Original Article

Role of Excess Glycogenolysis in Fasting Hyperglycemia Among Pre-Diabetic and Diabetic Zucker (*fa/fa*) Rats

Eunsook S. Jin,[1] Byung-Hyun Park,[2] A. Dean Sherry,[1,3] and Craig R. Malloy[1,4]

Sources of plasma glucose and glucose turnover were investigated in 8-week-old (pre-diabetic) and 13-week-old (diabetic) Zucker (*fa/fa*) rats after a 24-h fast. Intraperitoneal ^2H_2O was administered and [3,4-$^{13}C_2$]glucose and [U-$^{13}C_3$]propionate were infused into conscious active rats. ^{13}C nuclear magnetic resonance analysis of monoacetone glucose derived from blood glucose indicated that glucose production was increased significantly in 8- and 13-week-old *fa/fa* rats compared with age-matched Zucker (+/+) rats, and hepatic glycogen was dramatically higher among *fa/fa* animals regardless of age. Glycogenolysis, essentially 0 in +/+ rats after a 24-h fast, was significant in *fa/fa* rats (11 ± 6 and 17 ± 7% of glucose production in 8- and 13-week-old rats, respectively), even after a 24-h fast. Tricarboxylic acid (TCA) cycle flux and efflux of carbon skeletons from the cycle (cataplerosis) were both significantly higher in *fa/fa* rats compared with controls, but net gluconeogenesis from the TCA cycle was not higher because products leaving the cycle were returned to the cycle via a pyruvate cycling pathway. Thus, pyruvate cycling flux increased in proportion to TCA cycle flux, leaving net gluconeogenesis unchanged in *fa/fa* animals compared with control animals. The distribution of ^2H in skeletal muscle glycogen suggested that at least a fraction of glucose molecules entering glycogen pass through phosphomannose isomerase. *Diabetes* 56:777–785, 2007

The obese Zucker rat (*fa/fa*) is thought to model many aspects of human type 2 diabetes. At an early age, hyperphagia and obesity are associated with insulin resistance, and, with time, the animals become overtly diabetic (1–3). Because there is ample evidence of excess hepatic glucose production and abnormal hepatic glycogen metabolism among humans with type 2 diabetes (4–6), glucose production and storage in these animals have been studied extensively. Perhaps the most dramatic findings relate to abnormal glycogen synthesis and storage. The rate of glycogen synthesis was higher in hepatocytes from fasted obese *fa/fa* rats compared with hepatocytes from lean Zucker rats (7). Among *fa/fa* rats after a 6-day fast, hepatic glycogen content was paradoxically higher compared with animals after a 2- to 4-day fast (8). Excess liver glycogen could contribute to fasting hyperglycemia because endogenous glucose production is sensitive to the amount of hepatic glycogen available for hydrolysis (9). Both glycogenolytic flux and hepatic glycogen content have been measured in rats under general anesthesia. In that study, increased hepatic glycogen in *fa/fa* rats correlated with excess hepatic glycogenolysis after a 24-h fast (10). Together, these observations suggest that in this animal model after a 24-h fast, hepatic glucose output may be higher because excess glycogen stores permit continued glycogenolysis. In parallel, gluconeogenesis persists at inappropriately high levels, despite hyperinsulinemia and hyperglycemia.

This picture, however, depends on measurements in isolated hepatocytes or in animals under general anesthesia and may not be directly relevant to metabolism in the conscious awake animal. The objective of this study was to measure systemic glucose turnover in fasted conscious *fa/fa* rats to determine the rate of hepatic glycogenolysis and to determine whether hepatic glycogen stores are correspondingly increased. It is generally believed that conversion to overt type 2 diabetes is preceded by resistance of skeletal muscle to insulin-stimulated glucose transport and storage in skeletal glycogen (11). For this reason the pathways of glucose production and glycogen storage in both liver and skeletal muscle were evaluated in pre-diabetic and overtly diabetic *fa/fa* rats. Metabolic fluxes in conscious active animals were estimated using three stable isotope tracers: [3,4-$^{13}C_2$]glucose for glucose turnover measurement (12), ^2H_2O for the measurement of the relative contribution of glycogenolysis and gluconeogenesis to endogenous glucose production (13), and [U-$^{13}C_3$]propionate for measurement of fluxes related to the tricarboxylic acid (TCA) cycle (14). To test whether hepatic glycogenesis was active even in fasted animals, ^2H nuclear magnetic resonance (NMR) analysis was performed on glucosyl units of hepatic glycogen; a similar analysis was performed in skeletal muscle of the same animals. In both 8-week-old (pre-diabetic) and 13-week-old (diabetic) age-groups of *fa/fa* animals, hepatic glycogen content was dramatically increased, and glucose production was increased because of excess hepatic glycogenolysis. Although the contribution of carbon skeletons to glucose from the TCA cycle was not increased in *fa/fa* animals, pyruvate cycling and TCA cycle flux were

From the [1]Advanced Imaging Research Center, University of Texas Southwestern Medical Center, Dallas, Texas; the [2]Department of Biochemistry, Chonbuk National University Medical School, Chonju, Chonbuk, South Korea; the [3]Department of Chemistry, University of Texas at Dallas, Richardson, Texas; and the [4]Veterans Affairs North Texas Health Care System, Dallas, Texas.

Address correspondence and reprint requests to Eunsook S. Jin, PhD, Advanced Imaging Research Center, University of Texas Southwestern Medical Center, 5323 Harry Hines Blvd., Dallas, TX 75390-8568. E-mail: eunsook.jin@utsouthwestern.edu.

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FFA, free fatty acid; MAG, monoacetone glucose; NMR, nuclear magnetic resonance; PEP, phosphoenol pyruvate; PEPCK, PEP carboxykinase; PMI, phosphomannose isomerase; TCA, tricarboxylic acid.

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increased, indicating a generalized disruption of hepatic energy metabolism, even in the younger animals.

RESEARCH DESIGN AND METHODS

Male obese Zucker (*fa/fa*) and lean Zucker (+/+) rats were a generous gift from Dr. Roger H. Unger at the Veterans Affairs North Texas Health Care System (Dallas, Texas). [3,4-$^{13}C_2$]glucose (99%) was purchased from Omicron Biochemicals (South Bend, IN). [U-$^{13}C_3$]propionate (99%), ^2H_2O (99.9%), and deuterated acetonitrile (99.8%) were obtained from Cambridge Isotopes (Andover, MA). Deuterated N,N-dimethylformamide-d_7 (99.5%) and other common chemicals were purchased from Aldrich (Milwaukee, WI).

Protocol. This study was approved by the institutional animal care and use committee at the University of Texas Southwestern Medical Center. Four different groups of male Zucker rats were studied: 8- and 13-week-old *fa/fa* rats and age-matched +/+ rats. Immediately before surgery to place a chronic indwelling venous catheter, a urine sample was collected from 12-week-old *fa/fa* rats; development of glucosuria was checked by urine glucose level (DiastixR; Bayer, Munich, Germany). After the right jugular vein was cannulated under ketamine-xylazine anesthesia, animals were allowed to recover for 7 days with free access to water and standard chow containing 6% fat. Then, 1 day before the study, rats were fasted for 24 h with free access to water. At $t = -30$ min, rats received an intraperitoneal injection of ^2H_2O (20 μl/g rat), and at $t = 0$ they received a bolus infusion of [3,4-$^{13}C_2$]glucose (35 μmol/kg) followed by continuous infusion of [3,4-$^{13}C_2$]glucose and [U-$^{13}C_3$]propionate (1 and 13 $\mu mol \cdot kg^{-1} \cdot min^{-1}$, respectively) via the jugular vein catheter for 90 min. At the end of the 90-min infusion period, whole blood (~8–10 ml, the entire available blood volume) was drawn from the inferior vena cava under anesthesia with pentobarbital sodium (50 mg/kg body wt), and the liver and skeletal muscle tissue from the hind limbs were freeze-clamped and kept at −80°C for subsequent processing.

Sample processing for NMR analysis. Whole blood was immediately deproteinized by adding cold perchloric acid to a final concentration of 7% by volume. After neutralization with KOH and centrifugation, the supernatant was lyophilized. Dried glucose was converted into monoacetone glucose (MAG) (Fig. 1) as described previously (10,15). A 5- to 8-g portion of liver and skeletal muscle tissue was used for glycogen extraction and purification (16). Isolated glycogen was dissolved in ~5 ml of 10 mmol/l sodium acetate solution (pH 4.8) and incubated with amyloglucosidase (50 mg glycogen per 20 units) for 4 h at 50°C. After freeze-drying, the hydrolyzed glycogen was converted to MAG.

NMR spectroscopy and metabolic fluxes. All NMR spectra were collected using a Varian INOVA 14.1 T spectrometer (Varian Instruments, Palo Alto, CA) equipped with a 3-mm broadband probe as described previously (10). NMR spectra were analyzed using the curve-fitting routine of NUTS, a PC-based NMR spectral analysis program (Acorn, Fremont, CA). Glucose turnover was estimated from the dilution of infused [3,4-$^{13}C_2$]glucose using ^{13}C NMR to analyze MAG derived from blood glucose at the end of the infusion protocol (12). The contributions of glycogen, glycerol, and phosphoenol pyruvate (PEP) to blood glucose was obtained directly from the deuterium enrichment at positions 2, 5, and 6_S as determined from the ^2H NMR of MAG (13,17). A ^{13}C NMR isotopomer analysis—based on the ^{13}C-^{13}C spin-coupled multiplets of carbon 2 (C2) of MAG—that yields relative fluxes in the citric acid cycle has been reported previously (10,14).

Excess ^2H enrichment measurement in glucose. ^2H enrichment of the H2 position of glucose was measured using N,N-dimethylformamide-d_7 as an internal reference, as described previously (18). Also the natural abundance of methyl groups of MAG (Fig. 1) was used as internal references for the estimation of ^2H excess enrichment for low–enriched glycogen samples.

Metabolite assays. Plasma glucose was assayed by the glucose oxidase method (Sigma). Plasma free fatty acids (FFAs) were measured enzymatically using a commercially available kit (Wako Chemical, Richmond, VA). Plasma insulin was determined using a sandwich enzyme-linked immunosorbent assay kit (Alpco Diagnostics, Windham, NH). Freeze-clamped liver and muscle tissues were pulverized under liquid nitrogen, and a small portion (~0.5 g) of each tissue was used for glycogen assay (19).

Statistical analysis. The data are the means ± SE. Comparisons between groups were performed using one-way ANOVA. Differences in mean values were considered statistically significant at $P < 0.05$.

RESULTS

General metabolic features. Both 8- and 13-week-old *fa/fa* rats were heavier and had higher liver-to-body ratios than their age-matched controls (Table 1). Both age-groups of *fa/fa* rats were hyperglycemic after fasting and had higher levels of plasma FFAs and plasma insulin than their age-matched controls. Insulin levels in 13-week-old *fa/fa* rats tended to be lower than that of 8-week-old *fa/fa* rats, although this difference did not reach statistical significance. Liver glycogen was essentially depleted in controls, but it remained high in *fa/fa* rats, particularly the 13-week-old animals (24.0 ± 8.6 and 86.9 ± 15.4 μmol glucosyl unit/g wet weight in 8- and 13-week-old animals, respectively). Animals in all groups had similar low levels of skeletal muscle glycogen (~2 μmol glucosyl unit/g wet weight).

Glucose production by dilution of [3,4-$^{13}C_2$]glucose. Typical ^{13}C NMR spectra of MAG derived from blood glucose of an *fa/fa* rat and a +/+ rat are shown in Fig. 1. All six carbons originating from glucose are easily resolved, and consequently the tracer used for glucose turnover, [3,4-$^{13}C_2$]glucose, is detected at 75.3 ppm (signal from C3) and 80.5 ppm (signal from C4). The signal from each carbon is further split by spin-spin coupling, so the NMR signature of [3,4-$^{13}C_2$]glucose is unique. At a fixed rate of infusion of [3,4-$^{13}C_2$]glucose, the area of the characteristic doublets will decrease proportionally to the rate of hepatic glucose production (Fig. 1*C*). At steady state, [3,4-$^{13}C_2$]glucose was 1.79 ± 0.16% (8 weeks of age +/+), 1.52 ± 0.21% (8 weeks of age *fa/fa*), 2.00 ± 0.44% (13 weeks of age +/+), and 1.72 ± 0.17% (13 weeks of age *fa/fa*) of total plasma glucose. Glucose production ($v1$) (Table 2) was significantly higher (~18–27%) in *fa/fa* rats of both age-groups compared with their age-matched controls, despite higher levels of plasma insulin.

Sources of blood glucose by ^2H NMR analysis. The ratio of $H6_S$ to H2 in the ^2H NMR spectrum is a direct measure of the fraction of blood glucose produced by gluconeogenesis from the TCA cycle (17). By simple inspection of the ^2H NMR spectra in Fig. 2, it is easily seen that a significant fraction of blood glucose is derived from PEP in all groups because the $H6_S$ resonance is high compared with H2 resonance in all spectra. This is to be expected because the animals were fasting. Total gluconeogenesis from both glycerol and the TCA cycle is measured by H5/H2 (13). By inspection of Fig. 2, it is easily seen that the H5-to-H2 ratio is somewhat less than 1 in the 8-week-old *fa/fa* animals (Fig. 2*B*) and significantly less than 1 in the 13-week-old *fa/fa* animals (Fig. 2*D*). Because glycogenolysis is the difference between gluconeogenesis and glucose production, these spectra demonstrate higher glycogenolysis in both *fa/fa* groups. The higher hepatic glycogen content in *fa/fa* animals (Table 1) correlates nicely with a higher contribution from glycogenolysis to glucose production in these animals, as determined by ^2H NMR analysis (Table 2). The difference between H5 and $H6_S$ was modest in all four groups, indicating that the fractional contribution of glycerol to endogenous glucose production ranged from 16 to 19%.

The absolute rates of glucose production from glycogen, PEP, and glycerol were determined by multiplying the individual fractional contributions and the glucose production rates. These values are summarized in Table 2. Glycogenolysis was 6.3 ± 3.6 and 9.4 ± 4.0 $\mu mol \cdot kg^{-1} \cdot min^{-1}$ in 8- and 13-week-old *fa/fa* rats, respectively, but it was negligible in both +/+ rats. Endogenous glucose production from either glycerol or PEP was not significantly higher in *fa/fa* rats compared with age-matched controls.

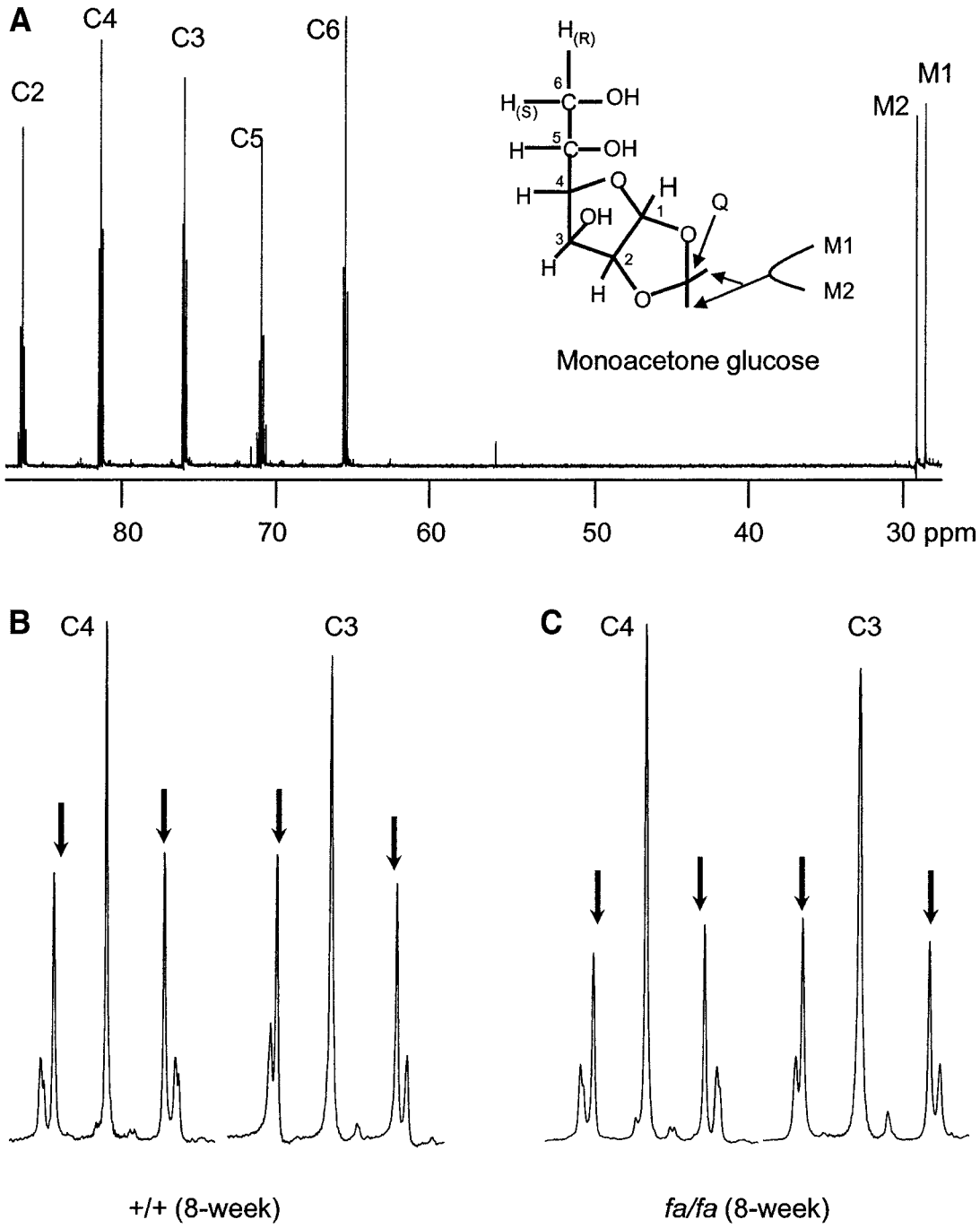

FIG. 1. ^{13}C NMR spectrum of MAG from blood glucose. *A*: The chemical structure and expanded spectrum of MAG from an 8-week-old +/+ rat. *B* and *C*: The spectrum around carbons 3 and 4 are further expanded from an 8-week-old +/+ rat and an 8-week-old *fa/fa* rat. Peak assignments for carbons 2–6 are shown plus the two ^{13}C natural abundance methyl resonances (M1 and M2). Arrows indicate doublets of carbon 3 and carbon 4 attributable to J_{34} of [3,4-$^{13}C_2$]glucose. *C*: The increase in the area of the singlet (due to endogenous glucose production) relative to the area of the doublet (due to tracer infusion) demonstrates higher rates of glucose production.

TCA cycle and related fluxes by ^{13}C NMR analysis of blood glucose. Entry of [U-$^{13}C_3$]propionate into the TCA cycle and subsequent turnover produces a mixture of ^{13}C isotopomers in all intermediates and eventually in glucose derived from the TCA cycle. Examples of the C2 multiplet pattern of the ^{13}C NMR spectrum of MAG from each group are shown in Fig. 3. In each case, these spectra are expansions of the C2 region shown in Fig. 1. Analysis of the C2 multiplets provides an estimate of flux through PEP carboxykinase (PEPCK), pyruvate cycling, and gluconeogenesis, all relative to citrate synthase flux. Because gluconeogenesis is commonly thought to be increased in type 2 diabetes, the finding that gluconeogenesis from the TCA cycle, relative to flux through citrate synthase, was ~30% lower among *fa/fa* rats compared with age-matched controls was unanticipated (Table 2).

The overall pattern of glucose production becomes much clearer once the individual measures of glucose production, fractional sources of glucose production by ^2H NMR, and TCA cycle fluxes by ^{13}C NMR are combined to measure fluxes through multiple pathways (Table 2 and Fig. 4). An interesting picture emerges. TCA cycle flux is

TABLE 1
General features, biochemical analysis, and NMR analysis of plasma, liver, and skeletal muscle tissue of 8-week-old +/+, 8-week-old *fa/fa*, 13-week-old +/+, and 13-week-old *fa/fa* rats

	+/+, 8 weeks	*fa/fa*, 8 weeks	+/+, 13 weeks	*fa/fa*, 13 weeks
n	6	6	7	6
Body weight (g)	205 ± 14	228 ± 21*	308 ± 22	398 ± 23†
Liver/body (mg/g)	19.6 ± 5.1	34.4 ± 2.7†	23.3 ± 1.8	40.0 ± 3.5†
Plasma glucose (mmol/l)	7.5 ± 1.1	9.8 ± 1.6*	6.4 ± 0.8	13.3 ± 1.2†
Plasma FFAs (μmol/l)	303 ± 68	691 ± 195†	282 ± 68	766 ± 351*
Plasma insulin (pmol/l)	90 ± 21	1178 ± 692*	88 ± 20	746 ± 530*
Liver glycogen (μmol glucosyl units/g wet wt)	1.7 ± 1.4	24.0 ± 8.6†	1.8 ± 0.7	86.9 ± 15.4†
Muscle glycogen (μmol glucosyl units/g wet wt)	2.1 ± 0.8	2.4 ± 1.0	1.8 ± 0.4	2.0 ± 0.7
^{2}H enrichment of H2 of blood glucose (%)	2.3 ± 0.2	3.3 ± 0.4†	2.7 ± 0.3	3.7 ± 0.7*
^{2}H enrichment of H2 of glucosyl unit of liver glycogen (%)‡	1.37 ± 0.93	0.15 ± 0.12*	1.26	0.18 ± 0.12†
^{2}H enrichment of H2 of glucosyl unit of skeletal muscle glycogen (%)§	0.21 ± 0.02	0.22 ± 0.17	0.15 ± 0.02	0.19 ± 0.08

Data are means ± SE. *Significantly different from age-matched +/+ rats ($P < 0.05$); †significantly different from age-matched +/+ rats ($P < 0.001$); ‡$n = 5$ for 8-week-old +/+ rats and $n = 1$ for 13-week-old +/+ rats because of negligible storage of liver glycogen in controls; §$n = 2$ for each +/+ rat group and $n = 4$ for each *fa/fa* rat group.

higher by ~40% in *fa/fa* animals in both age-groups compared with controls, and the flux through PEPCK ($v6$) was also higher by ~24–50%. However, despite these differences, gluconeogenesis through PEP ($v4$) was not higher in *fa/fa* animals compared with age-matched controls because of pyruvate cycling ($v5$) in *fa/fa* rats. Thus, the hepatic TCA cycle is significantly more active in *fa/fa* rats, but this does not translate into greater glucose production from the level of the TCA cycle because three carbon units leaving the cycle in the form of PEP are shunted back into cycle intermediates by pyruvate cycling mechanisms. This suggests that pyruvate cycling acts as a rheostat in modulating gluconeogenesis from the level of the TCA cycle, perhaps whenever glucose is available via glycogenolysis.

^{2}H NMR analysis of liver glycogen. In Fig. 5, ^{2}H NMR spectra were scaled so that the natural abundances of methyl signals are approximately equal. Therefore, each spectrum in Fig. 5 represents an equal number of glucose molecules. With this in mind, it is easy to appreciate that the fraction of hepatic glycogen enriched in ^{2}H is dramatically lower in *fa/fa* animals compared with controls, regardless of age (Fig. 5). Among 8-week-old animals, the ^{2}H enrichment in position 2 was nearly 10-fold lower in *fa/fa* animals (Fig. 5*B*). A similar pattern was evident in the 13-week-old animals. This marked difference between *fa/fa* and control animals is attributable largely to high glycogen content in *fa/fa* animals. For example, the mass of hepatic glycogen in 8-week-old controls was ~205 g

TABLE 2
Relative and absolute fluxes through pathways in glucose production

	+/+, 8 weeks	*fa/fa*, 8 weeks	+/+, 13 weeks	*fa/fa*, 13 weeks
Glucose production ($v1$) (μmol · kg^{-1} · min^{-1})	53.4 ± 5.3	63.3 ± 7.9*	44.6 ± 4.2	56.1 ± 6.2*
Fractional sources of blood glucose				
Glycogen	0.01 ± 0.01	0.11 ± 0.06*	0.01 ± 0.01	0.17 ± 0.07†
Glycerol	0.19 ± 0.04	0.17 ± 0.03	0.18 ± 0.06	0.16 ± 0.04
PEP	0.80 ± 0.03	0.72 ± 0.06*	0.81 ± 0.06	0.67 ± 0.08*
Fluxes relative to citrate synthase (μmol · kg^{-1} · min^{-1})				
Gluconeogenesis/citrate synthase ($v4/v7$)	1.71 ± 0.25	1.20 ± 0.23*	1.70 ± 0.31	1.28 ± 0.11*
(Pyruvate kinase + malic enzyme)/citrate synthase ($v5/v7$)	1.90 ± 0.27	2.54 ± 0.19†	1.97 ± 0.29	1.94 ± 0.41
PEPCK/citrate synthase ($v6/v7$)	3.61 ± 0.51	3.74 ± 0.22	3.67 ± 0.58	3.23 ± 0.47
Derived fluxes (μmol · kg^{-1} · min^{-1})				
Glycogenolysis ($v2$)	0.7 ± 0.8	6.3 ± 3.6*	0.3 ± 0.5	9.4 ± 4.0†
Glycerol → glucose ($v3$)	19.8 ± 3.3	22.2 ± 3.4	14.8 ± 5.2	18.0 ± 5.0
PEP → glucose ($v4$)	86.5 ± 11.2	91.9 ± 12.6	71.4 ± 6.0	75.4 ± 10.9
PEP or malate → pyruvate ($v5$)	97.2 ± 16.9	182.1 ± 62.1*	80.3 ± 6.7	112.7 ± 27.7*
Oxaloacetate → PEP ($v6$)	183.7 ± 27.8	271.2 ± 74.0*	151.7 ± 11.2	187.0 ± 35.1*
Oxaloacetate → citrate ($v7$)	52.5 ± 15.3	74.7 ± 18.8*	41.4 ± 8.7	58.5 ± 12.0*

Data are means ± SE. Glucose production was measured by detection of [3,4-$^{13}C_2$]MAG by ^{13}C NMR. Relative metabolic fluxes were determined on ^{2}H (H2, H5, and H6$_S$) and ^{13}C NMR (C2 resonance) analysis of MAG derived from blood glucose of 8-week-old +/+ ($n = 6$), 8-week-old *fa/fa* ($n = 6$), 13-week-old +/+ ($n = 7$), and 13-week-old *fa/fa* ($n = 6$) rats. Notations for fluxes, $v1$–$v7$, are illustrated in Figure 4. *Significantly different from age-matched +/+ rats ($P < 0.05$); †significantly different from age-matched +/+ rats ($P < 0.001$).

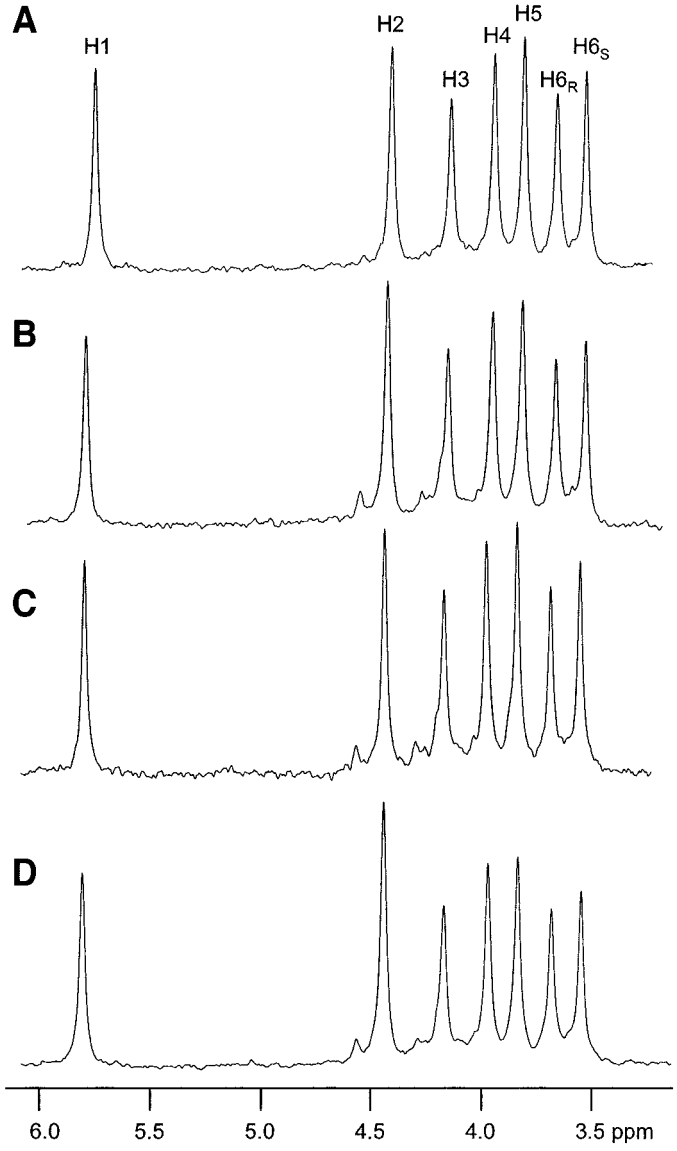

FIG. 2. ^{2}H NMR spectra of MAG derived from blood glucose. Results from an 8-week-old +/+ rat (*A*), an 8-week-old *fa/fa* rat (*B*), a 13-week-old +/+ rat (*C*), and a 13-week-old *fa/fa* rat (*D*) are shown. The relatively low ^{2}H enrichments in the H5 position of glucose from *fa/fa* rats in *B* and *D* indicate that a significant fraction of blood glucose was derived from glycogenolysis.

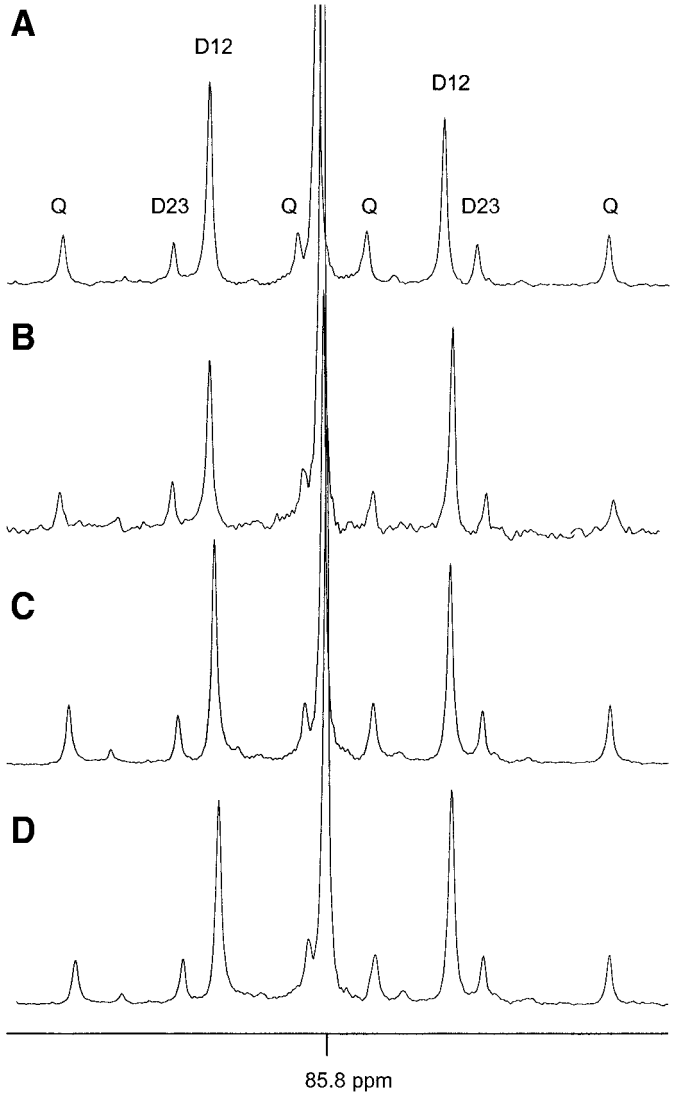

FIG. 3. C2 resonances from ^{13}C NMR spectra of MAG derived from blood glucose of an 8-week-old +/+ rat (*A*), an 8-week-old *fa/fa* rat (*B*), a 13-week-old +/+ rat (*C*), and a 13-week-old *fa/fa* rat (*D*). D12, doublet from coupling of carbon 1 with C2; D23, doublet from coupling of C2 with carbon 3; Q, doublet of doublets, or quartet, arising from coupling of C2 with both carbons 1 and 3.

body wt per rat × 19.6 mg liver/g body wt × 1.7 μmol glycogen/g liver, or 6.8 μmol hepatic glucosyl units per +/+ rat. Because the ^{2}H enrichment in H2 was 1.37% in glycogen compared with 2.3% in plasma glucose, ~60% of this hepatic glycogen mass was synthesized over the course of the experiment (2 h), or ~0.16 μmol · kg body wt^{-1} · min^{-1}. Similarly, the mass of hepatic glucosyl units was ~188 μmol per 8-week-old *fa/fa* rat, ~25 times more than the age-matched controls, whereas the rate of glycogen synthesis was ~0.31 μmol · kg body wt^{-1} · min^{-1}, roughly double the value seen in controls. In summary, higher levels of "old" glycogen are responsible for the obvious differences seen in Fig. 5 between *fa/fa* and control animals in ^{2}H spectra of hepatic glucosyl units.

^{2}H NMR analysis of glycogen from skeletal muscle. The ^{2}H enrichment patterns in MAG from hepatic glycogen were strikingly different between *fa/fa* and +/+ animals. In contrast, the ^{2}H enrichment patterns in MAG from skeletal muscle glycogen were indistinguishable between groups, and overall absolute ^{2}H excess enrichment of the H2 position of the glucosyl unit was low (~0.2%) (Table 1) in all groups. However, the ^{2}H distribution pattern was unusual compared with either blood glucose or hepatic glycogen: the highest ^{2}H enrichments were in H1 and H2, whereas the remaining H3–H6 positions were enriched at a much lower level (Fig. 6).

DISCUSSION

These observations demonstrate a profound age-dependent disruption in glycogen metabolism among *fa/fa* rats. Compared with age-matched controls, the *fa/fa* animals were heavier, had greater liver mass per gram body weight, and had far more glycogen per gram liver. After a 24-h fast, the hepatic glycogen content per *fa/fa* rat was >25 times that of control among the 8-week-old animals and >100 times that of control among the 13-week-old animals. Although the fraction of plasma glucose derived from glycogen was also increased, gluconeogenesis from

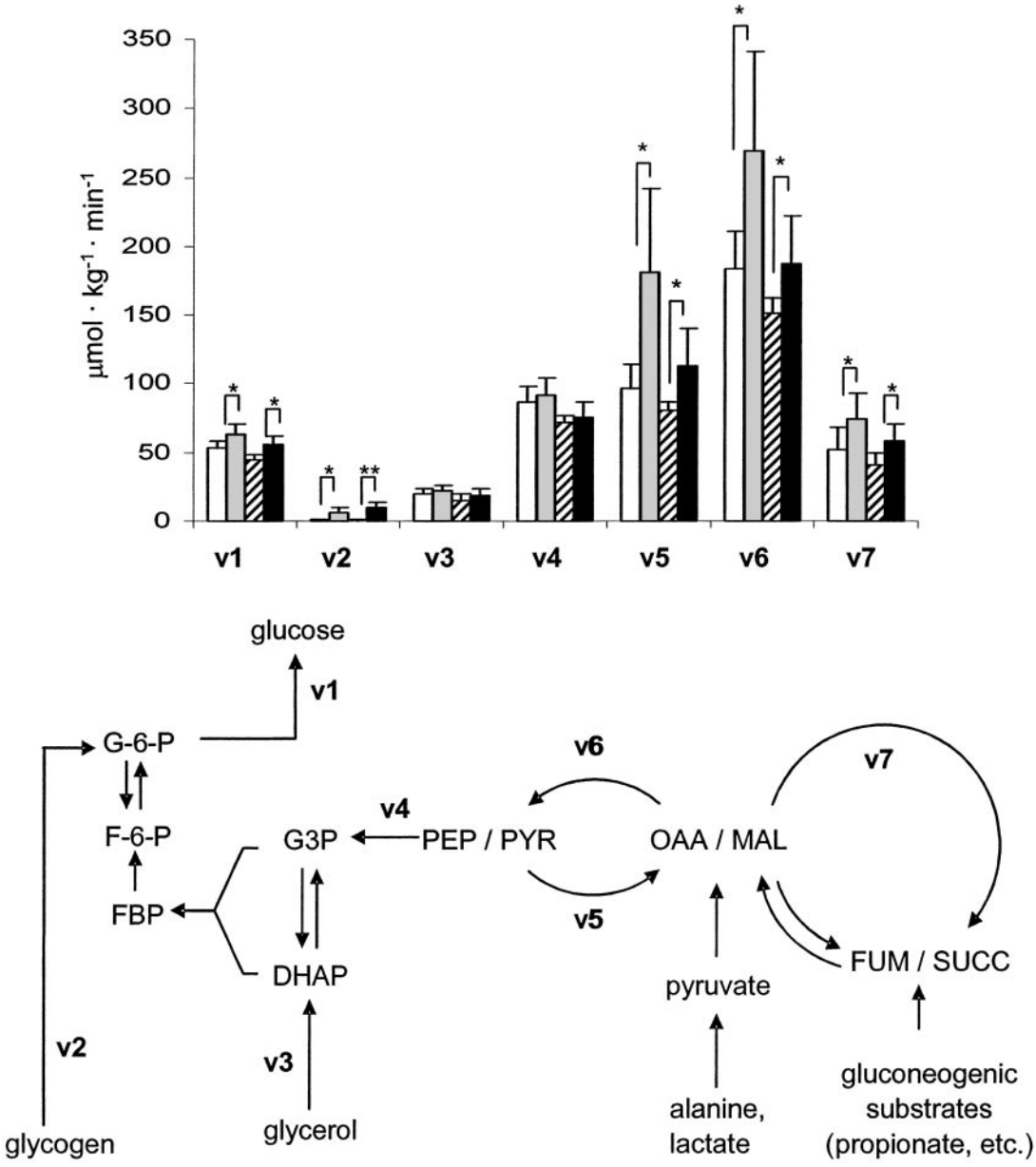

FIG. 4. Fluxes in the metabolic network supporting glucose production in 8-week-old +/+ rats (□), 8-week-old *fa/fa* rats (▩), 13-week-old +/+ rats (▨), and 13-week-old *fa/fa* rats (■). DHAP, dihydroxyacetone phosphate; F-6-P, fructose 6-phosphate; FUM, fumarate; FBP, fructose-1,6-bisphosphate; G-6-P, glucose 6-phosphate; G3P, glyceradehyde 3-phosphate; MAL, malate; OAA, oxaloacetate; PYR, pyruvate; SUCC, succinyl-CoA. *Significantly different from age-matched +/+ rats ($P < 0.05$); **significantly different from age-matched +/+ rats ($P < 0.001$).

glycerol and the citric acid cycle remained quite active, so the overall fraction of glucose derived from glycogen was <20%. In this widely used animal model of type 2 diabetes, fasting hyperglycemia is caused by a combination of continual gluconeogenesis and excess glycogenolysis.

Many features of type 2 diabetes are observed in the *fa/fa* rat, and for this reason it is a popular model for study (3). The current observations among these *fa/fa* animals, such as fasting hyperglycemia, hyperinsulinemia, elevated plasma FFAs, and elevated endogenous glucose production, are typical of human patients, particularly those with "severe" type 2 diabetes, as defined by Basu et al. (20) as fasting glucose >9 mmol/l. However, it should be emphasized that the data presented here highlight similarities but also substantial discrepancies between glucose metabolism measured in this rat model compared with measurements in patients with type 2 diabetes. For example, Clore et al. (4) measured the glycemic response to glucagon after 64 h of fasting, a time when gluconeogenesis should be maximally stimulated. With the assumption that the glycemic response represents glycogen, patients with type 2 diabetes had higher liver glycogen after a 3-day fast compared with an overnight fast, and glycogen after a 3-day fast was higher among patients with type 2 diabetes compared with nondiabetic individuals. Muller et al. (21) found that hepatic glycogen measured in liver biopsies was increased among obese human subjects. These observations are consistent with the elevated glycogenolysis and increased hepatic glycogen content reported here. In contrast, hepatic glycogen measured directly by ^{13}C NMR spectroscopy, considered highly reliable, was reduced among patients with diabetes in studies by Magnusson et al. (22), Hundal et al. (23), and Kunert et al. (24). The rate of glycogenolysis among patients with type 2 diabetes is

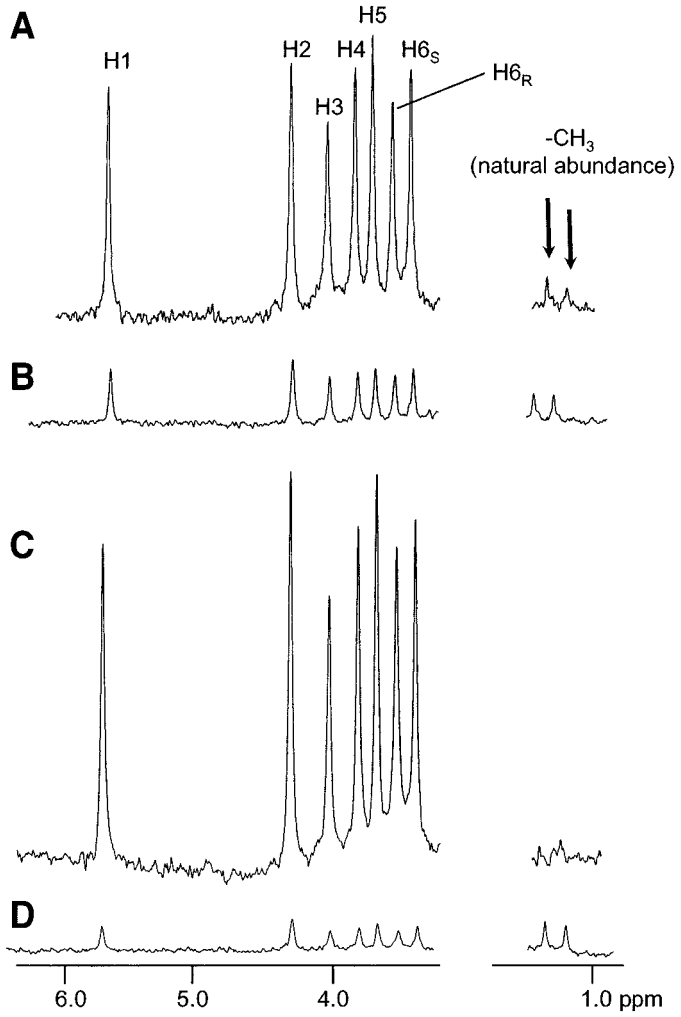

FIG. 5. ^{2}H NMR spectra of MAG derived from liver glycogen of an 8-week-old +/+ rat (*A*), an 8-week-old *fa/fa* rat (*B*), a 13-week-old +/+ rat (*C*), and a 13-week-old *fa/fa* rat (*D*). Seven hydrogens from glucose (hydrolyzed from glycogen) and hydrogens of methyl groups (natural abundance, indicated by arrows) of MAG are shown. Because each ^{2}H NMR spectrum is taken from approximately equal masses of glucose (because the methyl natural abundance signal is approximately equal), the ^{2}H enrichment in control animals (*A* and *C*) is far greater than in *fa/fa* rats (*B* and *D*).

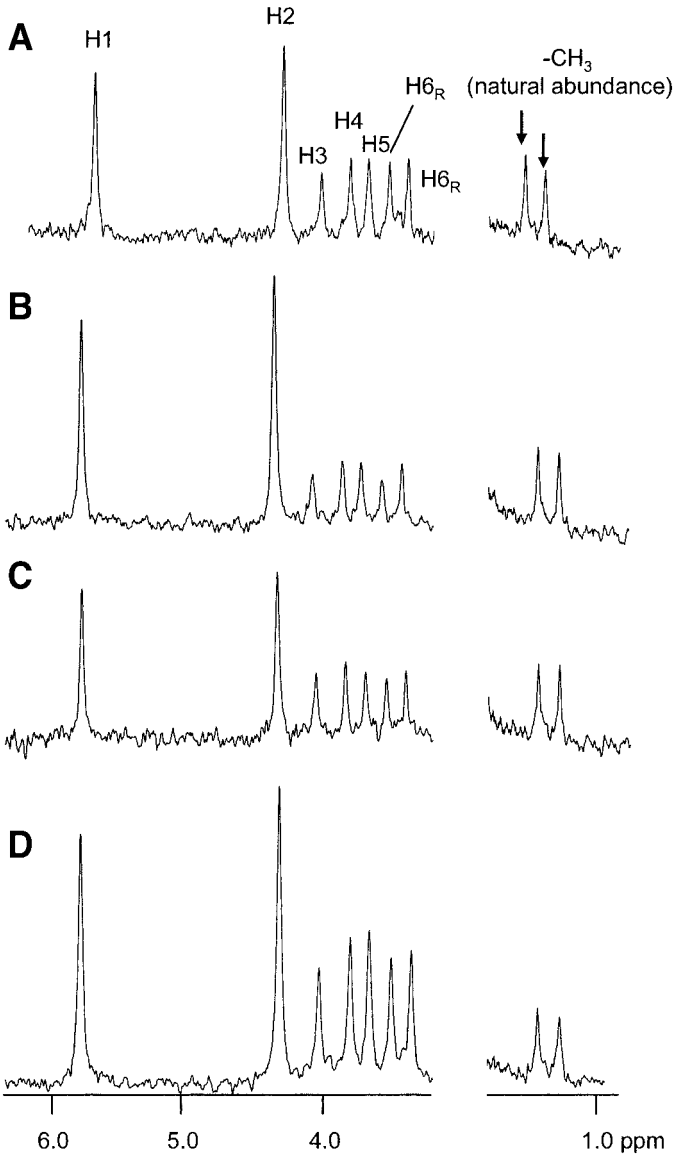

FIG. 6. ^{2}H NMR spectra of MAG derived from skeletal muscle glycogen of an 8-week-old +/+ rat (*A*), an 8-week-old *fa/fa* rat (*B*), a 13-week-old +/+ rat (*C*), and a 13-week-old *fa/fa* rat (*D*). Seven hydrogens from glucose (hydrolyzed from skeletal muscle glycogen) and hydrogens of methyl groups (natural abundance, indicated by arrows) 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).

also controversial. Reduced (22,25), unchanged (5), and increased (20) rates of glycogenolysis all have been reported. The associated metabolic features of diabetes may also play a role in glycogen metabolism because elevated fatty acids may inhibit glycogen mobilization (26). These discrepancies may be attributable to differences in methods for assessing glycogenolysis and gluconeogenesis, or to the study of patients at different stages of disease. It is conceivable that glycogen metabolism among humans with diabetes for several years may be quite different from metabolism in a rodent with insulin resistance for several weeks, at most.

Despite the relatively early reports of abnormal hepatic glycogen metabolism among subjects with diabetes, the cause of preserved glycogen stores in humans or rats during a modest fast is unclear. Clore et al. (4) proposed that increased gluconeogenesis could be responsible for glycogen sparing, but this possibility was discounted because suppressed gluconeogenesis did not deplete liver glycogen in type 2 diabetic patients after a 3-day fast (27). Previous studies of hepatocytes from *fa/fa* rats suggested increased glycogen synthesis (7,28). In the current study, the ^{2}H enrichment patterns of the glucosyl unit of glycogen provided valuable information about the sources of hepatic glycogen in two respects. First, ^{2}H enrichment in the H2 position was ~20-fold less than that of blood glucose in *fa/fa* rats (Table 1). This level of enrichment of liver glycogen in *fa/fa* animals is far too low to attribute preservation of liver glycogen to increased glycogen synthesis (glyconeogenesis) during the period of this study. Second, the ^{2}H NMR spectrum also demonstrated active glycogen synthesis through gluconeogenic pathways in fasted *fa/fa* rats and even among controls, though at a lower rate (Fig. 5). The latter finding is somewhat unexpected because glycogen synthesis is not thought to be active in fasted normal animals. The ^{2}H NMR data indicate that both glyconeogenesis and reduced glycogen turnover

relative to the mass of glycogen contribute to the preserved glycogen stores in *fa/fa* animals.

Citrate synthase flux ($v7$) and efflux ($v6$) of carbon skeletons from the TCA cycle of *fa/fa* rats were higher than controls. Nevertheless, glucose production from the TCA cycle was not increased because of increased reentry into the TCA cycle. Efflux of a four-carbon skeleton into a three-carbon intermediate followed by recarboxylation has been termed "pyruvate cycling." This phrase refers to combined flux through two distinct futile cycles: oxaloacetate → PEP → pyruvate → oxaloacetate, and oxaloacetate → malate → pyruvate → oxaloacetate. Thus, in pyruvate cycling, pyruvate kinase and malic enzyme are two key enzymes. NADPH is produced during the conversion of malate to pyruvate through malic enzyme, which is then consumed in fatty acid chain elongation as a part of lipogenesis. Thus, increased pyruvate cycling of *fa/fa* rats may be needed because of increased requirement for NADPH if energy reserves are to be stored as fat.

The unique ^2H distribution pattern of skeletal muscle glycogen suggests an unemphasized aspect of glyconeogenesis in skeletal muscle. Lactate, amino acids, muscle glycolytic intermediates, and glycerol are known or potential substrates for glyconeogenesis (29). However, those substrates and known pathways for glycogen synthesis would not result in high ^2H enrichment of both the H1 and H2 position of the glucosyl unit of muscle glycogen. Chandramouli et al. (30) reported ^2H labeling into the H1 and H2 positions of glucose from ^2H_2O via phosphoglucose isomerase and phosphomannose isomerase (PMI) activities, which interchanges mannose 6-phosphate, glucose 6-phosphate, and fructose 6-phosphate. The involvement of PMI in skeletal muscle glyconeogenesis has not been described, but mRNA levels of PMI were high in human skeletal muscle (31). Another possibility for the high enrichment of H1 and H2 is via glycogen cycling and intramolecular ^1H (or ^2H) exchange at the phosphoglucose isomerase level (32). In an earlier study, however, ^2H NMR analysis of liver glycogen with active glycogen cycling did not demonstrate exceptionally high enrichment in both H1 and H2 (10). Thus, the possibility of high enrichment through intramolecular ^2H exchange seems low in muscle.

In this study, steady-state metabolic conditions were not confirmed in each rat over the infusion time course because it was not practical due to the inherent low sensitivity of NMR. Previously, we reported that a primed 90-min infusion was sufficient to accurately measure endogenous glucose production in rats by comparing the 90-min results with those after a primed 150-min infusion (12). In the current study, to eliminate the possibility of overestimation of endogenous glucose production in hyperglycemic *fa/fa* rats because of a low priming dose or short infusion duration, steady state was confirmed by performing a 120-min infusion with 13-week-old *fa/fa* rats ($n = 3$). Endogenous glucose production of the rats was 59.6 ± 4.7 $\mu mol \cdot kg^{-1} \cdot min^{-1}$, essentially the same value as a 90-min infusion for the same group of rats (56.1 ± 6.2) (Table 2), satisfying the steady-state assumption. Isotopic steady state was also assumed for metabolism of [U-$^{13}C_3$]propionate in this study. The analysis of glucose C2 (or MAG C2) depends only on the multiplet patterns detected in the ^{13}C NMR spectrum, not on the absolute ^{13}C enrichment. The 13-week-old *fa/fa* rats with a 120-min infusion showed a similar multiplet pattern of glucose C2 (Fig. 3*D*) as the same group of rats with a 90-min infusion. This observation is consistent with an early study that indicated that a 45-min infusion is sufficient to reach a steady-state condition during infusion of [U-$^{13}C_3$]propionate in rats (33). Together, these data indicate that a primed 90-min infusion was sufficient to reach steady state.

The current results illustrate the need to develop practical methods that would allow clinical investigators to easily quantify glycogen content and turnover in patients. Our results indicate that hepatic glycogenolysis plays a significant role in glucose overproduction in *fa/fa* rats, especially in older animals. Both 8- and 13-week-old *fa/fa* rats had higher fluxes of hepatic pyruvate cycling, PEPCK flux, and TCA cycle compared with age-matched controls. These abnormal metabolic fluxes involve multiple pathways in hepatic metabolism, even in relatively young animals, suggesting that enzyme fluxes may be potential markers for predicting onset of overt type 2 diabetes as well as potential targets to prevent the transition from a pre-diabetic state into overt diabetes.

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