Sources of Hepatic Glucose Production by ^{2}H$_2$O Ingestion and Bayesian Analysis of ^{2}H Glucuronide Enrichment

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The contribution of gluconeogenesis to hepatic glucose production (GP) was quantified after ^{2}H$_2$O ingestion by Bayesian analysis of the position 2 and 5 ^{2}H-NMR signals (H2 and H5) of monoacetone glucose (MAG) derived from urinary acetaminophen glucuronide. Six controls and 10 kidney transplant (KTx) patients with cyclosporine A (CsA) immunosuppressant therapy were studied. Seven KTx patients were lean and euglycemic (BMI = 24.3 ± 1.0 kg/m^2; fasting glucose = 4.7 ± 0.1 mM) while three were obese and hyperglycemic (BMI = 30.5 ± 0.7 kg/m^2; fasting glucose = 7.1 ± 0.5 mM). For the 16 spectra analyzed, the mean coefficient of variation for the gluconeogenesis contribution was 10% ± 5%. This uncertainty was associated with a mean signal-to-noise ratio (SNR) of 79:1 and 45:1 for the MAG H2 and H5 signals, respectively. For control subjects, gluconeogenesis contributed 54% ± 7% of GP as determined by the mean and standard deviation (SD) of individual Bayesian analyses. For the lean/normoglycemic KTx subjects, the gluconeogenic contribution to GP was 62% ± 7% (P = 0.06 vs. controls), while hyperglycemic/obese KTx patients had a gluconeogenic contribution of 68% ± 3% (P < 0.005 vs. controls). These data suggest that in KTx patients, an increased gluconeogenic contribution to GP is strongly associated with obesity and hyperglycemia. Magn Reson Med 60:517–523, 2008. © 2008 Wiley-Liss, Inc.

Key words: Bayesian analysis; gluconeogenesis; glucuronide; posttransplant diabetes; glycogenolysis

Plasma glucose levels are maintained over the daily feeding/fasting cycle through tight coordination of glucose appearance and disposal. During overnight fasting, glucose appearance is entirely accounted for by endogenous glucose production (GP) once digestive absorption has ceased. The occurrence of hyperglycemia after overnight fasting is a hallmark of defective glucose metabolism and indicates a mismatch between GP and plasma glucose clearance. Impaired glucose uptake by peripheral tissues and inappropriately high levels of hepatic GP can both contribute to this condition. Among other things, resolving these underlying defects could prove useful for guiding and evaluating interventions of fasting hyperglycemia, since some antihyperglycemic medications act by inhibiting GP while others function by improving whole-body glucose uptake.

Systemic glucose metabolism is typically assessed in the clinical setting by quantifying plasma glucose levels before and after an oral glucose load, but this approach does not provide any information about hepatic glucose metabolism. Assessment of GP with stable-isotope tracers could provide useful information to complement measurements of plasma glucose clearance; however, current methods are poorly suited for routine clinical studies. This is due to several factors, including high tracer cost, the requirement of lengthy infusion times for administration of the tracer, and extensive sample processing and analysis.

Deuterated water (^{2}H$_2$O) is an inexpensive tracer of gluconeogenesis that can be administered orally and is therefore well suited for routine clinical study procedures. The metabolic information is derived from the analysis of the ^{2}H-enrichment distribution of plasma glucose (1–7). Current gas chromatography-mass spectrometry (GC-MS) methods, while highly sensitive and therefore applicable to small blood samples, are labor-intensive and poorly suited for high sample throughputs. Analysis by ^{2}H NMR spectroscopy of the monoacetone glucose (MAG) derivative of plasma glucose is less sensitive but far simpler to the extent that both sample preparation and NMR spectroscopy can be extensively automated with current technologies. Conversion of plasma glucose to MAG has been successfully performed with a commercially available robotic chemical synthesizer (8), and high-resolution ^{2}H NMR spectra of the product can be obtained with automated tuning/shimming routines and minimal technical oversight. The contribution of gluconeogenic and glycogenolytic fluxes to GP can be automatically obtained from the ^{2}H NMR signal by Bayesian/Markov chain Monte Carlo (MCMC) analysis of the free induction decay (FID) (9). The analysis also provides estimates of uncertainty levels for the flux parameters. Data modeling studies indicate that these uncertainty levels are heavily influenced by the signal-to-noise ratio (SNR) of the ^{2}H NMR signal. For practical and safety reasons, human body water ^{2}H-enrichment from ^{2}H$_2$O is limited to 0.3% to 0.5%. To obtain ^{2}H NMR signals of suitable SNRs for Bayesian analysis requires a large sample size (∼30 ml of whole blood) and/or long NMR
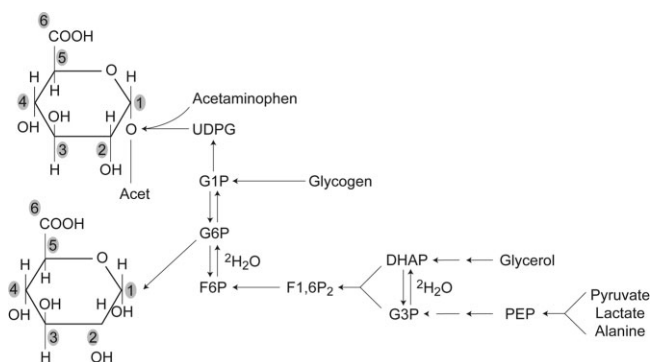
FIG. 1. Metabolic pathways evolved in fasting hepatic glucose metabolism after ^2H_2O and acetaminophen ingestion. Uridine diphosphoglucose (UDPG), glucose-1-phosphate (G1P), glucose-6-phosphate (G6P), fructose-1-phosphate (F6P), fructose-1,6-biphosphate (F1,6P), dihydroxyacetone phosphate (DHAP), phosphoenolpyruvate (PEP).

collection times, which limits the practicality and throughput of the method.

Due to rapid exchange between hepatic glucose-6-phosphate (G6P) and glucose-1-phosphate (G1P), the ^2H-enrichment distribution of plasma glucose is also reflected in hydrogens 1–5 of acetaminophen glucuronide (see Fig. 1) (10). In addition to eliminating the need to collect and rapidly process blood samples, the quantity of urinary glucuronide that is available for analysis is typically 5–10 times that of plasma glucose. Under these conditions, we anticipated that ^2H NMR spectra with sufficient SNR to yield reasonably precise estimates of gluconeogenesis by Bayesian analysis could be obtained without prohibitively long NMR collection times. The Bayesian method is ideal for analyzing data of this type. Experimental time constraints prevent repeated measurements upon the same sample to construct a more traditional statistical estimate of the uncertainty, i.e., the mean and standard deviation (SD) of repeated NMR spectra for a given sample. The Bayesian formulation of this problem uses prior knowledge regarding the shifts and amplitudes of the relevant resonances, as well as a model for the "nuisance" resonances that are not important for the metabolic measurements. A MCMC simulation makes multiple independent analyses of the posterior probability distribution that define the metabolic measurement. This provides an estimate of the most probable values of the metabolic parameters, as well as the uncertainty value for each parameter.

In this study the gluconeogenic contribution to GP after overnight fast was estimated by Bayesian analyses of the ^2H NMR signals of urinary acetaminophen glucuronide collected after overnight ingestion of ^2H_2O and acetaminophen followed by derivatization to MAG. The analysis was applied to healthy subjects and kidney transplant (KTx) recipients, including a subgroup diagnosed with posttransplant diabetes. The development of hyperglycemia and posttransplant diabetes is a frequent event following kidney transplantation (11,12). This may involve several factors, including increased adiposity, and glucose intolerance resulting from posttransplant weight gain, age, and ethnicity (11), as well as the direct action of immuno-

suppressive agents such as glucocorticoids and the calcineurin inhibitor, cyclosporine A (CsA), on insulin secretion and sensitivity (13,14). The activity of hepatic gluconeogenesis and its contribution to posttransplant hyperglycemia under these conditions is not known. In this study, sources of fasting GP derived by Bayesian analysis were collated with plasma metabolite and insulin sensitivity measurements.

MATERIALS AND METHODS

Human Studies

The study protocol was approved by the University Hospital of Coimbra Ethics Committee and was performed in accordance with the ethical standards laid down in the Helsinki Declaration. Subjects were studied in the Department of Endocrinology, Metabolism and Diabetes in the University Hospital of Coimbra after they provided informed consent. Three groups were studied as follows: Group I: six healthy, lean normoglycemic controls; Group II: seven lean, normoglycemic patients with a functional KTx with stable ongoing CsA-based therapy; and Group III: three obese and hyperglycemic patients with a functional renal transplant and CsA-based therapy. All KTx patients received 200–400 mg/day of CsA in conjunction with a low dose of corticoids (5 mg/day).

^2H_2O Administration and Sampling

Fasting was started at 20:00 (24-h clock) the previous evening after a normal dinner meal. At 01:00 and 03:00 the next day, patients were given two loading doses of ^2H_2O (2.5 g/kg of body water) to achieve a target body water enrichment of 0.5% ^2H_2O. Body water was assumed to be 60% of body weight for males and 50% of body weight for females. To improve palatability and decrease the risk of vertigo, each loading was diluted with bottled spring water to give a final enrichment of 35% ^2H_2O. Acetaminophen (1000 mg) was also taken at 03:00. At 06:00 the patients were asked to empty their bladder and all urine was collected from 06:00 to 08:00. Blood for clinical analysis was also drawn at 06:00 and 08:00.

Biochemical Analysis

Blood collected at 10 h of fasting was analyzed for plasma glucose, insulin, C-peptide, cholesterol, high-density lipoprotein (HDL) cholesterol, low-density lipoprotein (LDL) cholesterol, triglycerides, glycosylated hemoglobin, creatinine, and ureic nitrogen levels in the laboratories of University Hospital of Coimbra. Plasma free fatty acid (FFA) levels were determined independently, using an enzymatic colorimetric method kit from Wako Chemicals GmbH, in plasma collected at 12 h of fasting.

Sample Processing

Acetaminophen glucuronide from the 06:00 and 08:00 urine samples was derivatized to MAG as previously described (15,16). Briefly, urine was concentrated to 10% of its original volume and this portion was mixed with 9 volumes of 100% ethanol. The precipitate was centri-

fuged, the supernatant was evaporated to ~10 ml, and the pH was adjusted to 8.0–9.0 with 1 M NaOH. The supernatant was applied to an 18 × 1 cm diameter Dowex-1X8-200-acetate column. The column was washed with 35 ml of water, the glucuronide was eluted with 35 ml of 10 M acetic acid, and the acetic acid fraction was evaporated to dryness at 40–50°C. The residue was resuspended in 50 ml water and the pH was adjusted to 4.5–5.0. The acetaminophen glucuronide in this solution was derivatized to monoacetoneglucuronic lactone (MAGL). A total of 2000 units of β-glucuronidase (*H. Pomatia*; Sigma Chemical Company) were added and the solution was incubated at 45°C for 48 h. The solution was then passed through 10 ml Dowex-50X8-200 H^+ ion-exchange resin and evaporated to complete dryness at 40°C, resulting in the conversion of free glucuronic acid to glucuronolactone. The lactone was converted to MAGL by stirring for 24 h with 5 ml of anhydrous acetone and 0.1 ml concentrated H_2SO_4. The yellow solution was mixed with 5 ml water, the pH was adjusted to between 4 and 5 with 0.5 M Na_2CO_3, and the solution was evaporated to dryness at room temperature. MAGL was extracted from the salt products with 2–3 ml acetonitrile and reduced to MAG with lithium borohydride using tetrahydrofuran as the solvent (15).

NMR Spectroscopy

^{2}H NMR spectra were acquired at 11.75 T with a Varian Unity 500 Spectrometer equipped with a 5-mm broadband "switchable" probe with *z*-gradient (Varian, Palo Alto, CA, USA). MAG was dissolved in 90% acetonitrile/10% water, and shimming was performed on selected ^{1}H resonances of MAG. Proton-decoupled ^{2}H NMR spectra were acquired without field-frequency lock at 50°C using a 90° pulse and a 1.6-s acquisition time. Typically 6000-30,000 FIDs were collected (2.7–13.5 h total collection time). For the determination of urine water ^{2}H enrichment using the method of Jones et al. (17), spectra were obtained at 25°C using a 22.5° pulse and a 4-s acquisition time.

Bayesian/MCMC Analysis of Glucuronide Metabolic Fluxes

G6P derived from glycogenolysis has deuterium in position 2 only, as a result of exchange between G6P and fructose-6-phosphate (F6P), whereas G6P derived from any gluconeogenic precursor has deuterium incorporated in both positions 2 and 5 due to additional exchanges at the triose phosphate level. Hence, the ratios of ^{2}H enrichment in positions 5 and 2 (H5/H2) of G6P and plasma glucose reflect the fractional contribution of gluconeogenesis to fasting GP. The glucuronide moiety of acetaminophen is assumed to be derived from a hepatic uridine diphosphate (UDP)-glucose pool that is in isotopic steady state with hepatic G6P as a result of rapid G6P-G1P exchange (18). On this basis, glucuronide H5/H2 is identical to that of hepatic G6P, and the fractional contributions of glycogenolysis and gluconeogenesis to GP are given by the following equations:

Gluconeogenesis fraction

$$= \text{Glucuronide H5/Glucuronide H2} \quad [1]$$

Glycogenolysis fraction

$$= 1 - (\text{Glucuronide H5/Glucuronide H2}) \quad [2]$$

The H5/H2 parameter is thus obtained from the ratio of the glucuronide ^{2}H NMR signals for these positions. This parameter can be obtained in an operator-independent manner by Bayesian analysis of the FID, as previously described (9). The posterior probability distribution for the gluconeogenesis and glycogenolysis fractions was obtained from a MCMC simulation consisting of 50 independent Markov chains, from each of which 50 independent samples were drawn (a total of 2500 samples [9]). Estimates of the mean and SD for each flux parameter were derived from this sample set.

Data Analysis

Statistical differences were determined using one-way analysis of variance (ANOVA). $P < 0.05$ was considered to be statistical significant.

RESULTS

Plasma Metabolite and Hormone Levels

Table 1 provides a summary of plasma metabolite and insulin levels along with body mass index (BMI), age, and gender for all subjects. There were no significant differences in the plasma levels of insulin, C-peptide, cholesterol, HDL cholesterol, LDL cholesterol, triglycerides, and FFA between healthy controls and either of the KTx patient groups. All patients treated with CsA showed significant increases in plasma creatinine and ureic nitrogen relative to healthy controls. These observations likely reflect an impairment in kidney function due to the nephrotoxic effects of CsA (19). Hyperglycemic patients had significantly higher fasting plasma glucose levels on the morning of the study, as well as higher values of glycosylated hemoglobin. This indicates that the hyperglycemia in these patients was well established prior to the study. This group also had a significantly higher BMI and age average compared to both normoglycemic transplant patients and the healthy control group.

^{2}H-Enrichment Distribution of Body Water and Urinary Glucuronide

Steady-state body water enrichments were 0.53% ± 0.01% in the hyperglycemic obese KTx patients treated with CsA. This was significantly higher than that of controls (0.43% ± 0.01%, $P < 0.01$) and the normoglycemic KTx patients treated with CsA (0.48% ± 0.02%, $P < 0.05$). These results are consistent with a reduction in body water and an increased body fat fraction for the hyperglycemic post-transplant patients.

^{2}H NMR spectra of MAG derived from acetaminophen glucuronide generated well-resolved ^{2}H MAG resonances, with SNRs of the hydrogen 5 signal ranging from 20:1 to 60:1. Both the sample mass and the ^{2}H-NMR spectra derived from healthy controls were consistent with those recently reported in a study of overnight-fasted healthy controls (16). Enrichment of position 2, reflecting isotope

Table 1
Characteristics of the Different Groups Studied[†]

	Group I: Controls	Group II: Normoglycemic treated with CsA	Group III: Hyperglycemic treated with CsA
Gender (male/female)	3/3	7/0	3/0
Age (years)	39 ± 6	45 ± 2	58 ± 4*,***
BMI (kg/m²)	26.2 ± 1.6	24.3 ± 1.0	30.5 ± 0.7*,***
Glucose (mM)	4.8 ± 0.2	4.7 ± 0.1	7.1 ± 0.5*,****
Insulin (μUI/ml)	7.3 ± 2.9	10.7 ± 5.8	4.5 ± 0.2
C-peptide (nM)	2.6 ± 0.9	3.0 ± 0.7	2.7 ± 0.3
Cholesterol (mg/dl)	189.8 ± 14.8	217.7 ± 16.9	209.7 ± 18.7
HDL cholesterol (mg/dl)	51.8 ± 3.9	46.1 ± 5.6	65.0 ± 18.8
LDL cholesterol (mg/dl)	119.3 ± 13.3	141.6 ± 12.1	130.7 ± 29.5
Triglycerides (mg/dl)	179.2 ± 80.6	166.0 ± 25.3	171.7 ± 50.5
Glycosylated hemoglobin (%)	5.7 ± 0.2	5.6 ± 0.2	7.2 ± 0.4*
Creatinine (mg/dl)	1.0 ± 0.1	1.3 ± 0.1*	1.2 ± 0.0*
Ureic nitrogen (mg/dl)	14.8 ± 1.5	25.9 ± 2.4**	21.7 ± 3.2*
Plasma free-fatty acids (mmol/liter)	0.31 ± 0.07	0.39 ± 0.05	0.45 ± 0.12
HOMA-IR	1.7 ± 0.7	2.2 ± 1.2	1.4 ± 0.2

[†]Biochemical parameters corresponding to blood samples collected at 10-h fast and FFA plasma levels at 12-h fast. Results are shown as mean ± SEM.
*$P < 0.05$, relative to controls.
**$P < 0.01$ relative to controls.
***$P < 0.05$, relative to normoglycemic patients treated with cyclosporine.
****$P < 0.01$ relative to normoglycemic patients treated with cyclosporine.

equilibration with body water, was estimated to be 86% ± 12% that of body water. This indicates that the exchange between hydrogen 2 of hepatic G6P and that of body water via interconversion of G6P and F6P was essentially complete. This is in agreement with recent reports in which it was found that after 5 h of ^2H_2O incubation, plasma ^2H_2O and H2 were equilibrated (16). Other MAG ^2H-signals corresponding to positions 3, 4, and 5 of glucuronide were less intense, indicating dilution of ^2H-enrichment at these sites by G6P derived from unlabeled hepatic glycogen. As was previously observed with glucuronide enrichment distributions from ^2H_2O in healthy subjects and in Type 1 diabetic patients, the hydrogen 4 and 5 positions were enriched to similar levels (10,16). The ratio of hydrogen 3 enrichment relative to hydrogen 2 (H3/H2) was significantly lower than that of H5/H2 (0.26 ± 0.02 vs. 0.47 ± 0.05, $P < 0.005$ for controls; and 0.45 ± 0.04 vs. 0.62 ± 0.03, $P < 0.0002$ for posttransplant patients). The difference in enrichment between positions 3 and 5 could be the result of incomplete exchange of 1R-dihydroxyacetone phosphate (the precursor of G6P hydrogen 3) with body water deuterium via triose phosphate isomerase, possibly due to isotopic discrimination. If so, then hydrogen 3 enrichment would systematically underestimate the gluconeogenic contribution to GP. In addition, the difference in enrichment between hydrogens 3 and 5 could also reflect transaldolase activity, which catalyzes the exchange of the 456 carbon fragment of fructose-6-P with free glyceraldehyde-3-phosphate (20). Since transaldolase exchange results in the enrichment of unlabeled F6P (i.e., that derived from glycogenolysis) in positions 4, 5, and 6, this activity would contribute to hydrogen 5 enrichment of G6P independently of gluconeogenic flux, whereas hydrogen 3 enrichment would not be affected.

Gluconeogenesis and Glycogenolysis Contributions to GP by Bayesian/MCMC Analysis

A summary of the Bayesian/MCMC estimates of the fractional contributions of gluconeogenesis and glycogenolysis to GP for each subject, along with the SNR of the Fourier-transformed ^2H NMR spectrum of MAG, is shown in Table 2. Generally, the precision of the flux estimates, as determined by the size of the SDs, was highest for spectra with high SNRs (i.e., 40–60:1 for the position 5 resonance). The effects of improving the SNR of the ^2H NMR signal on the Bayesian output are illustrated in Fig. 2. The initial spectrum had SNRs of 40:1 and 21:1 for the hydrogen 2 and 5 signals, respectively. The resulting flux estimates had unacceptably high SDs, hence the NMR spectrum was reacquired with an increased number of FIDs, which resulted in an approximately twofold increase of the SNR. When these data were processed by Bayesian analysis, the SD range for the flux estimates was reduced by about one-half, in accord with the one-half reduction in spectrum SNR. The improved precision was also accompanied by a shift to a lower value for the fractional gluconeogenesis estimate. These experimental observations are highly consistent with the predicted effects of noise levels on the Bayesian output for gluconeogenic flux using simulated data (9). As noise levels were increased, the SD for gluconeogenic flux decreased while the estimate fractional gluconeogenic flux was shifted upwards of the theoretical value (9).

Our results also indicate that the precision of the Bayesian analysis is related to the relative intensities of the H5 and H2 signals independently of their individual SNRs. As seen in Table 2, ^2H NMR spectra derived from controls and from hyperglycemic KTx patients had comparable SNRs for H2 and H5. However, metabolic flux outputs from the spectra of the KTx patients had smaller SDs compared to

Table 2
Estimates of Fractional Gluconeogenesis and Glycogenolysis Contributions to GP as Determined by Bayesian Analysis and Represented as Mean ± SD of the Bayesian Posterior Probabilities (SDBPP) for Each Subject[†]

Subject	% GP from gluconeogenesis	% GP from glycogenolysis	H2 SNR	H5 SNR
Group I: controls				
AS	56 ± 10	44 ± 10	22.6	11.6
FP	55 ± 8	45 ± 8	66.4	33.1
IL	51 ± 7	49 ± 7	143.4	64.6
RC	43 ± 7	57 ± 7	69.7	35.7
RAC	55 ± 3	45 ± 3	130.9	63.5
TD	64 ± 9	36 ± 9	47.2	28.2
Average ± SD	54 ± 7	46 ± 7		
Group II: normoglycemic treated with CsA				
AB	54 ± 6	46 ± 6	80.2	46.5
AL	55 ± 6	45 ± 6	71.4	30.8
AM	69 ± 5	31 ± 5	80.6	52.8
AR	68 ± 4	32 ± 4	70.0	45.6
FO	70 ± 4	30 ± 4	55.1	44.0
LC	60 ± 5	40 ± 5	158.2	96.4
LS	58 ± 2	42 ± 2	86.2	51.7
Average ± SD	62 ± 7	38 ± 7		
Group III: hyperglycemic treated with CsA				
AM	69 ± 5	31 ± 5	30.9	22.2
AC	70 ± 4	30 ± 4	62.6	34.6
FS	65 ± 4	35 ± 4	95.2	59.6
Average ± SD	68 ± 3*	32 ± 3*		

[†]Also shown are the signal-to-noise ratios (SNRs) of the deuterium 2 and 5 signals (H2 and H5) of MAG as determined after Fourier transformation of the free-induction decay and applying of 1-Hz line-broadening using the NUTS NMR processing software.
*$P < 0.05$ relative to controls.

those of controls (5% ± 1% vs. 7% ± 2%). Aside from a higher H5/H2 intensity ratio (0.64 vs. 0.54), the spectra derived from the patients were similar to those of the control group in terms of signal linewidths and resolution. Our results suggest that when the gluconeogenic contribution is low (i.e., less than 50% of GP), the SNR of the H5 signal should be at least 60:1 to obtain reasonably low levels of uncertainty (i.e., a coefficient of variation of 10% or less). With higher gluconeogenic contributions (>65% of GP), a similar level of precision can be obtained from spectra with H5 SNRs of only ~30:1.
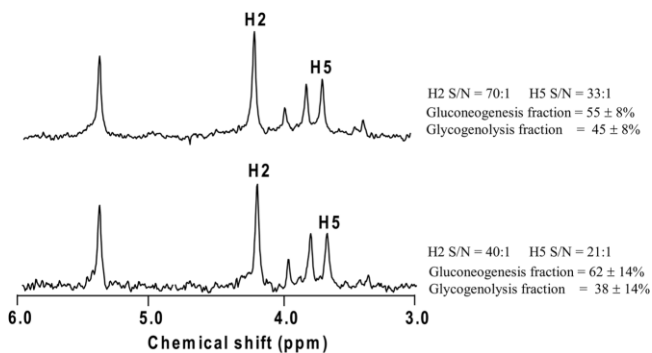
For the six control subjects, the mean contribution of gluconeogenesis and glycogenolysis to GP was 54% ± 7% and 46% ± 7%, respectively (Fig. 3). This is in good agreement with previous estimates of gluconeogenesis derived by the ^2H_2O method using ^2H NMR analysis of plasma glucose derived to MAG (3,4,10) as well as GC-MS analysis of plasma glucose (1,2). Our data are also in good agreement with values obtained by ^2H NMR analysis of MAG derived from urinary glucuronide (10,16).

The gluconeogenic and glycogenolysis contribution to GP for all the patients on CsA treatment was 64% ± 7%

FIG. 2. ^2H NMR spectra from a MAG sample prepared from a healthy control subject (FP) with the accumulation of 34,000 FIDs (bottom spectrum) and 66,500 FIDs (top spectrum). Also shown are the estimates for glycogenolysis and gluconeogenesis contribution to hepatic glucose production (±SD of the Bayesian posterior probabilities [SDBPP]) and the SNR of the position 2 (H2) and 5(H5) signal.

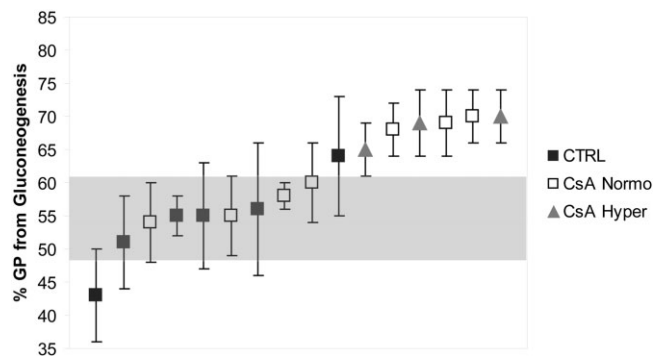

FIG. 3. Percentage of glucose production from gluconeogenesis for each subject studied represented as the mean ± SD of the Bayesian posterior probabilities (SDBPP). The dotted line corresponds to the average of the gluconeogenic contribution to GP of the subjects in the control group, and the gray zone to the upper and lower limits of the respective SD.

and 33% ± 7%, respectively ($P < 0.05$, relative to controls). The lean normoglycemic KTx patients had a wider range of gluconeogenic contributions to GP with a tendency towards a modestly increased gluconeogenic contribution to GP ($P = 0.06$ relative to controls). For the obese, hyperglycemic posttransplant patients, the gluconeogenic contribution to GP approached 70%. This estimate is somewhat higher than the value of 64% determined in healthy obese subjects by the ^2H_2O method, but is in excellent agreement with the value of 68% obtained from obese subjects with T2D (21). There was no significant relationship between the fractional contribution of gluconeogenesis and any of the reported clinical parameters.

DISCUSSION

Our work demonstrates the feasibility and some limitations of quantifying fractional gluconeogenesis from Landau's ^2H_2O method by Bayesian time-domain analysis of glucuronide enrichment in positions 5 and 2. As a result of the relatively large sample mass provided by glucuronide harvesting, ^2H NMR spectra with high SNR were obtained with a standard 11.75T spectrometer and 5-mm broadband probe. The accuracy and precision of the Bayesian analysis is largely dependent on the SNR of the ^2H NMR spectrum. For a given sample, improving the SNR proportionally reduces the SDs and improves the accuracy of the flux measurements, in accord with simulated noise data. Among other things, this is a valuable tool for objectively determining the relationship between the sample collection time and statistical power of the analysis.

From the patient's perspective, urinary glucuronide analysis is a more convenient and less invasive alternative to blood sampling. In addition, urinary glucuronide is far less susceptible than plasma glucose to degradation from delayed processing or storage above −80°C. In our experience, plasma glucose storage at −20°C for more than 1 month results in a marked decrease in glucose recovery as MAG, whereas urinary glucuronide is stable for at least 4 months under these conditions. However, there are several disadvantages to acetaminophen glucuronide analysis compared to that of plasma glucose. The derivatization of acetaminophen glucuronide to MAG involves several additional steps compared to that of plasma glucose, and is therefore far less amenable for routine automation. In principle, the Bayesian analysis could be applied to ^2H NMR data of alternative glucuronides such as menthol glucuronide, which can be more easily purified from urine and has fully resolved glucuronide ^2H signals in its original form (22). Compared to plasma glucose, the analysis of glucuronide ^2H enrichment is less informative since the hexose position 6 hydrogens are removed during the conversion of UDP-glucose to glucuronide. The absence of position 6 ^2H-enrichment information means that the contributions of phosphoenolpyruvate (PEP) and glycerol to gluconeogenesis, which relies on quantifying ^2H-enrichment in both positions 5 and 6 (1), cannot be resolved. Gluconeogenesis measurement cannot be performed if there are contraindications to acetaminophen, such as advanced cirrhosis, or if the subject is inherently unable to synthesize significant levels of glucuronide, such as patients with UDP-glucuronosyl transferase deficiency (23). There is additional uncertainty in the glucuronide sampling interval due to a 30- to 60-min lag time from its hepatic site of formation to reaching the bladder (24). Hepatic glucuronide and glucose may exhibit different enrichment patterns from certain tracers as a result of metabolic zonation (25). Glycogen metabolism and glucuronide synthesis are both located in the pericentral region of the hepatic lobule, whereas glucose secretion is predominantly in the periportal region (26,27). To the extent that there is a difference in the gluconeogenic contribution to G6P synthesis in periportal vs. pericentral hepatocytes, plasma glucose and urinary glucuronide could report different fractional contributions of gluconeogenesis to GP. For a small group of healthy subjects, plasma glucose and urinary glucuronide provided equivalent estimates of gluconeogenesis (10), but this has not been tested in larger subject populations or in patients with disorders of glucose metabolism.

Our results suggest that the gluconeogenic contribution to GP had a tendency to be modestly higher in lean normoglycemic KTx patients undergoing CsA-based immunosuppressant therapy compared to healthy controls, but this difference did not reach statistical significance in our study. Substrate selection for hepatic GP can be modified by changes in insulin action as well as by the availability of oxidizable substrates such as FFAs. Hepatic glycogenolysis is suppressed by insulin (28) and enhanced by glucagon (28), and is therefore sensitive to alterations in plasma insulin levels (28). FFAs can stimulate gluconeogenesis, possibly via augmentation of hepatic ATP and NADH production (29,30). They may also increase the rate of glycogenolysis by impairing its suppression by insulin (30). While CsA has been reported to reduce pancreatic insulin secretion (31), possibly by inducing β-cell apoptosis (32), neither the normo- nor the hyperglycemic CsA patients of our study had significantly different plasma insulin levels compared to healthy controls, at least under basal fasting conditions. Therefore, our data suggest that the observed alterations in GP sources are not due to defective insulin secretion during fasting. Moreover, homeostasis model assessment of insulin resistance (HOMA-IR) indices for the three subject groups were similar, suggesting that insulin sensitivity, as determined by this method, was not significantly modified in KTx patients. While plasma FFA levels were not significantly different between healthy, lean/normoglycemic and obese/hyperglycemic KTx patients, there was a tendency for FFA levels to be higher in the KTx patients, with the highest values found for the obese group.

In summary, for KTx patients undergoing CsA immunosuppressant therapy, the gluconeogenic contribution to GP is significantly increased in the setting of posttransplant diabetes. This metabolic alteration is most strongly associated with adiposity and BMI, but may also be associated with elevated plasma FFA. There was no association between the gluconeogenic contribution to GP and either fasting plasma insulin levels or insulin sensitivity as determined by HOMA-IR. The Bayesian analysis of urinary glucuronide ^2H-enrichment allows an objective evaluation of hepatic GP sources to be easily integrated with other simple and practical measurements of human glucose homeostasis.

ACKNOWLEDGMENTS

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