

Published in final edited form as:

Biochem J. 2012 May 1; 443(3): 829–839. doi:10.1042/BJ20112197.

Fasting induces ketoacidosis and hypothermia in PDHK2/ PDHK4-double-knockout mice

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Abstract

The importance of PDHK (pyruvate dehydrogenase kinase) 2 and 4 in regulation of the PDH complex (pyruvate dehydrogenase complex) was assessed in single- and double-knockout mice. PDHK2 deficiency caused higher PDH complex activity and lower blood glucose levels in the fed, but not the fasted, state. PDHK4 deficiency caused similar effects, but only after fasting. Double deficiency intensified these effects in both the fed and fasted states. PDHK2 deficiency had no effect on glucose tolerance, PDHK4 deficiency produced only a modest effect, but double deficiency caused a marked improvement and also induced lower insulin levels and increased insulin sensitivity. In spite of these beneficial effects, the double-knockout mice were more sensitive than wild-type and single-knockout mice to long-term fasting, succumbing to hypoglycaemia, ketoacidosis and hypothermia. Stable isotope flux analysis indicated that hypoglycaemia was due to a reduced rate of gluconeogenesis and that slightly more glucose was converted into ketone bodies in the double-knockout mice. The findings establish that PDHK2 is more important in the fed state, PDHK4 is more important in the fasted state, and survival during long-term fasting depends upon regulation of the PDH complex by both PDHK2 and PDHK4.

Keywords

glucose; heart; hypothermia; ketoacidosis; liver; pyruvate dehydrogenase complex (PDH complex); pyruvate dehydrogenase kinase (PDHK); skeletal muscle

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Author Contribution: Yasmeen Rahimi, Nam Ho Jeoung, Pengfei Wu and Robert Harris designed the experiments. Yasmeen Rahimi, Nam Ho Jeoung, and Pengfei Wu performed the experiments. Paul Lee analysed and helped to interpret the stable isotope studies. Yasmeen Rahimi, Nam Ho Jeoung, Paul Lee and Robert Harris wrote the paper.

Introduction

The PDH complex (pyruvate dehydrogenase complex) plays a pivotal role in controlling the concentrations of glucose in the fed and fasted state [1]. In the well-fed state, the PDH complex is highly active, promoting glucose oxidation by generating acetyl-CoA, which can be oxidized by the citric acid cycle or used for fatty acid and cholesterol synthesis. In the fasted state, the PDH complex is inactivated by phosphorylation by PDHKs (pyruvate dehydrogenase kinases) to conserve three carbon compounds for the production of glucose [2].

The four PDHK isoenzymes responsible for phosphorylating the PDH complex are expressed in a tissue-specific manner [3–5]. Among the four, PDHK2 and PDHK4 are most abundantly expressed in the heart [5–7], skeletal muscle [5,8–10] and liver [5,11–14] of fasted mice. Of these two, PDHK2 is of interest because of its greater sensitivity to activation by acetyl-CoA and NADH and inhibition by pyruvate [15]. However, PDHK4 has received greater attention because its expression is increased in many tissues by fasting and diabetes [12] and transcription of its gene is regulated by insulin, glucocorticoids, thyroid hormone and fatty acids [16,17]. Inactivation of the PDH complex by phosphorylation helps to maintain euglycaemia during fasting, but contributes to hyperglycaemia in Type 2 diabetics. The increase in PDHK activity in diabetes raises the question of whether the PDHKs should be considered therapeutic targets for the treatment of diabetes [18]. Support for this possibility has been provided by the finding that mice lacking PDHK4 are euglycaemic in the fasted state and are more glucose tolerant than wild-type mice fed on a high-fat diet [19–21].

In the present study, PDHK2-KO (knockout) mice were produced to determine the importance of this isoform in glucose homeostasis. In contrast with PDHK4-KO mice, blood glucose levels were not lowered in the fasting state and glucose tolerance was not improved in mice lacking PDHK2. This raised the question of whether the presence of PDHK4 compensates for the lack of PDHK2 and vice versa. To answer this question, PDHK2/PDHK4-DKO (double-knockout) mice were produced and characterized. In contrast with the relatively mild phenotypes of the single-KO mice, the DKO mice are unable to tolerate fasting for extended periods of time. The findings show that survival during fasting depends upon inactivation of the PDH complex by PDHK2 and/or PDHK4.

Materials and Methods

Generation of the PDHK2/PDHK4-DKO mice

The procedures used to generate *Pdk4*^{-/-} (homozygous PDHK4-KO mice) C57BL/6J black mice [19] and *Pdk2*^{-/-} (homozygous PDHK2-KO mice) C57BL/6J black mice [22] have been described previously. PDHK2-KO mice were crossed with PDHK4-KO mice to produce PDHK2/PDHK4-DKO mice. Age-matched wild-type mice were produced from the C57BL/6J black mice (The Jackson Laboratory, Bar Harbor, ME, U.S.A.) that were used to stabilize the genetic backgrounds of the PDHK2-KO and PDHK4-KO mice.

Animal protocols

Mice were housed in an AALAC (American Association for Laboratory Animal Care)-approved pathogen-free barrier facility (12 h light/12 h dark cycles with temperature maintained at $23 \pm 2^\circ\text{C}$) and *ad libitum* fed on a standard rodent chow diet (Harlan; #7071). Studies were conducted with the approval of the Institutional Animal Care and Use Committee of Indiana University School of Medicine. Blood was collected from either a tail vein or the submandibular vein for the determination of metabolite levels. To determine PDH complex activity and phosphorylation states, mice were anaesthetized by injecting pentobarbital (60 mg/kg of body weight) intraperitoneally. Blood was drawn from the inferior vena cava to measure metabolites. Gastrocnemius muscle, liver, heart, kidney, testes and brain were harvested as rapidly as possible followed by immediate freeze-clamping with Wollenberger tongs at the temperature of liquid nitrogen, powdered in liquid nitrogen and stored at -85°C for analysis. Body temperature was determined with a rectal temperature probe (MicroTherma 2T; Braintree Scientific).

Glucose-, insulin- and pyruvate-tolerance tests

Glucose-tolerance tests were performed after mice had been fasted overnight (17:00 h–09:00 h). Glucose (2 g/kg of body weight) was administered by intraperitoneal injection followed by measurement of glucose in tail blood by a glucometer (AccuCheck; Roche) at 0, 15, 30, 60 and 120 min after injection. Insulin-tolerance tests were conducted after 6 h of fasting (08:00 h–14:00 h). Insulin (0.5 unit/kg of body weight; Humulin R, Eli Lilly) was administered by intraperitoneal injection. Tail blood was taken for determination of glucose levels at 0, 15, 30, 60 and 120 min after injection. Mice were fasted overnight (17:00 h–09:00 h) for the pyruvate-tolerance test. Pyruvate (1.5 g/kg of body weight) was delivered by intraperitoneal injection followed by collection of tail blood for measuring glucose at 0, 15, 30, 60 and 120 min. Blood was collected from the jugular vein 30 min after pyruvate administration.

Measurement of metabolite concentrations in blood and liver

Serum was deproteinized with 7% perchloric acid followed by neutralization with KOH and precipitation of KClO_4 . Pyruvate [23], lactate [24], alanine [25], acetoacetate [26], β -hydroxybutyrate [26], and branched-chain amino acids and α -keto acids [27] were assayed by enzymatic methods. Triacylglycerols were extracted with isopropyl alcohol and determined with an L-type TG H assay kit (Wako Chemicals, Richmond, VA). NEFAs (non-esterified fatty acids) were assayed by the NEFA Half Micro Assay kit (Roche Diagnostics). Blood pH, $p\text{CO}_2$ and bicarbonate levels were measured with an I-STAT clinical analyser with EC8 + cartridges (Heska Corporation). Liver glycogen was measured by an enzymatic method [28].

Metabolic flux analysis in the fasting condition

Metabolic flux analysis was determined with stable-isotope-labelled glucose as described previously [29,30]. Mice that had been fasted for 3h (16:00 h–19:00 h) were anaesthetized with 5% isoflurane and surgically implanted subcutaneously with pre-activated mini-osmotic pumps (model 2001D; 8 $\mu\text{l/h}$; Alzet Osmotic Pumps) containing 50 mg of

[U-¹³C₆]glucose (99.9% enriched; Isotech) dissolved in 200 μ l of water. Blood was collected from the mice between 10:00 h and 11:00 h the next morning.

Mass isotopomer analysis using GC–MS

Lactate and β -hydroxybutyrate were extracted from plasma with ethyl acetate. The residue obtained by drying the extract was treated with bis-trimethylsilyl trifluoroacetamide and trimethylchlorosilane (99:1, v/v) (Sulpeco) before GC–MS analysis as described by Des Rosiers et al. [31]. Analyses were performed on a Hewlett Packard Mass Selective Detector (model 5973A) connected to a Hewlett Packard Gas Chromatograph (model 5890) using chemical ionization with a ZB5 capillary column (30 m length \times 250 μ m internal diameter \times 0.25 μ m film thickness) (Phenomenex), with methane as carrier gas with a flow rate of 1.0 ml/min, sample injector temperature 150°C, and oven temperature held at 70°C for 5 min and programmed at a ramp of 10°C/min to 150°C. The clusters around m/z 219 and m/z 233 were monitored for isotopomer calculation. For glucose analysis, the aqueous phase was deproteinized, deionized, dried and treated with hydroxylamine hydrochloride and acetic anhydride to form aldonitrile penta-acetate derivatives for GC–MS analysis as described by Szafranek et al. [32] with methane as carrier gas with a flow rate of 1.0 ml/min, sample injector temperature 250°C, and oven temperature programmed from 210 to 250°C at a ramp of 10°C/min. The ion clusters around m/z 328 were monitored (m/z 327 to m/z 336).

Mass isotopomer distribution was determined from the mass spectra as described previously [33,34]. The method corrects for the contribution of derivatizing agent and natural ¹³C abundance to mass isotopomer distribution of the compound of interest and also for the presence of small amounts of m4 and m5 in the infused [U-¹³C₆]glucose. Data for the mass isotopomers in glucose, lactate, or β -hydroxybutyrate are reported as molar fractions of m0, m1, m2, etc. according to the number of labelled carbons in the molecule [33,34]. The enrichment of a certain ¹³C-labelled molecule is defined as its molar fraction m_i , the fraction of molecules with i being the number of ¹³C substitutions. The sum of all isotopomers of the molecules, $\sum m_i$ for $i = 0$ to n ($n = 3, 4$ or 6 for lactate, β -hydroxybutyrate and glucose respectively), is equal to 1 or 100%. Substrate production or turnover rates were determined using the principle of tracer dilution. At isotopic steady state, the HGP (hepatic glucose production) rate was determined from the equation [35] $\text{HGP (mg/min/kg)} = (\text{working pump rate})/m_6 - \text{working pump rate}$, where m_6 = final substrate enrichment in glucose [29].

The PDH complex produces [1,2-¹³C₂]acetyl-CoA (m2-acetyl-CoA) from [U-¹³C₆]glucose which can be incorporated into β -hydroxybutyrate with the formation of [1,2-¹³C₂] β -hydroxybutyrate or [3,4-¹³C₂] β -hydroxybutyrate, designated hereafter as m2- β -hydroxybutyrate. The amount of the ketone bodies in the blood produced from glucose was determined from the equation: ketone bodies produced from glucose (mmol/l) = $[\text{KB}] \times m_2 / (m_6 \times 2)$, where $[\text{KB}]$ = sum of plasma concentrations of β -hydroxybutyrate and acetoacetate in mmol/l, m_2 = ¹³C isotopomer enrichment in β -hydroxybutyrate, m_6 = ¹³C isotopomer enrichment in glucose. The factor 2 in the equation corrects for the two acetyl-CoA units required to produce β -hydroxybutyrate. The sum of β -hydroxybutyrate and acetoacetate is used since both ketone bodies are assumed to be in equilibrium.

Measurement of enzyme activities

For the determination of PDH complex activity, tissues were pulverized in liquid nitrogen and homogenized with a motor-driven Teflon homogenizer in 5 vol. (w/v) of extraction buffer containing 30 mM Hepes/KOH (pH 7.5), 3% (v/v) Triton X-100, 2 mM EDTA, 2% (v/v) rat serum, 5 mM DTT (dithiothreitol), 10 μ M TPCK (tosylphenylalanylchloromethane), 10 μ g/ml trypsin inhibitor, and 1 μ M leupeptin. For the determination of actual PDH complex activity, i.e. the activity that exists in the intact tissue as a result of the phosphorylation state, an aliquot of tissue extract (16 μ l) was mixed with an equal volume of resuspension buffer containing 30 mM Hepes/KOH (pH 7.5), 1% Triton X-100, 0.2 mM EDTA, 2% (v/v) bovine serum, 1 μ M leupeptin, 5 mM DTT, 20 mM dichloroacetate and 100 mM KF. For the determination of total PDH complex activity, i.e. the activity of the complex after complete dephosphorylation, an aliquot of tissue extract (25 μ l) was mixed with an equal volume of an activation buffer (resuspension buffer containing 40 mM MgCl₂, 1.5 mM CaCl₂ and 1 μ g of recombinant pyruvate dehydrogenase phosphatase 1 protein). Complete activation of the PDH complex by dephosphorylation was achieved by incubating this mixture at 30°C for 20 min. Activity of the PDH complex was measured spectrophotometrically in a 96-well plate reader (Spectra Max 190, Molecular Devices) with a coupled assay based on the reaction catalysed by arylamine acetyltransferase as described previously [36]. One unit of PDH complex activity corresponds to the acetylation of 1 μ mol of *p*-(*p*-aminophenylazo)-benzenesulfonate per min at 30°C.

PEPCK (phosphoenolpyruvate carboxykinase) activity was measured as described by Rajas et al. [37]. Glucose-6-phosphatase activity was measured as described by Foster et al. [38].

Western blot analysis

Tissue powders (50–80 mg) prepared in liquid nitrogen were homogenized with 8–10 \times (v/w) RIPA extraction buffer [25 mM Tris/HCl (pH 7.4), 150 mM NaCl, 0.1 % SDS, 0.5 % deoxycholic acid and 1 % (v/v) Nonidet P40] containing HaltTM protease inhibitor (1 \times) and phosphatase protease inhibitor cocktails (1 \times) (Thermo Scientific). Protein was determined with a Bio-Rad Laboratories Protein Assay Kit using BSA as standard. Protein (10–30 μ g) was separated by SDS/PAGE (10% gels) [39] and transferred on to nitrocellulose membranes (Bio-Rad Laboratories) by the semi-dry electroblotting method [8] and probed with antibodies against phospho-Ser²⁹³-PDHE1 α (pyruvate dehydrogenase E1 α) (AP1062; EMD Chemicals), PDHK2 (SC-100534, Santa Cruz Biotechnology) and PDHK4 [19], HSP60 (heat-shock protein 60) (622562; Transduction Laboratories) and PDHK1 (KAP- PK112; Stressgen). Rabbit PDHK3 antiserum was generated by AbFrontier against the C-terminal 19 amino acids (RDASKYKAKQDKIKSNRTF) that are unique to PDHK3. By Western blot analysis, the antiserum against this peptide detected a protein with the expected molecular mass of 45 kDa in tissue extracts of organs (brain, kidney and testes) known to express PDHK3 [5]. That this protein corresponded to PDHK3 was confirmed by its absence in tissue extracts of brain, kidney, and testes harvested from PDHK3-KO mice (kindly provided by Dr Kirill M. Popov, University of Alabama at Birmingham, Birmingham, AL, U.S.A.). Images of the Western blots were processed with a Gel DockTM XR⁺ Imaging System (Bio-Rad Laboratories).

Statistical analysis

The statistical significance of differences between groups was determined by Student's *t* test or one-way ANOVA when appropriate. Results are either means \pm S.D. or means \pm S.E.M. for the indicated number of independent samples. $P < 0.05$ was considered statistically significant.

Results

Fed and fasting blood glucose levels in PDHK2-KO, PDHK4-KO and PDHK2/PDHK4-DKO mice

PDHK2-KO mice were generated to assess the importance of this kinase in glucose homeostasis during feeding and fasting. PDHK2-KO mice are viable and do not differ from wild-type mice in growth and body composition when fed on a standard rodent chow diet (results not shown). Interestingly, relative to wild-type mice, blood glucose levels of PDHK2-KO mice were reduced in the fed, but not the fasted, state (Table 1). In contrast, as we have reported previously [19], PDHK4-KO mice have lower blood glucose levels in the fasted, but not the fed, state (Table 1). Therefore, not surprisingly, knocking out both PDHK2 and PDHK4 to produce DKO mice resulted in significantly lower blood glucose levels in both the fed and the fasted state, with the effect greater in the fasted than the fed state (Table 1). To evaluate further the effect of PDHK deficiency on glucose homeostasis, glucose-tolerance studies were conducted. Previously, we found that glucose tolerance was mildly, but significantly, enhanced in PDHK4-KO mice compared with wild-type mice (area under the curve reduced by 17%, $n = 12$ per group, $P < 0.01$) [19]. In contrast, no difference in glucose-tolerance was found in direct comparison of PDHK2-KO mice with wild-type mice (Figure 1A). In spite of these findings, the tolerance of DKO mice to glucose was remarkably improved (area under the curve of 13413 ± 1176 compared with 26592 ± 2749 mg/dl per min for wild-type mice; means \pm S.E.M., $n = 4$ per group, $P < 0.05$) (Figure 1B). Insulin levels measured in blood collected 30 min after initiation of the glucose-tolerance test were also lower in the DKO mice (1.0 ± 0.2 for DKO mice compared with 2.5 ± 0.9 ng/ml for wild-type mice, means \pm S.E.M., $n = 4$ per group, $P < 0.05$), consistent with greater insulin-sensitivity in the DKO mice.

To determine the effect of prolonged fasting on blood glucose levels, mice of the four genotypes were concurrently fasted for 24 h. Relative to wild-type mice, 24 h of fasting significantly lowered blood glucose levels in the PDHK4-KO and DKO mice, but not in the PDHK2-KO (Figure 1C).

Since glucose and insulin levels were lower in the DKO mice during the glucose-tolerance test, an insulin-tolerance test was conducted to assess insulin-sensitivity. Insulin had a greater effect on blood glucose levels in the DKO mice (Figure 1D; area above the curve of 4800 ± 294 compared with 3634 ± 304 mg/dl per min for wild-type mice, means \pm S.E.M., $n = 6$ per group, $P < 0.05$), indicating greater insulin-sensitivity than in wild-type mice.

Effect of knocking out PDHK2 and PDHK4 on PDH complex activity state in the fed and fasted state

Consistent with lower blood glucose levels in the fed state, the actual activity and activity state of PDH complex were increased in the liver and skeletal muscle of PDHK2-KO and PDHK2/PDHK4-DKO mice (Table 2). Likewise, consistent with the lack of an effect on blood glucose level in the fed state, the actual activity and activity state of PDH complex were not increased in the liver of PDHK4-KO mice (Table 2). However, the actual activity of the complex was increased significantly in the skeletal muscle. Likewise, consistent with no effect on blood glucose levels in the fasted state, actual PDH complex activity in PDHK2-KO mice did not differ from that of wild-type mice in the liver and skeletal muscle (Table 3). In contrast, PDH complex actual activity was modestly enhanced in the liver and markedly increased in the skeletal muscle of fasted PDHK4-KO mice (Table 3). In spite of the absence of any effect of knocking out PDHK2, the combined deficiency of PDHK2 and PDHK4 provoked a 3- and 4-fold increase in PDH complex activity in the liver and skeletal muscle respectively. These findings suggest that PDHK2 is more important in the fed state, whereas PDHK4 is more important in the fasted state. This agrees with the higher level of PDHK2 expression in the fed state and the much greater induction of PDHK4 in the fasted state [12]. In this regard, it is noteworthy that double deficiency of PDHK2 and PDHK4 induced synergistic effects on PDH complex activity in both the liver and the muscle in the fasted state, but not in the fed state (Tables 2 and 3).

To confirm that increases in PDH complex activity are due to decreased phosphorylation of the E1 α subunit of the PDH complex, the relative amounts of phosphorylated protein for the primary phosphorylation site (Ser²⁹³) of the E1 α subunit of the PDH complex were determined by Western blot analysis with a site-specific phosphoprotein antibody [40]. Since the greatest increase in PDH complex activity was found in skeletal muscle of PDHK2/PDHK4-deficient mice, levels of phosphorylated E1 α -Ser²⁹³ were determined in the skeletal muscle of wild-type, PDHK2-KO, PDHK4-KO and DKO mice in the fed and fasted state (Figure 2). As anticipated, levels of the phosphorylated E1 α subunit of the PDH complex relative to wild-type mice were significantly reduced in PDHK2-KO and PDHK4-KO mice, and dramatically lowered in DKO mice, consistent with the actual activities of the PDH complex measured in the fed and fasted states.

Blood concentrations of gluconeogenic precursors and ketone bodies are greatly altered in PDHK2/PDHK4-DKO mice

Knocking out the PDHKs enhances PDH complex activity and thereby increases pyruvate oxidation, resulting in lower levels of pyruvate, lactate and alanine, the principal three-carbon substrates for gluconeogenesis [19,41]. As predicted, serum levels of lactate, pyruvate and alanine were significantly lowered in overnight-fasted DKO mice (Table 4). Since pyruvate is reduced more than lactate, the lactate/pyruvate ratio was greatly elevated in the DKO mice compared with wild-type mice. Branched-chain amino acids levels were elevated, but branched-chain α -keto acids were reduced, consistent with the reduced availability of the pyruvate required to recycle glutamate back to α -ketoglutarate for transamination of the branched-chain amino acids [19]. In contrast with the gluconeogenic precursors, β -hydroxybutyrate and acetoacetate were greatly increased in DKO mice (Table

4), in spite of no difference in serum levels of NEFAs and triacylglycerol. As a result of a greater increase in acetoacetate than in β -hydroxybutyrate, and in striking contrast with the more reduced state of the lactate/pyruvate ratio, the hepatic β -hydroxybutyrate/acetoacetate ratio was decreased (1.3 ± 0.2 in DKO mice compared with 1.68 ± 0.09 in wild-type mice, $n = 6$ mice per group, $P < 0.05$), indicating a more oxidized mitochondrial matrix space in the DKO mice.

Fed and fasting liver glycogen levels in PDHK2-KO, PDHK4-KO and PDK2/PDK4-DKO mice

In the fed state, liver glycogen levels of the DKO mice, but not the single-KO mice, were significantly lower (17%) than that of the wild-type mice (Figure 3A). After fasting for 24 h, liver glycogen remained higher in the wild-type mice than in the KO mice, higher in the PDHK2-KO mice than in the other KO mice, and virtually depleted in the PDHK4-KO and DKO mice (Figure 3B). When considered in combination with the effect of fasting on blood glucose levels (Figure 1C), these findings suggest that liver glycogen was used more rapidly to maintain blood glucose levels in PDHK-deficient mice. Retention of larger amounts of liver glycogen in the PDHK2-KO mice than in the PDHK4 and DKO mice is consistent with less effect of fasting on blood glucose levels.

Pyruvate tolerance and clearance is enhanced in DKO mice

To determine whether exogenous pyruvate would eliminate the difference in fasting blood glucose levels between the wild-type and DKO mice, pyruvate was administered according to the standard pyruvate-tolerance test. The DKO mice showed a greater tolerance to exogenous pyruvate than wild-type mice (area under the curves of 5725 ± 30 and 8460 ± 735 arbitrary units respectively, $n = 4$ mice/group, $P < 0.02$) (Figure 3C). Although blood glucose levels of the DKO mice were not restored to those of the wild-type, this can be explained by rapid clearance of pyruvate from the blood of DKO mice.

Whereas serum pyruvate concentrations measured 15 min after initiation of the test were high enough to maintain maximum rates of gluconeogenesis in both the wild-type (1.8 ± 0.3 mM) and DKO mice (0.7 ± 0.1 mM, means \pm S.E.M., $n = 5$ mice per group, $P < 0.05$), the levels measured after 30 min were too low (0.039 ± 0.002 in DKO mice compared with 0.53 ± 0.04 mM for wild-type mice, means \pm S.E.M., $n = 5$ mice per group, $P < 0.001$) and the lactate/pyruvate ratio was too high [42] in the DKO mice (106 ± 18 compared with 18 ± 4 for wild-type mice, means \pm S.E.M., $n = 5$ mice per group, $P < 0.01$). The alanine concentration measured at 30 min in the DKO mice (0.041 ± 0.009 compared with 0.32 ± 0.04 mM for wild-type mice, means \pm S.E.M., $n = 5$ mice per group, $P < 0.01$) was also too low for maximum rates of glucose synthesis. During the first 15 min of the test, glucose levels increased at comparable rates in wild-type and DKO mice (3.7 ± 0.3 and 3.4 ± 0.3 mg/dl per min respectively, $n = 4$ mice/group, not significantly different). As expected from the concentrations of pyruvate and alanine measured, the rate of increase in glucose during the second 15 min of the test remained the same in the wild-type mice, but fell off dramatically in the DKO mice (2.3 ± 0.5 compared with 3.9 ± 0.3 mg/dl per min for wild-type mice). Therefore, under conditions of saturating concentrations of gluconeogenic substrates, i.e. the first 15 min of the test, no difference in rates of increase in glucose

concentration were found between wild-type and DKO mice, suggesting comparable enzymatic capacity for gluconeogenesis in DKO and wild-type mice.

Activities of key gluconeogenic enzymes are not reduced in the liver of DKO mice

Consistent with the results of the pyruvate-tolerance test, the activity of hepatic PEPCK was not different between the wild-type and DKO mice (1.50 ± 0.2 compared with 1.25 ± 0.15 $\mu\text{mol}/\text{min}$ per g of wet weight for wild-type, mean \pm S.E.M., $n = 5$ mice per group, $P = 0.30$) and the activity of glucose-6-phosphatase was greater rather than reduced in the DKO mice (1.54 ± 0.13 compared with 1.01 ± 0.18 $\mu\text{mol}/\text{min}$ per g of wet weight for wild-type mice, $n = 5$ per group, $P < 0.05$), suggesting that reduced enzymatic capacity for hepatic gluconeogenesis is not responsible for lower blood glucose levels in the DKO mice.

Rate of glucose production is decreased in DKO mice

Stable-isotope-based metabolic flux measurements with $[\text{U}-^{13}\text{C}_6]\text{glucose}$ revealed a 52% lower rate of glucose production in DKO mice relative to wild-type mice (Figure 3D). Therefore the lower blood glucose levels seen in the DKO mice can be explained at least in part by a lower rate of glucose production, which is consistent with the hypothesis that substrate supply limits the rate of gluconeogenesis in PDHK-deficient mice.

Contribution of acetyl-CoA produced by the PDH complex to ketone body production in DKO mice

In the fasted state, ketone bodies are primarily derived from acetylCoA produced by the oxidation of fatty acids in the liver. Synthesis of ketone bodies from glucose and the three-carbon compounds derived from glucose is largely prevented by inactivation of the PDH complex by phosphorylation. Because the PDH complex remains partially active in the fasted state in DKO mice, glucose should contribute more carbon to the synthesis of ketone bodies in these mice. This was examined by measuring the incorporation of carbon from $[\text{U}-^{13}\text{C}_6]\text{glucose}$ into ketone bodies in wild-type and DKO mice. As anticipated, greater β -hydroxybutyrate enrichment with two ^{13}C carbons ($m2$ - β -hydroxybutyrate) was found in the plasma of the DKO mice, which, combined with the greater concentration of ketone bodies in the DKO mice, established that more ketone bodies were produced from glucose in the DKO mice than in the wild-type mice (Figure 4A). This finding is consistent with greater flux through the PDH complex with subsequent conversion of acetyl-CoA into ketone bodies. However, the relative contribution of glucose carbon to the formation of ketone bodies was minuscule ($<1\%$ in both the wild-type and DKO mice) relative to other carbon sources, which presumably were almost entirely fatty acids. Since serum levels of NEFAs were similar between DKO and wild-type mice (Figure 4B), greater availability of fatty acids for oxidation does not explain the increase in ketone bodies.

Fasting induces ketoacidosis and hypothermia in the DKO mice

PDHK4-KO mice tolerate fasting without evidence of metabolic decompensation [19–21]. Since preliminary studies suggested that DKO mice are more sensitive to fasting, the metabolic effects of fasting for various periods of time were determined with wild-type, single-KO and DKO mice (Figures 4C and 4D). Relative to wild-type mice, a modest, but

significant, increase in β -hydroxybutyrate occurred after 12 h, but not after 24 and 36 h, of fasting in PDHK2-KO mice (Figure 4D). In PDHK4-KO mice, acetoacetate was significantly increased after 24 and 36 h of fasting and β -hydroxybutyrate after 36 h of fasting. In the DKO mice, fasting induced much higher levels of both ketone bodies throughout the study than observed in the other genotypes. Fasting for 36 h induced nearly a 5-fold increase in acetoacetate in DKO mice compared with wild-type mice and a 2.5-fold increase compared with PDHK4 KO (Figure 4C). In addition, the concentration of β -hydroxybutyrate was elevated approximately 4-fold in the DKO mice compared with wild-type mice and 2-fold compared with PDHK4-KO mice (Figure 4D). Because ketosis can induce metabolic acidosis, the blood pH of the DKO mice was determined. Fasting for 4 h significantly lowered blood pH in the DKO mice compared with wild-type mice (7.08 ± 0.03 and 7.20 ± 0.06 respectively; Figure 5A). After 24 h of fasting, blood pH of the DKO mice reached dangerously low levels (6.87 ± 0.07 and 7.22 ± 0.03 respectively) owing to severe ketoacidosis. Unlike the response of the DKO mice, 36 h of fasting did not lower blood pH of PDHK2-KO and PDHK4-KO mice (Figure 5B). As expected, with the presence of acidosis, the concentration of bicarbonate was dramatically reduced in the DKO mice (9 ± 2 mmol/l) compared with wild-type mice (20.7 ± 0.7 mmol/l) (Figure 5C). Furthermore, $p\text{CO}_2$ was significantly reduced in DKO mice (25 ± 4 compared with 40 ± 6 mmHg for wild-type mice, means \pm S.E.M., $n = 4$ per group, $P < 0.05$). In addition to suffering from ketoacidosis, the DKO mice experienced hypothermia (27 ± 2 compared with $34.5 \pm 0.7^\circ\text{C}$ in wild-type mice, $n = 5$ mice per group, $P < 0.05$) after 36 h of fasting, leading ultimately to their death.

Expression of PDHK4 does not compensate for a lack of PDHK2 in PDHK2-KO mice and vice versa

PDHK2 and PDHK4 were measured by Western blot analysis to assess whether altered expression of these proteins compensates for the lack of PDHK2 and PDHK4 in the corresponding KO mice (Figure 6). Protein levels of PDHK2 were not changed in the tissues of the PDHK4-KO mice compared with wild-type mice. Protein levels of PDHK4 were likewise similar in heart, liver and skeletal muscle of PDHK2-KO mice and wild-type mice. These findings suggest that, in the fasted state, expression of PDHK2 does not compensate for the lack of PDHK4 and expression of PDHK4 does not compensate for the lack of PDHK2.

Expression of PDHK1 and PDHK3 does not compensate for the lack of PDHK2 and PDHK4 in the DKO mice

Protein levels of PDHK1 were similar in the liver and skeletal muscle of wild-type, PDHK2-KO and PDHK4-KO mice (results not shown), suggesting that PDHK1 does not compensate for the loss of PDHK2 or PDHK4. Furthermore, the amounts of PDHK1 expressed in the liver and skeletal muscle were similar for wild-type and DKO mice, but slightly reduced in the heart of DKO mice (Figure 6). Therefore the expression of PDHK1 does not compensate for the lack of PDHK2 and PDHK4 in the DKO mice. PDHK3 protein could not be detected in the liver, skeletal muscle and heart of wild-type and DKO mice with an antibody that readily detects this protein in brain, testes and kidney (results not shown).

Discussion

The present study shows that PDHK2 deficiency lowers blood glucose in the fed state without a large increase in ketone bodies, PDHK4 deficiency lowers blood glucose in the fasted state with a significant increase in ketone bodies, and a combined PDHK2 and PDHK4 deficiency lowers blood glucose in both the fed and fasted states with an increase in ketone bodies that culminates in metabolic acidosis and decompensation.

The finding that PDHK2 was effective in the fed state but not the fasted state was unexpected, but can be rationalized on the basis of the expression pattern and regulatory properties of PDHK2 relative to the other PDHKs. PDHK2 is constitutively expressed in tissues in the fed and fasted state [12,43], its activity is more sensitive than the other PDHKs to modulation by pyruvate, NADH and acetyl-CoA [15,44], and its expression level is only modestly increased by fasting in the liver and kidney of rats [8] and by diabetes in Otsuka Long–Evans Tokushima Fatty rats [43], and in Type 2 diabetes in humans [45]. Because these observations seemed to suggest an important role for PDHK2 in the regulation of glucose metabolism during fasting and in diabetes, we were surprised to find that blood glucose is maintained at normal fasting levels in PDHK2-KO mice. Consistent with this observation, knocking out PDHK2 had no effect upon the phosphorylation and activity state of the PDH complex in the fasted state in either the liver or the skeletal muscle. Because PDHK2 deficiency does not alter PDH complex activity, the rate of pyruvate oxidation is not increased and therefore the supply of three-carbon compounds for hepatic and renal glucose production is not affected in these mice. Since gluconeogenesis was not limited by substrate supply, glucose levels were not affected.

In wild-type mice, the PDH complex is normally less phosphorylated and more active in the fed state than in the fasted state. Nevertheless, a large portion of the complex remains phosphorylated and catalytically inactive in the fed state. Therefore a considerable reserve of PDH complex activity exists in the fed state that can be called into play by dephosphorylation when needed. The present study suggests that PDHK2 plays an important role in phosphorylating and therefore maintaining this reserve of PDH complex activity in the fed state. Rendering mice deficient in PDHK2 reduces this reserve, resulting in greater PDH complex activity and therefore a greater rate of glucose disposal and lower glucose levels in the fed state. Because gluconeogenesis is inhibited in the fed state, lower blood glucose levels are not the result of a reduced rate of glucose production, but, rather, increased glucose disposal.

PDHK4 has received most of the attention in studies on physiological regulation of the PDH complex because its expression is more sensitive to the metabolic stress of fasting and diabetes [8]. The expression of PDHK4 is remarkably sensitive to stimulation by glucocorticoids and PPAR (peroxisome-proliferator-activated receptor) α and δ ligands and to inhibition by insulin [16,46,47], consistent with an important role for PDHK4 in setting the phosphorylation and activity state of PDH complex in fasting and diabetes. In addition, fasting blood glucose levels are significantly reduced in PDHK4-KO mice relative to that of corresponding control mice [19,20]. In the present study, the phosphorylation state of the PDH complex was found to be reduced in PDHK4-KO mice, consistent with the increase in

the PDH complex activity state reported previously [17]. Lactate, pyruvate and alanine levels are therefore reduced in PDHK4-KO mice, resulting in lower blood glucose levels for want of three-carbon compounds for gluconeogenesis. In the fed state, PDHK4 deficiency has no effect on blood glucose because PDHK4 is not expressed at high enough levels to affect the activity state of PDH complex. PDHK4 is effective in the fasted state because of the higher level of its expression and therefore its greater importance in regulation of PDH complex.

Double deficiency of PDHK2 and PDHK4 greatly increased PDH complex activity and reduced blood glucose levels in both the fed and the fasted state. Whereas a greater rate of glucose oxidation can explain the reduced level of blood glucose in the fed state, the reduced concentrations of pyruvate, alanine and lactate suggest that a decreased supply of substrates needed for gluconeogenesis is the mechanism by which double deficiency of PDHK2 and PDHK4 reduces blood glucose levels in the fasted state. Consistent with this hypothesis, metabolic flux analysis with [U-¹³C₆]glucose indicated that DKO mice synthesized glucose at a lower rate than did wild-type mice. Furthermore, no difference in PEPCK activity, which limits the rate of gluconeogenesis under some conditions, was found between DKO and wild-type mice. Furthermore, glucose-6-phosphatase was slightly elevated rather than reduced in DKO mice. These findings suggest that altered enzymatic capacity for gluconeogenesis is not responsible for lower blood glucose levels in DKO mice. It is therefore more likely that reduced substrate supply for gluconeogenesis limits the rate of hepatic/renal glucose production in the DKO mice. These data support findings with the PDHK inhibitor dichloroacetate [41,48,49], which lowers blood glucose levels by reducing the rate of gluconeogenesis by decreasing the blood levels of pyruvate, lactate and alanine [41].

Even though PDHK deficiency results in lower blood glucose levels [19] and may therefore serve as a therapeutic target for Type 2 diabetes [18], lack of control of PDH complex activity by PDHK2 and PDHK4 greatly increases blood ketone body levels. Overnight fasting of DKO mice caused a 4-fold increase in β -hydroxybutyrate and a 10-fold increase in acetoacetate. Ketone bodies were not significantly affected in PDHK2-KO mice compared with wild-type mice, and, although elevated in the PDHK4-KO mice, ketone body levels in these mice remained much lower than in the DKO mice. Furthermore, acidosis was induced by the ketosis in DKO mice, resulting in fatally low blood pH after 24 h of food deprivation. Although PDHK4-KO mice have elevated ketone bodies, these animals maintain blood pH levels in the normal range. Therefore PDHK2 and PDHK4 are essential for preventing ketoacidosis during fasting. At least in part, this is because inhibition of the PDH complex by PDHK2 and PDHK4 prevents competition between pyruvate and ketone body oxidation in peripheral tissues [19]. Studies with the PDHK inhibitor dichloroacetate showed that increased glucose oxidation inhibits ketone body oxidation [41]. In addition, inhibition of the PDH complex by PDHK2 and PDHK4 limits the conversion of the carbon of glucose and gluconeogenic precursors into ketone bodies. Normally, the PDH complex is inactive in the fasted state and acetyl-CoA, which is derived primarily from fatty acid oxidation, produces ketone bodies. The greater enrichment of ketone bodies with m2 β -hydroxybutyrate originating from [U-¹³C₆]glucose shows that knocking out PDHK2 and

PDHK4 increases PDH complex activity, resulting in production of acetyl-CoA from pyruvate that can be used to synthesize ketone bodies, although in insignificant amounts relative to the ketone bodies formed from fatty acids.

In contrast with the single-KO mice, PDH complex activity is greatly increased in the liver and skeletal muscle of the PDHK2/PDHK4-DKO mice, resulting in mice that cannot survive an extended period of fasting because of hypoglycaemia, ketoacidosis and hypothermia. Since PDHK2-KO and PDHK4-KO mice have mild phenotypes compared with the DKO mice, we predicted that compensation by increased expression of the other PDHKs might protect single-KO mice from deleterious effects experienced by the DKO mice. However, the expression of PDHK2 does not compensate for the lack of PDHK4 and the expression of PDHK4 does not compensate for the lack of PDHK2. Therefore the modest phenotype of the PDHK2-KO and PDHK4-KO mice relative to the DKO mice is not due to up-regulation of the expression of PDHK2 or PDHK4. Instead, the intrinsic activity of PDHK4 and its sensitivity to stimulation by acetyl-CoA and NADH [15] may be sufficient to cover for the absence of PDHK2. Likewise, PDHK2 may be able to cover partially, but less effectively, for the absence of PDHK4 in PDHK4-KO mice. The phenotype of the DKO mice is much more severe because these compensatory effects are not possible, and also because PDHK1 and PDHK3 are unable to cover for the absence of PDHK2 and PDHK4.

The present study documents a precaution relative to the potential use of PDHK inhibitors for the treatment of diabetes. Double deficiency of PDHK2 and PDHK4 renders mice sensitive to hypoglycaemia, ketoacidosis and hypothermia during fasting. Previous studies have shown that knocking out PDHK4 inhibits fatty acid oxidation in isolated diaphragms, suggesting that PDHK deficiency impairs peripheral fatty acid oxidation, resulting in hypothermia in DKO mice [19]. From these findings, it is clear that compounds that totally inhibit PDHK2 and PDHK4 would have deleterious effects. Finally, it is noted that children with idiopathic ketoacidotic hypoglycaemia suffer fasting-induced hypoglycaemia and ketoacidemia as a result of reduced levels of the gluconeogenic precursors [50]. Since similar effects are observed in PDHK2/PDHK4-DKO mice, PDHK deficiency is a potential cause and these mice might serve as an animal model for this disorder.

Acknowledgments

Funding: This work was supported by a T32 Diabetes and Obesity National Institutes of Health predoctoral fellowship [grant number T32 DK0 64466], a Merit Review Award from the Veterans Administration (to R.A.H.), National Institutes of Health R01 [grant number DK47844], a Basic Science Research Program Award [grant number 2011-0004906 (to N.H.J.)], and the World Class University Program [grant number R32-10064 (to R.A.H. and N.H.J.)] administered through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology. The Mass Spectrometry Facility at Harbor-UCLA is partly supported by the Clinical and Translational Science Institute at the University of California (UCLA CTSI) [grant number 1UL1-RR033176].

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Abbreviations

DKO	double-knockout
DTT	dithiothreitol
HGP	hepatic glucose production
KO	knockout
NEFA	non-esterified fatty acid
PDH complex	pyruvate dehydrogenase complex
PDHK	pyruvate dehydrogenase kinase
PEPCK	phosphoenolpyruvate carboxykinase

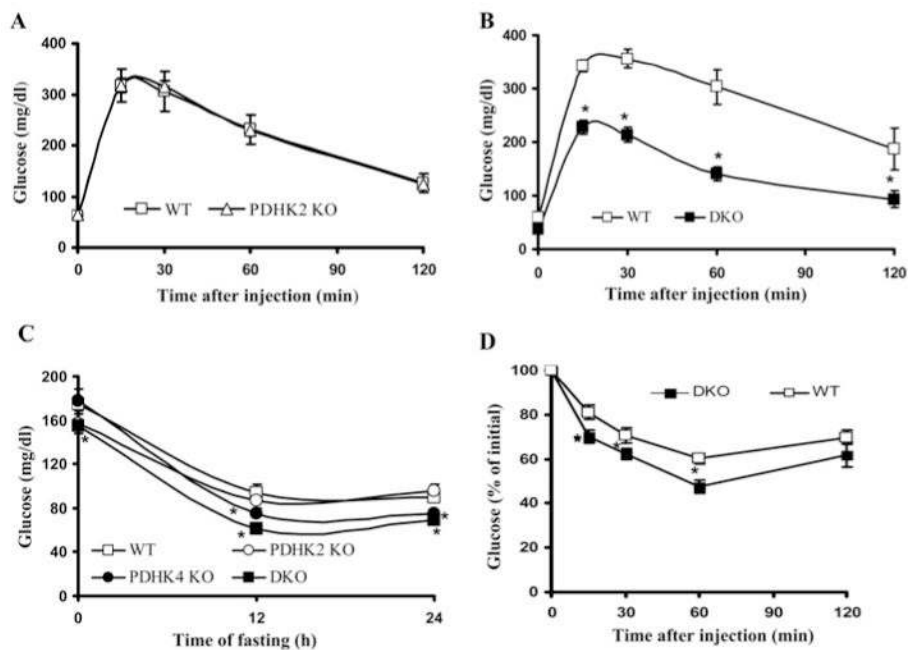


Figure 1. Improved glucose-tolerance and increased insulin-sensitivity in PDHK2/PDHK4-DKO mice, but not PDHK2-KO mice

(A) Glucose-tolerance tests were performed on wild-type (WT) and PDHK2-KO mice; $n = 4$ for both. (B) Glucose-tolerance tests were conducted with wild-type (WT) and PDHK2/PDHK4-DKO mice; $n = 4$ for both. (C) Food was removed from mice of the indicated genotypes for 24 h. Blood glucose levels were determined 0, 12 or 24 h after removal of food; $n = 6$ for wild-type (WT), PDHK2-KO and PDHK4-KO mice; $n = 5$ for DKO mice. (D) Insulin-tolerance tests were performed on wild-type (WT) and PDHK2/PDHK4-DKO mice; $n = 6$ for both. Results are means \pm S.E.M. * $P < 0.05$ relative to wild-type mice determined by Student's t test.

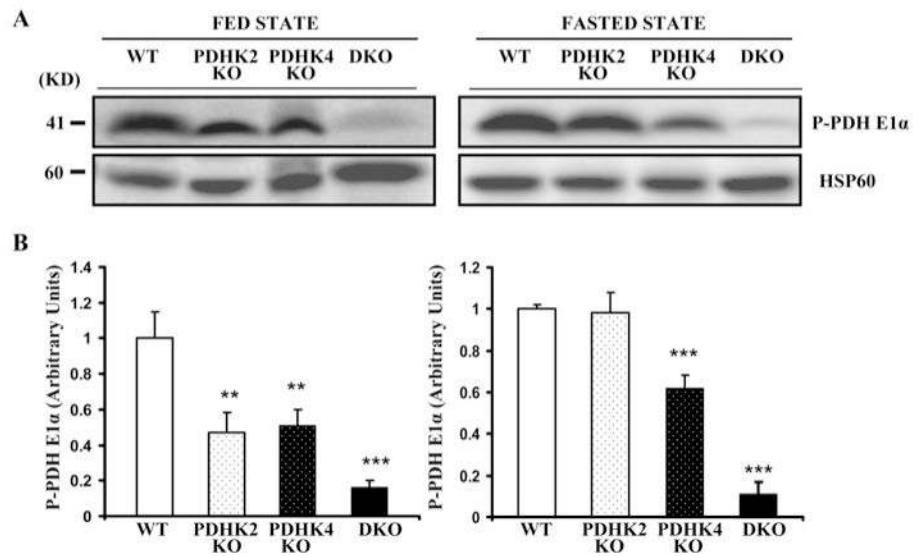


Figure 2. Decreased phosphorylation of the PDH complex E1 α subunit in the skeletal muscle of PDHK-KO mice

(A) Representative immunoblots of the Ser²⁹³-phosphorylated form of the PDH complex E1 α subunit of skeletal muscle from wild-type (WT), PDHK2-KO, PDHK4-KO and PDHK2/PDHK4-DKO mice. Molecular masses are indicated in kDa (KD). (B) Histograms constructed from data obtained by Western blot analysis. Results are means \pm S.D. with $n = 3$ mice per group. ** $P < 0.01$; *** $P < 0.001$ relative to wild-type mice determined by one-way ANOVA.

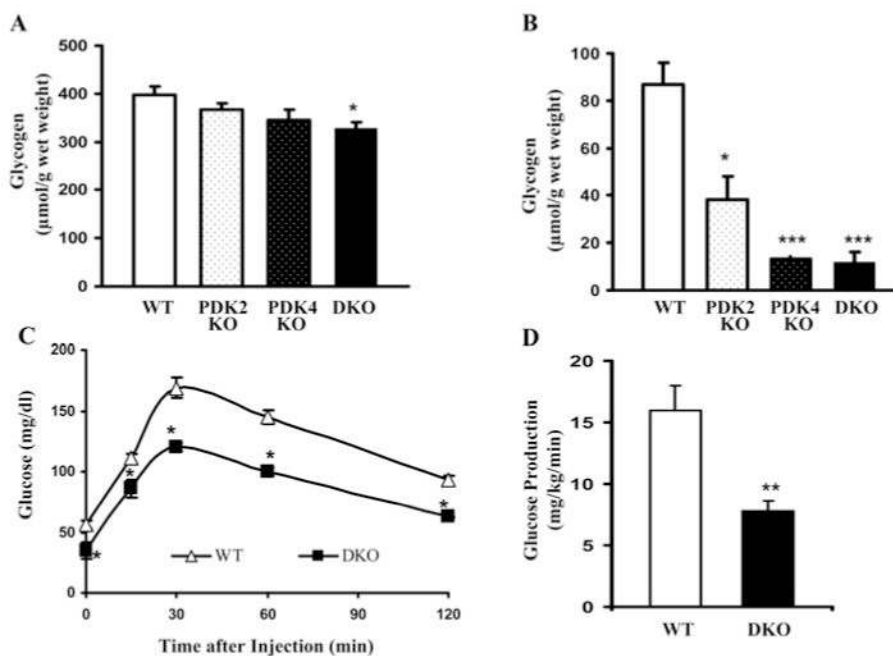


Figure 3. Glycogen levels are reduced, pyruvate clearance is increased and the rate of glucose production is reduced in PDHK2/PDHK4-DKO mice

Liver glycogen levels in wild-type (WT), PDHK2-KO, PDHK4-KO and DKO mice in the fed state (A) and 24 h fasted state (B); $n = 5$ mice per group. (C) Blood glucose levels during pyruvate-tolerance test with wild-type (WT) and PDHK2/PDHK4-DKO mice; $n = 4$ mice for both. (D) Glucose production rate in wild-type (WT) and DKO after 18 h of fasting was determined using constant infusion of $[U-^{13}C_6]$ glucose; $n = 5$ for wild-type; $n = 6$ for DKO. Results are means \pm S.E.M. * $P < 0.05$; ** $P < 0.01$ relative to wild-type mice determined by Student's t test.

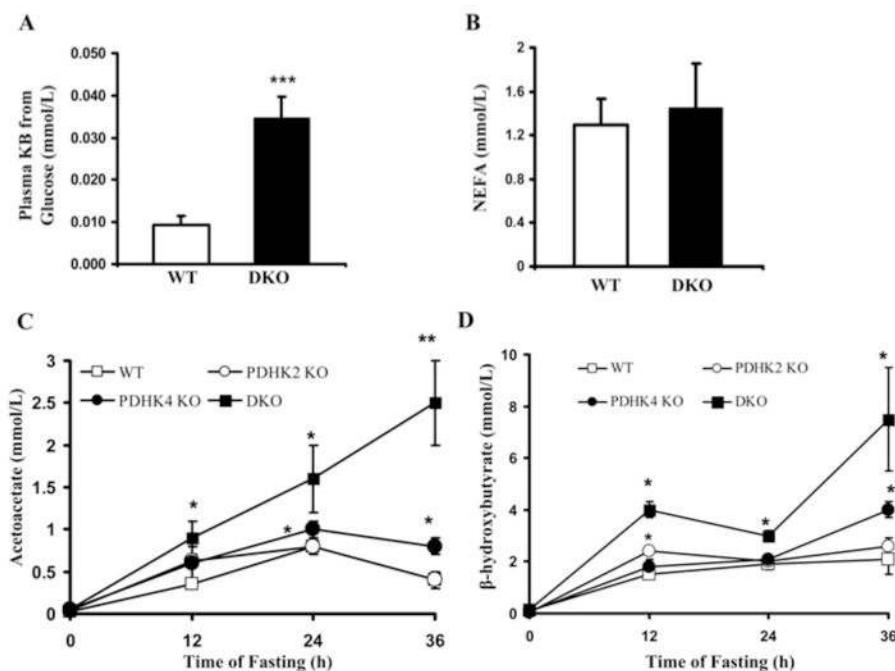


Figure 4. Conversion of glucose into ketone bodies and blood ketone bodies is increased in PDHK2/PDHK4-DKO mice

(A) Plasma ketone bodies (KB) (β -hydroxybutyrate + acetoacetate) produced from glucose in wild-type (WT) and DKO mice after 18 h of fasting was determined using constant infusion of $[U-^{13}C_6]$ glucose; $n = 5$ for wild-type; $n = 6$ for DKO. (B) Plasma NEFA levels were determined in male wild-type (WT) and PDHK2/PDHK4-DKO mice after overnight fasting; $n = 4$ for both. (C and D) Plasma acetoacetate (C) and β -hydroxybutyrate (D) levels were determined in male wild-type (WT), PDHK2-KO, PDHK4-KO and PDHK2/PDHK4-DKO mice 0, 12, 24 or 36 h after removal of food. Results are means \pm S.E.M. with $n = 5$ in each group for each time point. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ relative to wild-type mice determined by Student's t test.

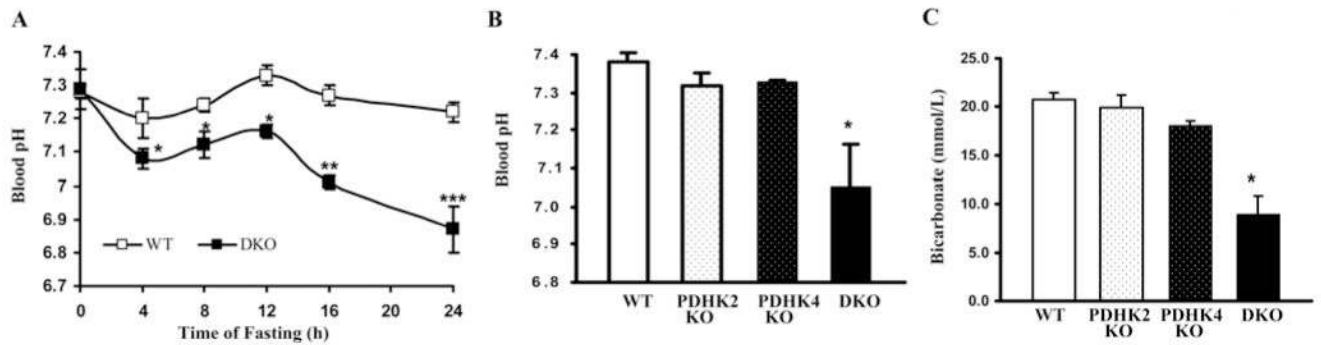


Figure 5. Fasting induces acidosis in PDHK2/PDHK4-DKO mice

(A) Blood pH was determined in wild-type (WT) and PDHK2/PDHK4-DKO mice during fasting; $n = 5$ for both. (B and C) Blood pH (B) and bicarbonate (C) were determined in wild-type (WT), PDHK2-KO, PDHK4-KO and PDHK2/PDHK4-DKO mice during fasting. Results are means \pm S.E.M. with $n = 4$ per group. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ relative to wild-type mice determined by Student's t test.

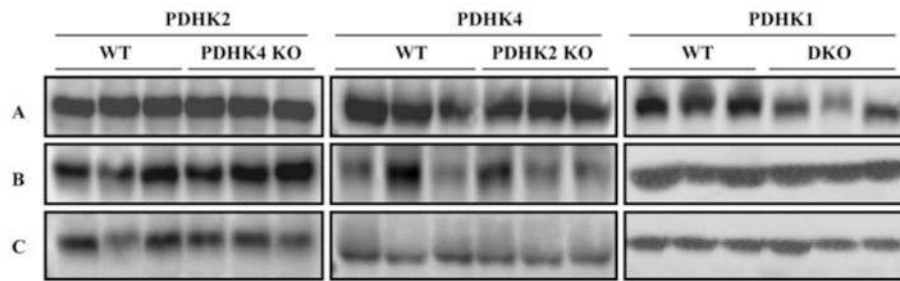


Figure 6. Deficiency of PDHK2, PDHK4, and both PDHK2 and PDHK4 does not increase expression of other PDHK isoforms in the heart, liver and skeletal muscle

Protein levels of PDHK2, PDHK4 and PDHK1 were determined by Western blot analysis in the heart (A), liver (B) and skeletal muscle (C) of wild-type (WT), PDHK4-KO mice, PDHK2-KO mice and PDHK2/PDHK4-DKO mice.

Table 1
Blood glucose levels in wild-type, PDHK2-KO, PDHK4-KO and PDHK2/PDHK4-DKO mice in the fed and fasted states

Genotype	Blood glucose (mg/dl)	
	Fed	Fasted
Wild-type	174 ± 6	94 ± 7
PDHK2-KO	149 ± 3*	87 ± 7
PDHK4-KO	177 ± 11	75 ± 2*
PDHK2/PDHK4-DKO	154 ± 4*	61 ± 2*#

Mice were fasted overnight (12 h).

* $P < 0.05$, compared with wild-type mice in the same nutritional state determined by Student's *t* test.

$P < 0.05$, compared with PDHK4-KO mice in the fasted state determined by Student's *t* test.

$n = 4$ mice per group, except for $n = 7$ for wild-type.

Table 2
**PDH complex activity in tissues of wild-type, PDHK2-KO, PDHK4-KO and PDHK2/
 PDHK4-DKO mice in the fed state**

Tissue	Genotype	Actual activity ($\mu\text{mol}/\text{min}$ per g of tissue wet weight)	Total activity ($\mu\text{mol}/\text{min}$ per g of tissue wet weight)	Activity state (% active)
Liver	Wild-type	0.7 ± 0.1	2.47 ± 0.08	30 ± 4
	PDHK2-KO	$1.3 \pm 0.1^*$	2.54 ± 0.08	$51 \pm 4^*$
	PDHK4-KO	0.8 ± 0.2	2.3 ± 0.1	32 ± 8
	PDHK2/PDHK4-DKO	$1.2 \pm 0.1^*$	2.28 ± 0.06	$56 \pm 5^*$
Skeletal muscle	Wild-type	0.4 ± 0.1	3.7 ± 0.2	9 ± 2
	PDHK2-KO	$1.98 \pm 0.04^*$	3.4 ± 0.3	$62 \pm 4^*$
	PDHK4-KO	$2.0 \pm 0.4^*$	4.2 ± 0.4	$49 \pm 10^*$
	PDHK2/PDHK4-DKO	$4.0 \pm 0.5^*$	$5.7 \pm 0.5^*$	$76 \pm 5^*$

* $P < 0.05$ compared with wild-type mice determined by Student's t test.

$n = 5$ mice per group.

Table 3
**PDH complex activity in tissues of wild-type, PDHK2-KO, PDHK4-KO and PDHK2/
 PDHK4-DKO mice in the fasted state**

Tissue	Genotype	Actual activity ($\mu\text{mol}/\text{min}$ per g of tissue wet weight)	Total activity ($\mu\text{mol}/\text{min}$ per g of tissue wet weight)	Activity state (% active)
Liver	Wild-type	0.21 \pm 0.01	2.31 \pm 0.09	9.2 \pm 0.8
	PDHK2-KO	0.17 \pm 0.02	2.15 \pm 0.04	8.1 \pm 0.8
	PDHK4-KO	0.28 \pm 0.01 *	2.28 \pm 0.09	12.2 \pm 0.8 *
	PDHK2/PDHK4-DKO	0.60 \pm 0.09 *	2.24 \pm 0.10	27 \pm 4 *#
Skeletal muscle	Wild-type	0.36 \pm 0.01	2.56 \pm 0.03	14.0 \pm 0.4
	PDHK2-KO	0.34 \pm 0.02	2.56 \pm 0.02	14.3 \pm 0.8
	PDHK4-KO	1.0 \pm 0.1 *	2.69 \pm 0.02	39 \pm 4 *
	PDHK2/PDHK4-DKO	1.85 \pm 0.03 *	2.73 \pm 0.02	67 \pm 1 *#

Mice were fasted for 24 h.

* $P < 0.05$ compared with wild-type mice.

$P < 0.05$ compared with PDHK4-KO mice determined by Student's t test.

$n = 4$ mice per group.

Table 4
Blood metabolic parameters of wild-type and DKO mice

Measurement	Wild-type	DKO
Lactate	2.64 ± 0.14*	1.49 ± 0.01
Pyruvate	0.107 ± 0.013*	0.029 ± 0.004
Alanine	0.21 ± 0.02*	0.11 ± 0.01
β -Hydroxybutyrate	0.92 ± 0.13*	3.85 ± 0.61
Acetoacetate (AcAc)	0.17 ± 0.02*	1.68 ± 0.03
Branched-chain amino acids	0.36 ± 0.03	0.87 ± 0.03
Branched-chain α -keto acids	0.060 ± 0.007*	0.041 ± 0.003
Triacylglycerol	103 ± 15	117 ± 32
[Lactate]/[pyruvate]	25 ± 2*	54 ± 7
[β -Hydroxybutyrate]/[acetoacetate]	5.5 ± 0.6*	2.3 ± 0.4

Metabolites are given in units of mmol/l, except for triacylglycerol which is given in mg/dl.

* $P < 0.05$ compared with DKO mice.

$n = 4$ mice per group.