Compartmentation of Hexokinase in Rat Heart
A Critical Factor for Tracer Kinetic Analysis of Myocardial Glucose Metabolism

Raymond R. Russell III, Joseph M. Mrus, Jane I. Mommessin, and Heinrich Taegtmeyer
Department of Medicine, Division of Cardiology, University of Texas Medical School at Houston, Texas 77030

Abstract
Radiolabeled analogues of 2-deoxyglucose are widely used to trace glucose metabolism in cell cultures, whole organs, and intact animals, although kinetic differences in transport and phosphorylation between these compounds and glucose exist. The present studies were undertaken to determine the effects of insulin stimulation on the phosphorylation of 2-deoxyglucose compared to glucose in the intact, saline-perfused working rat heart. Rates of glucose utilization determined from tritiated glucose differed from rates estimated from the accumulation of [14C]2-deoxyglucose in a nonconstant manner when comparing rates in the absence or presence of physiologic levels of insulin (13 μU/ml). The fraction of monophosphorylated hexoses that was accounted for by [14C]2-deoxyglucose 6-phosphate was dramatically decreased in hearts perfused in the presence of insulin. Additionally, hexokinase activity associated with the mitochondrial fraction of tissue extracts was increased in hearts stimulated by insulin. While this redistribution of hexokinase to the mitochondria did not affect the apparent affinity constant for glucose, hexokinase bound to mitochondria exhibited an 8.5-fold decrease in the affinity for 2-deoxyglucose when compared with hexokinase present in the cytosolic fraction. The findings are consistent with an insulin-mediated preferential uptake and phosphorylation of glucose compared to deoxyglucose. These results also imply that the redistribution of hexokinase and the differential effect of insulin on its affinity for tracer and tracee are responsible for changes in the "lumped constant" (i.e., the correction factor used to equate 2-deoxyglucose to glucose uptake). These changes must be taken into account when regional myocardial glucose metabolism is assessed by the 2-deoxyglucose method. (J. Clin. Invest. 1992. 90:1972–1977.) Key words: 2-deoxyglucose • positron emission tomography • hexokinase • insulin • lumped constant

Introduction
Myocardial glucose metabolism depends on many factors including fuel supply, the hormonal environment of the heart, and the energy demands placed on the heart. These factors act in concert to regulate both the rate of glucose transport across the sarcolemma and the intracellular fate of glucose. The control of glucose utilization may therefore shift between several different levels depending on the metabolic state of the heart. Since the validation of the 2-deoxy-d-glucose method for tracing brain glucose metabolism by Sokoloff and his co-workers (1), this method has been extended to the heart using positron emitting 2-[18F]fluoro-2-deoxy-D-glucose (2, 3). The development of a simplified graphical method to determine rates of glucose uptake based on the trapping of tracer in an irreversible compartment (4, 5), has allowed studies of the kinetics of glucose uptake by external detection in the intact heart both in vitro and in vivo. Initial work on the rapid kinetic analysis of glucose metabolism in the intact heart has been reported (6–8) and has suggested that the relationship between uptake of the tracer, 2-[18F]fluoro-2-deoxy-D-glucose, and uptake of the tracee, glucose, is not always constant.

Because glucose analogues based on 2-deoxyglucose are presumed to be trapped as 2-deoxyglucose 6-phosphate in the heart, they trace glucose metabolism through the steps of glucose transport and phosphorylation via hexokinase. In both of these steps, the tracer compounds may differ kinetically from glucose (9–12). It is for this reason that the lumped constant (i.e., the correction factor used to equate 2-deoxyglucose to glucose uptake) must be used to derive rates of glucose utilization from rates of 2-deoxyglucose uptake and phosphorylation (1). Therefore, changes in the kinetics of either 2-deoxyglucose transport or phosphorylation that do not parallel changes in the kinetics of glucose transport or phosphorylation will result in changes of the lumped constant.

Insulin stimulation, which acts to recruit glucose transporters to the sarcolemma, has been shown to increase the lumped constant in the heart (8). But because 2-deoxyglucose is favored over glucose by glucose transporters (9, 10), insulin stimulation would be expected to increase 2-deoxyglucose uptake relative to glucose and result in a decrease in the value for the lumped constant. It is therefore unlikely that the increase in the lumped constant caused by insulin is due to changes in 2-deoxyglucose transport.

Conversely, hexokinase has been shown to favor glucose (11, 12) over its analogues. In addition, it has been shown that one of the actions of insulin is to increase the binding of hexokinase to mitochondria in heart, skeletal muscle, and brain (13–16) and that binding decreases the ability of glucose 6-phosphate to inhibit the enzyme (17, 18). Because the association of hexokinase with mitochondria could alter the apparent affinity constant (Km) for either glucose or 2-deoxyglucose, it is necessary to determine if differences do indeed exist in the affinity constants for glucose and 2-deoxyglucose for hexokinase that is bound to mitochondrial particles. To this end, the present studies were undertaken to investigate the effects of insulin on the distribution of hexokinase between the soluble, cytosolic fraction and the particulate, mitochondrial fraction, and the resulting effects on the kinetic characteristics of the enzyme for the two substrates, glucose and 2-deoxyglucose. The results indicate that insulin leads to a significant increase
in hexokinase bound to mitochondria which favors phosphorylation of glucose over 2-deoxyglucose.

**Methods**

*Perfusion protocol.* Male Sprague-Dawley rats (300–350 g) were fasted overnight (16–20 h) with free access to water. The rats were anesthetized with sodium pentobarbital (15 mg/100 g body wt, i.p.). Before thoracotomy, heparin (200 IU, i.v.) was administered. The heart was rapidly removed and mounted on an anortic cannula to begin retrograde perfusion to flush residual blood out of the heart chambers and coronary vasculature. The isolated working heart perfusion apparatus described previously (19) was used to measure the physiologic performance of the heart in terms of the cardiac power (20). Hearts were perfused at a preload of 7.5 cm H₂O and an afterload of 70 cm H₂O. The perfusion medium consisted of Krebs-Henseleit buffer (21) which was gassed with 95% O₂/5% CO₂ ((Ca²⁺): 2.5 mM). Defatted BSA (fraction V, 1% wt/vol) was present in all of the experiments.

To determine rates of glucose utilization and changes in tissue metabolites, hearts were perfused for 30 min with perfusate containing glucose (10 mM) and various combinations of [2-³H]glucose (10 µCi/perfusion), [U-¹⁴C]2-deoxyglucose (5 µCi/perfusion) and [1,2-³H]2-deoxyglucose (5 µCi/perfusion). When present, 8 µM of regular human insulin (NovoRin; Novo-Nordisk, Bagsvaerd, Denmark) was added to 200 ml of recirculating perfusion medium. Radioimmunoassay of the perfusate for insulin using a commercially available kit (Pharmacia Fine Chemicals, Piscataway, NJ) demonstrated an insulin concentration of 13.38±2.72 µU/ml at the beginning of perfusion and 10.47±0.37 µU/ml at the end of perfusion.

Hearts were perfused for 30 min and then freeze clamped while still being perfused. Metabolites were subsequently extracted from the hearts in 6% perchloric acid and assayed. To compare rates of glucose metabolism through the glycolytic pathway with rates of 2-deoxyglucose accumulation, hearts were perfused with [2-³H]glucose and [U-¹⁴C]2-deoxyglucose. Hearts perfused with [1,2-³H]2-deoxyglucose were used to determine tissue metabolites, free intracellular [1,2-³H]2-deoxyglucose and [1,2-³H]2-deoxyglucose 6-phosphate. To determine the fate of intracellular 2-deoxyglucose 6-phosphate, a third set of perfusions was performed in which hearts were initially perfused with glucose (10 mM) and [1,2-³H]2-deoxyglucose (5 µCi/perfusion) without insulin for 30 min. The hearts were then switched to perfusate containing only glucose (10 mM) in either the presence or absence of insulin. The perfusions were continued for an additional 30 min and the hearts freeze clamped and assayed.

In a fourth set of experiments in which the distribution of hexokinase between the cytosol and the mitochondria was studied, hearts were perfused for 30 min with glucose (10 mM) in the presence or absence of insulin. Immediately after the perfusion period, the hearts were cut from the perfusion cannulae, minced, and mitochondria isolated using the method of Gots and Bessman (22). The mitochondrial pellet and postmitochondrial supernatant (i.e., cytosolic fraction), as well as a sample of whole heart extracted in 0.01 M Tris (pH 7.5) containing 0.5% Triton X-100, were assayed for hexokinase activity with radioassay (22).

*Biochemical methods.* Glucose utilization was determined using the [2-³H]glucose method of Katz and Dunn (23) in which tritiated water was separated from [2-³H]glucose by ion exchange chromatography (24). Rates of glucose uptake were also estimated from the intracellular accumulation of [U-¹⁴C]2-deoxyglucose. Rates of glucose uptake are reported as micromoles per minute per gram dry weight.

The distribution of [1,2-³H]2-deoxyglucose between its free and phosphorylated form was determined chromatographically as described previously (6) on samples used for metabolite extraction with 30% perchloric acid in a dry ice/aceton bath. Free intracellular [1,2-³H]2-deoxyglucose was determined from the total free [1,2-³H]2-deoxyglucose by subtracting the amount of radiolabeled 2-deoxyglucose present in the extracellular fluid space. The extracellular fluid space was determined by including [U-¹⁴C]sucrose (5 µCi/perfusion) for the final 10 min of the perfusion. The extracellular fluid space for hearts perfused without insulin was 0.62±0.08 µl/mg tissue vs. 0.55±0.03 µl/mg tissue for hearts perfused in the presence of insulin (not statistically significant). The relatively large value for the extracellular fluid space probably reflects the perfusate trapped in the ventricular cavities while the heart was freeze clamped. Glucose 6-phosphate was determined using a standard spectrophotometric assay (25) on a neutralized perchloric acid extract of the tissue. Tissue metabolites are reported as micromoles per gram dry weight.

The radioassay for hexokinase activity is based on the conversion of [U-¹⁴C]glucose to [U-¹⁴C]glucose 6-phosphate or [1,2-³H]2-deoxyglucose to [1,2-³H]2-deoxyglucose 6-phosphate and the separation of the two compounds by batch chromatography using Dowex 2X8-100 resin (22). The assay was normally carried out in Tris (0.107 M, pH 7.5) with magnesium chloride (30 mM), ATP (5 mM), and glucose (1 mM) except in the studies of the effect of the association of hexokinase with the mitochondria on the apparent Km for glucose and 2-deoxyglucose.

Determination of the activity associated with the mitochondrial fraction was performed on samples treated with Triton X-100 (0.5%) which solubilized hexokinase from membranes. In those studies of the affinity constant in which it was necessary to maintain the association of hexokinase with the mitochondrial membranes, the assay buffer consisted of Tris (0.01 M, pH 7.4), mannitol (0.35 M), MgCl₂ (0.5 mM), ATP (5 mM), EDTA (0.1 mM), atracylate (0.5 mM), and varying concentrations of [U-¹⁴C]glucose or [1,2-³H]2-deoxyglucose. Triton X-100 was omitted from these studies and atracylate was added to inhibit the mitochondrial adenosine nucleotide translocase. The apparent affinity constants of hexokinase for glucose and 2-deoxyglucose were determined by double-reciprocal plots of reaction velocity and substrate concentration. One unit of hexokinase activity represents that amount of hexokinase that can phosphorylate 1 µmol of glucose or 2-deoxyglucose per min at pH 7.4.

Data were analyzed using the Student's t test or analysis of variance when appropriate. Post hoc comparisons were performed if the analysis of variance demonstrated statistical differences. All values are reported as the mean±SEM.

**Results**

The addition of physiologic concentrations of insulin to hearts perfused as working heart preparations results in the expected increase in glucose uptake and flux through the glycolytic pathway as measured by the production of tritiated water from [2-³H]glucose (Table I). The increase in glucose uptake cannot be explained by an increase in cardiac work as evidenced by a lack of a difference in the pressure-volume work between the two groups during the 30-min perfusion period (cardiac work was 9.04±1.43 mW at t = 0 and 14.11±1.99 mW at t = 30 for hearts perfused without insulin, and 7.42±2.71 mW at t = 0 and 12.75±4.06 mW at t = 30 for hearts perfused in the presence of insulin).

<table>
<thead>
<tr>
<th>Glucose uptake</th>
<th>Ratio of [2-³H]glucose to [U-¹⁴C]2-deoxyglucose</th>
</tr>
</thead>
<tbody>
<tr>
<td>µmol/min per g dry wt</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.94±0.15</td>
</tr>
<tr>
<td></td>
<td>4.95±1.36</td>
</tr>
<tr>
<td></td>
<td>0.51±0.08</td>
</tr>
<tr>
<td></td>
<td>0.51±0.06</td>
</tr>
<tr>
<td></td>
<td>1.25±0.47*</td>
</tr>
</tbody>
</table>

*P < 0.05.*

Compartmentation of Hexokinase in Rat Heart 1973
ence of insulin. The values for the two groups are not statistically different). When glucose utilization was estimated by the accumulation of $^{14}$C-labeled 2-deoxyglucose in the heart, the rate of glucose utilization demonstrated a small and not statistically significant increase with insulin stimulation (Table I). When the rates of glucose uptake determined by $[2-^{3}$H]glucose were compared to rates determined by $[U-^{14}$C]2-deoxyglucose, a dramatic increase in the ratio of the former to the latter becomes apparent. This change is consistent with an insulin-mediated alteration in the ability of 2-deoxyglucose to trace glucose utilization and flux through the glycolytic pathway in the heart.

Associated with insulin stimulation is an increase of 184% in the tissue content of glucose 6-phosphate (Fig. 1, left panel). The $[1,2-^{3}$H]2-deoxyglucose 6-phosphate can be expressed in terms of the "specific activity" of the tissue glucose 6-phosphate as a measure of the contribution of 2-deoxyglucose to the glucose 6-phosphate pool. We define specific activity as disintegrations per minute per gram dry weight present as $[1,2-^{3}$H]2-deoxyglucose 6-phosphate divided by micromoles per gram dry weight of glucose 6-phosphate. A 48% decline in the specific activity of glucose 6-phosphate occurs with insulin stimulation (Fig. 1, right panel). This decline in the specific activity is consistent with the preferential phosphorylation of glucose over 2-deoxyglucose in hearts stimulated by insulin.

Recent $^{31}$P-NMR studies by Hoerter et al. (26) offer surprising evidence that the signal from "trapped" 2-deoxyglucose 6-phosphate decreases with the administration of insulin, suggesting that 2-deoxyglucose 6-phosphate may not represent a true irreversible component in the heart. The work of Hoerter and colleagues was performed with nontracer (0.25–20 mM) amounts of 2-deoxyglucose and may therefore not be applicable to the situation in which $[^{14}$C]2-deoxyglucose, $[^{3}$H]2-deoxyglucose, or $[2-^{18}$F]fluoro-2-deoxyglucose are used in trace amounts (in the present studies, radiolabeled 2-deoxyglucose was present at a concentration of ~5 nM). However, we still found it important to determine whether the fall in the specific activity of glucose 6-phosphate in the present studies was due to degradation of $[1,2-^{3}$H]2-deoxyglucose 6-phosphate. To determine the possible degradation of $[1,2-^{3}$H]2-deoxyglucose 6-phosphate, hearts were initially perfused with $[1,2-^{3}$H]2-deoxyglucose for 30 min and then switched to perfusate containing only glucose (10 mM) either with or without insulin (13 μU/ml). The data in Table II indicate that there is no statistical difference in the amount of $[1,2-^{3}$H]2-deoxyglucose 6-phosphate retained in hearts perfused in either the presence or the absence of insulin, and that the amount of $[1,2-^{3}$H]2-deoxyglucose 6-phosphate does not change after perfusion with buffer containing no radiolabeled 2-deoxyglucose. It is therefore highly unlikely that the insulin associated decrease in the specific activity of glucose 6-phosphate in the present studies can be attributed to degradation of $[1,2-^{3}$H]2-deoxyglucose 6-phosphate.

To elucidate the origin of the insulin-mediated change in the preferential phosphorylation of glucose over 2-deoxyglucose by hexokinase, studies were performed to determine whether the insulin-stimulated association of hexokinase with mitochondrial membranes is responsible for this phenomenon. Since the association of hexokinase with mitochondria is, in part, dependent on the magnesium concentration (27), mitochondria were isolated from hearts in a medium that contained a concentration of magnesium (0.5 mM) which is similar to that reported for the cytosolic magnesium concentration for the heart (28, 29). Under these conditions, the addition of physiologic levels of insulin to the perfusion medium resulted in an increase in the hexokinase activity associated with the mitochondria (Fig. 2). Studies indicated no change in the total myocardial hexokinase activity in these experiments (data not shown). In addition, kinetic measurements performed on the cytosolic fraction of hexokinase as well as the mitochondrial form of the enzyme demonstrated that the apparent $K_m$ of the enzyme for glucose does not change appreciably with association with mitochondria (Fig. 3). On the other hand, association of hexokinase with the mitochondrial fraction increases the apparent $K_m$ for deoxyglucose 8.5 times.

**Discussion**

Because the various isoenzymes of hexokinase can phosphorylate sugars other than glucose, specifically 2-deoxy analogues of glucose which are trapped in cells as their phosphorylated forms, 2-deoxyglucose analogues have been used to trace glu-

![Figure 1](image_url)

**Figure 1.** Tissue content of glucose 6-phosphate determined enzymatically (left panel) and specific activity of glucose 6-phosphate (glucose 6-phosphate SA, right panel) as defined by the ratio of $[1,2-^{3}$H]2-deoxyglucose 6-phosphate radioactivity (dpm/g dry wt) to glucose 6-phosphate content (μmol/g dry wt) and expressed as dpm/μmol. *P < 0.05 compared to same workload in the absence of insulin. †, No insulin; ‡, insulin.

<table>
<thead>
<tr>
<th>Table II. Changes in Glucose 6-Phosphate and $[1,2-^{3}$H]2-Deoxyglucose 6-Phosphate after 30 min of Washout Perfusion in either the Presence or Absence of Insulin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose 6-phosphate (μmol/g dry wt)</td>
</tr>
<tr>
<td>$[1,2-^{3}$H]2-Deoxyglucose 6-phosphate (dpm $\times 10^5/g$ dry wt)</td>
</tr>
</tbody>
</table>

Hearts were initially perfused during the control period for 30 min with $[1,2-^{3}$H]2-deoxyglucose and 10 mM glucose to load the hearts with tracer. *P < 0.05 compared to control and washout without insulin.
Glucose metabolism. While 2-deoxyglucose was originally used to trace glucose metabolism in the brain (1), the 2-deoxyglucose method has been expanded to include measurements of adipose tissue, skeletal muscle, and heart muscle (30–32). The 2-deoxyglucose method is now widely used and has been modified to use the positron-emitting compound [18F]2-fluoro-2-deoxyglucose (33). However, one concern with using 2-deoxyglucose to trace glucose metabolism exists which centers on the kinetic differences between the two hexoses with respect to cell membrane transport and phosphorylation and is expressed by the “lumped constant” correction factor. This concern is amplified by the possibility that these kinetic differences are not always constant in different metabolic situations. Although it has previously been asserted that the value for the lumped constant does not change for the heart (34), Ng and his co-workers recently demonstrated a change in the lumped constant (i.e., increased preference for glucose over 2-deoxyglucose) with insulin stimulation in the isolated working rat heart (8). In the studies by Ng et al., insulin was used in pharmacodynamic concentrations (10 mU/ml) to assure maximal stimulation. In the present study, insulin was used in physiologic concentrations and it not only stimulated glucose uptake but also indicated changes in the rates of glucose utilization determined by the [2-3H]glucose method and the 2-deoxyglucose method consistent with an insulin-mediated increase in the lumped constant. The lack of a change in the lumped constant in the early study by Krivokapich et al. may be explained in part by the fact that an externally paced, isolated interventricular septum was used with a low coronary flow instead of the more physiologic working heart preparation with a comparatively high coronary flow.

In further support of a change in the lumped constant in the current studies is the change in the specific activity of the glucose 6-phosphate with insulin stimulation. The decrease in the specific activity indicates that a smaller proportion of the radio-labeled 2-deoxyglucose is being phosphorylated with respect to glucose. The decrease in the specific activity of glucose 6-phosphate is in keeping with data indicating that glucose is preferred kinetically over 2-deoxyglucose by hexokinase in both plant and animal species (11, 12). The decreased specific activity cannot be attributed to a decrease in the available substrate because the intracellular nonphosphorylated [1,2-3H]2-deoxyglucose does not decrease with insulin stimulation but actually increases (65,900±5,419 dpm/g dry wt for hearts perfused without insulin compared with 133,548±8,038 dpm/g dry wt for hearts perfused in the presence of insulin, P < 0.005). In addition, it is unlikely that the increased unlabeled glucose 6-phosphate is coming from glycogen breakdown because insulin stimulates glycogen synthesis not degradation.

The results of the present studies suggest that compartmentation of hexokinase is responsible, at least in part, for determining the value for the lumped constant and also explains the mechanism by which insulin changes the lumped constant. Bessman already proposed in 1954 that the mitochondrial association of hexokinase could serve to compartmentalize the enzyme (35). He speculated that the association of hexokinase with the mitochondria increased oxidative energy metabolism by providing a phosphate acceptor, glucose, and thereby increasing the return of ADP to mitochondria. Work performed subsequently demonstrated that insulin mediated an acute redistribution between the soluble form and the particulate, or mitochondrial bound, form of hexokinase in skeletal muscle (14, 36–39). The results of the current studies indicate that, as in skeletal muscle, insulin stimulation increases the activity of hexokinase associated with mitochondria in heart muscle.

Association of hexokinase with mitochondria has been shown to decrease the $K_m$ for ATP and increase the $K_i$ for glucose 6-phosphate (18, 40–43). In addition, one study has suggested an increase in the affinity of mitochondrial-bound hexokinase for glucose (18). The present studies indicate that the association of hexokinase with mitochondria results in a significant increase in the $K_m$ for 2-deoxyglucose which is not paralleled by an increase in the value for the apparent affinity constant for glucose. This observation has far reaching implications, since the deoxyglucose method for the measurement of the rate of glucose utilization requires constancy of the lumped constant (LC), i.e., the factor correcting for the difference between tracer and tracee. The lumped constant is defined by the equation $LC = \frac{\lambda V_m}{K_m} / \Phi V_m K_m^2$ (1). In this equation, $\lambda$ represents the ratio of the distribution volumes for 2-deoxyglucose and glucose, which is believed to remain constant (1); $\Phi$ is a constant relating the glucose 6-phosphatase activity of the tissue, which in the case of the heart is negligible (44); and $K_m$ and $V_m$ are the Michaelis constants for glucose and 2-deoxyglucose (denoted by asterisks). The present studies demonstrate
that the association of hexokinase with the mitochondrial membrane does indeed change the kinetics of deoxyglucose phosphorylation with respect to glucose, and further, demonstrate that insulin increases the activity of hexokinase associated with mitochondrial membranes.

In light of these results, any metabolic perturbation that results in a change in the amount of hexokinase associated with the mitochondrial fraction of cardiac myocytes could potentially affect the lumped constant. Studies of rat, mouse, and chick brain indicate that ischemia causes a redistribution of hexokinase favoring association with the mitochondrial membrane (45). Further work is necessary to determine if redistribution of hexokinase occurs in the heart during ischemia. It is conceivable that other metabolic perturbations such as stimulation by thyroid hormone or epinephrine could also result in redistribution of hexokinase between the soluble and particulate fraction of the heart thereby affecting the fidelity of 2-deoxyglucose tracers of glucose metabolism. With respect to the role of 2-[18F] fluoro-2-deoxy-D-glucose-based measurements of glucose metabolism in the human heart using positron emission tomography, the present studies would argue strongly for a rational and consistent methodology in patient studies in which either the insulin concentration is monitored or clamped to insure reproducible interpretation of patient studies.

Acknowledgments

This work was supported in part by a grant-in-aid from the National Heart, Blood, and Lung Institute (RO1 HL-43133) and a grant-in-aid from the American Heart Association (National Center). J. M. Mrus was supported by the M.D./Ph.D. program cosponsored by the University of Texas Medical School at Houston and the University of Texas M. D. Anderson Cancer Center.

References


