Effect of murine strain on metabolic pathways of glucose production after brief or prolonged fasting

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The mouse has been emphasized in studies of type 2 diabetes and obesity because of genetic homologies with humans and the relative simplicity of generating targeted mutations. Because these disorders result from a complex interaction among multiple genes and the environment, it is attractive in principle to probe individual genes to determine the magnitude of their contribution to the disease phenotype. However, the potential for a single mutation to produce diabetes or obesity is exquisitely sensitive to the inbred strain background (8). For example, mutations have been described in both the leptin receptor gene and the leptin gene. In a B6 background, either mutation produces obesity and transient diabetes, but the same mutations in a C57BL/6J background are lethal (9). While limited data are available for specific metabolic fluxes in vivo that may be responsible for the differences in strain phenotypes, there are inherent differences in several pathways involving glucose metabolism of inbred strains of mice that may contribute to a phenotype after genetic manipulation in these animals. The techniques used here are amenable to use as a secondary or tertiary tool for studying mouse models with disruptions of intermediary metabolism.

nuclear magnetic resonance; tricarboxylic acid cycle; phosphoenolpyruvate carboxykinase; metabolic flux; stable isotope tracers; deuterium; mouse phenotype

The mouse has been emphasized in studies of type 2 diabetes and obesity because of genetic homologies with humans and the relative simplicity of generating targeted mutations. Because these disorders result from a complex interaction among multiple genes and the environment, it is attractive in principle to probe individual genes to determine the magnitude of their contribution to the disease phenotype. However, the potential for a single mutation to produce diabetes or obesity is exquisitely sensitive to the inbred strain background (8). For example, mutations have been described in both the leptin receptor gene and the leptin gene. In a B6 background, either mutation produces obesity and transient diabetes, but the same mutations produce a severe diabetes phenotype in the C57BL/6J strain (12). More recently, Colombo et al. (9) found that the depletion of adipose tissue and hyperinsulinemia in the A-ZIP/F-1 lipomatous mouse was similar in both the FVB and B6 inbred backgrounds, but on the B6 background, the hyperglycemia, hypertriglyceridemia, and mortality were all attenuated. It was suggested that the B6 strain has inherently higher triglyceride clearance in the liver, which may protect against adverse effects of increasing triglycerides. A related problem is the observation that disruption of critical features of glucose metabolism may fail to yield the expected result. For example, in two recent studies, muscle cell-specific deletion of the insulin receptor did not impair glucose tolerance (2), and hepatocyte-specific deletion of cytosolic phosphoenolpyruvate carboxykinase (PEPCK) did not reduce glucose turnover in starved animals (34). The only reason the latter mice were examined more extensively (4, 33) was the prior conviction that metabolism must be abnormal. Given the known sensitivity of metabolic phenotype to strain background, it is possible that the variant phenotype would have been more dramatic in a different inbred strain.

These observations suggest that aspects of glucose metabolism differ at baseline among strains, and a better understanding of these differences may aid in the design of genetic manipulations. It is difficult in general to evaluate metabolic fluxes in mice because of their size and attendant sampling limitations. Consequently, the more subtle aspects of metabolic pathways contributing to glucose production have not been investigated because of the difficulty of measuring systemic glucose turnover and of using classical radiotracer methods to measure metabolic fluxes in vivo. Recently, a number of laboratories have successfully demonstrated remarkable progress toward miniaturization of standard methods to measure glucose turnover in intact mice (24, 29).

Stable isotope tracers are attracting increasing interest for analysis of metabolic networks because of the high information content encoded in the labeling patterns of product molecules. Recently, we have described an integrated study of glucose production pathways in humans (21, 22, 38) and rats (18–20) involving the following three tracers: 1) ¹³C-labeled glucose to measure glucose turnover by conventional indicator dilution, 2) ²H₂O to measure the fractional contribution of glycolysis, glycero1, and the TCA cycle to glucose production, and 3) ¹³C-labeled propionate as a gluconeogenic tracer to measure fluxes through pathways associated with the TCA cycle. NMR was chosen for analysis of the ²H and ¹³C labeling patterns in vivo.
plasma glucose because, in contrast to mass spectrometry, $^2$H and $^{13}$C do not interfere with one another in the experiment, and NMR sensitivity appears to be adequate (33).

The present study was designed to evaluate whether flux in glucose production pathways differs across commonly used mouse strains. The strains studied included 1) ICR mice, an outbred strain, 2) FVB, an inbred strain of mouse with large pronuclei enabling easy microinjection of genetic material (35), 3) 129, an inbred strain of mouse popular because of availability of embryonic stem cell lines (11), and 4) B6, a popular inbred strain of mouse with the first published genome (37) and a known susceptibility to diet-induced diabetes (31). The data show relatively minor yet significant strain variation with regard to several metabolic fluxes related to in vivo glucose production/turnover. These differences may be important factors contributing to variable background strain phenotypes in response to identical genetic manipulations, pharmacological interventions, or environmental stresses.

METHODS

Chemicals. $[U-^{13}$C$]_3$propionate (99%) and $^2$H$_2$O (99%) were purchased from Cambridge Isotopes (Andover, MA). DSC-18 solid-phase extraction gel was obtained from Supelco (St. Louis, MO). Other common chemicals were purchased from Sigma (St. Louis, MO) unless otherwise noted. Ensure (Abbott Laboratories, Abbott Park, IL) was purchased at a local grocery store.

Experimental protocol for relative fluxes. The experimental protocol was approved by the Institutional Animal Care and Use Committee. ICR, FVB, B6, and 129 female mice (10 wk old) were purchased from Jackson Laboratory (Bar Harbor, ME) and used by 14 wk of age. They were maintained on standard chow and exposed to a 12-h dark cycle from 7:00 PM to 7:00 AM. The mice had free access to water throughout the study. Before each metabolic experiment, mice were fasted for either 24 or 4 h. The 24-h fast was achieved by moving animals to a cage without food for 12 h, followed by free access to water. The 4-h fast was achieved by moving animals to a cage without food for 12 h, followed by free access to a liquid diet (Ensure) given during the dark cycle for 2 h, followed by an additional 4-h fast. The liquid diet was chosen because it is absorbed quickly, whereas solid chow can remain in the stomach for hours. These two conditions were studied so that the mice could be studied under a setting where the importance of gluconeogenic flux is maximal (24-h fast) or moderate (4-h fast). Ensure contains 0.75 g/o fluid, 5 g/o carbohydrates, and 1.1 g/o protein.

Isotope solutions were administered via either an intraperitoneal injection or an intravenous injection as noted. The deuterium oxide ($^2$H$_2$O) dose was typically 27 $\mu$g mouse and resulted in ~4% body water enrichment. $[U-^{13}$C$]_3$propionate (0.28–4.22 $\mu$mol/g mouse) was dissolved in the $^2$H$_2$O. After intraperitoneal injection, the mice were returned to their cage for a time described below. After this equilibration period, the mice were anesthetized with ketamine-xylazine, and blood was collected from the vena cava. Plasma glucose was treated as described below, except that plasma samples from mice were not combined. Plasma tracer enrichment was determined by $^{13}$C NMR and used along with the known rate of infusion to calculate glucose turnover or endogenous glucose production (EGP) as previously detailed (18). EGP (v1) was then multiplied by the relative flux values described below to convert to absolute flux values.

Analytical procedures. Samples obtained from each mouse were separated into plasma and red blood cells by centrifugation. Plasma (10 $\mu$L) from each mouse was used to measure total glucose (1). The remaining plasma was extracted with perchloric acid and neutralized with potassium hydroxide, and the glucose was purified on ion-exchange resin (33). Purified glucose from two or three animals was combined and converted to the 1,2-isopropylidene glucosurano derivative (monooacetone glucose, MAG), as previously described (6, 21).

NMR spectroscopy was performed as previously reported (21). Briefly, MAG was dissolved in 150 $\mu$L of acetonitrile and 5–10 $\mu$L of water. $^1$H NMR spectroscopy was performed on a 14.1 T Varian spectrometer using a 3-mm inverse detection probe for $^1$H detection. A 90° excitation pulse, 1-s interpulse delay, 2-s acquisition time, and 256 acquisitions were used to collect $^1$H spectra. $^2$H NMR spectra were collected on a $^2$H-optimized probe using a 90° excitation pulse, no interpulse delay, 1-s acquisition time, and an average of 5,000–10,000 acquisitions over 2–4 h. Walfit-16 decoupling was used to remove proton-deuteron scalar coupling. $^2$H spectra were collected in blocks of 256 acquisitions without lock and later aligned. Proton-decoupled $^{13}$C NMR spectra were collected on the same samples after the addition of 20 $\mu$L of deuterated acetonitrile for locking. A 50° pulse, acquisition time of 1.5 s, and no further delay were found to give the highest sensitivity for MAG carbons-3 and -4 (18). A Walfit-16 sequence was used to decouple $^1$H from $^{13}$C. Resonance areas in the $^1$H and $^{13}$C NMR spectra were analyzed by using the line-fitting routine in the PC-based software NUTS (Acorn NMR, Martinez, CA).

Metabolic analysis. During in vivo synthesis, protons on the backbone of glucose become enriched with deuterium in proportion to total body water $^2$H enrichment and the activity of each pathway responsible for the exchange (7, 25, 26). Enrichments at the H-2, H-5, and H-8 positions have been shown to be quantitative markers of glucose production from glycerol, gluconeogenesis from glycerol (GNGglycerol), and gluconeogenesis from the TCA cycle, respectively.
This deuterated water method was pioneered by Landau and coworkers using mass spectroscopy as the analytical tool (7, 25, 26) and was recently shown to be equally effective when enrichments were measured by 2H NMR (5). The fraction of glucose originating from glycolgen, v2/v1, is reported as 1 – (H-5/H-2), where H-5 and H-2 are the resonance areas in the 2H NMR spectrum of MAG, the fraction of glucose originating from glycerol, v3/v1, is reported by (H-5 – H-6s)/H-2, and the fraction of glucose originating from the TCA cycle, v4/v1, is reported by 1 – (H-5 – H-6s)/H-2 (18).

[U-13C3]propionate was used as a vehicle to enrich all TCA cycle intermediates with 13C and thereby provide a means of quantifying fluxes through several pathways associated with the TCA cycle (18, 20). Propionate was chosen for this purpose because it is avidly extracted from the circulation by the liver and because there is only one pathway for entry in the TCA cycle (optional tracers like pyruvate can enter via at least 2 pathways). Because carbons-1, -2, and -3 of oxaloacetate (OAA) are transmitted to carbons-1, -2, and -3 of glucose, the multiplets in the C2 resonance of MAG reflect the 13C in the OAA pool, and this in turn is sensitive to fluxes in and out of the TCA cycle. Gluconeogenesis from the TCA cycle relative to citrate synthase flux, v4/v7, is reported by the multiplet area ratio (C2Q)/C2D23; and total cataplerotic flux (H-5/H-2) or 21% of the plasma glucose originated by 13C NMR. This commonality provides the connection needed to standardize all flux values in the same units as the value measured for EGP, v1 (18, 21). EGP was assigned a unitless value of one, and all other fluxes were normalized accordingly. It was assumed that the sum of v2, one-half v3, and one-half v4 equals EGP.

Plasma glucose and hormone determinations. B6 mice were anesthetized with midazolam (0.4 mg/mouse, Dormicum; Hoffman-La Roche, Basel, Switzerland) and a combination of fluanison (0.9 mg/mouse, Hypnorm; Janssen, Beerse, Belgium) after which an intraperitoneal injection of 0.4 mg/mouse, Dormicum; Hoffman-La Roche, Basel, Switzerland) and a combination of fluanison (0.9 mg/mouse, Hypnorm; Janssen, Beerse, Belgium) after which an intraperitoneal injection of 1.1 mg/kg propionate in 99% 2H2O was given. At the indicated time points (Fig. 1), plasma samples were drawn from the retroorbital plexus. Plasma glucose was determined by the glucose oxidase method (Sigma); insulin and glucagon were measured by RIA (Linco, St. Charles, MO).

Statistical analysis. All data are reported as means ± 1 SD. Statistical comparisons were made either as an unpaired t-test against the average of the other groups or performed using a Bonferroni t-test with correction for multiple comparisons in the SAS statistical software package, as indicated in the text.

RESULTS

Timing of plasma samples and physiological consequence of tracers. Experiments in ICR mice were performed to establish that the minimum time necessary to achieve steady-state 13C and 2H enrichment of blood glucose after intraperitoneal injection of deuterated water and [U-13C3]propionate in mice is ~30 min. A dose of 1.1 μmol/g [U-13C3]propionate/g mouse was chosen after several experiments were performed covering an array of doses from 0.28 to 4.22 μmol/g. This dosage has the advantage of maximizing 13C enrichment yet minimizing elevated plasma glucose concentrations resulting from stress of injection or gluconeogenic stimulation by propionate (data not shown). To determine if intraperitoneal injection of 1.1 μmol/g [U-13C3]propionate in 99% 2H2O has an effect on the whole body glucose handling, glucose, insulin, and glucagon levels were measured as a function of time after administration of the isotope cocktail (Fig. 1). There were small, but insignificant changes detected early after the injection, but all three concentrations returned to baseline by 30 min when plasma collection for the NMR experiments was performed.

Sources of plasma glucose from 2H NMR spectroscopy. Typical 2H NMR spectra of MAG derived from plasma glucose of three B6 mice after a 4-h fast vs. 24-h fast are shown in Fig. 2. The fraction of glucose derived from glycogen, glycerol, and the TCA cycle is simple to determine from the relative intensities of H-2, H-5, and H-6s. With the use of the 2H spectrum from a 4-h-fasted animal as an example, H-5/H-2 = 0.79, so 1 – H-5/H-2 or 21% of the plasma glucose originated in glycogen. The remainder was derived from glycero (H-5 – H-6s)/H-2 = 18%) and the TCA cycle (H-6s/H-2 = 61%). It is easy to appreciate that the H-5-to-H-2 ratio is closer to unity in the 2H spectrum from the 24-h-fasted animal, consistent with a decrease in glycogenolysis and an increase in gluconeogenic contributions. Table 1 summarizes these three contributions to EGP for the four strains of mice after a 4-h fast vs. a 24-h fast. In 24-h-fasted B6 mice, the fraction of glucose derived from glycogen was 11% compared with 6% for the average of the other strains (P < 0.07), whereas the fraction of glucose from the TCA cycle was less in the B6 (63%) compared with the other strains (77%, P < 0.03). However, after correction for multiple-group comparison, only the B6 (11%)

Fig. 1. Effect of ip propionate injection on glucose metabolism. Changes in plasma glucose (A), insulin (B), and glucagon (C) after ip injection of 1.1 μmol/g mouse propionate. Mean of 7 mice ± SD.
and the 129 strains (1%) differed significantly. As anticipated, 24-h-fasted mice had a lower contribution from glycogen than mice in the 4-h-fasted group. Among mice fasted for 24 h, the fraction of glucose from glycogen was 5% compared with 17% ($P < 0.003$) in mice fasted for 4 h.

**Liver flux profile.** [U-$^{13}$C$_3$]propionate was used as a tracer to measure flux through the TCA cycle and adjoining pathways. The original three-carbon backbone of [U-$^{13}$C$_3$]propionate is rearranged predictably, depending on the pathways it follows on its way to becoming a glucose molecule (20). By decoding the NMR multiplets formed from $^{13}$C-$^{13}$C spin-spin coupling in the C2 position of MAG derived from plasma glucose (Fig. 3), relative anaplerotic and cataplerotic fluxes were determined as shown in Table 2. The analysis gives total cataplerosis relative to TCA cycle flux ($v_6/v_7$), pyruvate cycling flux relative to TCA cycle flux ($v_5/v_7$), and total gluconeogenesis from phosphoenolpyruvate (GNG$_{PEP}$) relative to TCA cycle flux ($v_4/v_7$). Total anaplerosis from pyruvate/lactate/alanine plus a small percentage from propionate was assumed to be equal to total cataplerotic flux (PEPCK flux in Fig. 4). There were no differences between strains among these flux ratios in the 4-h-fasted group. In 24-h-fasted animals, FVB mice had significantly higher anaplerotic and gluconeogenic rates (relative to TCA cycle flux) compared with the other strains.

Because both the deuterated water method (Table 1) and the $^{13}$C NMR isotopomer analysis method (Table 2) provide independent measurements of GNG$_{PEP}$ ($v_4/v_1$ and $v_4/v_7$, respectively), the two sets of data can be normalized to EGP ($v_1$) to give an integrated description of glucose metabolism, as shown in Fig. 4. Figure 5A shows the flux values relative to EGP for the four strains of mice measured after a 4-h fast. In these data, and the 129 strains (1%) differed significantly. As anticipated, 24-h-fasted mice had a lower contribution from glycogen than mice in the 4-h-fasted group. Among mice fasted for 24 h, the fraction of glucose from glycogen was 5% compared with 17% ($P < 0.003$) in mice fasted for 4 h.

**Table 1. Effect of strain and fast duration on sources of plasma glucose by $^2$H NMR**

<table>
<thead>
<tr>
<th>Mouse Strain</th>
<th>Glycogen ($v_2/v_1$)</th>
<th>Glycerol ($v_3/v_1$)</th>
<th>TCA Cycle ($v_4/v_1$)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>24-h Fast</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ICR</td>
<td>8 ± 6</td>
<td>26 ± 3</td>
<td>66 ± 6</td>
</tr>
<tr>
<td>FVB</td>
<td>3 ± 3</td>
<td>20 ± 8</td>
<td>77 ± 8</td>
</tr>
<tr>
<td>B6</td>
<td>11 ± 6*</td>
<td>26 ± 9</td>
<td>63 ± 6*</td>
</tr>
<tr>
<td>129</td>
<td>1 ± 2</td>
<td>22 ± 4</td>
<td>77 ± 2</td>
</tr>
<tr>
<td><strong>4-h Fast</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ICR</td>
<td>15 ± 8</td>
<td>16 ± 18</td>
<td>69 ± 9</td>
</tr>
<tr>
<td>FVB</td>
<td>12 ± 4</td>
<td>20 ± 2</td>
<td>68 ± 2</td>
</tr>
<tr>
<td>B6</td>
<td>21 ± 6</td>
<td>18 ± 3</td>
<td>62 ± 8</td>
</tr>
<tr>
<td>129</td>
<td>17 ± 11</td>
<td>21 ± 8</td>
<td>62 ± 3</td>
</tr>
</tbody>
</table>

Values are means ± SD. The fraction of plasma glucose (%) derived from glycogen, glycerol, and the TCA cycle is shown. Nine mice were used to determine each value (3 plasma samples were pooled for 3 separate NMR observations). *$P < 0.05$ vs. lowest value in the group after Bonferroni correction.

Fig. 3. $^{13}$C NMR spectrum of MAG derived from plasma glucose. $^{13}$C enrichment (via incorporation of [U-$^{13}$C$_3$]propionate in the TCA cycle) originates from contribution of TCA cycle intermediates to glucose production. Multiplets in the C2 resonance of MAG derived from plasma glucose are diagnostic of fluxes into and out of the TCA cycle.

Fig. 4. Flow chart showing the metabolic pathways leading to glucose production. The two sets of data can be normalized to EGP ($v_1$) to give an integrated description of glucose metabolism.
there were no apparent differences in glucose metabolism between the different strains. After the fast was extended to 24 h, these measures remained similar between strains, but some differences became apparent. In particular, glycogenolysis was higher and the GNGPEP contribution to EGP was lower in the B6 strain compared with the other strains. Relative PEPCK flux also tended to be lower in the fasted B6 compared with the other strains of mice ($t$-test, $P < 0.07$). The data for the FVB mice matched the other strains in every parameter except for a slightly depressed TCA cycle flux relative to glucose production (0.40 vs. 0.67 average for the other strains combined, $P < 0.05$ and $P < 0.2$ after Bonferroni correction).

To investigate more closely the differences observed in the B6, FVB, and 129 strains after a 24-h fast, [3,4-13C2]glucose was infused in mice via a chronically implanted catheter to evaluate EGP by NMR analysis of plasma glucose (18). In control mice, where glucose turnover is high and the pool size is low, an infusion time of 75 min without a bolus injection was sufficient to reach steady-state conditions (data not shown). However, in mouse models of diabetes, a bolus and/or longer

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### Table 2. Effect of fast duration and strain on flux through TCA cycle-related pathways measured relative to flux through CS

<table>
<thead>
<tr>
<th>Mouse Strain</th>
<th>Flux Through Pyruvate Carboxylase and Equivalent Reactions, Relative to CS ($v6/v7$)</th>
<th>Flux Through Pyruvate Kinase and Malic Enzyme, Relative to CS ($v5/v7$)</th>
<th>Flux of TCA Intermediates in Glucose, Relative to CS ($v4/v7$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>24-h Fast</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ICR</td>
<td>3.86±0.68</td>
<td>1.41±0.46</td>
<td>2.46±0.22</td>
</tr>
<tr>
<td>FVB</td>
<td>5.76±1.7*</td>
<td>1.73±0.92</td>
<td>4.03±0.88*</td>
</tr>
<tr>
<td>B6</td>
<td>3.06±1.1</td>
<td>0.94±0.46</td>
<td>2.12±0.83</td>
</tr>
<tr>
<td>129</td>
<td>3.87±1.5</td>
<td>1.33±0.07</td>
<td>2.54±0.88</td>
</tr>
<tr>
<td>4-h Fast</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ICR</td>
<td>2.93±0.34</td>
<td>1.15±0.34</td>
<td>1.78±0.11</td>
</tr>
<tr>
<td>FVB</td>
<td>4.25±1.0</td>
<td>1.88±0.6</td>
<td>2.38±0.76</td>
</tr>
<tr>
<td>B6</td>
<td>3.78±0.44</td>
<td>1.40±0.52</td>
<td>2.38±0.10</td>
</tr>
<tr>
<td>129</td>
<td>2.75±0.43</td>
<td>1.08±0.45</td>
<td>1.68±0.43</td>
</tr>
</tbody>
</table>

Values are means ± SD. CS, citrate synthase. *$P < 0.05$ against average of all other strains within the fasted group.

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Fig. 4. Hepatic glucose metabolism. Pathways in bold are interrogated by combined deuterium and 13C NMR isotopomer analysis. The deuterated water method introduces deuterium label in all the positions of glucose, but enrichment at H-2, H-5, and H-6 occurs at phosphoglucose isomerase, triose phosphate isomerase, and fumarase, making those positions diagnostic of glycogenolysis ($v2$), GNGglycerol ($v3$), and GNGPEP ($v4$). [U-13C3]propionate is used as vehicle to introduce 13C label in the TCA cycle by conversion to succinyl-CoA. The resulting 13C isotopomers that form in glucose as a result of carbon flow from the TCA cycle in gluconeogenesis are used to determine anaplerosis/cataplerosis, pyruvate cycling ($v5$), and GNGPEP ($v4$) relative to TCA cycle turnover (citrate synthase; $v7$). Total anaplerosis via pyruvate carboxylase and propionate carboxylase was assumed to be equal to total cataplerosis via phosphoenolpyruvate carboxykinase (PEPCK; $v6$). Pyruvate cycling via pyruvate kinase cannot be distinguished from pyruvate cycling via the malic enzyme. All six fluxes ($v2$–$v7$) can be converted into absolute flux units given an independent measure of EGP ($v1$). G6P, glucose 6-phosphate; F6P, fructose 6-phosphate; G3P, glucose 3-phosphate; Pyr, pyruvate; OAA, oxaloacetate; Fum, fumarate; FBP, fructose bisphosphate; DHAP, dihydroxyacetone phosphate.
infusion periods such as those used in other laboratories may be necessary (3, 33, 40). Interestingly, FVB and B6 mice had very similar rates of EGP, whereas the 129 strain had an EGP 30% lower than either (Fig. 7, inset). These glucose turnover data were then used to convert the relative flux data of Fig. 5 into absolute flux values. These data show that FVB mice had 30% greater GNGPEP and PEPCK flux (P < 0.02 and P < 0.09 after Bonferroni correction, respectively) compared with the B6 and 129 strains, whereas all other flux values appeared to be consistent between strains (Fig. 7). Some of the pathways of the B6 strain that initially appeared divergent from the other strains in the relative flux measurements (Fig. 5) were normal in light of the glucose production data. However, absolute glycogenolysis in the B6 strain was still higher (12 μmol·kg⁻¹·min⁻¹) than the FVB and 129 strains (4 and 0.7 μmol·kg⁻¹·min⁻¹, respectively). Glycogenolysis was different from zero only in B6 mice.

**DISCUSSION**

In view of the cost, time, and effort devoted to generating a single mouse line with a variant genotype, there must be relatively simple but rigorous methods to probe intermediary metabolism in these mice. Numerous mouse lines have been generated with the hope that a single modification of one or more metabolic pathways would yield insight into disease phenotypes that model human metabolic disorders. This hope

**Fig. 5.** Liver flux profile relative to EGP in ICR, FVB, B6, and 129 strains. Pathways v1–v7 refer to the pathways depicted in Fig. 4. A: in the 4-h-fasted group, there were no differences in LFP relative to EGP in ICR, FVB, B6, or 129 strains. B: in the 24-h-fasted group, the B6 mice diverged from the other strains in glycogenolysis (Gly, v2), GNGPEP (v4), and PEPCK (v6) flux relative to EGP. †P < 0.05 vs. the highest and lowest value in the group after Bonferroni correction. ‡P < 0.05 with standard t-test vs. the highest and lowest values in the group without Bonferroni correction.

**Fig. 6.** Liver flux profile relative to EGP in 4-h-fasted vs. 24-h-fasted mice. Pathways v1–v7 refer to the pathways depicted in Fig. 4. Groups represent the average of all strains of mice after a 4- or 24-h fast. †P < 0.05 after Bonferroni correction.

**Fig. 7.** Absolute liver flux profile. Pathways v1–v7 refer to the pathways depicted in Fig. 4. Absolute fluxes through these pathways derived from the data in Figs. 5A and 6B (a combination of 2H and 13C NMR data) were multiplied by data in Fig. 6B (EGP). †P < 0.05 vs. the highest and lowest values in the group after Bonferroni correction. ‡P < 0.05 with standard t-test vs. the highest and lowest values in the group without Bonferroni correction.
has not always been fulfilled, in part because a close correspondence between modification of a single gene and a specific phenotype is generally an oversimplification (9, 14, 27). Another problem is a practical one. The effects of genomic manipulation may be masked by multiple compensatory pathways in the animal, making routine primary screens of metabolism uninformative. Even relatively sophisticated secondary screens of metabolism using current methods may yield confusing results. For example, She and colleagues (33, 34), generated a mouse with liver-specific deletion of cytosolic PEPCK (PEPCK-C), but this mouse maintained normal systemic glucose turnover during a fast. Furthermore, despite upregulation of all the enzymes of fatty acid oxidation and the TCA cycle, TCA cycle activity is impaired in the liver-specific PEPCK-C null mouse (4). This obvious discrepancy between the predicted and measured outcomes is only one of many that show both the plasticity of complex metabolic systems and the immediate need for practical analytical tools to probe metabolic fluxes in vivo.

Because the mouse is the most common species of genomic and pharmacological experimentation, one goal was to develop technology that would allow a quantitative evaluation of metabolic flux phenotype in mice. As a prelude to genetic manipulation, H and 13C NMR were used to evaluate and compare metabolic flux profiles related to glucose production in several strains of mice after either a 4- or 24-h fast. The primary difference between short-term and long-term fasting was, as expected, a decreased reliance on glycogenolysis for EGP. The resulting dependence on gluconeogenesis was satisfied by a decrease in the disposal of phosphoenolpyruvate through the pyruvate cycling pathway rather than an increased flux through PEPCK, suggesting an important role for pyruvate kinase in the regulation of gluconeogenesis (15, 23). Another striking observation of this work is that gluconeogenesis has a much greater contribution from glycerol in mice compared with humans. In humans, glycerol contributes ~5% to glucose synthesis (21, 38), approximately fourfold less than found here in mice. A large contribution from GNGglycerol is consistent with other reports that glycerol turnover is 118 μmol·kg⁻¹·min⁻¹ in mice (30) compared with 6.3 μmol·kg⁻¹·min⁻¹ in humans (16). Furthermore, the plasma concentration of glycerol is >400 μM in mice (30) compared with ~60 μM in humans (13). This approximately sevenfold increase in plasma glycerol concentration mirrors the increase in GNGglycerol flux compared with humans. From this conversion of glycerol to glucose (~50 μmol·kg⁻¹·min⁻¹; Fig. 7), we can estimate that about one-half of the glycerol turnover in mice may be related to GNGglycerol.

An important objective of this study was to determine whether differences in metabolic flux exist between strains of mice. These differences must be recognized to avoid misinterpretation of genetic interventions, especially since some data indicate simple differences in metabolite levels. A recent report by Naggert et al. (28) published in the Jackson Mouse Phenome Database demonstrates a remarkable strain variation in plasma glucose levels after a 4-h fast. The female 129 strain (170 mg/dl) has a 32% lower plasma glucose level than the B6 strain (224 mg/dl), whereas the FVB strain (190 mg/dl) lies between the two (28). Despite these differences, we found no evidence of variation in metabolic flux between strains after a regulated short-term fast. Differences in hepatic glucose metabolism among strains could have gone undetected in these experiments because of differences in glucose turnover, but such a hidden disparity would require a proportional increase or decrease in all of the fluxes of each strain. Other important processes, such as glucose disposal by peripheral tissue and its regulation by pancreatic islet function, could also affect plasma glucose levels without necessarily affecting metabolic flux in the liver.

After a 24-h fast, these mouse strains were again remarkably similar, yet there were some discreet differences. The B6 strain displayed a slightly decreased H-5-to-H-2 ratio in the deuterium NMR spectra after a 24-h fast, reflecting slightly elevated glycogenolysis compared with the other strains. Correspondingly, the B6 strain also had a significantly lower GNGPEP relative to EGP associated with a decrease in PEPCK flux, although the significance of that difference disappears after correction for multiple comparisons. The FVB mice tended toward lower citrate synthase flux, suggesting that the TCA cycle in livers of these animals requires less oxidation of acetyl-CoA per glucose molecule produced through gluconeogenesis. The origin of this higher “gluconeogenic efficiency” is unknown, although FVB mice have approximately threefold lower liver triglycerides and display lower triglyceride clearance than B6 mice (9). Decreased hepatic fatty acid depots could signal more tightly coupled mitochondria (39) and, as a consequence, decreased hepatic fatty acid oxidation and TCA cycle turnover. However, measures of absolute TCA cycle flux in FVB mouse livers tended to be lower but did not reach statistical significance compared with the B6 or 129 strains.

In an attempt to more fully understand the nature of the strain differences observed after a 24-h fast, EGP was measured in 24-h-fasted FVB, 129, and B6 mice by use of indwelling jugular vein catheters. EGP determined for all three strains of mice was within the range reported in the literature. Values as low as 46.7 μmol·kg⁻¹·min⁻¹ (40) to as high as 150 μmol·kg⁻¹·min⁻¹ (3) have been reported in fasted control mice generated on the B6 strain, demonstrating that mouse metabolism has an extraordinary dynamic range in response to even minor changes in environment. Nonetheless, under identical conditions, the 129 strain demonstrated ~30% lower EGP than either the FVB or B6 strains, consistent with lower plasma glucose levels in the 129 strain. The decreased EGP in 129 mice may be related to decreased basal physical activity levels in these mice compared with the FVB and B6 strains (32, 36), an observation that was qualitatively confirmed during our infusion experiments.

Despite less physical activity in the 129 strain, Seburn (32) reports that whole body oxygen consumption is the same or slightly elevated in these animals compared with FVB and B6, suggesting that the three strains are energetically quite similar. This is consistent with our observation that hepatic TCA cycle flux does not differ between these three strains of mice (Fig. 5). The significance of the 30% elevation in GNGPEP and PEPCK flux (Fig. 5) in the FVB compared with the B6 strain is unclear, but these two strains clearly respond to metabolic challenges differently. For instance, the B6 strain is known to be more susceptible to diet-induced diabetes and atherosclerosis than other strains of mice (31), and some genetic manipulations targeting intermediary metabolism have variable consequences, depending on whether the manipulation is expressed on an FVB or B6 background. For instance, both the lipoatro-
phic AZIP mouse (9) and the ob/ob mouse (14) have a milder whole body phenotype on the B6 background than the FVB background. The important difference between the two strains is apparently the result of increased triglyceride clearance in the B6 mouse facilitated by a high capacity for hepatic lipid storage (9, 14). Interestingly, PEPCk expression is similar in the fed livers of B6 and FVB mice, whereas levels of glucose-6-phosphatase are approximately threefold higher in B6 liver (9). This elevated level of glucose-6-phosphatase expression has very little impact on in vivo activity, since EGP measured here and elsewhere is not different between the two strains (9, 14).

The elevated flux through PEPCk in FVB mice measured here also does not result in elevated EGP because its impact is dissipated by increased pyruvate cycling, decreased GNGglycerol (both insignificant differences), and a significantly lower rate of glycogenolysis compared with the B6 mouse. An 11% contribution to glycogenolysis after a 24-h fast in the B6 is remarkable, since hepatic glycogen content is thought to be depleted rapidly in mice and the B6 was the only strain with glycogenolysis significantly different from zero. In general, insulin resistance is associated with reduced glycogen stores and a decrease in glycogen synthase and the protein targeting glycogen scaffolding (10, 41). Yet, residual glycogenolysis after long-term fasting in the B6 mouse is reminiscent of the diabetic ZDF rat model where it was recently demonstrated that 50% of EGP was derived from glycogen after a 24-h fast (17). Elevated glycogenolysis after long-term fasting, along with elevated liver triglyceride concentrations (9), may be a marker for a genetic/metabolic predisposition toward the development of hepatic insulin resistance in rodents.

Metabolic pathways related to glucose production were quantified in several strains of mice, and flux differences were identified. These results do not necessarily support the use of one background strain over another for the generation of a transgenic line but do highlight the need for a pure ground genotype on muscle and liver insulin sensitivity of lipoatrophic receptor-deficient mice. These studies were supported by funding from National Institutes of Health Grants RR-02584 and U24-DK-59632 and by an American Diabetes Association Junior Faculty Award (to S. C. Burgess).

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