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Inhibition of Pyruvate Dehydrogenase Kinase 2 Protects Against Hepatic Steatosis Through Modulation of Tricarboxylic Acid Cycle Anaplerosis and Ketogenesis

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Hepatic steatosis is associated with increased insulin resistance and tricarboxylic acid (TCA) cycle flux, but decreased ketogenesis and pyruvate dehydrogenase complex (PDC) flux. This study examined whether hepatic PDC activation by inhibition of pyruvate dehydrogenase kinase 2 (PDK2) ameliorates these metabolic abnormalities. Wild-type mice fed a high-fat diet exhibited hepatic steatosis, insulin resistance, and increased levels of pyruvate, TCA cycle intermediates, and malonyl-CoA but reduced ketogenesis and PDC activity due to PDK2 induction. Hepatic PDC activation by PDK2 inhibition attenuated hepatic steatosis, improved hepatic insulin sensitivity, reduced hepatic glucose production, increased capacity for β-oxidation and ketogenesis, and decreased the capacity for lipogenesis. These results were attributed to altered enzymatic capacities and a reduction in TCA anaplerosis that limited the availability of oxaloacetate for the TCA cycle, which promoted ketogenesis. The current study reports that increasing hepatic PDC activity by inhibition of PDK2 ameliorates hepatic steatosis and insulin sensitivity by regulating TCA cycle anaplerosis and ketogenesis. The findings suggest PDK2 is a potential therapeutic target for nonalcoholic fatty liver disease.

Hepatic steatosis is induced by a number of lipid metabolic abnormalities, including increased de novo lipogenesis, inhibited triacylglycerol (TG) release, enhanced fatty acid (FA) influx from adipose tissue, and reduced hepatic FA oxidation and ketogenesis (1). Recently, it was proposed that dysregulation of ketone body metabolism could potentially contribute to the pathogenesis of nonalcoholic fatty liver disease (NAFLD). Reduced ketogenesis exacerbates diet-induced hepatic steatosis and hyperglycemia (2), and a ketotic diet reduces the body weight of mice as much as a calorie-restricted diet (3). However, the precise mechanism by which impaired ketogenesis contributes to hepatic steatosis is still unclear.

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In the well-fed state, acetyl-CoA produced from glucose is converted to FAs for storage of excess energy or oxidized to CO_2 in the tricarboxylic acid (TCA) cycle to generate ATP by oxidative phosphorylation. In the fasting state, two-thirds of the free FAs entering the liver are converted to acetyl-CoA by β-oxidation and then further processed to ketone bodies, which act as an energy source for peripheral tissues. The other one-third of FAs is used by the TCA cycle to generate ATP to meet hepatic energy demands (4). Whether acetyl-CoA produced by β-oxidation forms ketone bodies or enters the TCA cycle is determined by anaplerotic influx of TCA cycle intermediates. The conversion of pyruvate to oxaloacetate (OAA) by pyruvate carboxylase (PC) is one of the most important sources of anaplerosis in the liver.

Pyruvate can also be converted to acetyl-CoA by oxidative decarboxylation mediated by the pyruvate dehydrogenase complex (PDC). PDC activity is inhibited via phosphorylation of the pyruvate dehydrogenase E1α subunit (PDHE1α), which is mediated by increased expression of pyruvate dehydrogenase kinases (PDKs) during fasting or in an insulin-resistant state (5–7). Four isoforms of PDK (PDK1–4) are expressed in a tissue-specific manner, with unique expression profiles in response to different physiological conditions (8). Among these isoforms, PDK2 is the major PDK responsible for regulation of PDC activity in the liver (8–10).

Recently, it was reported that decreased PDC activity and enhanced pyruvate carboxylation due to hepatic insulin resistance contribute to increased hepatic gluconeogenesis in obese subjects with hepatic steatosis (11,12). Because of competition for pyruvate, the balance between PDC and PC activity may play a critical role in metabolic dysfunction caused by obesity and insulin resistance. In this study, we examined the possibility that activation of PDC by PDK2 inhibition reduces the anaplerotic flux of pyruvate into the TCA cycle, which may increase ketogenesis and prevent hepatic steatosis induced by a high-fat diet (HFD). Indeed, we found that PDK2 inhibition ameliorates hepatic steatosis and insulin resistance, suggesting that PDK2 is a potential therapeutic target for NAFLD.

RESEARCH DESIGN AND METHODS

Animal Experiments

All experiments were approved by the Institutional Animal Care and Use Committee of Kyungpook National University. For the NAFLD mice model, 8-week-old male wild-type (WT) (C57BL/6J) and PDK2 knockout (KO) mice (13) were fed an HFD in which 20% of the calories were derived from carbohydrates and 60% from fat (D12492 pellets; Research Diets). Control WT and PDK2 KO mice were fed an isocaloric low-fat diet (LFD) in which 70% of the calories were derived from carbohydrates and 10% from fat (D12450B pellets; Research Diets). The mice were housed and maintained on a 12-h light/dark cycle at 22 ± 2°C. After the mice were sacrificed, tissues were rapidly collected and freeze-clamped with Wollenberger tongs at the temperature of liquid nitrogen and stored at −80°C for analysis.

Construction of PDK2 Dominant-Negative Recombinant Adenovirus and Its Infection In Vivo

pCMV6-Kan/Neo mouse PDK2 plasmid was purchased from OriGene. The PDK2 dominant-negative (DN) mutant R157A (14,15) was generated by site-directed mutagenesis with the QuickChange II site-directed mutagenesis kit (Stratagene) using the following primers: forward, 5′-CCA GTACTTCCTGGACGCCTTCTACCTCAGC-3′ and reverse, 5′-GCTGAGGTAGAAGGCGTCCAGGAAGTACTGG-3′. Recombinant adenovirus for PDK2 DN mutant was generated using pAd-Track-CMV shuttle vector as described previously (16). Recombinant PDK2 DN and green fluorescent protein (GFP) adenovirus were amplified in AD-293 cells and purified by CsCl gradient centrifugation. Adenovirus titers were determined using Adeno-X rapid titer (BD Biosciences) according to the manufacturer's instructions. After 12 weeks of HFD feeding in WT mice, recombinant adenoviruses (1.0×10^9 plaque-forming units) expressing PDK2 DN and GFP constructs were delivered by tail vein injection. On day 3 after adenovirus infection, glucose tolerance tests were performed in overnight-fasted mice by intraperitoneal injection of glucose (1 g/kg body weight). On day 7 after adenovirus infection, mice were sacrificed after overnight fasting. After the mice were sacrificed, tissues were rapidly collected and freeze-clamped with Wollenberger tongs at the temperature of liquid nitrogen.

Measurement of PDC Activity

PDC activity was spectrophotometrically measured in a 96-well plate reader by coupling to the reaction catalyzed by arylamineacetyltransferase, as described previously (7). One unit of PDC activity corresponds to the acetylation of 1 μmol of *p*-(*p*-aminophenyl azo) benzenesulfonate per min at 30°C.

Statistical Analysis

Statistical significance was determined by the unpaired Student *t* test when two groups were compared. Values are presented as the means ± SEM of the indicated number of independent samples. *P* values <0.05 were considered statistically significant.

See the Supplementary Data for additional details.

RESULTS

Upregulation of PDK2 Reduces Hepatic PDC Activity in HFD-Fed Mice

PDK2 expression is increased in the livers of obese animals (9,17), but its role in the development of hepatic metabolic diseases remains unclear. We examined PDC activity and the expression of PDK isoenzymes in the livers of WT mice fed an HFD. As shown in Fig. 1*A*, PDC activity was significantly lower in the livers of mice fed an HFD than in those of mice fed an LFD. The reduction in hepatic PDC activity was associated with significant increases in the phosphorylation of PDHE1α (Fig. 1*B*). *PDK2* mRNA levels were significantly higher in the livers of HFD-fed mice compared with LFD-fed mice (Fig. 1*C*). Conversely, the level of the *PDK4*, which is induced by an HFD in skeletal muscle and

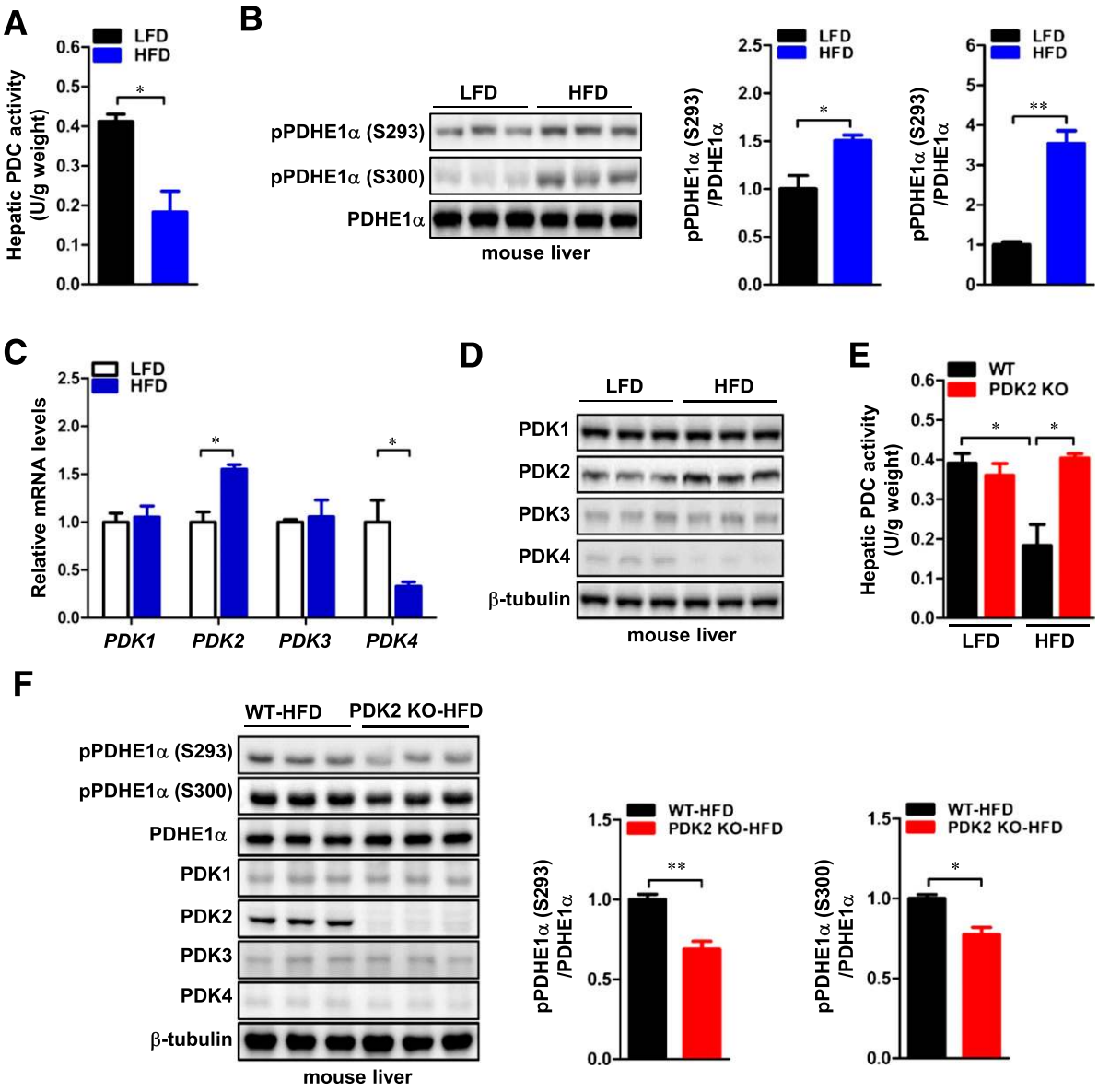

Figure 1—HFD reduces hepatic PDC activity by upregulation of PDK2. *A*: Hepatic PDC activity in overnight-fasted WT mice fed an LFD or HFD for 16 weeks ($n = 5$). *B*: Phosphorylation (p) state of PDHE1α in the livers obtained from the mice in *A*. The bar graphs on the right show quantification of PDHE1α phosphorylation. Hepatic expression of mRNA (*C*) and protein (*D*) of PDK isoenzymes in HFD-fed mice ($n = 5$). *E*: Hepatic PDC activity in overnight-fasted WT and PDK2 KO mice fed an LFD or an HFD for 16 weeks ($n = 5$). *F*: Phosphorylation of PDHE1α and expression of PDK isoforms in the liver of HFD-fed WT and HFD-fed PDK2 KO mice (*E*). The bar graphs on the right show quantification of PDHE1α phosphorylation. Data are presented as the mean ± SEM. $^*P < 0.05$; $^{**}P < 0.01$.

heart (5), was markedly decreased in the livers of HFD-fed mice (Fig. 1*C*). The protein levels of PDK2 and PDK4 showed the same pattern as their respective mRNAs (Fig. 1*D*). To confirm that PDK2 regulates hepatic PDC activity in HFD-fed mice, PDC activity and phosphorylation of PDHE1α were measured in the livers of WT and PDK2 KO mice. As expected from the data shown in Fig. 1*A*, the HFD reduced hepatic PDC activity in WT mice but not in PDK2 KO mice (Fig. 1*E*). Consistent with this finding, PDHE1α phosphorylation was decreased in PDK2 KO mice compared with WT mice (Fig. 1*F*). To examine PDC activity change by PDK2 deficiency in other tissue, we measured the phosphorylation state of PDHE1α in muscle, heart, and kidney. Phosphorylation of PDHE1α was similar between HFD-fed PDK2 KO mice and HFD-fed WT mice in these tissues (Supplementary Fig. 1). The serum levels of pyruvate, lactate, and free FA were not different between WT and PDK2 KO mice after HFD feeding (Supplementary Fig. 2). These findings indicate that PDK2 is a major regulator of PDC activity in the liver of HFD-fed mice.

PDK2 Deficiency Prevents HFD-Induced Hepatic Steatosis

To elucidate the role of PDK2 in the development of hepatic steatosis, WT and PDK2 KO mice were fed an

HFD to induce NAFLD. After 16 weeks of HFD feeding, body weight gain in HFD-fed PDK2 KO mice was significantly lower than that of HFD-fed WT mice (Fig. 2*A*). Even though the body weights of HFD-fed PDK2 KO mice were lower than those of WT mice, there were no differences in food consumption or physical activity (Supplementary Fig. 3*A* and *B*). Relative to HFD-fed WT mice, HFD-fed PDK2 KO mice exhibited greater energy expenditure (Supplementary Fig. 3*C*).

The liver/body weight ratio of PDK2 KO mice fed an HFD was significantly lower than that of WT mice (Fig. 2*B*). The number and the size of the lipid droplets were greatly decreased in livers of PDK2 KO mice fed an HFD (Fig. 2*C*). However, no difference in phenotype in epididymal adipose tissue was observed between WT and PDK2 KO mice on either diet (Fig. 2*C*). Consistent with the histological analysis, the hepatic TG content was significantly lower in PDK2 KO mice than in WT mice after HFD feeding (Fig. 2*D*). The level of hepatic glycogen was also significantly reduced in PDK2 KO mice fed an HFD compare with WT mice fed an HFD (Fig. 2*E*). Serum activities of aspartate aminotransferase and alanine aminotransferase, markers of liver injury, were also significantly elevated in WT mice fed an HFD, but not in PDK2 KO

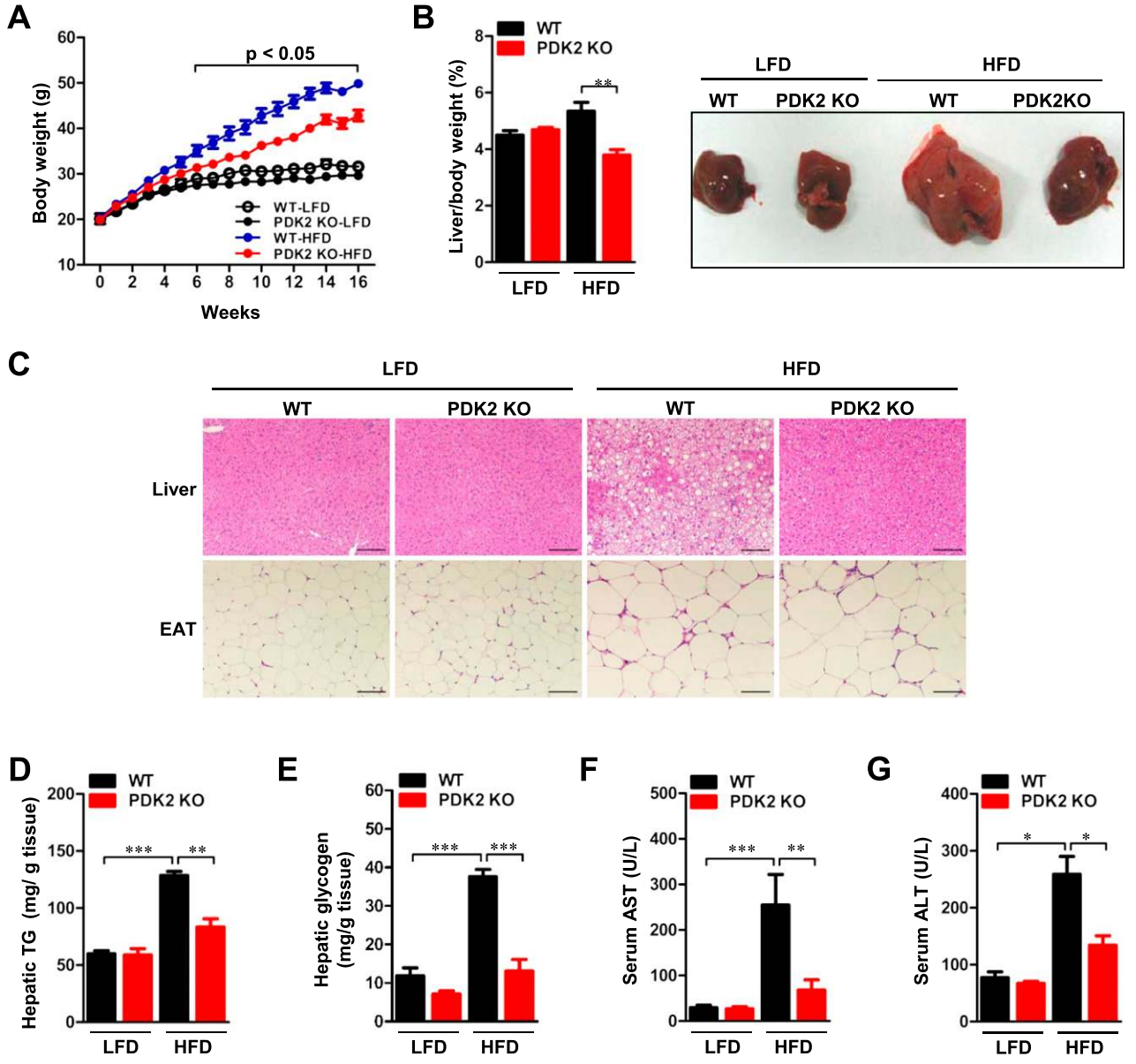

Figure 2—PDK2 deficiency ameliorates hepatic steatosis in mice fed an HFD. Body weight gain (n = 12) (*A*), liver/body weight ratio (n = 6) (*B*), and representative images of the livers (*B*) of WT and PDK2 KO mice fed an LFD or HFD. *C*: Representative histological appearance of H&E-stained liver and epididymal adipose tissue (EAT) obtained from the mice in *A*. Scale bars, 100 μm. Level of hepatic TG (*D*), hepatic glycogen (*E*), serum aspartate aminotransferase (AST) (*E*), and serum alanine aminotransferase (ALT) (*F*) activities in overnight-fasted WT and PDK2 KO mice (n = 6). Date are presented as the mean ± SEM. *P < 0.05; **P < 0.01; ***P < 0.001.

mice (Fig. 2*F* and *G*). The expression of genes involved in inflammation, including tumor necrosis factor-α, monocyte chemoattractant protein-1, and plasminogen activator inhibitor-1 were significantly lower in PDK2 KO mice than in WT mice fed an HFD (Supplementary Fig. 4*A*). These data indicate that PDK2 deficiency could ameliorate HFD-induced hepatic steatosis, injury, and inflammation.

PDK2 Deficiency Ameliorates Hepatic Insulin Resistance and Reduces Hepatic Glucose Production

In HFD-fed mice, PDK2 deficiency lowered fasting blood glucose and serum insulin levels by 15 and 63%, respectively, relative to WT mice (Fig. 3*A* and *B*). HFD-fed PDK2 KO mice processed glucose more efficiently than HFD-fed WT mice, as evidenced by a 30% decrease in the area under the curve during a glucose tolerance test (GTT) (Fig. 3*C*). However, with LFD-fed mice, the elimination of glucose was similar between PDK2 KO mice and WT mice (Fig. 3*C*), which is in agreement with previous data (18). These observations suggest that PDK2 deficiency prevents impaired glucose homeostasis induced by an HFD.

To better delineate the mechanism responsible for improved hepatic glucose homeostasis in HFD-fed PDK2 KO mice, we performed a hyperinsulinemic-euglycemic clamp study. In HFD-fed WT mice, the glucose infusion rate was lower, hepatic glucose production (HGP) was higher during the clamp, and whole-body glucose turnover was lower (Fig. 3*D*) than in LFD-fed WT mice. However, in HFD-fed PDK2 KO mice, the glucose infusion rate was increased twofold compared with HFD-fed WT mice (Fig. 3*D*). Furthermore, both basal and clamped HGP were significantly lower in HFD-fed PDK2 KO mice than in HFD-fed WT mice (Fig. 3*D*). Nevertheless, whole-body glucose turnover was not different between HFD-fed PDK2 KO mice and WT mice (Fig. 3*D*). HGP, measured by the pyruvate tolerance test (PTT), was lower in HFD-fed PDK2 KO mice than in HFD-fed WT (Fig. 3*E*). To evaluate insulin signaling, we examined the phosphorylation levels of AKT and forkhead box class O1 (FoxO1) in the liver and muscle of HFD-fed WT and PDK2 KO mice after insulin injection. Insulin-stimulated phosphorylation of AKT and FoxO1 were significantly increased in the livers of PDK2 KO mice compared with those of WT mice (Fig. 3*F*). Interestingly, in muscle, the levels of AKT and FoxO1 phosphorylation did not differ between HFD-fed PDK2 KO mice and HFD-fed WT mice (Supplementary Fig. 5), suggesting that the liver is the major organ affected by PDK2 deficiency in HFD-fed mice. Hepatic insulin resistance caused by obesity may be caused by increased intracellular diacylglycerol (DAG) and long-chain acyl-CoA esters, leading to activation of protein kinase Cε (PKCε), which negatively affects insulin signaling (19). To examine whether PDK2 deficiency ameliorates hepatic insulin resistance induced by an HFD, we measured DAG levels in the livers of HFD-fed PDK2 KO mice and HFD-fed WT mice. The amounts of 16:0, 18:1, 16:0–18:1, and total DAG were markedly lower in the livers of HFD-fed PDK2 KO mice than in those of HFD-fed WT mice (Fig. 3*G*). Furthermore, the phosphorylation of PKCε at Ser^{729} was significantly reduced in the livers of HFD-fed PDK2 KO mice compared with HFD-fed WT mice (Fig. 3*H*). These results suggest that activation of hepatic PDC by PDK2 deficiency ameliorates HGP and hepatic insulin resistance induced by an HFD.

PDK2 Deficiency Reduces TCA Cycle Intermediates and Malonyl-CoA in HFD-Fed Mice

Altered hepatic TCA cycle anaplerosis and ketogenesis are associated with hepatic steatosis and insulin resistance (20). We hypothesized that hepatic PDC activation by PDK2 deficiency shifts the fate of pyruvate from TCA cycle anaplerosis by PC to oxidative decarboxylation by PDC. This shift would reduce the levels of TCA cycle intermediates, thereby reducing TCA cycle flux. Thus, we measured hepatic pyruvate, lactate, and TCA cycle intermediates in WT and PDK2 KO mice. Levels of lactate and pyruvate in HFD-fed PDK2 KO mice were significantly lower than in HFD-fed WT mice (Supplementary Fig. 6*A* and *B*). However, the (lactate)/(pyruvate) ratio, an index of the cytosolic free (NADH)/(NAD) ratio, was not different between two groups (Supplementary Fig. 6*C*). Hepatic TCA cycle intermediates (OAA, citrate, and succinate) were significantly higher in HFD-fed WT mice than in LFD-fed WT mice (Fig. 4*A*). By contrast, the TCA cycle intermediates in HFD-fed PDK2 KO mice were dramatically lower than in HFD-fed WT mice (Fig. 4*A*). Despite the increase in hepatic PDC activity caused by PDK2 deficiency, there were no differences in the levels of hepatic acetyl-CoA and ATP in PDK2 KO mice compared with WT mice fed either diet (Fig. 4*B*). Most importantly, the increase in malonyl-CoA levels in the livers of WT mice fed an HFD was reduced significantly in the livers of HFD-fed PDK2 KO mice (Fig. 4*B*). Because malonyl-CoA is a major negative regulator of β-oxidation via inhibition of carnitine palmitoyltransferase 1 (21), we measured hepatic β-hydroxybutyrate (β-HB) levels as an index of ketogenesis. The levels of hepatic β-HB were significantly decreased in HFD-fed WT mice compared with LFD-fed WT mice (Fig. 4*C*), confirming that the reduction in ketogenesis is associated with hepatic steatosis induced by an HFD. However, β-HB levels were completely restored in HFD-fed PDK2 KO mice compared with HFD-fed WT mice (Fig. 4*C*). We then determined the rate of β-HB production in WT and PDK2 KO mice using a stable isotope ([^{13}U]β-HB) dilution method and found that the decreased rate of β-HB production in HFD-fed WT mice was restored in HFD-fed PDK2 KO mice (Fig. 4*D*).

Next, we examined the expression of genes involved in β-oxidation, ketogenesis, and lipogenesis in the livers of WT and PDK2 KO mice. As expected, the expression of genes involved in β-oxidation and ketogenesis, including peroxisome proliferator-activated receptor γ coactivator 1-α, peroxisome proliferator-activated receptor γ coactivator 1-β, carnitine palmitoyltransferase 1-liver, peroxisome proliferator-activated receptor α, and 3-hydroxy-3-methylglutaryl-CoA

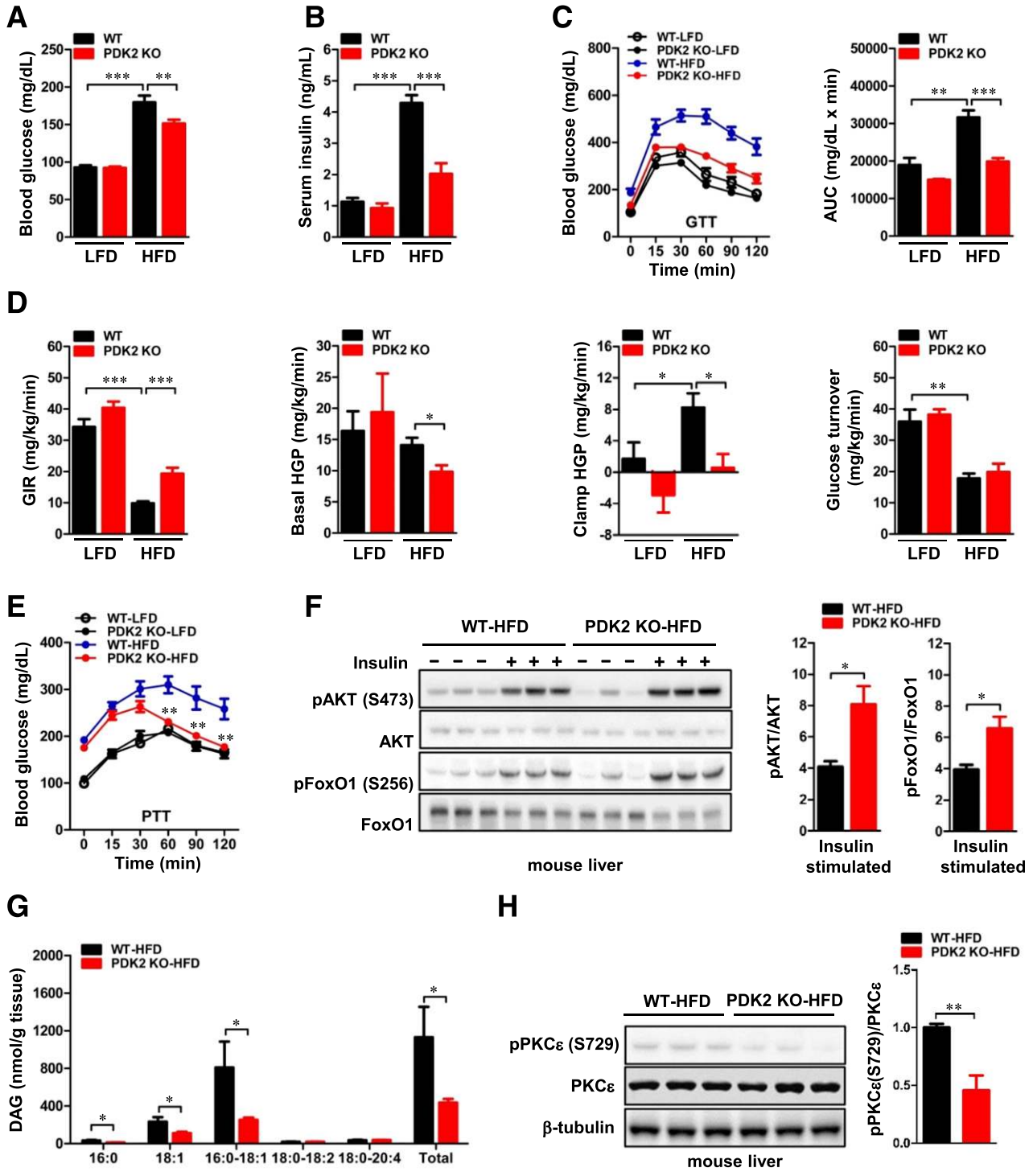

Figure 3—PDK2 deficiency improves glucose homeostasis and ameliorates HGP and insulin resistance induced by an HFD. Blood glucose (*A*) and serum insulin (*B*) levels of overnight-fasted WT mice and PDK2 KO mice fed an HFD or LFD ($n = 6$). GTT (*C*), hyperinsulinemic-euglycemic clamp study (*D*), and PTT (**$P < 0.01$ WT-HFD vs. PDK2 KO-HFD) (*E*) with overnight-fasted WT and PDK2 KO mice fed an LFD or HFD ($n = 6$). *F*: Hepatic insulin signaling in WT and PDK2 KO mice fed an HFD ($n = 5$). Levels of hepatic DAG (*G*) and PKCε phosphorylation (p) (*H*) in WT and PDK2 KO mice fed an HFD for 16 weeks ($n = 5$). The bar graphs on the right show quantification of PKCε phosphorylation. Data are presented as the mean ± SEM. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. AUC, area under the curve; GIR, glucose infusion rate.

synthase 2, were markedly downregulated in the livers of WT mice fed an HFD compared with an LFD. However, the expression levels of these genes were either partially or completely restored in the livers of PDK2 KO mice (Fig. 4*E*). The expression of genes involved in lipogenesis, such as sterol regulatory element-binding protein 1c, FA synthase, acetyl-CoA carboxylase 1, and stearoyl-CoA desaturase 1, was reduced in HFD-fed PDK2 KO mice relative to

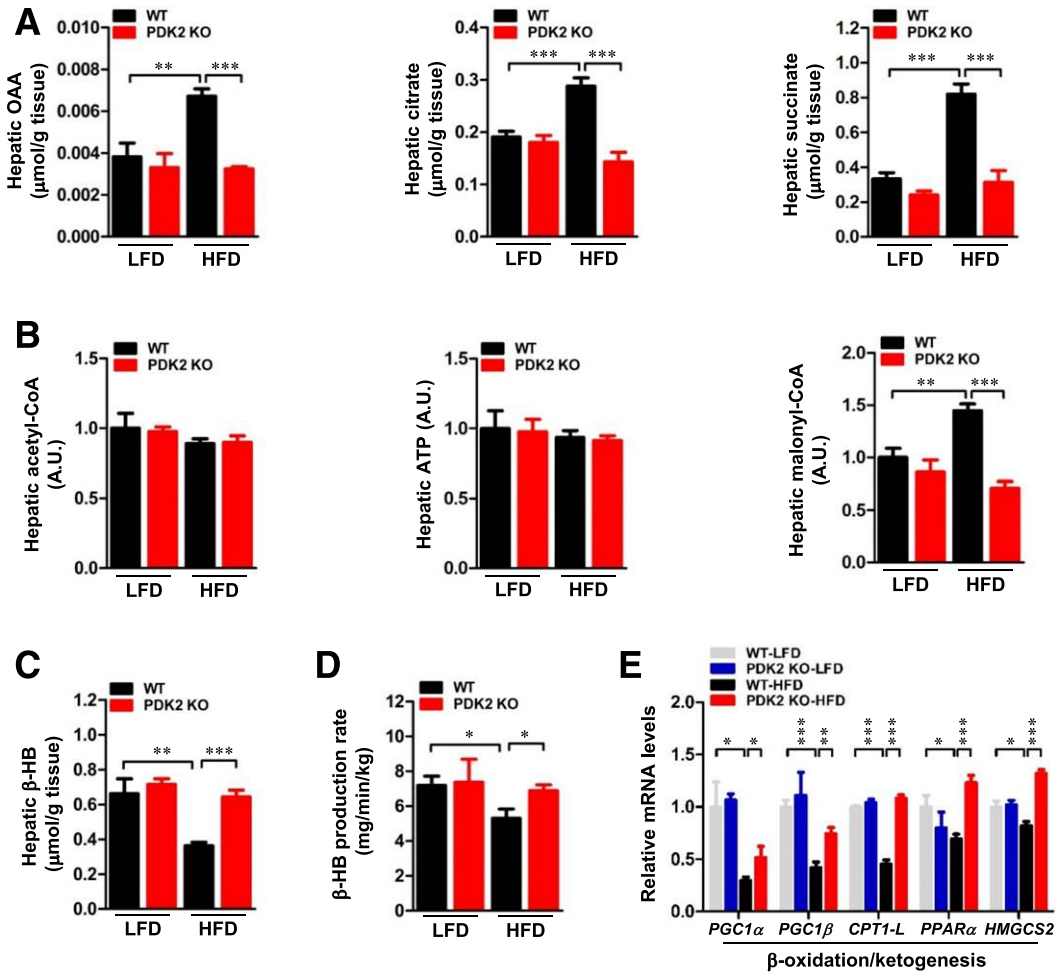

Figure 4—Enhanced hepatic PDC activity reduces TCA cycle intermediates and malonyl-CoA and increases ketogenesis. *A*: Levels of hepatic TCA cycle intermediates such as OAA, citrate, and succinate in overnight-fasted WT and PDK2 KO mice fed an LFD or an HFD for 16 weeks ($n = 6$). Level of hepatic metabolites such as acetyl-CoA (*B*), ATP (*B*), malonyl-CoA (*B*), and β-HB (*C*) obtained from the mice in *A*. *D*: β-HB production rates in WT and PDK2 KO mice after 18 h of fasting was measured using constant infusion of [U-$^{13}C_4$]sodium DL-3-hydroxyburyrate ($n = 6$). *E*: Hepatic gene expression of β-oxidation/ketogenic enzymes obtained from the mice in *A*. Data are presented as the mean ± SEM. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. A.U., arbitrary units.

HFD-fed WT mice (Supplementary Fig. 7). These findings suggest that activation of hepatic PDC reduced TCA cycle activity by decreasing the anaplerotic influx of OAA. This resulted in reduction of malonyl-CoA in liver of PDK2 KO mice fed an HFD.

Alteration of Anaplerotic Flux Into the TCA Cycle by PDK2 Deficiency Promotes Ketogenesis

In the previous results, a decrease in TCA cycle intermediates and an increase in ketone body were observed in the liver of PDK2 KO mice fed an HFD (Fig. 4). These results suggest that increased PDC activity by PDK2 deficiency might diminish hepatic PC flux and TCA cycle flux. To examine this hypothesis, we used stable isotopomer flux study with $^{13}C_6$-glucose supplement with 2 mmol/L octanoate as an FA substrate in primary hepatocytes isolated from WT and PDK2 KO mice. The PDC flux (measured by acetyl-CoA[M+2] enrichment) in primary hepatocytes of PDK2 KO mice was significantly increased compared with that of WT hepatocytes (Fig. 5*A*). However, PC flux (measured by aspartate[M+3] and citrate[M+3]) and TCA flux (measured by citrate[M+2], glutamate[M+2], succinate[M+2], and aspartate[M+2]) in primary hepatocyte of PDK2 KO mice were significantly lower than that of WT mice (Fig. 5*A*). These results indicate that TCA flux is decreased due to reduced anaplerotic PC flux even though the PDC flux is increased, which suggests that PDK2 deficiency leads to the production of ketone bodies from FA rather than complete oxidation to CO_2 by TCA cycle.

In order to examine this hypothesis, we measured the production of β-HB and CO_2 with octanoate in the primary hepatocytes obtained from WT mice and PDK2 KO mice. Oxidation of [$^{14}C_1$]-octanoate to $^{14}CO_2$ was significantly reduced and the rate of β-HB production increased in primary hepatocytes from PDK2 KO mice compared with WT mice (Fig. 5*B* and *C*). On the contrary, in the

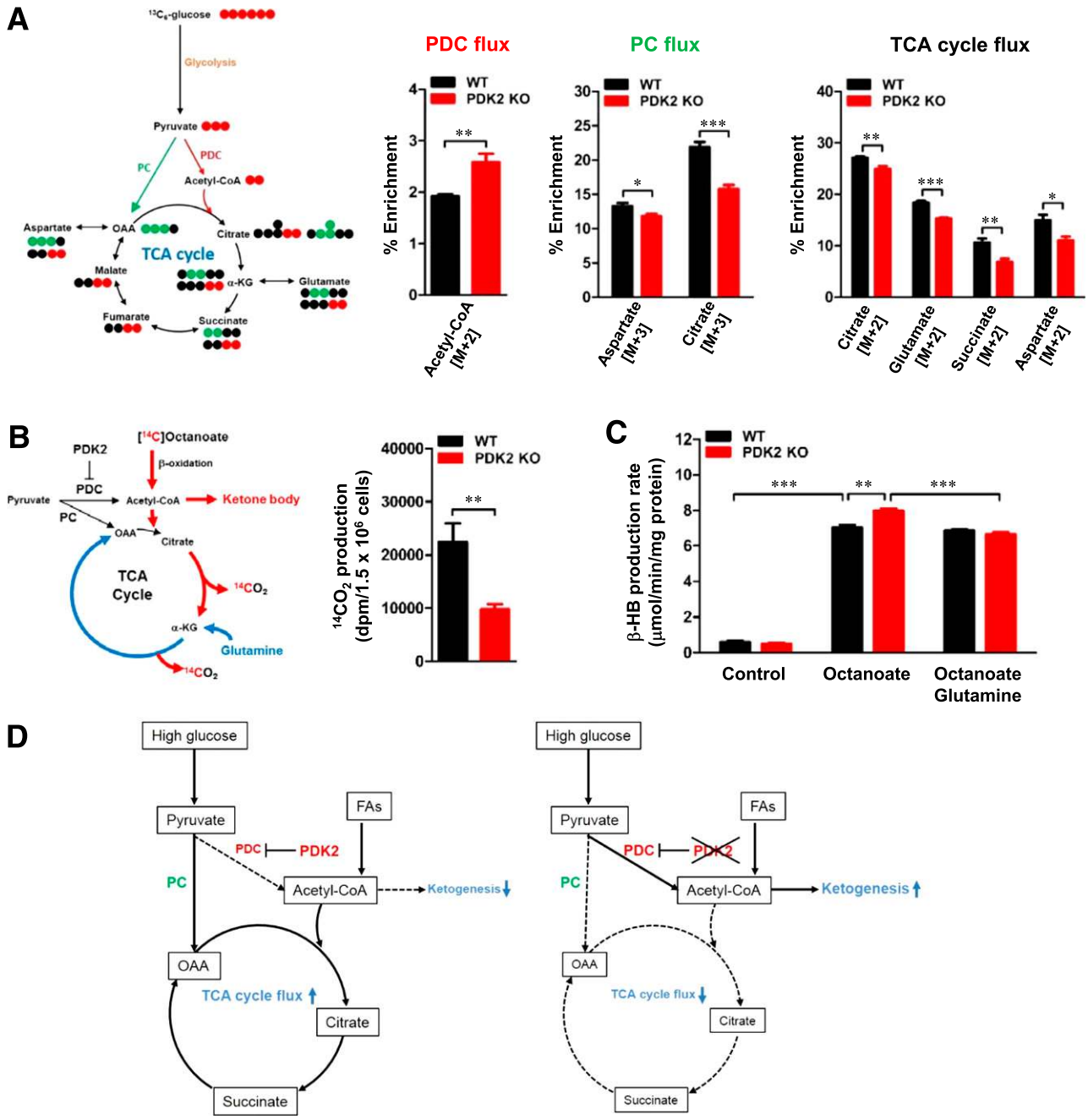

Figure 5—PDK2 deficiency enhances ketogenesis by decreasing TCA cycle anaplerosis in mouse primary hepatocytes. *A*: The incorporation of ^{13}C atoms from $^{13}C_6$-glucose to acetyl-CoA[M+2] (PDC flux, red), aspartate[M+3], citrate[M+3] (PC flux, green), citrate[M+2], glutamate[M+2], succinate[M+2], and aspartate[M+2] (TCA cycle flux). WT and PDK2 KO primary hepatocytes were treated with 25 mmol/L $^{13}C_6$-glucose and 2 mmol/L sodium octanoate for 2 h (n = 5). The enrichment of ^{13}C isotopomer was determined by liquid chromatography–mass spectrometry. α-KG, α-ketoglutarate. *B*: Oxidation of [1-^{14}C] sodium octanoate to $^{14}CO_2$ in WT and PDK2 KO primary hepatocytes (n = 6). *C*: WT and PDK2 KO primary hepatocytes were treated in control or ketogenic media (2 mmol/L octanoate) with or without 4 mmol/L glutamine for 4 h. Levels of β-HB in the culture media were determined enzymatic methods (n = 6). *D*: Schematic models for induction of ketogenesis in the primary hepatocytes of PDK2 KO mice. All data are presented as the mean ± SEM. *P < 0.05; **P < 0.01; ***P < 0.001.

presence of glutamine, another anaplerotic precursor of the TCA cycle, increased ketogenesis in PDK2-deficient hepatocytes was reduced to a level similar to that of WT hepatocytes (Fig. 5*C*), indicating that limited TCA anaplerotic influx is responsible for induction of ketogenesis in PDK2 KO hepatocytes (Fig. 5*D*).

Liver-Specific PDK2 Inhibition Prevents Hepatic Steatosis and Restores Glucose Homeostasis in HFD-Fed Mice

It has been reported that a mutation in the DW motif in the carboxy-terminal tails of the PDKs and the reciprocal DW motif (R157A) in the amino-terminal domain of the

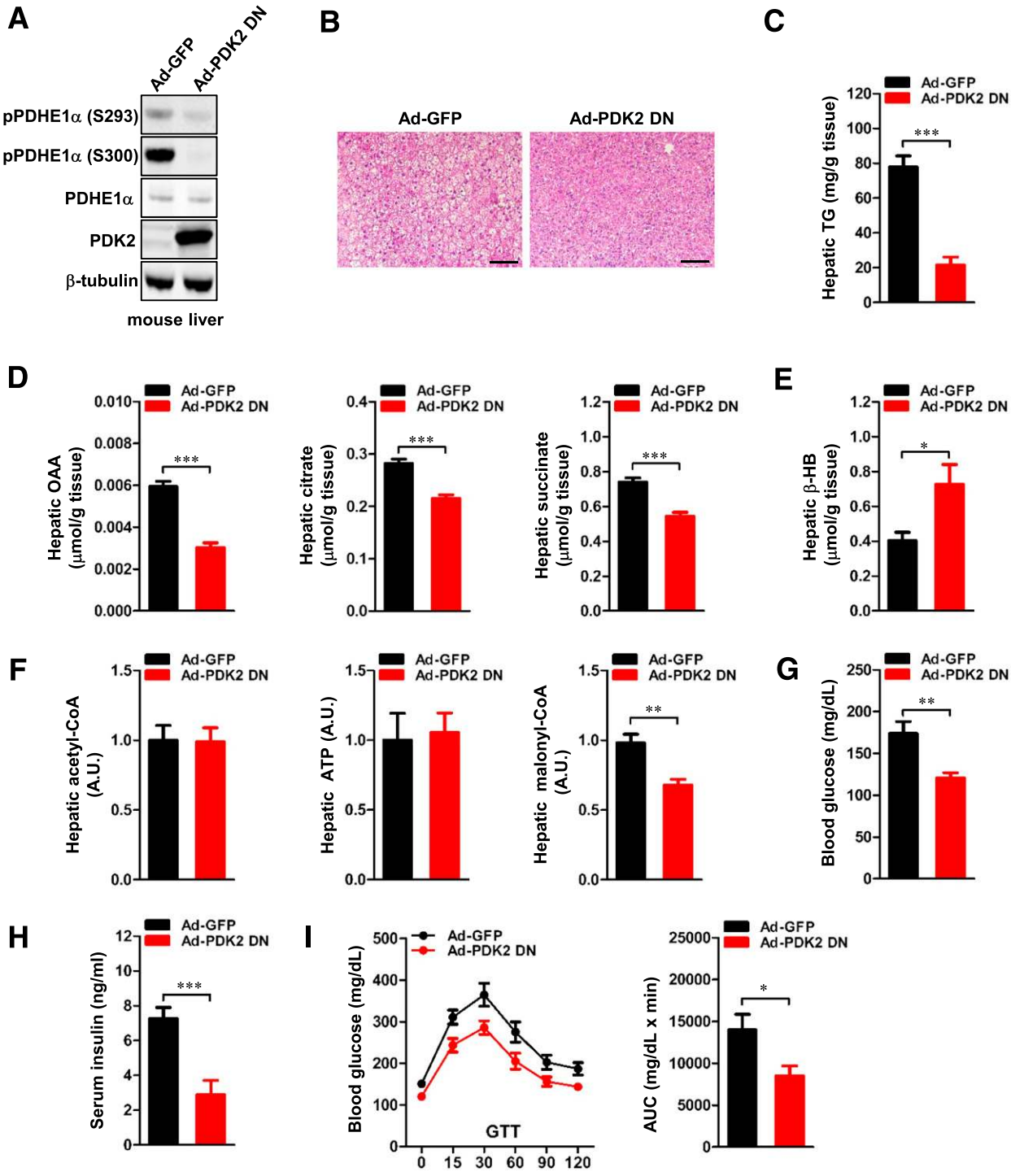

Figure 6—Liver-specific PDC activation by Ad-PDK2 DN prevents hepatic steatosis and improves glucose homeostasis in HFD-fed mice. Representative hepatic PDHE1α phosphorylation (p) (*A*) and histological appearance of liver tissue (*B*) in HFD-fed mice infected with Ad-GFP or Ad-PDK2 DN. Scale bars: 100 μm. The levels of hepatic TG (*C*), TCA cycle intermediates (*D*), β-HB (*E*), acetyl-CoA (*F*), ATP (*F*), and malonyl-CoA (*F*) in HFD-fed mice infected with Ad-GFP or Ad-PDK2 DN (n = 5). Blood glucose (*G*) and serum insulin (*H*) levels in HFD-fed mice infected with Ad-GFP or Ad-PDK2 DN (n = 5). *I*: GTT in HFD-fed mice infected with Ad GFP or Ad-PDK2 DN (n = 5). All data are presented as the mean ± SEM. *P < 0.05; **P < 0.01; ***P < 0.001. A.U., arbitrary units; AUC, area under the curve.

PDKs inactivates their enzymatic activity, showing these motifs are indispensable for kinase activity (14,15).

To determine the liver-specific effect of PDK2 inhibition, HFD-fed WT mice were infected with PDK2 R157A DN mutant adenovirus (Ad-PDK2 DN) or GFP adenovirus (Ad-GFP). PDHE1α phosphorylation was significantly decreased by Ad-PDK2 DN (Fig. 6*A*), indicating that PDC is activated in the livers of HFD-fed WT mice (Fig. 6*A*).

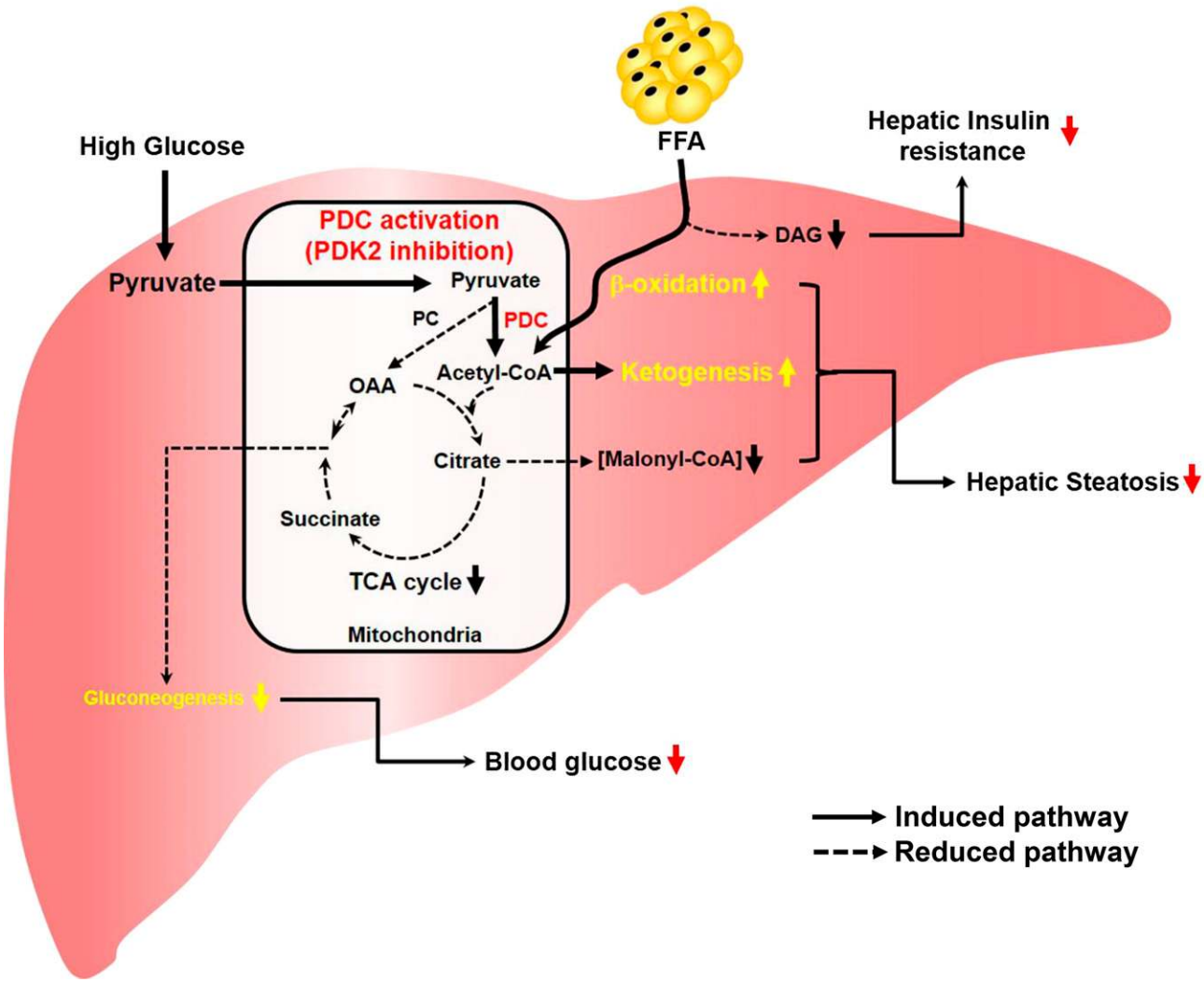

Figure 7—Schematic model of changes in hepatic glucose and lipid metabolism via PDC activation induced by PDK2 inhibition in liver of HFD-fed mice. FFA, free FA.

The number and the size of the lipid droplets and TG levels were reduced in the livers of HFD-fed mice infected with Ad-PDK2 DN compared with those infected with Ad-GFP (Fig. 6*B* and *C*). Similar to PDK2 KO mice, hepatic TCA cycle intermediates (OAA, citrate, and succinate) were also significantly decreased in HFD-fed mice infected with Ad-PDK2 DN (Fig. 6*D*). There were no differences in the levels of acetyl-CoA and ATP in the livers of the two groups of mice (Fig. 6*F*). Malonyl-CoA levels were reduced in Ad-PDK2 DN–treated mice (Fig. 6*F*), whereas the levels of hepatic β-HB were significantly increased (Fig. 6*E*). These results suggest that hepatic PDC activation by Ad-PDK2 DN prevents hepatic steatosis by decreasing TCA cycle anaplerosis and increasing ketogenesis.

To examine whether hepatic PDC activation by Ad-PDK2 DN ameliorates dysregulation of glucose metabolism induced by an HFD, fasting blood glucose and serum insulin were measured, and a GTT was performed. In HFD-fed mice, liver-specific PDK2 inhibition improved hyperglycemia, hyperinsulinemia, and glucose intolerance (Fig. 6*G–I*, respectively). These results indicate that inhibition of PDK2 in the liver is able to attenuate the impaired glucose homeostasis induced by an HFD.

DISCUSSION

As the link between glycolysis and the TCA cycle and a precursor for gluconeogenesis and lipogenesis, pyruvate plays a critical role in both anabolic and catabolic metabolism depending upon the tissue and the physiological condition of the organism (22). In the insulin-resistant condition, pyruvate is used for gluconeogenesis as well as de novo fat synthesis rather than ATP generation in the TCA cycle, resulting in the induction of hyperglycemia and hepatic steatosis (23). Although abnormal regulation of pyruvate metabolism has long been recognized in diabetes and metabolic disease (2,24,25), much remains to be elucidated about the molecular mechanisms that are involved. The fate of pyruvate is decided by the relative

activity of PC to PDC. In this study, we provide evidence that decreased PDC activity by increased PDK2 expression contributes to the development of hepatic steatosis in HFD-fed mice. Conversely, inhibition of PDK2 prevents the development of HFD-induced hepatic steatosis via increased ketogenesis due to decreased anaplerotic influx. Furthermore, HFD-fed PDK2 KO mice are resistant to impaired glucose metabolism and weight gain compared with HFD-fed WT mice.

The large decrease in TCA cycle intermediates in the livers of PDK2 KO mice may promote β-oxidation and ketogenesis. The reduction in TCA intermediates is presumably caused by decreased availability of OAA due to reduced anaplerotic influx from pyruvate, which would reduce the rate of ATP production in the TCA cycle. Complete oxidation of palmitate to CO_2 and water generates 129 ATPs, whereas only 16 ATPs are produced when palmitate is converted into β-HB. Surprisingly, hepatic ATP levels were not different between PDK2 KO mice and WT mice. To compensate for reduced ATP production by the TCA cycle, FA consumption via ketogenesis must be greatly enhanced in the liver of HFD-PDK2 KO mice, leading to less fat accumulation. Consistent with this, the enzymatic capacity for β-oxidation and ketogenesis was greater in HFD-PDK2 KO mice than in HFD-WT mice. The observed reduction in malonyl-CoA levels and increase of β-HB production rate also supports the promotion of β-oxidation and ketogenesis in PDK2-deficient mice.

Recently, it was reported that increased anaplerotic flux into the TCA cycle (from pyruvate to OAA by PC) is correlated with NAFLD in human and rodents (11,12). Furthermore, the relative PDC to TCA flux (V_{PDH}/V_{TCA}) is diminished in the livers of rats with chronic lipid-induced hepatic insulin resistance (11). The reduction in V_{PDH}/V_{TCA} is likely due to increased PDK2 activity in HFD feeding. PDK2 deficiency may increase PDC activity and thereby restore V_{PDH}/V_{TCA}. To examine this hypothesis, we measured PC, PDC, and TCA cycle flux using $^{13}C_6$-glucose isotopomer in primary hepatocytes obtained from WT and PDK2 KO mice. As shown in Fig. 5, increased PDC flux by PDK2 deficiency significantly reduced PC flux and TCA cycle flux. PC activity is allosterically regulated by acetyl-CoA. However, the allosteric effect of acetyl-CoA on PC activity may not be different between WT mice and PDK2 KO mice because hepatic acetyl-CoA levels of WT mice and PDK2 KO mice were comparable to each other. Instead, the lowered PC flux may be due to pyruvate limitation induced by increased PDC flux caused by PDK2 deficiency. In addition, the reduced PC flux by PDK2 deficiency decreased the availability of OAA, which affect the decreased TCA cycle flux in PDK2 KO hepatocytes. Recently, Satapati et al. (20) also showed that induction of the TCA cycle promotes hepatic oxidative stress and inflammation. Together with our results showing reduced expression of inflammatory markers in the livers of PDK2 KO mice, these findings are in agreement with the role of oxidative TCA cycle flux as a major source of electrons for oxidative stress in cells. Although testing the effect of PDK2 deletion on oxidative stress under conditions of nutritional overburden was beyond the limits of the current study, decreased oxidative metabolism by the TCA cycle is a likely mechanism for the protective effect of PDK2 deficiency.

Importantly, PDK2 deficiency decreased HGP and improved glucose tolerance in the HFD condition, as assessed by the GTT, PTT, and the hyperinsulinemic-euglycemic clamp. As OAA is a main precursor for gluconeogenesis in the liver, it is not surprising that activation of PDC causes a decrease in gluconeogenesis. In agreement with our data, it has been demonstrated that hepatic PC activity correlates with gluconeogenesis in vivo and ex vivo in lipid-induced obesity models (20,26). In addition, we found that hepatic levels of pyruvate and OAA in PDK2 KO mice were lower than in WT mice after HFD challenge, which may reduce HGP as well as lipogenesis in PDK2 KO mice.

DAG-mediated activation of PKCε can cause hepatic steatosis-associated insulin resistance (27,28). Consistent with these findings, mice lacking PDK2 were resistant to HFD-induced hepatic DAG accumulation and PKCε activation and therefore had significantly improved hepatic insulin signaling. This improvement was apparent in vivo, as evidenced by decreased serum fasting insulin levels and decreased HGP in the HFD-PDK2 KO mice.

In conclusion, we confirmed that hepatic PDC activation by inhibition of PDK2 could prevent obesity-induced hepatic steatosis and improve hepatic insulin sensitivity and glucose homeostasis, most likely by augmentation of FA consumption associated with reduction of TCA cycle anaplerosis, resulting in a reduction in OAA availability and induction of ketogenesis in the livers of HFD-mice (Fig. 7). These findings may open up a new avenue of treatment for NAFLD and its complications.

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