PDK4 Deficiency Suppresses Hepatic Glucagon Signaling by Decreasing cAMP Levels

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Diabetes 2018;67:2054–2068 | https://doi.org/10.2337/db17-1529

In fasting or diabetes, gluconeogenic genes are transcriptionally activated by glucagon stimulation of the cAMP-protein kinase A (PKA)–CREB signaling pathway. Previous work showed pyruvate dehydrogenase kinase (PDK) inhibition in skeletal muscle increases pyruvate oxidation, which limits the availability of gluconeogenic substrates in the liver. However, this study found upregulation of hepatic PDK4 promoted glucagon-mediated expression of gluconeogenic genes, whereas knockdown or inhibition of hepatic PDK4 caused the opposite effect on gluconeogenic gene expression and decreased hepatic glucose production. Mechanistically, PDK4 deficiency decreased ATP levels, thus increasing phosphorylated AMPK (p-AMPK), which increased p-AMPK–sensitive phosphorylation of cyclic nucleotide phosphodiesterase 4B (p-PDE4B). This reduced cAMP levels and consequently p-CREB. Metabolic flux analysis showed that the reduction in ATP was a consequence of a diminished rate of fatty acid oxidation (FAO). However, overexpression of PDK4 increased FAO and increased ATP levels, which decreased p-AMPK and p-PDE4B and allowed greater accumulation of cAMP and p-CREB. The latter were abrogated by the FAO inhibitor etomoxir, suggesting a critical role for PDK4 in FAO stimulation and the regulation of cAMP levels. This finding strengthens the possibility of PDK4 as a target against diabetes.

The liver is an important organ for maintenance of glucose homeostasis throughout the starve-feed cycle. Hepatic gluconeogenesis is tightly regulated by the interwoven actions of insulin and glucagon, the hormones mainly affected by nutritional status. In the fasted state, glucagon is responsible for the increase in cAMP level and protein kinase A (PKA) activity (1). PKA, in turn, activates the transcription factor CREB by phosphorylation, which promotes expression of key regulatory gluconeogenic genes, including peroxisome proliferator–activated receptor γ coactivator 1α (*Pgc-1α*), *Pepck*, and glucose-6-phosphatase (*G6pase*) (2,3). Because increased glucagon secretion plays a pivotal role in the pathophysiology of type 2 diabetes (4), this signaling cascade is of considerable therapeutic interest.

Pyruvate dehydrogenase complex (PDC) plays a central role in cellular energy metabolism by catalyzing the conversion of pyruvate to acetyl-CoA via oxidative decarboxylation (5,6). This process is critically important

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Received 15 December 2017 and accepted 11 July 2018.

This article contains Supplementary Data online at http://diabetes.diabetesjournals.org/lookup/suppl/doi:10.2337/db17-1529/-/DC1.

B.-Y.P. and J.-H.J. contributed equally to this work.

PATHOPHYSIOLOGY

in the maintenance of hepatic glucose homeostasis, as evidenced by changes in its activity according to the starve-feed cycle (7–9). In the fed state, for example, activated PDC increases glucose oxidation for the provision of acetyl-CoA for the citric acid cycle, fatty acid synthesis, and cholesterol synthesis. In the fasted state, however, PDC is inactivated by pyruvate dehydrogenase kinase (PDK)–induced phosphorylation, which prevents the conversion of the glucogenic compounds alanine, lactate, and pyruvate to acetyl-CoA, a nonglucogenic compound.

Four isoenzymes of PDKs (PDK1-4) are expressed in mammalian tissues. Among these, PDK2 and PDK4 are highly expressed in the liver, and especially PDK4 is known to be increased in diabetic conditions as well as in the fasting condition when glucagon action is augmented (10,11). Thus, several efforts have been made to unveil whether the hyperglycemia of pathologic conditions can be alleviated by modulating PDK4 activity. Indeed, PDK-knockout (KO) mice exhibit lower fasting blood glucose levels, which is generally believed to be due to reduced availability of precursors for hepatic gluconeogenesis (12,13). Likewise, the well-known PDK inhibitor dichloroacetate (DCA) is believed to lower blood glucose by the same mechanism (14).

To the best of our knowledge, however, the possibility that PDK4 deficiency or inhibition might also reduce expression of gluconeogenic enzymes has not been properly evaluated. Here we show evidence that besides its effect on limitation of substrate for gluconeogenesis previously observed in global PDK4-KO mice, liver-specific inhibition of PDK4 is sufficient to modulate glucagon-simulated hepatic gluconeogenesis by decreasing cellular cAMP level and, therefore, gluconeogenic gene expression.

RESEARCH DESIGN AND METHODS

Mouse Primary Hepatocyte Experiments

Primary hepatocytes were prepared as described previously (15). For virus infection experiments, cells were infected with adenoviruses for 2–3 days in 10% complete 199 media and then treated with glucagon (G2044; Sigma-Aldrich) or 8-bromo (Br)-cAMP (B5386; Sigma-Aldrich). For DCA (347795; Sigma-Aldrich) experiments, cells were incubated with different doses of DCA in complete medium for 16 h and then treated with glucagon.

Animal Experiments

C57BL/6J male mice, *db*/*db* mice and control *db*/+ mice (8–10 weeks old) were acclimated in our animal facility for 1 week before experiments were initiated. To compare mRNA and protein levels, mice were fasted for 16 h and refed for 4 h. For DCA experiments with diet-induced obese mice, 4-week-old male mice were fed a high-fat–high-sucrose (HFHS) diet (D12231; Research Diets). DCA (300 mg/kg) and vehicle were delivered by intraperitoneal injection every day for the indicated number of days, and blood glucose levels were measured after 6-h fasting at 10-day intervals during the 50-day experiment. For liver-specific PDK4 overexpression or knockdown experiments, the indicated adenoviruses were injected via the tail vein. Body composition was measured with a minispec plus Live Mice Analyzer (LF50; Bruker). Livers were harvested after the mice were fasted for 16 h. All experiments were approved by the Deagu-Gyeongbuk Medical Innovation Foundation Institutional Animal Care and Use Committee (DGMIF-10630802-00).

Pyruvate and Glucose Tolerance Tests

After indicated adenovirus (target templates sequence: short hairpin green fluorescent protein [shGFP], 5′-GCATCAAGGTGAACTTCAAGA-3′; shPdk4, 5′-GGAAGGAATCAAAGCACTTTA-3′) (16) or 7-day DCA treatment, 16-h fasted mice were intraperitoneally injected with 2 g/kg of sodium pyruvate (P2256; Sigma-Aldrich). For glucose tolerance test, mice fasted for 16 h were intraperitoneally injected with 1.5 g/kg of D-(+)-glucose (G7528; Sigma-Aldrich). Tail vein blood was collected at 0, 15, 30, 60, and 120 min for the measurement of blood glucose.

Measurement of Liver Triglyceride and Plasma Glucagon

Liver triglyceride was measured with a kit according to the manufacturer's instructions (K622-100; BioVision), and plasma glucagon was measured with the Wako Glucagon ELISA Kit (297-57101; Wako).

In Vivo Imaging

G6Pase (−231/+57) promoter (provided by S.-H.K.) was prepared as previously described (17). Adenoviral G6Pase-luc (1.5×10^9/plaque-forming units [pfu]) was delivered by i.v. injection to C57BL/6J male mice for 4–7 days. Mice fasted for 15 h were injected with 30 mg/mL firefly D-luciferin intraperitoneally 5 min before imaging. Mice were anesthetized with 2% isoflurane gas and imaged with an IVIS bioluminescence imaging system (Xenogen).

Promoter Assay

Transfections were conducted in AML-12 cells with Lipofectamine 2000 (52887; Invitrogen) with Plus Reagent (10964; Invitrogen), according to the manufacturer's instructions. Reporter plasmids encoding the human G6Pase promoter (−1,227/+57) and rat Pepck promoter (−2,000/+73) were used as previously described (18). Wild-type CREB vector (15221; Addgene) and mutant CREB vector (15222; Addgene) were purchased from Addgene.

Quantitative Real-time PCR

Mouse liver tissue and primary hepatocyte RNA were isolated using QIAzol (79306; Qiagen) and cDNA was synthesized from 2 μg total RNA using oligo dT primer (K1622; Thermo Scientific). Quantitative real-time PCR was performed on a Viia7 instrument (Applied Biosystems) using SYBR green reagent (4367659; ABI). Mouse primer

sequences for real-time PCR are reported in Supplementary Table 1.

Western Blot Analysis

Western blot assay was performed using antibodies specific for PDK4 and PDC (provided by R.A.H.), phosphorylated (p)–PDH-E1α ser293 (AP1062; Calbiochem), p-CREB (9198; Cell Signaling), p-PKA substrate (5045; Cell Signaling), p-AMPK (2532; Cell Signaling), total CREB (9197; Cell Signaling), Hsp90 (4874; Cell Signaling), p-PDE4B (provided by M.H. Rider, Université catholique de Louvain and de Duve Institute, Brussels, Belgium), and total PDE4B (sc-25812; Santa Cruz Biotechnology).

Quantification of p-CREB by Immunofluorescence

Primary mouse hepatocytes were fixed on collagen type I–coated cover slips (GG-22-collagen; neuVitro) with 4% paraformaldehyde for 30 min. Cells were washed with filtered PBS, followed by incubation for 15 min with 0.1% Triton X-100 containing permeable solution. After permeabilization, cells were incubated overnight at 4°C with anti–p-CREB at a dilution of 1:400. Cells were washed with filtered PBS three times and then incubated for 2 h with secondary antibody (Alexa Fluor 488 or 568). The cells were further washed three times with PBS before the cover slips were mounted with mounting solution containing DAPI.

Hepatic Glucose Production Assay

For glucose production assay, hepatocyte growth medium was replaced with Krebs-Ringer bicarbonate (KRB) buffer including 0.5% BSA, 10 mmol/L sodium lactate, and 1 mmol/L sodium pyruvate, with or without glucagon. The culture medium was collected after incubation of the plates for 4–6 h. Glucose formed by the cells was determined with a hexokinase/glucose-6-phosphate dehydrogenase (H4502 and G7877; Sigma-Aldrich) assay and normalized to total protein concentrations and incubation times.

Intracellular cAMP Measurement

cAMP was determined in primary hepatocytes with a kit from ABI (T1500) and in mouse livers with an ELISA kit (ab65355).

Isotopomer Labeling Studies and Metabolite Measurement by Liquid Chromatography–Tandem Mass Spectrometry

To establish steady-state labeling, cells were incubated for 2 h with 200 μmol/L BSA-conjugated U-[^{13}C]palmitic acid (605573; Sigma-Aldrich) or 1 mmol/L U-[^{13}C]sodium pyruvate (490717; Sigma). Before collection, cells were treated with glucagon for 15 min.

The liquid chromatography–tandem mass spectrometry analysis was performed as described previously (19). Briefly, analytes were separated on a Mastro C18 (3 μm particle size, length 150 mm, and inner diameter 2.0 mm) column by gradient elution using an HPLC Nexera instrument coupled with an LCMS-8060 mass spectrometer (Shimadzu, Japan).

RESULTS

Hepatic PDK4 Expression Is Positively Correlated With Gluconeogenic Signaling

Compared with the refed state, the expression of the mRNA for *Pdk4* was greatly increased in the liver in the fasted state, along with increased expression of the gluconeogenic genes *Pgc-1α*, *G6pase*, and *Pepck* (Fig. 1*A*). Accordingly, the PDK4 protein level was higher in the livers of fasted mice (Fig. 1*B*), and compared with *db/+* mice, hepatic *Pdk4* mRNA and its protein were highly induced in diabetic *db/db* mice where gluconeogenesis is aberrantly reinforced (Supplementary Fig. 1*A* and *B*). We therefore, we checked whether cAMP and glucagon, which are augmented in the fasting condition and diabetes, affect PDK4 expression in primary mouse hepatocytes. As expected, gluconeogenic gene expression was significantly increased by the glucagon and 8-Br-cAMP challenge (Fig. 1*C*). *Pdk4* mRNA expression was also markedly increased without increases in the mRNAs for the other PDK isoenzymes (Fig. 1*C*). The protein level of PDK4 and p-PDHE1α were increased by 8-Br-cAMP, suggesting that hepatic PDK4 expression is positively regulated by cAMP stimulation (Fig. 1*D*).

Next, hepatocytes were pretreated with PKA inhibitor H89, followed by cAMP stimulation. H89 markedly suppressed levels of p-PKA substrates, p-CREB , and PDK4 protein, suggesting that the hepatic PDK4 level is tightly coupled with the gluconeogenic signaling pathway (Fig. 1*D*). To further confirm PKA-CREB signaling is responsible for PDK4 transcription, we transfected wild-type and mutant CREB, which is mutated at serine 133 to alanine for cytosolic retention, and examined PDK4 promoter activity (Fig. 1*E*). Unlike wild-type CREB, mutant CREB failed to increase PDK4 transcription (Fig. 1*E*). This finding is also concordant with a recent report that direct binding of p-CREB to the PDK4 promoter is responsible for cAMP/PKA induction of PDK4 transcription in stromal fibroblasts (20).

Ablation of Hepatic PDK4 Decreases Hepatic Gluconeogenesis In Vivo

To gain better insight into the role of PDK4 in hepatic gluconeogenesis, adenoviral-mediated shRNA against PDK4 (shPDK4) was injected into HFHS-fed mice. Successful knockdown of PDK4 in the liver was confirmed by a substantial decrease in *Pdk4* mRNA level, whereas the mRNAs for other PDK isoenzymes remained unchanged (Fig. 2*A*). The amount of PDK4 protein in the liver was markedly decreased in shPDK4 mice, whereas PDK4 protein in the adipose tissue was preserved, suggesting specific targeting of the liver (Fig. 2*B*). Surprisingly, the expression levels of the mRNAs for *Pgc-1α*, *G6pase*, and *Pepck* were decreased by liver-specific PDK4 knockdown (Fig. 2*A*). Furthermore, luciferase activity driven by the G6Pase promoter was decreased (Fig. 2*C*). The pyruvate tolerance test confirmed that hepatic gluconeogenesis was attenuated in PDK4-knockdown

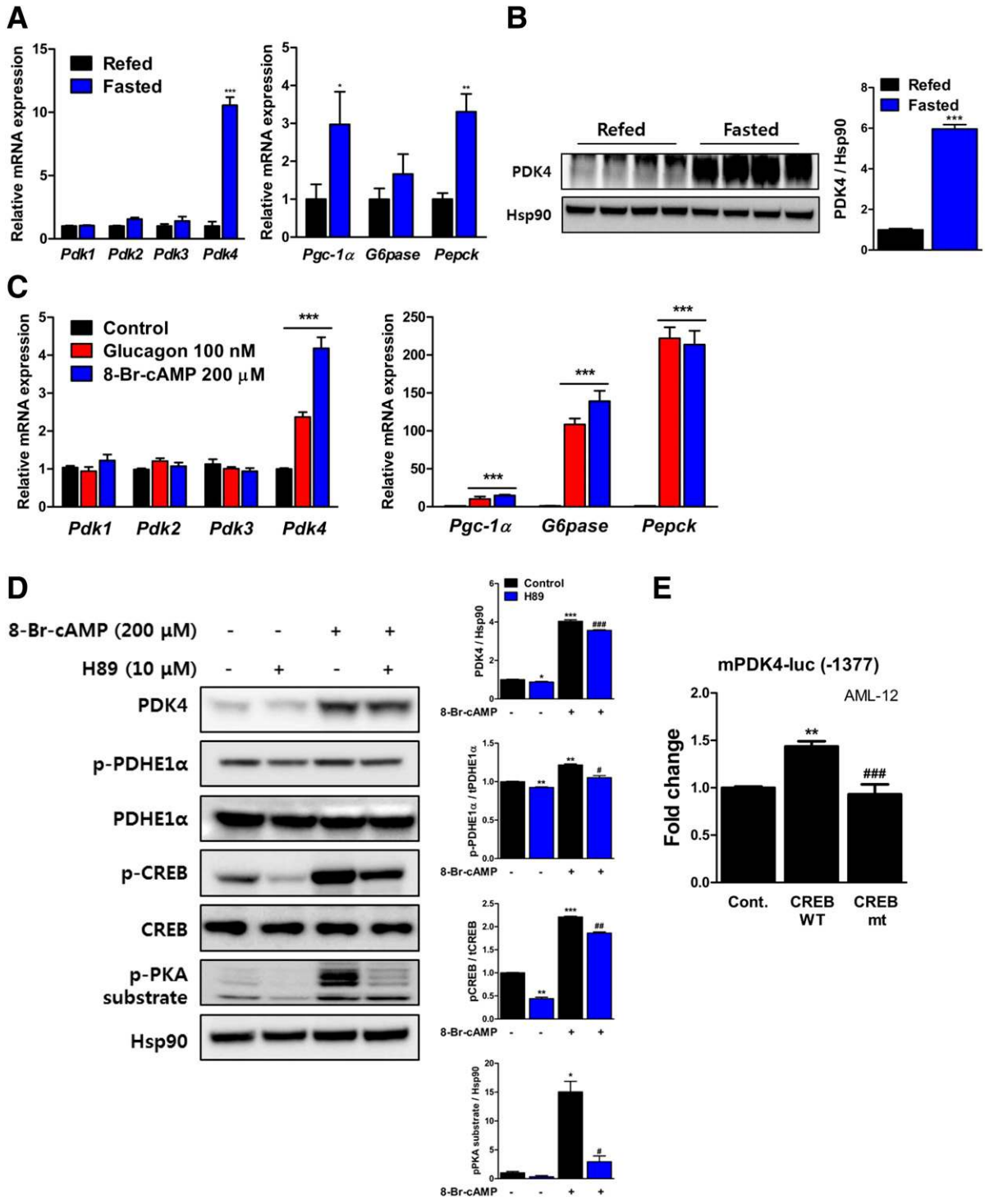

Figure 1—Hepatic PDK4 expression is positively correlated with gluconeogenic signaling. *A*: Relative mRNA expression for PDK isoforms and gluconeogenic genes (*Pgc-1α*, *G6Pase*, and *Pepck*) in the livers of 4-h refed mice and 18-h fasted mice ($n = 6$). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared with refed control. *B*: Western blot data showing PDK4 protein level for the two groups of mice. Hsp90 served as a loading control. $^{***}P < 0.001$ compared with refed control. *C*: mRNA expression for PDK isoforms and gluconeogenic genes (*Pgc-1α*, *G6Pase*, and *Pepck*) in primary mouse hepatocytes exposed to 100 nmol/L of glucagon or 200 μmol/L of 8-Br-cAMP for 4 h. Results are from three independent experiments. The data are given as means ± SEM. $^{***}P < 0.001$ compared with control. *D*: Western blot analysis bar graph showing the effect of PKA inhibitor H89 on protein expression of p-CREB, PDK4, p-PDHE1α (ser 293), and p-PKA substrate in mouse primary hepatocytes treated with or without 8-Br-cAMP for 3 h. H89 was present at a concentration of 10 μmol/L for 2 h before harvest. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared with control. $\#P < 0.05$, $\#\#P < 0.01$, $\#\#\#P < 0.001$ compared with glucagon treated hepatocytes. *E*: PDK4 promoter activity in AML-12 cells transfected with CREB wild type (WT) or CREB mutant (mt). $^{**}P < 0.01$ vs. control, $\#\#\#P < 0.001$ vs. CREB WT.

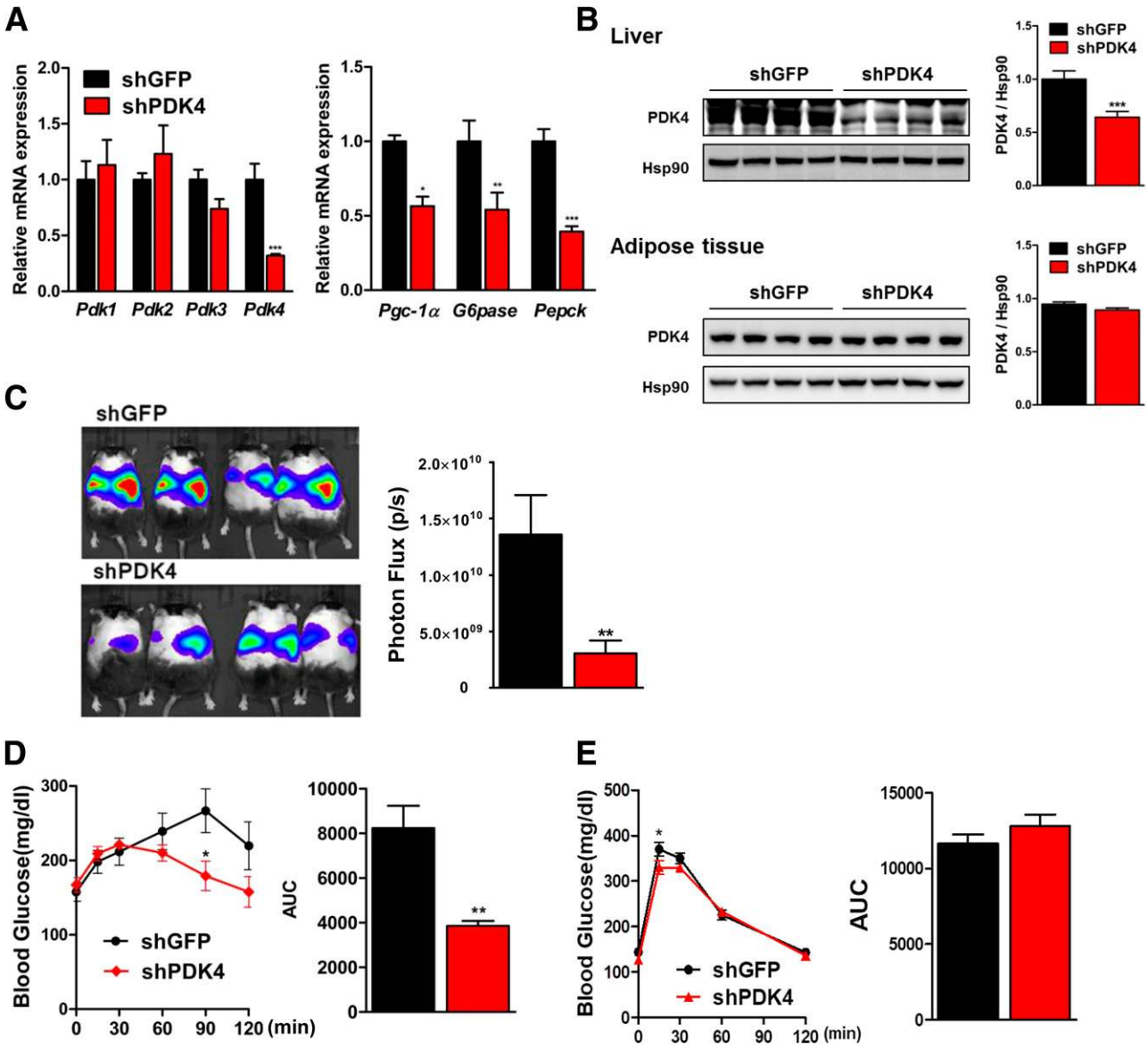

Figure 2—Knockdown of hepatic PDK4 attenuates hepatic gluconeogenesis in HFHS-fed mice. *A*: Relative mRNA expression of PDK isoenzymes and gluconeogenic genes in the livers from mice expressing shGFP and shPDK4. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared with livers from control shGFP mice. HFHS-fed mice were infected with an adenovirus expressing shGFP or shPDK4 at 1×10^9 pfu ($n = 10$). *B*: Protein expression of PDK4 in the liver and adipose tissue in mice expressing shGFP and shPDK4. $^{***}P < 0.001$ compared with control shGFP mice. *C*: In vivo imaging of luciferase activity driven by G6Pase promoter activity in livers. Ad-G6Pase (1.5×10^9 pfu) together with shGFP or shPDK4 was injected, and images were acquired 7 days later. $^{**}P < 0.01$ compared with control shGFP mice. Pyruvate tolerance test (*D*) and glucose tolerance test (*E*) at day 5 after adenovirus injection. Glucose was measured at the indicated times after an intraperitoneal injection of 2 g/kg pyruvate or of 1.5 g/kg glucose into 16-h fasted mice ($n = 6$). AUC, area under the curve. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with control shGFP mice.

mice (Fig. 2*D*). Glucose tolerance test results were not altered by PDK4 deficiency (Fig. 2*E*), ruling out the possibility that improved glucose tolerance contributed to reduced glucose levels in the pyruvate tolerance test. Furthermore, food intake and the lean-to-fat mass ratio were not altered by hepatic PDK4 deficiency (Supplementary Fig. 2*A*).

PDK4 Deficiency Reduces Hepatic Glucose Production by Decreasing cAMP Level

To delineate the underlying molecular mechanism by which PDK4 deficiency inhibits hepatic gluconeogenesis, primary mouse hepatocytes were infected with shPDK4. PDK4-deficient cells showed decreased glucose production (Fig. 3*A*) and blunted gluconeogenic gene expression in response to glucagon (Fig. 3*B*). The amount of p-CREB was diminished by PDK4 knockdown (Fig. 3*C*). Moreover, unlike control (shGFP) hepatocytes, p-CREB did not merge with DAPI staining in shPDK4 hepatocytes under glucagon stimulation, indicating that PDK4 deficiency blocked p-CREB entrance into the nucleus (Fig. 3*D*). Because PKA and CREB are regulated by cAMP, we measured

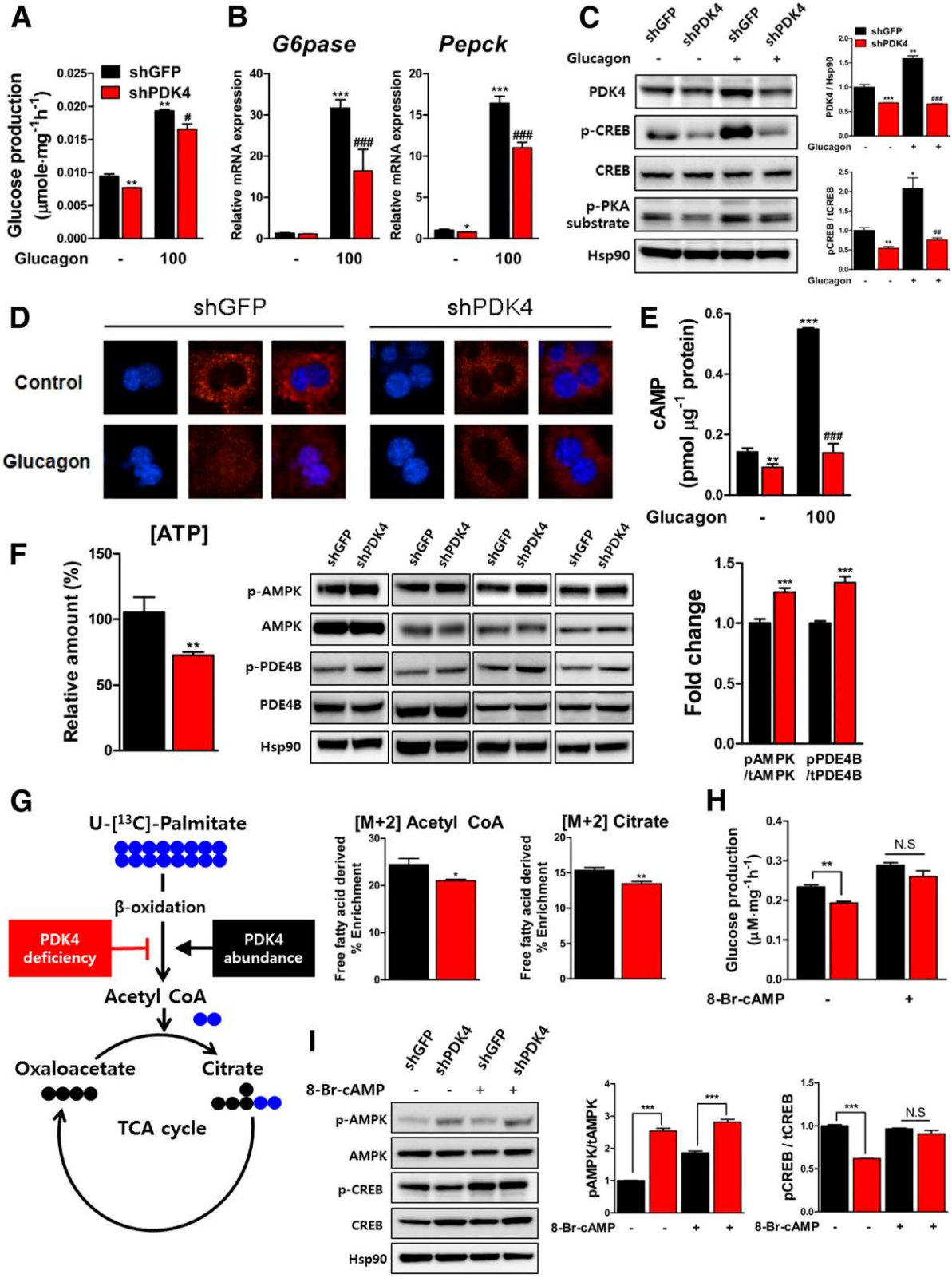

Figure 3—PDK4 deficiency suppresses hepatic gluconeogenesis in mouse primary hepatocytes by attenuating cAMP-PKA-CREB signaling cascade. *A*: Effect of PDK4 suppression on hepatic glucose production in mouse primary hepatocytes exposed to 100 nmol/L glucagon in KRB buffer with 10 mmol/L sodium lactate and 1 mmol/L sodium pyruvate for 4 h. *B*: mRNA expression for gluconeogenic genes in shGFP- or shPDK4-infected primary mouse hepatocytes exposed to 100 nmol/L glucagon for 4 h. *C*: Western blot showing the effect of shPDK4 on CREB phosphorylation in mouse primary hepatocytes exposed to 100 nmol/L of glucagon for 6 h. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared with shGFP control; $\#P < 0.05$, $\#\#P < 0.01$, $\#\#\#P < 0.001$ compared with glucagon-treated shGFP. *D*: Immunofluorescence staining showing the effect of shPDK4 on cytoplasm translocation of CREB in mouse primary hepatocytes exposed to glucagon (100 nmol/L)

cAMP levels and found them decreased in PDK4-deficient primary mouse hepatocytes with or without glucagon stimulation (Fig. 3*E*). PDK4 deficiency also decreased ATP levels and increased p-AMPK levels (Fig. 3*F*). Given that hepatic ATP is primarily synthesized by fatty acid oxidation (FAO), followed by oxidation of acetyl-CoA in the citric acid cycle (21), we asked whether FAO is altered by the amount of PDK4. U-[^{13}C]palmitate-driven acetyl-CoA [M+2] and citrate [M+2] enrichment, which reflect FAO flux (Fig. 3*G*), were significantly decreased by PDK4 deficiency in the cultured primary hepatocyte (Fig. 3*G*). Taken together, these findings suggest that inhibition of PDK4 in the liver is sufficient to inhibit hepatic gluconeogenesis by decreasing cAMP levels and increasing AMPK phosphorylation.

A recent study reported that AMPK activation results in the activation of PDE4B by phosphorylation (22). To examine whether this mechanism should be considered in our working model, we measured Ser304 p-PDE4B by Western blot analysis. p-PDE4B and p-AMPK were positively correlated, suggesting activation of PDE4B by phosphorylation may be responsible for the decrease in cAMP levels in PDK4-deficient hepatocytes (Fig. 3*F*). Furthermore, when hepatocytes were challenged with 8-Br-cAMP rather than glucagon, the reduction in p-CREB signaling and the reduction in hepatic glucose production by PDK4 deficiency were cancelled (Fig. 3*H* and *I*). This finding suggests that the effect of hepatic PDK4 ablation on gluconeogenesis relies on regulation of cAMP levels.

Pharmacologic Inhibition of PDK Attenuates Gluconeogenesis via Downregulation of the cAMP-PKA-CREB Pathway

To examine whether inhibition of hepatic gluconeogenesis could be observed by pharmacologic inhibition of PDK, HFHS-fed mice were treated with 300 mg/kg DCA by daily intraperitoneal injection for 50 days. DCA-treated mice showed significantly decreased 6 h-fasted blood glucose level (Fig. 4*A*). Of note, short-term (7-day) DCA administration was sufficient to decrease blood glucose (Fig. 4*A* and *D*) without affecting body weight and body composition (Supplementary Fig. 2*B*). We therefore conducted the rest of the experiments after the administration of DCA for 7 days. DCA downregulated mRNAs for *Pgc-1α* and *Pepck* but, paradoxically, not *G6pase* (Fig. 4*B*). Concordant with the findings with shPDK4-injected mice, DCA-treated mice showed marked attenuation of luciferase activity driven by the G6Pase promoter (Fig. 4*C*). Pyruvate tolerance test analysis showed that gluconeogenesis was markedly suppressed by DCA (Fig. 4*D*). Unlike shPDK4 mice, DCA treatment also improved glucose tolerance (Fig. 4*E*), presumably by increased PDH-mediated glucose oxidation in the muscle (12), which is consistent with previous findings noted in global PDK2/4 double-KO and PDK4-KO mice (10,23). The beneficial result on pyruvate tolerance by DCA may therefore reflect the combined effects of inhibition of gluconeogenesis in the liver and increased glucose oxidation in muscle.

In primary mouse hepatocytes, DCA inhibited glucose production in a dose-dependent manner (Fig. 5*A*). Increased gluconeogenic gene expression in response to glucagon was attenuated by DCA (Fig. 5*B*). This correlated with decreased levels of p-CREB and the phosphorylation of PKA substrates (Fig. 5*C*) and perturbed nuclear *trans*-localization of p-CREB (Fig. 5*D*). Furthermore, glucagon-stimulated production of cAMP was significantly abrogated by DCA in a dose-dependent manner (Fig. 5*E*). In line with the increased p-AMPK level in response to PDK4 deficiency, DCA treatment increased the p-AMPK level in a dose-dependent fashion (Fig. 5*F*). Furthermore, DCA also increased the p-PDE4B level concordantly with p-AMPK (Fig. 5*F*). We then determined the effects of the PDE4B inhibitor rolipram on DCA-treated hepatocytes. Inhibition of PDE4B not only increased cAMP levels but also restored downregulated PKA-CREB signaling of hepatic glucose production by DCA (Supplementary Fig. 3*A*–*C*).

Forced Upregulation of PDK4 Stimulates Hepatic Gluconeogenesis by Induction of Gluconeogenic Genes

Given that PDK4 expression was increased in *db/db* mice (Supplementary Fig. 1*A*), we generated adenoviruses for overexpression of PDK4 (Ad-PDK4) and a control mock adenovirus (Ad-Mock) to gain further insight into the pathologic role of PDK4 in hepatic gluconeogenesis. Successful hepatic PDK4 overexpression was achieved by tail vein injection of the adenovirus, as evidenced by increased *Pdk4* mRNA (Fig. 6*A*). These mice showed

for 30 min. Red, CREB; blue, DAPI. *E*: Effect of shPDK4 on intracellular cAMP concentration in mouse primary hepatocyte exposed to 100 nmol/L glucagon for 15 min. *F*: The effect of shPDK4 on intracellular ATP level as measured by liquid chromatography–tandem mass spectrometry and AMPK phosphorylation and phosphodiesterase 4B (PDE4B) phosphorylation in mouse primary hepatocytes exposed to glucagon 100 nmol/L for 15 min. All experiments were conducted with three independent tests in four independent samples. shGFP control. Data given in figures correspond to the means ± SEM. $^{**}P < 0.01$, $^{***}P < 0.001$ compared with shGFP control. $^{###}P < 0.001$ compared with glucagon-treated shGFP. *G*: (Left) Schematic diagram of fatty acid flux study. [M+2] Acetyl CoA and [M+2] citrate were driven from U-[^{13}C]sodium palmitate. (Right) The effect of PDK4 suppression on the tricarboxylic acid cycle (TCA) cycle intermediates levels from FAO. Representative TCA cycle intermediates, [M+2] acetyl CoA and [M+2] citrate, production from U-[^{13}C]-palmitate 200 μmol/L and for 3 h before glucagon treatment for 15 min. All experiments were three independent tests, and data are shown as means ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with shGFP control. *H*: Hepatic glucose production of shGFP and shPDK4 mouse primary hepatocyte either unexposed or unexposed with 200 μmol/L of 8-Br-cAMP for 24 h. Data given in figures correspond to the means ± SEM. $^{**}P < 0.01$ compared with control shGFP. *I*: Ratios of p-CREB to total (t)-CREB, p-AMPK to t-AMPK, and p-PDE4B to t-PDE4B levels of shGFP and shPDK4 in mouse primary hepatocytes exposed or unexposed to 200 μmol/L of 8-Br-cAMP for 30 min. $^{***}P < 0.001$ compared with shGFP.

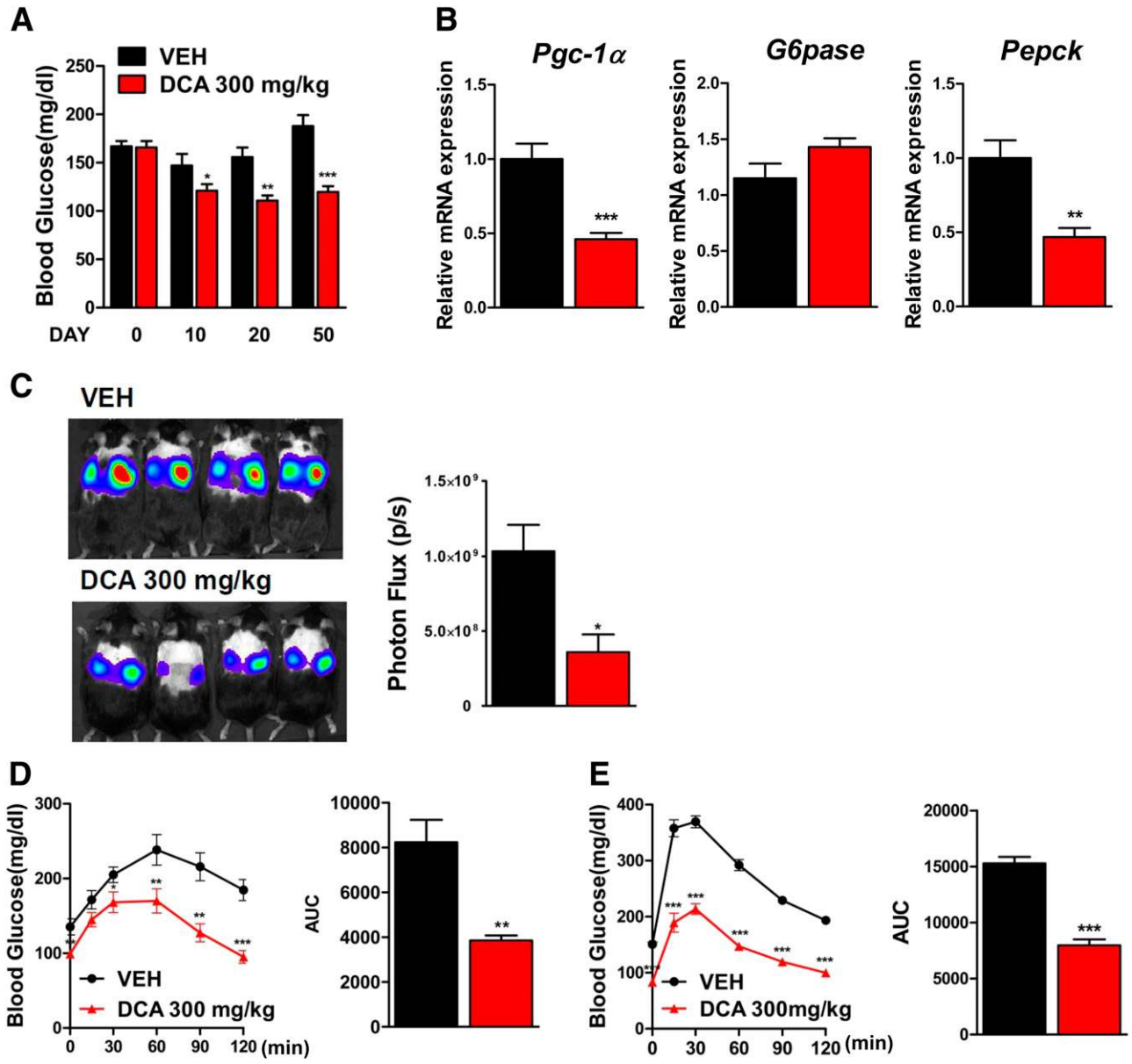

Figure 4—Pharmacologic inhibition of PDK4 ameliorates hyperglycemia in DIO mice. *A*: Blood glucose levels of HFHS-fed mice fasted 6 h and intraperitoneally injected daily with DCA or vehicle (VEH) for 50 days ($n = 7$). *B*: mRNA levels of gluconeogenic genes (*Pgc-1α*, *G6Pase*, and *Pepck*) of HFHS-fed mice intraperitoneally injected daily with DCA or vehicle for 7 days. *C*: In vivo imaging of hepatic G6Pase promoter activity. Ad-G6Pase promoter (1.5×10^9 pfu) was injected, followed by daily DCA injection at 300 mg/kg for 7 days. Images were acquired after 6-h fasting on the last day of DCA injection. Pyruvate tolerance test (*D*) and glucose tolerance test (*E*) at 7 days after of daily intraperitoneal vehicle (VEH) or DCA injection. Mice were fasted for 16 h before the test. Glucose was measured at the indicated times after intraperitoneal injection of pyruvate (2 g/kg) or glucose (1.5 g/kg) ($n = 9$). Data given in figures correspond to means ± SEM. AUC, area under the curve. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared with vehicle control.

significant upregulation of gluconeogenic gene expression (Fig. 6*B*). Importantly, luciferase expression driven by the G6Pase promoter was significantly upregulated in Ad-PDK4–injected mice relative to control mice (Fig. 6*C*). p-PDHE1α–to–total PDHE1α and CREB ratios were increased by PDK4 overexpression as well as phosphorylation of PKA substrates (Fig. 6*D*). These hepatic PDK4-overexpressed mice manifested no appreciable difference compared with control mice in body weight, body composition, or food intake (Supplementary Fig. 2*C*).

PDK4 overexpression in primary mouse hepatocytes also resulted in a significantly higher rate of glucose production (Fig. 7*A*) and gluconeogenic gene expression (Fig. 7*B*). Because FAO flux is decreased by PDK4 deficiency, we checked the carnitine palmitoyltransferase-1 (*Cpt-1*) mRNA level, which was significantly increased by PDK4 overexpression (Fig. 7*B*). In addition, phosphorylation of PKA substrates and p-CREB levels were increased (Fig. 7*C*). Ad-PDK4 hepatocytes showed increased p-CREB nuclear localization as analyzed by immunofluorescence staining

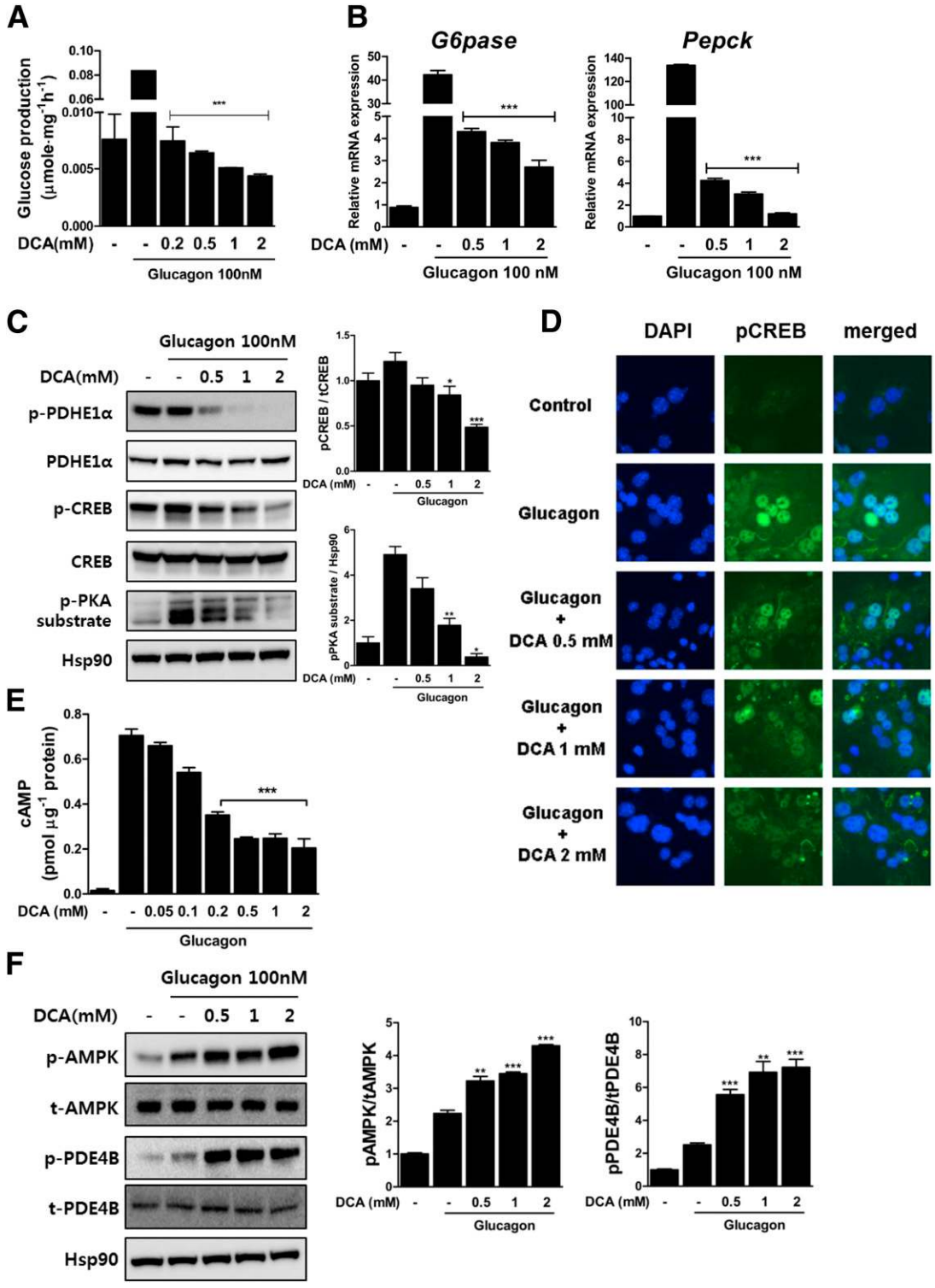

Figure 5— Pharmacologic inhibition of PDK4 attenuates hepatic gluconeogenesis in mouse primary hepatocytes by reduction of the cellular cAMP level. *A*: Effect of DCA on hepatic glucose production exposed to glucagon (100 nmol/L) for 4 h in KRB buffer with 10 mmol/L lactate and 1 mmol/L pyruvate. *B*: Effect of a 16-h pretreatment with DCA on gluconeogenic gene expression in mouse primary hepatocytes exposed to glucagon (100 nmol/L) for 4 h. *C*: Western blot showing the effect of a 16-h pretreatment with DCA on PKA-CREB signaling in mouse primary hepatocytes exposed to glucagon (100 nmol/L) for 30 min. *D*: Immunofluorescence staining showing the effect of a 16-h pretreatment with DCA on subcellular localization of p-CREB in mouse primary hepatocytes exposed to glucagon (100 nmol/L) for 30 min. Green, CREB; blue, DAPI. *E*: Effect of DCA on intracellular cAMP concentration in mouse primary hepatocytes exposed to glucagon (100 nmol/L) for 15 min. *F*: Western blot showing p-AMPK and p-PDE4B compared with total (t)-AMPK and t-PDE4B. Indicated dose of DCA was treated for 16 h before glucagon (100 nmol/L) treatment in mouse primary hepatocyte. All experiments were conducted with three independent tests. Data given in figures correspond to means ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared with glucagon-treated hepatocytes.

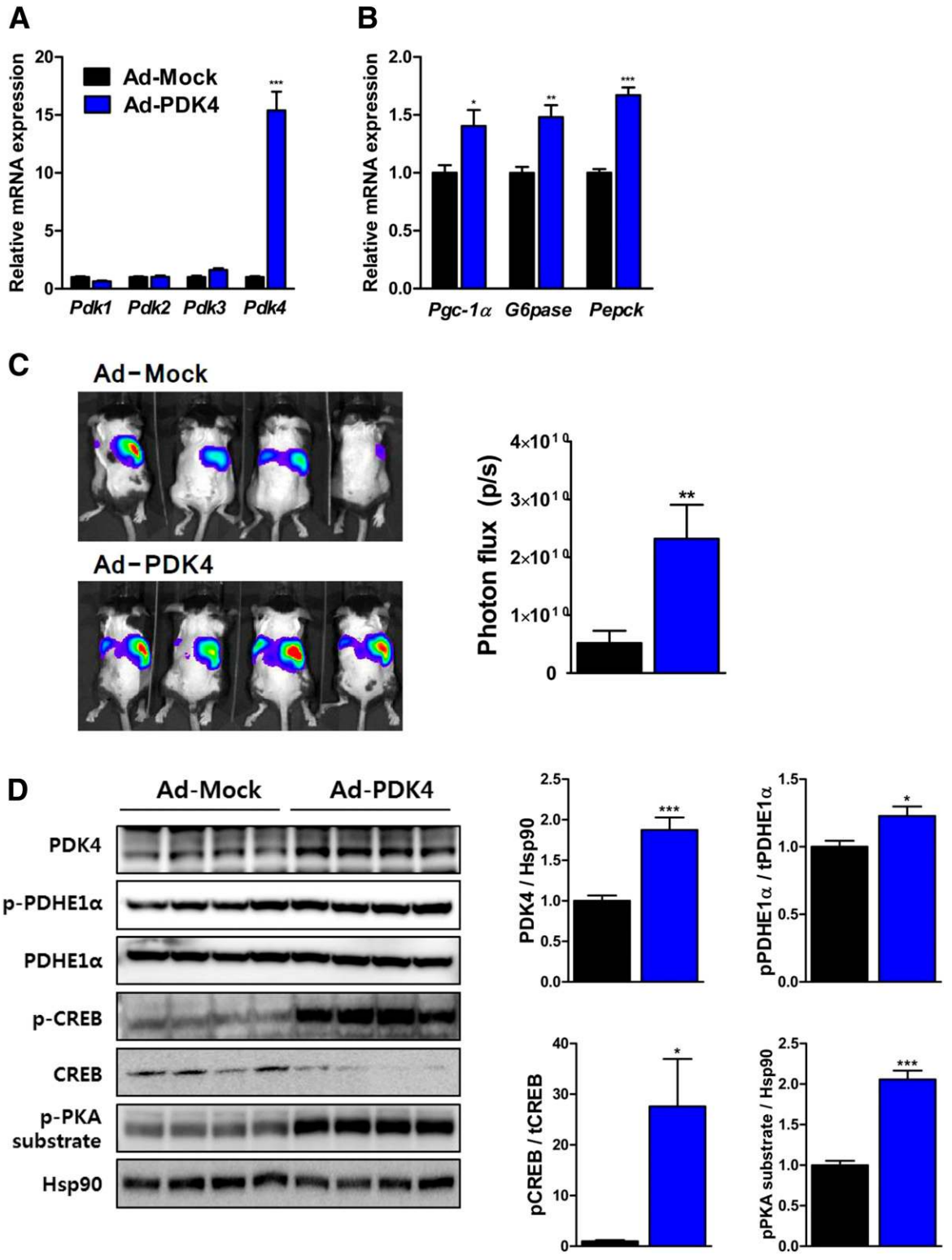

Figure 6—Overexpression of PDK4 in the liver increases hepatic gluconeogenesis in vivo. Relative mRNA expression of PDK isoenzymes (*A*) and gluconeogenic genes (*B*) in the livers of Ad-Mock– or Ad-PDK4–injected wild-type mice fed a chow diet. Ad-Mock or Ad-PDK4 (1.5×10^9 pfu) was injected daily for 5 days. (n = 10–11). *C*: In vivo imaging of hepatic G6Pase promoter activity. Ad-G6Pase promoter and Ad-Mock or Ad-PDK4 (1.5×10^9 pfu) was injected, and images were acquired 7 days later. *D*: Western blot data of PKA-CREB signaling in the liver of Ad-Mock– or Ad-PDK4–injected wild-type mice fed a chow diet. t, total. Data given in figures correspond to means ± SEM. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared with Ad-Mock control.

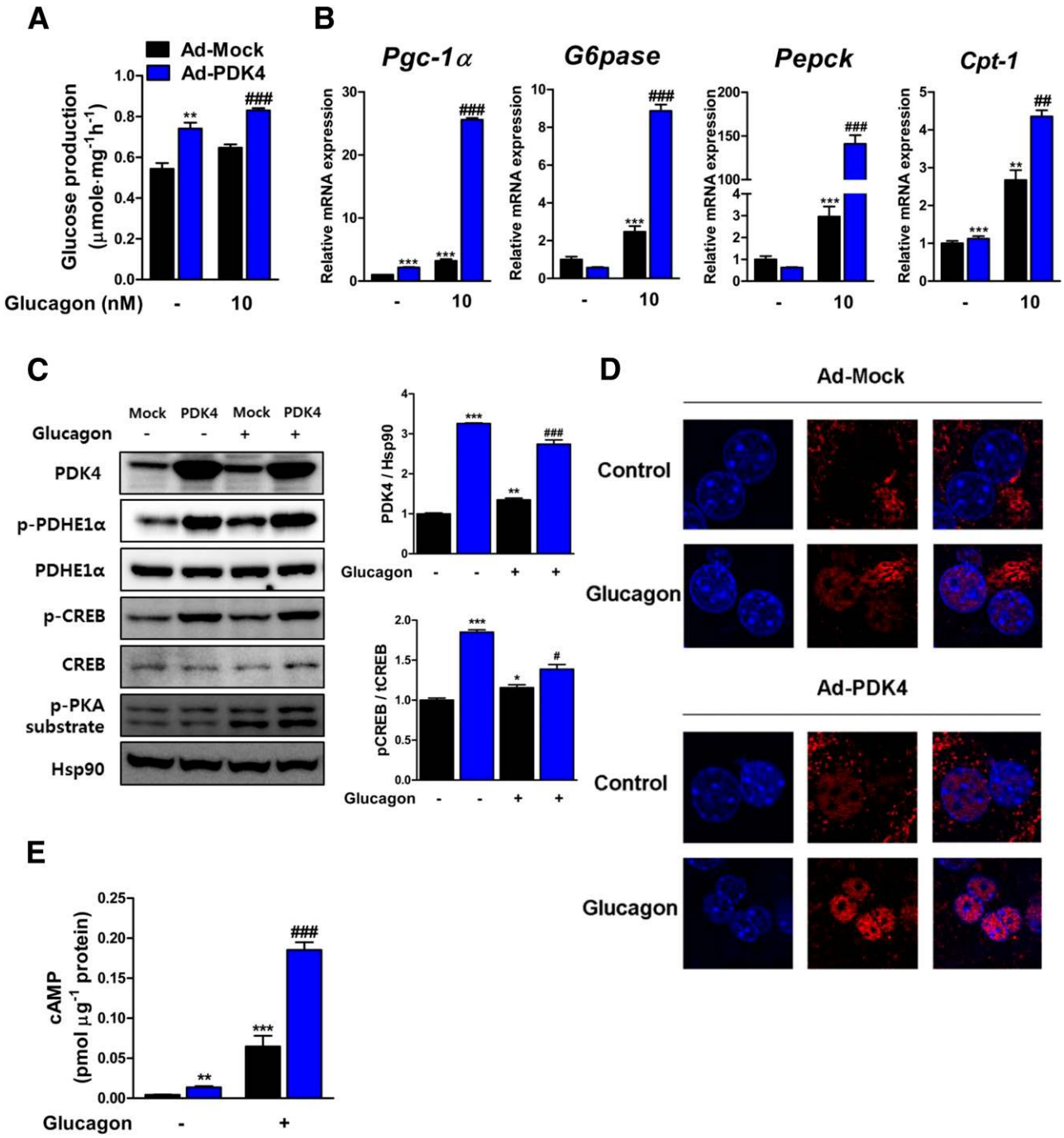

Figure 7—Forced expression of PDK4 promotes gluconeogenesis in mouse primary hepatocytes. *A*: The effect of PDK4 on hepatic glucose production in mouse primary hepatocytes, with or without exposure to 10 nmol/L of glucagon in KRB buffer, together with 10 mmol/L of sodium lactate, 1 mmol/L of sodium pyruvate, and 200 μmol/L sodium palmitate for 4 h. *B*: Real-time PCR analysis showing the effect of Ad-PDK4 against Ad-Mock (control) on gluconeogenic genes (*G6pase, Pepck*) and FAO gene (*Cpt-1*) expression in mouse primary hepatocytes after glucagon treatment for 4 h. *C*: Western blot data showing the effect of Ad-PDK4 on PKA-CREB signaling in mouse primary hepatocytes with or without exposure to glucagon (10 nmol/L) for 6 h. t, total. *D*: Immunofluorescence staining showing the effect of Ad-PDK4 on subcellular localization of p-CREB in mouse primary hepatocytes exposed to glucagon 10 nmol/L for 30 min. Red, CREB; blue, DAPI. *E*: The effect of PDK4 on intracellular cAMP concentration in mouse primary hepatocytes with or without exposure to 10 nmol/L glucagon for 15 min. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ compared with Ad-Mock control. #$P < 0.05$, ##$P < 0.01$, ###$P < 0.001$ compared with glucagon-treated Ad-Mock.

(Fig. 7*D*). These findings were a consequence of the increased cAMP level by PDK4 (Fig. 7*E*). Intriguingly, PDK4-induced cAMP increment and subsequent glucose production were also observed without glucagon stimulation, suggesting that PDK4 overexpression overrides the need for hormonal stimuli (Fig. 7*A* and *E*). To further

address the pathologic role of facilitated cAMP-PKA-CREB signaling, we compared this signaling pathway in *db/+* and *db/db* mice. Livers from *db/db* mice showed augmented PKA-CREB signaling (Supplementary Fig. 1*B*) and contained higher cAMP concentrations (Supplementary Fig. 1*C*), wherein PDK4 levels were increased (Supplementary Fig. 1*A*). To further address the role of hepatic PDK4, we knocked down PDK4 in *db/db* mice (Supplementary Fig. 1*D* and *E*). PDK4 deficiency in the liver decreased the cAMP level in the liver without affecting the plasma glucagon level per se. Taken together with Figs. 3 and 5, the findings suggest that the glucagon antagonizing effect of PDK4 deficiency is achieved by acceleration of cAMP degradation without modulation of plasma glucagon level.

PDK4 Modulates cAMP Level and Gluconeogenic Signaling by Promoting FAO

Because *Cpt-1* mRNA expression was increased by PDK4 (Fig. 7*B*), and FAO flux was decreased by PDK4 deficiency in the cultured primary hepatocytes (Fig. 3*G*), we hypothesized that the effect of PDK4 on gluconeogenesis might be dependent on its ability to modulate FAO. Acetyl-CoA [M+2] and citrate [M+2] from U-[^{13}C]palmitate were enriched by PDK4 overexpression in the cultured primary hepatocyte, confirming that increasing PDK4 abundance increases the FAO rate (Fig. 8*A*). The increase in FAO flux caused by PDK4 overexpression was normalized in the presence of the CPT-1 inhibitor etomoxir (Fig. 8*A*). Indeed, the glucose production rate correlated with FAO; that is, it was blocked by etomoxir (Fig. 8*B*). The PDK4-induced increase in ATP was cancelled by etomoxir, suggesting that increased ATP production in PDK4-overexpressed hepatocytes was driven by increased FAO (Fig. 8*C*). As expected, etomoxir completely eliminated the effect of PDK4 overexpression on the cAMP increment (Fig. 8*D*). These effects were also observed in control hepatocytes, suggesting that FAO-driven energy production is required for hepatic gluconeogenesis (Fig. 8*B–D*). We further investigated the causal relationship between FAO flux and the cAMP level by detecting changes in p-AMPK and p-PDE4B levels after CPT-1 inhibition. Inhibition of FAO restored p-AMPK and p-PDE4B levels as a result of the decreased ATP level and the corresponding increase in the AMP-to-ATP ratio (Fig. 8*E*). Collectively, these findings suggest that the effect of PDK4 on potentiation of cAMP-PKA-CREB signaling and inhibition of AMPK phosphorylation is largely dependent upon its ability to increase FAO flux and ATP synthesis during gluconeogenesis.

DISCUSSION

In the current study, we identified a novel and unexpected role of PDK4 in hepatic gluconeogenesis. Our results show that hepatic PDK4 expression is enhanced by glucagon or cAMP treatment of hepatocytes and that overexpression of PDK4 stimulates the cAMP-PKA-CREB pathway and, therefore, glucose production. Conversely, pharmacologic inhibition of PDK4 in vitro and in vivo reduces the expression of gluconeogenic genes and, therefore, hepatic glucose production. Importantly, we demonstrate that the effect of PDK4 on hepatic gluconeogenesis is largely dependent on modulating the cytosolic cAMP concentration. These changes were tightly coupled with the rate of FAO. Given that FAO coupled to the citric acid cycle is the primary source of ATP for hepatic gluconeogenesis, it can be concluded that hepatic PDK4 plays a critical role in fuel selection during gluconeogenesis, which controls the overall rate of the gluconeogenic pathway, including cAMP levels, PKA-CREB signaling, and gluconeogenic enzyme expression (Supplementary Fig. 4; graphical summary). Interestingly, inhibition of FAO by hepatic PDK4 deficiency did not affect the liver triglyceride level (Supplementary Fig. 2). In addition to the decrease in *Cpt-1* expression, mRNA expression of lipogenesis-related genes, such as *Pparγ*, *Scd1*, and *Acc* was simultaneously downregulated in PDK4-deficient hepatocytes (data not shown).

The role of PDK4 in diabetes or insulin resistance was previously discussed in the context of its role in shutting down the pyruvate dehydrogenase complex to conserve the gluconeogenic substrates alanine, pyruvate, and lactate (6,24). Remarkable upregulation of PDK4 in skeletal muscle in response to starvation or diabetes increases the delivery of these gluconeogenic substrates to the liver. Here, however, we found that PDK4 also affects the rate of gluconeogenesis by modulating the enzymatic capacity for gluconeogenesis and FAO.

A dramatic decrease in the phosphorylated form of CREB in response to PDK4 deficiency was the most surprising and important observations of this study. The significance of CREB in the regulation of hepatic gluconeogenesis is well established (3). Liver-specific deficiency of p-CREB reduces blood glucose levels, an apparent consequence of reduced expression of gluconeogenic genes (2). Our results show that besides the known effects on the expression of gluconeogenic genes, inhibition of PKA-CREB signaling reduced PDK4 protein levels in hepatocytes (Fig. 1*D*). Because p-CREB has been shown to bind and activate the PDK4 promoter in fibroblasts (20), this finding is presumably due to reduced transcription of the PDK4 gene by p-CREB deficiency. Conversely, in tumor cells, PDK4 was reported to physically bind to CREB and maintain CREB stability (25). However, no effect of PDK4 deficiency on the amount of CREB was found in this study with normal cells, and mutant CREB did not increase promoter activity of PDK4 in the hepatocytes, suggesting a critical role of p-CREB in PDK4 gene transcription (Fig. 1*E*).

Glucagon stimulates hepatic gluconeogenesis by increasing cAMP levels. Metformin antagonizes glucagon signaling by inducing the accumulation of AMP, which lowers cAMP independently of AMPK by direct inhibition of adenylyl cyclase (26) and dependently on

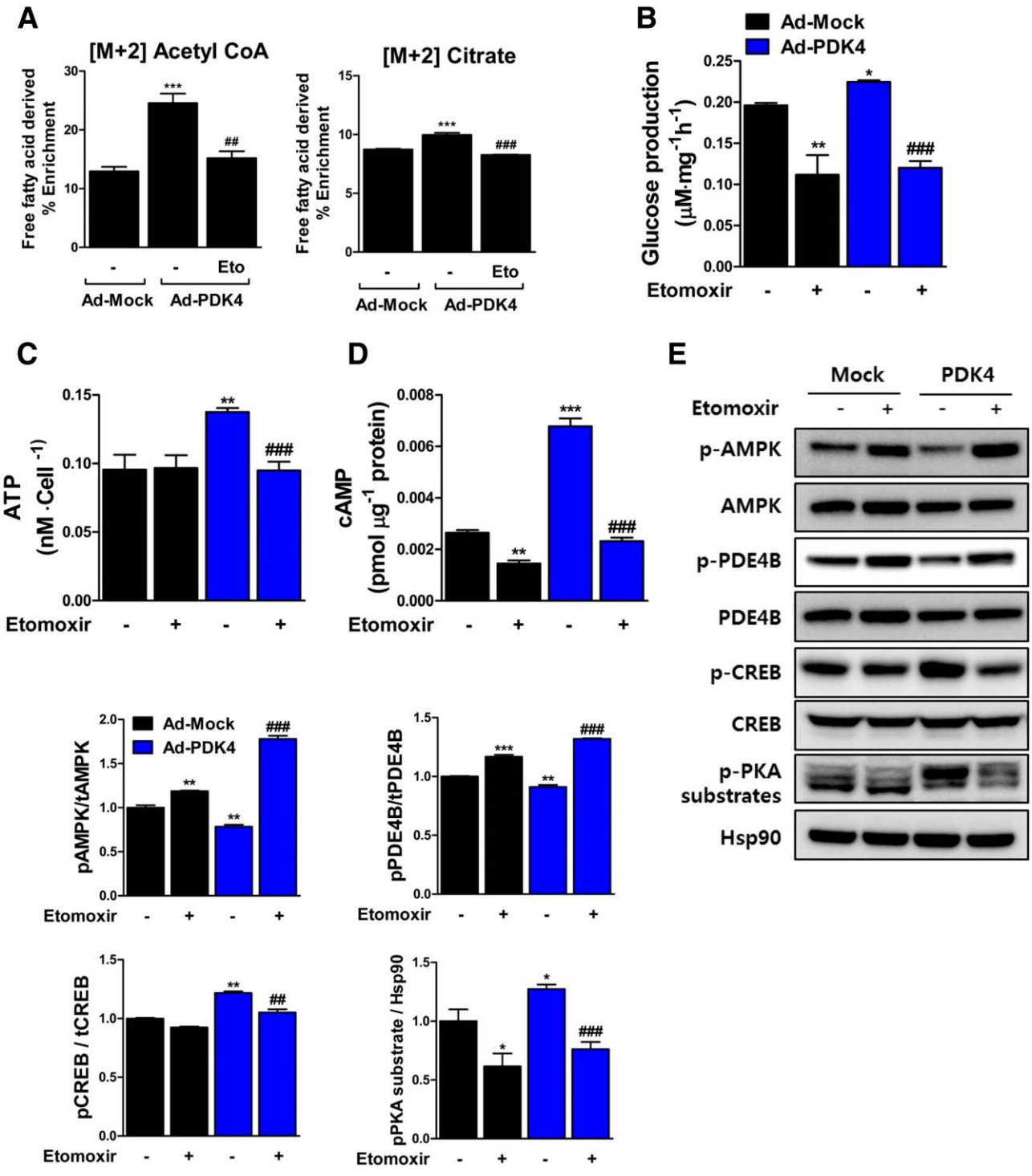

Figure 8—PDK4 mediates FAO rate during gluconeogenesis to modulate cAMP level and gluconeogenic signaling. *A*: The effect of PDK4 on tricarboxylic acid cycle (TCA) cycle intermediates levels from FAO. Representative TCA cycle intermediates, [M+2] acetyl-CoA (left) and [M+2] citrate (right), enriched by U-[^{13}C]palmitate (200 μmol/L) and etomoxir (Eto) (30 μmol/L) cotreatment for 3 h before glucagon treatment for 15 min. *B*: The effect of etomoxir on hepatic glucose production in mouse primary hepatocytes cultured in KRB buffer with sodium lactate (10 mmol/L), sodium pyruvate (1 mmol/L), and glucagon (10 nmol/L) for 4 h. Treatment with etomoxir (30 μmol/L) for 3 h before cell harvest. Quantification using total protein amounts. Intracellular ATP level (*C*) and cAMP level (*D*) in mouse primary hepatocytes. Pretreatment with etomoxir (30 μmol/L) for 3 h before exposed to glucagon (10 nmol/L) for 15 min. *E*: Western blot data showing AMPK (at Thr172 site), PDE4B (at Ser304 site), PKA substrates, and CREB phosphorylation in mouse primary hepatocyte. Pretreatment with etomoxir (30 μmol/L) for 3 h before exposure to glucagon (10 nmol/L) for 30 min. Data corresponds to means ± SEM. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ compared with Ad-Mock control; ##$P < 0.01$, ###$P < 0.001$ compared with Ad-PDK4 control.

AMPK by activation of PDE4B (22).We show here that PDK4 deficiency likewise antagonizes glucagon signaling by inducing the accumulation of AMP, which lowers cAMP. Although direct inhibition of adenylyl cyclase by AMP may be involved, we document activation of PDE4B.

Altered production of ATP by FAO in response to the level of PDK4 expression is responsible for changes in AMP levels, given that FAO and glucose oxidation reciprocally regulate each another (27) and that PDK is an important negative regulator of glucose oxidation. This is further supported by the recent finding that acetamiprid, the insecticide, suppresses testosterone synthesis by decreasing testicular ATP level, and in turn, cAMP level and StAR expression, which is downstream of CREB (28). A manipulation that restored ATP synthesis also recovered cAMP level (28). This finding, in accordance with our finding, suggests that the cAMP level can be tightly coupled with the rate of ATP synthesis, and in turn, affect the PKA-CREB pathway.

One of the unexpected findings was the relationship between PDK4 and AMPK. Others have noted that intensification of the cAMP-PKA pathway by cAMP treatment increases ATP production and thereby decreases AMPK phosphorylation in Leydig cells (29). The 8-Br-cAMP dose dependently increased cellular ATP levels, and p-AMPK was reciprocally decreased in this model (29). This finding strongly suggests that the gluconeogenic and steroidogenic pathways, both of which require activation of PKA-CREB signaling, positively affect ATP synthesis and thereby negatively affect phosphorylation of AMPK.

We show preclinical evidence that PDK4 is an important therapeutic target of diabetes. Although novel small molecules targeting PDK2 are under development (30), no drug that specifically targets PDK4 is available.

Acknowledgments. The authors thank Dr. Xiaocheng C. Dong (Indiana University School of Medicine, Indianapolis, IN) for providing shGFP- and shPDK4-expressing adenovirus and Dr. Mark H. Rider (Université catholique de Louvain and de Duve Institute, Brussels, Belgium) for providing the Ser304 p-PDE4B antibody.

Funding. This research was supported by the Korea Health Technology R&D Project through the Korea Health Industry Development Institute, funded by the Ministry of Health and Welfare (MOHW), Republic of Korea (HI16C1501); Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Science and ICT (NRF-2017R1A2B3006406 and NRF-2016R1D1A1B03935408); and the Bio & Medical Technology Development Program of NRF and funded by the Korean government (Ministry of Science, ICT and Future Planning and MOHW) (NRF-2016M3A9B6902872).

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

Author Contributions. B.-Y.P., J.-H.J., Y.G., R.A.H., and I.-K.L. generated the hypothesis, designed the experiments, and wrote the manuscript. B.-Y.P., Y.G., H.J.H., J.-E.K., E.K.Y., W.H.K., and Y.H.J. performed the experiments. N.-H.J., S.-H.K., B.-G.K., L.H., K.-G.P., R.A.H., and I.-K.L. analyzed and discussed the data. I.-K.L. 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.

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