DE NOVO SYNTHESIS OF GLUCOKINASE IN HEPATOCYTES ISOLATED FROM NEONATAL RATS

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1. Introduction

Hepatic glucokinase (EC 2.7.1.2) is first detectable around the 16th day in the neonatal rat [1–3]. Precocious appearance of the enzyme is induced by intubation of glucose; the resulting activities are enhanced by prior treatment of the animals with triiodothyronine (T_3) [4,5]. The factors that regulate synthesis of glucokinase in neonatal rat liver are complex [6] and it would be advantageous to be able to study its synthesis in a liver preparation in isolation from extra-hepatic influences.

We now report that rapid de novo synthesis of glucokinase can be induced in vitro by glucose plus insulin in hepatocytes isolated from 13-day-old rats. The synthesis is glucose-concentration dependent.

2. Experimental

Rats were from our departmental strain of specific-pathogen-free Wistar-derived animals and were maintained and treated as appropriate as in [5]. Hepatocytes were isolated from 13-day-old animals by a preperfusion with Ca^{2+}-free Krebs-Ringer bicarbonate (KRB) buffer and then a re-circulating perfusion [7] with KRB containing 0.025% (w/v) collagenase (Sigma, type IV), 5 mM glucose and the amino acid mixture of [8] but with 0.09 mM alanine, 0.01 mM arginine and 0.4 mM ornithine [9]. About 9×10^7 cells were obtained/liver (which weighs ~0.65 g in a 13-day-old rat). The preparations were essentially free of non-parenchymal cells as revealed by light microscopy. Some 90–95% of the freshly-prepared hepatocytes excluded trypan blue and ~85% of those subsequently incubated (see below) for 4 h maintained this facility. Leakage of lactate dehydrogenase activity from freshly-prepared cells was only 5–8% of the total cell activity; this low leakage was directly attributable to the presence of the amino acid supplement in the perfusion medium.

Medium 199 (Wellcome Reagents), a completely-defined tissue culture medium containing no serum supplement, to which was added 0.4 mM ornithine [9] was found to be a suitable incubation medium and, with additions as given in section 3, has been used as follows. About 3×10^6 cells were incubated in 3 ml medium in open 25 ml siliconized-glass conical flasks with shaking at 37°C under a gassing hood through which 95% O_2–5% CO_2 (v/v) was passed. The cells were collected by centrifugation, washed once in KRB buffer (to remove the glucose prior to later enzyme assays) and then homogenized in a Dounce glass hand homogenizer. The homogenate was centrifuged at 105 000 × *g* for 1 h. Glucokinase and hexokinase (EC 2.7.1.1) were assayed as described and critically evaluated in [5]. Supernatant protein was assayed by the Lowry method [10]. The antibody used was a sheep antiserum raised against pure glucokinase [11].

3. Results and discussion

Any glucokinase activity present in liver is retained during the isolation of hepatocytes by perfusion with collagenase. Glucokinase activity of the order of 0.5 unit/g liver was induced in vivo in 13-day-old rats by oral intubation of glucose [5]. Hepatocytes prepared from such animals using the fortified perfusion

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medium (see section 2) contained similar glucokinase activities to those measured following direct homogenization of the livers from other induced animals. Omission of either the glucose or amino acid mixture from the perfusion medium resulted in considerable loss of glucokinase activity during cell preparation. When hepatocytes isolated from 13-day-old rats in which glucokinase had been induced [5] were incubated for 4 h in the medium 199 no glucokinase activity was lost.

Hepatocytes prepared from both normal untreated and T_3-treated 13-day-old rats the livers of which contain no glucokinase activity [5], were incubated in medium 199 in the presence of L-[4,5-^{3}H]leucine. Incorporation of the radioactivity into trichloroacetic acid-precipitable protein in the supernatant fraction of homogenates of the cells was proportional to time over 2 h and was at 2.2–2.7%/h [12]. This result is higher than that obtained for [^{14}C]valine incorporation by adult hepatocytes in an unsupplemented medium [12]; it may reflect in part a greater overall rate of protein synthesis in the neonatal cells. Incorporation was not affected by the addition of 10^{-7} M insulin. The insulin-sensitivity of the cells was assessed by incubation in medium 199 for 15 min at either 10.5 or 20.5 mM glucose and in the presence or absence of 10^{-7} M insulin. The increases, ranging from 40–110%, in the percentage of the total glycogen synthetase activity that was in the I-form [13] found in cells from both normal and T_3-treated cells were not dissimilar to those in control experiments on adult hepatocytes.

Initial experiments showed that glucokinase activity could be detected in hepatocytes from 13-day-old rats after incubations for 4 h in medium 199 (which contains 5.5 mM glucose) supplemented with additional glucose and insulin. The concentration dependence of the effect upon these 2 agents (table 1) was explored first in cells from animals 48 h pre-treated with T_3 (1 μg/g body wt). No glucokinase appeared in the presence of 5.5 mM glucose. When the glucose concentration was increased over a range that may occur in portal blood as a result of ingestion of glucose, increasing glucokinase activity resulted (table 1). The presence of insulin was essential and varied in a manner similar to other insulin-dependent phenomena in isolated hepatocytes [14]. Table 2 shows that lower glucokinase activities were induced in cells from normal 13-day-old rats but the dependence upon both elevated glucose concentrations and insulin was essentially the same as in cells from T_3-treated animals.

Taking into account the fact that 1 g liver contains ~100 mg cytosolic protein, division of the values given in tables 1 and 2 by 10 gives an approximate indication of the glucokinase activity/g liver. The

Table 1
Effects of glucose and insulin concentrations upon glucokinase synthesis in hepatocytes isolated from day 13 rats pre-treated with triiodothyronine

Expt. no.	Insulin (M)	Glucose (mM)	No. of results	Glucokinase activity (units/g protein)	Hexokinase activity (units/g protein)
1	10^{-7}	5.5	8	0	2.72 ± 0.54
	10^{-7}	10.5	8	1.40 ± 0.51	2.48 ± 0.48
	10^{-7}	15.5	8	2.28 ± 1.00	2.54 ± 0.32
	10^{-7}	20.5	12	5.30 ± 1.03	2.64 ± 0.16
	10^{-7}	25.5	4	3.90 ± 2.02	2.72 ± 0.16
	10^{-7}	30.5	4	3.78 ± 1.27	2.64 ± 0.08
	10^{-7}	35.5	4	3.61 ± 1.36	3.04 ± 1.0
2	0	30.5	8	0	2.50 ± 0.02
	10^{-11}	30.5	4	0.37 ± 0.37	2.42 ± 0.36
	10^{-10}	30.5	4	0.37 ± 0.25	2.38 ± 0.20
	10^{-9}	30.5	4	1.09 ± 0.62	2.08 ± 0.41
	10^{-8}	30.5	8	6.91 ± 0.92	2.45 ± 0.80
	10^{-7}	30.5	4	6.53 ± 2.56	1.98 ± 0.72
	10^{-6}	30.5	4	6.36 ± 0.78	2.30 ± 0.60

Hepatocytes were incubated for 4 h in medium 199 with added insulin and/or glucose adjusted to the final concentration indicated. Other details are in the text. Results are means ± SE

Table 2
Effects of glucose and insulin concentrations upon glucokinase synthesis in hepatocytes isolated from normal day 13 rats

Expt. no.	Insulin (M)	Glucose (mM)	No. of results	Glucokinase activity (units/g protein)	Hexokinase activity (units/g protein)
3	10^{-7}	5.5	4	0	1.55 ± 0.24
	10^{-7}	10.5	4	0.60 ± 0.29	1.59 ± 0.08
	10^{-7}	15.5	4	1.56 ± 0.59	1.65 ± 0.36
	10^{-7}	20.5	4	3.59 ± 0.87	2.02 ± 0.42
4	0	20.5	2	0	1.71 ± 0.16
	10^{-10}	20.5	3	0	1.60 ± 0.01
	10^{-9}	20.5	4	1.74 ± 0.18	1.50 ± 0.12
	10^{-8}	20.5	4	2.70 ± 0.54	1.64 ± 0.36
	10^{-7}	20.5	4	3.84 ± 0.27	1.60 ± 0.02

Hepatocytes were incubated for 2 h in medium 199 with additions, as in table 1. The insulin concentration indicated was added at 0 and 1 h. Other details are in the text. Results are means ± SE

activities being induced in vitro are therefore very similar to those recorded in [5,6] for induction in vivo.

A time-course study of the effects obtained in table 1 revealed that most of the glucokinase synthesis occurred during the first 2 h of incubation. Because the tailing-off might be due to destruction of insulin by the hepatocytes [15], insulin was added at each hour during the incubation. As a result, more linear rates of glucokinase synthesis over 4 h were observed and the cells from T_3-treated animals synthesized more glucokinase than hepatocytes from normal litter-mates (table 3); the analogy with in vivo induction is again apparent.

Table 3 highlights another phenomenon, which can also be seen by comparison of results in tables 1 and 2, that was recorded earlier for the in vivo situation [5], namely, the presence of somewhat higher hexokinase activity in hepatocytes from the T_3-treated animals. We conclude from this data that low-K_m hexokinase activity is present in the parenchymal cells (see also the electrophoretic evidence below). Hexokinase activity was also observed spectrophotometrically and electrophoretically in paren-

Table 3
Time-course of glucokinase synthesis in hepatocytes isolated from day 13 rats

Status of animals	Time (h)	Glucokinase (units/g protein)	Hexokinase (units/g protein)
Normal	0	0	1.63 ± 0.03
	1	1.85 ± 0.25	1.61 ± 0.27
	2	2.65 ± 0.29	1.24 ± 0.33
	3	3.10 ± 0.28	1.25 ± 0.20
	4	4.60 ± 0.88	1.50 ± 0.39
Triiodothyronine-treated	0	0	2.10 ± 0.17
	1	3.81 ± 1.0	1.98 ± 0.17
	2	4.67 ± 0.59	1.98 ± 0.24
	3	6.59 ± 0.52	1.98 ± 0.21
	4	9.19 ± 0.87	2.35 ± 0.19

Hepatocytes were incubated for the time indicated in medium 199 at 20.5 mM final glucose conc. Insulin at 10^{-7} M was added at 0 time and each subsequent hour as appropriate. Other details are in the text. Results are means ± SE for 4 results in each case

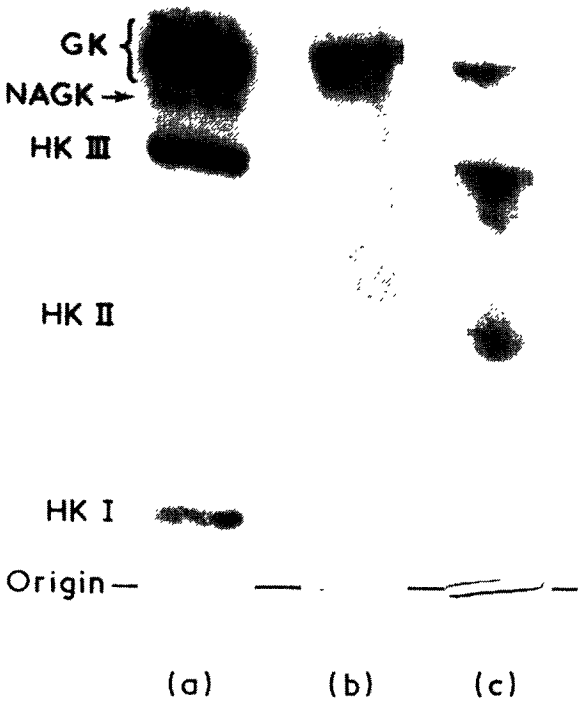

Fig.1. Electrophoretic evidence of glucokinase synthesis. A discontinuous buffer system was employed with agarose as the supporting medium [25]. The samples were: (a) normal adult liver; (b) extract of neonatal hepatocytes incubated with 20.5 mM glucose and 10^{-7} M insulin for 2 h as in table 2; (c) neonatal rat liver in which glucokinase had been induced in vivo. Samples (b,c) had been concentrated by ultrafiltration. Electrophoretograms were stained for enzyme activity [25] at 100 mM glucose. HKI, HKII and HKIII are the 3 low K_m hexokinase isoenzymes; GK, glucokinase; NAGK, *N*-acetyl-D-glucosamine kinase [25]. The glucokinase bands did not show up when similar gels were stained with 0.5 mM glucose or without ATP.

chymal cells from adult rats. This distribution of hexokinase activity in rat liver had been anticipated [16] but has been the subject of controversy (see [17] for refs).

Figure 1 provides electrophoretic evidence that neonatal hepatocytes incubated in vitro in the presence of glucose and insulin did contain glucokinase activity, as did the liver of a 13-day-old rat in which glucokinase had been induced in vivo [5]. Electrophoretic examination of cells from control experiments in which either insulin or an increased glucose concentration was not included showed no glucokinase band (not illustrated). When the glucokinase activity in a pooled extract from 6 incubation flasks was incubated with a small excess of an antiserum to glucokinase, all the glucokinase activity was removed while the hexokinase activity was unchanged; this was confirmed by electrophoresis (not shown). Finally, when either actinomycin D or cycloheximide was included in incubations at concentrations that prevent glucokinase synthesis in adult hepatocytes [18] no glucokinase activity appeared while hexokinase activity remained constant (table 4). These experiments support the conclusion that de novo synthesis of glucokinase in vitro is occurring.

It has been suggested that suspensions of hepatocytes are not suitable for experiments on enzyme induction [19]. Glucokinase activity is lost rapidly when hepatocytes are kept in culture [20,21] and attempts to overcome such problems have failed [22]. However, changes in glucokinase activity resulting from hormonal treatment of adult hepatocytes maintained in primary culture over a much longer time-period than in our experiments and, in particular,

Table 4
Inhibition of glucokinase synthesis

Additions	No. of results	Glucokinase activity (units/g protein)	Hexokinase activity (units/g protein)
20.5 mM Glucose only	2	0	2.0 ± 0.08
20.5 mM Glucose			
+ 10^{-7} M insulin each hour	5	5.24 ± 0.68	1.94 ± 0.20
+ actinomycin D[a]	4	0	2.05 ± 0.08
+ cycloheximide[b]	4	0	2.05 ± 0.16

[a] Final conc. 0.4 μg/ml
[b] Final conc. 10 μg/ml

Hepatocytes were incubated for 4 h in medium 199 with added glucose, insulin and inhibitors as indicated. The insulin was added at 0, 1, 2 and 3 h. Other details are in the text. Results are means ± SE

an effect of glucocorticoids were reported [18,23]. It will be interesting to know whether glucocorticoids exert any effect upon de novo synthesis of glucokinase in cells from neonatal rat liver especially as circulating glucocorticoid concentrations are low during this period [24] and it has been claimed [3] that glucocorticoid treatment can facilitate the precocious induction of glucokinase by glucose in vivo. This report shows that neonatal cells provide a system for the study of the factors and mechanism by which glucokinase synthesis is regulated. The results in this paper provide an in vitro demonstration of the requirement for an increased glucose concentration and for insulin in glucokinase synthesis recognised in vivo [22].

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