Insulin stimulates pancreatic–duodenal homoeobox factor-1 (PDX1) DNA-binding activity and insulin promoter activity in pancreatic β cells

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Pancreatic–duodenal homoeobox factor-1 (PDX1) is a homoeo-domain transcription factor that plays an important role in linking glucose metabolism in pancreatic β cells to the regulation of insulin gene transcription. Our previous results indicated that glucose activates PDX1 DNA-binding activity and insulin promoter activity via a stress-activated signalling pathway involving phosphatidylinositol 3-kinase (PtdIns 3-kinase) and stress-activated protein kinase 2 (SAPK2/p38). The present study was undertaken to determine the effects of other metabolizable and non-metabolizable nutrients. The results indicate that non-metabolizable nutrients, with the exception of 2-deoxyglucose, had no effect. Metabolizable nutrients that could stimulate calcium uptake and insulin release were shown to activate both PDX1 and the insulin promoter. The possible role of insulin acting via an autoregulatory loop was therefore examined. Insulin

was shown to potently activate PDX1 DNA-binding activity and insulin promoter activity. The effects of insulin were inhibited by the PtdIns 3-kinase inhibitors wortmannin and LY294002 and by the SAPK2 inhibitor SB203580, suggesting that its effects were mediated via activation of PtdIns 3-kinase and SAPK2. Further support for the insulin-mediated activation of SAPK2 came from the observation that both glucose and insulin stimulated the phosphorylation of SAPK2. These results suggest that both glucose and insulin stimulate PDX1 DNA-binding activity and insulin promoter activity via a pathway involving PtdIns 3-kinase and SAPK2.

Key words: diabetes mellitus, insulin gene, islets of Langerhans, transcription.

INTRODUCTION

Insulin plays a pivotal role in the maintenance of glucose homeostasis. It is produced in the pancreatic islets of Langerhans, from where it is secreted in response to feeding-induced sympathetic neural and hormonal signals, as well as in response to changes in the circulating blood glucose concentration [1]. In order to replenish insulin stores between meals, nutrients also stimulate an increase in insulin mRNA levels over a period of hours and an increase in insulin biosynthesis over a period of minutes [2]. The effects on insulin mRNA levels result from a combination of a stabilizing effect on pre-existing mRNA [3], increased processing of pre-RNA [4], and an increased rate of insulin gene transcription [5].

Transcription of the insulin gene is dependent on sequences located within a region up to 350 bp from the transcription start site, although in the case of the human insulin gene an adjacent hypervariable region and further upstream sequences may also be important [6]. Within the promoter proximal region of the human insulin gene, a number of *cis*-acting regulatory sequences have been identified [7], of which the E boxes located at –104 and –232, and the A boxes located at –76, –130, –210 and –312, are particularly important. The E1 box (–104) binds a factor IEF1 (insulin enhancer factor 1), which is a heterodimer of the basic helix–loop–helix proteins Beta2/NeuroD and E47 [8,9]. The E2 box (–232) is a complex site that binds a number of transcription factors, including upstream stimulatory factor and

a RIPE3B1-like factor [10]. The A boxes bind a number of homoeodomain transcription factors [11], of which PDX1 (pancreatic–duodenal homoeobox factor-1; also known as insulin promoter factor 1, IDX1, STF1 and IUF1) is the most abundant in β cells [12]. PDX1 plays an important role in development of the pancreas and islet cell ontogeny [13,14]. It may function by virtue of its DNA-binding properties, thus bringing other DNA-bound regulatory proteins into juxtaposition with the pre-initiation complex [15].

Several transcription factors have been shown to bind, and in some instances transactivate, the insulin promoter in response to glucose. These include the uncharacterized factor RIPE3B1 that binds to the C1 element of the rat II insulin promoter [16], a factor that binds to the negative regulatory element of the human insulin gene [17], and PDX1 [18–21]. PDX1 is an essential component of the mechanisms whereby glucose modulates both insulin promoter activity and endogenous insulin mRNA levels. Thus glucose has no effect on these parameters in a human β cell line (NES2Y) that lacks PDX1, whereas overexpression of PDX1 in these cells re-instates a normal response of the promoter and of endogenous insulin mRNA levels to glucose (W. M. Macfarlane, R. M. Shepherd, K. E. Cosgrove, R. F. L. James, M. J. Dunne and K. Docherty, unpublished work). PDX1 DNA-binding activity is modulated by glucose via a pathway involving stress-activated protein kinase 2 (SAPK2) [22]. SAPK2, which is also known as RK/p38, is a member of an expanding family of kinases, related to mitogen-activated protein kinase, that are

Abbreviations used: DMEM, Dulbecco's modified Eagle's medium; EMSA, electrophoretic mobility shift assay; IRS, insulin receptor substrate; PDX1, pancreatic–duodenal homeobox factor-1; SAPK2, stress-activated protein kinase 2.

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activated in response to adverse stimuli, such as heat, osmotic shock, UV light and DNA-damaging reagents, and by pro-inflammatory cytokines that are produced under conditions of stress [23]. In pancreatic β cells, glucose activates SAPK2 via phosphatidylinositol 3-kinase (PtdIns 3-kinase), whereas stress activation of SAPK2 (via, for example, arsenite) is not dependent on PtdIns 3-kinase [22]. Activation of SAPK2 leads to phosphorylation in the cytoplasm of an inactive 31 kDa form of PDX1, which is modified to a 46 kDa active form that is translocated to the nucleus [24].

The aim of the present study was to investigate the effects of various nutrients on PDX1 DNA-binding activity and on insulin promoter activity. The results show that all nutrients that acted as insulin secretagogues could activate both PDX1 and the insulin promoter. Insulin was further shown to stimulate PDX1 DNA-binding activity and insulin promoter activity via PtdIns3-kinase and SAPK2.

EXPERIMENTAL

Materials

[γ - ^{32} P]ATP was purchased from Amersham International (Slough, Berks., U.K.). Wortmannin was purchased from Sigma (Poole, Dorset, U.K.), sodium arsenite from Fisons (Loughborough, Leics., U.K.) and oligonucleotide B [25] from Alta Bioscience (University of Birmingham, Birmingham, U.K.). SB203580 was kindly provided by Professor P. Cohen (University of Dundee, Dundee, U.K.). All other chemicals and reagents were obtained from Sigma.

Human islets of Langerhans

Human islets of Langerhans were obtained from the British Diabetic Association Centralised Human Islet Facility at the University of Leicester (Leicester, U.K.). Purified islets were maintained in RPMI 1640 medium (Sigma) supplemented with glucose (11 mM), 10% (v/v) foetal calf serum (Sigma), 100 units/ml sodium penicillin and 100 μ g/ml streptomycin sulphate at 37 °C in a humidified atmosphere of 95% air/5% CO₂ for 24 h before use.

Preparation of nuclear extracts

Islets were centrifuged for 10 s in a microcentrifuge at 12000 *g* and resuspended in 400 μ l of ice-cold buffer containing 10 mM Hepes, pH 7.9, 10 mM KCl, 0.1 mM EGTA, 0.1 mM EDTA, 1 mM dithiothreitol and 1 $\times$ Protease Inhibitor Cocktail Solution (Boehringer Mannheim). Islets were allowed to swell for 15 min on ice before adding 25 μ l of 10% (v/v) Nonidet P-40. The islets were then vortexed for 15 s and centrifuged for 30 s at 12000 *g* and 4 °C. The pellets were resuspended in 40 μ l of ice-cold buffer containing 20 mM Hepes, pH 7.9, 0.4 M NaCl, 1 mM EGTA, 1 mM EDTA, 1 mM dithiothreitol, 1 $\times$ Protease Inhibitor Cocktail Solution and 5% (v/v) glycerol. Protein concentrations were determined using the Bio-Rad Protein Assay II System. Nuclear extracts were then divided into small volumes and stored at -70 °C.

Electrophoretic mobility shift assays (EMSAs)

EMSAs were performed using the A3 PDX1 binding site in oligonucleotide B [25], which corresponds to sequence from positions -210 to -230 of the human insulin promoter. Nuclear extracts (2 μ g of protein) were incubated with the ^{32} P-labelled probe for 18 min at room temperature in binding buffer con-

taining 10 mM Tris/HCl (pH 7.5), 50 mM KCl, 500 ng of poly(dI-dC)/poly(dI-dC), 5 mM dithiothreitol, 1 mM EDTA and 5% (v/v) glycerol. Gel loading buffer was added and the mixtures were loaded on to a non-denaturing 6% (w/v) polyacrylamide gel (acrylamide/bisacrylamide, 29:1) which was run in 0.5 $\times$ TBE (50 mM Tris, 50 mM boric acid and 1 mM EDTA) at 10–15 V/cm for 2.5 h. The gel was dried and subjected to autoradiography.

Western blotting

Cell extracts (0.5 μ g/sample) were prepared and Western blotting was performed as previously described [24], using primary anti-SAPK2 and anti-phospho-SAPK2 antibodies (New England Biolabs, Cambridge, U.K.) at 1:1000 and 1:500 dilutions respectively. Detection was performed using a 1:1000 dilution of horseradish peroxidase-conjugated anti-(rabbit IgG) secondary antibody, using the ECL® detection system (Amersham), as previously described [24].

Cell culture

The glucose-responsive MIN6 cell line [26] was cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma) supplemented with 10% (v/v) foetal bovine serum (Sigma), 5 mM D-glucose and 2 mM L-glutamine. All experiments were performed with MIN6 cells between passages 27 and 32.

Insulin promoter studies

Vector pGL-LUC200 contained a -60 to -260 bp *HincII*/*PvuII* human insulin gene promoter fragment upstream of the thymidine kinase promoter of Herpes simplex virus driving the firefly luciferase gene. The control vector pGL-LUC contained the thymidine kinase promoter upstream of the firefly luciferase gene. Plasmid DNAs were prepared by the Maxiprep method using a QIAGEN endotoxin-free kit. MIN6 cells were transfected using liposomes as previously described [22].

Luciferase reporter gene assay

Following treatment, transfected cells were harvested, washed in ice-cold PBS and resuspended in 70 μ l of extraction buffer containing 100 mM potassium phosphate (pH 7.8) and 1 mM dithiothreitol. Cells were lysed by three cycles of freezing/thawing, and cell debris was removed by centrifugation at 4 °C and 10000 *g* for 1 min. The amount of protein in the cell extract was determined using the Bio-Rad Protein Assay II Kit. Luciferase enzyme activity was measured [22] using a Berthold Luma LB 9501 luminometer.

RESULTS

The following nutrients were shown to stimulate PDX1 DNA-binding activity in isolated human islets of Langerhans: D-glucose, D-mannose, fructose, pyruvate, D-glyceraldehyde, oxo-isocaproate (ketoisocaproate) and xylitol (Figure 1A). When studied over a range of concentrations, D-glucose and oxo-isocaproate exhibited a threshold effect on PDX1 DNA-binding activity at 5 mM, while xylitol and D-mannose exhibited a threshold at 0.5–1 mM in the presence of 3 mM glucose (results not shown). The non-metabolizable sugars L-glucose, D-galactose and 3-O-methyl-D-glucose had no effect (Figures 1B and 1C). However, 2-deoxyglucose, which undergoes phosphorylation but no further metabolism, was found to stimulate PDX1 DNA-binding activity (Figure 1C).

Figure 1 Effects of metabolizable and non-metabolizable nutrients on PDX1 DNA-binding activity

Batches of 200 human islets of Langerhans were pre-incubated for 3 h in DMEM containing 3 mM glucose. They were then incubated for 1 h in DMEM containing 3 mM glucose and metabolizable or non-metabolizable sugars at the indicated final concentrations. PDX1 DNA-binding activity was measured by EMSA using oligonucleotide B (A3 site) as probe. The results are representative of similar experiments performed on at least three separate occasions. Ketoisocaproate = oxoisocaproate.

Figure 2 Effects of nutrients on insulin promoter activity in transfected MIN6 cells

MIN6 cells were transfected with pGL-LUC200 or pGL-LUC control plasmid. After 48 h the transfected cells were incubated for 1 h in DMEM containing 0.5 mM glucose alone or 0.5 mM glucose plus the indicated nutrients at 18 mM. Cell extracts were prepared and firefly luciferase activity measured. The data are representative of three experiments, and show means $\pm$ S.D. for three separate determinations. Ketoisocaproate = oxoisocaproate.

To determine the effect of nutrients on insulin promoter activity, the construct pGL-LUC200, along with a control construct lacking the -60 to -260 insulin promoter fragment (pGL-LUC), was transfected into MIN6 β cells. Then 48 h later the cells were incubated in media supplemented with various nutrients. All nutrients, including 2-deoxyglucose, that stimulated PDX1 DNA-binding activity also stimulated the activity of the insulin promoter fragment in pGL-LUC200, while exhibiting no significant effect on the control construct (pGL-LUC) (Figure 2). The non-metabolizable sugars 3-*O*-methyl-D-glucose, L-glucose

and D-galactose had no effect on the activity of pGL-LUC200 (results not shown).

We had shown previously that arsenite activation of PDX1 DNA-binding activity and insulin promoter activity was inhibited by SB203580, but not by wortmannin or LY294002, whereas the effects of glucose on PDX1 and the insulin promoter were inhibited by all three reagents [22]. This suggested that stress and metabolic effects could be differentiated; the former signalling through SAPK2 (inhibited by SB203580) and the latter signalling through PtdIns 3-kinase and SAPK2 (inhibited by wortmannin and LY294002 as well as SB203580). To investigate the relative contributions of PtdIns 3-kinase and SAPK2 to the nutrient effects on PDX1 and the insulin promoter, human islets were treated with selected nutrients in the absence or presence of wortmannin, LY294002 or SB203580. The results indicated that the four sugars tested, i.e. D-glucose, 2-deoxyglucose, glyceraldehyde and xylitol, increased PDX1 DNA-binding activity via a cell signalling pathway that was inhibited by wortmannin (50 nM), LY294002 (50 μ M) and SB203580 (10 μ M) (Figures 3A–3D). This indicated that PtdIns 3-kinase and SAPK2 were involved. As reported previously [22], the calmodulin-dependent protein kinase II inhibitor KN62 had no effect, and nor did the p70^{rsk} inhibitor rapamycin (results not shown).

The observation that the effects of nutrients were mediated via PtdIns 3-kinase was intriguing. PtdIns 3-kinase is involved in insulin-, growth factor- and integrin-dependent signalling. There is no evidence for a direct effect of nutrients on its activity. One possibility was that nutrients were mediating their effects in part by stimulating the exocytosis of insulin, which was then activating PDX1 DNA-binding activity and insulin promoter activity via an autoregulatory loop involving β cell insulin receptors signalling via insulin receptor substrate (IRS)-1/2 and subsequently to PtdIns 3-kinase. To test this, human islets incubated in low (3 mM) concentrations of glucose were treated with insulin at various concentrations. Insulin, at 1–10 ng/ml, was shown to stimulate PDX1 DNA-binding activity. The half-maximal effect of insulin was observed at 4 ng/ml (Figure 4), and was inhibitable by wortmannin (50 nM), LY294002 (50 μ M) and SB203580

Figure 3 Effects of wortmannin, LY294002 and SB203580 on nutrient-stimulated PDX1 DNA-binding activity

Batches of 200 human islets of Langerhans were pre-incubated for 30 min in DMEM containing 3 mM glucose alone, or 3 mM glucose plus the indicated concentration of wortmannin, LY294002 or SB203580. They were then incubated for 1 h in media containing 3 mM glucose alone (G3) or 3 mM glucose and the indicated inhibitors plus 16 mM D-2-deoxyglucose (**B**), 16 mM D-glyceraldehyde (**C**), 20 mM xylitol (**D**) or 16 mM glucose (**A**). PDX1 DNA-binding activity was measured by EMSA using oligonucleotide B (A3 site) as probe. Results are representative of similar experiments performed on two separate occasions.

Figure 4 Insulin stimulates PDX1 DNA-binding activity in a dose-dependent manner

Batches of 200 human islets of Langerhans were incubated for 3 h in DMEM containing 3 mM glucose. They were then incubated for 60 min in DMEM containing 3 mM glucose alone (G3) or 3 mM glucose plus the indicated final concentration of bovine insulin. PDX1 DNA-binding activity was then measured by EMSA using oligonucleotide B (A3 site) as probe. The results are representative of similar experiments performed on at least three separate occasions.

Figure 5 Insulin stimulates PDX1 DNA-binding activity via PtdIns 3-kinase and SAPK2

Batches of 200 human islets of Langerhans were pre-incubated in DMEM containing 3 mM glucose for 3 h; the indicated concentration of inhibitor was added for the final 30 min of this period. They were then incubated in DMEM containing 3 mM glucose (G3), 16 mM glucose (G16) or insulin (10 ng/ml) plus the indicated concentration of wortmannin, LY294002 or SB203580 for 30 min. The results are representative of similar experiments performed on at least three separate occasions.

(10 μM) (Figure 5), suggesting a signalling pathway involving both PtdIns 3-kinase and SAPK2. The IC₅₀ for SB203580, 8 μM (results not shown), was similar to that observed for its inhibitory effect on glucose-stimulated PDX1 DNA-binding activity [22]. Further support for an effect of insulin on SAPK2 was provided by experiments in which insulin (10 ng/ml) and glucose were shown to stimulate the phosphorylation of SAPK2 (Figure 6).

The effects of insulin and glucose on the phosphorylation of SAPK2 were inhibited by wortmannin, LY294002 and SB203580, whereas the effect of arsenite was inhibited only by SB203580. Insulin was further shown to stimulate pGL-LUC200, but not the control vector pGL-LUC, in transfected MIN6 cells (Figure 7).

Figure 6 Insulin stimulates phosphorylation of SAPK2

Shown is Western blot analysis of SAPK2 in cell extracts prepared from batches of 200 human islets of Langerhans. Islets were pre-incubated in 3 mM glucose for 2 h, and SB203580 (SB; 10 μ M) or wortmannin (Wtm; 50 nM) was added 30 min before further stimulation of the islets with 20 mM glucose (G), 10 ng/ml insulin (I) or 1 mM sodium arsenite (A) for 30 min, as indicated. Portions of 0.5 μ g of total protein were added to each well, and duplicate filters were probed with antibodies specific for SAPK2 (upper panel) or phospho-SAPK2 (lower panel). Detection was performed using a horseradish peroxidase-conjugated secondary antibody with ECL methodology, with exposure times of 1 min (upper panel) and 10 s (lower panel). SAPK2 has a molecular mass of 38 kDa. The results shown have been reproduced in three separate experiments, and in similar experiments on the β cell line MIN6.

Figure 7 Effects of glucose and insulin on the activity of the insulin promoter in transfected MIN6 cells

MIN6 cells were transfected with pGL-LUC200 or pGL-LUC control plasmid. After 48 h, cells were pre-incubated for 3 h in DMEM containing 0.5 mM glucose, and were then incubated in 0.5 mM glucose, 16 mM glucose or 0.5 mM glucose plus 10 ng/ml insulin for 3 h. The results shown are means $\pm$ S.D. for three separate experiments.

DISCUSSION

The present study was undertaken to determine the effects of a range of nutrients on PDX1 DNA-binding activity and on the activity of an insulin promoter construct. The results confirm that metabolism is required [19]. Thus non-metabolizable nutrients such as L-glucose, 3-O-methylglucose and galactose had no effect. The ability of pyruvate, glyceraldehyde and oxoisocaproate to stimulate PDX1 DNA-binding and insulin promoter activity emphasized the importance of mitochondrial metabolism. Those nutrients that activated PDX1 DNA-binding and insulin promoter activity all (with the exception of 2-deoxyglucose) shared the ability to act as insulin secretagogues. This prompted an investigation of the role of exogenous insulin and led to the observation that insulin activated PDX1 DNA-

binding and insulin promoter activity via activation of PtdIns 3-kinase and SAPK2.

Insulin mediates its cellular effects by binding to a plasma membrane receptor that undergoes autophosphorylation on tyrosine residues. The activated receptor then phosphorylates downstream effector molecules, such as IRS-1, IRS-2 and SHC [27]. β cells express the insulin receptor and IRS-1 [28,29]. However, the role of insulin in regulating β cell function, and insulin secretion in particular, is unclear. Although insulin has a feedback inhibitory effect on isolated islets of Langerhans and perfused pancreas [30–32], other studies have shown that insulin stimulates exocytosis (as measured amperometrically) and calcium uptake, suggesting a positive effect of insulin on insulin secretion [33]. Similarly, although previous studies indicate a negative feedback effect of insulin on insulin biosynthesis and gene expression [34], recent studies suggest that insulin exerts a stimulatory effect on insulin biosynthesis and endogenous insulin mRNA levels in β cells overexpressing the insulin receptor and in HIT-T15 β cells [35–37]. Particularly strong evidence for a role for insulin in regulating insulin secretion from β cells comes from studies in which the insulin receptor was specifically inactivated in the β cells of transgenic mice. This gene knockout resulted in a selective loss of insulin secretion in response to glucose [38].

Leibiger et al. [37] demonstrated that insulin stimulates the activity of a rat insulin I promoter construct (positions –411 to +1) and increases endogenous insulin mRNA levels in isolated rat islets of Langerhans and HIT-T15 cells. The effects of insulin were inhibited by LY294002, KN62 and rapamycin, but not by SB203580. This led to the conclusion that PtdIns 3-kinase, p70 S6 kinase and calmodulin-dependent protein kinase II, but not SAPK2, were involved. There are some similarities between the study of Leibiger et al. [37] and the results reported in the present paper, e.g. the effects of insulin are mediated via PtdIns 3-kinase in both studies. However, there are marked differences, in that the present study reports no effects of rapamycin, KN62 or calcium (results not shown). This discrepancy may be explained if, as reported by others [39], the effects of nutrients (and hormones) on the insulin promoter are dependent on the combined activity of a number of DNA sequences. These elements bind factors that, depending on the context, act in either a positive or a negative manner [40]. The net effect may be the consequence of several signalling pathways acting on different factors. The present study was designed to investigate specific effects on PDX1 and a human insulin upstream promoter fragment that contains four PDX1 binding sites placed adjacent to a heterologous promoter (thymidine kinase promoter). This experimental design clearly demonstrates that nutrients and insulin act on PDX1 DNA-binding activity and insulin promoter activity. The study of Leibiger et al. [37] was designed to address effects on endogenous insulin mRNA levels and more global effects on the insulin promoter. In this respect it is significant that, in the study of Leibiger et al. [37], insulin acted through E box-binding factors and not PDX1.

Interestingly, 2-deoxyglucose, which enters the cell and is phosphorylated but is not metabolized further, stimulated both PDX1 DNA-binding activity and insulin promoter activity. Similar effects of 2-deoxyglucose on transcription of the fatty acid synthase gene in adipose tissue and β cells have been reported previously [41,42]. On the basis of these findings, and of a direct correlation between glucose 6-phosphate concentration and mRNA levels, it was proposed that glucose 6-phosphate is the metabolic signal for the glucose-induced transcription of fatty acid synthase, L-type pyruvate kinase, acetyl-CoA carboxylase and S14 [43]. The present data suggest that glucose 6-phosphate may also be a metabolic signal for the insulin gene.

Similarly, the effects of xylitol could be explained by increased pentose phosphate pathway activity, with perhaps the intermediate xylulose 5-phosphate acting as a signalling metabolite [44], or through increased glycolytic metabolism resulting from the non-oxidative branch of the pathway. Studies are under way to determine whether the effects of insulin and glucose are additive [45], and to elucidate the mechanisms whereby glucose and other nutrients might activate PtdIns 3-kinase independently of insulin.

In conclusion, we show here that nutrients and insulin stimulate PDX1 DNA-binding activity and insulin promoter activity in isolated human islets of Langerhans and in MIN6 β cells. These results may explain the impaired ability of β cells to replenish insulin stores in type II diabetes resulting from reduced insulin or nutrient signalling in the β cell.

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