

Involvement of Per-Arnt-Sim Kinase and Extracellular-Regulated Kinases-1/2 in Palmitate Inhibition of Insulin Gene Expression in Pancreatic β -Cells

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OBJECTIVE—Prolonged exposure of pancreatic β -cells to simultaneously elevated levels of fatty acids and glucose (glucolipotoxicity) impairs insulin gene transcription. However, the intracellular signaling pathways mediating these effects are mostly unknown. This study aimed to ascertain the role of extracellular-regulated kinases (ERKs)1/2, protein kinase B (PKB), and Per-Arnt-Sim kinase (PASK) in palmitate inhibition of insulin gene expression in pancreatic β -cells.

RESEARCH DESIGN AND METHODS—MIN6 cells and isolated rat islets were cultured in the presence of elevated glucose, with or without palmitate or ceramide. ERK1/2 phosphorylation, PKB phosphorylation, and PASK expression were examined by immunoblotting and real-time PCR. The role of these kinases in insulin gene expression was assessed using pharmacological and molecular approaches.

RESULTS—Exposure of MIN6 cells and islets to elevated glucose induced ERK1/2 and PKB phosphorylation, which was further enhanced by palmitate. Inhibition of ERK1/2, but not of PKB, partially prevented the inhibition of insulin gene expression in the presence of palmitate or ceramide. Glucose-induced expression of PASK mRNA and protein levels was reduced in the presence of palmitate. Overexpression of wild-type PASK increased insulin and pancreatic duodenal homeobox-1 gene expression in MIN6 cells and rat islets incubated with glucose and palmitate, whereas overexpression of a kinase-dead PASK mutant in rat islets decreased expression of insulin and pancreatic duodenal homeobox-1 and increased C/EBP β expression.

CONCLUSIONS—Both the PASK and ERK1/2 signaling pathways mediate palmitate inhibition of insulin gene expression. These findings identify PASK as a novel mediator of glucolipotoxicity on the insulin gene in pancreatic β -cells. *Diabetes* 58: 2048–2058, 2009

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Received 28 April 2008 and accepted 21 May 2009.

Published ahead of print at <http://diabetes.diabetesjournals.org> on 5 June 2009. DOI: 10.2337/db08-0579.

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The glucolipotoxicity hypothesis posits that abnormalities in lipid metabolism, in conjunction with chronic hyperglycemia, contribute to the deterioration of β -cell function in type 2 diabetes (1,2). In vitro, prolonged exposure of isolated islets and insulin-secreting cells to simultaneously elevated levels of fatty acids and glucose leads to inhibition of glucose-induced insulin secretion, impairment of preproinsulin gene (hereafter referred to as "insulin gene") expression, and cell death by apoptosis (rev. in 1). Our observation (3) that inhibition of insulin gene expression also occurs in islets from rats chronically infused with glucose and intralipid confirmed the relevance of this phenomenon to in vivo situations.

Studies in our laboratory have attempted to define the cellular and molecular mechanisms of fatty acid inhibition of the insulin gene. First, we showed that the mechanisms by which fatty acids impair insulin secretion and insulin gene expression are distinct (4,5). Thus, both saturated (e.g., palmitate) and unsaturated (e.g., oleate) long-chain fatty acids inhibit glucose-induced insulin secretion (5), whereas only palmitate impairs insulin gene expression (4). This is because inhibition of insulin gene expression is mediated by de novo synthesis of ceramide, for which only palmitate can serve as a substrate (4). Second, palmitate inhibits glucose-induced insulin promoter activity (4) through decreased glucose-induced MafA expression and nuclear exclusion of pancreas-duodenum homeobox-1 (PDX-1), thereby reducing the binding activities of both transcription factors (6).

Signaling pathways implicated in palmitate inhibition of insulin gene expression are mostly unknown. Several members of the mitogen-activated protein kinase family are activated by phosphorylation in response to glucose. Upon activation, the extracellular-regulated kinases (ERK)1/2 promote nuclear translocation and/or binding of PDX-1, MafA, and β 2/NeuroD1 to the insulin promoter, and in turn, these transcription factors synergistically activate insulin gene transcription (7–11). On the other hand, glucose activates the phosphatidylinositol-3 kinase (PI3K)-dependent insulin signaling pathway and increases protein kinase B (PKB) phosphorylation in β -cells (12), although the role of the PI3K pathway in the regulation of insulin gene expression remains controversial (10,13,14). Finally, induction of mRNA and protein expression of the Per-Arnt-Sim kinase (PASK) in MIN6 cells and isolated

islets mediates glucose-induced insulin gene expression (15).

The aims of this study were 1) to examine the effects of glucose and palmitate on the phosphorylation state and expression levels of ERK1/2, PKB, and PASK in MIN6 insulin-secreting cells and isolated rat islets; and 2) to ascertain, using pharmacological and molecular tools, whether these signaling pathways mediate palmitate inhibition of insulin gene expression.

RESEARCH DESIGN AND METHODS

Reagents and solutions. RPMI-1640 and FBS were from Invitrogen (Burlington, ON). Dulbecco's modified Eagles's medium (DMEM) was from Wisent (Saint Bruno, QC) and bovine growth serum was from HyClone (Logan, UT). Fatty acid-free BSA was from Equitech-Bio (Kerrville, TX). LY 294002, PD98059, U0126, and thapsigargin were from Calbiochem (EMD Biosciences, San Diego, CA). Propidium iodide was from Invitrogen. C2-ceramide was from Biomol (Plymouth Meeting, PA). Palmitate (sodium salt) and all other reagents (analytical grade) were from Sigma (St. Louis, MO) unless otherwise noted. Rabbit anti-PKB, anti-phospho-Ser⁴⁷³-PKB, anti-phospho-p44/p42 MAP kinase (Thr²⁰²/Tyr²⁰⁴), and anti-cleaved caspase 3 antibodies were from Cell Signaling (New England Biolabs, Beverly, MA). Rabbit anti-ERK1/2 antibody was from Promega (Madison, WI). Interleukin (IL)-1 β was from Cedarlane Laboratories (Burlington, ON). Rabbit anti-p85 subunit of PI3K and anti-PDX-1 antibodies were from Upstate Biotechnology (Lake Placid, NY). Rabbit anti-hPASK (U2501), anti-mrPASK (U2508) antibodies, and hPASK adenoviruses were generated as described (16). Insulin radioimmunoassay kits were from Linco Research (St. Charles, MI). Palmitate and C2-ceramide solutions were prepared as described (4). The final molar ratio of palmitate:BSA was 5:1. All control conditions included a solution of vehicle (ethanol:H₂O) mixed with fatty acid-free BSA.

Male Wistar rats (225–275 g) (Charles River Laboratories, Wilmington, MA) were housed on a 12-h light/dark cycle with free access to standard laboratory rat diet and water. All procedures were approved by the Institutional Committee for the Protection of Animals at the Centre Hospitalier de l'Université de Montréal.

Rat islet isolation and cell culture. Rat islets were isolated as described (4,17). Isolated islets were precultured for 16 h at 2.8 mmol/l and then exposed to 2.8 or 16.7 mmol/l glucose in various experimental conditions as described in RESULTS. MIN6 cells (passage 28–40) were maintained as previously described (18). Cells were seeded in six-well plates at a density of 1×10^6 cells per well for 3–5 days. One day before the experiment, cells were cultured in medium containing 5.5 mmol/l glucose for 16 h. Cells were serum-starved in medium containing 0.5% BSA for 2 h and then exposed to various experimental conditions as described in RESULTS.

Acridine orange and propidium iodide fluorescent staining. Islets were incubated for 30 min with 1 μ mol/l acridine orange and 15 μ mol/l propidium iodide. Stained islets were examined under a Leica TCS SP5 confocal microscope (Leica Microsystems [Canada], Richmond Hill, ON). Living cells were identified by green staining (Acridine orange) and dead cells by brown-red staining (propidium iodide). For immunohistochemistry, MIN6 cells were fixed with 3.7% formaldehyde for 30 min and permeabilized in cold methanol for 20 min. After saturation with 5% normal horse serum, cells were incubated with rabbit anti-PDX-1 antibody, followed by fluorescein isothiocyanate goat anti-rabbit (Vector, Burlingame, CA). Cells were observed under a fluorescent microscope (Leica Microsystems, Bannockburn, IL).

Adenoviral infection of islets and MIN6 cells. The appropriate titer of recombinant adenovirus (Adv) encoding luciferase (Adv-Luc) or human PASK (Adv-hPASK [16]) was determined in pilot experiments. MIN6 cells were subcultured in six-well plates (1×10^6 cells per well). After 3 days of culture to reach ~60% confluence, MIN6 cells were infected with 4×10^5 plaque-forming unit/cell of Adv-Luc or Adv-hPASK for 2 h, after which the medium was replaced and the cells further cultured for 16 h before serum starvation and exposure to various experimental conditions as described in RESULTS. For arterial delivery of adenoviral vectors in rat pancreas, Wistar rats were anesthetized and subjected to laparotomy. After ligation of the portal vein, the superior aorta, and the hepatic, mesenteric, and right and left renal arteries, adenoviruses (4.10^{10} pfu/pancreas) were injected in the celiac trunk of the inferior aorta as described (19).

Protein extraction and immunoblotting. Proteins from cells or islets were extracted and resolved by SDS-PAGE as described (6). Membranes were blotted with antibodies against pERK1/2 (Thr²⁰²/Tyr²⁰⁴) or total ERK1/2, pSer⁴⁷³-PKB or total PKB, hPASK, mrPASK, or the p85 subunit of PI3K. Signals were detected using a horseradish peroxidase-labeled anti-rabbit IgG

(BioRad, Richmond, CA) and enhanced chemiluminescence (ECL; PerkinElmer Las Canada, Woodbridge, ON) on Kodak BioMax XAR films (Kodak, Rochester, NY). The bands were quantified by G Box Chemi 16 using Gene Tools from Syngene software (Syngene, Frederick, MD).

Real-time PCR. Total RNA was extracted from aliquots of 100 islets each using the RNeasy Qiagen micro-kit (Qiagen, Mississauga, ON). MIN6 cell RNA was extracted by the Trizol method, reverse transcribed, and RT-PCR was carried out using the Faststart DNA Master PLUS SYBR Green Kit (Roche, Indianapolis, IN), as previously described (3). All mouse, rat, and human primer sets, listed in supplementary Table 1 (available in the online appendix at <http://diabetes.diabetesjournals.org/cgi/content/full/db08-0579/DC1>), were designed using Primer3 (20). To amplify preproinsulin pre-mRNA (pre-INS2), a forward primer was designed against a sequence in exon two and a reverse primer designed against a sequence in intron 2, as described (21).

Insulin content. Intracellular insulin content was determined by radioimmunoassay after acidified-ethanol extraction.

Statistical analysis. Data are expressed as means \pm SE and were analyzed by one-way ANOVA with Bonferroni post hoc adjustment for multiple comparisons, using GraphPad InStat (GraphPad Software, San Diego, CA); $P < 0.05$ was considered significant.

RESULTS

Palmitate inhibits glucose-induced expression of insulin pre-mRNA and mRNA species in MIN6 cells and rat islets. The long half-life of mature insulin mRNA species makes it difficult to examine early changes in insulin gene expression induced by palmitate (4). To circumvent this, we used a set of primers against the short-lived pre-mRNA (pre-INS2), as described by Evans-Molina et al. (21). We used primers specific for the insulin 2 gene containing intron 2 because its short half-life reflects earlier changes in transcriptional rates (22). Glucose induced a larger increase in pre-INS2 mRNA than in mature INS2 mRNA after 24 h of exposure in both MIN6 cells ($n = 3$; $P < 0.05$; Fig. 1A) and rat islets ($n = 3$; $P < 0.001$; Fig. 1B). Palmitate significantly blocked the stimulatory effect of glucose on pre-INS2 mRNA within 24 h in both MIN6 cells ($n = 3$; $P < 0.001$) and rat islets ($n = 3$; $P < 0.001$), whereas at that time point mature INS2 mRNA levels were not yet significantly decreased (Fig. 1A and B). Because palmitate inhibition of insulin gene expression is, at least in part, due to nuclear exclusion of PDX-1 (6), we measured PDX-1 target genes in islets exposed to glucose \pm palmitate for 24 h (Fig. 1C). We observed that during this period glucose induced the expression of GLUT2 mRNA and islet amyloid polypeptide (IAPP) pre-mRNA, but not INS1, glucokinase, or IAPP mature mRNA levels. Palmitate significantly decreased INS1 mRNA levels at both low and high glucose, as well as GLUT2 and glucokinase mRNA and IAPP pre-mRNA levels at high glucose (Fig. 1C). To exclude a possible effect of palmitate on cell viability, we first measured caspase 3 cleavage in MIN6 cells. As expected, a 24-h exposure to IL-1 β or thapsigargin induced caspase 3 cleavage, whereas palmitate had no effect (supplemental Fig. 1). Similarly, culture of islets for 24 h with palmitate did not lead to an increase in PI-positive cells, in contrast to the positive control H₂O₂ (supplemental Fig. 2).

Prolonged exposure of MIN6 cells and isolated rat islets to high glucose and palmitate increases ERK1/2 and PKB phosphorylation. We first assessed the phosphorylation state and expression levels of ERK1/2 and PKB in MIN6 cells and isolated islets. As shown in Fig. 2A–C, exposure of MIN6 cells to 11 mmol/l glucose time-dependently increased both ERK1 and ERK2 phosphorylation ($n = 4$; $P < 0.01$; Fig. 2B and C, respectively), which was further enhanced by addition of palmitate. The effect of palmitate was detected at 4 h (data not shown), further increased after

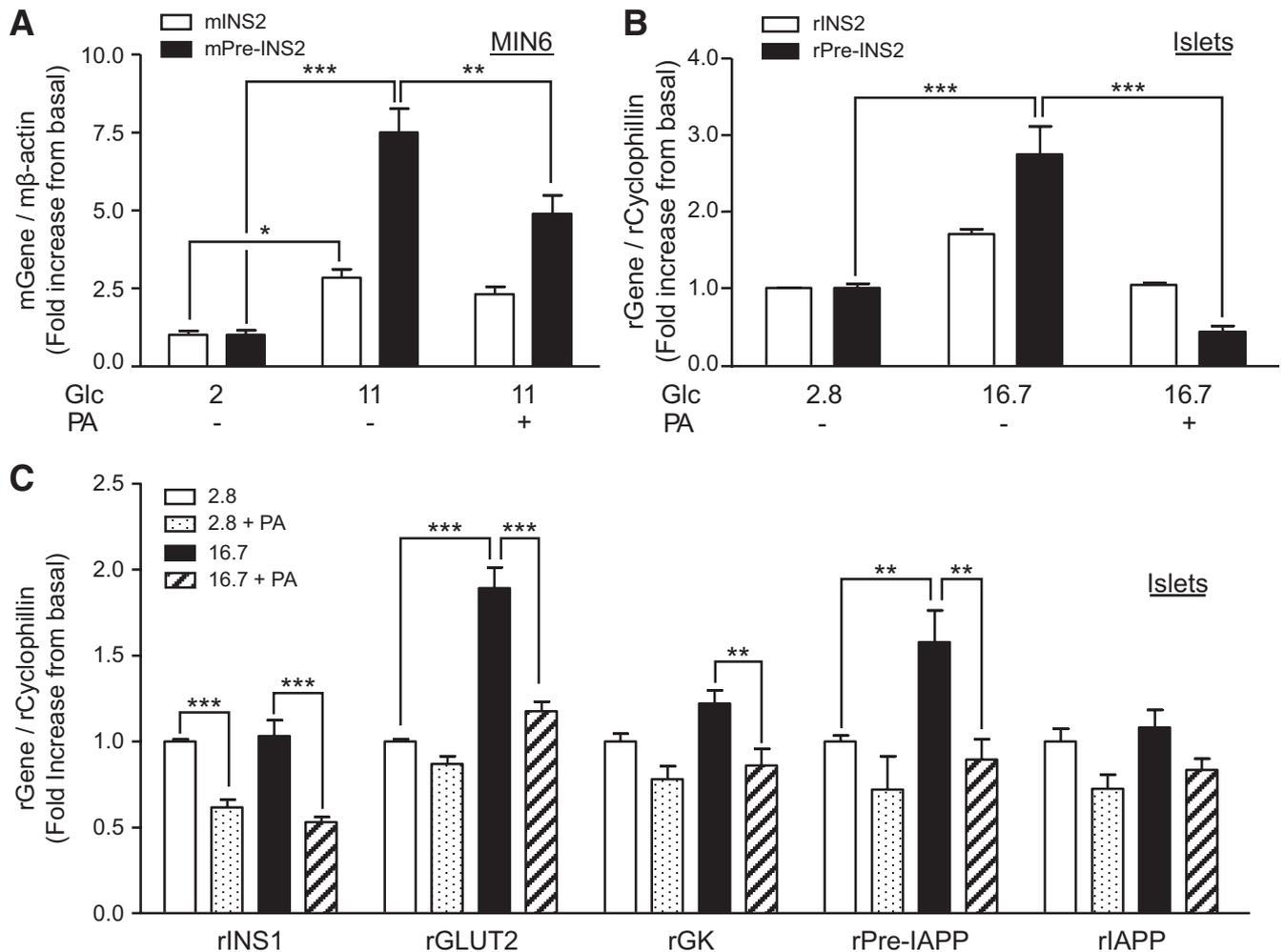


FIG. 1. Palmitate inhibits preproinsulin pre-mRNA and mRNA levels. **A:** MIN6 cells were cultured for 24 h with 2 or 11 mmol/l glucose, \pm 0.5 mmol/l palmitate. Mature (mINS2) or pre- (mPre-INS2) preproinsulin mRNA levels were measured by RT-PCR and normalized by β -actin mRNA levels. **B:** Rat islets were cultured for 24 h with 2.8 or 16.7 mmol/l glucose \pm 0.5 mmol/l palmitate. Mature (rINS2) or pre- (rPre-INS2) preproinsulin RNA levels were measured by RT-PCR and normalized by cyclophilin mRNA levels. **C:** Insulin 1, GLUT2, glucokinase, and IAPP mRNA as well as IAPP pre-mRNA levels were measured by RT-PCR and normalized by cyclophilin mRNA levels. Results are expressed as fold increase of the ratio of gene/ β -actin or cyclophilin mRNA over the control value and are means \pm SE of three replicate experiments. * P < 0.05, ** P < 0.01, *** P < 0.001; NS, not significant.

12 h and was sustained over a 24-h period ($n = 4$; $P < 0.001$; Fig. 2*B* and *C*). Ser⁴⁷³-phosphorylation of PKB was slightly but not significantly enhanced by glucose and strongly augmented by palmitate after 12 h but not 24 h ($n = 5$; $P < 0.01$; Fig. 2*D* and *E*). Similar effects of palmitate on ERK1/2 and PKB phosphorylation were observed in isolated rat islets after an 18-h exposure (Fig. 2*F* and *G*).

Inhibition of MEK1/2, but not PI3K, partially prevents palmitate inhibition of insulin gene expression.

To determine whether phosphorylation of ERK1/2 and PKB by palmitate plays a role in the inhibition of insulin gene expression, pharmacological inhibitors of their respective signaling pathways were used. As expected, glucose-induced ERK1/2 phosphorylation with or without palmitate was blocked by the MEK1/2 inhibitors U0126 (Fig. 3*A*) and PD98059 (not shown). PKB phosphorylation was blocked in the presence of LY294002 (Fig. 3*B*). In MIN6 cells, both U0126 and LY294002 significantly potentiated the stimulatory effect of glucose on insulin pre-mRNA ($n = 4$ – 7 ; $P < 0.001$; Fig. 3*C*), suggesting some degree of tonic inhibition of insulin gene expression by the ERK1/2 and PKB pathways in MIN6 cells, but not in islets, in which neither U0126 (Fig. 3*D*) or LY294002 (Fig. 3*F*) had

any effect on basal or glucose-stimulated insulin pre-mRNA expression. Palmitate inhibited insulin pre-mRNA levels in MIN6 cells at 11 mmol/l glucose ($n = 4$; $P < 0.001$; Fig. 3*C*) and rat islets at both 2.8 and 16.7 mmol/l glucose ($n = 8$; $P < 0.001$; Fig. 3*D* and *E*). This effect was partially reversed in the presence of U0126 in MIN6 cells (Fig. 3*C*) and in the presence of either U0126 (Fig. 3*D*) or PD98059 (Fig. 3*E*) in rat islets. In contrast, LY294002 did not prevent palmitate inhibition of insulin pre-mRNA levels in either MIN6 cells (Fig. 3*C*) or rat islets (Fig. 3*F*). Altogether, these data indicate that blockade of the ERK1/2 pathway, but not of PKB, partially prevents palmitate inhibition of insulin gene expression.

C2-ceramide enhances ERK1/2 phosphorylation and inhibits insulin gene expression in MIN6 cells.

We have previously shown that de novo ceramide synthesis mediates palmitate inhibition of insulin gene expression in islets (4). To address whether the effect of ceramide is mediated by ERK1/2 phosphorylation, we exposed MIN6 cells to the cell-permeable analog C2-ceramide in the absence or presence of MEK1/2 inhibitors (Fig. 4). First, we observed that C2-ceramide further enhanced ERK1/2 phosphorylation in response to glucose (Fig. 4*A*), similar

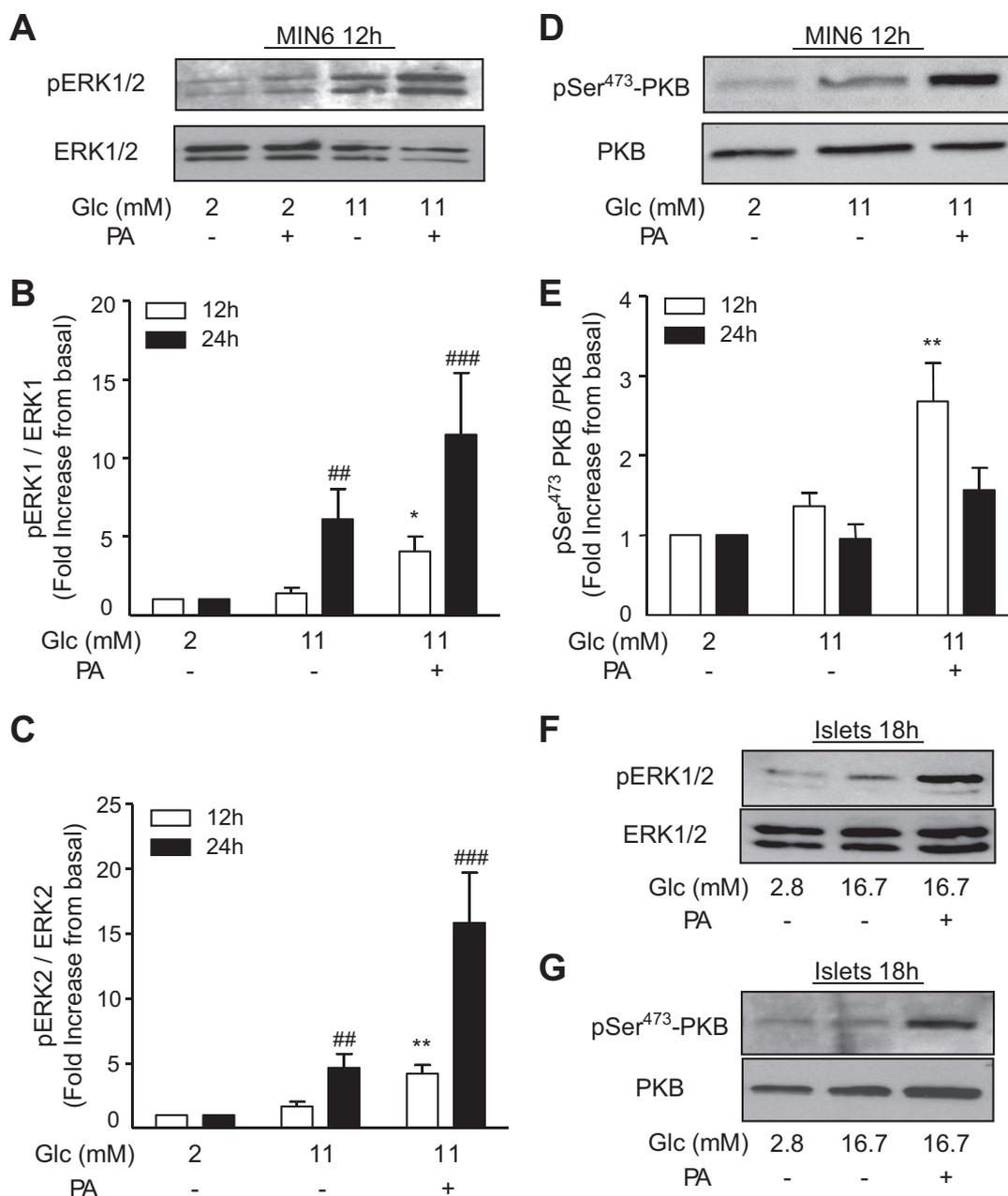


FIG. 2. Palmitate enhances ERK1/2 and PKB phosphorylation. Representative immunoblots of phospho- and total ERK1/2 (**A** and **F**) and phospho-Ser⁴⁷³ and total PKB (**D** and **G**) in MIN6 cells or rat islets, respectively. **A**: MIN6 cells were exposed for 12 h to 2 or 11 mmol/l glucose \pm 0.5 mmol/l palmitate, and phospho- and total ERK1/2 were examined by immunoblotting. **B** and **C**: Quantitative measurements of ERK1 (**B**) and ERK2 (**C**) phosphorylation after a 12- and 24-h culture. Data are means \pm SE of four replicate experiments and are normalized to control values at 2 mmol/l glucose and the same time point. * P < 0.05, ** P < 0.01, *** P < 0.001. **D**: MIN6 cells were exposed for 12 h to 2 or 11 mmol/l glucose \pm 0.5 mmol/l palmitate, and phospho- and total PKB were examined by immunoblotting. **E**: Quantitative measurements of PKB phosphorylation after a 12- or 24-h culture. Data are means \pm SE of four replicate experiments and are normalized to the control values at 2 mmol/l glucose. ** P < 0.01. **F**: Rat islets were exposed for 18 h to 2.8 or 16.7 mmol/l glucose \pm 0.5 mmol/l palmitate, and phospho- and total ERK1/2 were examined by immunoblotting. **G**: Rat islets were exposed for 18 h to 2.8 or 16.7 mmol/l glucose \pm 0.5 mmol/l palmitate, and phospho- and total PKB were examined by immunoblotting.

to the effects of palmitate shown in Fig. 2. As expected, this was blocked in the presence of U0126 or PD98059 (Fig. 4A). C2-ceramide significantly inhibited glucose induction of insulin pre-mRNA levels, and this effect was totally prevented in the presence of U0126 (Fig. 4B). The nonmetabolizable analog C2-dihydroceramide had no effect on insulin gene expression (not shown).

Inhibition of ERK1/2 does not alter the expression levels of MafA, PDX-1, and C/EBP β . We have previously shown that the molecular mechanisms whereby palmitate inhibits insulin gene expression involve decreased MafA expres-

sion and PDX-1 nuclear exclusion (6). On the other hand, Lawrence et al. (9) have shown that the transcription factor C/EBP β inhibits insulin promoter activity. We examined whether ERK1/2 was involved in the changes in MafA, PDX-1, and C/EBP β expression in response to palmitate. As expected, a 24-h exposure of MIN6 cells to elevated glucose increased MafA and PDX-1 mRNA and decreased C/EBP β mRNA (Fig. 5A, B, and C, respectively). Addition of palmitate decreased MafA mRNA (Fig. 5A) but did not alter PDX-1 (Fig. 5B) or C/EBP β mRNA (Fig. 5C), and these effects of palmitate were unchanged in the presence of the MEK1/2 inhibitor U0126.

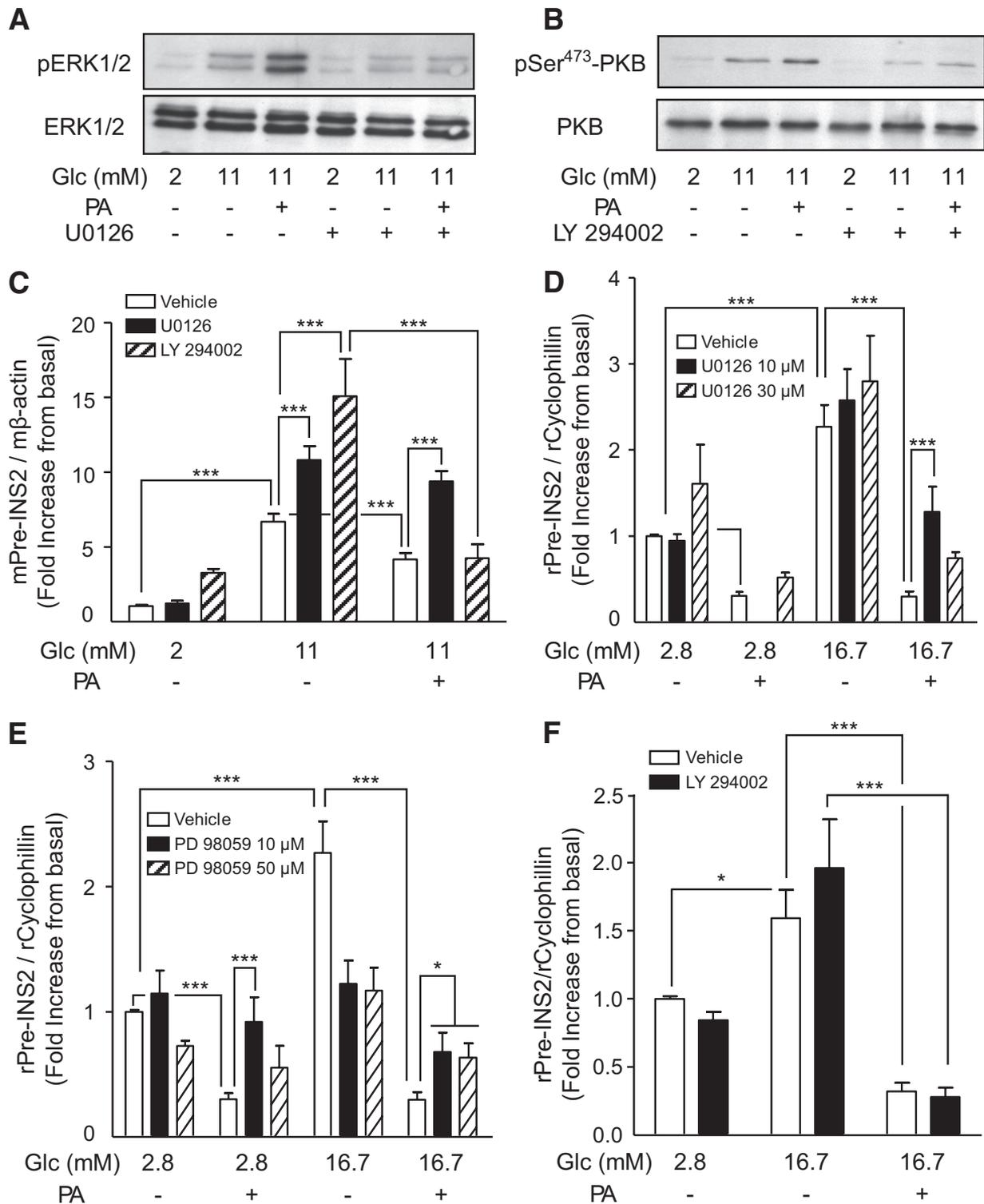


FIG. 3. Inhibition of ERK1/2 pathway, but not PKB, partially prevents palmitate inhibition of insulin gene expression in MIN6 cells and rat islets. **A:** Representative immunoblots of phospho- and total ERK1/2 in MIN6 cells exposed for 24 h to 2 or 11 mmol/l glucose \pm 0.5 mmol/l palmitate, in the presence or absence of U0126 (10 μ mol/l). **B:** Representative immunoblots of phospho- and total PKB in MIN6 cells exposed for 24 h to 2 or 11 mmol/l glucose \pm 0.5 mmol/l palmitate, in the presence or absence of LY 294002 (20 μ mol/l). **C:** Effects of U0126 (10 μ mol/l) and LY 294002 (20 μ mol/l) on insulin pre-mRNA levels in MIN6 cells. Results are expressed as fold increase of the ratio of pre-INS2/ β -actin mRNA over the control value and are means \pm SE of four to seven replicate experiments. **D–F:** Insulin pre-mRNA levels in rat islets, exposed for 24-h to 2.8 mmol/l glucose or 16.7 mmol/l glucose, \pm 0.5 mmol/l palmitate, with 0, 10, or 30 μ mol/l U0126 (**D**); 0, 10, or 50 μ mol/l PD98059 (**E**); or 0 or 20 μ mol/l LY 294002 (**F**). Results are expressed as fold increase of the ratio of pre-INS2/cyclophilin mRNA over control value and are means \pm SE of eight replicate experiments. ** P < 0.01, *** P < 0.001.

PASK is implicated in palmitate inhibition of insulin gene expression. PASK has been demonstrated to play a role in metabolic sensing and in the control of insulin gene

expression by glucose in pancreatic β -cells (15,23,24). A 6-h exposure to high glucose induced a significant increase in PASK mRNA levels in MIN6 cells ($n = 3$; $P < 0.05$; Fig.

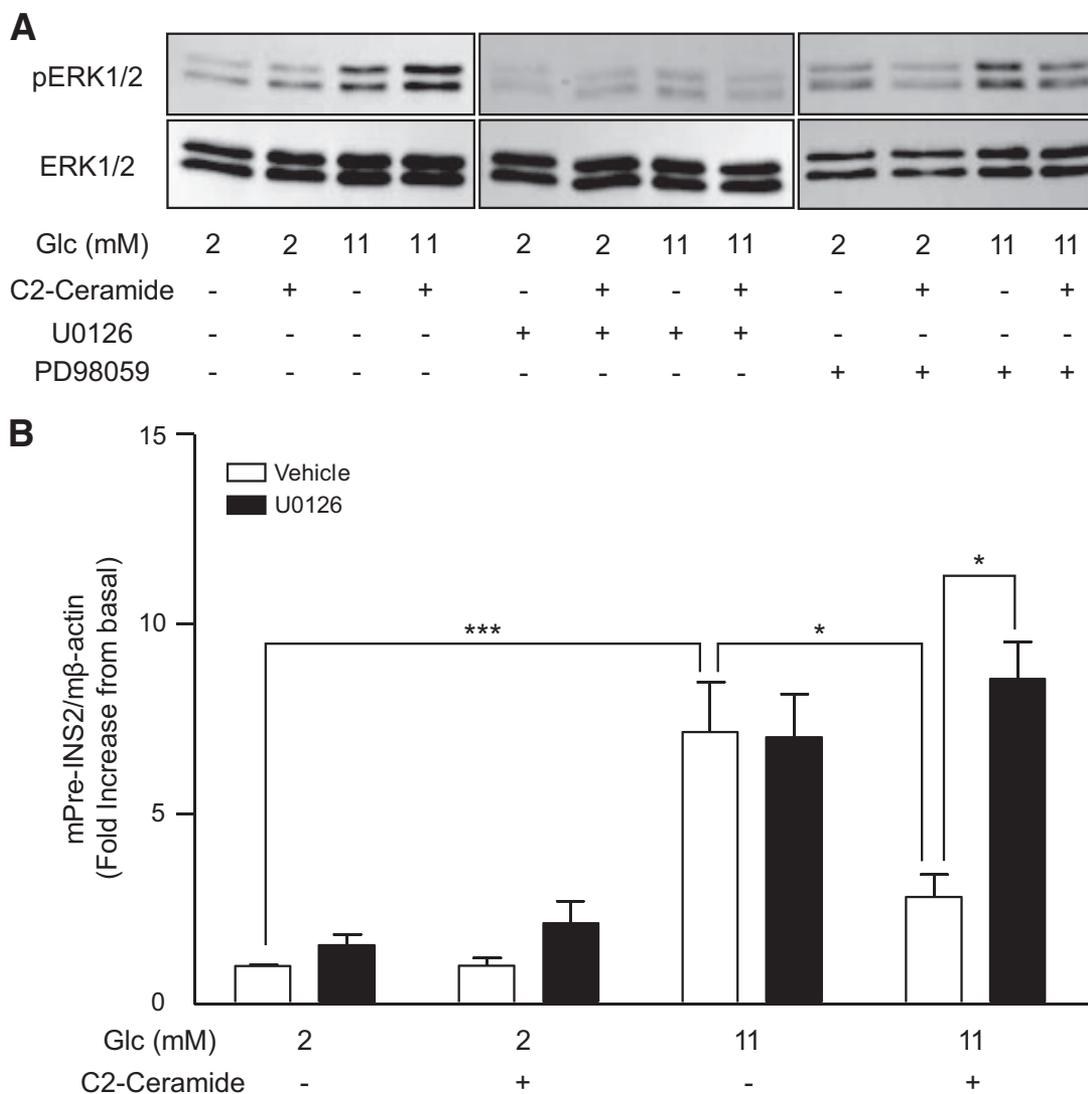


FIG. 4. Inhibition of insulin gene expression by C2-ceramide is prevented by ERK1/2 inhibition. **A:** Representative immunoblots of phospho- and total ERK1/2 in MIN6 cells exposed for 24 h to 2 or 11 mmol/l glucose \pm 50 μ mol/l C2-ceramide, in the presence or absence of U0126 (30 μ mol/l) or PD98059 (50 μ mol/l). **B:** Effects of U0126 (30 μ mol/l) on insulin pre-mRNA levels in MIN6 cells. Results are expressed as fold increase of the ratio of pre-INS2/ β -actin mRNA over control value and are means \pm SE of three replicate experiments.

6A) and in rat islets ($n = 3$; $P < 0.001$; Fig. 6B), consistent with a previous report (15). The concomitant presence of palmitate nearly abrogated glucose-induced PASK mRNA expression in both MIN6 cells (Fig. 6A) and rat islets (Fig. 6B). A similar pattern was observed at the protein level in MIN6 cells (Fig. 6C and D). To determine whether palmitate inhibition of PASK expression mediates its effects on the insulin gene, we first infected MIN6 cells with an adenovirus encoding for wild-type hPASK (Fig. 7A). As previously reported (15), overexpression of hPASK significantly increased insulin gene expression at low glucose ($P < 0.001$; Fig. 7B). In MIN6 cells infected with a control adenovirus expressing luciferase (Luc), palmitate inhibited glucose-induced insulin pre-mRNA levels after 24 h of exposure ($n = 7$; $P < 0.05$; Fig. 7B). In contrast, palmitate had no effect on insulin pre-mRNA in MIN6 cells overexpressing hPASK ($n = 7$; NS; Fig. 7B). Overexpression of PASK did not affect ERK1/2 phosphorylation (not shown). These data suggest that PASK mediates palmitate inhibition of insulin gene expression in MIN6 cells. To confirm these observations in islets, we overexpressed the wild-type or a kinase-dead mutant of hPASK in islets using

arterial adenoviral delivery (Fig. 7C). After isolation, islets were exposed to 16.7 mmol/l glucose and 0.5 mmol/l palmitate. We verified that the adenoviral infection did not affect cell viability, as indicated by the absence of propidium iodide-positive cells (supplemental Fig. 2C–E). In islets exposed to elevated glucose and palmitate, overexpression of wild-type hPASK increased insulin pre-mRNA levels (Fig. 7D), consistent with the observation in MIN6 cells (Fig. 7B). The kinase-dead hPASK mutant was previously shown to abrogate PASK activity and to block glucose-induced insulin promoter activity in MIN6 cells (15,16). Accordingly, overexpression of the kinase-dead hPASK mutant in MIN6 cells decreased intracellular insulin content by $\sim 28\%$ ($1,060 \pm 53$ vs. $1,484 \pm 61$ ng/ml in Luc-expressing cells; $P < 0.05$; $n = 4$). In islets, overexpression of kinase-dead hPASK decreased insulin pre-mRNA and PDX-1 mRNA levels and increased C/EBP β expression (Fig. 7D). To confirm the effects of PASK on PDX-1 expression, we examined PDX-1 protein levels by immunohistochemistry (Fig. 8). PDX-1 protein expression was induced by high glucose (Fig. 8B) and reduced in the presence of palmitate (Fig. 8C). Wild-type hPASK overex-

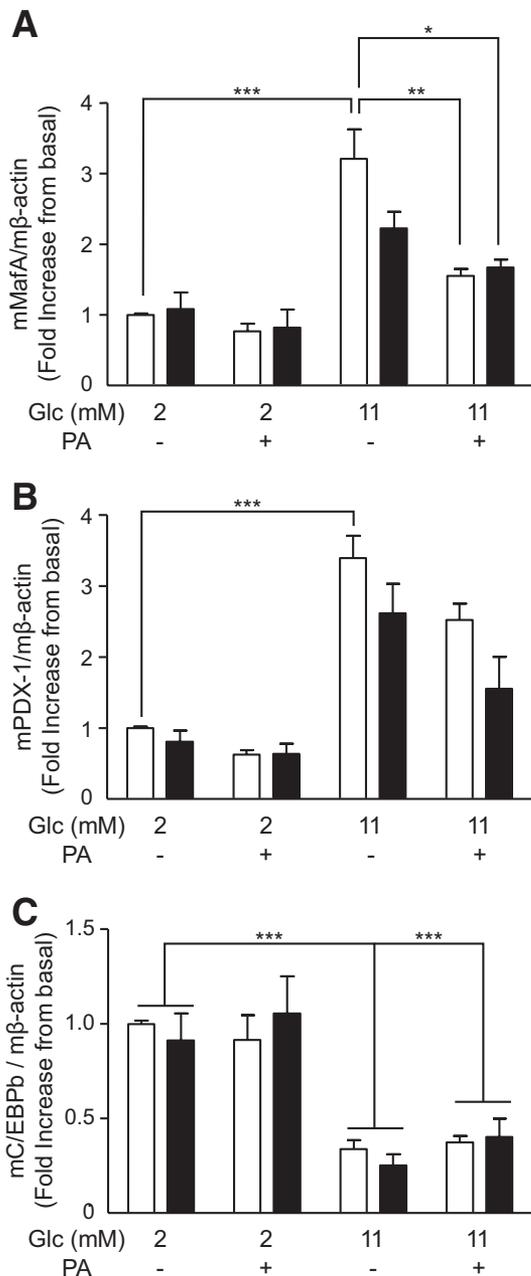


FIG. 5. Inhibition of ERK1/2 does not prevent palmitate inhibition of MafA expression and has no effect on PDX-1 and C/EBP β expression. MIN6 cells were cultured for 24 h with 2 mmol/l or 11 mmol/l glucose, \pm 0.5 mmol/l palmitate, in the presence or absence of U0126 (30 μ mol/l). MafA (A), PDX-1 (B), and C/EBP β (C) mRNA levels were measured by RT-PCR and normalized to β -actin mRNA. Results are expressed as fold increase of the ratio of gene/ β -actin mRNA over control value and are means \pm SE of three replicate experiments. ** P < 0.01, *** P < 0.001 vs. vehicle. Vehicle, \square ; U0126, \blacksquare .

pression enhanced PDX-1 expression at low glucose (Fig. 8D) as well as in the presence of palmitate (Fig. 8F). In contrast, overexpression of the kinase-dead hPASK mutant blocked the stimulation of PDX-1 expression by glucose (Fig. 8I).

DISCUSSION

This study aimed to identify the intracellular signaling pathways mediating palmitate inhibition of insulin gene expression in pancreatic β -cells. We found that palmitate enhances glucose-induced PKB and ERK1/2 phosphoryla-

tion and decreases glucose-induced PASK expression. Both ERK1/2 and PASK are implicated in the inhibitory effects of palmitate on insulin gene expression. Further, modulation of ERK1/2 phosphorylation seems to occur downstream of ceramide synthesis, whereas inhibition of PASK expression decreases PDX-1, but not MafA, expression.

Our observation that glucose enhances PKB phosphorylation is consistent with a number of previous studies in pancreatic β -cells (12,25–28). This is believed to represent a protective mechanism by which glucose exerts anti-apoptotic effects (29). In contrast, the observed effect of palmitate enhancing PKB phosphorylation in both MIN6 cells and rat islets is in contradiction with most (12,26,30), but not all (31) previous studies in pancreatic β -cells, C2C12 muscle cells (32), and 3T3-L1 adipocytes (33). However, most of these studies investigated the proapoptotic action of fatty acids (12,30,34–36), whereas in our experimental conditions we did not observe any detectable cell death. Therefore, it is possible that under non-proapoptotic conditions, fatty acids enhance PKB phosphorylation, whereas when either the concentration or the length of exposure is increased, fatty acid-induced cell death is associated with a secondary decrease in PKB phosphorylation. Martinez et al. (30) demonstrated in MIN6 cells an early enhancement of PKB phosphorylation by palmitate at 4 h, followed by a decrease at 24 h associated with marked cell death. Although the time-course of changes in PKB phosphorylation is clearly different in our study in islets than in the Martinez et al. (30) study in MIN6 cells, their results also suggest that the effects of palmitate on PKB phosphorylation might be biphasic.

The observed stimulation of ERK1/2 phosphorylation by glucose is consistent with several previous studies (8,9,37–40). As reported (38), PD98059 inhibited glucose-stimulation of insulin mRNA expression. Importantly, both palmitate and ceramide enhanced ERK1/2 phosphorylation in MIN6 cells and isolated islets, and blockade of ERK1/2 phosphorylation by U0126 or PD98059 increased insulin pre-mRNA expression in the presence of palmitate. This suggests that the ERK1/2 pathway is implicated in both the stimulation of insulin gene expression by glucose and its inhibition by palmitate, consistent with a dual role of ERK1/2 in the regulation of insulin gene expression by glucose as reported under glucotoxic conditions (9). Further, the inhibitory effect of ceramide on insulin gene expression was completely reversed upon inhibition of ERK1/2 in MIN6 cells. In view of our previous findings that blockade of de novo ceramide synthesis prevents palmitate inhibition of the insulin gene in islets (4), this observation suggests that ceramide generated de novo from palmitate inhibits the insulin gene via ERK1/2 activation. Because ERK1/2 inhibitors did not affect PDX-1 expression, we hypothesize that ERK1/2 induces posttranslational modifications of PDX-1 directly or indirectly, thereby regulating its nuclear translocation, which we have shown to be implicated in palmitate inhibition of insulin gene transcription (3,6).

PASK is an evolutionarily conserved serine/threonine protein kinase, containing a PAS domain sensitive to the intracellular environment that regulates the kinase domain to transduce the signal (41). In budding yeast, it coordinates sugar storage and protein synthesis with carbohydrate availability (42). In mammals, it has been demonstrated to be an important regulator of glycogen

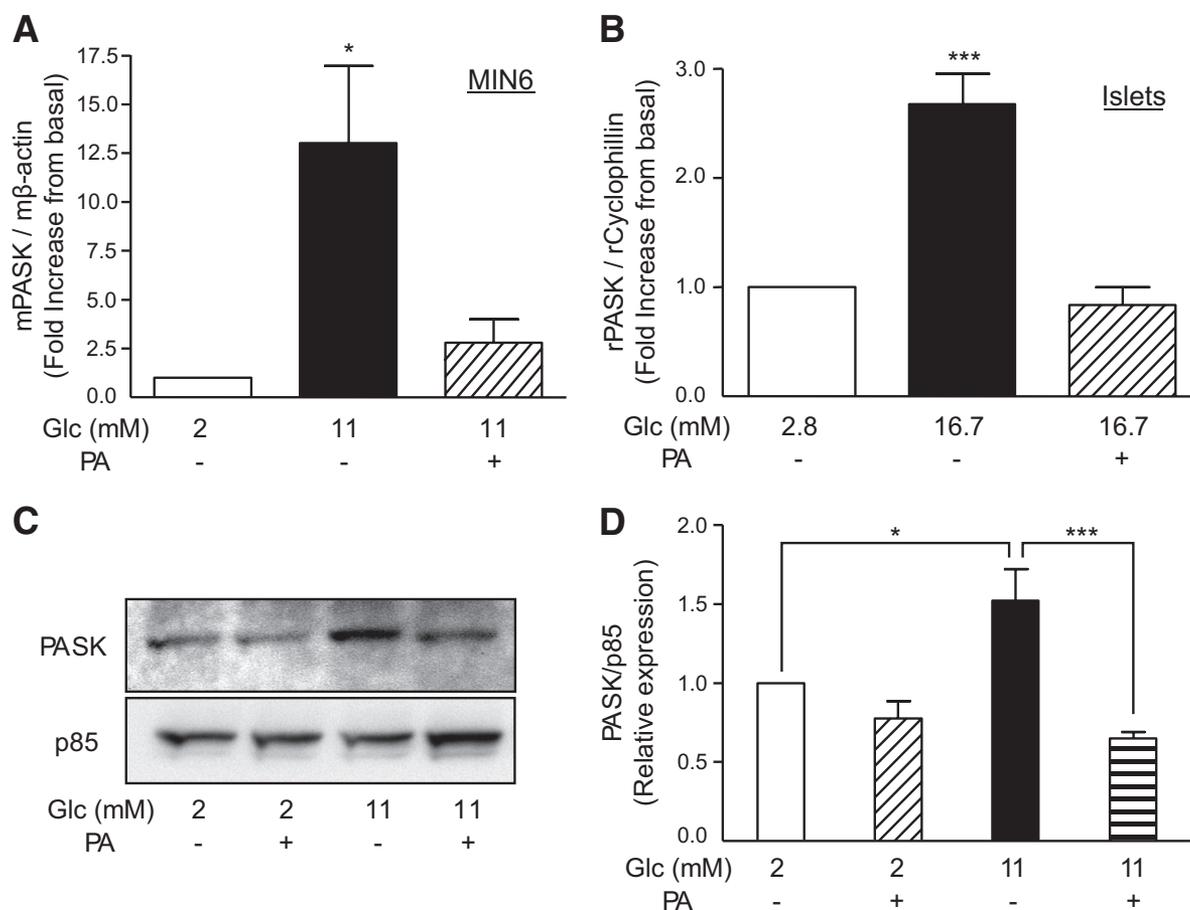


FIG. 6. Palmitate decreases glucose-induced PASK expression in MIN6 cells and rat islets. **A:** MIN6 cells were cultured for 6 h with 2 or 11 mmol/l glucose \pm 0.5 mmol/l palmitate. Results are expressed as fold increase of the ratio of mPASK/ β -actin mRNA over control value and are mean \pm SE of three replicate experiments. **B:** Rat islets were cultured for 6 h with 2.8 or 16.7 mmol/l \pm 0.5 mmol/l palmitate. Results are expressed as fold increase of the ratio of rPASK/cyclophilin mRNA over control value and are means \pm SE of three replicate experiments. * P < 0.05; *** P < 0.001. **C:** Representative immunoblots for PASK in MIN6 exposed to 2 or 11 mmol/l glucose for 24 h \pm 0.5 mmol/l palmitate. **D:** Quantification of PASK expression in MIN6 cells after a 24-h exposure to 2 or 11 mmol/l glucose \pm 0.5 mmol/l palmitate. Results are expressed as fold increase of the ratio of PASK/p85 protein over control value and are means \pm SE of three replicate experiments. * P < 0.05; *** P < 0.001.

synthase and cellular energy balance (43). The role of PASK in vivo remains unclear, because two different studies using PASK^{-/-} mice exhibited different phenotypes. Although the findings of Borter et al. (44) did not support a role for PASK in the regulation of insulin expression or secretion, Hao et al. (45) found that PASK^{-/-} mice had impaired glucose-induced insulin secretion and were protected from high-fat diet-induced obesity and insulin resistance. In pancreatic β -cells, PASK was shown to be required for glucose-induced insulin gene transcription (15). In the present study, we demonstrated that palmitate blocks glucose-induction of PASK expression, and that overexpression of hPASK prevents the inhibitory effect of palmitate on insulin mRNA and PDX-1 mRNA and protein expression in MIN6 cells. In addition, adenoviral-mediated overexpression of wild-type PASK increased, whereas a kinase-dead mutant of PASK decreased, insulin mRNA and PDX-1 protein expression in islets. The partial restoration of insulin pre-mRNA levels in islets exposed to glucose and palmitate is presumably because of a lower transfection efficiency than in MIN6 cells, in which restoration was complete.

To better understand the links between the two signaling pathways identified as implicated in palmitate inhibition of insulin gene expression (namely ERK1/2 and PASK) and the transcription factors MafA and PDX-1 that we

previously reported to be involved in this process (3,6), we measured the expression of these transcription factors upon ERK1/2 inhibition or PASK overexpression. In addition, we measured the expression of C/EBP β , which was recently shown to be increased in MIN6 cells exposed to palmitate (46) and is known to repress insulin gene transcription (9). First, we observed that expression of neither MafA nor PDX-1 was augmented in the presence of U0126 in cells exposed to glucose and palmitate, suggesting that the ERK1/2 pathway does not affect expression of these transcription factors. This, of course, does not rule out the possibility that ERK1/2 regulates the activity of these factors at the posttranslational level (9,47). Second, expression of PDX-1 was inhibited in islets overexpressing the kinase-dead hPASK mutant, suggesting that PASK regulates PDX-1 mRNA expression, as previously shown by da Silva Xavier et al. (15) under high-glucose conditions. Whether or not this is associated with changes in PDX-1 binding activity remains to be examined. Contrary to a recent observation by Plaisance et al. (46), we did not observe an increase in C/EBP β expression in MIN6 cells exposed to high glucose and palmitate. This difference might be because of a lower concentration of palmitate and shorter exposure in our study. Interestingly, overexpression of the kinase-dead hPASK mutant resulted in a marked increase in C/EBP β expression, which could con-

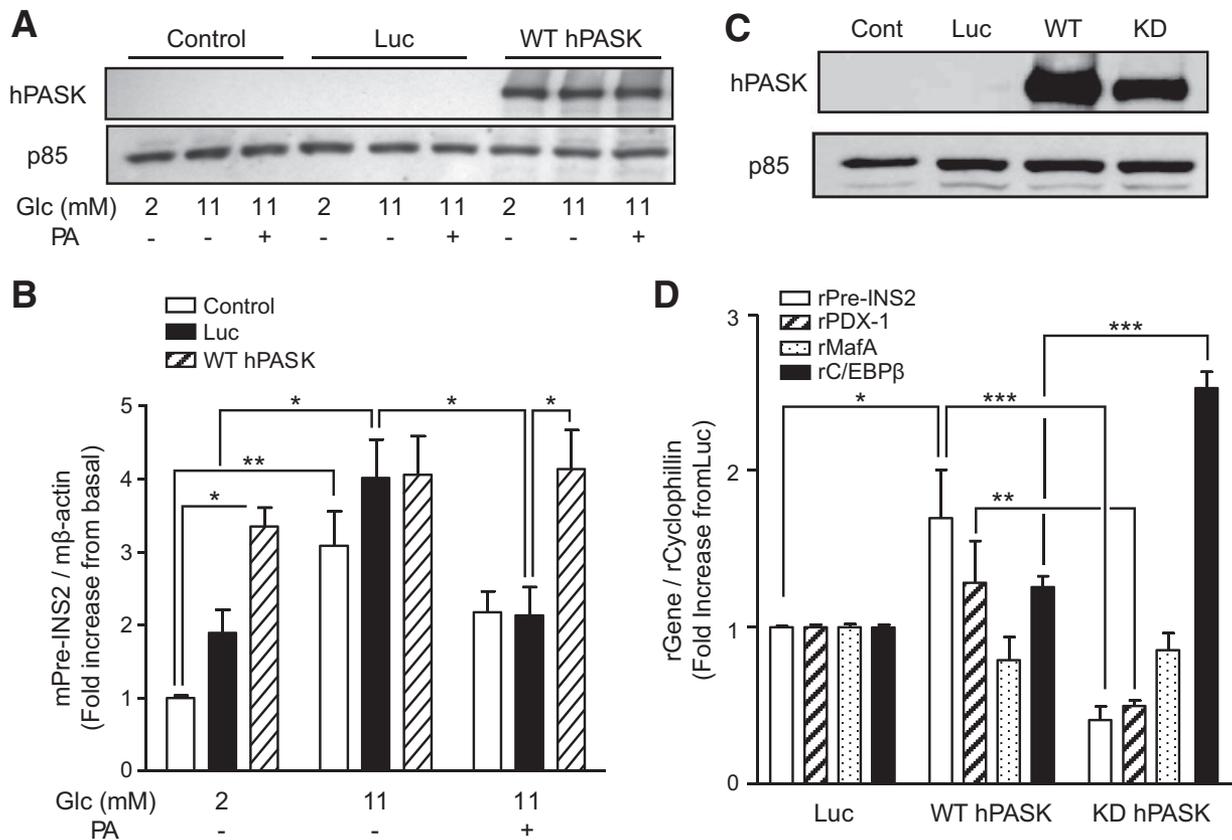


FIG. 7. Overexpression of PAS kinase prevents the inhibitory effects of palmitate on insulin gene expression. **A:** Representative hPASK and p85 immunoblots of control, Adv-Luc-infected, or Adv-hPASK wild-type infected MIN6 cells cultured for 24 h in 2 or 11 mmol/l glucose \pm 0.5 mmol/l palmitate. **B:** Insulin pre-mRNA levels. Results are expressed as fold increase of the ratio of mPre-INS2/ β -actin mRNA over control value and are mean \pm SE of seven replicate experiments. * P < 0.05, ** P < 0.01. **C:** Representative hPASK and p85 immunoblots of control, Adv-Luc-infected, Adv-hPASK wild-type or Adv-hPASK kinase-dead infected rat islets cultured for 24 h in the presence of 16.7 mmol/l glucose + 0.5 mmol/l palmitate. **D:** Pre-INS2, MafA, PDX-1, and C/EBP β mRNA levels were measured by RT-PCR and normalized to cyclophilin mRNA. Results are expressed as fold increase of the ratio of rGene/rCyclophilin mRNA over control value (Adv-Luc) and are means \pm SE of three replicate experiments. * P < 0.05; ** P < 0.01; *** P < 0.001. Cont, control; KD, kinase dead; Luc, luciferase; WT, wild type.

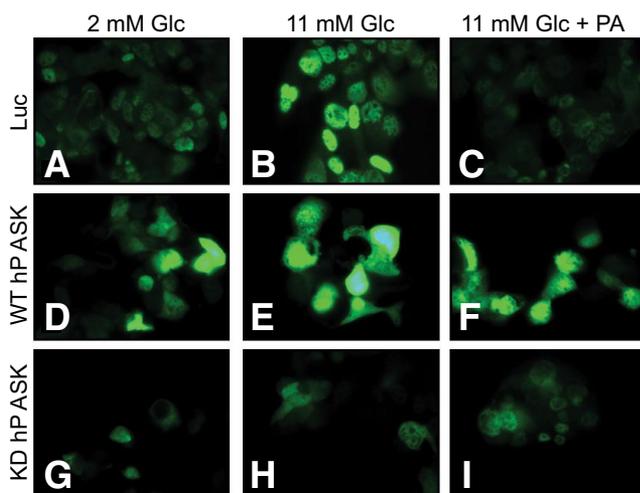


FIG. 8. PDX-1 protein expression in MIN6 cells overexpressing hPASK wild-type or kinase-dead. MIN6 cells were infected with adenoviruses expressing Luc (**A-C**), wild-type hPASK (**D-F**), or kinase-dead hPASK (**G-I**), and cultured for 24 h with 2 mmol/l glucose (**A**, **D**, and **G**), 11 mmol/l glucose (**B**, **E**, and **H**), or 11 mmol/l glucose + palmitate (**C**, **F**, and **I**). Cells were immunostained for PDX-1 and examined under a fluorescence microscope. Images are representative of three replicate experiments. KD, kinase dead; Luc, luciferase; WT, wild type. (A high-quality digital representation of this figure is available in the online issue.)

tribute to the decrease in insulin gene expression under these conditions. We hypothesize that under our relatively mild conditions of exposure to glucose and palmitate, which are not associated with cell death, C/EBP β expression is not yet increased, whereas under more drastic conditions, such as kinase-dead hPASK overexpression or longer times in culture with palmitate (46) or glucose (9,48), C/EBP β expression is enhanced and contributes to the decrease in insulin gene expression and eventual cytotoxicity (46). A similar opposite pattern of expression for PDX-1 and C/EBP β was also found in islets from animal models of type 2 diabetes (48), suggesting that perhaps PASK regulates these two transcription factors and, thereby, insulin gene expression under conditions of fuel surfeit. As we previously observed (6), the concomitant presence of palmitate in culture blocked glucose-induced of MafA mRNA expression. However, this effect was prevented neither by ERK1/2 inhibition nor by hPASK overexpression, suggesting that it is mediated by another, yet to be identified, signaling pathway. We acknowledge that the inherent caveats of pharmacological tools and the fact that we have not examined transcription factor binding activities limit the interpretation of our findings. However, our results suggest that palmitate inhibits insulin gene expression by three distinct signaling arms. First, de novo ceramide synthesis from palmitate activates ERK1/2, which in turn might affect, directly or indirectly, transcrip-

tion factor binding activity. Second, palmitate inhibits glucose-induced PASK expression, which leads to decreased PDX-1 expression and, presumably, binding activity. Third, palmitate inhibits glucose-induced MafA expression, via mechanisms that do not appear to involve ERK1/2 or PASK.

In conclusion, we have demonstrated a role for both the ERK1/2 and PASK pathways in mediating fatty acid inhibition of glucose-induced insulin gene expression in rodent β -cells. These findings uniquely identify PASK as a novel mediator of glucolipototoxicity on the insulin gene. Whether or not similar mechanisms are operative in human islets remains to be examined.

ACKNOWLEDGMENTS

This work was supported by the National Institutes of Health (R01 DK-58096 to V.P. and R01 DK-50610 and DK-55267 to C.J.R.). G.F. is supported by a Post-Doctoral Fellowship from the Canadian Diabetes Association. V.P. holds the Canada Research Chair in Diabetes and Pancreatic β -Cell Function.

No potential conflicts of interest relevant to this article have been reported.

We thank Drs. Thierry Alquier, Marc Prentki, and Erik Joly for critical input, as well as Grace Ferguson, Mélanie Ethier, Annie Tardif, and Mélanie Guévremont for valuable technical help.

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