). However, fenofibrate treatment (50 and 100 μM for 24 h) failed to reduce TG accumulation in Huh.8 cells (Fig. 6C). These results indicate that fenofibrate-mediated augmentation of AdipoR2 levels in Huh.8 cells is independent of TG content.

DISCUSSION

These present studies suggest that exposure to saturated fatty acids may contribute to ER stress-related downregulation of AdipoR2 and loss of pAMPK in the liver in addition to hepatic steatosis. Our results suggest that ER stress is an important pathological feature associated with downregulation of AdipoR2 and pAMPK levels in human hepatoma cells exposed to fatty acids. In Huh7 cells, palmitate significantly reduced AdipoR2 protein levels, while treatment with fenofibrate blocked fatty acid-mediated loss of AdipoR2 levels and reduced levels of ER stress indicators phospho-eIF2α and pJNK. We also show the chemical inducers of ER stress TPG and TUN reduced pAMPK and AdipoR2 levels. To further demonstrate that reversing ER stress had the opposite effect, we treated Huh7 cells with PBA, an agent that clears misfolded proteins from the ER and lowers ER stress in cells and animals (46). PBA prevented both palmitate- and TPG-induced loss of AdipoR2 and pAMPK levels in Huh7 cells (Fig. 4), suggesting that relieving ER stress with either PBA or fenofibrate can restore AdipoR2 and pAMPK.

We also observed a restoration in AdipoR2 and pAMPK levels in HCV-infected Huh.8 cells treated with fenofibrate despite no change in TG content, indicating that fenofibrate-mediated increase in AdipoR2 levels in Huh.8 cells is independent of TG content. These results imply a different mechanism(s) other than simply reducing TG could be involved in the effect of fenofibrate to restore levels of AdipoR2 and pAMPK. Huh.8 cells have defects in several pathways of fatty acid metabolism [oxidation, export, de-novo lipogenesis, and uptake (47–49)] that may cause TG levels to be unchanged with fenofibrate. Although the mechanism for the fenofibrate effect on restoring AdipoR2 and ER stress without changing TG levels in Huh.8 cells is unclear, it is possible that such mechanisms as reducing the impairment in protein folding or membrane trafficking may be important contributors to the restoration of AdipoR2. However, more studies are necessary for clarification.

While ER stress activity is acutely induced by fatty acids, this process is adaptive; in Huh.8 cells exposed to the ER stress agent TPG, there was a further loss of AdipoR2 (Fig. 5A). These results suggest that other virally induced events in addition to ER dysfunction may play an important role in the downregulation of AdipoR2 expression in hepatitis C. ER stress also has been reported to induce reactive oxygen species production (46). It has been shown that PBA reduces inducible nitric oxide synthase and tumor necrosis factor α expression (50), suggesting that PBA could indirectly regulate pathways that target AdipoR2 for degradation. Further studies are necessary to understand the mechanisms that regulate AdipoR2 protein turnover in HCV-induced fatty liver disease.

Recent studies have demonstrated that exogenous fatty acids, especially palmitate, cause ER stress and are associated with increased expression of the proapoptotic transcription factor CHOP (51). Certain pathological stress conditions disrupt ER homeostasis and lead to accumulation of unfolded or misfolded proteins in the ER lumen (52, 53). Among these are abnormal lipid accumulation and lipid biosynthesis (54, 55). Ozcan et al. (23) demonstrated an association among obesity, ER stress, and insulin resistance through the JNK pathway. More recently,

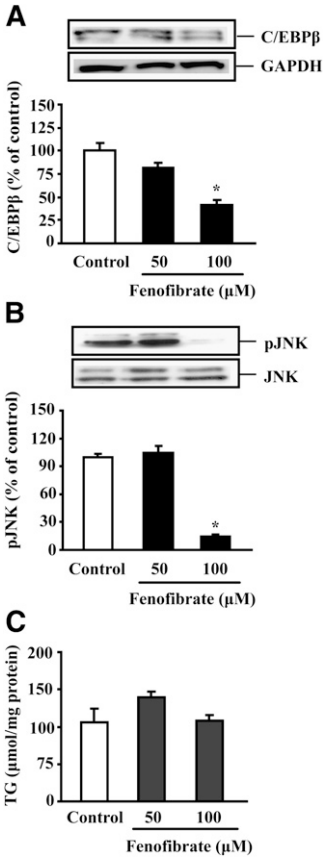

Fig. 6. Fenofibrate treatment reduced pJNK activation and C/EBPβ expression in Huh.8 cells without affecting TG levels. A–C: Huh.8 cells were grown in high glucose medium, serum-starved overnight, and treated without (control, with 0.1% ethanol) or with 50 or 100 μM fenofibrate for 24 h. Immunoblots and densitometric values for C/EBPβ (A) and pJNK (B). Representative blots are shown. Values are means ± SE of two independent experiments run with three replicates and expressed as % change over control (setting control to 100) after normalization to GAPDH for C/EBPβ and JNK for pJNK. C: Extraction of lipid and measurements of TG were described in Materials and Methods. Values are means ± SE of two independent experiments run with three replicates and expressed as μmol/mg protein. A–C: Statistical significance was estimated using a nonpaired Student's *t*-test. $^*P < 0.05$ versus control.

this group has shown that relieving ER stress using a ER chemical chaperone prevented the detrimental effects of obesity on insulin resistance and inflammation with no change in body weight (22), suggesting that attenuating ER stress may be an important therapeutic target to prevent the loss of adiponectin receptor expression associated with steatosis.

In addition to a loss of AdipoR2, palmitate treatment was associated with a profound reduction in basal AMPK activation, as judged by reduced pAMPK levels. Several mechanisms may be responsible for palmitate-mediated reduction of pAMPK activity. First, palmitate treatment might enhance free fatty acid availability to increase TG synthesis by increasing malonyl-CoA levels, an inhibitor of AMPK activation (56, 57), and thereby reducing AMPK phosphorylation. In addition, alterations in glycerol phosphate acyltransferase, malonyl-CoA decarboxylase, PPARα, and PPARγ coactivator-1 α have all been observed in obesity-related hyperlipidemic conditions in liver (58–61), raising the possibility that excess saturated fatty acids may impair multiple genes and enzymes related to fatty acid oxidation, a condition that also raises malonyl-CoA levels and would inhibit pAMPK. The reduced basal AMPK phosphorylation and AdipoR2 levels, potentially with different signaling activities (9), may provide an explanation for fatty acid-induced attenuation of adiponectin responses at the postreceptor level. However, further studies are necessary to confirm this hypothesis.

Hepatitis C infection can lead to hepatic steatosis and insulin resistance (62). Here, we have documented a reduction of basal AMPK phosphorylation and AdipoR2 protein expression in Huh.8 cells, along with a substantial increase in TG accumulation under basal conditions. Regarding the mechanism of HCV-induced hepatic steatosis, a loss of PPARα, a nuclear receptor regulating several genes responsible for fatty acid oxidation and reduced expression of carnitine palmitoyltransferase 1A in the liver of chronic hepatitis C patients, has been reported (47). Another study has shown that HCV core protein stimulates gene expression of lipogenic enzymes and fatty acid uptake-associated proteins (48). Secretion of apolipoprotein B via lipoproteins is also drastically reduced in cells containing the HCV subgenomic replicon (49), suggesting there may also be a defect in lipoprotein secretion in addition to increased synthesis and/or impaired fatty acid oxidation associated with chronic hepatitis C. Recently, it has been shown that serum adiponectin levels are low in some (63) but not all (64) chronic hepatitis C patients, suggesting a role for reduced circulating adipokines. Interestingly, fenofibrate reversed HCV-mediated impairment of AMPK and AdipoR2 expression in Huh.8 cells but did not affect TG levels. Fenofibrate reduced the palmitate-induced activation of C/EBPβ and pJNK while increasing PPARα protein expression and this supports the role of fenofibrate in reducing ER stress and activation of inflammatory genes found in earlier studies (33, 65–67). While chronic high-fat feeding in mice has been shown to both decrease (49) and increase (68) hepatic AdipoR1 and AdipoR2 mRNA expression, hepatocytes incubated with insulin reduced the expression of AdipoR1/R2 mRNA in vitro (69). Recently, Lin et al. (9) noted that hyperglycemia in mice decreased AdipoR2 mRNA in liver, as well as decreasing PPARα target gene expression, and was associated with increased liver TG. Tomita et al. (17) found that enhancement of AdipoR2 expression in the liver improved NASH at every stage, from the early stage to the progression of fibrosis, while inhibition of AdipoR2 signaling in the liver diminished hepatic PPARα signaling, leading to an increase in lipid peroxidation, suggesting that controlling the levels of AdipoR2 might be an important therapeutic target for the treatment of NASH.

In summary, we have shown that AdipoR2 expression and basal AMPK phosphorylation are suppressed in palmitate-loaded Huh7 cells and in HCV subgenome replicon Huh.8 cells, a model for HCV-induced steatosis. Fenofibrate treatment in both palmitate-loaded Huh7 cells and Huh.8 cells restored basal pAMPK and AdipoR2 expression by reducing ER stress and inflammatory proteins. Although the mechanism underlying ER stress-induced loss of AdipoR2 protein and pAMPK remains to be elucidated, these results suggest that fenofibrate and PBA might be useful therapeutic agents to correct lipid- and stress-mediated metabolic abnormalities.

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