Role of pyruvate dehydrogenase kinase isoenzyme 4 (PDHK4) in glucose homoeostasis during starvation

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The PDC (pyruvate dehydrogenase complex) is strongly inhibited by phosphorylation during starvation to conserve substrates for gluconeogenesis. The role of PDHK4 (pyruvate dehydrogenase kinase isoenzyme 4) in regulation of PDC by this mechanism was investigated with PDHK4$^{-/-}$ mice (homozygous PDHK4 knockout mice). Starvation lowers blood glucose more in mice lacking PDHK4 than in wild-type mice. The activity state of PDC (percentage dephosphorylated and active) is greater in kidney, gastrocnemius muscle, diaphragm and heart but not in the liver of starved PDHK4$^{-/-}$ mice. Intermediates of the gluconeogenic pathway are lower in concentration in the liver of starved PDHK4$^{-/-}$ mice, consistent with a lower rate of gluconeogenesis due to a substrate supply limitation. The concentration of gluconeogenic substrates is lower in the blood of starved PDHK4$^{-/-}$ mice, consistent with reduced formation in peripheral tissues. Isolated diaphragms from starved PDHK4$^{-/-}$ mice accumulate less lactate and pyruvate because of a faster rate of pyruvate oxidation and a reduced rate of glycolysis. BCAAs (branched chain amino acids) are higher in the blood in starved PDHK4$^{-/-}$ mice, consistent with lower blood alanine levels and the importance of BCAAs as a source of amino groups for alanine formation. Non-esterified fatty acids are also elevated more in the blood of starved PDHK4$^{-/-}$ mice, consistent with lower rates of fatty acid oxidation due to increased rates of glucose and pyruvate oxidation due to greater PDC activity. Up-regulation of PDHK4 in tissues other than the liver is clearly important during starvation for regulation of PDC activity and glucose homoeostasis.

Key words: branched chain amino acid, fatty acid oxidation, glucose homoeostasis, pyruvate dehydrogenase complex, pyruvate dehydrogenase kinase isoenzyme 4 (PDHK4) deficiency, starvation.

INTRODUCTION

Regulation of the activity of the PDC (pyruvate dehydrogenase complex) is achieved in large part by phosphorylation of its dehydrogenase component by PDHKs (pyruvate dehydrogenase kinases) and dephosphorylation by PDPs (pyruvate dehydrogenase phosphatases) [1,2]. In the well-fed state, PDC is relatively dephosphorylated and therefore active to promote oxidation of three-carbon compounds. In the starved state, PDC is inactivated as a consequence of phosphorylation of its dehydrogenase component, which helps conserve three-carbon compounds for gluconeogenesis [3].

Rapid regulation of PDC activity can be achieved by allosteric activation of PDHK activity by acetyl-CoA and NADH formed during fatty acid oxidation [4], inhibition of PDHK activity by pyruvate produced from glucose by glycolysis [5] and activation of PDP activity by Ca^{2+} released from the sarcoplasmic reticulum and taken up by the mitochondrion during muscle contraction [6]. Longer-term regulation of PDC that occurs in response to starvation and diabetes involves stable increases in PDHK activity that are independent of short-term activation mechanisms [7,8]. Much of the stable increase in PDHK activity occurring in response to starvation and diabetes is a consequence of an increase in the amount of PDHK protein [8,9].

Four PDHK isoenzymes are expressed in a tissue-specific manner in mammals [10]. Two of them, PDHK2 (PDHK isoenzyme 2) and PDHK4, increase in response to starvation and diabetes in a tissue-specific manner in the rat [9,11,12]. PDHK2 increases significantly in liver and kidney and only slightly or not at all in other tissues. PDHK4 increases greatly in heart, skeletal muscle and kidney, but only slightly in the liver. These observations suggest that control of the amount of PDHK4, and to a lesser extent the amount of PDHK2, is important in long-term regulation of PDC activity and therefore glucose homoeostasis. Findings of the present study with mice lacking PDHK4 indicate that up-regulation of PDHK4 in peripheral tissues is indeed important for glucose homoeostasis during starvation. Inactivation of PDC by phosphorylation as a consequence of increased expression of PDHK4 helps maintain glucose levels by conserving substrates for gluconeogenesis.

EXPERIMENTAL

Materials

A BAC (bacterial artificial chromosome) clone (no. 19893) containing the mouse PDHK4 gene was purchased from Incyte Genomics (St. Louis, MO, U.S.A.). C57BL/6J black mice for backcrossing were purchased from The Jackson Laboratory (Bar Harbor, ME, U.S.A.). Enzymes and reagents were purchased from Promega (Madison, WI, U.S.A.) and Sigma (St. Louis, MO, U.S.A.). Ultra-sensitive mouse insulin ELISA kit was obtained

Abbreviations used: BCAA, branched chain amino acid; DCA, dichloroacetate; DTT, dithiothreitol; PEG, poly(ethylene glycol); PDC, pyruvate dehydrogenase complex; PDHK2, pyruvate dehydrogenase kinase isoenzyme 2; PDHK4$^{-/-}$ mice, homozygous PDHK4 knockout mice; PDP, pyruvate dehydrogenase phosphatase; Tos-Phe-CH_2Cl, tosylphenylalanylchloromethane.

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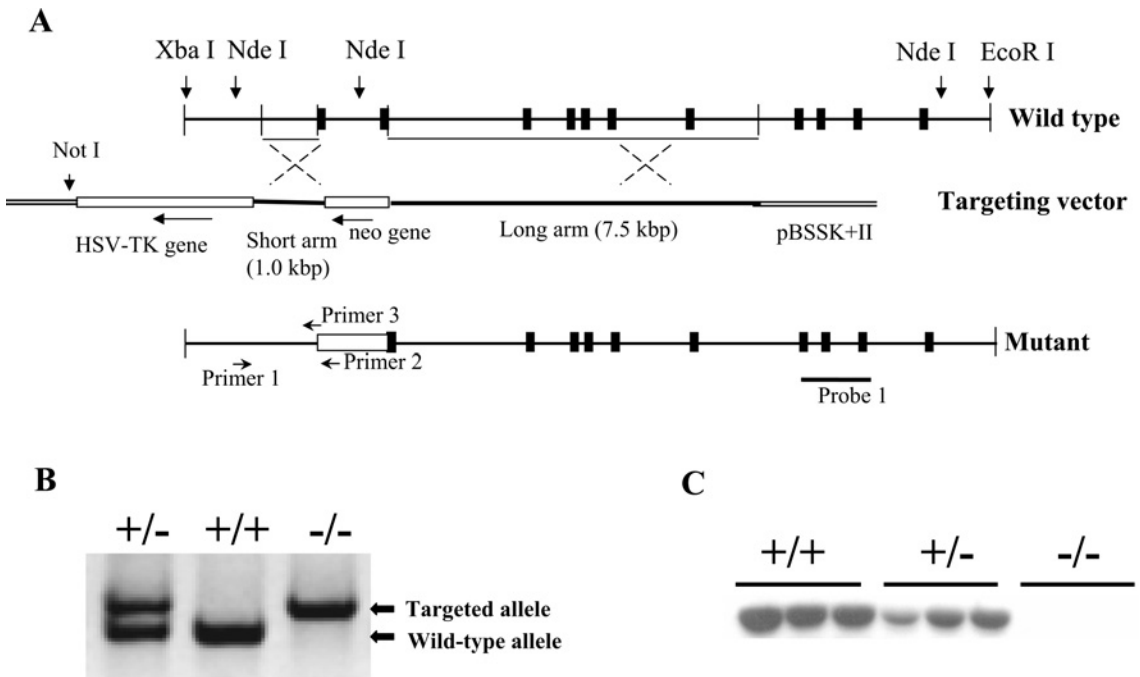

Figure 1 Targeting strategy for disruption of the mouse PDHK4 gene

(**A**) A 1.0 kb DNA fragment of the mouse PDHK4 gene promoter was used as the short arm; a 7.5 kb DNA fragment extending from exon 2 to intron 7 as the long arm. The targeting vector was linearized at a NotI site for electroporation into embryonic stem cells. After homologous recombination, the endogenous PDHK4 gene was disrupted by the neomycin gene of the targeting vector (labelled 'Mutant' in the diagram). Probe 1 was used to screen targeted embryonic stem cells by Southern-blot analysis. (**B**) Mice were genotyped for disruption of the PDHK4 gene by PCR with primers P1, P2 and P3. Template DNA was purified from toe clips. (**C**) Western-blot analysis was carried out with muscle extracts obtained from 24 h fasted mice [wild-type (+/+), heterozygous (+/–) and homozygous (–/–)]. Protein (50 μg) was separated by SDS/PAGE (12.5 % gel) and then transferred on to a nitrocellulose membrane. Western-blot analysis was conducted with a rabbit antiserum for PDHK4.

from Mercodia (Winston Salem, NC, U.S.A.). All radiochemicals were purchased from Amersham Biosciences (Piscataway, NJ, U.S.A.).

Targeting of the mouse PDHK4 gene

To construct the targeting vector (Figure 1A), a PCR-amplified 0.9 kb DNA fragment located upstream of exon 1 of the PDHK4 gene was inserted into the SalI site of a pBluescript II/neo-TK vector [13] that contains the phosphoglycerate kinase promoter driving the neomycin-resistant gene (PGK-neo) and the herpes virus thymidine kinase gene. A 7.5 kb DNA fragment containing a part of exon 2 and extending through intron 7 of the gene was inserted at the XhoI site. The vector was linearized at NotI and used for electroporation into R1 embryonic stem cells (129SvJ origin) by the Transgenic Facility of the Duke Comprehensive Cancer Center (Durham, NC, U.S.A.). The disrupted allele was confirmed by Southern-blot analysis with a radiolabelled PCR-amplified DNA fragment from exon 8 to exon 10. Genotyping of mice was performed by PCR (40 cycles at 95 °C for 30 s, 57 °C for 30 s and 65 °C for 4 min). Primer 1 (5′-gactttcataacacccagtctcc-3′) and primer 2 (5′-cgcttttctggattcatcgactgtggc-3′) were used to amplify a 1.4 kb fragment from the targeted allele. Primers 1 and 3 (5′-ggtgctcgagcctgggtgaagg-3′) produced a 1.2 kb fragment from the wild-type allele. To stabilize the genetic background, heterozygous mice were backcrossed with C57BL/6J wild-type mice for six generations.

Design of experiments

Mice were housed in an AALAC (Association for Assessment and Accreditation of Laboratory Animal Care)-approved pathogen-free barrier facility (12 h light/dark cycles with temperature maintained at 23 $\pm$ 2 °C) and fed *ad libitum* a standard rodent chow diet (Harlan; no. 7071). Studies were conducted with the approval of Institutional Animal Care and Use Committee of Indiana University School of Medicine. Tail blood was collected for glucose determination. To compare blood and tissue metabolite levels of fed and starved mice, groups of male mice [wild-type and PDHK4$^{-/-}$ mice (homozygous PDHK4 knockout mice)] were either fed a chow diet or deprived of food for up to 48 h. Mice were killed by injection of Nembutal. Liver, heart, kidney, diaphragm and gastrocnemius muscle were freeze-clamped at the temperature of liquid nitrogen.

Measurement of metabolite concentrations in blood and liver

Glucose and lactate were measured in tail blood by an Accu-Chek advantage glucometer (Roche, Indianapolis, IN, U.S.A.) and a Lactate Prolactate test meter (Arkray, Shiga, Japan) respectively. Acetoacetate and β-hydroxybutyrate [14], pyruvate [15] and BCAAs (branched chain amino acids) [16] were assayed by enzymatic methods with deproteinized serum. Serum triacylglycerol and non-esterified fatty acids were determined enzymatically with the INFINITY triacylglycerol reagent (Sigma; 343-25P) and the NEFA (non-esterified fatty acid) Half Micro Assay (Roche) respectively. Glycogen was measured by a colorimetric assay [17]. Metabolites were measured in $HClO_4$ extracts of liver by enzymatic methods [18].

Glucose and insulin tolerance tests

Overnight-fasted mice were injected intraperitoneally with either a sterile solution of glucose (2 g/kg of body mass) or human

recombinant insulin (0.75 unit/kg of body mass; Eli Lilly, Indianapolis, IN, U.S.A.). Tail blood was collected as a function of time for glucose determination.

Measurement of PDC activity

For the determination of actual PDC activity, pulverized tissue was homogenized in 5 vol. (w/v) of extraction buffer [19] containing 30 mM Hepes/KOH (pH 7.5), 0.5 mM thiamin pyrophosphate, 3 % (v/v) Triton X-100, 5 mM EDTA, 2 % (v/v) bovine serum, 5 mM DTT (dithiothreitol), 10 μM Tos-Phe-CH_2Cl (tosylphenylalanylchloromethane; 'TPCK'), 10 μg/ml trypsin inhibitor, 1 μM leupeptin, 2 mM DCA (dichloroacetate) and 50 mM KF. The supernatant obtained by centrifugation at 10000 ***g*** for 10 min at 4 °C was made 9 % (w/v) in PEG [poly(ethylene glycol)] 6000 to precipitate PDC. Pellets produced by centrifugation at 12000 ***g*** for 10 min were suspended in a suspension buffer containing 30 mM Hepes/KOH (pH 7.5), 1 % Triton X-100, 0.2 mM EDTA, 2 % bovine serum, 1 μM leupeptin and 5 mM DTT. For the determination of total PDC activity, tissues were homogenized in 5 vol. of the extraction buffer described above but without DCA and KF. The supernatant (200 μl) obtained by centrifugation of the homogenate at 10000 ***g*** for 10 min at 4 °C was added to 100 μl of an activation buffer (suspension buffer containing 25 mM $MgCl_2$, 1.5 mM $CaCl_2$ and 1 μg of recombinant PDP1 protein). After incubation for 20 min at 30 °C to dephosphorylate and activate PDC, samples were treated with PEG 6000 (final 9 %) to precipitate PDC. An aliquot of an extract of the pellet was used to assay total PDC activity. PDC activity was measured spectrophotometrically in a 96-well plate reader (Spectra Max 190; Molecular Devices, Sunnyvale, CA, U.S.A.) with a coupled assay based on the reaction catalysed by arylamine acetyltransferase [19,20]. One unit of PDC activity corresponds to the acetylation of 1 μmol of *p*-(*p*-aminophenylazo)benzenesulphonate per min at 30 °C.

Western-blot analysis

Tissue powders (50 mg) prepared under liquid nitrogen were homogenized with 0.5 ml of extraction buffer (10 mM Tris/HCl, pH 7.5, 1 mM EDTA, 0.5 % Triton X-100, 2 % bovine serum, 1 μM leupeptin, 10 μM Tos-Phe-CH_2Cl and 10 μg/ml trypsin inhibitor). Protein concentrations of tissue extracts were determined with a Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA, U.S.A.) using BSA as the standard. Proteins (50 μg) were separated by SDS/PAGE (12.5 % gel) [21] and transferred on to a nitrocellulose membrane (Schleicher and Schuell, Keene, NH, U.S.A.) by the semi-dry electroblotting method. Western-blot analysis was carried out as described previously [22] using an enhanced chemiluminescence immunodetection system (Roche Diagnostics). Immunoblotting was performed with polyclonal rabbit antisera against mouse PDHK4 peptide (HQENRPSLTPVEAT) and mouse PDHK2 peptide (EIQEVNATNANQPIH) custom-produced by Sigma-Genosys (The Woodlands, TX, U.S.A.).

Glucose utilization and fatty acid oxidation by isolated diaphragms

Diaphragms were removed from 48 h starved mice, rinsed in Krebs–Henseleit bicarbonate buffer [23], blotted, weighed and placed in 10 ml Erlenmeyer flasks containing 1.5 ml of Krebs–Henseleit bicarbonate buffer (pH 7.4), 5 mM glucose and 0.2 % (w/v) BSA. The flasks were flushed with 95 % O_2/5 % CO_2, sealed with rubber stoppers, placed in a shaking (60 cycles/min) water bath at 37 °C and pre-incubated for 30 min. Diaphragms were removed from the flasks, blotted and transferred on to new flasks containing 1.5 ml of Krebs–Henseleit bicarbonate buffer supplemented with 5 mM glucose containing 20 μCi/mmol [U-^{14}C]glucose and 80 μCi/mmol of [5-^{3}H]glucose and 1 m-unit/ml insulin (Novo-Nordisk, Princeton, NJ, U.S.A.) as described by Clark et al. [24]. Flasks were flushed with 95 % O_2/5 % CO_2, sealed with rubber serum caps fitted with hanging centre wells (Kontes, Vineland, NJ, U.S.A.) and incubated for 1 h with shaking at 37 °C. Reactions were terminated by the injection of 0.25 ml of phenethylamine/methanol (1:1, v/v) into the centre wells and 0.1 ml of 60 % (w/v) $HClO_4$ into the contents of the flask. The rate of glucose oxidation was determined from the production of $^{14}CO_2$; the rate of glycolysis was determined from the difference between the rate of ^3H_2O formed [25] and the rate of substrate recycling; the rate of substrate recycling was determined from the difference between the rates of glycogen synthesis from [U-^{14}C]glucose and [5-^{3}H]glucose [26]. Glycogen was purified [17] to determine the amount of glycogen synthesized from [U-^{14}C]glucose and [5-^{3}H]glucose incorporation.

To measure fatty acid oxidation, diaphragms obtained from 48 h starved mice were rinsed, weighed and pre-incubated for 30 min in Erlenmeyer flasks as described above. Diaphragms were removed from the flasks, blotted and transferred to new flasks containing 1.5 ml of Krebs–Henseleit bicarbonate buffer (pH 7.4), 5 mM glucose, 1 m-unit/ml insulin, 0.15 mM palmitate containing 500 μCi/mmol [1-^{14}C]palmitate and 0.2 % BSA. $^{14}CO_2$ was collected after 1 h incubation as described above. Acid-soluble radioactive products formed during the incubation were measured in aliquots (100 μl) of the $HClO_4$-treated medium.

Statistical analysis

The statistical significance of differences between groups was determined with Student's *t* test. All values are presented as means $\pm$ S.E.M. for the indicated number of independent samples.

RESULTS

Generation of PDHK4$^{-/-}$ mice

A null mutation of the PDHK4 gene was generated by exchange of exon 1 and a part of exon 2 with a neomycin gene through homologous recombination (Figure 1A). Genotyping of progeny mice was determined by PCR with DNA prepared from toe clips (Figure 1B). PDHK4 protein could not be detected by Western-blot analysis in skeletal-muscle extracts of 24 h fasted PDHK4$^{-/-}$ mice (Figure 1C). The amount of PDHK4 protein expressed in skeletal muscle of heterozygotic (PDHK4$^{+/-}$) mice was approximately half of that of wild-type mice.

Characteristics of PDHK4$^{-/-}$ mice

The genotype distribution (56:87:45) of PDHK4$^{+/+}$/PDHK4$^{+/-}$/PDHK4$^{-/-}$ progeny of heterozygous mating was close to the 1:2:1 ratio expected for Mendelian inheritance. Body masses at weaning (results not shown) and at 9 weeks were not different between wild-type and PDHK4$^{-/-}$ mice (24.0 ± 0.3 and 23.6 ± 0.2 g respectively; means $\pm$ S.E.M.; $n = 20$ in each group). Food consumption measured over 6 days was not different for 8–9-week-old mice (3.6 ± 0.1 and 3.4 ± 0.2 g $\cdot$ day^{-1} $\cdot$ mouse^{-1} respectively; means $\pm$ S.E.M.; $n = 10$ in each group). Liver, heart, kidney, epididymal fat pad and brain weights also were not significantly different (results not shown).

Effects of PDHK4 deficiency on blood analyte levels in the fed state

No difference was found in blood glucose levels between fed PDHK4$^{-/-}$ and wild-type mice. For example, blood glucose levels in wild-type mice were 140 ± 8 mg/dl versus 133 ± 10 mg/dl in

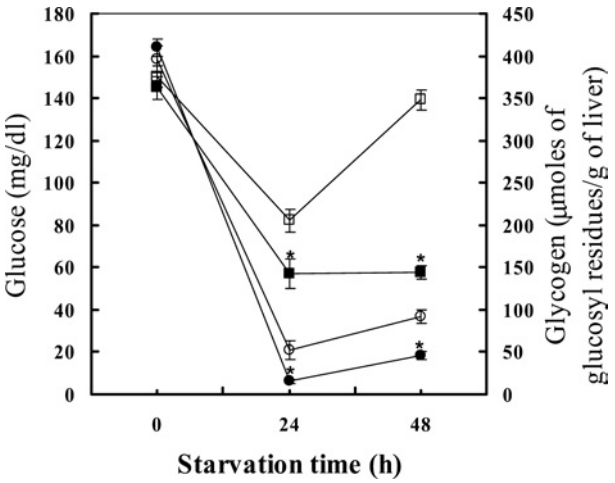

Figure 2 Effect of starvation on blood glucose and hepatic glycogen in wild-type and PDHK4$^{-/-}$ mice

Blood glucose for wild-type (□) and PDHK4$^{-/-}$ mice (■), with $n = 14$ and 12 in the groups respectively. Liver glycogen for wild-type (○) and PDHK4$^{-/-}$ (●) mice, with $n = 6$ for each group. All data points are means ± S.E.M. *Significantly different between wild-type and PDHK4$^{-/-}$ mice ($P < 0.01$).

PDHK4$^{-/-}$ mice (means ± S.E.M.; $n = 20$ in each group). Levels of blood lactate, non-esterified fatty acids, triacylglycerol, β-hydroxybutyrate, acetoacetate and insulin were likewise not significantly different between PDHK4$^{-/-}$ and wild-type in the fed state (results not shown).

Effects of PDHK4 deficiency on blood glucose levels after overnight fasting

Blood glucose levels were invariably lower in overnight-fasted PDHK4$^{-/-}$ mice (45 ± 1 mg/dl) than in overnight-fasted wild-type mice (64 ± 3 mg/dl; means ± S.E.M.; $n = 12$ in each group; $P < 0.0001$).

Effect of PDHK4 deficiency on blood analyte levels in the starved state

Blood glucose levels of PDHK4$^{-/-}$ mice were 30 % lower than that of wild-type mice after starvation for 24 h (Figure 2). A rebound back to a higher level of blood glucose occurred between 24 and 48 h of starvation in wild-type mice but not in PDHK4$^{-/-}$ mice (Figure 2). Blood glucose levels of PDHK4$^{-/-}$ mice were 58 % lower than that of wild-type mice after starvation for 48 h (Table 1).

Serum concentrations of lactate, pyruvate and alanine in PDHK4$^{-/-}$ mice (Table 1) were also lower in PDHK4$^{-/-}$ mice, consistent with either a higher rate of oxidation or a reduced rate of production of these compounds in PDHK4$^{-/-}$ mice. In contrast, higher serum concentrations of non-esterified fatty acids, acetoacetate, β-hydroxybutyrate, BCAAs and triacylglycerol were induced by starvation in PDHK4$^{-/-}$ mice relative to wild-type mice (Table 1). A small increase in serum concentration of insulin was also observed (Table 1).

Glycogen levels in the livers of PDHK4$^{-/-}$ and wild-type mice were high and almost identical in the fed state (Figure 2). However, more glycogen was lost from the livers of PDHK4$^{-/-}$ mice during 24 h of starvation, and analogous to what was observed for blood glucose, less rebound in the amount of liver glycogen occurred between 24 and 48 h of starvation in mice lacking PDHK4

Table 1 Blood metabolic parameters of wild-type (PDHK4$^{+/+}$) and PDHK4$^{-/-}$ mice after 48 h starvation

Data are means ± S.E.M., with $n = 20$ for each group for glucose; $n = 5$ for each group for other measurements. All of metabolites except glucose and lactate were measured with enzymatic methods in serum as described in the Experimental section. Glucose and lactate were measured with a glucometer and a lactate test meter in blood.

Measurement	Source of blood . . .	PDHK4$^{+/+}$ mice	PDHK4$^{-/-}$ mice
Glucose (mg/dl)		139 ± 5	58 ± 3*
Lactate (mmol/l)		1.93 ± 0.15	1.10 ± 0.10*
Pyruvate (mmol/l)		0.071 ± 0.011	0.035 ± 0.008*
Alanine (mmol/l)		0.26 ± 0.01	0.18 ± 0.01*
Non-esterified fatty acid (mmol/l)		1.44 ± 0.13	2.20 ± 0.07*
β-Hydroxybutyrate (mmol/l)		1.90 ± 0.60	6.38 ± 1.81*
Acetoacetate (mmol/l)		0.14 ± 0.04	1.28 ± 0.11*
BCAAs (mmol/l)		0.36 ± 0.02	0.58 ± 0.06*
Triacylglycerol (mg/dl)		38 ± 6	61 ± 9*
[β-Hydroxybutyrate]/[acetoacetate]		13.6 ± 3.5	5.0 ± 1.3*
Insulin (ng/ml)		0.11 ± 0.01	0.17 ± 0.02*

* Significantly different from wild-type (PDHK4$^{+/+}$) mice ($P < 0.01$).

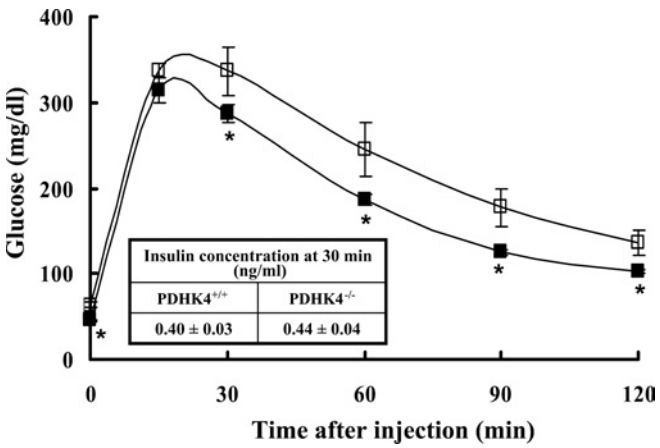

Figure 3 Glucose tolerance test in wild-type (□) and PDHK4$^{-/-}$ mice (■)

Glucose (2 g/kg of body mass) was administrated to overnight-fasted mice by intraperitoneal injection. All data points are means ± S.E.M. with $n = 12$ in each group. Insulin values, measured 30 min after glucose injection, are means ± S.E.M. with $n = 6$ in each group. *Significantly different between wild-type (PDHK4$^{+/+}$) and PDHK4$^{-/-}$ mice ($P < 0.01$).

(Figure 2). Skeletal-muscle glycogen levels of PDHK4$^{-/-}$ mice and wild-type mice were also the same in the fed state (20.3 ± 1.6 versus 20.6 ± 1.5 μmol of glucose/g wet weight; means ± S.E.M.; $n = 6$ in each group). Starvation for 48 h reduced skeletal-muscle glycogen levels to the same extent (40 %) in both groups of mice (results not shown).

Effect of PDHK4 deficiency on glucose tolerance test and blood insulin levels

PDHK4$^{-/-}$ mice were slightly but significantly more glucose-tolerant than PDHK4$^{+/+}$ mice (Figure 3). Serum insulin concentrations measured during the glucose tolerance test did not differ in the two groups of mice (Figure 3), suggesting that PDHK4$^{-/-}$ mice may be slightly more sensitive to insulin than PDHK4$^{+/+}$ mice. No difference in insulin sensitivity between the two groups of mice was detected, however, by intraperitoneal insulin tolerance test (results not shown).

Table 2 Levels of liver metabolites in wild-type (PDHK4$^{+/+}$) and PDHK4$^{-/-}$ mice after 48 h starvation

Data are means ± S.E.M., with $n = 6$ in each group.

Metabolite or ratio	Source of liver . . .	Metabolite concentration (μmol/g wet weight) PDHK4$^{+/+}$ mice	PDHK4$^{-/-}$ mice
Glucose		6.24 ± 0.50	3.60 ± 0.28*
Glucose 6-phosphate		0.19 ± 0.03	0.05 ± 0.01*
Fructose 1,6-bisphosphate		0.045 ± 0.001	0.036 ± 0.003*
Dihydroxyacetone phosphate		0.031 ± 0.002	0.034 ± 0.002
Glyceraldehyde 3-phosphate		0.045 ± 0.003	0.045 ± 0.006
Phosphoenolpyruvate		0.14 ± 0.01	0.13 ± 0.01
Pyruvate		0.059 ± 0.006	0.040 ± 0.007*
Lactate		0.79 ± 0.06	0.68 ± 0.04*
Malate		0.27 ± 0.03	0.26 ± 0.03
Citrate		0.19 ± 0.03	0.10 ± 0.02*
Alanine		0.32 ± 0.04	0.26 ± 0.06
Glutamate		2.29 ± 0.23	1.93 ± 0.25
Acetyl-CoA		0.090 ± 0.004	0.071 ± 0.004*
β-Hydroxybutyrate		1.71 ± 0.31	2.85 ± 0.20*
Acetoacetate		0.26 ± 0.04	0.69 ± 0.13*
[β-Hydroxybutyrate]/[acetoacetate] . . .		6.6 ± 1.0	4.1 ± 0.9*
[Lactate]/[pyruvate] . . .		13 ± 8	17 ± 6

* Significantly different from wild-type (PDHK4$^{+/+}$) mice ($P < 0.05$).

Effect of PDHK4 deficiency on metabolic intermediates in the liver in the starved state

The livers of starved PDHK4$^{-/-}$ mice were found to contain 42 % less free glucose than livers of starved wild-type mice (Table 2). Concentrations of intermediates of the gluconeogenesis pathway were likewise lower (glucose 6-phosphate, fructose 1,6-bisphosphate, pyruvate, lactate and citrate) in the livers of PDHK4$^{-/-}$ mice or not different (dihydroxyacetone phosphate, glyceraldehyde 3-phosphate and phosphoenolpyruvate) compared with that of wild-type mice (Table 2). No significant differences were found in concentrations of malate, glutamate and alanine. In agreement with the difference found in the blood, ketone bodies were elevated and the ratio of [β-hydroxybutyrate] to [acetoacetate] was more oxidized in the liver of PDHK4$^{-/-}$ mice. Acetyl-CoA was lower in the liver of PDHK4$^{-/-}$ mice. Lactate and pyruvate were both lower in concentration without a significant change in their ratio in the livers of PDHK4$^{-/-}$ mice.

Effect of PDHK4 deficiency on rates of glucose oxidation and glycolysis by diaphragms from starved mice

Glucose oxidation rate, measured by $^{14}CO_2$ production from [U-^{14}C]glucose, was 36 % greater in diaphragms from PDHK4$^{-/-}$ mice relative to diaphragms from wild-type mice (Table 3). However, rates of lactate release and net glycolysis, measured by ^3H_2O production from [5-^{3}H]glucose, were 37 and 35 % less respectively in diaphragms from PDHK4$^{-/-}$ mice. This was accompanied by a trend for a greater rate of glycogen synthesis in diaphragms from PDHK4$^{-/-}$ mice that did not reach statistical significance (Table 3).

Effect of PDHK4 deficiency on rates of fatty acid oxidation by the diaphragms from starved mice

Rates of fatty acid oxidation, measured either by $^{14}CO_2$ production or by acid-soluble product formation from [1-^{14}C]palmitate, or by the sum of the two, were approximately 50 % lower in diaphragms obtained from PDHK4$^{-/-}$ mice than in diaphragms from wild-type mice (Table 4).

Table 3 Effect of PDHK4 deficiency on glucose metabolism by isolated diaphragm

Diaphragms were obtained from mice starved for 48 h. Data are means ± S.E.M., with $n = 5$ in each group.

Process	Source of diaphragm . . .	Rate [μmol.h^{-1} · (g wet weight)$^{-1}$] PDHK4$^{+/+}$ mice	PDHK4$^{-/-}$ mice
Glycolysis		20.7 ± 2.8	13.4 ± 0.5*
Lactate release		28.7 ± 3.4	17.9 ± 0.3*
Pyruvate release		2.08 ± 0.40	0.49 ± 0.09*
Glucose oxidation		1.13 ± 0.10	1.54 ± 0.11*
Net glycolysis		20.7 ± 2.8	13.4 ± 0.5*
Glycogen synthesis		2.40 ± 0.17	3.28 ± 0.58

* Significantly different from wild-type (PDHK4$^{+/+}$) mice ($P < 0.05$).

Table 4 Effect of PDHK4 deficiency on fatty acid oxidation by isolated diaphragm

Diaphragms were obtained from mice starved for 48 h. Fatty acid oxidation rate refers to the sum of the rate of production of CO_2 and acid-soluble radioactive products. Data are means ± S.E.M., with $n = 3$ in each group.

Product	Source of diaphragm . . .	Production rate [μmol of palmitate · h^{-1} · (g wet weight)$^{-1}$] PDHK4$^{+/+}$ mice	PDHK4$^{-/-}$ mice
CO_2		1.22 ± 0.10	0.61 ± 0.04*
Acid-soluble products		0.47 ± 0.01	0.19 ± 0.04*
Fatty acid oxidation		1.69 ± 0.09	0.80 ± 0.06*

* Significantly different from PDHK4$^{+/+}$ mice ($P < 0.002$).

Effects of PDHK4 deficiency on PDC activity

Since PDHK4 expression is relatively low in tissues of fed rats [11], lack of PDHK4 was expected to have little or no effect on actual PDC activities (measured as extracted from the tissue without activation by dephosphorylation) and PDC activity states (percentage of total PDC that is active) in tissues of mice in the fed state. Indeed, this was found to be the case for kidney and the gastrocnemius muscle (Table 5). However, a significantly greater actual PDC activity and PDC activity state were found in the diaphragms of fed PDHK4$^{-/-}$ mice. Trends towards greater actual PDC activities and PDC activity states that did not make statistical significance were also found for the heart and liver. These findings suggest that PDHK4 contributes to the PDC activity state in the diaphragm and possibly the heart and liver in the fed state.

Since there is little data on PDC activity in the literature for the mouse, it is interesting to note that the highest total PDC activities were observed in the heart, whereas the lowest total activities were observed in the liver. The lowest activity states were found in the diaphragm and heart from starved mice, whereas the highest activity states were found in the diaphragm and liver of fed mice.

As expected from many studies with rats [7,9,11,22], starvation markedly decreased actual PDC activities in wild-type mice without significantly affecting total PDC activity, thereby decreasing the activity states of PDC to values typical of those induced in tissues of starved rats (Table 5). Since it is well established in studies with rats that starvation decreases the activity state of PDC and increases PDHK4 expression [11], we expected that the activity states of PDC would be maintained at

Table 5 PDC activity in tissues of wild-type (PDHK4$^{+/+}$) and PDHK4$^{-/-}$ mice

Data are means ± S.E.M., with $n = 4$ for each group. Fed mice were killed at 07:00 h. Starved mice were killed at 09:00 h. Food had been removed from cages of starved mice for a total of 48 h.

		Actual PDC (units/g wet weight)		Total PDC (units/g wet weight)		PDC activity state (%)	
Tissue	Nutritional state	PDHK4$^{+/+}$	PDHK4$^{-/-}$	PDHK4$^{+/+}$	PDHK4$^{-/-}$	PDHK4$^{+/+}$	PDHK4$^{-/-}$
Heart	Fed	2.46 ± 0.23	3.68 ± 0.67	13.30 ± 0.28	13.16 ± 0.17	18.5 ± 1.6	28.5 ± 5.3
	Starved	0.25 ± 0.03*	0.40 ± 0.10†‡	14.49 ± 0.83	12.75 ± 0.29	1.7 ± 0.1*	3.1 ± 0.8†‡
Liver	Fed	1.09 ± 0.18	1.39 ± 0.06	2.45 ± 0.06	2.50 ± 0.10	44.2 ± 6.7	55.8 ± 1.8
	Starved	0.16 ± 0.02*	0.19 ± 0.01†	2.22 ± 0.19	2.35 ± 0.07	7.1 ± 0.5*	8.1 ± 0.5†
Skeletal muscle	Fed	0.53 ± 0.04	0.54 ± 0.03	3.65 ± 0.19	3.96 ± 0.19	14.4 ± 0.7	13.8 ± 1.5
	Starved	0.25 ± 0.02*	0.38 ± 0.02†‡	3.39 ± 0.18	3.43 ± 0.15	7.5 ± 0.7*	11.0 ± 0.7‡
Kidney	Fed	1.25 ± 0.13	1.46 ± 0.06	6.20 ± 0.21	6.67 ± 0.08	20.1 ± 1.7	21.9 ± 0.7
	Starved	0.25 ± 0.01*	0.56 ± 0.03†‡	6.12 ± 0.17	6.28 ± 0.23	4.2 ± 0.3*	9.0 ± 0.4†‡
Diaphragm	Fed	2.46 ± 0.16	3.35 ± 0.13*	5.67 ± 0.01	5.76 ± 0.10	43.3 ± 3.4	58.1 ± 1.3*
	Starved	0.09 ± 0.01*	0.60 ± 0.13†‡	5.56 ± 0.09	5.64 ± 0.06	1.7 ± 0.2*	10.7 ± 2.4†‡

* Significantly different from fed wild-type mice ($P < 0.05$).
† Significantly different from fed PDHK4$^{-/-}$ mice ($P < 0.05$).
‡ Significantly different from starved wild-type mice ($P < 0.05$).

higher levels during starvation in tissues of mice lacking PDHK4 than in the tissues of wild-type mice. To our surprise, the activity state of PDC was still markedly reduced by starvation in all tissues of PDHK4$^{-/-}$ mice examined (Table 5). Tissue-specific expression of PDHK1 may contribute to the very low activity state induced in the heart by starvation. Nevertheless, a significantly higher PDC activity state was observed in the heart, muscle, kidney and diaphragm of PDHK4$^{-/-}$ mice (Table 5), consistent with a role for PDHK4 in inactivation of PDC in these tissues. The differential in activity states between wild-type and PDHK4$^{-/-}$ mice was the greatest for diaphragm, followed by kidney, skeletal muscle and then heart. No difference in PDC activity states was observed in the livers of wild-type and PDHK4$^{-/-}$ mice. It is noteworthy that starvation did not induce a significant decrease in PDC activity state in gastrocnemius muscle of PDHK4$^{-/-}$ mice, whereas it did in wild-type mice, suggesting that much of the decrease in PDC activity induced in skeletal muscle by starvation is caused by increased expression of PDHK4.

Effect of starvation on PDHK2 and PDHK4 expressions

The amount of PDHK4 protein in gastrocnemius muscle of wild-type mice was very low in the fed state, but markedly increased by starvation (Figures 4A and 4B). The amounts of PDHK2 protein were similar in gastrocnemius muscle in the two groups of mice in the fed state (Figure 4C). Starvation had no effect in wild-type mice but, surprisingly, caused a decrease in PDHK2 protein in PDHK4$^{-/-}$ mice. PDHK4 protein in heart of wild-type mice was very low in the fed state, but markedly increased by starvation (Figures 5A and 5B). PDHK2 protein in heart was greater in PDHK4$^{-/-}$ mice, perhaps partially compensating for the absence of PDHK4 (Figure 5C). Starvation induced an increase in PDHK2 protein of wild-type mice, but had no effect on the already elevated amount of PDHK2 protein in the PDHK4$^{-/-}$ mice. The response of the kidney was similar to that of the heart (Figure 6). PDHK4 protein was markedly increased in response to starvation in the wild-type mice (Figures 6A and 6B). PDHK2 protein was greater in the heart of fed PDHK4$^{-/-}$ mice, but in contrast with the heart, starvation caused an additional increment in PDHK2 protein in the PDHK4$^{-/-}$ mice as well as the PDHK4$^{+/+}$ mice (Figure 6C). The response of the liver to starvation was qualitatively the same as the other tissues (Figure 7). Starvation also increased PDHK4 protein in the liver of wild-type mice (Figures 7A and 7B). PDHK2

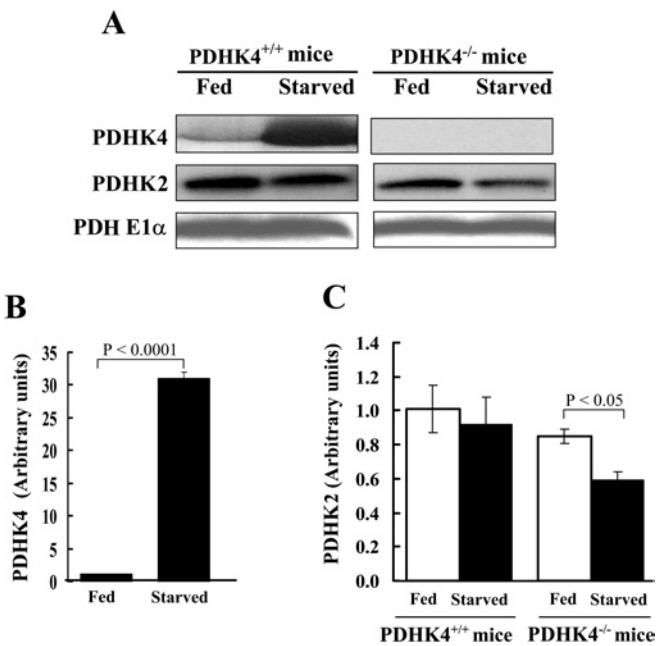

Figure 4 Effect of starvation on PDHK protein expression in skeletal muscle of wild-type and PDHK4$^{-/-}$ mice

(**A**) Representative Western blots of PDHK4 and PDHK2 protein in gastrocnemius muscles of fed and 48 h starved wild-type (PDHK4$^{+/+}$) and PDHK4$^{-/-}$ mice. (**B**) Histograms constructed from data obtained by Western-blot analysis, showing relative amounts of PDHK4 protein expressed in gastrocnemius muscle of fed and 48 h starved wild-type (PDHK4$^{+/+}$) mice. $n = 4$ in each group. (**C**) Histograms constructed from data obtained by Western-blot analysis, showing relative amounts of PDHK2 protein expressed in gastrocnemius muscle of fed and starved wild-type (PDHK4$^{+/+}$) and PDHK4$^{-/-}$ mice. $n = 4$ in each group.

protein was similar in amount in the livers of fed PDHK4$^{+/+}$ mice and PDHK4$^{-/-}$ mice (Figure 7C). Starvation increased the amounts of PDHK2 protein in both groups of mice. The amount of PDHK4 protein in the diaphragm of fed wild-type mice was low but detectable by Western-blot analysis (Figure 8A). Starvation also markedly increased the amount of PDHK4 in the diaphragm of wild-type mice (Figure 8B). In contrast with the other tissues, similar amounts of PDHK2 protein were found in the diaphragms of both types of mice and starvation had no effect (Figure 8C).

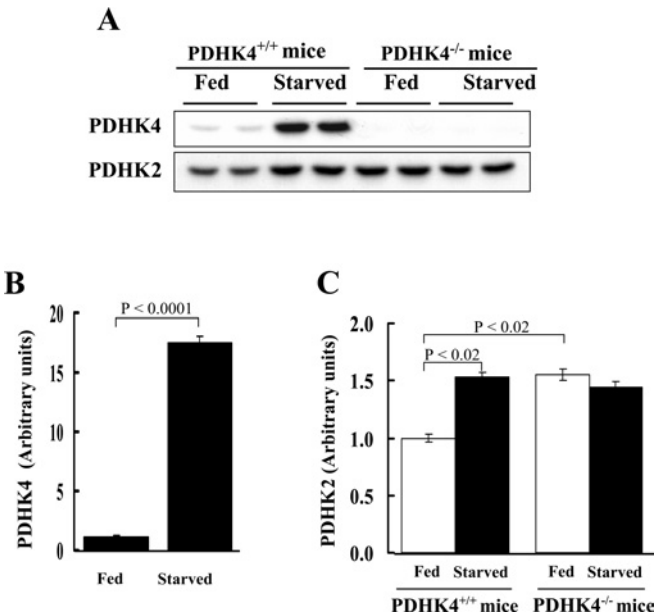

Figure 5 Effect of starvation on PDHK protein expression in heart of wild-type and PDHK4$^{-/-}$ mice

(**A**) Representative Western blots of PDHK4 and PDHK2 protein in heart of fed and 48 h starved wild-type (PDHK4$^{+/+}$) and PDHK4$^{-/-}$ mice. (**B**) Histograms constructed from data obtained by Western-blot analysis, showing relative amounts of PDHK4 protein expressed in heart of fed and 48 h starved wild-type (PDHK4$^{+/+}$) mice. $n = 4$ in each group. (**C**) Histograms constructed from data obtained by Western-blot analysis showing relative amounts of PDHK2 protein expressed in heart of fed and starved wild-type (PDHK4$^{+/+}$) and PDHK4$^{-/-}$ mice. $n = 4$ in each group.

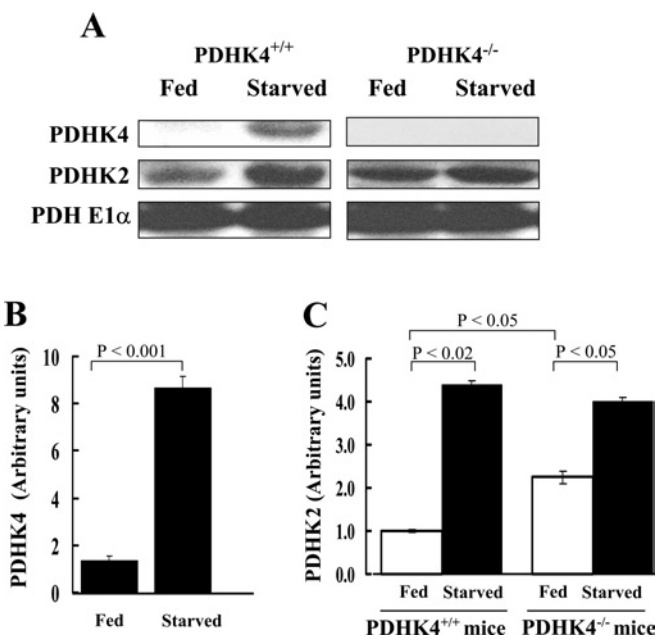

Figure 6 Effect of starvation on PDHK protein expression in kidney of wild-type mice and PDHK4$^{-/-}$ mice

(**A**) Representative Western blots of PDHK4 and PDHK2 protein in kidney of fed and 48 h starved wild-type (PDHK4$^{+/+}$) and PDHK4$^{-/-}$ mice. (**B**) Histograms constructed from data obtained by Western-blot analysis, showing relative amounts of PDHK4 protein expressed in kidney of fed and 48 h starved wild-type (PDHK4$^{+/+}$) mice. $n = 4$ in each group. (**C**) Histograms constructed from data obtained by Western-blot analysis, showing relative amounts of PDHK2 protein expressed in kidney of fed and starved wild-type (PDHK4$^{+/+}$) and PDHK4$^{-/-}$ mice. $n = 4$ in each group.

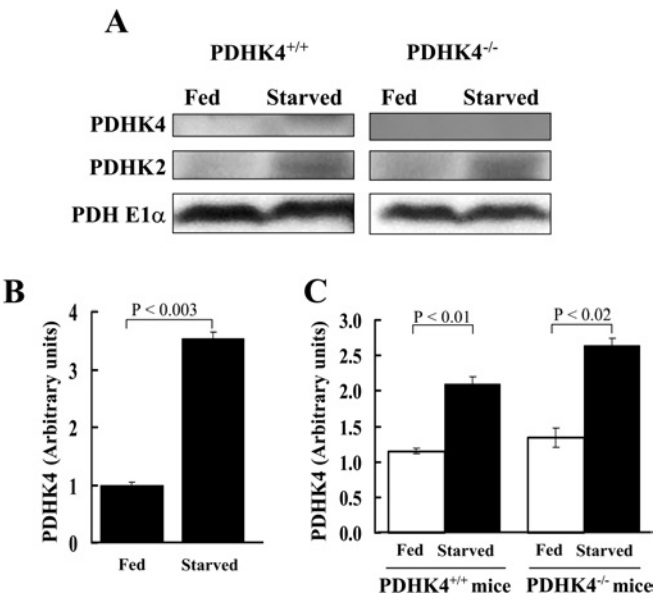

Figure 7 Effect of starvation on PDHK protein expression in liver of wild-type and PDHK4$^{-/-}$ mice

(**A**) Representative Western blots of PDHK4 and PDHK2 protein in liver of fed and 48 h starved wild-type (PDHK4$^{+/+}$) and PDHK4$^{-/-}$ mice. (**B**) Histograms constructed from data obtained by Western-blot analysis, showing relative amounts of PDHK4 protein expressed in liver of fed and 48 h starved wild-type (PDHK4$^{+/+}$) mice. $n = 4$ in each group. (**C**) Histograms constructed from data obtained by Western-blot analysis, showing relative amounts of PDHK2 protein expressed in liver of fed and starved wild-type (PDHK4$^{+/+}$) and PDHK4$^{-/-}$ mice. $n = 4$ in each group.

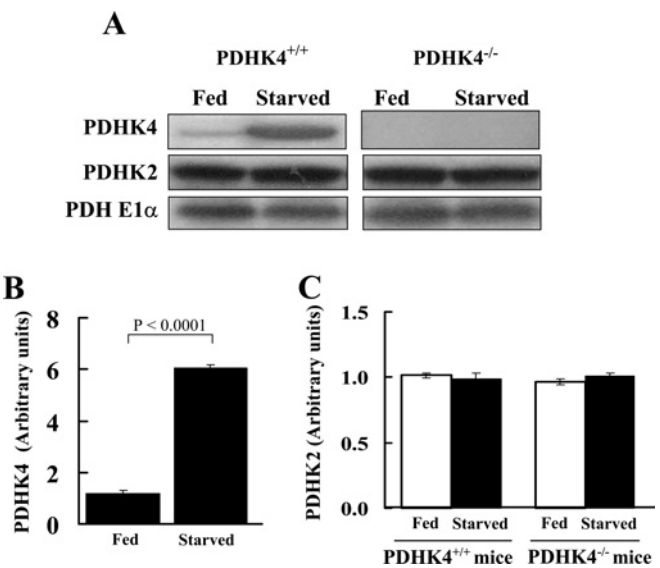

Figure 8 Effect of starvation on PDHK protein expression in diaphragm of wild-type and PDHK4$^{-/-}$ mice

(**A**) Representative Western blots of PDHK4 and PDHK2 protein in diaphragm of fed and 48 h starved wild-type (PDHK4$^{+/+}$) and PDHK4$^{-/-}$ mice. (**B**) Histograms constructed from data obtained by Western-blot analysis, showing relative amounts of PDHK4 protein expressed in diaphragm of fed and 48 h starved wild-type (PDHK4$^{+/+}$) mice. $n = 4$ in each group. (**C**) Histograms constructed from data obtained by Western-blot analysis, showing relative amounts of PDHK2 protein expressed in diaphragm of fed and starved wild-type (PDHK4$^{+/+}$) and PDHK4$^{-/-}$ mice. $n = 4$ in each group.

DISCUSSION

Starvation causes nearly complete inhibition of PDC by phosphorylation in most tissues [3]. PDC becomes highly phosphorylated in the starved state because acetyl-CoA and NADH produced by fatty acid oxidation stimulate PDHK activity [3] and also because a stable increase occurs in PDHK activity [27], which is primarily due to an increase in PDHK4 expression [28]. PDHK4$^{-/-}$ mice were produced to investigate the importance of PDHK4 in PDC regulation in the starved state.

PDHK4$^{-/-}$ mice are viable, fertile and grow normally. PDHK4 protein is absent and no induction occurs upon starvation. With the exception of the liver, the activity state of PDC under food-deprived conditions is higher in tissues as a result of PDHK4 deficiency. Therefore PDHK4 contributes to the regulation of PDC activity during starvation.

PDHK4 deficiency has no effect on blood glucose levels in the fed state. This is consistent with the low level of PDHK4 expression in tissues in the fed state and, therefore, less effect on the activity state of PDC. After overnight fasting, blood glucose levels are significantly lower in PDHK4$^{-/-}$ mice than in wild-type mice, suggesting that the increase in PDHK4 protein is important for maintaining euglycaemia during fasting. A more pronounced difference in blood glucose levels occurs after 24 h of starvation, and an even greater difference occurs after 48 h of starvation. The magnitude of the difference at 48 h results in large part from a rebound to a higher glucose level in the wild-type mice. An increase in blood glucose beyond 24 h of starvation [29] may reflect adaptations that increase the capacity for glucose synthesis and decrease the capacity for glucose utilization. This does not occur in PDHK4$^{-/-}$ mice, presumably because up-regulation of PDHK4 is required. Overall, these findings are consistent with the hypothesis that inhibition of PDC caused by up-regulation of PDHK4 is essential for glucose homoeostasis during starvation.

We had anticipated that larger differences would be found in PDC activity states in tissues of wild-type and PDHK4$^{-/-}$ mice. Differences in the expected direction were found in heart, gastrocnemius muscle, diaphragm and kidney of starved PDHK4$^{-/-}$ mice but not in liver. PDC was 11 % active in diaphragm of starved PDHK4$^{-/-}$ null mice, whereas it was 2 % active in the diaphragm of starved wild-type mice. Less difference was found for the kidney, gastrocnemius muscle and the heart. Mechanisms other than increased expression of PDHK4 must therefore contribute to decreased activity of PDC in these tissues during starvation. No evidence was found for up-regulation of PDHK2 to compensate for lack of PDHK4. Indeed, the amount of PDHK2 protein in gastrocnemius muscle of PDHK4$^{-/-}$ mice was decreased rather than increased in response to starvation. This exacerbates rather than compensates for the absence of PDHK4. The lack of any difference in PDC activity states in the liver between wild-type and PDHK4$^{-/-}$ mice suggests that PDHK4 is less important for regulation of PDC in this tissue than in other tissues during starvation. Although PDHK4 expression is increased in the liver during starvation, the magnitude was less than in other tissues, consistent with PDHK4 being less important for regulation of PDC activity in the liver.

Blood glucose levels were better maintained in PDHK4$^{-/-}$ mice during starvation than we had anticipated. Levels were lower than that of wild-type mice, but not low enough to be life-threatening. Greater use of ketone bodies because of the higher levels in PDHK4$^{-/-}$ mice may contribute to the maintenance of blood glucose levels and the survival of these mice during starvation.

Why are blood glucose levels lower in PDHK4$^{-/-}$ mice in the starved state?

PDHK4 deficiency induces effects similar to those induced by the PDHK inhibitor DCA [30]. PDHK4 deficiency and treatment with DCA [31–33] lower blood glucose levels in starved rats but not in fed rats. PDHK4 deficiency and treatment of starved rats with DCA [31,33] lower blood levels of gluconeogenic precursors. Studies with isolated hepatocytes [34] and intact dogs [35] have shown the importance of substrate supply for hepatic gluconeogenesis. Gluconeogenesis normally operates below maximal capacity at the concentrations of the precursors present in the blood [34]. Induction of a lower concentration of precursors as a consequence of higher PDC activity further limits gluconeogenesis by the liver. Consistent with this mechanism, PDHK4 deficiency and DCA [31] cause either no change or a decrease in steady-state concentrations of the intermediates of gluconeogenesis in the liver. Lack of a crossover point in the intermediates of gluconeogenesis suggests a limitation of substrate supply rather than a specific site of inhibition.

Why are gluconeogenic precursors reduced in the blood of PDHK4$^{-/-}$ mice?

DCA decreases the concentration of lactate, pyruvate and alanine in the blood by stimulation of pyruvate oxidation [31,36–38] and inhibition of glycolysis [39] in peripheral tissues. Decreased pyruvate availability in muscle inhibits transamination of BCAAs by limiting the availability of α-oxoglutarate [40], resulting in an elevation in BCAAs and a reduction in alanine in DCA-treated animals [41]. PDHK4 deficiency likewise resulted in lower than normal levels of alanine and higher than normal levels of BCAAs in the blood. Studies with isolated diaphragms are also consistent with a DCA-like mechanism for PDHK4$^{-/-}$ mice. Diaphragms from PDHK4$^{-/-}$ mice produce less lactate, oxidize more glucose, but utilize less glucose by the glycolytic pathway. These are the same effects produced by incubation of skeletal muscle with DCA [24]. As found for DCA, inhibition of glycolysis accounts for most of the decrease in lactate production by diaphragms from PDHK4$^{-/-}$ mice. Generation of ATP by oxidation of glucose means less has to be generated by anaerobic metabolism of glucose, but like inhibition of glycolysis by DCA [39], the exact mechanism by which greater ATP production by oxidative metabolism results in less ATP production by glycolysis remains uncertain.

Why are the levels of ketone bodies greater in the blood of starved PDHK4$^{-/-}$ mice?

Starvation induces much greater increases in non-esterified fatty acids and ketone bodies in the blood of PDHK4$^{-/-}$ mice than in wild-type mice. Whether high ketone bodies levels in starved PDHK4$^{-/-}$ mice are caused by greater hepatic ketogenesis or less ketone bodies utilization by peripheral tissues of PDHK4$^{-/-}$ mice is not known. Perhaps both are involved. Elevated non-esterified fatty acids should promote fatty acid oxidation and the reduced supply of three-carbon compounds should limit oxaloacetate availability in the liver and direct acetyl-CoA into ketone body formation. However, acetyl-CoA levels would be expected to be increased by a greater rate of fatty acid oxidation and reduced flux into the tricarboxylic acid cycle. The opposite was found: acetyl-CoA levels are lower in the liver of PDHK4$^{-/-}$ mice than in the liver of wild-type mice. Furthermore, the ratio of β-hydroxybutyrate to acetoacetate was found lower in the blood of PDHK4$^{-/-}$ mice than in the blood of wild-type mice. Since a greater rate of ketone body production by the liver would be

expected to increase rather than decrease the β-hydroxybutyrate/acetoacetate ratio [31], tissues other than the liver must be involved in increasing the ketone body concentration in starved PDHK4$^{-/-}$ mice. Therefore inhibition of fatty acid and ketone body oxidation as a consequence of greater PDC activity in peripheral tissues likely contributes to the higher blood levels of fatty acids and ketone bodies. Indeed, diaphragms from PDHK4$^{-/-}$ mice oxidize palmitate at a lower rate than diaphragms from wild-type mice. A difference in sensitivity of acetoacetate oxidation versus β-hydroxybutyrate oxidation may account for the lower β-hydroxybutyrate/acetoacetate ratio. Why greater PDC activity induces these effects has not been explained in a satisfactory way. Competition between pathways for CoA has been suggested [36] and is currently being investigated as a possible cause.

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REFERENCES

1 Linn, T. C., Pettit, F. H. and Reed, L. J. (1969) α-Keto acid dehydrogenase complex, X: regulation of the activity of the pyruvate dehydrogenase complex from beef kidney mitochondria by phosphorylation and dephosphorylation. Proc. Natl. Acad. Sci. U.S.A. **62**, 234–241

2 Yeaman, S. J. (1989) The 2-oxo acid dehydrogenase complexes: recent advances. Biochem. J. **257**, 625–632

3 Holness, M. J. and Sugden, M. C. (2003) Regulation of pyruvate dehydrogenase complex activity by reversible phosphorylation. Biochem. Soc. Trans. **31**, 1143–1151

4 Roche, T. E., Baker, J. C., Yan, X., Hiromasa, Y., Gong, X., Peng, T., Dong, J., Turkan, A. and Kasten, S. A. (2001) Distinct regulatory properties of pyruvate dehydrogenase kinase and phosphatase isoforms. Prog. Nucleic Acid Res. Mol. Biol. **70**, 33–75

5 Behal, R. H., Buxton, D. B., Robertson, J. G. and Olson, M. S. (1993) Regulation of the pyruvate dehydrogenase multienzyme complex. Annu. Rev. Nutr. **13**, 497–520

6 Chen, G., Wang, L., Liu, S., Chuang, C. and Roche, T. E. (1996) Activated function of the pyruvate dehydrogenase phosphatase through Ca^{2+}-facilitated binding to the inner lipoyl domain of the dihydrolipoyl acetyltransferase. J. Biol. Chem. **271**, 28064–28070

7 Denyer, G. S., Kerbey, A. L. and Randle, P. J. (1986) Kinase activator protein mediates longer-term effects of starvation on activity of pyruvate dehydrogenase kinase in rat liver mitochondria. Biochem. J. **239**, 347–354

8 Sugden, M. C., Fryer, L. G., Orfali, K. A., Priestman, D. A., Donald, E. and Holness, M. J. (1998) Studies of the long-term regulation of hepatic pyruvate dehydrogenase kinase. Biochem. J. **329**, 89–94

9 Wu, P., Sato, J., Zhao, Y., Jaskiewicz, J., Popov, K. M. and Harris, R. A. (1998) Starvation and diabetes increase the amount of pyruvate dehydrogenase kinase isoenzyme 4 in rat heart. Biochem. J. **329**, 197–201

10 Bowker-Kinley, M. M., Davis, W. I., Wu, P., Harris, R. A. and Popov, K. M. (1998) Evidence for existence of tissue-specific regulation of the mammalian pyruvate dehydrogenase complex. Biochem. J. **329**, 191–196

11 Wu, P., Blair, P. V., Sato, J., Jaskiewicz, J., Popov, K. M. and Harris, R. A. (2000) Starvation increases the amount of pyruvate dehydrogenase kinase in several mammalian tissues. Arch. Biochem. Biophys. **381**, 1–7

12 Sugden, M. C., Kraus, A., Harris, R. A. and Holness, M. J. (2000) Fibre-type specific modification of the activity and regulation of skeletal muscle pyruvate dehydrogenase kinase (PDK) by prolonged starvation and refeeding is associated with targeted regulation of PDK isoenzyme 4 expression. Biochem. J. **346**, 651–657

13 Askew, G. R., Doetschman, T. and Lingrel, J. B. (1993) Site-directed point mutations in embryonic stem cells: a gene-targeting tag-and-exchange strategy. Mol. Cell. Biol. **13**, 4115–4124

14 Williamson, D. H., Mellanby, J. and Krebs, H. A. (1962) Enzymic determination of D(−)-β-hydroxybutyric acid and acetoacetic acid in blood. Biochem. J. **82**, 90–96

15 Czok, R. and Lamprecht, W. (1974) Pyruvate, phosphoenolpyruvate and D-glycerate-2-phosphate. In Methods of Enzymatic Analysis (Bergmeyer, H. U., ed.), 2nd edn, pp. 1446–1451, Academic Press, New York

16 Livesey, G. and Lund, P. (1988) Determination of branched-chain amino and keto acids with leucine dehydrogenase. Methods Enzymol. **166**, 3–10

17 Lo, S., Russell, J. C. and Taylor, A. W. (1970) Determination of glycogen in small tissue samples. J. Appl. Physiol. **28**, 234–236

18 Bergmeyer, H. U. (1974) Methods in Enzymatic Analysis, 2nd edn, Academic Press, New York

19 Nakai, N., Sato, Y., Oshida, Y., Fujitsuka, N., Yoshimura, A. and Shimomura, Y. (1999) Insulin activation of pyruvate dehydrogenase complex is enhanced by exercise training. Metab. Clin. Exp. **48**, 865–869

20 Coore, H. G., Denton, R. M., Martin, B. R. and Randle, P. J. (1971) Regulation of adipose tissue pyruvate dehydrogenase by insulin and other hormones. Biochem. J. **125**, 115–127

21 Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**, 680–685

22 Wu, P., Inskeep, K., Bowker-Kinley, M. M., Popov, K. M. and Harris, R. A. (1999) Mechanism responsible for inactivation of skeletal muscle pyruvate dehydrogenase complex in starvation and diabetes. Diabetes **48**, 1593–1599

23 Krebs, H. A. and Henseleit, K. (1932) Untersuchungen uber die Harnstoff-bildung im Tierkorper. Hoppe-Seyler's Z. Physiol. Chem. **210**, 33–66

24 Clark, A. S., Mitch, W. E., Goodman, M. N., Fagan, J. M., Goheer, M. A. and Curnow, R. T. (1987) Dichloroacetate inhibits glycolysis and augments insulin-stimulated glycogen synthesis in rat muscle. J. Clin. Invest. **79**, 588–594

25 Ashcroft, S. J. H., Weerasinghe, L. C. C., Bassett, J. M. and Randle, P. J. (1975) The pentose cycle and insulin release in mouse pancreatic islets. Biochem. J. **126**, 525–532

26 Katz, J., Wals, P. A., Golden, S. and Rognstad, R. (1975) Recycling of glucose by rat hepatocytes. Eur. J. Biochem. **60**, 91–101

27 Hutson, N. J. and Randle, P. J. (1978) Enhanced activity of pyruvate dehydrogenase kinase in rat heart mitochondria in alloxan-diabetes or starvation. FEBS Lett. **92**, 73–76

28 Harris, R. A., Huang, B. and Wu, P. (2001) Control of pyruvate dehydrogenase kinase gene expression. Adv. Enzyme Regul. **41**, 269–288

29 Croniger, C. M., Millward, C., Yang, J., Kawai, Y., Arinze, I. J., Liu, S., Harada-Shiba, M., Chakravarty, K., Friedman, J. E., Poli, V. and Hanson, R. W. (2001) Mice with a deletion in the gene for CCAAT/enhancer-binding protein β have an attenuated response to cAMP and impaired carbohydrate metabolism. J. Biol. Chem. **276**, 629–638

30 Whitehouse, S., Cooper, R. H. and Randle, P. J. (1974) Mechanism of activation of pyruvate dehydrogenase by dichloroacetate and other halogenated carboxylic acids. Biochem. J. **141**, 761–774

31 Blackshear, P. J., Holloway, P. A. and Alberti, K. G. (1974) The metabolic effects of sodium dichloroacetate in the starved rat. Biochem. J. **142**, 279–286

32 Stacpoole, P. W. and Felts, J. M. (1970) Diisopropylammonium dichloroacetate (DIPA) and sodium dichloracetate (DCA): effect on glucose and fat metabolism in normal and diabetic tissue. Metab. Clin. Exp. **19**, 71–78

33 Crabb, D. W. and Harris, R. A. (1979) Mechanism responsible for the hypoglycemic actions of dichloroacetate and 2-chloropropionate. Arch. Biochem. Biophys. **198**, 145–152

34 Groen, A. K., van Roermund, C. W., Vervoorn, R. C. and Tager, J. M. (1986) Control of gluconeogenesis in rat liver cells: flux control coefficients of the enzymes in the gluconeogenic pathway in the absence and presence of glucagon. Biochem. J. **237**, 379–389

35 Chu, C. A., Sindelar, D. K., Neal, D. W., Allen, E. J., Donahue, E. P. and Cherrington, A. D. (1997) Comparison of the direct and indirect effects of epinephrine on hepatic glucose production. J. Clin. Invest. **99**, 1044–1056

36 McAllister, A., Allison, S. P. and Randle, P. J. (1973) Effects of dichloroacetate on the metabolism of glucose, pyruvate, acetate, 3-hydroxybutyrate and palmitate in rat diaphragm and heart muscle *in vitro* and on extraction of glucose, lactate, pyruvate and free fatty acids by dog heart *in vivo*. Biochem. J. **134**, 1067–1081

37 Gao, J., Islam, M. A., Brennan, C. M., Dunning, B. E. and Foley, J. E. (1998) Lactate clamp: a method to measure lactate utilization *in vivo*. Am. J. Physiol. **275**, E729–E733

38 Evans, O. B. and Stacpoole, P. W. (1982) Prolonged hypolactatemia and increased total pyruvate dehydrogenase activity by dichloroacetate. Biochem. Pharmacol. **31**, 1295–1300

39 Shangraw, R. E. and Jahoor, F. (2004) Mechanism of dichloroacetate-induced hypolactatemia in humans with or without cirrhosis. Metab. Clin. Exp. **53**, 1087–1094

40 Snell, K. and Duff, D. A. (1984) Branched-chain amino acid metabolism and alanine formation in rat diaphragm muscle *in vitro*: effects of dichloroacetate. Biochem. J. **223**, 831–835

41 Goodman, M. N., Ruderman, N. B. and Aoki, T. T. (1978) Glucose and amino acid metabolism in perfused skeletal muscle: effect of dichloroacetate. Diabetes **27**, 1065–1074

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