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Version-2**A role for PFK-2/FBPase-2 as distinct from fructose 2,6-bisphosphate in regulation of insulin secretion in pancreatic beta-cells**

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Abbreviations

Ad, adenoviral vector; AICAR, 5-aminoimidazole-4-carboxamide 1-beta-d-ribofuranoside; AMPK, AMP-activated protein kinase; fructose 2,6-P₂, fructose-2,6-bisphosphate; glucose 6-P, glucose 6-phosphate; PFK-2/FBPase-2, 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase; PFK-1, phosphofructokinase-1; KA, kinase activr; KD, kinase deficient; ZMP, 5-aminoimidazole-4-carboxamide 1-beta-d-ribofuranotide.

6-Phosphofructo 2-kinase/fructose 2,6-bisphosphatase (PFK-2/FBPase-2) catalyses the formation and degradation of fructose 2,6-bisphosphate and is also a glucokinase-binding protein. The role of fructose 2,6-bisphosphate in regulating glucose metabolism and insulin secretion in pancreatic beta-cells is unresolved. We down regulated the endogenous isoforms of PFK-2/FBPase-2 with siRNA and expressed kinase-active and kinase-deficient variants to distinguish between the role of PFK-2/FBPase-2 protein as distinct from its product fructose 2,6-bisphosphate in regulating beta-cell function. Human islets expressed the PFKFB2 and PFKFB3 isoforms and mouse islets expressed PFKFB2 at mRNA level (RT-PCR). Rat islets expressed PFKFB2 lacking the C-terminal phosphorylation sites. The glucose-responsive MIN6 and INS1E cell lines expressed PFKFB2 and PFKFB3. PFK-2 activity and the cell content of fructose 2,6-bisphosphate were increased by elevated glucose concentration and during pharmacological activation of AMP-activated protein kinase which also increased insulin secretion. Partial down-regulation of endogenous PFKFB2 and PFKFB3 in INS1E by siRNA decreased PFK-2/FBPase-2 protein, fructose 2,6-bisphosphate content, glucokinase activity and glucose-induced insulin secretion. Selective down-regulation of glucose-induced fructose 2,6-bisphosphate in the absence of downregulation of PFK-2/FBPase-2 protein, using a kinase-deficient PFK-2/FBPase-2 variant resulted in sustained glycolysis and elevated glucose-induced insulin secretion indicating an over-riding role of PFK-2/FBPase-2 protein, as distinct from its product fructose 2,6-bisphosphate, in potentiating glucose-induced insulin secretion. Whereas down-regulation of PFK-2/FBPase-2 decreased glucokinase activity, overexpression of PFK-2/FBPase-2 only affected glucokinase distribution. It is concluded that PFK-2/FBPase-2 protein rather than its product fructose 2,6-bisphosphate is the over-riding determinant of glucose-induced insulin secretion through regulation of glucokinase activity or subcellular targeting.

INTRODUCTION

6-Phosphofructo 2-kinase/fructose 2,6-bisphosphatase (PFK-2/FBPase-2) is a bifunctional enzyme that catalyses the synthesis and the degradation of fructose 2,6- P_2 , a potent allosteric activator of the glycolytic enzyme, phosphofructokinase-1 (PFK-1) [1,2]. The N-terminal half acts as a kinase to catalyse the ATP-dependent phosphorylation of fructose 6-phosphate to fructose 2,6- P_2 whilst the C-terminal half acts as a bisphosphatase [2]. An unequivocal role for fructose 2,6- P_2 as a regulator of glycolysis is established for a number of tissues including liver and heart [1,3,4], but whether fructose 2,6- P_2 has a physiological role in control of glycolysis and insulin secretion in pancreatic islets remains unclear [5-7].

There are 4 isozymes of PFK-2/FBPase-2 designated liver, heart, brain and testis, encoded by genes PFKFB 1 to 4, respectively [2]. Differential splicing of these genes gives rise to isoforms that share conservation of the catalytic core but differ in the length of the N and C-terminal regions that contain phosphorylation sites that regulate the kinase or bisphosphatase activity. The PFKFB1 gene encodes the liver and skeletal muscle isoforms, which differ in the length of the N-terminus. The liver isoform is regulated by phosphorylation of ser-32 by cAMP-dependent protein kinase, which causes inhibition of the kinase and activation of the bisphosphatase, resulting in depletion of fructose 2,6- P_2 and suppression of glycolysis [8]. Dephosphorylation of P-ser-32 is catalysed by a type-2A phosphatase that is activated by xylulose 5-P. The increase in fructose 2,6- P_2 caused by elevated glucose is explained by an increase in xylulose 5-P which activates the phosphatase and dephosphorylates PFK-2/FBPase-2 [9]. The muscle isoform lacks the first 32 residues and is not regulated by cAMP [2]. The heart-type isoform encoded by PFKFB2 and the brain/inducible isoform encoded by PFKFB3 are activated (increase in kinase/ bisphosphatase activity) by phosphorylation of Ser-466 (heart) or Ser-461 (brain) by AMP-activated protein kinase resulting in increased fructose 2,6- P_2 [10-12]. The heart isoform also has additional phosphorylation sites at the N-terminus (ser-94) and C-terminus (ser-475, ser-483) that can be phosphorylated by protein kinase C, calmodulin-dependent kinases and kinases activated by insulin and growth factor signalling [2]. Various splice variants have been reported for both PFKFB2

and PFKFB3 in human and rat tissue which differ in the C-terminal domain and therefore in their regulatory properties [13-16].

Two independent studies on rat pancreatic islets have reported the expression of an isoform of the PFKFB2 gene that does not contain the Ser-466 phosphorylation site [17,18]. The more recent study by Baltrusch and colleagues also reported the presence of a novel splice variant of PFKFB2, although as a minor form [18]. Malaisse and colleagues reported that glucose caused a large increase in fructose 2,6- P_2 in rat islets similar to the increment in hepatocytes except that the effect was more rapid [5,6]. Since islets do not express the liver isoform of PFK-2/FBPase-2, the glucose effect has been suggested to be due to an increase in the concentration of 6-phosphogluconate, which inhibits the biphosphatase activity [17]. A later study on rat islets, reported much more modest effects of glucose on fructose 2,6- P_2 [7].

Work by Baltrusch and colleagues identified PFK-2/FBPase-2 as a glucokinase binding protein [18] and subsequent work in beta-cells focused on the possible role of PFK-2/FBPase-2 in regulating glucokinase activity [19,20]. However, the question whether fructose 2,6- P_2 has a role in pancreatic beta-cells in control of glucose metabolism and insulin secretion through either activation of PFK-1 or other mechanisms as reported for other tissues [21] has not been addressed.

In this study we investigated the role of endogenous PFK-2/FBPase-2 in control of insulin secretion by use of si-RNA to down-regulate the endogenous isoforms and we used kinase-active and kinase-deficient variants to distinguish between a role for fructose 2,6- P_2 as distinct from a non-catalytic role for PFK-2/FBPase-2 protein in regulating beta cell function.

EXPERIMENTAL

Isolation of pancreatic islets Pancreatic islets were isolated from male C57/BL6 mice (age 12-16 weeks) and male Wistar rats (body weight 180-220g). The pancreas was injected with 1.5mg/ml liberase (Roche) in Ringer Phosphate Buffer (10mM Hepes (pH 7.4), 90mM NaCl, 5mM NaHCO₃, 4.8mM KCl, 0.7mM KH₂PO₄, 0.6mM MgSO₄ 2.5mM CaCl₂, 0.1% BSA, 5.5mM glucose) and shaken in the same medium for 6 min with further digestion with 0.75mg/ml liberase for 2-6min. After washing in enzyme-free buffer and islets were picked and mRNA extracted from 50 (rat) or

100 (mouse) islets using micro-spin columns (Qiagen). Human islets were isolated from pancreases retrieved from heart-beating deceased human donors following ethical approval and informed consent from donor relatives. Cold ischemic time was < 9 h. Islets were isolated at King's College Islet Isolation Facility, London [22] and transported to Newcastle University in Islet Transport Medium (CMRL 1066-Supplemented, (Cellgro, Herndon, VA) containing 5% human serum albumin). Islet viability was > 90% and purity > 70%. Islet RNA was extracted using GeneElute Mammalian Total RNA extraction kit (Sigma-Alrich) and RNasin(r) Plus RNase Inhibitor (Promega) to prevent RNA degradation.

Cell lines and hepatocyte culture MIN6 (p20-27) and INS1E (p110-153) were cultured as described previously [23]. Hepatocytes were isolated from male Wistar rats and were suspended in MEM containing 5% (v/v) newborn calf serum (Invitrogen) and seeded in multi-well plates [24].

Treatment with adenoviral vectors Adenoviral vectors for expression of kinase-deficient (KD) and kinase-active (KA) variants of PFK-2/FBPase-2 and glucokinase were described previously [21,25]. Adenoviral stocks were prepared by replication in 293 cells and tested for their effects on enzyme activity (glucokinase) or fructose 2,6-P₂ (PFK-2/FBPase-2 variants). They were then used at titres that altered glucokinase or fructose 2,6-P₂ by up to 4 fold relative to endogenous levels. Cells were treated with adenoviral vectors (2-2.5 fold increments) at either 2 h (hepatocytes) or 24 h (MIN6) after plating, for 2 h (hepatocytes) or 6 h (MIN6). Cells were then cultured for 24 h to allow protein expression.

Treatment with siRNA INS1E cells were transfected either with scrambled siRNA (Allstar negative control, Qiagen) or with siRNA targeted against PFKFB2 and PFKFB3 isoforms (Rat PFKFB2 (SI01960014), Rat PFKFB3 (SI01960035), Qiagen) using lipofectamine 2000 (Invitrogen). Cells were incubated for 72h to allow for silencing.

Real time RT-PCR Cellular RNA was extracted using TRIzol (Invitrogen) and treated with DNaseI (Roche, East Sussex, UK). Single strand cDNA was synthesized from 1µg of total RNA with random hexamers and Superscript II (Invitrogen). Real-

time RT-PCR was performed in a total volume of 10 μ l containing 50ng of reverse transcribed total RNA and 5ng of forward and reverse primers (Table 1). The reactions were carried out in capillaries in a Light Cycler (Roche) with initial denaturation at 95°C for 10min followed by 40 cycles consisting of 95°C for 15sec, 60°C for 7sec and 72°C for 15sec. The relative amount of PFKFB mRNA was corrected relative to the 18S RNA. The amplification of the correct PCR fragments was confirmed by DNA sequencing.

Insulin secretion and glycolysis MIN6 and INS1E were cultured in 24-well plates and washed with Krebs-Ringer Buffer [26]. They were then preincubated for 30min at 37°C in glucose-free Krebs Ringer buffer, followed by a 1h incubation at the indicated glucose concentration with [3-³H]glucose (2 μ Ci/ml) for determination of glycolysis and insulin secretion. Insulin was determined in the medium using a Rat Insulin Elisa Kit (Mercodia). For hepatocytes, glycolysis was determined as described previously [24].

Determination of metabolites and enzyme activity For determination of fructose 2,6-P₂, MIN6 and INS1E were extracted into 0.05M or 0.15M NaOH and hepatocytes in 0.1 M NaOH and heated for 5 min at 80°C. Fructose 2,6-P₂ was determined as in [27]. For determination of ATP cells were extracted in 3% (w/v) perchloric acid and ATP was determined fluorimetrically [28]. Glucokinase activity in MIN6 was determined in 13,000g supernatants of sonicated extracts as described in [29]. For elution in digitonin, MIN6 were incubated with 0.04 mg/ml digitonin in 150mM KCl, 3mM Hepes, 2 mM DTT for 4 minutes and glucokinase activity was determined on the digitonin extract [29]. PFK-2 activity was determined as in [30].

Western Blotting Proteins were fractionated by SDS-PAGE and transferred to Nitrocellulose [26]. Membranes were then blotted for: glucokinase immunoreactivity (rabbit antibody against human glucokinase residues 318-405 (Santa Cruz)) and PFK-2/FBPase-2 immunoreactivity with an antibody to the bisphosphatase domain raised in chicken [18] and AMPK-Thr-172(P) (New England Biolabs).

Statistical Analysis Results are expressed as means $\pm$ SEM based on 3-6 individual

experiments except for Figure 8D, which represent replicates within a single experiment. Statistical analysis was by either ANOVA followed by the Bonferroni test or paired t-test using the Prism analysis programme.

RESULTS

mRNA expression of PFK-2/FBPase-2 isoforms in islets and beta-cell lines

Previous studies on rat islets reported the expression of either the heart [17] or brain [18] isoforms of PFK-2/FBPase-2 lacking the C-terminal phosphorylation sites. However, the isoforms expressed in human and mouse islets or the MIN6 and INS1E glucose-responsive beta-cell lines have not been reported. Using isoform-specific primers we determined the expression of PFKFB1-3 in the cell lines and in rat (Fig. 1A-C), mouse (Fig. 1D-F) and human (Fig. 1H) islets. RNA from rat and mouse liver, heart and brain was used as positive controls for the rat and mouse primers. Rat islets expressed PFKFB2, with isoforms PFKFB1 and PFKFB3 being almost undetectable (Fig. 1A-C), whilst mouse islets also expressed predominantly PFKFB2, with low levels of PFKFB1 and PFKFB3 (Fig. 1D-F). Human islets expressed PFKFB2 and PFKFB3, with isoform PFKFB1 being almost undetectable (Fig. 1H). Using primers to the C-terminal phosphorylation site ser-466 of PFKFB2 we confirmed that both mouse and human islets express this C-terminal region. However, rat islets did not express the region around the ser-466 phosphorylation site (Fig. 1G, H), consistent with previous findings [17,18]. INS1E (rat, Fig. 1A-C) and MIN6 (mouse, Fig. 1D-F) expressed mainly PFKFB2 and PFKFB3. In MIN6 as in mouse islets, the PFKFB2 C-terminal ser-466 phosphorylation site was expressed at high levels (compared with heart). INS1E expressed high levels of PFKFB2 mRNA compared to heart tissue (6-8 fold, Fig. 1B) but low levels of the C-terminal ser-466 region. Increasing passage number of INS1E (p110-p153) and MIN6 (p19-p35) had negligible effect on the PFKFB expression profile.

Increase in fructose 2,6-P₂ by pharmacological activation of AMPK

Tissues that express the heart or brain isoforms show elevation of fructose 2,6-P₂ during pharmacological activation of AMPK with oligomycin (which inhibits ATP synthetase) or AICAR (which is phosphorylated to ZMP, an AMP analogue) as a result of phosphorylation of ser-466 (heart isoform) or ser-461 (brain isoform) which

increases the kinase activity of PFK-2/FBPase-2 [10,11]. We therefore tested whether these pharmacological activators of AMPK affect fructose 2,6-P₂ levels in MIN6 and INS1E. Preliminary studies testing the effects of AICAR (100 – 500 μ M) showed a concentration-dependent increase in fructose 2,6-P₂ (control 100%; 100 μ M, 114 ± 14 ; 200 μ M 133 ± 9 ; 500 μ M 200 ± 19). At the highest concentration tested, AICAR (500 μ M) also increased cellular ATP (untreated, 6.5 ± 0.3 ; AICAR, 8.4 ± 0.6 nmol/mg protein, $n=5$, $P < 0.005$). However, 500 μ M AICAR decreased glycolysis ($80 \pm 11\%$ relative to control). Subsequent experiments on glycolysis and insulin secretion were therefore performed at AICAR concentrations up to 200 μ M. Incubation of MIN6 with 0.5 μ M oligomycin for 2-10 min or with 200 μ M AICAR for 1 h increased the phosphorylation of AMPK-Thr-172 consistent with activation of the enzyme (Fig. 2A and 2B). This was associated with a biphasic increase in fructose 2,6-P₂ after addition of oligomycin (Fig. 2A) and a sustained increase during incubation with AICAR (Fig 2B). AICAR (200 μ M) increased insulin secretion at 25 mM glucose but had no effect on glycolysis (Fig. 2C,D). The increase in fructose 2,6-P₂ by AICAR in MIN6 was associated with an increase in kinase activity of PFK-2/FBPase-2 (Fig. 2E). This is consistent with expression of the C-terminal phosphorylation sites of the heart and / or brain isoforms and contrasts with the suppression of PFK-2/FBPase-2 kinase activity by AICAR in hepatocytes (Fig. 2F), which express PFKFB1. The fructose 2,6-P₂ content was also increased in mouse islets by AICAR (Fig. 3A) and in INS1E cells with AICAR (1.4 fold) and with oligomycin (1.2 fold at 2 min, $P < 0.05$). .

Glucose-dependent regulation of fructose 2,6-P₂ in MIN6 and INS1E cells

The fructose 2,6-P₂ content was glucose-responsive in islets, MIN6 and INS1E cells. In rat and mouse islets it was increased by 57-69 % at 25 mM compared with 5 mM glucose (Fig. 3A). Fructose 2,6-P₂ was 2-fold higher in MIN6 than in INS1E at 5 mM glucose and it was increased by 50% and 3-fold, respectively at 35mM glucose (Fig. 3B). In MIN6 cells the kinase activity of PFK-2/FBPase-2 was slightly but significantly ($P < 0.05$) higher at 25 mM compared with 5 mM glucose (5mM, 4.0 ± 0.3 ; 25 mM 4.9 ± 0.3 , $n=8$) suggesting that the effect of glucose is at least in part due to covalent modification of PFK2/FBPase-2.

Because 6-phosphogluconate is an inhibitor of the bisphosphatase activity of PFK-

2/FBPase-2 and is elevated by high glucose concentration [17], we next tested the effects of epiandrosterone, an inhibitor of glucose 6-phosphate dehydrogenase. Epiandrosterone caused a concentration-dependent suppression of fructose 2,6-P₂ in MIN6 and INS1E (Fig. 4A-B), consistent with a possible role for 6-phosphogluconate or downstream metabolites of the pentose phosphate pathway in increasing fructose 2,6-P₂, and it also suppressed glucose-induced insulin secretion (Fig. 4C). At 25 mM glucose, epiandrosterone suppressed but did not totally abolish the increase in fructose 2,6-P₂ caused by AICAR (Fig. 4D). Kinase activity of PFK-2/FBPase-2 measured in parallel conditions as in Fig. 4D (control, 4.2 ± 0.2 ; epiandrosterone, 3.9 ± 0.2 ; AICAR 6.0 ± 0.4 ; AICAR + epiandrosterone 4.2 ± 0.4 , n=4) was decreased by the inhibitor in the presence ($P < 0.05$) but not in the absence of AICAR. The latter is consistent with a role for 6-phosphogluconate in suppressing fructose 2,6-P₂ by competitive inhibition of the bisphosphatase activity of PFK-2/FBPase-2 [17].

The role of endogenous PFK-2/FBPase-2 in pancreatic beta cells

To date, studies investigating the role of PFK-2/FBPase-2 in regulating glucokinase activity in beta-cells have relied on PFK-2/FBPase-2 overexpression in cells expressing either endogenous or overexpressed glucokinase [19,20]. To determine the role of endogenous PFK-2/FBPase-2 in pancreatic beta cells, we used siRNA against PFKFB2 and PFKFB3 to downregulate endogenous enzyme. Silencing was confirmed by a 50% decrease in the mRNA levels of PFKFB2 and PFKFB3 (Fig. 5A) and by a 40-50% decrease in immunoreactivity to PFK-2/FBPase-2 and in fructose 2,6-P₂ content (Fig. 5B-C). Combined down-regulation of PFKFB2 and PFKFB3 suppressed insulin secretion at 25 mM glucose and also glucokinase activity. Rates of glycolysis and glucokinase immunoreactivity were not significantly changed (Fig. 5D-F).

The role of fructose 2,6-P₂ in pancreatic beta cells

To distinguish between a role for PFK-2/FBPase-2 protein as distinct from fructose 2,6-P₂ in regulating glucokinase activity and insulin secretion we used adenoviral vectors expressing either kinase-active (KA) or kinase-deficient (KD) variants of PFK-2/FBPase-2 to up-regulate and down-regulate fructose 2,6-P₂, respectively. We validated these variants in hepatocytes, where the role of fructose 2,6-P₂ in the control glycolysis is well established. Titrated expression of KA- PFK-2/FBPase-2 resulted

in a 2-fold increase in fructose 2,6-P₂ and in a small increase in glycolysis (Fig. 6A-B), whereas expression of KD- PFK-2/FBPase-2 decreased fructose 2,6-P₂ and glycolysis (Fig. 6C-D) similarly to glucagon (Fig. 6E). This confirms that suppression of glycolysis by glucagon is due to suppression of fructose 2,6-P₂. Titrated expression of KA- PFK-2/FBPase-2 in MIN6 increased fructose 2,6-P₂ during incubation with 5 mM glucose and it increased glycolysis and insulin secretion (Fig. 7A-C). Cellular ATP was unchanged in cells expressing KA-PFK-2/FBPase-2 (n=2, results not shown). Titration with the KD-PFK-2/FBPase-2 lowered fructose 2,6-P₂ by 50% at 15mM glucose (Fig. 7D). However, unlike in hepatocytes, glycolysis showed a small apparent increase (Fig. 7E). Insulin secretion was similarly increased by the two variants despite the converse changes in fructose 2,6-P₂ (Fig. 7F).

Activation of glucokinase by PFK-2/FBPase-2 in pancreatic beta cells

We determined whether an increase in glucokinase activity could explain the increase in glucose-induced insulin secretion by the KA and KD variants of PFK-2/FBPase-2. Glucokinase activity in the cytosol fraction (13,000g supernatant of sonicated extracts) was not increased in cells overexpressing KA-PFK-2/FBPase-2 (Fig. 8A) or KD- PFK-2/FBPase-2 (results not shown). However, using a digitonin permeabilisation assay which elutes the free or unbound enzyme, there was a higher glucokinase activity eluted from cells overexpressing KA-PFK-2/FBPase-2 (Fig. 8B). Since expression of PFK-2/FBPase-2 increases glucokinase activity in cytosol extracts of MIN6 expressing GK-GFP chimaeras but not in untransfected MIN6 [26], we determined the effects of separate and combined overexpression of glucokinase and KA- PFK-2/FBPase-2 in MIN6 cells (Fig. 8C). Whereas overexpression of either enzyme alone had little effect on glucose-induced insulin secretion, combined overexpression of both proteins stimulated insulin secretion, similar to the effect of a glucokinase activator (Fig. 8D).

DISCUSSION

Three key findings emerged from this study. First, the cell content of fructose 2,6-P₂ in beta cells is regulated by glucose and by activation of AMPK. The latter was associated with activation of the kinase activity of PFK-2/FBPase-2 and is consistent with the expression of PFKFB2 and PFKFB3 isoforms. Second, in

pancreatic beta-cells endogenous PFK-2/FBPase-2 has a critical role in regulation of cell function as shown by the decrease in total glucokinase activity and glucose-induced insulin secretion during partial down regulation of endogenous PFK-2/FBPase-2 isoforms by siRNA. Third, by using kinase-deficient and kinase-active variants of PFK-2/FBPase-2 we demonstrate an over-riding role of PFK-2/FBPase-2 protein as distinct from its catalytic activity in regulating glucokinase activity. This does not rule out an additional role for fructose 2,6-P₂ in the control of metabolic oscillations and / or pulsatile insulin secretion.

Several allosteric effectors are involved in the regulation of PFK-1 and thereby glycolysis. They include both inhibitors (ATP, citrate, phosphoenolpyruvate) and activators (ADP, AMP, fructose 1,6-P₂, glucose 1,6-P₂, 6-phosphogluconate, fructose 2,6-P₂). Of these fructose 2,6-P₂ is the most potent effector with an activation constant in the nanomolar or micromolar range depending on the concentrations of other effectors [1]. The relative sensitivity of PFK1 to these effectors is isoform dependent, with the M (muscle) isoform, having a relatively higher affinity for fructose 1,6-P₂ and lower affinity for fructose 2,6-P₂ compared with the L (liver) and C (platelet) isoforms [31,32]. Rat pancreatic islets express predominantly the C-isoform with lower levels of M and L isoforms [33], and both deficiency in M and overexpression of the L-isoform are associated with impaired insulin secretion suggesting a critical role for the different isoforms in islets [34,35].

The regulation of the cell content of fructose 2,6-P₂ is dependent on the PFKFB isoforms expressed. In liver which expresses PFKFB1, the fructose 2,6-P₂ content is regulated by cAMP [8] and by glucose through a xylulose 5-phosphate dependent type 2A phosphatase [9], whereas in heart and monocytes which express PFKBP2 and PFKBP3, it is regulated by AMPK activity [10,11]. In the latter tissues the stimulation of glycolysis by anoxia could be attributed to activation of PFK1 either by AMP or by the elevated fructose 2,6-P₂. The specific contribution of the latter mechanism has not been determined [11]. We show in this study that in mouse islets and pancreatic beta-cell lines the fructose 2,6-P₂ content is increased by glucose and by pharmacological activation of AMPK. The latter was associated with activation of the kinase activity of PFK-2/FBPase-2 and is consistent with expression in mouse islets and beta-cell lines of PFKFB2 and PFKFB3. The lack of concomitant stimulation of glycolysis during incubation with AICAR despite activation of AMPK, could be due to either inhibition of PFK1 by ZMP as shown for the FTO2B hepatoma

[36], or to inhibition of glucose phosphorylation [37]. In hepatocytes AICAR inhibits glucose phosphorylation by an AMPK-independent mechanism involving inhibition of glucokinase translocation from the nucleus to the cytoplasm [38] and / or inhibition of glucokinase activity by ZMP [37]. In pancreatic beta cells glucokinase does not shuttle between the nucleus and cytoplasm as in hepatocytes. However, inhibition of glucokinase by ZMP [37] cannot be firmly excluded.

In pancreatic beta cells both stimulation and inhibition of insulin secretion during activation of AMPK with AICAR has been reported [39-42]. In this study AICAR stimulated insulin secretion and increased cellular ATP, despite the lack of stimulation of glycolysis. It is possible that the variable effects of AICAR on insulin secretion in different studies may reflect the net changes in ATP/ADP. Differences in cell culture conditions such as the folate content of the medium, which affects the metabolism of ZMP may account for variability in cellular ATP with AICAR treatment [36].

We found that rat islets express PFKFB2 lacking the C-terminal AMP-kinase motif, consistent with previous findings of a truncated heart isoform [17,18]. The apparent species difference between rat islets and murine and human islets is surprising. We cannot rule out age-dependent differences in isoform expression as shown for PFK-1 [43]. This might in part explain the wide variation in the rat islet content of fructose 2,6-P₂ reported previously [5-7]. Although the truncated PFK2FB2 lacks the terminal AMPK motif (ser-466), it has two putative AMPK motifs (ser-58 and ser-86) with a hydrophobic residue at P-5 and a basic residue at P-3/-4, consistent with these sites being potential substrates for both AMPK and cAMP dependent protein kinase [44], which causes activation of the heart isoform [45].

The present finding that the fructose 2,6-P₂ content of beta-cells is elevated during activation of AMPK, raises the question of the possible role of this mechanism in beta-cells. A tentative hypothesis is that the increase in fructose 2,6-P₂ may have a role in pulsatile insulin secretion which is attributed to oscillations in glycolysis and in the ATP/ADP ratio [46-48]. Pulsatile insulin secretion occurs both *in vivo* and in perfused islets and clonal beta-cells *in vitro* and is attributed to oscillations in the ATP/ADP ratio and in metabolites of glycolysis. Metabolic oscillations are not unique to beta-cells but also occur in other cell types and in muscle and heart extracts [49]. The oscillations in cell extracts involve converse fluctuations in hexose monophosphates (glucose 6-P and fructose 6-P) and fructose 1,6-P₂, indicating

repetitive activation and inactivation of PFK1. The inactivation phase is preceded by the rise in ATP/ADP, whereas the activation of PFK1 is preceded by the decline in ATP/ADP ratio. The latter has been conventionally explained in skeletal muscle by the rise in fructose 1,6-P₂ which is a high-affinity activator of the muscle PFK1 isoform [49]. It can be hypothesized that in tissues expressing the PFKFB2 and PFKFB3 isoforms such as islets (which also express the C and L PFK1 isoforms), the metabolic oscillations may involve activation of AMPK during the decline in ATP/ADP resulting in an increase in fructose 2,6-P₂ and activation of the C/L isoforms of PFK1. Loss of pulsatile insulin secretion in both human type 2 diabetes and animal models is well documented [46], and may be due to age-dependent changes in the expression of either PFK-1 [34,35,43] or PFK-2/FBPase-2 isoforms.

This study provides evidence for control of fructose 2,6-P₂ by glucose (25mM vs 5 mM) in pancreatic islets and in MIN6 and INS1E cell lines. Glucose may impact the fructose 2,6-P₂ content by covalent modification of PFK-2/FBPase-2 or through changes in concentrations of metabolites that act as activators or inhibitors of either the kinase or bisphosphatase activity. Elevated glucose may cause a decrease in AMPK activity [40,41], which would be expected to decrease the kinase activity of PFK-2/FBPase-2 and thereby the cell content of fructose 2,6-P₂. However, in this study elevated glucose caused an increase in kinase activity of PFK-2/FBPase-2. This suggests that glucose causes covalent modification of PFK-2/FBPase-2, by a mechanism that over-rides any effect resulting from glucose-induced suppression of AMPK [40,41]. The increase in fructose 2,6-P₂ caused by glucose can be explained by both covalent modification of PFK-2/FBPase-2, as shown by the increase in kinase activity and by an increased concentration of 6-phosphogluconate (a competitive inhibitor of the bisphosphatase activity) as a result of increased flux through the pentose phosphate pathway [17]. The marked suppression of fructose 2,6-P₂ with epiandrosterone, in the absence of a corresponding decrease in kinase activity of PFK-2/FBPase-2 is consistent with a role for 6-phosphogluconate as an inhibitor of the bisphosphatase.

The higher fructose 2,6-P₂ content in the cell lines compared with islets (10-25 vs 3 pmol/mg) may be related to the elevated flux through the pentose phosphate pathway in proliferating cells. Since 6-phosphogluconate is an activator of PFK1 [31,32] as well as an inhibitor of the bisphosphatase [17], the apparent refractoriness of glycolysis to fluctuations in fructose 2,6-P₂ with the kinase-deficient variant in

MIN6 could be due to the elevated concentration of 6-phosphogluconate which may substitute for fructose 2,6-P₂ in the regulation of PFK1 or to altered expression of PFK1 isoforms [33].

By using siRNA to down-regulate endogenous PFKFB2 and PFKFB3 this study demonstrates for the first time the critical role for PFK-2/FBPase-2 in beta-cell function. Down-regulation of PFK-2/FBPase-2 protein by 50% resulted in a decrease in fructose 2,6-P₂, in total glucokinase activity and glucose-induced insulin secretion. The lack of suppression of glucokinase activity or insulin secretion during down-regulation of fructose 2,6-P₂ with the kinase-deficient variant rules out a role for fructose 2,6-P₂ in explaining the effects of down-regulation of PFK-2/FBPase-2. Two mechanisms can be considered for the role of PFK-2/FBPase-2 in insulin secretion: either it determines subcellular targeting of glucokinase or it enhances (or stabilises) glucokinase catalytic activity. The increase in glucokinase activity in the digitonin elutable fraction (though not in the cytosol fraction) suggests that overexpressed PFK-2/FBPase-2 may affect the sub-cellular distribution of glucokinase. In pancreatic beta-cells glucokinase associates with insulin granules and microtubules [23,50]. The co-localisation of glucokinase and PFK-2/FBPase-2 with insulin granules as shown by both subcellular fractionation and fluorescence imaging [20,23], may play a role in determining the subcellular distribution of glucokinase. The lack of increase in total glucokinase activity when kinase active PFK-2/FBPase-2 was overexpressed can be explained by a saturating role for the endogenous PFK-2/FBPase-2 protein in stabilising glucokinase. Previous work showed that overexpression of PFK-2/FBPase-2 increases glucokinase activity in MIN6 cells expressing GFP-GK chimaeras but not in non-transfected cells expressing only endogenous glucokinase [26]. This was explained by a role for endogenous PFK-2/FBPase-2 in stabilising endogenous glucokinase as in hepatocytes [24]. This study demonstrates that overexpression of either glucokinase or PFK-2/FBPase-2 alone has negligible effect on insulin secretion. However, combined overexpression of both proteins stimulates insulin secretion (similar to a glucokinase activator) and also enhances glucokinase activity relative to overexpression of glucokinase alone. This supports a model for co-ordinate control of insulin secretion by glucokinase and PFK-2/FBPase-2, consistent with the findings from the siRNA experiments, where suppression of PFK-2/FBPase-2 led to a decrease in total glucokinase activity and in glucose-induced insulin secretion.

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Species	Gene	Primer	Sequence
Mouse	PFKFB1	FWD: TCC ATG TCA CAC CTC GAT CT REV: GGA GCT GAT GCT TTG AGA CC	NM_00824 (808-943bp)
	PFKFB2	FWD: ATG GAG CTA GAG CGT CAA GG REV: GGG GCA CCT CAA GTA TGG TA	NM_008825 (1252-1349bp)
	PFKFB2 (Ser ⁴⁶⁶)	FWD: TCT TGG ATA AAG GCG CAG AT REV: GAT TAG TGG CAA GGG CAA GA	NM_008825 (1325-1698bp)
	PFKFB3	FWD: AGA ACT TCC ACT CTC CCA CCC REV: AGG GTA GTG CCC ATT GTT GAA	NM_133232 (1600-1999bp)
Rat	PFKFB1	FWD: GTT TAC CAG CTC GAG GCA AG REV: TGT TGT CTG GGC GAA AGA AT	NM_12621 (348-478bp)
	PFKFB2	FWD: CCG AGG AAC TGG ATC TAC CA REV: ATG TTC CTG TTG GGC ATG AG	S67900 / NM_080477 (1638-1769bp)
	PFKFB2 (Ser ⁴⁶⁶)	FWD: TCT TGG ATA AAG GCG CAG AT REV: TGT CCA TTT GCA AAC TCC AG	NM_001033965 (1403-1774bp)
	PFKFB3	FWD: CAC GGC GAG AAT GAG TAC AA REV: TTC AGC TGA CTG GTC CAC AC	NM_057135 (1165-1291bp)
Human	PFKFB1	FWD: CCT GCA AAT CAG GAA GCA GT REV: TTT GCA AAC TGC AGG ATC AG	NM_002625 (382-503bp)
	PFKFB2	FWD: TAC GAC TTC TTT CGG CAT GA REV: CTC CTC TCC CGG GTT GTA TT	NM_006212 (324-453bp)
	PFKFB2 (Ser ⁴⁶⁶)	FWD: CTT AAC GTG GAG GCT GTG AA REV: TTG GAC GCC TTA TTG TAT TCG	NM_006212 (1361-1470bp)
	PFKFB3	FWD: CCG TGT GGA ATC CAT CTA CC REV: CTG TTG ATG CGA GGC TTT TT	NM_004566 (1619-1752bp)

Table 1. Primer sequences for real-time RT-PCR.

LEGENDS TO FIGURES

Figure 1. Expression of PFKFB isoforms in rat, mouse and human islets and MIN6 and INS1E. RNA was extracted from islets and cell lines and expression of PFKFB1, PFKFB2 and PFKFB3 mRNA was determined by real-time RT-PCR. A-C. Primers against rat PFKFB1 (A), PFKFB2 (B), and PFKFB3 (C). D-F. Primers against mouse PFKFB1 (D), PFKFB2 (E), and PFKFB3 (F). Results are expressed as PFKFB/18S ratio. $n = 3$. G. Primers against the C-terminus of rat and mouse PFKFB2. Results are representative of 2 experiments. H. Primers against human PFKFB1, PFKFB2, PFKFB2(C-terminus) and PFKFB3. Results represent 4 human islet preparations.

Figure 2. Effects of oligomycin and AICAR on fructose 2,6-P₂ content.

A. MIN6 were incubated with 50 μ M oligomycin for 2, 5 or 10 min at 5mM glucose. Immunoblot for AMPK-Thr172(P) (upper panel) and fructose 2,6-P₂ content (F26P₂), expressed as percentage control. $n = 5$. * $P < 0.05$, relative to no oligomycin. B-D. MIN6 were incubated without or with 50, 100 and 200 μ M AICAR for 1h at 5 or 25mM glucose. B. AMPK-Thr172(P) and fructose 2,6-P₂. C. Glycolysis; D. Insulin secretion. Results are expressed as percentage of 5 mM glucose control. Control values are: fructose 2,6-P₂, 6.9 ± 1.26 pmol/mg protein; glycolysis, 128 ± 16 nmol/h/mg protein and insulin secretion, 1.5 ± 0.4 μ g/mg protein, $n=4$, * $P < 0.05$. ** $P < 0.01$, relative to 5 mM glucose control. # $P < 0.05$, relative to 25mM glucose control. E,F. PFK2 activity determined after 1 h incubation without or with 500 μ M AICAR. E. MIN6; F. hepatocytes, $n = 4$, * $P < 0.05$. ** $P < 0.01$ effect of AICAR.

Figure 3. Effects of glucose on fructose 2,6-P₂.

A. Mouse and rat islets were incubated with 5 or 25 mM glucose and without or with μ M AICAR for 1 h. $n = 3$ islet preparations. Fructose 2,6-P₂ (F26P₂) is expressed as fmol/islet. B. MIN6 and INS1E were incubated with varying glucose concentration (5-35mM) for 1h. Fructose 2,6-P₂ is expressed as pmol/mg protein. $n = 4$. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.005$ relative to 5mM glucose.

Figure 4. Epiandrosterone suppresses fructose 2,6-P₂ in MIN6 and INS1E.

A-B. MIN6 (A) and INS1E (B) were incubated for 1 h with varying epiandrosterone concentrations at 5 or 25mM glucose. Fructose 2,6-P₂ (F26P₂) is expressed as pmol/mg protein. n = 1 (A), or 2 (B). C. Insulin secretion was determined in MIN6 incubated for 1 h without or with 50μM epiandrosterone at 5 or 25mM glucose and is expressed as percentage of 5mM glucose. Control values: 1.5 ± 0.2 μg/mg protein. n = 2. D. Fructose 2,6-P₂ was determined in MIN6 after 1 h incubation without or with 25μM epiandrosterone or 500μM AICAR at 25 mM glucose, n=4, * P < 0.05, *** P < 0.005, effect of AICAR; ### P < 0.005, effect of epiandrosterone.

Figure 5. Silencing of PFK-2/FBPase-2 in INS1E.

INS1E cells were transfected with either scrambled (-ve) or siRNA against PFKFB2 and PFKFB3 (FB2 + FB3) and cultured for 72h. A. PFKFB2 and PFKFB3 mRNA. B. PFK-2/FBPase-2 protein determined by immunoblotting and densitometry. C. Fructose 2,6-P₂ at 5 and 25mM glucose. D. Insulin secretion. E. glycolysis. F. glucokinase activity and protein determined on the 13,000g supernatant after sonication. Results are expressed as percentage of -ve siRNA control. Control values: fructose 2,6-P₂, 5.8 ± 1.0 pmol/mg protein; insulin secretion, 1.3 ± 0.2 μg/mg protein; glycolysis, 102 ± 18 nmol/h/mg protein; glucokinase activity, 2.7 ± 0.2 mU/mg protein. n = 4. Immunoblots are representative of 4 experiments. * P < 0.05, ** P < 0.01, *** P < 0.005 relative to 5mM control. # P < 0.05, ## P < 0.01, ### P < 0.005 relative to -ve siRNA control.

Figure 6. Alteration of fructose 2,6-P₂ in hepatocytes by expression of KA-PFK-2/FBPase-2 and KD-PFK-2/FBPase-2. KA-PFK-2/FBPase-2 (KA, A,B) and KD-PFK-2/FBPase-2 (KD, C,D) were expressed in hepatocytes by incubation for 2h with increasing titres of adenoviral vectors (1-3). After overnight culture, fructose 2,6-P₂ (A,C) and glycolysis (B,D) were determined after 1h incubation with 25 mM glucose with glucagon (100nM) where indicated in C,D. E. Fructose 2,6-P₂ content (A,C) versus glycolysis (B,D). Results are expressed as percentage control. Control values: fructose 2,6-P₂, 132 ± 21 pmol/mg protein; glycolysis, 98 ± 26 nmol/h/mg protein. n = 4 (A,B) or 5 (C,D). * P < 0.05, *** P < 0.005 relative to control.

Figure 7. Alteration of fructose 2,6-P₂ in MIN6 by expression of KA-PFK-2/FBPase-2 and KD-PFK-2/FBPase-2. KA-PFK-2/FBPase-2 (A-C) and KD-PFK-2/FBPase-2 (D-F) were expressed in MIN6 by incubation with increasing titres (1-3) of adenovirus for 6h. After overnight culture, cells were incubated for 1 h with the glucose concentration indicated (A-C, 25 and 5mM; D-F, 5 and 15 mM) for determination of fructose 2,6-P₂ (A,D), glycolysis (B,E) and insulin secretion (C,F). Results are expressed as percentage control. Control values: 5mM (A-C): fructose 2,6-P₂, 7.1 ± 0.8 pmol/mg protein; glycolysis, 133 ± 22 nmol/h/mg protein; insulin secretion, 1.8 ± 0.4 μ g/mg protein. 15mM (D-F): fructose 2,6-P₂, 17.6 ± 3.3 pmol/mg protein; glycolysis, 208 ± 43 nmol/h/mg protein; insulin secretion, 2.6 ± 0.2 μ g/mg protein. n = 5 (A-C) or 6 (D-F). * P < 0.05, ** P < 0.01, *** P < 0.005 vs control.

Figure 8. Effect of expression of PFK-2/FBPase-2 on glucokinase activity.

A,B. KA-PFK-2/FBPase-2 was expressed in MIN6 as in Fig. 7. After overnight culture, cells were extracted either by sonication and centrifugation at 13,000g (A) or by permeabilisation with 0.04mg/ml digitonin for 4min (B) and glucokinase activity determined. n = 5 (A) or 4 (B). C,D. KA-PFK-2/FBPase-2 and rat liver glucokinase were expressed in MIN6 cells by incubation with adenoviral vectors for 6h. After overnight culture glucokinase activity was determined on the sonicated supernatant (C) and insulin secretion determined at 5 or 25mM glucose in the absence or presence of 10 μ M GKA [27]. Results are expressed as percentage control. Control values: A. glucokinase activity = 1.49 ± 0.3 mU/mg protein, C. glucokinase activity = 1.02 mU/mg protein, D. insulin secretion = 0.51 ± 0.06 μ g/mg protein. n = 1 based on 3 replicates within an individual experiment representative of 2. ** P < 0.01 relative to control.

Figure 1

Figure 2

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Figure 3

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Figure 4

Figure 5

Figure 6

THIS IS NOT THE FINAL VERSION - see doi:10.1042/BJ20070962

Figure 7

Figure 8