Differing mechanisms of hepatic glucose overproduction in triiodothyronine-treated rats vs. Zucker diabetic fatty rats by NMR analysis of plasma glucose

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Submitted 11 August 2004; accepted in final form 17 November 2004

ALTHOUGH THE PATHWAYS CONTRIBUTING to glucose production are well established, less is known about the effects of disease on hepatic glucose output in humans. Earlier studies examined selected aspects of glucose production in the Zucker diabetic fatty (ZDF) rat (fa/fa) and the 3,3′-5-triiodo-3′-thyronine (T3)-treated rat. Studies in T3-treated animals focused on gluconeogenesis after a 24-h fast.2H2O and [U-13C3]propionate were administered intraperitoneally, and [3,4-13C2]glucose was administered as a primed infusion for 90 min under ketamine-xylazine anesthesia. 13C NMR analysis of monoacetone glucose derived from plasma glucose indicated that hepatic glucose production was twofold higher in both T3-treated rats and ZDF rats compared with controls, yet the sources of glucose overproduction differed significantly in the two models by 2H NMR analysis. In T3-treated rats, the hepatic glycogen content and hence the contribution of glycogenolysis to glucose production was essentially zero in this case, excess glucose production was due to a dramatic increase in gluconeogenesis from TCA cycle intermediates. 13C NMR analysis also revealed increased phosphoenolpyruvate carboxykinase flux (4X), increased pyruvate cycling flux (4X), and increased 13C flux (5X) in T3-treated animals. ZDF rats had substantial glycogen stores after a 24-h fast, and consequently nearly 50% of plasma glucose originated from glycogenolysis; other fluxes related to the TCA cycle were not different from controls. The differing mechanisms of excess glucose production in these models were easily distinguished by integrated 2H and 13C NMR analysis of plasma glucose.

although the pathways contributing to glucose production are well established, less is known about the effects of disease on hepatic glucose output in humans. Earlier studies examined selected aspects of glucose production in the Zucker diabetic fatty (ZDF) rat (fa/fa) and the 3,3′-5-triiodo-3′-thyronine (T3)-treated rat. Studies in T3-treated animals focused on gluconeogenesis from the TCA cycle, particularly flux through phosphoenolpyruvate carboxykinase (PEPCK) and cycling pathways associated with phosphoenolpyruvate (PEP). PEP can undergo dephosphorylation to pyruvate by pyruvate kinase (PK) rather than conversion to glycogen, an apparent energy-wasting process. Rognstad (26) and Cohen et al. (5) found that flux through PK was significantly higher in hepatocytes isolated from T3-treated rats. Later, Petersen et al. (22) showed that direct conversion of malate to pyruvate via malic enzyme (ME) also contributes to this futile cycle and cannot be distinguished from the oxaloacetate (OAA) → PEP → pyruvate → OAA cycle using isotope methods (22). Flux through these combined pathways, referred to as pyruvate recycling (7) or simply pyruvate cycling (17), is increased in livers of T3-treated rats in vivo (23).

Most investigations of glucose production in ZDF rats have focused on glycogenolysis. Among patients with poorly controlled type 2 diabetes mellitus (T2DM), glycogenolysis has a causative role in hepatic glucose overproduction in the fasted state, and glycogenolysis has been reported to be similar or less than in control human subjects (10, 18, 19). A male ZDF rat is an animal model of obesity and T2DM, yet studies of ZDF rats suggest that glycogenolysis may play an important role in hyperglycemia in the fasted state. Hepatocytes from fasted ZDF rats have higher rates of glycogen synthesis than hepatocytes from lean Zucker rats (20), and glycogen stores are preserved to a greater extent in ZDF rats compared with lean animals during starvation (30). Consequently, glycogenolysis may provide a source of glucose not available in lean animals after fasting.

These data suggest that metabolic fluxes associated with glucose production of T2DM and hyperthyroidism may differ substantially. However, only a subset of relevant pathways was examined in each study, and the requirement of most methods for liver tissue restricts use of these methods in humans. 13C and 2H NMR offer a flexible, comprehensive, and essentially noninvasive approach to analysis of metabolic fluxes in vivo. One important advance was the ability to monitor glycogen stores in vivo by 13C NMR, which provides a direct measure of glycogenolysis (10, 18, 25). Other methods to characterize glucose production require only analysis of blood samples (15). Jin et al. (14) recently introduced the use of 13C NMR coupled with [3,4-13C2]glucose to measure glucose turnover. This approach is convenient because the characteristic 13C-13C spin-spin coupling in tracer carbons 3 and 4 is easily distinguished from 13C enrichment in carbon positions 1, 2, 5, or 6 that arises from the 13C label entering the TCA cycle from labeled glycogen precursors. Furthermore, the presence of low levels of 13C enrichment in plasma glucose does not interfere with analysis of deuterium exchange in the same experiment using 2H NMR. Thus this triple-tracer method allows multiple aspects of systemic glucose production to be probed in a single experiment.

The objective of this study was to measure fluxes in relevant pathways using only 13C and 2H NMR of plasma glucose in Zucker diabetic fatty rats and the 3,3′-5-triiodo-3′-thyronine (T3)-treated rats.
two previously studied rat models of excess glucose production. We tested the hypothesis that the distribution of $^{13}$C and $^2$H in plasma glucose would be sensitive to these two disease states and that the sources of excess glucose production in these two models would differ. Although glucose production was identical in both ZDF and T3-treated rats and significantly elevated compared with normal controls, the metabolic networks supporting glucose production in T3-treated and ZDF animals differed substantially. Because these measurements may be obtained from a single sample of plasma glucose, extension of the technique to humans is straightforward.

MATERIALS AND METHODS

**Materials.** ZDF rats (fa/fa) were a generous gift from Dr. Roger H. Unger of the Veterans Affairs North Texas Health Care System (Dallas, TX). [3,4-$^{13}$C$_2$]glucose (99%) was purchased from Omicron Biochemicals (South Bend, IN). [U-$^{13}$C$_3$]propionate (99%), $^2$H$_2$O (99.9%), and deuterated acetonitrile (99.8%) were obtained from Cambridge Isotopes (Andover, MA). DSC-18 solid-phase extraction (SPE) gel was obtained from Supelco (St. Louis, MO). Protein assay reagents were purchased from Bio-Rad (Hercules, CA). Other common chemicals were purchased from Sigma (St. Louis, MO).

**Protocol.** The study was approved by the Institutional Animal Care and Use Committee at the University of Texas Southwestern Medical Center. Three different groups of rats were studied: male Sprague-Dawley rats (220 ± 31 g), T3-treated male Sprague-Dawley rats (236 ± 16 g after T3 treatment), and 19- to 20-wk-old male ZDF rats (482 ± 68 g). T3-treated rats were given T3 in their drinking water for 5 days before initiation of the experiment. One liter of drinking water was prepared by dissolving 2 mg of T3 into 200 ml of NaOH (50 mM) followed by dilution to 1 liter. T3-treated rats lost 20.7 ± 8.4 g during 5 days of T3 treatment, including the 24-h fasting period. All rats were fasted for 24 h, with free access to water, after which the jugular vein was cannulated under ketamine-xylazine anesthesia. Rats received an intraperitoneal injection (20 µl/rat) containing $[^{13}$C$_2$]propionate (5 mg/ml) dissolved in $^2$H$_2$O. Immediately after this injection, each rat received a bolus infusion of 3.5 mg of [3,4-$^{13}$C$_2$]glucose (99%), followed by continuous infusion of [3,4-$^{13}$C$_2$]glucose (4.0 mg/ml water) at a rate of 1.0 ml/h for 90 min. At the conclusion of the 90-min infusion period, blood was drawn from the descending aorta and the liver was freeze-clamped and stored at −80°C until processed.

**Sample processing for NMR analysis.** Blood was immediately centrifuged, and plasma supernatant was deproteinized by adding cold perchloric acid to a final concentration of 7% by volume. After neutralization with KOH and centrifugation, the supernatant was lyophilized. To convert plasma glucose into monoacetone glucose (MAG; Fig. 1), the dried residue was suspended in 3.0 ml of acetone containing 120 µl of concentrated sulfuric acid. The mixture was stirred for 4 h at room temperature to yield diacetone glucose. After filtering off particulates and adding 3 ml of water, we adjusted the pH to 2.0 by dropwise addition of 1.5 M Na$_2$CO$_3$. The mixture was stirred for 24 h at room temperature to convert diacetone glucose into MAG. The pH was then further increased to approximately 8.0 by dropwise addition of Na$_2$CO$_3$. Acetone was evaporated under a vacuum, and the sample was freeze-dried. MAG was extracted into 3 ml (5X) of hot ethyl acetate, the solutions were combined, and the ethyl acetate was removed by vacuum evaporation. The resulting MAG was further purified by passage through a 3-ml DSC-18 cartridge, using 5% acetonitrile as eluant. The effluent was freeze-dried and stored dry before NMR analysis.

A 5- to 10-g portion of liver tissue was used for glycogen extraction and purification (21). Isolated liver glycogen was dissolved in 5 ml of 10 mM sodium acetate solution (pH 4.8) and incubated with amylo-
glucosidase (50 mg glycogen/20 U amylglucosidase) for 4 h at 50°C. After freeze-drying, the glucose was converted to MAG as described above.

NMR spectroscopy. MAG was dissolved in 90% acetonitrile-10% water. 2H NMR spectra were collected with a Varian INOVA 14.1 T spectrometer (Varian Instruments, Palo Alto, CA) equipped with a 3-mm broadband probe with the observe coil tuned to 2H (92.1 MHz). Shimming was performed on selected 1H resonances of MAG detected via the decoupler coil. Proton-decoupled 2H NMR spectra were acquired at 50°C, using a 90° pulse and a repetition rate of 1 s. Typically, 6,000–20,000 scans were acquired for plasma samples and 60,000–70,000 scans for liver samples. Proton decoupling was performed using a standard WALTZ-16 pulse sequence. 2H NMR spectra were analyzed with curve-fitting and no delay between pulses. Typically, 15,000–40,000 scans were acquired at 25°C, using a 52° observe pulse, a 1.5-s acquisition time, spectroscopy, the MAG samples were dried and resuspended in 60,000–70,000 scans for liver samples. Proton decoupling was performed via the decoupler coil. Proton-decoupled 2H NMR spectra were detected via the decoupler coil. Proton-decoupled 2H NMR spectra were acquired at 52°C, using a 52° observe pulse, a 1.5-s acquisition time, and no delay between pulses. Typically, 15,000–40,000 scans were required. 13C NMR spectra were analyzed with the curve-fitting routine supplied with NUTS. 2H2O enrichment in plasma of ZDF rats was determined as described previously (16).

Glucose turnover and metabolic fluxes. Glucose turnover was estimated from the dilution of infused [3,4-13C2]glucose, using 13C NMR to analyze MAG derived from plasma glucose at the end of the infusion protocol (14). Briefly, the fraction of [3,4-13C2]glucose in plasma glucose was determined from the ratio of the areas of the doublet due to J34 (13C-13C spin-spin couplings in carbons 3 and 4) in carbon 3 [at 75.3 parts/million (ppm)] and in carbon 4 (at 80.5 ppm) compared with the total area of the two methyl resonances (26.1 and 26.7 ppm). Because the methyl resonances reflect natural abundance 13C, the fraction of [3,4-13C2]glucose in MAG was evaluated based on standard curves (14). Glucose production was calculated from the known infusion rate (Ri), the fraction of infused glucose that was [3,4-13C2]glucose (Lp), and the fraction of plasma glucose that was [3,4-13C2]glucose (Lp) at the end of the infusion period

\[ v_1 = \text{glucose production} = R_i \cdot (L_i - L_p)/L_p \]  

Flux from glycogen, glycerol, and PEP into plasma glucose was estimated from the deuterium enrichment at positions 2, 5, and 6 (H2, H5, and H6, respectively), as determined from the 2H NMR of MAG

\[ v_2 = \text{flux from glycogen} = v_1 \cdot (H_2 - H_5)/H_2 \]  

\[ v_3 = \text{flux from glycerol} = 2 \cdot v_1 \cdot (H_5 - H_6)/H_2 \]  

\[ v_4 = \text{flux from PEP} = 2 \cdot v_1 \cdot H_6/H_2 \]  

A 13C NMR isopomer analysis based on the 13C-13C spin-coupled multiplets of carbon 2 of MAG has been reported previously that yields relative fluxes in the citric acid cycle as follows

\[ v_5/v_1 = (C_2Q - C_2D_23)/C_2D_23 \]  

\[ v_6/v_1 = (C_2D_12 - C_2Q)/C_2D_23 \]  

\[ v_7/v_1 = (C_2D_12 - C_2D_23)/C_2D_23 \]  

where the variables C2Q, C2D12, and C2D23 are the areas of the quartet, doublet due to J132, and doublet due to J23, relative to the area of the carbon 2 resonance. The assumptions in the metabolic model include the following: 1) all glucose originates from the liver, 2) hydrolytic conversion of glycogen to glucose via amylol-1.6-glucosidase is negligible, 3) there is insignificant labeling of acetyl-CoA entering the TCA cycle from either tracer [3,4-13C2]glucose or [U-13C3]propionate, 4) there is complete equilibration of all 13C-enriched four-carbon intermediates in the TCA cycle, and 5) steady-state metabolic conditions apply (17).

[3,4-13C2]glucose is an ideal tracer of glucose turnover when used in combination with [U-13C3]propionate as a tracer of the TCA cycle. It is assumed that any metabolism of [3,4-13C2]glucose to the level of a triose followed by resynthesis to glucose yields only [3-13C]glucose or [4-13C]glucose, since the absolute enrichment of the triose pool is low and de novo generation of [3,4-13C2]glucose is negligible. Another important aspect of this glucose tracer is that any metabolism of [3,4-13C2]glucose to pyruvate followed by carboxylation to OAA, continued metabolism in the TCA cycle, and resynthesis to glucose can only produce [3-13C]glucose or [4-13C]glucose. This feature means that 13C enrichment patterns in glucose carbons 1 and 2 (or 5 and 6) can only be derived from metabolism of [U-13C3]propionate.

Each variable used to describe the metabolic results (v1–v7) generally describes the combined actions of multiple enzyme-catalyzed reactions. For example, v2 indicates the rate of generation of glucose 6-phosphate from glycogen, without implying measurement of flux in a specific reaction. Similarly, v5 is used to describe the flux of carbon skeletons from the PEP/pyruvate pool through carboxylation and regeneration of OAA. Flux from OAA to pyruvate to OAA can occur via the combined effects of either malate dehydrogenase, the malic enzyme and pyruvate carboxylase, or PEPCk, pyruvate kinase and pyruvate carboxylase. Hence, v5 simply designates the sum of fluxes through both pathways. Pyruvate carboxylase is common to both pathways, so v5 is also a lower limit on total flux through pyruvate carboxylase.

Metabolic assays. Plasma glucose was assayed enzymatically (2). Freeze-clamped livers were pulverized under liquid nitrogen, and a small portion (<0.5 g) was extracted with perchloric acid (6%) and subsequently used for glycogen and lactate assays (2). The remaining portion of each pulverized liver (~5 g) was suspended in 25 ml of 20 mM Tris-HCl (pH 7.4) buffer containing 0.25 M sucrose, 0.1 mM dithiothreitol, and 0.1 mM EDTA; homogenized; and centrifuged at 800 g for 10 min to remove cell debris. The supernatant was further centrifuged at 8,500 g for 15 min to separate a mitochondrial pellet. The pellet was washed (3×) with the buffer and resuspended in 1.5 ml of buffer. This represented the mitochondrial fraction, whereas the supernatant represented the cytosolic fraction. Total proteins in the mitochondrial and cytosolic fractions were measured with Bio-Rad assay reagents (3). The activities of ME and PK were determined on the cytosolic fraction (4, 11). Malate in cytosolic fraction was measured enzymatically (2).

Statistical analysis. Data are expressed as means ± SD. Comparisons between groups were performed using one-way ANOVA. A P value < 0.05 was considered significant.

RESULTS

Glucose production by dilution of [3,4-13C2]glucose. Plasma glucose was higher in ZDF rats after a 24-h fast than in T3-treated rats and controls (Table 1). Typical 13C NMR spectra of MAG from a T3-treated rat, a ZDF rat, and a control rat are shown in Fig. 1. The doublets of [3,4-13C2]glucose were well resolved from the singlet and from other multiplets of glucose isotopeomers originated through gluconeogenic pathways from [U-13C3]propionate. A comparison of the doublet component areas of the carbon 3 (75.3 ppm) and carbon 4 (80.5 ppm) resonances relative to the natural abundance methyl resonances provided a direct readout of [3,4-13C2]glucose enrichment in plasma. These were 3.77 ± 0.65, 1.66 ± 0.38, and 0.82 ± 0.16% for control, T3-treated, and ZDF rats, respectively. Given the known infusion rates of [3,4-13C2]glucose, this corresponds to glucose production rates of 40.4, 94.5, and 86.5 μmol·kg⁻¹·min⁻¹, respectively (Table 2). This indicates that hepatic glucose production was approximately twofold
higher in both T3-treated rats and ZDF rats compared with controls.

**Glycogen stores and sources of plasma glucose by 2H NMR.** Liver glycogen was essentially depleted in control animals and in T3-treated rats after a 24-h fast but remained high (112.7 ± 9.3 μmol glucosyl U/g wet wt; Table 1) in ZDF rats after a similar period of fasting. This was also reflected in the contribution of hepatic glycogen to plasma glucose as determined by 2H NMR (Fig. 2), where the H5/H2 ratio was close to 1 in controls and T3-treated rats but only 0.53 ± 0.04 (see Fig. 2C) in ZDF rats. This value indicates that approximately one-half of the plasma glucose in ZDF rats is derived from glycogen, with the remainder coming from either glycerol or the TCA cycle. The difference between H5 and H6 was modest for all three groups, with the fractional contribution of glycogen to glucose ranging from 13 to 17% (Table 2). The H6/H2 ratio, an index of the contribution of PEP to glucose production, was ~80% in controls and T3-treated rats but significantly lower (40%) in ZDF rats (Table 2).

The absolute rates of glucose formation from glycerogen, PEP, and glyceral were determined by multiplying the individual fractional contributions with the glucose production rates. These values are summarized in Table 2. Glycogenolysis was ~41 μmol·kg⁻¹·min⁻¹ in ZDF rats but negligible in controls and T3-treated rats. Gluconeogenesis from glycogen was significantly higher in both T3-treated rats (P < 0.05) and ZDF rats (P < 0.01) compared with controls, and gluconeogenesis from PEP was approximately twofold higher in T3-treated rats than in controls and ZDF rats.

**13C labeling in plasma glucose.** Entry of [U-13C3]propionate into the TCA cycle via propionyl-CoA, methylmalonyl-CoA, and succinyl-CoA and subsequent turnover produce a mixture of 13C isotope homoplasms in all intermediates and in molecules in exchange with those intermediates. Because glucose is derived from OAA, one would anticipate that MAG derived from glucose would show extensive couplings in its 13C NMR spectrum (Fig. 3). To confirm this, plasma glucose had achieved isotopic steady-state enrichment from [U-13C3]propionate, an additional experiment was performed with 24-h-fasted Sprague-Dawley rats (n = 3 at each time point, 251 ± 15 g). The same amount of [U-13C3]propionate-[2H2]O was injected intraperitoneally, and blood was drawn from the descending aorta at different time points (60, 120, and 150 min). Plasma glucose at each time point was converted into MAG and scanned by 13C NMR. As illustrated in Fig. 3A, the carbon 2 multiplet ratios Q/D23 and D12/D23 seen in the spectra of MAG were relatively constant and similar to the values measured in spectra of MAG derived from control animals at 90 min. Over the entire study period (60–150 min), the D12/D23 and Q/D23 ratios varied no more than 5.3–5.6 and 2.5–2.7, respectively (Fig. 3A). Conversely, the absolute 13C enrichment of carbon 2 of plasma glucose as estimated from 1H

Table 1. Biochemical analysis of plasma and liver tissue from control, ZDF, and T3-treated rats

<table>
<thead>
<tr>
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<th>Control</th>
<th>ZDF (fafa)</th>
<th>T3Treated</th>
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</thead>
<tbody>
<tr>
<td>Plasma glucose, mM</td>
<td>6.2 ± 1.0</td>
<td>17.6 ± 0.9*</td>
<td>8.7 ± 1.6†</td>
</tr>
<tr>
<td>Liver glycogen, μmol glucosyl U/g wet wt</td>
<td>1.8 ± 0.5</td>
<td>112.7 ± 9.3*</td>
<td>1.2 ± 0.2†</td>
</tr>
<tr>
<td>Liver lactate, μmol/g wet wt</td>
<td>1.0 ± 0.2</td>
<td>3.8 ± 0.4*</td>
<td>1.7 ± 2.2‡</td>
</tr>
<tr>
<td>Mitochondrial protein, mg/g wet wt</td>
<td>13.3 ± 2.9</td>
<td>10.7 ± 4.2</td>
<td>25.5 ± 4.7†</td>
</tr>
<tr>
<td>Cytosolic malate, μmol/g wet wt</td>
<td>0.6 ± 0.1</td>
<td>1.1 ± 0.5*</td>
<td>0.5 ± 0.3</td>
</tr>
<tr>
<td>Mitochondrial pellet volume, ml/g wet wt</td>
<td>0.19 ± 0.06</td>
<td>0.19 ± 0.07</td>
<td>0.35 ± 0.06‡</td>
</tr>
<tr>
<td>Malic enzyme activity, μU/mg protein</td>
<td>2.2 ± 0.4</td>
<td>6.3 ± 2.9*</td>
<td>3.1 ± 0.4†</td>
</tr>
<tr>
<td>Pyruvate kinase activity, μU/mg protein</td>
<td>41.6 ± 6.1</td>
<td>85.2 ± 26.9*</td>
<td>29.8 ± 5.3†</td>
</tr>
</tbody>
</table>

Values are means ± SD. Glucose production was measured by detection of [3,4-13C2]glucose by 13C NMR. Relative metabolic fluxes were determined by 2H (H2, H5, and H6) and 13C NMR (C2 resonance) analysis of monocarboxamide glucose (MAG) derived from plasma glucose of controls (n = 6), T3-treated rats (n = 6), and ZDF rats (n = 7). Notations for fluxes v1–v7 are illustrated in Fig. 4. PEP, phosphoenolpyruvate; PYR, pyruvate; OAA, oxaloacetate; MAL, malate.

Table 2. Relative and absolute fluxes through key reactions in glucose production

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>ZDF (fafa)</th>
<th>T3Treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose production, (v1), μmol·kg⁻¹·min⁻¹</td>
<td>40.4 ± 2.9</td>
<td>86.5 ± 14.6*</td>
<td>94.5 ± 21.4*</td>
</tr>
<tr>
<td>Fractional sources of plasma glucose</td>
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<td>Glycogen</td>
<td>0.03 ± 0.02</td>
<td>0.47 ± 0.04*</td>
<td>0.03± 0.05†</td>
</tr>
<tr>
<td>Glycerol</td>
<td>0.14 ± 0.04</td>
<td>0.13 ± 0.04</td>
<td>0.17 ± 0.06</td>
</tr>
<tr>
<td>PEP</td>
<td>0.83 ± 0.03</td>
<td>0.40 ± 0.04*</td>
<td>0.79 ± 0.08†</td>
</tr>
<tr>
<td>Fluxes relative to citrate synthase</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gluconeogenesis/citrate synthase (v9/v7)</td>
<td>1.46 ± 0.32</td>
<td>1.21 ± 0.27</td>
<td>0.60 ± 0.14†</td>
</tr>
<tr>
<td>v7/v9</td>
<td>3.19 ± 0.44</td>
<td>2.68 ± 0.27*</td>
<td>2.46 ± 0.27*</td>
</tr>
<tr>
<td>v9/v7</td>
<td>4.65 ± 0.62</td>
<td>3.89 ± 0.45*</td>
<td>3.06 ± 0.38‡</td>
</tr>
<tr>
<td>Derived fluxes, μmol·kg⁻¹·min⁻¹</td>
<td></td>
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<tr>
<td>Gluconeogenesis (v2)</td>
<td>1.0 ± 0.8</td>
<td>40.7 ± 8.7*</td>
<td>2.9 ± 4.0</td>
</tr>
<tr>
<td>Glycolate → glucose (v3)</td>
<td>11.5 ± 3.5</td>
<td>22.4 ± 5.1*</td>
<td>34.2 ± 17.5†</td>
</tr>
<tr>
<td>PEP → glucose (v4)</td>
<td>67.3 ± 5.6</td>
<td>69.5 ± 15.2</td>
<td>148.9 ± 31.9†</td>
</tr>
<tr>
<td>PEP/PYR → OAA/MAL (v6)</td>
<td>154.2 ± 43.4</td>
<td>159.9 ± 52.0</td>
<td>646.1 ± 203.6‡</td>
</tr>
<tr>
<td>OAA/MAL → PEP/PYR (v8)</td>
<td>221.7 ± 47.6</td>
<td>229.7 ± 65.4</td>
<td>795.0 ± 232.6†</td>
</tr>
<tr>
<td>OAA → citrate (v9)</td>
<td>49.1 ± 16.8</td>
<td>60.7 ± 20.4</td>
<td>268.3 ± 106.0†</td>
</tr>
</tbody>
</table>

Values are means ± SD. Glucose production measured by detection of [3,4-13C2]glucose by 13C NMR. Relative metabolic fluxes were determined by 2H (H2, H5, and H6) and 13C NMR (C2 resonance) analysis of monocarboxamide glucose (MAG) derived from plasma glucose of controls (n = 6), T3-treated rats (n = 6), and ZDF rats (n = 7). Notations for fluxes v1–v7 are illustrated in Fig. 4. PEP, phosphoenolpyruvate; PYR, pyruvate; OAA, oxaloacetate; MAL, malate. *Significantly different from control rats (P < 0.05). †Significantly different from ZDF rats (P < 0.05).
NMR spectra of MAG (15) was 3.5 ± 0.1% at 60 min, 4.4 ± 0.3% at 90 min, 3.1 ± 0.4% at 120 min, and 2.3 ± 0.4% at 150 min. This indicates that, while the total contribution of [U-13C3]propionate to plasma glucose reaches a maximum at 90 min, the multiplet ratios used to derive the metabolic fluxes reported here were indeed constant over this study period.

Figure 3 shows that the multiplet pattern of the MAG carbon 2 resonance from a T3-treated rat differed from that of a control and a ZDF rat. In particular, the doublet due to J23 is larger than that of quartet in MAG from a T3-treated rat compared with control and ZDF animals. An analysis of the carbon 2 multiplets using Eqs. 5–7 provides a measure of flux through PEPCK, pyruvate cycling, and gluconeogenesis, all relative to citrate synthase flux. These data indicate that all three flux ratios (v4/v7, v5/v7, v6/v7) were significantly smaller in T3-treated rats than in ZDF or control rats (Table 2). However, upon conversion of these flux ratios into absolute flux values via the metabolic network shown in Fig. 4, a somewhat different picture emerges. Specifically, absolute flux from PEP → glucose (v4) was approximately twofold greater, PEPCK flux (v6) and pyruvate cycling (v5) fluxes were approximately fourfold greater, and TCA cycle flux (v7) was approximately fivefold greater in T3-treated animals than in either ZDF rats or controls, whereas these same absolute fluxes did not vary significantly between ZDF and control rats. It was also interesting that the total mitochondrial protein content and the mitochondrial pellet volume in livers of T3-treated rats were approximately twofold higher than in either ZDF or control rats (Table 1), consistent with an increased TCA cycle capacity in these animals.

2H NMR analysis of liver glycogen in conscious ZDF rats. The fact that glycogen stores in ZDF rats were preserved after a 24-h fast (Table 1) suggests reduced glycogenolysis coupled with perhaps increased gluconeogenesis. However, the distribution of 2H in plasma glucose (Fig. 2) indicates that substantial glycogenolysis does occur in livers of ZDF rats. To evaluate this apparent discrepancy, additional experiments were performed with a similar group of 24-h-fasted ZDF rats. The same amount of 2H2O-[U-13C3]propionate was injected intraperitoneally into the conscious rats without measurement of glucose turnover. Animals were killed at 90 min under ketamine-isoflurane anesthesia, hepatic glycogen was isolated and converted to MAG, and 2H spectra were collected (Fig. 5). The 2H2O enrichment of plasma water in these ZDF rats was 2.8 ± 0.3%. If liver glycogen synthesis was active during the 90-min period after 2H2O injection, a significant 2H enrichment in glycogen would have been expected. Experimentally, the 2H enrichment in H2 of MAG was ~0.27%, showing that glycogen synthesis was indeed active in conscious ZDF rats in the fasted state. Liver glycogen cycling was also substantial, as confirmed by low a H5/H2 ratio in Fig. 5.

Fig. 2. 2H NMR spectra of MAG derived from plasma glucose. Results from a control rat (A), a T3-treated rat (B), and a ZDF rat (C) are shown. The relatively low enrichment in H5 of glucose from a ZDF rat in C indicates that a significant fraction of plasma glucose was derived from glycogenolysis. ppm, Parts per million.
This study demonstrates that patterns of glucose production may be distinguished by a simple NMR analysis of tracers in plasma glucose. The general features of abnormal glucose metabolism established in prior invasive studies were confirmed using only plasma samples, including the preserved capacity for glycogen release in ZDF animals and increased flux in the pyruvate cycling in T3-treated animals. To our knowledge, this is the first report of abnormal pyruvate cycling in the liver using only plasma samples, and it is the first demonstration that preserved glycogen stores in ZDF animals are associated with excess glucose production from glycogen. The study also illustrates the advantages of judicious selection of tracers. The distinct advantage of \([3,4-13C_2]\)glucose as a tracer of glucose turnover is that it does not interfere with the simultaneous use of \([U-13C_3]\)propionate as a tracer of TCA cycle activity to measure pyruvate cycling or the use of \(2H_2O\) as a tracer to measure the relative contributions of glycerol, glycogen, and the TCA cycle to glucose production by \(^2H\)NMR. This approach may be easily extended to humans, and the comparable spectral quality obtained on samples of glucose collected from rodents vs. humans further supports the potential for the integration of stable isotopes and NMR analysis into routine clinical exams.

**Role of glycogen in excess glucose turnover in ZDF rats.** The role of hepatic glucose production in the hyperglycemia of T2DM is controversial. Recent studies suggested that excess hepatic gluconeogenesis plays a causative role in hyperglycemia (10, 18 –19). Although the current study involves only an animal model of T2DM, it illustrates the value of simultaneous measurement of all sources of glucose. In fed ZDF rats, glycerol was reported to be a major substrate for hepatic glucose production (29). Although statistically significant in-
In these studies, the animal was injected with $^{2}$H$_2$O and $[^{13}$C$_3$]propionate. The natural-abundance $^2$H signal in the methyl groups of MAG (see Fig. 1 for structure). After 90 min, the animal was anesthetized and killed to obtain hepatic glycogen. Seven hydrogens from MAG (derived from glucose hydrolyzed from glycogen) and hydrogens of methyl groups (natural-abundance $^2$H) of MAG are shown. The $^2$H enrichment in position 2 was calculated from the natural-abundance $^2$H signal in the methyl groups of MAG (see Fig. 1 for structure).

Fig. 5. $^2$H NMR spectrum of MAG derived from liver glycogen of a ZDF rat. In these studies, the animal was injected with $^{2}$H$_2$O and $[^{13}$C$_3$]propionate. After 90 min, the animal was anesthetized and killed to obtain hepatic glycogen. Seven hydrogens from MAG (derived from glucose hydrolyzed from glycogen) and hydrogens of methyl groups (natural-abundance $^2$H) of MAG are shown. The $^2$H enrichment in position 2 was calculated from the natural-abundance $^2$H signal in the methyl groups of MAG (see Fig. 1 for structure).

Increases in gluconeogenesis from glycerol were confirmed in ZDF animals in this study, these changes were modest in the context of total glucose production, and the overall impact of this change was small (Fig. 4).

The main observations were that the glycogen content was higher in ZDF animals after a 24-h fast compared with controls, which correlated with the noninvasive $^2$H NMR analysis, and that increased glycogenolysis rather than excess gluconeogenesis was the source of increased glucose production under these conditions. Earlier reports demonstrated that the glycogen content is higher in obese ZDF animals and, paradoxically, that glycogen stores are higher after a 6-day fast compared with a 2- to 4-day fast (30). Earlier reports also indicated that hepatocytes from obese ZDF rats had a higher rate of glycogen synthesis compared with hepatocytes from lean ZDF rats (20). However, others have reported impaired glycogen synthesis in ZDF animals (1, 31). The mechanism of preserved glycogen stores in these animals could be the result of reduced glycogenolysis, increased glycogen synthesis, or a combination of both. The observation that the $^2$H of glycogen glucosyl units is enriched with $^2$H suggests continued glycogen synthesis in fasted animals, yet a role for reduced glycogenolysis cannot be excluded. Because the hyperglycemia of ZDF rats may be an important inhibitor of glycogenolysis (24), the mechanism of preserved glycogen stores in these animals is unclear. However, there is no doubt (see the $^2$H NMR spectra in Fig. 2) that the preserved glycogen stores can be mobilized, at least during the conditions used in the present experiments.

The activities of two key enzymes that may be involved with pyruvate cycling, PK and ME, were increased significantly in ZDF animals (Table 1), as was the concentration of malate, the substrate for ME. Together these measurements suggest a role for reduced glycogenolysis as the result of pyruvate cycling, increased oxygen consumption, and protein and glycogen content and longer and more numerous cristae (12, 27, 32). These prior observations are entirely consistent with an enhanced TCA cycle flux as found here for T$_3$-treated rats. One might anticipate that such a dramatic increase in TCA cycle flux as found here for T$_3$-treated rats might lead to proportional excess glucose production in these animals. However, pyruvate cycling flux was also dramatically higher in T$_3$-treated animals, and this levels much of the excess catecholergic flux from the cycle ($v_0$) by redirecting a larger percentage of those carbons back into the TCA cycle. The net effect is that gluconeogenesis from PEP is only 120% higher in T$_3$-treated rats compared with ZDF or control animals, even though TCA cycle flux ($v$) and total catecholergic outflow ($v_0$) are 380 and 250% higher, respectively, in T$_3$-treated rats compared with ZDF or control animals. These results suggest that pyruvate cycling may play a functional role in protecting against overproduction of glucose production in liver by modulating gluconeogenesis from the TCA cycle during alterations in mitochondrial activity.

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1. tcSIM is a shareware software package (http://www4.utsouthwestern.edu/rogersmrn) that accepts operator-determined relative fluxes in the TCA cycle and gluconeogenesis as well as $^{13}$C enrichment patterns in the input molecules. For example, the model accepts flux through PK, flux through pyruvate carboxylase (YPC), and flux through combined anaplerotic reactions feeding succinyl-CoA (YS), all relative to TCA cycle flux. The package calculates all $^{13}$C isomers of all relevant intermediates. To evaluate the model by Petersen and colleagues (22, 23), a 3-carbon gluconeogenic precursor pool (alanine + lactate) was assumed enriched in the 3 position. Unlabeled carbon skeletons were allowed to enter via the succinate pool (YS > 0). Both PK flux and YPC flux were allowed to vary. The fraction of alanine and glucose molecules enriched in carbon 2 was calculated at steady state. Petersen and colleagues (22, 23) reported that pyruvate cycling relative to YPC flux was equal to $((^{2}$H$_2$-alanine)/(alanine)) + $((^{1}$C$_3$-glucose)/(glucose)). In tcSIM, the flux ratio is PK/YPC. The ratio of $^{13}$C fractional enrichment in position 2 of alanine relative to $^{13}$C fractional enrichment in glucose is ala2$^{13}$C/gra2$^{13}$C. Across a wide range of YS, PK, and YPC, tcSIM found that ala2$^{13}$C/gra2$^{13}$C was equal to PK/YPC as predicted by Petersen and colleagues (22, 23).
This study compared metabolic fluxes in controls, T3-treated rats, and ZDF rats after a 24-h fast and during ketamine-xylazine anesthesia. Both T3-treated rats and ZDF rats showed enhanced gluconeogenesis from glycerol compared with control animals. The distinguishing feature of T3-treated rats was enhanced gluconeogenesis from the TCA cycle and excess pyruvate cycling compared with both ZDF rats and controls. For ZDF rats, the characteristic metabolic pattern was excess glycolysis, possibly stimulated by surgical stress or anesthesia (9). The important observation was that the distribution of 2H in plasma glucose correctly reported the preserved glycogen stores. On the basis of the 2H labeling in glucosyl units of glycogen (Fig. 5), it appears that glycogen synthesis continues in livers of ZDF rats even during fasting.

It is also interesting that, despite increased ME and PK activities and higher steady-state levels of malate in ZDF rats, the measured pyruvate cycling flux (v3) did not differ from that in control animals. However, this observation should be interpreted with caution, because it is based on fluxes normalized to body mass. ZDF rats would have higher fluxes than the values in Table 2 if the values were reported per rat. For example, the pyruvate cycling would be ~36 μmol·rat⁻¹·min⁻¹ (154 μmol·kg⁻¹·min⁻¹ × 0.236 kg) for controls and ~77 μmol·rat⁻¹·min⁻¹ (159.9 μmol·kg⁻¹·min⁻¹ × 0.482 kg) for ZDF rats without normalization to body mass. With that perspective, the measured activities of ME and PK are fully consistent with measured pyruvate cycling fluxes in controls and ZDF rats. If we expand the unit into gluconeogenesis, again ZDF rats would have higher gluconeogenesis than controls. Conversely, cytosolic malate levels were no different in controls vs. T3-treated animals, and ME and PK activities differed only slightly, yet pyruvate cycling flux was approximately fourfold higher in T3-treated animals compared with controls. These observations underscore the importance of combining flux measurements with assays of enzyme activity or metabolite levels for highlighting metabolic phenotype differences.

Previously, we reported that a primed 90-min infusion was sufficiently long to accurately measure glucose turnover in rats by comparing the 90-min results with those after a primed 150-min infusion (14). Ideally, steady state should be confirmed in turnover measurements by serial blood samples in each rat over the infusion time course, but this was not practical in the current study due to the inherently low sensitivity of NMR, especially 2H NMR. Previous studies from other labs have also indicated that steady state was achieved after primed 60-min infusion in rats (28). An earlier report found that steady state in plasma glucose specific radioactivity during infusion of [3H]glucose was achieved very quickly and was maintained throughout a 15- to 120-min infusion period in ZDF rats (29). On the basis of these previous observations, a 90-min infusion period was considered sufficient to reach steady state in the present experiments for both control and ZDF rats.

The constant ratios of D12/D23 and Q/D23 of carbon 2 resonances from rats at different time points (Fig. 3) also indicate that isotopic steady-state enrichment of plasma glucose from [U-13C3]propionate was achieved at 90 min. Because [U-13C3]propionate was administered as a bolus, the absolute 13C enrichment was not constant throughout the study period, it is important to underscore the fact that analysis of glucose carbon 2 (or MAG carbon 2) depends only on the multiplet patterns detected in the 13C NMR spectrum, not on the absolute 13C enrichment.

Glucose production was assumed to originate from the liver in these experiments, even though one cannot exclude production from other organs such as kidney. The role of the kidney in glucose production during disease states is essentially unknown. Approximately 20% of glucose production is considered to come from kidney in the postabsorptive state of normal humans (6, 8). If gluconeogenesis from the kidney (or small intestine) is significant in the current studies, the estimates of systemic production of glucose from glycerol, gluconeogenesis from glycerol, and gluconeogenesis from the TCA cycle would remain unaltered. However, gluconeogenesis reported here as occurring in the liver would be decreased in proportion to the flux through gluconeogenesis occurring elsewhere. The model used to interpret the spectra, of course, is independent of the observation that 2H and 13C distribution in plasma glucose was altered in these models of disease.

In summary, two animal models of excess systemic glucose turnover were chosen based on earlier reports that suggested glucose production may be increased via different mechanisms. The metabolic patterns observed in these animals using only stable-tracer distribution in plasma glucose corresponded well with those reported by more invasive methods. Obvious clinical applications are suggested by these experiments. For example, T2DM is a heterogeneous disorder where the contributions of excess gluconeogenesis from the TCA cycle and gluconeogenesis from glycerol vs. glycogenolysis to glucose homeostasis remain controversial, and the impact of drug therapy is poorly understood. In other disorders, for example fatty liver, measurement of hepatic TCA cycle flux is critical to understanding the balance between fat storage and fatty acid oxidation. This study increases confidence in this analysis using only plasma samples of glucose.

ACKNOWLEDGMENTS

We thank Charles Storey and Angela Milke for outstanding technical support.

GRANTS

This study was supported in part by the National Science Foundation and National Institutes of Health Grants RR-02584 and HL-34557.

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