A novel $^{13}$C NMR method to assess intracellular glucose concentration in muscle, in vivo

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Cline, Gary W., Beat M. Jucker, Zlatko Trajanoski, Alexander J. M. Rennings, and Gerald I. Shulman. A novel $^{13}$C NMR method to assess intracellular glucose concentration in muscle, in vivo. Am. J. Physiol. 274 (Endocrinol. Metab. 37): E381–E389, 1998.—Intracellular glucose concentration in skeletal muscle of awake rats was determined under conditions of hyperglycemic (10.2 ± 0.6 mM) hyperinsulinemia (~1.200 pM) and hyperglycemic (20.8 ± 1.5 mM) hypoinsulinemia (~12 pM) by use of $^{13}$C nuclear magnetic resonance (NMR) spectroscopy during a prime-constant infusion of [1-13C]glucose and [1-13C]mannitol with either insulin (10 µU·kg$^{-1}$·min$^{-1}$) or somatostatin (1.0 µg·kg$^{-1}$·min$^{-1}$). Intracellular glucose was calculated as the difference between the concentrations of total tissue glucose (calculated from the in vivo $^{13}$C NMR spectrum with mannitol as an internal concentration standard) and extracellular glucose, corrected by the ratio of intra- and extracellular water space. Extracellular concentration was calculated for an interstitial fluid-to-plasma glucose concentration gradient of 0.83 ± 0.07, determined by open-flow microperfusion. The mean ratio of intra- to extracellular glucose space, determined from the relative NMR signal intensities and concentrations of mannitol and total creatine, was 9.2 ± 1.1 (hyperglycemic hyperinsulinemia, $n = 10$), and 9.0 ± 1.7 (hyperglycemic hypoinsulinemia, $n = 7$). Mean muscle intracellular glucose concentration was <0.07 mM under hyperglycemic-hyperinsulinemic conditions ($n = 10$) and 0.32 ± 0.06 mM under hyperglycemic hypoinsulinemic conditions ($n = 7$). This method is noninvasive and should prove useful for resolving the question of whether glucose transport or phosphorylation is responsible for the reduced rate of muscle glycogen synthesis observed in diabetic subjects.

nuclear magnetic resonance spectroscopy

Because there was no buildup of G-6-P associated with the reduced rates of glycolysis synthesis, it was concluded that either glucose transport or glucose phosphorylation is impaired in the skeletal muscle of the diabetic subjects.

To determine which of the two metabolic steps, transport or phosphorylation, is rate controlling, it is sufficient to measure the intracellular glucose concentration under different glycemic conditions. Although it is not possible to directly resolve the intracellular and extracellular glucose signals in the NMR spectrum, by using metabolites confined to either the intra- or extracellular compartments as internal spectroscopic standards, we can determine all the parameters necessary to accurately calculate intracellular glucose. The glucose NMR signal that is observed in vivo is dependent on 1) the intra- and extracellular concentrations of glucose and 2) the relative volumes of the two spaces. These parameters were assessed by comparing the in vivo tissue NMR spectra acquired during infusions of [1-13C]glucose and [1-13C]mannitol with the NMR spectra of plasma sampled during the clamps. By use of mannitol as an internal calibration standard for extracellular space and the combined signal from creatine and phosphocreatine as a calibration standard for intracellular space, the contribution of intracellular glucose to the observed in vivo glucose signal was determined. We have tested the utility of this method for application to human investigation by using the conscious rat model under conditions of hyperglycemia, with and without insulin-stimulated glucose uptake and glycogen synthesis.

METHODS

Animals

Male Sprague-Dawley rats (Charles River, Raleigh, NC), weighing between 250 and 350 g, were maintained on standard rat chow (Ralston Purina, St. Louis, MO) and housed in an environmentally controlled room with a 12:12-h light-dark cycle. Rats were chronically catheterized in the right jugular vein and carotid artery, as previously described (14). The catheters were externalized through a skin incision at the back of the head. The rats were allowed to recuperate after surgery until they were at least of preoperative weight (5–10 days). All rats were fasted 24 h before the infusion experiment. The rats were transiently anesthetized (<30 s) with a
low dose (2.5 mg) of thiopental (Sigma Chemical, St. Louis, MO) and placed in a restraining tube modified to allow one hindlimb to be secured to the outside of the tube. The restraining tube was then mounted to the radio frequency (RF) coil assembly such that the rat hindlimb was centered over the surface coils. The rat and RF coil assembly were then placed in the bore of the magnet, with the hindlimb positioned at magnet isocenter. Infusion lines were extended to syringes and pumps outside the RF enclosure of the magnet. After collection of a 15-min baseline spectrum, acquisition of spectra was made continuously during one of the two infusion protocols described below for the determination of intracellular glucose.

Protocol 1

To assess intracellular glucose concentration under conditions of near maximal muscle glycogen synthesis, rats were infused with a prime-constant infusion of [1-13C]glucose (prime, 0.6 g/kg rat; constant, 45 mg·kg⁻¹·min⁻¹; 99% 13C) and [1-13C]mannitol (prime, 0.2 g/kg rat; constant, 10 mg·kg⁻¹·min⁻¹; 99% 13C). Simultaneously, a prime (190 mU/kg) constant (10 mU·kg⁻¹·min⁻¹) infusion of insulin was begun.

Protocol 2

To assess intracellular glucose concentration during periods of hyperglycemia in the absence of insulin, rats were infused with a prime-constant infusion of [1-13C]glucose (prime, 0.4 g/kg rat; constant, 30 mg·kg⁻¹·min⁻¹; 99% 13C) and [1-13C]mannitol (prime, 0.2 g/kg rat; constant, 10 mg·kg⁻¹·min⁻¹; 99% 13C). At the same time, a constant (1 µg·kg⁻¹·min⁻¹) infusion of somatostatin was begun and maintained for the duration of the clamp.

In Vivo NMR Spectroscopy

All in vivo NMR experiments were performed on a Bruker Biospec 7.0T system (horizontal 22-cm-diam bore magnet). Concentric 13C (18-mm)/1H (30-mm) coils were used to collect 1H (300.68 MHz) decoupled 13C (75.65 MHz) observed NMR spectra, as previously described (6). NMR spectra were acquired with a nonselective 13C hard pulse (−70° flip angle, 5 mm from surface coil), with broadband 1H Waltz-16 decoupling applied during acquisition. Additional nuclear Overhauser enhancement (nOE) was achieved using low-power decoupling (0.4 W) during the pulse delay [repetitive time (TR) = 0.5 s, sweep width (SW) = 20 KHz, 4 K data]. A 15-min baseline spectrum was followed by 15-min acquisitions throughout the duration of the experiment. All data were processed using a Gaussian filter followed by Fourier transformation. Relative signal intensities of glucose, mannitol, and total creatine were corrected for T₁ relaxation differences and nOE from comparison of spectra collected with TR = 0.5 s and TR = 120 s (without low-power decoupling during the relaxation delay) of the metabolites in vivo and in phantom solutions.

The 31P NMR spectra were acquired with 31P/H coils of the same design and geometry as the 13C/H coil assembly. Direct 1H (300.68 MHz) decoupled 31P NMR spectra were acquired at 121.72 MHz. A hard pulse (45° flip angle) was optimized 5 mm from the surface coil (TR = 1.4 s), with broadband 1H Waltz-16 decoupling applied during acquisition. Data were accumulated with a time resolution of 15 min. Data were processed using a Gaussian filter followed by Fourier transformation. Processing of G-6-P and phosphocreatine data was performed as previously described (15), with the assumption of an ATP concentration of 7 µmol/g wet wt (9).

Glucose and Mannitol in Interstitial Fluid and Muscle

Glucose and mannitol concentrations were determined in the interstitial fluid by open flow microperfusion (21). Briefly, chronically catheterized rats were anesthetized for ~30 min with thiopental, and a perforated double lumen catheter (prepared from a conventional iv cannula, 24-gauge × 19 mm, diam 0.6 mm; Neoflon, Viggo AB, Helsingborg, Sweden) was inserted into the hindlimb muscle with a steel mandrin. Then the mandrin was removed and replaced by the inner tube (steel tube length 16 mm, OD 0.2 mm, ID 0.1 mm). A perfusate of (135 mM NaCl, 3 mM KCl, 1 mM MgCl₂, 1.2 mM CaCl₂, 200 µM ascorbate, and Na phosphate buffer of 2 mM adjusted to pH 7.4) was continuously perfused through the inner lumen, and an effluent, i.e., perfusate and a certain amount of interstitial fluid, was collected through the outer lumen at a flow rate of 0.5 µl/min.

Thirty minutes after the insertion of the catheter, hyperglycemic clamps (natural abundance of glucose and mannitol) after protocols 1 (n = 5) and 2 (n = 5) were started and maintained for 6 h. Two hours after the start of the clamps, a calibration procedure [no net flux method (12)] was used to determine the absolute concentration of substrates in the interstitial space. The calibration procedure is based on the principle that measurement of substrate in the samples with differing concentrations of that substrate in the perfusate will enable estimation of the absolute concentration in the interstitial fluid at the point where net exchange across the catheter is zero (the perfusate and the interstitial concentration are equal). For different concentrations of glucose and mannitol were added to the perfusate, and the net increase of these substances was recorded in the samples. At each stage, the effluents were collected at 30-min intervals, with 30-min washout periods between each change in the perfusate to allow equilibration with the new perfusate concentration. Regression analysis was than applied to calculate the concentration resulting in no net change (i.e., the point where net increase equals zero).

At the end of the clamp, the rats were anesthetized with thiopental (50 mg/kg), and the gastrocnemius and quadriceps muscles were freeze-clamped in situ for determination of tissue glucose and mannitol concentrations. The muscle was excised and stored at −70°C until extraction with perchloric acid (PCA). Rats were euthanized with a lethal dose of thiopental.

In Vitro Phantom for Method Validation

Phantoms were fabricated from glass tubing (2 mm ID), with length cut to fit tightly within a 45 × 45 × 15-mm plastic box. The glass tubing was filled with solutions (−180 µl) of [1-13C]glucose (0–5 mM) and creatine (Cr) (75.6 mM) and sealed at both ends with silicon sealant. The higher Cr concentration was chosen to replicate the signal intensities in the phantoms relative to those observed in vivo. Forty tubes containing a fixed concentration of glucose were tightly packed in the box, and the external volume (~6 ml) was filled with a solution of [1-13C]glucose (5 mM) and [1-13C]mannitol (5 mM). Similar volumes for the internal and external compartments were chosen to test the limits of the methodology under a worst-case scenario. Because the NMR signal of the glucose is directly proportional to its concentration and the compartment volume, in vivo the intracellular glucose NMR signal will increase as the proportion of intra- to extracellular volume increases. 13C NMR spectra of the
phantoms were collected with the identical parameters used to collect the in vivo spectra.

Analytic Procedures

Plasma glucose concentrations were measured by the glucose oxidase method (GlucoseAnalyzer II, Bedmann, Fullerton, CA). Plasma immunoreactive free insulin was measured with a double-antibody radioimmunoassay technique (Linco Research, St. Charles, MO). 13C enrichment of plasma glucose and mannitol (as acetate derivatives) was determined by gas chromatography-mass spectrometry (GC-MS) with a Hewlett-Packard 5971A MSD (methane chemiluminescence). Relative concentrations of plasma [1-13C]glucose and [1-13C]-mannitol were determined from 13C NMR spectra acquired at 125.76 MHz (AM 500, Bruker Instruments) by use of a 30° pulse width, quadrature detection, digital resolution of 2.7 Hz/pixel, and with a pulse program for inverse-gated heteronuclear WALTZ decoupling with TR = 1 s. Relative signal intensities of glucose and mannitol were corrected for relaxation differences in T1 and mOe from comparison of spectra collected with TR = 1.0 s and TR = 120 s. Absolute concentrations of mannitol were calculated from the absolute concentration of plasma glucose, the 13C enrichments of each, and their relative 13C concentrations. Alternatively, in the glucose clamp studies using natural abundance, the relative glucose-to-mannitol concentrations were determined by GC-MS.

Total muscle glucose was determined in the extract of the freeze-clamped muscle as follows. The combined quadriceps and gastrocnemius muscle (1 g) was homogenized over ice with a variable high-speed electric homogenizer with 0.9% perchloric acid (3 vol/wt) and centrifuged (3,400 rpm, at 4°C for 10 min). The supernatant was lyophilized and reconstituted in 200 µl water. The total muscle glucose concentration was then determined by the glucose oxidase method.

Calculations

Glycogen synthesis rate. The incremental change in muscle glycogen concentration was calculated from the increase in the [1-13C]glycogen signal intensity at 101 ppm and the plasma [1-13C]-glucose atom percent enrichment, as previously described (1, 18). Net rates of glycogen synthesis were calculated from the slope of the least-squares linear fit to the glycogen concentration curve beginning 30 min after the start of either the insulin or the somatostatin infusion.

Intracellular glucose. The in vivo NMR signal intensity of mannitol (Maninvivo) is the product of the concentration of mannitol in the extracellular space ([Man]e), the mole fraction of [1-13C]Man ([13C]Man) and the volume of the extracellular space (E).

\[
\text{Man}_{\text{invivo}} = [\text{Man}]_e \times [13C]\text{Man} \times E \tag{1}
\]

The in vivo NMR signal intensity of glucose (Glicinvivo) is the sum of the product of the concentration of glucose in the extracellular space ([Glc]e), mole fraction of [1-13C]Glc ([13C]Glc) and E, and of the product of the concentration of glucose in the intracellular space ([Glc]i), [13C]Glc, and the volume of the intracellular space (I).

\[
\text{Glc}_{\text{invivo}} = ([\text{Glc}]_e \times [13C]\text{Glc} \times E) + ([\text{Glc}]_i \times [13C]\text{Glc} \times I) \tag{2}
\]

The in vivo NMR signal of G-6-P (G6Pinvivo) coresonates with glucose and contributes to the observed glucose signal intensity (*Glcinvivo). Thus

\[
*\text{Glc}_{\text{invivo}} = \text{Glc}_{\text{invivo}} + \text{G6P}_{\text{invivo}} \tag{3}
\]

where G6Pinvivo is the product of the concentration of G-6-P in the extracellular space ([G6P]e), mole fraction of [1-13C]G-6-P ([13C]G6P) and I

\[
\text{G6P}_{\text{invivo}} = [\text{G6P}]_e \times [13C]\text{G6P} \times I \tag{4}
\]

Taking the ratio of Maninvivo to *Glcinvivo and solving for [Glc], we derive

\[
[Glc]_i = E/I \times ([*\text{Glc}_{\text{invivo}}/\text{Man}_{\text{invivo}}] - [\text{Glc}]_e) \times ([13C]\text{Man}/[13C]\text{Glc}) \times [\text{Man}]_e \times [\text{Glc}]_e \times (E/I + E) \tag{5}
\]

Tissue glucose concentration from NMR. The equation for calculating tissue glucose concentration ([Glc]i) from the NMR spectra is derived to yield a concentration value equivalent to the concentration of glucose in the PCA extract

\[
[Glc]_i = ([Glc]_i \times (E/I + E) + [Glc]_e \times (E/I + E) \tag{6}
\]

Extracellular-to-intracellular volume. The creatine signal observed in the 13C NMR spectrum is from both Cr and phosphocreatine (PCr) and is referred to here as total Cr (Crt). With the assumption that the Crt signal is compartmentalized to the intracellular muscle space and is natural abundance 13C, the in vivo NMR signal intensity of Crt to mannitol is

\[
\text{Crt}_{\text{invivo}}/\text{Man}_{\text{invivo}} = (0.011 \times [\text{Crt}] \times I) / ([13C]\text{Man} \times [\text{Man}]_e \times E) \tag{7}
\]

Solving for I/E, we have

\[
I/E = ([\text{Crt}]_{\text{invivo}}/([13C]\text{Man} 	imes 0.011)) \times ([\text{Man}]_e/\text{Crt}) \tag{8}
\]

where [Crt] is assumed to be 33 mM, which is the concentration of Crt found in the plantaris muscle of the rat, representative of a mixture of low- and high-oxidative fiber types (9). Crt concentration ranged from 40 mM in white gastrocnemius muscle (low-oxidative fiber) to 23 mM in soleus muscle (high-oxidative fiber) (9).

Statistical Analysis

All data are reported as means ± SE. Student’s two-tailed t-test or the Mann-Whitney rank-sum test (nonparametric analysis) was performed on data to determine significance at a minimum threshold of P < 0.05.

RESULTS

Hyperglycemic-Hyperinsulinemic Conditions

During infusion protocol 1, plasma glucose concentration plateaued at ~10 mM and 13C enrichment at 85 ± 3%, and plasma mannitol reached a steady-state concentration of ~4 mM within 1 h after start of the clamp (Table 1 and Fig. 1A). Mean steady-state plasma insulin concentration reached supraphysiological levels. A series of baseline-subtracted spectra (Fig. 2) clearly resolves the C-1 resonances of mannitol, α-glucose, β-glucose, and glycogen. Crt C-2 is resolved in the spectra before
Table 1. Substrate concentrations in rat hindlimb determined by 13C NMR and biochemical assay

<table>
<thead>
<tr>
<th>Protocol 1: Hyperglycemic hyperinsulinemia</th>
<th>Biochemical</th>
<th>NMR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma glucose concentration, mmol/l</td>
<td>10.1±0.3 (5)</td>
<td>10.2±0.6 (10)</td>
</tr>
<tr>
<td>Plasma insulin concentration, pmol/l</td>
<td>&lt;12 (5)</td>
<td>&lt;12 (7)</td>
</tr>
<tr>
<td>Total muscle glucose, mmol/l</td>
<td>0.75±0.13 (5)</td>
<td>1.02±0.10 (10)</td>
</tr>
<tr>
<td>Intracellular glucose, mmol/l</td>
<td>-0.23±0.14 (5)</td>
<td>-0.09±0.10 (10)</td>
</tr>
<tr>
<td>G-6-P, mmol/l</td>
<td>0.24±0.05 (5)</td>
<td></td>
</tr>
<tr>
<td>Basal</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Δ Above baseline</td>
<td>0.09±0.02 (3)</td>
<td></td>
</tr>
<tr>
<td>Intracellular/extracellular glucose space</td>
<td>9.2±1.1 (10)</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Protocol 2: Hyperglycemic hypoinsulinemia</th>
<th>Biochemical</th>
<th>NMR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma glucose concentration, mmol/l</td>
<td>19.9±0.2* (5)</td>
<td>20.8±1.5* (7)</td>
</tr>
<tr>
<td>Plasma insulin concentration, pmol/l</td>
<td>&lt;12 (5)</td>
<td>&lt;12 (7)</td>
</tr>
<tr>
<td>Total muscle glucose, mmol/l</td>
<td>2.68±0.55* (5)</td>
<td>2.63±0.71* (7)</td>
</tr>
<tr>
<td>Intracellular glucose, mmol/l</td>
<td>0.70±0.57 (5)</td>
<td>0.32±0.06* (7)</td>
</tr>
<tr>
<td>G-6-P, mmol/l</td>
<td>0.24±0.05 (5)</td>
<td></td>
</tr>
<tr>
<td>Basal</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Δ Above baseline</td>
<td>0.04±0.04 (2)</td>
<td></td>
</tr>
<tr>
<td>Intracellular/extracellular glucose space</td>
<td>9.0±1.7 (7)</td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± SE. Nos. in parentheses are nos. of rats used for each measurement. NMR, nuclear magnetic resonance; G-6-P, glucose 6-phosphate. *P < 0.05 vs. protocol 1.

baseline subtraction, as shown in Fig. 3. The signal-to-noise ratio (S/N) of the smallest relevant resonance was typically greater than 20:1, which gives a precision in the measurement within ±5%. The muscle glycogen synthetic rate was 0.31 ± 0.03 mmol·l⁻¹·min⁻¹.

From the 31P spectrum, we measured a PCr concentration of 23.7 ± 0.6 mmol/l, which is similar to the concentration of 20.7 ± 1.1 mmol/l reported for the rat muscle with mixed muscle fiber type by use of standard biochemical assay (9). Because both Cr and PCr are observed in the 13C NMR spectra as a single peak, we use the reported value of 33 mmol/l for the Cr concentration in this muscle fiber type to calculate the ratio of intracellular glucose concentration, however, it is necessary to consider the impact of any glucose gradients between the sampling site (in this case, venous plasma) and the arterial plasma and interstitial fluid, as discussed below.

Hyperglycemic-Hypoinsulinemic Conditions

During infusion protocol 2, plasma glucose plateaued at concentrations about two times those observed during protocol 1. However, plasma glucose 13C enrichment (87 ± 2%) and plasma mannitol concentration were similar to values observed during protocol 1 (Table 1 and Fig. 1B). Mean steady-state plasma insulin fell to concentrations below basal levels. Nonoxidative glucose disposal in the form of muscle glycogen synthesis, as assessed by 13C NMR, was negligible. Under these conditions, we observed no change in the ratio of intracellular glucose plus G-6-P concentration of 0.15 ± 0.07 mM (n = 7). The mean change above basal G-6-P concentration was similar to that observed and alanine, we estimate that the enrichment of the G-6-P pool is ~70% of plasma glucose under similar hyperglycemic-hypoinsulinemic clamp conditions (6). Therefore, we estimate a maximum correction for G-6-P of 0.23 mM. To arrive at a final value for the intracellular glucose concentration, however, it is necessary to consider the impact of any glucose gradients between the arterial plasma and interstitial fluid, as discussed below.

Fig. 1. Plasma glucose (○) and mannitol (●) concentrations under hyperglycemic-hypoinsulinemic (A, n = 10) or hyperglycemic-hypoinsulinemic (B, n = 7) conditions. Values are means ± SE.
during protocol 1. Assuming the same $^{13}$C enrichment of G-6-P as in protocol 1, we estimate that G-6-P is contributing 0.20 mM to the observed intracellular glucose concentration.

Plasma vs. Interstitial Glucose and Mannitol

Plasma and interstitial concentrations of glucose and mannitol are given in Table 2. Under both experimental protocols, interstitial glucose concentration tended to be ~80% of the venous plasma concentration. Although the difference did not reach statistical significance within either group, a paired t-test of the combined glucose data of both groups was significant, with the ratio of interstitial to plasma glucose concentration of $0.83 \pm 0.07 \ (P < 0.05)$. Interstitial and plasma mannitol concentrations were no different in either protocol, or with the combined data from both protocols. This interstitial-plasma glucose gradient will reduce the extracellular tissue glucose concentration by ~15% relative to the plasma glucose concentration in both protocols [if we assume a ratio of 6 to 1 for the interstitial to plasma volumes (5)]. Because we observed no mannitol concentration gradient, the calculated total tissue glucose remains unchanged. Correcting for the interstitial-plasma glucose concentration increases the intracellular glucose by 0.10 and 0.37 mM in protocols 1 and 2, respectively. With both the G-6-P and the glucose concentration gradient correction factors accounted for, we calculate an intracellular glucose concentration of $-0.09 \pm 0.10$ mM under hyperglycemic-
Validation of Method: NMR Phantoms

NMR measurement of the glucose, mannitol, and Cr ratios in the series of phantoms was performed on three different days to assess the reproducibility and accuracy of our method. The ratio of glucose to mannitol signal intensity was directly proportional to the change in the glucose concentration in the inner compartment of the phantoms. From the mannitol-to-Cr concentration we calculated a mean ratio of intra- to extracompartment volumes of 1.15 ± 0.18, compared with a value of 1.2 calculated from the measured volumes of solutions in the intra- and extracompartments. There was excellent correlation (r = 0.9966) between the NMR and biochemical determinations of the intracompartmental glucose concentrations (Fig. 4). Using the NMR technique, we were able to confidently assess an intracompartmental glucose concentration of 0.31 mM determined biochemically.

The lower limit of reliable detection of the intracellular glucose from the glucose-to-mannitol ratio is dependent on the S/N of the signals for these two metabolites and the ratio of intracellular to extracellular space. Because the NMR signal intensity of the intracellular glucose is directly proportional to its concentration and the intracellular volume, the sensitivity of our calculation for any given intracellular glucose concentration increases proportionally with the relation of intra- to extracellular space. The I/E volumes of the phantoms were approximately equal; thus the change in the

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Table 2. Glucose concentrations in rat plasma and hindlimb interstitial fluid

<table>
<thead>
<tr>
<th></th>
<th>Venous Plasma</th>
<th>Interstitial Fluid</th>
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<tbody>
<tr>
<td><strong>Protocol 1:</strong> Hyperglycemic hyperinsulinemia (n = 5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose concentration, mmol/l</td>
<td>10.1 ± 0.3</td>
<td>8.1 ± 1.0</td>
</tr>
<tr>
<td>Mannitol concentration, mmol/l</td>
<td>4.2 ± 0.6</td>
<td>4.2 ± 0.5</td>
</tr>
<tr>
<td><strong>Protocol 2:</strong> Hyperglycemic hypoinsulinemia (n = 5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose concentration, mmol/l</td>
<td>19.9 ± 0.2*</td>
<td>17.0 ± 2.2</td>
</tr>
<tr>
<td>Mannitol concentration, mmol/l</td>
<td>4.0 ± 0.2</td>
<td>4.4 ± 0.6</td>
</tr>
</tbody>
</table>

Values are means ± SE; n, no. of rats/protocol. *P < 0.05 vs. protocol 1.
Validation of Method: NMR vs. Biochemical Assay

Biopsy measurements of muscle glucose concentration were made in the undisturbed hindlimb of the rats used in the microperfusion studies. Biopsy measurements were made in these rats because it was possible to take the biopsy much more quickly, with less stress to the rat, and required no interruption of the glucose-mannitol infusions, in contrast to the delays associated with removal of the rats from the bore of the spectrometer after the NMR measurements. Using standard biochemical assays, we determined total muscle glucose concentration and calculated the intracellular glucose concentration by use of the parameters measured using the NMR technique (Table 1). The agreement of the results obtained by the two methods demonstrates that our in vivo NMR method can measure intracellular glucose with similar or better precision than the classical biopsy-biochemical assay technique (7, 8, 10).

DISCUSSION

In this study, we demonstrate the feasibility of using a noninvasive $^{13}$C NMR technique to measure intracellular glucose concentration in vivo. We have developed the method for muscle tissue; however, the technique is applicable to any tissue amenable to NMR observation. The strength of this method lies in the use of mannitol as an extracellular marker and in comparison of the ratio of glucose to mannitol in the tissue, determined by $^{13}$C NMR, with that of the plasma.

Methods used previously to measure intracellular glucose fall into two basic categories: 1) biopsy and biochemical assay (7, 8, 10) and 2) kinetics of the uptake of glucose and nonmetabolized glucose analogs (4, 22). Estimating intracellular glucose in muscle by biopsy is complicated by the need to use microdissection to remove nonmuscle constituents and by the significant impact that contamination of the tissue by small volumes of plasma can have on the mass of glucose present in the biopsy. Because of the small mass of glucose actually present in the muscle tissue and the relatively high concentration of glucose in blood, any change in the blood volume of the tissue during the extraction procedure (either addition from bleeding or loss during freeze-clamping) can add (or subtract) a substantial proportion of glucose to the tissue. For example, a typical assay of 1 g of muscle tissue (I/E ~ 9), with intracellular glucose concentration of 0.1 mM and plasma glucose concentration of 10 mM, has a total glucose content of only 1.1 µmol. Contamination of this muscle with just 20 µl of plasma is sufficient to raise the total glucose concentration by ~20% and the calculated intracellular glucose concentration to 0.22 mM. With 20 mM plasma glucose, the calculated intracellular glucose would be increased by ~0.4 mM. Furthermore, the correction factor for water space cannot be directly determined. In regard to the kinetic approach, although much can be learned from sophisticated kinetic analysis of metabolite transport, the conclusions reached are highly dependent on the model used to analyze the data (4, 22).

NMR offers a more direct measurement of intracellular glucose. Disregarding the practical difficulties of biopsy measurements, the advantages of NMR over biopsy techniques are primarily that the measurements are made in vivo. Thus the intracellular glucose concentration can be measured at multiple time points throughout the experiment, thereby allowing responses to physiological perturbations to be measured. Roussel and co-workers (17) demonstrated the feasibility of NMR to measure intracellular glucose by calibrating the relation of muscle glucose NMR intensity to concentration during a control period for determination of total glucose (and intracellular glucose) during a subsequent hyperglycemic clamp. Recently, they modified their method to calibrate the relation of glucose NMR intensity to concentration by use of an external standard (16). Advantages of using the ratio of glucose to mannitol, as opposed to successive glucose intensity measurements, are that mannitol acts as an extracellu-
nal concentration standard, thereby minimizing assumptions regarding initial concentrations of intracellular glucose and relative volumes of the glucose spaces, and avoids errors due to spectrometer instability (16-18).

In muscle, with Cr serving as an intracellular marker, the relative volumes of intracellular and extracellular space can also be determined directly during the course of the experiment. Our NMR measurements are nonlocalized, and signals arise from a mixture of low- and high-oxidative fiber types. We therefore based our calculations on use of a total Cr concentration from a hindlimb muscle with mixed fiber types. Our ratio of ~9 for the volume of intracellular to extracellular water volume can be compared with ratios of Streeter and Woo (20), who used insulin to determine cell water in rat skeletal muscle. They found that the ratio of intra- to extracellular water volume in the various leg muscles ranged from 6.1 (soleus) to 9.2 (tibialis anterior), with an average of 7.3 (20). These values are quite similar to those found in resting human muscle, with values ranging from 5.8 in soleus muscle to 9.3 in gastrocnemius muscle (19).

A potential source of error in calculating intracellular glucose from the ratio of glucose to mannitol in the tissue with that in plasma arises from the magnitude of the arteriovenous (a-v) and plasma-interstitial glucose gradients. A reasonable estimate for the a-v glucose gradient is that the arterial glucose concentration is ~10% higher than the venous plasma concentration (11). Using open-flow microperfusion, we determined that the glucose concentration in the interstitial fluid was ~80% of the plasma concentration. This is in agreement with results obtained in humans under fasting and euglycemic-hyperinsulinemic clamp conditions, in which interstitial glucose concentrations were ~70% of arterialized plasma values (13). Because mannitol is not metabolized, there should be no a-v or plasma-interstitial fluid concentration gradients. This was confirmed in our microperfusion studies, where we found no difference in the plasma and interstitial fluid mannitol concentrations. The impact of the glucose gradients will be to lower the extracellular glucose concentrations by ~15% relative to the venous plasma glucose concentrations under both conditions and to increase the calculated values for intracellular glucose concentrations by 0.10 and 0.37 mM in protocols 1 and 2, respectively.

Katz et al. (8) reported a significant accumulation of intracellular glucose in human muscle under hyperglycemic-hypoinsulinemic conditions. After 2 h of hyperglycemia (~26 mM), when insulin was suppressed with an infusion of somatostatin, the estimated concentration of intracellular glucose was 9.5 mmol/kg dry weight, or ~2.2 mM with their assumption that skeletal muscle is 77% water. We used our NMR method to test whether we could detect any significant elevation of intracellular glucose in the rat muscle during similar conditions of hyperglycemic hypoinsulinemia (protocol 2). We raised glucose levels above 20 mM while suppressing endogenous insulin release with a somatostatin infusion. There was a slight but statistically significant (P < 0.05) increase in the intracellular glucose concentration (0.32 ± 0.06) compared with conditions of insulin-stimulated glycogen synthesis (protocol 1, 0.09 ± 0.10). However, the accumulation of intracellular glucose was about sevenfold lower than that observed under similar conditions in humans (8). It is unclear whether these divergent results can be attributed to methodological or species differences.

In summary, under hyperglycemic clamp conditions, we observed low (<0.1 mM) concentrations of intracellular glucose in muscle, resulting in a more than 100-fold extracellular-intracellular glucose gradient. This method is noninvasive and should prove useful for resolving the questions of whether glucose transport or phosphorylation is responsible for the reduced rate of muscle glycogen synthesis observed in insulin-resistant states.

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