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Palmitate Induces mRNA Translation and Increases ER Protein Load in Islet β-Cells via Activation of the Mammalian Target of Rapamycin Pathway

Saturated free fatty acids (FFAs) have complex effects on the islet β-cell, acutely promoting adaptive hyperplasia but chronically impairing insulin release. The acute effects of FFAs remain incompletely defined. To elucidate these early molecular events, we incubated mouse β-cells and islets with palmitate and then studied mRNA translation by polyribosomal profiling and analyzed signaling pathways by immunoblot analysis. We found that palmitate acutely increases polyribosome occupancy of total RNA, consistent with an increase in mRNA translation. This effect on translation was attributable to activation of mammalian target of rapamycin (mTOR) pathways via L-type Ca^{2+} channels but was independent of insulin signaling. Longer incubations led to depletion of polyribosome-associated RNA, consistent with activation of the unfolded protein response (UPR). Pharmacologic inhibition of mTOR suppressed both the acute effects of palmitate on mRNA translation and the chronic effects on the UPR. Islets from mice fed a high-fat diet for 7 days showed increases in polyribosome-associated RNA and phosphorylation of S6K, both consistent with activation of mTOR. Our results suggest that palmitate acutely activates mRNA translation and that this increase in protein load contributes to the later UPR.

Consumption of foods high in saturated fat is associated with obesity and insulin resistance. Obese, "metabolically healthy" individuals maintain normoglycemia in the face of insulin resistance by augmenting insulin release from islet β-cells. Failure to maintain the necessary state of augmented β-cell mass and/or function leads to the development of type 2 diabetes (1,2). The specific signals that cause the initial increases in β-cell mass and function in obesity and the later loss of these parameters in type 2 diabetes have not been entirely elucidated, but hormonal and cytokine signals emanating from distant sources such as the liver and bone have been variably implicated (3–6). In addition to these organ-derived signals, diet-derived factors such as free fatty acids (FFAs) have also been shown to directly impact β-cell responses (6,7). FFAs appear to have a duality of effects on the β-cell, either augmenting in the short-term or limiting in the long-term insulin release and cellular replication (7–9).

The molecular mechanisms underlying the dichotomous responses of the β-cell to FFAs have not been fully elucidated. It has been postulated that the effect of FFAs to augment β-cell function (i.e., glucose-stimulated insulin secretion) may be important for the early hypersecretion

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of insulin seen in insulin resistance. This effect of FFAs is thought to occur via several mechanisms. One is through the interaction of FFAs with FFA receptor 1 (GPR40), which signals through Gαq/11 to augment glucose-stimulated insulin secretion (10,11). A second mechanism is through the cellular metabolism of FFAs (to generate lipid-derived signaling molecules) and glycerolipid/FFA cycling (12). More recently, elegant studies of Zarrouki et al. (6) suggest growth-promoting effects of FFAs in rats in vivo may in part be secondary to growth factor signaling and activation of mammalian target of rapamycin (mTOR). Studies have also shown deleterious effects of FFAs on β-cell function, a finding that is thought to be a more chronic effect and that is frequently observed in the presence of hyperglycemia ("glucolipotoxicity"). It has been suggested that these lipotoxic effects on the β-cell are mediated in part by endoplasmic reticulum (ER) stress (13–15). However, the specific mechanisms by which saturated FFAs influence ER protein load and mRNA translation in the β-cell have never been investigated.

In this study, we sought to clarify the effect and mechanisms of the major circulating saturated FFA palmitate on mRNA translation in a mouse β-cell line and isolated mouse islets. Polyribosome profile (PRP) analysis during short- and long-term incubations revealed that palmitate acutely triggers mRNA translation via mTOR and increases ER protein load; longer incubations caused activation of the ER stress cascade and a block in mRNA translational initiation. Our results suggest a model whereby the activation of mTOR in a dose-dependent manner by palmitate in β-cells may contribute to an early hyperplastic response, and that these effects impose increased protein load on the ER, activating the unfolded protein response (UPR) in the long-term.

RESEARCH DESIGN AND METHODS

Animals
Male C57BL/6J mice were purchased from The Jackson Laboratory. All mouse experiments were approved by the Indiana University Institutional Animal Care and Use Committee. Eight-week-old mice were fed a rodent diet containing either 10 or 60% kcal from fat (Research Diets D12450B or D12492, respectively). Glucose tolerance tests in mice were performed after 6 days of diet using 2 g/kg glucose injected intraperitoneally (16). Body composition was measured using DEXA after 6 days of diet using a PIXImus DEXA scanner. Mouse islets were isolated from 8-week-old chow-fed animals as previously described (17).

Antibodies
Antibodies were commercially acquired as follows: p-Akt (Thr308) (no. 4056; Cell Signaling Technology), p-Akt (Ser473) (no. 9271; Cell Signaling Technology), Akt (no. 2920; Cell Signaling Technology), p-4E-BP1 (Thr70) (no. 9455; Cell Signaling Technology), p-p70 S6K (Thr389) (no. 9206; Cell Signaling Technology), p70 S6K (no. 2708;

Cell Signaling Technology), p-eIF2α (no. 9721; Cell Signaling Technology), β-actin (no. 691002; MP Biomedicals), 4E-BP1 (sc-6024; Santa Cruz Biotechnology), and eIF2α (sc-133132; Santa Cruz Biotechnology). Fluorophore-labeled secondary antibodies IRDye 800 and IRDye 700 were from LI-COR Biosciences.

Cell Isolation and Culture
MIN6 β-cells, β-cell insulin receptor knockout (βIRKO) cells, and LOX cells were maintained in 25 mmol/L glucose as previously described (18). On the evening prior to experimentation, cells were incubated in medium containing 5.5 mmol/L glucose, and on the morning of experimentation, glucose concentration was increased to 20 mmol/L. Mouse islets were cultured in 11 mmol/L glucose as previously described (16) and were allowed to recover overnight prior to experimentation. Human islets were obtained from the Integrated Islet Distribution Program and cultured in 11 mmol/L glucose as previously described (19). For PRP experiments, islets were transferred into cold RPMI medium containing 5 mmol/L glucose after isolation and used immediately. Sodium palmitate, sodium oleate, fatty acid–free BSA, thapsigargin, wortmannin, and rapamycin were purchased from Sigma-Aldrich. Torin1 was from Tocris Bioscience. FFA and fatty acid–free BSA conjugates (at an 8:1 molar ratio of FFA to BSA, unless otherwise stated) were prepared as described previously (20). Control cells were identically treated in terms of glucose concentrations and timing of inhibitors, except that BSA was added without FFA.

PRP and RT-PCR Experiments
PRP experiments using MIN6 cells and islets proceeded as described previously (21,22), wherein cell lysates were passed through a 10–50% sucrose gradient and fractionated using a BioComp piston gradient fractionator. RNA absorbance at 254 nm was recorded using an in-line UV monitor, and fractions were collected. Total RNA from the fractions was reverse transcribed and subjected to quantitative real-time PCR. Polysome-to-monosome (P/M) ratios were quantitated by calculating the area under the curve corresponding to the polyribosome peaks (more than two ribosomes) divided by the area under the curve for the monosome (80S) peak. Reverse-transcribed RNA was analyzed by real-time PCR using SYBR Green I-based methodology (23). Primers for *Bip*, *Xbp1*, spliced *Xbp1*, *Chop*, *Atf4*, *Pcna*, *Ki67*, and *Tbp* were described previously (24–27). All samples were corrected for input RNA by normalizing to *Tbp* message.

Immunoblot Analysis
Whole-cell extracts from MIN6 cells and mouse islets were prepared as described previously (28). Immunoblot analyses of MIN6 cells and islet extracts were performed after resolution of protein extracts by 4–20% gradient SDS-PAGE. Immunoblots were visualized using fluorescently labeled secondary antibodies (LI-COR Biosciences) and were quantified using LI-COR software.

[35]S-Met/Cys Incubation Studies

MIN6 cells or mouse islets were cultured in 12-well plates in the presence or absence of palmitate or cycloheximide for 1 h. For the pulse label, the cells or islets were washed and incubated with culture media lacking Met and Cys, supplemented with 125 μCi of a mixture of ^{35}S-Met and ^{35}S-Cys (PerkinElmer) for the final 15 min of the incubation. Cellular lysates were loaded on a 10% SDS-polyacrylamide gel and subjected to electrophoresis. The gel was dried and exposed to X-ray film overnight and quantitated using ImageJ Software (National Institutes of Health).

Statistical Analyses

All data are presented as the mean ± SEM. Statistical significance of differences between two groups was evaluated using a two-tailed Student t test. Prism 5 software was used for all statistical analyses. Statistical significance was assumed at $P < 0.05$.

RESULTS

Chronic Palmitate Incubation Reduces Polyribosome-Associated RNAs in β-Cells

To study mRNA translation, we used PRP analysis. PRP analysis involves the isolation of total cellular RNA and then subjecting the RNA to sedimentation through a sucrose gradient, which separates RNAs based on the nature and number of associated ribosome units (29,30). The normal PRP of MIN6 β-cells is shown in Fig. 1A (*solid line*), which identifies the positions of 40S and 60S ribosome-associated RNAs, as well as monoribosome (80S)- and polyribosome (more than two monoribosomes)-associated RNA species. To study the effects of saturated FFAs on mRNA translation in β-cells, we incubated MIN6 β-cells with 0.5 mmol/L palmitate (at an 8:1 molar ratio of palmitate to BSA)/20 mmol/L glucose and subsequently performed PRP analysis. Within 1 h of palmitate addition, there was an increase in the fraction of RNAs associated with polyribosomes (Fig. 1A, *dashed line*), which was reflected by an increased P/M ratio (Fig. 1A). The P/M ratio subsequently normalized by 24 h of palmitate incubation (Fig. 1B), and by 72 h of incubation, a decrease in the P/M ratio was observed (Fig. 1C).

Decreases in P/M ratios are characteristically associated with the UPR, wherein relative reductions in translation initiation, a result of phosphorylation of eukaryotic translation initiation factor 2α (eIF2α), cause "runoff" of ribosomes (29). As shown in Fig. 1D, palmitate incubation of MIN6 cells led to increases in phospho-eIF2α (p-eIF2α) with time. Interestingly, the basal level of p-eIF2α also increased with increasing time of incubation, suggestive of an effect of cellular growth. Incubation of MIN6 β-cells for 4 h with 1 μmol/L thapsigargin, a very potent inducer of p-eIF2α and the UPR, resulted in a similar (though more dramatic) reduction in the P/M ratio (Fig. 1E), accompanied by increased p-eIF2α (Fig. 1F). The results in Fig. 1 collectively indicate that palmitate causes a progressive reduction in global translation initiation that is consistent with activation of the UPR.

Acute Palmitate Incubation Leads to Activation of the mTOR Pathway to Promote mRNA Translation

Although the decreased P/M ratio after 72 h palmitate incubation was consistent with activation of the UPR, the increase in P/M ratio observed at 1 h was unexpected and suggestive of a different process. Increases in P/M ratios can be caused by either an increase in total mRNA translation (i.e., an increase in global or selected protein synthesis) or by a block in mRNA elongation (31,32). To distinguish these possibilities, we performed ^{35}S labeling of proteins in MIN6 cells and mouse islets treated with palmitate. As shown in Supplementary Fig. 1A and B, ^{35}S incorporation into total protein was unchanged (though a tendency to increase) in MIN6 cells and mouse islets, respectively, treated with palmitate for 1 h. By contrast, treatment of cells with cycloheximide (an inhibitor of translation elongation) completely blocked ^{35}S incorporation in both cell types (Supplementary Fig. 1A and B). These results suggest that the increased P/M ratios in response to palmitate are not caused by translational blockade, but more likely from increased mRNA translation.

mTOR exists as two distinct protein complexes (mTOR complex 1 and complex 2 [mTORC1 and mTORC2]) that couple the sensing of the nutritional/energy status of the cell to protein synthesis and growth (33). We tested the possibility that palmitate activates mTOR complexes to enhance mRNA translation. As shown in Fig. 2A, incubation of MIN6 cells for 1 h with increasing concentrations of palmitate (0.1–0.5 mmol/L) led to increasing phosphorylation of two major translation-promoting targets of mTORC1, S6 kinase (S6K) at T389 and eIF4E binding protein 1 (4E-BP1) at T70. In accordance with a causative role of the mTOR pathway in palmitate-induced increases in mRNA translation, Fig. 2B and C show that coincubation with palmitate and the mTORC1 inhibitor rapamycin or with the mTORC1/2 inhibitor Torin1 resulted in reversal of the palmitate-induced increase in polyribosome-associated RNAs in MIN6 cells.

Activation of mTORC1 by Palmitate Is Mediated Through the Phosphoinositide-3 Kinase/Akt Pathway

To interrogate the signaling mechanisms linking acute palmitate effects to mTORC1/2 activation, we next assessed the phosphoinositide-3 kinase (PI3K)/Akt pathway. Figure 3A and B show that palmitate acutely activates Akt in MIN6 cells and mouse islets within 1 h, as reflected by phosphorylation of Akt residues T308 and S473. These Akt phosphorylation events (and, indeed, even basal levels of phosphorylation) were abrogated by the selective PI3K inhibitor wortmannin but not by the selective mTORC1 inhibitor rapamycin (Fig. 3A and B), a result consistent with an upstream dependency of Akt activation on PI3K. The mTORC1/2 inhibitor Torin1 blocked the Akt phosphorylation events in MIN6 cells, in agreement with prior observations that mTORC2 lies upstream of Akt phosphorylation (34).
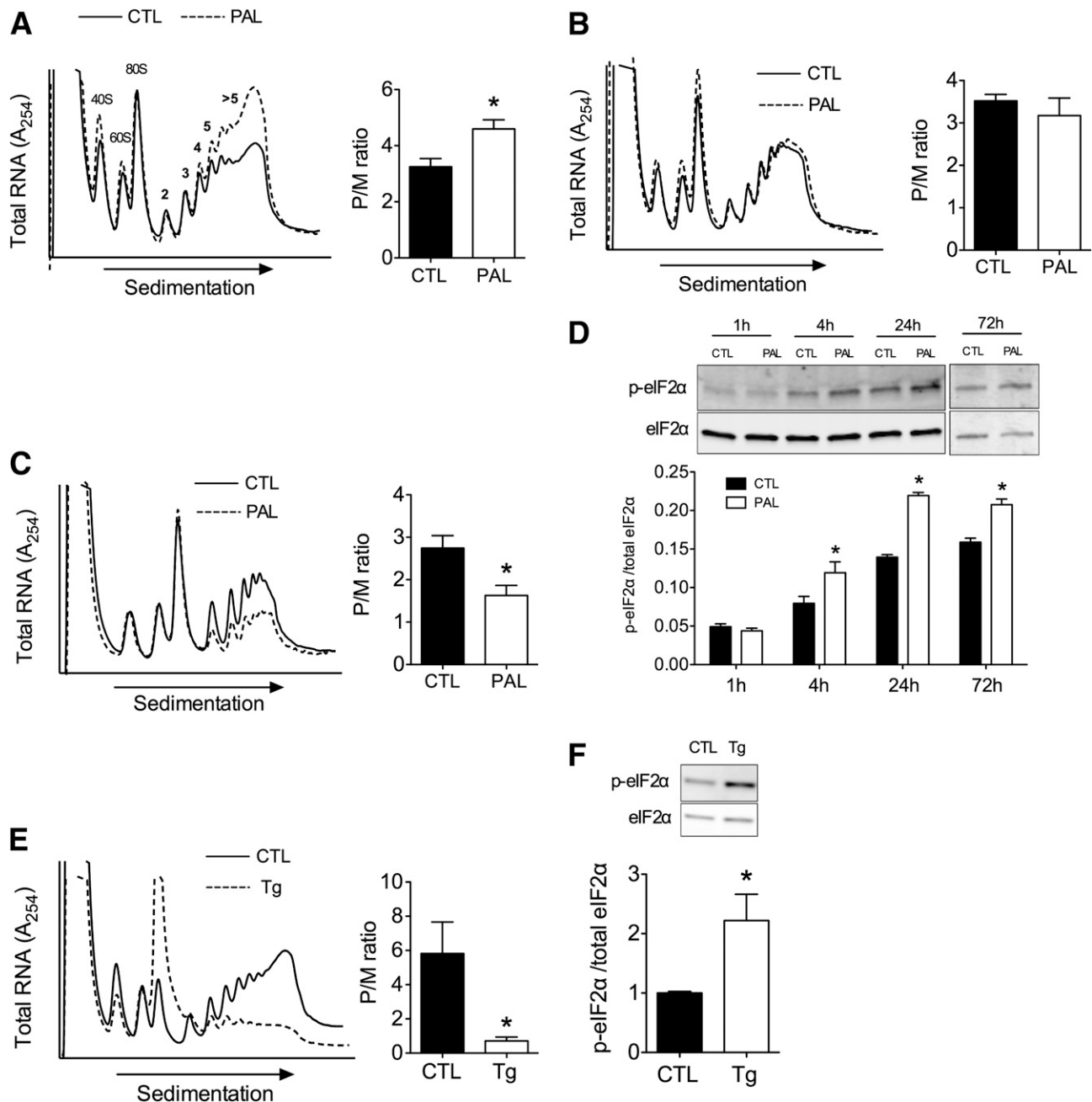
Figure 1—Acute and chronic effects of palmitate on mRNA translation. *A*: PRP analysis (*left panel*) and associated P/M ratio (*right panel*) of MIN6 cells after incubation under control (CTL) or palmitate (PAL, 0.5 mmol/L) conditions for 1 h. Numbers above the peaks indicate numbers of ribosomes. *B*: PRP analysis (*left panel*) and associated P/M ratio (*right panel*) of MIN6 cells after incubation under control and palmitate conditions for 24 h. *C*: PRP analysis (*left panel*) and associated P/M ratio (*right panel*) of MIN6 cells after incubation under control and palmitate conditions for 72 h. *D*: Representative immunoblot (*top panel*) and corresponding quantitation (from $n = 4$) (*bottom panel*) of p-eIF2α and total eIF2α after incubation of MIN6 cells with control or palmitate for the indicated times. *E*: PRP analysis (*left panel*) and associated P/M ratio (*right panel*) of MIN6 cells after incubation under control and thapsigargin (Tg, 1 μmol/L) conditions for 4 h. *F*: Representative immunoblot (*top panel*) and corresponding quantitation (from $n = 4$) (*bottom panel*) for p-eIF2α and total eIF2α after incubation of MIN6 cells with control or thapsigargin for 4 h. In all panels, control cells were incubated at the same glucose concentrations as palmitate-treated cells, and BSA was added in place of palmitate-BSA conjugates. Quantitative data represent mean $\pm$ SEM of at least three independent determinations. *$P < 0.05$.

Major downstream effects of mTORC1 action include the phosphorylations of S6K and 4E-BP1, events that promote mRNA translation (33). As shown in Fig. 3*C* and *D*, palmitate incubation led to phosphorylations of S6K and 4E-BP1 within 1 h. These phosphorylation events were blocked by wortmannin, rapamycin, and Torin1 (Fig. 3*C* and *D*), placing the effects of palmitate on S6K and 4E-BP1 downstream of mTOR signaling. Importantly,
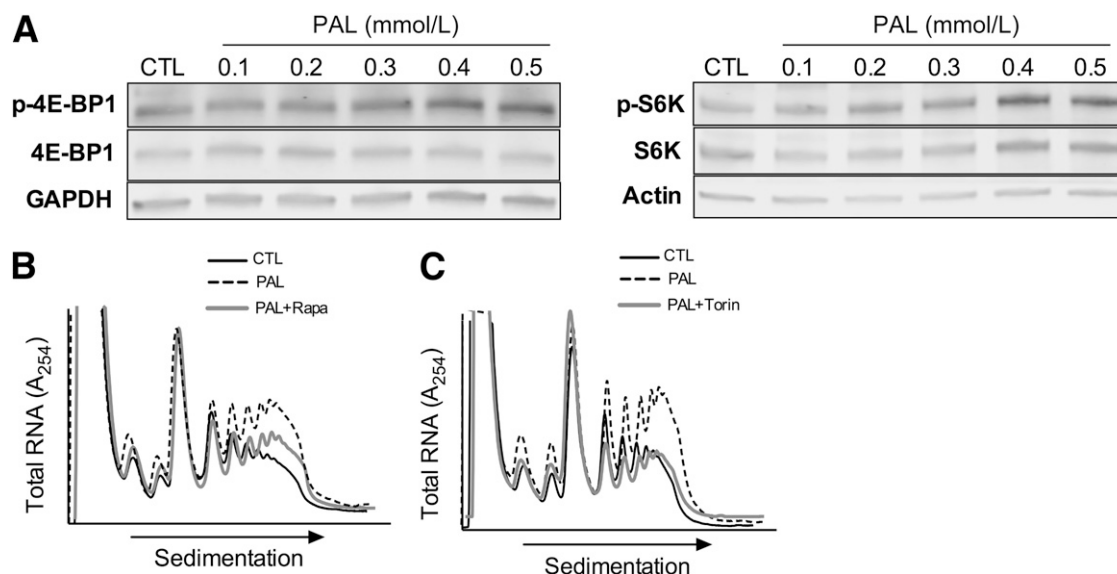
Figure 2—Palmitate activates mTOR signaling. *A*: Representative immunoblot for the indicated proteins after incubation of MIN6 cells under control (CTL) or with varying concentrations of palmitate (PAL) for 1 h. *B*: Representative PRP analysis of MIN6 cells after incubation under control, palmitate (0.5 mmol/L), or palmitate + rapamycin (Rapa, 50 nmol/L) conditions for 1 h. *C*: Representative PRP analysis of MIN6 cells after incubation under control, palmitate (0.5 mmol/L), or palmitate + Torin1 (Torin, 250 mmol/L) conditions for 1 h. In all panels, control cells were incubated at the same glucose concentrations as palmitate-treated cells, and BSA was used in place of palmitate-BSA conjugates.

the effects of palmitate on S6K and Akt phosphorylation events were also observed in isolated human islets (Supplementary Fig. 1*C*). Additionally, the effects of palmitate on Akt and S6K phosphorylations and on the PRP in MIN6 cells occurred at a lower molar ratio of palmitate to BSA (5:1), and the effects on the PRP were also observed (but with lower magnitude) at a 2.5:1 molar ratio (Supplementary Fig. 2*A–C*). Supplementary Fig. 2*C* and *D* show that phosphorylations of Akt and S6K, and their downstream effects on the PRP, were also seen at low glucose concentrations (3 mmol/L).

To determine if the effects on mTOR activation are specific to saturated FFAs, we performed experiments using the monounsaturated FFA oleate. As shown in Fig. 4*A* and *B*, 0.5 mmol/L oleate caused no significant increase in S6K phosphorylation and a reduced phosphorylation of Akt (S473) compared with palmitate. Consistent with these findings, oleate did not alter the P/M ratio in the PRP (Fig. 4*C*).

Palmitate Activation of mTOR Is Independent of Insulin Signaling but Dependent Upon L-Type Calcium Channels

To interrogate the mechanisms linking saturated FFAs to activation of PI3K/Akt/mTOR, we next examined the insulin signaling pathway, a major growth factor pathway known to activate mTOR. We performed experiments using a βIRKO cell line described previously (35). Figure 5*A* confirms the knockout of the insulin receptor in the βIRKO line (*upper panel*) and shows that palmitate still induced Akt (S473) and S6K phosphorylations in βIRKO cells (*lower panels*). These findings were also confirmed in

the absence of serum in the medium (data not shown). Together, these findings suggest that palmitate activation of mTOR in vitro does not involve insulin signaling or signaling by other growth factor components in the serum.

Palmitate mobilizes intracellular Ca^{2+}, and Ca^{2+}/calmodulin has been shown to activate the p85 subunit of PI3K (36). Figure 5*B* and *C* show that palmitate-induced phosphorylation of Akt (T308 and S473) and S6K in MIN6 cells is blocked by the L-type Ca^{2+} channel blocker nifedipine. By contrast, nifedipine does not block the same phosphorylation events induced by insulin. These data suggest that the activation of the PI3K/Akt/mTOR pathway by palmitate is dependent upon flux through L-type Ca^{2+} channels.

Activation of mTOR by Palmitate Precedes and Contributes to the UPR

Saturated FFAs activate the UPR, leading to ER stress and β-cell dysfunction (13). Because the UPR is triggered in part by increased ER protein load, we asked if the activation of the mTOR pathway by palmitate temporally and causally precedes the UPR. As shown in Fig. 6*A*, mRNA parameters of UPR activation, including spliced *Xbp1*, *Bip*, *Atf4*, and *Chop* are increased at 72 h after palmitate incubation in MIN6 cells (trending similarly with 4 h of thapsigargin incubation). A feature of the UPR is the activation of translation initiation of *Atf4* mRNA, wherein *Atf4* mRNA is actively engaged by ribosomal translational machinery to increase ATF4 protein levels (37). Figure 6*B* shows that the positioning of the *Atf4* mRNA is unaltered in the PRP of MIN6 cells treated with palmitate for 1 h. By contrast, Fig. 6*C* shows that *Atf4* mRNA shifts from
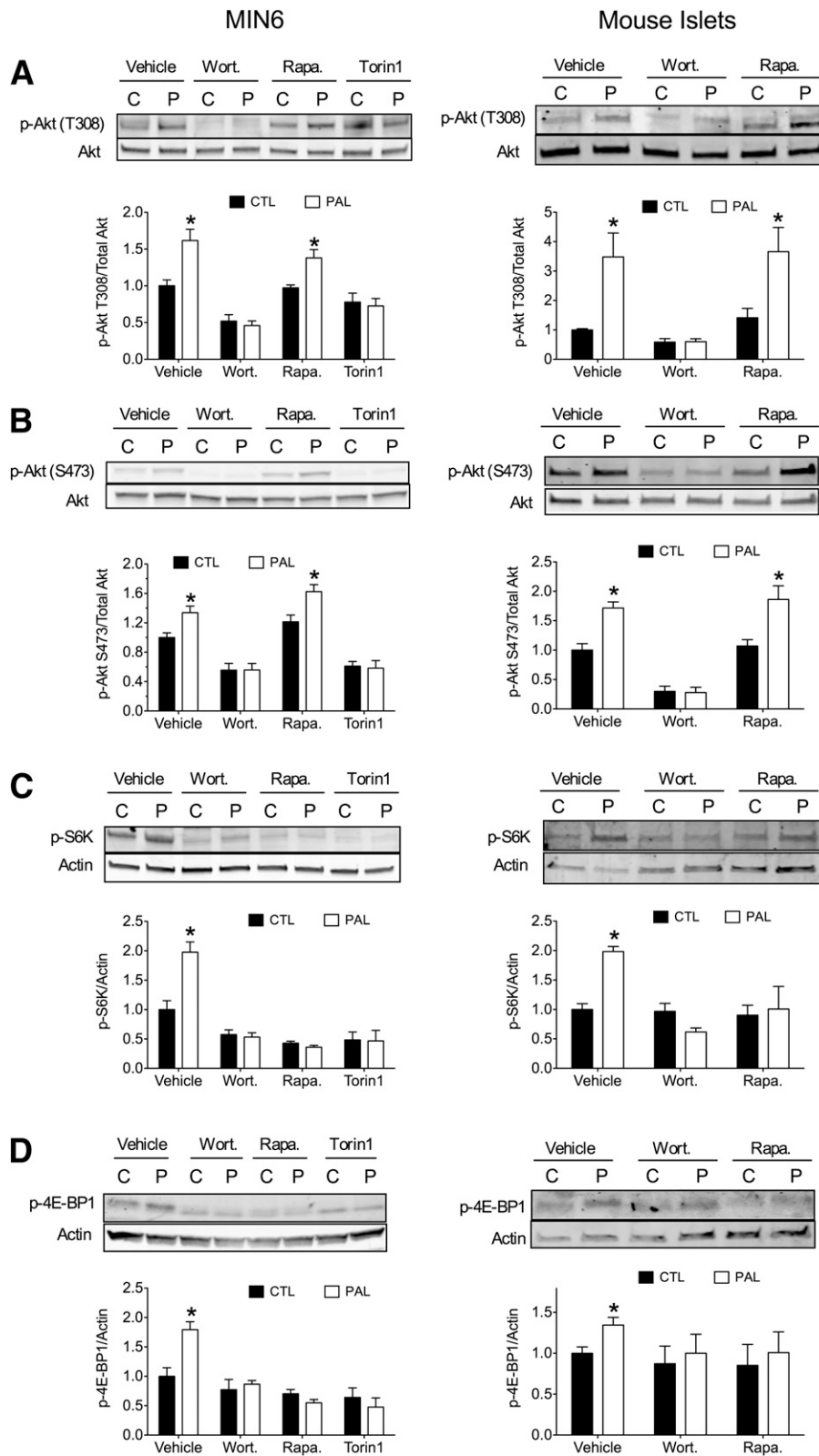
Figure 3—Palmitate activates mTOR via PI3K/Akt. MIN6 cells (*left panels*) and mouse islets (*right panels*) were incubated under control (C), palmitate (P, 0.5 mmol/L), or palmitate + inhibitor conditions for 1 h (MIN6) or 30 min (islets), and then subjected to immunoblot analysis for the indicated proteins. Inhibitors are wortmannin (Wort., 200 nmol/L), rapamycin (Rapa., 50 nmol/L), and Torin1 (250 nmol/L). *A*: Immunoblot for p-Akt (T308) and Akt. *B*: Immunoblot for p-Akt (S473) and Akt. *C*: Immunoblot for p-S6K and actin. *D*: Immunoblot for p-4E-BP1 and actin. $n = 3–6$. *$P < 0.05$ compared with control conditions. CTL, control; PAL, palmitate.
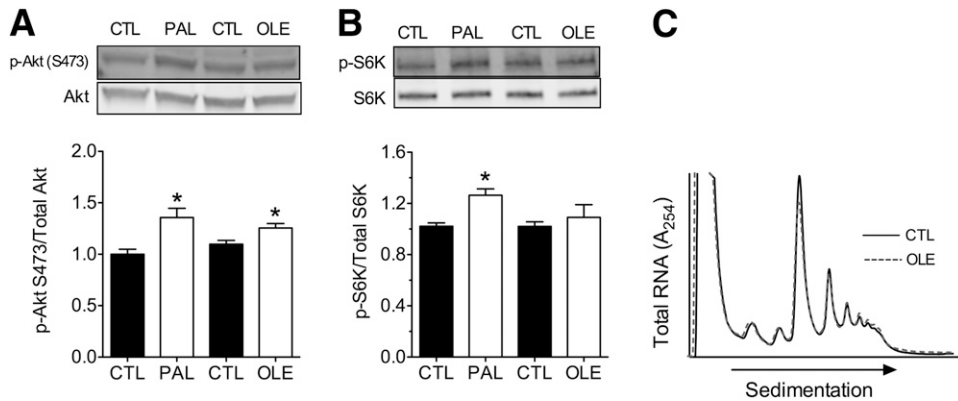
Figure 4—Oleate does not activate mTOR signaling. MIN6 cells were incubated under control (CTL), palmitate (PAL, 0.5 mmol/L), or oleate (OLE, 0.5 mmol/L) for 1 h. *A*: Representative immunoblot (*top panel*) and corresponding quantitation (from *n* = 6) (*bottom panel*) of p-Akt (S473) and Akt. *B*: Representative immunoblot (*top panel*) and corresponding quantitation (from *n* = 3) (*bottom panel*) of p-S6K and S6K. *C*: Representative PRP analysis of MIN6 cells after incubation under control and oleate conditions for 1 h. *P < 0.05 for the value compared with the corresponding control conditions.

relative monosome fractions to polysome fractions in re-sponse to 72-h incubation with palmitate, in a manner similar to that seen with thapsigargin treatment (Fig. 6D).

We hypothesized that blockade of mTOR would reduce the protein load upon the ER in response to saturated FFAs, and thereby reduce the propensity to trigger the UPR. To test this hypothesis, we performed

studies using the mTORC1/2 inhibitor Torin1. As shown in Fig. 7A and B, when MIN6 cells are treated with pal-mitate in the presence of Torin1, phosphorylation of eIF2α at 4 h is blocked, as is the later increase in UPR-related genes (*Chop, Atf4, Bip,* and *Xbp1s*) at 10 h. Sim-ilar inhibition of palmitate-induced UPR gene activation with Torin1 was also observed in mouse islets (Fig. 7C).
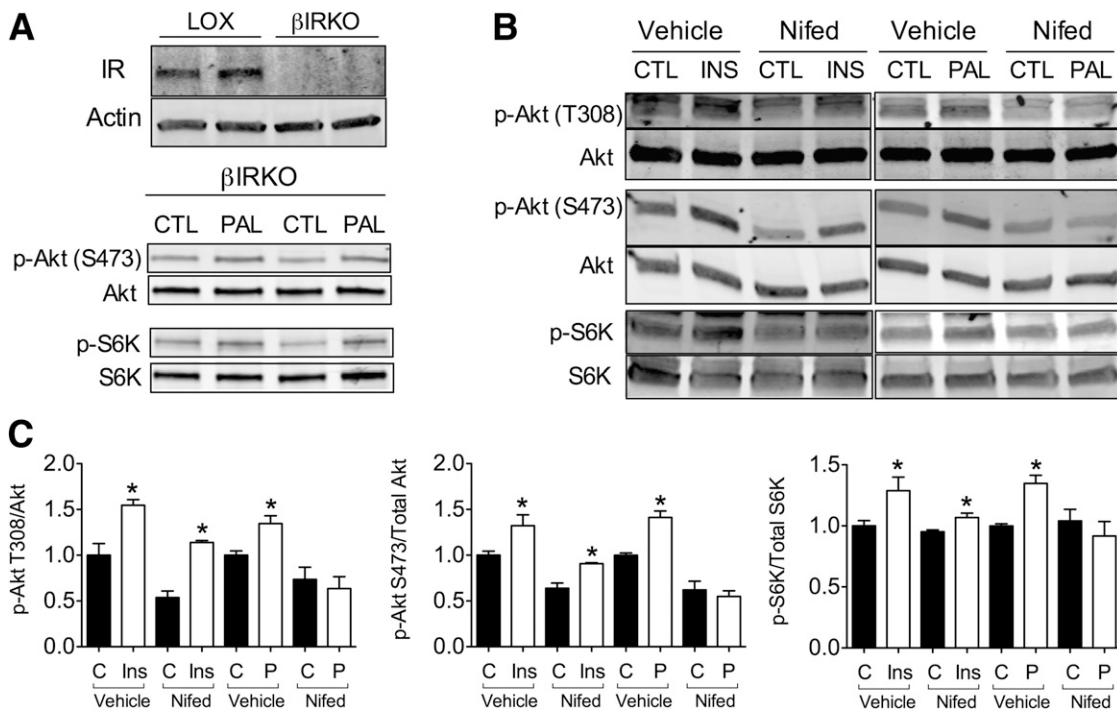

Figure 5—Palmitate activation of mTOR is not dependent on insulin signaling but requires L-type Ca^{2+} channels. *A*: The βIRKO cell line was incubated under control (CTL) or palmitate (PAL, 0.5 mmol/L) conditions at 20 mmol/L glucose, and then subjected to immunoblotting for the proteins indicated. *Upper panel* shows representative immunoblot of control (LOX cells) and βIRKO cells for insulin receptor (IR). *Lower panel* shows two independent immunoblots in βIRKO cells. *B*: Representative immunoblots from MIN6 cells treated under CTL, insulin (INS, 200 nmol/L), or PAL (0.5 mmol/L) conditions, with or without nifedipine (Nifed, 10 μmol/L). *C*: Quantitation of the immunoblot data (for *n* = 4) from *B*. *P < 0.05 for the comparison. C, control; P, palmitate.
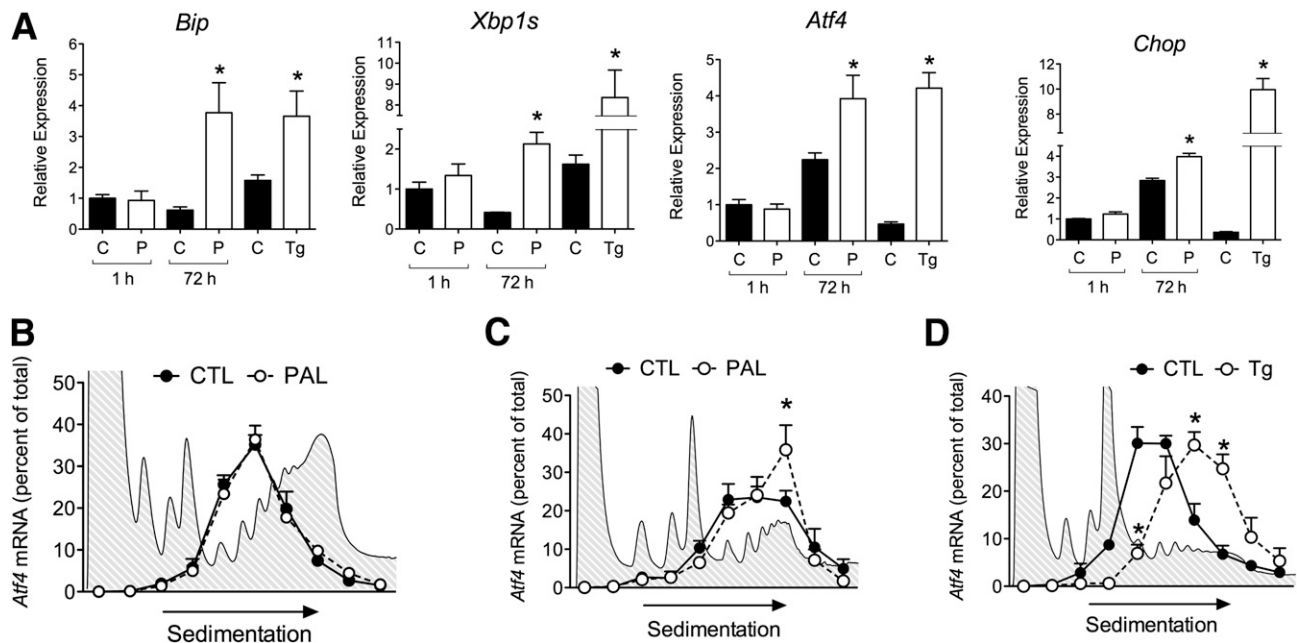
Figure 6—Activation of the UPR by palmitate. *A*: MIN6 cells were incubated under control (C) or palmitate (P, 0.5 mmol/L) conditions for 1 or 72 h or with thapsigargin (Tg, 1 μmol/L) for 4 h, and then subjected to RT-PCR for the indicated genes (*n* = 4 independent determinations). MIN6 cells were treated under control or palmitate conditions for 1 (*B*) and 72 h (*C*) or under control and thapsigargin conditions for 4 h (*D*), and then subjected to PRP analysis followed by real-time RT-PCR for *Atf4* of fractions from the PRP (*n* = 3 independent determinations). The shaded regions in *B–D* show the representative PRP, and the lines represent mRNA levels. **P* < 0.05 for the value compared with the corresponding control value in all panels. CTL, control; PAL, palmitate.

High-Fat Feeding of C57BL/6J Mice Increases Phosphorylation of Akt and S6K in Islets and Increases mRNA Translation

To study the physiological relevance of mTOR activation in vivo, we examined the short-term effects of a high-fat diet (HFD; 60% kcal from fat, mostly saturated fat) versus a low-fat diet (LFD; 10% kcal from fat) on mouse islets. Prior studies showed that 60% HFD feeding for 1 week increased β-cell replication and mass (27). As shown in Fig. 8A and B, respectively, 1 week of HFD feeding led to increases in body weight and fat mass compared with animals on an LFD. Figure 8C shows that glucose tolerance was worsened upon HFD feeding (when glucose was administered intraperitoneally based on total body weight). Islets from HFD-fed mice revealed increases in phosphorylations of Akt (T308) and S6K, but without changes in phosphorylation of eIF2α (Fig. 8D). In accordance with the activation of mTOR and mRNA translation, islets from HFD-fed mice displayed an increase in genes encoding proteins involved in proliferation (*Ki67* and *Pcna*) and an increase in the P/M ratio in PRP analysis (Fig. 8E and F). These results suggest that 1 week of HFD feeding may be more akin to the short-term incubations of isolated cells with palmitate.

DISCUSSION

By use of PRP analysis, we show that β-cells acutely respond to saturated FFAs (typified by palmitate) by increasing association of RNA species with polyribosomes,

a process reflective of increased mRNA translation. The increased mRNA translation in response to palmitate emanates from activation of the mTOR pathways, which link nutrient sensing to cellular growth. The growth and function-promoting effect of saturated FFAs on β-cells has been the topic of studies in both humans and rodents. In adult humans, short-term (<24 h) elevations in FFA levels (via intralipid infusions) result in exaggerated glucose-stimulated insulin secretion (38–40). In rats, elegant studies by Poitout and colleagues (6,8) showed that 6-month-old rats receiving just 72 h of intralipid infusion exhibit ~50% expansion in β-cell mass that was attributed to enhanced mTOR signaling and β-cell replication. These latter studies suggest that mTOR may be a crucial pathway in response to FFA signaling.

The mTOR complexes (mTORC1 and mTORC2) are sensors of ambient nutritional status and link growth factor signaling and availability of nutrients to protein synthesis and cellular proliferation and size in β-cells and other cell types (33). Of the two complexes, mTORC1 is characterized better in β-cells. mTORC1 includes mTOR, Raptor, and mLST8 and is sensitive to inhibition by rapamycin. Secondary activation of mTORC1 by expression of constitutively active Akt levels in β-cells increases cell expansion and results in enhancement of cell cycle proteins (41). Major downstream effects of mTORC1 activation are the phosphorylations of S6K and 4E-BP1. These events are seminal triggers of global translational initiation and elongation of mRNAs, which promote accrual of
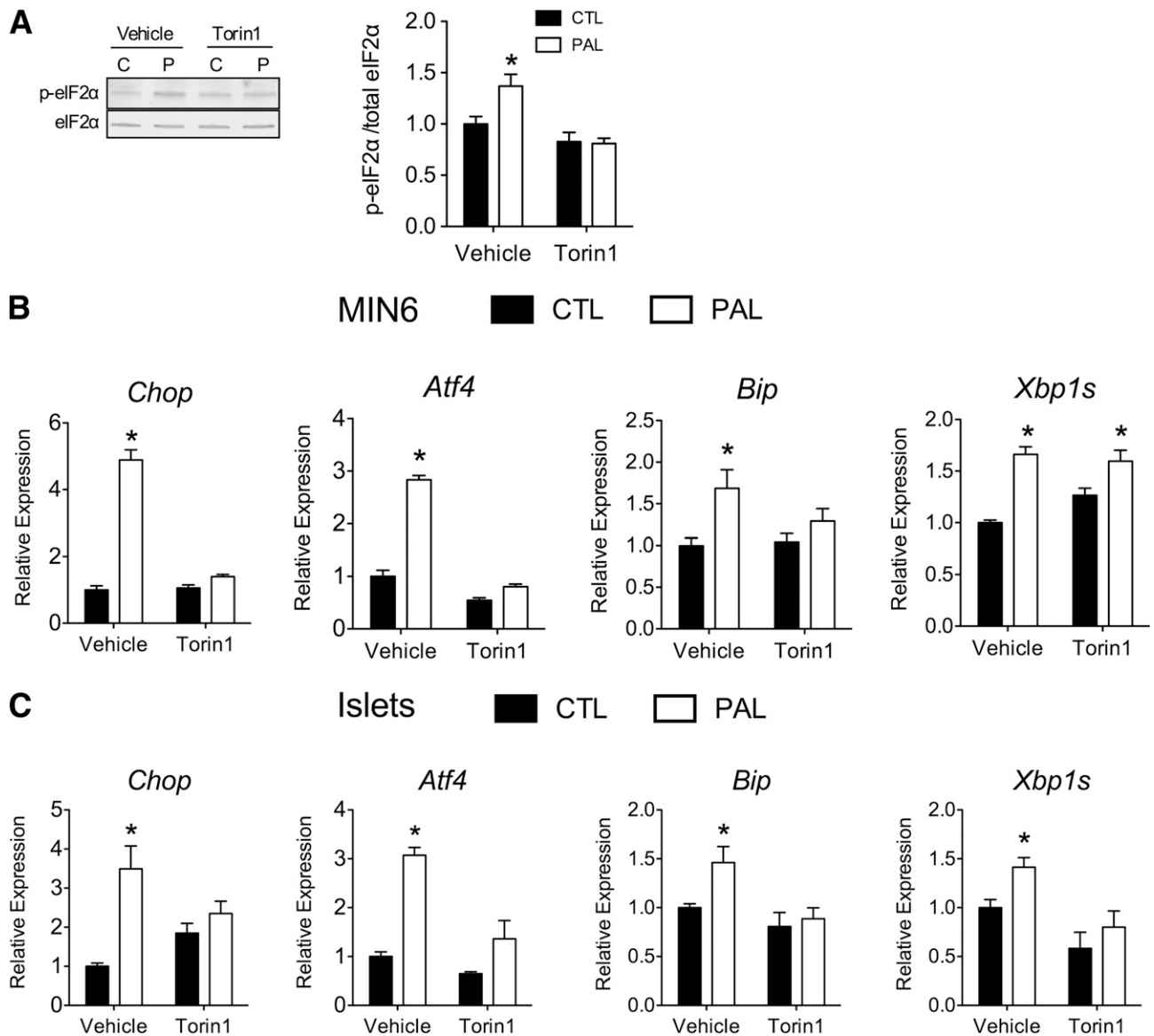
Figure 7—Inhibition of mTOR inhibits activation of the UPR by palmitate. A: Representative immunoblot (*left panel*) of p-eIF2α and eIF2α from MIN6 cells under control (CTL) and palmitate (PAL, 0.5 mmol/L) conditions with or without Torin1 (250 nmol/L) for 4 h, with corresponding quantitation ($n = 6$) (*right panel*) of the immunoblots. C, control; P, palmitate. B: Real-time RT-PCR data from MIN6 cells under control and palmitate conditions with or without Torin1 for 10 h. C: Real-time RT-PCR data from mouse islets under control and palmitate conditions with or without Torin1 for 10 h. All data represent mean ± SEM of at least three independent determinations. *$P < 0.05$ for the value compared with the corresponding control.

β-cell size and number (33). Our studies are the first to show that palmitate acutely induces an increase in polyribosome-associated RNAs in β-cells, a finding consistent with an increase in global mRNA translation. This effect of palmitate is concordant with an increased activity of the PI3K/Akt signaling pathway and the phosphorylations of S6K and 4E-BP1. We note that the effect of palmitate on global mRNA translation is dependent upon the fraction of free palmitate, since lower palmitate-to-BSA ratios have attenuated effects. Because inhibitors of mTORC1 appear to completely block S6K and 4E-BP1 phosphorylation events and polyribosome-associated RNA increases,

we propose that a direct effect of saturated FFAs is to activate mTOR signaling. The linkage between saturated FFAs, mTOR, and mRNA translation is supported by recent studies in rats in vivo, which revealed that rapamycin blocks the increase in β-cell mass, size, and proliferation in response to intralipid infusion in rats (6). Our studies in 1 week HFD-fed mice also support a role for FFAs activating mRNA translation in islets in vivo.

In contrast to the increases in polyribosome-associated RNAs with acute palmitate incubation, more prolonged incubations resulted in reductions in polyribosome-associated RNAs. Such reductions are suggestive of a decline in
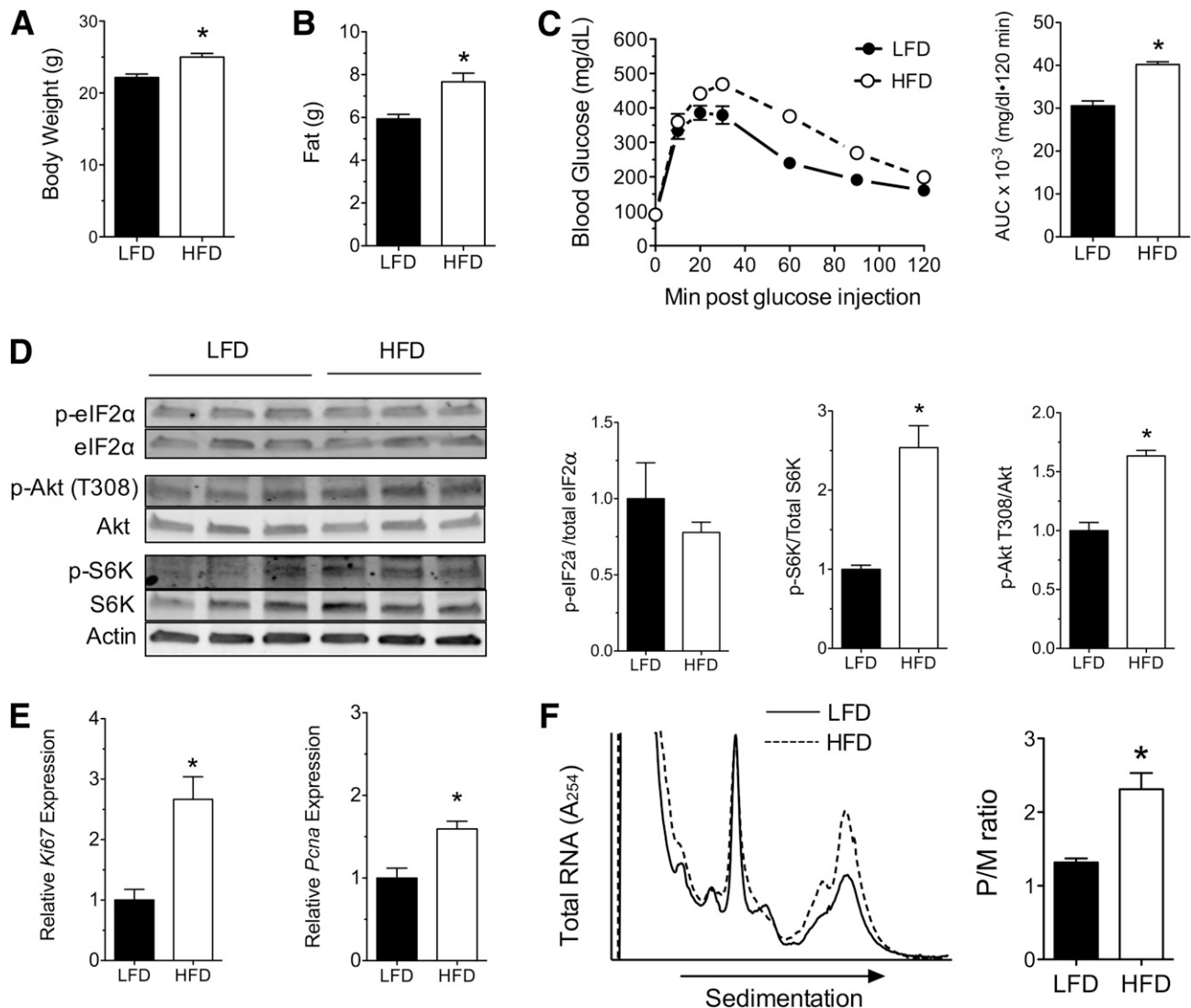
Figure 8—Activation of mTOR signaling in islets of HFD-fed mice. C57BL/6J mice (n = 10 per group) were fed an LFD (10% kcal from fat) or HFD (60% kcal from fat) for 7 days. A: Body weight of mice. B: Fat mass of mice, as determined by DEXA analysis. C: Results of glucose tolerance test (left panel) and corresponding area under the curve (AUC) analysis (right panel). D: Results of immunoblot analysis for the indicated proteins from islets isolated from LFD- and HFD-fed mice (n = 3 mice per group) and corresponding quantitations. E: Results of RT-PCR analysis from islets isolated from LFD- and HFD-fed mice (n = 3 mice per group) for the Ki67 and Pcna genes. F: Representative PRP analysis and corresponding P/M ratio (n = 3 mice per group) of islets isolated from LFD- and HFD-fed mice. For all panels, *P < 0.05 for the HFD compared with the LFD.

translation initiation rate relative to elongation rate, resulting in a paucity of ribosomes associated with RNA (29). In agreement with these findings, we observed the onset of the UPR, in which p-eIF2α blocks general (cap-dependent) mRNA translation. The induction of the UPR by palmitate has been observed in a number of studies and has been variably attributed to several processes, including derangement of cellular calcium homeostasis, induction of mitochondrial oxidative stress, posttranslational modification of proteins, and inhibition of splicing factors and transcription factors (13,42–44). An important factor that contributes to UPR and eventual ER stress is increased protein load on the ER (45,46). Because we found an early increase in mTOR activation and mRNA translation with

palmitate incubation, we tested the possibility that this early increase might contribute to later induction of the UPR. Accordingly, inhibition of mTOR with Torin1 blocked the increase in p-eIF2α levels and Bip, spliced Xbp1, and Chop mRNA levels. These results temporally link the early increase in mRNA translation with the later development of ER stress. However, the contribution of ER protein synthesis versus other effects of mTOR is unclear from our studies; we note that other studies have linked Torin1 treatment with increases in autophagy, a process that could also help to mitigate ER stress (47).

Activation of the PI3K/Akt/mTOR pathway is linked to growth factor signaling and/or nutrient (e.g., glucose, amino acid) abundance. Although palmitate can acutely

augment insulin secretion in the presence of glucose, we show that insulin signaling does not account for mTOR activation, since the phenomenon is observed in β-cells lacking insulin receptor. Studies of Poitout and colleagues (6) suggest that EGF signaling in response to intralipid/glucose infusion in rats is important in the activation of mTORC1 in β-cells, a possibility that may be more relevant in vivo. In this regard, we note that the intralipid infusions use mixtures of saturated and unsaturated FFAs, which likely activate release of growth factors from other organs, secondarily affecting β-cell growth (3,4). We cannot rule out the possibility in our high-fat feeding studies in vivo that mTOR activation may have been an indirect consequence of FFAs.

Some prior studies have implicated a role for FFAs in activating mTOR. In hepatocytes, FFAs were shown to increase mTOR signaling, but in an inverse relationship with Akt phosphorylation. In those studies, it was felt that mTOR activation by FFAs leads to insulin resistance (whereas inhibition of mTOR by metformin reduces it) (48). By contrast, studies in cell-free systems suggest that palmitate inhibits protein tyrosine phosphatase 1B, resulting in increases in Akt phosphorylation (49). Our studies in β-cells point to a role for L-type Ca^{2+} channels in the activation of Akt. Palmitate acutely increases intracellular Ca^{2+} in human and mouse β-cells (50), and increases in intracellular Ca^{2+}, via calmodulin, have been shown to associate with and activate PI3K (51). What remains unclear is how precisely palmitate is linked to L-type Ca^{2+} channels, and links to FFA transporters (e.g., CD36) and receptors (e.g., GPR40) remain possibilities.

Our results suggest a model whereby palmitate induces pathways that promote increased mRNA translation and ER workload in the β-cell. This early effect of palmitate appears to contribute to activation of the UPR, which when prolonged, can lead to β-cell apoptosis. We acknowledge some limitations to our studies, the most notable that they involve effects of a single saturated FFA on cells cultured in vitro. Effects in vivo likely are more complex and involve mixtures of FFAs and signaling from different organs. Nevertheless, our studies identify an important effect of palmitate on a key β-cell pathway that has heretofore been unappreciated. Future studies focusing on conditional knockouts of mTOR signaling molecules in mice will allow for more definitive conclusions in vivo on the role of mTOR in FFA signaling.

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