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SEX SPECIFIC REGIONAL DIFFERENCES IN HEPATIC GLUCOKINASE ACTIVITY

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SUMMARY: Using microdissection and microchemical analysis, the distribution of glucokinase and hexokinase was studied in six adjacent segments of the rat liver sinusoid. Glucokinase activity was significantly higher in males than in females with a different sinusoidal distribution. Hexokinase was only demonstrable at the beginning and the end of the sinusoid. High values at the beginning might be attributed to Kupffer cells. The reciprocal distribution of glucokinase and glucose-6-phosphatase activity in female rats indicates that spatial separation of antagonistic catalytic steps might be a basic principle of the functional organization of liver parenchyma.

INTRODUCTION: Although hepatocytes are still considered by most investigators to be metabolically homogeneous (1,2,3,4), regional differences of enzyme activity have been found, using microdissection and microchemical analysis (5,6,7,8,9). Glucose-6-phosphatase activity has been shown to decrease along the liver sinusoid (9), the functional unit of the liver (10). Because it is not this enzyme alone which determines glucose release, but rather the activity ratio of this phosphatase and the antagonistic glucokinase (1,2,11) it was decided to measure the distribution of glucokinase activity as a first step toward the elucidation of hepatic glucose uptake and release. Both male and female animals were studied, since sex differences were to be expected on the basis of earlier reports (9).

MATERIALS AND METHODS: Preparation of Materials - Adult Wistar rats (obtained from Charles Rivers) were maintained at constant temperature (21°C), with a constant light-dark rhythm (light from 5 a.m.-7 p.m.) and fed a diet of Purina Chow and water ad lib. They were anesthetized by intraperitoneal injection of sodium pentobarbital (5 mg/100 g body weight). Livers were frozen in situ with liquid nitrogen and stored at -70°C until use. Ten pairs of adjacent 10 µm cryostat sections were studied from each of four

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animals. One section from each pair was used for histochemical demonstration of glucose-6-phosphatase activity (12), the other section was vacuum dried from the frozen state (13). Microdissection was carried out in a room with controlled humidity and temperature, using equipment previously described (13). 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 terminal branches of the portal vein and hepatic artery to its end at the functionally allied tributary of the hepatic vein). These strips were subdivided into six adjacent samples. In order to correlate chemical analyses with the micro architecture of the liver parenchyma, 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 (20-50 ng) was determined on a quartz fiber balance (13).

Analytical Methods - Quantitative assessment of glucokinase (EC 2.7.1.2) and hexokinase (EC 2.7.1.1) activity was based on the procedure of Trus et al. (8), in which the product of the reaction, glucose-6-P, is oxidized by NAD^+ in the presence of glucose-6-P dehydrogenase from Leuconostoc mesenteroides. Some problems with this method were circumvented by adding a further analytical step in which 6-P-gluconate was determined with 6-P-gluconate dehydrogenase and $NADP^+$. NADPH formed in this step was measured after amplification with the enzymatic cycling procedure of Chi et al. (14). Because of the small sample size, initial analytical steps were carried out in small droplets under oil (13).

Reagent A: 40 mM imidazole base, 10 mM imidazole-HCl (pH about 7.6), 10 mM $MgCl_2$, 170 mM KCl, 0.02% bovine serum albumin, 0.2 mM dithiothreitol, and 0.2 mM EDTA. Standards consisted of 5 µM glucose-6-P (enzymatically standardized) prepared in this reagent. Reagent B: The same as Reagent A with omission of $MgCl_2$ and KCl and addition of 1.8 mM NAD^+, 17 mM ATP, 6 µg (1.6 U) per ml of glucose-6-P dehydrogenase (Leuconostoc mesenteroides) and either 1.7 or 350 mM glucose (Reagent B1 and B2) for assessing hexokinase or glucokinase plus hexokinase, respectively. ATP was omitted for tissue blanks (Reagents B3 and B4). Reagent C: 60 mM imidazole base, 40 mM imidazole acetate (pH about 7.0), 60 mM ammonium acetate, 10 mM $MgCl_2$, 2 mM EDTA, 1 mM dithiothreitol, 30 µM $NADP^+$ and 30 µg (0.4 U) per ml of yeast 6-P-gluconate dehydrogenase.

Procedure: Each tissue sample was added through the oil into 1.3 µl of Reagent A and incubated about 30 min at 20°C; 1.3 µl volumes of standards and of extra Reagent A for blanks were included in other wells. From each well, four 0.25 µl aliquots were distributed into new oil wells. The 60 min incubation for the specific glucokinase and hexokinase reactions and tissue blanks was made at 20° to 22°C, by adding 0.1 µl of either Reagent B1, B2, B3 or B4 at timed intervals and then adding 0.1 µl of 0.2 N NaOH at the same time intervals, starting 60 min after the first addition. The oil well rack was then heated 30 min at 60°C. After cooling to room temperature, 0.55 µl of Reagent C was added; the rack was incubated 15 min at room temperature, 2.3 µl of 0.1 N NaOH were added and the rack heated 30 min at 60°C to destroy excess $NADP^+$.

Amplification of the NADPH 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 (14). With 13 U/ml of glucose-6-P dehydrogenase from baker's yeast (substituted for that from Leuconostoc-see below) and 80 U/ml glutamate dehydrogenase from beef liver, amplification of about 24,000 fold was achieved with a 90 min incubation at 37°C. The activities measured at 20°-22°C were converted to 37°C rates on the basis of temperature coefficients (Q_{10}) determined to be 1.76 and 1.66 between 20° and 37°C for hexokinase and glucokinase, respectively.

Comments on the procedure: A major problem arises if glucokinase and hexokinase are measured in liver by the NADPH produced with glucose-6-P dehydrogenase and $NADP^+$. This is because 6-P-gluconate dehydrogenase is so active in liver that the 6-P-gluconate is partially oxidized further to yield an uncertain extra amount of NADPH. Since 6-P-gluconate dehydrogenase is specific for $NADP^+$, Trus et al. (8) were able to circumvent this problem by substituting NAD^+ for $NADP^+$ and using Leuconostoc glucose-6-P dehydrogenase, which reacts with either nucleotide (unlike the $NADP^+$ specific enzyme from baker's yeast). This, however, introduced another problem: troublesome tissue blanks due to NADH formation from other tissue components (a particularly bothersome problem with hexokinase because of its low activity). Moreover, it does not get around the high reagent blanks with glucokinase assay, caused by the substantial rate of oxidation by glucose-6-P dehydrogenase of glucose at the high level required. The modified procedure described here solves both problems: the NADH produced during the specific step is ignored; instead, the 6-P-gluconate produced in that step is oxidized with $NADP^+$ in an additional step, after killing tissue enzymes. The NADPH can now be measured (and amplified) with an enzymatic cycle in which one of the enzymes (baker's yeast glucose-6-P dehydrogenase) is completely specific for NADP. Preincubation for 30 min at room temperature in Reagent A proved to be sufficient to dissolve both enzymes without loss of activity. This step allowed measurement in tissue samples of all four parameters of interest (phosphorylation at both 0.5 and 100 mM glucose concentration, and the blanks contributed by the tissue in the absence of ATP). Both kinase reactions proved to be linear with time (up to 90 min at room temperature) and with tissue (dry weights from 20-120 ng). Recovery of glucose-6-P standards in the hexokinase assay were close to 100%. The glucose-6-P standard in the assay for glucokinase activity was only 4% lower than that in the hexokinase assay (compare 8). The contributions of the tissue to the blank were equivalent to 25% of average glucokinase activity. The high reagent blank observed with the procedure of Trus et al. (8), which was found to be due to a fluorescent compound formed when the high level glucose is heated in alkali, was considerably reduced by using a smaller volume of the specific step reagent (0.4 instead of 2 µl) and cycling of a small aliquot at a high rate rather than cycling the total volume at a low rate. Enzymes, coenzymes and substrates were obtained from Sigma Chemical Co. and Boehringer Corp. All other chemicals were of reagent grade.

RESULTS AND DISCUSSION: Evaluation of liver cell heterogeneity has so far usually been made through analysis of tissue samples from the beginning and the end of sinusoid, i.e. from the periportal zone and the perivenous zone (5,6,7,8). But considerable evidence for the limitation of this approach has emerged from a recent study (9) where it was shown that enzyme activity changes throughout the whole extent of the liver sinusoid. From this it follows that values obtained will be greatly influenced by the sample size: with small samples of 5-20 ng dry weight (8) information is restricted to small, nonrepresentative segments, whereas with large samples of 200-400 ng dry weight (6,7) there is little opportunity for the recognition of regional differences. A sampling procedure was therefore developed which

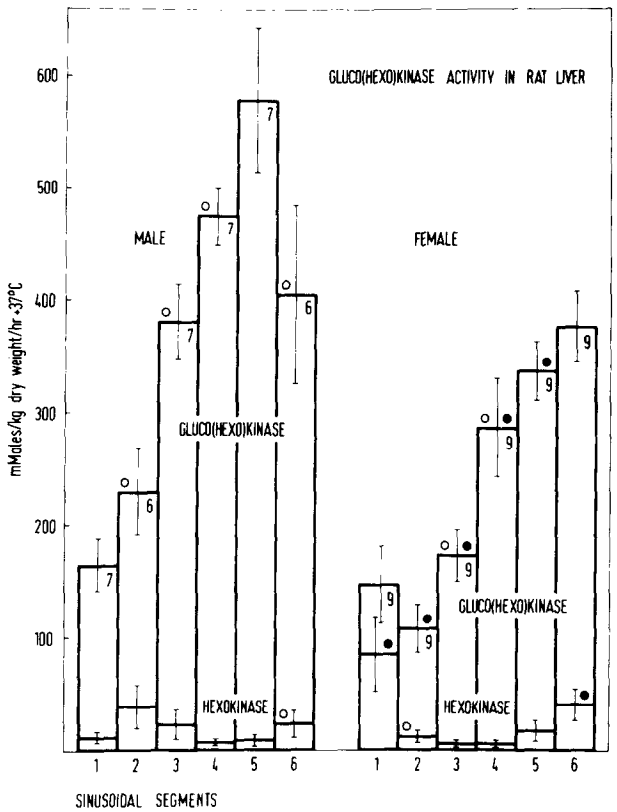

Figure 1. Distribution of the activity of glucokinase and hexokinase along the liver sinusoid in two male and two female rats. Each bar is the average ± SE for the number of samples indicated by the numerals. Segment 1 is located at the beginning, segment 6 at the end. The average size of sinusoidal segments (and of samples) is: 70x120x10 μm. Open circles indicate a significant difference ($P<.05$) between this and the previous segment. Closed circles indicate a significant difference between corresponding segments in the male and female.

allowed assessment of the distribution of enzyme activity along the whole extent of the sinusoid.

Distribution of glucokinase activity now reveals a progressive increase along the sinusoid almost from beginning to end, both in male and female rats (Fig. 1). Activities in segments 2-5 are significantly higher in male than in female rats. In addition, in the male activity is significantly lower in segment 6 than in segment 5, while there is a slight increase from segment 5 to 6 in female rats. The contribution of low Km hexokinase activity to glucokinase activity in both sexes is higher at the beginning and at the end of the sinusoid (Segments 2,3,6 in the male, segments 1,2,5,6

in the female) than in the intermediate segments. It is known that low Km hexokinases are mainly restricted to sinusoidal cells (15,16,17,18,19), among which Kupffer cells could be partly responsible for the activity at the beginning of the sinusoids (9,20). Nevertheless, the regional and sex specific differences are difficult to interpret, since it is not known to what extent the different cell types contribute to the total activity. In any event, the contribution of low Km hexokinases to total hexokinase activity does not exceed 15% (except in segment 1 in female rats). It can therefore be anticipated that low Km hexokinases will exert only a minor effect on sinusoidal glucose concentration. A comparison of the sinusoidal distribution of glucokinase activity with that of glucose-6-phosphatase (9) (suitable data are at present available only for female rats) reveals a complete regional reciprocity of these antagonistic enzymes. Since the rate of the substrate cycle between glucose and glucose-6-P is (apart from the concentration of these substrates themselves) determined by the ratio of the two responsible enzymes (1,2,11,21), it can be expected that both the cycling rate and the amount of ATP lost through this process will change along the sinusoid. On the basis of the results reported here and in earlier studies (9) it seems reasonable to conclude that in addition to intracellular compartmentation (22), spatial separation of antagonistic catalytic steps along the liver sinusoid is a basic principle of the functional organization of liver parenchyma. This clearly challenges the assumption that hepatocytes are metabolically homogeneous.

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