Regulation of liver glucokinase activity in rats with fructose-induced insulin resistance and impaired glucose and lipid metabolism

Flavio Francini, María C. Castro, Juan J. Gagliardino, and María L. Massa

Abstract: We evaluated the relative role of different regulatory mechanisms, particularly 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase (PFK2/FBPase-2), in liver glucokinase (GK) activity in intact animals with fructose-induced insulin resistance and impaired glucose and lipid metabolism. We measured blood glucose, triglyceride and insulin concentration, glucose tolerance, liver triglyceride content, GK activity, and GK and PFK2 protein and gene expression in fructose-rich diet (FRD) and control rats. After 3 weeks, FRD rats had significantly higher blood glucose, insulin and triglyceride levels, and liver triglyceride content, insulin resistance, and impaired glucose tolerance. FRD rats also had significantly higher GK activity in the cytosolic fraction (18.3 ± 0.35 vs. 11.27 ± 0.34 mU/mg protein). Differences in GK protein concentration (116% and 100%) were not significant, suggesting a potentially impaired GK translocation in FRD rats. Although *GK* transcription level was similar, *PFK2* gene expression and protein concentration were 4- and 5-fold higher in the cytosolic fraction of FRD animals. PFK2 immunological blockage significantly decreased GK activity in control and FRD rats; in the latter, this blockage decreased GK activity to control levels. Results suggest that increased liver GK activity might participate in the adaptative response to fructose overload to maintain glucose/triglyceride homeostasis in intact animals. Under these conditions, PFK2 increase would be the main enhancer of GK activity.

Key words: fructose-rich diet, liver glucokinase, liver 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase, insulin resistance, glucose/lipid homeostasis.

Résumé : Nous avons évalué durant 3 semaines le rôle relatif de divers mécanismes régulateurs de l'activité de la glucokinase (GK) hépatique, en particulier la 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase (PFK2/FBPase-2), chez des animaux intacts présentant une insulinorésistance induite par le fructose (FRD) et un métabolisme glucose/lipides défaillant. Nous avons mesuré la concentration de glucose sanguin, de triglycérides et d'insuline, la tolérance au glucose, la teneur en triglycérides et l'activité de la GK hépatiques, ainsi que l'expression génique et protéique de la GK et de la PFK2 chez des rats FRD et témoins. Les rats FRD ont eu des taux de glucose sanguin, d'insuline et de triglycérides, une teneur en triglycérides hépatiques, une résistance à l'insuline et un défaut de tolérance au glucose significativement plus élevés. Les rats FRD ont eu une activité GK nettement plus élevée dans la fraction cytosolique (18,3 ± 0,35 et 11,27 ± 0,34 mU/mg de protéine; $p < 0,05$). Les différences dans la concentration de la protéine GK n'ont pas été significatives (116 et 100 %), laissant croire à une translocation potentiellement défaillante de la GK chez les rats FRD. Le taux de transcription de la *GK* a été similaire, mais l'expression génique et la concentration protéique de la *PFK2* ont été d'un facteur 4 et 5 plus élevées dans la fraction cytosolique des rats FRD; le blocage immunologique de la PFK2 a diminué significativement l'activité de la GK chez les rats témoins et FRD; chez les FRD, ce blocage a diminué l'activité de la GK aux taux témoins. Les résultats donnent à penser qu'une augmentation de l'activité de la GK hépatique pourrait participer à la réponse adaptative à une surcharge de glucose afin de maintenir l'homéostasie glucose/triglycérides chez les animaux intacts; dans ces conditions, l'augmentation de la PFK2 serait le principal activateur de l'activité de la GK.

Mots-clés : diète riche en fructose, glucokinase hépatique, 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase hépatique, insulinorésistance, homéostasie glucose/lipides.

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Introduction

Liver glucokinase (GK) plays a pivotal role in glucose homeostasis under normal conditions, increasing or decreasing glucose output and uptake and thus helping to ensure an appropriate glucose provision to peripheral tissues (Iynedjian 2009).

Several extracellular signals and intracellular mechanisms constitute a complex net that controls GK activity to optimize its homeostatic function in a coordinated manner. In

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F. Francini,[1] M.C. Castro, J.J. Gagliardino, and M.L. Massa. CENEXA / Center for Experimental and Applied Endocrinology (UNLP-CONICET, PAHO/WHO Collaborating Center for Diabetes), National University of La Plata, School of Medicine, 1900 La Plata, Argentina.

[1]Corresponding author (e-mail: f_francini@yahoo.com).

the postprandial period, there is a rise in blood glucose levels accompanied by a similar increase in serum insulin levels, both of which induce an increase in GK activity due to the combination of increased protein production, changes in cellular compartmentation, and positive interaction with 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase (PFK2/FBPase-2) (Baltrusch and Tiedge 2006; Iynedjian 1993; Iynedjian 2009). Impairment of any of these regulatory components, particularly for a long period, can change and even reverse their effect upon GK activity. For example, in Zucker *fa/fa* rats—which portray insulin resistance and a modest increase in blood glucose concentration—the nucleus-to-cytoplasm GK translocation is refractory to glucose, and overexpression of the *PFK2* gene can correct such a defect (Payne et al. 2007). It has also been shown that hyperglycemia in Zucker diabetic rats results in an impaired regulation of liver GK activity (glucotoxic effect) (Fujimoto et al. 2006).

In the case of overt diabetes, glucose metabolism in the liver presents 3 major defects: inappropriately high fasting glucose production (DeFronzo et al. 1992; Magnusson et al. 1992; Puhakainen et al. 1992), lower glucose-induced suppression of endogenous glucose production (DeFronzo et al. 1992; Firth et al. 1986; Kelley et al. 1994), and impaired glucose uptake (Mitrakou et al. 1990). Evidence has shown the important role that changes in GK activity play in the pathogenesis of these defects: (*i*) animals with streptozotocin (STZ)-induced diabetes have a markedly decreased hepatic GK activity (Ferre et al. 1996; Iynedjian et al. 1988; Burcelin et al. 1992), (*ii*) liver-specific *GK*-knockout mice exhibit mild hyperglycemia and defective glycogen synthesis during the hyperglycemic clamp (Postic et al. 1999), (*iii*) *GK* mutations in humans causes maturity-onset diabetes of the young (MODY 2) (Byrne et al. 1994), and (*iv*) defective GK activity has been reported in a group of people with type 2 diabetes mellitus (T2DM) (Caro et al. 1995). Paradoxically, increased GK activity has been described in obese diabetic people (Belfiore et al. 1989) and in obese hyperinsulinemic Zucker rats (Huupponen et al. 1989). Moreover, long-term GK overproduction in the liver of normal mice induces insulin resistance, increased triglyceride levels, and impaired glucose tolerance (Ferre et al. 2003). On the basis of these data, it can be assumed that inadequate GK activity, rather than increasing or decreasing activity, is a main contributing factor in the abnormal liver glucose handling reported under these conditions.

Although this assumption is based on the available experimental evidence, most of this evidence was obtained using heterogeneous experimental models—human and animal studies, in vitro experiments, and settings with knockout or overexpressed genes—that are difficult to observe under nonexperimental conditions. Therefore, it is difficult to be assured that the described GK regulatory players can display identical effects in a single intact animal model. In this regard, many key components of the GK regulatory net of signals and intracellular mechanisms are altered in normal rats fed with a fructose-rich diet (FRD), namely, impaired liver glucose handling, glucose tolerance, and insulin resistance (Bizeau and Pagliassotti 2005). Moreover, we have recently reported changes in islet GK activity and regulation in FRD rats (Maiztegui et al. 2009). On the other hand, many authors suggest that the increased consumption of fructose observed in many societies is responsible for the world epidemics of obesity and diabetes (Bray et al. 2004; Elliott et al. 2002; Gross et al. 2004; Basciano et al. 2005). Consequently, the present study attempted to acquire (*i*) a deeper insight into the control mechanisms of liver GK activity in intact animals with altered glucose homeostasis induced by experimental conditions (FRD intake) that can seemingly occur in human beings, and (*ii*) evidence of the relative role played by different regulators of GK activity under this condition, particularly PFK2. Therefore, we measured GK activity in the liver of normal rats fed with a FRD for 3 weeks.

Materials and methods

Chemicals and drugs

Reagents of the purest available grade were obtained from Sigma Chemical (St. Louis, USA). GK antibody (sheep anti-GST – glucokinase fusion protein antibody) was kindly provided by Dr. Mark Magnusson, Vanderbilt University, Nashville, USA, and PFK2 polyclonal antibody (IgY-FBPase-2) by Prof. Sigurd Lenzen and Dr. Simone Baltrusch, Hannover Biomedical Research School, Hannover, Germany.

Animals

Normal male Wistar rats (150–180 g body weight) were maintained in a temperature-controlled room (23 °C) with a fixed 12 h light:dark cycle (0600–1800). Animals were housed and cared for according to institutional guidelines and approved protocols.

One group of 15 animals had free access to a standard commercial diet and tap water (control), and another group of 15 had access to the same diet but with 10% fructose in the drinking water (FRD). Treatment continued for 21 days. Water intake was measured daily, and individual body weight was recorded once a week.

After 21 days, all animals were fasted for 4 h and lightly anesthetized with halothane. Blood samples were drawn from the retroorbital plexus and collected into heparinized tubes to measure plasma glucose, triglyceride, and immunoreactive insulin levels. The animals were killed by decapitation and a portion of the liver was removed and processed as described below.

Plasma measurements

Glucose was measured with the glucose oxidase GOD-PAP method (Roche Diagnostics, Mannheim, Germany). Triglyceride levels were assayed enzymatically with a commercial kit implemented in an automated clinical analyzer. Immunoreactive insulin levels were determined by radioimmunoassay (RIA) (Herbert et al. 1965) using an antibody against rat insulin, rat insulin standard (Linco Research, USA), and highly purified porcine insulin labeled with ^{125}I (Linde et al. 1980). Serum insulin and fasting blood glucose values were used to estimate insulin resistance by the homeostasis model assessment (HOMA-R) index using the following formula: [serum insulin (μU/mL) × fasting blood glucose (mmol/L)] / 22.5 (Matthews et al. 1985).

Glucose tolerance

On day 20, glucose tolerance was measured in 12 h-fasted

rats after i.p. glucose injection (1 g/kg in saline). Blood samples were obtained from the orbital plexus at 0, 15, 30, 60, and 120 min under pentobarbital anesthesia (48 mg/kg). Results were expressed as the area under the glucose curve (AUC) in millimoles per litre (mmol/L) per minute.

Liver triglyceride content

Liver triglyceride extraction was performed following the protocol described by Schwartz and Wolins (2007), and the enzymatic assay of triglyceride levels was carried out with a commercial kit, as described for serum measurements.

Liver measurements

The liver portion removed from each animal was immediately suspended in ice-cold phosphate saline buffer containing 0.1 mmol/L PMSF, 0.1 mmol/L benzamidin, 2 mmol/L DTT, 4 μg/mL aprotinin and 0.3 mol/L sucrose adjusted to pH 7.5 and homogenized in a hand-held homogenizer (20 times). The homogenate was then passed through a 23-gauge needle syringe (5 times) to ensure appropriate sample mixing. Aliquots of these homogenates were centrifuged at 600*g* to separate the nuclear fraction. The supernatant was centrifuged twice at 8 000*g* and 100 000*g* at 4 °C (Lenzen et al. 1988*a*, 1988*b*), and the resulting supernatant was collected and identified as the cytosolic fraction (Lenzen et al. 1988*a*, 1988*b*; Jetton and Magnuson 1992; Davidson and Arion 1987). Thereafter, the nuclear fraction was resuspended and incubated for 12 min at 20 °C in a permeabilizating medium containing 150 mmol/L KCl, 3 mmol/L Hepes, 2 mmol/L DTT, and 0.04 mg/mL digitonin, pH 7.2, following a modification of the procedure described by de la Iglesia et al. (2000). At the end of this incubation, samples were centrifuged (600*g*) and the digitonin supernatant was removed and collected for further determinations. GK activity was finally measured in aliquots of both liver cytosolic fraction and digitonin supernatant.

Glucokinase assay

Rates of glucose phosphorylation in the 100 000*g*-soluble cytosolic fraction and the digitonin supernatant were measured at 37 °C, pH 7.4, by recording the increase in absorbance at 340 nm in a well-established enzyme-coupled photometric assay containing glucose-6-phosphate dehydrogenase, ATP, and NADP (Massa et al. 2004; Lenzen et al. 1988*a*, 1988*b*; Tiedge et al. 2000; Lenzen et al. 1987; Baltrusch et al. 2005). For each assay, 5 independent experiments were done in triplicate. GK activity was obtained by subtracting the activity measured at 1 mmol/L glucose (hexokinase) from that measured at 100 mmol/L glucose. These concentrations were selected after fitting different curves obtained using a range of glucose concentrations (3.1, 6.25, 12.5, 25, 50, 100, and 200 mmol/L). The $S_{0.5}$ and Hill coefficient values for GK were calculated from Hill plots (Baltrusch et al. 2005; Cornish-Bowden et al. 1979). GK activity was also measured after addition of increasing concentrations of a PFK2-specific antibody to the cytosolic fraction with a procedure previously validated by Massa et al. (2004), as described in Figure 3. Nonimmune serum was used as negative control.

The ratio of nuclear to cytosolic GK was calculated by dividing the activity measured in the digitonin supernatant by that of cytosolic fraction.

Enzyme activities were expressed as milliunits per milligram (mU/mg) of protein. One unit of enzyme activity was defined as 1 μmol glucose-6-phosphate formed from glucose and ATP per minute at 37 °C.

Isolation of total RNA

Total liver RNA was isolated from FRD and control rats using Trizol reagent (Gibco, Rockville, USA) (Chomczynski and Sacchi 1987). The integrity of isolated RNA was checked by 1% agarose-formaldehyde gel electrophoresis. Possible contamination with protein or phenol was tested by measuring the 260/280 nm absorbance ratio, while DNA contamination was avoided using DNAase I digestion (Gibco). Reverse transcription (RT)-PCR was performed using the SuperScript III (Gibco) and total RNA (50 ng) from FRD and control liver as a template.

Analysis of gene expression by real-time quantitative PCR

Real-time quantitative PCR (qPCR) was performed with a MiniOpticon real-time PCR detector (BioRad), using SYBR Green I as a fluorescent dye that binds only double-stranded DNA. A 10 ng aliquot of cDNA was amplified in a 25 μL qPCR mixture containing 0.6 μmol/L final concentrations of each primer (3 mmol/L $MgCl_2$, 0.3 mmol/L dNTPs, and 0.2 μL of a 6 U/μL Platinum *Taq* DNA polymerase (Invitrogen)). Samples were first denatured at 94 °C for 3 min followed by 40 PCR cycles. Each cycle comprised a melting step at 94 °C for 30 s, an annealing step at 63 °C for 45 s, and an extension step at 72 °C for 30 s, followed by a final extension at 72 °C for 10 min. The optimal parameters for the PCRs were empirically defined. Each PCR amplification was performed in triplicate. The following oligonucleotide primers (Invitrogen) were used: *β-actin* gene (GenBank accession No. NM019130), 5′-AGAGGGAAATCGTGCGTGAC-3′ and 5′-CGATAGTGATGACCTGACCGT-3′; *GK* gene (GenBank accession No. NM012565), 5′-GTGTACAA GCTGCACCCGA-3′ and 5′-CAGCATGCAAGCC TTC TTG-3′; *PFK2* liver isoform gene (GenBank accession No. Y00702), 5′-CGATCTATCTACCTATGCCGCCAT-3′ and 5′-ACACCCGCATCAATCTCATTCA-3′. All amplicons were designed in a size range of 90 to 250 bp. β-Actin was used as a housekeeping gene. SYBR Green fluorescence emission was determined after each cycle. The purity and specificity of the amplified PCR products were verified by melting curves generated at the end of each PCR. Product length and PCR specificity were further checked by 2% (*w*/*v*) agarose gel electrophoresis and ethidium bromide staining. Data were expressed as relative gene expression after normalization to the β-actin housekeeping gene using Qgene96 and LinRegPCR software (Muller et al. 2002).

Western blot analysis

Immunodetection of GK and PFK2 was performed on the cytosolic fraction. Protein concentration was quantified by the Bio-Rad protein assay (Bradford 1976). Thereafter, dithiothreitol and bromophenol blue were added to a final concentration of 100 mmol/L and 0.1%, respectively. Aliquots of the cytosolic fraction containing 20 μg for GK and

100 μg for PFK2 of whole protein were placed in reducing 10% SDS-PAGE and electroblotted to polyvinylidene difluoride membranes. The amount of protein loaded was confirmed by the Bradford (1976) method, and the uniformity of protein loading in each lane was assessed by staining the blot with Ponceau S. Nonspecific binding sites of the membranes were blocked by overnight incubation with nonfat dry milk at 4 °C. Enzyme identification and quantification were performed using specific primary antibodies against GK (final dilution of 1:2000) (Tiedge et al. 1997) for 90 min or PFK2 antibody (final dilution of 1:10 000) (Massa et al. 2004) for 2 h. At the end of the incubation period, the membranes were rinsed in Tris-buffered saline and further incubated for 1 h with the corresponding secondary antibody: anti-sheep IgG streptavidin – horseradish peroxidase conjugate or anti-chicken IgY peroxidase-labeled, respectively. Diaminobenzidine (DAB, Sigma) was used for colour development. Finally, the bands were quantified by densitometry using the Gel-Pro Analyzer software.

Statistical analysis

Data were expressed as means ± SE unless specifically stated otherwise. Statistical analyses were performed using ANOVA followed by Dunnett's test for multiple comparisons or *t* test for correlations using the Prism analysis program (GraphPad, San Diego, USA). Differences were considered significant when $p < 0.05$.

Results

Body weight and water intake

Similar body weights were recorded in FRD (294.2 ± 5.4 g) and control animals (289.4 ± 14.6 g) after the 3-week period. FRD animals drank a larger volume of water than controls (41.6 ± 2.5 vs. 31.8 ± 3.1 mL/day, $p < 0.02$). Conversely, control rats ate a significantly larger amount of solid food than FRD rats (21.2 ± 1.4 vs. 15.5 ± 1.8 g/animal per day, $p < 0.001$). Consequently, while the daily intake of nutrients (percentage) was different in both experimental groups (control vs. FRD, carbohydrates:proteins:lipids, 45:43:12 vs. 60:31:9, respectively), they had a comparable intake of calories (control 55.6 ± 4.5 kcal/day, FRD 50.8 ± 3.4 kcal/day).

Blood glucose, serum insulin, and triglyceride levels

FRD rats evinced significantly higher plasma glucose (8.3 ± 0.2 vs. 7.2 ± 0.3 mmol/L, $p < 0.05$), insulin (4.7 ± 0.6 vs. 2.7 ± 0.5 ng/mL, $p < 0.02$) and triglyceride (1.3 ± 0.1 vs. 0.8 ± 0.1 mmol/L, $p < 0.001$) concentrations than control rats. Although higher, plasma glucose levels in FRD were still within normal range.

The higher insulin:glucose molar ratio measured in FRD rats (0.57 ± 0.04 vs. 0.37 ± 0.02, $p < 0.001$) and the HOMA-R index (43 ± 3 vs. 26 ± 2, $p < 0.001$) demonstrate the existence of an insulin-resistant state in these rats.

Glucose tolerance

Plasma glucose AUC values after i.p. glucose administration were significantly higher in FRD than control rats (4.31 ± 0.5 vs. 1.57 ± 0.63 mmol/L per minute, $p < 0.01$).

Liver triglyceride content

Triglyceride content was higher in FRD rats compared with control animals (756.2 ± 43.1 vs. 549.5 ± 17.6 μg/100 mg tissue, $p < 0.001$).

Glucokinase activity

GK activity measured in the liver cytosolic fraction of control animals showed a V_{max} of 11.3 ± 0.3 mU/mg protein, an $S_{0.5}$ of 6.0 ± 0.6 mmol/L, and a Hill coefficient of 2.0 ± 0.2 for glucose. These values were all comparable with those reported by other authors (Lenzen et al. 1988*a*, 1988*b*; Agius et al. 1996; Fernández-Novell et al. 1999). FRD rats had significantly higher GK activity in the cytosolic fraction (V_{max} 18.3 ± 0.4 mU/mg protein, $p < 0.05$) than controls, while no significant differences between groups were recorded in $S_{0.5}$ (6.7 ± 0.7 mmol/L) and Hill coefficient (1.9 ± 0.2) values. Conversely, GK activity in the digitonin supernatant of controls was significantly higher (V_{max} 22.6 ± 0.7 vs. 9.15 ± 0.3 mU/mg protein, $p < 0.001$) than that of FRD animals. Consequently, the ratio of nuclear to cytosolic GK activity was 2 for controls and 0.5 for FRD rats.

qPCR

FRD rats showed a significant increase in the level of *PFK2* relative gene expression (385.6% ± 42.3% vs. 100% ± 24.3%, $p < 0.001$). In contrast, no significant differences were measured in *GK* gene expression level (control 100% ± 22.3%, FRD 77.5% ± 17.7%). In both cases, values were expressed as a percentage of the housekeeping β-actin gene (Figs. 1A and 1B).

Western blot analysis

Western blots performed in samples of the cytosolic fraction from control and FRD animals using specific GK and PFK2 antibodies showed single bands of about 50 and 55 kDa, respectively, compatible with the molecular weight of the corresponding enzymes. The intensity of the specific bands increased as a function of the protein concentration used, supporting the reliability and specificity of the immune measurements (data not shown).

GK protein production in the cytosolic fraction of FRD animals (116% ± 14.7%) was higher but not significantly different from that measured in control animals (100% ± 14.0%) (Figs. 2A and 2C).

The intensity of the PFK2 band measured in the cytosolic fraction was markedly higher in FRD than control rats (500 ± 22.9 vs. 100 ± 2.2%, $p < 0.001$) (Figs. 2B and 2D).

Effect of PFK2 antibody on GK enzyme activity

GK activity was measured in the cytosolic fraction from both control and FRD animals in the presence of different concentrations of a specific PFK2 antibody raised against the FBPase-2 domain. GK activity decreased significantly as a function of the antibody concentration in the media (Fig. 3). This inhibition was not observed when nonimmune serum was used as a negative control (data not shown).

Discussion

This study found that FRD-fed rats presented insulin re-

Fig. 1. *GK* (A) and *PFK2* (B) gene expression in liver homogenates from control and FRD animals. Values are means ± SE of the relative gene expression of 5 independent experiments run in triplicate. **, significant at $p < 0.001$. GK, glucokinase; PFK2, 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase; C, control; FRD, fructose-rich diet.

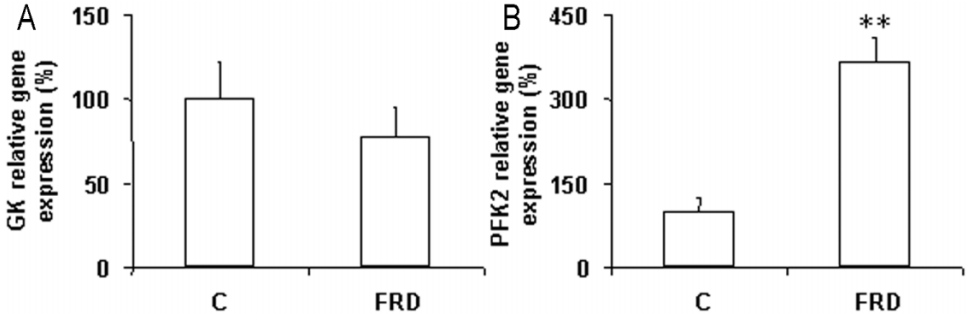
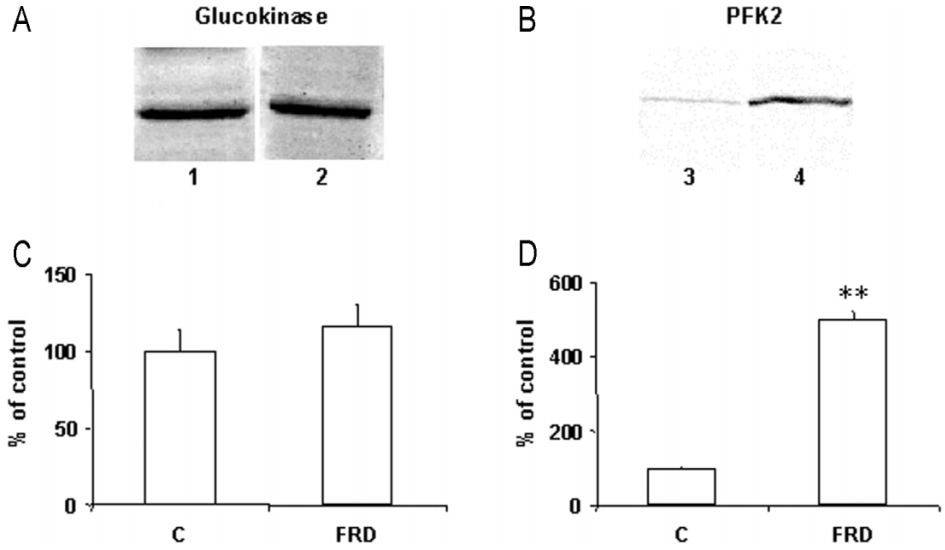


Fig. 2. GK (A) and PFK2 (B) protein production in liver cytosolic fraction from control (lines 1 and 3) and FRD (lines 2 and 4) rats. GK (C) and PFK2 (D) band intensities were measured in control and FRD rats. Representative blots of 5 independent experiments are shown. **, significant at $p < 0.001$. GK, glucokinase; PFK2, 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase; C, control; FRD, fructose-rich diet.



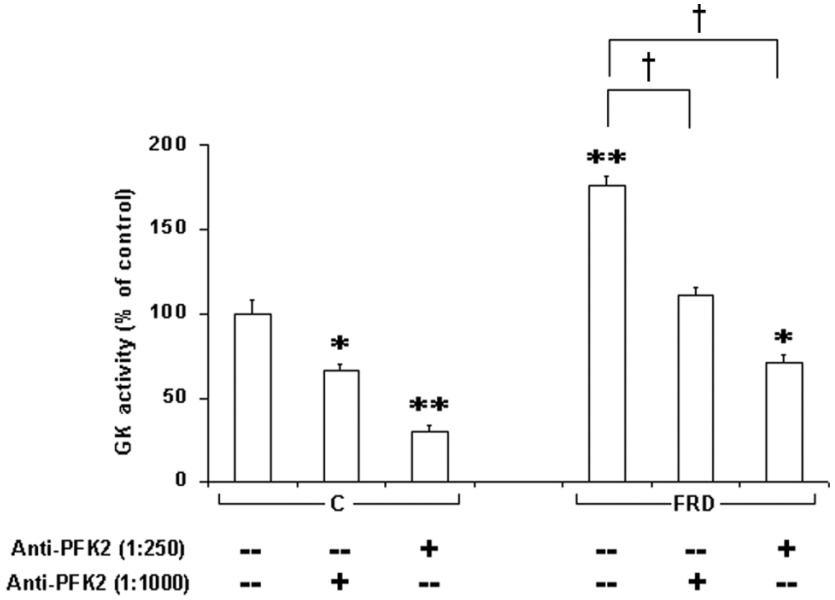
sistance (demonstrated by hyperinsulinemia with normoglycemia, high insulin:glucose molar ratio, and HOMA-R index), significantly increased serum triglyceride levels, and impaired glucose tolerance. These results are consistent with findings previously reported by Thorburn et al. (1989); Rebolledo et al. (2008); and Maiztegui et al. (2009) using this model.

Fructose is metabolized in the liver (Hallfrisch 1990), the main organ responsible for the conversion of excess dietary carbohydrates into triglycerides. Fructose is metabolized to pyruvate, which is either oxidized to provide energy or channelled into pathways for the synthesis of fatty acids which are thereafter incorporated into triglycerides. In the latter process, glycerol-3-phosphate acyltransferase (GPAT) catalyzes the first committed step in phospholipid and triglyceride synthesis. The importance of GPAT is supported by the fact that overproduction of this enzyme in rat hepatocytes diverts fatty acyl coenzymes away from β-oxidation and toward triglyceride synthesis (Lindén et al. 2004; Lewin et al. 2005). In our study, FRD rats showed an increase in both liver triglyceride content and GPAT activity (data not shown). The oxidative stress present in FRD-fed rats (Rebolledo et al. 2008; Alzamendi et al. 2009) would contribute to this shift of carbohydrates towards lipid metabolism and to the development of insulin resistance (Brownlee 2005).

GK activity was significantly higher in the liver cytosolic fraction of FRD rats as a result of a significant increase in its V_{max} without changes in either the $S_{0.5}$ for glucose or the Hill coefficient.

The lower ratio of nuclear to cytosolic GK activity measured in FRD rats (0.5 vs. 2.0 in controls) shows an uneven enzyme compartmentation and could apparently explain the higher GK activity measured in these rats. In cultured hepatocytes, high levels of fructose cause a parallel and dose-dependent increase in fructose 1-phosphate formation and glucokinase translocation (Niculescu et al. 1997). Thus 3 weeks of FRD intake would be expected to produce a similar increase in liver fructose 1-phosphate concentration and a consequent increase in translocation of GK from the nucleus to cytosol. In our FRD rats, however, the high and significant GK activity increment measured in the cytosolic fraction—the active form of the enzyme (Van Schaftingen et al. 1994)—was not accompanied by a proportional increase in the amount of enzyme protein. Since under normal

Fig. 3. Effect of a PFK2-specific antibody on GK enzyme activity in liver homogenates of FRD rats. Homogenates were incubated for 1 h in the presence of the PFK2 antibody at the final dilutions shown. Enzyme activity was measured spectrophotometrically as described in Methods. Data are expressed as the percentages of enzyme activity measured in control animals in the absence of PFK2 antibody and at 100 mmol/L glucose. Results are means ± SE from 5 independent experiments run in triplicate. *, significant at $p < 0.01$ and **, $p < 0.001$ compared with untreated control; †, $p < 0.001$ compared with untreated FRD. Absolute values for control and FRD animals without the antibody were 11.43 ± 0.88 and 20.1 ± 0.6 mU/mg protein, respectively.



conditions insulin has a dramatic effect on hepatocyte *GK* gene expression (Iynedjian 2009), the insulin-resistant state and the lack of increase of *GK* mRNA in FRD rats may indicate an impaired transcription process. On the basis of these data, we can assume that (*i*) although still operating, nuclear–cytosolic translocation of GK protein in FRD animals is not fully effective, similar to the situation in Zucker diabetic fatty rats (Fujimoto et al. 2004), and (*ii*) the increased GK activity measured in FRD rats could be partly due to its interaction with an enhancing cytosolic partner.

In the cytoplasm, GK may interact with binding partners other than glucokinase regulatory protein, such as PFK2. Payne et al. (2005, 2007) have reported that overexpression of the *PFK2* gene increased GK translocation from the nucleus to the cytoplasm in a dose-dependent manner, and also corrected its refractoriness to glucose effect. PFK2 positively and complementarily interacts with GK in insulin-producing cells (Baltrusch et al. 2001), and this interaction potentially stabilizes the GK catalytic state resulting in an increase of the enzyme activity due to an increase in its V_{max} with no changes in the $S_{0.5}$ value for glucose (Massa et al. 2004). The current data show all these changes: we recorded a significant increase of *PFK2* mRNA (approximately 4-fold) and its protein (5-fold) in the liver of FRD rats. Further, incubation of the cytosolic fraction with a specific anti-PFK2 antibody raised against the FBPase-2 domain, which is directly involved in GK–PFK2 interaction (Massa et al. 2004), significantly decreased GK activity (60%–70%). The absence of such inhibition when the samples were incubated with nonimmune serum suggests that the antibody inhibition was a specific rather than a nonspecific or general effect. Interestingly, this immunological blockage affects GK activity in both control and FRD rats, thus suggesting that PFK2 plays a specific enhancing role on GK activity under both normal and pathological conditions. The larger quantity of PFK2 protein measured in FRD rats can explain why after immunological blockage (obtained with a fixed antibody dilution) the remaining GK activity was significantly higher in these rats than in controls.

Liver PFK2/FBPase-2 activity is modulated by a phosphorylation/dephosphorylation process (Okar et al. 2001). Promotion of its phosphorylation by forskolin blocks GK activation, thus suggesting that a high FBPase-2 activity of the phosphorylated liver PFK2/FBPase-2 may significantly affect its binding to GK through an unfavourable conformation of the PFK2/FBPase-2 protein. The cellular stoichometry between the phosphorylated and dephosphorylated PFK2/FBPase-2 enzyme may be crucial for GK activation (Massa et al. 2004). To date, however, we do not know whether in our model this shift from the dephosphorylated to the phosphorylated isoform occurs, thus affecting its binding to and activation of GK.

Given these results, we can suggest that the enhanced GK activity measured in the FRD rats results from an eventually impaired, although still operating, enzyme traslocation, and is mainly caused by an interaction of GK with the increased PFK2 rather than by an increase in GK protein concentration. Experimental evidence supports this assumption because (*i*) liver GK protein interacts in vitro with the liver PFK2 isoform (Baltrusch et al. 2001), and (*ii*) overproduction of PFK2 in insulin-producing RINm5F-GK and rat insulinoma (INS-1) cells stimulates GK activity without promoting measurable changes in its protein levels (Massa et al. 2004).

The current data demonstrate that the FRD-induced in-

crease in PFK2 liver concentration mimics the results previously obtained in PFK2-transgenic mice. In our case, the increase in hepatic PFK2 protein levels and GK activity would represent an adaptative response to the state of insulin resistance and impaired glucose and lipid metabolism induced by a fructose overload that serves to maintain glucose homeostasis. This assumption is supported by the fact that in mice with STZ-induced diabetes, overproduction of liver PFK2 isoform reduced hepatic glucose production by enhancing glycolysis and inhibiting gluconeogenesis, thereby lowering blood glucose (Wu et al. 2001). Further, we observed that in FRD rats, there is a significant increase in glycogen deposits as well as in glucose-6-phosphatase activity; this latter change would lead to an increased rate of the futile cycle (F. Francini, M.C. Castro, G. Schinella, M.E. García, B. Maiztegui, M.A. Raschia, J.J. Gagliardino, and M.L. Massa, manuscript submitted for publication). Although these changes have probably prevented a more marked elevation of blood glucose levels in FRD rats, they were not sufficient to maintain normal glucose homeostasis, as shown by the appearance of impaired glucose tolerance.

Altogether, these data show for the first time that the regulatory mechanisms of GK activity associated with *PFK2* overexpression and PFK2 overproduction reported in vitro or in culture of primary hepatocytes are fully operative in normal intact animals. The data also support the concept that inadequate, rather than simply increasing or decreasing, GK activity may be a main contributing factor to the abnormal liver glucose handling and overall glucose homeostasis.

The knowledge gained from the in vivo GK–PFK2 interaction may help us to understand the process for upregulation of GK under pathological conditions (i.e., insulin-resistance and impaired glucose and triglyceride metabolism) and to find new prospective therapeutic strategies to activate or preserve the glucose sensor function of the enzyme in T2DM (Grimsby et al. 2003; Brocklehurst et al. 2004; Matschinsky et al. 2006). It may further point out the need for increased public awareness of the risks associated with high fructose consumption and the importance of reducing supplementation of packaged foods with high fructose additives (Basciano et al. 2005).

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