The contributions of glucokinase and phosphofructokinase-2/fructose bisphosphatase-2 to the elevated glycolysis in hepatocytes from Zucker fa/fa rats

Victoria A. Payne¹, Catherine Arden¹, Alex J. Lange² and Loranne Agius¹#

¹Institute of Cellular Medicine, Newcastle University, Newcastle upon Tyne, NE2 4HH, UK
²Department of Biochemistry, Molecular Biology and Biophysics, University of Minnesota, Minneapolis, MN55455, USA

Running head: Control of glucose metabolism by glucokinase and PFK2

(#) Contact information:
Institute of Cellular Medicine,
The Medical School,
Leech Bld Level 4,
Newcastle University,
Newcastle upon Tyne, NE2 4HH, UK
Tel. 044-191-2227033
Fax. 044-191-2220723
E-mail Loranne.Agius@ncl.ac.uk

Abbreviations
Ad, adenoviral vector; GKRP, glucokinase regulatory protein; fructose 2,6-P₂, fructose-2,6-bisphosphate; glucose 6-P, glucose 6-phosphate; N/C, nuclear / cytoplasmic ratio; PFK2, 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase; PFK2-M, PFK2-S32A/H258A; PFK2-W, wild-type PFK2.
ABSTRACT

The insulin-resistant Zucker fa/fa rat has elevated hepatic glycolysis and activities of glucokinase and phosphofructokinase-2/fructose bisphosphatase-2 (PFK2). The latter catalyses the formation and degradation of fructose 2,6-P₂ and is a glucokinase-binding protein. The contributions of glucokinase and PFK2 to the elevated glycolysis in fa/fa hepatocytes were determined by overexpressing these enzymes individually or in combination. Metabolic Control Analysis was used to determine enzyme coefficients on glycolysis and metabolite concentrations. Glucokinase had a high control coefficient on glycolysis in all hormonal conditions tested whereas PFK2 had significant control only in the presence of glucagon, which phosphorylates PFK2 and suppresses glycolysis. Despite the high control strength of glucokinase the elevated glycolysis in fa/fa hepatocytes could not be explained by the elevated glucokinase activity alone. In hepatocytes from fa/fa rats, glucokinase translocation between the nucleus and the cytoplasm was refractory to glucose but responsive to glucagon. Expression of a kinase-active PFK2 variant reversed the glucagon effect on glucokinase translocation and glucose phosphorylation confirming the role for PFK2 in sequestering glucokinase in the cytoplasm. Glucokinase had a high control on glucose 6-phosphate content, but like PFK2 it had a relative modest effect on the fructose 2,6-P₂ content. However combined overexpression of glucokinase and PFK2 had a synergistic effect on fructose 2,6-P₂ levels suggesting that interaction of these enzymes may be a pre-requisite for formation of fructose 2,6-P₂. Cumulatively, this study provides support for co-ordinate roles for glucokinase and PFK2 in the elevated hepatic glycolysis in fa/fa rats.

Key words: Liver, glucose metabolism, glycolysis, fructose 2,6-bisphosphate
INTRODUCTION

6-Phosphofructo-2-kinase/fructose-2,6-bisphosphatase (PFK2), is a bifunctional enzyme that catalyses both the formation of fructose 2,6-P₂ and its degradation (26,27,35). Fructose 2,6-P₂ is a regulator of glycolysis because it is a potent activator of phosphofructokinase-1 and inhibitor of fructose 1,6-bisphosphatase-1. Various tissue specific isoforms of PFK2 encoded by four genes are expressed in mammals. They differ in their relative kinase and bisphosphatase activities and also in their regulatory mechanisms (36). The liver isoform is regulated by phosphorylation of a serine residue at the N-terminus (ser-32) by cAMP-dependent protein kinase which leads to an increase in the bisphosphatase / kinase activity ratio. This mechanism accounts for the lowering of fructose 2,6-P₂ and inhibition of glycolysis caused by glucagon (26).

Recent studies by Baltrusch and co-workers showed that PFK2 binds to glucokinase through the bisphosphatase domain (8). Putative roles for this heterodimeric interaction have been proposed for both the hepatocyte and pancreatic β-cell which expresses different isoforms of PFK2 (8,31,34). In pancreatic β-cells binding of PFK2 activates glucokinase by post-translational mechanisms (31), whereas in hepatocytes PFK2 has a dual role in regulating glucokinase expression and its subcellular compartmentation (34). Hepatic glucokinase shuttles between the cytoplasm and the nucleus depending on the substrate and hormonal conditions (1,13). At low glucose, glucokinase is sequestered in the nucleus, through high-affinity binding to a 68kDa protein (GKRP) that is both a specific inhibitor for glucokinase and a nuclear receptor (13,42). Binding of glucokinase to GKRP is counteracted by fructose 1-P and accordingly precursors of fructose 1-P cause translocation of glucokinase from the nucleus to the cytoplasm (2,33). Translocation is also induced by elevated glucose concentrations by a mechanism that is synergistic with the effects of fructose 1-P (4). Glucagon has the reverse effect and induces translocation of glucokinase from the cytoplasm to the nucleus (2,13,34). The role of PFK2 in mediating this effect of glucagon was shown by expression of a PFK2 variant lacking ser-32 that functions as a constitutively active kinase (34). This PFK2 variant when expressed at high levels, reverses the inhibitory effects of glucagon on glycolysis, fructose 2,6-P₂ and glucokinase translocation indicating a role
for either PFK2 protein or its product fructose 2,6-P$_2$ in mediating the effects of glucagon. It remains as yet undetermined whether retention of glucokinase in the cytoplasm at elevated glucose or low cAMP levels is mediated by binding of glucokinase to the dephosphorylated form of PFK2 or to independent cytoplasmic receptors through fructose 2,6-P$_2$-dependent mechanisms (34).

Translocation of glucokinase between the nucleus and the cytoplasm has been studied in isolated hepatocytes during incubation with various substrates (2,4,33) and in vivo after fasting and refeeding (19) or after a glucose and insulin infusion (14). Defects in glucokinase translocation have been reported in animal models of Type 2 diabetes including the Goto-Kakizaki rat and the OLETF rat (41) and the Zucker diabetic fatty rat (20,21). The Zucker diabetic rat had impaired glucokinase translocation during a glucose and insulin infusion (21). The mechanistic defect that accounts for the impaired translocation in these models of Type-2 diabetes is not known. A tentative hypothesis is that metabolic conditions associated with the diabetic or insulin-resistant state may alter the interaction of glucokinase with either nuclear or cytoplasmic receptors. In this study we tested the hypothesis that PFK2 is a possible candidate for the impaired glucokinase translocation in a model of insulin resistance and type 2 diabetes.

The hepatic activity of PFK2 is elevated in the insulin-resistant Zucker fa/fa rat (30) which is homozygous for a mutation in the leptin receptor gene. This model is characterised by hyperphagia, hyperinsulinaemia and mild hyperglycaemia and is considered a good model for insulin resistance and type 2 diabetes (40). The hepatic defect in this animal model is associated with increased partitioning of glucose 6-P towards glycolysis and lipogenesis as opposed to glycogenesis and with various enzyme abnormalities including elevated activities of glucokinase and glycolytic enzymes (5,6,39). In this study we determined the contributions of glucokinase and PFK2 to the elevated glycolysis in this model by overexpressing these enzymes individually or in combination. We used Metabolic Control Analysis to determine the sensitivity of glycolytic flux to changes in activity of these enzymes. We tested the role of PFK2 in the control of glucokinase translocation in this model of insulin resistance.
MATERIALS AND METHODS

Hepatocyte isolation: Male Zucker Fa/?, and fa/fa rats aged 11-13 weeks (body wt: Fa/?, 311 ± 5; fa/fa 461 ± 10 g) were obtained either from AstraZeneca, Alderley Park, Cheshire or from Harlan Olac, Bicester, UK. They were housed under standard conditions and fed ad libitum. All experiments were carried out in accordance with EC Council Directive (86/609/EEC). Hepatocytes were isolated by collagenase perfusion of the liver (34) and suspended in Minimum Essential Medium containing 5% newborn calf serum and seeded in multiwell plates at a cell density of 8 x 10^4 cells / cm^2.

Hepatocyte monolayer culture and enzyme overexpression: After cell attachment (2 h) the medium was replaced by serum-free Minimum Essential medium containing adenoviral vectors for overexpression of rat liver glucokinase (Ad-LGK), the wild-type liver isoform of PFK2 (Ad-PFK2-W) or a kinase-active double mutant (S32A/H258A) variant of PFK2 (Ad-PFK2-M) described previously (7,10). After a further 2 h, the medium was replaced by serum-free MEM containing 10 nM dexamethasone and 5 mM glucose and the cells were cultured for 18 h to allow for enzyme expression (34).

Metabolic incubations: For determination of rates of glucose phosphorylation or glycolysis the hepatocyte monolayers were incubated for 3 h in MEM containing either [2-3H]glucose or [3-3H]glucose and the concentrations of glucose indicated without or with 100 nM glucagon or 10 nM insulin. Rates of glucose metabolism were linear during 3 h (16). At the end of the incubation, the medium was collected for determination of 3H2O and lactate (16). For determination of fructose 2,6-P2, the hepatocyte monolayer was extracted in 0.1 M NaOH and the plates were heated for 5 min at 80 C. Fructose 2,6-P2 was determined as described previously (43). For determination of glucose 6-P the plates were snap-frozen in liquid nitrogen and glucose 6-P was determined fluorimetrically in neutralised perchlorate extracts (25).

Enzyme analysis: Glucokinase (free and bound) was determined spectrometrically after permeabilisation of hepatocyte monolayers with digitonin (3) and free
glucokinase activity is expressed as a percentage of total activity. Total activity was expressed as munits / mg protein, where 1 munit represents the amount converting one nmol of substrate per min. PFK2 activity was determined from the rate of conversion of fructose 6-P to fructose 2,6-P₂ during a 15 min incubation as in (9) and activity is expressed as pmol/min per mg (34).

**Metabolic Control Analysis:** Flux control coefficients of glucokinase or PFK2 on glucose metabolism, which are a measure of the fractional change in flux that results from a fractional change in enzyme activity (18,28), were determined from experiments with titrated glucokinase or PFK2 overexpression using adenoviral vectors as described previously (16). The control coefficients were determined from the initial slopes of the double log plots of rates of glucose metabolism (glucose phosphorylation or glycolysis) against the total activity for glucokinase or PFK2, respectively. Concentration control coefficients of glucokinase on glucose 6-P or fructose 2,6-P₂, representing the fractional change in cellular metabolite content that results from a fractional change in enzyme activity (28), were determined from experiments with titrated glucokinase overexpression from the slope of the double log plot of metabolite content against enzyme activity (16,25). The latter does not take into account possible subcellular compartmentation of these metabolites.

**Western Blotting:** Immunoreactivity to glucokinase and PFK2 was determined by Western Blotting using an antibody against human recombinant liver glucokinase (a kind gift from K. Brocklehurst, AstraZeneca, Macclesfield, Cheshire, UK) and an antibody to the bisphosphatase domain of rat liver PFK2 raised in chicken (31,34).

**Immunofluorescence staining:** After incubation of the hepatocyte monolayers on coverslips with the conditions indicated they were rinsed in phosphate-buffered saline (PBS) and fixed in 4% paraformaldehyde/PBS (12). They were treated with NaBH₄, pre-blocked and stained as described previously (32). Antibody dilutions were: glucokinase (1:100 dilution, rabbit IgG against human glucokinase residues 318-405, Santa Cruz) and PFK2 (1:50 dilution, chicken IgG against the bisphosphatase domain (31). The secondary antibodies (donkey: anti rabbit or anti-chicken IgG, Jackson Immunoresearch) were FITC-labelled (12). The cells were imaged using a Nikon Eclipse E400 epifluorescence microscope with narrow-band filters for FITC (B-2EC)
and Nikon DXM1200 digital camera. Three representative fields were selected for each condition comprising between 50 and 90 nuclei and the mean pixel intensity of the nuclei and cytoplasmic areas was analysed from Gray images using Lucia G/F Analysis Software. For each incubation condition the mean value for the nuclei and cytoplasm was determined and the results expressed as a nuclear / cytoplasmic ratio. Expression of results as a ratio corrects for drifts in fluorescence intensity (32).

RESULTS

Increased expression of glucokinase and PFK2 in fa/fa hepatocytes
Hepatocytes from Zucker fatty fa/fa rats have altered activities of various enzymes involved in glucose metabolism (39). In this study we confirmed that hepatocytes from fa/fa rats had a higher (P < 0.05) activity of glucokinase compared with Fa/? controls (10.6 ± 0.8 vs 6.0 ± 0.6 munits/mg, means ± SE, n=12). They also had a higher immunoreactivity to glucokinase (1.47 ± 0.04 vs 1 relative densitometry units, n=6) and to PFK2 (1.81 ± 0.04 vs 1 relative densitometry units, n=6).

Control of metabolic flux and metabolite concentrations by glucokinase
To determine the contribution of glucokinase to the metabolic defect, hepatocytes from fa/fa and Fa/? rats were treated with four titres of recombinant adenovirus (Ad-LGK) to achieve titrated glucokinase overexpression by between 2 and 7 fold above endogenous activity. The flux control coefficients of glucokinase on glucose phosphorylation and glycolysis were determined from the slopes of double log plots of flux against total glucokinase activity (Fig. 1A) and are summarised in Table 1. Hepatocytes from fa/fa rats had higher (46-68%, P < 0.05) flux control coefficients of glucokinase on glucose phosphorylation (determined from metabolism of [2-3H]glucose) and on glycolysis, determined both from metabolism of [3-3H]glucose and formation of lactate than Fa/? controls (Table 1). However, the concentration control coefficients of glucokinase on glucose 6-P and fructose 2,6-P2 (determined from double log plots of metabolite content against glucokinase activity) were similar in fa/fa compared with Fa/? hepatocytes (Fig. 1B,Table 1). The concentration control coefficients of glucokinase on fructose 2,6-P2 were much lower than on glucose 6-P.
Correlation between elevated glycolysis and fructose 2,6-P₂

The divergence in slope of metabolic flux against glucokinase activity (Fig. 1A) indicates that the higher rates of glycolysis in hepatocytes from fa/fa rats cannot be explained by the elevated glucokinase activity alone. In addition, the higher rates of glycolysis in fa/fa hepatocytes also cannot be explained by a higher glucose 6-P content (Fig. 2A). However, they showed an apparent correlation (Fa/?, r, 0.97, P < 0.01; fa/fa r 0.97, P < 0.01) with the cell content of fructose 2,6-P₂ (Fig. 2B), suggesting a possible contribution of either fructose 2,6-P₂ or the dephosphorylated form of PFK2 protein.

Sub-cellular location of glucokinase and effects of glucose and glucagon

The flux control coefficient of glucokinase on glucose metabolism is markedly dependent on its interaction with binding proteins (16,25). The higher control coefficient of glucokinase on glycolysis in hepatocytes from fa/fa rats compared with controls (Table 1) could be due to differences in sub-cellular location and/or interaction with binding proteins. In hepatocytes from Fa/? rats, the N/C distribution of glucokinase was lower (P < 0.05) at 25 mM than 10 mM glucose and it was increased (P < 0.05) by glucagon at 25 mM glucose (Fig. 3), in agreement with previous findings on hepatocytes from Wistar rats (34). In fa/fa hepatocytes there was no significant effect of glucose (25mM vs 10mM) on the sub-cellular distribution of glucokinase. However, glucagon increased the N/C ratio at both 10mM (P < 0.005) and 25 mM glucose (P < 0.005). This shows that hepatocytes from fa/fa rats are refractory to glucose but responsive to glucagon at both low and high glucose concentration. Hepatocytes from fa/fa rats incubated at 10 mM glucose also had a higher free glucokinase activity (determined by the digitonin release assay) compared with Fa/? controls (49 ± 2 vs 43 ±2 free GK % total activity) as shown previously (5), indicating a greater distribution of glucokinase in the cytoplasm at low glucose.

Expression of PFK2-M counteracts the effect of glucagon on translocation

Since PFK2 mediates the effects of glucagon on the nuclear / cytoplasmic (N/C) distribution of glucokinase (34) we determined the N/C distribution of glucokinase in hepatocytes after pre-treatment without or with a recombinant virus for expression of a kinase-active PFK2 variant (PFK2-M: S32A,H258A). Expression of PFK2-M by
3-fold relative to endogenous PFK2 activity, counteracted the effects of glucagon on translocation in hepatocytes from both fa/fa and Fa/? rats (Fig. 3) consistent with a role for PFK2 in mediating the effects of glucagon on glucokinase translocation in hepatocytes from fa/fa rats.

**Regulation of glucose metabolism by glucagon: role of PFK2**

Because hepatocytes from fa/fa and Fa/? rats showed differences in glucose-induced but not glucagon-induced glucokinase translocation (Fig. 3) we evaluated the effects of glucagon and of expression of PFK2-M by 3-fold relative to endogenous activity on glucose metabolism. Rates of glucose phosphorylation and glycolysis were determined at 10 mM glucose in similar incubation conditions as in Fig. 3. In hepatocytes from both fa/fa and Fa/? rats glucagon inhibited glycolysis by 42-45% and it caused a small but significant suppression of glucose phosphorylation (Fig. 4A,B). The latter is consistent with the translocation of glucokinase to the nucleus with glucagon (Fig. 3). Expression of PFK2-M by 3-fold relative to endogenous activity reversed the inhibition of glucose phosphorylation caused by glucagon (Fig. 4A) and it caused partial reversal (42-45 to 29-30 %) of the inhibition of glycolysis (Fig. 4B) but did not increase the fructose 2,6-P2 content (Fig. 4C). The latter is consistent with previous findings that expression of PFK2-M by about 10-fold above endogenous activity is necessary to counteract the suppression of fructose 2,6-P2 caused by glucagon (34). The counteraction by PFK2-M of the inhibition of glucose phosphorylation by glucagon despite sustained suppression of fructose 2,6-P2 implicates a role for PFK2 protein as distinct from fructose 2,6-P2 in reversing the effects of glucagon on glucose phosphorylation and subcellular location.

**Flux control coefficients of PFK2 on glucose metabolism**

In incubations with glucagon, the flux control coefficients of PFK2 on glucose phosphorylation (fa/fa: 0.18 ± 0.05 vs Fa/?: 0.14 ±0.05) and glycolysis (fa/fa: 0.36 ± 0.10 vs Fa/?: 0.38 ±0.05) were lower than the control coefficients for glucokinase (glucose phosphorylation: 0.52 ± 0.07 and 0.39 ±0.03; glycolysis, 0.89 ± 0.14 and 0.68 ±0.08). The latter coefficients of glucokinase were significantly higher (< P < 0.05) than when flux was measured in the absence of glucagon (0.69 and 0.44, Table 1).
Effects of combined expression of glucokinase and PFK2-W on fructose 2,6-P₂

To determine the control of fructose 2,6-P₂ concentration by glucokinase and PFK2 we overexpressed glucokinase and wild-type-PFK2 separately and in combination by treatment with recombinant adenoviruses Ad-LGK and Ad-PFK2-W. Expression of either glucokinase or PFK2 alone by 5-fold above endogenous levels had small effects (< 50% increase) on the fructose 2,6-P₂ content at both 10 mM glucose (Fig. 5A) and at 25 mM glucose (results not shown). In contrast, combined expression of glucokinase and PFK2 had a greater than additive effect on fructose 2,6-P₂ levels which were increased by 3-fold and 4-fold in fa/fa and Fa/⁻ hepatoocytes, respectively (Fig.5A). Similar synergism was observed in incubations with 25 mM glucose (results not shown).

During combined treatment with Ad-LGK and Ad-PFK2-W, glucokinase was overexpressed by 10-12 fold compared with 4-5 fold during treatment with Ad-LGK alone because PFK2 protein potentiates glucokinase expression (34). To normalise for differences in glucokinase activity between treatments with Ad-LGK alone or with additional Ad-PFK2, the effects of glucokinase on fructose 2,6-P₂ were expressed as concentration control coefficients (as in Table 1). During combined PFK2 and glucokinase overexpression the concentration control coefficients for glucokinase were greater (P < 0.01) than in cells overexpressing glucokinase alone in both Fa/⁻ (10mM glucose: 0.38 ± 0.04 vs 0.28 ±0.05; 25mM glucose: 0.55 ± 0.06 vs 0.16 ±0.05) and fa/fa hepatoocytes (10mM glucose: 0.48 ± 0.05 vs 0.27 ±0.07; 25mM glucose: 0.33 ± 0.04 vs 0.18 ±0.054) confirming synergistic effects by combined enzyme overexpression.

Glucokinase overexpression overrides the inhibition of glycolysis by glucagon

Overexpression of PFK2-W (wild-type) by 6-fold had a negligible effect (< 15%) on glycolysis (Fig. 5A), similar to the lack of effect of expression of a kinase-active variant (PFK2-M) in the absence of glucagon (Fig 4A). However, overexpression of glucokinase (4-5 fold) increased glycolysis by 2-3 fold both in the absence and presence of glucagon (Fig. 5B), even though it did not over-ride the suppression of fructose 2,6-P₂ caused by glucagon (Fig. 5A). The stimulation of glycolysis caused
by glucokinase overexpression correlated with the rate of glucose phosphorylation (Fig. 5C).

**DISCUSSION**

The Zucker fa/fa rat which is homozygous for a mutation in the leptin receptor gene and is widely used as a model for insulin resistance and type 2 diabetes is characterised by high rates of glycolysis and lipogenesis and elevated activities of glucokinase and PFK2 (30,39). In this study we determined the contributions of these enzymes to the elevated glycolysis in hepatocytes from fa/fa rats by overexpressing glucokinase and PFK2 either individually or in combination. Three main findings emerged from this study. First, that glucokinase had a much higher control strength on glycolysis than PFK2 in all experimental conditions tested, to the extent that glucokinase overexpression can over-ride the inhibition of glycolysis caused by glucagon, despite sustained suppression of fructose 2,6-P2. Secondly, that the elevated activity of glucokinase alone cannot explain the higher rate of glycolysis in hepatocytes from fa/fa compared with control rats indicating the involvement of additional mechanisms which together impart a higher control strength of glucokinase on glycolysis in fa/fa hepatocytes. Thirdly, that the cell content of fructose 2,6-P2 in liver cells is co-ordinately controlled by glucokinase and PFK2.

**Major but not exclusive role for glucokinase in the control of glycolysis**

Titrated glucokinase overexpression by up to 7-fold above endogenous levels resulted in a progressive increase in glycolysis both when this was determined in the absence of hormones and also in the presence of insulin or glucagon. These hormones affect the phosphorylation state of PFK2 and thereby the kinase / bisphosphatase ratio (26,27). This is reflected by changes in fructose 2,6-P2 which was increased by insulin and decreased by glucagon. Overexpression of PFK2 as either a kinase-active variant (Fig. 4) or the wild-type enzyme (Fig. 5) did not significantly increase glycolysis when this was determined in the absence of glucagon. However, the kinase-active variant PFK2-M, increased glycolysis in the presence of glucagon. This indicates that the endogenous level of PFK2 sustains near-maximal rates of glycolysis when it is in its dephosphorylated state (high kinase / bisphosphatase ratio). In these conditions control of glycolysis resides predominantly
at glucokinase. However, in the presence of glucagon when PFK2 is phosphorylated control is shared between glucokinase and PFK2. It is noteworthy, that glucokinase overexpression increased glycolysis in the presence of glucagon, despite sustained suppression of fructose 2,6-P2 content, indicating the over-riding role of glucose phosphorylation in controlling glycolysis in conditions of suppressed fructose 2,6-P2 but elevated glucose phosphorylation.

Despite the high control strength of glucokinase on glycolysis, the elevated rate of glycolysis in hepatocytes from fa/ fa rats cannot be explained by the elevated activity of glucokinase alone. This is supported by the upward shift in the correlation between glycolysis and glucokinase activity in hepatocytes from fa/ fa rats compared with controls (Fig. 1A). The higher control strength of glucokinase on glycolysis in fa/ fa hepatocytes (represented by the slope of the double log plot in Fig. 1A), also indicates involvement of additional factors that act synergistically with glucokinase in fa/ fa hepatocytes.

**Impaired glucose-induced glucokinase translocation in fa/ fa hepatocytes**

Previous studies have shown that glucose-induced translocation of glucokinase from the nucleus to the cytoplasm is impaired in models of insulin resistance and diabetes such as the Goto Kakizaki, OLETF (41) and ZDF-rats (20,21). However, the underlying mechanisms have not been determined. We show in this study that glucokinase translocation in hepatocytes from fa/ fa rats was refractory to glucose (25 mM vs 10mM) but responsive to glucagon at both 25 mM and 10 mM glucose. The refractoriness to glucose could be due to either impaired translocation at 25 mM glucose or to increased accumulation of glucokinase in the cytoplasm at 10 mM glucose. We cannot unequivocally exclude the former possibility. However, the increase in free glucokinase activity at 10mM glucose, in fa/ fa hepatocytes compared with controls supports the latter hypothesis. A higher cell content of the dephosphorylated form of PFK2 in hepatocytes from fa/ fa rats, as suggested by the higher cell content of fructose 2,6-P2 could explain the sequestration of glucokinase in the cytoplasm at low glucose.

**Expression of a kinase-active PFK2 variant counteracts glucagon-induced glucokinase translocation independently of fructose 2,6-P2**
Previous work showed that when a kinase-active variant of PFK2 (lacking ser-32 and His-258) is expressed by 10-fold excess over endogenous PFK2 activity it reverses the suppression of fructose 2,6-P$_2$ caused by glucagon (34). The requirement for such a high level of expression of this variant was tentatively explained by the high activity of the endogenous bisphosphatase at saturating glucagon concentration (34). This study shows that expression of the kinase-active PFK2 variant by 3-fold relative to endogenous enzyme, had negligible effect on the cell content of fructose 2,6-P$_2$ particularly in hepatocytes from fa/fa rats (Fig. 4C), but nonetheless reversed both the translocation of glucokinase from the cytoplasm to the nucleus and the suppression of glucose phosphorylation caused by glucagon (Fig. 4A). This supports a role for PFK2 protein as distinct from its product fructose 2,6-P$_2$ in controlling glucokinase location and glucose phosphorylation. At this level of PFK2 expression there was only partial reversal of the inhibition of glycolysis by glucagon, consistent with the dual control of glycolysis by the rate of glucose phosphorylation (determined by glucokinase) and by allosteric activation of phosphofructokinase-1 by fructose 2,6-P$_2$.

**Synergistic control of fructose 2,6-P$_2$ by glucokinase and PFK2**

Overexpression of glucokinase had a large effect on the cell content of glucose 6-P in agreement with previous findings (25,37) but it had a comparatively modest effect on fructose 2,6-P$_2$ levels as shown by the lower concentration control coefficients (0.3 vs 1.1). This indicates that neither glucose 6-P, nor fructose 6-P which is in equilibrium with glucose 6-P, are major determinants of the cell content of fructose 2,6-P$_2$. Expression of PFK2 like expression of glucokinase also had a modest effect on fructose 2,6-P$_2$ levels (Fig. 5A). However, combined overexpression of glucokinase and PFK2 had a synergistic effect on fructose 2,6-P$_2$ levels. This is supported by the higher concentration control coefficient of glucokinase on fructose 2,6-P$_2$ when PFK2 was overexpressed. This clearly establishes co-ordinate roles of glucokinase and PFK2 in regulating the fructose 2,6-P$_2$ content. A tentative hypothesis is that in the absence of phosphorylation of ser-32, glucokinase can bind to PFK2 and facilitate formation of fructose 2,6-P$_2$ through either inhibition of the bisphosphatase, since glucokinase binds to the bisphosphatase domain (8) or through channelling involving phosphoglucoisomerase. A further possibility is that glucokinase may increase the kinase activity of PFK2 as suggested
from a study on liver homogenates (38). The higher cell content of fructose 2,6-P$_2$
with titrated glucokinase overexpression in hepatocytes from fa/fa compared with
Fa/? controls (Fig. 1B) is consistent with the higher expression of PFK2 in fa/fa
hepatocytes and with co-ordinate regulation by glucokinase and PFK2.
Glucokinase translocates from the nucleus to the cytoplasm in response to a rise in
extracellular glucose concentration in the absorptive state (1,14,19). Based on the
present and previous findings (34) it is proposed that glucokinase binding to the
dephosphorylated form of PFK2 through the bisphosphatase domain (8), sequesters
glucokinase in the cytoplasm. As glucose absorption from the gut declines, the rise
in glucagon results in phosphorylation of PFK2 (35), dissociation of glucokinase and
translocation to the nucleus. Pathophysiological states associated with increased
expression of PFK2 or with its dephosphorylation would favour sequestration of
glucokinase in the cytoplasm and increased glycolysis.

ACKNOWLEDGEMENTS
The work was supported by Project Grant support from the Wellcome Trust and
equipment grants from the Medical Research Council (JREI, G0100348) and Diabetes
UK (RD01/0002364) to LA and by NIH RO1-38354 to AJL.
REFERENCES


LEGENDS TO FIGURES

Fig. 1. Effects of glucokinase overexpression on glucose metabolism and metabolite concentrations in hepatocytes from fa/fa and Fa/? rats. Hepatocytes from Fa/? (open symbols) and fa/fa (filled symbols) rats were treated with 4 titres of Ad-LGK for titrated glucokinase overexpression. After 18 h culture they were incubated for 3 h in medium with 10 mM glucose for determination of glucose phosphorylation, glycolysis, glucose 6-P and fructose 2,6-P2. Rates of glucose metabolism (A) and concentrations of metabolites (B) are expressed as double log plots relative to the respective glucokinase activity. Values are means for 5-6 experiments. The flux control coefficients and concentration control coefficients, determined from the slopes of the double log plots from the individual 5 experiments are summarised in Table 1.

Fig. 2. Glycolysis correlates with the cell content of fructose 2,6-P2. Data are from the experiments in Fig. 1 and Table 1. A. Glycolysis vs glucose 6-P. B. Glycolysis vs fructose 2,6-P2. Open symbols, Fa/?; closed symbols, fa/fa.

Fig. 3. Glucagon but not glucose causes translocation of glucokinase in hepatocytes from fa/fa rats. Hepatocytes from Fa/? (open bars) and fa/fa (filled bars) rats were either untreated or treated with Ad-PFK2-M and then cultured for 18 h. They were then incubated for 3 h with 10 mM or 25 mM glucose without or with 10 nM glucagon and fixed and stained for glucokinase. The nuclear / cytoplasmic (N/C) ratio for glucokinase was determined as described in the Methods Section. Results are means ± SE for 5 experiments, * P < 0.05; ** P < 0.005 presence vs absence of glucagon; + P < 0.05; ++ P < 0.01 25 mM vs 10 mM glucose.

Fig. 4. Expression of PFK2-M counteracts the inhibition of glucose phosphorylation caused by glucagon. Hepatocytes from fa/fa and Fa/? rats were either untreated or treated with Ad-PFK2-M for expression of PFK2-M by 2.5 fold relative to endogenous activity. After 18 h culture they were incubated for 3 h in medium with 10mM glucose without or with 10 nM insulin (Ins) or 10 nM glucagon (Glu) for determination of glucose phosphorylation (A), glycolysis (B) and fructose 2,6-P2 (C).
Results are means ± SE for 6 experiments. # P < 0.05, ## P < 0.005 presence vs absence of glucagon or insulin; * P < 0.05 effect of PFK2-M expression.

Fig. 5. Synergistic effects of combined overexpression of glucokinase and PFK2-W on hepatocyte fructose 2,6-P_2 content. Hepatocytes from fa/fa and Fa/? rats were either untreated (C) or treated with Ad-LGK (GK) and/or Ad-PFK2-W (W). After 18 h culture they were incubated for 3 h in medium with 10 mM glucose in either the absence (open bars) or presence (closed bars) of 10 nM glucagon for determination of fructose 2,6-P_2 (A), glycolysis (B) and glucose phosphorylation. C. Glycolysis vs glucose phosphorylation. Results are means ± SEM for 4 experiments, * P < 0.05; ** P < 0.005 relative to corresponding untreated controls; ## P < 0.005 relative to treatment with Ad-LGK (GK) or Ad-PFK2-W (W) alone.
Table 1. *Flux control coefficients and concentration control coefficients of glucokinase.*

<table>
<thead>
<tr>
<th></th>
<th>Hepatocytes</th>
<th>Fa/?</th>
<th>fa/fa</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Flux control coefficients</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose phosphorylation</td>
<td>0.30 ± 0.03</td>
<td>0.44 ± 0.02 *</td>
<td></td>
</tr>
<tr>
<td>Glycolysis ([3-3H]glucose)</td>
<td>0.44 ± 0.05</td>
<td>0.69 ± 0.08 *</td>
<td></td>
</tr>
<tr>
<td>Lactate formation</td>
<td>0.26 ± 0.02</td>
<td>0.55 ± 0.09 *</td>
<td></td>
</tr>
<tr>
<td><strong>Concentration control coefficients</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose 6-P</td>
<td>1.12 ± 0.10</td>
<td>1.09 ± 0.12</td>
<td></td>
</tr>
<tr>
<td>Fructose 2,6-P₂</td>
<td>0.27 ± 0.04</td>
<td>0.31 ± 0.06</td>
<td></td>
</tr>
</tbody>
</table>

For experimental details see legend to Figure 1. Flux control coefficients were determined from the slope of double log plots of rates of glucose phosphorylation (detritiation of [2-3H]glucose), glycolysis (detritiation of [3-3H]glucose) or lactate formation against glucokinase activity (Fig. 1A). Concentration control coefficients were determined from the slope of double log plots of glucose 6-P or fructose 2,6-P₂ against glucokinase activity (Fig. 1B). Values and means ± SE for 5-6 hepatocyte preparations. * P < 0.05 relative to Fa/?
Fig. 1. Effects of glucokinase overexpression on glucose metabolism and metabolite concentrations in hepatocytes from fa/fa and Fa/? rats.
Fig. 1. Effects of glucokinase overexpression on glucose metabolism and metabolite concentrations in hepatocytes from fa/fa and Fa/? rats.
Fig. 2. Glycolysis correlates with the cell content of fructose 2,6-P2.

71×78mm (300 x 300 DPI)
Fig. 2. Glycolysis correlates with the cell content of fructose 2,6-P2.

Log Glycolysis

Log Fructose 2,6-P₂

Fig. 2. Glycolysis correlates with the cell content of fructose 2,6-P₂.
70x71mm (300 x 300 DPI)
Fig. 3. Glucagon but not glucose causes translocation of glucokinase in hepatocytes from fa/fa rats.

91x50mm (300 x 300 DPI)
Fig. 4. Expression of PFK2-M counteracts the inhibition of glucose phosphorylation caused by glucagon.
Fig. 4. Expression of PFK2-M counteracts the inhibition of glucose phosphorylation caused by glucagon.

92x70mm (300 x 300 DPI)
Fig. 4. Expression of PFK2-M counteracts the inhibition of glucose phosphorylation caused by glucagon.
Fig. 5. Synergistic effects of combined overexpression of glucokinase and PFK2-W on hepatocyte fructose 2,6-P2 content.

93x77mm (300 x 300 DPI)
Fig. 5. Synergistic effects of combined overexpression of glucokinase and PFK2-W on hepatocyte fructose 2,6-P2 content.

93x68mm (300 x 300 DPI)
Fig. 5. Synergistic effects of combined overexpression of glucokinase and PFK2-W on hepatocyte fructose 2,6-P2 content.

97x71mm (300 x 300 DPI)