Quantitative histochemical assessment of regional differences in hepatic glucose uptake and release

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Summary. As a further step in the investigation of the heterogeneity of liver cells in general and regionality of glucose metabolism in particular, requirements for isolation of appropriate tissue samples were defined and procedures for measurement of the biochemical parameters responsible for measurement of the biochemical parameters responsible for glucose uptake and release developed and tested. By using enzymatic cycling for chemical amplification, in conjunction with the oil-well technique, sufficient analytical sensitivity was provided to assay samples averaging 20 ng dry weight. Microchemical data on the distribution of glucokinase and glucose-6-phosphatase and of their substrates, glucose and glucose-6-P, were used to, first calculate in vivo rates of these catalytic steps by means of the Michaelis-Menten equation, and then, to determine the direction and rate of net glucose flux, as well as, the rate of substrate cycling between glucose and glucose-6-P.

Calculations from the results indicated a reciprocal distribution of in vivo glucokinase and glucose-6-phosphatase velocities, as well as, sex-specific differences. The distribution of in vivo activities results in a spatial separation of these antagonistic steps. Separation is incomplete, but nevertheless appears to lead to regionally different rates in futile substrate cycling. Glucose gradients permit differentiation between net glucose uptake and release and were, therefore, used as a test of the validity of the calculations of in vivo activities. The observed discrepancies between glucose gradients and calculated in vivo enzyme activities illustrate the power of this approach: it provides a way to compare changes in glucose along the sinusoid with what would be predicted from the levels of enzymes which liberate and tie up glucose and of their respective substrates.

Introduction

The remarkable stability of blood glucose levels, despite large changes in the supply of glucose, indicates precise regulatory mechanisms which allow the liver to oscillate between balanced uptake and release of glucose. This is achieved by the integrated action of opposing enzyme systems. Enzyme differences in the hepatocytes along the sinusoid, indicated in recent microchemical studies (Teutsch 1981; Teutsch and Lowry 1982) could be of considerable regulatory importance, especially in cases, such as glycolysis and gluconeogenesis, where opposing catalytic steps are known to proceed simultaneously and to result in futile cycling (Katz et al. 1978). The extent to which functional heterogeneity reduces or prevents futile cycling in opposing catalytic steps, however, remains unclear and the characteristics of such heterogeneity are not yet established. It was the aim of this study to: a) develop microchemical techniques for quantitative assessment of the distribution of the two enzymes responsible for glucose uptake and release, glucokinase and glucose-6-phosphatase, and of related substrates; b) test these techniques by assessing the distribution of the activities of these enzymes and of glucose and glucose-6-P levels in liver from male and female rats, and to c) use the microchemical data to estimate in vivo rates of the glucokinase and glucose-6-phosphatase reactions, the rate of net glucose uptake and release, as well as the rate of futile cycling.

Materials and methods

Preparation of materials

Adult Wistar rats (obtained from Charles Rivers) were maintained at a constant temperature (21 °C) and a light/dark cycle (lights from 5 a.m. – 7 p.m.) and fed a diet of Purina Chow and water ad lib. After two weeks of adaptation, animals were anesthetized (9 p.m.) by intraperitoneal injection of sodium pentobarbital (5 mg/100 g body weight). Livers were frozen in situ with liquid nitrogen (Teutsch 1984), removed and stored at −70 °C until use. Ten pairs of adjacent 10 μm cryostat sections were cut from the livers of one male and one female rat. One section of each pair was used for histochemical demonstration of glucose-6-phosphatase activity (Teutsch 1978a), the other section was vacuum dried from the frozen state (Lowry and Passonneau 1972). Microdissection was carried out in a room with controlled humidity and temperature, using equipment previously described (Lowry and Passonneau 1972). The stained section served as a guide for the isolation from the other section of narrow strips, comprising tissue from the whole extent of the sinusoid (i.e. from its beginning at the portal vessels to its end at the functionally allied central venule). These strips were subdivided into six adjacent samples with an average size of $70 \times 100 \times 10 \mu m$. In order to correlate chemical analyses with the microarchitecture of the liver, the size and location of samples within the strip of tissue were recorded, using a calibrated drawing tube, attached to a Wild dissecting microscope. The dry weight of samples was determined on a quartz fiber balance (Lowry and Passonneau 1972).

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Analytical methods

Because of the small sample size, most analytical steps were carried out in small droplets under a mixture of mineral oil and hexadecane in one of 80 wells drilled into a Teflon block (Lowry and Passonneau 1972). The methods are all based on pyridine-nucleotide reactions. Sensitivity was amplified by enzymatic cycling (Kato et al. 1973; Chi et al. 1978; Hintz et al. 1983); cycling was carried out in 5 μl volumes under oil, or directly in the fluorometer tube. Buffers are described by acid-base ratio rather than pH. This is to ensure that the required acid-base balance is achieved at successive steps, e.g., when the solution is strongly acidic or basic at one step, but must be brought to a suitable pH for an enzyme action at the next. All final measurements were made in a Farrand optical filter fluorometer at 340 nm excitation and 460 nm emission wavelengths.

The method for Glucokinase (E.C.2.7.1.2) has been described (Teutsch and Lowry 1982).

Glucose-6-phosphatase (E.C.3.1.3.9)

The procedure is based on that described by Burch et al. (1978) and has been used in previous studies (Teutsch 1978b, 1981) as a direct fluorometric assay.

Principle (enzymes not shown)

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glucose-6-P \rightarrow \text{glucose + Pi}
\]

(1)

\[
glucose + NADH \rightarrow \text{pyruvate + ADP + NAD}^+
\]

(2)

The major problem is that glucose-6-P, pyruvate and ATP are contaminated with variable amounts of glucose, pyruvate and ADP, respectively, thus leading to high reagent blanks. The solution to this problem was to pretreat the step 2 reagent first with pyruvate kinase (E.C.2.7.1.40) to convert ADP into ATP and then to destroy pyruvate with H₂O₂ and heat; excess H₂O₂ was eliminated with catalase (Hinz et al. 1982). Possible interference from glucose-6-P contamination with glucose was minimized by reducing the amount of glucose-6-P in the step 1 reagent (i.e. incubation of samples in a volume of 0.2 μl). NAD formed in step 2 was amplified using the cycle of Kato et al. (1973).

Step 1 reagent: 200 mM imidazole-HCl buffer (acid:base ratio:1:1.9:1), 2 mM EDTA, 0.08% BSA. 20 mM glucose-6-P prepared in aqua dest. Standards consisted of 450 μM glucose (enzymatically standardized).

Step 2 reagents: 100 mM imidazole-HCl buffer (acid:base ratio:1:4:1), 75 mM KCl, 2 mM MgCl₂, 0.04% BSA, 150 μM pyruvate, 50 μM ATP, 1 μg (0.25 IU)/ml of pyruvate kinase from rabbit muscle and 5 mM H₂O₂. After 15 min incubation at RT and 20 min at 60 °C, 0.1 μg (6.5 IU)/ml of catalase (E.C.1.11.1.6) from beef liver were added and further incubated 15 min at RT. Immediately before use, NADH (75 μM final concentration) and 5 μg (0.7 IU)/ml of hexokinase (E.C.2.7.1.1) from yeast, 5 μg (1 IU)/ml pyruvate kinase from rabbit muscle and 2 μg (0.5 IU)/ml of lactate dehydrogenase (E.C.1.1.27) from beef liver were added.

Procedure: Each sample was added through the oil into 0.1 μl of step 1 reagent and the reaction started by adding 0.1 μl of 20 mM glucose-6-P at timed intervals. 0.2 μl volumes of standards (225 μM glucose, final concentration) and reagent (0.1 μl step 1 reagent plus 0.1 μl H₂O) were included in other wells. For tissue blanks, samples were incubated in a reagent from which the substrate had been omitted. After 60 min at 20 °C the oil well rack was heated 30 min at 60 °C. The rack was cooled to RT, 2 μl of step 2 reagent were added to each oil well and the reaction was incubated 30 min at RT. The reaction was stopped with 10 μl of 0.2 N HCl and heating the rack 20 min at 60 °C. Amplification of NAD was carried out directly in the fluorometer tube with 0.5 μl aliquots from the previous step and 50 μl of the enzymatic cycling reagent (Kato et al. 1973). With 5 μg (6 IU)/ml of malate dehydrogenase (E.C.1.1.1.37) from pig heart and 60 μg (18.5 IU)/ml of alcohol dehydrogenase (E.C.1.1.1.1) from yeast (treated with Norite to remove NAD (Kato et al. 1973)) amplification of about 20,000 fold was achieved with 60 min incubation at 25 °C. The activities measured at 20 °C were converted to 37 °C rates using a factor of 3, based on the temperature coefficient determined by Burch et al. (1978). Tissue blanks were 8 ± 1% SEM (n = 21) of the average glucose-6-phosphatase activity.

Glucose and glucose-6-P

The procedures of Hintz et al. (1982) were adapted for measuring glucose and glucose-6-P. Both metabolites were measured in the same sample. Glucose and glucose-6-P were first converted to 6-P-glucose with hexokinase and/or glucose-6-P dehydrogenase (E.C.1.1.49). NADPH formed in the specific step was measured after amplification with the enzymatic cycling procedure of Chi et al. (1978) and Hintz et al. (1983). Because of the small sample size, all analytical steps (except for the last step) were carried out in small droplets under oil.

Glucose-6-P reagent: 100 mM Tris-HCl buffer (acid:base ratio:1:1), 15 mM NADP, 0.3 mM 1.4 dithiothreitol, 0.075% BSA, 5 μg (1.5 IU)/ml glucose-6-P dehydrogenase from Leuconostoc mesenteroides.

Glucose reagent: 100 mM Tris-HCl buffer (acid:base ratio:1:1), 200 μM ATP, 2 mM MgCl₂, 10 μM NADP, 5 μg (0.7 IU)/ml hexokinase from yeast. Standards consisted of 0.1 μl volumes of 0.02 N HCl containing both 5, 10 and 25 μM glucose and 0.05, 0.1 and 0.2 μM glucose-6-P. These were carried through all steps of both procedures.

Procedure: Each tissue sample was added through the oil into 0.1 μl of 0.02 N HCl. After heating the oil well rack 20 min at 80 °C, 0.25 μl of glucose-6-P reagent were added to all oil wells, for samples, standards and reagent blanks. The rack was incubated 20 min at RT, then 0.1 μl aliquots were transferred to a second oil well rack, 0.25 μl of glucose reagent were added and the rack was incubated 20 min at RT. Reactions were stopped and excess NADP was destroyed in both racks by adding 0.25 μl of 0.1 N NaOH and heating the racks 20 min at 80 °C. Amplification was carried out in the oil wells by adding 5 μl of enzymatic cycling reagent (Hintz et al. 1983). For assessment of the sum of glucose and glucose-6-P, an amplification of 12,000 fold was achieved with 40 μg (12 IU)/ml of glucose-6-P dehydrogenase from Leuconostoc mesenteroides and 260 μg (31 IU)/ml of glutamate dehydrogenase (E.C.1.4.1.13) from beef liver with 60 min incubation at RT. For assessment of glucose-6-P the concentration of both enzymes was kept the same, but incubation was extended to 15 h at 15 °C, and an amplification of 130,000 fold was achieved. Reactions were stopped with 1 μl of 0.75 N NaOH and heating the racks 20 min at 80 °C. For final measurement of 6-P-glucose, 2.5 μl (sum of glucose and glucose-6-P) and 5 μl (glucose-6-P) were added to 1 ml of 6-P-glucose reagent. Glucose was calculated by difference.

Calculation of in vivo rates (v)

The Michaelis-Menten equation (V = Vmax×S)/(Km + S) was used to calculate in vivo activities of glucokinase and glucose-6-phosphatase from data on the distribution of maximal velocities (Vmax) of these enzymes and their substrates for various half-saturating substrate concentrations (Km) of these enzymes, i.e. 5 and 9 mM glucose and 1 and 3 mM glucose-6-P. Km values for glucose of 5, 12 and 10-15 mM were reported by Storer and Cornish-Bowden (1976), Salas et al. (1965) and Colowick (1973) respectively. Km values for glucose-6-P of 1, 1.8 and 2-3 mM were published by