THE ROLE OF PYRUVATE DEHYDROGENASE KINASE IN GLUCOSE AND KETONE BODY METABOLISM

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DEDICATION

I dedicate my thesis to my inspirational mother, Mariam Rahimi, and loving brother, Haroon Rahimi. The support and love of my family has provided me with the drive to become a scientist.

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ABSTRACT

Yasmeen Rahimi

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The expression of pyruvate dehydrogenase kinase (PDK) 2 and 4 are increased in the fasted state to inactivate the pyruvate dehydrogenase complex (PDC) by phosphorylation to conserve substrates for glucose production. To assess the importance of PDK2 and PDK4 in regulation of the PDC to maintain glucose homeostasis, PDK2 knockout (KO), PDK4 KO, and PDK2/PDK4 double knockout (DKO) mice were generated. PDK2 deficiency caused higher PDC activity and lower blood glucose levels in the fed state while PDK4 deficiency caused similar effects in the fasting state. DKO intensified these effects in both states. PDK2 deficiency had no effect on glucose tolerance, PDK4 deficiency produced a modest effect, but DKO caused a marked improvement, lowered insulin levels, and increased insulin sensitivity. However, the DKO mice were more sensitive than wild-type mice to long term fasting, succumbing to hypoglycemia, ketoacidosis, and hypothermia. Stable isotope flux analysis indicated that hypoglycemia was due to a reduced rate of gluconeogenesis. We hypothesized that hyperglycemia would be prevented in DKO mice fed a high saturated fat diet for 30 weeks. As expected, DKO mice fed a high fat diet had improved glucose tolerance, decreased adiposity, and were euglycemic due to reduction in the rate of gluconeogenesis. Like chow fed DKO mice, high fat fed DKO mice were unusually sensitive to fasting because of ketoacidosis and hypothermia. PDK deficiency resulted in

greater PDC activity which limited the availability of pyruvate for oxaloacetate synthesis. Low oxaloacetate resulted in overproduction of ketone bodies by the liver and inhibition of ketone body and fatty acid oxidation by peripheral tissues, culminating in ketoacidosis and hypothermia. Furthermore, when fed a ketogenic diet consisting of low carbohydrate and high fat, DKO mice also exhibited hypothermia, ketoacidosis, and hypoglycemia. The findings establish that PDK2 is more important in the fed state, PDK4 is more important in the fasted state, survival during long term fasting depends upon regulation of the PDC by both PDK2 and PDK4, and that the PDKs are important for the regulation of glucose and ketone body metabolism.

Robert A. Harris, Ph.D., Chair

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INTRODUCTION

1. Mechanisms for regulation of blood glucose levels

1.1. Regulation of blood glucose levels in the fed state

Glucose is an important nutrient for the body by serving as a major energy source for many cells. Maintaining blood glucose levels are crucial for different nutritional states. During the well fed state, blood glucose levels rise when a meal is digested and the glucose is absorbed. To reduce blood glucose levels back to normal, the beta cells of the pancreas secrete insulin. Insulin increases blood flow in the skeletal and cardiac muscle by activating nitric oxide generation which dilates blood vessels [1-3]. Increased blood flow enhances glucose delivery in the muscle. The abundance of glucose is removed from the circulation by the high affinity glucose transporter, GLUT4, which is highly expressed in muscle and fat cells [4]. Insulin stimulates glucose transport by GLUT4 across the cell membranes through a facilitative diffusion mechanism [5]. Insulin binds to the insulin receptor (IR) tyrosine kinase on the surface of muscle and adipose cells (Figure1).

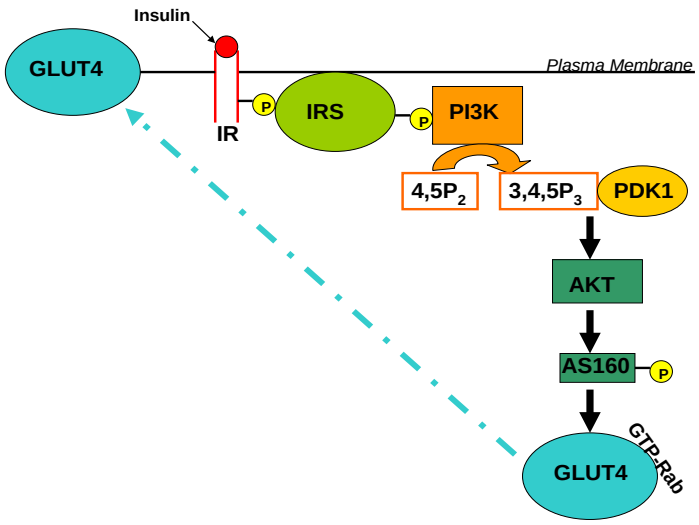


Figure 1. Insulin-stimulated signaling pathway leads to GLUT4 translocation.

This binding induces a conformational change in the receptor leading to tyrosine phosphorylation of insulin receptor substrate proteins (IRS) which in turn recruit an effector molecule, PI 3-kinase (PI3K) (Figure 1). PI3K converts phosphatidylinositol (4,5) P_2 to phosphatidylinositol (3,4,5) P_3 known as PIP_3 which stimulates the kinase activity of Akt through the interaction of phosphatidylinositol-dependent kinase-1 (PDK1) [6]. The active Akt phosphorylates the Akt 160 kDa substrate (AS160), which inhibits the GTPase-activating domain associated with AS160 and promotes Rab proteins to exchange from its GDP to GTP bound state. Increasing the active GTP bound state stimulates the recruitment of intracellular GLUT4 storage vesicle to the plasma membrane [4]. At the cell surface, GLUT4 facilitates the diffusion of glucose down its concentration gradient within the muscle and fat cells.

Insulin also promotes the storage of glucose as glycogen in liver and muscle cells [7]. Insulin activates glycogen synthase (GS), the enzyme that converts glucose to glycogen, by inhibiting glycogen synthase kinase 3 (GSK3) [8, 9] and stimulating protein phosphatase 1 (PPA1) [10, 11]. Dephosphorylated GS is active and catalyzes the formation of glycogen from glucose. Insulin stimulated glycogen synthesis and glucose uptake by GLUT4 removes glucose from the circulation and reduces blood glucose levels to normal.

1.2. Regulation of blood glucose levels in the fasted state

When blood glucose levels fall below normal, insulin secretion by beta cells of the pancreas is reduced while glucagon secretion is increased by alpha cells of the pancreas. Glucagon, a counter regulatory hormone to insulin, accelerates glycogenolysis,

the process by which liver glycogen is broken down into glucose [11, 12]. Glucagon binds to the glucagon receptor on liver cells and induces a conformational change which leads to the activation of G coupled proteins [13]. These G coupled proteins stimulate adenylate cyclase which in turn increases the level of cAMP. This second messenger activates protein kinase A (PKA). Subsequently, PKA phosphorylates and activates glycogen phosphorylase kinase which in turn phosphorylates glycogen phosphorylase, leading to its activation. Glycogen phosphorylase is the key regulatory enzyme in glycogen degradation. When glycogen is broken down, glucose is made available for cells to maintain blood glucose levels from falling lower. The second mechanism by which blood glucose levels are replenished in the fasted state is gluconeogenesis, the process by which cells synthesize glucose from metabolic precursors [14]. Gluconeogenesis occurs primarily in the liver and to a lesser extent in the kidney during periods of fasting and starvation. Since the brain and red blood cells are dependent on glucose, it is essential to synthesize glucose from precursors such as lactate, alanine, pyruvate, and glycerol. Although gluconeogenensis consists of eleven enzyme-catalyzed reactions, phosphoenolpyruvate carboxylase (PEPCK) has long been considered the most important regulatory enzyme [15]. Glucagon regulates the transcription of PEPCK by increasing cAMP which activates protein kinase A (PKA) that phosphorylates CREB [16]. Phospho-CREB binds to cAMP response element (CRE) and recruits the coactivators CBP and p300 which attract additional coactivators to initiate PEPCK gene transcription [17].

Another key enzyme in gluconeogenesis is the mitochondrial enzyme, pyruvate carboxylase (PC). This enzyme is highly expressed in liver and kidney but it is also

present in adipose tissue, pancreas and brain, showing that PC is involved in other metabolic pathways [18]. In the liver and kidney, PC synthesizes oxaloacetate from pyruvate for gluconeogenesis. In adipose tissue, PC provides oxaloacetate for condensation with acetyl-CoA for formation of citrate for de novo fatty acid synthesis [19]. In the pancreas, PC enhances glucose-stimulated insulin release [20, 21]. In brain, PC is responsible for producing oxaloacetate to replenish α-ketoglutarate for the synthesis of glutamate, the precursor for γ-aminobutyric acid (GABA) [22, 23]. The highest activity of PC is found in the fasted state primarily in gluconeogenic tissues, indicating that pyruvate carboxylase is essential for supplying oxaloacetate for PEPCK for the production of glucose. Similar to PEPCK, transcription of PC is increased by glucagon. Dissimilar to PEPCK, PC is subject to allosteric regulation [24]. It has been shown that PC is positively regulated by acetyl-CoA, enhancing the production of oxaloacetate. Regulating expression of PC and PEPCK is an essential mechanism for controlling gluconeogenesis in the fasted state to prevent blood glucose levels from falling to low levels.

It has also been shown that glucose production in the liver is controlled by substrate supply of gluconeogenic precursors (also denoted as 3 carbon compounds), lactate, pyruvate, and alanine. Metabolic flux studies in isolated hepatocytes [25] and in dogs [26] have shown that limiting the supply of 3 carbon compounds reduces the rate of gluconeogenesis. In summary, regulation of gluconeogenesis as well as glycogenolysis by various mechanisms is essential in the fasted state to maintain glucose homeostasis.

1.3. Regulation of blood glucose levels by counter-regulatory hormones

When glucose levels fall during fasting, blood levels of counter-regulatory hormones, glucagon, epinephrine, growth hormone, and cortisol, increase. Epinephrine binds to β-adrenergic receptors and causes a conformational change, leading to activation of adenylate cyclase which activates cAMP. Similar to glucagon, epinephrine stimulates glycogen breakdown. Furthermore, activated PKA phosphorylates the bifunctional enzyme 6-phosphofructo-2-kinase/fructose-2,6-biaphosphatase (PFK-2/FBPase), subsequently the kinase is inactivated and the biphosphatase is activated [27, 28]. Active FBPase catalyzes the dephosphorylation of fructose-2,6-biaphosphate to fructose-6-phosphate, resulting in lower concentrations of fructose-2,6-biaphosphatase, the positive allosteric regulator of phosphofructokinase and negative allosteric regulator of fructose-1,6-biaphosphatase. Reduction in fructose-2,6-biaphosphate stimulates fructose-1,6-biaphosphatase activity and thus, promotes glucose production by the liver. The induction of glycogenolysis and gluconeogenesis by epinephrine helps to restore blood glucose levels to normal.

Growth hormone, another counter-regulatory hormone, decreases glucose oxidation and muscle glucose uptake, although the mechanism by which growth hormone mediates these effects remain unknown [29]. Nevertheless, increased secretion of growth hormone is needed for sustaining glucose homeostasis in the fasted state. Glucocorticoid is an additional counter-regulatory hormone. It restores blood glucose levels by promoting glucose production through transcriptional activation of key gluconeogenic enzymes, PEPCK and PC [15, 30]. Glucocorticoids bind to a glucocorticoid receptor (GR) which dimerizes to form a homodimer. The GR complex enters the nucleus and

binds to the glucocorticoid response element (GRE) of the PEPCK and PC gene and induces PEPCK and PC transcription to promote glucose production [30].

1.4. Importance of anaplerosis and cataplerosis in regulation of blood glucose levels

Glucose production is mediated by key gluconeogenic enzymes, PEPCK and PC, which in turn catalyze cataplerotic and anaplerotic reactions. Anaplerosis is the process by which metabolic intermediates of the TCA cycle are replenished [31, 32]. Normally, the pool of TCA cycle intermediates is sufficient to sustain the carbon flux over a wide range, so that concentrations of TCA cycle intermediates remain constant. However, many biosynthetic pathways utilize the TCA cycle intermediates as substrates. One of these pathways is gluconeogenesis which uses oxaloacetate, the recycling TCA cycle intermediate, to produce glucose. The process by which TCA cycle intermediates are disposed is termed cataplerosis. While the major anaplerotic enzyme, pyruvate carboxylase, sustains the pool of oxaloacetate for the TCA cycle, the cataplerotic enzyme, PEPCK, utilizes oxaloacetate as substrate in gluconeogenesis [31]. Therefore, anaplerosis is coupled with cataplerosis to sustain the supply of oxaloacetate for glucose production in the liver.

1.5. Role of PDC in maintaining blood glucose levels

The mechanisms regulating hepatic glucose production are not solely ascribed to changes in key gluconeogenic enzymes but also by the availability of substrates that can be converted to glucose. Regulation of substrate availability is determined by many factors including the pyruvate dehydrogenase complex (PDC). PDC is a mitochondrial

enzyme that catalyzes the irreversible oxidative decarboxylation of pyruvate to form acetyl-CoA, CO_2, and NADH. In the well fed state, PDC is active and promotes glucose oxidation and the disposal of three carbon compounds (lactate, pyruvate, and alanine). In contrast to fed conditions, PDC is turned off in the fasted state. As a result of an inactivated PDC, pyruvate oxidation is inhibited and three carbon compounds are conserved. Preserving these compounds is indispensable for sustaining gluconeogenesis. If the complex remains totally active in the starved state, pyruvate oxidation would deplete the three carbon compounds needed for gluconeogenesis.

Switching between an active and inactive PDC is not only important in glucose production but also in transition of glucose oxidation to fatty acid oxidation as proposed by the Randle cycle [33]. A series of experiments in cardiac and skeletal muscle conducted by Randle and colleagues showed that increased fatty acid oxidation increases the ratio of [acetylCoA]/[CoA] and [NADH]/[NAD^+], both of which inhibit PDC activity. Accumulation of acetyl-CoA in the mitochondria results in increased citrate formation which in turn inhibits 6 phosphofructo-1-kinase (PFK-1), leading to increased levels of glucose-6-phosphate [34]. Glucose-6-phosphate inhibits hexokinase, leading to reduced glucose oxidation. This mechanism by which fatty acid oxidation inhibits glucose oxidation through PDC inactivation is known as the Randle cycle. Earlier the Randle cycle was proposed as a mechanism to explain insulin resistance in type 2 diabetes since the hallmark of this disease is increased fatty acid oxidation and reduced glucose oxidation [33]. However, human and rodent studies of type 2 diabetes suggest high concentrations of fatty acids cause insulin resistance by decreasing glucose uptake rather than reducing glucose oxidation [35, 36]. Increased levels of fatty acids promote

the synthesis of diacylglycerol (DAG) and ceramide. DAG activates protein kinase C (PKC) which phosphorylates and inhibits tyrosine kinase activation of the insulin receptor and tyrosine phosphorylation of insulin receptor substrate (IRS-1) [37-40]. Ceramide, a sphingolipid derivative of palmitate, on the other hand, inhibits Akt/protein kinase B [41]. Both of these lipid derivatives turn off the insulin signaling cascade and prevent insulin stimulated glucose uptake, resulting in less glucose disposal and greater insulin resistance. Although the current knowledge of insulin signaling producing insulin resistance interferences with the Randle's cycle as possible explanation, this cycle is needed to explain the transition of glucose oxidation to fatty acid oxidation which is highly dependent on the activity of PDC.

2. Mechanisms responsible for regulation of pyruvate dehydrogenase complex

2.1. Regulation of pyruvate dehydrogenase complex

The PDC is inactivated by phosphorylation by pyruvate dehyrogenase kinases (PDKs) and activated by deposphorylation by pruvate dehyrogenase phosphatases (PDPs) [42, 43]. There are four isoforms of the PDKs and two isoforms of PDPs. The multiple isoforms of the PDKs and the PDPs are distinguished by differences in tissue distribution, specific activities toward the phosphorylation sites, kinetic properties, and sensitivity to regulatory molecules [44, 45]. Phosphorylation of serine residues of the E1α subunit by the PDKs inactivates the PDC. Activation of the complex, on the other hand, is associated with a dephosphorylated state. Beyond these regulations, the PDC activity is sensitive to allosteric regulations (Figure 2).

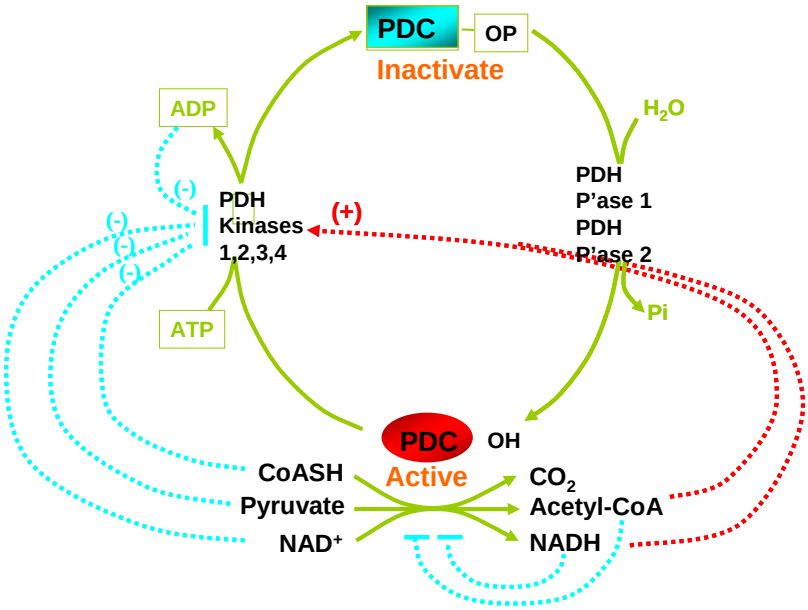


Figure 2. Regulation of pyruvate dehydrogenase complex by phosphorylation and allosteric effectors [46].

The products of the PDC reaction, acetyl-CoA and NADH, indirectly inhibit the activity of the complex by activating the PDKs. A high NADH to NAD^+ ratio reduces the lipoyl moieties of E2 while a high acetyl-CoA to CoA ratio favors the acetylations of the reduced lipoyl moieties of E2. The reduced and acetylated lipoyl moieties of E2 subunit attract the binding of the PDKs and ensure maximum kinase activity, resulting in a greater phosphorylation state and less PDC activity [47, 48]. The sensitivity to allosteric regulation by acetyl-CoA and NADH has the order of PDK2>PDK1>PDK4>PDK3 [44]. Meanwhile, pyruvate inhibits PDK activity by binding to PDK [47]. In addition, an activated state of PDC is induced by the substrates (pyruvate, NAD^+, and CoA) of the reaction [48]. These positive allosteric molecules inhibit the PDKs, resulting in activation of PDC by the PDPs.

2.2. Regulation of pyruvate dehydrogenase kinases expression and activity

Allosteric mechanisms account for short term regulation of the PDKs, while long term regulation is achieved by altered expression of the levels of PDKs, which occurs in a tissue specific manner in starvation, diabetes, and cancer. Starvation and diabetes induce the expression of PDK2 in liver and kidney [49] and the expression of PDK4 in heart [49-51], skeletal muscle [52, 53], kidney [49], and liver [44, 49, 53]. Starvation and diabetes are marked by high levels of glucocorticoids and free fatty acids and low levels of insulin. Glucocorticoids activate the glucocorticoid receptor (GR), which cooperates with the transcriptional factors Fork head members of the O class (FOXO) to recruit the co-activators p300/CBP that catalyze histone acetylation to induce PDK4 gene expression [54]. Free fatty acids stimulate peroxisome proliferator-activated receptor α (PPARα) which in turn activates PDK4 expression [55, 56]. While fasting conditions induce the expression of PDK2 and PDK4 in various of tissues, the fed state suppresses this induction. Insulin inhibits PDK4 transcription by activating the protein kinase B which phosphorylates FOXO [46, 54, 55, 57]. Phospho-FOXO leaves the nucleus and can no longer bind to p300/CBP which in turn can not foster acetylation of histones, resulting in suppression of PDK4 transcription [58]. Insulin has also been shown to repress the induction of PDK2 in hepatoma cells [55].

PDK1 expression is induced in some tumors [59, 60]. Cancer cells rely on aerobic glycolysis to generate energy, known as the Warburg effect [61]. Survival of tumor cells in a low oxygen environment requires hypoxic-induced factor (HIF) signaling [62]. HIF induces the transcription of PDK1 in tumor cells to decrease flux through the PDC and promote conversion of pyruvate to lactate. Among the four pyruvate

dehydrogenase kinases, PDK3 has a limited tissue distribution. PDK3 is expressed in testes, kidney, and brain [44] and is not subject to long term regulation in starvation and diabetes. HIF-1 induces the expression of PDK3 in some solid tumors [63].

2.3. Metabolic effect of inhibiting PDKs by dichloroacetate

The expression of PDK2 and PDK4 are induced in diabetes while PDK1 and PDK3 are induced in cancer. Since the PDKs are important in prevalent diseases, it is reasonable to target PDK inhibition as a therapeutic target for diabetes and cancer. A well-studied PDK inhibitor is dichloroactate (DCA) which was initially proposed as a treatment for lactic acidosis [64, 65]. It was anticipated that DCA lowers lactic production by increasing PDC activity through PDK inhibition to divert pyruvate into the TCA cycle instead of the synthesis of lactate. A controlled clinical trial of DCA showed a marginal reduction in blood lactate levels without diminishing acidosis [66]. Nevertheless, DCA treatment lowers the blood levels of lactate, pyruvate, and alanine in rats [67]. The reduction of these gluconeogenic precursors limits the rate of glucose production in the liver, resulting in lower blood glucose levels [64, 68]. Even though DCA has glucose lowering effects, DCA has been excluded for treatment of type 2 diabetes due to conversion of DCA to toxic metabolites, glyoxylate and oxalate [69] and causing peripheral neuropathy [64]. Additional PDK inhibitors, 3-chloroproprionate [70] and AZD7545 [71-73], lowered blood glucose levels but are not being pursued clinically.

2.4. Metabolic effect of knocking out PDK4

Although PDK inhibitors lower blood glucose levels, they lack specificity for PDK2 and PDK4 which are induced in starvation [49]. In order to better understand the role of PDK2 and PDK4 in glucose homeostasis, our group generated PDK4 knockout (KO) mice [74] and PDK2 KO mice. Initially, our group focused primarily on the PDK4 KO mice. First, we hypothesized that knocking out PDK4 would prevent the phosphorylation of PDC, resulting in increased PDC activity. As anticipated, PDC activity was significantly increased in heart, liver, skeletal muscle, kidney, and diaphragm of 48 h fasted PDK4 KO mice. In the fed state, on the hand, PDC activity was similar between PDK4 KO and wild-type mice, suggesting that regulation of PDC by PDK4 is minimal in the fed state. As a result of increased PDC activity, pyruvate oxidation was enhanced in PDK4 KO mice in the fasted state. Lower pyruvate levels reduce the supply of gluconeogenic precursors in the fasted state, yielding lower blood glucose levels in PDK4 KO mice. In the fed state, blood glucose levels are not different between PDK4 KO and wild-type mice. Reduced blood glucose levels found in PDK4 KO mice may be the result of increased glucose oxidation in peripheral tissues. Diaphragms isolated from PDK4 KO mice oxidize glucose at higher rates than diaphragms from wild-type mice. Lower fatty acid oxidation rate in PDK4 KO mice may be the result of increased glucose oxidation. The phenomenon of increased glucose oxidation inhibiting fatty acid oxidation is known as the “reverse-glucose-fatty acid cycle”. Indeed, the PDK4 inhibitor, dichloroacetate, also inhibits fatty acid oxidation and stimulates glucose oxidation in rat muscle, heart, and liver [67, 75, 76].

Since a significant up regulation of PDK4 occurs in type 2 diabetic humans [77], in genetic type 2 diabetic animal models [68], in animals fed a high fat diet [78], and in humans consuming a high fat diet [79], PDK4 KO mice and wild-type mice were fed a high fat diet rich in unsaturated fatty acids [74] and saturated fatty acids [74, 80]. On both diets, PDK4 KO mice had lower blood glucose levels compared to wild-type mice. Nevertheless, 8 months of high fat diet feeding induced hyperinsulinemia in both groups of mice even though body weight, percentage of body fat, and fat accumulation in liver and skeletal muscle were significantly lower in PDK4 KO mice compared to wild-type mice [80]. These findings support PDK4 as a viable target for the treatment of type 2 diabetes.

Although PDK4 inhibition has potential as a therapeutic target for type 2 diabetes due to its glucose lowering effect, our findings with PDK4 KO mice raise concern about side effects that may limit the use of PDK inhibitors for the treatment of diabetes. In the fasted state, blood ketone bodies (acetoacetate and β-hydroxybuyrate) are elevated more in PDK4 KO mice than in wild-type mice.

3. Mechanism responsible for regulation of ketone body levels

3.1. Regulation of ketone body production

Ketone bodies (acetoacetate and β-hydroxybutyrate) are produced by the liver to provide an alternative substrate to glucose for the production of energy in brain and other peripheral tissues during fasting. Ketone bodies are primarily derived from fatty acids which are stored in adipose tissue as triacylglycerols [81]. The rate of ketogenesis, the process by which ketone bodies are produced, is determined by the flux of fatty acids

(FA) to the liver which in turn is governed by the rate of lipolysis in the adipose tissue. During fasting, lipolysis is enhanced when insulin levels are low and catecholamine levels are high, promoting the release of fatty acids from the adipose tissue [82].

Catecholamines include the hormones ephinephrine, norepinephrine, and dopamine. Epinephrine increases cAMP by stimulation of β-adrenergic receptors which promote adenylate cyclase activity. The second messenger, cAMP, then activates protein kinase A which phosphorylates hormone sensitive lipase (HSL) and perilipin, resulting in recruitment of HSL to the surface of the lipid droplet after removal of perilipin [83, 84]. Phosphorylation of perilipin also promotes the activation of adipose tissue triglyceride lipase (ATGL) which converts triacylglycerol (TAG) to diacylglycerol (DAG) and the release of one free fatty acid. On the other hand, Hormone sensitive lipase, catalyzes the conversion of DAG to monoacylglycerol and one free fatty acid. Although HSL is capable of breaking down TAG to DAG, it performs this conversion to a lesser extent than ATGL [85]. These free fatty acids are transported to the liver where they enter the mitochondria for conversion to ketone bodies with the help of a mitochondrial membrane protein, carnitine palmityl transferase 1 (CPT1) [86], the second point of control of ketogenesis.

CPT1 is inhibited by malonyl-CoA, the product by acetyl-CoA carboxylase (ACC) [87]. AMP activated kinase (AMPK) phosphorylates ACC, leading to inactivation. Subsequently, less acetyl-CoA is converted to malonyl-CoA [88]. Suppression of malonyl-CoA levels diminishes the inhibition of CPT1 and promotes the transport of fatty acids to the mitochondria for oxidation to acetyl-CoA. Normally, acetyl-CoA combines with oxaloacetate for further oxidation to CO_2 and H_2O by the

TCA cycle. However, if oxaloacetate levels are low and acetyl-CoA levels are high, acetyl-CoA is directed to the synthesis of acetoacetyl-CoA catalyzed by thiolase [89, 90]. Acetoacetyl-CoA is further condensed with acetyl-CoA to form hydroxyl-β-methylglutaryl-CoA (HMG-CoA) and free CoA by HMG-CoA synthase, the third key regulatory enzyme for ketogenesis (Figure 2) [91]. HMG-CoA is cleared by HMG-CoA lyase to acetoacetate (AcAc), which is converted to β-hydroxybutyrate (β-HB) by reduction with NADH (Figure 2) [91-93].

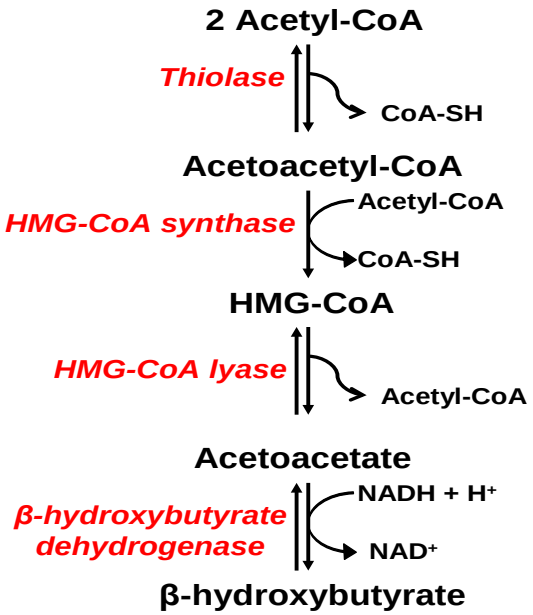


Figure 2. Key enzymes and reactions in ketogenesis. Reactions are shown in black and enzymes catalyzing the reactions are indicated in red.

HMG-CoA synthase, is highly expressed in the liver and is induced in fasting, high fat feeding, and diabetes [92]. Fasting and high fat feeding elevates the levels of fatty acids which in turn activate the peroxisome proliferator-activated receptors (PPAR) [94]. This nuclear receptor forms a heterodimer with cis-retinoid receptor (RXR) and binds to the promoter region of HMG-CoA synthase gene to induce transcription.

Hepatocyte nuclear factor 4 (HNF4) represses HMG-CoA synthase by competing for the same binding site as PPAR [95]. Besides transcriptional regulation, HMG-CoA synthase is subject to succinylation, leading to inhibition of enzyme activity [96]. Succinylation is an important control mechanism in the fed state to reduce HMG-CoA synthase activity to prevent the synthesis of ketone bodies. Glucagon has been shown to lower the mitochondrial succinyl-CoA content in perfused livers and isolated hepatocytes, thereby, stimulating ketogenesis [97]. Glucagon increases flux through HMG-CoA synthase by preventing inhibition by succinylation.

3.2. Regulation of ketone body utilization

Blood levels of ketone bodies are determined by their rates of production (ketogenesis) and utilization (ketolysis). Ketone bodies are synthesized by the liver but oxidized in heart, kidney, brain, and skeletal muscle [98]. Within the mitochondria of ketolytic organs, β-HB is oxidized back to AcAc in a reaction catalyzed by β-hydroxybutyrate dehydrogenase [81]. Acetoacetate receives a CoA moiety from succinyl-CoA, generating AcAc-CoA in a reaction catalyzed by succinly-CoA: 3-oxo-acid CoA-transferase (SCOT) [99]. Mitochondrial AcAc-CoA thiolase catalyzes the conversion of AcAc-CoA to acetyl-CoA, which enters the TCA cycle to be further oxidized to CO_2 and H_2O. SCOT is an important enzyme in ketolysis and is induced in peripheral tissues and suppressed in the liver.

3.3. Metabolic acidosis due to increased ketone bodies

Increased ketogenesis and decreased ketolysis yield an increase in blood ketone body levels which in turn provoke metabolic acidosis. Metabolic acidosis is a condition that is characterized by a fall in blood pH due to increased production of hydrogen ions by the body or the inability of the body to produce bicarbonate [100]. The causes of metabolic acidosis are lactic acidosis, ketoacidosis, renal failure, or intoxication with methanol, salicylate, propylene glycol, ethylene glycol, and 5-oxoproline [100, 101]. Lactic acidosis occurs due to increased buildup of lactic acid. When intracellular lactate is released into the blood, maintenance of electroneutrality requires the release of protons. The accumulation of protons causes the reduction of blood pH. Ketoacidosis, on the other hand, is associated with high concentrations of ketone bodies. It occurs when the body produces large amounts of ketone bodies which are accompanied by protons. While oxidation of ketone bodies disposes the protons, an inability to oxidize the ketone bodies leaves the protons in the blood while ketone bodies are excreted into the urine with sodium as the counter ion. Currently, the successful treatments for metabolic acidosis involve the administration of sodium bicarbonate and administrating insulin to decrease ketone body production and enhance ketone body utilization.

3.4. Conditions leading to increased ketoacidosis

Ketoacidosis is highly prevalent in type 1 diabetics caused by insulin deficiency and abundance of counterregulatory hormones which increase lipolysis and subsequently ketogenesis [102]. Ketoacidosis also occurs in pyruvate carboxylase (PC) deficient patients [103]. PC deficiency is a very rare autosomal recessive disease, characterized by

impaired synthesis of oxaloacetate for TCA cycle activity [104, 105]. Decreased oxaloacetate availability leads to failure of the TCA cycle with diversion of acetyl-CoA into ketone body production [89, 90]. Other conditions such as high fat feeding and ketogenic diets also induce ketosis but usually without acidosis because of the regulation of the process [106, 107]. The ketogenic diet, featuring a diet rich in fat and low in carbohydrate, increases blood ketone bodies far greater than a high fat diet, a diet rich in fat and carbohydrates. Since both diets contain an abundant amount of fat, the increased availability of free fatty acids results in increased production of ketone bodies. Since a low-carbohydrate, high fat diet is one of many dietary regimens for the treatment of obesity, individuals on these diets need to aware of the precautions such as ketosis associated with it. The body produces ketone bodies in the fasted state to be used as an energy source but when production rates exceed the capacity of utilization in these conditions, then a rise in ketone bodies can result in ketoacidosis which can be fatal.

4. Use of stable isotope tracers to study glucose and ketone body metabolism

Production of ketone bodies and glucose is essential in the fasted state to provide tissues with substrates for energy production. Although many *in vitro* studies have been conducted to understand the mechanisms by which glucose and ketone body are produced, *in vivo* studies have been limited. *In vivo* measurements of the synthesis rates of glucose and ketone bodies can be measured by utilization of stable isotope tracers in metabolic flux studies. A tracer is a compound that is chemically and functionally identical to the tracee, the naturally occurring compound of interest, but differs in the mass number due to differences in the number of neutrons. For example, ^{12}C is the most

common isotope of carbon with 98.89 % mass abundance while ^{13}C is the less common with 1.11 % mass abundance. While ^{12}C is the naturally occurring carbon, ^{13}C is known as the stable isotope. The position within a molecule of an enriched stable isotope of mass 13 carbon is referred to by the appropriate carbon number. For example, [1-$^{13}C_1$] glucose refers to a molecule of glucose in which the 1 position is labeled with carbons enriched with stable isotope of mass 13 and the subscript following the C refers to the number of specifically enriched atoms of carbon in the molecule.

To measure *in vivo* production rates of glucose in mice, for example, [U-$^{13}C_6$] glucose is delivered at a constant flow rate by a subcutaneously implanted Alzet mini osmotic pump (Model 2001D, Alzet, Palo Alto, CA) which dispenses [U-$^{13}C_6$] glucose at a constant rate of 8 µL/hour (Figure 4).

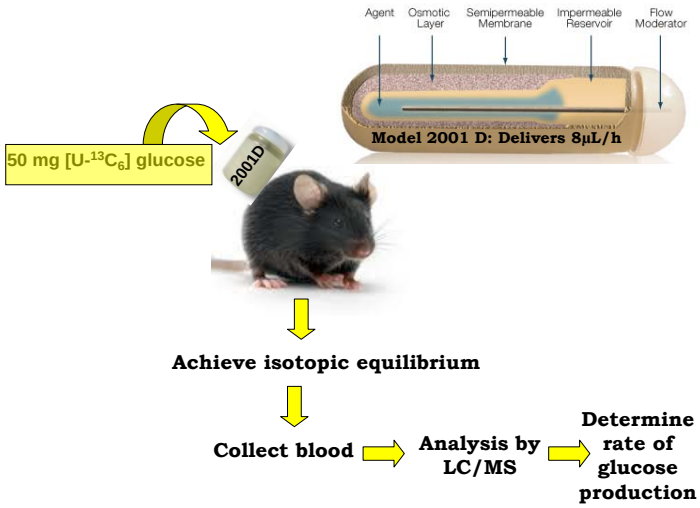


Figure 4. Utilization of stable isotope, [U-$^{13}C_6$] glucose, to determine rate of glucose production. Stable isotope [U-$^{13}C_6$] glucose is placed into the miniosmotic pump (as shown in the right corner) which is subcutaneously implanted in to the mice. After 16 h of continuous delivery, isotopic equilibrium is achieved and blood collected for measuring rate of glucose production.

Important for this method is the establishment of isotopic equilibrium which occurs when the rate of appearance of [U-$^{13}C_6$] glucose (inflow) is the same as the disappearance of the [U-$^{13}C_6$] glucose (outflow) at constant infusion rate. Isotopic equilibrium for [U-$^{13}C_6$] glucose was determined by Lee and colleagues by generating an isotope enrichment versus time curve where the plateau in the enrichment is defined as the isotopic enrichment at isotopic equilibrium (E_p) [108]. Blood is collected after 16 h of continuous delivery of [U-$^{13}C_6$] glucose to the mice. Enrichment of glucose in the blood is detected by GC/MS since $^{13}C_-$ glucose produces a separate mass signature that differentiates it from the molecular weight of naturally existing glucose. The glucose production rate (GP) is determined by the following equation:

$$GP\ (mg\ kg^{-1}min^{-1}) = \frac{[U\text{-}^{13}C_6]\ glucose\ infusion\ rate\ (mg\ kg^{-1}min^{-1})}{E_p\ (mg\ [U\text{-}^{13}C_6]\ glucose/mg\ glucose)}$$

$$-\ [U\text{-}^{13}C_6]\ glucose\ infusion\ rate\ (mg\ kg^{-1}min^{-1})$$

This equation was derived by making the assumption that at equilibrium, the rate of appearance of glucose (R_a) is the same as the disappearance of glucose (R_d) at constant infusion rate. Therefore, $R_a = R_d$ and $R_a = R_d$ at isotopic equilibrium, where

R_a = inflow of ^{12}C glucose (µmol/min)

R_d = outflow of ^{12}C glucose (µmol/min)

R_a = inflow of ^{13}C glucose (µmol/min)

R_d = outflow of ^{13}C glucose (µmol/min)

The equation above can be rearranged as follows:

$$R_a/\ R_a\ (inflow) = R_d/\ R_d\ (outflow)$$

$$\frac{^{13}C\ glucose\ (\mu mol/min)}{^{12}C\ glucose\ (\mu mol/min)}\ (inflow) = \frac{^{13}C\ glucose\ (\mu mol/min)}{^{12}C\ glucose\ (\mu mol/min)}\ (outflow)$$

Dividing out the minute term from the numerator and denominator of the outflow term

$$\frac{^{13}\text{C glucose (}\mu\text{mol/}\cancel{\text{min}}\text{)}}{^{12}\text{C glucose (}\mu\text{mol/}\cancel{\text{min}}\text{)}} \text{ (outflow)}$$

gives ^{13}C glucose (μmol)/^{12}C glucose (μmol) which corresponds to the isotopic equilibrium (E_p). We can substitute E_p for outflow:

$$\frac{^{13}\text{C glucose (}\mu\text{mol/min)}}{^{12}\text{C glucose (}\mu\text{mol/min)}} \text{ (inflow)} = E_p$$

Rearranging the equation yields:

$$^{12}\text{C glucose (}\mu\text{mol/min) (inflow)} = \frac{^{13}\text{C glucose (}\mu\text{mol/min) (inflow)}}{E_p}$$

Since ^{13}C glucose is infused in relatively large amounts, a correction has to be made for the amount infused:

$$^{12}\text{C glucose (}\mu\text{mol/min)} = \frac{^{13}\text{C glucose (}\mu\text{mol/min)}}{E_p} - {}^{13}\text{C glucose (}\mu\text{mol/min)}$$

The latter equation can be used to calculate the rate of hepatic glucose production [^{12}C glucose (μmol/min)] at isotopic equilibrium (E_p) from the rate of glucose infusion by the osmotic pump [^{13}C glucose (μmol/min)].

This method of metabolic flux analysis can also be utilized to determine rate of ketone body production. For this purpose, mice receive subcutaneously implantation of mini osmotic Alzet pump containing [U-$^{13}C_4$] β-hydroxybutytrate. After implantation, mice are fasted overnight followed by analysis of enrichment by GC/MS. Production of β-hydroxybutyrate (β-HPR) can be calculated by the equation:

$$\beta\text{-HPR (mg kg}^{-1}\text{ min}^{-1}) = \frac{[U\text{-}^{13}C_4]\ \beta\text{-hydroxybutytrate infusion rate (mg kg}^{-1}\text{ min}^{-1})}{E_p(\text{mg } [U\text{-}^{13}C_4]\ (\beta\text{-hydroxybutytrate/mg } \beta\text{-hydroxybutytrate}) - [U\text{-}^{13}C_4]\ \beta\text{-hydroxybutytrate (mg kg}^{-1}\text{ min}^{-1})} \quad [109]$$

Isotopic enrichment of β-hydroxybutyrate at isotopic equilibrium (E_p) is obtained by measuring the plateau of an enrichment versus time curve.

5. Specific aims of this study

Stable isotope tracer studies provide a useful tool for determining production rates of blood metabolites in the fed and fasted states. In the fasted state, PDK4 has been shown to regulate blood glucose and ketone body levels. PDK4 KO mice have lower blood glucose levels and increased levels of ketone bodies in the fasted state. At the beginning of this study it was not known whether PDK2 deficiency had an effect on glucose and ketone body metabolism. To answer this question, PDK2 KO mice were examined. In addition, PDK2 KO mice and PDK4 KO mice were crossed to produce the PDK2/PDK4 double knockout (DKO) mice.

Our first aim was to determine the effects of PDK2, PDK4, and PDK2/PDK4 deficiency on glucose and ketone body metabolism in mice. We hypothesized that the combined PDK2/PDK4 deficiency would have greater effects on glucose and ketone body metabolism as a result of greater increase in PDC activity compared to partially increased PDC activity in PDK2 KO and PDK4 KO mice.

Our second aim was to determine the mechanism by which PDKs regulate blood glucose levels. We hypothesized that knocking out PDK2 and PDK4 increases PDC activity which in turn limits pyruvate for gluconeogenesis. Subsequently, reduced

substrate supply to the liver would limit the rate of glucose production by the liver. We proposed that this mechanism could be tested by flux measurements with stable isotopes.

Our third aim was to determine whether PDK2/PDK4 double knockout mice maintain lower blood glucoses levels in a diet-induced diabetes mouse model. To answer this question, DKO mice and wild-type mice were fed a high saturated fat diet for 30 weeks. Our working hypothesis is that DKO mice would be protected from hyperglycemia by reduction in gluconeogenesis.

Our fourth aim was to determine the mechanism by which PDKs regulates ketone body levels and the effects of ketogenic diet in PDK deficient mice. We hypothesized that knocking both PDK2 and PDK4 would elevate ketone bodies by increasing the rate of ketogenesis and/or decreasing the rate of ketolysis. To test this hypothesis, we will perform *in vivo* and *in vitro* studies with stable and radioactive isotopes to determine the rates of keogenesis and ketolysis.

CHAPTER I: FASTING INDUCES KETOACIDOSIS AND HYPOTHERMIA IN PDK2/PDK4 DOUBLE KNOCKOUT MICE

1. Overview

The importance of pyruvate dehydrogenase kinases (PDK) 2 and 4 in regulation of the pyruvate dehydrogenase complex (PDC) was assessed in single and double knockout (DKO) mice. PDK2 deficiency caused higher PDC activity and lower blood glucose levels in the fed but not the fasted state. PDK4 deficiency caused similar effects but only after fasting. Double deficiency intensified these effects in both the fed and fasted states. PDK2 deficiency had no effect on glucose tolerance, whereas PDK4 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 DKO mice were more sensitive than wild-type and single KO mice to long term fasting, succumbing to hypoglycemia, ketoacidosis, and hypothermia. Stable isotope flux analyses indicated that hypoglycemia was due to a reduced rate of gluconeogenesis and that slightly more glucose was converted to ketone bodies in the DKO mice. The findings establish that PDK2 is more important in the fed state, whereas PDK4 is more important in the fasted state. These results indicate that survival during long periods of fasting depend upon regulation of the PDC by both PDK2 and PDK4.

2. Introduction

The pyruvate dehydrogenase complex (PDC) plays a pivotal role in controlling the concentrations of glucose in the fed and fasted state [46]. In the well fed state, PDC

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, PDC is inactivated by phosphorylation by pyruvate dehydrogenase kinases (PDKs) to conserve three carbon compounds for the production of glucose [110].

The four PDK isoenzymes responsible for phosphorylating the PDC are expressed in a tissue specific manner [44, 111, 112]. Among the four isoenzymes, PDK2 and PDK4 are most abundantly expressed in the heart [44, 50, 51], skeletal muscle [44, 52, 53, 78], and liver [44, 49, 113-115] of fasted mice. PDK2 is of interest because of its greater sensitivity to activation by acetyl-CoA and NADH and inhibition by pyruvate [116]. However, PDK4 has received greater attention because its expression is increased in many tissues by fasting and diabetes [49] and transcription of its gene is regulated by insulin, glucocorticoids, thyroid hormone, and fatty acids [58, 117]. Inactivation of the PDC by phosphorylation helps maintain euglycemia during fasting but contributes to hyperglycemia in type 2 diabetics. The increase in PDK activity in diabetes begs the question of whether the PDKs should be considered as therapeutic targets for treatment of diabetes [118]. Support for this idea is provided by the finding that mice lacking PDK4 are euglycemic in the fasted state and are more glucose tolerant than wild-type mice fed a high-fat diet [74, 80, 119].

In this study, PDK2 KO mice were produced to determine the importance of this isoform in glucose homeostasis. In contrast to PDK4 KO mice, blood glucose levels were not lowered in the fasting state and glucose tolerance was not improved in mice lacking PDK2. This raised the question of whether the presence of PDK4 compensates for the lack of PDK2 and vice versa. To answer this question, PDK2/PDK4 DKO mice

were produced and characterized. In contrast to 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 PDC by PDK2 and/or PDK4.

3. Materials and Methods

3.1. Animal protocol

Mice were housed in an *AALAC* approved, pathogen-free barrier facility (12 h light/dark cycles with temperature maintained at 23 ± 2 °C) and *ad libitum* fed a standard rodent chow diet (Harlan; #7071). Studies were conducted with approval of 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 PDC activity and phosphorylation states, mice were anesthetized by injecting pentobarbital (60 mg/kg) 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).

3.2. Generation of the PDK2/PDK4 DKO mice

The procedures used to generate $PDK4^{-/-}$ (homozygous PDK4 KO mice) C57BL6J black mice [119, 120] and $PDK2^{-/-}$ (homozygous PDK2 KO mice) C57BL6J

black mice [121] were described previously. PDK2 KO mice were crossed with PDK4 KO mice to produce PDK2/PDK4 DKO mice. Aged-matched wild type mice were produced from the C57BL6J black mice (The Jackson Laboratory, Bar Harbor, ME, U.S.A.) that were used to stabilize the genetic backgrounds of the PDK2 KO and PDK4 KO mice.

3.3. Glucose and insulin tolerance tests

Glucose tolerance tests were performed after mice had been fasted overnight (5 p.m. to 9 a.m.). Glucose (2 g/kg of body weight) was administered by intraperitoneal injection followed by measurement of glucose in tail blood by a glucometer (Accu-Check; Roche) at 0, 15, 30, 60, and 120 min after injection. Insulin tolerance tests were conducted after 6 h of fasting (8 a.m. to 2 p.m.). 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 (5 p.m. to 9 a.m.) 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.

3.4. Measurement of metabolites concentrations in blood and liver

Serum was deproteinized with 7% perchloric acid followed by neutralization with KOH and precipitation of $KClO_4$. Pyruvate [122], lactate [123], alanine [123], acetoacetate [124], β-hydroxybutyrate [124], and branched chain amino acids and α-

ketoacids [125] 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). Free Fatty acids were assayed by the NEFA Half Micro Assay kit (Roche Diagnostics, Indianapolis, IN). Blood pH, pCO_2 and bicarbonate levels were measured with an I-STAT clinical analyzer with EC8+ cartridges (Heska Corporation, Loveland, CO). Liver glycogen was measured by an enzymatic method [126].

3.5. Metabolic flux analysis in the fasting condition

Metabolic flux analysis was determined with stable-isotope-labeled glucose as described previously [127, 128]. Mice that had been fasted for 3 h (4:00 PM to 7:00 PM) were anesthetized with 5% isoflurane and surgically implanted subcutaneously with pre-activated Alza miniosmotic pumps (model 2001D; 8 μL/h; Alzet Osmotic Pumps, Palo Alto, CA) containing 50 mg of [U-$^{13}C_6$]glucose (99.9% enriched; Isotech, Miamisburg, OH) dissolved in 200 μL of water. Blood was collected from the mice between 10:00 and 11:00 AM the next morning.

3.6. 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) (Sulpelco Inc. Bellefonte, PA) before GC/MS analysis as described Des Rosiers et al. [129]. 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 meter × 250 μM (i.d.) × 0.25 μM film thickness (Phenomenex, Torrance, CA), 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 pentaacetate derivatives for GC/MS analysis as described by Szafranek et al [130] 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 [131, 132]. The method corrects for the contribution of derivatizing agent and natural ^{13}C 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 [$U^{13}C_6$]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 labeled carbons in the molecule [131, 132]. The enrichment of a certain ^{13}C-labeled molecule is defined as its molar fraction mi, the fraction of molecules with i being the number of ^{13}C substitutions. The sum of all isotopomers of the molecules, Σmi 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 hepatic glucose production (HGP) rate was determined from the equation [133]:

HGP (mg/min/kg) = (working pump rate)/m6 – working pump rate, where m6 = final substrate enrichment in glucose [127].

The PDC produces [1,2-$^{13}C_2$]-acetyl-CoA (m2-acetyl-CoA) from [U$^{13}C_6$]glucose which can be incorporated into β-hydroxybutyrate with the formation of [1,2-$^{13}C_2$]- or [3,4-$^{13}C_2$]-β-hydroxybutyrate, designated herein 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) = [KB] x m2/(m6 x 2) where [KB] = sum of plasma concentrations of β-hydroxybutyrate and acetoacetate in mmol/L, m2 = ^{13}C isotopomer enrichment in β-hydroxybutyrate, m6 = ^{13}C 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.

3.7. Measurement of enzyme activities

For the determination of PDC activity, tissues were pulverized in liquid nitrogen and homogenized with a motor-driven Teflon homogenizer in 5 volumes (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 dithiothreitol (DTT), 10 µM tosyl phenylalanyl chloromethyl ketone(TPCK), 10 µg/ml trypsin inhibitor, and 1 µM of leupeptin. For the determination of actual PDC activity, i.e. the activity that exists in the intact tissue as a result of the phosphorylation state, an aliquot of tissue extract was mixed with an equal volume of re-suspension buffer containing 30 mM HEPES/KOH (pH 7.5), 1% Triton X-100, 0.2 mM EDTA, 2% bovine serum, 1 µM leupeptin, 5 mM DTT, 20 mM

dichloroacetate, 100 mM KF. For the determination of total PDC activity, i.e. the activity of the complex after complete dephosphorylation, an aliquot of tissue extract (25 µL) was mixed with an equal volume of an activation buffer (re-suspension buffer containing 40 mM $MgCl_2$, 1.5 mM $CaCl_2$ and 1µg of recombinant pyruvate dehydrogenase phosphatase 1 protein). Complete activation of PDC by dephosphorylation was achieved by incubating of this mixture at 30 °C for 20 min. Activity of the PDC was measured spectrophotometically in a 96 well plate reader (Spectra Max 190, Molecular Devices, Sunnyvale, CA) with a coupled assay based on the reaction catalyzed by arylamine acetyltransferase as described previously [134]. One unit of PDC activity corresponds to the acetylation of 1 µmol of *p*-(*p*-aminophenylazo)-benzenesulphonate per min at 30 °C.

Phosphoenolpyruvate carboxykinase (PEPCK) activity was measured as described by Rajas et al. [135]; glucose-6-phosphatase activity as described by Foster et al. [136].

3.8. Western blot analysis

Tissue powders (50 - 80 mg) prepared in liquid nitrogen were homogenized with 8-10x (v/w) RIPA extraction buffer containing Halt™ protease inhibitor (1x) and phosphatase protease inhibitor cocktails (1x) (Thermo Scientific, Rockford, IL). Protein was determined with a Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA) using bovine serum albumin as standard. Ten to 30 µg of protein were separated on 10% (w/v) SDS-PAGE [137] and transferred to nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA) by the semidry electroblotting method [53] and probed with antibodies against phospho-Ser293-PDHE1α (AP1062; EMD Chemicals), PDK2 (SC-

100534, Santa Cruz Biotechnology) and PDK4 antiserum [119], HSP-60 (622562; Transduction Laboratories), and PDK1 (KAP-PK112; Stressgen). Rabbit antiserum for PDK3 was generated by AbFRONTIER (Seoul, Korea) against the C-terminal 19 amino acids (rdaskykakqdkiksnrtf) that are unique to PDK3. By Western blot analysis, the antiserum against this peptide detected a protein with the expected molecular weight of 45 kDa in tissue extracts of organs (brain, kidney, and testes) known to express PDK3 [44]. That this protein corresponded to PDK3 was confirmed by its absence in tissue extracts of brain, kidney, and testes harvested from PDK3 KO mice (kindly provided by Dr. Kirill M. Popov, University of Alabama at Birmingham). Images of the Western blots were processed with a Gel Dock^{TM} XR^{+} Imaging System (Bio-Rad Laboratories, Hercules, CA).

3.9. Statistical analysis

The statistical significance of differences between groups was determined by the Student's *t*-test or one–way ANOVA when appropriate. Values are presented as either means ± SD or means ± SEM with the indicated number of independent samples. P values less than 0.05 were considered statistically significant.

4. RESULTS

4.1. Fed and fasting blood glucose levels in PDK2 KO, PDK4 KO, and PDK2/PDK4 DKO mice

PDK2 KO mice were generated to assess the importance of this kinase in glucose homeostasis during feeding and fasting. PDK2 KO mice are viable and do not differ

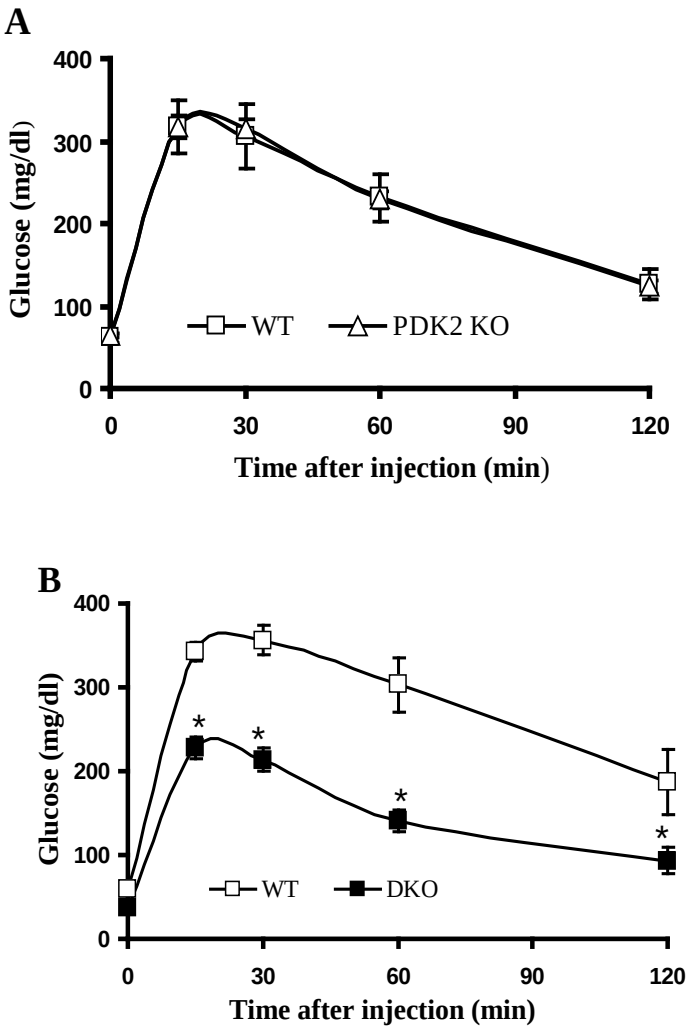
from wild-type mice in growth and body composition when fed a standard rodent chow diet (Harlan; #7071) (data not shown). Interestingly, relative to wild-type mice, blood glucose levels of PDK2 KO mice were reduced in the fed but not the fasted state (Table 1). In contrast, as we reported previously [119], PDK4 KO mice have lower blood glucose levels in the fasted but not the fed state (Table 1). Not surprisingly, therefore, knocking out both PDK2 and PDK4 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).

	Blood Glucose	
Genotype	**Fed**	**Fasted**
	mg/dl	
WT	**174 ± 6**	**94 ± 7**
PDK2 KO	**149 ± 3***	**87 ± 7**
PDK4 KO	**177 ± 11**	**75 ± 2***
PDK2/PDK4 DKO	**154 ± 4***	**61 ± 2*** #

Table 1. Blood glucose levels in WT, PDK2 KO, PDK4 KO, and PDK2/PDK4 DKO mice in the fed and fasted states. Mice were fasted overnight (12 hours). * $P < 0.05$, compared to wild-type in the same nutritional state determined by Student's *t* test. # $P < 0.05$, compared to PDK4 KO in the fasted state determined by Student's *t* test. All values are the mean ± S.E.M. with $n = 4$ mice per group except for $n = 7$ for wild-type.

To further evaluate the effect of PDK deficiency on glucose homeostasis, glucose tolerance studies were conducted. Previously we found glucose tolerance was mildly but significantly enhanced in PDK4 KO mice compared to wild-type mice (area under the curve reduced by 17 %, n=12 per group, $P < 0.01$) [119]. In contrast, no difference in

glucose tolerance was found in direct comparison of PDK2 KO mice with wild-type mice (Figure 5A). 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·min for wild-type mice; mean ± SEM, n = 4 per group, $P < 0.05$) (Figure 5B). 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 to 2.5 ± 0.9 ng/mL for wild-type mice; mean ± SEM, n = 4 per group, $P < 0.05$), consistent with greater insulin sensitivity in the DKO mice.



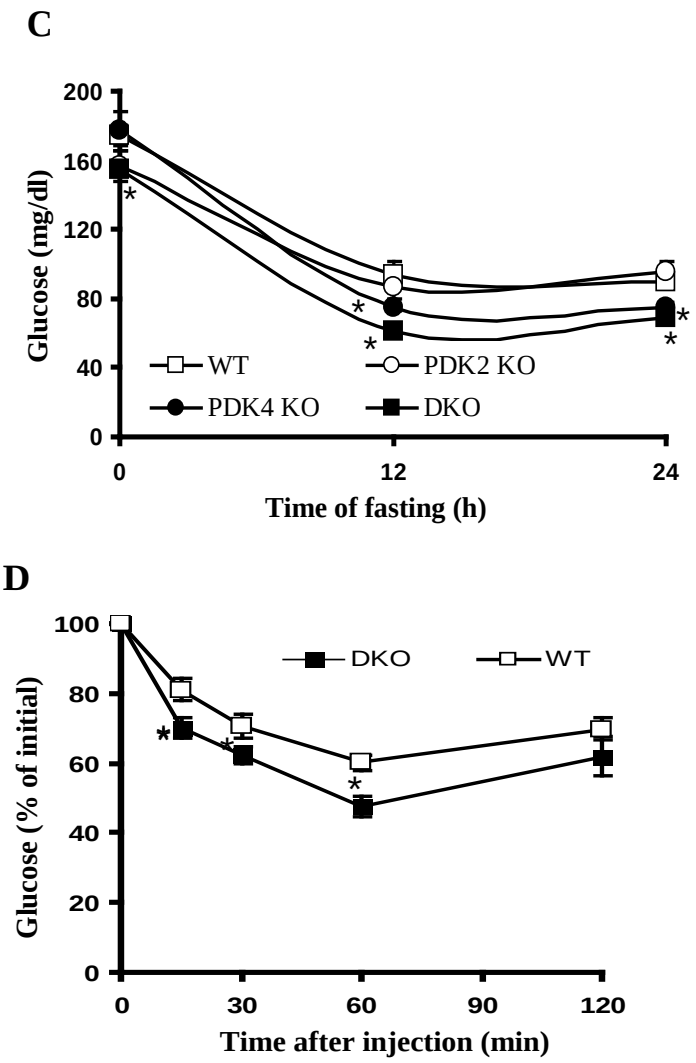


Figure 5. Improved glucose tolerance and increased insulin sensitivity in PDK2/PDK4 DKO mice but not PDK2 KO mice. (A) Glucose tolerance tests (GTTs) were performed on wild-type and PDK2 KO mice. WT, *n* 4; PDK2 KO, *n* = 4. **(B)** GTTs were conducted with wild-type and PDK2/PDK4 DKO mice. WT, *n* = 4; DKO, *n* = 4. **(C)** Food was removed from mice of the indicated genotypes for 24 h. Blood glucose levels were determined at 0, 12, and 24 h after removal of food. WT, n = 6 wild-type mice; PDK2 KO, *n* = 6; PDK4 KO, *n* = 6; DKO, *n* = 5. **(D)** Insulin tolerance test was performed on wild-type and PDK2/PDK4 DKO mice. WT, *n* = 6; DKO, *n* = 6. All

values are the mean ± S.E.M. * $P < 0.05$ relative to wild-type mice determined by Student's *t* test.

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-fasting significantly lowered blood glucose levels in the PDK4 KO and DKO mice but not in the PDK2 KO (Figure 5C). 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 5D; area above the curve of 4800 $\pm$ 294 compared to 3634 $\pm$ 304 mg/dL·min for wild-type mice; mean $\pm$ SEM, n =6 per group, $P < 0.05$), indicating greater insulin sensitivity than wild-type mice.

4.2. Effect of knocking out PDK2 and PDK4 on PDC 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 PDC were increased in the liver and skeletal muscle of PDK2 KO and PDK2/PDK4 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 PDC were not increased in the liver of PDK4 KO mice (Table 2). However, the actual activity of the complex was significantly increased in the skeletal muscle.

Tissue	Genotype	PDH Complex		
		Actual activity	Total activity	Activity State
		μmol/min/g wet weight tissue		% active
Liver	WT	0.7 ± 0.1	2.47 ± 0.08	30 ± 4
	PDK2 KO	1.3 ± 0.1*	2.54 ± 0.08	51 ± 4*
	PDK4 KO	0.8 ± 0.2	2.3 ± 0.1	32 ± 8
	PDK2/PDK4 DKO	1.2 ± 0.1*	2.28 ± 0.06	56 ± 5*
Skeletal muscle	WT	0.4 ± 0.1	3.7 ± 0.2	9 ± 2
	PDK2 KO	1.98 ± 0.04*	3.4 ± 0.3	62 ± 4*
	PDK4 KO	2.0 ± 0.4*	4.2 ± 0.4	49 ± 10*
	PDK2/PDK4 DKO	4.0 ± 0.5*	5.7 ± 0.5*	76 ± 5*

Table 2. PDC activity in liver and skeletal muscle of wild-type mice, PDK2 KO , PDK4 KO, and PDK2/PDK4 DKO mice in the fed state. * $P < 0.05$ compared to wild-type determined by Student's *t* test. Values are the mean ± S.E.M. with $n = 5$ mice per group.

Likewise, consistent with no effect on blood glucose levels in the fasted state, actual PDC activity in PDK2 KO mice did not differ from that of wild-type mice in the liver and skeletal muscle (Table 3). In contrast, PDC actual activity was modestly enhanced in the liver and markedly increased in the skeletal muscle of fasted PDK4 KO mice (Table 3). In spite of the absence of any effect of knocking out PDK2, the combined deficiency of PDK2 and PDK4 led to a three- and four-fold increase in PDC activity in the liver and skeletal muscle, respectively. These findings suggest PDK2 is more important in the fed state whereas PDK4 is predominant in the fasted state. This

agrees with the higher level of PDK2 expression in the fed state and the much greater induction of PDK4 in the fasted state [49].

		PDH Complex		
Tissue	**Genotype**	**Actual activity**	**Total activity**	**Activity State**
		μmol/min/g wet weight tissue		**% active**
Liver	**WT**	**0.21 ± 0.01**	**2.31 ± 0.09**	**9.2 ± 0.8**
	PDK2 KO	**0.17 ± 0.02**	**2.15 ± 0.04**	**8.1 ± 0.8**
	PDK4 KO	**0.28 ± 0.01***	**2.28 ± 0.09**	**12.2 ± 0.8***
	PDK2/PDK4 DKO	**0.60 ± 0.09***	**2.24 ± 0.10**	**27 ± 4*#**
Skeletal muscle	**WT**	**0.36 ± 0.01**	**2.56 ± 0.03**	**14.0 ± 0.4**
	PDK2 KO	**0.34 ± 0.02**	**2.56 ± 0.02**	**14.3 ± 0.8**
	PDK4 KO	**1.0 ± 0.1***	**2.69 ± 0.02**	**39 ± 4***
	PDK2/PDK4 DKO	**1.85 ± 0.03***	**2.73 ± 0.02**	**67 ± 1*#**

Table 3. PDC activity in tissues of wild-type mice, PDK2 KO, PDK4 KO, and PDK2/PDK4 DKO mice in the fasted state. Mice were fasted for 24 hours. * $P < 0.05$ compared to wild-type. # $P < 0.05$ compared to PDK4 KO determined by Student's *t* test. Values are the mean ± S.E.M. with $n = 4$ mice per group.

In this regard, it is noteworthy that double deficiency of PDK2 and PDK4 induced synergistic effects on PDC 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 PDC activity are due to decreased phosphorylation of the E1α subunit of PDC, the relative amounts of phosphorylated protein for the primary phosphorylation site (serine293) of the E1α subunit of the PDC were determined by western blot analysis with a site-specific phosphoprotein antibody [138]. Since the

greatest increase in PDC activity was found in skeletal muscle of PDK2/PDK4 deficient mice, levels of phosphorylated E1αSer293 were determined in the skeletal muscle of wild-type, PDK2 KO, PDK4 KO, and DKO mice in the fed and fasted state (Figure 6).

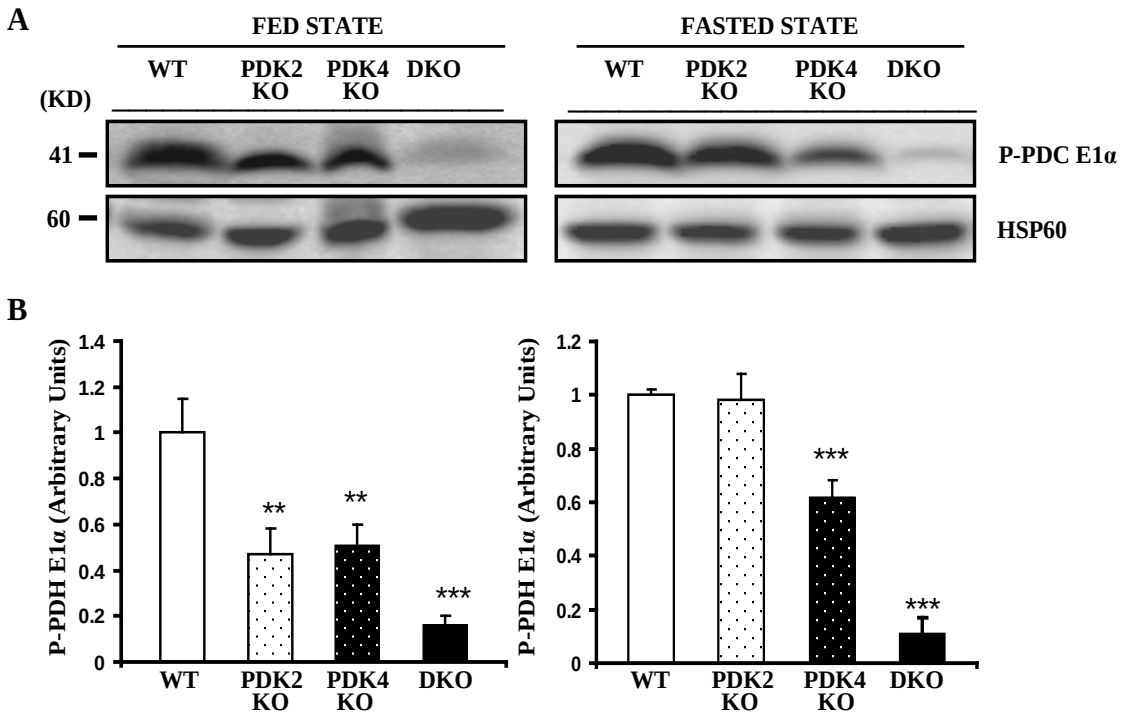


Figure 6. Decreased phosphorylation of the PDH complex E1α subunit in the skeletal muscle of PDK KO mice. (A) Representative immunoblots of the Ser293 phosphorylated form of the PDH complex E1α subunit of skeletal muscle from wild-type, PDK2 KO, PDK4 KO, and PDK2/PDK4 DKO mice. **(B)** Histograms constructed from data obtained by Western blot analysis. Results are presented as mean ± S.D. with $n = 3$ mice per group. **$P < 0.01$; *** $P < 0.001$ relative to wild-type mice determined by one–way ANOVA.

As anticipated, levels of the phosphorylated E1α subunit of PDC relative to wild-type mice were significantly reduced in PDK2 KO and PDK4 KO mice, and dramatically lowered in DKO mice, consistent with the actual activities of the PDC measured in the fed and fasted states.

4.3. Blood concentrations of gluconeogenic precursors and ketone bodies are greatly altered in PDK2/PDK4 DKO mice

Knocking out the PDKs enhances PDC activity and thereby increases pyruvate oxidation, resulting in lower levels of pyruvate, lactate, and alanine, the principal three-carbon substrates for gluconeogenesis [67, 119]. As predicated, serum levels of lactate, pyruvate, and alanine were significantly lowered in overnight fasted DKO mice (Table 4).

Measurement	WT	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 (HB)	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- ketoacids	0.060 ± 0.007	0.041 ± 0.003*
Triacylglycerol	103 ± 15	117 ± 32
[Lactate]/[Pyruvate]	25 ± 2	54 ± 7*
[HB]/[AcAc]	5.5 ± 0.6	2.3 ± 0.4*

Table 4. Blood metabolic parameters of wild-type (WT) and DKO mice.

Metabolites are given in units of mmol/L except triacylglycerol which is given in mg/dl.

* P < 0.05 for wild-type versus DKO mice. Values are the mean ± S.E.M. with *n* = 4 mice per group.

Since pyruvate is reduced more than lactate, the lactate to pyruvate ratio was greatly elevated in the DKO mice compared to wild-type mice. Branched-chain amino acids levels were elevated but branched-chain α-ketoacids were reduced, consistent with the lowered availability of the pyruvate required to recycle glutamate back to α-ketoglutarate for transamination of the branched-chain amino acids [119]. In contrast to the gluconeogenic precursors, β-hydroxybutyrate and acetoacetate were greatly increased in DKO mice (Table 4), in spite of there being no difference in serum levels of free fatty acids and triacylglycerol. As a result of a greater increase in acetoacetate than in β-hydroxybutyrate, and in striking contrast to the more reduced state of the lactate to pyruvate ratio, the hepatic β-hydroxybutyrate to acetoacetate ratio was decreased (1.3 ± 0.2 in DKO mice compared to 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.

4.4. Fed and fasting liver glycogen levels in PDK2 KO, PDK4 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 7A). After fasting for 24 h, liver glycogen remained higher in the wild-type mice than in the KO mice, higher in the PDK2 KO mice than in the other KO mice, and virtually depleted in the PDK4 KO and DKO mice (Figure 7B).

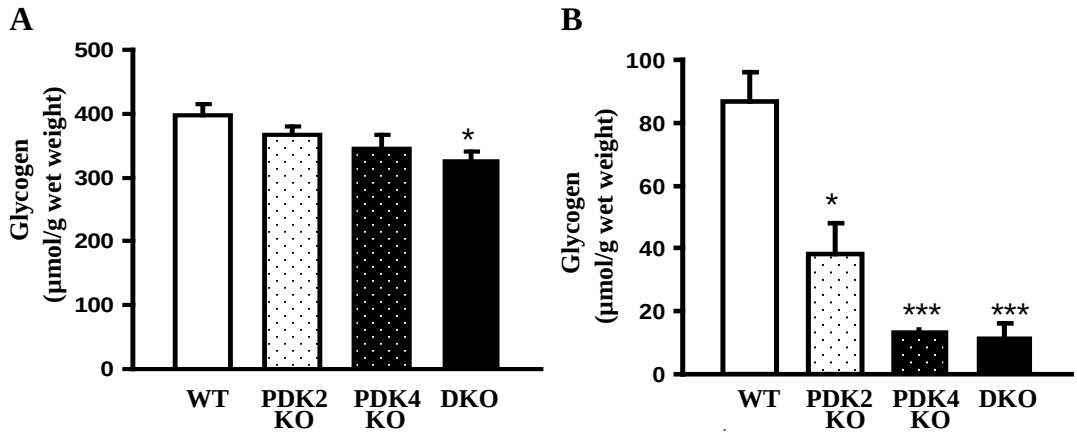


Figure 7. Glycogen levels are reduced in PDK2/PDK4 DKO mice. Liver glycogen levels in wild-type, PDK2 KO, PDK4 KO, and DKO mice in the fed state **(A)** and 24 hr fasted state **(B)**. All values are mean ± S.E.M. with $n = 5$ mice per group. * $P < 0.05$; *** $P < 0.001$ relative to wild-type mice determined by Student's *t* test.

When considered in combination with the effect of fasting on blood glucose levels (Figure 8), these findings suggest liver glycogen was used more rapidly to maintain blood glucose levels in PDK deficient mice. Retention of larger amounts of liver glycogen in the PDK2 KO mice than in the PDK4 and DKO mice is consistent with less effect of fasting on blood glucose levels.

4.5. 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 5725 $\pm$ 30 and 8460 $\pm$ 735 arbitrary units, respectively; n = 4 mice/group; $P < 0.02$) (Figure 8).

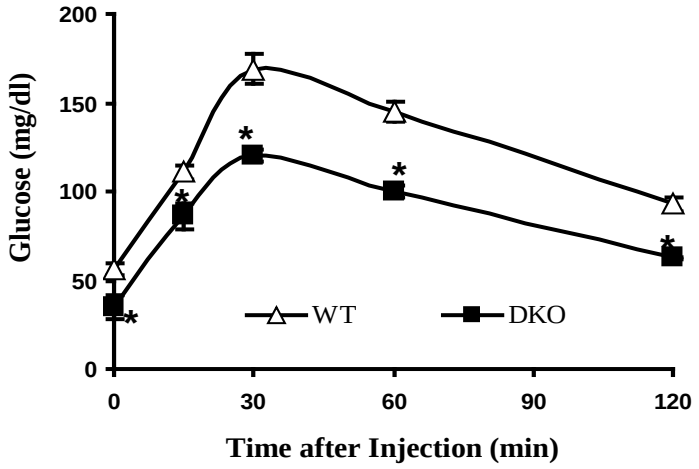


Figure 8. Pyruvate clearance is increased in PDK2/PDK4 DKO mice. Blood glucose levels during pyruvate tolerance test with wild-type and PDK2/PDK4 DKO mice. WT, *n* = 4; DKO, *n* = 4. All values are mean ± S.E.M. * $P < 0.05$; ** $P < 0.01$ relative to wild-type mice determined by Student's *t* test.

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; mean ± SEM, n = 5 mice per group, $P < 0.05$), the levels measured after 30 min were too low (0.039 ± 0.002 mM in DKO mice compared to 0.53 ± 0.04 mM for wild-type mice; mean ± SEM, n = 5 mice per group, $P < 0.001$) and the lactate to pyruvate ratio was too high [139] in the DKO mice (106 ± 18 compared to 18 ± 4 for wild-type mice; mean ± SEM, n = 5 mice per group, $P < 0.01$). The alanine concentration measured at 30 min in the DKO mice (0.041 ± 0.009 mM versus 0.32 ± 0.04 mM for wild-type mice; mean ± SEM, 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/min, respectively; n = 4 mice/group; not significantly different). As expected from the measured concentrations of pyruvate and alanine, 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 mg/dl/min compared to 3.9 ± 0.3 mg/dl/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.

4.6. 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 versus 1.25 ± 0.15 μmol/min·g of wet weight for wild-type; mean $\pm$ SEM, 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 $\pm$ 0.13 versus 1.01 $\pm$ 0.18 μmol/min·g of wet weight for wild-type mice, n = 5 per group, $P < 0.05$), suggesting reduced enzymatic capacity for hepatic gluconeogenesis is not responsible for lower blood glucose levels in the DKO mice.

4.7. Rate of glucose production is reduced in DKO mice

Stable-isotope based metabolic flux measurements with [U-$^{13}C_6$]glucose revealed a 52% lower rate of glucose production in DKO mice relative to wild-type mice (Figure 9).

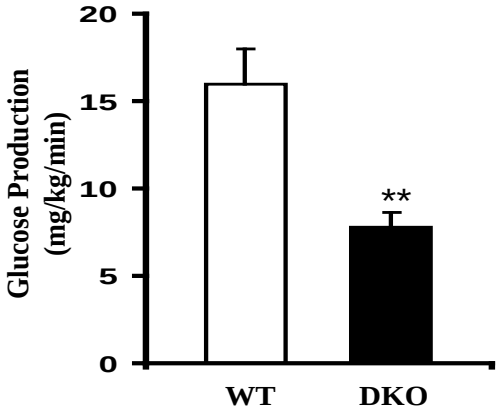


Figure 9. Rate of glucose production is reduced in PDK2/PDK4 DKO mice. Glucose production rate in wild-type and DKO after 18 h fast was determined using constant infusion of [U-$^{13}C_6$] glucose. WT, n = 5; DKO, n = 6. All values are mean ± S.E.M. ** $P < 0.01$ relative to wild-type mice determined by Student's t test.

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 PDK deficient mice.

4.8. Contribution of acetyl-CoA produced by PDC to ketone body production in DKO mice

In the fasted state, ketone bodies are primarily derived from acetyl-CoA 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 PDC by phosphorylation. Because the PDC 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 [U-$^{13}C_6$]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 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 10A).

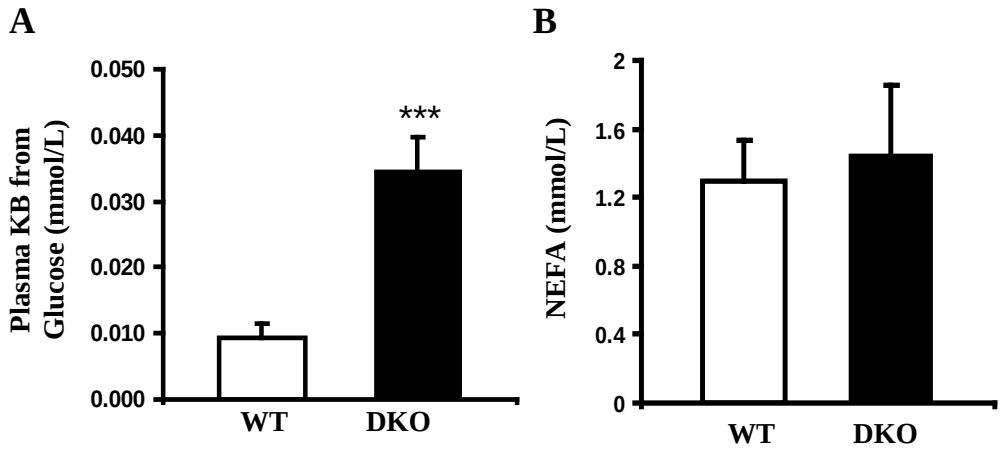


Figure 10. The conversion of glucose into ketone bodies are increased in PDK2/PDK4 DKO mice. (A) Plasma ketone bodies (β-hydroxybutyrate + acetoacetate) produced from glucose in wild-type and DKO mice after 18 h fast was determined using constant infusion of [U-$^{13}C_6$] glucose. WT, *n* = 5; DKO, *n* = 6. **(B)** Plasma NEFA levels were determined in male wild-type and PDK2/PDK4 DKO mice after overnight fast. WT, *n* = 4; DKO, *n* = 4. *** $P < 0.001$ relative to wild-type mice determined by Student's *t* test.

This finding is consistent with greater flux through the PDC with subsequent conversion of acetyl-CoA into ketone bodies. However, the relative contribution of glucose carbon to the formation of ketone bodies was miniscule (<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 free fatty acids were similar between DKO and wild-type mice (Figure 10B), greater availability of fatty acids for oxidation does not explain the increase in ketone bodies.

4.9. Fasting induces ketoacidosis and hypothermia in the DKO mice

PDK4 KO mice tolerate fasting without evidence of metabolic decompensation [74, 80, 119]. Since preliminary studies suggested 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 (Figure 11A & B). Relative to wild-type mice, a modest but significant increase of β-hydroxybutyrate occurred after 12 h but not after 24 and 36 h of fasting in PDK2 KO mice (Figure 11B). In PDK4 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. Thirty-six h of fasting induced nearly a five-fold increase of acetoacetate in DKO mice compared to wild-type mice and two and a half-fold increase compared to PDK4 KO (Figure 11A). In addition, the concentration of β-hydroxybutyrate was elevated about four-fold in the DKO mice compared to wild-type mice and two-fold compared to PDK4 KO mice (Figure 11B).

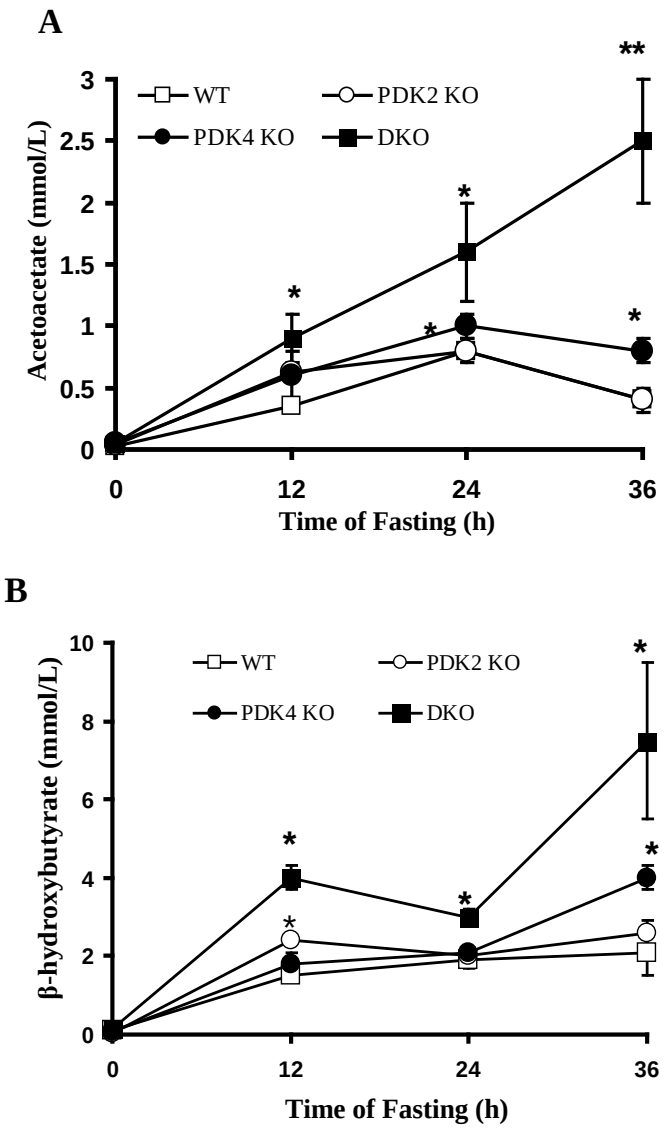
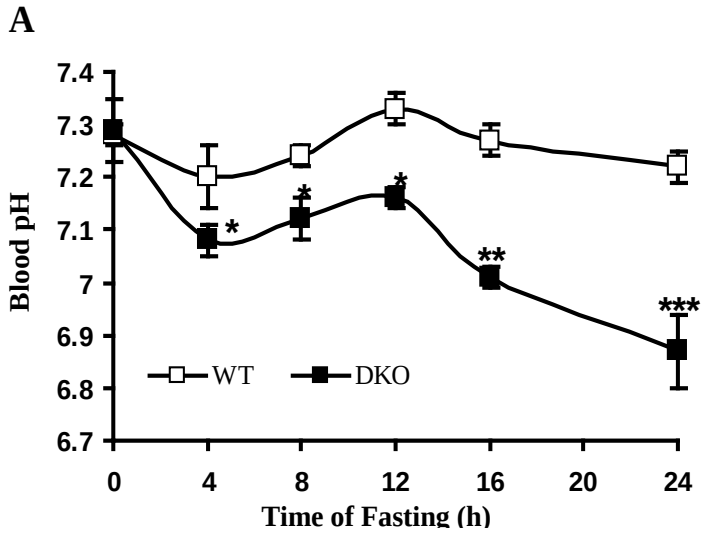
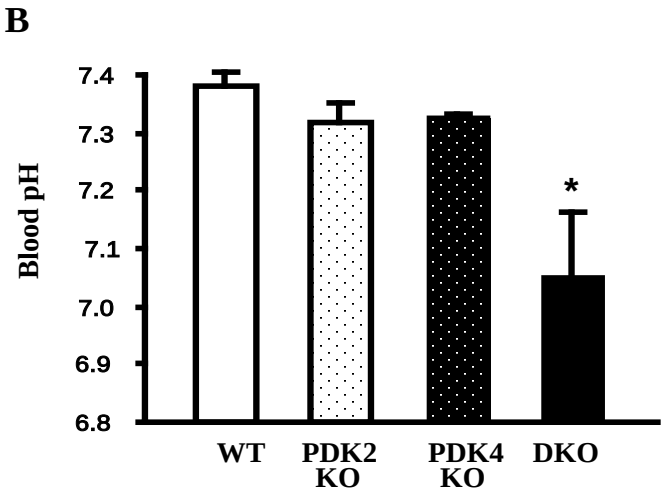


Figure 11. Blood ketone bodies are increased in PDK2/PDK4 DKO mice. (A) Plasma Acetoacetate and β-hydroxybutyrate. **(B)** levels were determined in male wild-type, PDK2 KO, PDK4 KO, and PDK2/PDK4 DKO mice after 0, 12, 24, and 36 h of food removal. Values are mean ± S.E.M. with $n = 5$ in each group for each time point. * $P < 0.05$; ** $P < 0.01$ relative to wild-type mice determined by Student's *t* test.

Because ketosis can induce metabolic acidosis, blood pH of the DKO mice was determined. Four hours of fasting significantly lowered blood pH in the DKO mice compared to wild-type mice (7.08 $\pm$ 0.03 and 7.20 $\pm$ 0.06, respectively; Figure 12A). After 24 h of fasting, blood pH of the DKO mice reached dangerously low levels (6.87 $\pm$ 0.07 and 7.22 $\pm$ 0.03, respectively) due to severe ketoacidosis. Unlike the response of the DKO mice, 36 h of fasting did not lower blood pH of PDK2 KO and PDK4 KO mice (Figure 12B).





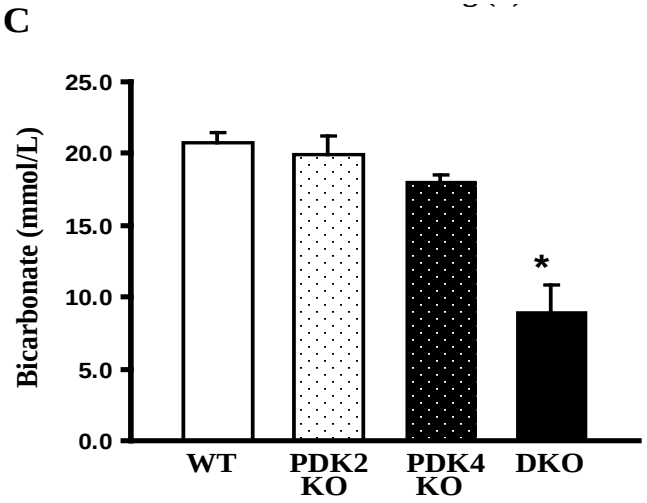


Figure 12. Fasting induces acidosis in PDK2/PDK4 DKO mice. (A) Blood pH were determined in wild-type and PDK2/PDK4 DKO mice during fasting. WT, *n* = 5; DKO, *n* = 5. **(B)** Blood pH and bicarbonate **(C)** were determined in WT, PDK2, PDK4 KO, PDK2/PDK4 DKO mice during fasting. Values are the mean ± 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.

As expected, with the presence of acidosis, the concentration of bicarbonate was dramatically reduced in the DKO mice (9 $\pm$ 2 mmol/L) compared to wild-type mice (20.7 $\pm$ 0.7 mmol/L) (Figure 12C). Furthermore, pCO_2 was significantly reduced in DKO mice (25 $\pm$4 mm Hg ° versus 40 $\pm$ 6 mm Hg °for wild-type mice; mean $\pm$ SEM, n = 4 per group, $P < 0.05$). In addition to suffering from ketoacidosis, the DKO mice experienced hypothermia (27 ± 2 °C compared to 34.5 ± 0.7 °C in wild-type mice, n = 5 mice per group, $P < 0.05$) after 36 h of fasting, leading ultimately to death.

4.10. Expression of PDK4 does not compensate for lack of PDK2 in PDK2 KO mice and vice versa

PDK2 and PDK4 were measured by western-blot analysis to assess whether altered expression of these proteins compensates for the lack of PDK2 and PDK4 in the corresponding KO mice (Figure 13).

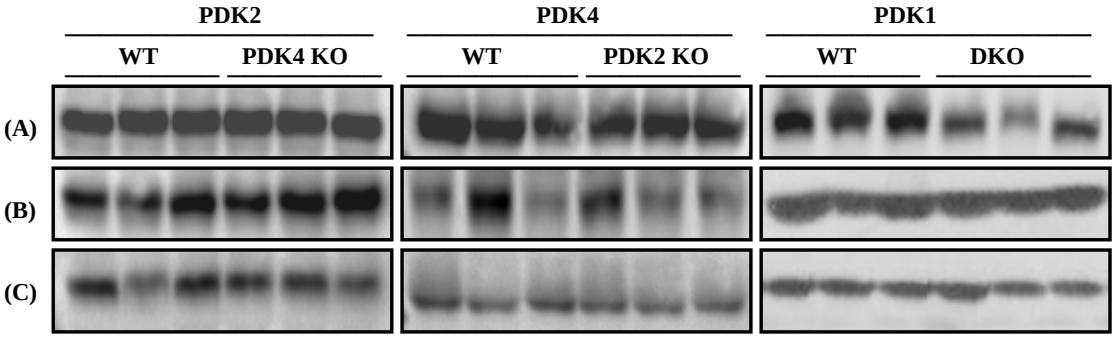


Figure 13. Deficiency of PDK2, PDK4, and both PDK2 and PDK4 does not increase expression of other PDK isoforms in the heart, liver, and skeletal muscle. Protein levels of PDK2, PDK4, and PDK1 were determined by western blot analysis in the heart **(A)**, liver **(B)**, and skeletal muscle **(C)** of PDK4 KO mice, PDK2 KO mice, and PDK2/PDK4 DKO mice.

Protein levels of PDK2 were not changed in the tissues of the PDK4 KO mice compared to wild-type mice. Protein levels of PDK4 were likewise similar in heart, liver, and skeletal muscle of PDK2 KO mice and wild-type mice. These findings suggest that in the fasted state expression of PDK2 does not compensate for the lack of PDK4 and expression of PDK4 does not compensate for the lack of PDK2.

4.11. Expression of PDK1 and PDK3 does not compensate for the lack of PDK2 and PDK4 in the DKO mice

Levels of PDK1 protein were similar in the liver and skeletal muscle of wild-type mice, PDK2 KO mice and PDK4 KO mice (data not shown) suggesting that PDK1 does not compensate for the loss of PDK2 or PDK4. Furthermore, the amounts of PDK1 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 13). Therefore, the expression of PDK1 does not compensate for the lack of PDK2 and PDK4 in the DKO mice. PDK3 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 (data not shown).

5. Discussion

This study shows PDK2 deficiency lowers blood glucose in the fed state without a large increase in ketone bodies. PDK4 deficiency lowers blood glucose in the fasted state, with a significant increase in ketone bodies, and a combined PDK2 and PDK4 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 PDK2 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 PDK2 relative to the other PDKs. PDK2 is constitutively expressed in tissues in the fed and fasted state [49, 68], its activity is more sensitive than the other PDKs to modulation by pyruvate, NADH, and acetyl-CoA [116, 140], and its expression

level is only modestly increased by fasting in the liver and kidney of rats [53] and by diabetes in Otsuka Long-Evans Tokushima Fatty rats [68], and in type 2 diabetes in humans [141]. Because these observations seemed to suggest an important role for PDK2 in regulation of glucose metabolism during fasting and in diabetes, we were surprised to find blood glucose is maintained at normal fasting levels in PDK2 knockout mice. Consistent with this observation, knocking out PDK2 had no effect upon the phosphorylation and activity state of the PDC in the fasted state in either the liver or the skeletal muscle. Because PDK2 deficiency does not alter PDC activity, the rate of pyruvate oxidation is not increased and therefore the supply of 3 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 PDC 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 PDC activity exists in the fed state that can be called into play by dephosphorylation when needed. This study suggests PDK2 plays an important role in phosphorylating PDC, and therefore maintaining this reserve of PDC activity in the fed state. Rendering mice deficient in PDK2 reduces this reserve, resulting in greater PDC 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 reduced rate of glucose production but rather increased glucose disposal.

PDK4 has received most of the attention in studies on physiological regulation of the PDC because its expression is more sensitive to the metabolic stress of fasting and diabetes [53]. The expression of PDK4 is remarkably sensitive to stimulation by glucocorticoids and PPARα and δ ligands and to inhibition by insulin [58, 142, 143], consistent with an important role for PDK4 in setting the phosphorylation and activity state of PDC in fasting and diabetes. In addition, fasting blood glucose levels are significantly reduced in PDK4 KO mice relative to that of corresponding control mice [74, 119]. In the present study, the phosphorylation state of PDC was found to be reduced in PDK4 KO mice, consistent with the increase in PDC activity state reported previously [17]. Lactate, pyruvate, and alanine levels are therefore reduced in PDK4 mice, resulting in lower blood glucose levels for want of 3 carbon compounds for gluconeogenesis. In the fed state PDK4 deficiency has no effect on blood glucose because PDK4 is not expressed at high enough levels to impact the activity state of PDC. PDK4 is effective in the fasted state because of the higher level of its expression and therefore its greater importance in regulation of PDC.

Double deficiency of PDK2 and PDK4 greatly increased PDC 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 PDK2 and PDK4 reduces blood glucose levels in the fasted state. Consistent with this hypothesis, metabolic flux analysis with [U-$^{13}C_6$] glucose indicated DKO mice synthesized glucose at a lower rate than wild-type mice. Furthermore, no difference in

phosphoenolpyruvate carboxykinase (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 altered enzymatic capacity for gluconeogenesis is not responsible for lower blood glucose levels in DKO mice. It is more likely, therefore, that reduced substrate supply for gluconeogenesis limits the rate of hepatic/renal glucose production in the DKO mice. These data support findings with the PDK inhibitor dichloroacetate, [67, 69, 144], which lowers blood glucose levels by reducing the rate of gluconeogenesis by decreasing the blood levels of pyruvate, lactate, and alanine [67].

Even though PDK deficiency results in lower blood glucose levels [119] and, therefore may serve as a therapeutic target for type 2 diabetes [118], lack of control of PDC activity by PDK2 and PDK4 greatly increases blood ketone body levels. Overnight fasting of DKO mice caused a four-fold increase in β-hydroxybutyrate and ten-fold increase in acetoacetate. Ketone bodies were not significantly affected in PDK2 KO mice compared to wild-type mice, and although elevated in the PDK4 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 PDK4 KO mice have elevated ketone bodies, these animals maintain blood pH levels in the normal range. Therefore, PDK2 and PDK4 are essential for preventing ketoacidosis during fasting. At least in part, this is because inhibition of the PDC by PDK2 and PDK4 prevents competition between pyruvate and ketone body oxidation in peripheral tissues [119]. Studies with PDK inhibitor DCA showed that increased glucose oxidation inhibits ketone body oxidation [67]. In addition,

inhibition of the PDC by PDK2 and PDK4 limits the conversion of the carbon of glucose and gluconeogenic precursors to ketone bodies. Normally the PDC 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-hydroxybuytrate originating from [U-$^{13}C_6$]g lucose shows that knocking out PDK2 and PDK4 increases PDC activity, resulting in production of acetyl-CoA from pyruvate that can be used to synthesize ketone bodies, although in amounts insignificant relative to the ketone bodies formed from fatty acids.

In contrast to the single KO mice, PDC activity is greatly increased in the liver and skeletal muscle of the PDK2/PDK4 DKO mice, resulting in mice that cannot survive an extended period of fasting because of hypoglycemia, ketoacidosis, and hypothermia. Since PDK2 KO and PDK4 KO mice have mild phenotypes compared to the DKO mice, we predicted that compensation by increased expression of the other PDKs might protect single KO mice from deleterious effects experienced by the DKO mice. However, the expression of PDK2 does not compensate for the lack of PDK4 and the expression of PDK4 does not compensate for the lack of PDK2. Therefore, the modest phenotype of the PDK2 KO and PDK4 KO relative to the DKO is not due to up regulation of the expression of PDK2 or PDK4. Instead, the intrinsic activity of PDK4 and its sensitivity to stimulation by acetyl-CoA and NADH [116] may be sufficient to cover for the absence of PDK2. Likewise, PDK2 may be able to cover partially but less effectively for the absence of PDK4 in PDK4 KO mice. The phenotype of the DKO mice is much more severe because these compensatory effects are not possible, and also because PDK1 and PDK3 are unable to cover for the absence of PDK2 and PDK4.

This study documents a precaution relative to the potential use of PDK inhibitors for the treatment of diabetes. Double deficiency of PDK2 and PDK4 renders mice sensitive to hypoglycemia, ketoacidosis, and hypothermia during fasting. Previous studies showed that knocking out PDK4 inhibits fatty acid oxidation in isolated diaphragms, suggesting that PDK deficiency impairs peripherial fatty acid oxidation, resulting in hypothermia in DKO mice [119]. From these findings it is clear that compounds that totally inhibit PDK2 and 4 would have deleterious effects. Finally, it is noted that children with idiopathic ketoacidotic hypoglycemia suffer fasting-induced hypoglycemia and ketoacidema as a result of reduced levels of the gluconeogenic precursors [145]. Since similar effects are observed in PDK2/PDK4 DKO mice, PDK deficiency is a potential cause and these mice might serve as an animal model for this disorder.

CHAPTER II: PDK2/PDK4 DOUBLE KNOCKOUT MICE FED A HIGH FAT DIET REMAIN EUGYLEMIC BUT ARE PRONE TO KETOACIDOSIS

1. Overview

The pyruvate dehydrogenase kinases (PDKs) inactivate the pyruvate dehydrogenase complex (PDC) to conserve substrates for gluconeogenesis. Since excessive gluconeogenesis contributes to hyperglycemia in type 2 diabetics, decreasing the supply of glucoeneogenic substrates by inhibiting the PDKs is an attractive mechanism for lowering excessive hepatic glucose production. To investigate whether PDK inhibition prevents hyperglycemia in a diet induced diabetic animal model, PDK2/PDK4 double knockout (DKO) mice were fed a high saturated fat diet for 30 weeks. The DKO mice remained euglycemic, accumulated less body fat, and were more glucose tolerant than wild type mice. Stable isotope flux analysis indicated that euglycemia was due to a reduced rate of gluconeogenesis. PDK deficiency resulted in greater PDC activity which limited the availability of pyruvate for oxaloacetate synthesis. Since oxaloacetate is essential for TCA cycle activity, suppression of oxaloacetate in DKO mice limited acetyl-CoA from entering the TCA cycle. Subsequently, ketone body and fatty acid oxidation was suppressed, provoking fasting induced ketoacidosis and hypothermia in DKO mice. When fed a very high fat, low carbohydrate diet, DKO mice also exhibited hypothermia, ketoacidosis, and hypoglycemia. These effects of PDK deficiency plus elevated lactate to pyruvate ratios, reduced β-hydroxybutyrate to acetoacetate ratios, and increased blood citrulline levels are similar to the metabolic imbalance induced by suppression of oxaloacetate in pyruvate carboxylase deficiency in humans.

2. Introduction

The pyruvate dehydrogenase complex (PDC) links glycolysis to the citric acid cycle by transforming pyruvate to acetyl-CoA [46]. The PDC is subject to phosphorylation by pyruvate dehydrogenase kinases (PDKs). Under fasting conditions, the expression of PDK2 and PDK4 is elevated, which in turn phosphorylate and inactivate the PDC. As a consequence of an inactive PDC, the conversion of pyruvate to acetyl-CoA is reduced, resulting in conservation of pyruvate needed for gluconeogenesis. Inactivating PDC benefits the starved state by conserving gluconeogenic substrates for hepatic glucose production to maintain blood glucose levels [110]. Since excessive hepatic glucose production accounts for a significant proportion of the hyperglycemia in type 2 diabetes, prevention of the inactivation of the PDC should lower blood glucose in the diabetic state by inhibiting gluconeogenesis. A significant up regulation of PDK2 and PDK4 occurs in type 2 diabetic humans [77], in genetic type 2 diabetic animal models [68], in animals fed a high fat diet [78], and in humans consuming high fat diet [79]. Therefore, we placed PDK2/PDK4 double knockout mice on a high saturated fat diet to investigate whether PDK inhibition protects against hyperglycemia in a diet-induced diabetic animal model. On the high fat diet, DKO mice maintained normal blood glucose levels and were protected from adiposity and fasting induced hepatic steatosis compared to wild-type mice. However, similar to DKO mice fed the chow diet [146], these mice suffered from ketoacidosis and hypothermia under fasting conditions. This raised the question whether elevated levels of blood ketone bodies are caused by increased ketogenesis or ketolysis and whether hypothermia is a consequence of reduced fatty acid oxidation. To answer these questions, rates of ketogenesis, ketolysis, and fatty acid oxidation were determined. DKO mice were unable to utilize ketone bodies and fatty

acids, two essential substrates for energy production in fasted state. Without these substrates, survival of DKO mice in the fasted state was jeopardized.

3. Materials and Experimental Procedures

3.1. Animals

Mice were housed in an *AALAC* approved, pathogen-free barrier facility (12 h light/dark cycles) with temperature maintained at (23 ± 2 °C). The procedure to generate homozygous PDK2/PDK4 double knockout (DKO) mice and C57BL6J mice were described previously [146]. At 6 weeks of age, groups of 24 male wild-type and 24 male DKO mice were housed with three mice per cage and fed a high fat diet (D12330; Research Diets) that by calories was 58% fat, 16.4% protein, and 25.5% carbohydrate. According to the supplier, the fat composition of the high saturated fat diet was 93.3% saturated fat, 2.4% monounsaturated fat, and 4.3% polyunsaturated fat. Body weights of the mice were determined once a week. The experiment was terminated after 30 weeks of feeding. Food was removed from half of the mice in each group in the evening (between 7 and 8 p.m.). The next morning (between 7 a.m. and 8 a.m.) mice were sacrificed by cervical dislocation and tissues were harvested and immediately freeze-clamped with Wollenberger tongs at the temperature of liquid nitrogen, and later powdered under liquid nitrogen with a mortar and pestle, and stored at -85°C for analysis. Small pieces of tissue were also collected without freezing for histological analysis.

To determine the response to a ketogenic diet, mice were fed a low carbohydrate-high fat diet (TD963555; Harlan Tekland Premier Laboratory Diets) that by calories was 90.5% fat, 9.5% protein, and 0.4% carbohydrate. At eight weeks of age, groups of 12 wild-type and 12 DKO mice were fed either the high saturated fat diet (D12492) or

ketogenic diet (TD963555). After 2 days on the diets, mice were killed in the morning (between 9 a.m. and 10 a.m.) by cervical dislocation followed by collection of blood and harvesting of tissues. Body temperature was measured by a rectal temperature probe (MicroTherma 2T, Braintree Scientific Inc, Braintree, MA).

3.2. Exercise Protocol.

To determine the response to exercise, the mice were trained for 4 days (3 min/day) on a treadmill designed for mice (Exer6M, Columbus Instruments). On the first day, the treadmill incline was set to 5° with an intial speed of 8 m/min which was increased by 1 m/min during the training session. Each day during the training session, the incline was increased by 5° and the initial speed to 10, 11, and 12 m/min. For the exercise performance test, mice were run on a treadmill (20° incline with a starting speed of 12 min/min) followed by gradual increase in speed by 1 m/min at 2, 5, 10, 20, 30, 40, and 60 min after initiation of the exercise. Mice that stepped off the belt experienced a mild electrical stimulus (16-28 V). Exhaustion was defined as the point at which the mice were unable to continue running.

3.3. Measurement of body fat

Percentage body fat of the mice was determined by DEXA (dual-energy X-ray absorptiometry) with a Lunar PIXImus Mouse Densitometer (GE Healthcare). Mice were anaesthetized with 1.5% isoflurane [2-chloro-2-(difluoromethoxy)-1,1,1-trifluoroethane] during the measurement.

3.4. Glucose and insulin tolerance tests

At 26th week of feeding, glucose tolerance tests were performed with mice that were fasted for twelve hours (7 p.m. to 7 a.m.). Glucose (1 g/kg of body weight) was administered by intraperitoneal injection followed by measurements of glucose in tail blood with a glucometer (Lifescan) at 0, 15, 30, and 60, and 120 min after injection. At the end of the 27rd week of feeding, insulin tolerance tests were conducted after 6 h of fasting (8 a.m. to 2 p.m.). Insulin (1 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.

3.5. Measurements of metabolite concentrations in blood, skeletal muscle, and liver

Serum and tissue extracts were deproteinized with perchloric acid followed by neutralization with KOH and precipitation of $KClO_4$. Pyruvate [122], lactate [123], alanine [123, 147], acetoacetate [124], β-hydroxybutyrate [124], oxaloacetate [148, 149], malate [149], citrate [150], α-ketoglutarate [149] 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). Free Fatty acids were assayed by a NEFA Half Micro Assay kit (Roche Diagnostics, Indianapolis, IN). Free Fatty acids were assayed by a NEFA Half Micro Assay kit (Roche Diagnostics, Indianapolis, IN). Blood pH and bicarbonate levels were measured with an I-STAT clinical analyzer with cartridge EC8+ cartridges (Heska Corporation, Loveland, CO).

3.6. Glucose and ketone body utilization by isolated diaphragms

Diaphragms were removed from 16 h starved mice and pre-incubated as previously described by Jeoung et al. [119]. After pre-incubations, diaphragms were removed from the flasks, blotted, and transferred into to new flasks containing 1.5 mL of Krebs-Henseleit bicarbonate buffer supplemented with 5 mM glucose containing 25 μCi/mmol [U-^{14}C]glucose and 1 mU/mL Insulin (Eli Lilly, Indianapolis, IN). Flask were flushed with 95 % O_2 / 5 % CO_2, sealed with rubber serum caps which were fitted with hanging center wells (Kontes, Vineland, NJ) and incubated for 1 h shaking at 37 °C. Reactions were determined by the injection of 0.250 mL of phenethylamine/methanol (1:1, v/v) into the center wells and 0.1 mL of 60% (w/v) $HClO_4$ into the contents of the flasks. The rate of β-hydroxybutyrate was determined from the production of $^{14}CO_2$. To measure β-hydroxybutrate oxidation, diaphragms obtained from 16 h fasted mice were rinsed, weighted, and preincubated for 30 min as previously described [119]. Diaphragms were removed from the flasks, blotted, weighed, and transferred into new flasks containing 1.5 mL of Krebs-Henseleit bicarbonate buffer (pH 7.4), 5 mM glucose, 1mU/mL insulin, 50 μCi/mmol [3-^{14}C]β-hydroxybutyrate. $^{14}CO_2$ was collected after 1 h incubation as described above.

3.7. Metabolic flux analysis in the fasting condition

Metabolic flux analysis was determined with stable-isotope-labeled glucose as described previously [127, 128]. Mice that had been fasted for 3 h (4:00 PM to 7:00 PM) were anesthetized with 5% isoflurane and surgically implanted subcutaneously with pre-activated Alzet miniosmotic pumps (model 2001D; 8 μL/h; Alzet Osmotic Pumps)

containing 50 mg of [U-$^{13}C_6$]glucose or 50 mg of [U-$^{13}C_4$]β-hydroxybuytrate (99.9% enriched; Isotech, Miamisburg, OH) dissolved in 200 µL of water. Blood was collected from the mice between 10:00 and 11:00 AM the next morning. Mass isotopomer analysis using GC/MS was determined as described previously [146]. Substrate production or turnover rates were determined using the principle of tracer dilution. At isotopic steady state, the hepatic glucose production (HGP) rate was determined using the following equation [133]:

HGP (mg/min/kg) = (working pump rate)/m6 – working pump rate, where m6 = final isotope substrate enrichment in glucose [127]. Similarly, β-hydroxybutyrate production rate (mg/min/kg) can be calculated using the following equation:

β-HBP = (working pump rate)/m4 – working pump rate, where m4 = final isotope enrichment in β-hydroxybutyrate.

3.8. Oxygen consumption, energy expenditure, and fatty acid oxidation

Measurements of oxygen consumption and carbon dioxide production were performed using a TSE indirect calorimetry system (Lab Master, TSE systems, Midland, MI). Mice were individually housed in the chamber for 48 h with lights on from 7 a.m. to 7 p.m. in an ambient temperature of 22-24 °C. Food was removed during the dark cycle (7 p.m. to 7 a.m. the next following morning) for 24 h. The TSE systems measured carbon dioxide produced and oxygen consumed over a 1-min period six times per hour. These values were averaged to determine the rate of carbon dioxide produced (VCO_2) and oxygen consumed (VO_2) in mL/h. Energy expenditure (EE), respiratory quotient

(RQ), and fatty acid oxidation (FA) were calculated using the following equation [151, 152]:

$$RER = VCO_2/VO_2$$

$$EE\ (kcal/h) = [3.815 + 1.232 \text{ X RER}) \text{ x } VO_2] / 1000$$

$$FA\ oxidation\ (kcal/h) = EE \text{ X } (1 - RER/0.3)$$

3.9. Determination of nucleotides in the liver and skeletal muscle

Analyses were performed with a Shimadzu Prominence HPLC System equipped with the LC20 AD Solvent Delivery System and SPD-M20A Photodiode Array Detector, using absorbance at 259 nm for quantification. Nucleotides were separated using a Phenomenex Gemini reverse phase column (250 x 4.6 mm, 5 µm particle size) and a Phenomenex Security Guard C18 precolumn (4 x 3.0 mm), with gradient elution. Mobile phase A was prepared by adjusting the pH of 0.1 M KH_2PO_4 to 6.0 with 0.1 M K_2HPO_4. Mobile phase B consisted of mobile phase A and methanol (80:20). Extracts were diluted (20:60) with mobile phase B and 10 µL was injected onto the HPLC column for analysis. Linear gradients of mobile phase, flow and temperature were utilized as follows: 0-7.5 min, 0-1 % B; 7.5-10 min, 1-3 % B; 10-17 min, 3-10 % B; 17-28 min, 10-100% B. The initial flow rate of 0.5 mL/min was held constant for 10 min. From 10-12 min the flow was increased from 0.5 to 1.0 mL/min and held constant until the end of the run. From 0-6 min the temperature was increased from 25 to 29 ºC, from 6-15 min the temperature was returned to 25 ºC. Peaks of interest eluted within 27 min, and the system was equilibrated for an additional 15 min prior to the next injection. The concentration of the highest AMP standard solution was determined using absorbance at

259 nm with a Cary 50 Bio UV-Visible Spectrophotometer (Varian) and the molar absorption coefficient of AMP (15,400 M^{-1} cm^{-1} at 259 nm). Peak areas of the samples were quantified relative to an AMP standard curve then multiplied by the ratio of the molar absorption coefficient of AMP to that of the compound of interest. The molar absorption coefficient of ADP and ATP is the same as AMP. The molar absorption coefficient of NAD and NADP is 18,000 M^{-1} cm^{-1}, and that of GMP, GDP and GTP was determined to be 12,300 M^{-1} cm^{-1} at 259 nm.

3.10. Histochemistry of the livers

Histological examinations and preparation of H&E staining of the liver were performed by the Immunohistochemistry Laboratory of Indiana University School of Medicine (Indianapolis, IN, U.S.A.).

3.11. Statistical analysis

The statistical significance of differences between groups was determined with the Student's *t*-test. All values are presented as means ± SEM with the indicated number of independent samples. P values less than 0.05 were considered statistically significant.

4. Results

4.1. Body weight gain, body fat and liver fat accumulation are attenuated in DKO mice fed a HSF diet.

Wild-type and DKO mice were fed a high saturated fat (HSF) diet for 30 weeks. During the first eleven weeks, DKO mice and wild-type mice experienced the same weight gain (Figure 14).

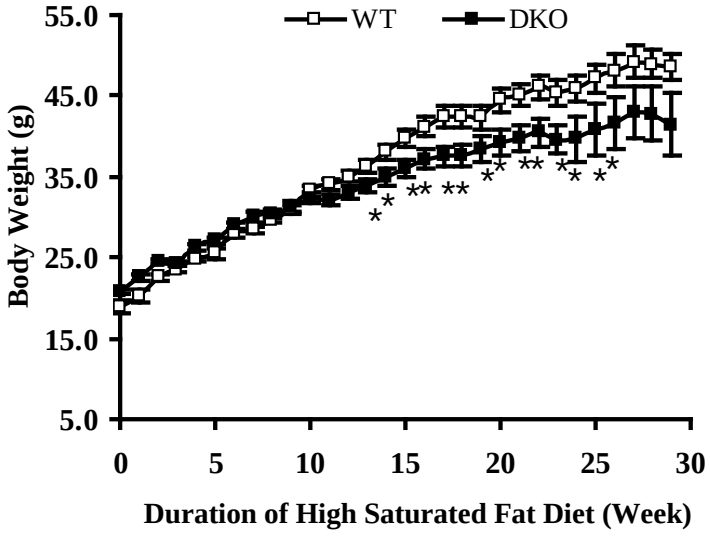
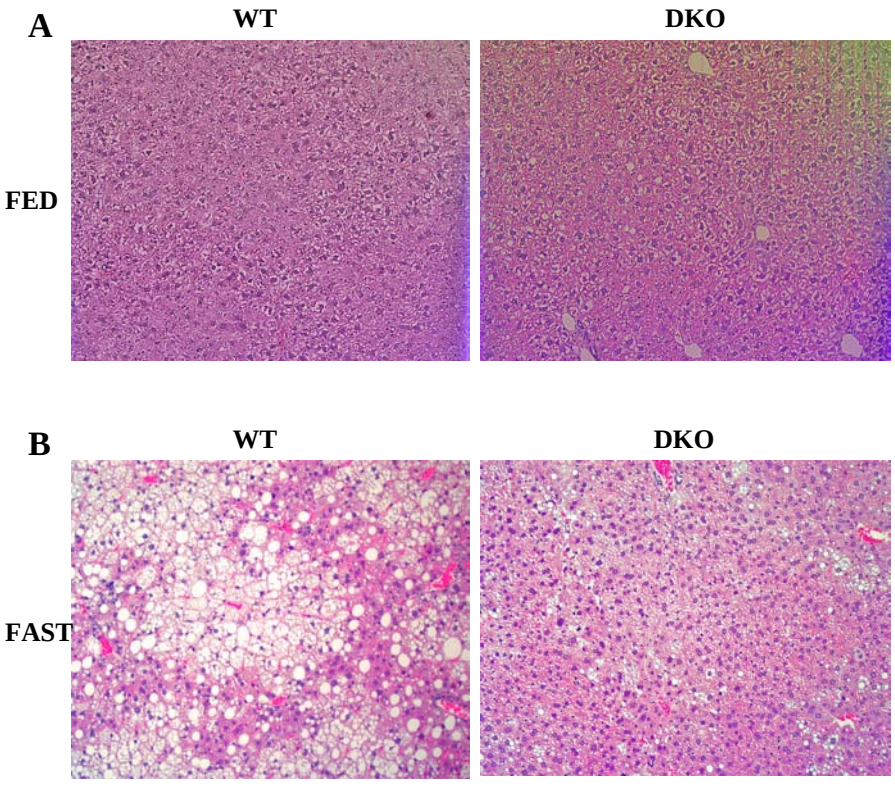


Figure 14. Body weight gain is attenuated in DKO mice fed a HSF diet for 30 weeks. Wild-type and DKO mice were fed a high saturated fat (HSF) diet for 30 weeks. The body weights of the mice were measured at the beginning of every week from the beginning of the feeding period. WT, $n = 24$; DKO, $n = 24$. All values are the mean ± S.E.M. * $P < 0.05$ relative to wild-type mice determined by Student's *t* test.

However, by the 14th week DKO mice weighed significantly less than wild-type mice, a trend that continued through the 25th week. After that, no difference in body

weight was apparent between the two groups. In spite of this, the body fat percent measured by DEXA was significantly less in the DKO mice (32 ± 2%) compared to the wild-type mice (39 ± 1%; mean ± SEM, n = 6 mice/group, $P < 0.05$). In agreement with these findings, relative percentage of epididiymal fat pad to body weight was less in the DKO mice (4.9 ± 0.3 %) compared to wild-type mice (6.0 ± 0.1 %; mean ± SEM; n = 8 mice/group, $P < 0.05$). Although DKO mice accumulated less body fat, fat content of the livers of fed DKO mice was similar to that of fed wild-type mice (Figure 15A). In the fasted state, however, the fat content of the liver of DKO mice was less than that of wild-type mice (Figure 15B). As observed previously with PDK4 KO mice [80], fat accumulated in microvesicles in the DKO mice rather than macrovesicles which is characteristic of HFD fed wild-type mice (Figure 15B).



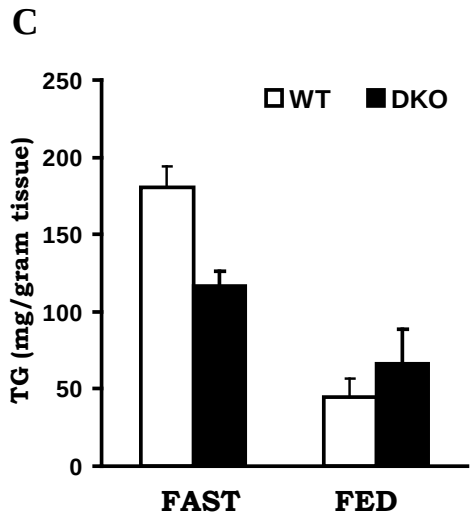


Figure 15. Fasting induced hepatic steatosis is reduced in DKO mice fed a HSF diet. Microscopic photographs demonstrating histological appearance of livers of wild-type and DKO mice fed the high HSF diet for 30 weeks. **(A)** Livers were obtained from wild-type and DKO mice in fed state. **(B)** Livers were obtained from wild-type and DKO mice after overnight fast. Tissue sections were stained with hematoxylin and eosin (H&E) stain. The material stained white corresponds to fat droplets. **(C)** Triacylglycerol (TG) was determined in the fed and fasted livers of wild-type and DKO mice. WT, n = 12; DKO, n = 12. All values are the mean ± S.E.M. * $P < 0.05$ relative to wild-type mice determined by Student's t test.

In agreement with the histology data, liver triacylgycerol content was similar between the groups of mice in the fed state (Figure 15C). In the starved state, however, liver triacylglycerol levels were significantly reduced in DKO mice relative to wild-type mice (Figure 15C), confirming the histological evidence that fasting induced less hepatic steatosis in DKO mice relative to wild-type mice. Although DKO mice accumulates less

fat in the liver as well as the whole body, DKO mice consumed similar amounts of food as wild-type mice.

4.2. Hyperglycemia is attenuated in DKO mice fed the HSD

In the fed state, DKO maintained similar blood glucose levels as wild-type mice (198 ± 13 mg/dL for wild-type mice and 186 ± 19 mg/dL for DKO mice; n = 8 mice/group, $P > 0.05$). However, in the fasted state, wild-type mice remained hyperglycemic (147 $\pm$ 11 mg/dL; n = 10) whereas the DKO mice lowered their blood glucose into the normal range of fasting blood glucose concentrations (87 $\pm$ 7 mg/dL; n = 10; $P < 0.001$).

4.3. DKO mice have improved glucose tolerance

To further test the effect of PDK2/PDK4 deficiency on glucose, glucose tolerance studies were conducted after feeding the mice the HSF diet for 27 weeks. In agreement with our previous findings with chow diet fed mice [146], DKO mice had a better glucose tolerance than wild-type mice fed HSF diet (Figure 16A). The area under the curve for DKO mice (8859 $\pm$ 601 $mg \cdot dl^{-1} \cdot min^{-1}$; n=10) was significantly reduced compared to wild-type mice (11284 $\pm$ 601 $mg \cdot dl^{-1} \cdot min^{-1}$; n = 10; $P<0.02$). In contrast to the glucose tolerance, DKO mice responded similar to wild-type mice in the insulin tolerance test (Figure 16B) as evidenced by similar areas above the curve for DKO and wild-type mice (area above the curve of 19 ± 4 mg/dL ·min compared to 9 ± 5 for wild-type mice; mean $\pm$ SEM, n =6 per group, $P > 0.05$).

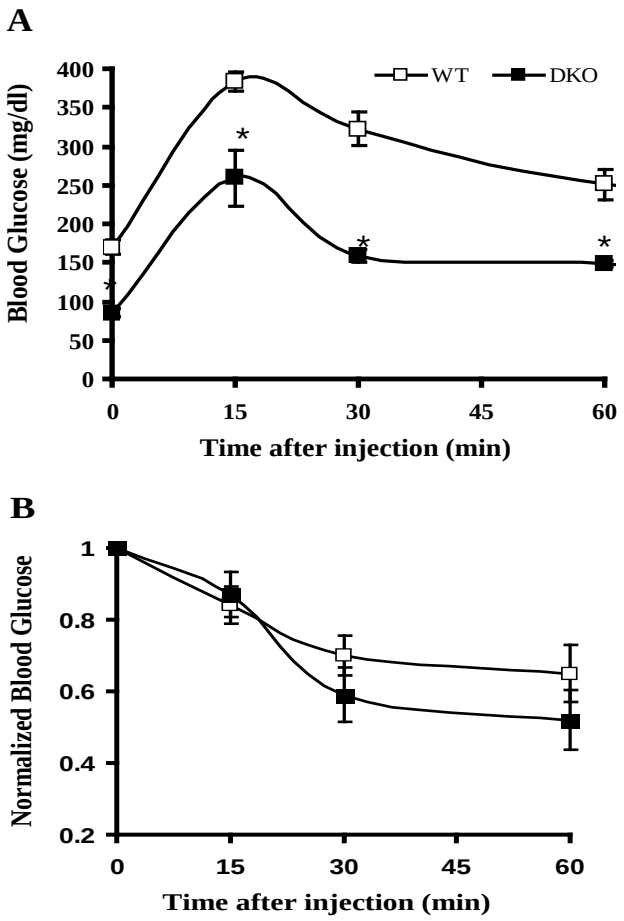


Figure 16. Improved glucose tolerance without improved insulin sensitivity in DKO mice fed a HSF diet. (A) Glucose tolerance tests (GTTs) were performed on wild-type and DKO mice fed the HSF diet for 27 weeks. WT, *n* = 8; DKO, *n* = 8. **(B)** Insulin tolerance tests (ITTs) were performed on wild-type and DKO mice fed the HSF diet for 28 weeks. WT, *n* = 8; DKO, *n* = 8. All values are the mean ± S.E.M. * $P < 0.05$ relative to wild-type mice determined by Student's *t* test.

Insulin levels were similar between DKO (13 ± 2 µU/mL, n = 5) and wild-type mice (16 ± 1 µU/mL, n = 5, $P > 0.05$) at 12 weeks. At 30 weeks, insulin levels were greatly increased in both groups of mice but insulin levels were found lower in DKO

mice compared to wild-type mice, leading to reduced HOMO-IR scores for DKO mice suggesting greater insulin sensitivity (Table 5).

Measurement	Units	WT	DKO
Glucose	mg/dL	155 ± 18	81 ± 9*
Insulin	μU/mL	29 ± 6	12 ± 2*
HOMO-IR		11 ± 3	2.4 ± 0.4*
Lactate	mmol/L	2.2 ± 0.3	0.9 ± 0.3*
Pyruvate	mmol/L	0.13 ± 0.02	0.017 ± 0.004*
β-HB	mmol/L	1.2 ± 0.02	5.2 ± 0.3*
Acetoacetate	mmol/L	0.2 ± 0.03	3.2 ± 0.2*
pH		7.32 ± 0.02	7.08 ± 0.04*
BUN	mg/dL	32 ± 3	45 ± 5
Creatinine	mg/dL	0.23 ± 0.03	0.43 ± 0.03*
FFA	mmol/L	1.2 ± 0.1	1.5 ± 0.01*
TG	mg/dL	72 ± 14	108 ± 28
ALT	U/mL	284 ± 68	287 ± 73

Table 5. Blood metabolic parameters of wild-type (WT) and DKO mice. Mice were fasted for 12 h. * $P < 0.05$ compared to wild-type mice. Values are the mean ± S.E.M. with $n = 4$ mice per group.

4.4. DKO mice have lower blood concentrations of gluconeogenic substrates but higher levels of ketone bodies

To test the hypothesis that PDK2/PDK4 deficiency reduces blood glucose by the lowering supply of gluconeogenic substrates needed for glucose production, serum levels of pyruvate and lactate, were measured (Table 5). DKO mice had lower levels of pyruvate and lactate than wild-type mice (Table 5). In contrast, greater levels of β-hydroxybutyrate, acetoacetate, and free fatty acids were found in the DKO mice fed the

HSF diet (Table 5). Additionally, blood pH in the DKO mice were significantly reduced compared to wild-type mice. High levels of ketone bodies in conjunction with decline in pH confirmed the presence of ketoacidosis in HSF diet fed DKO mice. The dramatic rise of ketone bodies overwhelmed the capacity of the kidney for reabsorption of ketone bodies, resulting in larger excretion of β-hydroxybutyrate in the urine of DKO mice (19 $\pm$ 8 mmol/L DKO mice versus 1.6 $\pm$ 0.2 mmol/L in wild-type mice after 24 h of fasting; n = 4 mice/group; $P < 0.05$). The amount of blood nitrogen as measured by blood urea nitrogen (BUN) and creatinine levels were elevated in DKO mice (Table 5). Levels of triacylglycerol (TG) and alanine transaminase (ALT), an enzyme that is released to the blood as a result of liver injury, were similar between DKO and wild-type mice (Table 5).

4.5. DKO mice suffer from fasting induced hypothermia

In addition to suffering from ketoacidosis, DKO fed the HSF diet experienced severe hypothermia (24.9 ± 0.4 °C compared to 36.5 ± 0.3 °C in wild-type mice, n = 6 mice per group, $P < 0.05$) after 24 hours of fasting, leading ultimately to their death.

4.6. Plasma essential amino acids and key gluconeogenic amino acids are reduced while citrulline is elevated in DKO mice

Amino acid analysis was performed to determine whether gluconeogenic or ketogenic amino acids were altered in DKO mice. Alanine, a key gluconeogenic amino acid, was reduced in DKO mice both in the fed and fasted state (Table 6).

	Fed State		24 h Fasted State	
	WT	DKO	WT	DKO
Asn	110 ± 7	86 ± 14*	119 ± 22	115 ± 20
His	95 ± 4	81 ± 7*	187 ± 32	168 ± 35
Ala	423 ± 131	282 ± 84*	777 ± 172	403 ± 89*
Pro	202 ± 13	153 ± 13*	229 ± 45	173 ± 25
Thr	235 ± 9	109 ± 13*	370 ± 52	476 ± 94
Tyr	22 ± 1	22 ± 1	51 ± 5	45 ± 7
Met	136 ± 5	88 ± 6*	212 ± 49	147 ± 38
Phe	43 ± 1	37 ± 2*	111 ± 18	84 ± 11
Val	359 ± 25	258 ± 8*	415 ± 53	831 ± 275*
Ile	141 ± 14	91 ± 2*	169 ± 25	353 ± 125*
Leu	269 ± 24	188 ± 7*	529 ± 96	834 ± 94*
Lys	674 ± 14	458 ± 11*	990 ± 277	885 ± 272
Cit	107 ± 2	123 ± 13	120 ± 20	199 ± 32*

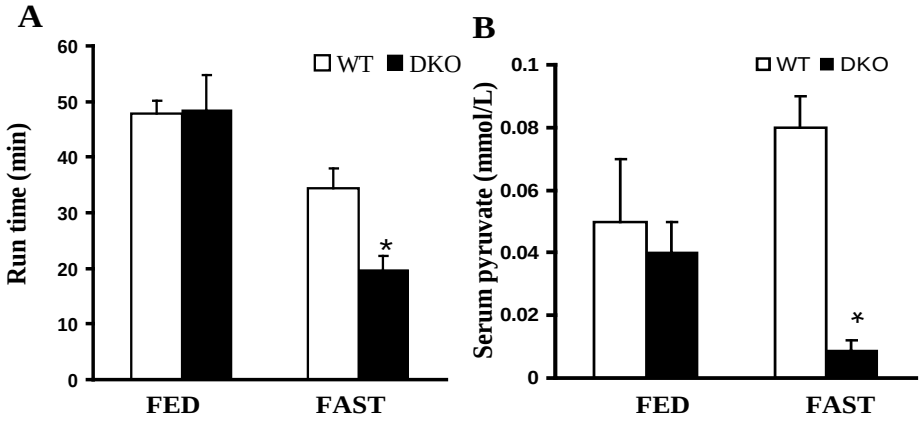
Table 6. Plasma amino acid levels in WT and DKO mice. Amino acid levels are given in nmol/mL. Blood was obtained for analysis in the fed state (9:00 a.m.) and after 24 hr fast (6:00 p.m.). * $P < 0.05$ compared to wild-type mice. Values are the mean ± S.E.M. with $n = 6$ mice per group except for $n = 4$ wild-type mice in the fed state.

Branched chain amino acids (valine, isoleucine, and leucine), were lower in DKO mice compared to wild-type mice in the fed state, whereas in the fasted state the DKO mice showed elevated amount of these amino acid. Lysine, another ketogenic amino acid, was reduced in the fed state but not in the fasted state in DKO mice (Table 6). Amino acid analysis also revealed that essential amino acids were lowered in the fed state and unchanged except for BCAAs in the fasted state in DKO mice. The decrease in essential amino acids in the fed state may be associated with increased protein synthesis, suggesting that PDK2/PDK4 deficiency promotes protein synthesis. Furthermore, levels

of citrulline, an intermediate in the urea cycle, was increased in DKO mice after 24 hr fasting (Table 6). In the fed state, however, citrulline levels were similar between DKO and wild-type mice.

4.7. DKO mice exhibit reduced capacity to sustain exercise under fasting conditions

We hypothesized that DKO mice would not tolerate exercise due to ketoacidosis. DKO mice were exercised on a motorized treadmill apparatus using a run-to-exhaustion protocol. DKO mice fasted for 12 h exhibited a markedly reduced capacity to sustain running exercise, whereas the running capacity of wild-type mice modestly reduced between the fed and fasting conditions (Figure 17). Serum levels of pyruvate, lactate, bicarbonate, β-hydroxybutyrate, and blood pH were similar in fed DKO and wild-type mice after exhaustion (Figure 17B - 17F). In contrast, significant reductions in pyruvate, lactate, bicarbonate, and blood pH occurred in fasted DKO mice compared to wild-type mice after exhaustion. These findings suggest that sustaining exercise capacity under fasting conditions depend upon the presence of PDK2 and PDK4 to prevent ketoacidosis and prolong exercise endurance.



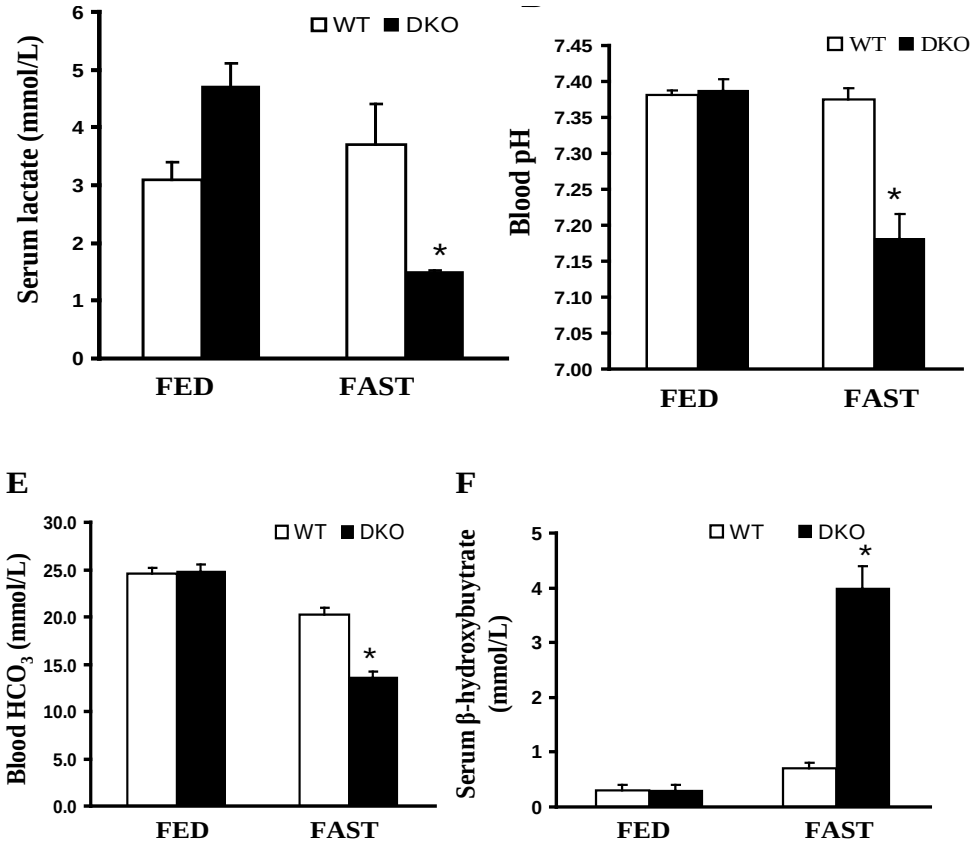
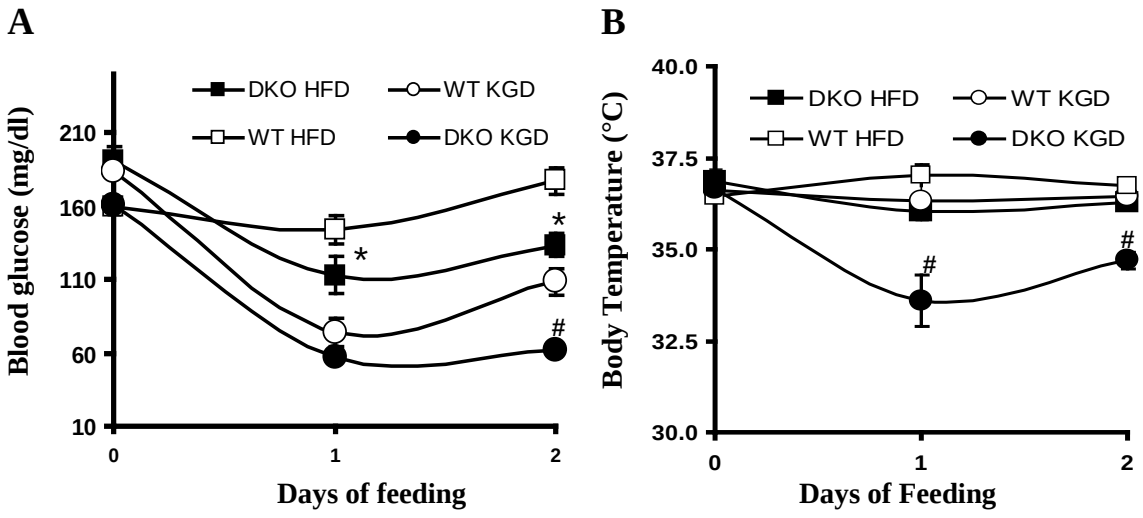


Figure 17. Effect of exercise on wild-type and DKO mice fed a HSF diet. (A) Run time, defined as the time point when mice were unable to run any longer, was determined in wild-type and DKO mice fed a HSF diet for 16 weeks. Half of the mice in each group were fasted for 12 h and the remaining were in the fed state followed by exercise assessment. Serum pyruvate **(B)**, lactate **(C)**, blood pH **(D)**, blood bicarbonate **(E)**, and serum β-hydroxybutyrate **(F)** were monitored after exercise. All values are the mean ± S.E.M. with WT, $n = 10$; DKO, $n = 10$. * $P < 0.05$ relative to wild-type mice in the fasted state determined by Student's *t* test.

4.8. DKO mice exhibit hypothermia and ketoacidosis when fed a ketogenic diet

We hypothesized that ketogenic conditions induced by a high-fat/low-carbohydrate intake state would lower blood glucose levels and provoke ketoacidosis and hypothermia as occurs under fasting conditions. To examine this possibility, 8-week old wild-type and DKO mice were fed a low carbohydrate/high fat diet (0.4% carbohydrate, 90.5% fat, and 9.1% protein from calories) or high carbohydrate/high fat diet (25.5% carbohydrate, 58.0 % fat, and 16.4 protein). On the ketogenic diet, wild-type mice exhibited lower blood glucose compared to wild-type mice fed HC/HF diet while DKO mice experienced severe hypoglycemia compared to HC/HF fed DKO mice (Figure 18A). Additionally, DKO mice on the ketogenic diet exhibited lower body temperature (Figure 18B), whereas wild-type and DKO mice maintained body temperatures at 37 °C on the HC/HF.



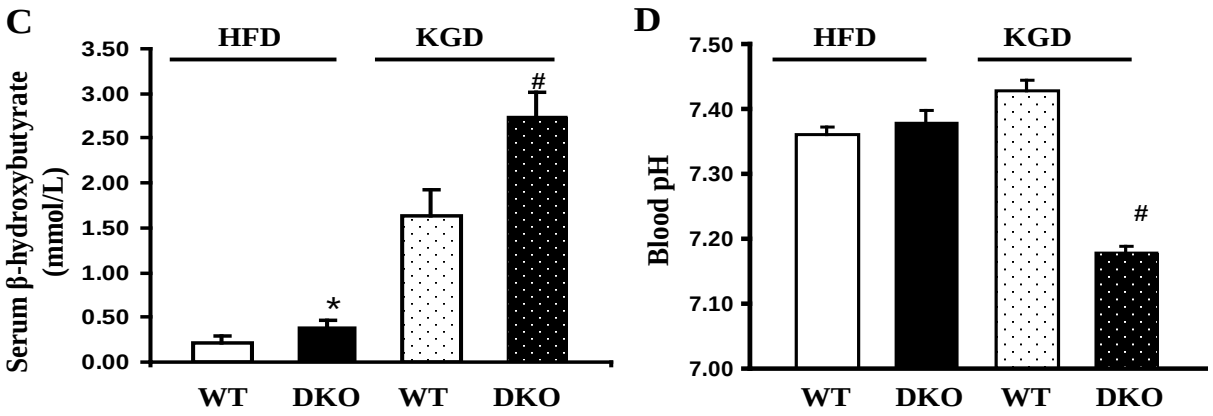


Figure 18. Ketogenic diet induces hypoglycemia, hypothermia and ketoacidosis in DKO mice. Blood glucose levels **(A)** and body temperature **(B)** were determined at 0, 1, and 2 d after addition of a high saturated fat diet (HFD) and ketogenic diet (KGD). WT on HFD, *n* = 8; DKO on HFD, *n* = 8; WT on KGD, *n* = 8; DKO on KGD, *n* = 8. Serum β-hydroxybutyrate **(C)** and blood pH **(D)** were determined after 2 d of feeding with HSD and KGD. WT on HFD, *n* = 8; DKO on HFD, *n* = 8; WT on KGD, *n* = 8; DKO on KGD, *n* = 8. All values are the mean ± S.E.M. * $P < 0.05$ relative to wild-type mice on the HFD; $^{\#}$ $P < 0.05$ relative to wild-type mice on the KGD determined by Student's *t* test.

Blood β-hydroxybutyrate levels were highly elevated (Figure 18C) while blood pH significantly reduced in DKO mice fed the ketogenic diet (Figure 18D). Blood alanine transaminase (ALT) levels were reduced in DKO mice (41 ± 5 U/L, n = 12) compared to wild-type mice fed the high fat diet (64 ± 7 U/L; n = 12 mice per group, $P < 0.05$). DKO mice fed the ketogenic diet had lower ALT levels (23 ± 9 U/L, n = 12) than wild-type mice (43 ± 10 U/L, n = 12, $P < 0.05$). These data indicated that regulation of blood glucose levels, ketone bodies, and thermogenesis mediated by PDK2 and PDK4 is

essential for fuel usage not only under fasting conditions but when mice are fed a high-fat/low-carbohydrate diet.

4.9. Rate of glucose production is reduced in DKO mice

To test the hypothesis that PDK2/PDK4 regulates blood glucose levels in fasted state by controlling the rate of glucose production through substrate supply, stable-isotope-based metabolic flux measurements were utilized to measure in vivo glucose production rates.

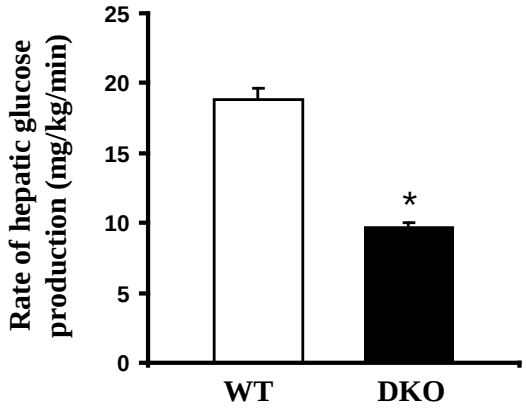


Figure 19. Rate of glucose production is reduced in DKO mice fed a HSF diet. Glucose production rate in wild-type and DKO after 18 h fast was determined using constant infusion of [U-$^{13}C_6$]glucose. WT, n = 4; DKO, n = 4. The number following m indicates the number of ^{13}C each molecule contains. WT, n = 4; DKO, n = 4. All values are the mean ± S.E.M. * $P < 0.05$ relative to wild-type mice determined by Student's t test.

As expected, glucose production rate was reduced two fold in DKO mice (Figure 19), suggesting that that rate of gluconeogenesis is limited by reduced supply of gluconeogenic substrates in the DKO mice. Furthermore, protein level of a key

regulatory enzyme for gluconeogenesis, phosphoenolpyruvate carboxylase (PEPCK), was measured to determine whether an alteration in the amount of this enzyme was responsible for the lower blood glucose levels in DKO mice. Protein levels of PEPCK were similar in the fasted livers of DKO and wild-type mice (Figure 20).

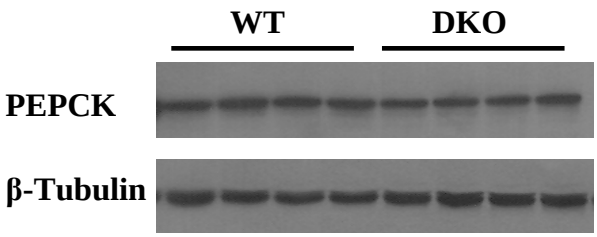


Figure 20. Expression of key gluconeogenic enzyme is not altered in the liver of DKO mice. Protein levels of phosphoenolpyruvate carboxylase (PEPCK) and β-tubulin were determined in the liver obtained from 12 h fasted wild-type and DKO mice. WT, *n* = 4; DKO, *n* = 4.

4.10. DKO mice synthesize more but oxidize less ketone bodies

We hypothesized that PDK2/PDK4 regulates ketone body levels by influencing the rate of ketone body production in the liver. To test this hypothesis, metabolic flux analysis with stable isotope labeled β-hydroxybutyrate was conducted. Measurements of β-hydroxybutyrate turnover rate using [U-$^{13}C_4$]β-hydroxybutyrate revealed a 30 % increase in β-hydroxybutyrate production rate in DKO mice (Figure 21A).

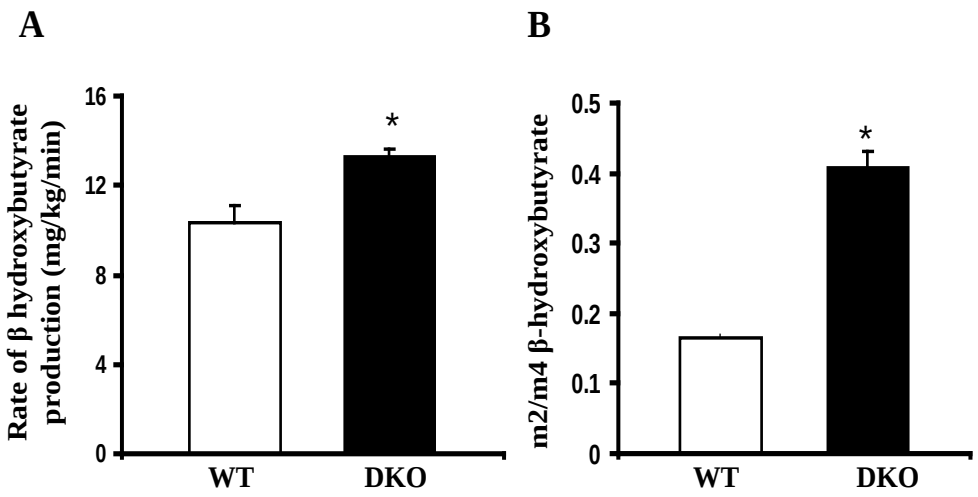


Figure 21. Rate of β-hydroxybutyrate production is increased in DKO mice fed a HSF diet. (A) β-hydroxybutyrate production rate in wild-type and DKO after 18 h fast was determined using constant infusion of [U-$^{13}C_4$]β-hydroxybutyrate. WT, *n* = 4; DKO, *n* = 4. **(B)** Enrichment of β-hydroxybutyrate was produced from β-hydroxybutyrate in wild-type and DKO mice using constant infusion of [U-$^{13}C_4$]β-hydroxybutyrate. The number following m indicates the number of ^{13}C each molecule contains. WT, *n* = 4; DKO, *n* = 4. All values are the mean ± S.E.M. * $P < 0.05$ relative to wild-type mice determined by Student's *t* test.

Additionally, the ratio of m2/m4 β-hydroxybutyrate which reflects the ratio of labeled acetyl-CoA (m2) to β-hydroxybutyrate (m4) was elevated by 2.5 fold in DKO mice (Figure 21B). These findings suggests greater rate of re-cycling the ketone bodies by pseudo ketogenesis, presumably because of the high ketone body concentrations in the DKO mice.

To evaluate whether ketone body oxidation was altered in DKO mice, diaphragms from 20 weeks HSF diet fed DKO and wild-type mice were incubated with [3-^{14}C]β-hydroxybutyrate followed by collection of $^{14}CO_2$.

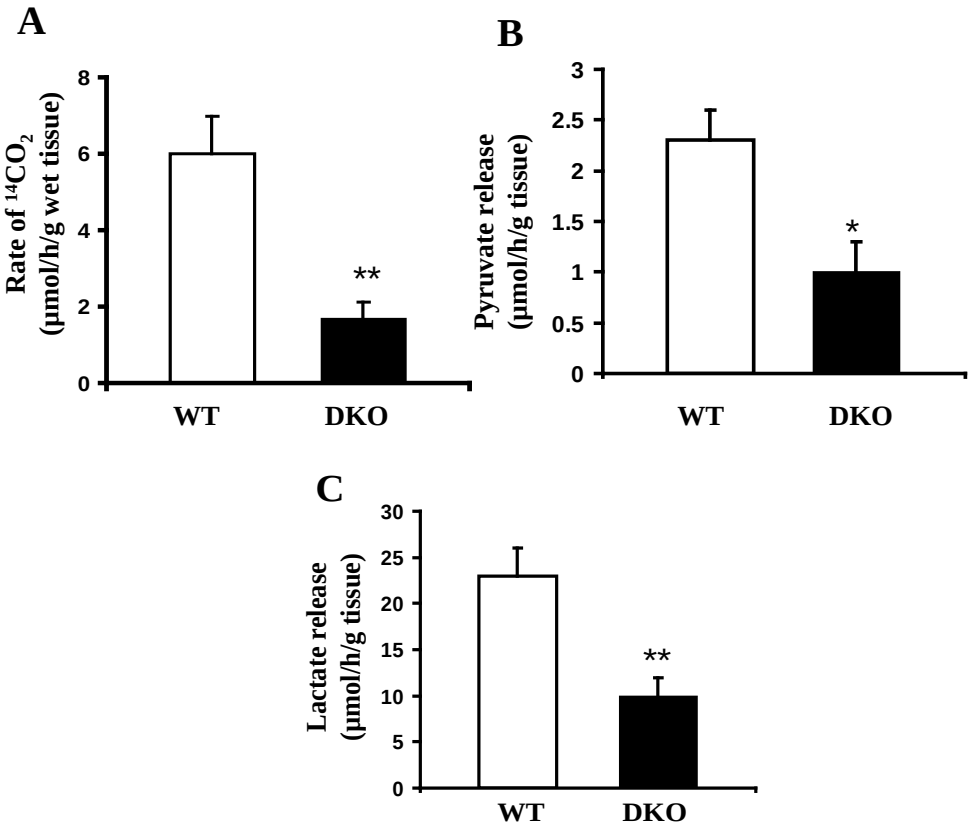


Figure 22. Ketone body oxidation is reduced in the diaphragms obtained from DKO mice. (A) Rate of β-hydroxybutyrate oxidation, measured by $^{14}CO_2$, was determined in hemi-diaphragms incubated with [3-^{14}C]β-hydroxybutyrate. Diaphragms were obtained from WT, *n* = 10; DKO, *n* = 10. Pyruvate release **(B)** and Lactate release **(C)** were determined in hemi-diaphragms incubated with [3-^{14}C]β-hydroxybutyrate. Diaphragms were obtained from WT, *n* = 10; DKO, *n* = 10. All values are the mean ± S.E.M. * $P < 0.05$; ** $P < 0.01$ relative to wild-type mice determined by Student's *t* test.

Beta-hydroxybutyrate oxidation rate, measured by $^{14}CO_2$ production, was 72% less in diaphragms from DKO mice relative in diaphragms from wild-type mice (Figure 22A). Furthermore, pyruvate (Figure 22B) and lactate release (Figure 22C) were 58% less respectively in diaphragms from DKO mice relative to diaphragms from wild-type mice. Since ketone body oxidation has been shown to inhibit glucose oxidation, we determined glucose oxidation rates in diaphragms obtained from 16 weeks HSF fed DKO mice and wild-type mice by incubating with [U-^{14}C] glucose followed by collection of $^{14}CO_2$.

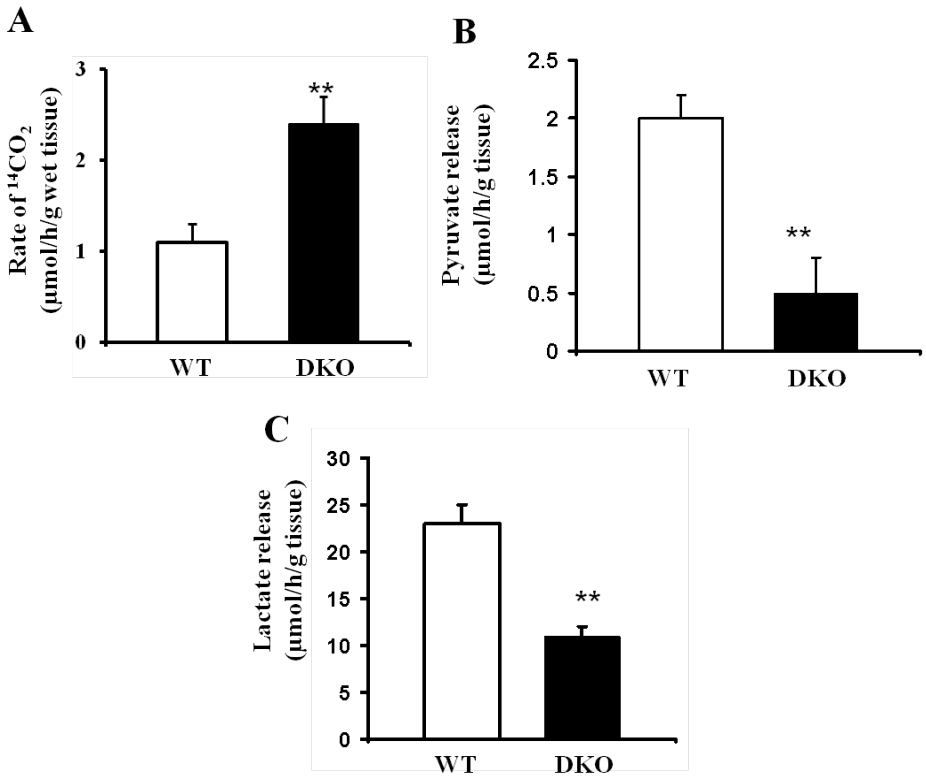


Figure 23. Glucose oxidation is not altered in the diaphragms obtained from DKO mice. (A) Rate of glucose oxidation, measured by $^{14}CO_2$, was determined in hemi-diaphragms incubated with [U-$^{14}C_6$] glucose. Diaphragms were obtained from WT, *n* = 10; DKO, *n* = 10. Pyruvate release **(B)** and Lactate release **(C)** were determined in hemi-

diaphragms incubated with [U-$^{14}C_6$] glucose. Diaphragms were obtained from WT, *n* = 10; DKO, *n* = 10. All values are the mean ± S.E.M. ** P < 0.01 relative to wild-type mice determined by Student's *t* test.

Glucose oxidation rate, measured by $^{14}CO_2$ production, was 46% greater in diaphragms from DKO mice relative from wild-type mice (Figure 23A). Pyruvate release (Figure 23B) and lactate release (Figure 23C) were reduced 2 fold in diaphragms from DKO mice relative to wild-type mice. These findings suggest that PDK2/PDK4 deficiency increases levels of ketone bodies by enhancing ketone body production and suppressing ketone body oxidation. In contrast, PDK2/PDK4 deficiency decreases blood glucose levels by decreasing glucose production and reducing glucose oxidation.

4.11. DKO mice oxidize less fatty acids

Using indirect calorimetry, we determined locomotor activity, the rate of oxygen consumption, energy expenditure, and fatty acid oxidation during 24 h fasting in 30 weeks of HSF fed DKO and wild-type mice (Figure 24). Locomotor activity was greater in DKO and wild-type mice at night consisting of the first 12 hrs of fasting (Figure 24A). At day time, locomotor activity was greatly reduced in both groups of mice but DKO mice remained less active than wild-type mice as fasting prolonged. Rate of oxygen consumption (Figure 24B) were similar between DKO and wild-type mice during the first 12 h of fasting but both vital parameters for DKO mice started to rapidly decline after 13 h of fasting. Energy expenditure (Figure 24C) and fatty acid oxidation rates (Figure 24D) were not different between wild-

type and DKO mice during the first 11 h while progressively reduced for DKO mice as the fasting continued, reaching a dangerously low plateau at 18 hrs of fasting.

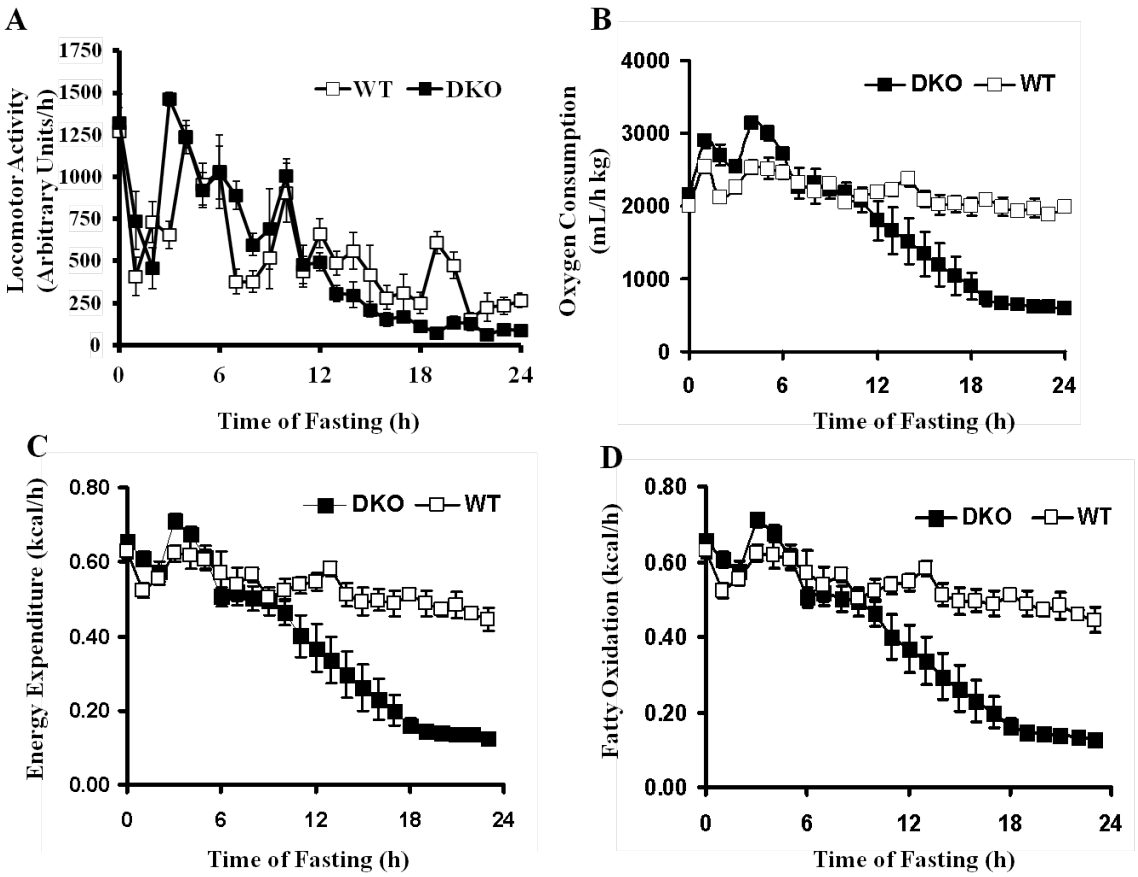


Figure 24. Rates of oxygen consumption, carbon dioxide production, energy expenditure, and fatty acid oxidation are reduced in HSF diet fed DKO mice in the fasted state. Rate of Locomotor activity **(A)**, oxygen production **(B)**, energy expenditure **(C)**, and fatty acid oxidation **(D)** were determined by TSE indirect calorimetry systems. Each parameter was recorded 6 times/h and then averaged to give hourly rates. WT, n = 8; DKO, n = 8. All values are the mean ± S.E.M. * $P < 0.05$ relative to wild-type mice determined by Student's t test.

Body temperature taken after 24 h of fasting was dramatically lowered in DKO mice (24.7 ± 0.3 °C, n = 7 mice) relative to wild-type mice (37.0 ± 0.5 °C; n = 6 wild-type mice; $P < 0.01$). These findings suggest that reduction in metabolic rate results in less production of heat and therefore lower body temperature. Since brown adipose tissue is a thermogenic tissue in which oxidation of fatty acids generates heat through uncoupling proteins (UCPs), we determined whether expression of UCPs were altered in the BAT obtained from hypothermic DKO mice (Figure 25A).

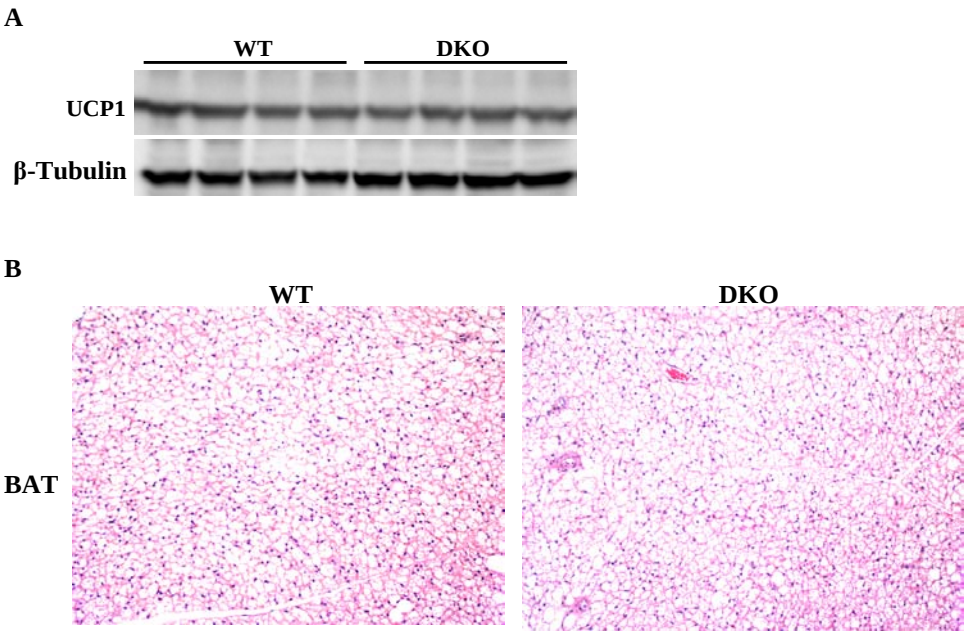


Figure 25. Expression of uncoupling protein (UCP1) and morphology of brown adipose tissue (BAT) are not altered in DKO mice. (A) Protein levels of UCP1 and β-tubulin of wild-type and DKO mice were determined by western blot analysis in the BAT obtained after 24 h fasting. WT, *n* = 4; DKO, *n* = 4. **(B)** Microscopic photographs demonstrating histological appearance of BAT of wild-type and DKO mice fed a high HSF diet for 30 weeks. BAT were obtained from 24 hr fasted wild-type and DKO mice.

Tissue sections were stained with H&E stain. The material stained white corresponds to fat droplets.

The protein levels of UCP1 in the BAT did not differ between wild-type and DKO mice (Figure 26A). During 24 h of fasting when DKO mice have lower body temperature, the quantity and morphology of BAT are indistinguishable between wild-type and DKO mice (Figure 25B).

4.12. PDK2/PDK4 deficiency suppresses levels of hepatic citric acid cycle intermediates

We hypothesized that PDK2/PDK4 deficiency lowers pyruvate which in turn limits the formation of oxaloacetate needed for TCA cycle activity in the liver. Subsequently, acetyl-CoA is directed to the production of ketone bodies instead of oxidized by the citric acid cycle to CO_2 and water. To test this hypothesis, levels of pyruvate, oxaloacetate and other citric acid cycle intermediates were measured in the liver of wild-type and DKO mice fed HSF for 30 weeks. In the fed state, liver concentrations of pyruvate (0.08 ± 0.01 μmol/g in DKO mice compared to 0.10 ± 0.03 μmol/g in wild-type mice, n = 6 DKO mice; n = 5 wild-type mice; $P > 0.05$) and oxaloacetate (0.04 ± 0.02 μmol/g in DKO mice compared to 0.03 ± 0.01 μmol/g in wild-type mice; n = 5 mice per group: $P > 0.05$) were similar between the wild-type and DKO. In the fasted state, on the other hand, oxaloacetate levels were reduced by 2 fold in the liver of DKO mice relative to wild-type mice (Table7). Measured oxaloacetate levels were similar to calculated oxaloacetate levels based on the malate dehydrogenase

equilibrium constant (Table 7). Since oxaloacetate is essential for citric acid cycle activity, level of malate, citrate, and α-ketoglutarate were determined in the liver of DKO and wild-type mice (Table 7). Malate concentration was not different in the liver of DKO and wild-type mice (Table 7) whereas the concentrations of citrate and α-ketoglutarate were lower in DKO mice.

Measurement	WT	DKO
Pyruvate	0.047 ± 0.007	0.010 ± 0.003*
OAA measured	0.015 ± 0.002	0.009 ± 0.002*
OAA calculated	0.012 ± 0.002	0.004 ± 0.003
Malate	0.35 ± 0.06	0.26 ± 0.1
Citrate	0.26 ± 0.04	0.13 ± 0.02*
α-ketoglutarate	0.016 ± 0.001	0.011 ± 0.001*
ATP	2.4 ± 0.1	2.25 ± 0.07
ADP	1.3 ± 0.1	1.14 ± 0.04*
AMP	0.47 ± 0.05	0.41 ± 0.02
Lactate	0.34 ± 0.05	0.20 ± 0.05*
β-Hydroxybutyrate (HB)	1.6 ± 0.1	4.5 ± 0.4*
Acetoacetate (AcAc)	0.26 ± 0.06	1.21 ± 0.05*
[Lactate]/[Pyruvate]	8 ± 1	32 ± 9*
[HB]/[AcAc]	5 ± 1	3.7 ± 0.02*

Table 7. Liver metabolic parameters of wild-type (WT) and DKO mice. Metabolites are given in units of μmol/g tissue. Mice were fasted for 12 h. * $P < 0.05$ compared to wild-type mice. Values are the mean ± S.E.M. with $n = 6$ mice per group.

These data suggest that PDK2/PDK4 deficiency reduces oxaloacetetate concentrations, as well as of the citric acid cycle intermediates. No differences in hepatic ATP and AMP concentrations were found between wild-type and DKO mice while ADP

was reduced in the liver of DKO mice (Table 7). Furthermore, reduction of hepatic pyruvate resulted not only in suppression of oxaloacetate but as well in suppression of lactate. The lactate/pyruvate ratio was elevated in the liver of DKO mice. In contrast, the β-hydroxybutyrate levels were increased by two fold while acetoacetate levels were increased 5 fold in the liver of DKO mice, yielding a reduced hepatic β-hydroxybutyrate/acetoacetate ratio.

4.13. OAA evels are reduced in the skeletal muscle of DKO mice

Since oxaloacetate levels were reduced in the liver DKO mice, we investigated whether low pyruvate concentrations in DKO mice may limit the formation of oxaloacetate in peripherial tissues, shunting acetyl-CoA away from citric acid cycle. As anticipated pyruvate and lactate levels were reduced in the skeletal muscle of DKO mice (Table 8).

Measurements	WT	DKO
Lactate	1.8 ± 0.3	0.7 ± 0.1*
Pyruvate	0.14 ± 0.05	0.05 ± 0.01*
Malate	0.3 ± 0.1	0.08 ± 0.03*
OAA	0.06 ± 0.02	0.022 ± 0.003*
ATP	4.8 ± 0.2	3.4 ± 0.2*
ADP	0.5 ± 0.1	0.3 ± 0.1
AMP	0.06 ± 0.01	0.03 ± 0.01

Table 8. Effect of PDK2/PDK4 deficiency on muscle metabolite levels. Metabolites are given in units of μmol/g of wet weight tissue. Mice were fasted for 12 hrs. * $P < 0.05$ compared to wild-type mice. Values are the mean ± S.E.M. with $n = 5$ mice per group.

As a consequence of low pyruvate, oxaloacetate levels were reduced remarkably in the skeletal muscle. Additionally, malate concentrations were reduced in DKO mice. Surprisingly, muscle ATP levels were significantly lowered with no differences in ADP and AMP in the muscle of DKO mice fasted for 12 h. Similar reduced ATP levels were also found in the muscle of DKO mice (3.6 ± 0.3 μmol/g; means ± S.E.M.; n = 6 mice) and wild-type mice (5.4 ± 0.3 μmol/g; n = 6 mice; $P < 0.05$) after 24 h of fasting. These data indicate that PDK2 and PDK4 plays a pivotal role in supplying pyruvate for pyruvate carboxylase to produce oxaloacetate needed to initiate TCA cycle activity for ATP production during starvation.

4.14. OAA levels are reduced in the liver of DKO mice fed the ketogenic diet

We hypothesized that a low carbohydrate-high fat diet such as the ketogenic diet lowers pyruvate levels which in turn will limit the production of oxaloacetate for TCA cycle activity and glucose production.

	High Fat Diet		Ketogenic Diet	
	WT	**DKO**	**WT**	**DKO**
Pyruvate	**0.04 ± 0.01**	**0.015 ± 0.002***	**0.016 ± 0.005**	**0.008 ± 0.002#**
Lactate	**0.8 ± 0.1**	**0.4 ± 0.1***	**0.16 ± 0.05**	**0.10 ± 0.04#**
β-hydroxybutyrate	**0.19 ± 0.05**	**0.42 ± 0.05***	**1.5 ± 0.2**	**2.25 ± 0.2#**
Oxaloacetate	**0.014 ± 0.003**	**0.012 ± 0.003**	**0.017 ± 0.002**	**0.010 ± 0.002#**

Table 9. Liver metabolites of WT and DKO mice fed a high fat diet and a ketotic diet. Metabolites are given in units of μmol/g tissue. Mice were killed in the fed state. * $P < 0.05$ compared to wild-type mice on high fat diet. # $P < 0.05$ compared to wild-type mice on ketogenic diet. Values are the mean ± S.E.M. with *n* = 12 mice per group.

Hepatic pyruvate and lactate concentrations were reduced in wild-type mice fed the ketogenic diet compared to wild-type fed the high fat diet (Table 9). But pyruvate and lactate levels were reduced in the liver of DKO mice on both diets compared to wild-type mice. Similar to blood concentrations, β-hydroxybutyrate levels were elevated in the liver of DKO fed either diet and in wild-type mice fed the ketogenic diet compared to wild-type mice fed the high fat diet. The concentrations of oxaloacetate was reduced in DKO mice fed the ketogenic diet (Table 9). These data suggest that PDK2 and PDK4 are essential in conserving pyruvate needed for pyruvate carboxylase to produce oxaloacetate when the dietary supply of pyruvate is lowered by low carbohydrate-high fat diet. As a consequence of low oxaloacetate, glucose synthesis is reduced and ketogenesis in increased resulting in hypoglycemia and ketoacidosis in DKO mice fed a low carbohydrate/high fat diet.

5. Discussion

This study shows that PDK2/PDK4 deficiency partially prevents high-fat diet induced hyperglycemia, adiposity, and fasting induced hepatic steatosis. Wild-type mice maintained for 30 weeks on a high saturated fat diet were grossly obese, hyperglycemia, hyperinsulinaemic, glucose intolerant, and insulin resistant. PDK2/PDK4 double knockout mice fed the same diet for the same length of time gained less weight, remained euglycemic, and were less glucose intolerant than wild-type mice. Although PDK2/PDK4 deficiency attenuated some of the negative effects caused by a high saturated fat diet, it induced ketoacidosis during fasting.

PDK deficiency is believed to lower fasting blood glucose levels by decreasing the supply of substrates needed for gluconeogenesis [67]. Consistent with this hypothesis, the blood concentrations of pyruvate, alanine, and lactate were reduced in PDK2/PDK4 double knockout mice. Since the availability of gluconeogenic substrates is limited in PDK2/PDK4 deficient mice, DKO mice synthesize glucose at a lower rate than wild-type mice as shown in this study by metabolic flux analysis with [U-$^{13}C_6$]glucose. The euglycemia of DKO mice is due to reduced rate of glucose production. Hyperglycemia in wild-type mice is due to increased rate of glucose production. Furthermore, no difference in the expression of PEPCK, which can limit the rate of gluconeogenesis in some situations [146], was found between DKO and wild-type mice. These findings suggest that altered expression of gluconeogenic enzyme is not responsible for euglycemia in DKO mice. These findings are consistent with recent studies that demonstrated that gluconeogenic enzymes are not elevated in hyperglycemic patients with type 2 diabetes and in 2 rodent models of hyperglycemia [153]. Our work suggests that hyperglycemia is dictated by the rate of glucose production which in turn depends on substrate supply regulated by the PDKs. We believe that induction of PDK2 and PDK4 in type 2 diabetic patients promotes the phosphorylation and inactivation of PDC to conserve gluconeogenic substrates, leading to enhanced substrate availability and increased glucose production. Enhanced glucose synthesis contributes to elevation of blood glucose levels resulting in hyperglycemia.

Although our study supports the idea that PDK inhibition may serve as a good target for treatment for type 2 diabetic patients, double deficiency of PDK2 and PDK4 greatly increases blood ketone body levels. Although this is only observed in the fasted

state, it is undesirable effect of PDK deficiency because it invokes ketoacidosis, which can be fatal. Stable isotope metabolic flux analysis with [U-$^{13}C_4$]β-hydroxybutyrate indicated DKO mice synthesize ketone bodies at a higher rate than wild-type mice while in vitro incubation studies with [3-^{14}C]β-hydroxybutyrate indicated that DKO mice oxidize ketone bodies at a lower rate than wild-type mice. It appears, therefore, that both an increase in ketogenesis and a decrease in ketolysis contribute to the accumulation of ketone bodies in the blood of DKO mice. We hypothesized that reduced supply of three carbon compounds limits availability of oxaloacetate in the liver which directs acetyl-CoA into ketone body formation instead of oxidation by the citric acid cycle. Consistent with this hypothesis, oxaloacetate and citric acid cycle intermediates were reduced in the liver of DKO mice. In the absence of PDK2 and PDK4, PDC is highly active to promote rapid oxidation of pyruvate, resulting in low pyruvate levels. Pyruvate is needed for synthesis of oxaloacetate which in turn is essential for condensation with acetyl-CoA to form citrate to initiate the TCA cycle activity. Low pyruvate levels limit oxaloacetate synthesis which in turn suppresses TCA cycle activity and shunts acetyl-CoA into ketone synthesis. Without citric acid cycle activity and without the enzymatic capacity for ketone body synthesis, extrahepatic tissues of DKO mice are unable to oxidize ketone bodies. Furthermore, indirect calorimetric measurements indicated that DKO mice oxidize fatty acids at a lower rate than wild-type mice. In the absence of oxaloacetate and reduced TCA cycle activity, complete oxidation of fatty acids is inhibited. Inhibition of fatty acid and ketone body oxidation, results in less heat produced to maintain body temperature, provoking hypothermia in DKO mice.

Depletion of oxaloacetetate is responsible for the ketoacidotic and hypothermic phenotype of PDK2/PDK4 knockout mice. Similar detrimental effects of oxaloacetate deficiency are also manifested in patients with pyruvate carboxylase (PC) deficiency, which is characterized by metabolic acidosis, ketosis, lactic acidemia, and delayed neurological development [103]. This disorder spans from the most severe (Type B) to less severe forms (Type A, C) [105]. Type B patients present with an elevated ratio of lactate to pyruvate (L/P) and reduced ratio of β-hydroxybutyrate to acetoacetate (B/A) ratios in contrast to those seen in patients with type A and type C. Since these ratios are determined by the redox state of NAD^+ in the cytoplasm and the mitochondria, respectively, it is apparent that the enzymatic capacity for the formation of OAA is a major determinant of the NAD^+ redox state. Reduced availability of oxaloacetate in the mitochondria as a result of PC deficiency decreases the activity of the malate-aspartate shuttle that moves reducing equivalents from the cytoplasm to the mitochondrial matrix space. As a result, the ratio of NADH to NAD^+ is high in the cytoplasm, favoring the conversion of pyruvate to lactate and resulting in a high L/P ratio. Less transport of NADH into the mitochondria leads to a low ratio of NADH to NAD^+ ratio in the mitochondrial matrix space, resulting in less conversion of acetoacetate to β-hydroxybutyrate and a low B/A ratio [104]. Similar alterations in the L/P and B/A ratios occur in PDK2/PDK4 double knockout mice. While lack of functional enzyme activity is responsible for low oxaloacetate levels in PC deficient patients, lack of substrate (pyruvate) is responsible for reduced pyruvate carboxylase flux in PDK deficient mice. Not surprisingly, therefore, the phenotype of PDK2/PDK4 double knockout mice resembles that of type B PC deficient patients.

This study demonstrates that the PDKs are essential in regulating pyruvate carboxylase flux by substrate supply to maintain blood glucose levels, ketone bodies, and body temperature. The PDKs phosphorylate the PDC to conserve pyruvate for glucose synthesis and for oxaloacetate formation which is needed for TCA cycle activity. Disruption of this control mechanism mediated by the PDKs has detrimental effects resulting in ketoacidosis and hypothermia.

GLOBAL CONCLUSION

The primary goal of my research was to establish the role of pyruvate dehydrogenase kinase (PDKs) in regulating glucose and ketone body metabolism. Our work determined that PDK2/PDK4 inactivates the pyruvate dehydrogenase complex (PDC) by phosphorylation to conserve substrate supply for the liver for glucose production. Furthermore, inactivation of PDC by the PDKs preserves pyruvate for oxaloacetate synthesis for TCA cycle activity. Proper TCA cycle activity in the liver promotes acetyl-CoA oxidation and prevents the diversion of acetyl-CoA into ketone bodies synthesis. Inhibition of PDK2/PDK4 disrupts this control mechanism and results in less glucose production and increased ketone body synthesis. Consequently, PDK2/PDK4 deficient mice experience lower blood glucose levels and increased ketone bodies.

My work also explored whether the glucose lowering effects of PDK inhibition would be maintained in a diet induced diabetic animal model. Inhibition of PDK2 and PDK4 in mice fed a high fat diet improved insulin sensitivity, decreased adiposity, reduced fat accumulation in the liver, and prevented hyperglycemia. These desirable effects of knocking out PDKs provide convincing evidence that PDK inhibition is a good target for treatment of type 2 diabetes. Nevertheless, my study revealed that the use of PDK inhibitors is accompanied by a potentially serious side effect. PDK2/PDK4 inhibition can induce severe ketoacidosis and hypothermia.

Currently, a synthetic PDK inhibitor, dichloroacetate (DCA) has been used experimentally to reduce growth of solid tumors in non-small cell lung cancer, glioblastoma, breast, endometrial and prostate cancer [154]. Glioblastoma patients have

been treated with DCA for 15 months without experiencing any toxicity of the compound, at least according to the investigators who carryout out this study [155]. Although based on my work with the PDK2/PDK4 deficient mice, I predict that patients treated with large doses of DCA may experience ketoacidosis and hypothermia. In the future, it may become important to design new inhibitors that partially inhibit the PDKs without causing ketoacidosis. Since complete inhibition of PDK2 and PDK4 results in severe ketoacidosis in the PDK2/PDK4 double knockout mice, we would predict that partially inhibiting the PDKs would not provoke such severe phenotype. Additionally, we believe that designing a liver specific PDK inhibitor will have glucose lowering effects without inducing ketoacidosis.

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Publications:

1. **Y. Rahimi**, S. Shrestha, T. Banerjee, S.K. Deo "Study of Metal Binding to DsRed-Monomer", **2007**, Proceeding of International Society of Chemilumescence.
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11. **Y. Rahimi**, N.H. Jeoung, P. Wu, Lee, R.A. Harris, "Fasting induces ketoacidosis and hypothermia in PDHK2/PDHK4 double knockout mice", Biochemical Journal, **2011**, 443, pg. 829 - 39.
12. **Y. Rahimi**, P. Wu, N.H. Jeoung, M. Kuntz, W. Davis, R.A. Harris, "PDK2/PDK4 deficient mice develop euglycemia, ketoacidosis, fasting induced hypothermia and reduced hepatic steatosis on a high saturated fat diet", *manuscript in preparation.*
13. G. Novarino, P. El-Fishaway, H. Kayserili, M. Kara, J. Schroth, J. L. Silhavy, S. Gabriel, L. Sweetmena, **Y. Rahimi**, R. A. Harris, M. W. State, J. G. Gleeson, "Mutations in the BCKD-kinase lead to a potentially treatable form of autism with epilepsy", *manuscript in preparation.*