Insulin does not change the intracellular distribution of hexokinase in rat heart

Doenst, Torsten, Qiuying Han, Gary W. Goodwin, Patrick H. Guthrie, and Heinrich Taegtmeyer. Insulin does not change the intracellular distribution of hexokinase in rat heart. Am. J. Physiol. 275 (Endocrinol. Metab. 38): E558–E567, 1998.—Preliminary evidence has suggested that hexokinase in rat heart changes its kinetic properties in response to insulin through translocation to the outer mitochondrial membrane. We reexamined this hypothesis in light of tracer kinetic evidence to the contrary. Our methods were as follows. Working rat hearts were perfused with Krebs-Henseleit buffer containing glucose (5 mmol/l) and sodium oleate (0.4 mmol/l). Dynamic glucose uptake was measured with [2-3H]glucose and with 2-deoxy-2-[18F]fluoroglucose (2-[18F]DG). Hexokinase activity was determined in the cytosolic and mitochondrial fractions. Our results are as follows. Uptake of glucose and uptake of 2-[18F]DG were parallel. Insulin (1 mU/ml) increased glucose uptake threefold but had no effect on 2-[18F]DG uptake. The tracer-to-tracee ratio decreased significantly. The Michaelis-Menten constant of hexokinase for 2-deoxyglucose was up to 10 times higher than for glucose. There was no difference in maximal reaction velocity. Two-thirds of hexokinase was bound to mitochondria. Insulin neither caused translocation nor changed Michaelis-Menten constant or maximal reaction velocity. In conclusion, the insulin-induced changes in the tracer-to-tracer ratio are due to a shift of the rate-limiting step for glucose uptake from transport to phosphorylation by hexokinase. Insulin does not affect the intracellular distribution or the kinetics of hexokinase.

isolated working rat heart; deoxyglucose; Percoll density-gradient centrifugation; isolated mitochondria; citrate synthase

HEXOKINASE (EC 2.7.1.1) catalyzes the conversion of glucose to glucose 6-phosphate, a step required for all glucose-metabolizing pathways. Hexokinase II is the isoform that predominates in adult mammalian myocardium. The enzyme is either located in the cytosol or associated with the mitochondria (3, 33). Interchange of the enzyme between these two compartments is regulated by the concentrations of glucose 6-phosphate and intracellular ions such as Mg2+. The effects of insulin on the translocation of hexokinase are a controversial topic (2, 3, 27), and the effects of such translocation on the kinetic properties of hexokinase for glucose and its analog 2-deoxyglucose (2-DG) are unknown.

An important concern regarding the use of 2-DG as tracer for glucose uptake is the lack of quantitation of myocardial glucose uptake (14). The accurate measurement of glucose transport and phosphorylation is important for two reasons: first, for the kinetic assessment of regulators of glucose metabolism, since glucose is an essential fuel for the heart (31); and second, for the identification of glucose-dependent, chronically ischemic (“viable”) myocardium. The lack of quantitation of glucose uptake by 2-DG may be due to differential changes in the kinetic properties of hexokinase for glucose and 2-DG upon translocation.

In a preliminary study, we suggested that the insulin-induced decrease in the ratio of 2-DG over glucose uptake (tracer-to-tracee ratio or lumped constant [LC]) (14, 26, 27) is due to the translocation of hexokinase from the cytosol to the mitochondria with a concomitant increase of the Michaelis-Menten constant (Km) of hexokinase for 2-DG (27). In contrast, evidence from tracer kinetic modeling of glucose uptake suggests that the kinetics of hexokinase are constant (5, 20).

Because of the preliminary nature of our earlier study on the mechanism responsible for changes in the tracer-to-tracee ratio (27), we devised extensive experiments to examine the effects of insulin on the location and the kinetic properties of hexokinase. We developed a new assay for hexokinase, and we selected perfusion conditions that were closer to the physiological conditions in vivo. We also controlled for the nutritional state because of differences in glucose metabolism in hearts from fed and fasted animals (28). In contrast to our preliminary study, we found that the location of hexokinase inside the cell and the kinetics of the enzyme were unaffected by insulin. The results of the present study invalidate the conclusions of our preliminary investigation. We provide evidence in support of the notion that the insulin-induced decrease in the tracer-to-tracee ratio is the result of a shift of the rate-limiting step for glucose uptake from transport to phosphorylation by hexokinase, which has a lower affinity for 2-DG than the glucose transporters.

METHODS

Animals

Male Sprague-Dawley rats (275–300 g) were obtained from Harlan (Indianapolis, IN). Animals were either fed ad libitum or fasted overnight (16–20 h) with free access to water. The use of animals and the experimental protocol were approved by the Animal Welfare Committee of the University of Texas-Houston Health Science Center.
HEXOKINASE AND MYOCARDIAL GLUCOSE UPTAKE

Materials

Chemicals were obtained from Fisher Scientific (Lexington, MA) or Sigma Chemical (St. Louis, MO). Enzymes and cofactors for metabolite assays were obtained from Boehringer Mannheim (Indianapolis, IN) or Sigma. Regular human insulin (Humulin R) was obtained from Eli Lilly (Indianapolis, IN) or Sigma Chemical (St. Louis, MO). Enzymes and cofactors for metabolite assays were obtained from Boehringer Mannheim (Indianapolis, IN) or Sigma.

Radioisotopes

The positron-emitting glucose analog 2-deoxy-2-[18F]fluoroglucose (2-[18F]DG, specific activity > 5,000 Ci/mmol) was prepared by the method of Hamacher et al. (13) at the University of Texas-Houston Health Science Center Cyclotron Facility (Houston, TX). HPLC-purified [2-3H]glucose, 2-deoxy-[1,2-3H]glucose (2-[1,2-3H]DG), and [U-14C]glucose were obtained from Amersham (Arlington Heights, IL). The purity of the 3H-labeled tracers was ascertained by measuring the intrinsic 3H2O content.

Working Heart Preparation

The preparation has been described in detail previously (32). Briefly, rats were anesthetized with pentobarbital sodium (5 mg/100 g body wt, ip). After injection of heparin (200 IU) into the inferior vena cava, the heart was rapidly removed and placed in ice-cold Krebs-Henseleit buffer. The aorta was freed of excess tissue and cannulated. A brief period of retrograde perfusion (~5 min) with oxygenated buffer containing glucose (5 mmol/l) was necessary to wash out any blood from the heart and to perform left atrial cannulation. Hearts were then perfused as working hearts at 37°C with recirculating Krebs-Henseleit buffer (200 ml) containing glucose (5 mmol/l) and sodium oleate (0.4 mmol/l) and both 2-[3H]glucose and 2-[18F]DG as tracers. Insulin was added to reach concentrations of 10, 50, 200, and 1,000 µU/ml at 20, 35, 50, and 65 min, respectively. At 80 min, the hearts were freeze-clamped.

Assessment of Contractile Performance

Mean aortic pressure (cmH2O) was calculated as (systolic – diastolic pressure × 2)/3. Heart rate was measured as beats per minute, and cardiac output was measured as milliliters per minute. Cardiac power was determined as (8)

\[ \text{Power (mW)} = \frac{\text{cardiac output (ml/min)} \times \text{mean aortic pressure (cmH}_2\text{O)}}{\text{conversion factor (612)}} \]

Measurement of Radioactivity

2-[18F]DG. Tissue accumulation of the positron-emitting 2-[18F]DG was measured on a second-to-second basis by a pair of coincidence detectors placed on opposite sides of the heart. Positron annihilation between the detectors was detected with a fast/slow coincidence system connected to a personal computer for data acquisition. 2-[18F]DG radioactivity in the perfusate was continuously counted by positron counting of a portion of the arterial side of the recirculating perfusate. All counts were decay corrected to the time at which 2-[18F]DG was added. The system was calibrated with a heart-shaped model (bar phantom) containing a known amount of radioactivity. A calibration factor (counts·s⁻¹·µCi⁻¹) was obtained from the decay curve of the bar phantom that was used to calculate glucose uptake rates (µmol·min⁻¹·g dry wt⁻¹) from the slopes of the time-activity curves. Slopes of fluorodeoxyglucose uptake were measured by 2-[18F]DG accumulation and 3H2O release from 2-[3H]glucose and ratio of tracer uptake over tracee uptake (lumped constant) of isolated working rat hearts from fasted animals. *P < 0.05 vs. 0; †P < 0.05 vs. all others.

Table 1. Influence of insulin on myocardial glucose uptake

<table>
<thead>
<tr>
<th>Insulin Concentration, µU/ml</th>
<th>Uptake rate by 2-[18F]DG, µmol·min⁻¹·g dry wt⁻¹</th>
<th>Uptake rate by 3H2O release, µmol·min⁻¹·g dry wt⁻¹</th>
<th>Tracer-to-tracee ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1.06 ± 0.17</td>
<td>0.95 ± 0.15</td>
<td>1.16 ± 0.20</td>
</tr>
<tr>
<td>10</td>
<td>1.02 ± 0.10</td>
<td>1.09 ± 0.18</td>
<td>1.08 ± 0.21</td>
</tr>
<tr>
<td>50</td>
<td>1.58 ± 0.20</td>
<td>2.05 ± 0.18</td>
<td>0.79 ± 0.09</td>
</tr>
<tr>
<td>200</td>
<td>1.67 ± 0.20</td>
<td>2.64 ± 0.18</td>
<td>0.70 ± 0.17</td>
</tr>
<tr>
<td>1,000</td>
<td>1.27 ± 0.16</td>
<td>4.11 ± 0.88</td>
<td>0.34 ± 0.04</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 4. Influence on myocardial glucose uptake as measured by both 2-deoxy-2-[18F]fluoroglucose (2-[18F]DG) accumulation and 3H2O release from 2-[3H]glucose and ratio of tracer uptake over tracee uptake (lumped constant) of isolated working rat hearts from fasted animals. *P < 0.05 vs. 0; †P < 0.05 vs. all others.
cose retention in the presence of insulin were obtained after a new steady state was reached (~10 min after addition of insulin).

3H and 14C. Dual-label counting of these isotopes was performed on a Packard 1900 TR liquid scintillation analyzer by the method of spectral index analysis, as described by the manufacturer (Packard Instruments, Meriden, CT).

Glucose uptake was determined by the rate of 3H2O production from [2-3H]glucose, as described by Katz and Dunn (17) and validated by us earlier (32). Release of 3H2O was separated from [2-3H]glucose in the perfusate by anion exchange chromatography on AG-1X8 resin (Bio-Rad Laboratories, Hercules, CA) (23). The amount of 3H2O in the perfusate was plotted against time, and the slopes of the desired intervals were used to calculate glucose uptake rates (expressed as µmol·min⁻¹·g dry wt⁻¹). 2-[1,2-3H]DG and [U-14C]glucose were used for the hexokinase assay as described in Hexokinase.

Tissue Analysis

The frozen tissue, ground under liquid nitrogen, was extracted with 6% perchloric acid. The tissue extracts were neutralized. A small portion of the pulverized tissue was dried in an oven (70°C) to constant weight, and the wet-to-dry ratio was calculated.

Perfusate Samples

Samples of the coronary effluent (1 ml) were withdrawn every 5 min. Samples were stored on ice until assayed for glucose and lactate with the use of a glucose/lactate analyzer (2300 STAT, YSI, Yellow Springs, OH). The samples were analyzed for the specific activity of [2-3H]glucose and for 3H2O content.

Subcellular Fractionation

Heart muscle homogenization. The cooled hearts were blotted dry, weighed, minced on ice, suspended in two volumes (vol/wt) of isolation medium (180 mmol/l KCl, 10 mmol/l Tris, and 0.5 mmol/l MgCl2, pH 7.4), and homogenized in a Polytron tissue homogenizer (Brinkmann Instruments, Westbury, NY) for 10 s at maximum speed. The pH was adjusted to 7.4 before the second addition of isolation medium (4 ml/g heart, vol/wt). The homogenization was completed with one downpass of a loosely fitting Teflon pestle in a Potter-Elvehjem glass homogenizer.

Density-gradient centrifugation. We modified the method of Chemnitius et al. (6) to isolate subsarcolemmal and interfibrillar mitochondria. All preparation steps were carried out at 4°C. Percoll (Pharmacia, Uppsala, Sweden) step gradients were prepared with isolation medium in polycarbonate tubes (Du Pont, Wilmington, DE). Heart muscle homogenates (600 µl) were suspended in isolation medium (180 mmol/l KCl, 10 mmol/l Tris, and 0.5 mmol/l MgCl2, pH 7.4) and Percoll to give a 2-ml fraction containing 65% (vol/vol) Percoll at the bottom of the tube. Lighter layers containing 50% (2 ml), 30% (3 ml), and 5% (1 ml) Percoll in isolation medium, respectively, were successively layered on top. Gradients were spun for 60 s at 20,000 rpm (50,000 g maximal gravitational force) in a Sorvall RC-5B centrifuge with an SS-34 rotor. The shape of density gradients was monitored using color-coded density marker beads (Pharmacia). Eight fractions (1 ml each) were collected through a small hole melted in the bottom of the centrifugation tube. Subsarcolemmal mitochondria were recovered in gradient fraction 4 (buoyant density 1.068 g/ml), and interfibrillar mitochondria were recovered in fraction 7 (buoyant density 1.040 g/ml). The location of the mitochondria had been confirmed by electron microscopy earlier (6). The cytosol was recovered in fractions 1 and 2.

Enzyme Assays

Citrate synthase. Citrate synthase (EC 4.1.3.7) was determined by the method of Srere (30). Assays were performed at 25°C. Total and free citrate synthase activities of homogenate and gradient fractions were determined after preincubation with or without 2.5% (vol/vol) Triton X-100. The free citrate synthase activity is considered an indicator for structurally damaged mitochondria. Latent citrate synthase activity was calculated as the difference between total and free activity and represents structurally intact mitochondria (6).

Hexokinase. Hexokinase was determined by a modification of the method of Gots and Bessman (11). The method is based on the conversion of [U-14C]glucose to [U-14C]glucose 6-phosphate or 2-[1,2-3H]DG to 2-deoxy-[1,2-3H]glucose 6-phosphate and the separation of the two compounds by batch chromatography with the use of Dowex 2-X8 resin (Bio-Rad). All assays were performed at room temperature.

Maximal reaction velocity of hexokinase was determined in all eight fractions and the homogenate. For the determination of the Kₘ values of hexokinase associated with mitochondria, equal volumes of fractions 4 (subsarcolemmal mitochondria) and 7 (interfibrillar mitochondria) were combined. The sample with the cytosolic hexokinase was obtained by passing 600 µl of fraction 1 (which was free of latent citrate synthase) through a Sephadex G25 column (5 ml) to remove any endogenous glucose. The sample was eluted from the column with 4 volumes of isolation medium.

For the assays using purified enzyme (beef heart hexokinase, Sigma), 0.125 mg/ml BSA was added to the reaction medium (see below). The concentration of hexokinase in the assay ranged from 5 to 60 ng/ml depending on the glucose 6-phosphate concentration, which ranged from 0 to 100 µmol/l.

The reaction medium consisted of Tris (134 mmol/l), MgCl₂ (40.3 mmol/l), and ATP (6.7 mmol/l), pH 7.5. One hundred microliters of sample were mixed with 400 µl of reaction medium. The samples were diluted so that not more than 5% of substrate was phosphorylated. The reaction was started with the addition of substrate solution. For the determination of the maximal reaction velocity of hexokinase on the gradient and in the homogenate, 100 µl of a 10 mmol/l glucose solution with 0.2 µCi/µmol [U-14C]glucose were added to start the reaction. For the determination of the Kₘ values in the cytosolic and mitochondrial fractions, 100 µl of glucose or 2-DG solutions of five different concentrations and specific activities were added. The glucose concentrations varied from 0.30 to 1.02 mmol/l, with specific activities of 0.22 to 0.40 µCi/µmol [U-14C]glucose. The 2-DG concentrations varied from 0.38 to 1.80 mmol/l, with specific activities of 0.22 to 0.50 µCi/µmol 2-[1,2-3H]DG. For the determination of the Kₘ values of the purified enzyme, the above glucose solutions were used with 2-[1,2-3H]DG present at the same time in tracer amounts.

The reaction was stopped after 10 min by transferring 500 µl of the reaction mixture into 4 ml of 1 mol/l glucose in 0.17 mol/l NH₄OH containing 250 mg Dowex 2-X8 resin. The samples were vortexed vigorously, and the resin was washed four times with 4 ml of 0.17 mol/l NH₄OH. Glucose 6-phosphate or 2-deoxyglucose 6-phosphate was eluted from the resin by addition of 1 ml HCl (1 mol/l). Five hundred microliters of the eluent were counted in 10 ml of scintillation fluid. All assays were performed in duplicate. A reagent blank was run for every substrate solution. Analysis of enzyme
kinetics was performed according to Eadie and Hofstee (see Ref. 10). We determined maximal reaction velocity ($V_{\text{max}}$), $K_m$, the specificity constant ($V_{\text{max}}/K_m$), and the ratio between the specificity constant of hexokinase for 2-DG and glucose ($R_p$; Ref. 20).

Statistical Analysis

All data are presented as means ± SE. Statistical comparison was by paired or unpaired Student's $t$-test or by single-factor ANOVA, with post hoc comparison by Newman-Keuls test as appropriate (34). Differences were considered statistically significant when $P < 0.05$. NS indicates no significance.

RESULTS

Cardiac Performance

Cardiac power was between 6.5 and 10 mW and was stable throughout all experiments. The addition of insulin did not change cardiac power (see Fig. 1B for a representative tracing).

Glucose Uptake

Figure 1A shows the tissue time-activity curve for 2-[18F]DG accumulation and myocardial $^3$H$_2$O release from [2-3H]glucose of a heart from a fasted animal. 2-[18F]DG accumulation in the tissue and release of $^3$H$_2$O were linear both before and after the addition of insulin. 2-[18F]DG radioactivity in the perfusate (input function) remained constant. Before the addition of insulin, the slopes of 2-[18F]DG retention and of cumulative $^3$H$_2$O production paralleled each other. The tracer-to-tracer ratio was 1.12. After the addition of insulin, the curves diverged. The cumulative release of $^3$H$_2$O increased significantly, whereas the pattern of 2-[18F]DG retention showed a biphasic response. During the adjustment of a new steady state, the slope of 2-[18F]DG retention increased temporarily, but accumulation remained unchanged for the rest of the perfusion compared with before the addition of insulin. The tracer-to-tracer ratio decreased to 0.44.

Figure 2 shows the rates of glucose uptake obtained by the two tracer methods for the experimental groups before and after insulin stimulation. Before the addition of insulin, rates of glucose uptake by hearts from fed rats were $1.67 ± 0.43$ µmol·min$^{-1}$·g dry wt$^{-1}$ as measured by release of $^3$H$_2$O and $1.80 ± 0.33$ µmol·min$^{-1}$·g dry wt$^{-1}$ as measured by 2-[18F]DG accumulation ($P = 0.815$). In the fasted state, uptake rates were slightly smaller, with rates of $1.05 ± 0.12$ µmol·min$^{-1}$·g dry wt$^{-1}$ as measured by release of $^3$H$_2$O and $1.40 ± 0.12$ µmol·min$^{-1}$·g dry wt$^{-1}$ as measured by 2-[18F]DG accumulation (NS compared with hearts from fed animals; $P = 0.289$ and 0.372, respectively). The rates obtained by the two different methods were also not different. With insulin stimulation, glucose uptake, as measured by release of $^3$H$_2$O, increased to $4.31 ± 0.93$ µmol·min$^{-1}$·g dry wt$^{-1}$ in hearts from fed animals and to $2.99 ± 0.27$ µmol·min$^{-1}$·g dry wt$^{-1}$ in hearts from fasted animals. This increase was not paralleled by an increase in 2-[18F]DG accumulation. Hence the tracer-to-tracer ratio decreased from $1.15 ± 0.15$ to $0.41 ± 0.06$ ($P < 0.001$) in the fed state and from $1.34 ± 0.07$ to $0.66 ± 0.08$ ($P < 0.001$) in the fasted state.

We obtained dose-response curves for the effects of insulin on 2-[18F]DG and glucose uptake on hearts from fasted rats. Figure 3 shows the tissue time-activity curve and the release of $^3$H$_2$O from a representative experiment. Rates of glucose uptake for the group, as determined by release of $^3$H$_2$O and 2-[18F]DG accumulation, and the tracer-to-tracer ratio for the different perfusion periods are shown in Table 1. Insulin increased glucose uptake more than 2-[18F]DG uptake, and, consequently, the tracer-to-tracer ratio decreased in a dose-dependent manner. In the absence of insulin, both 2-[18F]DG and glucose uptake remained un-
changed for the 80-min perfusion period (data not shown).

**Hexokinase and Citrate Synthase**

Subcellular fractionation of homogenates prepared from the perfused heart muscle was performed by Percoll density-gradient centrifugation. We obtained eight fractions with densities between 1.095 and 1.027 g/ml. Both hexokinase and citrate synthase activities were almost completely recovered from the gradient. The recovery of hexokinase in the groups ranged from 77.3 ± 7.11 to 88.4 ± 1.76%, and the recovery of citrate synthase ranged from 89.1 ± 3.19 to 98.7 ± 4.30% of the activity added.

The distribution of hexokinase and citrate synthase on the gradient is shown in Fig. 4 for a representative experiment. Figure 4A shows that free citrate synthase is mainly located in the first two fractions of the gradient. This activity represents completely solubilized enzyme. The free activity in fractions 3–8 represents particle-associated activity (6). The latent citrate synthase is mainly located in fractions 4 and 7, which have been classified as the fractions containing subsarcolemmal (fraction 4, 1.068 g/ml) and interfibrillar (fraction 7, 1.040 g/ml) mitochondria (6). Fractions 1 and 2 are termed the cytosolic fractions (see METHODS). Fractions 3, 5, and 6 contain mitochondria of intermediate density. The distribution of citrate synthase on the gradient was the same in all groups and was not influenced by the nutritional state or insulin addition. Between 56.6 ± 4.14 and 64.1 ± 4.58% of the latent citrate synthase was recovered in fractions 4 and 7. The enrichment of latent citrate synthase in these fractions varied from 2.30 ± 0.17 to 2.56 ± 0.18 among groups (NS).

Figure 4B shows hexokinase activity in the same fractions. The highest activities are found in fractions 1, 2, 4, and 7. Fractions 1 and 2 contain the cytosolic enzyme. Fractions 4 and 7 are the same as the fractions with the highest latent citrate synthase activity and represent hexokinase bound to mitochondria.

Table 2 shows hexokinase and citrate synthase activity of isolated hearts from fed or fasted animals before or after stimulation with insulin. Activities in cytosolic and particulate fractions are shown separately. In both the fed and the fasted state, between 30 and 35% of the...
hexokinase activity was found in the cytosolic fractions (fractions 1 and 2), and 65–70% was associated with subcellular particles. Insulin addition changed neither the pattern of hexokinase distribution nor the pattern of citrate synthase distribution.

Table 3 shows mitochondria-associated hexokinase expressed as activity per latent citrate synthase. Latent citrate synthase is a marker for structurally intact mitochondria. There was no difference in the hexokinase-to-latent citrate synthase ratio between the fed and the fasted state. Addition of insulin did not change this ratio.

Table 4 shows the $K_m$ of hexokinase for glucose and 2-DG as well as the $V_{\text{max}}$ in the cytosolic and in the mitochondrial fractions. The sample for the determination of the kinetics of hexokinase in the cytosol came from fraction 1 of the density gradient (which is free of latent citrate synthase) and was subjected to a Sephadex G25 column to remove all endogenous glucose before performance of the assays. To test the influence of remaining fragments of cell or mitochondrial membrane in fraction 1, we centrifuged the cytosolic samples of selected experiments for 30 min at 450,000 $g$ and measured $K_m$ values for hexokinase in the supernatant. No significant changes were detectable (data not presented).

The sample representing the mitochondrial part of the hexokinase activity came in equal volumes from fractions 4 and 7. Prior experiments in the presence or absence of insulin did not reveal any differences in the kinetics of hexokinase associated with subcellular mitochondria (fraction 4) or interfibrillar (fraction 7) mitochondria (data not presented).

The $K_m$ of cytosolic hexokinase for glucose was $40.7 \pm 9.12$ $\mu$mol/l in hearts from fed and $37.6 \pm 12.3$ $\mu$mol/l in hearts fromfasted animals ($P = 0.965$, Table 4). Similar values were obtained for hexokinase associated with mitochondria. The $K_m$ of hexokinase for 2-DG was 5–10 times higher than that for glucose in both the cytosolic and the mitochondrial fractions. The $K_m$ values of hexokinase in the cytosol were not different from the $K_m$ values of hexokinase associated with mitochondria. Insulin stimulation did not result in a change of the measured values.

Table 3. Hexokinase activity associated with structurally intact mitochondria

<table>
<thead>
<tr>
<th>Nutritional State</th>
<th>Hexokinase/Latent Citrate Synthase ($1 \times 10^3$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fed No insulin</td>
<td>14.2 $\pm$ 2.61</td>
</tr>
<tr>
<td>Fed Insulin</td>
<td>14.2 $\pm$ 2.61</td>
</tr>
<tr>
<td>Fasted No insulin</td>
<td>13.4 $\pm$ 2.04</td>
</tr>
<tr>
<td>Fasted Insulin</td>
<td>13.4 $\pm$ 2.04</td>
</tr>
</tbody>
</table>

Values are means $\pm$ SE; $n = 4–5$ in each group. Hexokinase activity associated with structurally intact mitochondria, expressed as ratio of hexokinase over latent citrate synthase activity in combined mitochondrial fractions (fractions 4 plus 7) of Percoll density gradient. Hearts came from fed or fasted animals and were perfused in presence or absence of insulin (1 $\mu$U/ml). See Perfusion Protocol for details. There are no significant differences among groups.
for hexokinase in the cytosol and in the absence of glucose 6-phosphate, the value was 0.12 ± 0.01. The Rp values were the same in all groups (ratios varied from 0.10 ± 0.03 to 0.14 ± 0.04; NS, P = 0.791 for hexokinase in the cytosol and P = 0.809 for hexokinase associated with mitochondria). The overall mean value was 0.12 ± 0.02.

Table 4. K_m and V_max of cytosolic hexokinase and of hexokinase associated with mitochondria

<table>
<thead>
<tr>
<th></th>
<th>K_m of HK, µmol/l</th>
<th>V_max of HK, µU/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>For glucose 2-DG</td>
<td>For glucose 2-DG</td>
</tr>
<tr>
<td>No insulin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cytosol</td>
<td>40.7 ± 9.12</td>
<td>237 ± 83.1*</td>
</tr>
<tr>
<td>Mitochondria</td>
<td>43.5 ± 9.01</td>
<td>327 ± 63.9*</td>
</tr>
<tr>
<td>Insulin</td>
<td></td>
<td></td>
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<tr>
<td>Cytosol</td>
<td>65.2 ± 7.46</td>
<td>316 ± 107</td>
</tr>
<tr>
<td>Mitochondria</td>
<td>38.2 ± 4.75*‡</td>
<td>248 ± 19.4*</td>
</tr>
<tr>
<td>Fasted</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No insulin</td>
<td>40.7 ± 9.12</td>
<td>237 ± 83.1*</td>
</tr>
<tr>
<td>Cytosol</td>
<td>37.6 ± 12.3</td>
<td>304 ± 56.7*</td>
</tr>
<tr>
<td>Mitochondria</td>
<td>43.6 ± 2.57</td>
<td>246 ± 27.2†</td>
</tr>
<tr>
<td>Insulin</td>
<td>47.4 ± 4.48</td>
<td>650 ± 263</td>
</tr>
<tr>
<td>Cytosol</td>
<td>53.9 ± 12.2</td>
<td>451 ± 105†</td>
</tr>
<tr>
<td>Mitochondria</td>
<td>47.4 ± 4.48</td>
<td>650 ± 263</td>
</tr>
</tbody>
</table>

The V_max values are means ± SE; n = 4–5 in each group. Michaelis-Menten constant (K_m) and maximal enzyme activity (maximal reaction velocity, V_max) of cytosolic hexokinase (HK) and of HK associated with mitochondria for glucose and 2-deoxyglucose (2-DG) after Percoll density-gradient centrifugation. Analysis was performed with the use of Eadie-Hofstee analysis (see Ref. 10). Hearts came from fed or fasted animals and were perfused at physiological workload with Krebs-Henseleit buffer containing glucose (5 mmol/l) and oleate (0.4 mmol/l). See Perfusion Protocol for details. *P < 0.05 and †P < 0.01 compared with "for glucose." ‡P < 0.05 and §P < 0.01 compared with cytosol.

The V_max was slightly higher for glucose than for deoxyglucose in all groups (significant only in the mitochondrial fractions before and after insulin stimulation of hearts from fed animals). The mitochondrial fractions contained more activity than the cytosolic fractions, which is also reflected in the distribution of hexokinase on the gradient. Stimulation with insulin did not influence V_max.

The specificity constants (U/mmol) of hexokinase for glucose ranged between 0.18 ± 0.04 and 0.24 ± 0.04 in the cytosolic and between 0.40 ± 0.09 and 0.56 ± 0.06 in the mitochondrial fractions. These values were 10 times higher than those for 2-DG (cytosol, 0.02 ± 0.005 to 0.03 ± 0.01; mitochondria, 0.04 ± 0.01 to 0.06 ± 0.01). The R_p values were the same in all groups (ratios varied from 0.10 ± 0.03 to 0.14 ± 0.04; NS, P = 0.791 for hexokinase in the cytosol and P = 0.809 for hexokinase associated with mitochondria). The overall mean value was 0.12 ± 0.02.

For 2-DG). Addition of glucose 6-phosphate resulted in a noncompetitive pattern of inhibition. At a concentration of 100 µmol/l glucose 6-phosphate, the V_max was reduced by 95%. The inhibition constant of hexokinase for glucose 6-phosphate was 2.1 µmol/l. The R_p was the same at all times (0.13 ± 0.004; n = 4), independent of the degree of hexokinase inhibition by glucose 6-phosphate.

DISCUSSION

The results of this study can be summarized as follows: the insulin-induced changes of the LC in rat heart in the presence of a physiological mixture of substrates can neither be explained by changes of the affinity of substrates to hexokinase nor by translocation of the enzyme. Instead, our data suggest that the observed changes of the LC are the result of a shift of the rate-limiting step for glucose uptake from transport to phosphorylation by hexokinase, which has a lower affinity for 2-DG than the glucose transporters. These results have direct implications for the quantitative assessment of myocardial glucose uptake in vivo because they validate an important prerequisite for the quantitative assessment of myocardial glucose uptake by tracer kinetic modeling.

Experimental Model

The simultaneous dynamic assessment of myocardial glucose and 2-DG uptake can only be performed in the isolated heart perfused with crystalloid buffer. In the present working rat heart model, both workload and metabolic environment are strictly controlled. Pressure-volume work performed in this model is comparable to the work performed in vivo (1). At the same time, glucose uptake can be measured simultaneously with the glucose tracer [2-3H]glucose and the glucose tracer analog 2-[18F]DG. As a result, the tracer-to-tracee ratio (LC) can be determined directly.

We chose a perfusate composition including physiological concentrations of the two main substrates for the heart in vivo, long-chain fatty acids (0.4 mmol/l oleate) and glucose (5 mmol/l). We investigated hearts from fed or fasted animals separately because glucose metabolism and insulin sensitivity of the heart differ between the two nutritional states (28). Our results suggest that the nutritional state neither influences hexokinase activity nor the effects of insulin on the tracer-to-tracee ratio of 2-DG and glucose.

Insulin Dose-Response Curves

To increase myocardial 2-[18F]DG uptake, insulin is commonly administered to patients undergoing positron emission tomography studies (18). The standard hyperinsulinemic clamp technique used for positron emission tomography studies usually increases insulin concentration to ~100–200 µU/ml (18, 21). Insulin and glucose concentrations are also clamped in the perfusate of the present isolated working rat heart preparation. At insulin concentrations comparable to those achieved in vivo, we also observed an increase in
2-[18F]DG uptake in vitro (Table 1, Fig. 3; between 50 and 200 µU/ml insulin). However, the effect cannot be observed at high insulin concentrations, which we chose for this study to elicit maximal insulin effects on the tracer-to-tracee ratio (Table 1, Fig. 2). We reasoned that if translocation of hexokinase is responsible for the decrease in the tracer-to-tracee ratio, the effects of insulin on hexokinase (distribution and kinetics) should be most pronounced at the concentration that also affects the tracer-to-tracee ratio the most.

It appears that at high insulin concentrations, insulin suppresses the tracer-to-tracee ratio more than it stimulates glucose uptake. Therefore, 2-[18F]DG uptake may actually decrease despite an increase in glucose uptake. This phenomenon has been previously observed with insulin dose-response studies on 2-deoxy-[14C]glucose uptake of rat heart in vivo (15). The investigators found that uptake of 2-deoxy-[14C]glucose increased with the addition of low concentrations of insulin (50–150 µU/ml) and returned to baseline values at high concentrations (670–7,600 µU/ml), whereas insulin stimulated glycogen synthesis at all concentrations.

The dose-response curves may not be fully representative for this phenomenon, since steady-state conditions may not have been reached during the short perfusion periods at each insulin concentration. Nevertheless, it is important to keep in mind that the tracer-to-tracee ratio is already decreased at low concentrations of insulin, and true glucose uptake will therefore be underestimated by 2-[18F]DG unless the insulin-induced changes of this ratio are taken into account.

Methodological Considerations

Our findings are in contradiction with results published earlier (27), in which we reported translocation of hexokinase from the cytosol to the mitochondrial membrane with a concomitant increase of the K_m of hexokinase for 2-DG. This decrease in affinity of hexokinase for 2-DG seemed to explain the observed changes in the tracer-to-tracee ratio but is in contradiction with the concept of the mathematical model described above because R_p would not remain constant.

There are several reasons for the differences between our present and our earlier study on hexokinase (27). First, in the present study, we removed all glucose from the samples before the hexokinase assays were performed. The glucose contained in the extracellular fluid space can give rise to free glucose concentrations in the homogenate of up to 400 µmol/l (with the assumption of a perfusate concentration of 10 mmol/l, a final homogenate volume of 10 ml, an extracellular fluid and vascular space of 40%, and a heart weight of 1 g (27)). A correction for the glucose content in the sample is not possible when the kinetics of hexokinase for 2-DG are investigated. These results are therefore affected most and have to be considered invalid. Prior studies (4, 11, 29), including our own (27), did not include the removal of glucose from the sample. Second, we chose to use a KCl-based isolation medium in contrast to a mannitol-based medium used in the last study. Although mannitol has been used for the isolation of mitochondria and the determination of hexokinase activity (11, 19), the chemical similarity of mannose to glucose (both are substrates for hexokinase) and a possible oxidation of mannitol to mannose by enzymes in the homogenate introduce another factor that may interfere with the assay. Third, the composition of the perfusion medium was different. Glucose was present at 5 mmol/l (not 10 mmol/l), oleate (0.4 mmol/l) was added as a second substrate, and the insulin concentration was 1 µU/ml (not 40 µU/ml). Fourth, we used an ultrafast isolation technique to separate mitochondria from the cytosol. With the use of Percoll density-gradient centrifugation, we were possible to increase the recovery of mitochondria from 20–40% to almost 100% (6). Hexokinase recovery was also almost complete. It is possible that the increase in hexokinase activity associated with mitochondria that we observed with the conventional isolation technique (27) is due to differences in mitochondrial recovery. In the present study we do not see any change in the distribution of hexokinase on the gradient that would be consistent with translocation. Our findings are in agreement with those of other investigators who used different centrifugation to isolate mitochondria from rabbit heart (2).

The localization of hexokinase in the myocardial cell is influenced by several factors. Hexokinase is bound to a voltage-dependent anion channel on the outer membrane of the mitochondrion. Glucose 6-phosphate causes a release and neutral ion solutions promote binding of the enzyme (9). Of all ions, Mg2+ has the strongest binding potential. Because the K+ ions promote binding only insignificantly (9), we chose to add an intracellular concentration of MgCl2 [0.5 mmol/l (16)] to the isolation medium. Under the described conditions, we find between 65 and 70% of the hexokinase associated with mitochondria, which is in agreement with other reports in the literature (4).

Kinetics of Hexokinase

The half-saturation constants (K_m) of hexokinase for glucose and 2-DG presented in this study are in agreement with results from other studies (2, 12, 22). Manchester et al. (22) report a K_m of hexokinase for glucose in extracts from isolated myocytes of 30 µmol/l. With insulin stimulation, the authors report a 30-fold increase in glucose uptake and measure an intracellular free glucose concentration of 300 µmol/l. The investigators concluded that under these conditions, hexokinase is nearly saturated, since the substrate concentration is 10 times higher than the K_m. The same argument would apply to our results. If insulin increases glucose uptake threefold (as in this study), and if one assumes that hexokinase is saturated, hexokinase activity should be <35% of the V_max in the absence of insulin. With a K_m of hexokinase for glucose between 30 and 45 µmol/l (as reported here by us and elsewhere by Manchester et al. (22)), the intracellular glucose concentration should be <30 µmol/l. Manchester et al. overlooked their own finding that the intracellular glucose concentration in the absence of insulin was...
100–200 μmol/l. If insulin doubles this concentration, a 30-fold increase in phosphorylation activity is only explainable when the enzyme is strongly inhibited in vivo and insulin alleviates this inhibition. We observed intracellular free glucose concentrations of 1.0 mmol/l in the presence of β-hydroxybutyrate (4.0 mmol/l) as cosubstrate. This concentration doubled with insulin (unpublished observations). It seems unlikely that the K_m or the V_{max} values of hexokinase in vivo are close to the values obtained in vitro, and it is conceivable that, in vivo, the enzyme is strongly inhibited. This line of reasoning is supported by other investigators who estimated that hexokinase in vivo operates at ≈10–15% of its potential V_{max} (24).

Specificity Constants

The process of glucose or 2-DG uptake measures the combined effects of transport and phosphorylation. The transporter protein prefers the transport of 2-DG over glucose (7), whereas hexokinase phosphorylates glucose in preference to 2-DG (12). The mathematical model proposed to predict the tracer-to-tracee ratio (20) is based on these concepts, i.e., it is dependent on the relative contributions of transport and phosphorylation to total glucose or 2-DG uptake (20). In other words, under conditions in which transport is rate limiting, the tracer-to-tracee ratio will be high, whereas under conditions in which phosphorylation is rate limiting, the ratio will be low. However, it could be argued that the proposed shift in the rate-limiting step of glucose uptake is due to an artificial concept because of the use of 2-DG, since 2-DG is preferred by the transporters and glucose is preferred by hexokinase. This possibility is unlikely to be the case because a steady state could never be reached under these conditions. In contrast, a continuous downcurving of the fluorodeoxyglucose tracings would be expected, while glucose uptake remains unchanged. In addition, the concept that insulin shifts the rate-limiting step from glucose transport to phosphorylation has also been proposed by other investigators who did not use 2-DG (22, 25).

One of the prerequisites for the prediction of the tracer-to-tracee ratio by the mathematical model is the assumption that the ratio of 2-DG to glucose phosphorylation (R_p) is constant. Here we provide strong support for the validity of this assumption. R_p can be expressed as the ratio of the specificity constants of hexokinase for the two hexoses, K_m or V_{max}. The ratio of the specificity constants of hexokinase for the two hexoses remained unchanged. These findings were corroborated with experiments using purified hexokinase. With glucose at substrate concentration and 2-DG at tracer concentration, a constant value for R_p was obtained that was in agreement with the values measured in the cytosolic and mitochondrial fractions of the heart. Furthermore, the values for R_p with purified hexokinase were not changed under widely varying conditions and inhibition by glucose 6-phosphate. Although all of these results were obtained from in vitro experiments, the heterogeneity of the experimental conditions and the similarity of the results make it reasonable to assume that similar results would be obtained from in vivo studies if the determination was possible. The mathematical model also assumes that the ratio of 2-DG to glucose transport is constant. Although the effects of insulin on the kinetics of the glucose transporters have not been assessed in this study, it is of importance to investigate this field because glucose uptake in heart muscle is governed by two transporter isoforms that may have different kinetic properties.

Conclusions

Insulin neither affects the localization nor the kinetic properties of hexokinase in rat heart. The results support the notion that the insulin-induced decrease in the tracer-to-tracee ratio is caused by a shift in the rate-limiting step of glucose uptake from transport to phosphorylation.

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Address for reprint requests: H. Taegtmeyer, Dept. of Internal Medicine, Div. of Cardiology, Univ. of Texas-Houston Medical School, 6431 Fannin, MSB 1.246, Houston, TX 77030.

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