The hypothesis that PDHK4 (pyruvate dehydrogenase kinase isoenzyme 4) has potential as a target for the treatment of type 2 diabetes was tested by feeding wild-type and PDHK4 knockout mice a high saturated fat diet that induces hyperglycaemia, hyperinsulinaemia, glucose intolerance, hepatic steatosis and obesity. Previous studies have shown that PDHK4 deficiency lowers blood glucose by limiting the supply of three carbon gluconeogenic substrates to the liver. There is concern, however, that the increase in glucose oxidation caused by less inhibition of the pyruvate dehydrogenase complex by phosphorylation will inhibit fatty acid oxidation, promote ectopic fat accumulation and worsen insulin sensitivity. This was examined by feeding wild-type and PDHK4 knockout mice a high saturated fat diet for 8 months. Fasting blood glucose levels increased gradually in both groups but remained significantly lower in the PDHK4 knockout mice. Hyperinsulinaemia developed in both groups, but glucose tolerance was better and body weight was lower in the PDHK4 knockout mice. At termination, less fat was present in the liver and skeletal muscle of the PDHK4 knockout mice. Higher amounts of PGC-1α [PPARγ (peroxisome proliferator-activated receptor γ) coactivator 1α] and PPARα and lower amounts of fatty acid synthase and acetyl-CoA carboxylase isoenzyme 1 were present in the liver of the PDHK4 knockout mice. These findings suggest PDHK4 deficiency creates conditions that alter upstream signalling components involved in the regulation of lipid metabolism. The findings support the hypothesis that PDHK4 is a viable target for the treatment of type 2 diabetes.

Key words: hepatic steatosis, obesity, PDHK4 knockout mice, pyruvate dehydrogenase complex, pyruvate dehydrogenase kinase isoenzyme 4 (PDHK4), Type 2 diabetes.

INTRODUCTION

Regulation of the activity of the PDC (pyruvate dehydrogenase complex) is important for glucose homoeostasis and the control of fuel selection by tissues [1]. When blood glucose levels are elevated by carbohydrate intake, PDC is relatively dephosphorylated and active to promote glucose disposal and the synthesis and storage of fatty acids as triacylglycerols. When glucose levels are low because of fasting, PDC is highly phosphorylated and inactive to inhibit oxidation of gluconeogenic substrates (pyruvate, lactate, and alanine) and promote glucose synthesis and fatty acid oxidation. The phosphorylation state and therefore the activity of the PDC [2,3] are set by the relative activities of the four PDHKs (pyruvate dehydrogenase kinases) and two PDPs (pyruvate dehydrogenase phosphatases) expressed in tissues. Of the enzymes that modulate PDC activity, PDHK4 is of special interest because its expression is markedly increased by fasting [4] and PDHK4 has been shown to be important for maintaining fasting blood glucose levels in studies with PDHK4 knockout mice [5].

Regulation of PDC by phosphorylation is defective in diabetes. When blood glucose levels are high, as in diabetes, PDC should be dephosphorylated and active. However, PDC is highly phosphorylated and inactive in diabetes [6], at least in part because of upregulation of PDHK4 expression [7–9] due to insulin deficiency or resistance. Lack of PDC activity worsens hyperglycaemia in diabetes by promoting glucose synthesis. That upregulation of PDHK4 contributes to the hyperglycaemia in diabetes has been shown in studies with PDHK4 knockout mice fed a high-fat, diabeticogenic diet [10].

Previously, we determined the effects of PDHK4 deficiency in mice fed a high-fat diet that was relatively rich in saturated fatty acids [10]. In the present study, mice were fed a high-fat diet rich in saturated fatty acids to induce greater obesity, insulin resistance, hyperglycaemia and ectopic fat accumulation. As expected from our previous studies [5,10], PDHK4 knockout mice maintained lower blood glucose levels throughout the study. Surprisingly, the PDHK4 knockout mice gained less weight, accumulated less body fat, and exhibited less hepatic steatosis than wild-type mice. Western blot analysis of transcription factors and regulatory enzymes suggests that PDHK4 deficiency protects mice from the deleterious effects of a HSF (high saturated fat) diet by altering the expression of key enzymes involved in lipid metabolism.

EXPERIMENTAL

Animals

Experimental protocols were approved by the Institutional Animal Care and Use Committee of Indiana University School of Medicine. The procedure used to generate PDHK4−/− (homozygous PDHK4 knockout) C57BL/6J black mice was...
described previously [5]. At 5 weeks of age, groups of 16 male wild-type and 16 male PDHK4 knockout mice were housed with two mice per cage under controlled temperature (23 ± 2°C) and a 12 h light/dark cycle (lights on at 7 a.m. and off at 7 p.m.). Half of the mice in each genotype group were fed a rodent standard chow diet (catalogue number 7017; Harlan) that was high in carbohydrate and low in fat (4% by calories). The other half of the mice were fed a HSF diet (catalogue number D12330; Research Diets) that was low in carbohydrate and high in fat (58% by calories). The fat composition of the HSF diet was analyzed by the supplier (93.3% saturated fat, 2.4% monounsaturated fat, and 4.3% polyunsaturated fat). According to the supplier, the hydrogenation process used to produce the hydrogenated coconut oil was complete; therefore, little or no trans fatty acids were present in the diet. Body weights of the mice were determined biweekly. Food consumption was monitored during the 16th week of the feeding period. The experiment was terminated after 32 weeks of feeding. Half of the mice in the four groups were killed in the fed state (between 8 a.m. and 9 a.m.), the other half after overnight fasting (from 3 p.m. to 8 a.m.). After blood had been taken from the tail for the measurement of glucose, the mice were anaesthetized by injecting pentobarbital (60 mg/kg of body mass) intraperitoneally. Blood was drawn from the inferior vena cava to measure serum metabolites. Gastrocnemius muscle, liver, heart, and epididymal fat were harvested as rapidly as possible in the order given, immediately freeze-clamped with Wollenberger tongs at the temperature of liquid nitrogen, powdered under liquid nitrogen with a mortar and pestle, and stored at −85°C for analysis. Small pieces of the liver were also collected and snap-frozen in liquid nitrogen for histological analysis. Since more tissue was needed for the analyses, the experiment was repeated in part with groups of 6 male wild-type and 6 male PDHK4 knockout mice fed the high saturated fat diet for 27 weeks. The mice were killed after overnight fasting.

Measurement of body composition

Body composition 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.

Measurement of metabolite concentrations

Serum extracts deproteinized with perchloric acid were neutralized with KOH. Pyruvate, lactate and alanine were measured by enzymatic methods as described previously [11–13]. Triacylglycerols were extracted with isopropyl alcohol and determined with an L-type TG H assay kit (Wako Chemical). At the 26th week of feeding, food was withheld from the mice for 2 h (7 a.m. to 9 a.m.) and blood was collected from the tail. Serum insulin levels were determined with an Ultrasensitive Mouse insulin ELISA kit (Mercodia, Winston-Salem, NC).

GTT (glucose tolerance test), ITT (insulin tolerance test) and PTT (pyruvate tolerance test)

At the 27th week of feeding, GTTs were performed after mice had been fasted overnight (5 p.m. to 9 a.m.). Glucose (1 g/kg of body weight) was given by intraperitoneal injection. Tail blood glucose was measured at 0, 15, 30, 60 and 120 min with a glucometer (Accu-Chek; Roche). At the 22nd week of feeding, ITTs were performed after food had been withheld from the mice for 5 h (9 a.m. to 2 p.m.). Insulin (1 unit/kg of body weight; Humulin R, Eli Lilly) was given by intraperitoneal injection. Tail blood glucose levels were measured at 0, 15, 30 and 60 min. PTTs were performed with 16 week old male, chow-diet fed mice that had been fasted overnight (5 p.m. to 9 a.m.). Pyruvate (1.5 g/kg of body weight) was given by intraperitoneal injection. Tail blood glucose was measured at 0, 15, 60 and 120 min. Serum pyruvate was measured at 0, 15 and 30 min.

Histochemistry of the livers

Histological examination of the liver was performed by the Immunohistochemistry Laboratory of Indiana University School of Medicine (Indianapolis, IN, U.S.A.).

Western blot analysis

Tissue powder (60 mg) prepared under liquid nitrogen was homogenized with RIPA buffer containing 50 mM Tris/HCl, pH 7.4, 150 mM NaCl, 0.25% (w/v) deoxycholic acid, 1% (v/v) Nonidet P-40, 1 mM EDTA, 10 μM tosyl phenylalanyl chloromethyl ketone, 10 μg/ml trypsin inhibitor, 1 mM PMSF, 2 μg/ml aprotinin, 3.5 mM bis-benzamidine, 50 mM potassium fluoride and 0.4 mM sodium orthovanadate. Tissue extracts were obtained by centrifugation for 10 min at 4°C and 13400 g. Protein concentration was determined by the Bio-Rad protein assay. Equal amounts of protein were separated on SDS/polyacrylamide gels, transferred to a PVDF membrane by wet blotting, and probed with antibodies directed against FAS (fatty acid synthase; 610962, BD Biosciences, San Jose, CA, U.S.A.), ACC1 (acetyl-CoA carboxylase isoenzyme 1; 3662, Cell Signaling, Danvers, MA, U.S.A.), PGC-1α [PPARγ (peroxisome proliferator-activated receptor γ) coactivator 1α; sc-13067, Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.], PPARα (sc-9000, Santa Cruz Biotechnology), FAT/CD36 (fatty acid translocase; sc-9154, Santa Cruz Biotechnology), MCAD (medium chain acyl-CoA dehydrogenase; sc-50587, Santa Cruz Biotechnology), PEPCCK (phosphoenolpyruvate carboxykinase; sc-32879, Santa Cruz Biotechnology), CPS1 (carbamoyl-phosphate synthase 1; sc-30060, Santa Cruz Biotechnology), tubulin (ab6046, Abcam, Cambridge, MA, U.S.A.) and PDHK4 [5]. The amounts of bound antibodies were accessed by the peroxidase activity of horseradish peroxidase-conjugated secondary antibody as detected by chemiluminescence with Lumi-light Western blotting substrate (Roche Diagnostics).

Statistical analysis

Values are presented as means ± S.E.M. with the indicated number of independent samples. The statistical significance of differences between groups was determined by the Student’s t test. P values less than 0.05 were considered statistically significant.

RESULTS

PDHK4 knockout mice gained less weight and accumulated less body fat than wild-type mice fed the HSF diet

Wild-type mice fed the HSF diet gained more weight and appeared more obese than PDHK4 knockout mice fed the HSF diet (Figure 1) (50.0 ± 1.1 compared with 44.9 ± 1.6 g respectively, after 27 weeks on the HSF diet; P < 0.02; n = 14 per group). In contrast, growth curves of wild-type and PDHK4 knockout mice fed the chow diet were identical (Figure 1). No difference in weight gain occurred between wild-type and PDHK4 knockout mice in the first 8 weeks on the HSF diet. Thereafter, the PDHK4 knockout mice gained less weight. Consistent with this finding,
Table 1 Body composition of wild-type (PDHK4+/+) and PDHK4−/− mice fed a chow or a HSF diet for 29 weeks

Results are presented as means ± S.E.M. with n = 8 for wild-type and PDHK4−/− mice fed a HSF diet; n = 4 for wild-type and PDHK4−/− mice fed a chow diet. The body composition of the mice was measured as described in the Experimental section. *P < 0.01 relative to wild-type mice fed an HSF diet.

<table>
<thead>
<tr>
<th>Diet</th>
<th>Genotype</th>
<th>Body lean mass (%)</th>
<th>Body fat mass (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chow</td>
<td>PDHK4+/+</td>
<td>82.2 ± 0.7</td>
<td>17.7 ± 0.7</td>
</tr>
<tr>
<td></td>
<td>PDHK4−/−</td>
<td>76.3 ± 1.6</td>
<td>21.7 ± 1.6</td>
</tr>
<tr>
<td>HSF</td>
<td>PDHK4+/+</td>
<td>57.6 ± 0.5</td>
<td>42.4 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>PDHK4−/−</td>
<td>62.1 ± 1.2*</td>
<td>37.9 ± 1.2*</td>
</tr>
</tbody>
</table>

Table 2 Weights of various tissues of overnight-fasted wild-type (PDHK4+/+) and PDHK4−/− mice fed either a chow or a HSF diet

Results are presented as means ± S.E.M. with n = 10 for wild-type and PDHK4−/− mice fed on a HSF diet; n = 9 for wild-type and PDHK4−/− mice fed on a chow diet. Indicated tissues were harvested from the overnight-fasted mice fed each diet. *P < 0.02 relative to wild-type mice fed a HSF diet. †P < 0.02 relative to wild-type mice fed a chow diet.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Chow</th>
<th>HSF</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PDHK4+/+ (g)</td>
<td>PDHK4−/− (g)</td>
</tr>
<tr>
<td></td>
<td>PDHK4+/+ (g)</td>
<td>PDHK4−/− (g)</td>
</tr>
<tr>
<td>Liver</td>
<td>1.23 ± 0.05</td>
<td>1.22 ± 0.08</td>
</tr>
<tr>
<td></td>
<td>2.16 ± 0.20</td>
<td>1.46 ± 0.15*</td>
</tr>
<tr>
<td>Epididymal fat</td>
<td>0.51 ± 0.08</td>
<td>0.19 ± 0.20*</td>
</tr>
<tr>
<td></td>
<td>3.26 ± 0.15</td>
<td>2.67 ± 0.10*</td>
</tr>
<tr>
<td>Gastrocnemius</td>
<td>0.35 ± 0.02</td>
<td>0.36 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>0.37 ± 0.02</td>
<td>0.37 ± 0.02</td>
</tr>
<tr>
<td>Heart</td>
<td>0.16 ± 0.01</td>
<td>0.14 ± 0.00</td>
</tr>
<tr>
<td></td>
<td>0.17 ± 0.00</td>
<td>0.16 ± 0.01</td>
</tr>
</tbody>
</table>

PDHK4 deficiency prevented fasting hyperglycaemia but not hyperinsulinaemia in mice fed the HSF diet

As reported previously [5], PDHK4 deficiency had no effect on blood glucose levels in chow-diet fed mice in the fed state but always caused lower fasting blood glucose levels. Similar results were obtained with mice fed the HSF diet. PDHK4 deficiency had no effect on blood glucose levels in the fed state (157 ± 6 mg/dl for wild-type mice; 154 ± 6 mg/dl for PDHK4 knockout mice after 19 weeks on the HSF diet; n = 8 mice/group), but always produced lower fasting blood glucose levels than wild-type mice fed the HSF diet (Figure 2A). Blood glucose level of the two groups of mice increased in parallel between 14 and 22 weeks of feeding but was followed by a large jump in the wild-type mice after 19 weeks on the HSF diet (P < 0.02). Weights of the heart and gastrocnemius muscle were not affected by PDHK4 deficiency in mice fed the chow diet or the HSF diet.

PDHK4 deficiency lowered the concentrations of gluconeogenic substrates but had no effect on pyruvate tolerance

To test the hypothesis that lower levels of gluconeogenic substrates in PDHK4 knockout mice reduce blood glucose levels by limiting gluconeogenesis, the serum levels of pyruvate, lactate, and alanine were measured (Table 3). As expected from previous studies [5], PDHK4 knockout mice had much lower pyruvate...
level than wild-type mice fed the HSF or chow diet. PDHK4 deficiency also significantly lowered alanine levels in mice fed the HSF diet. Although not statistically significant, a trend towards lower lactate levels occurred in PDHK4 knockout mice fed the HSF diet (1.82 ± 0.43 compared with 0.88 ± 0.24 mmol/l for wild-type and PDHK4 knockout mice respectively; n = 9 mice/group; P < 0.08). To confirm that three-carbon compounds limit glucose synthesis in PDHK4 knockout mice, pyruvate tolerance tests were conducted with overnight fasted wild-type and PDHK4 knockout mice that had been maintained on chow diet (Figure 3A). As expected, blood glucose levels were lower in the PDHK4 knockout mice before administration of pyruvate (70 ± 5 compared with 52 ± 3 mg/dl for wild-type and PDHK4 knockout mice respectively; n = 4 mice/group; P < 0.05) and remained lower following the administration of pyruvate. Nevertheless, the areas under the curves for the increase in glucose were not significantly different between the two groups of mice (4470 ± 610 compared with 3860 ± 430 mg·dl⁻¹·min⁻¹ for wild-type and PDHK4 knockout mice respectively; n = 4 mice/group; P = 0.44). Also as expected, there was a much lower pyruvate concentration in the blood of PDHK4 knockout mice before its administration (0.125 ± 0.006 compared with 0.045 ± 0.005 mmol/l for wild-type and PDHK4 knockout mice respectively; n = 4 mice/group; P < 0.01) and it was cleared much faster from the blood of PDHK4 knockout mice as shown by levels measured after 15 and 30 min (Figure 3B).

PDHK4 deficiency improved glucose tolerance but not insulin sensitivity in mice fed the HSF diet

To test the effect of PDHK4 deficiency on insulin sensitivity, ITTs and GTTs were performed after feeding the mice with the HSF diet or the chow diet for 22 or 27 weeks respectively. In agreement with our previous findings [5], PDHK4 knockout mice had better glucose tolerance than wild-type mice fed the chow diet (Figure 4A). The area under the curve for PDHK4 knockout mice fed the chow diet was less than that for wild-type mice (8080 ± 680 compared with 11000 ± 1020 mg·dl⁻¹·min⁻¹ respectively; n = 4 mice/group; P < 0.05). As expected, both wild-type and PDHK4 knockout mice fed the HSF diet showed impaired glucose tolerance compared with chow-diet fed mice. Nevertheless, PDHK4 knockout mice fed the HSF diet were more glucose tolerant than the wild-type mice (Figure 4A). The area under the curve for PDHK4 knockout mice fed the chow diet was less than that for wild-type mice (35 mg·dl⁻¹·min⁻¹ compared with 41 mg·dl⁻¹·min⁻¹ respectively; n = 4 mice/group; P < 0.01). Consistent with glucose tolerance, PDHK4 knockout mice fed the chow diet were more sensitive to insulin than wild-type mice (Figure 4B). The area above the curve for chow diet-fed PDHK4 knockout mice was greater than that of wild-type mice fed the chow diet (1333 ± 113 compared with 891 ± 61 mg·dl⁻¹·min⁻¹ between 0 and 30 min respectively; n = 4 mice/group; P < 0.05). Indeed, glucose had to be administered after 30 min to prevent the PDHK4 knockout mice from dying of hypoglycaemia. On the other hand, the PDHK4 knockout mice fed the HSF diet were not clearly more insulin sensitive than the wild-type mice (Figure 4B). Although insulin caused a greater reduction in blood glucose levels of the PDHK4 knockout mice at the 60 min time point (91 ± 13 compared with 58 ± 7 mg/dl for wild-type and PDHK4 knockout mice respectively; n = 4 mice/group; P < 0.05), the areas above the curves were not significantly different between the two groups of mice (1397 ± 191 compared with 1875 ± 212 mg·dl⁻¹·min⁻¹).
Figure 3  
Effect of PDHK4 deficiency on pyruvate tolerance

(A) Blood glucose levels during the PTT with wild-type (open squares) and PDHK4−/− (closed squares) mice fed the chow diet. Pyruvate (1.5 g/kg of body weight) was administered to overnight-fasted mice by intraperitoneal injection. The inset shows the area under the curve for the respective mice. (B) Serum pyruvate levels during the PTT with wild-type (open bars) and PDHK4−/− (closed bars) mice. Results are presented as means ± S.E.M. with n = 4 mice per group. *P < 0.01 relative to wild-type mice.

Figure 4  
Effect of PDHK4 deficiency on glucose tolerance and insulin sensitivity during the HSF diet feeding

GTT and ITT with wild-type (squares) and PDHK4−/− (triangles) mice fed the chow diet (open symbols) or the HSF diet (closed symbols). (A) GTT. At the 27th week of feeding, glucose (1 g/kg of body weight) was administrated to overnight-fasted mice by intraperitoneal injection. Blood glucose levels were measured at the indicated times. Data are presented as means ± S.E.M. with n = 4 mice per group. *P < 0.05 relative to wild-type mice fed the HSF diet. **P < 0.05 relative to wild-type mice fed the chow diet. (B) ITT. At the 22nd week of feeding, insulin (1 unit/kg of body weight) was administrated to 5 h-fasted mice by intraperitoneal injection. Blood glucose levels were measured at the indicated time points. Initial blood glucose levels after 5 h of fasting were 135 ± 6 mg/dl for chow-fed wild-type mice, 120 ± 14 mg/dl for chow-fed PDHK4 knockout mice, 151 ± 9 mg/dl for HSF-fed PDHK4 knockout mice, and 140 ± 7 mg/dl for HSF fed wild-type mice. Results are presented as means ± S.E.M. with four mice per group. *P < 0.05 relative to wild-type mice fed the HSF diet. **P < 0.05 relative to wild-type mice fed the chow diet.

PDHK4 deficiency reduced hepatic steatosis induced by the HSF diet

Larger amounts of fat were present in the livers of mice fed the HSF diet compared with mice fed the chow diet (compare Figures 5C and 5D with Figures 5A and 5B). However, the amount of fat accumulation induced by the HSF diet was attenuated by PDHK4 deficiency (compare Figure 5C with Figure 5D). The accumulated fat was primarily macrovesicular in the wild-type mice and microvesicular in the PDHK4 knockout mice. The amounts of triacylglycerol in liver, gastrocnemius muscle and heart were also measured by an enzymatic method (Table 4). In agreement with the histological analysis, less triacylglycerol was found in the livers of PDHK4 knockout mice fed the HSF diet. Conversely, more triacylglycerol was found in the liver of PDHK4 knockout mice relative to wild-type mice fed the chow diet (Table 4), a finding also apparent by histological analysis (Figures 5A and 5B). Similar to fat accumulation in liver, HSF diet feeding increased the amount of triacylglycerol in gastrocnemius muscle and PDHK4 deficiency attenuated the increase (Table 4). In contrast, neither HSF feeding nor PDHK4 deficiency affected the amount of triacylglycerol present in the heart (Table 4).

PDHK4 deficiency suppressed the increase in fatty acid synthesis enzymes in liver caused by long-term HSF diet feeding

To gain insight into the mechanism by which PDHK4 deficiency reduces the amount of liver fat in mice fed the HSF diet, the protein amounts of enzymes involved in fatty acid synthesis were measured (Figure 6). In wild-type mice, the amounts of both
Figure 5  Effect of PDHK4 deficiency on hepatic steatosis induced by the HSF diet

Micrographs demonstrating the histological appearance of the livers of wild-type (A) and PDHK4−/− (B) mice fed the chow diet or wild-type (C) and PDHK4−/− (D) mice fed the HSF diet for 27 weeks. Tissue sections were prepared from the livers of the overnight-fasted mice and stained with Oil Red O. The material stained red corresponds to fat droplets. Original magnification was ×200. Four liver samples from different mice were analysed in each group.

Table 4  Concentration of triacylglycerol in various tissues of overnight-fasted wild-type (PDHK4+/+) and PDHK4−/− mice fed either a chow or a HSF diet for 27 weeks

Results are presented as means ± S.E.M. with n = 6 for wild-type and PDHK4−/− mice fed a HSF diet, n = 5 for wild-type and PDHK4−/− mice fed a chow diet. Tissue extracts were obtained from overnight-fasted mice fed each diet for 27 weeks. Concentration of triacylglycerol was measured as described in the Experimental section. *P < 0.05 relative to wild-type mice fed a HSF diet; †P < 0.03 relative to wild-type mice fed a chow diet.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Chow</th>
<th>HSF</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PDHK4+/+ (mg/g of wet weight)</td>
<td>PDHK4−/− (mg/g of wet weight)</td>
</tr>
<tr>
<td>Liver</td>
<td>111 ± 9</td>
<td>137 ± 2†</td>
</tr>
<tr>
<td>Gastrocnemius</td>
<td>11 ± 2</td>
<td>33 ± 6†</td>
</tr>
<tr>
<td>Heart</td>
<td>11 ± 2</td>
<td>16 ± 1</td>
</tr>
</tbody>
</table>

FAS and ACC1 were increased by HSF diet feeding compared to mice fed the chow diet. However, the amounts of FAS and ACC1 in livers of PDHK4 knockout mice fed the HSF diet were less than the amounts present in wild-type mice fed the HSF diet. The expected decrease in FAS in response to fasting occurred in mice fed the chow diet but not mice fed the HSF diet (results not shown), suggesting that metabolic flexibility was reduced by the HSF diet in both wild-type and PDHK4 knockout mice. These findings suggest that higher amounts of fatty acid synthesis enzymes may contribute to the development of hepatic steatosis in wild-type mice fed the HSF diet and lack of PDHK4 may reduce fat accumulation in the liver by decreasing the fatty acid synthesis enzymes.

PDHK4 deficiency prevented the decrease of PGC-1α, PPARα, PEPCK and CPS1 induced by the HSF diet

HSF diet feeding decreased the expression of PGC-1α and PPARα (Figure 6), transcription factors that promote expression of enzymes involved in fatty acid oxidation. The reduction in expression of these factors by the HSF diet was attenuated by PDHK4 deficiency (Figure 6). However, expression of MCAD and FAT/CD36 was not affected by either the HSF feeding or PDHK4 deficiency (Figure 6). Since expression of PEPCK, a key gluconeogenic gene, is stimulated by PGC-1α and PPARα, we investigated the expression level of PEPCK. The HSF diet decreased PEPCK expression in the wild-type mice, but not in the PDHK4 knockout mice (Figure 6). Conditions that induce steatosis have been shown to decrease expression of CPS1 [14], the rate-limiting enzyme of the urea cycle. In agreement with those studies, wild-type mice fed the HSF diet expressed less CPS1 protein compared with wild-type mice fed the chow diet. As found for other metabolically important enzymes (Figure 6), PDHK4 deficiency attenuated this effect of HSF diet on CPS1 expression (Figure 6).

DISCUSSION

Mice fed a calorically dense, highly palatable, saturated-fat rich diet eat more calories than needed, become obese, develop
Figure 6  Effect of PDHK4 deficiency on expression of enzymes involved in lipid metabolism, gluconeogenesis and urea cycle

(A) Representative Western blots are shown for the amounts of FAS, ACC, PGC-1α, PPARα, MCAD, FAT/CD36, PEPCK, PDHK4 and CPS1 in the livers of wild-type or PDHK4$^{-/-}$ mice fed the chow or HSF diet. Tubulin served as the loading control. The Western blot analysis was performed as described in the Experimental section. Lane 1, chow diet-fed wild-type mice; lane 2, chow diet-fed PDHK4$^{-/-}$ mice; lane 3, HSF diet-fed wild-type mice; lane 4, HSF diet-fed PDHK4$^{-/-}$ mice. FAS and ACC were measured in livers harvested from fed mice; all other proteins were measured in livers harvested from overnight fasted mice. Loading control for tubulin for fed mice is not shown. (B) Histograms constructed from data obtained by the Western blot analysis. Results are presented as means ± S.E.M. with n = 4 or 10 mice per group. *P < 0.05 relative to wild-type mice fed the chow diet. **P < 0.05 relative to wild-type mice fed the HSF diet. #P < 0.05 relative to PDHK4$^{-/-}$ mice fed the chow diet.
symptoms characteristic of the metabolic syndrome, and, if left on the diet long enough, would most likely develop type 2 diabetes. Wild-type C57BL/6J mice maintained for 27 weeks on such a diet were grossly obese, hyperglycaemic (fasting blood glucose > 140 mg/dl), hyperinsulinaemic, glucose intolerant and insulin resistant. PDHK4 knockout mice fed the same diet for the same length of time gained less weight, remained euglycaemic (fasting blood glucose < 90 mg/dl), and had not become as glucose intolerant as wild-type mice. Thus, under the conditions of these experiments, PDHK4 deficiency attenuated some of the negative effects induced by a HSF diet.

Mice gain more weight on a diet rich in fat, regardless of whether the fat is saturated or unsaturated. In previous studies, we found no differences in weight gain between wild-type and PDHK4 knockout mice fed either a chow diet [5] or a high unsaturated fat diet [10]. In the present study, weight gain was again the same in wild-type and PDHK4 knockout mice fed the chow diet. As expected, mice of both genotypes fed the HSF diet gained considerably more weight than mice fed the chow diet. However, the PDHK4 knockout mice gained less weight and accumulated less total body fat than wild-type mice on the HSF diet. No difference in food consumption was found between the two groups, suggesting the genetically altered mice may be more physically active or less metabolically efficient than the wild-type mice.

A diet high in fat and rich in saturated fatty acids (93.3% saturated fatty acids, 2.4% monounsaturated fatty acids and 4.3% polyunsaturated fatty acids) was chosen because such a diet promotes the accumulation of greater amounts of fat in tissues than the more commonly used high unsaturated fat diet (39.7% saturated fatty acids, 49.8% monounsaturated fatty acids and 10.4% polyunsaturated fatty acids) [10]. Polyunsaturated fatty acids ameliorate hepatic steatosis in patients with NAFLD (non-alcoholic fatty liver disease) [15] and obese mice [16]. Hepatic steatosis is frequently associated with obesity and Type 2 diabetes [17], and ectopic fat accumulation is characteristic of insulin-resistant tissues [18]. Whether PDHK4 deficiency exacerbates the accumulation of fat in tissues is therefore an important issue. Since the increase in PDC activity resulting from PDHK4 deficiency increases fasting levels of FFAs [free (non-esterified) fatty acids], most likely by inhibiting fatty acid oxidation at the level of carnitine palmitoyltransferase 1 by increasing the concentration of malonyl-CoA [19,20], it can easily be seen how PDHK4 deficiency might promote the synthesis and therefore increase the triacylglycerol content of tissues. However, less fat was found in the liver and muscle of PDHK4 knockout mice fed the HSF diet. The appearance of the fat in the liver was different between the two groups of mice. Large fat droplets, i.e. macrovesicular steatosis, were apparent in the livers of wild-type mice, whereas smaller fat droplets, i.e. microvesicular steatosis, were present in the livers of PDHK4 knockout mice. Since increased fatty acid synthesis can contribute to the accumulation of fat in the liver, the relative amounts of FAS and ACC, key lipogenic enzymes, were measured. Surprisingly, much higher levels of FAS and ACC were found in the livers of mice fed the HSF diet relative to livers of mice fed the high carbohydrate chow diet. This fits with the fact that saturated fatty acids induce expression of lipogenic enzymes in the liver [21]. This stands in contrast with polyunsaturated fatty acids, which repress expression of these enzymes [16,22,23]. Thus de novo fatty acid synthesis probably contributed to the large amount of fat that accumulated in the livers of wild-type mice. Conversely, less capacity for de novo fatty acid synthesis because of reduced expression of FAS and ACC probably explains why livers of PDHK4 knockout mice were less fatty. An explanation for less expression of FAS and ACC in the livers of PDHK4 knockout mice was not found in the present study.

The level of expression of FAT/CD36, the protein responsible for transport of fatty acids into the liver, was measured with the expectation that it might be reduced in amount in the PDHK4 knockout mice. However, this was not found. Therefore, the lower amount of fat in the liver of the PDHK4 knockout mice was not due to decreased capacity for the transport of fatty acids into the liver.

We also measured the amount of PGC-1α, a transcription coactivator required for expression of genes involved in mitochondrial biogenesis [24], hepatic gluconeogenesis [25] and fatty acid oxidation [26]. PGC-1α was reduced in the livers of wild-type mice fed the HSF diet relative to mice fed the chow diet. Knocking out PDHK4 prevented this lowering of PGC-1α caused by feeding a HSF diet to wild-type mice. Although a high-fat diet has previously been reported to increase PGC-1α expression in the liver [21], that finding may not be relevant since the mice were only fed the high fat diet for two days, whereas the mice were fed the HSF diet for 8 months in the present study. Moreover, there are a number of reports in the literature that are consistent with our finding that a HSF diet decreases PGC-1α. These include studies that have shown PGC-1α is reduced in the skeletal muscle of Type 2 diabetic patients [27], reduced in the skeletal muscle of mice fed a HSF diet for 8 weeks [28], reduced in human fatty liver relative to normal human liver [29] and reduced in the liver of mice fed a HSF diet relative to a high polysaturated fat diet for 4 weeks [30]. Furthermore, knocking out PGC-1α induces fat accumulation in the liver [31], suggesting that the severe hepatic steatosis found in the mice fed the HSF diet may be due in part to the low levels of PGC-1α. Similarly, the accumulation of less fat in the liver of PDHK4 knockout mice may be explained in part by the expression of higher levels of PGC-1α.

Since PGC-1α controls expression of gluconeogenic enzymes, the amounts of PEPCK and PDHK4 were also measured. Consistent with the reduced amounts of PGC-1α and PPARα, PEPCK and PDHK4 were decreased in the livers of the wild-type mice fed the HSF diet compared with the mice fed the chow diet. In contrast, similar amounts of PEPCK were detected in the livers of the PDHK4 knockout mice fed the HSF diet and both genotypes of mice fed the chow diet. Reduced expression of PEPCK is surprising in the face of the insulin resistance and hyperglycaemia of the HSF fed mice. However, this is consistent with the low level of PGC-1α expression and increased capacity for de novo fatty acid synthesis. Furthermore, feeding a high fat diet induced hyperglycaemia and fatty liver in the strain of mice used in the present study (C57BL/6j) has been shown to strongly attenuate the increase in PEPCK caused by overnight fasting [32]. The same phenomenon was observed in the present study – less increase in PEPCK in response to fasting in mice fed a high fat diet relative to mice fed a chow diet. The finding of greater expression of PGC-1α and PEPCK in PDHK4 knockout mice fed the HSF diet (C57BL/6j) has been shown to strongly attenuate the increase in PEPCK caused by overnight fasting [32]. The same phenomenon was observed in the present study – less increase in PEPCK in response to fasting in mice fed a high fat diet relative to mice fed a chow diet. The finding of greater expression of PGC-1α and PEPCK in PDHK4 knockout mice fed the HSF diet appears inconsistent with the lower fasting blood glucose levels of these mice. However, the PTT of the present study plus the measurements of blood levels of gluconeogenic substrates [33] and intermediates of the gluconeogenesis pathway [5] suggest reduced substrate supply to the liver is primarily responsible for lower blood glucose levels in the PDHK4 knockout mice. Although PEPCK mRNA and protein are commonly taken as a direct index of the rate of hepatic gluconeogenesis, this view has long been controversial and was again strongly challenged in recent studies [34]. Since PEPCK is reduced in the wild-type mice fed the HSF diet, PEPCK activity may limit the rate of gluconeogenesis in these mice, whereas substrate supply limits the rate of glucose synthesis to a greater extent in PDHK4 knockout mice. If this is the case, the difference in glucose levels between wild-type and PDHK4 knockout mice might have been even
greater if PGC-1α and PEPCK were not downregulated in the wild-type mice.

Since PGC-1α and PPARα also control expression of fatty acid oxidation enzymes, the expression level of MCAD was also measured. No difference was observed between wild-type mice and PDHK4 knockout mice, suggesting altered capacity for fatty acid oxidation is not a factor in the difference in fat accumulation in the livers of these mice.

PDHK4 knockout mice were more insulin sensitive than wild-type mice fed the chow diet but not the HSF diet. We previously found PDHK4 knockout mice were more insulin sensitive than wild-type mice fed chow diet [5] or a high unsaturated fat diet [10]. These findings are surprising since it would seem that inhibition of fatty acid oxidation by activation of PDC should increase tissue levels of diacylglycerol and ceramide, which would be expected to activate stress kinases, which should reduce insulin sensitivity by serine phosphorylation of components of the insulin signalling pathway [35]. Perhaps PDHK4 deficiency helps conserve insulin sensitivity, because it minimized glucotoxicity by maintaining a lower steady-state concentration of glucose.

A higher than normal fasting blood glucose level (> 100 mg/dl) is an established risk factor for the development of Type 2 diabetes [36,37]. Since the rate of hepatic glucose production is the primary determinant of fasting blood glucose levels [38], it follows that overproduction of glucose by the liver contributes to the development of Type 2 diabetes [39]. Since obesity enhances hepatic glucose production, life style changes that prevent accumulation of excessive body fat or promote its loss have proven effective in preventing Type 2 diabetes [40]. Likewise, inhibition of hepatic glucose production by metformin and insulin provide the most effective therapy for Type 2 diabetes [41,42]. Based on these considerations, the PDHKs would appear to have potential as therapeutic targets for the prevention/treatment of Type 2 diabetes. Indeed, inhibition of PDHK activity with dichloacetate reduces fasting blood glucose levels in intact rats [43] by decreasing hepatic glucose production by decreasing the supply of gluconeogenic substrates (lactate, pyruvate and alanine) to the liver [33]. Unfortunately, toxic side effects [44] exclude the use of dichloacetate for the treatment of Type 2 diabetes. Regardless, the present study with PDHK4 knockout mice confirms the therapeutic potential of PDHK inhibitors for the treatment of type 2 diabetes and hepatic steatosis induced by a HSF diet.

**AUTHOR CONTRIBUTION**

Byounghoon Hwang, Nam Ho Jeoung and Robert Harris designed the experiments. Byounghoon Hwang and Nam Ho Jeoung performed the experiments and analysed the data. Byounghoon Hwang and Robert Harris wrote the manuscript.

**ACKNOWLEDGEMENTS**

We thank Dr R. V. Considine and Dr C. Turner of the Indiana University School of Medicine for access and training on the use of the DEKA instrument; Dr J. S. You and S. M. Hong at Monarch Life Sciences (Indianapolis, IN, U.S.A.) for confirmation of the identity of CPS1 protein by MS.

**FUNDING**

This work was supported by grants to R. A. H. from the U. S. Public Health Service [grant number DK47844] and the Grace M. Showalter Residency Trust.

**REFERENCES**


34 Burgess, S. C., He, T., Yan, Z., Lindner, J., Sherry, A. D., Malloy, C. R., Browning, J. D. and Magnuson, M. A. (2007) Cytoplasmic phosphoenolpyruvate carboxy kinase does not solely control the rate of hepatic gluconeogenesis in the intact mouse liver. Cell Metab. 5, 313–320


Received 9 March 2009/16 July 2009; accepted 23 July 2009
Published as BJ Immediate Publication 23 July 2009, doi:10.1042/BJ20090390