RESEARCH

Reducing hepatic PKD activity lowers circulating VLDL cholesterol

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Abstract

Protein kinase D (PKD) is emerging as an important kinase regulating energy balance and glucose metabolism; however, whether hepatic PKD activity can be targeted to regulate these processes is currently unclear. In this study, hepatic PKD activity was reduced using adeno-associated virus vectors to express a dominant-negative (DN) version of PKD1, which impairs the action of all three PKD isoforms. In chow-fed mice, hepatic DN PKD expression increased whole-body glucose oxidation, but had only mild effects on glucose and insulin tolerance and no effects on glucose homeostasis following fasting and refeeding. However, circulating VLDL cholesterol was reduced under these conditions and was associated with hepatic fatty acid accumulation, but not lipids involved in lipoprotein synthesis. The limited effects on glucose homeostasis in DN PKD mice was despite reduced expression of gluconeogenic genes under both fasted and refed conditions, and enhanced pyruvate tolerance. The requirement for PKD for gluconeogenic capacity was supported by *in vitro* studies in cultured FAO hepatoma cells expressing DN PKD, which produced less glucose under basal conditions. Although these pathways are increased in obesity, the expression of DN PKD in the liver of mice fed a high-fat diet had no impact on glucose tolerance, insulin action, pyruvate tolerance or plasma VLDL. Together, these data suggest that PKD signalling in the liver regulates metabolic pathways involved in substrate redistribution under conditions of normal nutrient availability, but not under conditions of overnutrition such as in obesity.

Key Words

- glucose metabolism
- signal transduction
- liver
- gene regulation
- cholesterol

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Introduction

Protein kinase D is a serine/threonine kinase that was originally termed PKCμ (Johannes *et al.* 1994). Similar to other PKCs, it has a N-terminal regulatory domain with cysteine-rich repeats that bind the lipid diacylglycerol (DG) (Valverde *et al.* 1994). However, due to sequence homology with the calcium/calmodulin-dependent protein kinases in its catalytic domain, it was renamed PKD (Manning *et al.* 2002). There are three PKD isoforms that differ slightly in structure and substrate specificity and it appears that some redundancy exists between isoforms (Matthews *et al.* 2006, Guo *et al.* 2011). In its inactive state, the regulatory domain of PKDs contain a pseudo-substrate region that engages with the catalytic domain, keeping the kinase in its inactive state (Valverde

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et al. 1994). Upon DG binding and phosphorylation of residues within both its regulatory and catalytic domains, PKD becomes active, resulting in auto-phosphorylation of serine 916 (Matthews *et al.* 1999). Growth factors and other systemic factors that activate tyrosine kinase receptors and G-protein coupled receptors can increase PKD activity through the actions of phospholipase C isoforms, which convert phosphatidylinositol 4,5-bisphosphate (PIP_2) to DG at the plasma membrane (Rozengurt 2011). Nutrient substrates such as saturated fatty acids that drive *de novo* DG accumulation can also increase PKD phosphorylation (Mayer *et al.* 2019). Furthermore, PKD can be activated through a DG-independent mechanism that is initiated in response to mitochondrial-derived reactive oxygen species, which involves phosphorylation of the regulatory domain by c-Abl and Src kinase (Storz *et al.* 2003, 2005). Once activated, PKD regulates a number of biological processes, including export of vesicles and their cargo from the Golgi network to the plasma membrane. PKD can also translocate to the nucleus to regulate gene transcription (Rozengurt 2011).

Consistent with PKD activation by DG and its sensitivity to cellular redox state, a number of regulatory roles for PKD in metabolic processes are starting to emerge. An important role for PKD in insulin secretion from pancreatic β-cells has been established, showing that PKD is involved in regulating whole-body glucose homeostasis (Sumara *et al.* 2009). We have also found that PKD can regulate glucose metabolism in muscle cells (McGee *et al.* 2014) and that PKD also regulates contraction-mediated glucose uptake in the heart (Dirkx *et al.* 2012). These findings suggest a broader role for PKD in the control of glucose metabolism in various tissues. The liver is critical for the control of systemic glycemia and glucose metabolism. Under pre-prandial and fasting conditions, the liver releases glucose into the systemic circulation through increases in glycogenolysis and gluconeogenesis to maintain glycemia (Herman & Kahn 2006). In post-prandial periods, hepatic glucose output is suppressed by insulin through inactivation of gluconeogenic enzymes such as glucose-6-phosphatase and increasing glycogen synthesis by inactivation of glycogen synthase kinases (Saltiel & Kahn 2001). These signalling mechanisms are mediated by the Akt kinase, which is a key component of the canonical insulin signalling pathway. Akt also reduces the expression of gluconeogenic enzymes (Saltiel & Kahn 2001).

A number of lines of evidence implicate PKD signalling in the regulation of hepatic glucose metabolism. In some cell types, PKD phosphorylates and inhibits phosphatidylinositol 3,4-kinase (PI3K) (Lee *et al.* 2011, Ni *et al.* 2013), a key component of the canonical insulin signalling pathway that is immediately upstream of PDK and Akt. This suggests that PKD could, therefore, inhibit the effect of insulin on hepatic glucose metabolism. This has been examined in a recent study, which found that constitutive knockout of PKD3, the most highly expressed PKD isoform in the liver, mildly enhanced whole-body glucose homeostasis and insulin action (Mayer *et al.* 2019). However, hepatic triglyceride accumulation occurred as a consequence of enhanced hepatic insulin action (Mayer *et al.* 2019). The purpose of this study was to determine whether a tissue-specific reduction in PKD activity, rather than constitutive knockout of PKD isoforms, could enhance whole-body glucose homeostasis, insulin action and lipid metabolism. This approach will determine whether manipulating PKD activity is a viable therapeutic approach for metabolic diseases. To reduce PKD signalling, an adeno-associated virus (AAV) 8 vector with liver-specific thyroxine-binding globulin (TBG) was used to express dominant-negative (DN) PKD1 exclusively in the liver. Overexpression of this inactive PKD1 mutant acts in a DN fashion against all three PKD isoforms (Czondor *et al.* 2009, Remillard-Labrosse *et al.* 2009) and reduced total PKD activity by ~45%. In chow-fed mice, expression of DN PKD had only mild effects on glucose and insulin tolerance and no effect on glucose homeostasis in response to fasting and refeeding, despite the reduced expression of gluconeogenic genes in both the fasted and refed state. Expression of DN PKD also reduced circulating LDL/VLDL cholesterol and enhanced pyruvate tolerance, suggesting the reduced capacity for gluconeogenesis. However, hepatic DN PKD expression in diet-induced obese mice had no effect on blood glucose homeostasis or circulating lipids, suggesting that, while lowering PKD activity regulates pathways involved in substrate redistribution in states of limited nutrient availability, its effects are lost under conditions of nutrient oversupply.

Materials and methods

Adeno-associated virus production

Green fluorescent protein (GFP) and human PKD1 cDNA with a mutation in the ATP-binding domain (K612W) that renders it an inactive, DN enzyme (Storz *et al.* 2003) were subcloned into an AAV expression plasmid containing the liver-specific TBG promoter (Bond *et al.* 2017).

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AAV vectors were packaged and purified at the Penn Vector Core, University of Pennsylvania.

Animal studies

Ethics approval was granted by the Deakin University Animal Ethics Committee (application G04-2013), which is subject to the Australian Code for the Responsible Conduct of Research. C57BL6 male mice were obtained from the Animal Resource Centre (Perth, Australia) at 5 weeks of age. At 7 weeks of age, mice were administered GFP or DN PKD AAV8-TBG vector via the lateral tail vein. For AAV dose titration experiments, mice were administered GFP and DN PKD AAV8-TBG vectors at 5×10^{10}, 1×10^{11} or 5×10^{11} vector genomes (v.g.) in HBSS. Tissues were collected 2 weeks later and were analysed for hepatic GFP and PKD protein expression to determine an appropriate AAV dosing regimen. The expression of GFP and PKD in the liver was similar for all doses tested (data not shown). Therefore, a dosing regimen of 5×10^{10} v.g. was selected for further experiments. One cohort of mice were administered either GFP or DN PKD AAV8-TBG vectors ($n=7$/group) and were humanely killed 5 weeks later by cervical dislocation, following a 5 h fast. The liver was collected and weighed, and other tissues were collected and immediately frozen in liquid nitrogen. All tissues were examined for GFP expression, while the liver was also analysed for PKD protein expression and activity. A separate cohort of mice ($n=6$/group) were used in metabolic experiments and body weight was assessed every 3–7 days throughout the experiment. Nine weeks after AAV administration, body composition was determined (EchoMRI) and mice underwent an insulin tolerance test (ITT; 0.7 U/kg lean mass i.p.) after a 5 h fast. Blood glucose obtained from the tail tip was measured using AccuChek glucose monitors (Roche) prior to insulin administration and 20, 40, 60, 90 and 120 min after insulin administration. Ten weeks after AAV administration, mice underwent indirect calorimetry, using the Fusion Metabolic system (AccuScan Instruments, USA) that uses open circuit calorimetry, as previously described (Gaur *et al.* 2016). Twelve weeks after AAV administration, mice underwent a glucose tolerance test (GTT; 2 g/kg lean mass i.p.) after a 5 h fast. Blood glucose from the tail tip was measured prior to glucose administration and 15, 30, 45, 60 and 90 min after glucose administration. An additional 30 µL of blood was obtained at 0, 15, 30 and 60 min for determination of plasma insulin. Thirteen weeks after AAV administration, mice underwent a pyruvate tolerance test (PTT; 2 g/kg lean mass i.p.) after a 5 h fast. Blood glucose from the tail tip was measured prior to glucose administration and 15, 30, 45, 60, 90 and 120 min after pyruvate administration. Fourteen weeks after AAV administration, following a final body composition measurement, mice were fasted overnight for 16 h and blood glucose and insulin measures were made on all mice, before half the mice from both groups were humanely killed by cervical dislocation. The remaining mice were again given access to food for 4 h before being humanely killed by cervical dislocation. In both cohorts of animals, plasma was collected via cardiac puncture and stored for later analysis of plasma lipid measures. The liver and other tissues were collected and immediately frozen in liquid nitrogen for later analysis. In another cohort of animals ($n=7$–8/group), mice underwent a dietary intervention 2 weeks after AAV administration. Mice remained on standard chow or were placed on a high-fat diet (HFD) consisting of 43% of calories from fat (23.5% by weight; SF04-001 Rodent Diet, Research Diets D12451 Equivalent; Specialty Feeds) for 12 weeks. Mice underwent the same experimental procedures as described for the previous cohort of mice at the same time points post-AAV administration.

Biochemical and plasma analyses

PKD activity was measured in liver homogenates using modifications to the Cyclex CaM Kinase II Assay kit (MBL Research), which uses an immobilised Syntide 2 peptide as substrate, which is also phosphorylated by PKD (Rozengurt *et al.* 1995). Briefly, ~20 mg of liver was homogenised in ~200 µL lysis buffer (50 mM Tris pH 7.5, 1 mM EDTA, 1 mM EGTA, 10% glycerol, 1% Triton X-100, 50 mM NaF, 5 mM sodium pyrophosphate, 1 mM DTT, 1× protease inhibitor cocktail (Sigma)). The total protein concentration of the lysates was determined using the BCA total protein kit (Pierce). A total of 30 ng of liver homogenate was added to immobilised Syntide 2 in a 96-well plate, in the absence or presence of 25 µM CID755673, a specific PKD inhibitor. Kinase assay and ATP buffers (supplied) were added and the reaction was allowed to proceed for 30 min at 30°C. Wells were washed with wash buffer before being incubated with a HRP conjugated anti-phospho Syntide 2 antibody (MS-6E6) at room temperature for 60 min. Absorbance at 450/540 nm was measured after incubations with substrate reagent and stop solution. Total PKD activity was determined as the difference between measures with and without CID755673 (Santa Cruz) and was normalised to PKD protein, which was determined by Western blot analysis (as described subsequently). Liver glycogen was

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determined as previously described (Howlett *et al.* 2013). Briefly, ~20 mg of liver tissue was freeze-dried and then dissected of connective tissue and blood. Glycogen content was determined by the method of Passonneau and Lauderdale (Passonneau and Lauderdale 1974). Plasma free fatty acids (FFA), LDL/VLDL cholesterol and HDL cholesterol were measured using commercially available kits (Sigma-Aldrich). Plasma insulin was determined using a Mouse Ultrasensitive Insulin ELISA (Alpco Diagnostics).

Molecular analyses

Proteins were extracted from approximately 50 mg of frozen liver tissue following homogenisation in protein lysis buffer as described previously. All protein homogenates were spun at 1000 ***g*** at 4°C. The supernatant was assayed for total protein content and then separated by SDS-PAGE and transferred onto PVDF membrane using a standard protocol. Membranes were blocked in TBST (TBS with 0.05% Tween 20 pH 7.4) containing 1% BSA for 1 h at room temperature. Membranes were incubated overnight in the relevant primary antibody diluted 1:1000 in TBST, including GFP, α-Tubulin (Sigma), pS133 CREB, total CREB, pS916 PKD, pS473 Akt, total Akt (Cell Signaling Technology) and total PKD (Abcam). Membranes were then washed with TBST and incubated at room temperature for 1 h with the appropriate HRP conjugated secondary antibody at a dilution of 1:10,000 in TBST. Membranes were then exposed to the chemiluminescent substrate (Life Technologies) and imaged using the ChemiDoc™ xRS+ with ImageLab™ software. For determining PKD abundance from samples used for PKD activity assays, liver lysates were run on gels with 0, 12.5, 25 and 50 ng of recombinant GST-PKD (Abcam) to generate a standard curve. Total PKD was quantified using this standard curve and all lysate samples fell within the curve (Supplementary Fig. 1, see section on supplementary materials given at the end of this article). Total RNA was extracted from 5 to 10 mg of liver using the Agilent total RNA Isolation Mini Kit (Agilent Technologies). RNA was then reverse transcribed using SuperScript III reverse transcriptase and Oligo (dT) primers. cDNA quantity was determined using Quant-iT™ OliGreen ssDNA Reagent. qPCR was performed using SYBR (Thermo) and the following primer pairs: *Fasn* Fwd: 5′-TCG GGT GTG GTG GGT-3′, Rev 5′-GCG TGA GAT GTG TTG-3′; *G6pase* Fwd 5′-ACG CCT TCT ATG TCC TCT TTC CC-3′, Rev 5′-TGT TGC TGT AGT AGT CGG TGT CC-3′; *Pgc-1a* Fwd 5′-CCT GCC ATT GTT AAG ACC-3′, Rev 5′-TGC TGC TGT TCC TGT TTT C-3′. Endogenous PKD was measured with primers towards mouse *Pkd1* Fwd 5′-CAT CAG CCG ACC CTT TCC-3′, Rev 5′-CTT CAG GTG CCA GGT ATG-3′. Exogenous PKD was measured with primers towards human *PKD1* Fwd 5′-ACG GCA CTA TTC GAG ATT GG-3′, Rev 5′-GCT GGA AGG ATT GAC CAC AT-3′. Relative gene expression was calculated as $2^{-\Delta Ct}$, including normalisation to cDNA concentration. Liver lipids were quantified with liquid chromatography, electrospray ionisation-tandem mass spectrometry, as we have previously described (Czeczor *et al.* 2018). Liver FFA, phosphatidic acid (PA), triglycerides (TG), cholesterol esters and free cholesterol were normalised to total phosphatidylcholine (PC) levels. The quantities of all PC species, phosphatidylethanolamine species, phosphatidylglycerol, phosphatidylinositol species and phosphatidylserine were summed to determine total phospholipid levels, which were normalised to sample protein concentration.

Cell culture

Rat FAO immortalised hepatoma cells were cultured in 5% CO_2 at 37°C in growth media consisting of RPMI 1640 media containing 2 g/L glucose (Life Technologies) and 10% foetal bovine serum (FBS, Thermo Scientific). Cells were transfected with DN PKD or empty plasmids using Lipofectamine (Life Technologies) and were assayed for glucose production under basal or insulin-stimulated conditions, as previously described (Martin *et al.* 2014). For protein analyses, cells were stimulated with vehicle or 0.1 nM insulin for 30 min prior to collection in lysis buffer.

Statistical analysis

All values are expressed as mean ± S.E.M. Data were analysed for statistical significance with GraphPad Prism 8 using unpaired *t*-tests or two-way ANOVA with Tukey multiple comparison testing. Statistical significance was designated as $P < 0.05$.

Results

Expression of DN PKD in the liver with an AAV8-TBG vector

AAV8 vectors have been reported to show high tropism towards the liver, by over three orders of magnitude compared with other tissues, for up to 6 months post-vector administration (Chen *et al.* 2013, Hinderer *et al.*

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2014). The TBG promoter also provides further tissue-specificity for transgene expression (Yan *et al.* 2012). To determine the tropism of the AAV8-TBG vector to the liver in our own hands, GFP or DN PKD expressing AAV8-TBG vectors were administered to mice via tail vein injection. Analysis of various tissues in GFP AAV8-TBG mice showed that GFP expression was restricted to the liver (Fig. 1A). There was a trend for endogenous PKD expression to be increased (Fig. 1B) in DN PKD mice, while there was no exogenous PKD expression (Fig. 1C), in mice administered GFP AAV8-TBG vectors. Mice administered DN PKD AAV8-TBG vectors had an approximate doubling of total PKD protein (Fig. 1D), which resulted in a reduction in basal PKD activity of ~45% (Fig. 1E). Unlike hepatic PKD3 knockout, which resulted in a dramatic increase in liver weight due to triglyceride accumulation (Mayer *et al.* 2019), expression of DN PKD had no effect on liver weight (Fig. 1F). These data show that expression of DN PKD via an AAV8-TBG vector is a viable model to test the effect of reducing hepatic PKD activity.

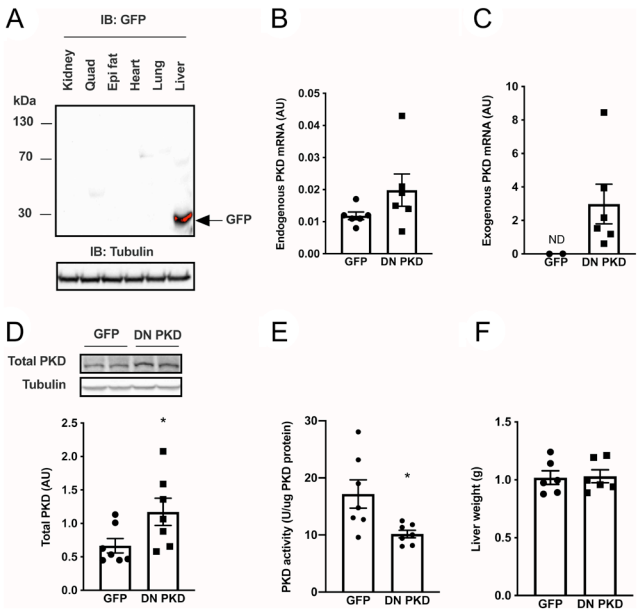

Figure 1
Expression of DN PKD in the liver with an AAV8-TGB vector. (A) GFP expression in the liver of mice after 2 weeks of i.v. administration of AAV8-TGB vectors. (B) Endogenous PKD mRNA; (C) exogenous PKD mRNA; (D) total PKD expression; (E) PKD activity; and (F) liver weight in mice administered AAV8-TGB vectors expressing GFP or dominant-negative (DN) PKD for 5 weeks. Data are mean ± S.E.M., $n = 6$–7/mice per group and were analysed by unpaired *t*-test. *Significantly different from GFP mice ($P < 0.05$). ND, not detected. A full color version of this figure is available at https://doi.org/10.1530/JOE-19-0548.

Hepatic DN PKD expression had only mild effects on glucose and insulin tolerance

To explore the effect of reducing hepatic PKD activity on glucose homeostasis and insulin action, a variety of metabolic parameters were assessed. Expression of DN PKD had no effect on body weight (Fig. 2A) or composition (Fig. 2B and C). Although there was no effect of DN PKD expression on energy expenditure (Supplementary Fig. 2A), the respiratory exchange ratio was higher (Supplementary Fig. 2B) and carbohydrate oxidation was higher in the darkness phase (Fig. 2D) in DN PKD mice. Although lipid oxidation tended to be lower in DN PKD mice, this was not statistically significant (Supplementary Fig. 2C). There was a main effect for blood glucose levels to be lower in mice expressing DN PKD throughout an i.p. GTT (Fig. 2E); however, there was no difference in the blood glucose incremental area under the curve (iAUC) for the test (Fig. 2F), indicating no difference in overall glucose tolerance. Furthermore, there were no differences in plasma insulin (Supplementary Fig. 2D) or plasma insulin AUC throughout the GTT (Fig. 2G). Similarly, there was a main effect for blood glucose levels to be lower in DN PKD mice throughout an ITT (Fig. 2H); however, there was no difference in the blood glucose AUC throughout the test (Fig. 2I), indicating no difference in insulin tolerance. These data suggest that reducing PKD activity in the liver has only a minor impact on glucose and insulin tolerance.

Hepatic DN PKD expression does not alter the glycaemic response to fasting and refeeding but reduces plasma lipoprotein-associated cholesterol levels

Although reducing hepatic PKD activity had only mild effects on glucose and insulin tolerance, the glucoregulatory responses under more complex metabolic transitions following fasting and refeeding were examined. Furthermore, as constitutive knockout of hepatic PKD3 enhanced lipogenesis and led to hypercholesterolemia under both fasting and fed conditions (Mayer *et al.* 2019), the effect of fasting and refeeding on lipid metabolism was also examined. Blood glucose levels were increased 4 h after refeeding following a 16 h fast, but there was no effect of genotype (Fig. 3A). However, the main effects for both feeding state and genotype were observed in the plasma insulin response to fasting and refeeding (Fig. 3B). Furthermore, plasma insulin was significantly higher in DN PKD mice when compared with GFP mice in the refed state

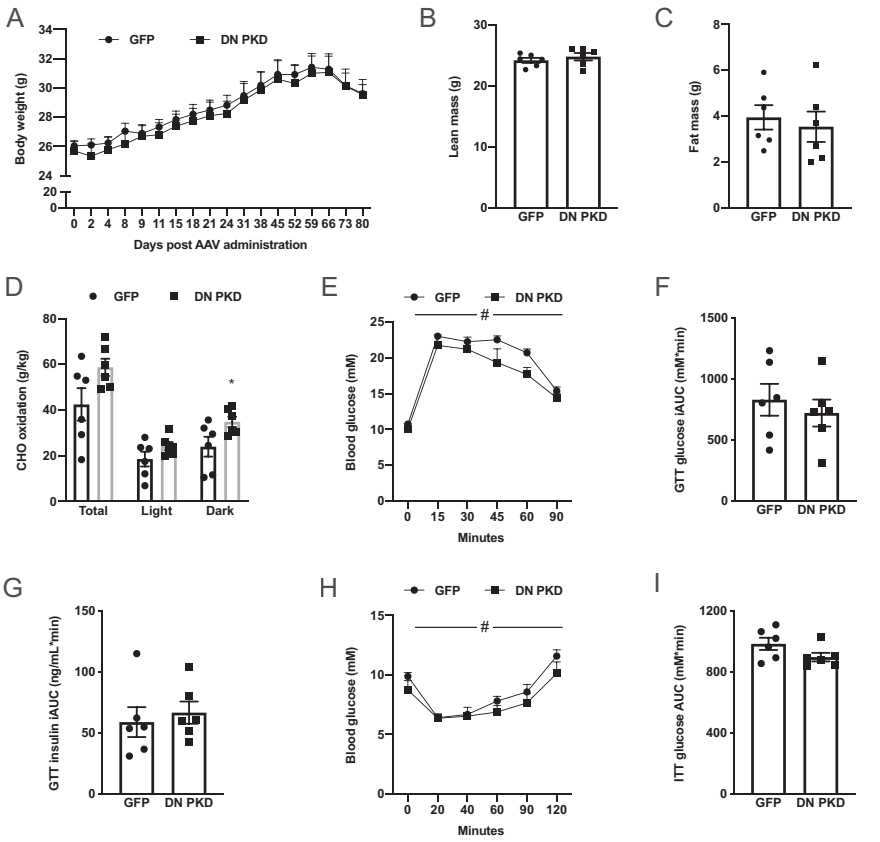

Figure 2
Hepatic DN PKD expression has no effect on glucose homeostasis or insulin action. (A) Body weight; (B) lean mass; (C) fat mass; (D) total, light and darkness phase carbohydrate (CHO) oxidation; (E) blood glucose throughout a glucose tolerance test (GTT; 2 mg/kg glucose i.p.); (F) GTT blood glucose incremental area under the curve (iAUC); (G) GTT plasma insulin iAUC; (H) blood glucose throughout an insulin tolerance test (ITT; 0.7 U/kg insulin); and (I) ITT blood glucose AUC in mice administered AAV8-TBG vectors expressing GFP or dominant-negative (DN) PKD. Data are mean ± S.E.M., n = 6/mice per group and were analysed by unpaired t-test and two-way ANOVA. *Significantly different from GFP mice in the same period ($P < 0.05$). #Main effect for genotype ($P < 0.05$).

(Fig. 3B). However, there were no genotype differences in liver glycogen (Fig. 3C), a measure of hepatic insulin action. Unlike constitutive knockout of PKD3 that increased insulin action (Mayer *et al.* 2019), these data suggest that reducing hepatic PKD activity under these conditions might impair physiological insulin action. The effects on circulating cholesterol were also assessed. Plasma LDL/VLDL (Fig. 3D) and HDL (Fig. 3E) cholesterol were significantly lower in DN PKD mice. To understand this further, plasma and liver free fatty acids (FFA) and lipids were assessed. There were no genotype differences in plasma FFA (Fig. 3F); however, liver FFA were increased in DN PKD mice (Fig. 3G). This was associated with a reduction in the liver levels of PA (Fig 3H), a key biosynthetic lipid intermediate for the synthesis of the acyl-glycerol lipids required for lipoprotein assembly. However, there were no differences in other lipids required for lipoprotein assembly, including TG (Fig. 3I), cholesterol esters (Fig. 3J), free cholesterol (Fig. 3K) or total phospholipids (Fig. 3L). Collectively, these data suggest that PKD activity is required for normal lipoprotein secretion, but the absence of PKD activity does not result in hepatic lipid accumulation, despite an increase in liver FFA.

Hepatic DN PKD expression reduces the expression of gluconeogenic genes, improves pyruvate tolerance in mice and reduces basal glucose production *in vitro*

In an effort to explain the higher carbohydrate oxidation in DN PKD mice, mechanisms regulating glucose metabolism in response to fasting and refeeding were also examined. There was no difference in the phosphorylation of E1-alpha subunit of pyruvate dehydrogenase (PDH) at serine 293, a key regulatory point that inhibits pyruvate oxidation (Supplementary Fig. 3A). However, the expression of the gluconeogenic genes *Pgc-1α* (Fig. 4A) and *G6pase* (Fig. 4B) were reduced in DN PKD mice, while there was no difference in the expression of *Pepck* gene (Fig. 4C), which has a role not only in gluconeogenesis, but also in other metabolic pathways (Yang *et al.* 2009). PKD is a known kinase of CREB, a transcription factor that regulates the expression of these gluconeogenic genes (Herzig *et al.* 2001), at its activation site at S133 (Ozgen *et al.* 2008). However, we did not observe any difference in CREB phosphorylation at S133 (Supplementary Fig. 3B) that might explain these gene expression changes. Nonetheless, gluconeogenic capacity was assessed using a pyruvate tolerance test. There was a main effect for DN PKD mice to have lower blood glucose

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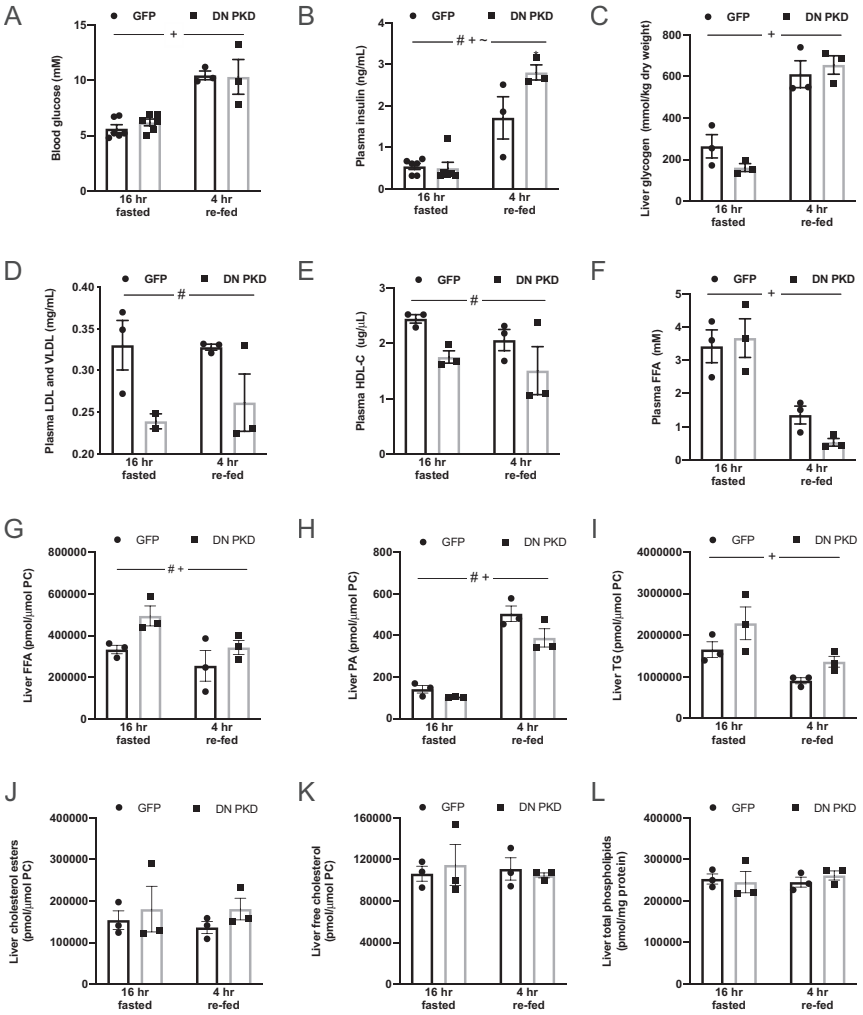

Figure 3
Hepatic DN PKD expression does not alter the glycaemic response to fasting and refeeding but reduces plasma lipoprotein cholesterol. (A) Blood glucose; (B) plasma insulin; (C) liver glycogen; (D) plasma LDL and VLDL cholesterol; (E) plasma HDL cholesterol; (F) plasma free fatty acids (FFA); (G) liver FFAs; (H) liver phosphatidic acid (PA); (I) liver triglycerides (TG); (J) liver cholesterol esters; (K) liver free cholesterol; and (L) liver total phospholipids in mice administered AAV8-TBG vectors expressing GFP or dominant-negative (DN) PKD after a 16-h fast or 4 h of refeeding. Data are mean ± S.E.M., $n = 3$–6/mice per group and were analysed by two-way ANOVA. #Main effect for genotype ($P < 0.05$). +Main effect for feeding state ($P < 0.05$). ~Significant interaction ($P < 0.05$). *Significantly different from GFP mice in the same feeding state.

throughout the test (Fig. 4D) and lower blood glucose AUC (Fig. 4E), suggesting that PKD could regulate gluconeogenic capacity. To further understand this, FAO hepatoma cells were transfected with a plasmid expressing DN PKD or an empty plasmid (control). Expression of DN PKD increased total PKD levels and reduced its activation, assessed by auto-phosphorylation at serine 916, in response to platelet-derived growth factor (PDGF; Fig. 4F). Expression of DN PKD in these cells reduced basal glucose production (Fig. 4G), which is principally due to gluconeogenesis as these cells do not store glycogen (Prip-Buus *et al.* 1992). Insulin reduced glucose production to similar absolute levels in both control and DN PKD cells (Fig. 4G), which resulted in a significant decrease in insulin action in DN PKD cells (Fig. 4H). Consistent with this observation, insulin-stimulated Akt phosphorylation appeared to be reduced in DN PKD cells (Fig. 4I). The idea that hepatic insulin action on glucose metabolism is impaired by DN PKD expression is consistent with our *in vivo* data where insulin levels were significantly higher in DN PKD mice following refeeding (Fig. 3B). Together, these data suggest that reducing hepatic PKD activity reduces the capacity for gluconeogenesis and also impairs the ability of insulin to suppress gluconeogenesis.

Hepatic DN PKD expression in mice fed a high-fat diet has no effect on glucose homeostasis

Although reducing hepatic PKD activity had no effect on glucose homeostasis and insulin action in chow-fed animals, given that PKD appears to contribute to gluconeogenic capacity and lipoprotein secretion, we next assessed whether reducing hepatic PKD activity impacts glucose and lipid homeostasis in obesity. Mice were fed with a high-fat diet for 12 weeks and while endogenous PKD mRNA was unchanged (Fig. 5A), the overexpression of exogenous DN PKD and total PKD protein was maintained throughout this time period (Fig. 5B and C). There were

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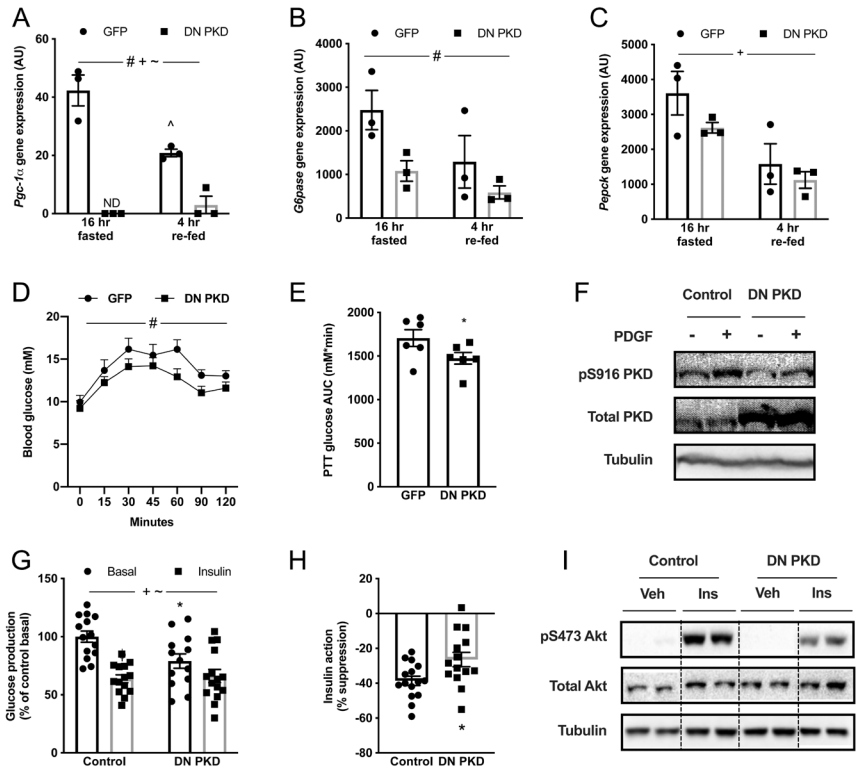

Figure 4
Hepatic DN PKD expression reduces the expression of gluconeogenic genes, improves pyruvate tolerance in mice, and reduces basal glucose production *in vitro.* (A) Peroxisome proliferator-activated receptor gamma coactivator 1 alpha (*Pgc-1α*) gene expression; (B) glucose-6-phosphatase (*G6pase*); and (C) phosphoenolpyruvate carboxykinase (*Pepck*) mRNA in mice administered AAV8-TBG vectors expressing GFP or dominant-negative (DN) PKD after a 16-h fast or 4 h of refeeding. (D) Blood glucose throughout a pyruvate tolerance test (PTT; 2 g/kg pyruvate i.p.); (E) PTT blood glucose area under the curve (AUC); (F) DN PKD expression in FAO hepatoma cells, pS916 PKD and PKD in control treated and PDGF treated cells; (G) basal and insulin-suppressed (10 nM) glucose production; (H) insulin action expressed as insulin-mediated suppression of glucose production; and (I) pS473 Akt and total Akt in FAO hepatoma cell transfected with control or dominant-negative (DN) PKD plasmids. Data are mean ± S.E.M., $n = 3$–6/mice per group or $n = 14$ biological replicates/group and were analysed by unpaired *t*-test and two-way ANOVA. #Main effect for genotype ($P < 0.05$). ⁺Main effect for feeding state/treatment ($P < 0.05$). ⁻Significantly interaction ($P < 0.05$). *Significantly different from GFP mice in the same feeding state or from control/control basal cells. ^Significantly different from GFP fasted. ᵠSignificantly different from Control Basal.

no differences in body weight (Supplementary Fig. 4A) or composition (Supplementary Fig. 4B and C) between GFP and DN PKD mice over this time period. No differences in energy expenditure (Supplementary Fig. 4D) or respiratory exchange ratio (Supplementary Fig. 4E) were observed between groups. Similarly, there were no differences in glucose tolerance (Fig. 5D and E) or plasma insulin throughout the GTT (Fig. 5F and G). There was a main effect for DN PKD mice to have lower blood glucose throughout an ITT (Fig. 5H); however, there was no difference in ITT AUC (Fig. 5I). Unlike in chow-fed mice, there was no difference in pyruvate tolerance between groups (Fig. 5J). Biochemical analyses were also performed in a random subset of samples from these mice. There were no differences in liver TG (Fig. 5K) and plasma LDL and VLDL cholesterol (Fig. 5L) between GFP and DN PKD mice when fed with a high-fat diet. These data show that reducing hepatic PKD activity in mice made obese through high-fat feeding has no effect on glucose or lipid homeostasis.

Discussion

The purpose of this study was to determine whether reducing hepatic PKD activity, rather than constitutive knockout of specific PKD isoforms, could enhance whole-body glucose homeostasis and insulin action, to establish whether targeting PKD activity is a viable target for metabolic diseases. Liver-specific overexpression of DN PKD reduced total hepatic PKD activity by ~45% and resulted in a number of metabolic alterations. These included increased whole-body glucose oxidation, altered transcriptional responses to fasting and refeeding and evidence of reduced capacity for gluconeogenesis. Furthermore, the expression of hepatic DN PKD reduced plasma LDL/VLDL cholesterol. However, reducing hepatic PKD activity did not alter glucose tolerance or induce lipid accumulation in chow-fed animals. These two points contrast to a recent study of mice with constitutive PKD3 knockout, which resulted in improved whole-body glucose homeostasis and hepatic steatosis as a result of enhanced insulin action. Our data suggest that reducing PKD activity, as might be attained by therapeutic PKD inhibition, does not influence glucose tolerance or hepatic lipid accumulation, despite alterations in the transcriptional program controlling gluconeogenesis and reduced circulating LDL/VLDL cholesterol. The present study also suggests that PKD has a role in nutrient redistribution under conditions of normal nutrient availability, but not under conditions of high nutrient availability such as obesity.

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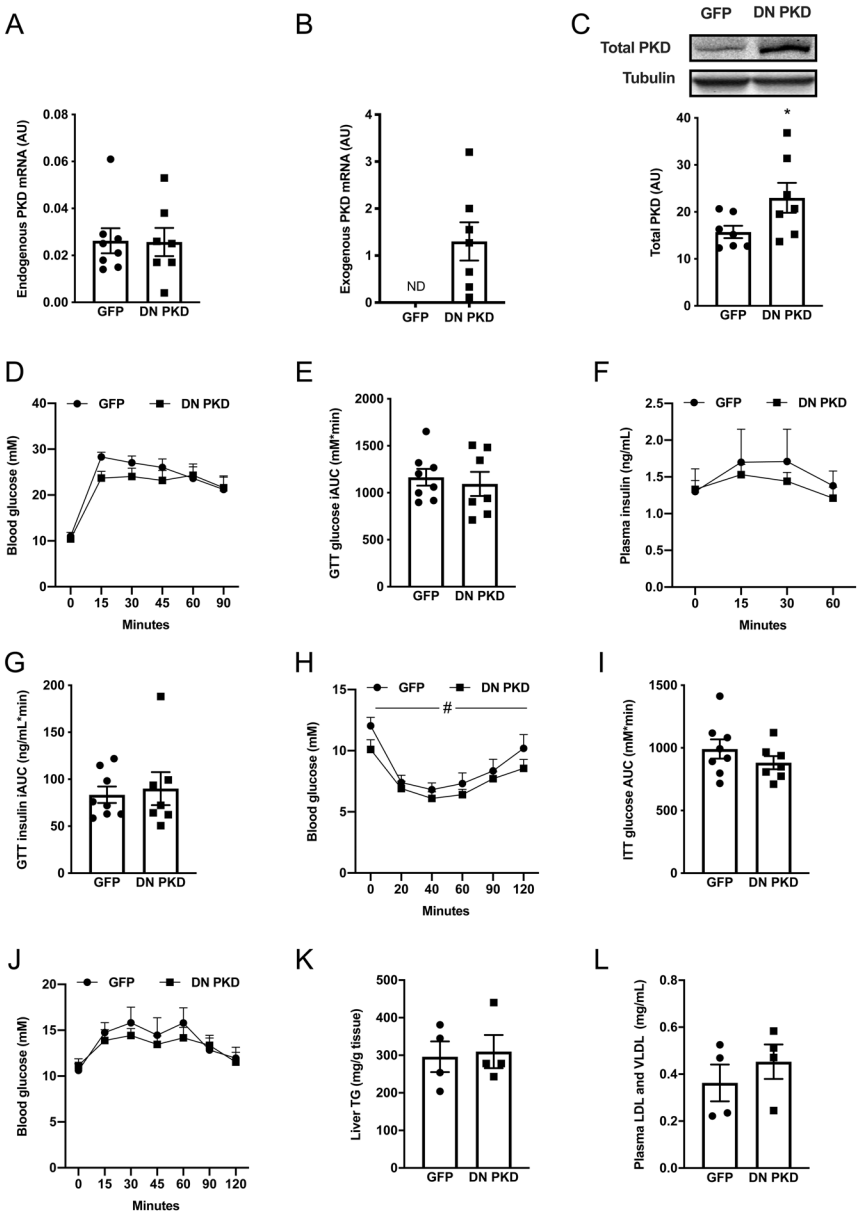

Figure 5
Hepatic DN PKD expression in mice fed a high-fat diet has no effect on glucose homeostasis. (A) Endogenous PKD mRNA; (B) exogenous PKD mRNA; (C) total PKD protein; (D) blood glucose throughout a glucose tolerance test (GTT; 2 mg/kg glucose i.p.); (E) GTT blood glucose incremental area under the curve (iAUC); (F) GTT plasma insulin; (G) GTT plasma insulin iAUC; (H) blood glucose throughout an insulin tolerance test (ITT; 0.7 U/kg insulin); (I) ITT blood glucose AUC; (J) blood glucose throughout a pyruvate tolerance test (PTT; 2 g/kg pyruvate i.p.); (K) liver triglycerides (TG); and (L) plasma LDL and VLDL cholesterol in mice administered AAV8-TBG vectors expressing GFP or dominant-negative (DN) PKD fed a high-fat diet. Data are mean ± S.E.M., $n = 7$–8/mice per group and were analysed by unpaired *t*-test and two-way ANOVA. #Main effect for genotype ($P < 0.05$).

Our data highlight a role for PKD in the transcriptional control of gluconeogenic genes under fasting and refeeding conditions, with *Pgc-1α* and *G6pase* expression reduced in DN PKD mice. Fasting typically increases the expression of *Pgc-1α*, which is a transcriptional coactivator of the gluconeogenic transcriptional program that includes *G6pase* (Puigserver *et al.* 2003). Despite the lower expression of these gluconeogenic genes in DN PKD mice, there were no differences in blood glucose levels under these same conditions. In contrast, there was a decrease in pyruvate tolerance, a proxy measure of gluconeogenic capacity, in DN PKD mice. These data suggest that, under these conditions, the transcriptional control of gluconeogenesis appears to make only very subtle contributions to glucose homeostasis. However, PKD-mediated control of the gluconeogenic transcriptional program might be more important in response to extended fasting or low nutrient availability situations. The mechanism by which PKD might regulate the gluconeogenic transcriptional program remains unknown. *Pgc-1α* and other gluconeogenic genes have conserved CREB binding sites within their promoter regions (Herzig *et al.* 2001) and PKD1 is known to phosphorylate CREB at S133, which increases its transcriptional activity (Ozgen *et al.* 2008). We analysed the phosphorylation state of CREB in the transition from fasting and refeeding and although there was a trend for CREB phosphorylation to be reduced in DN PKD mice, there were no significant differences observed. A number

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of kinases are known to phosphorylate the CREB S133 site, not least of all PKA, which is thought to be the most important kinase in the regulation of gluconeogenesis (Yang & Yang 2016). This suggests that other mechanisms beyond the regulation of CREB are important for the transcriptional regulation of *Pgc-1α* and gluconeogenic genes by PKD.

An interesting observation from the present study was that plasma LDL/VLDL cholesterol and HDL cholesterol were reduced in DN PKD mice. LDL/VLDL lipoproteins are secreted from the liver to redistribute lipids to peripheral tissues (Tiwari & Siddiqi 2012), while HDL lipoproteins return lipids to the liver (Ouimet *et al.* 2019). The synthesis of TG, cholesterol and phospholipids that form LDL/VLDL lipoproteins occurs in the endoplasmic reticulum (ER) before being transported to the trans-Golgi network (TGN), before entering secretory pathways that deliver the lipoproteins to the plasma membrane for export into the extracellular space and circulation (Tiwari & Siddiqi 2012). One of the best characterised functional roles for PKD signalling is the regulated transport of exocytotic vesicles from the TGN to the plasma membrane (Liljedahl *et al.* 2001, Yeaman *et al.* 2004). PKD is required for vesicle fission from the TGN, the maintenance of TGN structure and for the function of secretion pathways (Bossard *et al.* 2007). While the transport of VLDL lipoproteins from the TGN to the plasma membrane appears to involve specialised TGN-derived vesicles (Hossain *et al.* 2014), the specific mechanisms involved remain to be completely elucidated. The mechanism by which PKD regulates hepatic secretion of LDL/VLDL remains to be determined; however, DN PKD expression did not result in accumulation of hepatic lipids, suggesting that feedback mechanisms prevented flux through these pathways. It is possible that such a feedback mechanism occurred early in the lipogenic process, as there was an accumulation of hepatic FFAs. It is unclear whether the increase in total carbohydrate oxidation in DN PKD mice during the darkness phase is related to reduced circulating LDL/VLDL and lipid availability for peripheral tissues. However, the reduced expression of *Pgc-1α* could also contribute, as hepatic PGC-1α also drives fatty acid oxidation (Morris *et al.* 2012) and was consistent with the hepatic accumulation of FFAs in DN PKD mice. It should be noted that, although there was a trend for lipid oxidation to be reduced in DN PKD mice, this was not statistically significant. It is also interesting that the phenotypic effects of PKD inactivation that were observed in chow-fed mice, including enhanced pyruvate tolerance and reduced LDL- and VLDL-cholesterol, were lost in mice fed a high-fat diet. Adrenergic signalling via PKA-dependent phosphorylation of PKD at serine 427 inhibits G_q-receptor linked activation of PKD in cardiomyocytes (Nichols *et al.* 2014). This suggests that PKD signalling might be desensitised in high-catecholamine conditions, such as in obesity. A greater understanding of the signals that control PKD activity in this context will aid in understanding this aspect of PKD biology.

The data collected throughout this study differed markedly from those observed in mice with constitutive PKD3 knockout and a number of factors might contribute to these differences. The constitutive nature of PKD3 knockout could exert a number of effects on cell differentiation and tissue development that might have effects on metabolism. Various loss-of-function models of the various PKD isoforms report opposing data on the effects of the three PKD isoforms on developmental processes (Maier *et al.* 2007, 2019). Furthermore, the overt effects of PKD3 deletion might only become apparent when PKD3 activity is absent, rather than reduced. The dominant-negative approach used in the present study also inhibits the activity of all three PKD isoforms (Czondor *et al.* 2009, Remillard-Labrosse *et al.* 2009). Although redundancy between these isoforms has been noted, they also appear to have isoform-specific functions and regulatory mechanisms. For example, PKD3 is not activated by oxidative stress, as it lacks the critical threonine residue (T463 in human PKD1 and T438 in human PKD2) phosphorylated by the Abl kinase in response to reactive oxygen species (Cobbaut & Van Lint 2018). The isoform-specific functions of PKD may be masked in the inhibitory strategy used in the present study; however, this activity profile is one that might be expected if systemic PKD inhibition were to be adopted therapeutically, as many specific PKD inhibitors have little selectivity over the three PKD isoforms. For example, CRT0066101 inhibits PKD1-3 with IC_{50} values of 1, 2 and 2.5 nM, respectively (Harikumar *et al.* 2010). Similarly, CID755673, which is a non-ATP domain PKD kinase inhibitor, inhibits PKD1-3 with IC_{50} values of 182, 280 and 227 nM, respectively (Sharlow *et al.* 2008). Given the widespread roles of the three PKD isoforms in various aspects of normal biology, it might be anticipated that inhibition of PKD substrates that mediate specific aspects of PKD function might be a more efficacious approach.

In conclusion, the present study showed that reducing PKD activity in the liver did not improve glucose tolerance, despite increasing whole-body glucose oxidation, reducing the expression of genes in the transcriptional program

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controlling gluconeogenesis and improving pyruvate tolerance. An unexpected finding from the present study was that decreasing liver PKD activity reduced circulating LDL/VLDL cholesterol without leading to hepatic lipid accumulation, which we speculate could be due to the role of PKD in regulating secretory pathways from the TGN to the plasma membrane. Our data suggest that PKD is involved in regulating substrate redistribution under conditions of normal nutrient availability, but not under conditions of overnutrition such as in obesity.

Supplementary materials

This is linked to the online version of the paper at https://doi.org/10.1530/JOE-19-0548.

Declaration of interest

The authors declare that there are no conflicts of interest that could be perceived as prejudicing the impartiality of the reported research.

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