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PRECOCIOUS INDUCTION OF GLUCOKINASE IN PRIMARY CULTURES OF POSTNATAL RAT HEPATOCYTES[1]

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SUMMARY: Hepatocytes of 14-day-old rats have no detectable glucokinase activity in vivo, but it was induced by insulin (10^{-8}M) in primary cultures of these hepatocytes. The glucokinase induced by insulin was separated by electrophoresis on a cellulose acetate membrane and identified by its low affinity for glucose. This precocious induction of glucokinase was completely prevented by the presence of either actinomycin D or cycloheximide. Glucagon also inhibited its induction by insulin. Dexamethasone and testosterone, which alone had no inductive effect, strongly enhanced the induction by insulin. When hepatocytes of 14-day-old rats were cultured with 10^{-7}M insulin, 10^{-6}M dexamethasone and 10^{-7}M testosterone for 48 hr, their glucokinase activity increased to the non-induced level in hepatocytes of adult rats. Estrogen, thyroxine or growth hormone did not induce glucokinase precociously. Testosterone did not enhance induction of glucokinase by insulin in cultured hepatocytes of adult rats.

Glucokinase[EC 2.7.1.2] is a key enzyme in glucose utilization in the liver and it is induced by insulin and glucose in adult rats (1). The enzyme activity in hepatocytes of adult rats in primary culture disappeared rapidly (2-4), but it could be induced by insulin plus dexamethasone (5,6). Glucokinase has been used as a marker of terminal differentiation of liver parenchymal cells. In rat liver its activity first appears about 16 days after birth,and then rises rapidly reaching the adult level 10-12 days later (7,8). Exogenous glucose and insulin are apparently necessary for increase of glucokinase during development, since it is prevented by starvation, a carbohydrate-free diet or alloxan diabetes (7). However, attempts to induce glucokinase prematurely by infusion of glucose

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or insulin, or both, have been unsuccessful (2). There are reports that corticoid and thyroxine are necessary for precocious induction of glucokinase, but findings were ambiguous (8-10). This ambiguity may be due to the complexity of the in vivo system used in these studies. To avoid this complexity, we used hepatocytes of 14-day-old rats in primary culture to study the terminal differentiation of immature hepatocytes, as reflected by synthesis of glucokinase.

The present paper provides evidence that the physiological stimuli for postnatal development of glucokinase are insulin, glucocorticoid and testosterone.

MATERIALS and METHODS

Materials --- The rats and materials used for cell isolation and culture were as reported previously (11). Yeast glucose-6-phosphate dehydrogenase, NADP and ATP were obtained from Boehringer Mannheim, Mannheim; actinomycin D was from Schwazmann, New York; cycloheximide was from P-L Biochemicals, Milwaukee; and other chemicals were from Wako Pure Chemicals, Osaka.

Isolation and monolayer culture of parenchymal cells from 14-day-old rat liver --- Parenchymal hepatocytes were isolated from 14-day-old rats and adult rats and cultured as monolayers as reported previously (11). Suspensions of 5 x 10^6 cells were plated in 10 cm plastic dishes in 10 ml of Williams medium E supplemented with 10% fetal bovine serum and 10^{-6}M dexamethasone, and cultured in a humidified incubator at 37°C under 45% O_2, 5% CO_2 and 50% N_2. The medium was changed 24 hr after plating.

Assay of glucokinase --- The cells were harvested with a rubber policeman and homogenized in 0.5 ml of 20 mM K-phosphate buffer (pH 7.5) containing 10 mM glucose, 0.1 mM dithiothreitol, 5 mM EDTA and 150 mM KCl in a Polytron homogenizer for 0.5 min. The homogenate was centrifuged and the supernatant was used as the enzyme preparation. Glucokinase was assayed by the method of DiPietro and Weinhouse (12). Glucokinase activity was calculated by subtracting the hexokinase activity from the total activity of glucose-ATP phosphotransferase. One unit of glucokinase is defined as the amount forming 1 μmole of product per min. Protein was measured by the method of Lowry et al. (13).

Electrophoresis --- Electrophoresis was carried out on a cellulose acetate membrane (Gelman, 1 x 6.7 in) in Veronal buffer (pH 8.6, I=0.05) containing 5 mM EDTA, 10 mM 2-mercaptoethanol and 10 mM glucose at 100 Volts for 90 min in a cold room. After electrophoresis the membrane was stained for hexokinase and glucokinase activity by the method of Sato et al.(14).

RESULTS and DISCUSSION

No glucokinase activity was detectable in freshly isolated hepatocytes from 14-day-old rats, and it did not appear in hepa-

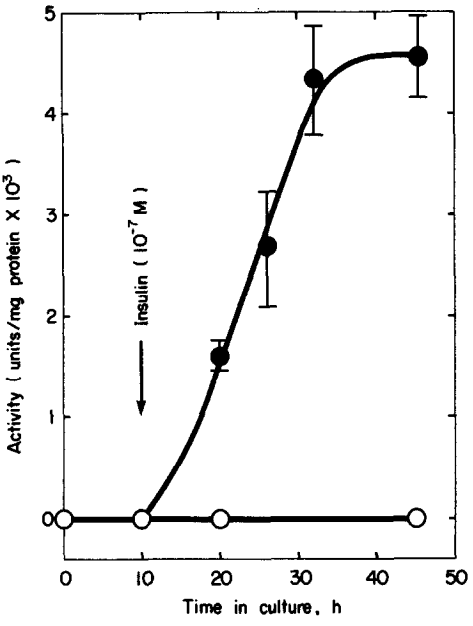

Fig. 1. Precocious induction of glucokinase by insulin in 14-day-rat hepatocytes in culture. Dexamethasone was added from start of culture and insulin was added 10 hr later. Values are means ± S.D. for 4 experiments. o, with insulin; o, without insulin.

tocytes cultured without insulin for up to 48 hr. It was reported in organ culture of fetal mouse liver that glucokinase activity increased moderately without added hormone (15). As shown in Fig. 1, however, in medium containing 10^{-7}M insulin, glucokinase appeared in the cells within 10 hr, and then increased linearly for 20 hr. We have shown that freshly isolated hepatocytes have greatly impaired functions (4). The number of insulin receptors on freshly isolated cells is only about half that on intact liver cells (unpublished data). However, their impaired functions and reduced insulin receptors are restored during culture. Therefore, to attain the maximum induction of glucokinase, insulin was added 10 hr after the start of culture.

The glucokinase induced in this way was extracted from the cells and identified by electrophoresis on a cellulose acetate membrane, as shown in Fig. 2. The band with the highest mobility toward the

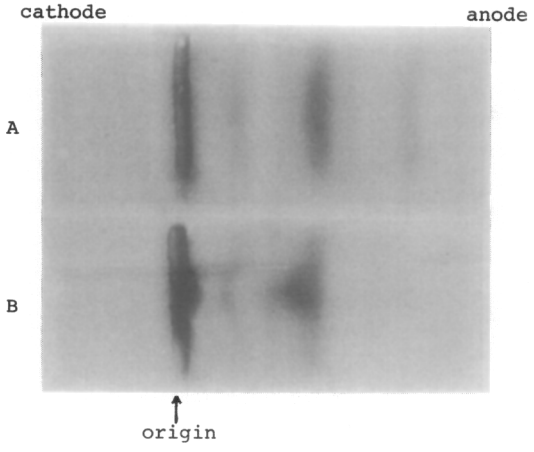

Fig. 2. Electrophoretic identification of glucokinase in 14-day-old rat hepatocytes in culture. The enzyme extract, prepared from hepatocytes cultured as shown in Fig. 1 for 48 hr, was concentrated in a small dialysis bag using Sephadex G-200. A sample of 3 µl of the concentrated enzyme extract was placed on the cathode side of a cellulose acetate membrane. Bands of enzyme were stained with 0.5 mM (A) or 100 mM glucose (B).

anode was identified as glucokinase, because it could be stained with 100 mM glucose, but not with 0.5 mM glucose. Induction of glucokinase by insulin was detectable with a physiological concentration of 10^{-9}M insulin (Fig. 3). The induced activity increased with the dose of insulin up to 10^{-8}M insulin.

The precocious induction of glucokinase was completely inhibited by addition of cycloheximide (10^{-5}M) or actinomycin D (0.3 µg/ml) (data not shown).

Dexamethasone alone did not induce glucokinase in cultures of 14-day-old rat hepatocytes, but it enhanced the induction by insulin (Table I). Dexamethasone may have a permissive effect (5, 6), or it may stimulate cellular activity in general as shown previously (11). Glucagon inhibited the induction of glucokinase by insulin, as shown in vivo (8). Since the enzyme activity induced by insulin plus dexamethasone was only about half that of mature hepatocytes, we next examined the effects of other

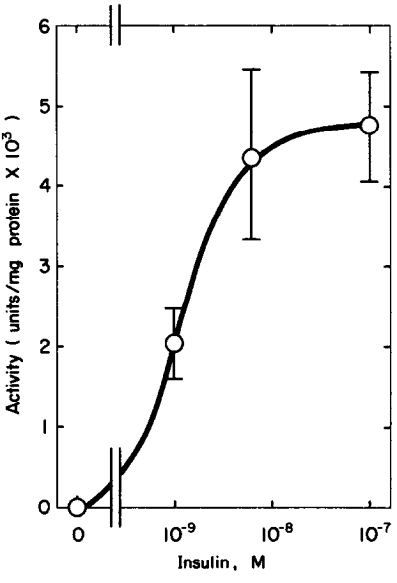

Fig. 3. Dose-response curve for the induction of glucokinase by insulin in 14-day-old rat hepatocytes in culture. Culture conditions were as for Fig. 1. Values are means ± S.D. for 4 experiments.

Table I. Effects of Various Hormones on Induction of Glucokinase in Primary Cultures of Hepatocytes from Adult and 14-Day-Old Rats

Hormones	Glucokinase (mU/mg protein)	
	14-day-old hepatocytes	Adult hepatocytes
Dexamethasone (10^{-6}M)	0.11 ± 0.07	7.28 ± 0.72
Insulin (10^{-7}M)	2.66 ± 0.25	12.30 ± 0.95
Dexamethasone and insulin	4.27 ± 0.14	17.91 ± 1.01
Dexamethasone, insulin and glucagon (10^{-7}M)	1.10 ± 0.10	11.32 ± 1.34
Dexamethasone and testosterone (10^{-7}M)	0.56 ± 0.22	8.78 ± 1.21
Dexamethasone, insulin and testosterone	8.29 ± 0.58	15.14 ± 1.52

Dexamethasone was present from the start of culture, except in experiments on addition of insulin alone. Ten hr later various hormones were added and glucokinase was assayed 48 hr after the start of culture. Values are means ± S.D. for 2-4 experiments.

hormones on its precocious induction. Testosterone plus dexamethasone did not induce glucokinase much, but addition of insulin to these hormones caused the highest induction, which was comparable to the non-induced level in adult rat liver (Table I). Thyroxine (10^{-7}M) and human growth hormone (0.1 μg/ml) did not induce glucokinase, or enhanced induction by insulin and dexamethasone (data not shown). Estrogen also did not induce the enzyme, or counteract the effect of testosterone (data not shown). It is interesting that testosterone did not enhance insulin-dependent induction of glucokinase in adult hepatocytes in primary culture (Table I). The reason why testosterone is effective in neonatal liver but not in adult liver is unknown. It should be mentioned that hexokinase activity did not change at all in these experiments.

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