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Fructose 2,6-bisphosphate in isolated foetal hepatocytes

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Fru 2,6-P_2 was present in isolated foetal hepatocytes at a concentration of 1.6 nmol per g cells. When foetal hepatocytes were exposed to glucagon no changes were observed either in the concentration of Fru 2,6-P_2 and lactate release or in the activities of 6-phosphofructo-2-kinase and pyruvate kinase. Incubation of purified 6-phosphofructo-2-kinase with the catalytic subunit of protein kinase did not change the enzyme activity. The inhibition by *sn*-glycerol 3-phosphate was much lower for the foetal than for adult enzyme. These results suggest that an isoenzyme of 6-phosphofructo-2-kinase in foetal hepatocytes different from that of adult hepatocytes may be present.

6-Phosphofructo-2-kinase; Fructose 2,6-bisphosphate; Glucagon; (Fetal hepatocyte)

1. INTRODUCTION

One of the characteristics of the perinatal period is the presence of hepatic isoenzymes for carbohydrate metabolism different from those prevailing in adult tissues. As a result, foetal liver actively carries out glycolysis and fails to achieve gluconeogenesis [1].

In adult liver, fructose 2,6-bisphosphate plays an important role in the control of the metabolic fluxes through 6-phosphofructo-1-kinase and fructose-1,6-bisphosphatase. Both enzymes are present in the 22-day-old foetus showing activities similar to those found in adult hepatocytes [2].

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Abbreviations: Fru 2,6-P_2, fructose 2,6-bisphosphate; PFK-2, 6-phosphofructo-2-kinase (EC 2.7.1.-); PFK-1, 6-phosphofructo-1-kinase (EC 2.7.1.11); PK, pyruvate kinase (ATP:pyruvate O^2-phosphotransferase, EC 2.7.1.40)

Changes in Fru 2,6-P_2 have been described in liver homogenates during the perinatal period [3]. However, foetal liver contains large amounts of haematopoietic cells, and recently it has been reported [4] that erythrocytes may interfere in the measurement of Fru 2,6-P_2. In order to eliminate these interferences we have used isolated foetal hepatocytes in this study.

The influence of hormonal changes on the control of Fru 2,6-P_2 concentration and on 6-phosphofructo-2-kinase is little understood. The present work suggests that foetal hepatocytes contain an isoenzyme of PFK-2 different from that of adult hepatocytes.

2. MATERIALS AND METHODS

2.1. *Animals*

Albino Wistar pregnant rats (300–350 g) fed on a standard laboratory diet were killed for the experiments between 09:00 and 10:00 h. Gestational age was confirmed by standard criteria [5]. Newborn rats were delivered by caesarean section in the morning of day 22 of gestation and were used immediately.

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2.2. *Isolation of hepatocytes*

Hepatocytes from foetal rat liver were prepared by a non-perfusion collagenase dispersion method that involved incubation with Ca^{2+}-free Krebs bicarbonate buffer [6], containing 0.5 mM EGTA for 30 min at 37°C under continuous gassing (O_2/CO_2, 19:1). The cell suspension was centrifuged (50 × *g* for 2 min), incubated for 60 min in the presence of 5 mM Ca^{2+} and 0.5 mg/ml of collagenase, centrifuged (50 × *g* for 4 min) and filtered through nylon of different meshes (500–50 μm). Separation of parenchymal from haematopoietic cells was carried out by washing the suspension at very low speed. Haematopoietic cell contamination was shown by microscopic observation to be less than 5%. Cells obtained (approx. 1.5×10^7 cells/g liver) resulted in about 15% recovery. Cell viability (trypan blue exclusion and lactate dehydrogenase activity) was always higher than 90%. Hepatocytes from adult rats were prepared as in [7]. Hepatocytes ($5–7 \times 10^6$ cells/flask) were incubated for 30 min at 37°C in Krebs bicarbonate in a final volume of 3 ml in the presence or absence of glucagon.

2.3. *PFK-2 purification*

Foetal and adult hepatocytes (2–3 g) were homogenized in a buffer containing 100 mM KCl/5 mM $MgCl_2$/2 mM DTT/50 mM Hepes (pH 7.4). After centrifugation (100000 × *g* for 1 h) the supernatant was fractionated with polyethylene glycol. The 6–15% pellet was resuspended in 5 ml homogenising medium supplemented with 20% (v/v) glycerol and passed through a column (2 × 0.5 cm) of Blue Sepharose. PFK-2 was eluted with a gradient (50–1000 mM) of KCl as described by Van Schaftingen et al. [8]. Both enzymes eluted as approx. 500–600 mM KCl.

2.4. *Measurements of enzymes and metabolites*

Fructose 2,6-bisphosphate was measured as described by Van Schaftingen et al. [9] and lactate as in [10]. cAMP was assayed using binding protein with the Amersham (England) kit. For the measurement of enzymes, samples were immediately frozen in a solid CO_2/acetone bath. Cells were homogenized at 4°C with an Ultraturrax in a medium containing 20 mM potassium phosphate/1 mM EDTA/100 mM KCl/1 mM DTT/50 mM NaF (pH 7). After centrifugation (20000 × *g* for 20 min at 4°C) the supernatant was assayed for pyruvate kinase at pH 7.4 in the presence of 1 mM ADP and 0.15 or 5 mM phospho*enol*pyruvate as described by Felíu et al. [11]. Fructose-1,6-bisphosphatase was assayed with 0.1 mM Fru 1,6-P_2 as described by Van Schaftingen and Hers [12]. 6-Phosphofructo-2-kinase activity was measured after precipitation with 15% polyethylene glycol [13] and resuspension in the extraction medium without NaF. The 'active' form of PFK-2 was measured at pH 6.6 in the presence of 1 mM fructose 6-phosphate. The total activity was measured at pH 8.5 with 5 mM fructose 6-phosphate [14]. The sensitivity of PFK-2 activity to *sn*-glycerol 3-phosphate inhibition was determined in a Blue Sepharose-purified PFK-2 fraction at pH 7.1 and in the presence of 0.1 mM Fru 6-P and 0.5 mM MgATP [13]. PFK-2 (0.1 mU Blue Sepharose fraction) was incubated in 0.1 ml at 30°C for 10 min with 0.1 mU catalytic subunit of protein kinase in the presence of 100 mM KCl/5 mM $MgCl_2$/1 mM DTT/20 mM Hepes (pH 7.0) and 1 mM MgATP [13]. Samples (20 μl) were used to assay PFK-2 activity at pH 6.6 and 8.5 as previously indicated.

Fructose-2,6-bisphosphatase was measured in this fraction by the production of $^{32}P_i$ from 10 μM Fru 2,6-[2-^{32}P]P_2. The assay was performed according to Rider et al. [13]. Fru 2,6-[2-^{32}P]P_2 was prepared by the method of Van Schaftingen et al. [8]. The reaction was linear with time and protein concentration.

The total activity of cAMP-dependent protein kinase (PKA) was assayed in supernatants from control hepatocytes after filtration through Sephadex G-25 by using histone H2 as substrate and in the presence or absence of 10 μM cAMP, according to Corbin and Reimann [15]. The reaction was performed under the linear range with time and protein concentration. One unit of PKA activity was defined as the incorporation of 1 pmol $^{32}P_i$ into histone H2 per min. Protein concentration was determined by the method of Bradford [16] using bovine serum albumin as standard.

3. RESULTS

To determine the relative content of Fru 2,6-P_2 in foetal liver vs isolated foetal hepatocytes, livers

were immediately homogenized with 50 mM NaOH (1:20, w/v). The content of Fru 2,6-P_2 was 1.5 ± 0.2 (n = 11) nmol per g liver and 1.6 ± 0.2 (n = 9) nmol per g foetal hepatocytes.

Foetal hepatocytes contain receptors for glucagon and adenylate cyclase activity [17,18]. To investigate whether glucagon-receptor occupancy affected the activity of PFK-2 and the concentration of Fru 2,6-P_2 as occurred in the adult, isolated foetal hepatocytes were exposed to 10^{-7} M glucagon for 5 or 30 min and assayed for their content of Fru 2,6-P_2, lactate release and PFK-2 and PK activities. As shown in table 1 glucagon was ineffective at changing any of these parameters; however, it was effective when adult hepatocytes were used.

To determine whether glucagon is able to increase the intracellular concentration of cAMP, foetal hepatocytes were incubated with different concentrations of the hormone (10^{-10}–10^{-6} M) and samples were collected to measure cAMP and PFK-2 activity at 5 and 30 min. Fig.1 shows that cAMP increased because of the effect of glucagon either after 5 or 30 min of incubation. However, PFK-2 activity expressed as the activity ratio at pH 8.5/pH 6.6 did not change at glucagon concentrations even as high as 1 μM. The maximal increase in cAMP (4-fold) was obtained at hormone concentrations in the physiological range (10^{-7} M). Moreover, the activity of the cAMP-dependent protein kinase, assayed with histone H2 as substrate, was similar in control adult and foetal hepatocytes resulting in 170 ± 35 and 215 ± 40 U/mg protein, respectively. These results sug-

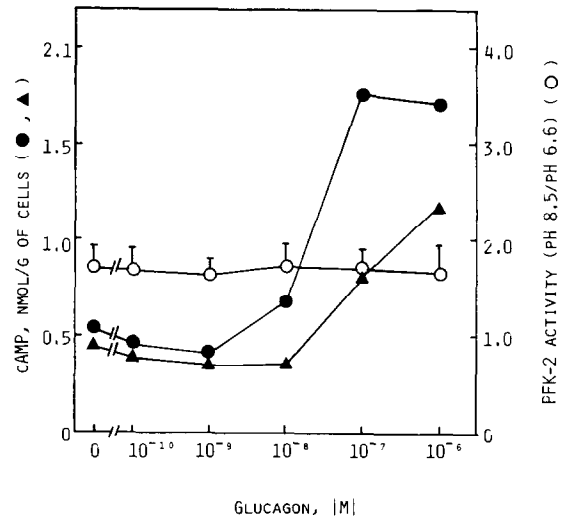

Fig.1. Effect of glucagon on cAMP concentration and PFK-2 activity in foetal hepatocytes. Foetal hepatocytes (2–3 × 10^6 cells/ml) were incubated with different concentrations of glucagon and aliquots (1.5 ml) were collected at 5 and 30 min. cAMP concentration and PFK-2 activity were assayed as described in section 2. Total PFK-2 activity (pH 8.5) was 53 ± 11 pmol Fru 2,6-P_2/min per mg protein for control hepatocytes. Results for cAMP are means of duplicates of two different experiments. (●, ▲) cAMP at 5 and 30 min, respectively; (○) PFK-2 activity at 30 min.

Table 1

Fru 2,6-P_2 content, lactate release, PFK-2 and PK activities in foetal and adult rat hepatocytes exposed to glucagon

Hepatocytes	Addition	Fru 2,6-P_2 (nmol/g cells) (5 min)	Fru 2,6-P_2 (nmol/g cells) (30 min)	Lactate (μmol/g cells) (30 min)	PFK-2 (pH 8.5/6.6) (30 min)	PK ($V_{0.15}/V_5$ mM) (30 min)
Foetal	none	1.4 ± 0.3	1.6 ± 0.2	11.5 ± 1.6	1.7 ± 0.3	0.66 ± 0.07
	10^{-7} M glucagon	1.4 ± 0.2	1.3 ± 0.2	10.5 ± 1.3	1.7 ± 0.3	0.57 ± 0.11
Adult	none	9.0 ± 0.4	8.5 ± 0.6	2.6 ± 0.4	1.6 ± 0.1	0.45 ± 0.08
	10^{-7} M glucagon	1.7 ± 0.3[a]	1.1 ± 0.1[a]	1.1 ± 0.2[a]	4.2 ± 0.6[a]	0.15 ± 0.02[a]

[a] Significance of the differences $p < 0.001$

Numbers in parentheses indicate times of incubation. Isolated hepatocytes (5–7 × 10^6 cells) were exposed to 10^{-7} M glucagon for 30 min and analysed for their content of Fru 2,6-P_2, lactate release and PFK-2 and PK activities. PFK-2 was measured at pH 6.6 and 8.5 and is expressed as the activity ratio pH 8.5/6.6. The total activities (pH 8.5) were 53 ± 11 and 155 ± 30 pmol Fru 2,6-P_2/min per mg protein for control foetal and adult hepatocytes and 52 ± 10 and 147 ± 13, respectively, when glucagon was present. Results are means ± SE of at least six different experiments

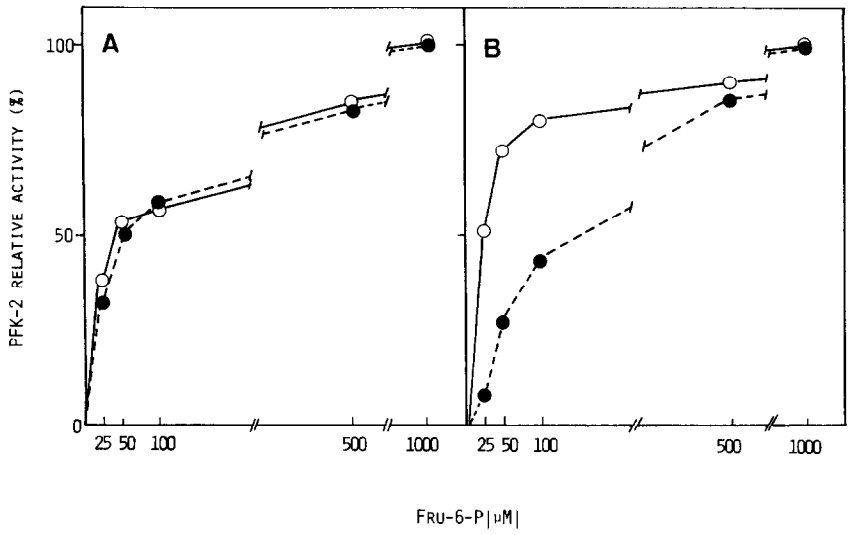

Fig.2. Effect of glucagon on the affinity of PFK-2 for Fru-6P in foetal and adult hepatocytes. The enzyme was partially purified by PEG fractionation and assayed at pH 7.1. Results are expressed as a percentage of the maximal activitiy. (A) Foetal hepatocytes, (B) adult hepatocytes. (○) No additions, (●) 10^{-7} M glucagon.

gest that foetal liver (22 days), despite exhibiting a glucagon-dependent rise in cAMP concentration and an activity of cAMP-dependent protein kinase similar to that found in adult hepatocytes, lacks the hormonal response induced by glucagon on PK, PFK-2 and Fru 2,6-P_2 that is present in adult tissue.

To characterize better the nature of PFK-2, the enzyme was partially purified (15% polyethylene glycol pellet) from foetal and adult hepatocytes incubated in either the absence or presence of 10^{-7} M glucagon. As shown in fig.2, the enzyme activity assayed at pH 7.1 had no response to glucagon in the foetus, whereas the adult tissue exhibited the reported change [19] in affinity for Fru 6-P (5-fold) as well as a decrease in maximal activity (from 173 to 82 pmol/min per mg protein).

The incubation of Blue Sepharose-purified PFK-2 with the catalytic subunit of cAMP-dependent protein kinase could not modify the activity of the foetal enzyme (table 2). The adult enzyme was inhibited when assayed at pH 6.6

Table 2

Effect of treatment of PFK-2 with the catalytic subunit of protein kinase

Hepatocytes	Addition	PFK-2 activity (mU/mg of protein)	
		pH 6.6	pH 8.5
Foetal	none	16 ± 2	22 ± 5
	catalytic subunit + ATP (1 mM)	18 ± 3	25 ± 3
Adult	none	28 ± 3	38 ± 5
	catalytic subunit + ATP (1 mM)	10 ± 2[a]	35 ± 4

Blue Sepharose-purified PFK-2 from foetal and adult hepatocytes was incubated with 0.1 mU catalytic subunit of protein kinase at 30°C for 10 min. Samples were used to assay PFK-2 activity at pH 6.6 and 8.5 as indicated in section 2.4

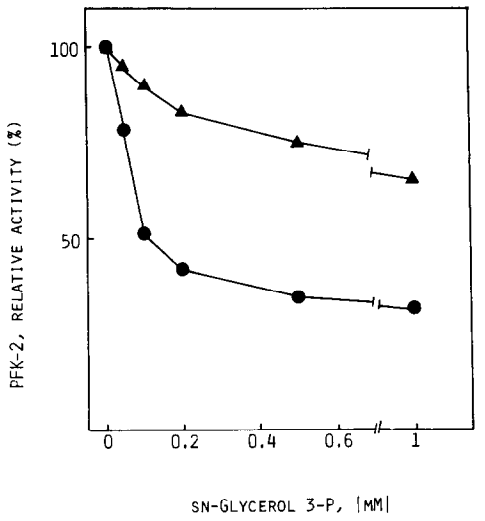

Fig.3. Effect of *sn*-glycerol 3-phosphate on PFK-2 activity in foetal and adult hepatocytes. Blue Sepharose-purified PFK-2 activity was assayed with 0.1 mM Fru 6P and 0.5 mM MgATP at pH 7.1. The specific activities of adult (●) and foetal (▲) PFK-2, corresponding to 100% values, were 22 and 38 mU/mg protein.

whereas no change was observed at pH 8.5 as described in [12].

It has been reported that hepatic PFK-2 is very sensitive to inhibition by *sn*-glycerol 3-phosphate whereas the heart enzyme is less sensitive [13]. PFK-2 from foetal hepatocytes was inhibited by *sn*-glycerol 3-phosphate markedly less than was the adult enzyme, K_i 2.5 and 0.1 mM respectively (fig.3).

Fructose-2,6-bisphosphatase was measured after Blue Sepharose purification. Fractions from foetal and adult hepatocytes containing similar PFK-2 activity (20 mU/mg protein) were assayed for fructose-2,6-bisphosphatase activity. Adult hepatocytes contained 4.1 mU/mg protein, whereas the activity of the foetal fraction was lower than 0.1 mU/mg protein.

In the 22-day-old foetuses we found a fructose-1,6-bisphosphatase activity of 3.6 U/g and 7.7 U/g in adult hepatocytes. This activity was inhibited by micromolar concentrations of Fru 2,6-P_2 and AMP. These results suggest that changes in the concentration of Fru 2,6-P_2 may play an important role in the control of the Fru 6-P/Fru 1,6-P_2 futile cycle allowing glycolysis to proceed for biosynthetic and energetic purposes.

4. DISCUSSION

In the adult liver glucagon plays an important role in the regulation of glycogenolysis and gluconeogenesis by activating adenylate cyclase and increasing cAMP concentration [20]. The foetal hepatic adenylate cyclase system is very responsive to glucagon [18] and it has been suggested that the development of sensitivity to glucagon may be important in the establishment of neonatal glucose homeostasis [21]. All of these findings are in agreement with our results showing that foetal hepatocytes respond to glucagon increasing cAMP concentration and that the activity of the cAMP-dependent protein kinase is similar in adult and 22-day-old foetal hepatocytes.

Here, we report that Fru 2,6-P_2 is present in isolated foetal hepatocytes at a concentration (1–2 nmol/g cells) similar to that found in foetal liver. This relatively low concentration of Fru 2,6-P_2 in foetus as compared to that prevailing in adult hepatocytes incubated under similar conditions is enough to activate PFK-1 allowing glycolysis to proceed. According to our results, the lactate released by foetal hepatocytes was much higher than in the adult. This high glycolytic capacity of foetal liver has been previously reported [2].

Because foetal hepatocytes contain receptors for glucagon, we investigated the possible existence of hormonal control of the concentrations of Fru 2,6-P_2 and of the activity of PFK-2. Our data clearly show that foetal PFK-2 was unresponsive to glucagon (assayed up to 10^{-6} M).

The analysis of PFK-2 activity showed differences between the adult and foetal enzymes. Incubation of Blue Sepharose-purified PFK-2 with the catalytic subunit of the protein kinase failed to modify the enzyme activity in foetal hepatocytes. Moreover, the sensitivity to *sn*-glycerol 3-phosphate inhibition was markedly lower for the foetal enzyme (more than 10-fold). The activity ratio between PFK-2/fructose-2,6-bisphosphatase under our conditions was 5 and 200 for adult and foetal preparations, respectively. Taken together, these results suggest the presence of a PFK-2 enzyme in foetal hepatocytes different from that present in adult liver.

Further work is in progress to characterize the isoenzymatic form of PFK-2 present in foetal liver.

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