Increased Hepatic Fructose 2,6-Bisphosphate after an Oral Glucose Load Does Not Affect Gluconeogenesis*

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The generally accepted metabolic concept that fructose 2,6-bisphosphate (Fru-2,6-P₂) inhibits gluconeogenesis by directly inhibiting fructose 1,6-bisphosphatase is based entirely on in vitro observations. To establish whether gluconeogenesis is indeed inhibited by Fru-2,6-P₂ in intact animals, a novel NMR method was developed using [U-13C]glucose and 2H₂O as tracers. The method was used to estimate the sources of plasma glucose from gastric absorption of oral [U-13C]glucose, from gluconeogenesis, and from glycogen in 24-h fasted rats. Liver Fru-2,6-P₂ increased 10-fold shortly after the glucose load, reached a maximum at 60 min, and then dropped to base-line levels by 150 min. The gastric contribution to plasma glucose reached ∼50% at 30 min after the glucose load and gradually decreased thereafter. Although the contribution of glycogen to plasma glucose was small, glucose formed from gluconeogenesis was substantial throughout the study period even when liver Fru-2,6-P₂ was high. Liver glycogen repletion was also brisk throughout the study period, reaching ∼30 μmol/g at 3 h. These data demonstrate that Fru-2,6-P₂ does not inhibit gluconeogenesis significantly in vivo.

Plasma glucose is preserved by gluconeogenesis after exhaustion of glycogen stores during a moderate fast. Following an oral glucose load, gluconeogenesis is thought to be modulated by allosteric regulation of fructose-1,6-bisphosphatase (1). This is an eminently satisfying model because a key regulatory site in glycolysis and gluconeogenesis occurs at level of fructose-2,6-P₂ and fructose-1,6-P₂ (Fig. 1). Phosphofructokinase-1, the glycolytic enzyme, is potently activated by fructose-2,6-P₂, whereas fructose-1,6-bisphosphatase, the gluconegenic enzyme, is thought to be inhibited by this same effector molecule (2, 3). Thus, by regulating the activities of phosphofructokinase-1 and fructose-1,6-bisphosphatase in a reciprocal manner, Fru-2,6-P₂ is thought to serve as an elegant regulator of glucose usage/production by the liver after an oral glucose load.

This generally accepted model is based on kinetic analysis of fructose-1,6-bisphosphatase in vitro, which shows that Fru-2,6-P₂ competes with Fru-1,6-P₂ for the active site of fructose-1,6-bisphosphatase and that both molecules have similar affinity constants, 1–5 μM (4–6). However, the in vivo concentrations of Fru-1,6-P₂ in fasted and fed livers are 20 and 35 μM, respectively, whereas those of Fru-2,6-P₂ are 1 and 8 μM, respectively (7–9). This suggests that it would be difficult for Fru-2,6-P₂ to have a significant direct effect on fructose-1,6-bisphosphatase activity in vivo based simply upon concentration differences. Some evidence has been presented that suggests Fru-2,6-P₂ is not a potent inhibitor of gluconeogenesis in intact animals. For example, Kuwajima et al. (10) reported continual production of liver glycogen in sucrose-fed rats despite high levels of Fru-2,6-P₂, and Hue and Bartrons (11) observed stimulated glucose production by glucagon in isolated hepatocytes regardless of the levels of Fru-2,6-P₂. Levels of Fru-2,6-P₂ have also been manipulated by recombinant adeno-virus overexpression of the bifunctional enzyme phosphofructo-2-kinase/fructose-2,6-bisphosphatase (the enzyme that catalyzes both synthesis and degradation of Fru-2,6-P₂) in mice and rats in vivo (12–14). Here, increased hepatic Fru-2,6-P₂ in vivo actually resulted in increased glycogen synthesis from [1-13C]glucose via the indirect pathway (14), thereby suggesting that activation ofglycolysis by Fru-2,6-P₂ is more important than inhibition ofglyconeogenesis in vivo. The relationship between glucose production by liver and hepatic Fru-2,6-P₂ after an oral glucose load typical of that used in a tolerance test (OGTT) is even less well defined. It has also been shown that hepatic glycogen output in 24–30-h fasted rats is not suppressed after an oral glucose load (15, 16), but [Fru-2,6-P₂] was not measured.

Continual production of glucose by the liver may play a role in diabetes, and so a simple method to detect persistent gluconeogenesis after an oral glucose load or after administration of a hypoglycemic agent may assist in therapy of this epidemic disease (17). Numerous methods to monitor sources of plasma glucose have been described. Classical metabolite balance studies across the liver or across the entire splanchnic circulation are not optimal because measuring hepatic glucose production requires access to the portal vein, and analysis of splanchnic glucose balance is limited by uncertainties about glucose uptake in the gut. Detection of hepatic glycogen by 13C NMR offers direct, noninvasive, and serial measurements of hepatic glycogen mobilization, but the method is not widely available and is difficult to apply to small animals without prelabeling of hepatic glycogen. Sophisticated isotope tracer studies rely on

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† The abbreviations used are: OGTT, oral glucose tolerance test; J-HSQC, J-resolved heteronuclear single quantum coherence; MAG, monooctate glucose.
incorporation of \(^{13}\)C gluconeogenic precursors into plasma glucose, incorporation of deuterium or tritium from body water into specific sites in plasma glucose, or the redistribution of \(^{13}\)C label within plasma glucose molecules (18). These methods assume metabolic steady state and require metabolic models of variable sophistication.

The present study had two purposes: 1) to distinguish the sources of plasma glucose (gastric absorption, gluconeogenesis, glycogenolysis) in fasted rats during an oral glucose load using a simple combination of \(^{13}\)C and \(^2\)H tracers; and 2) to determine whether hepatic Fru-2,6-P\(_2\), elevated after an oral glucose load, alters the contribution of gluconeogenesis to plasma glucose. Here, a combination of \(^1\)H and \(^J\)-resolved heteronuclear single quantum coherence (J-HSQC) spectroscopy was used to evaluate the contributions of hepatic versus gastric glucose over time following an oral glucose load, whereas \(^2\)H enrichments at the H5 versus H2 positions of plasma glucose as determined by \(^2\)H NMR gave a direct measure of the glycogenolysis versus gluconeogenesis contributions to plasma glucose. The data show that gluconeogenesis is not inhibited significantly in vivo following a glucose load in 24-h starved rats and that gluconeogenesis remains active even when Fru-2,6-P\(_2\) is elevated 10-fold.

**MATERIALS AND METHODS**

**Protocol**—The study was approved by the Institutional Animal Care and Use Committee at the University of Texas Southwestern Medical Center. Male Sprague-Dawley rats weighing 100–140 g (120 ± 9 g) were fasted for 24 h with free access to water. To initiate the study (t = 0), a bolus of glucose (2 g/kg; enriched with \([U-^{13}\)C\]) was administered by oral gavage, and 2 ml of \(^2\)H\(_2\)O (99.9%; Cambridge Isotopes) along with a few grains of sodium bicarbonate (NaHCO\(_3\)). 2H NMR spectra were collected at 50 °C using a Varian INOVA 14.1 T spectrometer (Varian Instruments, Palo Alto, CA) equipped with 3-mm broadband probe tuned to \(^3\)H (92.1 MHz). Shim...
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Glucose turnover was determined by 

1H NMR as the fraction of the glucose that originated from the stomach (Fgsugar) was (g x h)/0.05 or 48%. Because total plasma glucose measured 15.8 mM, then 7.6 mM originated from oral glucose.

Plasma Glucose Originating from Glycogenolysis or Gluconeogenesis: 2H NMR Analysis—From the combined information in the 1H and 13C NMR spectra, one can conclude that about half of plasma glucose arose from gastric absorption of oral glucose. The remaining glucose resulted from either degradation of glycogen or gluconeogenesis, and this is reported in the 2H NMR spectrum of MAG (23) as

Glucose from glycogenolysis = (H5/H2) (Eq. 3)
Glucose from glycogen = 1 - (H5/H2) (Eq. 4)

Integration of 13C and 1H Trace Observations—Together, these spectra allow a measure of the sources of plasma glucose.

Fraction from the stomach = Fgsugar = g/h/0.05 (Eq. 5)
Fraction from glycogenolysis = Fglycogenolysis

= (H5/H2) x (1 - g/h/0.05) (Eq. 6)
Fraction from glycogen = Fglycogen

= (1 - (H5/H2)) x (1 - g/h/0.05) (Eq. 7)

where Fgsugar + Fglycogenolysis + Fglycogenolysis = 1. For example, the 1H spectrum in Fig. 5 indicates that the contribution of glycogen to hepatic glucose production was 1 - (H5/H2) = 0.18. Thus, the fraction of plasma glucose that originated from glycogen 30 min after the oral glucose load was (0.18)(1-0.48) or 9.4%. The assumptions that underlie this calculation are reviewed under “Discussion.”

Metabolite Assays—Freeze-clamped liver was homogenized in 0.1 M NaOH solution, and the supernatant was incubated at 80 °C for 5 min after centrifugation. Fructose-6-phosphate phosphotransferase to Fructose-2,6-P2 was assayed by Van Schaftingen et al. (24). Liver glycogen was assayed enzymatically (25), and blood glucose was measured using a HemoCue glucose analyzer (HemoCue AB, Angelholm, Sweden).

Statistical Analysis—The data are expressed as means ± S.D. using Microsoft Excel. Linear regression analysis for standard curves was also performed with the same program.

RESULTS

Influence of an Oral Glucose Load on Liver Fructose-2,6-P2 and Other Metabolites—The influence of the oral glucose load on hepatic glycogen, Fructose-2,6-P2, and plasma glucose is shown in Fig. 4. Plasma glucose peaked at 30 min after the glucose load and decreased gradually thereafter. Liver glycogen was low in 24-h-fasted animals but was gradually replenished throughout the study period, reaching a maximum of 31.5 μmol/g at 3 h. This was a significant increase compared with time zero but considerably below the normal level in fed rats (~200 μmol/g) (26). Fructose-2,6-P2 also increased significantly after the glucose load (Fig. 4). The initial concentration prior to glucose administration was 0.4 ± 0.3 nmol/g of liver, but this was followed by a ~10-fold increase by 30 min after the glucose load. The time courses of hepatic Fructose-2,6-P2 and plasma glucose were roughly parallel. Finally, the amount of 2H2O in plasma water (1.6–1.8%) did not change significantly throughout the study period (Fig. 4). The plasma glucose concentration was somewhat higher than expected for a typical OGTT. Earlier studies (27) used indwelling venous catheters for blood drawing in conscious animals, in contrast to our study, in which the liver was removed from anesthetized animals. Therefore, in a smaller group of 24-h-fasted rats, glucose (2 g/kg body weight) containing natural abundance levels of 13C1 was dissolved in H2O and administered by oral gavage, 2 ml of 2H2O was injected into the intraperitoneal cavity, and blood was withdrawn at various time points via the tail vein in the complete absence of anesthesia. In this group, plasma glucose peaked at 7.5 mM at 30
Glucose injection of 2H2O.

Satellite wings in these spectra were typically /H11011

Spectra

High resolution 1H NMR spectra of monoacetone glucose showed a fully resolved H1 resonance with well resolved 13C satellite peaks (not shown). The total areas of the 13C multiplets of the respective C-1, C-2, and C-5, and thus it represents [U-13C]glucose from oral glucose that had been taken either to the level of a triose or the citric acid cycle before being resynthesized to glucose. The singlet (S) represents glucose isotopomers with 13C only at C-1. This peak has contributions from naturally abundant glucose and from singly enriched isotopomers derived from the citric acid cycle. As illustrated in Fig. 2, the Q fraction in these spectra was maximal at 30 min and decreased gradually thereafter, whereas the D12 fraction was minimal at 30 min and increased thereafter.

Intraperitoneal injection of 2H2O resulted in rapid equilibration (within 30 min) of 2H into plasma water to a level of 1.6–1.8% excess enrichment (Fig. 4). 2H incorporation into glucose has been reported to occur at various steps along the gluconeogenic and glycogenolytic pathways (Fig. 1), and the 2H spectrum of MAG provides a convenient readout of those exchanges. In the 24-h fasted animals prior to oral glucose, an H5/H2 ratio of 0.79 ± 0.17 indicated that 79% of all plasma glucose containing 2H was produced via gluconeogenesis, whereas the remaining ~21% came from glycogen. Somewhat surprisingly, the deuterium spectrum did not change significantly after the oral glucose load. The H5/H2 ratio remained relatively constant (0.8 ± 0.2) throughout the study period (Fig. 3, inset), again suggesting that ~80% of all plasma glucose containing 2H was produced via gluconeogenesis. That portion of plasma glucose arising from the oral load would not be detected by 2H NMR in this experiment unless glucose cycling between plasma and liver was active. The 2H NMR spectra of MAG derived from liver glycogen isolated at 120–180 min also confirmed that hepatic glycogen was derived via gluconeogenesis (H5/H2 = 0.90 ± 0.05).

Sources of Plasma Glucose—The contributions of oral glucose, gluconeogenesis, and glycogenolysis to plasma glucose pools are summarized in Fig. 5. At 30 min, about half of the plasma glucose originated from oral glucose, and its contribution decreased gradually thereafter. The gluconeogenic contribution to plasma glucose was substantial throughout the study period (~5.5–6.7 mM), whereas the contribution of glycogenolysis to plasma glucose was ~2 mM and constant throughout the study period.

DISCUSSION

The purpose of this study was to measure sources of plasma glucose after a gastric load and to determine whether the contribution of gluconeogenesis to hepatic glucose production is suppressed by increased hepatic Fru-2,6-P2. Based on early in vitro reports that Fru-2,6-P2 inhibits fructose-1,6-bisphosphatase, it is now standard teaching (30) that this inhibition holds true in vivo as well. The 2H NMR results of Fig. 3 show that gluconeogenesis continues even in the presence of substantially increased levels of hepatic Fru-2,6-P2 (Fig. 4), and thus inhibition of gluconeogenesis by Fru-2,6-P2 does not appear to be important in vivo. This result is consistent with a recent report showing that increasing hepatic levels of Fru-2,6-P2 by overexpression of the kinase isoform of phosphofructo-2-kinase resulted in increased glycogen synthesis from [1-13C]glucose via the indirect pathway (14). This result indicates that Fru-2,6-P2 stimulates glycolysis in vivo but does not inhibit production of glycogen via the indirect pathway.

Effects of an Oral Glucose Load on the 1H, J-HSQC, and 2H Spectra—High resolution 1H NMR spectra of monoacetone glucose showed a fully resolved H1 resonance with well resolved 13C satellite peaks (not shown). The total areas of the 13C satellite wings in these spectra were typically ~3–4% or ~7–8% for rats given an oral glucose load containing either 5% [U-13C]glucose or 10% enriched glucose, respectively. The H1 projection of typical J-HSQC spectra is shown in Fig. 2. Here, the peak labeled quartet (Q) is the dominant multiplet. At a minimum, this glucose isotopomer must be enriched in C-1, C-2, and C-5, and thus it represents [U-13C]glucose from oral glucose that has not undergone metabolism. The doublet (D12) is the signal from glucose isotopomers with 13C at C-1 and C-2 representing [1,2,3-13C3]- or [1,2-13C2]glucose. D12 reflects oral glucose that had been taken either to the level of a triose or the citric acid cycle before being resynthesized to glucose. The doublet (D12) represents glucose isotopomers with 13C only at C-1. This peak has contributions from naturally abundant glucose and from singly enriched isotopomers derived from the citric acid cycle. As illustrated in Fig. 2, the Q fraction in these spectra was maximal at 30 min and decreased gradually thereafter, whereas the D12 fraction was minimal at 30 min and increased thereafter.

Plasma glucose reached higher levels than expected at 30 min post-oral glucose and did not return to pre-oral glucose
levels even at 180 min. Thus, the curve shown in Fig. 4 is somewhat elevated compared with that observed for an OGTT in conscious animals (27). Dohm et al. (31) reported that both methoxyflurane (inhalation) and Innovar (intramuscular injection) induce glycogenolysis in rats, with the effect being higher in fed animals than in fasted animals. Thus, the somewhat enhanced levels of plasma glucose found here during the OGTT likely resulted from increased glycogenolysis during exposure of the animals to ether prior to collection of plasma glucose. Increased glycogenolysis, however, does not detract from the primary conclusion of this study because any glycogen degraded in response to anesthesia was synthesized de novo during the OGTT (see Fig. 4, bottom panel). The observation that the H5/H2 ratio in MAG derived from liver glycogen at 180 min was similar to that of plasma glucose demonstrates that gluconeogenesis contributed equally to both. Thus, any glycogenolysis that may have occurred during the short period of anesthesia would have reported the same H5/H2 ratio.

The NMR method reported here for detecting persistent gluconeogenesis requires three reasonable assumptions. First, all glucose isotopomers enriched in 13C at carbons 1, 2, and 5 reflect only [U-13C]glucose from oral glucose. The chance that this group of isotopomers could arise from [U-13C]glucose re-synthesized after metabolism to a triose is small. If the entire oral glucose load was metabolized to a triose and resynthesized, the chances of [U-13C]glucose reforming would be at best 0.25%, if oral glucose consisted of 5% [U-13C]glucose, and at most 1%, if oral glucose consisted of 10% [U-13C]glucose. This lower limit would be reduced even further in vivo because of dilution of the triose pools by endogenous gluconeogenic precursors. It was also assumed that plasma glucose only arises from three possible sources: gastric absorption, liver glycogenolysis, or liver gluconeogenesis. This assumption excludes other organs as origins of endogenous glucose production. The kidney is also a gluconeogenic organ, but its contribution to blood glucose is not considered significant except during unusual circumstances such as prolonged fasting or acidosis (32, 33). A third assumption was that the 2H labeling in glucose reflected recent glucose synthesis. Within 30 min after an oral glucose load, glucose turnover increases from about 15 mg/kg/min at base line to more than 50 mg/kg/min (15). Although glucose turnover was not measured in this study, the gluconeogenic contribution to plasma glucose at 30 min after the oral glucose and 2H2O loads was already significant. Consequently, the gluconeogenic contribution at later time points must also reflect the metabolic activity of each individual time point rather than an accumulated result over the previous periods. Further evidence for rapid glucose turnover is shown by the rapid decline in [U-13C]glucose (originating in the oral load) observed in plasma (Fig. 5), consistent with the high turnover of glucose reported in earlier studies of oral glucose loading in rats (15).

Perturbation of glucose metabolism with an oral load continues to attract interest because it is thought that the post-prandial state accounts for much of the duration of hyperglycemia in patients with diabetes, and because the OGTT is a standard method for diagnosis of abnormalities in carbohydrate metabolism. Despite intensive work, the fate of oral glucose remains surprisingly controversial. Reports of the cumulative appearance of oral glucose in plasma have varied from about 70% (34, 35) to nearly 100% (36, 37), and the maximal rates of glucose appearance have varied about 2-fold. Livesey et al. (36) reported a study of glucose kinetics in 12-h fasted humans after an oral glucose load, using stable isotopes and mass spectrometry to detect gastric absorption of [13C6]glucose oral glucose. They report that the contribution of hepatic glu-
cose to plasma glucose began to decrease shortly after the oral load from an initial value of near 5 mM to a nadir of 1.1 mM (36), suggesting that gluconeogenesis may be more highly regulated in humans after an oral glucose load. However, that study differed from ours in two respects. First, the contribution of hepatic glucose production to plasma glucose was not measured directly but rather was obtained by difference between total plasma glucose (measured analytically) and plasma glucose derived from the oral load (measured as M/H110016 by mass spectrometry). It is important to point out that this method cannot distinguish hepatic glucose production from glycogenolysis versus gluconeogenesis, and it is well known that substantial liver glycogen remains after a short 12-h fast in humans. In our experiments with rats, liver glycogen was low after a 24-h fast, and gluconeogenesis was measured directly using the combined 13C and 2H tracers. This has allowed us to demonstrate that gluconeogenesis is not altered after an oral glucose load in this animal model. These study differences emphasize the need to apply simple tracer methods such as the method demonstrated here to assess post-prandial glucose metabolism in humans. The method reported here could easily be applied during an OGTT in humans. A dual isotope technique commonly used for the measurement of oral or endogenous glucose appearance (38–40) requires intravenous infusion at a constant rate of glucose tracer ([3H]glucose) and a glucose load of the other tracer ([14C]glucose). In comparison, the approach presented here requires only ingestion of [U-13C]glucose (and 2H2O), yet provides detailed information about the sources of plasma glucose. Such a study in humans could be especially timely because persistent hepatic glucose production after a meal may be an attractive therapeutic target for diabetic patients (17).

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FIG. 4. Liver glycogen (μmol/g wet weight), liver Fru-2,6-P2 (nmol/g wet weight), plasma glucose (mM), and 2H enrichment in plasma water as a function of time after an oral glucose load in 24-h fasted rats. At t = 0, animals were given an oral glucose load and injected intraperitoneally with a bolus of 2H2O. Animals were then sacrificed at each time point to collect the data shown. Each point represents the mean ± S.D. for three measurements.

FIG. 5. Changes in contributions of oral glucose (open bar), gluconeogenesis (cross-hatched), and glycogenolysis (shaded) to plasma glucose based upon analysis of the C-1 projections of J-HSQC and 2H NMR spectra. The height of each bar represents the mean ± S.D. for 3–5 measurements.
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