Lipid-Induced Endoplasmic Reticulum Stress in Liver Cells Results in Two Distinct Outcomes: Adaptation with Enhanced Insulin Signaling or Insulin Resistance

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Chronically elevated fatty acids contribute to insulin resistance through poorly defined mechanisms. Endoplasmic reticulum (ER) stress and the subsequent unfolded protein response (UPR) have been implicated in lipid-induced insulin resistance. However, the UPR is also a fundamental mechanism required for cell adaptation and survival. We aimed to distinguish the adaptive and deleterious effects of lipid-induced ER stress on hepatic insulin action. Exposure of human hepatoma HepG2 cells or mouse primary hepatocytes to the saturated fatty acid palmitate enhanced ER stress in a dose-dependent manner. Strikingly, exposure of HepG2 cells to prolonged mild ER stress activation induced by low levels of thapsigargin, tunicamycin, or palmitate augmented insulin-stimulated Akt phosphorylation. This chronic mild ER stress subsequently attenuated the acute stress response to high-level palmitate challenge. In contrast, exposure of HepG2 cells or hepatocytes to severe ER stress induced by high levels of palmitate was associated with reduced insulin-stimulated Akt phosphorylation and glycogen synthesis, as well as increased expression of glucose-6-phosphatase. Attenuation of ER stress using chemical chaperones (trimethylamine *N*-oxide or tauroursodeoxycholic acid) partially protected against the lipid-induced changes in insulin signaling. These findings in liver cells suggest that mild ER stress associated with chronic low-level palmitate exposure induces an adaptive UPR that enhances insulin signaling and protects against the effects of high-level palmitate. However, in the absence of chronic adaptation, severe ER stress induced by high-level palmitate exposure induces deleterious UPR signaling that contributes to insulin resistance and metabolic dysregulation. **(*Endocrinology* 153: 2164–2177, 2012)**

Insulin action in the liver plays a central role in the regulation of whole-body energy homeostasis. Insulin suppresses hepatic gluconeogenesis and stimulates the liver to store glucose in the form of glycogen. These actions are initiated by the binding of insulin to its receptor, which leads to the autophosphorylation of tyrosine residues and the recruitment and activation of insulin receptor intracellular substrates, such as insulin receptor substrate 1 (IRS1). This leads to the recruitment of PI3K (phosphatidylinositol 3-kinase) and ultimately to the activation of the downstream protein kinase effector, Akt. Akt activation then promotes glycogen synthesis and inhibits gluconeogenesis (1–3). Impaired insulin action in liver plays a crucial part in the pathogenesis of metabolic disorders, such as metabolic syndrome, nonalcoholic fatty liver disease, and type 2 diabetes (4–7). Lipid oversupply is a likely cause of obesity-associated hepatic insulin resistance (8). Increased plasma fatty acids are associated with insulin resistance in humans (9), and *in vivo* infusion of lipids leads to impaired insulin signaling in rodents and humans (8, 10–12). However, the underlying mechanisms by which fatty acids impair insulin action are not fully understood. The suggested mechanisms include synthesis of ceramide (13, 14), accumulation of diacylglycerol and as-

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Abbreviations: ATF, Activation transcription factor; CHOP, C/EBP homologous protein; DMSO, dimethylsulfoxide; eIF2α, eukaryotic initiation factor 2 α subunit; ER, endoplasmic reticulum; FBS, fetal bovine serum; IRE1, inositol requiring enzyme-1; IRS1, insulin receptor substrate 1; PERK, protein kinase RNA-like ER kinase; PI3K, phosphatidylinositol 3-kinase; siRNA, small interfering RNA; Tg, thapsigargin; Tm, tunicamicyn; TMAO, trimethylamine *N*-oxide; TUDCA, tauroursodeoxycholic acid; UPR, unfolded protein response; *XBP1*, X-box binding protein 1.

sociated activation of novel protein kinase C (15, 16), generation of reactive oxygen species (17), and activation of the IκB kinase/nuclear factor-κB pathway (18). Recently, endoplasmic reticulum (ER) stress has emerged as a potential mechanism for hepatic insulin resistance. ER stress is implicated in obesity-associated insulin resistance in animals (19–22) and humans (23), and direct activation of severe ER stress in Fao liver cells can impair insulin signaling (19). However, whether ER stress is required for fatty acid-mediated insulin resistance has not yet been established.

ER stress is caused by various insults that lead to the accumulation of misfolded proteins in the ER lumen. Upon initiation of ER stress, cells activate a signaling cascade known as the unfolded protein response (UPR). The role of the UPR is both to alleviate the ER stress and, paradoxically, to activate apoptosis, depending on the nature and severity of the stressor (24). ER stress is sensed by three transmembrane ER proteins: PERK (protein kinase RNA-like ER kinase), ATF6 (activation transcription factor-6) and IRE1 (inositol requiring enzyme-1). In an inactive state, the luminal domain of these proteins binds to the ER chaperone BiP (GRP78). Under stress conditions, BiP dissociation from the sensor proteins leads to their activation (25). PERK and IRE1 are activated by dimerization and autophosphorylation, whereas ATF6 translocates from the ER to the Golgi where it is activated by proteolysis, releasing an active transcription factor (26–28). Activated PERK phosphorylates eIF2α (eukaryotic initiation factor 2 α subunit), which leads to the inhibition of protein translation and the production of the transcription factor ATF4. Phosphorylation of IRE1 activates its endoribonuclease activity, which leads to splicing of *XBP1* (X-box binding protein 1) mRNA and the translation of a mature XBP1 transcription factor. These transcription factors facilitate adaptation to ER stress by increasing the protein-folding capacity of the ER via upregulation of chaperones, foldases, and components of the ER-associated degradation pathway. However, there are also multiple apoptotic pathways emanating from UPR activation, including ATF4-mediated transcriptional activation of C/EBP homologous protein (CHOP) (29) and IRE1-mediated activation of JNK (30).

Recent studies have demonstrated a critical influence of chronic low-level ER stress on the subsequent UPR signaling induced by an acute ER stress challenge (31). The suggestion from these studies is that the nature of the cellular response to an ER stress insult depends on the underlying status of the adaptive UPR. This may have important implications for cell function and dysfunction, because the UPR has been proposed to play both physiological and pathophysiological roles in several cell systems (32, 33). Whether the adaptive UPR induced by chronic low-level ER stress influences insulin action has not been tested.

In this study, we investigated the effects of ER stress on insulin action in liver cells. We examined the effects of variable periods of both mild and severe ER stress on insulin signaling to recapitulate the broad range of conditions that are likely to be encountered physiologically. Our studies found that mild and severe ER stress activation differentially regulates insulin signaling in hepatocytes: severe ER stress was required for the insulin resistance induced by the common saturated fatty acid, palmitate, whereas chronic mild ER stress actually enhances insulin signaling and protects against the deleterious effects of palmitate. These studies provide novel insight into the interface between the UPR and the insulin-signaling pathway in liver cells.

Materials and Methods

Materials

Cell culture media, buffers, and antibiotics, as well as NuPAGE reagents and gels, were from Invitrogen (Carlsbad, CA). Fetal bovine serum (FBS) was from Thermo Scientific (Scoresby, Victoria, Australia). Fatty acid-free BSA, sodium palmitate, thapsigargin (Tg), tunicamicyn (Tm), trimethylamine *N*-oxide (TMAO), tauroursodeoxycholic acid (TUDCA) were from Sigma-Aldrich (St. Louis, MO). Insulin (Actrapid) was obtained from Novo Nordisk Pharmaceuticals (Baulkham Hills, New South Wales, Australia). Transfection reagent and ON-TARGET*plus* SMARTpool small interfering RNA (siRNA) were purchased from Dharmacon (Lafayette, CO). Antibody details are provided in Supplemental *Materials and Methods* published on The Endocrine Society's Journals Online web site at http://endo.endojournals.org.

Cell culture and treatments

HepG2 human hepatoma cells were maintained in MEM containing 5.6 mM glucose, supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin. For short-term treatments, cells were seeded at a density of 6×10^4 cells/cm^2 and grown in MEM supplemented with 10% FBS and 25 mM HEPES, pH 7.5. Treatments were performed in medium containing 10% FBS or 2% FBS before stimulation with insulin. For ER stress induction, cells were incubated with Tg (1–50 nM) or Tm (50–1000 ng/ml) for 8 to 24 h. Dimethylsulfoxide (DMSO) alone was used as a control treatment. For lipid treatment, cells were incubated in the presence of 100–750 μM palmitate coupled to BSA, as described previously (34). Uncoupled BSA (0.9%) was used in control conditions. Cells were treated with TMAO (50–100 mM) or TUDCA (0.5 mg/ml) to alleviate ER stress. For siRNA transfection, HepG2 cells were transfected with XBP1 ON-TARGET*plus* SMARTpool siRNA or Non-Targeting siRNA using DharmaFECT 4 (Dharmacon) according to the manufacturer's instruction. For chronic experiments, cells were seeded at a density of $4–6.5 \times 10^4$ cells/cm^2 and grown in MEM supplemented with 10% FBS, 50 U/ml penicillin, 50 μg/ml strep-

tomycin, and 25 mM HEPES, pH 7.5. Cells were cultured for 3–6 d (one passage) in the presence of 2.5 nM Tg or 100 ng/ml Tm, or for 3–8 d (one or two passages) in the presence of 100 μM palmitate. DMSO or BSA (0.2%) alone were used as control treatments. Medium was changed daily. In some experiments, chronic treatments were followed by incubation with 750 μM palmitate for 18 h in MEM containing 2% FBS before stimulation with insulin.

Hepatocytes isolation, culture, and treatment

Primary hepatocytes were isolated from adult mice (C57BL/6) by a modified previously described method (35). See Supplemental *Materials and Methods*, for details. Procedures were approved by the Garvan Institute/St.Vincent's Hospital Animal Ethics Committee. Hepatocytes were seeded at a density of 7.5×10^4 cells/cm^2 and grown in M199 medium supplemented with 50 U/ml penicillin, 50 μg/ml streptomycin, 0.1% BSA, 2% dialyzed FBS (Invitrogen, Carlsbad, CA), 100 nM dexamethasone, and 100 nM insulin. 4 h after seeding, the medium was replaced with M199 medium supplemented with 50 U/ml penicillin, 50 μg/ml streptomycin, and 100 nM dexamethasone. When hepatocytes were kept in culture for more than 24 h before treatment, 1 nM insulin was added to the medium. Cells were treated with palmitate, TMAO, and TUDCA as described for HepG2 cells. Cells were insulin starved for 18 h before insulin stimulation.

Protein analysis

Protein was extracted in lysis buffer containing 50 mM HEPES (pH 7.5), 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM Na_3VO_4, 10 mM $Na_4P_2O_7$, 100 mM NaF, 1 mM EGTA, 1% (vol/vol) Triton × 100, 10% (vol/vol) glycerol, 0.2 mM phenylmethylsulfonyl fluoride, Complete Protease Inhibitor, and PhosSTOP Phosphatase Inhibitor Cocktail Tablets (Roche Diagnostics). Total protein concentrations were assessed using Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA). For IRS1 immunoprecipitation, 0.8 μg IRS1 antibody was added in 500 μl lysis buffer containing 400 μg total protein, and the samples were rocked gently overnight at 4 C. The complexes were precipitated in the presence of protein G-Sepharose (Invitrogen) for 1.5 h. Immunoprecipitated complexes were washed in lysis buffer and eluted in 60 μl loading buffer (NuPAGE LDS Sample Buffer completed with NuPAGE Reducing Agent, Invitrogen). After denaturation (70 C for 10 min), samples were subjected to SDS-PAGE. Alternatively, total lysates or immunoprecipitation supernatants were diluted in loading buffer and 10–30 μg of denatured proteins were separated on NuPAGE gels. Western blotting was performed as described previously (36). See Supplemental *Materials and Methods* for details. Densitometry analysis was performed using ImageJ software.

RNA analysis

Total RNA was extracted using the RNeasy mini kit, and cDNA was synthesized using the QuantiTect Reverse Transcription Kit according to manufacturer's protocols (QIAGEN, Doncaster, Victoria, Australia). For *XBP1* mRNA splicing analysis, fragments of spliced and unspliced *XBP1* cDNA were amplified by PCR and digested with *Pst*I (New England Biolabs, Ipswich, MA). The cDNA corresponding to the spliced form of XBP1 mRNA, which encode the active transcription factor, lacks the *Pst*I restriction site. Digested and undigested fragments were separated using 1.5% agarose gels and quantified by densitometry using a ChemiDoc System and Quantity One software (Bio-Rad Laboratories, Hercules, CA). The value obtained for spliced *XBP1* mRNA (undigested fragment) was expressed as a ratio of the total *XBP1* mRNA level (undigested + digested fragments) for each sample. For quantitative gene expression analysis, real-time PCR was performed using *Power* SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) and gene-specific primers (Supplemental Table 1) on a 7900HT Real-Time PCR System (Applied Biosystems). The values obtained for each specific gene were normalized to the control gene (*RPS9* for HepG2 cell extracts and *cyclophilin A* for hepatocyte extracts).

Glycogen synthesis assays

HepG2 cells in six-well plates were incubated for 1 h in 1 ml/well of MEM supplemented with 25 mM HEPES (pH 7.5) containing D-[U-^{14}C]glucose (2 μCi/ml) (PerkinElmer, Waltham, MA). Glycogen production was assayed as described previously (14). See Supplemental *Materials and Methods* for details.

Statistical analysis

All values are given as means ± SE. Statistical analyses were performed using unpaired, two-tailed *t* test, one-sample *t* test, or ANOVA with Bonferroni *post hoc* tests.

Results

Palmitate inhibits insulin-stimulated Akt phosphorylation in liver cells

Chronic exposure of liver cells to elevated fatty acids, particularly the saturated long-chain fatty acid, palmitate (C16:0), leads to abnormalities in insulin receptor-mediated signaling (17, 37). We examined the changes in insulin receptor-mediated signaling induced by 18 h exposure of human hepatoma HepG2 cells and mouse primary hepatocytes to elevated palmitate. In control HepG2 cells, acute insulin stimulation led to dose- and time-dependent increases in tyrosine phosphorylation of insulin receptor (Tyr 1162/1163) and IRS1 (Tyr 612) and serine phosphorylation of Akt (Ser 473) (Fig. 1A and Supplemental Fig. 1, A and B). Palmitate treatment of HepG2 cells dose-dependently reduced insulin-stimulated serine phosphorylation of Akt (Fig. 1A) in a dose-dependent manner; 750 μM palmitate treatment reduced Akt phosphorylation in response to 10 and 100 nM insulin stimulation by 65–70%. In contrast, insulin receptor-mediated signaling upstream of Akt was not affected by palmitate pretreatment. Insulin-stimulated tyrosine phosphorylation of insulin receptor and IRS1 were not altered after palmitate pretreatment (Fig. 1A, Fig. 2, and Supplemental Fig. 1, A and B), with the exception of a minor reduction in IRS1 (Tyr 612) phosphorylation after 750 μM palmitate pretreatment at the single maximal insulin dose of 100 nM insulin (Sup-

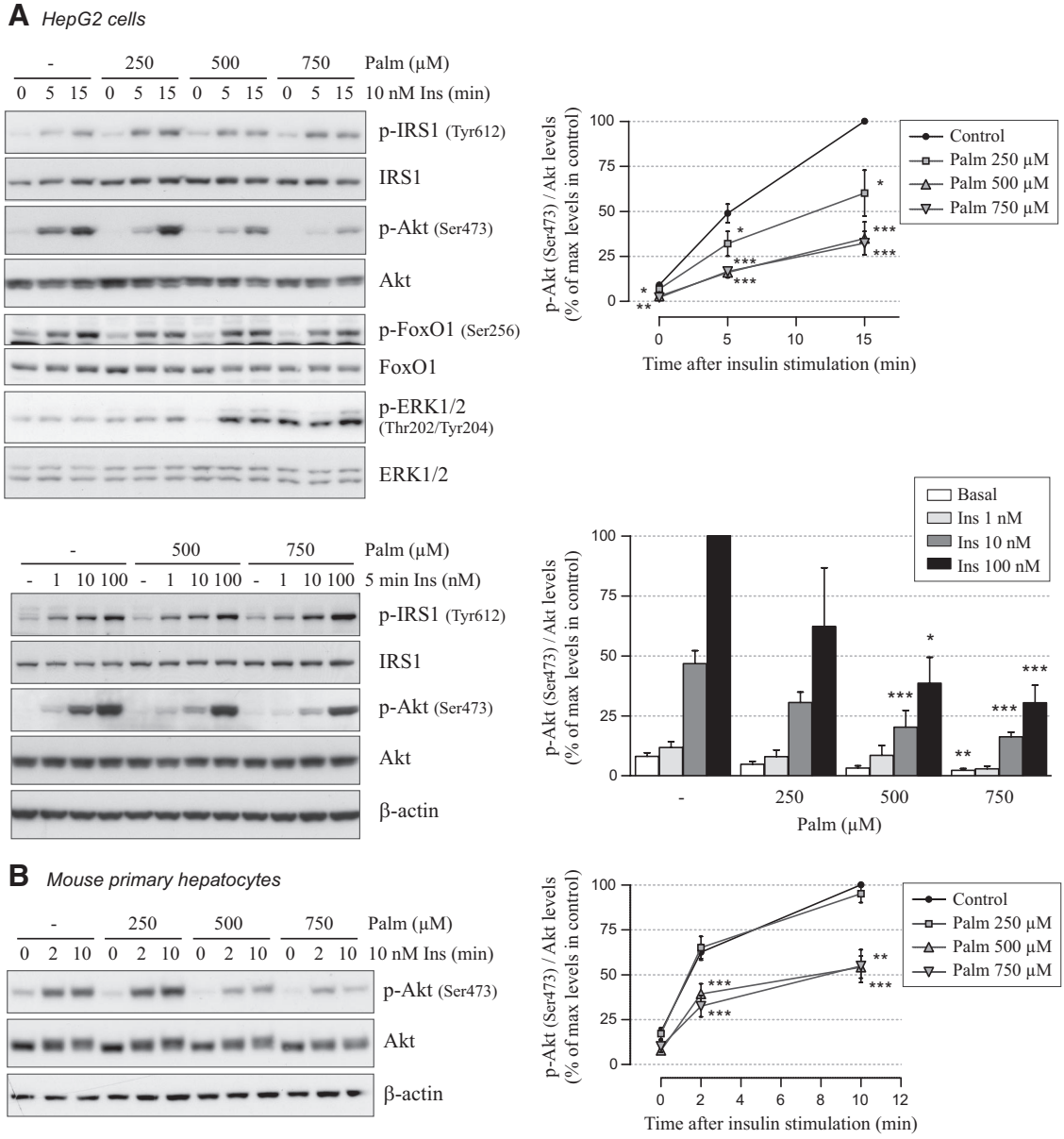

FIG. 1. Palmitate treatment inhibits insulin-stimulated phosphorylation of Akt in HepG2 cells and mouse primary hepatocytes. HepG2 cells (A) and isolated mouse primary hepatocytes (B) were pretreated with either BSA alone (Control) or BSA coupled to palmitate (Palm, 250, 500, or 750 μM) for 18 h before stimulation with insulin (Ins) as indicated (*upper panels* in A: 10 nM insulin for 5 or 15 min; *lower panels* in A: 1, 10, or 100 nM insulin for 5 min; B: 10 nM insulin for 2 or 10 min). Western blot analyses were performed to compare changes in IRS1 (Tyr 612), Akt (Ser 473), FoxO1 (Ser 256), and ERK1/2 MAPK phosphorylation (p). IRS1, Akt, FoxO1, and ERK1/2 levels were determined. β-Actin served as loading control. Representative blots and quantification of phosphorylated Akt are shown. p-Akt levels were normalized for Akt levels and expressed as a percentage (%) of the maximum (max) value in control. Values are means ± SE from at least three independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ *vs.* control at the same time point or dose of insulin.

plemental Fig. 1B). Total IR, Akt, and ERK1/2 levels were unaltered after palmitate pretreatment, whereas IRS1 and IRS2 levels were increased (Supplemental Fig. 2). Insulin-stimulated serine phosphorylation of FoxO1 was not altered after palmitate pretreatment (Fig 1A and Supplemental Fig. 1C), whereas ERK2 phosphorylation was increased (Fig 1A and Supplemental Fig. 1D). These findings suggest that 18 h exposure of HepG2 cells to elevated palmitate induces a specific defect in insulin-stimulated serine phosphorylation of Akt. A similar defect in insulin-stimulated Akt phosphorylation was observed in mouse primary hepatocytes treated with 500 or 750 μM palmitate (Fig. 1B).

Palmitate activates ER stress signaling pathways in HepG2 cells and mouse primary hepatocytes

The ER stress response has emerged as a potential signaling pathway involved in obesity-associated insulin re-

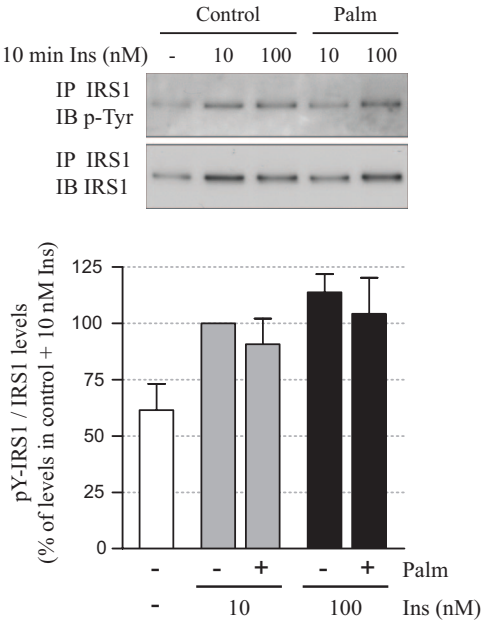

FIG. 2. Palmitate treatment does not affect insulin-stimulated IRS1 tyrosine phosphorylation in HepG2 cells. IRS1 was immunoprecipitated from lysates of HepG2 cells treated with BSA alone or BSA coupled to 750 μM palmitate (Palm) before 10 min stimulation with insulin (Ins) as indicated (10 or 100 nM). Immunoprecipitates (IP) were subjected to SDS-PAGE, and immunoblotting (IB) was performed to detect phosphotyrosine (p-Tyr) or IRS1. Representative blots and quantification by densitometry are shown. IRS1 tyrosine phosphorylation (pY-IRS1) levels were normalized for immunoprecipitated total IRS1 levels and expressed as a percentage (%) of the level in control cells stimulated with 10 nM insulin. Values are means ± SE from three independent experiments.

sistance (19). We found in HepG2 cells that direct activation of the ER stress response with two well-known ER stress inducers, Tg and tunicamycin (Tm), leads to a reduction in insulin-stimulated IRS1 and Akt phosphorylation (Supplemental Fig. 3, A and C). We next tested whether palmitate induces ER stress in our models. In HepG2 cells, palmitate treatment led to the activation, in a dose-dependent manner, of two arms of the ER stress response as indicated by detection of ER stress markers (Fig. 3). EIF2α phosphorylation was increased within 4 h of palmitate treatment in HepG2 cells (Fig. 3, A and C). *CHOP* mRNA levels were increased in HepG2 cells treated with palmitate (750 μM) compared with control (data not shown), whereas CHOP protein was only detected in Tg- and Tm-treated cells (Fig. 3B). Palmitate treatment increased the levels of phosphorylated IRE1 (Fig. 3, B and C) and spliced *XBP1* mRNA (Fig. 3D) in a dose-dependent manner. Interestingly, different patterns of ER stress signaling activation were observed among the three stressors (Fig. 3, B and D, and Supplemental Table 2). Tg and Tm treatments generated similar marked increases in IRE1 phosphorylation (Fig. 3B), whereas *XBP1* splicing was 42% lower after Tm (500 ng/ml) compared with Tg (25 nM) treatment (Fig. 3C). Moreover, palmitate (750 μM) and Tm (500 ng/ml) treatments led to similar increases in *XBP1* splicing (Fig. 3D) despite a difference in IRE1 phosphorylation (Fig. 3B). Phosphorylated JNK (Thr183/Tyr185) was increased after palmitate treatment of HepG2 cells, as was cleaved caspase 3, a marker of apoptosis (Fig. 4A). Phosphorylated JNK was also increased after Tg treatment but was not altered after Tm treatment (Supplemental Fig. 3B).

In mouse primary hepatocytes, palmitate treatment led to dose-dependent activation of the PERK pathway, as indicated by increased PERK phosphorylation and CHOP expression (Fig. 4B). The IRE1 pathway was also activated by palmitate, as indicated by increased levels of spliced *XBP1* mRNA (Fig. 4B). However, JNK phosphorylation levels were not altered (Fig. 4B). Furthermore, we examined by real-time PCR the palmitate-regulated expression of genes involved in the UPR in hepatocytes. Exposure to palmitate induced step-wise increases in levels of mRNA encoding the ER stress-inducible transcription factors ATF3, ATF4, CHOP, and TRIB3. mRNA levels of the ER chaperones BiP and Grp94 as well as the ER foldase Erp72 were also increased in hepatocytes exposed to 750 μM palmitate. In contrast, *EDEM1* and *FKBP11* mRNA levels were unchanged (Supplemental Fig. 4). These results demonstrate, for the first time, the widespread up-regulation of genes involved in the UPR in palmitate-treated primary hepatocytes.

We next tested whether these changes induced by palmitate were due to ER stress using the chemical chaperones trimethylamine *N*-oxide (TMAO, 100 mM), an osmolyte that improves ER-folding capacity and reduces the load of unfolded proteins in the ER (38), and tauroursodeoxycholic acid (TUDCA, 0.5 mg/ml), a bile acid derivative that improves ER function (21, 39). Pretreatment of HepG2 cells with TMAO attenuated palmitate-mediated changes in ER stress markers, as evidenced by reduced eIF2α and JNK phosphorylation as well as *XBP1* mRNA splicing (Fig. 4A). This was associated with reduced cleaved caspase 3, suggesting that the attenuation of ER stress protects against palmitate-induced apoptosis in HepG2 cells. Pretreatment of mouse hepatocytes with TUDCA attenuated palmitate-mediated ER stress, as indicated by reduced PERK phosphorylation, CHOP expression, and *XBP1* mRNA splicing (Fig. 4B). TUDCA pretreatment of hepatocytes also significantly reduced the palmitate-mediated changes in the expression of transcription factors, chaperones, and foldases (Supplemental Fig. 4). These data indicate that palmitate treatment induces the UPR in HepG2 cells and primary hepatocytes in

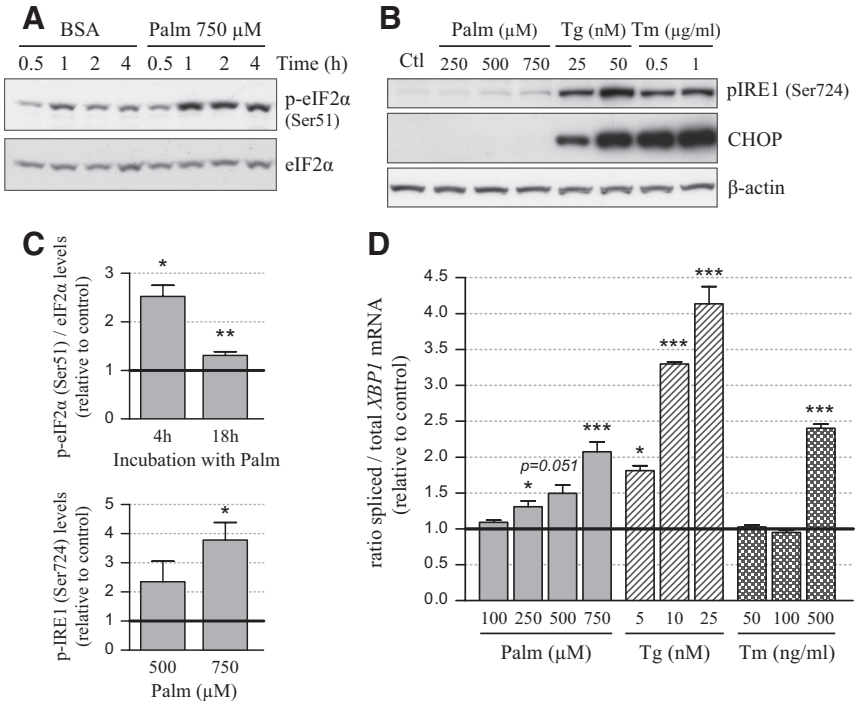

FIG. 3. Palmitate activates the ER stress response in HepG2 cells. HepG2 cells were treated with BSA alone, BSA coupled to palmitate (Palm), DMSO [control(Ctl)], Tg, or Tm for 18 h or as indicated. A and B, Western blot analyses were performed to compare changes in eIF2α (A) and IRE1 (B) phosphorylation (p) and CHOP protein expression (B). β-Actin served as loading control. C, Phosphorylated and total eIF2α, p-IRE1, and β-actin bands were quantified by densitometry. p-eIF2α and p-IRE1 levels were normalized for total eIF2α and β-actin levels, respectively, and expressed as fold changes of levels in control. D, Total RNA was extracted, and reverse transcribed, and *XBP1* cDNA was amplified by PCR. The ratios of spliced/total *XBP1* mRNA were quantified as described in *Materials and Methods* and are expressed as fold change of the ratio in control cells. Values are means ± SE *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ *vs.* control.

a dose-dependent manner and that pretreatment with two different chemical chaperones attenuates palmitate-induced ER stress.

ER stress activation is necessary for palmitate-mediated inhibition of insulin signaling in liver cells

The above studies demonstrate an association between deleterious UPR signaling and lipid-induced insulin resistance in liver cells, but whether ER stress activation makes a necessary contribution to the insulin signaling defects induced by palmitate has not yet been established. Here we demonstrated that the attenuation of ER stress with TMAO or TUDCA protected against the palmitate-mediated reduction in insulin-stimulated Akt phosphorylation in HepG2 cells (Fig. 5A and Supplemental Fig. 5A) and in mouse hepatocytes (Fig. 5B). These studies provide definitive evidence that ER stress activation is required for mediating the deleterious effects of palmitate on insulin-regulated Akt phosphorylation in liver cells. These changes in Akt phosphorylation occurred without significant alteration in IR or IRS1 phosphorylation, whereas ERK2 phosphorylation was partially restored after TMAO treatment (Supplemental Fig. 5, B and C).

ER stress activation plays an essential role in palmitate-mediated impairment of insulin-regulated glucose metabolism

To determine whether ER stress makes a necessary contribution to palmitate-mediated impairment of insulin action, we assessed glycogen synthesis and glucose-6-phosphatase (*G6PC*) expression in HepG2 cells (Fig. 5, C and D). Palmitate pretreatment of HepG2 cells led to inhibition of insulin-stimulated glycogen synthesis (Fig. 5C). This inhibitory effect of palmitate was partially prevented after the attenuation of ER stress with TMAO; insulin-stimulated glycogen synthesis was increased by 25% in HepG2 cells treated with palmitate in combination with TMAO compared with cells treated with palmitate alone (Fig. 5C). In control HepG2 cells, insulin stimulation led to the expected down-regulation of *G6PC* mRNA expression by 70%. (Fig. 5D). Palmitate pretreatment led to increased *G6PC* mRNA levels by 3.6- and 6.5-fold in basal and insulin-stimulated conditions, respectively. Attenuation of ER stress using TMAO partially prevented the palmitate-mediated up-regulation of *G6PC* mRNA levels; *G6PC* mRNA levels were reduced by 1.8-fold (basal) and 1.7-fold (insulin stimulated) in HepG2 cells treated with palmitate in combination with TMAO compared with cells treated with palmitate alone (Fig. 5D). Attenuation of ER stress also partially prevented the palmitate-mediated changes in *SREBF1* and *HMGCR* expression (Supplemental Fig. 6). *SREBF1* encodes the transcription factor sterol regulatory element-binding protein 1c, a major regulator of lipid metabolic processes, and *HMGCR* encodes 3-hydroxy-3-methylglutaryl-coenzyme A reductase, the rate-limiting enzyme for cholesterol synthesis. These findings suggest that ER stress plays an essential role in palmitate-mediated dysregulation of glycogen synthesis and hepatocyte gene expression. Taken together, the data demonstrate that the improvement in insulin-stimulated Akt phosphorylation after attenuation of ER stress in palmitate-treated cells is accompanied by

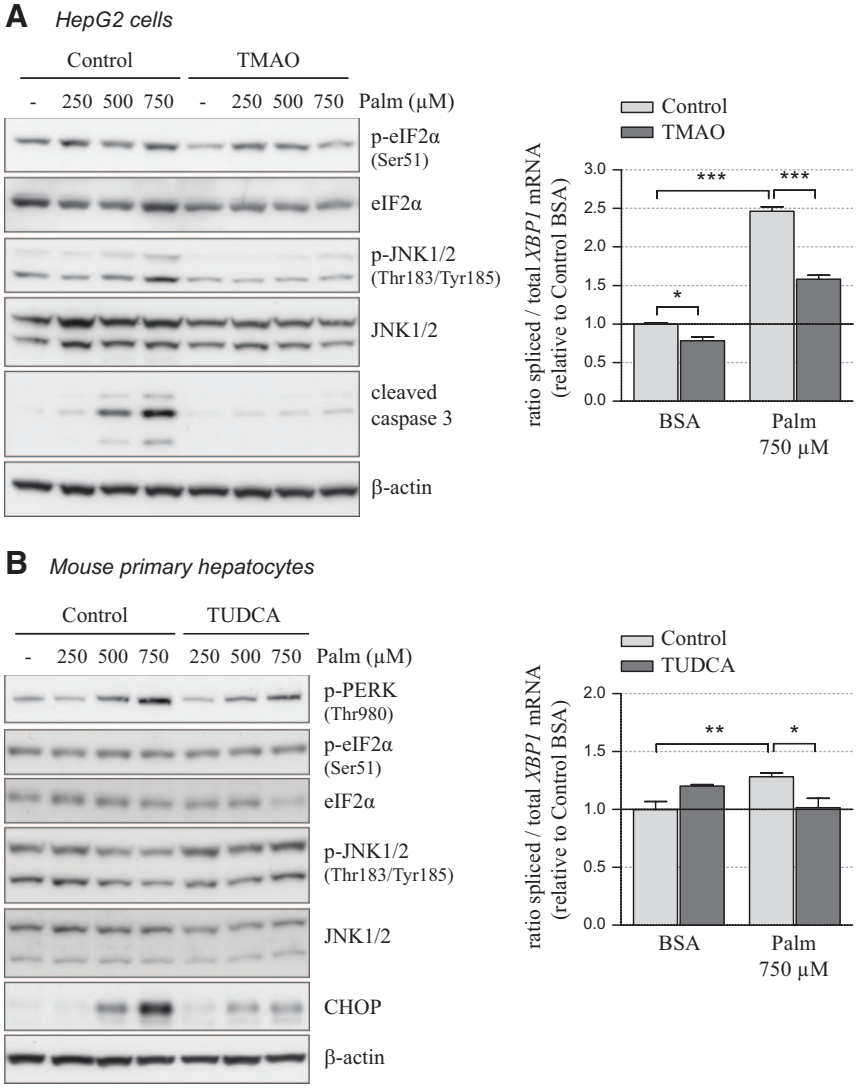

FIG. 4. The chemical chaperones, TMAO and TUDCA, attenuate palmitate-induced ER stress activation in HepG2 cells and mouse primary hepatocytes. HepG2 cells (A) and isolated mouse primary hepatocytes (B) were pretreated in the absence or presence of TMAO or TUDCA for 8 h and then incubated in combination with palmitate (Palm, 250, 500, or 750 μM) for 18 h as indicated. Western blot analyses were performed to compare changes in PERK, eIF2α, and JNK1/2 phosphorylation (p), and cleaved caspase 3 and CHOP expression. Representative blots are shown. β-Actin served as a loading control. The ratios of spliced/total *XBP1* mRNA were quantified as described in *Materials and Methods* and are expressed as fold change of the ratio in control cells. Values are means $\pm$ SE of three independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ for the indicated comparisons.

the partial restoration of insulin-regulated glucose metabolism.

XBP1 activation contributes to palmitate-mediated inhibition of insulin signaling in HepG2 cells

To assess the role of XBP1 as a potential mediator of palmitate-mediated inhibition of insulin signaling, HepG2 cells were transfected with XBP1 siRNA or control siRNA. XBP1 siRNA transfection led to reduced XBP1 expression and splicing compared with control siRNA-transfected cells (Supplemental Fig. 7). This resulted in partial restoration of insulin-stimulated Akt phosphorylation in palmitate-treated cells (Fig. 6A) and partial prevention of the palmitate-mediated up-regulation of *G6PC* mRNA levels (Fig. 6B). This evidence suggests that the activation of XBP1 contributes to lipid-induced insulin resistance.

Chronic mild ER stress is associated with enhanced insulin signaling in HepG2 cells

Previous studies examining the contribution of ER stress to hepatic steatosis and insulin resistance have adopted high pharmacological doses of ER stress agents, Tg (300–450 nM) and Tm (1–10 μg/ml) (21, 22, 40, 41). These high doses induce severe perturbations of ER function leading to the activation of the pro-apoptotic UPR. However, under physiological ER stress conditions, the first manifestation of the UPR is the activation of an adaptive response that is crucial for cell survival (31). We therefore examined the consequences of chronic mild ER stress and the adaptive UPR on insulin signaling in HepG2 cells. To determine the optimal conditions to model chronic mild ER stress, we performed dose-response and time-course analyses of ER stress markers exposed to low levels of Tg (1–50 nM) or Tm (0.1–1 μg/ml). Based on the observations of increased BiP expression and IRE1 phosphorylation combined with low levels of CHOP protein, we adopted concentrations of 2.5 nM Tg (Supplemental Fig. 8) and 100 ng/ml Tm (data not shown) to conduct the following studies examining the effects of mild ER stress. Importantly, incubation of HepG2 with these low levels of Tg and Tm allowed cells to proliferate and be passaged, whereas higher concentrations led to diminished growth rate and increased cell death.

We assessed the effects of chronic mild ER stress on insulin signaling in HepG2 cells that were exposed for 6 d (one passage) to low-level Tg and Tm treatment. Strikingly, insulin-stimulated Akt phosphorylation was increased in cells pretreated with low-level Tg and Tm compared with the control (Supplemental Fig. 8).

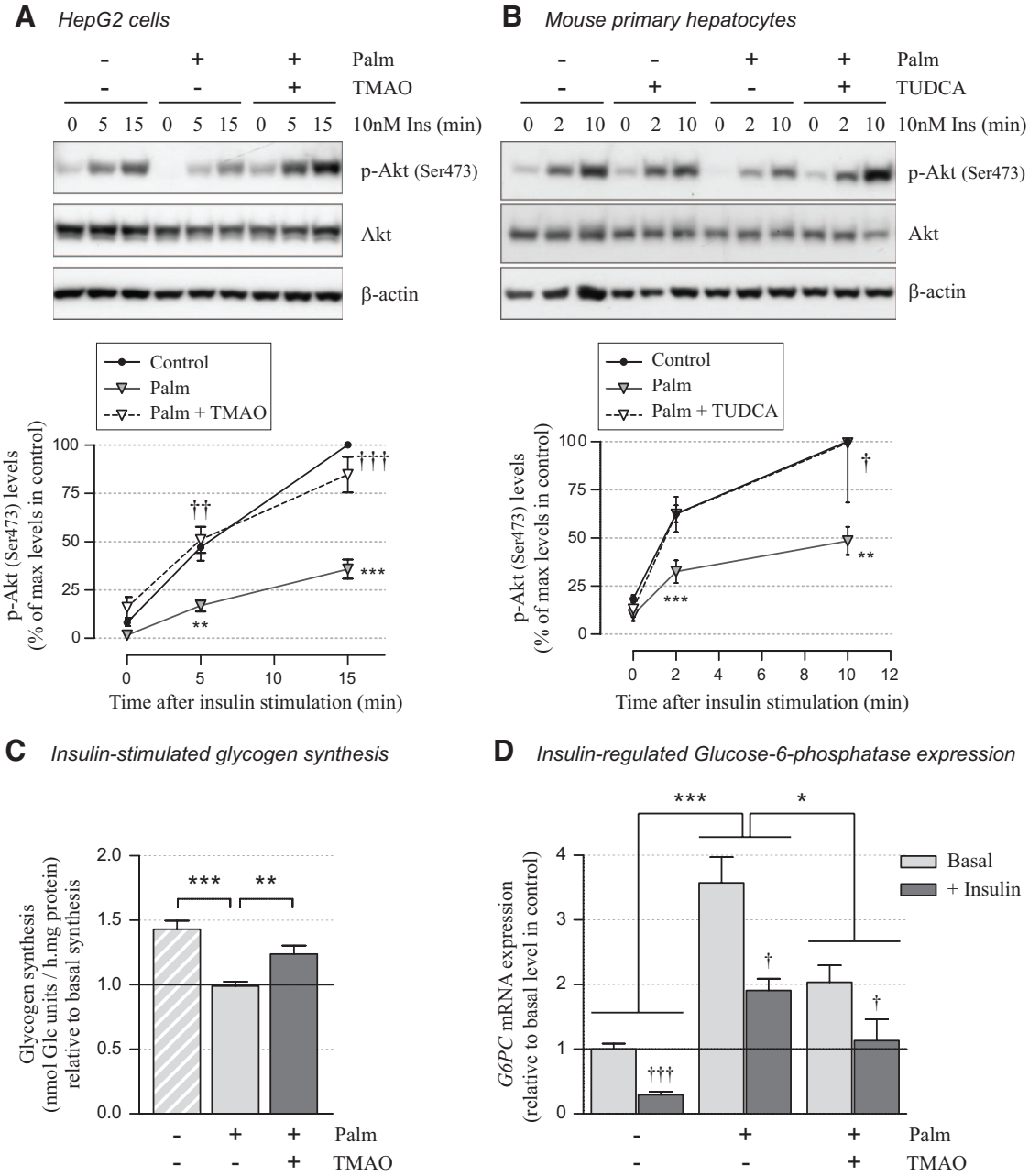

FIG. 5. Attenuation of ER stress protects against palmitate-mediated inhibition of insulin-stimulated Akt phosphorylation and impairment of insulin-regulated glycogen synthesis and glucose-6-phosphatase expression. HepG2 cells (A) and isolated mouse primary hepatocytes (B) were pretreated with TMAO or TUDCA for 8 h and then incubated in combination with palmitate (Palm) for 18 h. A, HepG2 cells were then stimulated with 10 nM insulin (Ins) for 5 or 15 min. B, Hepatocytes were treated with 10 nM insulin for 2 or 10 min. Western blot analysis was performed to compare changes in Akt (Ser 473) phosphorylation (p). Representative blots are shown. Phosphorylated and total Akt bands were quantified by densitometry. p-Akt levels were normalized for Akt levels and expressed as a percentage (%) of the maximum value in control. Values are means ± SE from three independent experiments. **, $P < 0.01$, ***, $P < 0.001$ *vs.* control cells; †, $P < 0.05$; ††, $P < 0.01$; †††, $P < 0.001$ effect of TMAO or TUDCA treatment in palmitate-treated cells. C, HepG2 cells were pretreated in the absence or presence of TMAO for 8 h and then incubated in combination or not with 750 μM palmitate for 18 h. Cells were then stimulated with 100 nM insulin for 1 h in the presence of D-[^{14}C(U)]glucose. Glycogen synthesis was determined in cell extracts as described in *Materials and Methods.* Results are means ± SE determined from five independent experiments, each carried out in triplicate, and expressed as fold change of basal levels. **, $P < 0.01$; *** $P < 0.001$ for the indicated comparisons. D, HepG2 cells were pretreated overnight in the absence or presence of TMAO and then incubated in combination or not with 750 μM palmitate for 38 h. For the last 8 h, cells were treated with 100 nM insulin. Total RNA was extracted and reverse transcribed, and the levels of *glucose-6-phosphatase* mRNA were quantified by real-time PCR. Results are means ± SE determined from two independent experiments, carried out in duplicate and triplicate, and are expressed as fold change of basal mRNA levels in control cells. *, $P < 0.05$; ***, $P < 0.001$ for the indicated comparisons; †, $P < 0.05$; †††, $P < 0.001$ effect of insulin stimulation in each group.

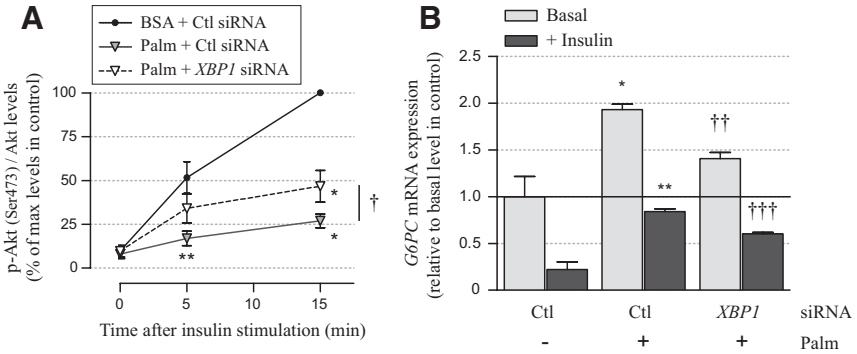

FIG. 6. Inhibition of XBP1 activation partially protects against palmitate-mediated inhibition of insulin-stimulated Akt phosphorylation and impairment of insulin-regulated glucose-6-phosphatase expression. HepG2 cells were transfected with ON-TARGET*plus* SMARTpool siRNA directed against *XBP1* mRNA (XBP1 siRNA) or ON-TARGET*plus* Non-Targeting Pool [control (Ctl) siRNA]. A, 48 h after transfection, cells were treated with either BSA alone or BSA coupled to 500 μM palmitate (Palm) for 18 h and then stimulated with 10 nM insulin (Ins) for 5 or 15 min. Western blot analysis was performed to compare changes in Akt (Ser 473) phosphorylation. p-Akt levels were normalized for Akt levels and expressed as a percentage (%) of the maximum value in control. Values are means ± SE from three independent experiments. B, Cells were pretreated with either BSA alone or BSA coupled to 500 μM palmitate (Palm) for 26 h. For the last 8 h, cells were treated with 100 nM insulin. Total RNA was extracted and reverse transcribed, and the levels of *glucose-6-phosphatase* mRNA were quantified by real-time PCR. Results are means ± SE determined from two independent experiments, carried out in triplicate, and are expressed as fold change of basal mRNA levels in control cells. *, $P < 0.05$; **, $P < 0.01$ *vs.* control cells; †, $P < 0.05$; ††, $P < 0.01$; †††, $P < 0.001$ effect of *XBP1* siRNA in palmitate-treated cells.

Chronic mild ER stress is associated with protection against palmitate-induced insulin resistance in HepG2 cells

We also assessed the effects of chronic mild ER stress on the sensitivity of HepG2 cells to palmitate-mediated inhibition of insulin-stimulated Akt phosphorylation. We found that chronic mild ER stress leads to increased insulin-stimulated Akt phosphorylation in HepG2 cells treated with palmitate; insulin (100 nM)-stimulated Akt phosphorylation was increased by 1.9-fold in cells that had been pretreated with low-level Tg for 3 d (Fig. 7). Thus, the findings suggest that chronic mild ER stress reduces the sensitivity of liver cells to palmitate-mediated inhibition of insulin signaling.

Chronic low-level palmitate exposure induces mild ER stress and enhanced insulin-stimulated Akt phosphorylation in HepG2 cells

We next investigated the consequences of chronic low-level palmitate exposure on ER stress activation in HepG2 cells. We used 100 μM palmitate, which allowed cell proliferation for at least two passages. After an 8-d period of exposure to low-level palmitate, *XBP1* mRNA splicing was significantly increased (Fig. 8A), but CHOP mRNA (Fig. 8B) and protein (data not shown) levels were not altered, suggesting the induction of mild ER stress. This mild ER stress induced by chronic low-level palmitate was associated with an attenuation of the severe ER stress response induced by subsequent acute high-level palmitate exposure; spliced *XBP1* and *CHOP* mRNA levels were reduced in cells pretreated with low-level palmitate followed by high-level palmitate treatment compared with cells treated with high-level palmitate alone (Fig. 8, A and B). This suggests that chronic mild ER stress induced by low-level palmitate exposure protects against the severe ER stress induced by high-level palmitate.

We also assessed the effects of chronic low-level palmitate exposure on insulin signaling in HepG2 cells. Strikingly, chronic exposure of HepG2 cells to low-level palmitate led to a marked increase in insulin-stimulated Akt phosphorylation (Fig. 8C); levels in 8-d-incubated cells were 2.5-fold higher after 5 min insulin stimulation (Fig. 8C). Furthermore, chronic low-level palmitate exposure partially protected against the inhibitory effects of acute high-level palmitate on insulin-stimulated Akt phosphorylation (Fig. 8D). Together these data suggest that mild ER stress induced by low-level palmitate is associated with an adaptive response that partially protects against the severe ER stress and insulin resistance induced by high-level palmitate.

Discussion

UPR signaling emanating from ER stress has been implicated in obesity-associated insulin resistance (19). However, chronic ER stress can also induce adaptations in UPR signaling that are required for the normal differentiation and maintenance of cell function. Our study examines the effects of varying degrees and durations of ER stress on hepatic insulin signaling. The main findings of this study are that 1) exposure of liver cells to the saturated fatty acid palmitate leads to specific inhibition of insulin-stimulated Akt phosphorylation; 2) palmitate exposure leads to dose-dependent activation of a comprehensive ER stress response; 3) severe ER stress induced by palmitate is required for the accompanying insulin resistance; 4) chronic mild ER stress induced by classic ER stressors or palmitate leads to increased insulin-stimulated Akt phosphorylation; and 5) this chronic mild ER stress protects against the deleterious effects of acute stressors on insulin signaling. These findings demonstrate for the first time that the ef-

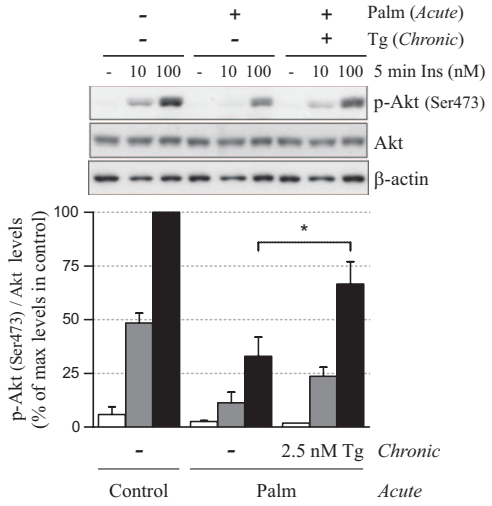

FIG. 7. Chronic mild ER stress enhances insulin-stimulated Akt phosphorylation in HepG2 cells. HepG2 cells were treated with low-level Tg for 3 d (Tg, 2.5 nM, chronic) to induce mild ER stress and then treated with 750 μM palmitate (Palm) for 18 h (acute). Cells were then stimulated with 10 or 100 nM insulin (Ins) for 5 min. Western blot analysis was performed to compare changes in Akt (Ser 473) phosphorylation (p). Representative blots of phosphorylated and total Akt are shown. Phosphorylated and total Akt bands were quantified by densitometry. p-Akt levels were normalized for Akt levels and expressed as a percentage (%) of the maximum (max) value in control. Values are means ± SE from three independent experiments. *, $P <$ 0.05 as indicated.

fects of ER stress on hepatic insulin action are critically dependent not only on the nature and severity of the ER stress insult, but also on the underlying status of the adaptive UPR.

Due to the inherent complexities of animal models, it is difficult to determine the contribution of fatty acids to hepatic insulin resistance or the mechanisms involved. Our *in vitro* studies of human HepG2 cells and isolated mouse hepatocytes indicate that the impaired insulin action induced by the saturated fatty acid palmitate is associated with a specific defect in Akt activation, which is independent of tyrosine phosphorylation of IRS1. However, we do not rule out the possibility that alterations in PI3K subunit contents and associations with IRS1, or XBP1 (42, 43), may be involved in the palmitate-mediated changes in Akt activation and insulin action. Interestingly, a different pattern of response was observed among the three ER stress inducers (see Supplemental Table 2 for summary of results). In contrast to palmitate treatment, high-dose Tg and tunicamycin treatments lead to reduced tyrosine phosphorylation of IRS1. These divergent responses may reflect the differing nature or severity of UPR activation among the three ER stress inducers. Tg and tunicamycin treatments are linked to stronger increases in IRE1 phosphorylation and CHOP levels. Perhaps the intensity or pattern of UPR activation required for inhibition of insulin-stimulated tyrosine phosphorylation was not evoked in our palmitate-treated cells. Previous studies in rat hepatoma cell lines (Fao and H4IIEC3) and HepG2 cells found that palmitate can inhibit tyrosine phosphorylation of insulin receptor and IRS1/2 (17, 37, 44, 45). However, in Fao cells, the palmitate-mediated reduction of Akt phosphorylation preceded the defect in IRS1 phosphorylation, and the reduction of insulin receptor phosphorylation corresponded to depleted levels of total insulin receptor protein, suggestive of it being a latter event (37). Thus, the studies suggest a strong correlation of palmitate-induced insulin resistance with an IRS-independent defect in Akt phosphorylation. More prolonged exposure to palmitate may result in defective signaling upstream of Akt.

The results of our studies establish the link between ER stress and palmitate-induced insulin resistance in hepatocytes. That a similar mechanism may operate *in vivo* is supported by studies in animal models of obesity. Attenuation of ER stress in rodents using transgenic approaches or chemical interventions is associated with improved insulin sensitivity (20, 21, 46). Moreover, interventions that enhance insulin sensitivity and improve diabetes are associated with reduced ER stress (47, 48). In humans, obesity is associated with increased ER stress in liver and adipose tissue (23, 49, 50), and the improved hepatic insulin sensitivity observed after weight loss is correlated with decreased expression of ER stress markers (23). Furthermore, ER stress has been implicated not only in the development of insulin resistance but also in lipid-induced apoptosis in liver cells (51, 52) and pancreatic β-cells (36, 53), highlighting the importance of ER stress in the progression of diseases, such as fatty liver disease and type 2 diabetes.

ER stress-induced hyperactivation of the JNK pathway has been proposed to mediate serine phosphorylation of IRS1, and thus its inactivation (19). JNK activation is found in mouse models of obesity and is implicated in the development of insulin resistance (54, 55). However, whether phosphorylation of IRS1 on serine 307 negatively regulates IRS1 activity (3, 19, 56, 57) has recently been challenged (58). Interestingly, tunicamycin-mediated inhibition of IRS1 tyrosine phosphorylation (Supplemental Fig. 3, A and 3C) occurred in the absence of JNK activation, as indicated by unchanged JNK phosphorylation in tunicamycin-treated cells (Supplemental Fig. 3B). This raises the possibility of a dissociation between JNK activation and defects in IRS1 phosphorylation in tunicamycin-treated cells. The association between ER stress-induced *TRIB3* (*tribbles* homolog 3) expression (59) and palmitate-mediated insulin resistance is of interest given the potential role of TRB3 as a negative modulator of Akt

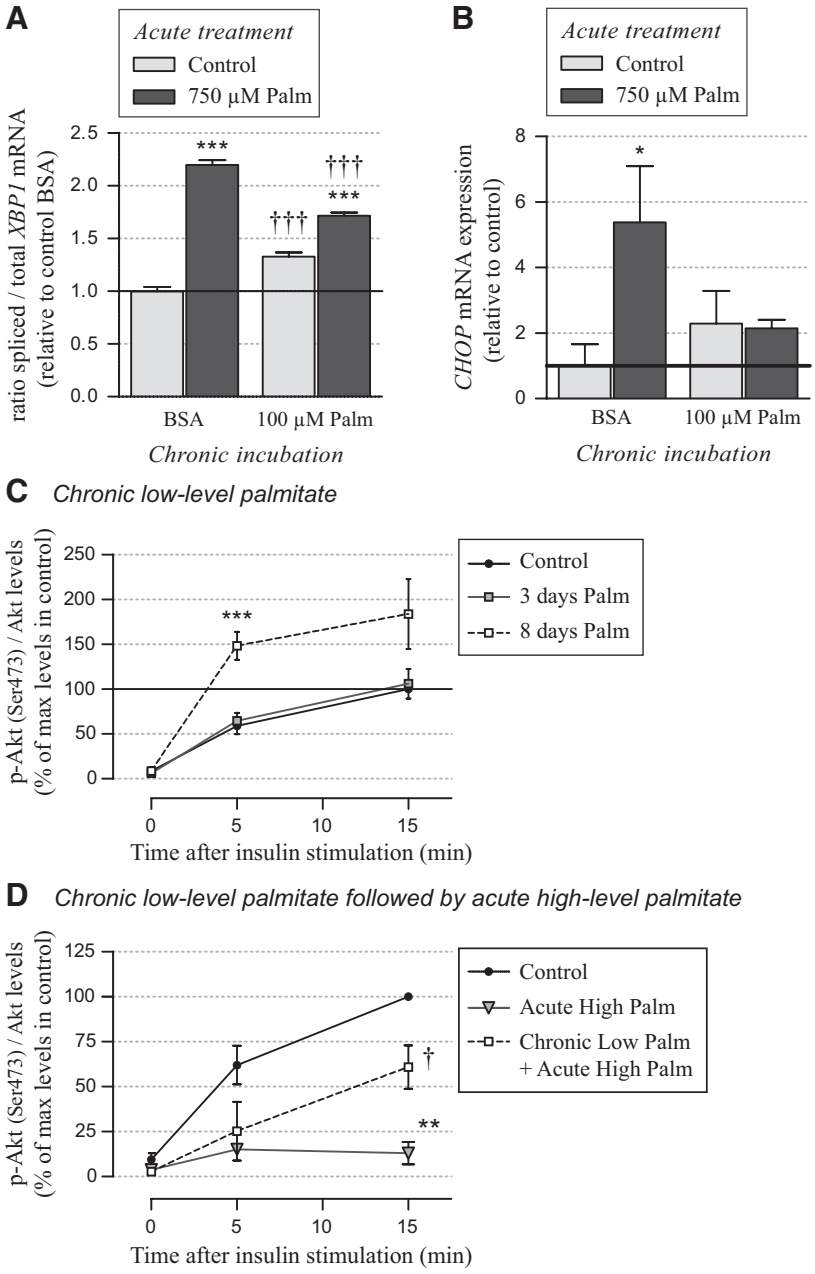

FIG. 8. Chronic low-level palmitate treatment increases insulin-stimulated Akt phosphorylation and protects against subsequent high-level palmitate exposure in HepG2 cells. HepG2 cells were treated with low-level palmitate (100 μM Palm) for 8 d (Chronic incubation) and then treated with high-level palmitate (750 μM Palm) for 18 h (Acute treatment). Total RNA was extracted and reverse transcribed. A, *XBP1* cDNA was amplified by PCR. The ratios of spliced/total *XBP1* mRNA were quantified as described in *Materials and Methods* and are expressed as fold change of the ratio in control cells. B, *CHOP* mRNA levels were assessed by real-time PCR. *, $P < 0.05$; ***, $P < 0.001$ effect of acute high-level palmitate treatment in chronic control and low-level palmitate-treated cells. †††, $P < 0.001$ effect of chronic low-level palmitate treatment in acute control and high-level palmitate-treated cells. C, HepG2 cells were treated with low-level palmitate (100 μM) for 3 or 8 d before stimulation with 10 nM insulin for 5 or 15 min. Western blot analysis was performed to compare changes in Akt (Ser 473) phosphorylation (p). p-Akt levels were normalized for Akt levels and expressed as a percentage (%) of the maximum values in control. ***, $P < 0.001$ *vs.* control cells at the same time point. D, HepG2 cells were treated in the absence or presence of low-level palmitate (100 μM) for 8 d (chronic low Palm) and then in the absence or presence of high-level palmitate (750 μM) for 18 h (acute high Palm). Cells were then stimulated with 10 nM insulin for 5 or 15 min. p-Akt levels were determined as above. All values are means ± SE from three experiments. **, $P < 0.01$ *vs.* control cells at the same time point. †, $P < 0.05$ *vs.* high-level palmitate-treated cells at the same time point.

(60). However, the role of TRB3 in the regulation of Akt has recently been questioned (61, 62). Alternatively, palmitate may cause the reduction in Akt phosphorylation levels by increasing phosphatase expression and/or activity. This is supported by findings in myotubes in which palmitate disrupts insulin signaling by promoting PP2A-like activity and the dephosphorylation of Akt (63). Our studies demonstrate a partial requirement of ER stress in palmitate-mediated inhibition of insulin signaling and action, suggesting that additional and/or interacting mechanisms are likely to be involved. Interactions between ER stress, mitochondrial dysfunction, and reactive oxygen species production (17, 37) would be facilitated by the close apposition of the mitochondria and ER membranes; stress in one of these organelles would be expected to communicate with and influence the function of the other. Indeed, mitochondrial dysfunction induced with oligomycin, an ATPase inhibitor, has been shown to induce an ER stress response in hepatoma cells, which results in potentiated up-regulation of gluconeogenic enzyme expression (64).

Various mechanisms may be responsible for the induction of ER stress by saturated fatty acids. In CHO cells, palmitate is rapidly incorporated into saturated phospholipid and TAG species in microsomal membranes, resulting in membrane remodeling, dilatation of the ER, and redistribution of chaperones to the cytosol (65). Other mechanisms may include intracellular accumulation of fatty acids or triglycerides (or associated metabolites) (66), a redistribution of calcium stores (67), or the generation of a trafficking defect between the ER and the Golgi (68).

The UPR is an important protective signaling pathway required for cell differentiation, function, and survival, as observed in secretory cells, such as B lymphocytes (33) and β-cells (32). Our studies demonstrate the striking differ-

ential effects of the adaptive and deleterious UPR on hepatic insulin signaling. The findings suggest that the adaptive UPR may play an important role in promoting insulin action in hepatocytes in addition to its potential involvement in lipid metabolism (22, 69–71). That the UPR is involved in the adaptation to low-level palmitate is supported by our findings of moderately increased *XBP1* mRNA splicing and the subsequent protection against the marked induction of *XBP1* splicing and *CHOP* expression with acute high-level palmitate. Furthermore, similar effects on insulin signaling were observed after the direct activation of chronic mild ER stress. The mechanisms by which the adaptive UPR leads to enhanced insulin-stimulated Akt phosphorylation in liver cells remain to be determined. XBP1 activity may play an important role in ER capacity to cope with stress as suggested in previous studies performed in mouse embryonic fibroblasts (19). Recent studies have also suggested that p38 MAPK (72) and the regulatory subunits of PI3K, p85α and p85β (42, 43), interact with, and increase the nuclear translocation of the spliced form of XBP1. This leads to an increase in the expression of chaperones and ER-associated degradation proteins that suppress feeding-induced ER stress and subsequently improve insulin sensitivity. It will be of interest to test whether similar mechanisms play a role in the enhanced insulin signaling observed in our models of chronic mild ER stress. Rutkowski *et al.* (24) suggested that the adaptive response to ER stress is a consequence either of selective activation of sensors or divergence in the activation of downstream effector molecules of the UPR.

In conclusion, the results of the present study demonstrated a direct connection between ER stress and the regulation of hepatic insulin action. Depending on the concentration and duration of exposure, elevated palmitate evokes either an adaptive UPR, which enhances insulin signaling and protects against subsequent palmitate challenges, or a deleterious UPR that causes insulin resistance. These findings raise the possibility that ER stress is a contributing factor in the normal adaptations that are required to meet changing nutritional demands for energy homeostasis, but also in the development of insulin resistance that is associated with metabolic diseases such as type 2 diabetes.

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