Reduced Milk Triglycerides in Mice Lacking Phosphoenolpyruvate Carboxykinase in Mammary Gland Adipocytes and White Adipose Tissue Contribute to the Development of Insulin Resistance in Pups\textsuperscript{1,2}

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Abstract

Obesity and type 2 diabetes are growing problems worldwide in adults and children. In this study, we focused on understanding the patterning of insulin resistance as a result of altered perinatal nutrition. We analyzed mice in which the binding site for PPAR\textsubscript{\gamma} was deleted from the promoter of the cytosolic phosphoenolpyruvate carboxykinase gene (Pck1) (PPARE\textsuperscript{-/-}). We analyzed pups from dams with the same genotype as well as fostered and cross-fostered pups. Pck1 expression and triglyceride concentration in the milk were measured. The PPARE mutation reduced Pck1 expression in white adipose tissue (WAT) to 2.2\% of wild type (WT) and reduced Pck1 expression in whole mammary gland tissue to 1\% of WT. The female PPARE\textsuperscript{-/-} mice had reduced lipid storage in mammary gland adipocytes and in WAT, resulting in a 40\% reduction of milk triglycerides during lactation. Pups from PPARE\textsuperscript{-/-} dams had insulin resistance as early as 14 d after birth, a condition that persisted into adulthood. WT pups fostered by PPARE\textsuperscript{-/-} dams had lower body weights and plasma insulin concentrations compared with WT pups reared by WT dams. PPARE\textsuperscript{-/-} pups fostered by WT dams had improved glucose clearance compared with pups raised by PPARE\textsuperscript{-/-} dams. PPARE\textsuperscript{-/-} and PPARE\textsuperscript{+/-} dams also patterned newborn pups for reduced growth and insulin resistance in utero. Thus, the in utero environment and altered nutrition during the perinatal period cause epigenetic changes that persist into adulthood and contribute to the development of insulin resistance. J. Nutr. 139: 2257–2265, 2009.

Introduction

Obesity and type 2 diabetes are growing problems worldwide, especially for children (1,2). The incidence of type 2 diabetes increased 10-fold from 1982 to 1994 (3). Although obesity levels did not increase significantly from 2005 to 2006, the BMI for adults and children remains high (4–6). Given their high incidence, it is important to determine the causes of obesity and type 2 diabetes and develop preventive strategies.

One theory addressing the development of obesity and insulin resistance is the thrifty phenotype hypothesis, which associates poor fetal and infant growth with increased risk for impaired glucose tolerance and metabolic syndrome (7,8). In previous studies, lactating rat dams that were fed a low-protein diet patterned their offspring for permanent growth restriction. The offspring become diabetic, develop insulin resistance, and are hypertensive (9–11). Overnutrition has also been shown to imprint metabolic changes in newborn pups. Rat pups from smaller litters develop hyperinsulinemia in conjunction with increased body growth, making them susceptible to obesity as adults (12). Thus, the early perinatal environment contributes to determining the susceptibility to future diseases (7,13).

In addition to environmental factors, there is evidence that genetics also contributes to the development of obesity and diabetes. Overexpression of phosphoenolpyruvate carboxykinase (Pck1)\textsuperscript{5} by a tissue-specific transgene for white adipose tissue (WAT) results in the development of obesity (14). The role of Pck1 in WAT has been clearly defined as glyceroneogenic.
Over 30 years ago, Ballard et al. (15) and Reshef et al. (16,17) demonstrated that, when the rat epididymal fat pad is incubated in vitro with pyruvate, the amount of FFA released is reduced by 65%. However, the rate of lipolysis remains unaffected. In WAT, glycerol is released during lipolysis, but it cannot be phosphor-ylated in preparation for triglyceride synthesis because the tissue manifests negligible glycerol kinase activity. Reshef et al. (17) determined that, during fasting, gluconeogenic precursors such as pyruvate and alanine are converted into the glycerol backbone of triglycerides. The pathway was named glyceroneogenesis. Glyceroneogenesis has also been described to occur in liver and brown adipose tissues (BAT) during times of food deprivation (18,19).

The promoter for Pck1 has been extensively studied. The region 1 kb upstream of the transcription start site is designated distal AF1 (also called PPARE) and binds PPAR region 1 kb upstream of the transcription start site is designated 2258 Hsieh et al.

resulting in heterozygous offspring. The mice were intercrossed (brother chimeric mice; these mice were mated with C57BL/6J female mice, WAT were generated as previously described (24). Briefly, E14 embry-mice (WT) and were used for controls throughout the study. Mice with a Mice containing wild-type promoter sequence were designated wild type either heterozygous (PPARE+/−)

Generation of PPARE−/− mice. We analyzed metabolic variables in pups from WT and PPARE−/− dams at d 14 after birth. The litters were culled to 6 pups/litter 1 d after birth to standardize the litters for all perinatal studies. We chose to test pups after glucose injection so that all of the pups would be in the same metabolic state (fed state).

For patterning studies, the body weights were measured on the first day after birth for the following mice: WT pups from WT dams, WT pups from PPARE−/− dams, PPARE−/− pups from WT dams, and PPARE−/− pups from PPARE−/− dams. We weighed pups from 3 separate litters for each condition. PPARE−/− pups generated from WT, PPARE−/−, and PPARE−/− dams were also tested. We chose to compare growth of the same genotype, PPARE−/−, so that the only variables would be nutrition during lactation and the in utero environment. To accomplish this, WT, PPARE−/−, and PPARE−/− females were mated to PPARE−/− males to eliminate paternal contribution to the pups.

For foster pup studies, WT pups were given to foster PPARE−/− dams and PPARE−/− pups were given to foster WT dams 1 d after birth. To verify that the differences were due to altered nutrition during lactation and not to the stress of fostering, we gave WT pups to foster WT dams and PPARE−/− pups to foster PPARE−/− dams.

Isolation of adipocytes and mammary epithelial cells. Epithelial cells and mammary gland adipocytes were isolated as previously described (26). Briefly, we dissected thoracic mammary glands from 8-wk-old virgin female mice (3 glands for each preparation), removed the lymph nodes, and minced the mammary glands with a razor blade. The tissue fragments were incubated at 37°C with gentle shaking for 1 h in DMEM:F12 medium containing 0.2% collagenase. Three batches of isolated epithelial cells and 3 batches of isolated adipocytes were prepared. The cells were isolated from the suspension by centrifugation at 176.08 × g for 10 min. The adipocytes and the epithelial cells were collected separately, washed extensively, and endothelial contaminants were removed by sedimentation. Mammary gland adipocytes and epithelial cells were collected and stored them in RNA later solubilization buffer (Qiagen) for isolation of RNA.

Adipocyte lipolysis. We harvested the thoracic mammary glands and isolated adipocytes from fat pads of WT and PPARE−/− female mice as described above. The cells were incubated at 37°C with constant shaking in Krebs-Ringer-Phosphate buffer (50 mmol/L HEPES, pH 7.4, 128 mmol/L NaCl, 4.7 mmol/L KCL, 1.25 mmol/L CaCl2, 1.25 mmol/L MgSO4, 10 mmol/L sodium phosphate, and 2.0 mmol/L pyruvic acid) and 2.5% fatty acid-free bovine serum albumin (BSA) in the absence (basal) or presence of epinephrine (10 μmol/L). Glycerol concentrations released from the adipocytes into the Krebs-Ringer-Phosphate buffer were measured using a commercial kit according to the manufacturer’s instructions [TG glycerol phosphate oxidase (GPO) reagent, Pointe Scientific]. The total cell protein was isolated from adipocytes using commercial lysis buffer (Promega). We estimated the protein concentration with a Bradford assay (Bio-Rad Protein assay) using BSA as a standard.

Materials and Methods

Generation of PPARE−/− mice. Mice with reduced Pck1 expression in WAT were generated as previously described (24). Briefly, E14 embry-onic stem cells (129/OLA/Hsd) were manipulated to generate male chimeric mice; 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 congenic strain. Mice containing wild-type promoter sequence were designated wild type mice (WT) and were used for controls throughout the study. Mice with a disrupted PPARY binding site in the Pck1 promoter were identified as either heterozygous (PPARE−/+) or homozygous (PPARE−/−) for the mutation. We screened for PPARE−/− mice by PCR analysis using primers spanning the PPARE site within the Pck1 promoter. Primer sequences were as follows: forward primer 5′-AGC CACTTC TTC TGT ACC-3′ and reverse primer 5′-GTA AGC TTT GTT CTG ACA GG-3′. We used the Xhol restriction endonuclease 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; diet no. 5P76). The proximate composition of the diet was 1.08 kJ protein, 0.58 kJ fat, and 14.33 kJ carbohydrate. The diet consisted of 4100 kJ/kg gross energy (25). Food intake was calculated as g food consumed/kg body weight over a 24-h period. All experimental protocols were approved by the Case Western Reserve University Institutional Animal Care and Use Committee (IACUC).
mammary gland, and isolated adipocytes from the mammary gland with the RNeasy Lipid Tissue Mini kit (Qiagen). We synthesized single-strand cDNA from 2 μg of total RNA with random hexamer primers and MMTV reverse transcriptase (Ambion) and amplified cDNA using Syber Green PCR Core reagent mix (Applied Biosystems). Real-time quantitative RT-PCR was performed in a Chromo4 Cycler (MJ Research) and relative amounts of mRNA were determined using a linear regression from the standard curves derived maximum method with Opticon Monitor 3 software (MJ Research) as previously described (27).

The observed mRNA expression levels were normalized to 185 rRNA levels. The utilized primer sequences (IDT) were as follows: FcKI (forward primer 5’-GTG CGT CAG TGC ATG TGC CGT GG-3’, reverse primer 5’-CTG GCT GAT TCT GGT CAG G-3’), glyceraldehyde-3-phosphate dehydrogenase (forward primer 5’-CCG AGA CCA GCC GTG TTA AGG-3’, reverse primer 5’-GTC CAC TGC TCC CAA TGG-3’), and 18s rRNA (forward primer 5’-AGA AAC GGC TAC CAC ATC CAA G-3’, reverse primer 5’-GAC TCA TTC CAA TTA CAG GGC C-3’).

Biochemical assays. Female mice were deprived of food overnight and then heart punctures were performed under anesthesia (249 mg/kg trilbromoethanol) to collect blood. We generated plasma using Microtainer plasma separator tubes (Becton Dickinson). Veterinary Diagnostic Services (Marshfield Laboratories) measured the levels of cholesterol, β-hydroxybutyrate, FFA, and triglycerides by an automated analyzer (Roche Modular Autoanalyzer). We assayed liver triglycerides using the triglyceride GPO reagent as previously described (28) (Pointe Scientific). Plasma insulin concentrations were measured with an Ultrasensitive Mouse Insulin ELISA according to the manufacturer’s instructions (Mercodia).

Insulin measurements and glucose tolerance tests. Female mice were deprived of food overnight for 18 h and 2 g glucose/kg body weight was injected intraperitoneally (IP) into the mice. Blood was collected from the tail vein and we measured the plasma insulin concentration at 0 and 30 min after glucose injection. With a separate group of female mice, we performed heart punctures as stated above to collect blood in the postprandial state (0800 h) and measured plasma insulin concentrations as described above.

Glucose tolerance tests (GTT) were performed on a separate group of female mice. Briefly, we deprived the mice (8 wk old) of food for 18 h and subsequently injected IP 2 g glucose/kg body weight and collected blood from the tail vein and measured glucose at 0, 15, 30, 60, and 120 min using an UltraTouch Glucose meter. For pup GTT and insulin measurements, we separated 14-d-old pups from dams for 4 h and glucose was subsequently injected IP 2 g glucose/kg body weight and collected blood as described above.

Characterization of dams’ milk. On d 14 postpartum, pups were separated from dams for 3 h. We anesthetized (249 mg/kg trilbromoethanol alcohol) adult female mice 10 min before milking the dams. Oxytocin (0.3 L of 200 IU/L, Sigma) was administered IP to the dams 10 min before milking the dams. Oxytocin was separated from dams for 3 h. We anesthetized (249 mg/kg tribromoethyl alcohol) and we collected blood in heparinized Micro-Hematocrit capillary tubes and stored them at −80°C until analysis. Case MMPC measured triglyceride concentrations and de novo lipogenesis as previously described (32). Briefly, triglyceride from tissues was isolated and labeled glycerol and palmitate were analyzed after derivatization by MS. The 2H label on triglyceride covalently linked to glycerol measures the amount of newly synthesized triglyceride, whereas the 3H label in triglyceride covalently attached to palmitate indicates the amount of new palmitate. In mice given 2H2O for 5 d, the contribution of de novo lipogenesis to the pool of triglyceride palmitate was calculated using the following equation:

\[ \% \text{ newly made palmitate} = \frac{\left[ \text{total} \ 2H\text{-labeled palmitate} \right] - \left[ \text{2H-labeled body water} \times n \right]}{\left[ \text{2H-labeled body water} \times n \right]} \times 100 \]

where \( n \) is the number of exchangeable hydrogens, assumed to equal 22 (33,34). The percentage of total newly made triglyceride glycerol was calculated using the following equation:

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Histology of mammary gland. We isolated thoracic mammary gland tissues from 8-wk-old female mice and fixed the tissues in 10% formalin (Sigma) at 4°C. The Pathology Core Facility (Case Western Reserve University, Cleveland, Ohio) embedded the tissues in paraffin and stained them with hematoxylin and eosin as previously described (35). The pictures were taken at 200× magnification.

Immunohistochemistry. Four percent buffered paraformaldehyde was perfused transcardially into female mice. Thoracic mammary glands were isolated and incubated in 30% sucrose + PBS at 4°C. Four percent buffered paraformaldehyde was perfused transcardially into female mice. Thoracic mammary glands were isolated and incubated in 30% sucrose + PBS at 4°C. Four percent buffered paraformaldehyde was perfused transcardially into female mice. Thoracic mammary glands were isolated and incubated in 30% sucrose + PBS at 4°C. The fixed tissues were cut into 10-μm cryosections and placed onto poly(I-Lysine) coated slides. The tissues were blocked and permeabilized with 10% donkey serum (SIGMA) in 0.05% PBS-Tween and immunolabeled overnight with sheep polyclonal anti-CK1 antibody (1:1000) in 3% donkey serum and 0.05% PBS-Tween. Negative controls were incubated with preimmune sheep serum instead of PCK antibody. The sections were incubated with Alexa Fluor 488 donkey anti-sheep antibody (1:250, Invitrogen) and TO-PRO 3 (1:5000, Invitrogen) in a solution of 3% donkey serum and 0.05% PBS-Tween and then washed and mounted using Mowiol. We detected the fluorescent signal by spectral confocal microscopy (Leica) using LCSLite software. The laser power, gain, and offset were set accordingly to obtain dark field images using negative controls (36).

Statistical analysis. The results are expressed as the mean ± SEM. For 3 or more comparisons, we analyzed the variables by 1-way ANOVA with Bonferroni’s post hoc test (Figs. 1 and 5A; Tables 1–4). For comparing various groups to WT mice, we analyzed the data by 1-way ANOVA and Dunnett’s post hoc test (Fig. 5B,C). We used Student’s t test to compare 2 means (Figs. 3A, B, and 4). For analysis of 2 × 2 factorial experiments, we used 2-way ANOVA with Bonferroni’s post hoc test (Fig. 3D; Table 2). Data that failed Bartlett’s test for homogeneous variances was analyzed using the Kruskal-Wallis test with Dunn’s multiple comparisons test.

Measurement of triglyceride concentration and synthesis by stable isotopes. The Case Mouse Phenotyping Center (MMPC) measured triglyceride content and newly synthesized triglyceride levels. To enrich body water to ~2% 3H, Case MMPC administered an IP injection of labeled water (2H2O) (20 μL/g body weight of 9 μL NaCl in 99% atomic percentage excess 2H2O) into adult female mice and they were returned to their cages and maintained on 5% 2H-labeled drinking water for 5 d. The mice were killed and blood and tissue samples were collected and flash-frozen in liquid nitrogen. The samples were stored at −80°C until analysis. Case MMPC measured triglyceride concentrations and de novo lipogenesis as previously described (32). Briefly, triglyceride from tissues was isolated and labeled glycerol and palmitate were analyzed after derivatization by MS. The 2H label on triglyceride covalently linked to glycerol measures the amount of newly synthesized triglyceride, whereas the 3H label in triglyceride covalently attached to palmitate indicates the amount of new palmitate. In mice given 2H2O for 5 d, the contribution of de novo lipogenesis to the pool of triglyceride palmitate was calculated using the following equation:

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variance were analyzed with the Kruskal Wallis test. For measurement of insulin at 0 and 30 min after glucose injection in adult female mice, the data were analyzed by the Mann Whitney test.

Results

Pck1 mRNA in the whole mammary glands and WAT. The PPARE mutation lowered Pck1 expression in WAT to 2.2% of WT and reduced Pck1 expression in whole mammary gland tissue to 1% of WT. The expression of Pck1 in BAT of PPARE−/− mice was reduced to 17% of WT. In WAT of PPARE−/+ female mice, Pck1 expression was reduced to 9% of WT (Fig. 1A).

To confirm that glycerol kinase (GyK) was not compensating for altered Pck1 expression, we measured GyK mRNA expression in WT and PPARE−/− mice and found no differences in GyK mRNA levels (data not shown).

Pck1 expression in mammary gland adipocytes. Pck1 mRNA expression was lowered to 21% of WT in mammary gland adipocytes from PPARE−/− mice (Fig. 1B); however, the expression levels of Pck1 were similar in epithelial cells from PPARE−/−, PPARE+/−, and WT mice.

We confirmed the reduction of Pck1 in mammary adipocytes by immunohistochemistry using an antibody specific for PCK1 (Fig. 2). The PCK1 antibody detected PCK1 in the epithelial cells and mammary gland adipocytes of WT mice. Importantly, staining was detected only in the epithelial cells of the PPARE−/− mice. The hematoxylin and eosin staining of the mammary gland indicated that stored triglycerides were greatly reduced in the adipocytes of the PPARE−/− mice (Fig. 2B). Triglyceride content and synthesis rates were measured by the 2H2O method (Fig. 3). The contribution of de novo lipogenesis to newly synthesized palmitate was less in the PPARE−/− tissues compared with those of WT mice (Fig. 3C). The PPARE−/− mice had reduced total triglyceride contents in the mammary gland and WAT (Fig. 3A); however, the amount of newly synthesized triglyceride was not altered in the WT or PPARE−/− mice (Fig. 3B). To determine whether the reduced triglyceride content in mammary gland adipocytes was due to increased lipolysis, we measured glycerol release from isolated mammary gland adipocytes (Fig. 3D). We found a significant interaction between genotype and epinephrine treatment (P < 0.05). Under basal conditions, isolated mammary gland adipocytes of PPARE−/− mice released 47% more glycerol than those from WT mice. After treatment with epinephrine, isolated mammary gland adipocytes from PPARE−/− mice released 40% more glycerol than those from WT mice. Thus, the PPARE−/− mammary gland adipocytes had greater rates of lipolysis than those from WT mice.

Metabolic consequences of the PPARE mutation in the Pck1 promoter. In food-deprived mice, plasma concentrations
of FFA and β-hydroxybutyrate were reduced in PPARE−/− and PPARE+/− female mice compared with WT mice (Table 1). To determine whether fed mice differed, we analyzed peripheral insulin resistance by GTT (Fig. 4). At 0 and 30 min, the glucose concentration of the PPARE−/− and WT mice differed (P < 0.05). The PPARE−/− mice had reduced glucose clearance as demonstrated by a greater area under the curve (24994 ± 926 mmol/L) compared with WT mice (20783 ± 962 mmol/L) (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. They had similar concentrations at 0 and 30 min after glucose injection (data not shown); however, when we analyzed mice in the postprandial state, PPARE−/− mice had lower plasma insulin concentrations than the PPARE−/− mice (226.2 ± 60.9 pmol/L) (P = 0.02).

**Milk triglycerides during lactation.** To establish the consequence of 78.9% less Pck1 in mammary gland adipocytes, we measured milk triglycerides in WT, PPARE+/−, and PPARE−/− dams. The volume of milk did not differ among the genotypes; however, milk triglyceride concentrations were ~40% lower in the PPARE−/− dams than in the other 2 groups (Fig. 5A). FFA, lactose, and protein concentrations also measured in extracted milk did not differ among the genotypes (data not shown).

**Consequences of the PPARE mutation in the perinatal period.** To determine the consequences of the PPARE mutation in the perinatal period, we analyzed metabolic variables in WT pups from WT dams and PPARE−/− pups from PPARE−/− dams 14 d after birth (Table 2). The PPARE−/− male and female pups were smaller than WT pups of the same gender. After an injection of glucose, PPARE−/− male and female pups had greater blood glucose concentrations than WT pups of the same gender (Table 2).

**Patterning of pups by altered milk triglycerides.** To determine the effects of both maternal genotypes and the genotypes of

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**TABLE 1** Concentrations of blood glucose and plasma metabolites in food-deprived WT, PPARE+/−, and PPARE−/− female mice

<table>
<thead>
<tr>
<th>Variable</th>
<th>WT</th>
<th>PPARE+/−</th>
<th>PPARE−/−</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose, mmol/L</td>
<td>4.63 ± 1.61</td>
<td>4.32 ± 0.28</td>
<td>5.53 ± 0.31</td>
</tr>
<tr>
<td>Insulin, pmol/L</td>
<td>60.9 ± 10.44</td>
<td>48.7 ± 15.66</td>
<td>53.9 ± 8.21</td>
</tr>
<tr>
<td>Cholesterol, mmol/L</td>
<td>1.35 ± 0.12</td>
<td>1.71 ± 0.13</td>
<td>1.71 ± 0.14</td>
</tr>
<tr>
<td>Triglyceride, mmol/L</td>
<td>0.50 ± 0.06</td>
<td>0.78 ± 0.11</td>
<td>0.64 ± 10.05</td>
</tr>
<tr>
<td>FFA, mmol/L</td>
<td>0.39 ± 0.03</td>
<td>0.29 ± 0.01</td>
<td>0.29 ± 0.03</td>
</tr>
<tr>
<td>β-Hydroxybutyrate, mmol/L</td>
<td>0.45 ± 0.08</td>
<td>0.27 ± 0.04</td>
<td>0.24 ± 0.01</td>
</tr>
</tbody>
</table>

1 Values are the mean ± SEM, n = 15–20. Means in a row with superscripts without a common letter differ, P < 0.05.
In CA we analyzed pup growth in PPARE+/− patterning in the perinatal period. Pups’ growth was affected in utero and may have contributed to being slightly heavier than the mice from WT dams (WT x H). Values are means ± SEM, n = 14–6 from 4 litters. Means in a row with superscripts without a common letter differ, P < 0.05.

The pups from PPARE+/− and PPARE−/− dams tended to have a higher blood glucose concentration 30 min after glucose injection compared with PPARE+/− pups from WT dams (P = 0.06) (Table 3).

We also analyzed the role of perinatal nutrition in the development of insulin resistance succeeding birth by using foster dams (Table 4). The blood glucose concentration in PPARE−/− pups cross-fostered by WT dams was lower than in PPARE+/− pups reared by foster PPARE−/− dams (P < 0.05). The WT pups cross-fostered by PPARE−/− dams had lower plasma insulin concentrations, higher FFA concentrations, and lower body weights compared with WT pups fostered by WT dams (P < 0.05) (Table 4).

Discussion

We hypothesized that alterations in perinatal nutrition contribute to the development of insulin resistance in newborn pups. In this study, we investigated the role of Pck1 in mammary gland adipocytes and determined the consequences of reduced milk triglycerides in the perinatal period with respect to the development of insulin resistance.

We determined whether alterations in Pck1 expression in the mammary gland resulted in altered perinatal nutrition. In this study, we have shown for the first time, to our knowledge, that Pck1 is present in both epithelial cells and mammary gland adipocytes. We found that PPARE−/− mice have a 40% lower milk triglyceride concentration at d 14 of lactation. Lactation induces major metabolic adaptations and there are substantial changes in substrate flux. The demands of milk production by the mammary gland increase overall energy output. In rats, lactation causes an ~70% loss of whole-body adipose tissue depots (37); however, the nutrient requirements of the lactating

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mammary gland exceed those of the whole-body nonlactating animals (38,39). In mammals, fatty acids for triglycerides originate from primarily 2 different sources: uptake from circulation and de novo synthesis within mammary epithelial cells (40). In ruminants (40) and mice (41), fatty acids C4–C14 and about one-half of the palmitate (C16) pool are synthesized de novo by the mammary gland from acetate. The liver also substantially contributes to de novo lipogenesis and thus to circulating fatty acids (41). Because the PPARE+/− mice have reduced plasma FFA, substrates for de novo lipogenesis in the mammary gland should be greatly diminished. We suggest that the maintenance of triglycerides in whole-body WAT and in the mammary gland is important for determining the concentration of milk triglycerides during lactation by contributing fatty acids to milk triglycerides in the mammary gland.

A number of factors in the gestational environment have important effects on offspring development. Cross-fostering studies in mice suggest that the prenatal environment accounts for 61–96% of variance in body weight gain in male pups and 35–92% in female pups (42). Maternal undernutrition, high-fat diet feeding, and maternal stress during gestation can predispose offspring to obesity (42). We found that PPARE+/− and PPARE−/− dams had smaller pups at birth compared with WT dams (Fig. 5B), suggesting that the gestational environment affected fetal development. This reduced growth is similar to that of infants with intrauterine growth retardation, who have a higher risk of insulin resistance, β-cell dysfunction, dyslipidemia, obesity, and type II diabetes (43).

Postnatal factors such as metabolic, hormonal, and behavioral interactions of pups with their dams can alter the development of neonates and predispose them to obesity and metabolic syndrome as adults (44). Rodent milk contains more lipid than carbohydrate. Therefore, neonates use fatty acids and ketone bodies as their primary energy substrate (45–47). Because the PPARE−/− dams had 40% reduced milk triglycerides and the same volume of milk, the pups reared by these dams were undernourished compared with pups of WT dams. The WT pups fostered by PPARE−/− dams had lower body weights and plasma insulin concentrations compared with WT pups reared by foster WT dams (Table 4). Plasma fatty acid concentrations in WT pups fostered by a PPARE−/− dam had elevated FFA (Table 4). The reason for this may be less secretion of insulin in response to a 40% reduction of milk triglyceride during the perinatal period. The PPARE−/− pups fostered by WT dams did not differ from PPARE−/− pups fostered by PPARE−/− dams in insulin release, but they did have slightly lower glucose (P = 0.01) concentrations, suggesting that the WT milk triglycerides moderately improve glucose homeostasis in PPARE−/− pups. The studies were performed in fed pups, but we have also analyzed the mice after 4 h of food deprivation (data not shown) and found greater variance of FFA concentrations within each group, so differences among the groups were not significant (P = 0.18). Deletion of the PPARE site in the promoter of Pck1 greatly reduced Pck1 expression in WAT, but it also affected BAT and may affect regulation of hepatic Pck1. It is possible that altered regulation of Pck1 by the deleted PPARE site in other tissues may contribute to the altered plasma FFA concentrations in food-deprived pups. The foster pup data also suggest that the patterning is more complex than just altered perinatal nutrition. Further analysis is necessary to answer these questions. In all, the reduced milk triglycerides in PPARE−/− dams during lactation affect the development of insulin resistance in new pups.

In adult female food-deprived PPARE−/− mice, the levels of plasma FFA and β-hydroxybutyrate were lower during food deprivation than in WT mice. This finding was unexpected, because these mice have 97% less Pck1 in WAT. We had anticipated that the PPARE−/− mice would have higher FFA and β-hydroxybutyrate concentrations. These differences may be explained by the triglyceride levels and palmitate synthesis (Fig. 3). In PPARE−/− mice, the total mammary gland and WAT triglyceride content was depressed, yet the amount of newly synthesized triglyceride did not differ. In isolated mammary gland adipocytes, PPARE−/− mice had increased lipolysis, as measured by glycerol released into the medium. The increased lipolysis contributed to the reduced total pool of mammary gland triglycerides (Fig. 3D). Because de novo fatty acid synthesis is decreased and triglyceride synthesis is unaffected, we suggest that glycerol-3-phosphate synthesis may be increased. If more glucose were shunted toward the synthesis of glycerol-3-phosphate for triglyceride synthesis in BAT, liver, mammary gland, muscle, and WAT, then less glucose would be available for de novo fatty acid synthesis in PPARE−/− mice in these tissues. In WT mice, both glucose and pyruvate, through the glycolytic and glyceroenogenic pathways, respectively, could be used for the synthesis of glycerol-3-phosphate for triglyceride formation in WAT and mammary gland tissues. Thus, more glucose could be used for de novo synthesis of palmitate in WT mice. More studies are needed to clarify this issue.

In summary, deletion of the PPARE region in the promoter of Pck1 resulted in an altered intrauterine environment and 40% lower milk triglyceride concentrations. These changes resulted in fetal programming of pups for the development of IUGR and insulin resistance. Fetal programming refers to changes during the perinatal period that result in altered gene expression and altered regulation of metabolic pathways with long-term effects. Future studies will focus on expression differences in the liver and pancreas for WT, PPARE+/− and PPARE−/− pups to determine the mechanism(s) for patterning a newborn for disease.

Acknowledgments
C.W.H and C.A.M. contributed equally to this work. C.W.H., C.A.M., D.D., S.P., J.M., and J.C.P. conducted research and

<table>
<thead>
<tr>
<th>Variable</th>
<th>WT pups</th>
<th>WT dams</th>
<th>PARE−/− pups</th>
<th>WT dams</th>
<th>WT pups</th>
<th>PARE−/− dams</th>
<th>WT pups</th>
<th>PARE−/− pups</th>
<th>WT dams</th>
<th>PARE−/− dams</th>
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<tbody>
<tr>
<td>Glucose, mmol/L</td>
<td>17.7 ± 1.71a</td>
<td>10.5 ± 1.30a</td>
<td>16.2 ± 1.92b</td>
<td>23.4 ± 3.98c</td>
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<td>Insulin, pmol/L</td>
<td>121.8 ± 10.42</td>
<td>109.6 ± 27.81</td>
<td>67.8 ± 26.13</td>
<td>156.6 ± 41.72</td>
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<td>FFA, mmol/L</td>
<td>0.39 ± 0.01a</td>
<td>0.46 ± 0.02b</td>
<td>0.45 ± 0.01b</td>
<td>0.42 ± 0.01c</td>
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<tr>
<td>Body weight, g</td>
<td>5.8 ± 0.2b</td>
<td>5.2 ± 0.11b</td>
<td>4.4 ± 0.12c</td>
<td>5.4 ± 0.11c</td>
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</tbody>
</table>

1 Values are the mean ± SEM, n = 4–6 pups from 4 litters from 4 dams. Means in a row with superscripts without a common letter differ, P < 0.05.
analyzed data. C.M.C. designed research, analyzed data, wrote the paper, and had primary responsibility for final content. All authors have read and approved the final manuscript. The PPARE−/− mice were a generous gift from Dr. Lea Reshef, Hebrew University-Hadassah Medical School, Jerusalem and Dr. Richard W. Hanson, Case Western Reserve University, OH. The polyclonal anti-PCK1 antibody was a kind gift from Dr. D.K. Granner, Vanderbilt University, Nashville, TN. We are grateful to Dr. Martin Snider for his careful review of the manuscript.

Literature Cited


