

# A Role for Mitochondrial Phosphoenolpyruvate Carboxykinase (PEPCK-M) in the Regulation of Hepatic Gluconeogenesis\*

Received for publication, December 19, 2013, and in revised form, January 21, 2014  
Published, JBC Papers in Press, February 4, 2014, DOI 10.1074/jbc.C113.544759

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**Background:** PEPCK-M is generally considered irrelevant for glucose production, although gluconeogenesis has never been characterized in its absence.

**Results:** PEPCK-M loss impaired gluconeogenesis from lactate, lowered plasma glucose, insulin, and triglycerides, reduced hepatic glycogen, and increased glycerol turnover.

**Conclusion:** Approximately a third of gluconeogenesis comes from PEPCK-M.

**Significance:** The nutrient-sensitive PEPCK-M has been overlooked and is potentially important for metabolic diseases such as diabetes.

Synthesis of phosphoenolpyruvate (PEP) from oxaloacetate is an absolute requirement for gluconeogenesis from mitochondrial substrates. Generally, this reaction has solely been attributed to the cytosolic isoform of PEPCK (PEPCK-C), although loss of the mitochondrial isoform (PEPCK-M) has never been assessed. Despite catalyzing the same reaction, to date the only significant role reported in mammals for the mitochondrial isoform is as a glucose sensor necessary for insulin secretion. We hypothesized that this nutrient-sensing mitochondrial GTP-dependent pathway contributes importantly to gluconeogenesis. PEPCK-M was acutely silenced in gluconeogenic tissues of rats using antisense oligonucleotides both *in vivo* and in isolated hepatocytes. Silencing PEPCK-M lowers plasma glucose, insulin, and triglycerides, reduces white adipose, and depletes hepatic glycogen, but raises lactate. There is a switch of gluco-

neogenic substrate preference to glycerol that quantitatively accounts for a third of glucose production. In contrast to the severe mitochondrial deficiency characteristic of PEPCK-C knock-out livers, hepatocytes from PEPCK-M-deficient livers maintained normal oxidative function. Consistent with its predicted role, gluconeogenesis rates from hepatocytes lacking PEPCK-M are severely reduced for lactate, alanine, and glutamine, but not for pyruvate and glycerol. Thus, PEPCK-M has a direct role in fasted and fed glucose homeostasis, and this mitochondrial GTP-dependent pathway should be reconsidered for its involvement in both normal and diabetic metabolism.

Inappropriately elevated gluconeogenic flux contributes to the increased fasting plasma glucose associated with poorly controlled type 2 diabetes mellitus (1–4). The decarboxylation of OAA<sup>3</sup> into PEP by PEPCK (E.C. 4.1.1.32) is obligate for mitochondria-derived gluconeogenesis (5). Mitochondrial gluconeogenic precursors such as lactate have an *absolute* requirement for PEPCK. Two isoforms of PEPCK in mammals, cytosolic (PEPCK-C) and mitochondrial (PEPCK-M), arise from distinct but homologous nuclear genes and depend upon GTP hydrolysis (6). The mitochondrial PEPCK isoform was the first to be discovered nearly 60 years ago, and 40% of hepatocyte PEP is in the mitochondria (mtPEP) (7, 8). Its highest level of expression is found in gluconeogenic tissues such as liver and kidney (9). Nevertheless, the cytosolic isoform is generally believed to account for all gluconeogenesis from mitochondria-dependent precursors in mammals (10). It was, therefore, somewhat surprising that endogenous glucose production (EGP) is largely unchanged in a genetic model of hepatic PEPCK-C deletion (11, 12). Similarly, in other genetic models of whole body PEPCK-C deletion (two with similar levels of tissue reduction as the hepatic knock-out), there was no effect on plasma glucose, insulin, glycerol, or lactate, and there was no significant reduction in hepatic glycogen, PEPCK flux, or gluconeogenesis from PEP (12, 13).

PEPCK-C gluconeogenesis has been studied almost to the complete exclusion of PEPCK-M (10, 14). PEPCK-M lacks the strong transcriptional regulation that is characteristic of PEPCK-C (9). An exception is in the chronically glucose-infused rat where augmented PEPCK-M expression accommodates the pathogenic increase in gluconeogenesis (15). The importance of PEPCK-C to human diabetes has recently come under additional scrutiny because PEPCK-C mRNA, protein, and activity from liver biopsy of humans with type 2 diabetes do not correlate with EGP (16). Nevertheless, although substantial indirect data support a diminished role for PEPCK-M, loss of function has never been directly assessed (14).

\* This work was supported, in whole or in part, by National Institutes of Health Grants R01 DK092606 (to R. G. K.), K08 DK080142 (to R. G. K.), R01 DK-40936 (to G. I. S.), R24 DK-085638 (to G. I. S.), U24 DK-059635, P30 DK79310-01, P30 DK34989, and P30 DK45735. This work was also supported by the American Diabetes Association (Grant 7-12-BS-092) (to R. G. K.), and Société Francophone de Néphrologie, Hospices Civils de Lyon (to F. G. E.). S. B. is employed by and holds stocks in Isis Pharmaceuticals.

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<sup>3</sup> The abbreviations used are: OAA, oxaloacetic acid; PEPCK, phosphoenolpyruvate carboxykinase; PEPCK-M, mitochondrial PEPCK isoform; PEPCK-C, cytosolic PEPCK-C isoform; PEP, phosphoenolpyruvate; mtPEP, mitochondrial phosphoenolpyruvate; mtGTP, mitochondrial GTP; ASO, antisense oligonucleotide; EGP, endogenous glucose production; LDH, lactate dehydrogenase; TCA, tricarboxylic acid;  $R_a$ , rate of appearance; Con, control.

The first prominent metabolic role for PEPCK-M was identified in the mechanism of glucose-stimulated insulin secretion (17). Beta-cell glucose metabolism generates mitochondrial GTP (mtGTP) via succinyl-CoA synthetase (E.C. 6.2.1.4) at a rate proportional to TCA cycle flux (18). The mtGTP, lacking a transporter, is confined to the mitochondrial matrix (19). Hydrolysis of mtGTP by PEPCK-M generates mtPEP from OAA. Glucose metabolism increases mtPEP synthesis that then escapes into the cytosol at rates as high as 40% of the glycolytic rate in islets (17). These large fluxes in beta-cells occur despite similar or lower PEPCK-M mRNA, protein, and activity as compared with rodent liver. Consequently, levels of PEPCK-M expression or enzyme activity may not be representative of intracellular metabolic flux. Here, we test the hypothesis that mitochondria of gluconeogenic tissues have an mtGTP-dependent flux-sensing pathway to generate mtPEP for gluconeogenesis.

## EXPERIMENTAL PROCEDURES

**PEPCK-M Silencing**—The Institutional Animal Care and Use Committee (IACUC) of Yale University School of Medicine approved all procedures in rats. Antisense oligonucleotides (ASOs) stabilized with a 2'-O-(2-methoxy)-ethyl modification and a phosphorothioate backbone have been widely used to acutely silence genes in liver, adipose, and proximal tubules but not skeletal muscle or pancreas of healthy, regular chow-fed adult male Sprague-Dawley rats as well as in humans (20). Twice weekly intraperitoneal injections were given for 4 weeks of 25 mg/kg of PEPCK-M ASO (PCK2<sup>ASO</sup>; ISIS421062:5'-GCACGCGCTCTTCCAGGGCC-3') as compared with a control ASO (Con<sup>ASO</sup>; ISIS141923:5'-CCTTCCCTGAAGGTTCTCC-3') (21, 22).

**Basal Turnover Studies**—Catheters were surgically inserted into the right internal jugular vein for sampling and left carotid artery into the proximal aorta for infusion. The rats recovered for 1 week prior to study. At the end of the studies, rats were anesthetized with intravenous pentobarbital, and tissues were freeze-clamped and stored at  $-80^{\circ}\text{C}$  for later analysis of hepatic glycogen, triglycerides, and mRNA (15, 21, 22). To minimize disturbance, rats were swivel-harnessed for the fasting studies and had free access to the cage (15). Glucose turnover rates were determined by primed continuous infusion of 99% D-[6,6-D<sub>2</sub>]glucose (21, 22). In a separate study, 98% L-[<sup>13</sup>C<sub>3</sub>]lactate ( $3\ \mu\text{mol kg}^{-1}\ \text{min}^{-1}$ ), 99% [1,1,2,3,3-<sup>2</sup>H<sub>5</sub>]glycerol ( $0.8\ \mu\text{mol kg}^{-1}\ \text{min}^{-1}$ ), 98% L-[2,3,3,3-<sup>2</sup>H<sub>4</sub>] alanine ( $2\ \mu\text{mol kg}^{-1}\ \text{min}^{-1}$ ), 97% L-[2,3,3,4,4-<sup>2</sup>H<sub>5</sub>]glutamine ( $1\ \mu\text{mol kg}^{-1}\ \text{min}^{-1}$ ), and 98% [<sup>13</sup>C, <sup>15</sup>N<sub>2</sub>]urea ( $1.2\ \mu\text{mol kg}^{-1}\ \text{min}^{-1}$ ) were used as a primed continuous infusion (Cambridge Isotope Laboratories Inc.). The choice of isotope labels minimized the exchange of label between the metabolites during the study. The experimental setup led to no change in plasma substrate levels, and all labels reached steady state at the time of their measurement. Glucose and glycerol enrichments were measured using GC/MS, and lactate, alanine, glutamine, and urea were measured by LC/MS/MS (17, 23). Samples were separated by isocratic elution (0.1% formic acid or 4 mM ammonium formate) from a Hypercarb column (3- $\mu\text{m}$  particle size,  $3 \times 150\ \text{mm}$ , Thermo Fisher Scientific) at  $700\ \mu\text{l}/\text{min}$  using a

Shimadzu Prominence UHPLC. Samples were ionized by electrospray into an AB SCIEX 5500 QTRAP equipped with a SelectION for differential mobility separation and acquired using multiple reaction monitoring simultaneously in positive mode (24). Samples were integrated using MultiQuant (AB SCIEX). Retention times of each of the metabolites were identified using known natural abundance and labeled standards. Atom percent excess of the metabolites was corrected for background, and concentrations were determined using standard curves of both unlabeled and labeled standards as described previously (17, 24). Unique parent daughter ions of alanine (90/44), glutamine (147/130), and urea (61/41) and their isotopologues were collected. Rates of basal turnover were determined as the ratio of the rate of the tracer infusion ( $\mu\text{mol}/\text{kg}/\text{min}$ ) to the plasma APE of the tracer minus the tracer infusion rate (corrected for its enrichment).

**Hepatocyte Studies**—Hepatocytes were isolated by the Yale University Liver Center from overnight fasted Con<sup>ASO</sup> and PCK2<sup>ASO</sup>. Isolated hepatocytes were suspended and washed two times in recovery medium containing DMEM high glucose (20 mM) (Sigma, D5648) with 10% FBS, 1 nM insulin, 1 nM dexamethasone, and antibiotics (10,000 units/ml penicillin and 10 mg/ml streptomycin, Invitrogen). Cell count and viability were estimated by trypan blue exclusion. The cells were plated at  $5 \times 10^5$  cells/cm<sup>2</sup> in collagen-I-coated (BD Biosciences) 6-well plates in recovery medium and were cultured under 5% CO<sub>2</sub> and 95% O<sub>2</sub> in air at  $37^{\circ}\text{C}$ . After 4 h, cells were washed with PBS, and the medium was changed to DMEM low glucose (5 mM) (Sigma, D5921) with supplemented antibiotics but no hormones prior to gluconeogenesis assays. Hepatocytes were also transfected using RNAiFect (Qiagen) reagent with a control or PEPCK-M siRNA and then cultured overnight before analysis as described previously for INS-1 cells (17). Silencing was confirmed at the time of gluconeogenesis by mRNA measurements. Hepatocytes were incubated in the presence of substrates as indicated in DMEM with 0.5 mM oleic acid without glucose. After 3 h the medium was removed for enzymatic analysis of glucose and normalized to the protein content. Oxygen consumption was measured from the indicated substrates in bicarbonate-free DMEM with 0.5 mM oleic acid using a Seahorse XF24 per the manufacturer's recommendations.

**Hormone and Metabolite Measurements**—Plasma glucose values for the animal experiments were determined by using 10  $\mu\text{l}$  of plasma by a glucose oxidase method (Beckman glucose analyzer II; Beckman Coulter). Plasma insulin, glucagon, adiponectin, and leptin concentrations were determined by a Multiplex assay (Millipore) and collected on ice in the presence of aprotinin. Corticosterone was measured via RIA (Millipore). Chemistry analysis to measure plasma triglyceride, nonesterified fatty acid, albumin, alanine aminotransferase, and ketone concentrations was performed using the COBAS MIRA Plus (Roche Diagnostics). Blood gases were determined immediately by a handheld clinical analyzer (i-STAT EC8, Abbott Laboratories).

**Statistics and Data Analysis**—The difference in glycogenolytic contribution to EGP for PCK2<sup>ASO</sup> was estimated assuming a uniform rate across the 12-h fast. Here, the difference in total liver glycogen between CON<sup>ASO</sup> and PCK2<sup>ASO</sup> in the basal

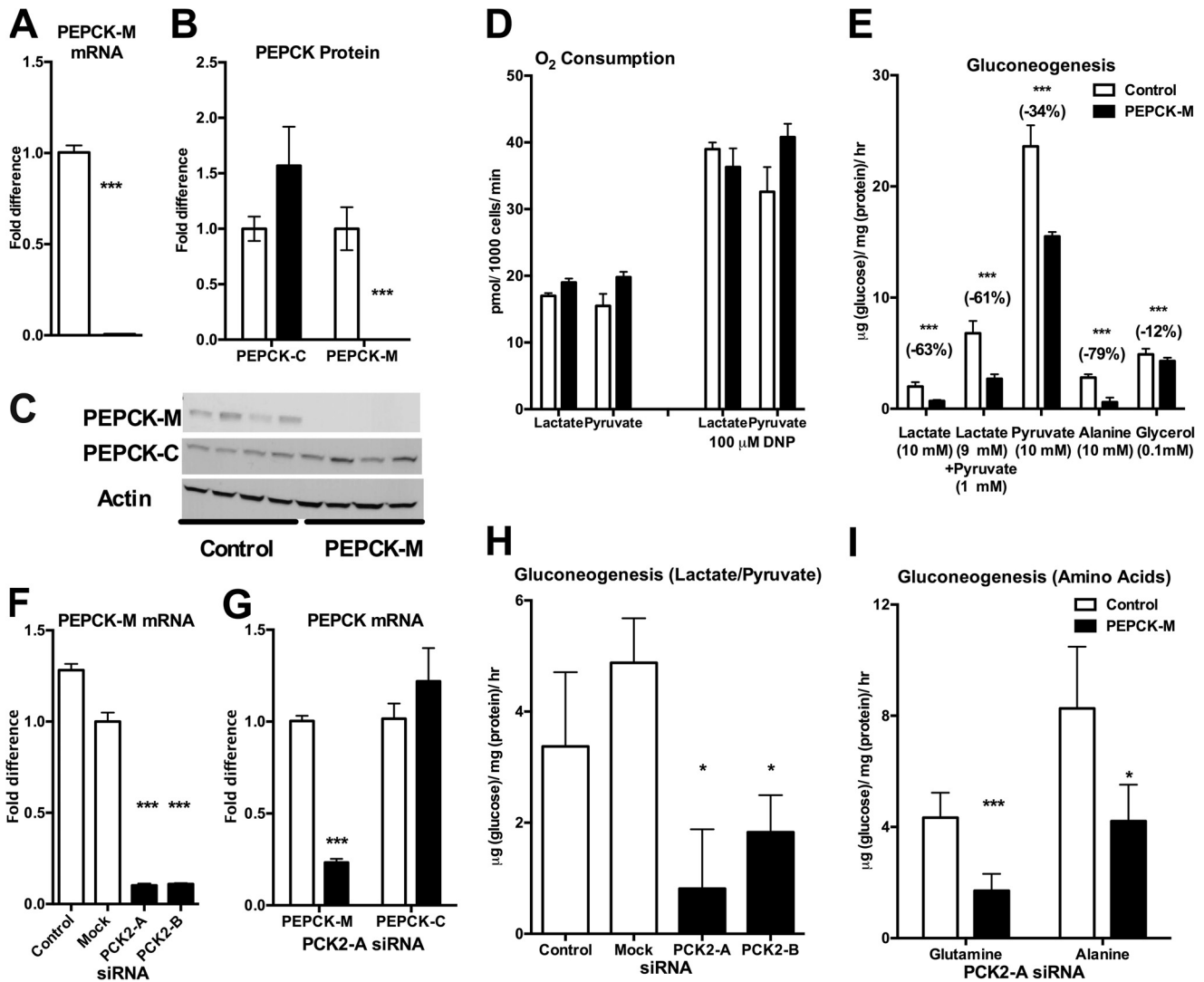


FIGURE 1. **Oxygen consumption and gluconeogenesis from hepatocytes.** *A*, PEPCK-M mRNA levels from hepatocytes isolated from Con<sup>ASO</sup> (white bars) and PCK2<sup>ASO</sup> (black bars). *B* and *C*, PEPCK-M and PEPCK-C protein expression quantified by Western blotting and normalized to actin. Knockdown levels in hepatocytes are indicative of PEPCK-M levels at the time of the *in vivo* and *in vitro* studies. *D*, oxygen consumption rates in hepatocytes incubated in the presence of either 10 mM lactate or pyruvate alone or uncoupled by 100  $\mu$ M dinitrophenol (DNP) ( $n = 5/5$ ) in isolated primary hepatocytes Con<sup>ASO</sup> (white bars) and PCK2<sup>ASO</sup> (black bars) rats ( $n = 6/6$ ). *E*, glucose production rates from hepatocytes treated with the indicated concentration of substrate. The percentage of decrease from Con<sup>ASO</sup> is indicated in parentheses for each substrate ( $n = 6/6$ ). *F* and *G*, PEPCK-M and PEPCK-C mRNA levels from isolated hepatocytes acutely transfected ( $n = 6/6$ ) with control (white bars) or two different siRNAs (PCK2-A and PCK2-B, black bars). *H* and *I*, glucose production from hepatocytes incubated with 9 mM lactate and 1 mM pyruvate (*H*) or 10 mM glutamine or alanine with siRNA PCK2-A (*I*). Data are presented as mean  $\pm$  S.E. *t* test: \*,  $p < 0.05$ ; \*\*\*,  $p < 0.001$ .

state was divided by the body weight and duration of the fast. This calculation could underestimate glycogenolytic contributions if fed glycogen was higher in the PCK2<sup>ASO</sup> or if glycogenolytic rates increased as the fast progressed. All data are reported as mean  $\pm$  S.E. Differences were considered to be significant at  $p < 0.05$  by unpaired two-tailed Student's *t* tests and one-way analysis of variance.

## RESULTS

**PCK2<sup>ASO</sup> Impairs Hepatic Mitochondrial Gluconeogenesis but Not Oxidative Respiration**—Hepatocytes were isolated from the livers of CON<sup>ASO</sup> and PCK2<sup>ASO</sup> rats to confirm whether PEPCK-M deletion directly affected hepatic gluconeogenesis. PEPCK-M mRNA and protein were silenced by the ASO treatment, although PEPCK-C protein was not significantly increased (Fig. 1, *A–C*). Preserved mitochondrial

oxidative function in PCK2<sup>ASO</sup> hepatocytes was indicated by preserved basal and uncoupled mitochondrial respiration (Fig. 1*D*).

Maximal gluconeogenic rates from lactate and alanine were severely impaired in hepatocytes from PCK2<sup>ASO</sup> rats with less impact for pyruvate or glycerol (Fig. 1*E*). The NADH made by LDH during lactate metabolism is consumed by glyceraldehyde-3-phosphate dehydrogenase (GAPDH) during gluconeogenesis and thus favors PEPCK-M to maintain a balanced cytosolic redox state (14, 25–27). In contrast, pyruvate depends on cytosolic malate dehydrogenase (rather than LDH) to supply GAPDH with NADH and thus favors PEPCK-C metabolism. Accordingly, pyruvate was a better gluconeogenic substrate than lactate in PCK2<sup>ASO</sup> rats. Glycerol is independent of both PEPCK isoforms and was largely unaffected. At higher glycerol concentrations, there were no differences in gluconeogenesis



## REPORT: Gluconeogenesis Relies on PEPCK-M

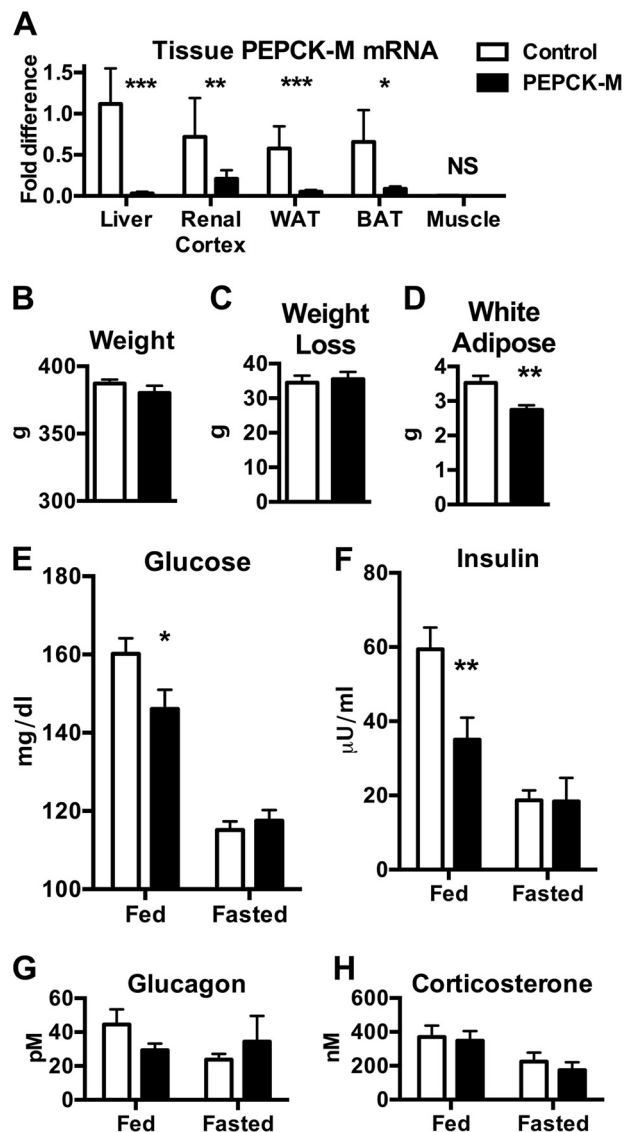
(not shown). Therefore, PEPCK-M silencing does not impair mitochondrial respiration or post-mitochondrial gluconeogenesis but severely impairs gluconeogenesis from mitochondrial substrates.

To rule out off-target effects of the ASO, PEPCK-M was acutely silenced with two different siRNAs in normal rat hepatocytes (Fig. 1, *F* and *G*). Here, maximal rates of gluconeogenesis from alanine and glutamine as well as lactate were also reduced (Fig. 1, *H* and *I*). Thus, despite its reported relatively low enzyme activity, at least *in vitro*, PEPCK-M loss appears to have a significant role in hepatic gluconeogenesis and particularly for gluconeogenesis from lactate.

**PEPCK-M Deficiency Is More Exposed during the Fed State**—ASO-treated rats were studied while fed and following a 36-h fast. The insulin to glucagon ratio largely determines the expression of PEPCK-C but not PEPCK-M. Thus, compensation by PEPCK-C should be minimal while fed and greatest following a prolonged fast. PEPCK-M was silenced in gluconeogenic/glyceroneogenic tissues but not in muscle (where its relative expression is very low) (Fig. 2*A*). Both groups started at the same weight and lost ~8% of initial body weight during the fast, although the epididymal fat pad mass was 20% lower in PCK2<sup>ASO</sup> (Fig. 2, *B–D*). Fed PCK2<sup>ASO</sup> glucose levels were 14 mg/dl lower, but that difference disappeared following the fast (Fig. 2*E*). Strikingly, fed insulin in PCK2<sup>ASO</sup> rats was also 40% lower, but this difference vanished at the end of the fast (Fig. 2*F*). Counter-regulatory hormones were similar at both times (Fig. 2, *G* and *H*). As would be predicted, the role of PEPCK-M in glucose homeostasis is more obvious during the fed state when insulin is high and PEPCK-C is suppressed.

**PCK2<sup>ASO</sup> Impacts Basal Metabolism**—Following an overnight fast, the difference in plasma glucose was intermediate between the fed and 36-h fasted state (-8 mg/dl), and the difference in plasma insulin was no longer statistically significant although ketones were elevated (Table 1). Glucagon, adiponectin, leptin, and corticosterone were similar, but hepatic glycogen content was ~70% lower in PCK2<sup>ASO</sup> rats. In contrast to the hepatic steatosis previously demonstrated with PEPCK-C deficiency (12, 28), hepatic triglycerides as well as other liver function tests were not elevated with PCK2<sup>ASO</sup>. In addition, there is a consistent reduction in epididymal fat and plasma triglycerides (-40%) despite similar insulin levels. Although plasma lactate was not elevated in PEPCK-C knock-out mice (12), it was 25% higher in PCK2<sup>ASO</sup>. Nevertheless, the other gluconeogenic precursors are unchanged, and pH balance was maintained. Increased amino acid deamination is suggested by a lower urine pH and higher blood urea nitrogen and ammonia. In previous studies there was no quantitatively significant difference in basal glucose turnover in hepatic PEPCK-C knock-outs (11, 12). Similarly, basal EGP was the same for CON<sup>ASO</sup> and PCK2<sup>ASO</sup>, suggesting that another metabolic pathway was compensating for PEPCK-M loss (Table 1).

**PEPCK-M Deficiency Promotes a Gluconeogenic Substrate Switch from Lactate to Glycerol**—Lower hepatic glycogen (Table 1) following an overnight fast could indicate either decreased glycogen synthesis or enhanced glycogenolysis. However, if equivalent glycogen stores at the beginning of the fast are assumed, then enhanced PCK2<sup>ASO</sup> glycogenolysis could



**FIGURE 2. Fed and fasted glucose homeostasis.** Rodents were allowed to eat *ad lib* overnight, and then food was withdrawn at 6 a.m. (*Fed*) before rodents underwent a 36-h fast (*Fasted*). *A*, PEPCK-M mRNA in indicated tissue CON<sup>ASO</sup> (white bars) and PCK2<sup>ASO</sup> (black bars) rats determined by quantitative PCR. Results are normalized to the control liver mRNA to demonstrate relative abundance of message, and significance is between CON<sup>ASO</sup> and PCK2<sup>ASO</sup>. WAT, white adipose tissue; BAT, brown adipose tissue. *B* and *C*, initial weights (*B*) and weight loss during and white adipose mass after the 36-h fast (*C*). *E–H*, glucose, insulin, glucagon, and corticosterone were measured. Data are shown as mean ± S.E. for *n* = 14/12 rats. \*, *p* < 0.05; \*\*, *p* < 0.01; \*\*\*, *p* < 0.001; NS, not significant.

only account for at most an additional estimated 3  $\mu\text{mol kg}^{-1} \text{min}^{-1}$  of glucose production. The elevated lactate also suggested dysfunctional Cori cycling. To assess whether compensation from other PEPCK-M-independent pathways helps maintain endogenous glucose production, the turnover rates of other gluconeogenic substrates were independently determined via tracer infusion. Basal turnover of glutamine ( $16.2 \pm 0.7$  versus  $15.9 \pm 0.4 \mu\text{mol kg}^{-1} \text{min}^{-1}$ ), alanine ( $21.3 \pm 1.1$  versus  $20.6 \pm 2.2 \mu\text{mol kg}^{-1} \text{min}^{-1}$ ), and urea ( $51.6 \pm 2.5$  versus  $52.3 \pm 2.3 \mu\text{mol kg}^{-1} \text{min}^{-1}$ ) suggest that amino acids individually or together are not providing significant additional gluconeogenic support. In contrast, the glycerol turnover is >25%

**TABLE 1**

Tissue weight, metabolites, and hormones from Con<sup>ASO</sup> and PCK2<sup>ASO</sup> rodents following an overnight (12-h) fast ( $n = 22/24$ )

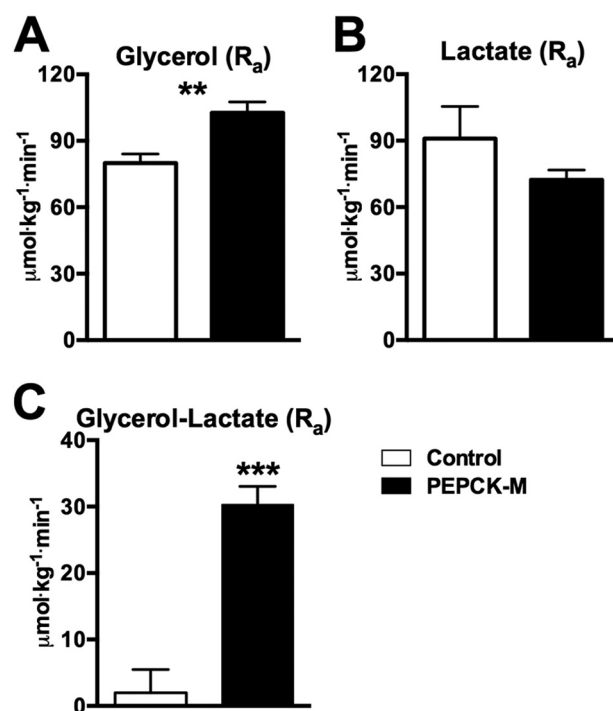
\*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ . The abbreviations are: NS, not significant; BUN, blood urea nitrogen; ALT, alanine aminotransferase; AST, aspartate aminotransferase.

	Control	PEPCK-M	<i>t</i> test
<b>Body</b>			
Weight (g)	356.2 ± 2.5	358.7 ± 2.4	NS
White adipose (g)	3.63 ± 0.36	2.78 ± 0.16	0.047*
<b>Metabolites</b>			
Glucose (mg/dl)	114.3 ± 1.8	106.8 ± 2.4	0.016*
Glucose $R_a$ ( $\mu\text{mol/kg/min}$ )	40.4 ± 1.1	38.8 ± 1.0	NS
Lactate (mM)	0.42 ± .02	0.53 ± 0.04	0.03*
Glycerol (mM)	0.27 ± 0.01	0.30 ± 0.01	NS
Glutamine (mM)	0.59 ± 0.02	0.59 ± 0.01	NS
Alanine (mM)	0.51 ± 0.02	0.49 ± 0.05	NS
BUN (mM)	4.3 ± 0.3	5.2 ± 0.2	0.022*
<b>Hormones</b>			
Insulin (microunits/ml)	11.1 ± 1.0	9.32 ± 1.0	NS
Glucagon (pg/ml)	53.1 ± 2.8	50.8 ± 1.9	NS
Adiponectin ( $\mu\text{g/ml}$ )	5.5 ± 0.4	5.6 ± 0.4	NS
Leptin (ng/ml)	1.7 ± 0.2	1.3 ± 0.1	NS
Corticosterone (ng/ml)	181.6 ± 37.5	180.7 ± 33.5	NS
<b>Plasma lipids</b>			
$\beta$ -Hydroxybutyrate (mM)	1.1 ± 0.05	1.3 ± 0.07	0.03*
Triglycerides (mg/dl)	44.0 ± 4.6	25.2 ± 2.8	0.0009***
Total cholesterol (mg/dl)	73.4 ± 3.6	73.7 ± 3.2	NS
HDL cholesterol (mg/dl)	13.5 ± 0.7	12.7 ± 0.5	NS
Nonesterified fatty acid (mM)	0.78 ± 0.04	0.83 ± 0.05	NS
<b>Liver function</b>			
Glycogen (mg/g of liver)	8.6 ± 2.2	2.9 ± 0.6	0.006**
Liver triglycerides (mg/g)	7.37 ± 0.54	6.01 ± 0.52	NS
ALT (units/liter)	40.3 ± 1.7	44.4 ± 2.4	NS
AST (units/liter)	93.8 ± 7.3	87.4 ± 8.8	NS
Bilirubin (mg/dl)	0.1 ± 0.008	0.1 ± 0.007	NS
<b>Chemistries</b>			
Potassium (meq/liter)	3.9 ± 0.1	4.1 ± 0.1	NS
Sodium (meq/liter)	142 ± 0.5	142 ± 0.3	NS
pH	7.46 ± 0.01	7.45 ± 0.01	NS
pCO <sub>2</sub> (mmHg)	38.2 ± 1.5	40.3 ± 1.0	NS
Bicarbonate (mM)	24.2 ± 0.8	22.9 ± 0.5	NS
Urine pH	6.0 ± 0	5.63 ± 0.1	0.001***
Ammonia ( $\mu\text{M}$ )	23.6 ± 0.7	28.7 ± 1.9	0.026*

( $22.7 \pm 4.9 \mu\text{mol kg}^{-1} \text{min}^{-1}$ ) higher than controls and mirrored by a reciprocal decrease in lactate turnover (Fig. 3, A and B). *In vivo*, lactate rapidly equilibrates across LDH, making accurate determinations of lactate turnover via tracer infusion more challenging (29). Nevertheless, the individual and overall differences between glycerol and lactate turnover rates identify a substantial shift to a PEPCK-independent gluconeogenic precursor at a rate that can account for a third of EGP (Fig. 3C). The reduction in white adipose (Fig. 2D and Table 1) is consistent with increased lipolysis supplying the glycerol rather than a glycerol-consuming defect in adipose glyceroneogenesis. Circulating glycerol must be phosphorylated by hepatic and/or renal glycerol kinase to be further metabolized. Increased glycerol turnover in the setting of lower plasma and hepatic triglycerides indicates that glycerol is fueling gluconeogenesis rather than being used to esterify fatty acids. Taken together, elimination of PEPCK-M from gluconeogenic tissues from otherwise healthy adult rats significantly impacts glucose homeostasis.

## DISCUSSION

Contemporary views of PEPCK-M would argue its elimination would be inconsequential to gluconeogenesis (14). Previously, strong arguments have favored a role for PEPCK-M in lactate gluconeogenesis, and repeated calls to further study



**FIGURE 3. Basal turnover studies from overnight fasted rats.** A and B, CON<sup>ASO</sup> (white bars) and PCK2<sup>ASO</sup> (black bars) rats were given tracer infusions of labeled (A) lactate and (B) glycerol ( $n = 7/8$ ) for basal turnover calculations. C, the individual differences between glycerol and lactate  $R_a$  are indicative of a substantial shift in gluconeogenic precursor preference. Data are presented as mean ± S.E., \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ .

PEPCK-M have been made (10, 14, 25–27). Nevertheless, PEPCK-M deletion was predicted to be “unlikely to have a relevant metabolic impact” at least in rodents (10). This apparent “tyranny of the species” may have arisen from a consistent underestimation of hepatic PEPCK-M activity in rodents by some assays (14, 17). Given the strong knock-out phenotype in rodents, this may indicate a much more important role in humans where its prevalent activity is not questioned. PEPCK-M silencing lowers plasma glucose, insulin, triglycerides, fat mass, and hepatic glycogen and raises lactate in normal healthy adult rats. Although the aggregate EGP did not change in this model, the relative contributions of lactate and glycerol did. There is a robust increase in glycerol turnover with a compensating reduction in lactate turnover accounting for nearly a third of EGP. Finally, silencing PEPCK-M in hepatocytes severely reduces glucose production from lactate but not glycerol. Unlike PEPCK-C deficiency, where resulting mitochondrial dysfunction affects *both* PEPCK isoforms (14), PEPCK-M loss leaves mitochondrial function intact but still impairs gluconeogenesis. In this setting, even normal PEPCK-C levels do not completely compensate for PEPCK-M loss. Thus, despite its reported scarcity as compared with PEPCK-C, PEPCK-M is identified as a key component of physiologic gluconeogenesis. It is important to clarify that the PCK2<sup>ASO</sup> data do not rule out contributions from PEPCK-C, but rather demonstrate for the first time a prominent and direct gluconeogenic role for PEPCK-M in mammals *in vivo*. Given the contribution of excessive gluconeogenesis to diabetes (1, 2), a heretofore untested possibility is that PEPCK-M is a nutrient-driven path-

ogenic contributor to excessive glucose production in diabetes that is potentially independent of insulin signaling.

This study is the first to directly address the role of PEPCK-M by elimination of the enzyme. The rats in these studies are otherwise healthy and not PEPCK-C-deficient. Basal glucose  $R_a$  is the same between the two groups *in vitro* despite severely impaired gluconeogenesis for individual substrates. This is similar to PEPCK-C knockouts with the exception that hepatic *PEPCK-C* deletion did not change glycerol or lactate turnover (11, 12). Glucose  $R_a$  is a composite of both PEPCK isoform fluxes plus net glycerol-3-phosphate dehydrogenase flux (into dihydroxyacetone phosphate) minus pyruvate kinase flux and glycogen synthesis. Still, it is often confused as a direct surrogate for PEPCK flux. In the absence of PEPCK-M, PEPCK-C was unable to fully compensate for PEPCK-M loss as indicated by a switch to glycerol to maintain glucose output. Indeed, the total physiologic contribution of PEPCK-M is likely underestimated given the possibility of enhanced glycogenolysis, other contributions from unmeasured gluconeogenic precursors, and/or partial PEPCK-C compensation.

Despite the impressive hormonal regulation of PEPCK-C, its prominent activity in rodent livers, and the finding that its knock-out impairs glucose production from perfused livers, the enthusiasm for PEPCK-C as sole regulator of gluconeogenic PEP synthesis may need to be recalibrated (14). In the past, a major argument against PEPCK-M in rodents has hinged upon relative enzyme activities of the isoforms. The cytosolic and mitochondrial isoforms are in different compartments, and PEPCK-M/PEPCK-C activity ratios can range anywhere from undetectable to 50% in rat liver depending on how the assay is performed (6, 14, 17, 30–38). Furthermore, expression levels are relevant only if PEPCK-M activity is rate-limiting. Pancreatic beta-cells are devoid of PEPCK-C, whereas PEPCK-M is present at comparable levels to liver (17). Here, PEPCK-M flux approaches 40% the glycolytic rate, indicating a high flux capacity.

Rodents should not be considered PEPCK-M knockouts especially when interpreting data from PEPCK-C knock-out mice (11–13, 39). It is important to point out that both isoforms depend on mitochondrial metabolism. Although stoichiometrically implausible, complete hepatic PEPCK-C loss reduces  $O_2$  consumption by 20% and also reduces [ $^{13}C$ ]propionate NMR estimates of TCA flux by 85% (13). Whole body loss of PEPCK-C increases hepatic TCA cycle intermediates and reduces oxidation of glucose, acetate, and glutamate between 65 and 80% (12, 28). Recently, 100-fold hepatic overexpression of PEPCK-M in liver-specific knockouts of PEPCK-C had no measurable consequence *in vivo* and only a marginal improvement (~8%) of PEP gluconeogenesis *in vitro* (39). The interpretation was that PEPCK-M has a minimal supportive role in gluconeogenesis. Another interpretation is that normal levels of PEPCK-M are sufficient for its metabolic role, but that complete PEPCK-C deletion impairs mitochondrial function severely enough to block PEPCK-M-mediated gluconeogenesis. The lack of hypoglycemia in hepatic PEPCK-C knockouts has been attributed to extra-hepatic sources, although this has not been experimentally tested. *Prima facie*, without a change in expression or activity of PEPCK-C in the kidney, without

changes in insulin, glucagon, or corticosterone, and without alterations in gluconeogenic substrate concentrations or turnover rates, this would appear a less likely explanation (11, 12).

The PEPCK isoforms consume GTP in compartmentalized but parallel reactions to generate PEP. The linkage of PEPCK-M to mtGTP production acts metaphorically like a mitochondrial “metabolic tachometer” coupling PEP production to TCA flux. In principle, this mechanism can sense and respond to acute changes within the local energetic environment (14, 17, 18). PEPCK-M provides a more efficient pathway from mitochondrial OAA to cytosolic PEP from lactate in terms of energetics (as much as 60% more energy-efficient), enzymatics (two *versus* four enzymes), and transport (two *versus* four transporters) that is independent of oxidative phosphorylation (14, 25–27). In rats lipopolysaccharide administration raises plasma lactate and lowers plasma glucose and is associated with a decreased PEPCK-M but increased PEPCK-C expression (40). Interestingly, whole body PEPCK-C reduction >90% did not significantly change basal glucose metabolism (12). This fits a model where constitutive PEPCK-M activity provides the foundation of gluconeogenesis that can be augmented by PEPCK-C in times of increased demand.

In summary, despite reports of low rodent enzyme activity *in vitro*, PEPCK-M has a crucial metabolic role in fed and fasted PEP production *in vivo* (6, 14, 17, 30–38). Even in the presence of normal PEPCK-C, PCK2<sup>ASO</sup> rats have lower plasma glucose and insulin but increased lactate. During fasting, a switch to glycerol bypasses the PEPCK reaction, and consequently, there is no hypoglycemia. Future studies are indicated for the role of PEPCK-M in lipid metabolism given the reduced plasma lipids, increased glycerol turnover, and reduced adipose. Notably, there is no mitochondrial impairment, disruption of pH, hepatic steatosis, or hepatitis. We hypothesize that PEPCK-M and succinyl CoA synthetase GTP isoform (SCS-GTP) serve as intermeshing metabolic gears that use the mtGTP cycle to sense TCA flux (14). Curiously, the same mitochondrial metabolic cycle could regulate both insulin secretion and gluconeogenesis. Incidentally, human liver has quantitatively more PEPCK-M activity than rodents (41). Taken together, gluconeogenesis from PEPCK-M is significant in rodents and may be an overlooked contributor to diabetes and/or a viable pharmaceutical target in humans.

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*Acknowledgments*—We thank Yanna Kosover and Lonnette Diggs of Yale University School of Medicine for their expert technical assistance with the studies and Aida Groszmann for performing hormone assays.

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