Phosphoenolpyruvate carboxykinase (Pck1) helps regulate the triglyceride/fatty acid cycle and development of insulin resistance in mice

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Abstract  The aim of this study was to investigate the role of the cytosolic form of phosphoenolpyruvate carboxykinase (Pck1) in the development of insulin resistance. Previous studies have shown that the roles of Pck1 in white adipose tissue (WAT) in glyceroneogenesis and reesterification of free fatty acids (FFA) to generate triglycerides are vital for the prevention of diabetes. We hypothesized that insulin resistance develops when dysregulation of Pck1 occurs in the triglyceride/fatty acid cycle, which regulates lipid synthesis and transport between adipose tissue and the liver. We examined this by analyzing mice with a deletion of the PPARY binding site in the promoter of Pck1 (PPARE−/−). This mutation reduced the fasting Pck1 mRNA expression in WAT in brown adipose tissue (BAT). To analyze insulin resistance, we performed hyperinsulinemic-euglycemic glucose clamp analyses. PPARE−/− mice were profoundly insulin resistant and had more FFA and glycerol released during the hyperinsulinemic-euglycemic clamp compared with wild-type mice (WT). Finally, we analyzed insulin secretion in isolated islets. We found a 2-fold increase in insulin secretion in the PPARE−/− mice at 16.7 mM glucose. Thus, the PPARE site in the Pck1 promoter is essential for maintenance of lipid metabolism and glucose homeostasis and disease prevention.—Millward, C. A., D. DeSantis, C-W. Hsieh, J. D. Heaney, S. Pisano, Y. Olswang, L. Reshef, M. Beidelschies, M. Puchowicz, and C. M. Croniger. Phosphoenolpyruvate carboxykinase (Pck1) helps regulate the triglyceride/fatty acid cycle and development of insulin resistance in mice. J. Lipid Res. 2010. 51: 1452–1463.

Supplementary key words  adipocytes • adipose tissue • diabetes • glucose • triglycerides

Obesity and type 2 diabetes are growing epidemics worldwide in adults and children. The incidence of type 2 diabetes increased by 10-fold from 1982 to 1994 and is expected to reach 366 million worldwide in 2030 (1, 2). Therefore, it is important to determine the causes of this disease and develop preventive strategies as well as more effective treatments. One risk factor for the development of type 2 diabetes is elevated free fatty acids (FFA) in the blood. Elevated FFAs have been shown to induce insulin resistance in peripheral tissues by inhibiting insulin-stimulated glucose uptake and glycogen synthesis (3–5).

In the fed state, triglycerides are synthesized using glyceral-3-phosphate generated from the oxidation of glucose. In the fasted state, however, insulin levels are lower and lipolysis occurs. A significant amount of FFA is reesterified to generate triglyceride in the fasted state in white adipose tissue (WAT) and liver. Since glucose is limited in the fasted state, the ability to generate glyceral-3-phosphate from glucose in adipose tissue is greatly reduced. Also, due to negligible levels of glyceral kinase (GyK) expression in adipose tissue, glyceral-3-phosphate cannot be generated from glyceral. Reshef et al. showed that gluconeogenic

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precursors, such as pyruvate, lactate, and alanine, are converted into the glycerol backbone of triglyceride by Pekl and the glyceroneogenic pathway (6) (7, 8).

In brown adipose tissue (BAT), glyceroneogenesis has been shown to generate as much as 81% of total glycerol-3-phosphate in rats. Hahn and Novak suggested that maintenance of triglyceride stores in the BAT is associated with activation of heat production. They proposed that FFA released from triglyceride were substrates for BAT mitochondria and increase the expression of uncoupling proteins (UCP) (9).

Glyceroneogenesis has also been described for the liver during fasting (10, 11). FFA released from adipose tissue is oxidized and converted to ketone bodies and carbon dioxide. However, a significant portion of the FFA (60%) is reesterified back to triglyceride and packaged into VLDL in the liver. VLDL can then be released for transport to muscle and adipose tissues (10, 12) (13). Therefore, the liver plays a central role in the triglyceride/fatty acid cycle, and the pathway of glyceroneogenesis is essential for regulation of the FFA flux in the triglyceride/fatty acid cycle (14).

The aim of this study was to examine the role of Pekl in the regulation of the triglyceride/fatty acid cycle in the liver and WAT. To accomplish this, we analyzed PPARE−/− mice (15) that have 0.2% Pekl mRNA expression of WT in WAT, 20% Pekl mRNA expression of WT in BAT, and altered regulation of Pekl mRNA expression in the liver.

METHODS

Generation of PPARE−/− mice

Mice with reduced Pekl mRNA expression in WAT were generated as previously described (15). Briefly, E14 embryonic stem (ES) cells (129/OlaHsd) were manipulated to generate male chimera mice, and these mice were mated with C57BL/6j female mice, resulting in heterozygous offspring. The mice were intercrossed (brother × sister matings) for over 20 generations to make a stable isogenic strain. Mice containing the wild-type promoter sequence were designated wild-type mice (WT) and were used as controls throughout the study. Mice with a disrupted PPARY binding site in the Pekl promoter were identified as homozygous (PPARE−/−) for the mutation. We screened the PPARE−/− mice by polymerase chain reaction (PCR) analysis using primers spanning the PPARE site within the Pekl promoter. Primer sequences were as follows: forward primer 5′-AGC CAC TTC TTC TGT ACC-3′ and reverse primer 5′-GTA AGC TTT GTT CTT AGC GG-3′. The XhoI restriction endonuclease was used to digest the PCR products and to identify the mutant allele. The mice were housed in microisolator cages and maintained on a 12-h light-dark cycle. Mice had free access to water and were fed a normal mouse diet (LabDiet, St. Louis, MO; Diet #5P76). The approximate composition of the diet was 1.08 kJ protein, 0.58 kJ fat, and 14.35 kJ carbohydrate. The diet consisted of 4100 kJ × kg body weight, collected blood from the tail vein, and measured glucose levels at 0, 15, 30, 60, and 120 min using an UltraTouch Glucose Meter.

Hyperinsulinemic-euglycemic clamp study

Clamp studies were performed at the Vanderbilt University Mouse Metabolic Phenotyping Core (MMPC) in Nashville, Tennessee. The clamp studies were conducted in WT and PPARE−/− mice at 12–14 weeks of age. The surgical procedures utilized for implanting chronic jugular vein and carotid artery catheters have been previously described (19, 20). Mice were allowed to recover from surgery for ≈5 days and were studied when body weight was restored to within 10% of the presurgery weight. The clamp was performed in mice fasted for 5 h. A continuous (0.05 µCi × min−1) infusion of HPLC-purified (21) 3H-glucose was initiated 120 min before the clamp. At t = 0 min, the basal glucose concentration and specific activity were determined, after which a constant infusion of regular human insulin (2.5 mU × kg−1 × min−1) and red blood cells (3 µl/min) was initiated. The 3H-glucose infusion rate to maintain euglycemia. Glucose levels were tested every 10 min from the carotid artery catheter at t = 80, 90, 100, and 120 min, the plasma glucose specific activity was assessed. At t = 120 min, a 12-µCi bolus of 2-deoxyglucose (DG) was administered (22). Blood (30 µl per mouse) was sampled at different times.
(t = 122, 125, 130, 135, and 145 min) to determine the arterial blood glucose and plasma DG concentrations (22). We also analyzed the concentrations of plasma FFA and glycerol under clamp conditions using the FFA and free glycerol kits (Zen-Bio Inc., Research Triangle Park, NC) following the manufacturer’s directions. At t = 145 min, a blood sample (100 µl) was obtained, and the mice were anesthetized with an infusion of sodium pentobarbital. Tissues (gastrocnemius, superficial vastus lateralis, and soleus muscles, as well as brain, epididymal fat, and liver) were removed, immediately frozen in liquid nitrogen, and stored at −70°C until further analysis. The rate of whole body glucose appearance (Rg) was calculated as the ratio of the $^{3}$H-glucose infusion rate (dpm × kg⁻¹ × min⁻¹) and plasma glucose specific activity (dpm/mg). The rate of endogenous glucose appearance during the hyperinsulinemic clamp was calculated as the difference between Rg and the exogenous glucose infusion rate. We isolated RNA from the liver (100 mg) and measured Pck1 expression as described above.

**Portal vein injection of insulin**

To observe the effects of the PPARE mutation in the Pck1 promoter on insulin signaling, insulin (1 mU × kg⁻¹ body weight) was administered to anesthetize mice by injection into the portal vein. Liver, skeletal muscle, and fat biopsies were removed before (basal) and 5 min after the insulin injection. We snap-froze the tissues for analysis by Western blot analysis.

**Islets isolation and insulin secretion**

Islets were isolated by the Islet Procurement Core Facility at Vanderbilt University (Nashville, TN) as previously described (23–25). The islets were isolated from WT and PPARE$^{-/-}$ mice by dissection of the splenic portion of the pancreas followed by digestion with collagenase P (Roche Molecular Biochemicals). After a purification procedure, islets were rinsed three times with 12 ml of RPMI-1640 media containing 10% fetal bovine serum, 100 µg/ml penicillin, 100 µg/ml streptomycin, and 5 mM glucose and then cultured in 10-cm untreated plates overnight at 37°C. As islets from WT and PPARE$^{-/-}$ mice varied in diameter, we hand-picked islets under a stereomicroscope and matched them by size. This common microscopic analysis is based on individual islet sizing, calculation of the frequency distribution, and conversion into an islet equivalent (IEQ), which is the volume of a spherical islet with a diameter of 150 mm (27). On the following day, 20 IEQs were incubated in 2 ml of RPMI-1640 media with 5 mM or 16.7 mM glucose. At the end of a static incubation, islets were collected in 1.5-ml tubes and washed three times with 1 ml of 1× PBS, and insulin was extracted in 0.2 ml of acid alcohol for 48 h at 4°C. The volume of a spherical islet with a diameter of 150 mm (27) was administered to anesthetize mice by injection into the portal vein. Liver, skeletal muscle, and fat biopsies were removed before (basal) and 5 min after the insulin injection. We snap-froze the tissues for analysis by Western blot analysis.

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**Protein isolation for Western blotting**

Proteins were isolated from the liver, kidney, WAT, BAT and skeletal muscle from PPARE$^{-/-}$ and WT mice. A piece of tissue was homogenized in 15 ml/g of tissue of ice-cold homogenizing buffer consisting of 20 mM Tris-HCl, pH 7.6, 0.1 mM EDTA, 0.5 mM EGTA, 1.0% Triton-X, 250 mM sucrose, and 50 µl/5 ml protease inhibitor mixture as described previously (28). The concentration of protein was measured with the Bio-Rad protein assay reagent using BSA as a standard.

**Electrophoresis and Western blotting**

We sonicated the protein samples for 20 s, and 10–20 µg of protein was diluted in Laemmli sample buffer (100 mM Tris-HCl, pH 6.8, 20% β-mercaptoethanol, 4% SDS, 0.2% bromophenol blue, and 20% glycerol) and separated by 10% SDS-PAGE. For analysis of PCK1, 0.8 mg of liver and kidney proteins and 10 mg of WAT and BAT proteins were loaded. For AKT phosphorylation analysis, 13 µg of liver, WAT, and skeletal proteins were loaded on 10% SDS-PAGE gels. Proteins were electrophoretically transferred to Immobilon® polyvinylidene difluoride membranes, and gels were stained with Coomassie stain (45% methanol, 10% acetic acid, and 2.5% Coomassie Blue R250). The polyvinylidene difluoride membrane was incubated with antibody to PCK1 (1.5 µg/ml) diluted 1:1000 in blocking buffer for 4 h at 22°C, followed by extensive washing with Tris-buffered saline (150 mM NaCl, 10 mM Tris, and 0.05% Tween 20). The primary PCK1 antibody was a generous gift from Richard W. Hanson (Case Western Reserve University). The blots were incubated with 1:10,000 secondary antibody linked to horseradish peroxidase (Santa Cruz Biotechologies, Inc., Santa Cruz, CA) in 10 ml of blocking buffer for 1 h at 22°C and washed again. Normalization for loading differences was done with an HSC70 antibody diluted at 1:16,000 (Santa Cruz Biotechnology) and anti-mouse IgG-HRP conjugated secondary antibody at 1:10,000 (Santa Cruz Biotechnology). Immunoreactive proteins were detected using the Super-Signal Chemiluminescent Substrate® Kit, and the density of the immunoreactive bands was measured by scanning densitometry using the UN-Scan-IT software (Silk Scientific, Inc., Orem, UT). For portal vein injection of insulin studies, the polyvinylidene difluoride membrane was incubated with antibodies to phosphorylated AKT-Ser$^{473}$, phosphorylated AKT-Thr$^{308}$ and total AKT (Cell Signaling Technology, Beverly, MA) diluted in blocking buffer for 4 h at 22°C. The blots were washed and incubated with secondary antibody, and immunoreactive proteins were detected as described above.

**Adipocyte lipolysis**

Wild-type and PPARE$^{-/-}$ male mice were sacrificed, and epididymal fat pads were removed and weighed. Adipose cells were isolated from gonadal fat pads (2–3 mice per experiment) by collagenase digestion (1 mg/ml, collagenase CLS Type I, Worthington Biochemical Corp., Lakewood, NJ). Cells were incubated at 37°C with constant shaking in Krebs-Ringer-Phosphate (KRP) buffer (50 mM HEPES, pH 7.4, 128 mM NaCl, 4.7 mM KCL, 1.25 mM CaCl₂, 1.25 mM MgSO₄, 10 mM sodium phosphate and 2.0 mM pyruvic acid) and 2.5% BSA (BSA)-FFA free and in the absence (basal) or presence of insulin (100 nM) or epinephrine (10 µM). Concentrations of FFA released from the adipocytes were measured using a commercial kit (Zen-Bio Inc., Research Triangle Park, NC) following the manufacturer’s directions. Total cell protein was prepared from adipocytes using a commercial lysozyme buffer (Promega, Madison, WI). The protein concentration was measured with the Bradford assay (Bio-Rad Protein Assay) using BSA as a standard.

**Determination of glycerol kinase activity**

GyK activity was measured as previously described (29, 30). Briefly, WAT, BAT, or liver was homogenized in extraction buffer (50 mM HEPES, pH 7.8, 40 mM KCl, 11 mM MgCl₂, 1 mM EDTA, and 1 mM DTT) on ice. The samples were centrifuged at 15,000 g for 15 min at 4°C, and 10 µg of protein was used for the enzymatic assay. The protein samples were incubated with 50 µl of assay buffer (50 mM Tris-HCl, pH 7.2, 5 mM ATP, 10 mM MgCl₂, 100 mM KCl, 2.5 mM DTT, 4 mM glycerol, and 500 µmol/µl $^{3}$H-glycerol) for 90 min at 37°C. The reaction was terminated with
100 µl stop solution (ethanol:methanol, 9:3). Equal amounts of samples (20 µl) were spotted onto DE81 Whatman filters (Whatman). The filters were air-dried and washed in water overnight. Radioactivity on the filters was measured by liquid scintillation counting.

Mitochondrial enzyme activity

BAT (20 mg) was homogenized in 1 ml of MSM-EDTA buffer (220 mM mannitol, 70 mM sucrose, 5 mM Mops, and 2 mM EDTA, pH 7.4) supplemented with 1 mg cholate/1 mg wet wt and 1 µl/ml mammalian protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO) (31). Mitochondrial content was assessed by measuring the succinate dehydrogenase (SCD) and citrate synthase (CS) activities by established spectrophotometric methods (31). Activities were normalized to the tissue wet weight (mg) and protein (µg/µl) and expressed as nmol/min/mg tissue or nmol/min/total protein.

Measurement of ATP concentrations in adipose tissues

In male PPARE−/− and WT mice, ATP tissue concentrations were measured in BAT and WAT. Samples were dissected from mice under anesthesia and flash-frozen. ATP tissue concentrations were analyzed enzymatically from 25–30 mg of tissue as previously described (32) and reported as nmol/mg wet tissue weight.

Measurement of triglyceride concentrations and synthesis by stable isotopes

The Mouse Metabolic Phenotyping Center (MMPC) of Case Western Reserve University (Cleveland, OH) measured the triacylglyceride and newly synthesized triglyceride levels. To Western Reserve University (Cleveland, OH) measured the triacylglycerides and 1 µl/ml mammalian protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO) (31). Mitochondrial content was assessed by measuring the succinate dehydrogenase (SCD) and citrate synthase (CS) activities by established spectrophotometric methods (31). Activities were normalized to the tissue wet weight (mg) and protein (µg/µl) and expressed as nmol/min/mg tissue or nmol/min/total protein.

Rectal body temperatures were taken at 0, 1.5, 3, and 4 h. At the conclusion of the study, the mice were killed, and plasma and tissues were collected. We measured plasma FFA and free glycerol as described above. RNA was isolated from BAT, and UCP-1 mRNA expression was measured by qRT-PCR as described above. The utilized primer sequences for UCP1 (IDT, Corabille, IA) are presented in supplementary Table I.

Measurement of triglyceride covalently linked to glycerol measures the amount of newly synthesized triglyceride-glycerol. We calculated the total triglyceride pool size factor (experimentally determined from the M2/M1 ratio of triglyceride-glycerol) was calculated using the following equation: % total newly made triglyceride-glycerol = [(total 3H-labeled triglyceride-glycerol x (14N-labeled body water x n))/100] x 100 where n is the number of exchangeable hydrogens, which is assumed to 22 (34, 35). The percentage of total newly made triglyceride-glycerol was calculated using the following equation: % total newly made triglyceride-glycerol = [(3H-labeled triglyceride-glycerol x (14N-labeled body water x n))/100] x 100 where 3H-labeled triglyceride-glycerol is the M1 isotopomer, 14N-labeled water is the average amount labeled in a given mouse, and α is the exchange factor (experimentally determined from the M2/M1 ratio of triglyceride-glycerol). We calculated the total triglyceride pool size (µmol/g tissue) in the tissues using the following equation: Total pool size of triglyceride = [(3H-labeled triglyceride-glycerol x (14N-labeled water x n))/100] x 100.

Cold exposure of mice

Adult male WT and PPARE−/− mice were cold-challenged by placing individually housed mice into a cold room (4°C) for 4 h.

Statistical analysis

Results are expressed as the means ± SEM. For analysis of two means, we used the Student t-test. For analysis of 2 × 2 parameters, we used two-way ANOVA with Bonferroni’s adjustment. For three comparisons, we analyzed the parameters by one-way ANOVA with Bonferroni’s adjustment. The response to GTT was measured by determining the areas under the curve above baseline (AUC) using GraphPad Prism 4. Data that failed Bartlett’s test for variance were analyzed with the Kruskal-Wallis test.

RESULTS

PEPCK-C mRNA expression in PPARE−/− mice

We measured Pck1 mRNA in various tissues from food-deprived WT and PPARE−/− adult male mice by quantitative real-time RT-PCR (Fig. 1A). The Pck1 mRNA levels were decreased in the liver (63% of WT), in WAT (0.2% of WT) and in BAT (6.0% of WT), yet the expression of renal Pck1 was unaffected. It has previously been shown that Pck1 mRNA levels reflect the PCK1 protein concentration and enzyme activity (36). To determine if the protein levels were also altered, we analyzed PCK1 by Western blot analysis (Fig. 1B). We found that PCK1 was ablated in WAT and greatly reduced (33% of WT) in BAT. The liver and kidney proteins, however, were equal to WT levels of PCK1.

Effect of altered PEPCK gene expression

To determine the metabolic consequences of altered Pck1 gene expression in WAT, we measured the fasting levels of glucose, triglycerides, free fatty acids, β-hydroxybutyrate, and cholesterol in the blood as well as triglyceride levels in the livers of fasted male mice (Table 1). Male PPARE−/− mice had elevated levels of plasma glucose (13% more than WT), FFA (26% more than WT), β-hydroxybutyrate...
PPARE−/− mice exhibit insulin resistance

To establish whether we could observe metabolic differences in the fed state, we analyzed peripheral insulin resistance by GTT (Fig. 2A). The means of each time point were compared with WT mice and were significantly different at several time points (*P < 0.05). The PPARE−/− mice had reduced glucose clearance as demonstrated by the greater AUC compared with WT mice (25020 ± 1202 and 19460 ± 1309 mg/dl × 120 min for PPARE−/− and WT mice, respectively). The AUCs were significantly different (P < 0.05). Reduced glucose clearance may be caused by reduced insulin secretion in response to glucose. Therefore, we measured plasma insulin concentrations in response to a bolus glucose injection in WT and PPARE−/− mice. WT and PPARE−/− mice had similar levels of insulin at 30 min after glucose injection (Fig. 2B); however, at 0 min, the PPARE−/− mice exhibited significantly reduced plasma insulin (P < 0.05) (Fig. 2B). This suggests that insulin secretion may be attenuated. To analyze islet function, β-cells were isolated and treated with 5 mM and 16.7 mM glucose. The β-cells from PPARE−/− mice contained and secreted more insulin at 16.7 mM glucose compared with WT mice (Fig. 2C, D).

Peripheral insulin resistance suggests reduced signaling in the AKT pathway. To determine if the insulin-signaling cascade was affected, we administered portal vein injection of insulin (Fig. 3). The insulin-signaling pathway was analyzed by phosphorylation of AKT-Ser173 and AKT-Thr308 and normalized to the total AKT protein. We found that the phosphorylation of AKT-Ser173 and AKT-Thr308 was greatly reduced in the skeletal muscle and liver (Fig. 3B, C), while phosphorylation of AKT-Thr308 was greatly reduced in WAT of PPARE−/− mice (Fig. 3A) (P < 0.05).

Reduced glucose uptake in muscle and adipose

To further characterize the insulin resistance, hyperinsulinemic-euglycemic clamp studies were conducted in male PPARE−/− and WT mice. The blood glucose was clamped at 120 mg/dl through glucose administration (Fig. 4A), and the glucose infusion rate (GIR) was monitored over 2 h. The PPARE−/− mice exhibited a 40% reduction in GIR on average over the course of the clamp (Fig. 4B). The difference in GIR was evident at 10 min, but compared with WT mice, insulin suppression of endogenous glucose Rg (endogenous Rg) was significantly
decreased, suggesting that the infused insulin did not suppress gluconeogenesis (Fig. 4F). We measured the concentration of the Pck1 mRNA in the liver from mice at the end of the clamp. The levels of Pck1 were greater in PPARE−/− mice (44% more than WT) even with the insulin infusion during the clamp (Fig. 5C). Therefore, PPARE−/− mice exhibited insulin resistance in the liver, muscle, and WAT compared with WT mice.

**Lipolysis in PPARE−/− and WT mice**

To ascertain the source of the increased fasting FFA, primary adipocytes were isolated from WT and PPARE−/− mice (Fig. 5A). The FFA released in response to insulin (65% more than WT) was greatly increased. Insulin failed to repress the release of FFA in the PPARE−/− mice. We also measured the concentration of glycerol and FFA under clamp conditions. We found that FFA release was greater in PPARE−/− mice (24% more than WT) and glycerol release was greater as well (45% more than WT) (Fig. 5B).

**Synthesis of triglyceride in PPARE−/− and WT mice**

As mutation of the PPARE region in the promoter of Pck1 alters its hepatic regulation, we analyzed its effect on triglyceride synthesis as well. Triglyceride and palmitate synthesis rates were measured with the 2H2O method (Fig. 6). Western blot analysis indicated that PCK1 was lower in PPARE−/− mice. The contribution of de novo lipogenesis to newly synthesized palmitate was equivalent in both genotypes (Fig. 6C). We found that PPARE−/− mice had 81% less ATP than WT levels. CS and SDH activities were measured and normalized by wet tissue weight (Table 2) and total protein (data not shown), and the activities were equal in WT and PPARE−/− mice. Thus the mitochondrial mass was unchanged, but there may be some perturbations in mitochondrial energetics because ATP levels were so dramatically reduced.

**Alterations in BAT metabolism**

Western blot analysis indicated that PCK1 was lower in BAT of PPARE−/− mice. To determine the metabolic consequence of altered Pck1 expression, we measured the ATP concentration in BAT (Table 2). We found that PPARE−/− mice had 81% less ATP than WT levels. CS and SDH activities were measured and normalized by wet tissue weight (Table 2) and total protein (data not shown), and the activities were equal in WT and PPARE−/− mice. The measured gapminder expression after fasting and cold-challenge and found that PPARE−/− mice had increased UCP-1 expression, we measured plasma FFA at 0 min and 30 min after glucose injection in male mice. Values are means ± SEM of n = 7-10 mice. *P < 0.05 compared with WT at that time. B: Plasma insulin concentrations at 0 min and 30 min after glucose injection in male mice. Values are means ± SEM of n = 7-10 mice. Subscripts without a common letter differ, P < 0.05. C: Insulin secretion and D: Percentage of insulin concentration from static isolation of WT and PPARE−/− islets. Values are the means ± SEM of n = 5-6 experiments. Subscripts without a common letter differ, P < 0.05. BAT, brown adipose tissue; KID, kidney; LIV, liver; WAT, white adipose tissue; WT, wild type.

**Insulin resistance in PPARE−/− mice**

Two hours after an i.p. glucose injection in male mice. Values are means ± SEM of n = 7–10 mice. Subscripts without a common letter differ, P < 0.05 compared with WT at that time. B: Plasma insulin concentrations at 0 min and 30 min after glucose injection in male mice. Values are means ± SEM of n = 7-10 mice. Subscripts without a common letter differ, P < 0.05. C: Insulin secretion and D: Percentage of insulin concentration from static isolation of WT and PPARE−/− islets. Values are the means ± SEM of n = 5-6 experiments. Subscripts without a common letter differ, P < 0.05. BAT, brown adipose tissue; KID, kidney; LIV, liver; WAT, white adipose tissue; WT, wild type.

To confirm that the alterations in BAT did not affect overall energy expenditure, we measured energy expenditure by indirect calorimetry over a 24-h period (light and dark cycle) and found relatively minor differences for oxygen consumption in the fed and fasted conditions (supplementary Figs. I and II). Food intakes were also measured and were found to be similar in WT and PPARE−/− mice (4.01 ± 0.14 and 4.2 ± 0.24 g/mouse/24 hr for WT and PPARE−/− mice, respectively; n = 8 mice). The calculated

**Fig. 2.** PPARE−/− mice are insulin resistant. A: Glucose tolerance tests (GTT) in WT and PPARE−/− mice. Values are the means ± SEM of n = 7-10 mice. *P < 0.05 compared with WT at that time. B: Plasma insulin concentrations at 0 min and 30 min after glucose injection in male mice. Values are means ± SEM of n = 7-10 mice. Subscripts without a common letter differ, P < 0.05. C: Insulin secretion and D: Percentage of insulin concentration from static isolation of WT and PPARE−/− islets. Values are the means ± SEM of n = 5-6 experiments. Subscripts without a common letter differ, P < 0.05. BAT, brown adipose tissue; KID, kidney; LIV, liver; WAT, white adipose tissue; WT, wild type.
The complex pathogenesis of type 2 diabetes is due to abnormalities that involve insulin action at the peripheral tissues and insulin production in cells (37). Genetic factors play key roles in the development of type 2 diabetes as well. Beale et al. found two C/T single nucleotide polymorphisms (SNP) in complete linkage disequilibrium at positions -1097 bp and -967 bp of the *Pck1* promoter that are associated with obesity and type-2 diabetes. Patients with T/T polymorphisms have higher HbA1c and higher fasting glucose levels (38). The region of the identified polymorphism in these patients is located within the same PPARE site of the *Pck1* promoter that was deleted in the PPARE/−/− mice. Our data in the PPARE/−/− mice suggest that dysregulation of *Pck1* in the liver and WAT contributes to the development of insulin resistance.

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**DISCUSSION**

We hypothesized that the PPARE region of the *Pck1* promoter is essential for the regulation of triglyceride/fatty acid flux between adipose and liver tissues and is important for the maintenance of glucose and lipid homeostasis for the prevention of insulin resistance.

**Fig. 3.** Reduced AKT phosphorylation in PPARE/−/− mice. Western Blot analysis of AKT phosphorylation in (A) WAT, (B) skeletal muscle, and (C) liver before (basal) and after portal vein injection of insulin (+INS) (n = 6 mice per group). The values from the quantified Western blots are shown in the bar graphs. The values are the means ± SEM of n = 3. *P < 0.05 compared with WT. WAT, white adipose tissue; WT, wild type.

respiratory quotient was similar in WT and PPARE/−/− mice, suggesting similar substrate utilization for energy. However, in the first light cycle, we found a slight decrease in heat production in PPARE/−/− mice, which indicates that mitochondrial function might be altered in PPARE/−/− mice, but further studies are needed.
In adult male PAPRE\textsuperscript{−/−} mice, the levels of plasma FFA acids and β-hydroxybutyrate were higher during food deprivation compared with WT mice. This finding was expected as these mice have 2% of the WT level of \textit{Pck1} mRNA in WAT and lack the ability to reesterify FFAs to triglycerides through glyceroneogenesis. The increased FFAs would be oxidized to ketone bodies in the liver. Previously, Olswang et al. established that PAPRE\textsuperscript{−/−} mice had a tissue-specific deletion of \textit{Pck1} in WAT only. They found only one out of four PAPRE\textsuperscript{−/−} mice had lipodystrophy (15) with mild insulin resistance. However, we found that the PAPRE region of the \textit{Pck1} promoter regulates \textit{Pck1} expression in BAT and WAT. After intercrossing (brother × sister mating) for over 20 generations to establish a stable isogenic background, the phenotype of lipodystrophy had been lost. Reshef also reported that backbreeding PAPRE\textsuperscript{−/−} mice into the C57Bl/6J background resulted in loss of the lipodystrophy phenotype (unpublished observation). Our data in the current study indicated that ablation of \textit{Pck1} in WAT and decreased \textit{Pck1} expression in BAT of PAPRE\textsuperscript{−/−} mice increased FFA release but did not result in lipodystrophy. This suggests that genetic modifier(s) from the genetic background (B6 × 129/OlaHsd) are required in addition to ablation of \textit{Pck1} expression in adipose tissue to produce lipodystrophy.
phenotypic consequences is the varied insulin resistance in the ir knockout mice, which encodes for the insulin receptor. Kido et al. have shown that the effect of the ir mutation is modified by genetic background (39). In B6 mice, the ir null mutation causes mild hyperinsulinemia. In contrast, in 129/Sv mice, the ir mutation causes severe hyperinsulinemia (39, 40). Thus mice with different genetic backgrounds can have different metabolic phenotypes as we found in our PPARE−/− in a different background.

The amounts of newly synthesized triglyceride in WAT and liver were higher in PPARE−/− mice (Fig. 6A). This was unexpected since the glycerol-3-phosphate backbone for triglyceride synthesis is usually generated from either glucose (through glycolysis) or lactate and alanine (through gluconeogenesis). The PPARE−/− mice had reduced glucose uptake in WAT during the hyperinsulinemic-euglycemic clamp and ablated PCK1 expression, so both glycolysis and gluconeogenesis would have attenuated production of glycerol-3-phosphate. However, we found that Gyk activity was significantly increased in WAT of PPARE−/− mice, thus increasing glycerol-3-phosphate synthesis from glycerol stored in lipid droplets. The increased labeling and packaging of VLDL in livers of PPARE−/− mice would also result in increased flux of triglyceride into adipose tissue.

Elevated FFA levels have been shown to cause insulin resistance by impairing insulin signaling through PI(3)K as measured by AKT phosphorylation (41, 42). During the hyperinsulinemic-euglycemic clamp, PPARE−/− mice have increased plasma FFA and glycerol levels, suggesting a lack
of response to insulin for repression of lipolysis (Fig. 5B). Thus, PPARE \(^{-/-}\) mice in both the fed and fasted states have increased plasma FFA concentrations. We found that insulin injections into the portal vein resulted in dramatically reduced hepatic and skeletal muscle AKT-Ser\(^{473}\) and AKT-Thr\(^{308}\) phosphorylation in PPARE \(^{-/-}\) mice; therefore, PPARE \(^{-/-}\) mice have reduced insulin signaling in the tissues tested. Cassuto et al. have shown that insulin treatment of isolated hepatocytes resulted in phosphorylation of FOXO1 and disassociation from the AF2, dAF1 sites in the promoter or that hepatic insulin regulation may require the PPARE region of the promoter (43). This suggests that in our study the reduced insulin signaling as well as a loss of the PPARE site contributed to dysregulation of Pek1 and the triglyceride–fatty acid cycle.

Insulin resistance in the liver also results in an inability to suppress hepatic glucose output. In the hyperinsulinemic-euglycemic clamp, endo \(R_\) is an indicator of glucose output in the liver. We found that suppression of hepatic glucose output by insulin infusion was reduced in the PPARE \(^{-/-}\) male mice, indicating hepatic insulin resistance. In the fed state, insulin inhibits gluconeogenesis by suppressing the hepatic transcription of Pek1 and glucose-6-phosphatase (44). The PPARE \(^{-/-}\) mice have increased hepatic Pek1 expression in response to the hyperinsulinemic-euglycemic clamp (Fig. 5C). This suggests that hepatic insulin regulation may require the PPARE region of the Pek1 promoter or that hepatic Pek1 regulation may be secondary to alterations in adipose tissue.

Alterations in BAT metabolism may also contribute to insulin resistance. In PPARE \(^{-/-}\) mice, the BAT had greatly reduced expression of Pek1 mRNA and protein. It has been established that BAT has significant gluconeogenesis activity, which regulates the rate of reesterification of fatty acids. Reduced reesterification of FFAs should increase UCP-1 expression (11, 45); however, we did not find a dramatic increase in UCP-1 in the fasted state for PPARE \(^{-/-}\) mice. One possible explanation is that BAT has significant Gyk activity, and the PPARE \(^{-/-}\) mice may depend more on Gyk activity for the generation of glycerol-3-phosphate to maintain triglyceride stores. We found that the food-deprived PPARE \(^{-/-}\) mice had a decreased ATP concentration in BAT despite having similar mitochondrial mass. Burgess et al. analyzed PCK flux and regulation of energy metabolism and gluconeogenesis by NMR analysis (46). They suggested that PCK1 flux correlates with energy generated in the TCA cycle. In mice with ablated hepatic PCK1, ATP levels were greatly reduced. Although their data was focused on the liver, this may explain the reduced ATP generation in BAT of fasted PPARE \(^{-/-}\) mice. The indirect calorimetry data suggested moderate alterations in overall energy expenditures, which support that mitochondrial function may be altered in PPARE \(^{-/-}\) mice. However, during cold exposure, the PPARE \(^{-/-}\) mice maintained their body temperatures for 4 h, while WT mice had a dramatic drop in body temperature. Since gluconeogenesis is compromised in the PPARE \(^{-/-}\) mice, they had increased plasma FFA release from WAT during the stress of cold exposure, which in turn induced UCP-1 expression in BAT to generate nonshivering thermogenesis. The increase in UCP-1 in PPARE \(^{-/-}\) mice would increase fatty acid oxidation and generate heat production in mitochondria to maintain the body temperature. More experiments are needed to determine if the PPARE \(^{-/-}\) mice...

### Table 2: ATP concentration and enzyme activity in WT and PPARE\(^{-/-}\) mice

<table>
<thead>
<tr>
<th>Tissue</th>
<th>ATP concentration (nmol/mg ww)</th>
<th>CS activity (nmol/min/mg ww)</th>
<th>SDH activity (nmol/min/mg ww)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BAT</td>
<td>7.91 ± 2.0</td>
<td>71.17 ± 2.54</td>
<td>0.57 ± 0.3</td>
</tr>
<tr>
<td>WT</td>
<td>1.58 ± 0.14</td>
<td>74.52 ± 1.00</td>
<td>0.46 ± 0.04</td>
</tr>
<tr>
<td>PPARE(^{-/-})</td>
<td></td>
<td></td>
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</tbody>
</table>

ATP concentration, CS activity, and SDH activity in BAT. Values are the mean ± SEM of n = 4. Abbreviations: BAT, brown adipose tissue; CS, citrate synthase; SDH, succinate dehydrogenase, WT, wild type. *P < 0.05 compared with WT.
have altered mitochondrial function, which will be the focus of future study.

Finally, we analyzed the consequence of alterations in the triglyceride/fatty acid cycle on insulin secretion from islets. We found that, in the isolated ß-islets from PPARE−/− mice, there was a 2-fold increase in insulin secretion at 16.7 mM glucose compared with WT mice. However, PPARE−/− mice had reduced insulin levels after fasting, but 30 min after IP glucose injection there were no differences in plasma insulin levels between PPARE−/− and WT mice. One possible explanation for this discrepancy is that isolation of islets from the pancreas severs the connections between the islet vasculature and the systemic circulation (47). In addition, as the isolated islets measure synthesis of insulin and insulin secretion while whole-body measurements of insulin assess the overall flux of insulin (synthesis, secretion, and degradation), it may be that PPARE−/− mice have increased clearance of insulin. Insulin clearance includes both first-pass hepatic and peripheral insulin uptake and degradation in all insulin-sensitive tissues (48). However, hepatic uptake is influenced by both physiological and pathophysiological factors that are not completely understood (49, 50). This process involves binding to a specific membrane receptor, internalization, intracellular compartmentalization of insulin-receptor complex, and proteolytic degradation by insulin degradation enzyme (IDE) (48). Further studies are needed to fully understand the mechanism of reduced fasting insulin in PPARE−/− mice.

Clearly the PPARE−/− islets have adapted to the insulin resistance. Several potential mediators have been suggested to be signals for the ß cells to respond to insulin resistance. These include glucose, FFA, autonomic nerves, fat-derived hormones, and the glut hormone glucagon-like peptide-1 (GLP-1). FFAEs have been shown to augment glucose-stimulated insulin secretion in perfused rat pancreas (51). Since we found elevated FFAs in PPARE−/− mice, it is possible that FFAs may be involved in the adaptation of insulin secretion to insulin resistance. Thus, regulation of the triglyceride–FFA cycle may also be critical for maintaining insulin sensitivity in the islets.

CONCLUSION

We have shown that the PPARE region of Pek1 contributes to the regulation of the triglyceride–FFA cycle in WAT and liver. The PPARE region of the Pek1 promoter is required for adipose tissue expression of Pek1. Ablation of Pek1 in WAT and reduced expression of Pek1 in BAT resulted in increased plasma FFA, thus contributing to the development of insulin resistance. In addition, hepatic insulin resistance may be a result of alterations in WAT that cause insulin resistance, or it may be caused by altered regulation of hepatic Pek1 due to the mutated PPARE region of the Pek1 promoter. Further studies are required to fully characterize these mechanisms. Therefore, investigation of Pek1 expression and its regulation in the triglyceride/fatty acid cycle is necessary for our understanding of maintenance of glucose and lipid homeostasis and disease prevention.

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