



Jayalakshmi Lakshmipathi,¹ Juan Carlos Alvarez-Perez,¹ Carolina Rosselot,¹ Gabriella P. Casinelli,² Rachel E. Stamateris,³ Francisco Rausell-Palamos,¹ Christopher P. O'Donnell,⁴ Rupangi C. Vasavada,¹ Donald K. Scott,¹ Laura C. Alonso,³ and Adolfo Garcia-Ocaña¹



PKC ζ Is Essential for Pancreatic β -Cell Replication During Insulin Resistance by Regulating mTOR and Cyclin-D2

Diabetes 2016;65:1283–1296 | DOI: 10.2337/db15-1398

Adaptive β -cell replication occurs in response to increased metabolic demand during insulin resistance. The intracellular mediators of this compensatory response are poorly defined and their identification could provide significant targets for β -cell regeneration therapies. Here we show that glucose and insulin in vitro and insulin resistance in vivo activate protein kinase C ζ (PKC ζ) in pancreatic islets and β -cells. PKC ζ is required for glucose- and glucokinase activator-induced proliferation of rodent and human β -cells in vitro. Furthermore, either kinase-dead PKC ζ expression (KD-PKC ζ) or disruption of PKC ζ in mouse β -cells blocks compensatory β -cell replication when acute hyperglycemia/hyperinsulinemia is induced. Importantly, KD-PKC ζ inhibits insulin resistance-mediated mammalian target of rapamycin (mTOR) activation and cyclin-D2 upregulation independent of Akt activation. In summary, PKC ζ activation is key for early compensatory β -cell replication in insulin resistance by regulating the downstream signals mTOR and cyclin-D2. This suggests that alterations in PKC ζ expression or activity might contribute to inadequate β -cell mass expansion and β -cell failure leading to type 2 diabetes.

Type 2 diabetes (T2D) results from insufficient functional β -cell mass to counteract the increase in insulin demand in the body (1,2). Before this failure occurs, the body

responds to an early increase in nutrient oversupply by enhancing compensatory β -cell proliferation and consequent β -cell expansion (3–6). Interest has been growing recently in identifying factors and signaling pathways that regulate β -cell expansion in acute nutrient oversupply and insulin resistance to leverage this knowledge into future therapies for β -cell regeneration in diabetes (6–10).

Genome-wide association studies have identified a number of gene sequence variants associated with T2D (11,12). Among them, several single nucleotide polymorphisms in the *PRKCZ* gene have been associated with increased risk of T2D development (13,14). Whether these variants are positively or negatively associated with the activity or expression of the encoded protein is still unknown. The *PRKCZ* gene encodes the atypical protein kinase C (PKC) ζ , a serine/threonine kinase activated by PI3K/PDK1 that is involved in cell replication, function, motility, and survival (15). Transfer of a constitutive active form of PKC ζ (CA-PKC ζ) to β -cells enhances their proliferation (16–18). However, the role of PKC ζ in β -cell homeostasis in physiological and pathological situations has not yet been deciphered.

Glucose is a well-known β -cell mitogen that regulates the induction of multiple signaling events (3,6,19). Among them, glucose induces the activation of the mammalian target of rapamycin (mTOR) and the upregulation of cyclin-D2

¹Diabetes, Obesity and Metabolism Institute, Division of Endocrinology, Diabetes and Bone Diseases, The Mindich Child Health and Development Institute, Icahn School of Medicine at Mount Sinai, New York, NY

²Division of Pediatric Hematology/Oncology and Blood and Marrow Transplantation, Department of Pediatrics, Children's Hospital of Pittsburgh, Pittsburgh, PA

³Department of Medicine, University of Massachusetts Medical School, Worcester, MA

⁴Division of Pulmonary, Allergy, and Critical Care Medicine, Department of Medicine, University of Pittsburgh, Pittsburgh, PA

Corresponding author: Adolfo Garcia-Ocaña, adolfo.g.ocana@mssm.edu.

Received 8 October 2015 and accepted 6 February 2016.

This article contains Supplementary Data online at <http://diabetes.diabetesjournals.org/lookup/suppl/doi:10.2337/db15-1398/-/DC1>.

J.L. and J.C.A.-P. contributed equally to this study.

© 2016 by the American Diabetes Association. Readers may use this article as long as the work is properly cited, the use is educational and not for profit, and the work is not altered.

in β -cells (3,6,20). Cyclin-D2 is essential for postnatal β -cell growth and the compensatory β -cell hyperplastic response to insulin resistance in rodents (21,22). mTORC1 activation regulates β -cell proliferation by increasing the expression of cyclin-D2 (6,20). Collectively, these studies suggest that the pathway mTOR–cyclin-D2 might be essential for compensatory β -cell growth. However, the upstream master regulator of the glucose-induced mTOR–cyclin-D2 signaling pathway in β -cells in the insulin resistance context is unknown. Here we report that blocking PKC ζ activity or expression impairs hyperglycemia/hyperinsulinemia/insulin resistance–induced β -cell proliferation. Furthermore, PKC ζ activity is required for the induction of the mTOR–cyclin-D2 pathway in this setting. To our surprise, the decrease in mTOR activity by kinase-dead PKC ζ (KD-PKC ζ) is independent of Akt activation. Glucose-induced human β -cell proliferation is also impaired by KD-PKC ζ , indicating the potential critical importance of this kinase in the early β -cell adaptive response to insulin resistance in humans. Taken together, these results highlight PKC ζ as key regulator of adaptive compensatory β -cell replication.

RESEARCH DESIGN AND METHODS

Genetically Modified Mice

Transgenic (TG) mice with KD-PKC ζ expression in β -cells (RIP-KD-PKC ζ TG mice) were generated and identified as described previously (23). The rat KD-PKC ζ (K281W) cDNA (1.8 kb) containing a hemagglutinin (HA) tag at the NH₂-terminal end for monitoring expression and a mutation in Lys-281 essential for kinase activity (24,25) was provided by Dr. Alex Toker (Harvard Medical School, Boston, MA). TG mice were generated and propagated in a C57Bl6 mouse background. β -Cell–specific inducible knockout mice of PKC ζ (β PKC ζ -KO mice) were generated by combining MIP-Cre-ERT mice provided by Dr. Louis Philipson (University of Chicago, Chicago, IL) (26) with PKC $\zeta^{\text{lox/lox}}$ mice (EUCOMM, Wellcome Trust Sanger Institute, Hinxton, U.K.), which have exon 5 flanked by loxP sites. Both mice were in a C57Bl6 mouse background. Induction of Cre-mediated recombination and disruption of PKC ζ expression was achieved by intraperitoneal injection for five consecutive days of 50 μ g/g body weight of tamoxifen (TM) (Sigma-Aldrich) dissolved in corn oil (27). All studies were performed with the approval of and in accordance with guidelines established by both the University of Pittsburgh and the Icahn School of Medicine at Mount Sinai Institutional Animal Care and Use Committees.

Glucose Infusion

Detailed protocols regarding mouse catheterization, tether system, housing, catheter maintenance, arterial blood sampling, and infusions were previously published (3,4). In brief, 8–10-week-old wild-type (WT) and RIP-KD-PKC ζ TG male mice were fed ad libitum, catheters were inserted in the left femoral artery and vein, and 0.9% sodium chloride or 50% dextrose was infused at a constant rate of 100 μ L/h for 4 days. Arterial blood glucose was measured

daily and plasma stored at -80°C for insulin measurement by radioimmunoassay (Millipore). After infusion, the pancreas was removed and processed for histological studies or islet isolation. Islets were isolated after collagenase P injection through the pancreatic duct, as previously reported (23).

Acute High-Fat Diet Feeding

β PKC ζ -KO and control mice were treated with TM for 5 days (see above), and 5 days later the high-fat diet (HFD) feeding experiment was started. Eight-week-old male RIP-KD-PKC ζ TG, β PKC ζ -KO, and their corresponding control littermate mice were randomly assigned to either a standard diet (SD) (13.1% kcal from fat; Purina PicoLab Rodent Diet no. 5053; LabDiet) or lard-based HFD (40.9% kcal from fat; TD 96001; Harlan Teklad). After 7 days, body weights were measured, nonfasting blood samples were collected for blood glucose and insulin, and then pancreata were harvested and processed for histological studies or islet isolation.

Immunohistochemistry and Analysis of β -Cell Proliferation in Pancreas

Paraffin-embedded pancreatic sections were immunostained with DAPI and antibodies for insulin (Dako), PKC ζ (Santa Cruz Biotechnology), or HA and phosphoS6 (Cell Signaling) as previously described (18). β -Cell proliferation was assessed by insulin and Ki67 (Thermo Fisher Scientific) staining, and at least 2,000 β -cells were blindly counted per mouse (28). β -Cell mass was measured in three insulin-stained pancreas sections per mouse using ImageJ (National Institutes of Health) as previously described (18,28).

Glucose Homeostasis

Blood glucose was determined by glucometer and plasma insulin by radioimmunoassay (18). Intraperitoneal glucose tolerance test was performed in 16–18 h–fasted mice injected intraperitoneally with 2 g D-glucose/kg, and insulin sensitivity test was performed in random-fed mice injected intraperitoneally with human insulin (1.5 units/kg), as previously reported (18).

Generation of Adenoviruses

Adv.KD-PKC ζ , Adv.LacZ, Adv.Cyclin-D2, Adv.Cre, and Adv.GFP were prepared as previously described (17). Multiplicity of infection (MOI) was determined by optical density at 260 nm and by plaque assay.

INS-1 Cells, Adenoviral Transduction, Small Interfering RNA, and [³H]thymidine Incorporation

INS-1 cells were transduced for 1 h in RPMI containing 11 mmol/L glucose, 100 units/mL penicillin, 100 μ g/mL streptomycin, 10 mmol/L HEPES, 2 mmol/L L-glutamine, 1 mmol/L sodium pyruvate, and 50 μ mol/L β -mercaptoethanol with 100 MOI of Adv.KD-PKC ζ , or Adv.LacZ and Adv.GFP as controls (18). Cells were then incubated for 24 h in this medium containing 10% FBS before they were refed with fresh medium with 2% FBS and containing 2 or 20 mmol/L glucose. In a set of experiments, [³H]thymidine (0.5 μ Ci/well; Amersham Pharmacia Biotech) or rapamycin and vehicle were also added. [³H]thymidine incorporation

was stopped 18–20 h later; radioactivity was corrected for protein levels measured by the Bradford method and the results were expressed as percentage of counts per minute per microgram protein in Adv-LacZ-transduced vehicle-treated cells (17). In some experiments, 100 nmol/L wortmannin was added 30 min prior to glucose addition. PKC ζ activation by insulin was tested in serum-depleted cells for 6 h in the presence of 2 mmol/L glucose.

Small interfering RNA (siRNA)-mediated decrease in PKC ζ and PKC λ expression was performed in INS-1 cells as previously performed (17). Twenty-four hours after transfection, cells were replated in 24-well plates and then serum depleted for [³H]thymidine incorporation studies or washed with ice-cold PBS containing 100 μ mol/L Na₃VO₄ for Western blot (17).

Western Blots

Islet or INS-1 cell extracts were separated on 7.5–12% SDS-PAGE, transferred to Immobilon-P membranes (Millipore), blocked in 5% nonfat dry milk, and then incubated with primary antibodies against p85 α -PI3K (B-9), PKC λ/ι (H-76), PKC ζ (C-20), and p-PKC ζ (T410) (SC-12894-R) from Santa Cruz Biotechnology; against p-PKC ζ (T560) (ab62372) and CDK4 (ab3112) from Abcam; against actin (A2066) and GAPDH (G9545) from Sigma-Aldrich; against α -tubulin (CPO6) from Calbiochem; against p-mTOR-S2448 (2971), p-p70S6K-T387 (9205), p-AKT-S473 (D9E), p-AKT-T308 (D25E6), p-4E-BP1-S65 (9451), and p-GSK3 α/β -S21/9 (9331) from Cell Signaling; and against cyclin-D1 (210-P1) and cyclin-D2 (221-P1) from Neomarkers. After several washes, blots were incubated with peroxidase-conjugated secondary antibodies followed by chemiluminescence detection (18).

β -Cell Proliferation in Human and Mouse Primary Islet Cell Cultures

Islet cell cultures were prepared as previously described (17). In brief, 400 adult human (Integrated Islet Distribution Program) or mouse islets were trypsinized and cells were plated on 12-mm glass coverslips placed in 24-well plates. Islet cells were either uninfected or transduced with 100 MOI of Adv.KD-PKC ζ , Adv.Cyclin-D2, Adv.Cre, or Adv.LacZ as control (17). Subsequently, 1 mL of RPMI medium containing 5 mmol/L D-glucose supplemented with 10% FBS, 100 units/mL penicillin, and 100 μ g/mL streptomycin was added, and cells were incubated for 24 h. Thereafter, cells were incubated overnight in fresh medium with 5% FBS containing 2, 7, or 20 mmol/L glucose. In some experiments, 100 nmol/L wortmannin was added 30 min prior to glucose addition, and in another set of experiments, the glucokinase activator (GKA) 3-[(1S)-2-hydroxy-1-methylethoxy]-5-[4-(methylsulphonyl)phenoxy]-N-1,3-thiazol-2-yl benzamide (Sigma-Aldrich) was added overnight in the presence of 7 mmol/L glucose. Then cells were rinsed with PBS and fixed in freshly prepared 4% paraformaldehyde for 30 min at room temperature. To analyze β -cell proliferation, Ki67 and insulin staining was performed as previously reported (29).

Statistical Analysis

The data are presented as means \pm SE. Statistical analysis was performed using unpaired two-tailed Student *t* test. *P* < 0.05 was considered statistically significant.

RESULTS

Glucose Increases PKC ζ Activity In Vitro and In Vivo

CA-PKC ζ increases β -cell replication (16–18). We previously showed that mild hyperglycemia and hyperinsulinemia in 4-day glucose-infused mice markedly increase β -cell replication (3,4). Based on this, we wondered whether glucose activates PKC ζ in mouse islets in vivo and β -cells in vitro. Islets from 4-day glucose-infused mice displayed increased PKC ζ phosphorylation (activity) at Thr410 and at the autophosphorylation site Thr560. This correlated with increased phosphorylation of mTOR and its downstream target p70S6K (Fig. 1A and Supplementary Fig. 1). No changes in p85-PI3K levels were observed (Fig. 1A and Supplementary Fig. 1). To address whether increased concentrations of glucose enhance PKC ζ phosphorylation specifically in β -cells, we performed dose-dependent and time-dependent studies in INS-1 cells. Glucose increased PKC ζ phosphorylation in a dose- and time-dependent manner (Fig. 1B and C). Glucose-mediated activation was dependent on PI3K activity since wortmannin completely eliminated the increase in PKC ζ phosphorylation (Fig. 1D). Since glucose increases insulin secretion from β -cells, we next determined whether insulin activates PKC ζ at 2 mmol/L glucose. As shown in Fig. 1E, insulin increased PKC ζ phosphorylation.

PKC ζ Is Required for Glucose- and GKA-Mediated β -Cell Proliferation In Vitro

To test whether PKC ζ activity is required for glucose-induced β -cell replication, we transduced INS-1 cells with Ad.KD-PKC ζ . INS-1 cell proliferation increased at 20 mmol/L glucose, but this effect was blocked in cells transduced with Ad.KD-PKC ζ (Fig. 1F). The effect of 20 mmol/L glucose was not observed with 20 mmol/L mannitol, suggesting that glucose-induced proliferation is not due to potential hyperosmotic effects (not shown). To determine the specificity of this atypical PKC in regulating glucose-induced INS-1 cell proliferation, we decreased PKC ζ and PKC λ expression using siRNA as previously reported (17) (Fig. 1G). PKC ζ downregulation, but not PKC λ , completely blunted glucose-induced proliferation (Fig. 1H). Taken together, these results indicate that glucose activates PKC ζ and this activation is required for glucose-induced INS-1 cell proliferation. However, these results do not address whether this is the case in primary β -cells as well.

To address this point, we first analyzed whether glucose increases PKC ζ phosphorylation in mouse islets. As shown in Fig. 2A, 20 mmol/L glucose significantly increased PKC ζ phosphorylation. We next measured β -cell proliferation by Ki67 and insulin costaining in primary cultures of mouse islet cells transduced with Ad.KD-PKC ζ and incubated with 2, 7, or 20 mmol/L glucose. Although there was a trend to

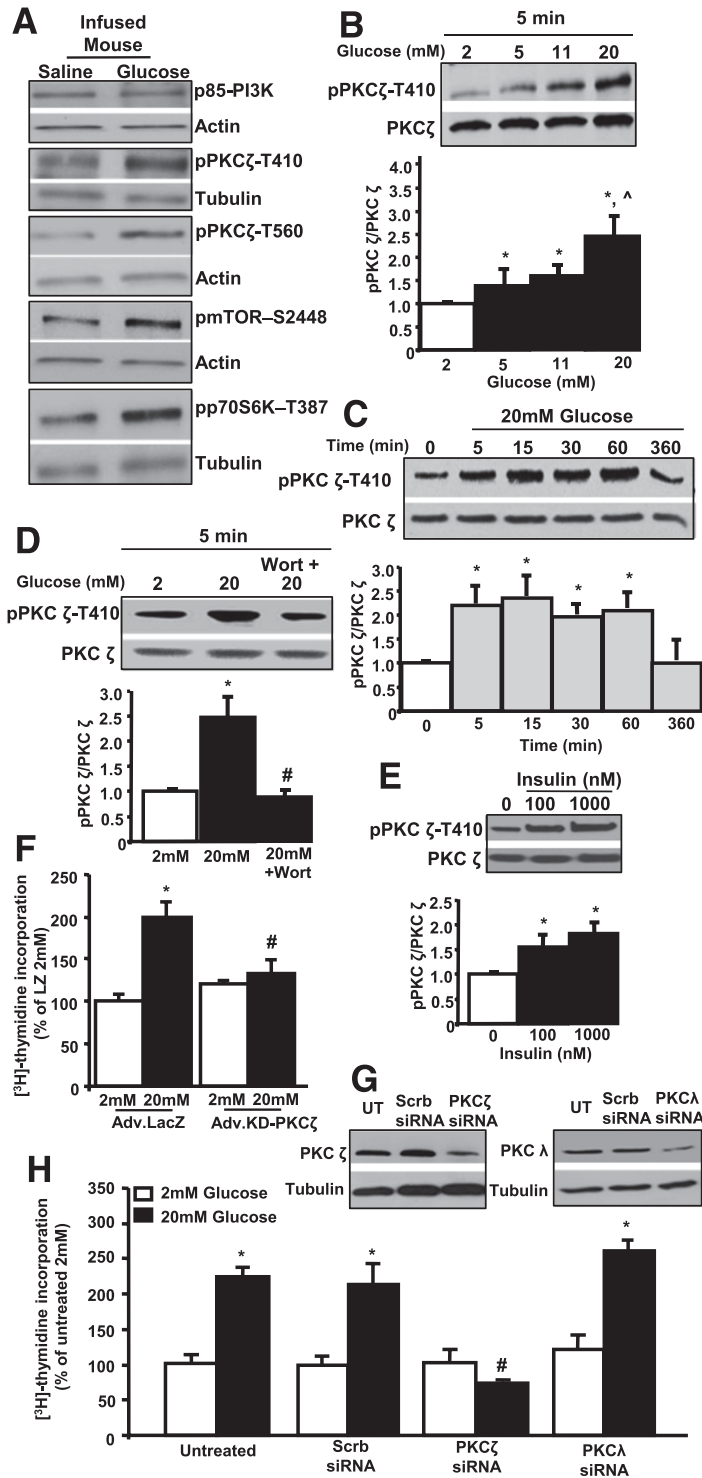


Figure 1—PKC ζ is activated by glucose and is required for glucose-mediated INS-1 cell replication. Representative images of Western blots showing levels and phosphorylation of different components of the PI3K/PKC ζ /mTOR signaling pathway in islets isolated from 4-day glucose- and saline-infused mice ($n = 4$ mice per condition) (densitometric quantitation shown in Supplementary Fig. 1) (A), dose-dependent (B) and time-dependent (C) effect of glucose on PKC ζ phosphorylation in INS-1 cells and the quantitation of $n = 4$ experiments, and effect of 100 nmol/L of the PI3K inhibitor wortmannin (Wort) on PKC ζ phosphorylation induced by 20 mmol/L glucose in INS-1 cells and the quantitation of $n = 4$ experiments (D). E: Effect of insulin (5 min) on PKC ζ phosphorylation in INS-1 cells incubated at 2 mmol/L glucose and the quantitation of $n = 4$ experiments. F: Effect of adenovirus KD-PKC ζ (Adv.KD-PKC ζ) compared with Adv.LacZ in glucose-induced proliferation in INS-1 cells measured by [3 H]thymidine incorporation. Representative images of Western blots showing downregulation of PKC ζ or PKC λ with siRNAs compared with untreated (UT) or scrambled siRNA (Scrb siRNA)-treated INS-1 cells ($n = 3$) (G), and its effect on glucose-induced INS-1 cell proliferation as in E (H). Results are means \pm SEM. * $P < 0.05$ vs. 2 mmol/L glucose, time 0 min or no insulin added; $^{\wedge}P < 0.05$ vs. 11 mmol/L glucose; # $P < 0.05$ vs. 20 mmol/L glucose control.

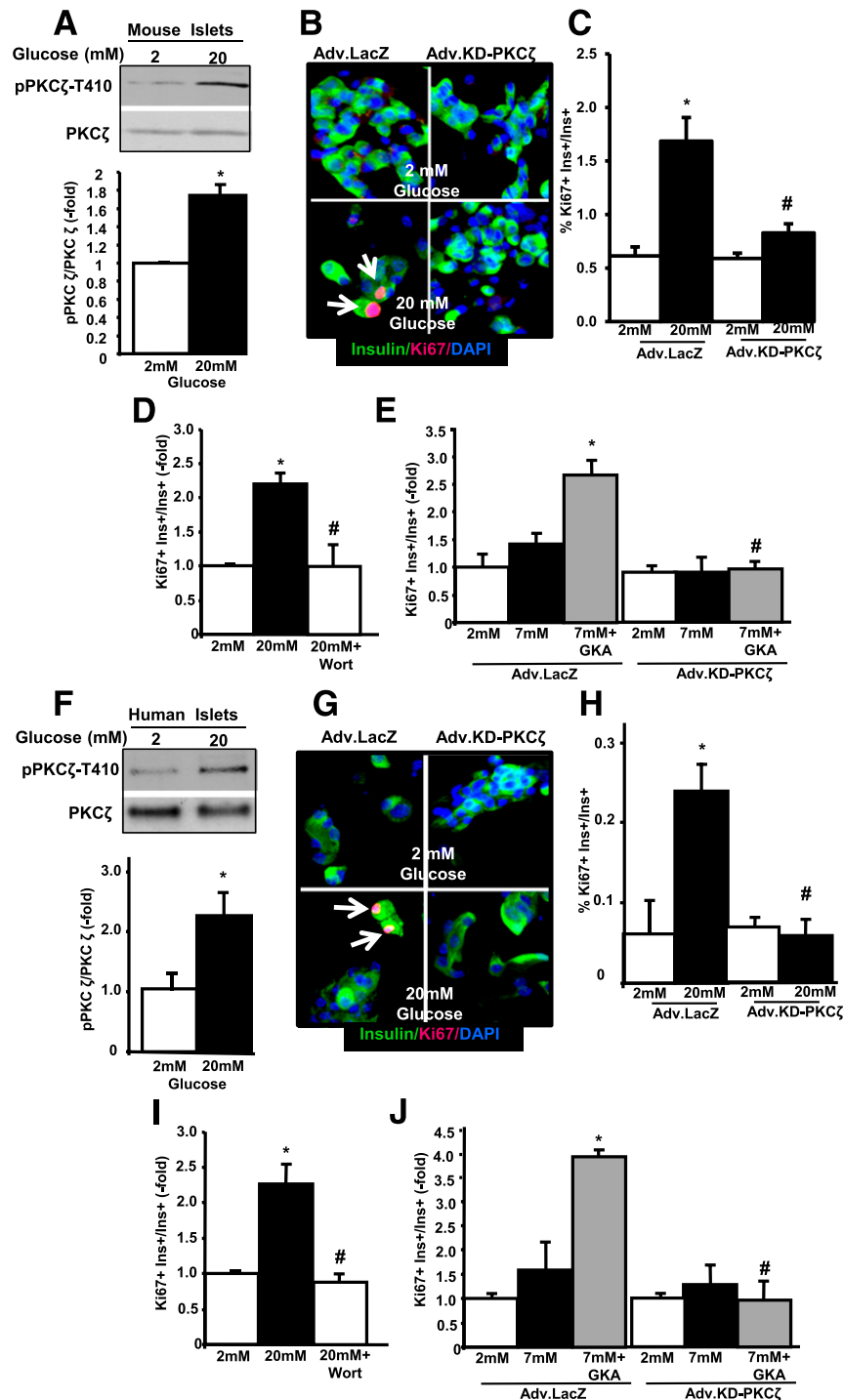


Figure 2—Glucose activates PKC ζ in mouse and human islets, and transfer of KD-PKC ζ completely blocks glucose-induced β -cell proliferation. **A**: Representative image of a Western blot showing the level and phosphorylation of PKC ζ in mouse islets treated with 2 or 20 mmol/L glucose for 5 min and the densitometric quantitation of $n = 4$ experiments. **B**: Representative photomicrographs of mouse islet cell cultures transduced with Adv.LacZ or Adv.KD-PKC ζ , treated with 2 or 20 mmol/L glucose for 24 h and stained for insulin (green), Ki67 (red), and DAPI (blue). Arrows indicate Ki67 $^{+}$ insulin $^{+}$ cells. **C**: Quantitation of $n = 5$ experiments in duplicate as in **B**. **D**: Effect of 100 nmol/L wortmannin (Wort) on mouse β -cell proliferation induced by glucose (24 h) in $n = 4$ experiments in duplicate. **E**: Effect of 7 mmol/L glucose with or without GKA on β -cell proliferation in mouse islet cell cultures transduced with Adv.LacZ or Adv.KD-PKC ζ in $n = 3$ experiments in duplicate. **F**: Representative image of a Western blot showing the level and phosphorylation of PKC ζ in human islets as in **A**, and the densitometric quantitation of $n = 5$ adult human islet preparations. **G**: Representative photomicrographs of adult human islet cell cultures as in **B**. Arrows indicate Ki67 $^{+}$ insulin $^{+}$ cells. **H**: Quantitation of $n = 5$ adult human islet preparations in duplicate. **I**: Effect of 100 nmol/L wortmannin on human β -cell proliferation induced by glucose (24 h) in $n = 5$ human islet preparations in duplicate. **J**: Effect of 7 mmol/L glucose with or without GKA on β -cell proliferation in human islet cell cultures transduced with Adv.LacZ or Adv.KD-PKC ζ in $n = 3$ adult human islet preparations in duplicate. Results are means \pm SEM. * $P < 0.05$ vs. 2 or 7 mmol/L glucose; # $P < 0.05$ vs. 20 mmol/L glucose or 7 mmol/L + GKA in cells transduced with Adv.LacZ.

enhanced β -cell proliferation with 7 mmol/L glucose (a postprandial glucose level), only 20 mmol/L glucose significantly increased mouse β -cell replication; however, this effect was not present in primary islet cells transduced with Ad.KD-PKC ζ (Fig. 2B–E). PI3K inhibition with wortmannin also eliminated glucose-mediated mitogenic effect (Fig. 2D). More importantly, glucose increased PKC ζ phosphorylation in human islets, and Ad.KD-PKC ζ completely blocked glucose-induced human β -cell replication (Fig. 2F–J). Equally, wortmannin inhibited glucose-induced human β -cell proliferation (Fig. 2I). GKA also increased mouse and human β -cell proliferation, and this effect was decreased in islet cells transduced with Ad.KD-PKC ζ , indicating that PKC ζ regulates GKA-induced β -cell replication (Fig. 2E and J).

PKC ζ Activity Is Required for Compensatory β -Cell Proliferation Induced by Hyperglycemia/Hyperinsulinemia in Glucose-Infused Mice

To determine whether PKC ζ is also required for glucose-mediated β -cell proliferation *in vivo*, we generated two lines of TG mice expressing KD-PKC ζ in β -cells. Islets from these TG mice display a similar (fourfold) increase in PKC ζ expression (endogenous + transgene) (Supplementary Fig. 2A). To assess the specificity of transgene expression in β -cells, we took advantage of the presence of the HA tag in the transgene and performed insulin and HA staining of pancreatic sections. Insulin-stained cells were also positively stained for HA, indicating transgene expression in β -cells (Supplementary Fig. 2B). No obvious increase in PKC ζ expression was observed in several organs of TG mice such as liver, fat, kidney, and hypothalamus (Supplementary Fig. 2C). Blood glucose, plasma insulin, glucose tolerance, insulin sensitivity, pancreatic weight, and β -cell mass were not different between WT and TG mice at 8 weeks of age (Supplementary Fig. 3), suggesting that PKC ζ activity is not required for glucose and β -cell homeostasis in basal conditions.

Next, we infused glucose or saline for 4 days in WT and TG male mice (Fig. 3A) (3,4). Similar mild hyperglycemia and hyperinsulinemia were achieved in both types of mice when infused with glucose (Fig. 3B–E). Although glucose-infused TG mice displayed slightly lower insulin levels, these were not significantly different compared with the values in WT mice (Fig. 3D and E). β -Cell proliferation assessed by Ki67 and insulin staining was increased in WT mice infused with glucose (Fig. 3F and G). In contrast, β -cell proliferation in TG mice infused with glucose was similar to saline-infused WT and TG mice (Fig. 3F and G). No changes in β -cell size were observed (data not shown). This indicates that compensatory β -cell replication induced by mild hyperglycemia and hyperinsulinemia requires PKC ζ activity.

PKC ζ Is Required for Compensatory β -Cell Replication Induced by HFD Feeding in Mice

To confirm the previous observations in a more pathophysiological setting with potential clinical implications, we used a recently reported mouse model of acute 7-day HFD feeding that leads to enhanced body weight, hyperglycemia,

hyperinsulinemia, and increased β -cell replication (5). PKC ζ phosphorylation was increased in islets from normal mice fed HFD compared with mice fed SD (Fig. 4A). Based on this, we next fed WT and TG mice with HFD. Body weight, blood glucose, and plasma insulin were similarly increased in both types of mice fed with HFD compared with SD-fed littermates (Fig. 4B–D). β -Cell proliferation was also enhanced in WT mice fed HFD compared with SD-fed mice; however, HFD feeding did not increase β -cell replication in TG mouse littermates (Fig. 4E and F).

RIP-KD-PKC ζ TG mice chronically express KD-PKC ζ in β -cells. To confirm the importance of PKC ζ in controlling acute β -cell proliferation in insulin resistance, we generated inducible β PKC ζ -KO mice in which we could acutely eliminate PKC ζ from β -cells. First, islets from PKC $\zeta^{\text{loxP/loxP}}$ mice transduced with Adv.Cre display significantly decreased levels of PKC ζ and are unable to increase β -cell replication when incubated with 20 mmol/L glucose *in vitro* (Fig. 5A–C). More importantly, mice with decreased expression of PKC ζ in islets and β -cells after TM injection (Fig. 5D and E) displayed diminished β -cell proliferation when fed HFD compared with WT mice ($\text{Cre}^-/\text{PKC}\zeta^{\text{loxP/loxP}}$ and $\text{Cre}^+/\text{PKC}\zeta^{+/+}$ mice) (Fig. 5F–I). Body weight, blood glucose, and plasma insulin levels were similar between these types of mice (Fig. 5F–H). No significant differences in these parameters were observed between $\text{Cre}^+/\text{PKC}\zeta^{+/+}$ and $\text{Cre}^-/\text{PKC}\zeta^{\text{loxP/loxP}}$ mice (not shown) and therefore were combined in the control group. These results confirm that adaptive β -cell proliferation in acute obesity, hyperglycemia, hyperinsulinemia, and insulin resistance requires PKC ζ .

Cyclin-D2 Upregulation by Glucose and HFD in β -Cells Requires PKC ζ Activation

The results reported thus far indicate that PKC ζ is required in insulin resistance-induced compensatory β -cell replication. However, they do not provide any insight into how PKC ζ might regulate this increased proliferation. To address this point, INS-1 cells transduced with Ad.KD-PKC ζ were treated with low and high glucose, and cell signaling molecules known to be activated in β -cells in this context were analyzed. Cyclin-D2, but not cyclin-D1 or cdk4, was upregulated by 20 mmol/L glucose, and this increase was not present in Ad.KD-PKC ζ -transduced cells (Fig. 6A). Equally, islets from HFD-fed WT mice displayed increased expression of cyclin-D2, but this increase was not present in TG mouse littermates (Fig. 6B). This indicates that PKC ζ regulates cyclin-D2 expression in the context of excessive nutrient supply. To address whether cyclin-D2 overexpression could rescue the inhibition of glucose-mediated β -cell proliferation induced by KD-PKC ζ , we transduced mouse islet cells with Ad.Cyclin-D2 and Ad.KD-PKC ζ and treated them with 2 and 20 mmol/L glucose. As shown in Fig. 6C and D, cyclin-D2 overexpression led to increased β -cell replication even at low glucose concentrations. This proliferation was highly exacerbated by 20 mmol/L glucose (Fig. 6D). In any case, KD-PKC ζ expression did not reduce the proliferation induced by cyclin-D2 overexpression, suggesting that cyclin-D2 is downstream of PKC ζ .

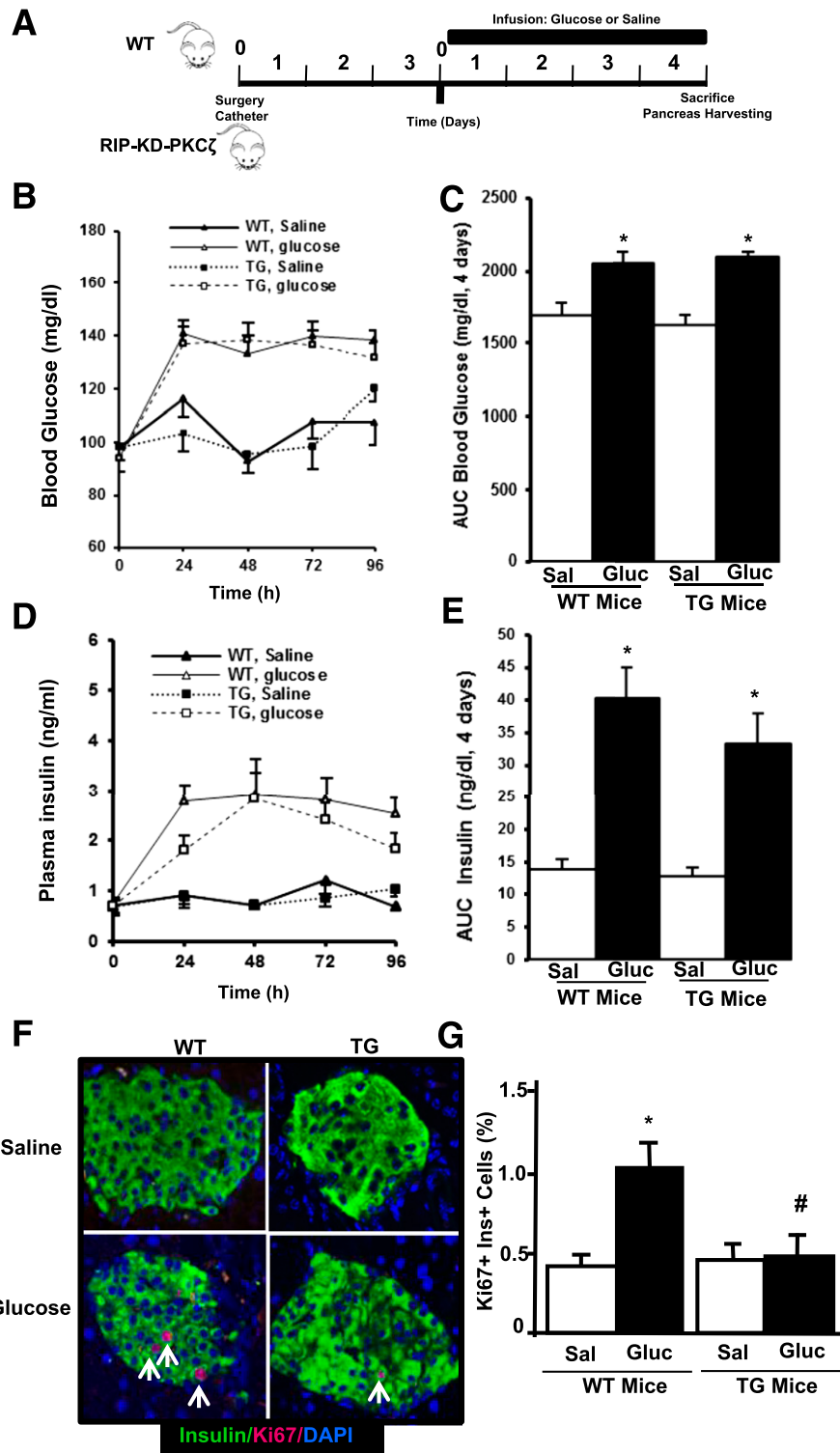


Figure 3—Inactivation of PKC ζ inhibits glucose-induced β -cell proliferation in 4-day glucose-infused RIP-KD-PKC ζ TG mice. **A**: Schematic representation of the experiments and timeline. Catheters were placed in 8-week-old male TG and WT mice and 3 days later glucose (Gluc) or saline (Sal) was infused for 4 days. Blood glucose and plasma insulin were measured daily until mice were sacrificed and pancreas harvested for immunohistochemical analysis. Blood glucose (**B**), area under the curve (AUC) for the blood glucose values (**C**), and plasma insulin (**D**) and AUC (**E**) for the plasma insulin values from saline-infused WT ($n = 7$) or TG ($n = 8$) mice and glucose-infused WT ($n = 17$) or TG ($n = 18$) mice. **F**: Representative photomicrographs of islets in mouse pancreas sections stained for insulin, DAPI, and Ki67 with arrows pointing to Ki67⁺ insulin⁺ cells. **G**: Quantitation of β -cell proliferation by measuring the number of Ki67⁺ insulin⁺ cells in the pancreas sections from the mice described in **F**. Results are means \pm SEM. * $P < 0.05$ vs. same type of mice infused with saline; # $P < 0.05$ vs. WT mice infused with glucose.

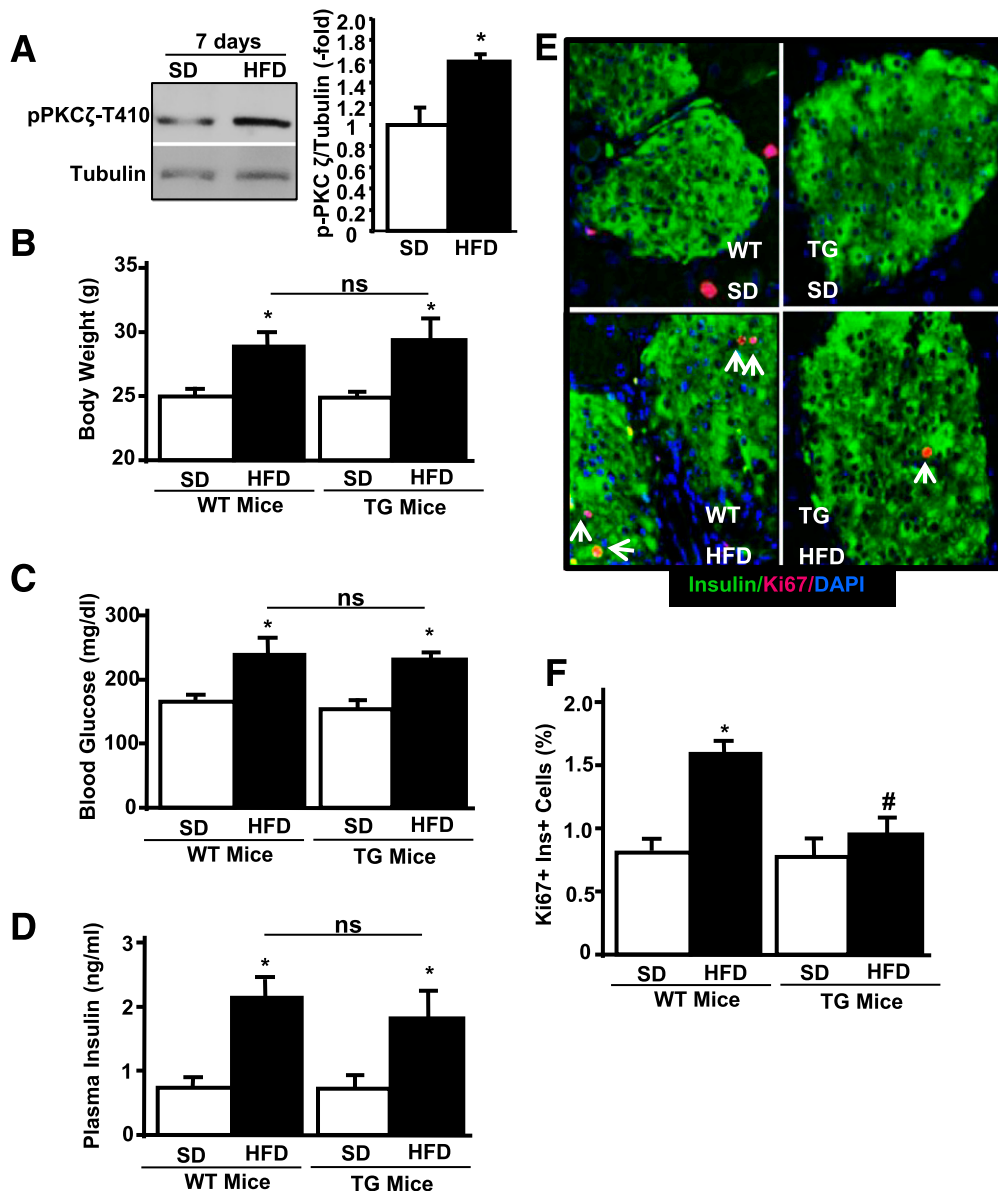


Figure 4—Inactivation of PKC ζ inhibits β -cell proliferation in 1-week HFD-fed RIP-KD-PKC ζ TG mice. **A**: Representative image of a Western blot showing PKC ζ phosphorylation in mouse islets from WT mice fed for 1 week with SD or HFD and the densitometric quantitation of islets from $n = 4$ mice. Body weight (**B**), blood glucose (**C**), and plasma insulin (**D**) of WT and TG male mice (9 weeks old) fed with SD or HFD for 7 days. **E**: Representative photomicrographs of islets in pancreas sections from these mice stained for insulin, DAPI, and Ki67 with arrows pointing to Ki67⁺ insulin⁺ cells. **F**: Quantitation of β -cell proliferation by measuring the number of Ki67⁺ insulin⁺ cells in the pancreas sections from the mice described in **D**. Results are means \pm SEM of SD-fed WT ($n = 6$) or TG ($n = 8$) mice and HFD-fed WT ($n = 8$) or TG ($n = 8$) mice. * $P < 0.05$ vs. same type of mice fed SD; # $P < 0.05$ vs. WT fed HFD.

mTOR Activation by Glucose and HFD Requires PKC ζ Activation

To address what signaling pathways could be modulated by PKC ζ in the context of nutrient oversupply, we performed Western blot analysis of the same cells and islets indicated in the previous section. Glucose increased mTOR, p70S6K, and 4E-BP1 phosphorylation in INS-1 cells, and this increase was significantly attenuated in KD-PKC ζ -transduced cells (Fig. 7A). mTOR activity was also increased in islets from HFD-fed WT mice, but this was not observed in islets from TG mice (Fig. 7B and C). Inhibition of mTOR with rapamycin

decreased cyclin-D2 expression, suggesting that PKC ζ could regulate cyclin-D2 levels by modulating mTORC1 (Fig. 7D). Interestingly, KD-PKC ζ did not alter glucose-mediated phosphorylation of AKT and GSK-3 α/β (Fig. 7E).

DISCUSSION

Insulin resistance leading to hyperglycemia and hyperinsulinemia is a known inducer of compensatory pancreatic β -cell replication and expansion (1–9). However, the molecular mechanisms involved in this adaptive effect in insulin-resistant states are poorly understood. Harnessing

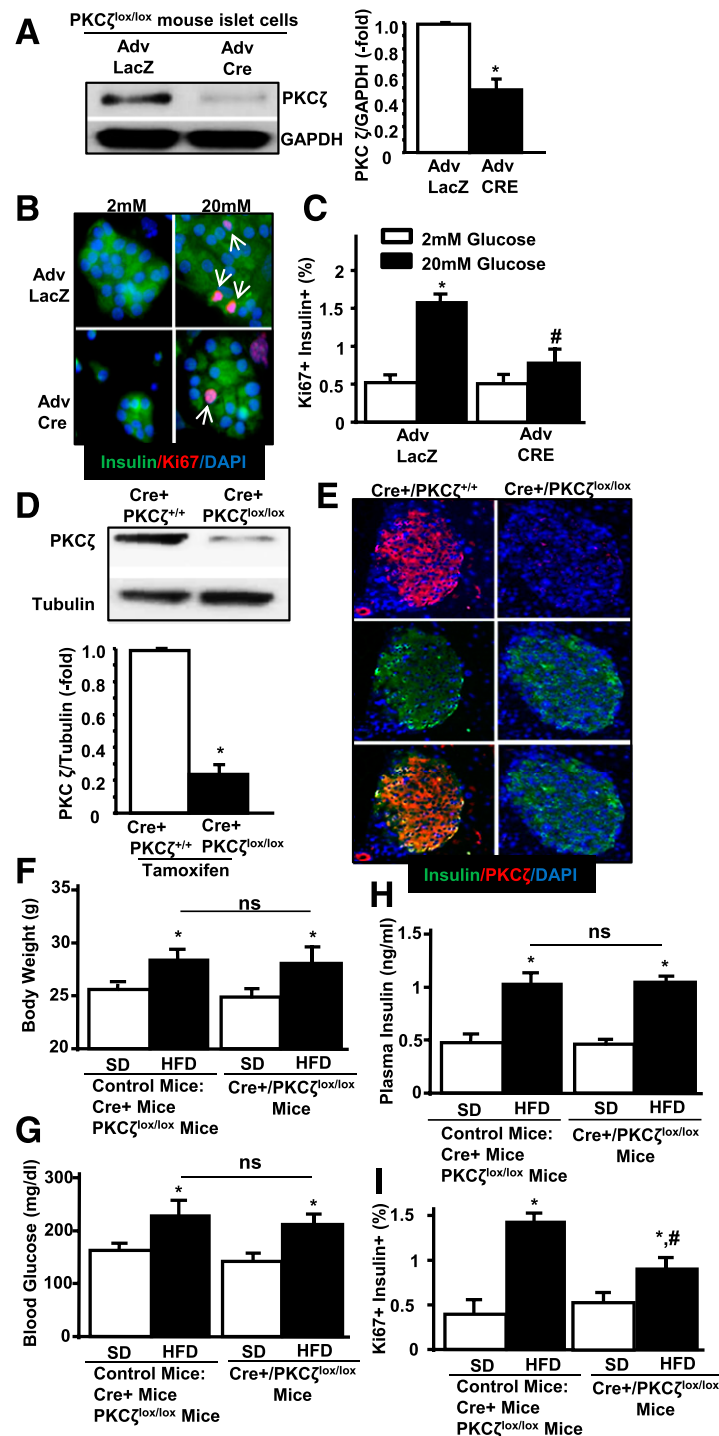


Figure 5—Downregulation of PKC ζ inhibits glucose-mediated β -cell replication in vitro and in 1-week HFD-fed β PKC ζ -KO mice. **A**: Representative image of a Western blot showing the expression of PKC ζ in protein extracts from islet cells from PKC $\zeta^{lox/lox}$ mice infected with Adv.LacZ or Adv.Cre and the densitometric quantitation of islets from $n = 3$ mice. **B**: Representative photomicrographs of PKC $\zeta^{lox/lox}$ mouse islet cell cultures transduced with Adv.LacZ or Adv.Cre, treated with 2 or 20 mmol/L glucose and stained for insulin, Ki67, and DAPI. Arrows indicate Ki67 $^{+}$ insulin $^{+}$ cells. **C**: Quantitation of $n = 5$ experiments as in **B** in duplicate. Results are means \pm SEM. * $P < 0.05$ vs. Adv.LacZ 2 mmol/L; # $P < 0.05$ vs. Adv.LacZ 2 mmol/L. **D**: Representative image of a Western blot showing the level of PKC ζ in mouse islets from Cre $^{+}$ /PKC $\zeta^{+/+}$ and Cre $^{+}$ /PKC $\zeta^{lox/lox}$ (β PKC ζ -KO) mice treated with TM for 5 days and the densitometric quantitation of $n = 3$ mice per condition. Results are means \pm SEM. * $P < 0.05$ vs. Cre $^{+}$ /PKC $\zeta^{+/+}$. **E**: Immunostaining for insulin, PKC ζ , and DAPI of pancreas sections from Cre $^{+}$ /PKC $\zeta^{+/+}$ and Cre $^{+}$ /PKC $\zeta^{lox/lox}$ mice. Body weight (**F**), blood glucose (**G**), and plasma insulin (**H**) of Cre $^{+}$ (Cre $^{+}$ /PKC $\zeta^{+/+}$) and PKC $\zeta^{lox/lox}$ (Cre $^{-}$ /PKC $\zeta^{lox/lox}$) (control) mice and Cre $^{+}$ /PKC $\zeta^{lox/lox}$ mice (9 weeks old) fed with SD or HFD for 7 days. Results are means \pm SEM of SD-fed control ($n = 9$) or Cre $^{+}$ /PKC $\zeta^{lox/lox}$ mice ($n = 5$) and HFD-fed control ($n = 10$) or Cre $^{+}$ /PKC $\zeta^{lox/lox}$ KO mice ($n = 5$). * $P < 0.05$ vs. same type of mice fed SD. **I**: Quantitation of β -cell proliferation by measuring the number of Ki67 $^{+}$ insulin $^{+}$ cells in the pancreas sections from the mice described in **F–H**. * $P < 0.05$ vs. same type of mice fed SD; # $P < 0.05$ vs. control mice fed HFD.

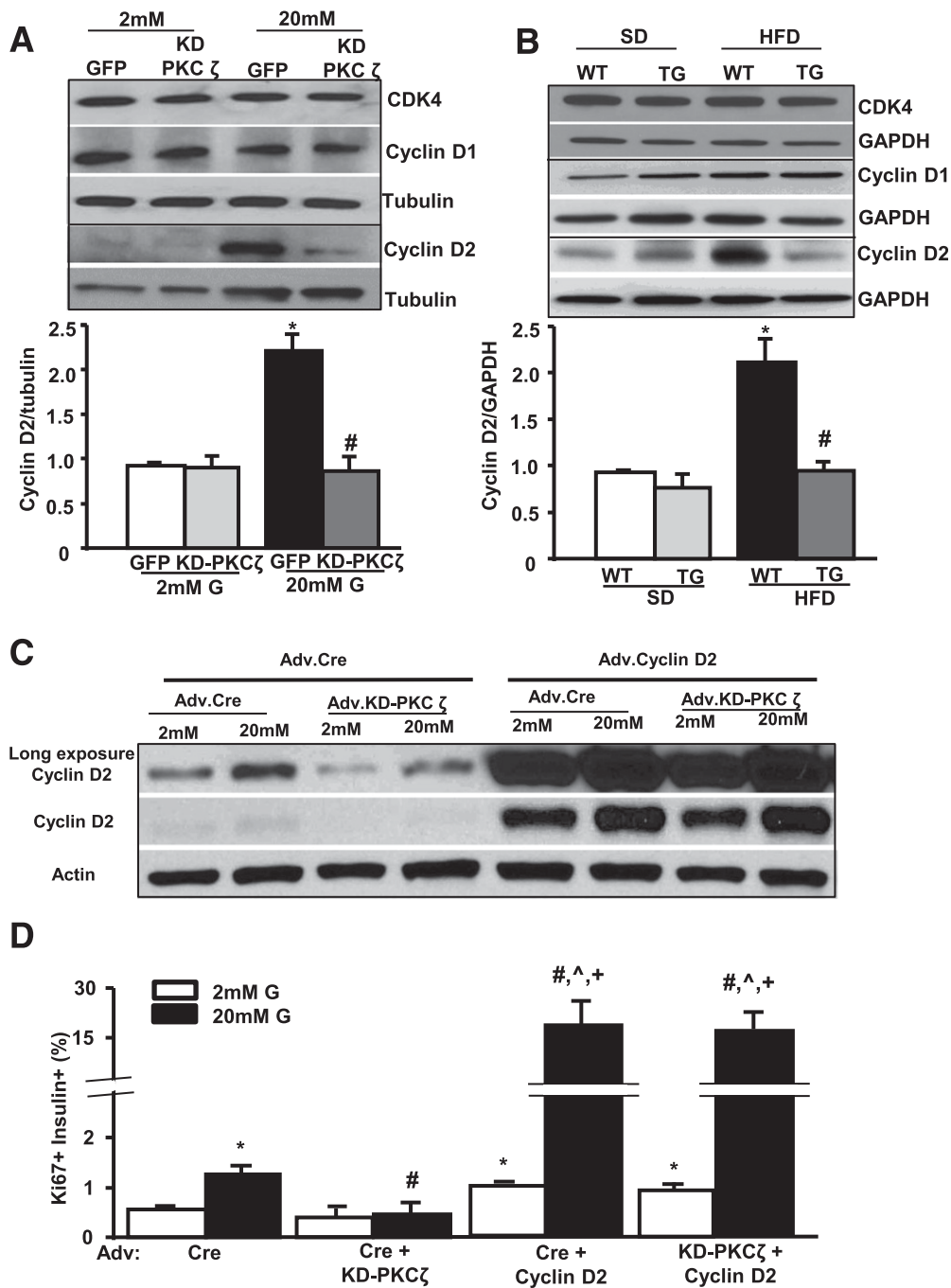


Figure 6—Glucose-mediated and HFD-induced upregulation of cyclin-D2 is impaired by inhibiting PKC ζ activity. Representative image of a Western blot showing the expression of cyclin-D1, cyclin-D2, and CDK4 in protein extracts from INS-1 cells transduced with Adv.GFP or Adv.KD-PKC ζ and treated with 2 or 20 mmol/L glucose (G) for 24 h (A) or islets from SD- or HFD-fed WT and RIP-KD-PKC ζ TG mice for 7 days (B) and the densitometric quantitation of cyclin-D2 in $n = 4$ experiments/mice. Results are means \pm SEM. * $P < 0.05$ vs. 2 mmol/L Adv.GFP or SD mice; # $P < 0.05$ vs. 20 mmol/L Adv.GFP or HFD-WT mice. C: Representative image of a Western blot showing the expression of cyclin-D2 in mouse islet cells transduced with Adv.Cre (control), Adv.KD-PKC ζ , and Adv.Cyclin-D2 at a total MOI of 200 and treated with 2 or 20 mmol/L glucose for 24 h. This Western blot was performed three times with identical results. D: β -Cell proliferation in mouse islet cell cultures transduced with the adenoviruses indicated in C, incubated with 2 or 20 mmol/L glucose for 24 h and assessed by insulin, Ki67, and DAPI immunostaining. Results are means \pm SEM. * $P < 0.05$ vs. 2 mmol/L Adv.LacZ; # $P < 0.05$ vs. 20 mmol/L Adv.LacZ; ^ $P < 0.05$ vs. 20 mmol/L Adv.KD-PKC ζ ; + $P < 0.05$ vs. 2 mmol/L Adv.Cyclin-D2 or Adv.Cyclin-D2 + Adv.KD-PKC ζ .

the factors and intracellular signals that control this increase in β -cell replication could provide therapeutic targets for β -cell regeneration in diabetes. Here we show

that high glucose and insulin in vitro and hyperglycemia, hyperinsulinemia, and insulin resistance in vivo activate a key intracellular kinase, atypical PKC ζ . Importantly,

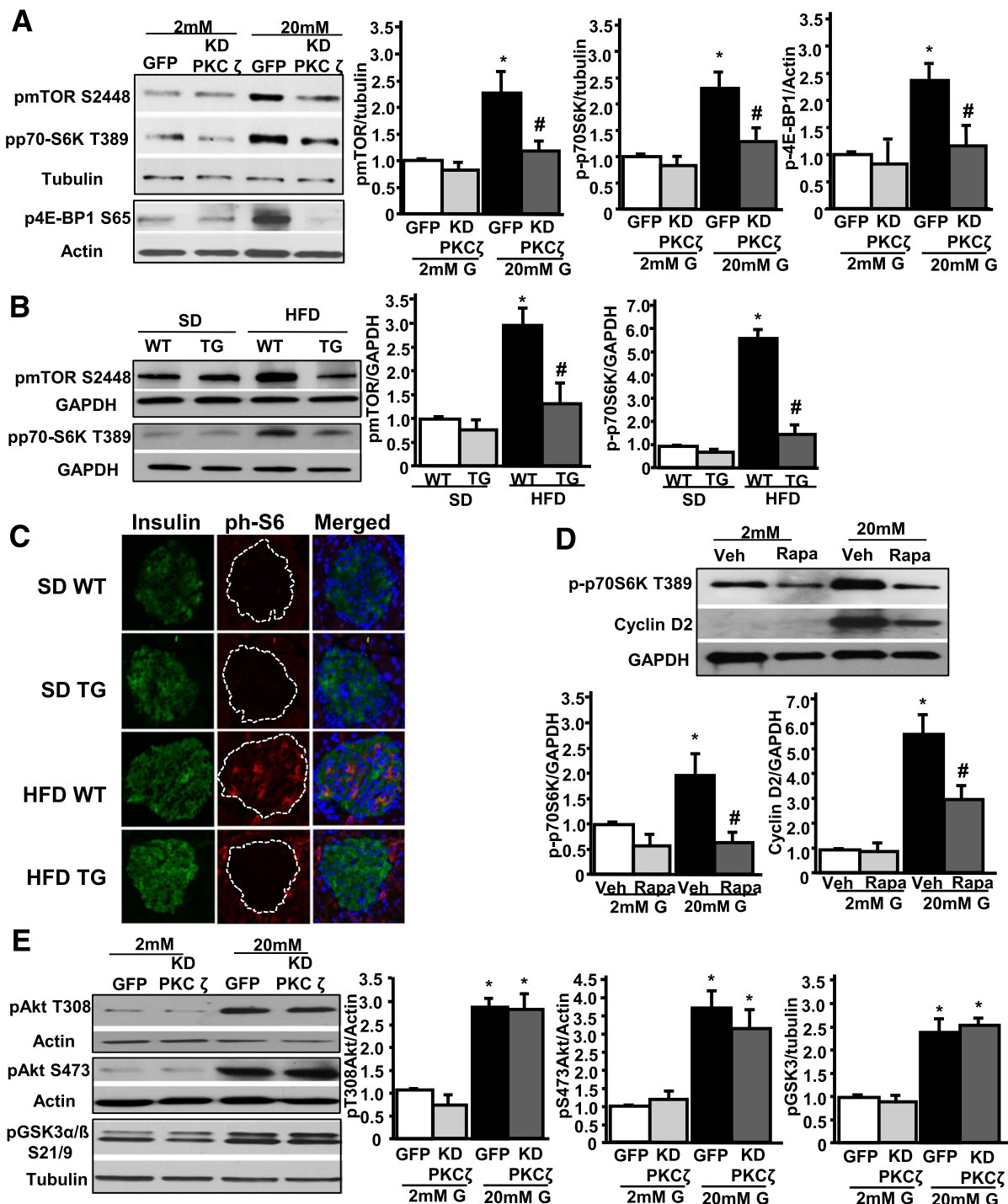


Figure 7—Glucose-mediated and HFD-induced activation of mTOR is impaired by inhibiting PKCζ activity. **A:** Representative image of a Western blot showing phosphorylation of Ser2448-mTOR, Thr389-p70S6K, and 4E-BP1 in protein extracts from INS-1 cells transduced with Adv.GFP or Adv.KD-PKCζ and treated with 2 or 20 mmol/L glucose (G) for 24 h. **B:** Representative image of a Western blot showing phosphorylation of Ser2448-mTOR and Thr389-p70S6K in protein extracts from islets from WT and TG mice fed for 7 days with SD or HFD and the densitometric quantitation of these signals in *n* = 4 experiments/mice. Results are means ± SEM. **P* < 0.05 vs. 2 mmol/L Adv.GFP or SD-fed mice; #*P* < 0.05 vs. 20 mmol/L Adv.GFP or HFD-WT mice. **C:** Representative microphotographs of pancreatic sections from WT and TG mice fed for 7 days with SD or HFD and stained for insulin (green), phosphoS6 (red), and DAPI (blue). Notice the increase in phosphoS6 staining in islets from WT mice fed HFD compared with TG mice fed HFD or SD-fed mice. Islets are outlined with a discontinuous white line. **D:** Representative image of a Western blot showing the expression of cyclin-D2 in INS-1 cells treated with vehicle (Veh) or rapamycin (Rapa) and 2 or 20 mmol/L glucose for 24 h and the densitometric quantitation of cyclin-D2 in *n* = 4 experiments. Results are means ± SEM. **P* < 0.05 vs. 2 mmol/L Veh; #*P* < 0.05 vs. 20 mmol/L Veh. **E:** Representative image of a Western blot showing the phosphorylation of Akt and GSK3α/β in protein extracts from INS-1 cells incubated with 2 or 20 mmol/L glucose for 24 h. Results are means ± SEM. **P* < 0.05 vs. 2 mmol/L Adv.GFP.

downregulation or inactivation of PKC ζ impairs compensatory β -cell replication in acute nutrient oversupply conditions. PKC ζ controls cyclin-D2 upregulation by modulating mTORC1 activity independent of Akt activation (Fig. 8). Therefore, PKC ζ is a master regulator of compensatory β -cell replication, suggesting both its potential as a therapeutic target for β -cell expansion in diabetes and its pathophysiological importance in the development of T2D.

Transfer of CA-PKC ζ to rodent and human islet cells enhances β -cell replication (16–18). However, the physiologic role of PKC ζ in situations of enhanced β -cell replication, such as acute hyperglycemia, hyperinsulinemia, and insulin resistance, was unknown. Here we demonstrate the need of PKC ζ activity for glucose to increase rodent β -cell proliferation using INS-1 cells as well as mouse islet cells in primary culture. Interestingly, this effect is specific for PKC ζ since downregulation of the other member of the atypical PKC family of proteins, PKC λ (15), did not affect glucose-mediated proliferation. It is important to note that PKC ζ also regulates glucose-mediated human β -cell proliferation in vitro, highlighting the significance of this kinase for the adaptation of human β -cells to situations of increased metabolic demand. In both rodent and human cells, PI3K inhibition with wortmannin eliminated glucose-mediated β -cell proliferation, suggesting that the PI3K-PKC ζ pathway controls glucose-mediated proliferation in vitro.

PKC ζ is activated by PI3K/PDK1 and also lipid components such as phosphatidic acid (30), arachidonic acid (31), and ceramide (32). In the current study, we found that glucose increases in dose- and time-dependent fashions the activity of PKC ζ through PI3K. We also observed that insulin increases PKC ζ activation, as previously shown in other non- β -cell types (33). On the other hand, whether PKC ζ activation requires glucose intermediary metabolites is unknown but warrants future studies. Indeed, glucokinase (Gck) haploinsufficiency leads to decreased β -cell replication in mice after HFD feeding and insulin resistance, suggesting that glucose metabolism is required for compensatory β -cell replication (34). Gck^{+/-} mice also display a decrease in insulin secretion (31,35), suggesting the involvement of the insulin/insulin receptor substrate-2 axis in the compensatory β -cell hyperplasia in insulin resistance. Insulin receptor substrate-2 haploinsufficiency or β -cell deletion of the insulin receptor, but not IGF1 receptor, completely eliminated β -cell hyperplasia in insulin resistance (34,36). In our studies, we found that GKA-mediated β -cell proliferation is inhibited by PKC ζ inactivation. In addition, mice with β -cell inactivation or disruption of PKC ζ display plasma insulin levels similar to WT mice after 7-day HFD feeding, ruling out deregulated insulin secretion as the cause of the impaired compensatory β -cell proliferation in these mice. However, it suggests that insulin might require PKC ζ activation to induce the compensatory growth response in insulin resistance.

Even though no changes in plasma insulin levels were observed in PKC ζ -deficient mice after 7-day HFD feeding, whether prolonged HFD feeding and sustained insulin resistance could lead to decreased β -cell expansion and inappropriate insulin production in these mice is unknown and warrants future studies.

It has been recently reported that TG mice carrying human growth hormone (hGH) gene sequences for transgene transcript stability express hGH in β -cells, leading to a pseudopregnancy phenotype in terms of glucose and β -cell homeostasis (37). However, RIP-KD-PKC ζ TG mice carrying hGH sequences in the transgene displayed normal glucose and β -cell phenotype in basal conditions. Nevertheless, to eliminate the uncertainty of whether this could contribute to the observed phenotype of decreased β -cell replication in TG mice in insulin resistance conditions, we also analyzed β -cell proliferation in β PKC ζ -KO mice fed with HFD. Importantly, in these studies, the corresponding littermates, including Cre⁻/PKC ζ ^{loxP/loxP} mice and Cre⁺/PKC ζ ^{+/+} mice carrying hGH gene sequences (38), were used as controls. As shown in our studies, mice with disruption of PKC ζ in β -cells displayed reduced β -cell replication when fed an HFD, an identical phenotype to the one observed in TG mice with KD-PKC ζ . Collectively, these studies truly describe PKC ζ as essential for compensatory β -cell replication in insulin resistance.

Cyclin-D2 is required for adult β -cell proliferation and mass expansion in mice (21,22). More recently, it has been demonstrated that cyclin-D2 is essential for the compensatory β -cell hyperplastic response to insulin resistance in mice (7). Previous studies have also shown that CA-PKC ζ in β -cells leads to decreased phosphorylation and increased expression of D cyclins potentially by enhancing their stability (18). This suggests that PKC ζ could regulate cyclin-D2

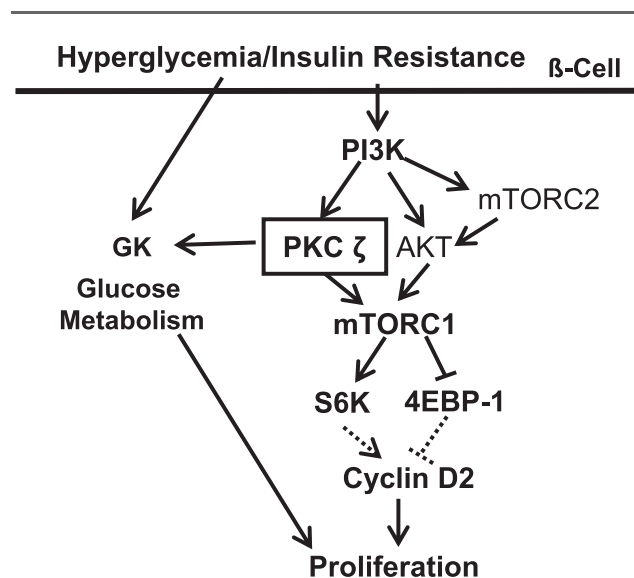


Figure 8—Schematic representation of the signaling network regulated by PKC ζ in glucose- and insulin resistance-mediated β -cell replication. GK, glucokinase.

expression in β -cells in insulin resistance. Indeed, in the current study, we observed that cyclin-D2 upregulation in mouse islets by glucose and insulin resistance is blunted by KD-PKC ζ and that exogenous overexpression of this cyclin bypasses the KD-PKC ζ -mediated inhibitory effect. Collectively, these studies suggest that PKC ζ regulates cyclin-D2 levels and hence compensatory β -cell proliferation that requires cyclin-D2 upregulation for cell cycle progression. It is important to note that cyclin-D1 and cdk4 are not regulated by glucose or insulin resistance in islets or β -cells in our hands. At this point it is unknown how glucose selectively regulates cyclin-D2 but not cyclin-D1 in rodent β -cells. This is highly relevant since human β -cells display minimal, if any, cyclin-D2 expression compared with cyclin-D1 (39). Equally, since cyclin-D2 seems negligible in human β -cells/islets, it will be important to decipher what cell cycle molecules are modulated by PKC ζ in glucose-mediated human β -cell replication.

Activation of mTOR by nutrients and growth factors is essential for cell growth and proliferation (40). mTOR forms two complexes: mTORC1 and mTORC2. mTORC1 is rapamycin sensitive and activates regulators of protein translation such as p70S6K and 4E-BP1, and its activation leads to increased β -cell replication and mass expansion (41). Hyperglycemia, hyperinsulinemia, and insulin resistance lead to mTOR activation (6). On the other hand, rapamycin inhibits β -cell proliferation in situations of increased metabolic demand, further highlighting the importance of mTORC1 in compensatory β -cell growth (42). In addition, mTORC1 activation regulates β -cell proliferation and mass by increasing the expression and stability of cyclin-D2 (20). CA-PKC ζ enhances mTORC1 activation and rapamycin blocks PKC ζ -induced β -cell proliferation (18). Taken together, these studies suggest that in a situation of nutrient oversupply and insulin resistance, activation of mTORC1 is essential for compensatory growth and that PKC ζ could regulate this effect. The results in this study clearly show that mTORC1 is activated by glucose and insulin resistance in β -cells and islets and that KD-PKC ζ blocks this activation. Importantly, glucose-induced Akt activation is unaltered by KD-PKC ζ . It is important to note that although Akt was suggested as mediator of compensatory β -cell growth in insulin-resistant states in rodents (43), evidence in genetically modified mice expressing KD-Akt rules out this possibility (44). Collectively, these studies clearly place PKC ζ as the key regulator of mTORC1 and compensatory β -cell replication when insulin demand rises. How PKC ζ regulates mTORC1 requires further studies, but a potential double role of p70S6K as target and kinase of PKC ζ and mTOR, respectively, could be implicated in this signaling event (25,45).

In summary, these studies underscore PKC ζ as a novel master regulator of adaptive β -cell replication in insulin resistance by controlling the activity of the nutrient-regulated kinase mTORC1 and the levels of the essential cell cycle component cyclin-D2. Future studies will determine

whether alterations in PKC ζ expression and/or activity could result in deficient β -cell compensation with nutrient oversupply and progression to T2D.

Acknowledgments. The authors thank Drs. Andrew F. Stewart and Nathalie Fiaschi-Taesch (Icahn School of Medicine at Mount Sinai) for helpful comments during the development of these studies and Dr. Karen Takane (Icahn School of Medicine at Mount Sinai), Dr. Varsha Sridhar, Taylor Rosa, Shelley Valle, Jordan Pascoe (University of Pittsburgh), and Kelly Hyles (Icahn School of Medicine at Mount Sinai) for technical help.

Funding. This work was supported in part by grants from the National Institute of Diabetes and Digestive and Kidney Diseases (DK-067351 and DK-077096 to A.G.-O., DK-065149 to D.K.S., DK-078060 to R.C.V., DK-095140 to L.C.A.) and the National Heart, Lung, and Blood Institute (HL-063767 and HL-111706 to C.P.O.).

Duality of Interest. No potential conflicts of interest relevant to this article were reported.

Author Contributions. J.L., J.C.A.-P., C.R., G.P.C., R.E.S., F.R.-P., C.P.O., and L.C.A. researched data, contributed to discussion, and reviewed and edited the manuscript. R.C.V. and D.K.S. contributed to discussion and reviewed and edited the manuscript. A.G.-O. researched data, contributed to discussion, and wrote the manuscript. A.G.-O. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

References

- Muoio DM, Newgard CB. Mechanisms of disease: molecular and metabolic mechanisms of insulin resistance and beta-cell failure in type 2 diabetes. *Nat Rev Mol Cell Biol* 2008;9:193–205
- Sachdeva MM, Stoffers DA. Minireview: meeting the demand for insulin: molecular mechanisms of adaptive postnatal beta-cell mass expansion. *Mol Endocrinol* 2009;23:747–758
- Alonso LC, Yokoe T, Zhang P, et al. Glucose infusion in mice: a new model to induce beta-cell replication. *Diabetes* 2007;56:1792–1801
- Levitt HE, Cyphert TJ, Pascoe JL, et al. Glucose stimulates human beta cell replication in vivo in islets transplanted into NOD-severe combined immunodeficiency (SCID) mice. *Diabetologia* 2011;54:572–582
- Stamateris RE, Sharma RB, Hollern DA, Alonso LC. Adaptive β -cell proliferation increases early in high-fat feeding in mice, concurrent with metabolic changes, with induction of islet cyclin D2 expression. *Am J Physiol Endocrinol Metab* 2013;305:E149–E159
- Zarrouki B, Benterki I, Fontés G, et al. Epidermal growth factor receptor signaling promotes pancreatic β -cell proliferation in response to nutrient excess in rats through mTOR and FOXM1. *Diabetes* 2014;63:982–993
- Georgia S, Hinault C, Kawamori D, et al. Cyclin D2 is essential for the compensatory beta-cell hyperplastic response to insulin resistance in rodents. *Diabetes* 2010;59:987–996
- El Ouaamari A, Kawamori D, Dirice E, et al. Liver-derived systemic factors drive β cell hyperplasia in insulin-resistant states. *Cell Reports* 2013;3:401–410
- Yi P, Park JS, Melton DA. Betatrophin: a hormone that controls pancreatic β cell proliferation. *Cell* 2013;153:747–758
- Imai J, Katagiri H, Yamada T, et al. Regulation of pancreatic beta cell mass by neuronal signals from the liver. *Science* 2008;322:1250–1254
- Wellcome Trust Case Control Consortium. Genome-wide association study of 14,000 cases of seven common diseases and 3,000 shared controls. *Nature* 2007;447:661–678
- Morris AP. Fine mapping of type 2 diabetes susceptibility loci. *Curr Diab Rep* 2014;14:549
- Qin L, Zhou L, Wu X, et al. Genetic variants in protein kinase C zeta gene and type 2 diabetes risk: a case-control study of a Chinese Han population. *Diabetes Metab Res Rev* 2008;24:480–485

14. Li YF, Sun HX, Wu GD, et al. Protein kinase C/zeta (PRKCZ) gene is associated with type 2 diabetes in Han population of North China and analysis of its haplotypes. *World J Gastroenterol* 2003;9:2078–2082
15. Hirai T, Chida K. Protein kinase Czeta (PKCzeta): activation mechanisms and cellular functions. *J Biochem* 2003;133:1–7
16. Buteau J, Foisy S, Rhodes CJ, Carpenter L, Biden TJ, Prentki M. Protein kinase Czeta activation mediates glucagon-like peptide-1-induced pancreatic beta-cell proliferation. *Diabetes* 2001;50:2237–2243
17. Vasavada RC, Wang L, Fujinaka Y, et al. Protein kinase C- ζ activation markedly enhances β -cell proliferation: an essential role in growth factor mediated β -cell mitogenesis. *Diabetes* 2007;56:2732–2743
18. Velazquez-Garcia S, Valle S, Rosa TC, et al. Activation of protein kinase C- ζ in pancreatic β -cells in vivo improves glucose tolerance and induces β -cell expansion via mTOR activation. *Diabetes* 2011;60:2546–2559
19. Porat S, Weinberg-Corem N, Tornovsky-Babaey S, et al. Control of pancreatic β cell regeneration by glucose metabolism. *Cell Metab* 2011;13:440–449
20. Balcazar N, Sathyamurthy A, Elghazi L, et al. mTORC1 activation regulates beta-cell mass and proliferation by modulation of cyclin D2 synthesis and stability. *J Biol Chem* 2009;284:7832–7842
21. Kushner JA, Ciemerych MA, Sicinska E, et al. Cyclins D2 and D1 are essential for postnatal pancreatic beta-cell growth. *Mol Cell Biol* 2005;25:3752–3762
22. Georgia S, Bhushan A. Beta cell replication is the primary mechanism for maintaining postnatal beta cell mass. *J Clin Invest* 2004;114:963–968
23. García-Ocaña A, Takane K, Syed MA, Philbrick WM, Vasavada RC, Stewart AF. Hepatocyte growth factor overexpression in the islet of transgenic mice increases beta cell proliferation, enhances islet mass, and induces hypoglycemia. *J Biol Chem* 2000;275:1226–1232
24. Chou MM, Hou W, Johnson J, et al. Regulation of protein kinase C zeta by PI 3-kinase and PDK-1. *Curr Biol* 1998;8:1069–1077
25. Romanelli A, Martin KA, Toker A, Blenis J. p70 S6 kinase is regulated by protein kinase Czeta and participates in a phosphoinositide 3-kinase-regulated signalling complex. *Mol Cell Biol* 1999;19:2921–2928
26. Wicksteed B, Brissova M, Yan W, et al. Conditional gene targeting in mouse pancreatic β -cells: analysis of ectopic Cre transgene expression in the brain. *Diabetes* 2010;59:3090–3098
27. Zhang H, Fujitani Y, Wright CV, Gannon M. Efficient recombination in pancreatic islets by a tamoxifen-inducible Cre-recombinase. *Genesis* 2005;42:210–217
28. Alvarez-Perez JC, Ernst S, Demirci C, et al. Hepatocyte growth factor/c-Met signaling is required for β -cell regeneration. *Diabetes* 2014;63:216–223
29. Wang P, Alvarez-Perez JC, Felsenfeld DP, et al. A high-throughput chemical screen reveals that harmine-mediated inhibition of DYRK1A increases human pancreatic beta cell replication. *Nat Med* 2015;21:383–388
30. Limatola C, Schaap D, Moolenaar WH, van Blitterswijk WJ. Phosphatidic acid activation of protein kinase C-zeta overexpressed in COS cells: comparison with other protein kinase C isotypes and other acidic lipids. *Biochem J* 1994;304:1001–1008
31. Müller G, Ayoub M, Storz P, Rennecke J, Fabbro D, Pfizenmaier K. PKC zeta is a molecular switch in signal transduction of TNF-alpha, bifunctionally regulated by ceramide and arachidonic acid. *EMBO J* 1995;14:1961–1969
32. Charruyer A, Jean C, Colomba A, et al. PKCzeta protects against UV-C-induced apoptosis by inhibiting acid sphingomyelinase-dependent ceramide production. *Biochem J* 2007;405:77–83
33. Mendez R, Kollmorgen G, White MF, Rhoads RE. Requirement of protein kinase C zeta for stimulation of protein synthesis by insulin. *Mol Cell Biol* 1997;17:5184–5192
34. Terauchi Y, Takamoto I, Kubota N, et al. Glucokinase and IRS-2 are required for compensatory beta cell hyperplasia in response to high-fat diet-induced insulin resistance. *J Clin Invest* 2007;117:246–257
35. Terauchi Y, Sakura H, Yasuda K, et al. Pancreatic beta-cell-specific targeted disruption of glucokinase gene. Diabetes mellitus due to defective insulin secretion to glucose. *J Biol Chem* 1995;270:30253–30256
36. Takamoto I, Terauchi Y, Kubota N, Ohsugi M, Ueki K, Kadowaki T. Crucial role of insulin receptor substrate-2 in compensatory beta-cell hyperplasia in response to high fat diet-induced insulin resistance. *Diabetes Obes Metab* 2008;10(Suppl. 4):147–156
37. Brouwers B, de Faudeur G, Osipovich AB, et al. Impaired islet function in commonly used transgenic mouse lines due to human growth hormone minigene expression. *Cell Metab* 2014;20:979–990
38. Oropeza D, Jouvret N, Budry L, et al. Phenotypic characterization of MIP-CreERT1Lphi mice with transgene-driven islet expression of human growth hormone. *Diabetes* 2015;64:3798–3807
39. Fiaschi-Taesch NM, Salim F, Kleinberger J, et al. Induction of human beta-cell proliferation and engraftment using a single G1/S regulatory molecule, cdk6. *Diabetes* 2010;59:1926–1936
40. Hay N, Sonenberg N. Upstream and downstream of mTOR. *Genes Dev* 2004;18:1926–1945
41. Rachdi L, Balcazar N, Osorio-Duque F, et al. Disruption of Tsc2 in pancreatic beta cells induces beta cell mass expansion and improved glucose tolerance in a TORC1-dependent manner. *Proc Natl Acad Sci U S A* 2008;105:9250–9255
42. Zahr E, Molano RD, Pileggi A, et al. Rapamycin impairs in vivo proliferation of islet beta-cells. *Transplantation* 2007;84:1576–1583
43. Jetton TL, Lausier J, LaRock K, et al. Mechanisms of compensatory beta-cell growth in insulin-resistant rats: roles of Akt kinase. *Diabetes* 2005;54:2294–2304
44. Bernal-Mizrachi E, Fatrai S, Johnson JD, et al. Defective insulin secretion and increased susceptibility to experimental diabetes are induced by reduced Akt activity in pancreatic islet beta cells. *J Clin Invest* 2004;114:928–936
45. Chiang GG, Abraham RT. Phosphorylation of mammalian target of rapamycin (mTOR) at Ser-2448 is mediated by p70S6 kinase. *J Biol Chem* 2005;280:25485–25490