Mechanisms by Which Liver-Specific PEPCK Knockout Mice Preserve Euglycemia During Starvation

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Liver-specific PEPCK knockout mice, which are viable despite markedly abnormal lipid metabolism, exhibit mild hyperglycemia in response to fasting. We used isotopic tracer methods, biochemical measurements, and nuclear magnetic resonance spectroscopy to show that in mice lacking hepatic PEPCK, *1*) whole-body glucose turnover is only slightly decreased; *2*) whole-body gluconeogenesis from phosphoenolpyruvate, but not from glycerol, is moderately decreased; *3*) tricarboxylic acid cycle activity is globally increased, even though pyruvate cycling and anaplerosis are decreased; *4*) the liver is unable to synthesize glucose from lactate/pyruvate and produces only a minimal amount of glucose; and *5*) glycogen synthesis in both the liver and muscle is impaired. Thus, although mice without hepatic PEPCK have markedly impaired hepatic gluconeogenesis, they are able to maintain a near-normal blood glucose concentration while fasting by increasing extrahepatic gluconeogenesis coupled with diminishing whole-body glucose utilization. *Diabetes* 52:1649–1654, 2003

Glucose homeostasis requires a precise balance between glucose production and utilization. The liver plays a key role in maintaining this balance during fasting by being the major site for both glycogenolysis and gluconeogenesis. During a prolonged fast, when glycogen stores are exhausted, hepatic gluconeogenesis is considered essential for maintaining glucose homeostasis. The conversion of oxaloacetate to phosphoenolpyruvate (PEP) by PEPCK has long been thought to play a key role in this process (1,2), as the enzyme provides the only known path whereby tricarboxylic acid (TCA) cycle intermediates can be converted to glucose. PEPCK is highly expressed in the liver, where it is adaptively regulated by a variety of different hormones and other agents in a manner that parallels gluconeogenic flux (2,3). Moreover, techniques of cross-organ substrate balance plus use of radioactive tracers in humans and dogs have clearly demonstrated that the liver is the major site of glucose production during a fast (4,5).

Previously, we examined the effects of either globally reducing PEPCK gene expression or eliminating PEPCK in a liver-specific manner on glucose homeostasis. We found that *1*) mice that totally lack PEPCK die within 3 days of birth, apparently from hypoglycemia; *2*) mice with a 50, 90, and 95% global reduction in PEPCK gene expression maintain a normal blood glucose concentration after a 24-h fast; *3*) resting mice with a liver-specific knockout of the enzyme also maintain fasting euglycemia; and *4*) mice with markedly diminished PEPCK gene expression develop profound abnormalities in lipid metabolism, including hepatic steatosis, after fasting (6).

Because our previous findings challenged current concepts of PEPCK's role in gluconeogenesis, as well as the exclusive role of the liver in maintaining glucose homeostasis during a fast, we sought to determine the mechanisms whereby mice with a liver-specific knockout of PEPCK are able to maintain fasting euglycemia. Here we describe studies that combined the use of isotopic tracers, biochemical analysis, and nuclear magnetic resonance (NMR) spectroscopy to measure the contributions from glycogenolysis, glycerol, PEP, and TCA cycle activity and fluxes of some pathways associated with the TCA cycle (7,8) to gluconeogenic flux, both in the presence and absence of hepatic PEPCK. Together, the results of these studies indicate the existence of multiple compensatory mechanisms, both intra- and extrahepatic, that act to preserve normal fasting blood glucose in the absence of hepatic PEPCK.

RESEARCH DESIGN AND METHODS

Animals. The liver-specific PEPCK knockout mice used in this study have been described previously (6). The knockout animals ($pck^{lox/lox}$/Alb$Cre^{+/-}$) contain two conditional (loxed) *pck* alleles and an albumin-cre transgene [TgN(AlbCre)21Mgn] that together selectively extinguish the expression of PEPCK in hepatocytes by age 6–8 weeks (6).

Liver perfusion. Isolated livers were perfused with Krebs-Henseleit bicarbonate buffer alone, buffer containing 5 mmol/l lactate and 0.5 mmol/l pyruvate, or buffer containing 5 mmol/l glycerol, as previously described (9). The flow rate into the cannulated portal vein was 8 ml · min^{-1} · $liver^{-1}$. Postperfusion samples were collected from inferior vena cava.

Basal in vivo isotopic kinetic experiments. All experiments were performed on awake, unrestrained, unstressed mice. Catheterizations and isotopic infusions were performed as previously described (6,10). Each experiment

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G6P, glucose-6-phosphate; G6Pase, glucose-6-phosphatase; GCMS, gas chromatography–mass spectrometry; HPLC, high-performance liquid chromatography; MAG, monoacetone glucose; NMR, nuclear magnetic resonance; PEP, phosphoenolpyruvate; SA, specific activity; TCA, tricarboxylic acid.

consisted of a 70-min tracer equilibration period (−100 to −30 min), a 30-min control period (−30 to 0 min), and a 120-min test period (0 to 120 min). Blood was collected from a carotid catheter at –30, 0, 30, 60, 90, and 120 min. Basal glucose turnover rates were determined by a 5-μCi bolus injection followed by a continuous infusion at 0.05 μCi/min of [3-^{3}H]glucose, purified by high-performance liquid chromatography (HPLC), for 220 min. At the end of each experiment, the livers were excised and immediately freeze-clamped in liquid nitrogen for analysis of [^{3}H]-specific activities of UDP-glucose and concentrations of hepatic metabolites.

To determine glucose cycling rates, HPLC-purified [2-^{3}H]glucose, [6-^{3}H]glucose, and [U-^{14}C]glucose were bolus-injected at 20, 5, and 3 μCi, respectively, then continuously infused at 0.2, 0.05, and 0.03 μCi/min, respectively, into a jugular catheter throughout the experiment. Deproteinized plasma was passed through two columns, a cation-exchange column (Dowex 50Wx8–200; Sigma, St. Louis, MO) and an anion-exchange column (Amberlite IRA-67; Sigma), and glucose was eluted with water. The elutes were treated with hexokinase, ATP, and phosphoglucose-isomerase to selectively remove ^{3}H from carbon 2 of [2-^{3}H]glucose. Glucose turnover rates measured using three types of tracer glucose were then calculated.

Glycerol turnover was determined by continuous infusion of [d_5]glycerol (Cambridge Isotope Laboratories, Woburn, MA) into the jugular vein at 0.48 $\mu mol \cdot kg^{-1} \cdot min^{-1}$ for 220 min. After a 70-min tracer equilibration period, six blood samples were collected from the carotid catheter at 30-min intervals. Two consecutive 30-μl samples were pooled, [d_5]-glycerol enrichment was determined using gas chromatography−mass spectrometry (GCMS; Hewlett-Packard, Palo Alto, CA), and the glycerol turnover rate was calculated, as previously described (11). HPLC-purified [6-^{3}H]glucose was also simultaneously infused in the experiment with [d_5]-glycerol infusion to obtain the glucose turnover rate in the presence of glycerol.

Determination of flux alterations in whole-body and hepatic glucose metabolism during hyperglycemic clamp. Hyperglycemic clamp studies were performed together with infusions of [3-^{3}H]glucose, as previously described (10). At the end of each experiment, whole liver and hindlimb muscle samples were collected for glycogen and UDP-glucose analysis. The total hepatic glycogen synthesis rate (micromoles per kilogram per minute) was calculated as (total tritium counts in hepatic glycogen)/(hepatic UDP-glucose tritium specific activity [SA] per body weight per 120 min). The rate of hepatic glycogen synthesis from the direct pathway was calculated as (total tritium counts in hepatic glycogen)/(plasma glucose tritium SA per body weight per 120 min). Muscle glycogen synthesis was expressed as glucose incorporation into glycogen (tritium counts per gram tissue per plasma glucose tritium SA).

Infusion of stable isotopes for metabolic flux profiling by NMR. Total body water was enriched to ~3% by direct intraperitoneal injection of 0.8 ml of D_2O 30 min before infusion of ^{13}C-labeled tracers (Cambridge Isotope Laboratories). ^{2}H enrichment in plasma water reaches a steady state in <30 min using this protocol (S.B., C.R.M., A.D.S., unpublished observations). A continuous infusion of [3-^{3}H]glucose dissolved in D_2O was started 10 min after the intraperitoneal injection of D_2O and continued for 80 min to determine glucose turnover rate. At 30 min, a mixture of unenriched glycerol and 99% [U-$^{13}C_3$]propionate (12 mg each substrate/30 g) dissolved in saline was infused (~1 μl/min adjusted for variations in body weight) into a jugular vein catheter for 60 min. At the end of the experiment, ~1 ml of blood was collected via the carotid artery catheter; 60 μl of plasma were used for determining ^{2}H enrichment and 10 μl were used to measure the SA of [^{3}H]glucose. Glucose was extracted from the remaining plasma by ion-exchange chromatography then converted to 1,2-isopropylidene glucofuranose (monoacetone glucose [MAG]), as described elsewhere (12)

NMR spectroscopy. ^{2}H and ^{13}C NMR spectra were collected using a 14.1 T Inova NMR spectrometer (Varian Instruments, Palo Alto, CA), 3-mm sample probes, and parameters previously described (7,13). Multiplet areas in the ^{13}C spectra were measured using the line fitting routine in the PC-based NMR program, NUTS (Acorn NMR, Fremont, CA).

Analytical procedures. Plasma glucose was measured using hexokinase and glucose-6-phosphate (G6P) dehydrogenase (14). Glucose in samples from the liver perfusion studies was measured using a glucose kit (Trinder; Sigma). Blood glucose was measured using a glucose meter (HemoCue, Mission Viejo, CA). Hepatic glucokinase and glucose-6-phosphatase (G6Pase) activities were measured as previously described (15). Hepatic UDP-glucose− and UDP-galactose−specific activities were measured with a chromatographic and HPLC method using a reverse-phase Supelcosil C18 column (Waters, Milford, MA). All results are presented as means ± SE. Statistical significance was determined by one-way ANOVA. $P < 0.05$ was considered statistically significant.

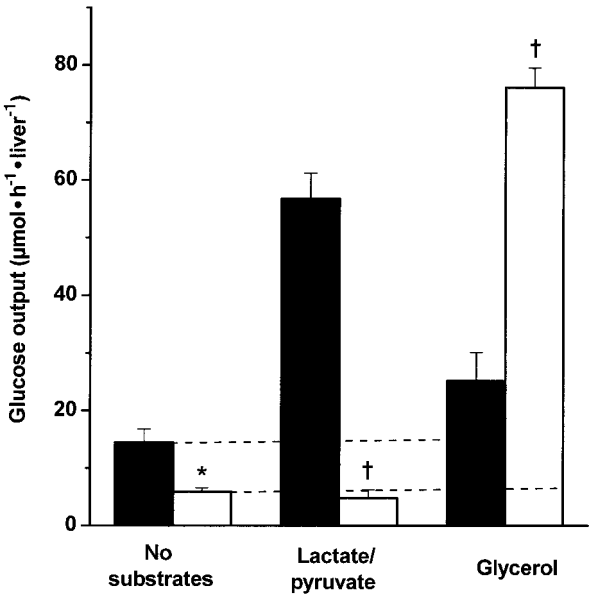

FIG. 1. Glucose production in perfused livers in the states of no gluconeogenic substrates, lactate/pyruvate (5 and 0.5 mmol/l, respectively), and glycerol (5 mmol/l) in the perfusion buffer. The dotted lines indicate increases in glucose production from basal levels after addition of gluconeogenic substrates in the control (■) and knockout (□) mice. Male mice ages 6–8 months were fasted for 26–30 h before perfusions. The experimental protocol consisted of a 25-min wash-out period of perfusion without addition of gluconeogenic substrates. Two basal samples were then collected at 25 and 30 min, gluconeogenic substrates were added, and four additional samples were collected at 35, 40, 45, and 50 min. *$P < 0.01$, †$P < 0.001$ vs. control values at each state ($n = 4–5$).

RESULTS

Substrate-specific in vitro gluconeogenic capacity of livers lacking PEPCK. To determine the impact of the lack of hepatic PEPCK on gluconeogenesis by the liver, we first performed an in situ perfusion study on livers from 26-h-fasted mice (Fig. 1). Before adding any gluconeogenic substrates, basal glucose production in livers without PEPCK was 40% of the control value. The addition of 5 mmol/l lactate and 0.5 mmol/l pyruvate to the perfusion buffer caused a fourfold increase in glucose production in control mouse livers, whereas there was no change in glucose production in livers lacking PEPCK. In contrast, the addition of 5 mmol/l glycerol to the perfusion buffer resulted in 6.5-fold more glucose synthesized by knockout mice livers compared to controls, suggesting an increased gluconeogenic capacity from glycerol in livers lacking PEPCK. These results indicate that glucose is not synthesized from either lactate or pyruvate in livers that lack PEPCK.

Glucose/glycerol metabolism in the basal fasted state. Measurements of the ratio of the SA of hepatic [^{3}H]UDP-glucose and plasma [^{3}H]glucose using samples obtained during our prior study of these mice (6) revealed a 6.3-fold greater value in the knockout mice (Table 1). This indicated that there was either a marked increase in hepatic glucose uptake into hepatic UDP-glucose or a marked decrease in gluconeogenesis in the knockout mice. Some hepatic enzyme and metabolite levels were also consistent with decreased gluconeogenesis in the knockout animals: hepatic UDP-glucose was 70% higher, hepatic G6P was 90% lower, and hepatic G6Pase and

TABLE 1
Parameters of hepatic glucose metabolism under basal conditions in conscious control and liver-specific PEPCK knockout mice

	Control	Knockout
G6P (nmol/g)	221 ± 18	23 ± 3†
UDP-glucose (nmol/g)	177 ± 15	303 ± 24*
Hepatic [^{3}H]UDP-glucose SA/plasma [^{3}H]glucose SA	0.095 ± 0.004	0.600 ± 0.087†
G6Pase V_{max} (nmol/·min^{-1}·mg^{-1} protein)	548 ± 22	369 ± 22†
Glucokinase activity (nmol·min^{-1}·mg^{-1} protein)	22.9 ± 1.3	12.3 ± 0.8†

Data are means ± SE. [3-^{3}H]glucose was infused for 220 and 120 min, respectively. Male mice ages 7–8 months were fasted for 26 h before infusions. *$P < 0.01$; †$P < 0.001$ versus control values at each condition (n = 4–7).

glucokinase activities were both lower (by 33 and 46%, respectively) (Table 1).

Glucose turnover rates were measured using three different tracers—[2-^{3}H]glucose, [6-^{3}H]glucose, and [U-^{14}C]glucose—and found to be either no different or slightly less in the knockout mice compared to controls. Glucose turnover determined from the [6-^{3}H]glucose data were 14% lower in the knockout mice than in the control mice ($P < 0.026$; n = 7) (Table 2). In these experiments, plasma glucose was 17% higher in knockout mice compared to that in controls (Table 2). Although the livers of knockout animals had an increased capacity for gluconeogenesis from glycerol, as shown by liver perfusion, whole-body glycerol turnover as measured by gas chromatography–mass spectrometry did not differ from controls (Table 2).

Glucose metabolism during a hyperglycemic clamp. The slightly diminished glucose turnover rate in unstressed, fasted knockout mice suggested a defect in glucose utilization in animals lacking hepatic PEPCK. To further unmask this small difference, whole-body glucose disposal rates were also measured during hyperglycemia. Blood glucose concentrations in 26-h-fasted control and knockout mice were clamped at 299 ± 9 and 292 ± 11 mg/dl, respectively. Plasma insulin concentrations were no different in knockout versus control mice (n = 4) under basal conditions (384 ± 40 vs. 412 ± 47 pg/ml), at 60 min (963 ± 139 vs. 1,209 ± 157 pg/ml), or at 120 min (1,350 ± 211 vs. 1,582 ± 269 pg/ml), whereas the plasma glucagon concentration at the end of the experiment was lower in the knockout mice compared to the controls (108 ± 14 vs. 176 ± 19 pg/ml; $P < 0.05$). Under these conditions, glucose turnover in the knockout mice was about half that in control mice (Table 3). Consistent with this, the glucose infusion rate in knockout mice was only 44% of that in control animals. The hepatic glycogen content and rate of glycogen synthesis at the end of the 2-h glucose infusion in knockout mice was only 9 and 19%, respectively, of those found in control mice. Hepatic glycogen, which in the control animals was synthesized mainly via the indirect pathway, was primarily formed from the direct pathway in the knockout mice (data not shown). In addition, muscle glycogen content was 30% less and glucose incorporation into the muscle glycogen during the hyperglycemic clamp was 85% less in the knockout compared to control mice (Table 3).

TABLE 2
Basal glucose and glycerol turnover rates in conscious control and knockout mice

	Control	Knockout
Plasma glucose (mmol/l)	5.9 ± 0.4	6.9 ± 0.2*
[2-^{3}H]glucose turnover (μmol·kg^{-1}·min^{-1})	61.7 ± 3.9	51.1 ± 2.3
[6-^{3}H]glucose turnover (μmol·kg^{-1}·min^{-1})	51.4 ± 2.1	44.2 ± 1.6*
[U-^{14}C]glucose turnover (μmol·kg^{-1}·min^{-1})	46.4 ± 3.3	39.8 ± 2.2
[d_5]glycerol turnover (μmol·kg^{-1}·min^{-1})	34.9 ± 2.3	31.4 ± 2.8

Data are means ± SE. In experiment 1, [2-^{3}H]glucose, [6-^{3}H]glucose and [U-^{14}C]glucose were infused for 220 min. In experiment 2, [d_5]glycerol with or without [6-^{3}H]glucose was infused for 220 min. Male mice ages 7–8 months were fasted for 26 h before infusions. *$P < 0.05$ versus control values. n = 4 for the [2-^{3}H]glucose and [U-^{14}C]glucose turnover rate; n = 7 for glucose, [6-^{3}H]glucose turnover rate, and [d_5]glycerol turnover rate.

TABLE 3
Whole-body and tissue parameters of glucose metabolism during a hyperglycemic clamp in conscious control and knockout mice

	Control	Knockout
Whole body		
Glucose infusion rate (μmol·kg^{-1}·min^{-1})	228.3 ± 9.4	100.6 ± 2.8**
Glucose turnover rate (μmol·kg^{-1}·min^{-1})	261.1 ± 11.1	137.7 ± 7.8**
Hepatic		
Glycogen content (μmol/g)	159.8 ± 7.3	14.4 ± 3.4†
Glycogen synthetic rate (μmol·kg^{-1}·min^{-1})	57.0 ± 6.4	10.7 ± 1.6†
Muscle		
Glycogen content (μmol/g)	17.4 ± 1.8	12.1 ± 0.6*
Glycogen synthesis (μmol/g)	10.3 ± 1.0	1.5 ± 0.3†

Data are means ± SE. [3-^{3}H]glucose was infused for 220 min, and blood glucose concentration was clamped at ~300 mg/dl for the last 120 min. Male mice ages 7–8 months were fasted for 26 h before infusions. *$P < 0.05$; †$P < 0.001$ versus control values at each condition (n = 4; **$P < 0.01$).

Metabolic profiling of plasma glucose using NMR and ^{2}H/^{13}C tracers. Because glucose production from pyruvate/lactate was abolished in the livers of knockout mice, whereas glucose production from glycerol may have been enhanced, NMR was used to evaluate the metabolic origin of glucose. In animals with total body water enriched to ~3% ^2H_2O and co-infused with unlabeled glycerol, [U-$^{13}C_3$]propionate, and [3-^{3}H]glucose, glucose turnover rates were 68 ± 14 and 56 ± 13 μmol·kg^{-1}·min^{-1} in the control and knockout mice, respectively. These values were similar to those obtained under basal conditions (Table 2).

High resolution ^{2}H and ^{13}C NMR of MAG derived from plasma glucose from control and knockout mice are compared in Fig. 2. Although the ^{2}H spectra were nearly identical, differences were readily apparent in the two ^{13}C spectra. As shown previously, the distribution of ^{2}H in H2, H5, and $H6_S$ provides a measure of glycogenolysis and gluconeogenesis from glycerol versus the TCA cycle (8). The H5/H2 ratio in spectra of MAG from control (n = 4) and knockout (n = 4) animals averaged 1.02 ± 0.09 and

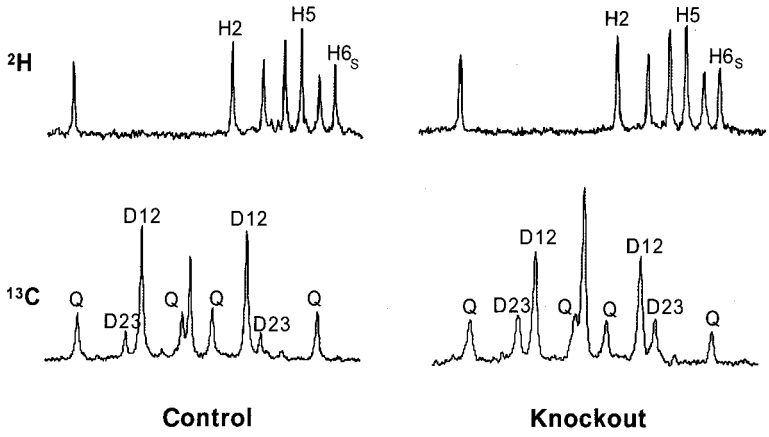

FIG. 2. ^2H and ^{13}C NMR spectra of MAG (derived from plasma glucose) from control (*left*) and knockout (*right*) mice after enrichment of total body water to ~3% ^2H_2O and infusion of glycerol and [U-$^{13}C_3$]propionate as a metabolic tracer. The resonance areas of H2, H5, and $H6_S$ in the ^2H spectra were used to determine glycogenolysis and gluconeogenesis from glycerol and the TCA cycle. The C2 resonance multiplet areas in the ^{13}C spectra of MAG were used to evaluate carbon flux through pathways associated with the TCA cycle (18).

0.94 ± 0.08, respectively. This confirmed that the contribution of glycogen to plasma glucose was insignificant in both 24-h-fasted control and knockout mice. Similarly, the gluconeogenic contribution coming from the level of the TCA cycle was given by the $H6_S$/H2 intensity ratios, 0.78 ± 0.15 in controls and 0.60 ± 0.15 in knockouts ($P \leq 0.128$), with the contribution of glycerol to glucose production being determined by the difference (0.24 ± 0.08 in controls and 0.34 ± 0.15 in knockouts; $P \leq 0.286$). Although the tissue origin of this gluconeogenic flux could not be determined from these data, the ^2H NMR results clearly indicated that the TCA cycle contributes somewhat more to overall glucose production in control versus knockout animals. Interestingly, the glycerol contribution to gluconeogenesis was only slightly higher in knockout animals in vivo, even though the perfusion experiments showed that livers from knockout mice have a much greater capacity to make glucose (Fig. 1). It is also interesting to note that glucose production from glycerol in control animals infused with the additional ~1 μmol/min glycerol did not differ from that in control animals without glycerol co-infusion (glycerol typically contributes ~25% in mice without infusion of any substrates; S.B., C.R.M., A.D.S., unpublished observations).

^{13}C NMR spectra of the same MAG samples showed that plasma glucose from control and knockout animals became enriched with ^{13}C that originated in [U-$^{13}C_3$]propionate (although all six MAG resonances were enriched with ^{13}C, only the C2 resonances are shown in Fig. 2). Unlike the ^2H spectra, the various multiplets in the ^{13}C spectra were more strikingly different in the controls versus knockouts. The ^{13}C satellite peaks in the corresponding ^1H NMR spectra (data not shown) indicated that more MAG was derived from [U-$^{13}C_3$]propionate in control animals (10%) than in knockouts (4%). The areas of the C2 multiplet components (labeled C2D12, C2D23, and C2Q in Fig. 2) were used to estimate anaplerosis, gluconeogenesis, and pyruvate recycling fluxes (Table 4), all relative to TCA cycle flux (7).

The relative metabolic fluxes determined by ^2H and ^{13}C NMR spectroscopy, when combined with the glucose turnover measurements in the same animals, enabled us to calculate the absolute fluxes, as summarized in Fig. 3. Although these fluxes reflect average values of all tissues contributing to glucose production, this metabolic profile reveals some important differences between the knockout and control animals in terms of glucose origin. First, absolute gluconeogenic flux from PEP (reaction 3) was 34% less in knockout animals than in controls ($P \leq 0.012$), whereas the absolute gluconeogenic flux from glycerol (reaction 2) did not differ. Second, these differences were amplified even further at the level of the TCA cycle where ^{13}C NMR reports that total anaplerosis was reduced by 37% (reaction 4; $P \leq 0.02$), pyruvate cycling was reduced by 52% (reaction 5; $P \leq 0.059$), and TCA cycle flux was increased by 49% (reaction 6; $P \leq 0.047$) in the knockout animals.

DISCUSSION

Whole-body glucose turnover in the absence of hepatic PEPCK. As shown in this study as well as in our previous study (6), the fasting blood glucose concentration is maintained at similar or slightly higher levels in mice lacking hepatic PEPCK. Moreover, the glucose turnover rate, which equals the endogenous rate of glucose production, is only slightly lower in knockouts compared to controls. Yet, despite the inability of the liver to make glucose in the absence of hepatic PEPCK, as clearly demonstrated in the in situ perfusion experiments, the NMR experiments showed that total body gluconeogenic flux from PEP was still 66% that of a normal mouse.

During prolonged starvation, when hepatic glycogenolysis is negligible, hepatic G6P is produced only from gluconeogenesis and by glucose phosphorylation, mostly via glucokinase. The six-fold higher [^3H] SA ratio of hepatic UDP-glucose (taken as reflection of the hepatic G6P pool) (16) to plasma glucose in the knockout mice indicates there was either a marked increase in the rate of

TABLE 4
Summary of flux ratios relative to TCA cycle as determined by analysis of the C2 multiplet areas in the ^{13}C NMR spectrum of MAG

	Control	Knockout
Gluconeogenesis (*g*)	2.08 ± 0.51	0.85 ± 0.04*
Pyruvate recycling (*pk* + *me*)	1.06 ± 0.20	0.36 ± 0.18*
Anaplerosis (*y*)	3.15 ± 0.42	1.21 ± 0.20*

Data are means ± SE. In this study, 0.8 ml of D_2O was injected intraperitoneally 30 min before continuous infusion of unenriched glycerol and 99% [U-$^{13}C_3$]propionate each at 12 mg/30 g body wt for 60 min. A prime-continuous infusion of [3-^3H]glucose was started at 10 min after intraperitoneal injection of D_2O and continued for 80 min to determine glucose turnover rate. *$P < 0.01$ versus control values ($n = 4$).

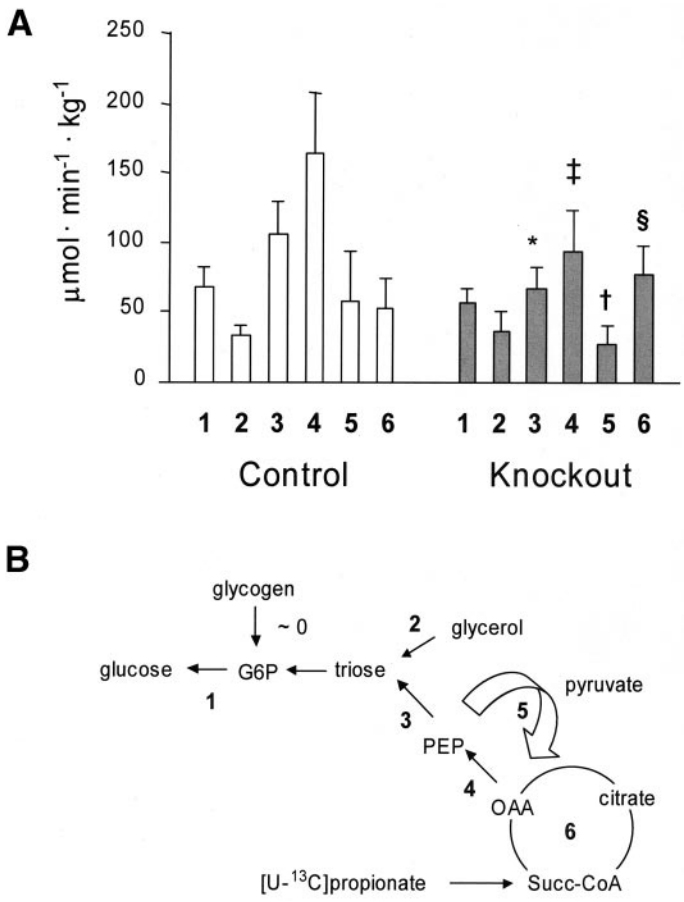

FIG. 3. Summary of total body fluxes (micromoles per kilogram per minute) in control and knockout animals. *A*: Fluxes were calculated for six different steps in gluconeogenesis. These data were obtained by measuring total body glucose turnover rates (using [^{3}H]glucose as tracer) in the same animals that were used to derive the NMR spectra. The peak areas of H2, H5, and H6S in ^{2}H NMR spectra and the multiplet areas in the C2 resonance of MAG in ^{13}C NMR spectra were used to calculate specific fluxes. *$P < 0.012$, †$P < 0.06$, ‡$P < 0.02$, and §$P < 0.047$ vs. control ($n = 4$). *B*: Diagram illustrating the six different metabolic reaction rates that were calculated. OAA, oxaloacetate; Succ-CoA, succinyl-CoA.

hepatic glucose phosphorylation, a marked decrease in the amount of gluconeogenesis, or both. The consistent differences in [2-^{3}H]glucose− and [6-^{3}H]glucose−derived glucose turnover rates between the knockout and control mice (Table 2) and the 50% decrease in glucokinase activity in the liver of the knockout animals (Table 1) strongly suggest that both glucose phosphorylation and G6P synthesis by gluconeogenesis was lower in the mice lacking hepatic PEPCK. Furthermore, the 33% decrease in G6Pase V_{max} and the 90% decrease in hepatic G6P concentration also point to a marked decrease in G6Pase-mediated flux to glucose. Taken together, these results indicate markedly diminished hepatic glucose production in these knockout mice, even though endogenous glucose production rates were near normal.

One might anticipate that hepatic gluconeogenesis from glycerol would be elevated in the knockout mice, especially as they have an elevated hepatic gluconeogenic capacity from glycerol, as shown by liver perfusion. Based on the glycerol turnover rate of 31.4 μmol · kg^{-1} · min^{-1} in the knockout mice (no different from control values) (Table 2), glycerol could contribute as much as 15.7 μmol · kg^{-1} · min^{-1} or 35% of total body glucose production, assuming that the glycerol turnover rate equals the gluconeogenic rate from glycerol. This value is consistent with the NMR-derived result (Fig. 3) that gluconeogenesis from glycerol in animals lacking hepatic PEPCK is 19 ± 7 μmol glucose μmol · kg^{-1} · min^{-1} or 34% of total glucose turnover. It is interesting that this consistency was maintained despite the fact that glycerol was included in the infusate in the NMR experiment, but not in the [d_5]-glycerol turnover experiment. Furthermore, the contribution of glycerol to gluconeogenesis as measured by ^{2}H NMR was the same in control and knockout animals. Thus, one can conclude that glucose production from glycerol in mice must occur largely in tissues other than liver and that, in the absence of hepatic PEPCK, the liver does not produce more glucose from glycerol in vivo, despite the increased metabolic capacity in vitro (Fig. 1).

Compensatory extrahepatic gluconeogenesis in mice lacking hepatic PEPCK. Given that the liver makes only a small amount of glucose without hepatic PEPCK, it follows that gluconeogenesis by extrahepatic tissues is responsible for a majority of glucose synthesized in mice lacking PEPCK. Although these data do not identify which organs are responsible for this compensatory gluconeogenesis, the kidney (17) and/or intestine (18) are likely sources as both express PEPCK and other gluconeogenic enzymes. Based on our finding of a twofold increase in plasma glutamine (892.2 ± 9.1 vs. 444.6 ± 6.9 μmol/l in knockout versus control mice, respectively; $P < 0.001$; $n = 7$), it is possible that glutamine serves as a major gluconeogenic substrate in the intestine and kidney of PEPCK knockout mice (18–20). Although the majority of glucose produced in these animals was derived from an unlabeled gluconeogenic substrate ([1,2,3-^{13}C]propionate was present only at tracer levels), the NMR experiment did not provide information about the identity of the gluconeogenic substrate.

The ^{13}C NMR analysis of plasma glucose revealed important differences in these animals: total anaplerosis from the TCA cycle was reduced by 37% ($P \leq 0.02$) and pyruvate cycling was reduced by 52% ($P \leq 0.059$), whereas TCA cycle flux was actually higher by 49% ($P \leq 0.047$) in knockout animals compared to controls. Thus, although mice lacking hepatic PEPCK were able to circumvent their inability to convert oxaloacetate to PEP in the liver, the compensatory mechanisms involved may have required additional energy expenditure. This observation is consistent with our previous finding that the expression of genes encoding hepatic pyruvate carboxylase, alanine aminotransferase, and enzymes in both the malate-aspartate shuttle and TCA cycle are increased in these animals (6). Moreover, the abundance of mRNA encoding the mitochondrial dicarboxylate carrier protein, a component of the inner mitochondria membrane responsible for transporting malate and succinate across the inner membrane in exchange for phosphate, sulfate, or thiosulfate (21), is increased 3.5-fold during fasting (data not shown). Because the operation of anaplerotic reactions requires the coexistence of cataplerotic reactions (22), it is likely that the elevated hepatic gene expression for the above proteins leads to a net release of glutamine from the liver to the blood as a response to the blockage of hepatic

gluconeogenesis from TCA cycle intermediates in these knockout animals. Thus, although the liver is incapable of producing much glucose in the absence of hepatic PEPCK, this does not mean that it ceases to be metabolically involved in maintaining glucose homeostasis.

Diminished glucose utilization in mice lacking hepatic PEPCK. The slight decrease in the basal glucose turnover and glucose clearance rates in the presence of similar levels of plasma insulin hint at a defect in glucose disposal. The hyperglycemic clamp study, which showed a marked decrease in glycogen synthesis from glucose in both liver and skeletal muscle, revealed that there are coexisting defects in glucose utilization in the knockout mice. In the case of the liver, our finding of a 46% decrease in hepatic glucokinase activity in the knockout mice is consistent with decreased hepatic glucose utilization, as glucose phosphorylation by this enzyme is essential (23).

Muscle glycogen synthesis was also found to be impaired in knockout mice, and thus is another indicator of impaired peripheral glucose utilization. This may reflect the markedly abnormal lipid metabolism that occurs in these mice during fasting. Even though the overall lipolytic rate was not altered, as suggested by similar glycerol turnover rates between the control and knockout mice, the fasting plasma free fatty acid and triglyceride concentrations were elevated. Furthermore, severe hepatic steatosis occurred in the knockout mice, which likely reflected an inhibition of hepatic fatty acid oxidation despite upregulated gene expression of fatty acid oxidation enzymes (6). Moreover, hepatic mRNAs for apolipoprotein CII, a specific activator of lipoprotein lipase, and CD36, a membrane fatty acid translocase, were increased 3.5- and 6-fold, respectively, in the fasted knockout mice (data not shown). The net effect of the changes would be elevated lipid delivery from secreted hepatic VLDL into the muscle by lipoprotein lipase, which would increase muscle concentrations of triglycerides and fatty acyl-CoA associated with impaired glucose utilization in muscle.

In summary, mice lacking hepatic PEPCK have a markedly impaired hepatic gluconeogenesis, but maintain fasting euglycemia through greatly increased extrahepatic gluconeogenesis coupled with mildly diminished peripheral glucose utilization. ^{2}H and ^{13}C NMR analysis of plasma glucose, reported here for the first time in mice, revealed that euglycemia in these fasted animals is achieved at the expense of marked perturbations in whole-body energy metabolism.

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