Intracellular distribution of hepatic glucokinase and glucokinase regulatory protein during the fasted to refed transition in rats

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Abstract We have studied the intracellular distribution in vivo of glucokinase (GK) and glucokinase regulatory protein (GKRP) in livers of fasted and refed rats, using specific antibodies against both proteins and laser confocal fluorescence microscopy. GK was found predominantly in the nucleus of hepatocytes from starved rats. GK was translocated to the cytoplasm in livers of 1- and 2-h refed animals, but returned to the nucleus after 4 h. GKRP concentrated in the hepatocyte nuclei and its distribution did not change upon refeeding. These results show that, in physiological conditions, GKRP is present predominantly in the nuclei of hepatocytes and that the translocation of hepatic GK from and to the nucleus is operative in vivo.

Key words: Glucokinase; Glucokinase regulatory protein; Liver; Refeeding; Translocation

1. Introduction

Glucokinase (GK, EC 2.7.1.1), also called hexokinase IV, catalyses glucose phosphorylation in hepatocytes, pancreatic islet cells and some neuroendocrine cells [1]. It is a key component of the glucose sensing machinery in mammals and plays a fundamental role in whole-body glucose homeostasis. Unlike the other hexokinases, GK has a low affinity for its substrate, with a $S_{0.5}$ in the physiological glucose concentration range, and it is not inhibited by glucose 6-phosphate (G6P), the product of the reaction [2].

The activity of GK in the liver is modulated by the glucokinase regulatory protein (GKRP). This protein binds to GK and inhibits enzyme activity competitively with respect to glucose [3]. In turn, GKRP can bind phosphate esters of fructose and some analogues, which modify its affinity for GK. Fructose 6-phosphate reinforces the inhibitory effect of GKRP, while fructose 1-phosphate abolishes binding and subsequent inhibition [3,4].

Using cultured rat hepatocytes, it has been shown that in response to high glucose concentrations or to micromolar concentrations of fructose or sorbitol, GK changes from a bound to a free state, in which it is more easily extractable with digitonin [5–7]. Translocation of GK by these substrates correlates with stimulation of glycogen synthesis [5,6].

Immunocytochemical studies of the subcellular distribution of hepatic GK in cultured rat hepatocytes [8] and in perfused rat livers [9,10] have been previously performed. In control conditions, GK was always detected in the nucleus of the hepatic cells. However, in the presence of fructose or high concentrations of glucose, the enzyme was mainly found in the cytoplasm. Whether GKRP also translocates in response to glucose is controversial. It has been reported that, in rat livers perfused with high glucose, GKRP moves together with GK to the cytoplasm [10]. However, in primary cultured hepatocytes, GKRP was always found in the nuclear compartment [8] and was not extractable with digitonin [11], even in the presence of fructose or high levels of glucose in the incubation medium.

No data are available about the intracellular distribution of GK and GKRP in the livers of animals in different nutritional conditions. In the present study we provide evidence that the translocation of GK from the hepatocyte nucleus to the cytosol takes place during the fasted to refed transition in rats, while GKRP remains in the nuclear compartment during this period.

2. Materials and methods

2.1. Animals

Male Wistar rats weighing 200–230 g were divided into two dietary groups: (A) starved animals: all food was removed from the cage 24 h before the experiment, but drinking water was available; (B) refed animals: 24-h starved rats were given access to food for the indicated periods of time.

2.2. Preparation of liver homogenates, GK activity and glycogen measurements

Rats were anaesthetised by inhalation of ethyl ether. Samples from livers were excised and immediately used for several measurements. Liver samples were homogenised in 10 volumes of ice-cold 50 mM KCl, 300 mM sucrose and 10 mM β-mercaptoethanol in a Polytron homogeniser. GK activity was determined as described [12].

Liver glycogen content was determined as previously described [13]. Briefly, frozen liver samples were homogenised with 10 volumes of 30% (w/v) KOH and boiled for 15 min. Glycogen was determined after ethanol precipitation. Statistical significance of differences was assessed by Student's *t*-test.

2.3. Immunofluorescence labelling

Starved and refed rats were anaesthetised by inhalation of ethyl ether and livers were then excised. Slices of livers were fixed in 3% paraformaldehyde in phosphate buffer 0.1 M (pH 7.4), cryoprotected for 10 h in 2.1 M sucrose, mounted on sample carriers and frozen in liquid nitrogen. Cryosections were obtained in a Reichert-Jung ultramicrotome equipped with an FL4 system for cryosectioning. For immunofluorescence labelling, sections were blocked with 20 mM glycine, 1% BSA in PBS (buffer A) for 10 min and incubated for 1 h at room temperature with a polyclonal antibody (kindly provided by Dr

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Abbreviations: GK, glucokinase; GKRP, glucokinase regulatory protein

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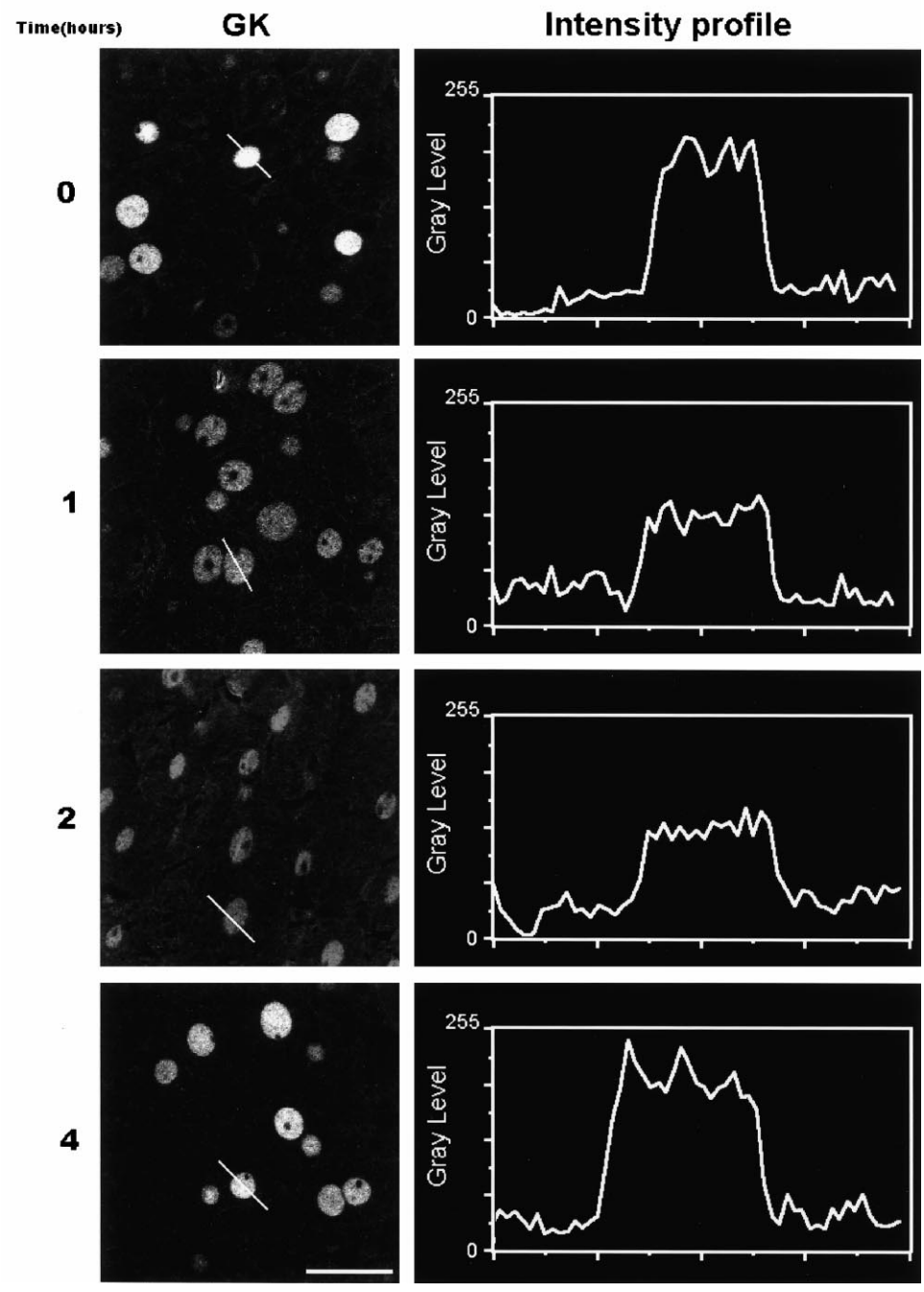

Fig. 1. Immunohistochemical localisation of GK in rat liver slices. Confocal images of livers from 24-h starved rats or from animals that had been fasted for 24 h and refed for 1, 2 or 4 h. Details of the procedure are described in Section 2. The intensity profiles along a random line across the cell are shown in the right column. Each confocal image is the average of four line scans at the standard scan rate. Scale bar = 10 μm.

M. Magnuson), raised in sheep against rat GK [14]. GKRP was detected using a polyclonal antibody (kindly provided by Dr E. Van Schaftingen), raised in rabbit [15]. After three washes in PBS, each for 10 min, sections were incubated for 45 min at room temperature with a secondary antibody anti-sheep FITC or anti-rabbit TRITC diluted in buffer A. After three washes in PBS, cryosections were mounted in hydrosoluble immunofluorescence medium (moviol).

2.4. Confocal microscopy

Confocal images were obtained with a Leica TCS 4D (Leica Lasertechnik GmbH, Heidelberg, Germany) confocal scanning laser microscope (CSLM) adapted to an inverted microscope (Leitz DMIRB). Images were taken using a 63× (NA 1.4 oil) Leitz Plan-Apochromatic or a 100× (NA 1.3) Leitz Plan Fluotar objective. For FITC (GK) and TRITC (GKRP) images, dual channel simultaneous acquisition exciting with the 488 and 568 nm lines of a krypton-argon laser were used. Image analysis and quantification were performed using the Metamorph analysis package (v. 3.5, Universal Imagining, PA, USA). The fluorescence density results of GK were calculated plotting random windows on the nucleus and cytoplasmic areas and dividing the total grey level values by the area (μm^2) [16]. Measurements were obtained from 15 cells for each condition of three independent experiments. Results are expressed as the ratio of nuclear to cytoplasmic fluorescence density. GK distribution is also shown as intensity profiles of grey levels, determined along a random line drawn across a cell in a single image.

3. Results and discussion

Livers from 24-h starved rats (control) or from animals that had been fasted for 24 h and refed for 1, 2 or 4 h were fixed and processed for laser confocal immunofluorescence detection of GK and GKRP, in order to study the subcellular distribution of these proteins in hepatic cells of animals at different nutritional conditions.

GK immunostaining was mainly detected in the hepatocyte nuclei of livers from starved animals (Fig. 1). After 1 h of refeeding, GK immunofluorescence in the nuclear compartment decreased and the cytoplasmic signal increased concomitantly. The nuclear GK signal was even weaker after 2 h of refeeding and the cytoplasmic GK signal had further increased. However, after 4 h of refeeding most GK immunostaining was found again in the nucleus. Intensity profiles along a random line drawn across a cell of each image clearly show the differences between the nucleus and the cytoplasm fluorescence intensities (Fig. 1). In order to quantify the extent of the translocation, the ratio of nuclear to cytosolic GK was calculated. This ratio was: 4.14, 2.58, 1.41 and 4.58, in starved rats and in 1-h, 2-h and 4-h refed animals, respectively. These changes were not due to variations in the total amount of GK, since GK activity measured in liver homogenates from starved and refed rats remained stable (Table 1).

The extent of GK translocation is not as extreme in vivo as reported in isolated hepatocytes, in which the nucleus can be completely depleted of GK [8]. This could be explained by the higher concentration of glucose used in the in vitro experiments, usually in the 20–30 mM range, compared to the levels attained in vivo. Serum glucose concentration was 6.5 ± 0.6 mM in starved rats, reached a maximum of 11.0 ± 0.7 mM after 2 h in refed rats, and decreased to 8.5 ± 0.6 mM after 4 h refeeding. Although in the refed condition, the glucose concentration in the portal vein may be somewhat higher [17], it is always lower than 20 mM. During this period there was also a marked increase in the amount of glycogen stored by the liver of these animals (Table 1).

In primary cultured hepatocytes, the glucose-induced translocation of GK out of the nucleus has been shown to be maximal after 2 h, and the return of GK to the nuclear compartment, upon lowering the concentration of glucose, to be essentially completed in 30 min [8]. Our results show that GK translocates from the nucleus to the cytosol in vivo and that there is a correlation between glycaemia and the distribution of GK in hepatic cells. In fasted conditions, when the blood glucose levels are low, GK is mainly localised to the nucleus. Upon refeeding, the blood glucose levels increase and GK moves partially to the cytoplasm. The maximum extent of translocation occurs after 2 h and coincides with the maximum level of glycaemia measured. Finally, when the blood glucose concentration decreases again, GK moves back to the nuclear compartment. Therefore, it appears that the level of blood glucose is fundamental in determining the in vivo intracellular distribution of hepatic GK.

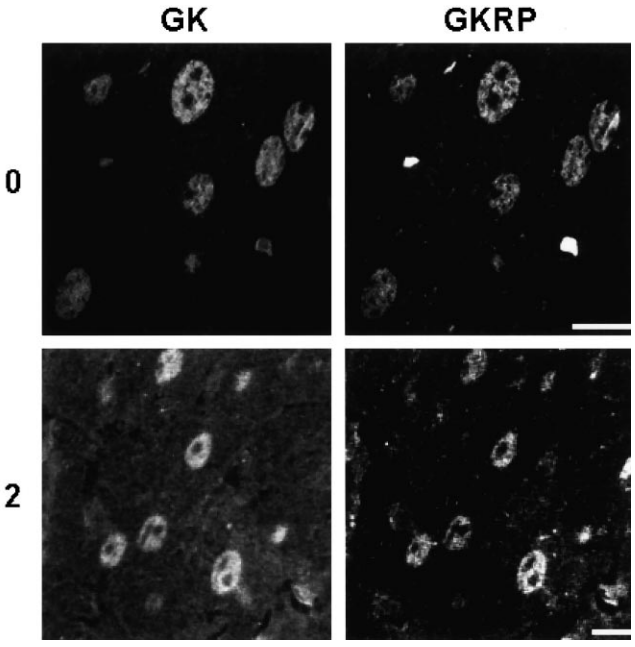

Fig. 2. Immunohistochemical localisation of GK and GKRP in rat liver slices. Confocal micrographs of livers from 24-h starved rats or from animals that had been fasted for 24 h and refed for 2 h. Each confocal image is the average of four line scans at the standard scan rate. Scale bar = 10 μm.

Immunostaining with antibodies against GKRP showed that this protein is mainly concentrated in the nuclear compartment, but a small fraction of the label appeared in the cytosol in all conditions examined (Fig. 2). Most importantly, the intracellular distribution of GKRP did not significantly change with the nutritional status of the animal, although small changes would remain undetected by the experimental approach followed. These results agree with what has been previously reported by us [18] and Brown et al. [8], using primary cultured hepatocytes. In this system, endogenous GKRP as well as GKRP fused to the green fluorescent protein always appeared concentrated in the nucleus, independently of the culture conditions, although a minor fraction of the protein was diffusely distributed throughout the cytosol. In contrast, Toyoda and collaborators [10] reported that in rat livers perfused with 5 mM glucose, GKRP was localised to the nucleus, but perfusion with 20 mM glucose led to the partial translocation of GKRP to the cytoplasm. However, in the same report [10], through Western blot analysis of subcellular fractions, GKRP was exclusively found in the nuclear fraction of normal-fed rat livers.

Table 1
Effects of refeeding on serum glucose, liver glycogen levels, and GK activity and subcellular distribution

State	Serum glucose (mM)	Glycogen (mg/g)	GK activity (U/g)	GK nucleus/cytosol ratio
Starved	6.5 ± 0.6	0.5 ± 0.1	4.2 ± 0.2	4.14 ± 0.28
1 h fed	10.2 ± 0.6*	7.2 ± 0.6*	4.2 ± 0.3	2.58 ± 0.23*
2 h fed	11.0 ± 0.7*	18.7 ± 1.2*	4.1 ± 0.2	1.41 ± 0.24*
4 h fed	8.5 ± 0.6*	34.8 ± 1.4*	4.3 ± 0.3	4.58 ± 0.40

Results are means ± S.E.M. for 7–9 independent experiments.
*$P < 0.001$ versus starved.

These contradictory observations about the behaviour of GKRP are difficult to explain. However, it has to be noted that Toyoda et al. observed the GKRP translocation in perfused livers [10], a preparation that is more prone to produce cellular hypoxia than the culture of hepatocytes in monolayers or the use of whole animals. Hypoxia causes a marked reduction in hepatic glucose metabolism and in this condition exogenous glucose is poorly utilised as an energy substrate by the liver [19,20]. It is tempting to speculate that hypoxia may lead to the translocation of GKRP from the nucleus to the cytosol, where it may remain bound to GK, thus inhibiting its activity in this compartment. Interestingly, fructose, which induces the rapid release of GK from GKRP and its translocation to the cytosol, greatly increases the rates of anaerobic glucose metabolism and protects the cell against anoxia-induced injury [20,21]. Furthermore, we have observed that in FTO2B cells, a hepatoma-derived cell line, GKRP is almost exclusively found in the cytosol (M. García-Rocha, J. Seoane and J.J. Guinovart, unpublished results). It is possible that GKRP may be continuously shuttling between the nucleus and the cytosol with an apparent constant distribution between these two compartments. Non-physiological or pathological states, such as anoxia or tumoural growth, could alter the rate of entrance to or exit from the nuclear compartment of GKRP, resulting in an anomalous cytosolic concentration of the protein.

The relevant role of GK in sensing the blood glucose levels has been recognised for a long time. A subtype of maturity-onset diabetes of the young has been shown to be associated with mutations of the GK gene [2]. Very recently, Magnuson and co-workers, through the use of cell-specific gene knockout mice, have demonstrated the important role of GK activity in the liver. Animals that are homozygous null for liver GK display defects in hepatic glucose turnover and also show impaired insulin secretion in response to glucose [22]. In this report, we provide for the first time evidence that the translocation of GK from and to the hepatocyte nucleus is physiologically relevant and occurs in the transition from the fasting to refed condition in live animals, while GKRP essentially remains in the nuclear compartment.

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