Glucose Production Pathways by $^2$H and $^{13}$C NMR in Patients With HIV-Associated Lipoatrophy

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Patients with HIV taking protease inhibitors were selected for the presence (five subjects) or absence (five subjects) of lipoatrophy. Following an overnight fast, subjects were given oral $^2$H$_2$O in divided doses (5 mL/kg body water), [U-$^{13}$C$_3$] propionate (10 mg/kg), and acetyaminophen (1000 mg). Glucose (from plasma) or acetyaminophen glucuronide (from urine) were converted to mono-acetone glucose for $^2$H NMR and $^{13}$C NMR analysis. The fraction of plasma glucose derived from gluconeogenesis was not significantly different between groups. However, flux from glycerol into gluconeogenesis relative to glucose production was increased from 0.20 ± 0.13 among subjects without lipoatrophy to 0.42 ± 0.12 (P < 0.05) among subjects with lipoatrophy, and the TCA cycle contribution was reduced. Lipoatrophy was associated with an abnormal profile of glucose production as assessed by $^{13}$C and $^2$H NMR of plasma and urine. Magn Reson Med 51:649–654, 2004. Published 2004 Wiley-Liss, Inc.†

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The lipodystrophies are a heterogeneous group of metabolic disorders characterized by subcutaneous fat wasting, redistribution of body fat, and abnormal glucose metabolism. One of the most distressing complications of otherwise successful HIV therapy is the loss of subcutaneous fat, also termed lipoatrophy (LA), which is one of the hallmarks of HIV-associated lipodystrophy. Sophisticated studies of body fat distribution and function (1,2) suggest that LA may be a marker of metabolic defects, since the amount and distribution of adipose tissue is a major determinant of glucose metabolism via its effects on insulin sensitivity. However, little is known about metabolism in specific pathways among patients with lipodystrophy. HIV protease inhibitors cause hypertriglycerideremia even in healthy HIV-negative volunteers (3). Since hypertriglycerideremia and accelerated lipolysis drive gluconeogenesis (4–6), the direct effects of protease inhibitors on adipose tissue metabolism may also drive flux in gluconeogenic pathways. In normal volunteers after an overnight fast, about 50% of plasma glucose is derived from gluconeogenesis, with the balance originating from glycogen (7,8), but patients with type 2 diabetes derive significantly more glucose from gluconeogenesis. If LA is a marker of insulin resistance and excessive triglyceride turnover, then increased gluconeogenesis relative to glycogenolysis would be anticipated.

Recently developed techniques based on the oral administration of $^2$H$_2$O may be used to evaluate the origins of plasma glucose by measuring enrichment in $^2$H at position 2 compared to position 5 of glucose (9,10). Intermediary metabolism has been examined (usually by mass spectrometry) in a wide range of subjects, including healthy adults (4), low birthweight infants (11), children (12), lactating women (13), obese subjects (14), patients with cirrhosis (15), and patients with or at risk for diabetes (6,16). $^{13}$C tracer methods have been used to probe other aspects of gluconeogenesis such as net production of glucose from the TCA cycle in normal volunteers (17) and among subjects with common disorders such as cancer (18) and diabetes (19).

These convenient methods, requiring only samples of blood or urine, have not been applied to patients with HIV perhaps because of perceived technical limitations. NMR detection of isotopes in glucose offers major advantages over mass spectrometry, but because of limited NMR sensitivity, relatively large blood volumes are required. An alternative might be to use the “chemical biopsy” technique of oral administration of acetyaminophen followed by collection of acetyaminophen glucuronide in the urine to measure the isotopic labeling pattern of intrahepatic glucose, but glucuronidation of acetyaminophen may be impaired among HIV-positive (HIV+) subjects on highly active antiretroviral therapy (HAART) (20,21). Rapid oral administration of $^2$H$_2$O is often complicated by nausea or vertigo, and it is not known if this method is acceptable to HIV+ subjects, particularly those with nausea on HAART.

This study was designed to determine whether adequate $^2$H and $^{13}$C NMR spectra could be obtained from plasma and urine of HIV+ subjects in an acceptable outpatient protocol, and to test whether the contribution of gluconeogenesis relative to total glucose production is increased among patients with LA, similar to the profile of type 2 diabetes.
MATERIALS AND METHODS

Materials

We obtained 70% 2H2O and 99% [U-13C3]propionate as the sodium salt from Cambridge Isotopes (Andover, MA). Acetaminophen (Tylenol, 500 mg) capsules were purchased over the counter. Other common reagents were purchased from Sigma (St. Louis, MO).

Protocol

The protocol and consent form were approved by the Institutional Review Board of VA North Texas Health Care System. Written informed consent was obtained prior to patient enrollment and prior to the day of the study. Five HIV+ subjects without lipoatrophy and five HIV+ subjects with lipoatrophy were studied. HIV+ patients with lipoatrophy (“patients with LA”) were selected by the staff of the Infectious Disease Clinic for prominent peripheral and facial subcutaneous fat wasting appearing after the initiation of PI therapy using criteria defined previously (2). Each patient with LA also reported appearance changes. HIV+ patients on HAART in the same clinic were approached for participation if they were on PI therapy for more than 6 months without apparent lipoatrophy (“patients without LA”). No subject had used alcohol for at least 6 months prior to enrolling, and no subject had been treated for an opportunistic infection or other major change in medical condition for 6 months prior to participation.

Following an early evening meal, subjects fasted overnight and reported to the Clinical Research Unit between 7:00 and 8:00 AM. Prescribed medications were taken on schedule with water. Each subject initially received oral acetaminophen, 1000 mg. Over the next 90 min, oral [U-13C3]propionate and 2H2O were given in three divided doses. The total propionate load was [U-13C3]propionate (10 mg/kg body weight, in rapid-release gel capsules). The administration of 2H2O was identical to the protocol described by Landau et al. (10) and Nielsen et al. (16), except that the 2H2O was given over 90 min instead of 2 or 4 hr. Briefly, all subjects received 2H2O (5 g/kg body weight, calculated as 60% of body weight for men and 50% of body weight for women). Half of the subjects reported no adverse effects throughout the protocol, but the other five reported disequilibrium with mild vertigo, presumably secondary to the 2H2O. All subjects denied overt nausea, but two subjects vomited abruptly.

Two hours after the final tracer administration, the subjects voided and the first 25 mL blood sample was drawn (hour 2 sample). Additional blood samples were drawn 3 hr and 4 hr after the final tracer administration (total blood collection was 75 mL), and urine was collected again at 4 hr. By the time of the last blood draw between noon and 1:00 PM, all subjects reported feeling normal. Each subject ate lunch and remained in the Clinical Research Unit for 1 hr after the final blood draw.

Sample Processing

Blood was collected in heparinized tubes, centrifuged immediately, deproteinized by mixing with perchloric acid, cooled on ice, and transferred immediately to the NMR lab, where it was centrifuged, neutralized with KOH, centrifuged, and freeze-dried. The monoacetone derivative of glucose (MAG) was synthesized as described by Landau et al. (10) and by Burgess et al. (22). Acetaminophen glucuronide in the urine was also converted to MAG (23). MAG was resuspended in 150 μL of HPLC-grade acetonitrile and 20 μL of deuterium-depleted H2O, loaded into a 3 mm NMR tube, and scanned at 14.1 T on a Varian Inova spectrometer. Plasma water 2H enrichment was measured as described previously (24).

2H NMR spectra from plasma MAG were collected on a deuterium-dedicated probe without field-frequency lock. Proton-decoupled 13C NMR spectra were collected with an additional 20 μL of deuterated acetonitrile for lock using a 3 mm broadband probe. 13C NMR tracer results were obtained from MAG derived from the urinary glucuronide because the signal in the proton-decoupled 13C NMR spectra from plasma MAG was inadequate for analysis. Resonance areas were determined using a PC-based NMR spectral analysis program (NUTS, Acorn NMR, Fremont CA) for 13C spectra, or a Bayesian statistical analysis for 2H spectra (25).

Analysis of 2H NMR Spectra of MAG From Plasma

In the presence of 2H in body water, plasma glucose becomes enriched in 2H because of reactions during gluconeogenesis and glycogenolysis that involve exchange with cell water (17). Relative 2H enrichment at H2, H5, and H6s depends on whether plasma glucose originates from glycogen, glycerol, or phosphoenol pyruvate (PEP) (Fig. 1). This information is easily obtained from the 2H NMR spectrum. For the subject without LA in Fig. 2, the relative areas were H2 (1.00), H5 (0.67), and H6s (0.57). Hence, for
this patient the rate of glucose production from each source relative to total glucose production was:

\[ \frac{v_2}{v_1} = \frac{1}{(H5/H2)} = 0.33 \]  
\[ \frac{v_3}{v_1} = 2 \times (H5 - H6(s))/H2 = 0.20 \]  
\[ \frac{v_4}{v_1} = 2 \times (H6(s))/H2 = 1.14, \]

where \( v_1 \) is the rate of glucose production. The rate of glycogenolysis relative to glucose production is \( v_2/v_1 \). The rate of glycerol conversion to glucose (\( v_3/v_1 \)) or the rate of PEP conversion to glucose (\( v_4/v_1 \)) must be corrected for the fact that two trioses are required to generate one glucose molecule.

Analysis of \(^{13}\text{C}\) NMR Spectra of MAG From Urine

\[^{13}\text{C}\] propionate is avidly extracted by the liver and metabolized exclusively through the citric acid cycle via succinyl-CoA. The \(^{13}\text{C}\) enrichment patterns in PEP and therefore in glucose synthesized from PEP depend on the relative rates of entry of unlabeled carbon (e.g., from alanine, lactate, or other pathways into the TCA cycle), pyruvate cycling, and citric acid cycle turnover. The pattern of\(^{13}\text{C}\) enrichment in glucose, in turn, can be measured by the relative intensity of multiplets in the \(^{13}\text{C}\) NMR spectrum of MAG. The carbon 2 resonance (see Fig. 3) is composed of four groups of resonances: the singlet (S) due to MAG enriched in C2 only, a doublet (D12) due to MAG enriched in both C1 and C2, a doublet (D23) due to MAG enriched in C2 and C3, and a quartet (Q) due to MAG enriched in C1, C2, and C3. In this case, the area of each set of resonances relative to the total area of the C2 resonance was: S (0.38), D12 (0.42), D23 (0.05), and Q (0.15). Since the area of each group of resonances is proportional to relative fluxes within the TCA cycle (26), it is a simple matter to calculate fluxes relative to citrate synthase:

\[ \frac{v_4}{v_7} = \frac{(Q - D23)/D23 = (0.15 - 0.05)/0.05 = 2.0 \]  
\[ \frac{v_5}{v_7} = \frac{(D12 - Q)/D23 = (0.42 - 0.15)/0.05 = 5.4 \]  
\[ \frac{v_6}{v_7} = \frac{(D12 - D23)/D23 = (0.42 - 0.05)/0.05 = 7.4, \]

where \( v_7 \) is flux through citrate synthase. Relative flux (net rate of flux) from the TCA cycle into glucose production is \( v_4/v_7 \), and relative flux through PEP carboxykinase is \( v_6/v_7 \). The sum of all fluxes from four-carbon TCA cycle

![FIG. 2. Plasma water enrichment in \(^{2}\text{H}\), and \(^{2}\text{H}\) NMR spectra of monacetone glucose derived from plasma glucose. The upper panel shows the percent enrichment in plasma water over time for all 10 subjects. The middle panel shows a typical \(^{2}\text{H}\) NMR spectrum of MAG from plasma of a subject without LA and the resonance assignments. (H1–H6S). A typical spectrum from a subject with LA is shown in the bottom panel. The ratios of \(^{2}\text{H}\) enrichment in positions 2, 5, and 6S indicate the sources of plasma glucose. Note that the difference in enrichment in position 5 and 6S is greater in the LA subject compared to the subject without LA, consistent with greater contribution of glycerol to plasma glucose.](image)

![FIG. 3. \(^{13}\text{C}\) NMR spectra of monacetone glucose derived from acetaminophen glucuronide in the urine. A typical proton decoupled \(^{13}\text{C}\) NMR spectrum showing all 6 carbon resonances of MAG from a subject without LA is shown with resonance assignments in the lower panel. The carbon 2 resonance is expanded, above, and labeled with the multiplet assignments as follows: quartet, Q; doublet due to J12, D12; doublet due to J23, D23; singlet, S. Notice that the ratio D12/D23 is much larger in MAG from the subject without LA than in MAG from the subject with LA. Since flux through PEPCK = (C2D12–C2D23)/C2D23, the spectrum indicates that flux through this pathway may be reduced in the LA subject.](image)
intermediates into pyruvate relative to citrate synthase, \( v_{2}/v_{7} \), is also referred to as pyruvate cycling. The malic enzyme and the combined effects of PEP carboxykinase and pyruvate kinase yield pyruvate, which reenters the TCA cycle via pyruvate carboxylase.

**Data Integration, Presentation, and Statistical Analysis**

The data from the \(^{2}H\) NMR spectra (relative fluxes of glycolysis, gluconeogenesis from glycerol, and gluconeogenesis from PEP) provided \( v_{2}/v_{1}, v_{3}/v_{1}, \) and \( v_{4}/v_{1} \). The \(^{13}C\) NMR analysis provided \( v_{2}/v_{7}, v_{3}/v_{7}, \) and \( v_{4}/v_{7} \). Together, this information allows calculation of fluxes in the entire network relative to glucose production, that is, \( v_{1} = 1 \).

Measurement of glucose turnover or other measures of absolute flux requires continuous intravenous infusion of a tracer or other more sophisticated studies. To limit this study to oral agents and simple blood and urine collection, absolute flux measures were not performed and all results are reported relative to glucose production (17). The underlying assumptions are discussed below.

The \(^{13}C\) NMR spectra from one subject with LA and one subject without LA were inadequate for analysis; therefore, all NMR-based results are from four subjects in each group. Data are mean $\pm$ 1 SD. Groups were compared using a two-tailed \( t \)-test, assuming unequal variances (Excel, Microsoft, Redmond, WA). No correction for multiple comparisons was performed.

**RESULTS**

Ten HIV+ subjects (nine men, one woman) participated in the study. Patients without LA were younger than patients with LA (47 $\pm$ 8 years vs. 59 $\pm$ 12 years, \( P < 0.05 \)), and plasma triglycerides were higher among LA subjects (393 $\pm$ 228 mg/dL) compared to subjects without LA (179 $\pm$ 107 mg/dL, \( P < 0.05 \)). One individual in the LA group developed diabetes during protease inhibitor therapy for which he was receiving treatment. All participants showed waist-to-hip ratios less than one. All subjects had undetectable viral load (<400 HIV RNA copies/mL) except one subject in the LA group with a stable viral load of 5000–10,000 HIV RNA copies/mL.

Plasma deuterium enrichment as a function of time after ingestion of \(^{2}H_{2}O\) is shown in Fig. 2 and ranged from 0.15–0.35%. The signal-to-noise in the \(^{2}H\) NMR spectra of MAG from samples at hour 2 was poor and these spectra were not analyzed. Spectra of MAG from samples at hour 3 and hour 4 were not significantly different from one another (data not shown).

The \(^{1}H\) NMR spectra of MAG from plasma glucose (Fig. 2) were satisfactory and generally similar to prior results. Relative to glucose production, glycolysis did not differ significantly between patients without LA (0.33 $\pm$ 0.11) and patients with LA (0.43 $\pm$ 0.09). The relative flux from glycerol to glucose was higher in subjects with lipatrophy (0.42 $\pm$ 0.12) compared to subjects without lipatrophy (0.20 $\pm$ 0.13, \( P < 0.05 \)). Conversely, the relative flux from the TCA cycle into plasma glucose (which necessarily provides the remainder of the gluconeogenic substrate) was decreased among the LA subjects (0.73 $\pm$ 0.24) compared to patients without LA (1.14 $\pm$ 0.16, \( P < 0.05 \)).

Multiplets due to \(^{13}C\)–\(^{13}C\) spin–spin coupling were readily observed (Fig. 3), which demonstrates that the orally administered [\(^{13}C\)]propionate had been metabolized through the TCA cycle and gluconeogenesis pathways, ultimately to form plasma glucose. Furthermore, sufficient acetyaminophen underwent glucuronidation to allow hepatic glucose to be trapped in the urine in sufficient quantity to provide very high-quality \(^{13}C\) NMR spectra.

The integrated results from \(^{2}H\) NMR of plasma glucose and \(^{13}C\) NMR of urinary acetaminophen glucuronide (Fig. 4) indicate that fluxes through PEP carboxykinase (\( v_{6}/v_{1} \)) and pyruvate cycling (\( v_{4}/v_{1} \)) were not significantly different between the two patient groups, although there was a trend towards lower flux through both pathways among LA subjects. Interestingly, in spite of these changes in gluconeogenic pathways, flux through citrate synthase (indexed to glucose production) was not significantly different between the groups: 0.56 $\pm$ 0.17 compared to 0.45 $\pm$ 0.15 (Fig. 4).

**DISCUSSION**

The first in vivo administration of a \(^{2}H\) tracer among HIV+ subjects was by McCune et al. (27), who used [\(^{6,6-2}H_{2}\)]glucose to analyze T-cell turnover. The current study describes the first application of either \(^{2}H\) or \(^{13}C\) tracers for analysis of intermediary metabolism among HIV+ subjects. The NMR spectra are adequate and the subjects tolerated the simple outpatient protocol reasonably well, despite transient side effects. This design avoids the ethical
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symmetric labeling of $^{13}$C in the oxaloacetate pool (as a result of scrambling in the symmetric 4-carbon intermediates of the TCA cycle), no significant $^{13}$C labeling in the acetyl-CoA pool, and steady-state isotopic and metabolic conditions. For the purpose of integrating the $^{2}$H and $^{13}$C NMR data, it was further assumed that all plasma glucose after an overnight fast is produced by the liver, and that the $^{13}$C labeling pattern of intrahepatic glucose is equal to the enrichment pattern of hepatic uridine diphosphate glucose, the substrate for glucuronidation of acetaminophen. None of these assumptions have been evaluated in HIV+ subjects, and to some degree each point is controversial, particularly the assumption that all glucose production is derived from the liver (31). Although the specifics of the analysis may be open to question, perhaps the most important observation is that the NMR spectra were different between the two groups, indicating differences in biochemical fluxes.

In this study a high priority was placed on minimizing the time commitment of the subject and the volume of blood drawn. Consequently, the protocol was designed to assure adequate $^{2}$H enrichment in plasma glucose, which requires administration of $^{2}$H$_{2}$O over a relatively brief period. The risk of mild nausea and vertigo due to $^{2}$H$_{2}$O, experienced by half of the patients, is roughly proportional to the rate of oral administration. As a single oral bolus, 200 mL of $^{2}$H$_{2}$O reliably causes vertigo among normal volunteers (32). Virtually the same dose administered over 4 hr was not associated with symptoms among any subject in an earlier study by Landau et al. (10); Nielsen et al. (16) did not report whether symptoms occurred among 37 subjects given the same amount of $^{2}$H$_{2}$O in three divided doses over 2 hr. In the current study, the underlying medical condition, concomitant drug therapy, or the simultaneous administration of propionate could each contribute to nausea. These unpleasant side effects would likely be eliminated if standard antinausea drugs such as promethazine are used, if the dose of $^{2}$H$_{2}$O could be reduced by 50%, or if the current dose is spread out over a longer period. Long-term administration of $^{2}$H$_{2}$O to humans at this level of enrichment has not been associated with adverse effects among experimental animals (33) or humans (34).

In summary, patients with LA did not have a significant increase in the proportion of plasma glucose derived from gluconeogenesis (unlike patients with type 2 diabetes), but gluconeogenesis from glycerol was increased and the TCA contribution was proportionately decreased. These observations are consistent with the hypothesis that excessive turnover of adipose tissue may be manifest both by LA and by increased glycerol gluconeogenesis. Further refinement and application of stable isotope tracer methods is warranted among HIV+ subjects with the goal of understanding the pathophysiology of metabolic disorders, and perhaps to assist in early detection, identification of patients at risk for metabolic complications of HAART, or identification of protease inhibitors or other agents which do not induce metabolic derangements.

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