

Function of phosphoenolpyruvate carboxykinase in mammary gland epithelial cells^S

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Abstract Previously, we have shown that *Pck1* expression in mammary gland adipocytes and white adipose tissue maintains triglyceride stores through glyceroneogenesis, and these lipids were used for synthesis of milk triglycerides during lactation. Reduced milk triglycerides during lactation resulted in patterning of the newborn for insulin resistance. In this study, the role of *Pck1* in mammary gland epithelial cells was analyzed. The developmental expression of *Pck1* decreased in isolated mouse mammary gland epithelial cells through development and during lactation. Using HC11, a clonal mammary epithelial cell line, we found that both Janus kinase 2 signal transducers and activators of transcription 5 and the AKT pathways contributed to the repression of *Pck1* mRNA by prolactin. These pathways necessitate three accessory factor regions of the *Pck1* promoter for repression by prolactin. Using [U-¹³C₆]glucose, [U-¹³C₃] pyruvate, and [U-¹³C₃]glycerol in HC11 cells, we determined that *Pck1* functions in the pathway for the conversion of gluconeogenic precursors to glucose and contributes to glycerol-3-phosphate synthesis through glyceroneogenesis. Therefore, *Pck1* plays an important role in both the mammary gland adipocytes and epithelial cells during lactation.—Hsieh, C-W., C. Huang, I. Bederman, J. Yang, M. Beidelschies, M. Hatzoglou, M. Puchowicz, and C. M. Croniger. **Function of phosphoenolpyruvate carboxykinase in mammary gland epithelial cells.** *J. Lipid Res.* 2011. 52: 1352–1362.

Supplementary key words adipocytes • gluconeogenesis • glyceroneogenesis

During lactation, there are profound modifications in the intermediary metabolism of different tissues to adequately supply nutrients for milk production (1, 2). There are decreases in both lipogenic capacity and activities of several lipogenic enzymes in adipose tissue (3–7). Prolactin is one of the key signals that integrates metabolism of

tissues during lactation, promoting lipogenesis over esterification in the adipose tissue (2, 3, 7, 8). These changes cause mobilization of lipid stores in adipose tissues to support milk production in the mammary gland.

One gene that is present in both adipocytes and epithelial cells of the mammary gland is the phosphoenolpyruvate carboxykinase gene *Pck1*. The role of *Pck1* in adipocytes of white adipose tissue (WAT) has been clearly defined as glyceroneogenic. Over 30 years ago, Ballard, Hanson, and Leveille (9) and Reshef, Niv, and Shapiro (10, 11) demonstrated that in vitro incubation of rat epididymal fat pad with pyruvate reduced the amount of FFAs released by 65% (10, 11). However, the rate of lipolysis remained unaffected. In WAT, glycerol is released during lipolysis, but it cannot be phosphorylated in preparation for triglyceride synthesis because the tissue manifests negligible glycerol kinase activity. Ballard and Hanson (12) and Reshef, Hanson, and Ballard (13) determined that during fasting, gluconeogenic precursors such as pyruvate and alanine are converted into the glycerol backbone of triglycerides through the glyceroneogenic pathway. Support for a glyceroneogenic role for *Pck1* in the mammary gland was established by Jimenez et al. (14), who established incorporation of labeled oleate into glycerol-3-phosphate in isolated acini from lactating Wistar rats. However, they suggested that the last steps of gluconeogenesis between triose-phosphate and glucose-6-phosphate are not operative in rat mammary gland acinar cells (14).

This work was supported by the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) Grant DK-075040 (CMC). The stable isotope studies were performed at the Case Western Reserve University (Cleveland, Ohio) Mouse Metabolic Phenotyping Center supported by NIDDK Grant DK-76769.

Manuscript received 8 November 2010 and in revised form 15 April 2011.

Published, JLR Papers in Press, April 19, 2011
DOI 10.1194/jlr.M012666

Abbreviations: AF, accessory factor; BAT, brown adipose tissue; CREB, cAMP response element binding; DI, dexamethasone and insulin; DIP, dexamethasone, insulin and prolactin; EGF, epidermal growth factor; GCMS, gas chromatography mass spectrometry; GR, glucocorticoid receptor; GRE, glucocorticoid response element; GRU, glucocorticoid response unit; MAPK, mitogen-activated protein kinase; MPE, mass percent enrichment; PPAR γ , peroxisome proliferator-activated receptor γ ; TCA, tricarboxylic acid; WAT, white adipose tissue; WT, wild type.

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^SThe online version of this article (available at <http://www.jlr.org>) contains supplementary data in the form of two figures and one table.

Previously, we have shown that the role for *Pck1* in mammary gland adipocytes is the formation of glycerol-3-phosphate through glyceroneogenesis (15). We analyzed mice in which the binding site for peroxisome proliferator-activated receptor γ (PPAR γ) was deleted from the promoter of *Pck1* (PPARE^{-/-}). *Pck1* expression and triglyceride content in the milk were measured. The *Pck1* mRNA expression was lowered to 2.2% that of wild-type (WT) mice in mammary gland adipocytes in PPARE^{-/-} mice; however, the expression levels of *Pck1* mRNA were similar in epithelial cells from PPARE^{-/-}, PPARE^{+/-}, and WT mice. The female PPARE^{-/-} mice presented reduced lipid storage in mammary gland adipocytes and in WAT, resulting in a 40% reduction of milk triglycerides during lactation. However, because the PPARE^{-/-} females had normal expression of *Pck1* in mammary gland epithelial cells, we wanted to determine the role of the *Pck1* gene in mammary gland epithelial cells during lactation. We hypothesized that *Pck1* provides glucose through gluconeogenesis in mammary epithelial cells and may contribute to lipid synthesis by the formation of glycerol-3-phosphate through glyceroneogenesis. In this study, we investigated the role of *Pck1* in mammary gland epithelial cells during lactation in mice and in HC11 cells.

EXPERIMENTAL PROCEDURES

Prolactin injection

Wild-type mice that were used previously for published studies on the role of *Pck1* in mammary gland (15) were further characterized in this study. The mice had free access to water and were fed a normal mouse diet ad libitum (LabDiet, St. Louis, MO; Diet # 5P76). The approximate composition of the diet was 1.08 kJ protein, 0.58 kJ fat, and 14.33 kJ carbohydrate. The diet consisted of 4,100 kJ \times kg⁻¹ gross energy (16). Four week-old virgin female mice were injected intraperitoneally with prolactin (1 μ g/g body weight) (7). Thirty minutes after the prolactin injection, the mice were euthanized, and thoracic mammary gland tissues were collected. We chose this time point because we found in a time course study that 30 minutes was adequate for repression of *Pck1* gene expression by prolactin (see supplementary Fig. 1). All experimental protocols were approved by the Case Western Reserve University Institutional Animal Care and Use Committee.

Isolation of adipocytes and mammary epithelial cells

Isolation of adipocytes and epithelial cells was performed as previously described (17, 18). Briefly, thoracic mammary glands were dissected from virgin female mice (three glands for each preparation). The lymph node was removed, and they were minced with a razor blade and 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 three batches of isolated adipocytes were prepared. After the dissociation was complete, the cell suspension was separated at 176.08 g for 10 min. The adipocytes and the epithelial cells were collected separately and washed extensively, and any endothelial contaminants were removed by sedimentation. The adipocytes and epithelial cells were collected and stored in RNAlater solubilization buffer (Qiagen; Valencia, CA) for isolation of RNA.

Plasmid constructs

The plasmid 490-Luc was generated by ligating the *Xba*I/*Pst*I fragment of the rat *Pck1* promoter into pGL3-basic (which contains the luciferase structural gene) that had been digested with restriction enzymes *Pst*I and *Bgl*II. The plasmids were isolated with Plasmid Mini Kits (Qiagen). Truncations of the gene promoter were created using restriction enzymes to delete segments of the promoter within p490-Luc to generate 402-Luc and 206-Luc. Heterologous constructs were made with the -490 bp to -402 bp fragment, and a -490 bp to -206 bp fragment of the rat *Pck1* promoter was ligated before the SV40 promoter in the pGL3 promoter vector (Promega; Madison, WI).

Site-directed mutagenesis

The AF1 (-451 bp to -439 bp), AF2 (-416 bp to -407 bp), GR1 (-391 bp to -371 bp), GR2 (-370 bp to -350 bp), and AF3 (-326 bp to -321 bp) were mutated using the following oligonucleotide (the mutated bases are in bold) primers for mutated AF1: forward: 5'-GCTGAATTCCTTCTCACT**ACG**ATTGGCCG-TGGGAGTGAC-3', reverse: 5'-GTCACCTCCACGGCCAAT**CG-TAG**TGAGAAAGGAATTCAGC-3'; primers for mutated AF2: forward, 5'-GACACCTCACAGCTGT**AGC**ATACTGACAACCAG-CAGCCAC-3'; reverse, 5'-GTGGCTGCTGGTTGT**CAG**TATGCTA-CAGCTGTGAGGTGTC-3'; primers for mutated GR1: forward, 5'-CAGCAGCCACTGGA**ATTCA**AGATCTAATGCCAGCAGCA TA-3'; reverse, 5'-TATGCTGCTGGC**ATTAG**ATCTTGA**ATTCCA**-GTGGCTGCTG-3'; primers for mutated GR2, forward: 5'-ATGTGCAGCCAGCAGCTGG**TAATCCG**TAAGAGGCGTCCCG-3'; reverse: 5'-CGGGACGCCTCT**TACG**GATTACCAGCTGCTG-GCTGCACAT-3'; primers for mutated AF3: forward, 5'-CCG-GCCAGCCCTGTCT**CGGT**ACCCACCTGACAATTAGGG-3'; reverse, 5'-CCTTAATTGTCAGGTGG**TACCG**AGGACAGGGC TGCCCG-3'. These sites were mutated in combination or individually to generate vectors using the QuickChange mutagenesis kit (Stratagene; La Jolla, CA). The plasmids resulting from the above mutations were isolated, and their sequence was confirmed by restriction digestion and DNA sequencing performed by the Case Western Reserve University Molecular Biology Core Laboratory.

Cell culture

HC11 mouse mammary gland epithelial cells were routinely maintained in RPMI 1640 medium supplemented with 10% FBS, 5 μ g/ml insulin, and 10 ng/ml epidermal growth factor (EGF) (19). For induction of differentiation, cells were grown to confluence and then kept in medium without EGF for 48 h to induce competence. Competent cells were incubated with differentiation medium (serum-free RPMI 1640 medium containing 5 μ g/ml insulin, 1 mM dexamethasone, and 1 μ g/ml ovine prolactin) for 72 h.

Transient transfection and luciferase assay

All plasmids were purified using the HiSpeed Plasmid Midi Kit (Qiagen). Cells were seeded in 12-well plates and transfected with plasmid DNA using FuGENE-HD transfection reagent (Roche Applied Science; Indianapolis, IN). Forty-eight hours after transfection, cells were incubated with serum-free RPMI 1640 medium containing lactogenic hormone mix [dexamethasone, insulin and prolactin (DIP)]: 1 mM dexamethasone, 5 mg/ml insulin, and 1 μ g/ml ovine prolactin (Sigma; St. Louis, MO) for 72 h. Controls were treated with dexamethasone and insulin only (DI). After 72 h treatment, the cells were washed with ice-cold 1 \times PBS, pH7.4, and lysed by the addition of 250 μ l of 1 \times passive lysis buffer. The insoluble material was pelleted by centrifugation in 1.5 ml eppendorf tubes for 6 min at 12,000 g, and the supernatant

was separated from the pellet for the measurement of luciferase activity. For luciferase activity, 20 μ l of the cell lysate was used to measure the integrated light units over 10 s. Firefly and Renilla luciferase activities were measured using dual-luciferase assay reagents (Promega) on a luminometer. Relative luciferase activity was calculated by dividing firefly luciferase activity by Renilla luciferase activity and expressed as relative *Pck1*-luciferase activity (relative activity). For the inhibition of signaling pathways in a separate experiment, cells were grown and transfected as described above. At 48 h after treatment with DI or DIP, the cells were treated with specific inhibitors Akt1/2 inhibitor (1 μ M) (Akt inhibitor VIII, Calbiochem/EMD Chemicals, Inc.; San Diego, CA) (20), MAPK/ERK inhibitor (1 μ M) (U0126, EMD Chemicals, Inc.) (21) and the STAT5 inhibitor (1 μ M) (Calbiochem/EMD Chemicals, Inc.) (22). After 24 h, relative activity was determined as described above. The assay was carried out in quadruplicate.

Real-time quantitative reverse-transcription PCR

Total RNA was prepared from 100 mg of liver, WAT, brown adipose tissue (BAT), and mammary gland from mice or HC11 cells with the RNeasy Lipid Tissue Mini Kit (Qiagen). The single-strand cDNAs were synthesized from 2 μ g total RNA with random hexamer primers and mouse mammary tumor virus reverse transcription (Ambion; Austin, TX). The cDNAs were amplified using Syber Green PCR Core Reagent Mix (Applied Biosystems; Foster City, CA). The real-time quantitative PCR was performed in the Chromo4 Cycler (MJ Research; Waltham, MA). The relative amounts of the mRNAs were determined by linear regression from standard curves derivative maximum method, with Opticon Monitor 3 software (MJ Research). RNA expression data were normalized to levels of 18S rRNA. The utilized primer sequences (Integrated DNA Technologies; San Diego, CA) are presented in supplementary Table I.

Stable isotope incubation conditions

Cells were incubated in glucose-free and 10% stripped FBS RPMI 1640 media [contains \sim 0.1–0.2 mM amino acid, 2 mM glutamine, without sodium pyruvate and without glucose (Invitrogen; Carlsbad, CA)] that contained 5 mM of $U^{13}C$ -labeled substrates: Condition 1, [$U^{13}C_6$]glucose-enriched 100% mass percent enrichment (MPE); Condition 2, [$U^{13}C_3$]glycerol-enriched 100% MPE; Condition 3, [$U^{13}C_3$]pyruvate-enriched 100% MPE. ^{13}C label incorporation into cells at 80–90% confluence was measured after switching the cells into fresh glucose-free medium containing 100% of one of the labeled substrates outlined in the above conditions, and cultured at 37°C for 6 h. The media were then removed and saved for measurement of MPE of the ^{13}C -labeled precursors and ^{13}C label incorporation into the respective “products.” The isolated cells were stored at $-80^\circ C$ and then analyzed for MPE (^{13}C label incorporation) of triglyceride-bound glycerol and palmitate. Measurement of MPE of glucose was also performed. All stable isotopomer analysis was completed at the Case Mouse Phenotyping Center. Briefly, total triglycerides were hydrolyzed and extracted from the cells, and the ^{13}C enrichments of glycerol and palmitate were determined by using gas chromatography mass spectrometry (GCMS) (23). The media were assayed for precursor MPE for the purpose of normalizing the product enrichments to the precursor enrichments.

^{13}C labeling of glucose, glycerol, and palmitate

Cells were saponified by performing complete hydrolysis using ethanol-hydroxide incubation at 70°C for 1 h and then extracted using the Folch procedure. Briefly, 3 ml of chloroform-methanol mix (2:1, v/v) was added, and cells were homogenized. Layers

were separated by water, and the chloroform layer was removed following centrifugation. Both organic and aqueous layers were evaporated to dryness. The aqueous layer from the previous step was reconstituted in 100 μ l water and divided into two equal parts. Total glycerol and palmitate MPEs were measured as the triglyceride-bound glycerol and palmitate and assayed from an aliquot containing either the organic extract or total palmitate. Also, the cell media (20 μ l aliquot) was sampled to determine precursor enrichment of glucose, glycerol, or pyruvate.

To determine ^{13}C labeling of glucose and glycerol in the media and/or cell extracts, aliquots containing the aqueous fraction were converted to their respective penta-acetate derivatives (24). This was done by derivatization accomplished by reacting it with 50 μ l of acetic anhydride solvent and further heated for 2 h at 70°C. To determine ^{13}C labeling by pyruvate or glycerol, an aliquot taken from the aqueous fraction (for media or cells) was first evaporated, and then pyruvate was reacted with 70 μ l of bis(trimethylsilyl) trifluoroacetamide + 10% trimethylchlorosilane and incubation for 1 h at room temperature. Using the GCMS system (Agilent 5973N-MSD equipped with an Agilent 6890 GC system and a DB-17 MS capillary column) all samples were analyzed for the respective ions corresponding to MO, M+1, M+2, and M+3 through M+6 for each of the analytes (25).

MPE calculations

The fractional contribution of the precursor to the product was based on the MPEs derived from the ^{13}C label incorporation into the total pool of each of the products (glycerol, palmitate, and glucose). Mass isotopomer analysis enables the measurements of unlabeled analyte (MO) to the labeled analyte (M+1, 2, or 3, etc.). The measured mass isotopomer distribution was corrected for natural enrichment of each mass (26, 27) by a technique that takes into account the skew of natural ^{13}C enrichment of molecules as increasing numbers of carbon atoms become fully labeled (26, 28). For example, the MPE calculated for glycerol following incubation with the precursor of [$U^{13}C_6$]glucose was calculated as the ratios of $[(M+3)/(MO+M3) \cdot 100]_{\text{glycerol}}$ to precursor MPE $[(M+6)/(MO+M6) \cdot 100]_{[U^{13}C_6]\text{glucose}}$ in the cells or media $\times 100$. The MPE calculated for glycerol following incubation with the precursor of [$U^{13}C_3$]pyruvate was calculated as the ratios of $[(M+2)/(MO+M2) \cdot 100]_{\text{glycerol}}$ to precursor MPE $[(M+3)/(MO+M3) \cdot 100]_{[U^{13}C_3]\text{pyruvate}}$ in the media $\times 100$ to account for the loss of label in the tricarboxylic acid (TCA) cycle (26, 29). We did not detect M+3 in our studies. Theoretically, since half of the phosphoenolpyruvate that emerges from the TCA cycle should have been randomized in the malate/fumarate exchange, we should have detected M+3. The lack of M+3 detection may be due to small sample size (too few cells) and thus M+3 was below the level of detection. The calculation of glycerol-bound triglyceride is a modified calculation of pyruvate to glucose as described (26).

Glycerol kinase activity

Glycerol kinase activity was measured as previously described (30, 31). Briefly, HC11 cells treated with or without prolactin were homogenized in extraction buffer (50 mM HEPES, pH 7.8, 40 mM KCl, 11 mM $MgCl_2$, 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 μ g of protein was used for the enzymatic assay. The protein samples were incubated with 50 μ l of assay buffer (50 mM Tris-HCl, pH 7.2, 5 mM ATP, 10 mM $MgCl_2$, 100 mM KCl, 2.5 mM DTT, 4 mM glycerol, and 500 μ mol/1 3H -glycerol) for 90 min at 37°C. The reaction was terminated with 100 μ l stop solution (ethanol-methanol, 97:3). Equal amounts of samples (20 μ l) were spotted onto DE81 Whatman filters (Whatman, Inc.; Piscataway, NJ).

The filters were air-dried and washed in water overnight. Radioactivity on the filters was measured by liquid scintillation counting.

Statistical analysis

The results were expressed as the mean \pm SEM. For three or more comparisons, we analyzed the parameters by one-way ANOVA with Bonferoni's correction. For analysis of three or more means compared with HC11 cells without prolactin, we analyzed the data by one-way ANOVA. For analysis of two means, we used Student's *t*-test, with $P < 0.05$.

RESULTS

Expression of *Pck1* in mammary gland

Pck1 mRNA is expressed in a variety of tissues. We compared the expression of *Pck1* mRNA in the mammary gland to its expression in the liver, WAT, and BAT in wild-type mice (Fig. 1A). The mammary gland had 25% the expression of *Pck1* mRNA compared with liver tissue. Previously, we established expression of *Pck1* protein in mammary gland adipocytes and epithelial cells by immunohistochemistry (15). To determine developmental expression of *Pck1* in mammary gland adipocytes and epithelial cells, we analyzed *Pck1* mRNA expression during development at 4 weeks and 8 weeks and during lactation (Fig. 1B). We found that the expression of *Pck1* in isolated mammary epithelial cells decreased over time in mice. However, in isolated adipocytes, we found increased expression of *Pck1* mRNA during lactation.

Because *Pck1* was greatly induced in the adipose tissue, whereas it was reduced in the epithelial cells of the mammary gland during lactation, we wanted to determine whether this was a direct response to prolactin, one of the key hormones present during lactation. In female mice, epithelial cells and adipose cells were isolated from the mammary gland 30 min after injection of prolactin. We found a reciprocal response to prolactin in epithelial cells compared with adipocytes. *Pck1* mRNA was reduced in mammary epithelial cells (Fig. 2), whereas mammary gland adipocytes and WAT have increased *Pck1* mRNA expression (Fig. 2). Two transcription factors that are known

regulators of *Pck1* expression are PPAR γ and forkhead box protein O1 (FoxO1) (32–36). We measured these in the same samples and found reduced levels of PPAR γ and FoxO1 after prolactin injection in isolated mammary gland epithelial cells (Fig. 3A). However, in isolated adipocytes, PPAR γ expression was induced, yet there was no effect on FoxO1 expression (Fig. 3B).

Mammary epithelial cell line HC11

To elucidate the mechanism for regulation of *Pck1* in mammary gland epithelial cells during lactation, we chose to study HC11 cells. HC11 cells are a mammary epithelial cell line derived from spontaneously immortalized COMMA-D epithelial cells, isolated from the mammary gland of pregnant BALB/c mice (37). HC11 cells can functionally differentiate and express milk proteins in vitro and can reconstitute ductal epithelium (37). HC11 cells have been used extensively to study the hormonal regulation of mammary epithelial cell differentiation and casein gene expression (38–40). When grown at confluence for several days after exposure to certain growth factors, such as EGF, and then treated with the lactogenic hormones prolactin, dexamethasone, and insulin, the cells produce β -casein milk protein.

We analyzed HC11 cells treated with or without prolactin. For a control and to ensure that the cells were responding to the lactogenic hormones, we measured β -casein gene expression. We found that β -casein gene expression was induced with prolactin treatment (see supplementary Fig. II). However, the expression of *Pck1* mRNA was reduced in response to prolactin (see supplementary Fig. II). Thus, these cells are responding to the lactogenic hormones and *Pck1* expression is responding in a similar manner as in vivo. We measured phosphorylation of FoxO1 in HC11 cells treated with prolactin (see supplementary Fig. II). We found increased phosphorylated FoxO1 in the cytoplasm of prolactin-treated cells. AKT (Akt/PKB) phosphorylates FoxO1, thus allowing it to migrate to the cytoplasm. Therefore, we tested whether AKT phosphorylation was altered. We found slightly increased levels of AKT phosphorylation in the prolactin-treated cells. The total amount of FoxO1 and AKT were not changed with prolactin treatment.

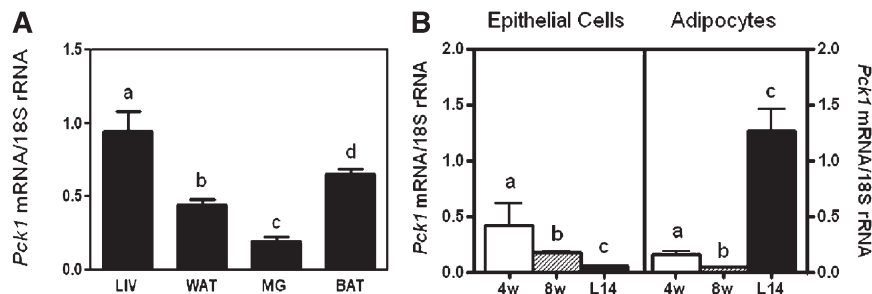


Fig. 1. *Pck1* expression in mammary gland. A: Expression of *Pck1* mRNA compared with liver (LIV), white adipose tissue (WAT), mammary gland (MG), and brown adipose tissue (BAT). Values are the mean \pm SEM, $n = 4$; subscripts with a different letter are statistically significant, $P < 0.05$. B: Mammary gland epithelial cells and mammary adipocytes were isolated from mice at 4 weeks (4w), 8 weeks (8w), and after 14 days of lactation (L14), and *Pck1* mRNA expression was measured by RT-PCR and normalized by 18S rRNA. Values are the mean \pm SEM, $n = 5$; subscripts with a different letter are statistically significant, $P < 0.05$.

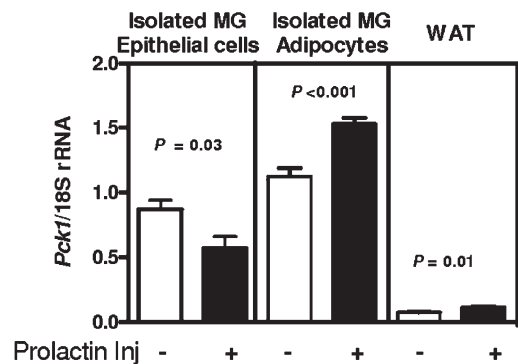


Fig. 2. The effect of prolactin injection on *Pck1* mRNA expression. Mice were treated with saline (–) or with 1 µg/g body weight of prolactin (+), and after 30 min, tissues were isolated. *Pck1* mRNA expression was measured by RT-PCR and normalized with 18S rRNA in isolated mammary gland (MG) epithelial cells, isolated mammary gland adipocytes, or isolated white adipose tissue (WAT). Values are the mean ± SEM; n = 5 mice per time point.

To confirm that the cytoplasmic and nuclear fractions were not contaminated with other cell fractions, we measured cAMP response element binding (CREB), a marker for nuclear proteins, and AKT, a marker for cytosolic proteins (see supplementary Fig. II). We found CREB in the nuclear cell fractions and AKT in the cytosolic cell fractions.

Region –490 bp to –402 bp in the *Pck1* promoter is required for repression by prolactin

We wanted to determine which region(s) of the *Pck1* promoter were required for repression by prolactin. Previous studies have shown that –490 bp of *Pck1* promoter are adequate for regulated expression of *Pck1* mRNA and is a minimal promoter construct (41–43). Thus we used –490-Luc (–490 bp to +72 bp *Pck1* promoter ligated to luciferase reporter gene) as a control. First, from deletion analysis of the *Pck1* promoter, we found that repression by prolactin was lost when the –402 bp-Luc construct was tested (Fig. 4A). However, the –490 bp-Luc construct had all of the necessary promoter elements for *Pck1* mRNA repression. Hence the region –490 bp to –402 bp in the *Pck1* promoter is required for repression by prolactin.

We mutated the regions within *Pck1* promoter that are known regulators of *Pck1* expression. Accessory factors (AFs) and glucocorticoid response elements (GREs) have been shown to regulate *Pck1* mRNA expression by glucocorticoids (41–44). Using single mutations of each AF (Fig. 4B, mutAF1, mutAF2, mutAF3) and combinations of these mutations (Fig. 4C, mutAF1 + mutAF2, mutAF1 + mutAF3, mutAF2 + mutAF3 and mutAF1 + mutAF2 + mutAF3), we found that AF1, AF2, and AF3 were required for the prolactin repression of *Pck1*. We also analyzed constructs that were mutated for GRE, but loss of GRE in the promoter still repressed luciferase expression with prolactin treatment; thus it was not required for repression of *Pck1* in HC11 cells.

To elucidate if AF1, AF2, and AF3 were acting as general repressors of gene expression or if this was specific to

the *Pck1* promoter, we made heterologous constructs with AF1+AF2 and AF1+AF2+AF3 before SV40 minimal luciferase promoter (Fig. 5). The promoter fragment –490 bp to –402 bp, which contains AF1 and AF2, recapitulated repression by prolactin. Because this repression was not complete, it suggested that AF1+AF2+AF3 were required for prolactin repression in the *Pck1* promoter only and not acting as a general repressor of gene expression.

AKT and STAT5 pathways contribute to repression of *Pck1* by prolactin

Upon prolactin binding to its receptor, three signaling pathways may mediate the activation of the receptor. These include the Janus kinase 2 (Jak2)/Signal transducers and activators of transcription 5 (STAT5) pathway, phosphatidylinositol-3-kinase (PI3K) pathway, and the mitogen-activated protein kinase (MAPK) pathway. To determine which pathway was necessary for the regulation of *Pck1* expression in HC11 cells, we used inhibitors of each pathway and then assessed whether *Pck1* mRNA expression was repressed after treatment with prolactin in –490 bp *Pck1* promoter constructs and in GR1 mutated *Pck1* promoter construct. We chose to test both constructs because the –490 bp *Pck1* promoter only had 25% repression by prolactin. The GR1 mutated version had a more robust response. We used AKT inhibitor (AKT1/2) to test the PI3K pathway, since it is downstream of PI3K. To test the Jak2/STAT5 signaling pathway, we used Stat5 inhibitor. To test the importance of

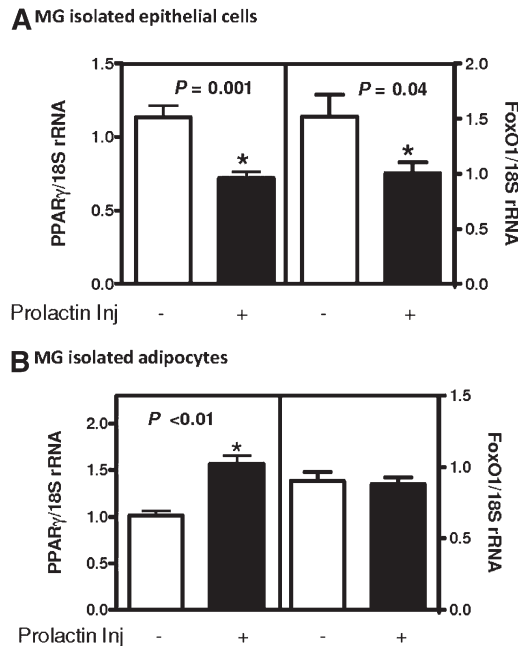


Fig. 3. PPARγ and FoxO1 expression in mammary adipocytes. Mice were treated with saline (–) or with 1 µg/g body weight of prolactin (+), and after 30 min, tissues were isolated. RNA was isolated, and gene expression was measured by RT-PCR and normalized by 18S rRNA. Expression of (A) PPARγ and FoxO1 in isolated mammary epithelium and (B) PPARγ and FoxO1 in isolated mammary gland adipocytes. Values are the mean ± SEM; n = 3–5 mice per time point, *P < 0.05.

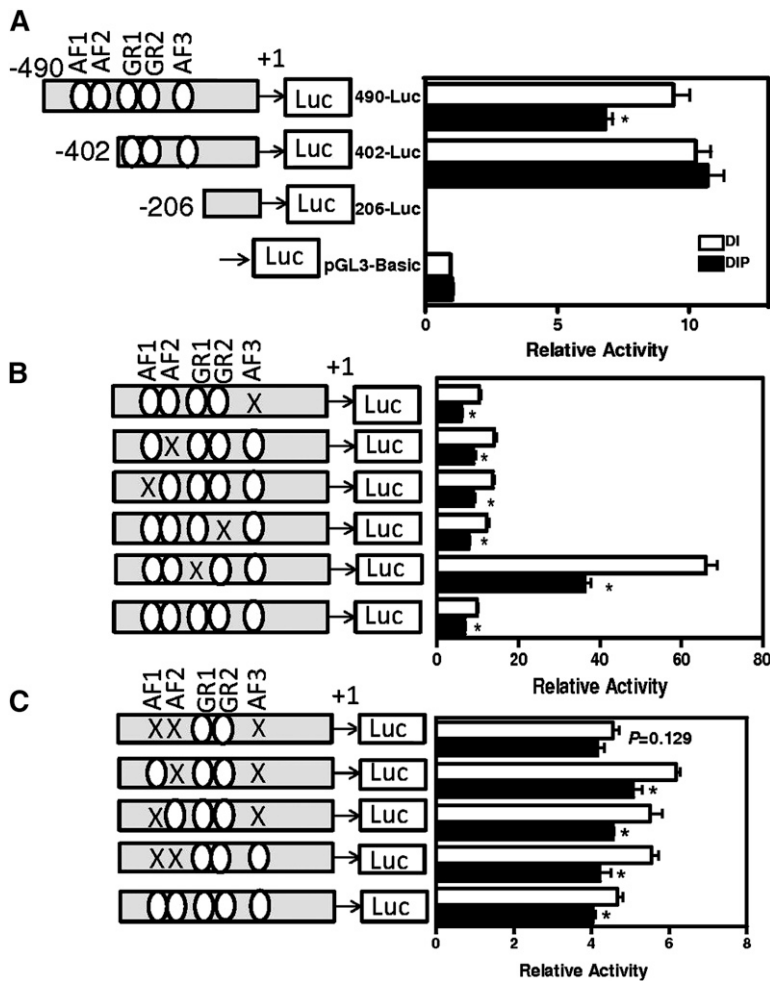


Fig. 4. Deletions and mutation of GRE and AF sites in *Pck1* promoter. A series of constructs were made that contained serial deletions of the *Pck1* promoter-firefly luciferase or contained mutations of GRE and AF sites (represented by X) and transfected into HC11 cells. Cells were incubated with the lactogenic hormones dexamethasone, insulin, and prolactin (DIP) or lactogenic hormones minus prolactin [dexamethasone and insulin (DI)] as described in Experimental Procedures, and relative luciferase activity was measured in cell extracts. A: For serial deletion analysis, portions of the *Pck1* promoter (-490 bp to +72 bp, -402 bp to +72 bp, -206 bp to +72 bp) were cloned into pGL3-basic promoter vector and analyzed, or (B) -490 bp to +72 bp of *Pck1* promoter was cloned into pGL3 basic vector with single site mutations of GRE and/or AF sites or (C) multiple site mutations of GRE and/or AF sites as described in Experimental Procedures. The data are expressed as the means \pm SEM, $n = 6$; $*P < 0.05$.

the MAPK signaling pathway, we used U0126 inhibitor, which is a highly selective inhibitor of MAPK/ERK kinase. We found that the AKT and Stat5 pathways, but not the MAPK pathway, were required for full repression of *Pck1* expression in HC11 cells for both constructs (Fig. 6).

Function of *Pck1* in mammary epithelial cells

Previously, we have shown that the function of *Pck1* in the adipocytes of the mammary gland was glyceroneogenesis (15). To determine the function of *Pck1* in HC11 epithelial cells, we first measured the genes in the gluconeogenic pathway, pyruvate carboxylase (*Pcx*), fructose-6-bisphosphatase (*Fbp*), *Pck1*, and glucose-6-phosphatase (*G6p*) in HC11 cells treated with or without prolactin. We

found that all of these gluconeogenic genes were expressed in HC11 cells. However, *Pcx* and *G6p* mRNA expression was not regulated by prolactin. *Fbp* had increased mRNA expression with prolactin treatment, whereas *Pck1* expression was repressed (Fig. 7).

To analyze the metabolic function of these genes in HC11 cells, we measured synthesis of triglyceride (synthesized glycerol bound to triglyceride) from either [U - $^{13}C_6$]glucose, [U - $^{13}C_3$]glycerol or [U - $^{13}C_3$]pyruvate using MS analyses (Fig. 8A). We established incorporation of [U - $^{13}C_6$]glucose into triglyceride-bound glycerol (Fig. 8A), suggesting that glycerol-3-phosphate was synthesized from glucose through glycolysis. We also found significant incorporation of label into triglyceride-bound glycerol using [U - $^{13}C_3$]glycerol (Fig. 8A),

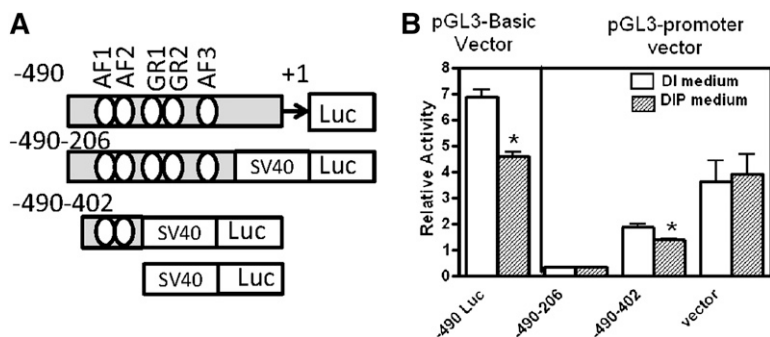


Fig. 5. AF1, AF2, and AF3 regions in the *Pck1* promoter. A: Portions of the *Pck1* promoter (-490 to -402, -490 to -206) were cloned into pGL3-promoter vector or -490 to +72 of *Pck1* promoter was cloned into pGL3 basic vector. B: The constructs and pGL3 promoter vector were transfected into HC11 cells. After transfection, cells were treated with the lactogenic hormones dexamethasone, insulin, and prolactin (DIP) or lactogenic hormones minus prolactin [dexamethasone and insulin (DI)] as described in Experimental Procedures, and luciferase relative activity was measured in cell extracts. The data are expressed as the means \pm SEM; $n = 6$, $*P < 0.05$.

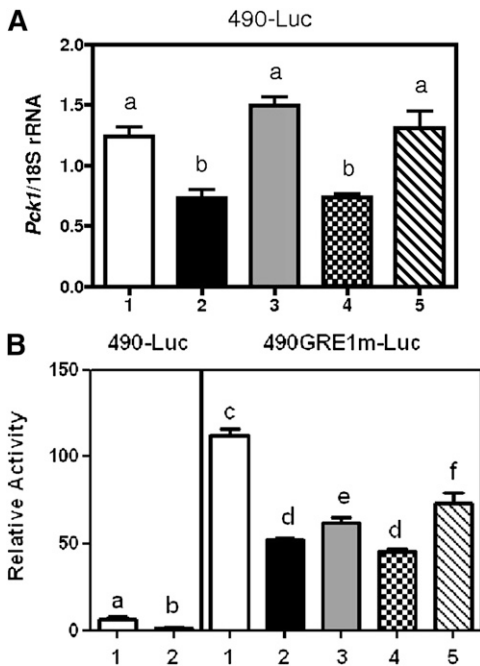


Fig. 6. Inhibitors of prolactin signaling pathways. A: -490 bp to $+72$ bp and (B) -490 GRE1m to $+72$ bp *Pck1* promoter were ligated to firefly luciferase and were transfected into HC11 cells. HC11 cells were treated without (open white bar) and with (shaded bars) prolactin ($1 \mu\text{g}/\text{ml}$) and inhibitor for prolactin pathway for 72 h. 1) Cells treated with dexamethasone and insulin (DI); 2) cells treated with dexamethasone, insulin and prolactin (DPI); 3) cells treated with DIP + AKT inhibitor ($1 \mu\text{M}$); 4) cells treated with DIP + Stat5 inhibitor ($1 \mu\text{M}$); 5) cells treated with DIP + MAPK inhibitor (U0126, $1 \mu\text{M}$). Total RNA was analyzed for *Pck1* expression and normalized with 18S rRNA. Values are the mean \pm SEM, $n = 4$; subscripts with a different letter are statistically significant, $P < 0.05$.

suggesting that glycerol is a precursor for glycerol-3-phosphate via glycerol kinase reaction. Finally, we found incorporation of $[\text{U-}^{13}\text{C}_3]$ pyruvate into triglyceride-bound glycerol, supporting the role of glyceroneogenesis through *Pck1*. We also measured incorporation of either glucose or pyruvate into the newly synthesized FA, palmitate (Fig. 8B). We found that both can contribute to FA synthesis.

Because the amount of newly made triglyceride-bound glycerol was significant from $[\text{U-}^{13}\text{C}_3]$ glycerol, we tested the expression of glycerol kinase (*GyK*) and its activity in HC11 cells. We found a 70% increase in *GyK* mRNA expression and a 50% increase in *GyK* activity with prolactin administration. Therefore *GyK* also contributes to the formation of the glycerol-3-phosphate backbone (Fig. 8C).

To further define the metabolic function of *Pck1* in HC11 cells, we measured synthesis of glucose from $[\text{U-}^{13}\text{C}_3]$ glycerol or $[\text{U-}^{13}\text{C}_3]$ pyruvate using MS analyses (Fig. 8). We found significant incorporation of $[\text{U-}^{13}\text{C}_3]$ pyruvate into glucose in cells and media that was reduced with prolactin treatment (Fig. 9A, B), suggesting that gluconeogenesis occurred through *Pck1*. We also found significant incorporation of $[\text{U-}^{13}\text{C}_3]$ glycerol into glucose in the media that was not regulated by prolactin treatment (Fig. 9B), suggesting that gluconeogenesis was generating glucose through *F6P* and bypassing the *Pcx* and *Pck1* steps in gluconeogenesis.

In this study, we investigated the role of *Pck1* in mammary gland epithelial cells during lactation in mice and in HC11 cells. Previously, mammary gland expression of *Pck1* mRNA has been studied using transgenic mice containing a chimeric gene with the promoter regulatory regions of rat *Pck1* linked to the bovine growth hormone structural gene. In these studies, expression of the transgene is detected in the mammary gland and requires the -355 bp to -460 bp region of the promoter for expression (19). However, our data indicated that the region from -490 bp to -202 bp in the promoter was required for *Pck1* expression in the HC11 cells. The discrepancy may be explained by the use of a different reporter gene. Although we used the same rat promoter as McGrane et al. (19), we used a different reporter gene. McGrane et al. used a growth hormone reporter gene, whereas we used a luciferase reporter gene. In the McGrane et al. studies, they analyzed transgenic mice, which also may be influenced by site of insertion and the enhancers near the transgene.

The presence of *Pck1* activity in the mammary gland of lactating rats and its coordinate regulation by prolactin, glucocorticoids, and insulin has been previously discussed by Lobato et al. (45). Lobato et al. administered 2-bromo- α -ergocryptine, which reduces serum prolactin levels in lactating rats. Prolactin was given back by injection of prolactin, and mammary gland tissue was isolated. Lobato et al. found a 50% increase of *Pck1* activity with administration of prolactin (38). The mammary gland tissue is composed of several cell types, including epithelial cells and adipocytes. We suggest that the increase of *Pck1* activity for the Lobato studies was due to changes in adipocyte expression of *Pck1*, because in our studies, we found prolactin increased *Pck1* mRNA expression in isolated adipocytes from the mammary gland after prolactin administration. Prolactin has been shown not only to regulate lactation in the mammary gland, but also to regulate adipocyte metabolism (46). In WAT, *Pck1* is increased during fasting to reesterify FA back to triglycerides after lipolysis. A key transcription factor that induces *Pck1* expression in WAT is PPAR γ . We found that prolactin induces PPAR γ expression in mammary gland adipose cells. Thus, we propose that prolactin induces glyceroneogenesis in the adipocytes to reesterify excess FAs acids back to triglycerides after lipolysis. We hypothesize that this is necessary to maintain lipid stores in the mammary gland adipocytes for triglyceride synthesis during lactation.

Previously Jimenez et al. (14) established a role for glyceroneogenesis in mammary gland epithelial cells. They analyzed incorporation of labeled acetate and oleate into glycerol-3-phosphate in isolated acini from lactating Wistar rats. However, they suggest that the last steps of glyconeogenesis between triose-phosphate and glucose-6-phosphate are not operative in rat mammary gland acinar cells (14). In our HC11 studies, we found that all of the key gluconeogenic genes were expressed (*Pcx*, *Pck1*, *F6p*, and *G6p*). We showed in our stable isotope studies that glucose was synthesized from labeled pyruvate and that

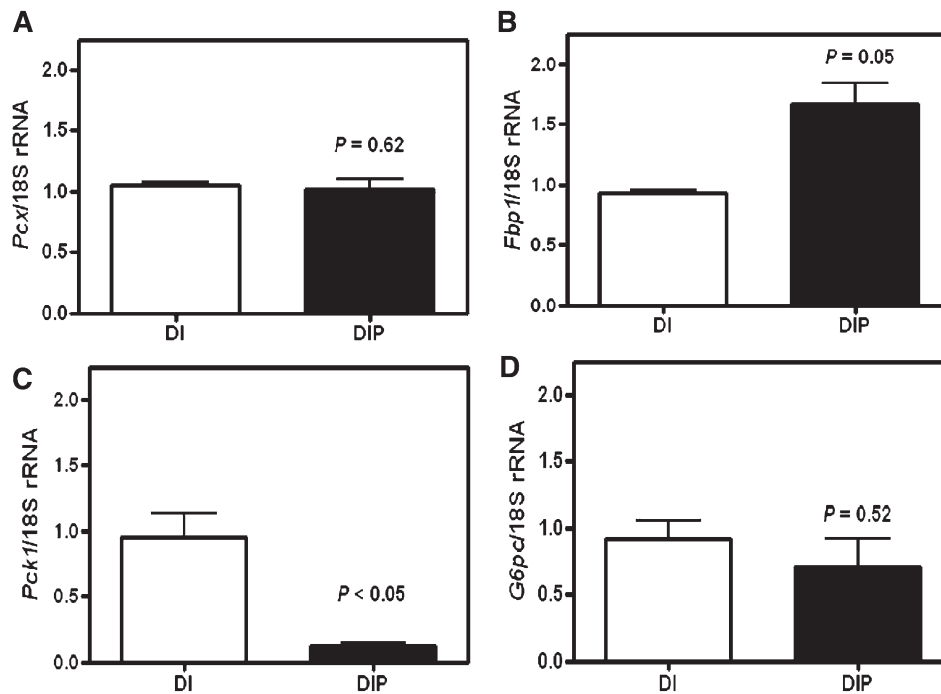


Fig. 7. Expression of gluconeogenic genes. RNA was isolated from HC11 cells treated with the lactogenic hormones dexamethasone, insulin, and prolactin (DIP) or lactogenic hormones minus prolactin [dexamethasone and insulin (DI)] as described in Experimental Procedures. Gene expression was measured by RT-PCR and normalized with 18S rRNA for A: pyruvate carboxylase, Pcx, B: fructose bisphosphatase, Fbp1, C: phosphoenolpyruvate carboxykinase, Pck1 and D: glucose-6-phosphatase, G6P. Values are the mean \pm SEM, $n = 4-6$.

glycerol and was released into the media. Thus, the gluconeogenic pathways were active in the HC11 epithelial cell line. Our isotope data also suggest that glucose and glycerol are the main substrates for glycerol-3-phosphate formation for triglyceride synthesis in HC11 cells. This supports earlier findings by Rao and Abraham (47) that found glucose oxidation for NADPH production and glycerol-3-phosphate production is required for FA synthesis. Our data also suggest that the role of *Pck1* in HC11 cells (epithelial cells) is glyceroneogenesis. We detected incorporation of labeled pyruvate into triglyceride-bound glycerol in HC11 cells.

The *Pck1* promoter has been well characterized (41, 48-53). During fasting and diabetes, cAMP, as well as glucocorticoids, is elevated and induces hepatic *Pck1* expression. The regions of the promoter responsible for glucocorticoid regulation span -455 bp to -321 bp in the *Pck1* promoter and include AF1, AF2, and AF3. The proper positions of AF1, AF2, and AF3 in the *Pck1* promoter are required for complete glucocorticoid response (44). In the current study, we found that these AF regions in the *Pck1* promoter are necessary for regulation of mRNA expression by prolactin in HC11 cells. In the mammary gland, prolactin binds to the extracellular domain of the prolactin receptor and causes dimerization and activation of Jak2, a tyrosine kinase (54-57). Phosphorylation of the intracellular domain recruits Stat5 through its SH2 domain to the receptor and phosphorylates Stat5, resulting in dimerization and nuclear localization. Specific DNA binding of Stat5 response element (Stat5-RE) TTCNNNGAA results in induction of gene transcription.

Glucocorticoids can bind to its receptor and translocate into the nucleus. The glucocorticoid receptor (GR) binds to its glucocorticoid response element (GRE) AGAA-CANNNTGTTCT in the promoter. In the presence of both hormones, Stat5 and the GR form a molecular complex that diminishes the glucocorticoid response of a GRE. Complex formation between Stat5 and the GR prevents binding to the consensus promoter sequence and therefore decreases transcription (58). The promoter of *Pck1* does not have a consensus Stat5-RE, but does have glucocorticoid response unit (GRU), which consists of two GREs (GR1, GR2) and three AF (AF1, AF2, AF3) regions in the -490 bp upstream of the start site. In our model, *Pck1* expression required all three AF sites but not the GRE sites for full repression by prolactin. From the inhibitor studies, we found that the Stat5 pathway is also required for *Pck1*. We propose that Stat5 is interacting with glucocorticoids bound to GR, thus inhibiting binding to the GRU and decreasing *Pck1* gene expression.

FoxO transcription factors are widely expressed (59) and are important targets of insulin action (60). FoxO proteins form a subgroup within the family of Forkhead box transcription factors (61). FoxO1 is expressed in the liver and has been shown to stimulate expression of genes in the gluconeogenic pathway (*Pck1* and glucose-6-phosphatase) (62, 63). FoxO1 interacts with insulin response sequences in the promoter of *Pck1* (64, 65). Inactivation of FoxO1 is through the action of insulin binding to its receptor and activating the insulin signaling cascade. AKT phosphorylates FoxO1, and the transcription factor then translocates into the cytoplasm. As a result of this

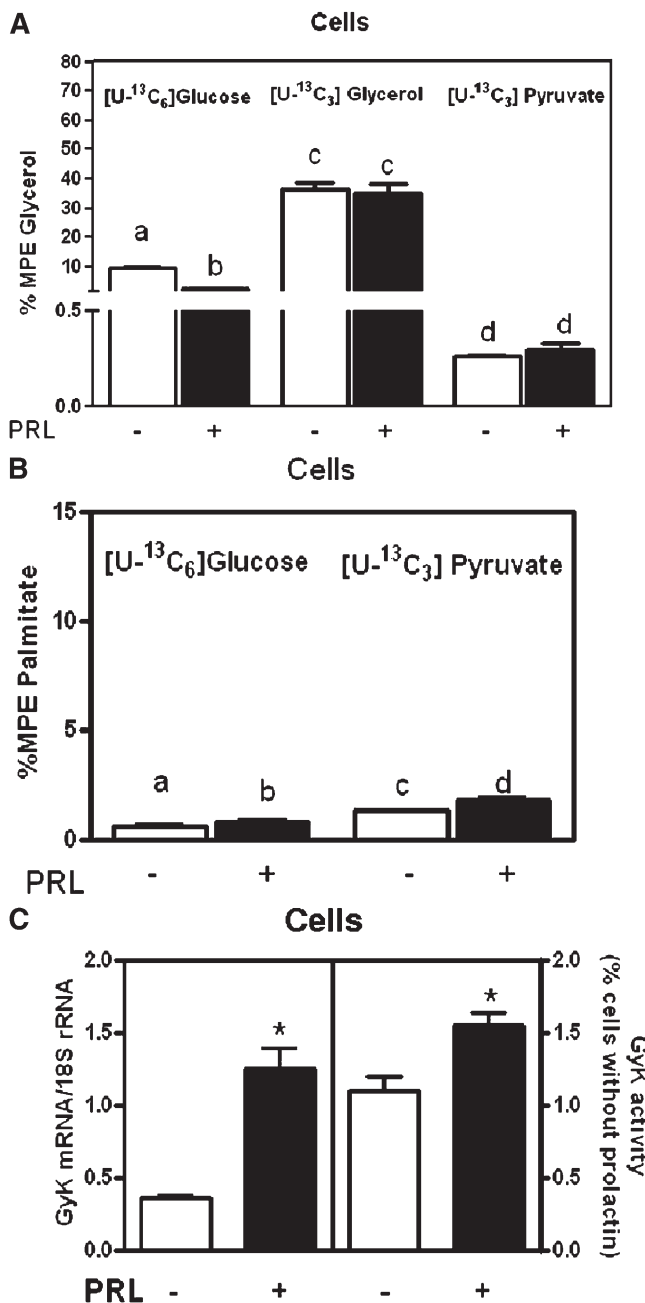


Fig. 8. Synthesis of glycerol-bond triglyceride and palmitate in HC11 cells. A: Percent MPE glycerol synthesis from labeled [U-¹³C₆]glucose, labeled [U-¹³C₃]glycerol, or [U-¹³C₃]pyruvate in HC11 cells treated with the lactogenic hormones dexamethasone, insulin, and prolactin [DIP (black bars)] or lactogenic hormones minus prolactin [dexamethasone and insulin (DI) open white bars] as described in Experimental Procedures. B: Percent MPE palmitate synthesis from labeled [U-¹³C₃]pyruvate and [U-¹³C₆]glucose in cells from HC11-treated cells. C: Glycerol kinase mRNA expression normalized to 18S rRNA and Gyk activity in HC11-treated cells. The data are expressed as the means ± SEM, n = 6; *P < 0.05. Subscripts that are different are statistically significant, P < 0.05.

translocation, when insulin is present, *Pck1* mRNA expression is reduced. In our study, we found reduced expression of FoxO1 mRNA in mammary epithelial cells after prolactin treatment. Using inhibitors for signaling

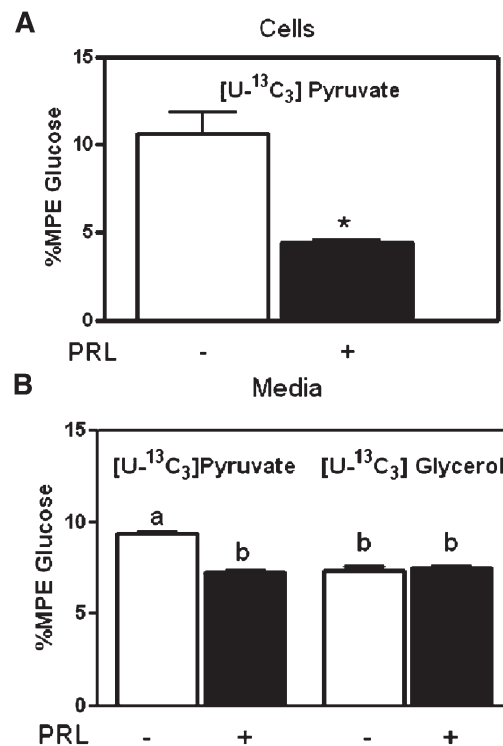


Fig. 9. Glucose synthesis in HC11 cells. A: Percent MPE glucose synthesis from labeled [U-¹³C₃]pyruvate in HC11 cells treated with the lactogenic hormones dexamethasone, insulin, and prolactin [(DIP) black bars] or lactogenic hormones minus prolactin [dexamethasone and insulin (DI) open white bars] as described in Experimental Procedures. B: Percent MPE glucose synthesis from labeled [U-¹³C₃]pyruvate and [U-¹³C₃]glycerol in media from HC11-treated cells. The data are expressed as the means ± SEM, n = 6; *P < 0.05. Subscripts that are different are statistically significant, P < 0.05.

pathways, we established that the AKT pathway is also required for *Pck1* repression by prolactin. Therefore, we propose that prolactin requires both the AKT and Stat5 pathways for repression of *Pck1* in HC11 cells. However, in the inhibitor studies, we did not fully restore 490GRE1m-Luc expression to untreated levels. This suggests that although AKT and Stat5 pathways contribute to regulation of *Pck1* mRNA expression, other factors are also required

During lactation, why is *Pck1* mRNA expression induced in mammary gland adipocytes while it is reduced in mammary gland epithelial cells? We propose that the increased *Pck1* mRNA expression in mammary gland adipocytes during lactation maintains mammary gland adipocyte lipid stores through reesterification of glycerol back to glycerol-3-phosphate. Thus, the adipocytes surrounding the epithelial cells, as well as the diet, would be important sources of FAs and glycerol for milk triglycerides. In the epithelial cells, we found that *Pck1* mRNA was reduced in response to prolactin and participated in gluconeogenesis and glyceroconeogenesis. Lobato et al. (45) have shown that hepatic *Pck1* mRNA is increased with prolactin administration. Thus, we hypothesize that the mammary gland relies on hepatic gluconeogenesis for glucose production and hepatic glyceroconeogenesis for triglyceride synthesis for milk

droplets during lactation. In addition *GyK* expression and activity within mammary gland epithelial cells also contributed to triglyceride production during lactation; we have shown an increase of *GyK* activity in HC11 cells with prolactin administration. This supports that the mammary epithelial cells use glycerol from either the diet or hydrolyzed triglycerides from surrounding adipocytes or other fat stores for glycerol-3-phosphate synthesis and triglyceride formation. Does *Pck1* have a different role in the mammary gland earlier in development? Because we found the highest expression of *Pck1* mRNA at 4 weeks in isolated mammary epithelial cells, it is possible that *Pck1* plays an important role in providing glucose and/or triglycerides during ductal elongation at puberty. Future studies will elucidate the metabolic role of *Pck1* in the developing mammary gland at this earlier time. **FIG**

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