Determination of muscle-specific glucose flux using radioactive stereoisomers and microdialysis

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THE MICRODIALYSIS TECHNIQUE was first introduced by Delgado et al. (7) and is based on the principle of diffusion through a semipermeable membrane. Briefly, a semipermeable fiber or microdialysis probe is inserted into the target tissue and perfused with a physiological solution. As the solution passes through the probe, compounds in the interstitial space, as well as in the perfusate, can diffuse into or out of the probe, respectively. The solution (dialysate) is collected after it has passed through the microdialysis probe and been analyzed for the compounds under investigation. The microdialysis technique has been applied to a number of different tissues, such as brain (2), adipose (20), and more recently skeletal muscle (13, 15, 16, 18), to study the interstitial concentrations of many different compounds, including glucose, lactate, glycerol, amino acids, and adenosine.

To take full advantage of the microdialysis technique, it is necessary to make calculations of actual interstitial concentrations. This is accomplished by making an estimate of the in vivo extraction fraction of the compound being measured from the interstitial space, termed “probe recovery.” A currently used method, introduced by Scheller and Kolb (19), allows the determination of probe recovery in each collected sample, thus allowing the continuous monitoring of probe recovery over time. This method, known as the “internal reference” method, requires the addition of a

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small amount of radioactive tracer, in the form of the compound being investigated, to be added to the perfusate. It has been shown that the relative loss of the isotope from the perfusate into the interstitial space represents probe recovery for that compound (10, 13, 19). Furthermore, a number of factors have been identified that influence the relative loss of the isotope (2, 9, 11, 13), including tissue uptake. Therefore, it may be possible to determine the relative change in tissue uptake by simultaneously perfusing the microdialysis probes with two compounds that are not equally affected by changes in tissue metabolism. For example, it is well established that the d-stereoisomer of glucose is readily taken up and metabolized by skeletal muscle, whereas the l-stereoisomer is not. Therefore, if radioactive tracers of both of the stereoisomers of glucose were simultaneously perfused through the microdialysis probes, then the l-stereoisomer would not be influenced by changes in skeletal muscle glucose uptake. As a result, it may be possible to examine and quantify changes in tissue glucose uptake by comparing the relative loss of these two stereoisomers.

Therefore, the purpose of the present study was to evaluate the feasibility of using radioactive glucose stereoisomers for investigating changes in glucose uptake directly at the tissue level. This was accomplished by applying the microdialysis technique to human skeletal muscle in conjunction with perfusion solutions containing radioactive D- and L-glucose, as well as varying concentrations of insulin.

METHODS

Subjects. The experimental protocol was approved by the Institutional Review Board of The Milton S. Hershey Medical Center. All subjects had the purposes and risks of the study explained to them, and written informed consent was obtained. Nine healthy fasted subjects (5 men, 4 women) with a mean age of 32 yr (range 24–40 yr) and body mass index of 26.1 ± 1.6 kg/m² participated in this study. The subjects were normotensive, and thus did not have any medication, and all refrained from the ingestion of caffeine-containing beverages for 24 h before the study.

Microdialysis probes. A complete description of the construction of the microdialysis probes has previously been given (14, 15). Briefly, a semipermeable fiber (artificial dialysis kidney, AM-UP-75) with a molecular mass cutoff of ~31,000 Da was glued between two nylon tubes (ID = 0.50 mm, OD = 0.63 mm). The actual probe length (distance between the two nylon tubes) was 4 cm (ID = 0.20 mm, OD = 0.22 mm).

Microdialysis probe insertion. Two microdialysis probes were inserted into the vastus lateralis muscle of the subject’s left leg. The skin and subcutaneous tissue at the probe’s entrance and exit sites was first anesthetized with a local injection (0.5–1.0 ml) of lidocaine + epinephrine (20 mg/ml + 12.5 g/ml). The probes were inserted into the muscle via a 17-gauge intravenous cannula (Venflon) in a direction parallel to muscle fiber orientation (i.e., 45º moving proximally and laterally). The distance between the entrance and exit sites of the probes was ~9 cm, and the distance between each probe was ~2–3 cm. After insertion, the microdialysis probes were attached to a perfusion pump (model 102, CMA) and perfused at a rate of 4 μl/min with a Ringer solution. In an effort to minimize the possibility of draining the interstitial space (6, 12), the perfusate contained 3.0 mM glucose and 0.5 mM lactate. The probes were perfused, and the subjects rested supine for 60 min before the experiment was initiated.

Determination of probe recovery. In the present study the internal reference method introduced by Scheller and Kolb (19) was used. With this method, a small amount of radioactive tracer, in the form of the compound being investigated, was added to the perfusate. The relative loss of the isotope is determined by a number of factors, including the rate of tissue uptake. Therefore, a second isotope in the form of the nonmetabolizable stereoisomer may be added to the perfusate, and thus the relative loss of this isotope represents all of the other factors that influence probe recovery except tissue uptake. As a result, any difference in the relative loss between these stereoisomers represents tissue uptake for that compound directly at the cellular level. In the present study very small amounts of D-[6-14C]glucose (500 dpm/10 μl) and L-[3-3H]glucose (2,000 dpm/10 μl) were added to the final perfusion solution as the internal reference markers for protocol I, and D-[U-14C]glucose and L-[3-3H]glucose were added for protocol II.

Experimental protocol I (6 subjects, 12 probes, D-[6-14C]glucose and L-[3-3H]glucose). Sixty minutes after insertion of the microdialysis probes, baseline (control) data collection was conducted. This length of time was allowed because it has been shown that, after microdialysis probe insertion, some cellular disruption occurs that transiently elevates the interstitial concentrations of several metabolites, including ATP (4). However, it has also been noted that the elevation in ATP declines to basal levels after only 30 min. Despite this observation, a 60-min equilibration period is used before the experiment is initiated to ensure that the extracellular environment surrounding the probe has returned to normal. Dialysate was then collected from the microdialysis probes for 15 min in microcentrifuge tubes, and then the perfusion solution was changed. The new solution contained the same compounds as those in the resting perfusion solution, as well as 1 μM insulin (Humulin). This solution was perfused at 4 μl/min for 60 min, followed by two 15-min collection periods. A schematic representation of protocol I is presented in Fig. 1. Experimental protocol II (3 subjects, 5 probes, D-[3-3H]glucose, and L-[U-14C]glucose). The same preconditioning and time line were followed in this protocol as those outlined in protocol I, and baseline (control) dialysate collection was conducted for 15 min. The perfusion solution was then changed, and the new solution contained 0.1 μM insulin and was perfused for 60 min, followed by a 15-min collection period. Two further perfusion solutions were used that contained 1 and 10 μM insulin, respectively. Each of these solutions was perfused for 60 min, followed by a 15-min collection period. A schematic representation of protocol II is presented in Fig. 1. It should be noted that, in one subject, one of the microdialysis probes ceased to function after insertion, and thus only five probes were used in this protocol.

The rationale for using D-[3-3H]glucose in this protocol instead of D-[6-14C]glucose is severalfold. First, by alternating radioactive isotopes we can control for differences and problems associated with the degradation of isotopes. Second, it is possible that some of the D-[3-3H]glucose taken up by the muscle may be converted to 3H2O by glycolysis and subsequently released back into the interstitial space. As a result, it is possible that some 3H2O may be diffused back into the microdialysis probe and be collected with the dialysate. Therefore, by drying the dialysate before scintillation count-
ing to eliminate all \(^3\text{H}_2\text{O}\) produced, it is possible to estimate all glucose metabolized by skeletal muscle.

**Analyses.** Ten microliters of dialysate and perfusate were pipetted into 5-ml scintillation vials, and 3 ml of scintillation fluid were added for the determination of the disintegrations per minute of D-[6-\(^{14}\text{C}\)]glucose and L-[3-\(^3\text{H}\)]glucose, respectively. For the determination of the disintegrations per minute of L-[U-\(^{14}\text{C}\)]glucose and D-[3-\(^3\text{H}\)]glucose, 10 μl of dialysate and perfusate were pipetted into 20-ml scintillation vials, 100 μl of \(^{2}\text{H}_2\text{O}\) were added, and the solutions were mixed. The samples were dried in an oven (70°C) for 45 min until all of the liquid had evaporated, and then 100 μl of \(^{2}\text{H}_2\text{O}\) were added, and the samples were mixed thoroughly. Ten milliliters of scintillation fluid were then added for the determination of the disintegrations per minute of L-[U-\(^{14}\text{C}\)]glucose and D-[3-\(^3\text{H}\)]glucose, respectively. The microcentrifuge collection tubes were weighed before and after dialysate collection, and the exact collection time was also recorded. These data were used to determine the average probe perfusion rate during each collection period.

**Calculations.** Probe recovery based on the internal reference method was calculated as follows

\[
\text{recovery} = \left( \frac{P_{dpm} - D_{dpm}}{P_{dpm}} \right) \times 100
\]

where \(P_{dpm}\) and \(D_{dpm}\) represent the disintegrations per minute in the perfusate and dialysate, respectively, for glucose. The change in tissue uptake of glucose was calculated as the ratio of D-glucose to L-glucose (D/L ratio). The magnitude of change in insulin-stimulated glucose uptake was calculated using the index \((D - L)/D\), where D and L represent the probe recoveries for these isotopes, respectively.

**Statistics.** The change from control in all variables was analyzed using a paired Student’s t-test. A Bonferroni correction factor for multiple comparisons was used to determine where the significant differences occurred. All values are expressed as means ± SE, and significance was accepted at \(P < 0.05\).

**RESULTS**

**Protocol I.** The microdialysis probe perfusion rate during the control period was \(4.21 ± 0.12\ \mu\text{l/min}\) and was not significantly different from that measured during perfusion with insulin (0–15 min = \(4.24 ± 0.04\ \mu\text{l/min}\) and 15–30 min = \(4.24 ± 0.07\ \mu\text{l/min}\)). There were no significant differences in probe recovery between the D- \((42.3 ± 3.5\%)\) and L- \((41.2 ± 3.5\%)\) stereoisomers during the control period (Fig. 2). However, during insulin (1 μM) perfusion, probe recovery for D-glucose increased 33 and 23% \((P < 0.05)\) for the two collection periods, respectively, and were significantly different from the corresponding L-glucose probe recoveries for the same time periods (Fig. 2). As discussed above, the difference between the D- and L-glucose probe recovery ratio from control to insulin perfusion represents the change due to insulin-stimulated glucose uptake. In the present study, the D/L ratio was \(1.04 ± 0.03\) during the control period, and it increased to \(1.62 ± 0.08\) and \(1.58 ± 0.07\) \((P < 0.05)\) during the two insulin perfusion periods, respectively (Fig. 3). It should be noted that a D/L ratio of 1 represents no net skeletal muscle glucose uptake; therefore, the net change in insulin-stimulated glucose uptake may be better reflected by using a second index, that is, \((D - L)/L\). Subsequently, the \((D - L)/L\) index during the con-
Control period was 0.03 ± 0.03 and increased (P < 0.05) to 0.36 ± 0.03 and 0.35 ± 0.03 during the two insulin perfusion periods, respectively (Fig. 4). From these data, one can see that the increase in the D/L ratio during insulin perfusion actually reflects a 12-fold increase (0.36/0.03) in insulin-stimulated glucose uptake.

**Protocol II.** The microdialysis D/L glucose ratio during the control period was 0.96 ± 0.03 and increased to 1.16 ± 0.12 (P > 0.05), 1.41 ± 0.06, and 1.40 ± 0.10 (P < 0.05) during the perfusion of 0.1, 1.0, and 10 μM insulin, respectively (Fig. 5). The change in insulin-stimulated glucose uptake [(D−L)/D] from control to perfusion with 1.0 and 10 μM insulin was 8.2- and 8.0-fold, respectively. There were no significant differences in the D/L glucose ratio or (D−L)/D glucose index between the perfusions of 1.0 and 10 μM insulin, respectively.

**DISCUSSION**

This study represents a unique method for determining tissue-specific glucose uptake by use of radioactive stereoisomers in conjunction with the microdialysis technique. In the present study it was observed that the probe recovery ratio for D- and L-glucose was ~1 during perfusion of the control solution and that it increased significantly after 60 min of 1 μM insulin perfusion. It was further determined that this elevation in D/L glucose ratio was mediated by an increase in D-glucose recovery, whereas L-glucose recovery remained unchanged. In addition, it was observed that the net increase in insulin-stimulated glucose uptake as indicated by the (D−L)/D glucose index was 12-fold. Similarly, it was observed that perfusion of the microdialysis probes with insulin concentrations >1 μM did not result in any further increase in the glucose ratio D/L or glucose index (D−L)/D.

The rationale for determining probe recovery is that, under most experimental conditions, there is incomplete recovery (i.e., <100% equilibration) of the substance being investigated from the interstitial space. Therefore, it is necessary to determine what percentage of the total amount of the substance in the interstitial space has diffused into the microdialysis probe. Unfortunately, the relatively simple in vitro calibration procedure, which is conducted in a quiescent medium, has been shown to yield dramatically different probe recoveries from those determined in vivo for the same substance (3, 12). This has been attributed to a more restricted diffusion path associated with a complex biological medium, such as interstitial fluid. Thus it is necessary to perform in vivo microdialysis probe calibration if accurate and reliable data are to be generated.

A wide variety of factors can influence probe recovery during the course of an experiment. These include 1) blood flow, 2) muscle uptake/release, 3) microdialysis probe length, 4) perfusion rate, 5) probe membrane characteristics, and 6) the rate of diffusion in the in-
terstitial space (2, 9, 11, 13). In this report we reasoned that it might be possible to determine the relative change or contribution of one of the factors to probe recovery by simultaneously perfusing the microdialysis probes with two compounds that are not equally affected by one of the factors. For example, it is well established that the D-stereoisomer of glucose is readily taken up and metabolized by skeletal muscle, whereas the L-stereoisomer is not. Therefore, when radioactive tracers of both stereoisomers of glucose are simultaneously perfused through the microdialysis probes, then the L-stereoisomer is not influenced by changes in skeletal muscle glucose uptake. As a result, it is possible to examine and quantify changes in tissue glucose uptake by comparing the relative loss of these two stereoisomers.

In the present study, all of the factors affecting probe recovery, with the exception of glucose uptake, would influence each stereoisomer equally, because both stereoisomers were perfused simultaneously. It has been shown in previous studies that resting skeletal muscle metabolic demand is low, and subsequently glucose uptake is quite small (1, 8). In addition, because serum insulin levels are also low in fasted subjects, one would expect basal glucose uptake to be low. In the present study the D/L ratio was ~1 and the (D−L)/D glucose index was approximately zero during the control perfusion period, indicating very little basal glucose uptake. These data support the contention that there is very little basal skeletal muscle glucose uptake, and this was accurately reflected by the D/L ratio and the (D−L)/D glucose index.

It is well established that insulin stimulates skeletal muscle glucose uptake in a dose-dependent fashion (5, 17). In the present study, when the microdialysis probes were perfused with a solution containing 1 μM insulin, the (D−L)/D glucose index reflected a 12-fold increase in glucose uptake. This change in the D/L ratio and the (D−L)/D glucose index was mediated solely by an increase in D-glucose recovery, as no significant change in L-glucose recovery was observed. This is a unique finding and suggests that insulin perfusion results in an increase in skeletal muscle glucose uptake independent of any other changes in the factors that influence probe recovery. These data further suggest that the use of radioactive stereoisomers is an accurate and reliable method for examining changes in skeletal muscle glucose uptake directly at the tissue level.

In the present study, an insulin dose-response protocol was performed (protocol II) in which D-[3-3H]glucose was used instead of [6-14C]glucose. The rationale for using D-[3-3H]glucose was that some of the 3H label taken up by the muscle is converted to 3H2O by glycolysis. As a result, it is possible that some of the 3H2O may have been released back into the interstitial space and diffused back into the microdialysis probe. Therefore, it is possible to estimate all of the glucose metabolized unequivocally by drying the dialysate before scintillation counting to eliminate all 3H2O produced. When this was performed, it was observed that the addition of 1.0 μM insulin to the perfusate resulted in an elevation in the D/L ratio similar to that observed when D-[6-14C]glucose was used. Furthermore, the perfusion of a solution containing 10 μM insulin did not result in any greater increase in the D/L ratio or the (D−L)/D glucose index compared with perfusion with 1 μM insulin. These data suggest that altering the type of stereoisomer used (i.e., D-[3-3H]glucose vs. D-[6-14C]glucose) does not influence the change in the D/L ratio or the (D−L)/D glucose index in response to insulin perfusion. Furthermore, D/L and the (D−L)/D glucose index are capable of accurately reflecting the effects that different doses of insulin have on skeletal muscle glucose uptake and whether these effects are maximal.

In summary, this study describes a novel method for determining tissue-specific glucose uptake using radioactive stereoisomers and the microdialysis technique. It was demonstrated that by comparing the ratio of the metabolizable D-glucose to the nonmetabolizable L-glucose, one can accurately examine changes in skeletal muscle glucose uptake. The advantages of this new method are that each probe acts as its own control, because both stereoisomers are perfused through the same microdialysis probe, and thus each is influenced to the same extent by factors other than tissue uptake that determine probe recovery. In addition, a wide variety of compounds and/or drugs can be added to the perfusate, as well as a number of different stereoisomers, thus allowing researchers to examine not only glucose metabolism but also a wide variety of other cellular events.

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