FEBS Letters 393 (1996) 60–64 FEBS 17440

Overexpression of either liver type or pancreatic β cell type glucokinase via recombinant adenovirus enhances glucose oxidation in isolated rat hepatocytes

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Received 25 June 1996

Abstract To elucidate a role of glucokinase in hepatic glucose metabolism, we overexpressed hexokinase I (HKI), liver type glucokinase (LGK), or β cell type glucokinase (βGK) in primary rat hepatocytes using a recombinant adenovirus vector system. Overexpression of HKI and LGK induced a 34- and 25-fold increase, respectively, in glucose phosphorylation activity measured in cell homogenates. While HKI overexpression induced only a 1.3-fold increase in glucose oxidation, LGK overexpression increased glucose oxidation by 2.9-fold. Overexpression of βGK had essentially the same effect as LGK. The results indicate that glucokinase does indeed regulate the rate of hepatic glucose oxidation and that the liver-specific sequence of this enzyme is not essential for this function.

Key words: Glucokinase; Hexokinase; Adenovirus; Glucose metabolism; Hepatocyte

1. Introduction

Glycolysis in the liver plays an important role in glucose metabolism in humans and other mammals. Although most glycolytic reactions are reversible, three are markedly exergonic and therefore considered physiologically irreversible. These reactions are those catalyzed by glucokinase (GK; EC 2.7.1.2), 6-phosphofructo-1-kinase (PFK), and pyruvate kinase (PK) and are the major sites of regulation of glycolysis in the liver [1]. The first step in glycolysis is catalyzed mainly by GK, hexokinase isozyme type IV, which can be distinguished from other hexokinases by a number of features including its low affinity for glucose and lack of inhibition by glucose 6-phosphate [2].

GK gene mutations were recently observed in diabetic patients, especially in a subset of diabetes mellitus, maturity onset diabetes of the young (MODY) [3–5]. In patients with GK gene mutations, the insulin secretory response to glucose was impaired [6,7], reflecting GK defects in pancreatic β cells, whereas the contribution of liver GK defects to impaired glucose metabolism has not been thoroughly investigated in patients. Very recent observations using mice lacking liver GK or both liver and β cell GK suggest that defects in this enzyme in pancreatic β cells play a more significant role in whole body glucose homeostasis than do defects in the liver enzyme [8,9].

To examine the role of liver type GK in liver glucose metabolism in vitro, it is preferable to use primary hepatocytes. Hepatoma cell lines do not necessarily represent normal hepatocytes in many respects including the levels of GLUT2 and GK expression, while hepatocytes isolated with collagenase retain many liver functions [10,11]. In the present study, using a recently developed adenovirus vector system, we overexpressed either liver type GK (LGK), β cell type GK (βGK), or hexokinase I (HKI) in primary cultured rat hepatocytes and investigated the effects of overexpression of these enzymes on glycolysis. Our results indicate that GK plays a crucial role in determining liver glucose oxidation capacity and that LGK can be replaced by βGK but not by HKI in the hepatic glycolytic pathway.

2. Materials and methods

2.1. Materials

Collagenase and ethylenediaminetetraacetic acid (EDTA) were purchased from Wako Pure Chemical Industries (Osaka, Japan). Leibovits L-15 medium and 5-bromo-4-chloro-2-indolyl-β-D-galactopyranoside were obtained from Sigma Chemical Co. (St. Louis, MO, USA), NADP, ATP and glucose 6-phosphate dehydrogenase from Boehringer Mannheim (Mannheim, Germany), *N*-(2-hydroxyethyl)-piperazine-*N'*-2-ethanesulfonic acid (HEPES), polyethylene glycol #300 and dithiothreitol from Nacalai Tesque Inc. (Kyoto, Japan). D-[6-^{14}C]Glucose was obtained from Amersham International plc (Buckinghamshire, UK), NaH[^{14}C]O_3 (74–370 MBq/mmol) and Aquasol-2 from Du Pont-New England Nuclear (Boston, MA, USA).

2.2. Preparation of recombinant virus containing rat liver type GK (Adex1CArLGK), rat β cell type GK (Adex1CArβGK), or hexokinase I (Adex1CArHKI)

Generation of recombinant adenoviruses was performed according to the method described by Miyake et al. [12]. Briefly, a 1.8 kb fragment containing the entire coding sequence of rat liver type GK cDNA was ligated into a cosmid, pAdex1pCA. The resulting cosmid was termed pAdex1pCArLGK. The recombinant adenovirus was then prepared by homologous recombination between the pAdex1pCArLGK cosmid and the parental adenovirus type 5 genomic DNA–terminal protein complex in the human kidney cell line 293, and was termed Adex1CArLGK. Similarly, rat β cell type cDNA and rat hexokinase I cDNA were used for preparation of Adex1CArβGK and Adex1CArHKI, respectively. Construction of Adex1CAlacZ was performed as described previously [13]. The recombinant adenovirus stocks of Adex1CArLGK, Adex1CArβGK, Adex1CArHKI, and Adex1CAlacZ had titers of 2.5×10^7, 2.7×10^7, 2.7×10^9, and 6×10^8 pfu/ml, respectively.

2.3. Hepatocyte isolation and adenovirus infection

Hepatocytes were isolated from a male Wistar rat (200 g) by collagenase digestion as described previously [14]. Isolated hepatocytes were seeded in 6 cm collagen-coated culture dishes at a density of 1.25×10^6 cells/5 ml in L-15 medium (11 mM glucose) and maintained at 37°C in humidified 5% CO_2 and 95% air. After the hepatocytes had attached to the dishes (4 h later), the medium was replaced with 1 ml of L-15 medium and the following amounts of recombinant adenovirus stocks were added to the cells: 100 μl of Adex1CArβGK, 50 μl of Adex1CArLGK, 2 μl Adex1CArHKI, or 50 μl Adex1CAlacZ. After incubation for 1 h with adenovirus at 37°C, the medium was removed and the hepatocytes were washed with phosphate-buffered

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PII S0014-5793(96)00833-2

saline (PBS). The hepatocytes were then incubated with 5 ml/dish of fresh L-15 medium. The experiments were performed 24 h after the isolation of hepatocytes.

2.4. Assays of GK, HKI and βGAL expression

GK and HKI expression were evaluated by direct measurement of enzyme activities. 20 h after virus administration, hepatocytes were collected and homogenized in 1 ml of a homogenization buffer (20 mM Tris, pH 7.4, 50 mM KCl, 5 mM $MgCl_2$, 2 mM EDTA, 1 mM dithiothreitol, 1% polyethylene glycol #300). Homogenates were centrifuged at $10000 \times g$ for 5 min in a microcentrifuge. HKI activity in the supernatant was measured at 0.5 mM glucose as described previously [15–17] using 20 μl of homogenate in the total volume of 1.5 ml of assay buffer (20 mM HEPES, pH 7.45, 0.1 M KCl, 0.5 mM NADP, 5 mM ATP, 7.5 mM $MgCl_2$, 0.4 units of glucose 6-phosphate dehydrogenase). GK activity was represented by the difference between glucose phosphorylating activity measured at 100 mM and 0.5 mM glucose.

For analysis of *lacZ* gene expression, hepatocytes were washed once with PBS, fixed in 3.4% formaldehyde in PBS, and then washed three times with PBS over 30 min. Expression of the introduced *lacZ* gene was demonstrated by staining the fixed cells at 37°C for 8 h in a chromogenic solution containing 5 mM $K_3Fe(CN)_6$/$K_4Fe(CN)_6 \cdot 3H_2O$, 2 mM $MgCl_2$, and 1 mg/ml 5-bromo-4-chloro-2-indolyl-β-D-galactopyranoside in PBS [18]. The efficacy of gene transfer was determined by counting clear and blue cells in three separate dishes in three independent experiments.

2.5. Glucose oxidation measurements

Hepatocytes transfected with GK, HKI or βGAL cDNA in the culture dishes were placed in sealed glass containers (7 cm in diameter × 10 cm in height) filled with 100% oxygen. D-[6-^{14}C]Glucose was added to 2 ml of L-15 medium and the cells were incubated with 11 mM glucose for 2 h at 37°C. At the end of incubation, 0.5 ml of 10% $HClO_4$ was added to the medium using a long 21-gauge syringe needle through rubber stoppers, allowing CO_2 gas (containing [^{14}C]O_2) to evaporate and be trapped in 2 ml of 10% KOH solution in a small glass cup suspended above the medium in the sealed glass container [19]. The glass containers were incubated for another 30 min, then KOH solution was transferred to the scintillation vials containing 10 ml of Aquasol-2, and the radioactivity was measured with a liquid scintillation counter. In this system, 92.5 ± 1.6% (mean ± S.D., $n = 3$) of CO_2 gas evaporated from the medium containing 0.5 μCi (18.5 kBq) NaH[^{14}C]O_3 was trapped in KOH solution.

2.6. Statistical analysis

Data are presented as means ± S.D. The statistical significance of differences between mean values was assessed using Student's t test.

3. Results

3.1. Efficient gene transfer and expression using the recombinant adenovirus system in primary rat hepatocytes

When the βGAL gene was transferred into primary hepatocytes by recombinant adenovirus (Adex1CAlacZ) infection at a multiplicity of infection (m.o.i) of 24.0, intense blue staining with X-gal was observed in the cytoplasm of all hepatocytes 20 h after viral infection (Fig. 1A). Essentially 100% efficiency of gene transfer was also demonstrated at a lower m.o.i. of 2.4 (data not shown). No blue staining with X-gal was observed in hepatocytes which had not been treated with Adex1CAlacZ (Fig. 1B). No apparent changes in either numbers or morphological features were observed in hepatocytes remaining attached to the culture dishes 20 h after viral infection with Adex1CAlacZ, as compared with untreated cells. The integrity of infected hepatocytes was also confirmed by the observation that the glucose phosphorylation activities measured at 0.5 and 100 mM, in Adex1CAlacZ-infected hepatocytes, were the same as those in untreated hepatocytes (Table 1).

3.2. The effects of adenovirus-mediated overexpression of LGK or HKI on glucose phosphorylation and glucose oxidation

In a series of experiments designed to compare the effects of hexokinase I overexpression with that of liver type GK overexpression, primary hepatocytes were infected with a 50 μl solution of Adex1CArLGK or a 2 μl solution of Adex1CArHKI. GK activity, which was represented by the difference between glucose phosphorylating activity measured at 100 mM and 0.5 mM glucose, was increased approximately 21-fold in the cell homogenates, as compared to the activity in untreated hepatocytes or Adex1CAlacZ-infected hepatocytes. Infection with Adex1CArHKI resulted in a marked increase, of 160-fold on average, in the HKI activity measured at 0.5 mM glucose (Table 1). In these infected hepatocytes, glucose oxidation measured by conversion of D-[6-^{14}C]glucose to [^{14}C]O_2 was increased 2.9-fold (2.94 ± 0.14, mean ± S.D., $n = 3$) in cells overexpressing liver type GK as compared to control Adex1CAlacZ-infected cells. In contrast, HKI overexpression induced only a very modest increase, 1.3-fold (1.25 ± 0.04, mean ± S.D., $n = 3$, $P < 0.05$), in glucose oxidation (Fig. 2). Glucose phosphorylating activity measured at 11 mM, the concentration used also to examine glucose oxidation, in a cell homogenate of hepatocytes overexpressing HKI was increased 34-fold as compared to that of controls (533 ± 52 vs. 16 ± 2 nmol/min mg protein, mean ± S.D., $n = 3$). This value was actually greater than that in hepatocytes overexpressing liver type GK (397 ± 37 nmol/min mg protein, $n = 3$, a 25-fold increase as compared to that of controls).

3.3. The effects of adenovirus-mediated overexpression of βGK or LGK on glucose phosphorylation and glucose oxidation

In another set of experiments, hepatocytes were infected with Adex1CArLGK or Adex1CArβGK to compare the effects of LGK overexpression with those of βGK overexpression. Infection with 100 μl of Adex1CArβGK solution induced an approximately 14-fold (13.5 ± 1.6, $n = 3$) increase in the GK activity and a 2.5-fold (2.51 ± 0.17, $n = 3$) increase in oxidation of 11 mM glucose. Similarly, infection with 50 μl of

Table 1
Glucose phosphorylating activity in hepatocytes

	Hexokinase activity (nmol/min mg protein)	Glucokinase activity (nmol/min mg protein)
Untreated	3.30 ± 0.67	25.8 ± 3.4
Adex1CAlacZ	3.10 ± 0.70	27.0 ± 3.7
Adex1CArHKI	502 ± 81	ND
Adex1CArLGK	ND	566 ± 51

Hepatocytes were isolated and treated with the indicated adenovirus or left untreated. Hexokinase activity was defined as the glucose phosphorylating activity measured at 0.5 mM glucose. Glucokinase activity was defined as the difference between glucose phosphorylating activity measured at 100 mM and 0.5 mM glucose as described in Section 2. Data are means ± S.D. of three independent experiments each performed in triplicate. ND: not determined.

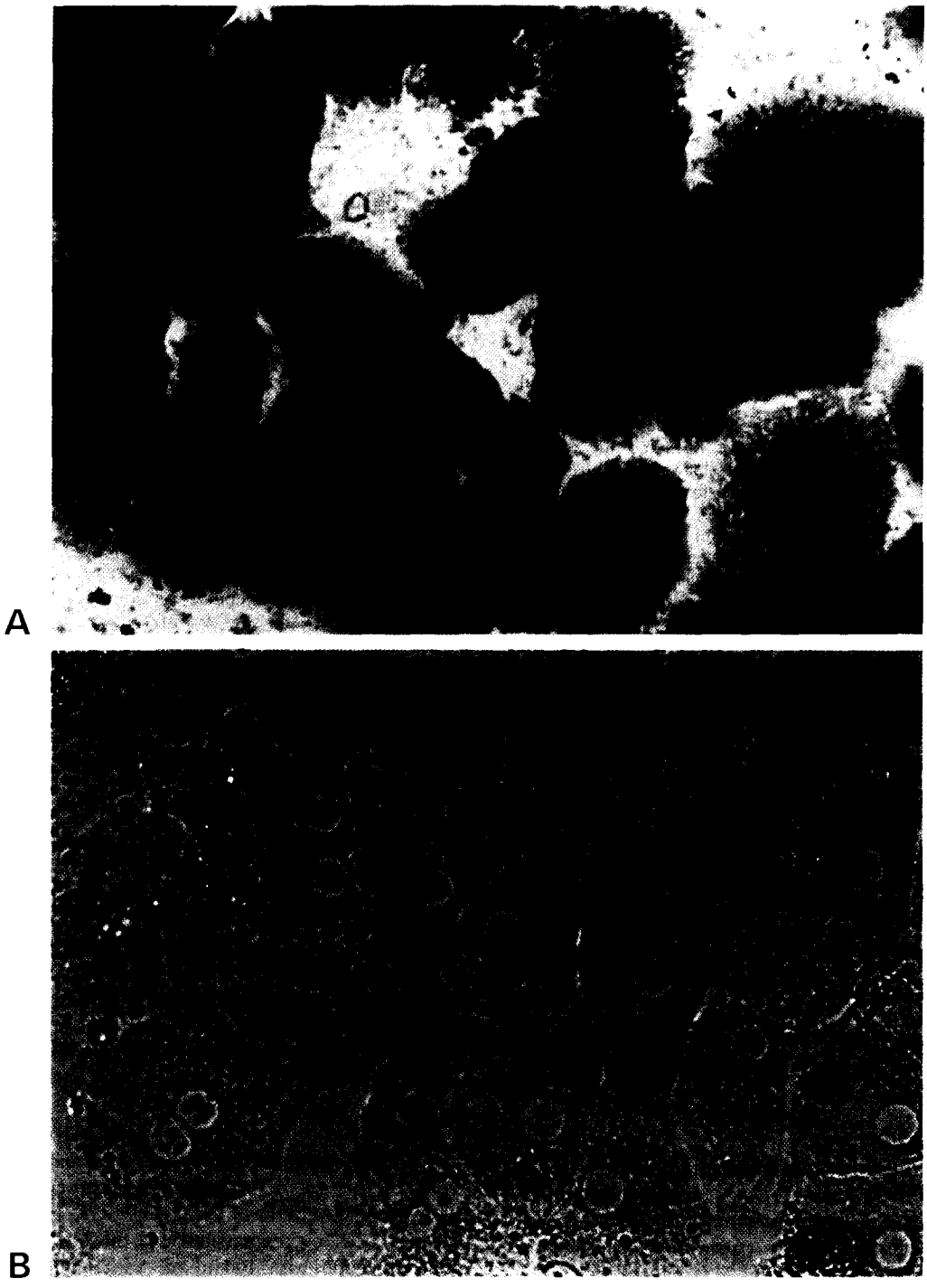

Fig. 1. β-Galactosidase expression in isolated hepatocytes treated with Adex1CAlacZ recombinant adenovirus. A: Light microscopic view of representative primary cultured hepatocytes 20 h after infection with Adex1CAlacZ (24 h after isolation). Hepatocytes were treated with chromogenic substrate as described in Section 2. B: Control hepatocytes similarly treated with a choromogenic substrate without previous exposure to Adex1CAlacZ recombinant adenovirus.

Adex1CArLGK solution induced an approximately 15-fold (14.5 ± 0.6, $n=3$) increase in GK activity and a 2.7-fold (2.65 ± 0.16, $n=3$) increase in glucose oxidation (Fig. 3).

The experiments were also performed using smaller amounts of virus. Infection with 8 μl of Adex1CArβGK solution induced a 3.4-fold increase in the GK activity and a 1.5-fold increase in oxidation of 11 mM glucose. Infection with 2 μl of Adex1CArLGK solution induced a 3.4-fold increase in GK activity and a 1.6-fold increase in glucose oxidation. Thus, the effects on GK activity apparently paralleled the effects on glucose oxidation regardless of whether phosphorylation activity was increased by overexpression of liver type GK or β cell type GK. It is not clear why the same volume of Adex1CArLGK stock solution exerted a smaller effect on GK activity in the latter than in the former series of experiments. However, the latter series of experiments were performed 2 months later than the former, such that the infective potency of the virus may have diminished during the storage period.

4. Discussion

Our results demonstrated clearly that gene transfer via recombinant adenovirus is highly effective in primary cultured rat hepatocytes. *LacZ* gene expression was observed in essentially 100% of primary hepatocytes infected with Adex1CA-lacZ. Similarly, a very high efficiency (86%) of muscle glycogen phosphorylase gene expression was previously obtained in isolated rat hepatocytes using an adenovirus vector [20]. Gene transfer via a retrovirus vector is not effective in non-proliferating cells including primary hepatocytes. Physical gene transfer methods such as those using liposomes and electroporation are at present rather inefficient methods of gene transfer, requiring subsequent cloning of the transfected cells which is not possible in primary hepatocytes. Infection with Adex1CAlacZ induced neither apparent morphological changes on light microscopic examination nor changes in glucose phosphorylating activity (Table 1) and the glucose oxidation rate (data not shown), as compared to uninfected cells. These observations indicate that adenovirus infection per se does not alter hepatocyte functions so long as virus titers are within the ranges used in this study.

Utilizing gene transfer and expression with an adenovirus vector, we were able to directly manipulate the GK gene expression in primary rat hepatocytes. We thereby demonstrated that GK is indeed a key enzyme determining the glucose oxidation rate in hepatocytes. This is consistent with very recent observations of hepatocytes isolated from the mice lacking GK gene expression [8,9]. In addition, we also demonstrated that an increase in GK gene expression no more than tripled overall glucose oxidation in primary rat hepatocytes. A discrepancy between the large increase, induced by overexpression of the enzyme, in glucose phosphorylation activity and the relatively small increase in glucose oxidation has also been reported in pancreatic islets and a pancreatic β cell line MIN6 [21,22]. We suggested that some step(s) other than glucose phosphorylation became rate-limiting for glucose utilization in MIN6 cells overexpressing hexokinase I [22]. The present results suggest that this is also the case in rat hepatocytes, although it should be kept in mind that the enzyme activity was assayed in vitro and CO_2 production was assessed in intact cells. It is possible that glucokinase regulatory protein present in hepatocytes inhibits GK activity, since the level of fructose 6-phosphate, a potent activator of glucokinase regulatory protein [23], may be increased by accelerated conversion of glucose to glucose 6-phosphate.

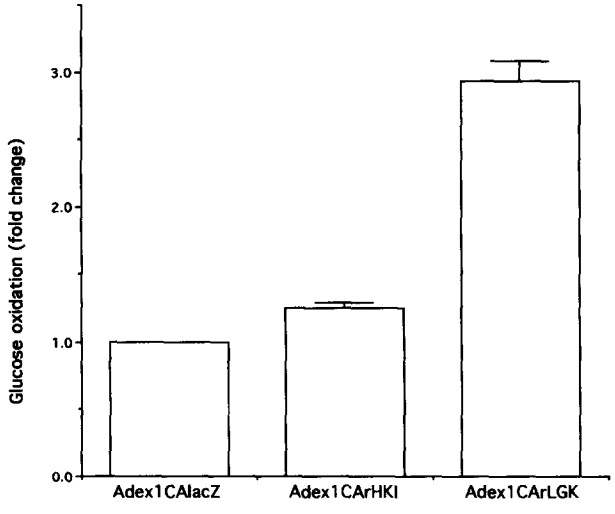

Fig. 2. Glucose oxidation in primary cultured hepatocytes overexpressing hexokinase I and liver type glucokinase. Glucose oxidation was measured by following the conversion of D-[6-^{14}C]glucose into [^{14}C]O_2 as described in Section 2. Data are expressed as the mean fold change ± S.D. of three independent experiments each performed in triplicate. The absolute values for the control ranged from 0.034 to 0.051 (nmol/min mg protein).

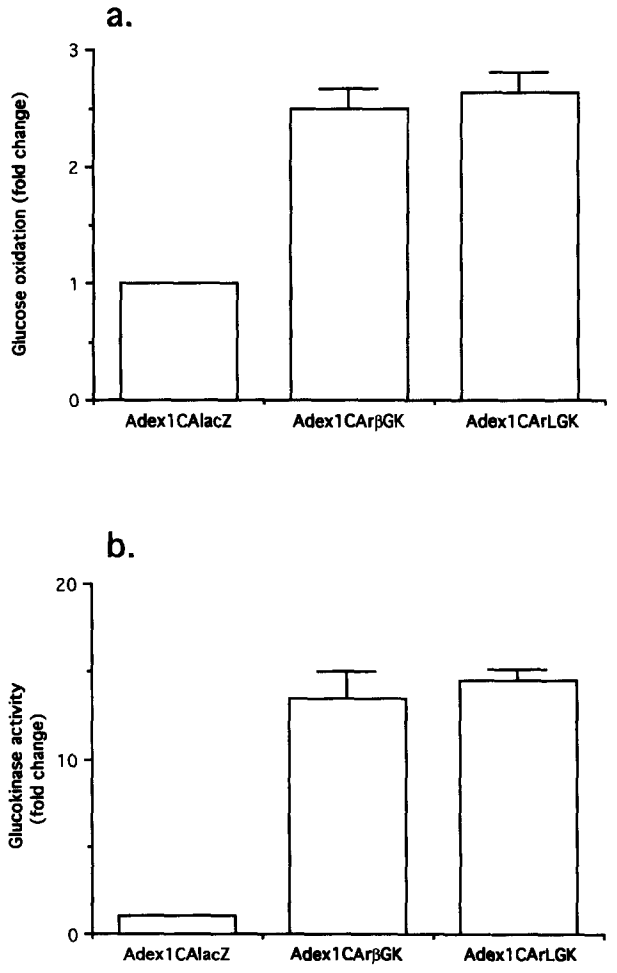

Fig. 3. Glucose oxidation and glucose phosphorylating activity in primary cultured hepatocytes overexpressing β cell type and liver type glucokinase. a: Glucose oxidation was measured 20 h after exposure to the indicated recombinant adenoviruses. b: Glucokinase activity was represented by the difference between glucose phosphorylating activity measured at 100 mM and 0.5 mM. Data are expressed as the mean fold change ± S.D. of three independent experiments each performed in triplicate.

The liver type and pancreatic β cell type GK differ only in the 14–16 amino acids of the amino-terminus which are encoded by tissue-specific leader exons. It has been speculated that the amino-terminal domain of glucokinase has a specific functional role [24]. However, these two glucokinases exerted essentially the same effect on glucose oxidation when overexpressed in primary rat hepatocytes, indicating that the liver-specific amino-terminal sequence of GK is not indispensable for its function in liver glucose metabolism.

GK overexpression exerted a greater facilitating effect on glucose oxidation than did HKI overexpression in primary

hepatocytes, even in the conditions where the effects of GK overexpression on glucose phosphorylating activity were smaller than those of HKI overexpression. This difference may be accounted for at least in part by HKI being allosterically inhibited by the product (glucose 6-phosphate) whereas liver GK is not. Very recently, Becker et al. reported that HKI overexpression has greater metabolic effects than GK overexpression in isolated rat islets [25]. The discrepancy of the effects between hepatocytes and islets is of great interest and needs further investigation.

In summary, this is the first report to our knowledge of gene transfer into and expression of glycolytic enzymes, HKI and GK, in primary rat hepatocytes. Our results indicate that GK regulates the rate of glucose metabolism in the liver and that the liver-specific N-terminal sequence is not essential for this function.

Acknowledgements: We are grateful to Drs. I. Saito and Y. Kanegae for their kind advice in preparing the recombinant adenoviruses. This work was supported by a grant-in-aid for scientific research from the Ministry of Education and Culture of Japan and a grant for diabetes research from Ohtsuka Pharmaceutical Co.

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