

# Alterations in Hepatic Glucose and Energy Metabolism as a Result of Calorie and Carbohydrate Restriction

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Carbohydrate restriction is a common weight-loss approach that modifies hepatic metabolism by increasing gluconeogenesis (GNG) and ketosis. Because little is known about the effect of carbohydrate restriction on the origin of gluconeogenic precursors (GNG from glycerol [GNG<sub>glycerol</sub>] and GNG from lactate/amino acids [GNG<sub>phosphoenolpyruvate {PEP}</sub>]) or its consequence to hepatic energy homeostasis, we studied these parameters in a group of overweight/obese subjects undergoing weight-loss via dietary restriction. We used <sup>2</sup>H and <sup>13</sup>C tracers and nuclear magnetic resonance spectroscopy to measure the sources of hepatic glucose and tricarboxylic acid (TCA) cycle flux in weight-stable subjects (n = 7) and subjects following carbohydrate restriction (n = 7) or calorie restriction (n = 7). The majority of hepatic glucose production in carbohydrate restricted subjects came from GNG<sub>PEP</sub>. The contribution of glycerol to GNG was similar in all groups despite evidence of increased fat oxidation in carbohydrate restricted subjects. A strong correlation between TCA cycle flux and GNG<sub>PEP</sub> was found, though the reliance on TCA cycle energy production for GNG was attenuated in subjects undergoing carbohydrate restriction. Together, these data imply that the TCA cycle is the energetic patron of GNG. However, the relationship between these two pathways is modified by carbohydrate restriction, suggesting an increased reliance of the hepatocyte on energy generated outside of the TCA cycle when GNG<sub>PEP</sub> is maximal. **Conclusion:** Carbohydrate restriction modifies hepatic GNG by increasing reliance on substrates like lactate or amino acids but not glycerol. This modification is associated with a reorganization of hepatic energy metabolism suggestive of enhanced hepatic  $\beta$ -oxidation. (HEPATOLOGY 2008;48:1487-1496.)

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Abbreviations: BMI, body mass index; EGP, endogenous glucose production; GCRC, General Clinical Research Center; GNG, gluconeogenesis; MAG, monoacetone glucose; NAFLD, nonalcoholic fatty liver disease; NMR, nuclear magnetic resonance; PEP, phosphoenolpyruvate; PEPCK, phosphoenolpyruvate carboxylase; RQ, respiratory quotient; TCA, tricarboxylic acid

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Since the seminal observation of Keys<sup>1</sup> in 1980, the recommended diet in the United States has been the low-calorie, low-fat diet. This diet originated primarily as an inexpensive approach to prevent cardiovascular disease but has now become the recommended treatment for overweight and obesity in clinical practice.<sup>2</sup> Despite the success of clinicians and the U.S. Public Health Service in reducing the U.S. population's fat intake and increasing its carbohydrate intake over the past 30 years, the prevalence of obesity has continued to rise.<sup>3,4</sup> During this same period, metabolic liver disease has become increasingly prevalent, taking the form of excess triglyceride accumulation in the liver that can result in inflammation, fibrosis, and cirrhosis.<sup>5,6</sup> The transition of this type of liver disease, known as nonalcoholic fatty liver disease (NAFLD), from relatively bland, inactive steatosis to a more morbid inflammatory condition, termed nonalcoholic steatohepatitis, occurs in a subset of individuals. The reason this transition takes place is unclear; however, some investigators have found a strong association between dietary carbohydrate intake and severity of both

steatosis and steatohepatitis.<sup>7,8</sup> Current evidence suggests that a high carbohydrate diet leads to increased hepatic de novo lipogenesis,<sup>9</sup> likely as the result of the molecular mediators carbohydrate and sterol response element binding protein.<sup>5</sup> Such an increase in hepatic fat synthesis would be anticipated to be associated with hepatic steatosis; however, the connection between carbohydrate intake and inflammatory activity remains elusive. The changes in hepatic metabolism and energy production that occur as a consequence of changes in dietary carbohydrate intake may be important in the pathogenesis and progression of NAFLD.

Several studies have used stable isotopes to investigate the impact of carbohydrate intake on hepatic glucose metabolism.<sup>10-12</sup> These studies clearly show that low-carbohydrate diets result in a reorganization of hepatic glucose production by changing the rate of glycogenolysis and, to a lesser degree, the rate of gluconeogenesis (GNG). However, little is known about the effect of carbohydrate restriction on the origin of gluconeogenic precursors (GNG from lactate/amino acids or glycerol) or its consequence on hepatic energy homeostasis. There is an empirical relationship between GNG and tricarboxylic acid (TCA) cycle flux<sup>13</sup>: suppressed rates of GNG result in impaired hepatic TCA cycle flux,<sup>14,15</sup> while increased GNG is accompanied by elevated TCA cycle flux.<sup>16</sup> This relationship appears to be an “energetic rheostat,” allowing the liver to match energy production with the requirements of GNG. If the increased rates of GNG observed during carbohydrate restriction are the result of increased conversion of lactate/amino acids to glucose, energy production at the level of the TCA cycle may be altered in a coordinated manner.

To gain a better understanding of hepatic energy production and its relationship to GNG under conditions of varied macronutrient intake, we used <sup>2</sup>H and <sup>13</sup>C tracers combined with nuclear magnetic resonance (NMR) spectroscopy<sup>17</sup> to simultaneously assess endogenous glucose production (EGP), glycogenolysis, GNG from lactate/amino acids (GNG<sub>phosphoenolpyruvate [PEP]</sub>), GNG from glycerol (GNG<sub>glycerol</sub>) pyruvate cycling, and TCA cycle flux in human subjects following a carbohydrate restricted or calorie restricted diet.

## Patients and Methods

**Materials.** Deuterium oxide (<sup>2</sup>H<sub>2</sub>O) (70%) and [U-<sup>13</sup>C]propionate (99%) were obtained from Cambridge Isotopes (Andover, MA). Sterility-tested and pyrogen-tested [3,4-<sup>13</sup>C]glucose was obtained from Omicron Biochemicals Inc. (South Bend, IN). Other common reagents were purchased from Sigma (St. Louis, MO).

**Participants.** The study was comprised of two groups of seven subjects aged 18 to 65 years that were matched for age, body mass index (BMI), gender, and ethnicity. To eliminate confounding variables, participants chosen were of stable health, using no medications known to alter hepatic glucose metabolism, participating in no weight loss diet or using no diet pills at enrollment or within 6 months prior to enrollment, and without baseline ketonuria. The study was restricted to subjects whose BMI was >25 kg/m<sup>2</sup> and <35 kg/m<sup>2</sup>. All subjects had a normal nutritional status, no ethanol intake, and did not participate in regular exercise above the activity required for daily living.

An additional group of seven subjects who were lean (BMI < 25 kg/m<sup>2</sup>) was also recruited to act as a weight stable comparison group. These subjects were all of normal health, had a normal nutritional status, no ethanol intake, and did not participate in regular exercise above the activity required for daily living.

The protocol and consent form were approved by the Institutional Review Board of the University of Texas Southwestern Medical Center and all participants provided written informed consent prior to enrollment.

**Design.** At enrollment, subjects were assigned to either a low-carbohydrate diet or a calorie restricted diet. Prior to initiating the diet, all subjects underwent a teaching session with the General Clinical Research Center (GCRC) dietician to explain the appropriate use of the diet history questionnaire, determine food preferences, and provide dietary instruction. At 2 weeks after enrollment, subjects were admitted to the inpatient GCRC for overnight metabolic studies. On admission (16:00; 24-hour clock), subjects were provided a dinner consistent with their assigned diet, after which they were fasted until completion of the study. Between 22:00 and 09:00, subjects received two stable isotope tracers orally: [U-<sup>13</sup>C]propionate (~1,200 mg) at 08:30 and divided doses of 70% <sup>2</sup>H<sub>2</sub>O (5 g/kg body water, calculated as 60% of body weight in men and 50% of body weight in women) at 22:00, 02:00, and 06:00. Subjects were allowed to drink 0.5% <sup>2</sup>H<sub>2</sub>O ad libitum during the study. Between 08:30 and 09:00 subjects underwent measurement of their respiratory quotient (RQ) using a Delta Trak II indirect calorimeter (SensorMedics, Yorba Linda, CA). An intravenous catheter was placed and subjects were given a 2.25 mg/kg bolus of [3,4-<sup>13</sup>C]glucose followed by a 2-hour infusion (0.0225 mg/kg/minute). At the end of the infusion period, a 50-cc blood draw was performed.

**Low-Carbohydrate Diet.** Subjects assigned to the low-carbohydrate diet were instructed by the GCRC dietician to limit carbohydrate intake to less than 20 g/day<sup>18</sup>

and were provided with instructional handouts detailing allowed and disallowed foods. Participants were encouraged to eat three regular-size meals and to consume eight 8-ounce glasses of water per day. Subjects initiated this diet on their own for the first 7 days of the study, keeping a detailed dietary record. Food for the final 7 days was provided to subjects as frozen meals preprepared by the GCRC kitchen in accordance with the caloric intake documented in the dietary record.

**Calorie Restricted Diet.** This diet was designed to place individuals in negative energy balance relative to their typically daily caloric intake. Subjects were asked to continue their regular diet without calorie restriction for the first 7 days of the study and record their food intake in a diet record to determine average daily caloric intake as well as dietary macronutrient composition. Based upon this diet record, the GCRC dietician and kitchen staff prepared all meals for the final 7 days of the study in accordance with the dietary composition recorded by the individual, but reduced in caloric content by 800 kcal/day.

**Weight-Stable Diet.** Subjects were asked to continue their regular diet, but were placed on a diet that was 40% carbohydrate, 30% fat, and 30% protein for 3 days prior to the study. The daily caloric value of the meals was 1,700 kcal for women and 2,000 kcal for men.

**Blood Samples.** Blood was collected in ethylene diaminetetraacetic acid (EDTA) tubes and centrifuged immediately to isolate plasma from red blood cells. Plasma was extracted with perchloric acid and the glucose was purified as described.<sup>19,20</sup> Purified plasma glucose was converted to 1,2-isopropylidene glucofuranose (also known as monoacetone glucose [MAG]) before NMR analysis as detailed.<sup>20-22</sup> The synthesized MAG was then resuspended in 150  $\mu$ L of high-performance liquid chromatography-grade acetonitrile and 20  $\mu$ L of deuterium-depleted H<sub>2</sub>O and loaded into a 3-mm NMR tube.

**Magnetic Resonance Spectroscopy.** Samples were analyzed on a 14.1-T Varian Inova spectrometer (Varian Instruments, Palo Alto, CA). Deuterium NMR spectra of MAG were collected on a 3-mm broadband probe tuned to 92 MHz with a 90-degree pulse width, 1-second acquisition time, and no additional delay. Deuterium spectra were performed without field frequency lock and were acquired in 250 scan blocks. These were combined at the end of the experiment by aligning the acetonitrile frequency. Proton-decoupled <sup>13</sup>C NMR spectra were collected after the addition of 20  $\mu$ L of deuterated acetonitrile (for frequency locking), using a 3-mm broadband probe tuned to 150 MHz. A 50-degree pulse width, 1.5-second acquisition time, and no further delay were used to acquire the <sup>13</sup>C spectra. Resonance areas were

determined using the one-dimensional NMR processor in ACD/Labs 9.0 (Advanced Chemistry Development, Toronto, Ontario, Canada).

**Isotopomer Analysis.** The relative deuterium enrichments in glucose H2, H5 and H6s were assessed by <sup>2</sup>H NMR and these values were used to determine the fractional contribution of GNG and glycogenolysis to EGP as detailed.<sup>17,19,20</sup> Pathways intersecting the TCA cycle were evaluated by <sup>13</sup>C isotopomer analysis of glucose C2. The <sup>13</sup>C NMR multiplets in glucose generated by the tracer [U-<sup>13</sup>C]propionate were evaluated to determine flux through anaplerosis, pyruvate cycling, and GNG<sub>PEP</sub> relative to the TCA cycle.<sup>23,24</sup> Here, we use anaplerosis as a measure of phosphoenolpyruvate carboxykinase (PEPCK) flux since this pathway is the predominant outlet of anaplerosis in the liver. This represents a maximal estimate of PEPCK flux since we are disregarding other minor pathways (e.g., malic enzyme) that may also contribute to the flux of intermediates out of the TCA cycle. This measurement is also an estimate of pyruvate carboxylase flux since pyruvate carboxylase is the main anaplerotic pathway in the liver. EGP was measured by <sup>13</sup>C NMR analysis of the multiplets of glucose C3 and C4 as described.<sup>25</sup> Fractional GNG and GNG measured by <sup>2</sup>H NMR was combined with PEPCK, pyruvate cycling, and GNG relative to TCA cycle flux measured by <sup>13</sup>C NMR and the rate of EGP ( $\mu$ mol/kg<sub>lean body weight</sub>/minute) to yield the absolute fluxes through each of these pathways.<sup>14,17,25</sup>

**Statistical Analysis.** Statistical analyses were performed using SigmaStat 3.0 (SPSS Inc., Chicago, IL). Differences between two groups were evaluated using unpaired *t* tests (means) or Mann-Whitney rank sums tests (medians). Differences between two repeated measures were analyzed using paired *t* tests. One-way analysis of variance was used for the comparison of more than two groups. Correlations between variables were determined using the Pearson product moment test. Statistical significance was taken at *P* < 0.05.

## Results

**Subjects.** Subjects in the low-carbohydrate group were matched to those in the low-calorie group (Table 1). The majority of participants (10/14) were obese (BMI > 30 kg/m<sup>2</sup>), with the remainder classified as overweight (25 kg/m<sup>2</sup> < BMI < 30 kg/m<sup>2</sup>). All subjects in the low-carbohydrate arm of the study demonstrated urinary ketones at the end of the study except for one. Despite the absence of ketosis, this individual had an RQ of 0.84 at study-end and experienced a 3.5-kg weight loss over the period of the study.

**Table 1. Treatment Group General Characteristics**

Characteristic	Low-Calorie (n = 7)	P Value	Low-Carbohydrate (n = 7)
Age (years)	43 ± 9	0.982	44 ± 13
Race/ethnicity (n)		1.000	
Caucasian	5		5
African-American	1		1
Hispanic	1		1
Gender ratio (female:male)	5:2	1.000	5:2
Body weight (kg)	90 ± 11	0.327	96 ± 12
Body mass index (kg/m <sup>2</sup> )	32 ± 4	0.618	33 ± 3
Total cholesterol (mg/dL)	201 ± 15	0.722	198 ± 18
Triglyceride (mg/dL)	102 ± 51	0.899	107 ± 75
HDL-c (mg/dL)	51 ± 17	0.599	47 ± 11
LDL-c (mg/dL)	131 ± 23	0.883	129 ± 16
AST (U/L)	22 ± 5	0.631	21 ± 5
ALT (U/L)	21 ± 10	0.839	20 ± 8
Fasting glucose (mg/dL)	94 ± 10	0.964	94 ± 13
HgbA1c (%)	5.0 ± 0.4	0.132	5.4 ± 0.5

Values are presented as mean ± standard deviation.

**Intervention.** Subjects in the low-carbohydrate arm experienced greater total weight loss over the 2-week period than those in the low-calorie arm (Table 2). There was no significant change in weight in the weight-stable group (data not shown). As expected, RQs were significantly lower and total plasma ketone bodies were significantly higher among carbohydrate-restricted subjects. Insulin, lactate, and triglyceride levels did not differ between the two dietary-restriction groups after 2 weeks. There was no significant difference in caloric intake between the two groups, although a trend toward lower caloric intake in the low-carbohydrate group was noted. The dietary macronutrient proportions were significantly different in the two arms of the study; however, absolute dietary macronutrient intake was only significantly different with regard to protein and carbohydrate, and absolute daily fat intake in the dietary groups was similar. Concordant with this, the absolute daily intake of saturated, monounsaturated, and polyunsaturated fats also did not differ between the two groups.

**Fractional Contribution of Glycogen, Glycerol, and PEP to Glucose Production: Analysis of Plasma Glucose <sup>2</sup>H Enrichment by <sup>2</sup>H-NMR.** The method for analyzing the <sup>2</sup>H spectra of plasma glucose is presented in Fig. 1. The fraction of glucose derived from glycogenolysis in the low-carbohydrate group was significantly higher than the low-calorie group (79 ± 9 versus 61 ± 8%; *P* = 0.001). As anticipated, the contribution of GNG to glucose production was significantly lower in carbohydrate-restricted subjects than that measured in calorie-restricted subjects (21 ± 9 versus 39 ± 8%; *P* = 0.001) (Table 3). Surprisingly, the fraction of GNG arising from glycerol (GNG<sub>glycerol</sub>) was similar between the low-carbohydrate and the low-calorie group. Thus, the observed increase in

the fractional contribution of GNG to glucose production in the carbohydrate-restricted group was solely the result of an increase in GNG originating from substrates such as lactate or amino acids that must pass through the TCA cycle and PEPCK (GNG<sub>PEP</sub>) (64 ± 5 versus 47 ± 11%; *P* = 0.002) (Table 3).

These data were also compared to a group of lean, weight-stable individuals. In this comparison, no differences were noted between the low-calorie group and the weight stable group with respect to fractional glycogenolysis or GNG<sub>PEP</sub> (Table 3). Interestingly, regardless of dietary composition or weight status, the fractional contribution of glycerol to GNG was similar for all groups studied (*P* = 0.799).

**TCA Cycle Flux: Isotopomer Analysis of Plasma Glucose <sup>13</sup>C Enrichment by <sup>13</sup>C-NMR.** To simultaneously measure hepatic TCA cycle turnover and fractional glucose production, subjects ingested [U-<sup>13</sup>C]propionate. Details regarding the analysis of the <sup>13</sup>C-NMR spectra of the glucose isotopomers produced as a result of the incorporation of the <sup>13</sup>C carbons from propionate into the TCA cycle are presented in Fig. 2. This analysis allowed the determination of maximal flux through PEPCK, pyruvate cycling, and GNG<sub>PEP</sub>, all relative to TCA cycle flux (Table 3). There was a trend toward a higher flux through PEPCK in carbohydrate-restricted subjects when compared to calorie-restricted subjects (*P* = 0.051). The combined flux through the malic enzyme (malate → pyruvate) and pyruvate kinase (phosphoenolpyruvate [PEP] → pyruvate), with the ultimate return of pyruvate to the TCA cycle via pyruvate carboxylase

**Table 2. Treatment Group Characteristics at Study End**

Characteristic	Low-Calorie (n = 7)	P Value	Low-Carbohydrate (n = 7)
Total weight loss (kg)	2.3 ± 1.7	<b>0.024</b>	4.4 ± 1.4
Respiratory quotient	0.90 ± 0.08	<b>0.006</b>	0.79 ± 0.04
Insulin (ng/mL)	3.4 ± 1.3	0.727	3.8 ± 1.9
Lactate (mmol/L)	1.3 ± 1.2	0.366	0.9 ± 0.2
Glucose (mg/dL)	90 ± 9	0.295	95 ± 8
Triglyceride (mg/dL)	99 ± 16	0.700	91 ± 36
Total ketones (μmol/L)	436 ± 146	0.002	1,280 ± 407
Caloric intake (kcal/day)	1,980 ± 727	0.153	1,520 ± 328
Diet composition			
Protein (%)	17 ± 3	<b>&lt;0.001</b>	34 ± 4
Fat (%)	36 ± 8	<b>&lt;0.001</b>	61 ± 5
Carbohydrate (%)	47 ± 6	<b>&lt;0.001</b>	5 ± 1
Protein (g/day)	81 ± 17	<b>0.001</b>	126 ± 20
Fat (g/day)	83 ± 50	0.347	104 ± 30
Carbohydrate (g/day)	228 ± 65	<b>&lt;0.001</b>	19 ± 1
Fat intake (g/day)			
Saturated	28 ± 15	0.184	39 ± 14
Monounsaturated	35 ± 24	0.822	37 ± 10
Polyunsaturated	18 ± 11	0.705	16 ± 8

Values are presented as mean ± standard deviation. **Bold** indicates statistical significance.

(pyruvate  $\rightarrow$  oxaloacetate) constitutes pyruvate cycling (Fig. 2); the flux through this cycle was similar between the two groups ( $P = 0.112$ ). The relative rate of  $\text{GNG}_{\text{PEP}}$  is simply the difference between the rate of production of PEP (PEPCK flux) and the rate of return of PEP to the TCA cycle (pyruvate cycling). Concordant with PEPCK flux, we found a trend toward an increase in  $\text{GNG}_{\text{PEP}}$  relative to the rate of TCA cycle flux in carbohydrate-restricted individuals ( $P = 0.063$ ) (Table 3).

A comparison of the  $^{13}\text{C}$  data from the low-carbohydrate group to the weight-stable group revealed that all

fluxes relative to TCA cycle flux were significantly higher in the low-carbohydrate group (Table 3). When compared to the weight-stable group, the measures in the low-calorie weight loss group were not significantly different.

**Integration of Relative Fluxes with EGP.** By combining the relative fluxes determined from  $^2\text{H}$  and  $^{13}\text{C}$  data with the rate of EGP determined by  $[3,4-^{13}\text{C}]$  glucose dilution, absolute flux rates of the metabolic pathways connecting GNG and the TCA cycle were calculated.<sup>17</sup> The absolute hepatic fluxes for the low-calorie and low-carbohydrate groups are expressed in terms of lean body weight (calculated) and are presented in Table 3. There was no difference in EGP between any of the groups studied. The absolute rate of glycogenolysis in the low-carbohydrate group was reduced 39% compared to the low-calorie group ( $P < 0.001$ ). This decrease in glycogenolysis in the low-carbohydrate group was offset by an increase in the absolute rate of  $\text{GNG}_{\text{PEP}}$  ( $P = 0.010$ ), while there was no significant difference with regard to  $\text{GNG}_{\text{glycerol}}$  ( $P = 0.421$ ).

Rates of glycogenolysis,  $\text{GNG}_{\text{PEP}}$ , and  $\text{GNG}_{\text{glycerol}}$  did not differ significantly between the weight-stable and low-calorie groups. Though the rate of glycogenolysis was lower in the low-carbohydrate group when compared to the weight stable group ( $P = 0.001$ ), the rate of  $\text{GNG}_{\text{PEP}}$  and  $\text{GNG}_{\text{Glycerol}}$  were similar (Table 3).

Contrary to the trend observed in the relative data above, absolute PEPCK flux and rates of pyruvate cycling did not differ between any of the three groups studied (Table 3). Of interest is the fact that TCA cycle flux was not different between the two weight-loss groups ( $P = 0.822$ ), indicating similar rates of energy production

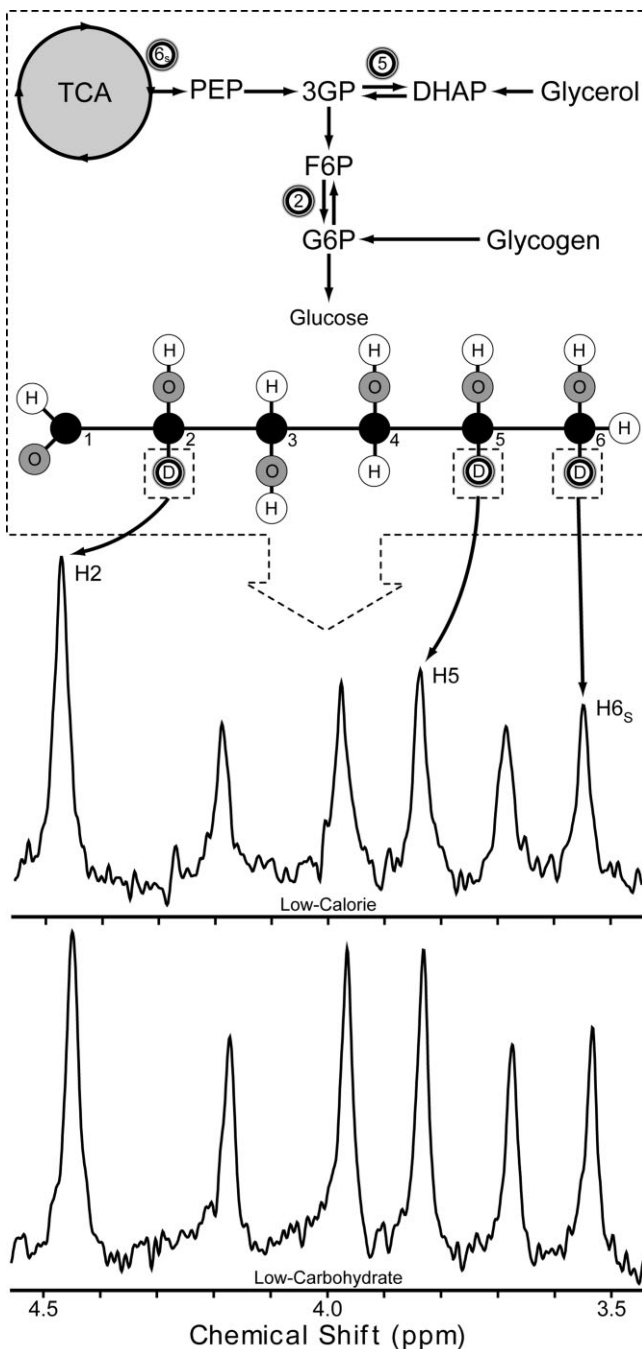


Fig. 1. Deuterium NMR analysis of the incorporation of deuterium oxide into glucose. Protons derived from water are incorporated into glucose at three distinct steps of its production. After enrichment of total body water by oral ingestion of deuterium oxide ( $^2\text{H}_2\text{O}$ ), deuterons are incorporated into glucose via the same mechanism. All glucose produced, whether from gluconeogenesis (GNG) or from glycogenolysis, becomes enriched at carbon 2 of glucose (H2). All glucose derived from GNG becomes enriched at carbon 5 of glucose (H5) while only GNG occurring from precursors originating in the TCA cycle (PEP) becomes enriched at carbon 6s of glucose (H6s). The relative enrichment of deuterium at each of the carbon positions of glucose, after conversion to its monoacetone derivative, is easily discerned by computing the peak areas obtained by  $^2\text{H}$ -NMR spectroscopy. The ratio of  $\text{H5}/\text{H2}$  gives the proportion of glucose that is derived from GNG while  $(\text{H5}-\text{H6s})/\text{H2}$  gives the proportion of glucose derived from PEP. The  $\text{H5}/\text{H2}$  ratio of the two depicted spectra are quite different: the low-calorie spectra indicates that GNG accounts for  $\sim 50\%$  of glucose production while the low-carbohydrate spectra indicates that  $\sim 80\%$  of glucose is derived from GNG. 3GP, 3-glycerophosphate; DHAP, dihydroxyacetone phosphate; F6P, fructose-6-phosphate; G6P, glucose-6-phosphate.

**Table 3. Metabolic Flux Data**

Relative Data	Weight-Stable (n = 7)	Low-Calorie (n = 7)	Low-Carbohydrate (n = 7)
Fractional glucose production (%)			
Glycogenolysis	37 ± 6	39 ± 8†	21 ± 9*
Gluconeogenesis <sub>(PEP)</sub>	50 ± 9	47 ± 11†	64 ± 5*
Gluconeogenesis <sub>(glycerol)</sub>	13 ± 6	14 ± 9	16 ± 8
TCA cycle (relative to citrate synthase)			
PEPCK	6.6 ± 1.4	7.9 ± 2.2	11.0 ± 4.1*
Pyruvate cycling	4.7 ± 1.0	5.3 ± 1.9	7.0 ± 2.2*
Gluconeogenesis <sub>(PEP)</sub>	1.9 ± 0.5	2.7 ± 0.8	4.1 ± 2.1*
Absolute data (μmol/kg <sub>L</sub> BW/minute)			
Endogenous glucose production	19.2 ± 3.3	17.1 ± 2.7	18.9 ± 4.2
Glycogenolysis	7.1 ± 1.8	6.7 ± 2.1†	3.7 ± 1.6*
Gluconeogenesis <sub>(PEP)</sub> triose units	19.5 ± 5.4	16.0 ± 4.0†	24.0 ± 5.3
Gluconeogenesis <sub>(glycerol)</sub> triose units	4.9 ± 1.9	4.8 ± 3.0	6.4 ± 4.9
PEPCK	69.2 ± 20.3	51.7 ± 25.9	70.0 ± 20.9
Pyruvate cycling	49.6 ± 16.1	35.8 ± 23.0	45.9 ± 18.0
TCA cycle	10.5 ± 2.0	6.9 ± 3.1*	7.2 ± 3.4*

Values are represented as mean ± standard deviation. \*Significantly different from weight-stable group. †Significantly different from low-carbohydrate group. LBW, lean body weight.

within the TCA cycle. However, both weight-loss groups had significantly lower TCA cycle flux rates when compared to the weight-stable group ( $P < 0.05$ ).

**Hepatic Energy Generation and GNG.** Energy generation in the hepatic TCA cycle has a powerful influence on flux through PEPCK and ultimately GNG.<sup>13,14,16,26,27</sup> A similar relationship is apparent in this present study (Fig. 3), with TCA cycle flux and PEPCK flux showing a strong correlation ( $r = 0.660$ ;  $P = 0.001$ ). To further examine this relationship, we compared the energy generated in the TCA cycle to that required to drive gluconeogenic flux in molar units of adenosine triphosphate (Table 4). In the fasted state, the major precursors for PEP formation are lactate (derived from the Cori Cycle) and alanine (derived from the nitrogen-exporting glucose-alanine cycle). Assuming GNG<sub>PEP</sub> was solely derived from lactate, significantly more energy was produced by the TCA cycle than could be consumed by GNG in both the weight-stable and low-calorie group. However, within the low-carbohydrate group, the rate of energy production in the TCA cycle and energy consumption of the measured pathways was nearly identical. Conversely, assuming alanine was the sole precursor for GNG<sub>PEP</sub>, energy production and consumption of the measured pathways were identical in the weight-stable and low-calorie group while energy consumption significantly outpaced energy production in the low-carbohydrate group ( $P = 0.028$ ; data not shown). While in reality, lactate and alanine are both important gluconeogenic precursors, these calculations suggest that regardless of the gluconeogenic substrate, there is an increased dependence on energy derived upstream of the

TCA cycle in individuals undergoing carbohydrate restriction (for example,  $\beta$ -oxidation/ketogenesis).

## Discussion

In this study, the effect of carbohydrate restriction on flux through the metabolic pathways of hepatic glucose production and the TCA cycle were simultaneously assessed by isotopomer analysis of glucose using <sup>2</sup>H and <sup>13</sup>C NMR spectroscopy. We found that carbohydrate restriction increased the rate of GNG and decreased the rate of glycogenolysis. However, the observed increase in GNG in the low-carbohydrate group was solely the result of increased GNG<sub>PEP</sub> rather than GNG<sub>glycerol</sub>. Despite the energetic investment required to increase GNG<sub>PEP</sub>, TCA cycle flux in the low-carbohydrate group was similar to the low-calorie group, indicating similar rates of energy generation. Interestingly, in the groups consuming carbohydrate as a significant proportion of their diet (weight-stable, low-calorie), the TCA cycle alone provided sufficient energy to drive GNG regardless of whether the gluconeogenic substrate was assumed to be lactate or alanine. This was not the case in individuals undergoing carbohydrate restriction, indicating that a reorganization of hepatic energy metabolism occurred in tandem with the changes in hepatic carbohydrate metabolism.

Among previous studies of carbohydrate restriction, it remained unclear which gluconeogenic precursors were primarily responsible for increased GNG. Evidence of a negative correlation between alanine conversion to glucose and dietary carbohydrate content suggests that anaplerosis and GNG<sub>PEP</sub> are increased with decreased di-

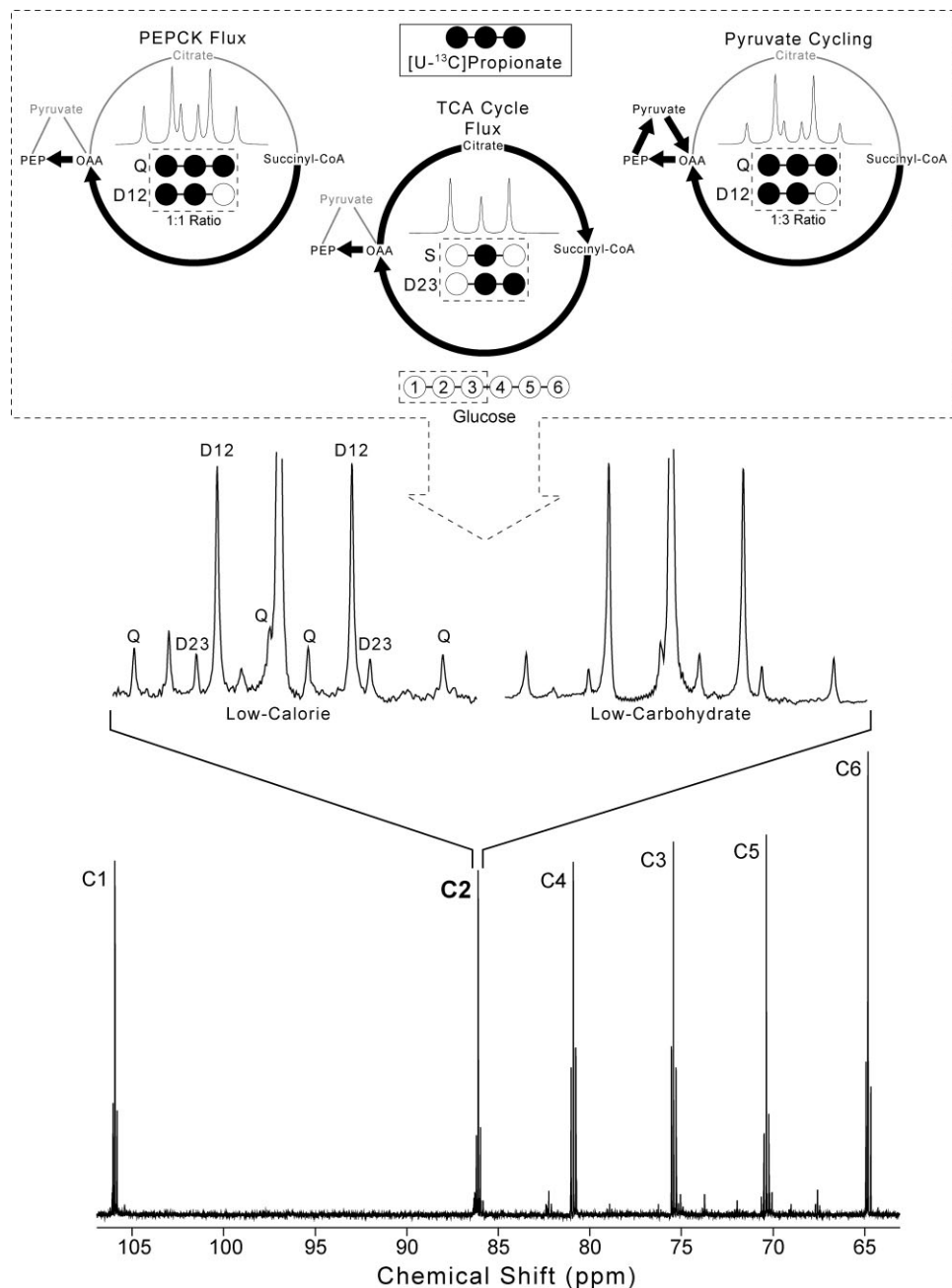


Fig. 2.  $^{13}\text{C}$ -NMR analysis of the incorporation of  $[\text{U-}^{13}\text{C}]$ propionate into glucose. After ingestion, propionate is avidly taken up by the liver and enters the TCA cycle as  $[1,2,3\text{-}^{13}\text{C}]$ succinyl-coenzyme A (CoA). Within the TCA cycle, this labeled intermediate can have one of three fates: (1) exit the TCA cycle as phosphoenolpyruvate (PEP) immediately upon conversion to oxaloacetate (OAA) via the action of phosphoenolpyruvate carboxykinase (PEPCK), ultimately leading to the formation of  $[1,2,3\text{-}^{13}\text{C}]$ glucose and  $[1,2\text{-}^{13}\text{C}]$ glucose in a 1:1 proportion; (2) proceed through a complete turn of the TCA cycle, passing through citrate synthase, and exiting the cycle as PEP, ultimately yielding  $[2\text{-}^{13}\text{C}]$ glucose and  $[2,3\text{-}^{13}\text{C}]$ glucose; and (3) exit the TCA cycle as PEP with subsequent conversion from PEP to pyruvate to OAA and back to PEP (so-called pyruvate cycling), ultimately leading to the formation of  $[1,2,3\text{-}^{13}\text{C}]$ glucose and  $[1,2\text{-}^{13}\text{C}]$ glucose in a 1:3 proportion. By examining the C2 of glucose using  $^{13}\text{C}$ -NMR after conversion to its monoacetone derivative, each of these glucose isotopomers is readily apparent:  $[1,2,3\text{-}^{13}\text{C}]$ glucose is denoted in the spectrum by the quartet (Q);  $[2,3\text{-}^{13}\text{C}]$ glucose is represented by the doublet D23;  $[1,2\text{-}^{13}\text{C}]$ glucose is represented by the doublet D12; and  $[2\text{-}^{13}\text{C}]$ glucose is represented by the singlet peak in the center of the C2 spectrum (not labeled). The areas under each of these peaks provide the information necessary to calculate PEPCK flux, pyruvate cycling, and the rate of production  $\text{GNG}_{\text{PEP}}$  relative to citrate synthase flux. These pathways and the isotopomers they produced can also be described by a full set of analytical equations.<sup>24</sup>

etary intake of carbohydrate.<sup>28,29</sup> However, increased fat oxidation during carbohydrate restriction might be expected to increase availability of the gluconeogenic pre-

cursor glycerol.<sup>10</sup> In the present study, we showed that the increase in GNG associated with carbohydrate restriction is due to the induction of  $\text{GNG}_{\text{PEP}}$ . This suggests that in

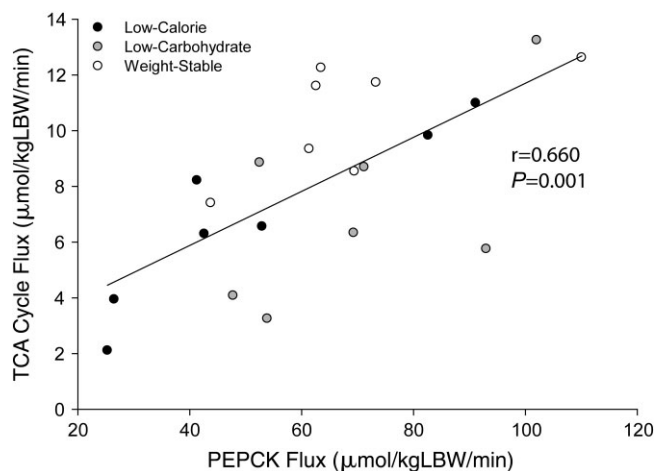


Fig. 3. Relationship between PEPCK flux and TCA cycle flux.

fasted human subjects undergoing weight loss, the elevated GNG associated with carbohydrate restriction is driven by substrates such as lactate or amino acids. While it seems likely this increase is due to amplified protein turnover, we could not rule out enhanced cycling of lactate from the periphery back to the liver (Cori Cycle) as a source of increased GNG. Plasma lactate levels were similar between the two weight loss groups (Table 2); however, these were static measurements and gave no insight into the rate of production of lactate by muscle and up-

take by liver. Likewise, protein turnover measurements were not performed so the contribution of amino acids also remains unknown. However, it is interesting to note that individuals on a low-carbohydrate diet increased their protein intake in favor of fat (Table 2), possibly as method to stave off nondietary protein breakdown for the formation of glucose.

The contribution of glycerol as a substrate for GNG was surprisingly unresponsive to dietary macronutrient composition. Though  $GNG_{glycerol}$  appeared to be numerically higher in the low-carbohydrate group, this failed to reach statistical significance. It is possible that the small sample size of the study and/or the sensitivity of our technique limited our ability to detect modest changes in this measure. However, prior data in fasting man suggests that  $GNG_{glycerol}$  occurs at a relatively fixed rate.<sup>30</sup> Our findings would further support this observation. Indeed, insulin levels were similar between the groups undergoing dietary restriction, suggesting that rates of peripheral lipolysis were also similar (Table 2). This was somewhat surprising as prior data in lean individuals clearly demonstrates a reduction in insulin levels and increase in free fatty acid levels as a result of carbohydrate restriction.<sup>31</sup> However, the present data is akin to that of Allick et al.<sup>32</sup> in which overweight/obese individuals with diabetes maintained similar insulin and free fatty acid levels regardless of di-

Table 4. Energetics of Measured Pathways

	ATP ( $\mu\text{mol}/\text{kg}_{\text{LBW}}/\text{minute}$ )		
	Weight-Stable	Low-Calorie	Low-Carbohydrate
Energy consuming			
Glucose production			
PEP $\rightarrow$ 1,3-PGA(-ATP)	-19.5 $\pm$ 5.4	-16.0 $\pm$ 4.0	-24.0 $\pm$ 5.3
OAA $\rightarrow$ PEP(-GTP)	-69.2 $\pm$ 20.3	-51.8 $\pm$ 25.9	-70.0 $\pm$ 20.9
Anaplerosis			
Pyruvate $\rightarrow$ OAA(-ATP)	-69.2 $\pm$ 20.3	-51.8 $\pm$ 25.9	-70.0 $\pm$ 20.9
Total ATP consumption	-157.8 $\pm$ 45.2	-119.5 $\pm$ 55.0	-163.9 $\pm$ 45.3
Energy producing			
Pyruvate cycling			
PEP $\rightarrow$ pyruvate( $\pm$ ATP)	+49.6 $\pm$ 16.1	+35.8 $\pm$ 23.0	+45.9 $\pm$ 18.0
$\beta$ -oxidation			
Fatty Acyl-CoA $\rightarrow$ Acetyl-CoA( $\pm$ NADH, $\pm$ FADH <sub>2</sub> )	+52.5 $\pm$ 10.2	+34.2 $\pm$ 15.7	+30.4 $\pm$ 20.7
TCA cycle			
citrate $\rightarrow$ $\alpha$ -KG( $\pm$ NADH)	+31.5 $\pm$ 6.1	+20.5 $\pm$ 9.4	+21.5 $\pm$ 10.2
$\alpha$ -KG $\rightarrow$ SUCC-CoA( $\pm$ NADH)	+31.5 $\pm$ 6.1	+20.5 $\pm$ 9.4	+21.5 $\pm$ 10.2
SUCC-CoA $\rightarrow$ SUCC( $\pm$ GTP)	+10.5 $\pm$ 2.0	+6.9 $\pm$ 3.1	+7.2 $\pm$ 3.4
SUCC $\rightarrow$ FUM( $\pm$ FADH <sub>2</sub> )	+21.0 $\pm$ 4.1	+13.7 $\pm$ 6.3	+14.3 $\pm$ 6.8
FUM $\rightarrow$ OAA( $\pm$ NADH)	+31.5 $\pm$ 6.1	+20.5 $\pm$ 9.4	+21.5 $\pm$ 10.2
Total ATP production	+228.1 $\pm$ 48.1	+152.2 $\pm$ 74.2	+167.8 $\pm$ 72.4
Consumption versus production	$P = 0.001$	$P = 0.013$	$P = 0.851$

Values are presented as mean  $\pm$  standard deviation. These calculations assume that lactate contributes 100% of the carbons required for gluconeogenesis. Further, the energetic comparison is in terms of ATP consumption and production; therefore, the reducing equivalents NADH and FADH<sub>2</sub> have been converted to their stoichiometric ATP values (3 and 2 ATP, respectively). PGA, phosphoglycerate; OAA, oxaloacetate;  $\alpha$ -KG,  $\alpha$ -ketoglutarate; SUCC-CoA, succinyl-coenzyme A; SUCC, succinate; FUM, fumarate; ATP, adenosine triphosphate; GTP, guanosine triphosphate; NADH, nicotinamide adenine dinucleotide; FADH<sub>2</sub>, flavin adenine dinucleotide, LBW, lean body weight.

etary macronutrient composition. Further studies are needed to verify this finding.

It should be noted that prior studies assessing the impact of carbohydrate restriction on hepatic glucose metabolism show that the main effect is a reduction in hepatic glucose output, predominantly via a reduction in glycogenolysis.<sup>31,32</sup> This is in contrast to the present study in which hepatic glucose production was similar between the dietary groups. The low-carbohydrate group was able to maintain hepatic glucose production at the levels observed for the weight-stable and low-calorie groups by increasing  $\text{GNG}_{\text{PEP}}$  to match the reduction in glycogenolysis. This observation is reminiscent of “hepatic auto-regulation” by which EGP remains unchanged in the setting of altered GNG or glycogenolysis because the two pathways tend to compensate for each other.<sup>28,33</sup> This finding may also be the result of the much larger intake of dietary protein in the low-carbohydrate group (~34%) as compared to prior studies (11%-15%),<sup>31,32</sup> possibly yielding an enhanced supply of gluconeogenic substrate.

The multitracer approach used in the present study allowed for the simultaneous assessment of both hepatic glucose production as well as the TCA cycle flux. Knowledge of metabolic flux through these pathways provided insight into the relationship between hepatic glucose and energy metabolism (Table 3). Energetic coupling of  $\text{GNG}_{\text{PEP}}$  and the TCA cycle was observed as a correlation between TCA cycle flux and PEPCK flux (Fig. 3), the metabolic pathway responsible for the delivery of substrate for GNG. It is, however, intriguing that the increased  $\text{GNG}_{\text{PEP}}$  in the low-carbohydrate group was not associated with increased TCA cycle flux (that is, energy production). Indeed, assuming net glucose synthesis predominated (i.e., alanine or other amino acids acted as the gluconeogenic substrate as opposed to lactate), energy production in the TCA cycle would be unable to meet the energetic demands of GNG in the low-carbohydrate group. This would suggest a greater reliance on sources of energy upstream from the terminal oxidation of fat in the TCA cycle in this group, possibly  $\beta$ -oxidation/ketogenesis. Prior data has demonstrated a relationship between  $\text{GNG}_{\text{PEP}}$  and ketogenesis: in general, ketogenesis parallels the rate of GNG under most circumstances.<sup>30</sup> Indeed, static measurements of ketone bodies were markedly higher in subjects undergoing carbohydrate restriction, indicating that the availability of acetyl-coenzyme A to 3-hydroxy-3-methylglutarate-coenzyme A synthase may have been greater. However, absolute rates of fatty acid delivery to liver as well as ketone body production were not measured, limiting our ability to further interpret the above findings.

Every attempt was made to equalize caloric intake between the two dietary restriction groups. However, a trend was noted toward decreased caloric intake in the group undergoing carbohydrate restriction (Table 2). It is possible that the differences observed between these two groups are solely the result of differences in caloric intake and weight loss. However, prior data obtained under weight-stable, isocaloric conditions showed similar changes in hepatic glucose metabolism in lean individuals.<sup>11</sup> Likewise, fractional and absolute glucose production was similar between the weight-stable and low-calorie group despite the difference in energy balance between the two. It should also be noted that the present study was not designed to determine the effectiveness of these two weight-loss diets in weight reduction, but was simply designed to assess hepatic metabolism under the differing macronutrient compositions during negative energy balance. Additionally, it was our desire to examine changes in hepatic metabolism under conditions likely to be encountered in a clinical setting; hence, dietary choices of the subjects were more varied than what would be encountered in a strict physiologic study.

In conclusion, we have shown that the sources from which endogenous glucose is produced are dependent upon dietary macronutrient composition. Carbohydrate restriction yields a decreased rate of glycogenolysis and an increased rate of GNG compared to calorie restriction. We have shown for the first time that this increased rate of hepatic GNG is the result of an increased rate of utilization of substrates like lactate and amino acids, but not glycerol. Additionally, the TCA cycle appears to be the energetic patron of  $\text{GNG}_{\text{PEP}}$ , as TCA cycle flux and PEPCK flux were highly correlated. Furthermore, it appears that the shift in glucose metabolism associated with a low carbohydrate diet leads to an increased contribution of energy generated outside of the TCA cycle to GNG. This shift is consistent with enhanced  $\beta$ -oxidation/ketogenesis, which could be beneficial in individuals with NAFLD due to enhanced disposal of hepatic triglyceride. These findings may explain, in part, the correlation between carbohydrate intake and severity of liver disease in individuals with NAFLD.<sup>7</sup> Understanding the alterations to cellular energetics that occur with simple macronutrient manipulation may be important for understanding and treating NAFLD and other metabolic disorders associated with obesity.<sup>34</sup>

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