Distinct roles for insulin and insulin-like growth factor-1 receptors in pancreatic β -cell glucose sensing revealed by RNA silencing

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The importance of the insulin receptor (IR) and the insulin-like growth factor-1 receptor (IGF-1R) for glucose-regulated insulin secretion and gene expression in pancreatic islet β -cells is at present unresolved. Here, we have used small interfering RNAs (siRNAs) to silence the expression of each receptor selectively in clonal MIN6 β -cells. Reduction of IR levels by > 90 % completely inhibited glucose (30 mM compared with 3 mM)-induced insulin secretion, but had no effect on depolarization-stimulated secretion. IR depletion also blocked the accumulation of preproinsulin (PPI), pancreatic duodenum homoeobox-1 (PDX-1) and glucokinase (GK) mRNAs at elevated glucose concentrations, as assessed by quantitative real-time PCR analysis (TaqMan[®]). Similarly, depletion of IGF-1R inhibited glucose-induced insulin secretion but, in contrast with the effects of IR silencing, had little impact on the regulation of gene expression by glucose. More-

over, loss of IGF-1R, but not IR, markedly inhibited glucose-stimulated increases in cytosolic and mitochondrial ATP, suggesting a role for IGF-1R in the maintenance of oxidative metabolism and in the generation of mitochondrial coupling factors. RNA silencing thus represents a useful tool for the efficient and selective inactivation of receptor tyrosine kinases in isolated β -cells. By inhibiting glucose-stimulated insulin secretion through the inactivation of IGF-1R, this approach also demonstrates the existence of insulin-independent mechanisms whereby elevated glucose concentrations regulate PPI, PDX-1 and GK gene expression in β -cells.

Key words: diabetes, glucose, insulin, islet β -cell, small interfering RNA (siRNA).

INTRODUCTION

Elevated glucose concentrations stimulate insulin release from pancreatic islet β -cells, in large part by enhancing ATP synthesis [1] and closing K_{ATP} channels (ATP-sensitive K^+ channels) [2]. Plasma membrane depolarization [3] and the subsequent opening of voltage-sensitive (L-type) Ca^{2+} channels [4] then causes insulin-containing vesicles to fuse at the plasma membrane [5].

Both insulin and insulin-like growth factor-1 receptors (IR and IGF-1R respectively) have been proposed to have an important role in maintaining the β -cell phenotype and responsiveness to nutrients [6]. Each receptor is a class II tyrosine kinase with a highly similar, disulphide-linked tetrameric structure [7]. Binding of either insulin or IGF-1 to the cognate receptor leads to the phosphorylation of downstream IR substrates 1–4 (IRS1–IRS4) [8], homologous downstream of kinases 1–4 (DOK1–DOK4) [9], or Shc (Src homology and collagen-like proteins). Recruitment of Grb2 (growth-factor-receptor-bound protein 2) or PI3K (phosphoinositide 3-kinase) [10] then activates signalling pathways, leading to multiple intracellular effects involved in both the acute regulation of metabolism and longer-term effects on gene expression, differentiation or proliferation [11]. Despite engaging overlapping signalling pathways, the effects of insulin and IGF-1 in pancreatic β -cells appear, at least to some extent, to be non-redundant. Thus targeted disruption of the IR gene selectively in β -cells causes impaired glucose tolerance in mice [12], whereas disruption of the mouse IGF-1R gene leads to a more complex

phenotype, characterized by resting hyperglycaemia and abnormal glucose tolerance, but no significant changes in preproinsulin (PPI) gene expression or islet insulin content [13]. Although these mice show normal β -cell growth and development, glucose-stimulated insulin secretion is inhibited *in vivo* and *in vitro* [13,14], and the expression of the glucose transporter Slc2a2 (also known as Glut2) and glucokinase (GK) is reduced [14].

Previous data have suggested that secreted insulin may also have an important role in mediating the effects of glucose on both insulin secretion [15] and PPI gene expression [12,16]. Thus addition of insulin to cultured β -cell lines stimulates transcription of the PPI [17,18] and other genes [18–20]. Conversely, pharmacological suppression of insulin release eliminates the effects of elevated glucose concentrations on the PPI gene [17]. The extent to which these findings reflect a requirement for IR or IGF-1 in mediating the effects of glucose acutely, compared with a longer-term role in maintaining the expression of key β -cell ‘glucose-sensing’ genes (such as those encoding Slc2a2, GK etc.), is, however, uncertain. Moreover, a detailed analysis of the relationship between medium insulin concentrations and the expression of the proposed target genes is currently lacking.

To address these questions, we develop here the use of RNA silencing with small interfering RNAs (siRNAs) [21,22] as a novel approach to explore the roles of these receptors in β -cell nutrient sensing using the glucose-responsive insulin-secreting cell line, MIN6 [23–25]. We examine the importance of each receptor for (a) glucose metabolism and glucose-stimulated insulin secretion,

Abbreviations used: ACC, acetyl-CoA carboxylase; ARNO, ADP-ribosylation factor nucleotide opener; CMV, cytomegalovirus; C_T , cycle threshold; eGFP, enhanced green fluorescent protein; DMEM, Dulbecco’s modified Eagle’s medium; GK, glucokinase; IGF-1(R), insulin-like growth factor 1 (receptor); IR, insulin receptor; KRB, Krebs–Ringer bicarbonate medium; L-PK, L-type pyruvate kinase; PDX-1, pancreatic duodenum homoeobox-1; PIP₃, phosphatidylinositol 3,4,5-trisphosphate; PI3K, phosphoinositide 3-kinase; PPI, preproinsulin; PH, pleckstrin homology; ROI, region of interest; RT-PCR, reverse transcription-PCR; siRNA, small interfering RNA.

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and (b) the regulation by glucose of PPI, pancreatic duodenum homoeobox-1 (PDX-1) and GK genes. Whereas the loss of IGF-1R causes marked perturbations in mitochondrial ATP synthesis and blocks glucose-stimulated insulin secretion, depletion of IR inhibits insulin release without affecting ATP production. On the other hand, IRs, but not IGF-1Rs, are indispensable for the stimulation by glucose of the PPI, PDX-1 and GK genes, and inhibition of insulin secretion with diazoxide blocks completely the effects of glucose on PPI and PDX-1, but not GK, gene expression.

In summary, these studies indicate that released insulin, acting via IR, is likely to be an important mediator of the effects of glucose on PPI and PDX-1 gene expression in normal MIN6 β -cells. However, insulin-independent mechanisms are sufficient to allow the control by glucose of GK gene expression in wild-type cells, and the regulation of PPI and PDX-1 genes in the absence of IGF-1R.

MATERIALS AND METHODS

Materials

Silencer™ siRNA Construction Kit was from Ambion (Huntingdon, Cambs., U.K.). Primers for siRNA construction were from Cruachem (Glasgow, Scotland, U.K.). The TransIT™-TKO transfection reagent was from Mirus Corp. (Madison, NY, U.S.A.). Rabbit polyclonal anti-IR α -subunit and mouse monoclonal anti-IGF-1R α -subunit antibodies were from Santa Cruz Biotechnology (Heidelberg, Germany). Plasmid cDNA encoding enhanced eGFP (enhanced green fluorescent protein) fused to full-length human ARNO (ADP-ribosylation factor nucleotide opener) and Grp-1 (general receptor for 3-phosphoinositides) was prepared as described previously [26]. Tissue-culture reagents were from Sigma-Aldrich (Poole, Dorset, U.K.) or Gibco-BRL (Paisley, Renfrewshire, Scotland, U.K.). LIPOFECTAMINE 2000™ was from Invitrogen (Paisley, Renfrewshire, Scotland, U.K.). LY 294002 was from Calbiochem-Novabiochem (Nottingham, U.K.). Other reagents were from Sigma or BDH.

MIN6 cell culture

Cells were cultured in a humidified atmosphere at 37 °C with 5 % CO₂. MIN6 β -cells [23] were used between passages #19 and #30, and grown in DMEM (Dulbecco's modified Eagle's medium) containing 15 % (v/v) heat-inactivated foetal-calf serum, 25 mM glucose, 5.4 mM KCl, 2 mM glutamine, 100 mM 2-mercaptoethanol, 100 units · ml⁻¹ penicillin and 100 μ g · ml⁻¹ streptomycin. MIN6 cells were seeded on to poly(L-lysine)-coated coverslips before microinjection or transfection with LIPOFECTAMINE 2000™ or TransIT™-TKO (siRNA). Transfected cells were cultured in normal medium for 48 h, and cultured further in medium containing 3 mM glucose for 16 h before experiments. For assays of insulin secretion, cells were seeded in six-well plates (Falcon, Lincoln Park, NJ, U.S.A.), and grown to 70 % confluence before transfection with 1.0 pg of siRNA using TransIT™-TKO transfection reagent, according to the manufacturer's instructions. Cell culture was continued for 48 h in DMEM containing 25 mM glucose, and then at 3 mM glucose for a further 16 h.

Construction of siRNAs

siRNAs were generated using the Ambion Silencer™ siRNA Construction Kit, according to the manufacturer's protocol. Starting primer pairs were designed as suggested in the Ambion proto-

col, and according to published guidelines [21,27] in order to optimize the GC content and to avoid regions rich in secondary-structure (hairpin loops etc.). Target sequences were derived from the complementary DNA sequences of mouse IR and IGF-1R (accession numbers NM010568 and AB006442 respectively). Control siRNAs were generated using primers derived by scrambling the target siRNA sequences. All primer sequences were subjected to BLAST searches to ensure that there were no matches with known sequences of other genes. Sense and antisense primers corresponding to the following target and control sequences were used: IR-target (5'-AACGAGGAATGTGGGGA-TGTC-3', corresponding to nt 583–603 of mouse IR mRNA), IR-control (5'-AACGAGTGGGTCGGAGGATAT-3'), IGF-1R-target (5'-AATCTCAAGGATATTGGCCTTT-3', corresponding to nt 415–436 of mouse IGF-1R mRNA) and IGF-1R-control (5'-AAGGAATCTCAGGACTTTTGT-3'). Each was extended with the sequence 5'-CCTGTCTC-3' at the 3' end. The designed IGF-1R target siRNA pair was found to decrease IGF-1R protein levels with a potency similar to that of a recently described RNA dimer, named 'R2', which was based on a 21-mer targeting nt 612 of human IGF-1R [28].

Western (immuno)blotting

Cells were washed twice in ice-cold PBS, scraped into ice-cold lysis buffer [PBS, 1 % (v/v) Triton X-100, 5 μ g · ml⁻¹ pepstatin, 5 μ g · ml⁻¹ antipain, 5 μ g · ml⁻¹ leupeptin, 2 mM benzamidin and 0.5 mM dithiothreitol] and vortex-mixed. Protein content was assayed using a BCA (bicinchoninic acid) protein assay kit (cat. no. 23225; Pierce, Rockford, IL, U.S.A.), against BSA Type V (Sigma) standards. Total protein extracts (25 μ g) were resolved by SDS/PAGE [10 % (w/v) acrylamide gels] and transferred to nitrocellulose membranes, followed by immunoblotting at the antibody concentrations shown in the Figure legends. Secondary antibodies (1:10 000 dilutions) were revealed using BM Chemiluminescence blotting substrate (Roche Diagnostics, Lewes, East Sussex, U.K.).

Laser-scanning confocal microscopy

To image cells transfected with the eGFP constructs, MIN6 cells were incubated in Krebs–Ringer bicarbonate buffer (KRB) comprising 132.5 mM NaCl, 3.6 mM KCl, 0.5 mM NaH₂PO₄, 0.5 mM MgSO₄, 1.5 mM CaCl₂, 10 mM Hepes and 2 mM NaHCO₃, pH 7.4, pre-equilibrated with 95 % O₂/5 % CO₂. Confocal microscopy was performed with a Leica DM IRBE inverted confocal microscope (488 nm excitation wavelength) controlled with TCS-NT4 software (Leica, Milton Keynes, U.K.). ARNO-eGFP and Grp-1-eGFP translocation to the plasma membrane was quantified in the regions of interest (ROIs) as described in legends. The level of eGFP intensity within ROIs was determined by TCS-NT software, the ratio of membrane-localized eGFP fluorescence was expressed as a percentage of total cellular eGFP fluorescence intensity. Mean data are presented from 20–100 individual cells (from three to six separate experiments in each case).

Measurement of total and free ATP concentrations

Total cellular ATP content was measured as described previously [29] after cell lysis in 10 % (v/v) HClO₃. Free ATP concentrations were measured after expression of recombinant targeted luciferases by microinjection of a plasmid driving humanized firefly luciferase expression under the CMV (cytomegalovirus) promoter

with no further targeting information (pCMV-Luc_{FF}) or bearing the mitochondrial targeting sequence of cytochrome *c* oxidase subunit VIII (pCMV-mitoLuc_{FF}) [1].

Single-cell promoter assays

Intranuclear microinjection of plasmids and siRNA was performed using an Eppendorf 5121/5246 micromanipulator [30,31] at plasmid concentrations of 0.1 (pINS-Luc_{FF}) and 0.05 (pCMV-RL) mg · ml⁻¹ [32], and siRNA at 0.5 pg · ml⁻¹. Individual experiments involved injection of 100–200 separate cells per condition, with an efficiency of 5–20 % productive injection, as assessed by expression of *Renilla reniformis* luciferase activity. MIN6 cells were imaged at 48 h and 6 h respectively, after microinjection and culture under the conditions described above. Photon-counting imaging of firefly and *R. reniformis* luciferase activities were performed in single living cells using an Olympus IX-70 inverted microscope (10× air objective, 0.4 numerical aperture) and a triply intensified charge-coupled device camera (Photek, Lewes, East Sussex, U.K.) as described previously [25,31,33].

Real-time PCR (TaqMan®)

Total RNA was isolated by cell lysis in TRI Reagent (Sigma) and first-strand cDNA synthesis was performed using 2 µg of total RNA using 2.5 µM random hexamer primers and Moloney-murine-leukaemia virus reverse transcriptase (2.5 units · µl⁻¹) in buffer containing 5 mM MgCl₂, 1 mM dNTPs and 20 µl of 10 × Buffer II, as supplied by Applied Biosystems (Warrington, Cheshire, U.K.). Primers for PCR were designed using Primer Express™ (Applied Biosystems) software. Real-time PCR was performed using 25 ng of reverse-transcribed total RNA with sense and antisense primers (5 µM), 5 µM TaqMan® probe labelled at the 5' and 3' ends respectively with 6-carboxyfluorescein (FAM) and 6-carboxy-*N,N,N',N'*-tetramethylrhodamine (TAMRA), 12.5 µl of a QuantiTect™ master mix containing Hot Star Tag® DNA polymerase, and Quantitect™ probe PCR buffer, as supplied by Qiagen (QuantiTect™; cat. no. 204343) in a total volume of 25 µl and using a DNA Engine Opticon™ 2 System (MJ Research Inc.). Standard curves were constructed by amplifying serial dilutions of untreated MIN6 cDNA (50 ng–0.64 pg) and plotting cycle threshold (C_T) values as a function of starting reverse-transcribed RNA, the slope of which was used to calculate relative expression of the target gene. The C_T value is the cycle number at which the reporter fluorescence generated by cleavage of the probe passes a fixed threshold above baseline, and is defined as the average intensity over a cycle range of 1–10, plus the standard deviation of this value.

Assay of insulin secretion

Cells were washed in PBS and incubated in KRB medium containing either low (3 mM) or high (30 mM) glucose concentrations. Incubations were performed for 20 min at 37 °C in a shaking water bath. Secreted and total insulin were measured by radioimmunoassay [24] using a commercially available kit (purchased from Linco, St Charles, MO, U.S.A.), with an identical affinity for mouse and pig insulin.

Statistical analysis

Data are given as means ± S.E.M. for at least three separate and independent experiments. Comparisons were performed by one-tailed Student's *t* test with Microsoft Excel.

Figure 1 Silencing of IR and IGF-1R expression in MIN6 cells

Cells were incubated with the indicated siRNAs before extraction, SDS/PAGE and immunoblotting with the primary antibodies indicated, each at 1:1000 dilution. Similar data were obtained in two further independent experiments (see the Materials and methods and Results sections for further details). No statistically significant effects of siRNAs on the levels of the non-cognate receptor were apparent (IR immunoreactivity in the presence of anti-IGF-1R siRNA, 99 ± 0.4 % of control; IGF-1R immunoreactivity in the presence of anti-IR siRNA, 98 ± 0.4 % of control; $n = 3$).

RESULTS

Silencing of the IR or the IGF-1R inhibits glucose-stimulated insulin secretion

Culture of MIN6 cells for 48–72 h with specific siRNAs (see the Materials and methods section) depleted IR protein levels by 91 ± 1.2 % ($n = 3$, where n refers to the number of independent experiments), but had no effect on IGF-1R levels (Figure 1). Similarly, treatment with siRNAs developed against the IGF-1R depleted protein levels of this receptor by 84 ± 2 % with respect to control cells ($n = 3$), without affecting IR protein levels (Figure 1).

In order to confirm the loss of ligand-stimulated signalling downstream of each receptor, we monitored the regulation of PI3K activity in control and siRNA-treated cells. Since PI3K activity in MIN6 cell extracts was below the level of detection by conventional biochemical assay [34] (I. Rafiq, K. Venkateswarlu and G. A. Rutter, unpublished work), we assessed PI3K activity in single MIN6 cells by following the recruitment to the plasma membrane of the phosphatidylinositol 3,4,5-trisphosphate (PIP₃)-binding protein ARNO fused to eGFP [26,35] (Figure 2). When imaged by laser-scanning confocal microscopy, ARNO-eGFP was distributed evenly throughout the cytoplasm in cells maintained at 3.0 mM glucose and in the absence of added insulin (Figure 2a). By contrast, treatment of MIN6 cells expressing ARNO-eGFP for 1 h (results not shown) or 6 h (Figure 2) with 20 nM insulin or 16.0 mM (compared with 3 mM) glucose significantly increased the ratio of plasma-membrane-bound compared with cytosolic ARNO-eGFP (Figures 2a and 2b), reflecting the accumulation of membrane-associated PIP₃. Complete blockade of the effects of either insulin or elevated glucose concentrations on PIP₃ production was observed in cells treated with IR siRNA (Figures 2a and 2b). In contrast, silencing of IGF-1R had no impact on the response to either stimulus (Figure 2c), but eliminated the effects of 10 nM IGF-1 (Figure 2d), as expected.

Culture of cells with siRNAs analysed with either the IR or IGF-1R had no effect on total cellular insulin content (see the legend to Figure 3). However, deletion of the IR led to a complete inhibition of glucose-stimulated insulin secretion when cells were incubated in full culture medium for 6 h (Figure 3a) with 30 mM compared with 3.0 mM glucose. By contrast, IGF-1R silencing (Figure 3a) led to a small but significant increase in basal insulin

Figure 2 IR and IGF-1R inactivation blocks downstream activation of PI3K only in response to the cognate ligand

(a) Translocation of ARNO-eGFP in response to glucose or insulin in single MIN6 cells treated with scrambled RNAs only (panels labelled 'Control'), or cells treated with interfering RNAs to diminish expression of either IR (panels labelled 'IR siRNA') or IGF-1R (panels labelled 'IGF-1R siRNA'). Cells expressing ARNO-eGFP were incubated for 6 h in DMEM-based medium containing the additions shown, before transfer to the confocal microscope and imaging (see the Materials and methods section). The scale bar represents 3 μ m. (b–d) Histograms show the quantification of data from three to five separate experiments involving at least 50 individual cells in each case. (c, d) As in (a), but after treatment with scrambled or anti-IGF-1R siRNAs. glc, glucose.

release (at 3.0 mM glucose), but completely suppressed the further stimulation of insulin secretion at 30 mM glucose (Figure 3a).

To determine whether a defect in a late stage of insulin exocytosis, reflecting a decrease in the number of vesicles in a 'secretion-competent' pool [5], might explain the loss of glucose-stimulated insulin secretion in IR-depleted cells, we monitored the effects of depolarizing the cells with a 20 min incubation in 30 mM KCl. Under these conditions, increases in intracellular free $[Ca^{2+}]$ occur independently of glucose metabolism, and lead to the fusion of secretory vesicles at the cell membrane [5]. The effects of receptor silencing on glucose-stimulated insulin secretion were fully evident during this shorter incubation period (Figure 3b), indicating that deletion of IR or IGF-1R affects both the first and the sustained phases of insulin release from these cells [36]. By contrast, KCl-stimulated insulin secretion was unaffected (Figure 3b), demonstrating that a marked change in the number

Figure 3 Regulation of insulin secretion in IR and IGF-1R siRNA-treated cells

(a) MIN6 cells previously treated with the siRNA duplexes shown were incubated in DMEM-based culture medium (see the Materials and methods section) for 6 h at either 3 or 30 mM glucose. The total cellular insulin contents were not significantly different under any of the conditions used (148 ± 2.98 , 145 ± 3.41 , 153 ± 2.75 ng $\cdot$ 300 000 cells $^{-1}$ for control, IR and IGF-1R siRNA treatments respectively; $n = 3$). (b) siRNA-treated cells were incubated in KRB medium containing the additions shown for 20 min, prior to the assay of released and total cellular insulin by radioimmunoassay (see the Materials and methods section). glc, glucose.

or exocytotic competence of secretory vesicles did not explain the effects of receptor deletion on secretion.

Released insulin has been proposed to stimulate the further exocytotic release of the hormone in a positive feedback loop [15,16]. To determine whether the impact of IR depletion may be due to a loss of insulin's ability to further potentiate its own release, we monitored the capacity of added insulin to stimulate release of the endogenous hormone from wild-type cells. We calculated the release of endogenous hormone by subtracting the contribution of the added hormone from the total measured insulin released. At 3 mM glucose, addition of 0.3 nM exogenous insulin slightly increased the calculated release of endogenous insulin (from ≈ 0.05 to ≈ 1.0 nM; Figure 4), whereas 1.0 nM added insulin had no effect (Figure 4). Similarly, addition of either 0.3 or 1.0 nM insulin had no effect on calculated insulin secretion at 30 mM glucose (Figure 4). Thus the glucose-stimulated (30 compared with 3 mM) increment in insulin release was similar (approx. 0.25, 0.15 and 0.25 nM) in the presence of 0, 0.3 and 1.0 nM exogenous insulin respectively. Extracellular insulin therefore seems likely to have only a minor role, if any, in controlling glucose-stimulated insulin secretion from MIN6 cells.

As expected, both basal and glucose-stimulated insulin secretion were eliminated in the presence of the opener of ATP-sensitive K^+ channels, diazoxide (Figure 4).

Effects of IR and IGF-1R depletion on intracellular [ATP]

To explore the effects of down-regulating IR and IGF-1R on glucose metabolism, we measured the total cellular ATP content

Figure 4 Effect of exogenously added insulin on the release of endogenous insulin

Cells were incubated in KRB medium at the indicated glucose and added pig insulin concentrations, in the presence or absence of diazoxide as shown. The total measured insulin concentration (added plus released) in the medium at the end of the 6 h incubation is given.

of cells incubated at low or high glucose concentrations for 60 min. As expected, the ATP content was significantly increased at 30 compared with 3 mM glucose in control cells. Whereas this increase was unaltered in IR-depleted cells (Figure 5a), in IGF-1R siRNA-treated cells basal ATP levels were significantly elevated, and 30 mM glucose caused a paradoxical decrease in ATP content (Figure 5a). These changes in total cellular ATP were also associated with alterations in free cytosolic and mitochondrial ATP concentrations, when these were analysed over a shorter time course in single cells expressing recombinant targeted firefly luciferases [1]. Thus bioluminescence imaging revealed that a step increase from 3 to 30 mM glucose caused an increase in free [ATP] both in subcellular compartments in control cells and in IR-depleted cells (Figures 5b and 5c). By contrast, IGF-1R siRNA treatment inhibited increases in cytosolic free ATP concentration provoked by 30 mM glucose (Figure 5b) and, in these cells, a marked decrease in mitochondrial ATP concentration to almost undetectable levels was observed after the addition of 30 mM glucose (Figure 5c).

Effects of IR and IGF-1R silencing on glucose-regulated PPI gene expression

To assess PPI gene transcription in single MIN6 cells we used a firefly luciferase reporter construct encoding the proximal 200 nt of the human insulin gene promoter and including A3, A2 and E-box regulatory elements (PPI-Luc_{FF}) [37]. This construct was introduced into MIN6 cells by plasmid microinjection [33], and its activity, normalized to that of the constitutive CMV promoter driving the expression of a distinct luciferase (*R. reniformis*), was followed by photon-counting imaging [31,33]. Exposure of untreated cells to 30 mM glucose or 20 nM insulin for 6 h strongly activated the PPI promoter with respect to cells maintained at 3 mM glucose (Figure 6a). In contrast, co-microinjection of cells with siRNAs directed against the IR led to a complete loss of the effect of added insulin, and a decrease in the stimulation of PPI promoter activity by 30 mM glucose (Figure 6a). By contrast, neither 30 mM glucose nor treatment with IR siRNA had any significant effect on the apparent activity of firefly luciferase expressed under the CMV promoter (Figure 6b), indicating that the small changes in total ATP content provoked by the sugar after 60 min (Figure 5a), and presumably the cytosolic free ATP concentration, were no longer significant after 6 h incubation. By

Figure 5 Effect of IR and IGF-1R inactivation on glucose-stimulated increases in (a) total or (b, c) free intracellular [ATP]

(a) Cells treated with the RNA duplexes indicated were incubated for 60 min in DMEM-based medium containing the additions shown, prior to extraction and assay of ATP as described (see the Materials and methods section). Changes in free ATP concentration were estimated by monitoring in real time the luminescence of individual cells expressing pCMV-Luc_{FF} (b) or pCMV-mitoLuc_{FF} (c) by photon-counting imaging. Cells were initially incubated in KRB medium containing 3 mM glucose prior to addition of 30 mM glucose, as indicated. Data in (b) and (c) are given as the fold increase in luminescence compared with pre-stimulatory values at 3 mM glucose, and are the means for three separate experiments involving at least 90 separate cells. Statistical differences between IGF-1R-treated and control cells are indicated, and were calculated by Student's *t* test.

contrast, large changes in ATP content and cytosolic free ATP concentration elicited by IGF-1R inactivation (Figures 5a and 5b) were apparent using the CMV luciferase report construct (results not shown), precluding the use of this firefly luciferase-based reporter assay for the measurement of IGF-1R-dependent changes in PPI promoter activity.

Effects of IR and IGF-1R depletion on the regulation by glucose and insulin of PPI mRNA levels

We next monitored the impact of IR and IGF-1R inactivation on the expression of the endogenous PPI gene. Assessed by quantitative real-time reverse transcription-PCR (RT-PCR) assay, depletion of the IR had no effect on basal levels of PPI mRNA (relative

Figure 6 Effect of IR inactivation on PPI promoter activity

Cells were microinjected with plasmid pINS-Luc_{FF} (a) or pCMV-Luc_{FF} (b) plus pCMV-RL (see the Materials and methods section) plus the indicated siRNAs or scrambled oligonucleotides ($1.0 \text{ pg} \cdot \text{ml}^{-1}$) prior to culture for 48 h at 25 mM glucose, and then 16 h at 3.0 mM glucose. Cells were then incubated further at the indicated glucose or added insulin concentrations for 6 h prior to imaging firefly and *R. reniformis* luciferase activities on a photon-counting microscope (as described in the Materials and methods section). The scale bar in (a) represents 20 μm .

to cyclophilin mRNA), but inhibited the approx. 5-fold increase in PPI mRNA levels provoked by a 6 h incubation of 30 mM compared with 3.0 mM glucose (Figure 7a), and fully reversed the increase in PPI mRNA provoked by 20 nM insulin in the presence of 3.0 mM glucose. (In three separate experiments, the PPI:cyclophilin mRNA ratio in control cells was 3.91 ± 0.01 and 10.97 ± 0.02 , $P < 0.01$, at 0 and 20 nM added insulin respectively; corresponding values for IR siRNA-treated cells were 3.35 ± 0.09 and 3.47 ± 0.10). By contrast, loss of IGF-1R had no effect on PPI mRNA levels at 3 mM glucose, and potentiated the increases at 30 mM glucose (Figure 7a). Under these conditions, addition of insulin (20 nM) augmented the PPI mRNA level at 3 mM glucose, but reduced the level of this mRNA at 30 mM glucose (Figure 7a). Similar, but more marked, effects were observed at each glucose concentration after the addition of 10 nM IGF-1 (Figure 7a).

Role of secreted insulin release in the effects of glucose on PPI gene expression

If secreted insulin is the principal mediator of the effects of glucose on the expression of the PPI and other β -cell genes, a close correlation should exist between the concentration of insulin in the medium and the PPI mRNA content under different conditions. As shown in Figure 7(b), an elevation of the glucose concentration from 3 to 30 mM caused a parallel increase in both PPI mRNA and the insulin concentration in the medium at the end of the incubation. Under the culture conditions used, the measured insulin concentrations were in the physiological range

of 60 to 300 pM. Inactivation of the IR or the addition of the ATP-sensitive K^+ channel-opener diazoxide (see Figure 4), which has no effect on glucose metabolism in MIN6 cells [25] but prevents glucose-stimulated insulin release [38], blocked the increment in PPI mRNA (Figure 7b). Thus under six different experimental conditions (control, IR siRNA, diazoxide; incubation in each case at either 3 or 30 mM glucose; shown in Figure 7b by the unbroken line), the insulin concentration in the medium and PPI mRNA levels were strongly correlated ($r^2 = 0.9933 \pm 0.0003$; unbroken line). By contrast, the deletion of the IGF-1R or the addition of IGF-1 led to a breakdown in the relationship between [insulin] in the medium and PPI mRNA content (Figure 7b, broken lines). Thus, in cells in which IGF-1R expression was silenced, PPI mRNA was strongly induced by 30 mM compared with 3 mM glucose, whereas the concentration of insulin in the medium was unchanged. Conversely, in control (IGF-1R positive) cells incubated in the presence of 10 nM IGF-1, the same elevation of glucose concentration led to a significant decrease in PPI mRNA levels, whereas insulin concentrations in the medium were slightly increased with respect to control cells at both 3 and 30 mM glucose (Figure 7b).

Effects of IR and IGF-1R depletion on glucose- and insulin-induced changes in PDX-1 and GK mRNA levels

PDX-1 is a homoeodomain transcription factor essential for normal pancreatic development [39] and the expression of the PPI

Figure 7 Effects of IR and IGF-1R depletion on glucose-, insulin- and IGF-1-dependent changes in PPI mRNA levels

(a) Cells were treated with the indicated siRNAs for 48 h prior to incubation for 16 h at 3 mM glucose, and then at the glucose concentrations shown for a further 6 h. Total RNA was extracted and PPI mRNA was measured by quantitative RT-PCR (as described in the Materials and methods section) and normalized to cyclophilin mRNA content. The latter was unaltered under any of the conditions tested (results not shown). (b) Correlation between insulin concentration in the medium and cellular PPI mRNA levels, normalized to cyclophilin mRNA content. Insulin was measured in the medium at the end of the incubation by radioimmunoassay. Data are taken from the experiments shown in (a) or from separate experiments, and the arrows indicate the effect of increasing the glucose concentration from 3 to 30 mM. Where added, the concentrations of IGF-1 and diazoxide were 10 nM and 200 μ M, respectively. The error bars show the S.E.M. in each case.

gene [40]. In common with the PPI gene, the induction of PDX-1 mRNA by 30 mM compared with 3 mM glucose was completely suppressed by deletion of IR, and mimicked by the addition of 20 nM insulin, but not by 10 nM IGF-1 (Figure 8a). PDX-1 mRNA levels were thus closely correlated to the concentration of insulin in the medium for control cells in the presence or absence of diazoxide, or after deletion of IR ($r^2 = 0.982 \pm 0.0027$; Figure 8a, unbroken line). In common with the PPI gene, induction of PDX-1 mRNA was unaffected by deletion of IGF-1R, indicating that glucose-stimulated insulin release was not required for the increase in gene expression under these conditions. However, in contrast with the PPI gene, induction of PDX-1 mRNA by 30 mM glucose was still observed in the presence of added IGF-1 (Figure 8a).

GK catalyses the first committed step in β -cell glycolysis, and is important in setting the flux of glucose carbon through this pathway [41]. Consequently, changes in GK expression may contribute to the effects of IGF-1R depletion on glucose-stimulated ATP accumulation (Figure 5). Since GK gene expression has recently been shown to be regulated by glucose in β -cells [17] via a mechanism involving the IR B-isoform [42], we explored here the impact of IR and IGF-1R depletion on the control of GK mRNA levels by glucose. The induction of GK mRNA by 30 mM compared with 3.0 mM glucose was eliminated by the deletion of IR, or the addition of diazoxide, and fully

Figure 8 Effects of IR and IGF-1R depletion on glucose-, insulin- and IGF-1-dependent changes in (a) PDX-1 and (b) GK mRNA levels

Measurements of PDX-1 and GK mRNA, and of the insulin content in the medium were performed on the same samples as those used to analyse PPI mRNA (see Figure 7, where other details are given). The unbroken line shows the fit of the correlation analysis for control, IR siRNA and diazoxide conditions (see the Results section for further details).

mimicked by the addition of 20 nM insulin (Figure 8b). However, the extent of the correlation between GK mRNA levels and released insulin in control, IR siRNA- or diazoxide-treated cells was lower than for PPI or PDX-1 mRNAs ($r^2 = 0.857 \pm 0.0017$). Furthermore, and in contrast with the former genes, the induction of GK mRNA was markedly suppressed by deletion of IGF-1R (Figure 8b). 10 nM IGF-1 had no effect on GK mRNA levels at either high or low glucose concentrations (Figure 8b).

Effects of insulin and glucose on gene expression in the presence of diazoxide

The persistence of glucose-regulated PPI and PDX-1 gene expression after the blockade of glucose-stimulated insulin secretion following IGF-1R deletion was unexpected, given that inhibition of secretion with diazoxide suppressed the induction by glucose of both genes (Figure 7b and Figure 8a). A possible explanation for this discrepancy is that insulin levels in the medium above cells depleted of IGF-1R are permissive for the regulation of

Figure 9 Regulation of endogenous PPI, PDX-1 and GK gene expression by glucose and insulin in the absence or presence of diazoxide

Cells previously cultured for 16 h at 3 mM glucose were incubated for 6 h in the presence (b, d and f) or absence (a, c and e) of 200 μ M diazoxide as shown, and at the indicated concentrations of glucose. Insulin (0.3, 1.0 or 20 nM) was added to give the final measured concentrations of hormone (added plus secreted) as shown. Extraction of RNA and quantification of PPI, PDX-1, GK and cyclophilin mRNAs by quantitative RT-PCR was performed as described in the Materials and methods section.

gene expression by glucose. To investigate this possibility, we inhibited both basal and glucose-stimulated insulin secretion with diazoxide (Figure 4), and measured the induction by 30 mM glucose of PPI, PDX-1 and GK mRNAs over a range of added insulin concentrations (Figure 9). In the absence of diazoxide, 30 mM glucose robustly stimulated expression of the PPI gene, as described above, and this effect was mimicked at the level of 3 mM glucose by adding exogenous insulin to give the total (added plus secreted) concentrations of the hormone shown (Figure 9a). However, in the presence of diazoxide, the effects of 30 mM glucose on PPI mRNA levels were eliminated, and were not restored by the addition of insulin over the range 0.3–20 nM (Figure 9b). Similar behaviour was also observed for the PDX-1 gene (Figures 9c and 9d), although some potentiation of the effects of 30 mM glucose was evident in the presence of 1.0 nM, but not 20 nM, insulin (Figure 9d). In contrast, GK gene expression was still significantly stimulated by 30 mM glucose in the presence of diazoxide, or in the presence of diazoxide plus approx. 300 pM insulin, but was lost at insulin concentrations > 1.0 nM (Figures 9e and 9f).

DISCUSSION

Expression of IR and IGF-1R is required for glucose-stimulated insulin secretion

We show here that RNA silencing can be used as an efficient and selective method for suppressing the expression of IR or IGF-1R in clonal MIN6 β -cells, consistent with findings using a range of other siRNA constructs in this cell type (G. A. Rutter, G. da Silva Xavier, A. Varadi and K. J. Mitchell, unpublished work), and with a recent assessment of the genome-wide impact of silencing stably expressed green fluorescent protein expression in human embryonic kidney cells [43]. In the present study, silencing of the IR or the IGF-1R by > 90% or approx. 85% respectively did not detectably affect the expression of the non-cognate receptor in MIN6 cells. The gene-silencing approach thus provides clear advantages with respect to the use of antisense RNAs, as used previously to reduce IGF-1R expression in human melanoma cells [44], in terms of both efficacy and selectivity. Importantly, we also show in the present study that downstream signalling by each receptor to PI3K was inhibited only in response to the corresponding ligand (Figure 2). Interestingly, the ability of elevated glucose concentrations to stimulate PI3K activity was lost after elimination of IR, implicating the activated release of insulin as the mechanism responsible for the effects of glucose. On the other hand, elevated glucose concentrations still stimulated PI3K activity in the absence of IGF-1R, a condition in which insulin secretion was not stimulated (see below). The latter findings suggests that IR activation is unlikely to explain the stimulation of PI3K activity by glucose in cells in which IGF-1R expression is suppressed, but instead implies the existence of an alternative, intracellular signalling pathway, or alternatively the secretion of an unknown regulatory factor.

The results shown in Figure 5 also demonstrate that the loss of IR has no impact on glucose-stimulated ATP synthesis, whereas IGF-1R depletion had marked effects on this parameter. Since the acute addition of IGF-1 had relatively little effect on insulin secretion when added acutely to cells (results not shown), the most likely explanation for these findings may be that the IGF-1R, but not IR, is required during the culture of cells for the preserved expression of key metabolic enzymes. However, IR, but not IGF-1R, silencing strongly suppressed the expression of PDX-1 mRNA examined at 30 mM glucose, a glucose concentration close to that used during the silencing of the receptor (25 mM; see the Materials and methods section). This loss of PDX-1 expression in IR-depleted cells was not associated with any apparent decrease in oxidative glucose metabolism (Figure 5), consistent with an effect distal to the generation of mitochondrial coupling factors. Correspondingly, suppression of PDX-1 activity has no effect on glucose metabolism in INS-1 β -cells [45], despite decreasing the expression of several key metabolic enzymes (e.g. GK, Slc2a2 and liver-type pyruvate kinase) and other β -cell genes in several models of β -cell PDX-1 deficiency [46–48].

At present, the identity of the gene products affected by IGF-1R silencing in MIN6 cells and responsible for the marked suppression of glucose metabolism is unknown. Whereas GK mRNA levels were diminished by approx. 80% in IGF-1R-depleted MIN6 cells (Figure 8b), consistent with the decrease observed in mice deleted for IGF-1R selectively in β -cells ($\approx$ 50%) [14], a reduction in GK activity seems unlikely to explain the effects on metabolism in these cells. Thus GK mRNA levels were reduced more markedly (> 90%; Figure 8b) in IR-depleted cells, whereas the latter showed no change in glucose-induced ATP increases compared with wild-type cells (Figure 5). Given the profound effect of IGF-1R silencing on glucose-induced changes in mitochondrial ATP production (Figure 5c),

it is conceivable that enzymes involved in oxidative metabolism may be affected by IGF-1R silencing. Other possibilities include changes in *Slc2a2* expression [14] or the expression of voltage-sensitive Ca^{2+} channel subunits, recently reported as a target for regulation by IGF-1 [49]. It might be noted that the expression of a number of candidate genes involved in the distal steps of exocytosis, including those encoding VAMP2 (vesicle-associated membrane protein 2), NSF (*N*-ethylmaleimide-sensitive fusion protein), Mss-4 and Munc18, were all unaffected in islets from mice deleted for IGF-1R [13], consistent with the absence of any effect on K^{+} -stimulated secretion, as observed in the present studies (Figure 3b).

Role of IR and IGF-1 in the regulation of gene expression by glucose

We show here that silencing of IR expression in β -cells has marked effects on the ability of glucose to regulate the expression of target genes. Moreover, we demonstrate that PPI mRNA levels are closely correlated with the concentration of insulin in the medium under three experimental conditions (control, IR suppression and diazoxide; Figure 7b). On the other hand, we noted that the concentrations of exogenously added insulin required to elevate PPI mRNA levels at 3 mM glucose (≥ 1 nM) exceed those which were found to maximally stimulate PPI gene expression at 30 mM glucose (Figure 9a). The apparent ability of 30 mM glucose to increase PPI mRNA levels to a greater extent than could be achieved by the addition of an equivalent concentration of insulin (≤ 300 pM; Figure 9a) might seem at first to suggest that the action of glucose cannot be explained by the release and rebinding of insulin. However, an alternative explanation is that there is an apparent 'left shift' in the dose-response to endogenous compared with exogenous insulin, since the concentrations measured in the bulk medium may not necessarily represent those at the cell surface. Such a scenario might result from the formation of a gradient of insulin concentration leading from sites of exocytosis at the cell surface. In practice, this gradient might only need to be a few molecules deep, given the likely close proximity (a few microns) between release sites at the cell surface, and IRs. Taken together, these results support the conclusion [12,17,18] that the effects of glucose on the induction of the PPI gene are likely, under many circumstances, to be attributable to the actions of secreted insulin.

Nevertheless, this study does reveal that, under certain experimental conditions, control of PPI gene expression by glucose can be achieved by mechanisms that are independent of secreted insulin. Thus inactivation of IGF-1R, and the abolition of glucose-stimulated insulin release, had no impact (or slightly potentiated) the effects of glucose on PPI mRNA levels (Figures 7a and 7b). Since addition of IGF-1, at a concentration (10 nM) unlikely to affect the IR, inhibited glucose-stimulated increases in the PPI mRNA level (Figure 6a) while having little effect on insulin secretion (Figure 6b), it seems possible that IGF-1R activity normally represses an intracellular signalling pathway through which glucose is able to transmit signals directly to the machinery controlling PPI gene transcription. The nature of this pathway is obscure, but may involve changes in the concentration of an intracellular metabolite of glucose, such as glucose 6-phosphate [50] or xylulose 5-phosphate [51,52].

Regulation of PDX-1 and GK gene expression by glucose and insulin

The responses to glucose and insulin of PDX-1 mRNA broadly paralleled those of PPI (Figures 7 and 8), consistent with glucose-

and insulin-induced increases in PDX-1 promoter activity reported recently in MIN6 cells ([53] and Q. Quan and G. A. Rutter, unpublished results). By contrast, the response of the GK gene to glucose and insulin revealed some interesting differences with respect to the other two genes examined in this study. First, the correlation between the concentration of released insulin and cellular GK mRNA levels was weaker than that for the PPI or PDX-1 genes, consistent with regulation by glucose via both released insulin and a more direct, intracellular signalling pathway. Correspondingly, elevated glucose concentrations provoked a significant increase in GK mRNA, even in the presence of diazoxide, and complete absence of added insulin ([insulin] in the medium < 100 pM; Figure 9f). Finally, the increase in GK mRNA in response to 30 mM glucose was still evident, though reduced in magnitude, after depletion of IGF-1R (Figure 8b).

Conclusions

These studies provide unexpected findings with respect to the role of the IR and IGF-1R in modulating the responses of pancreatic β -cells to glucose. Both the IR and IGF-1R are shown to be indispensable for glucose-stimulated insulin secretion, whereas only the IR is necessary for transcriptional responses to the sugar. Paradoxically, however, the acute release and rebinding of insulin to the IR was not essential for the actions of glucose on PPI or PDX-1 gene expression, as revealed by the conserved responses to glucose in cells that were depleted of IGF-1R, and thus were unable to respond to glucose with enhanced insulin release. Similarly, whereas the GK gene is responsive to insulin in MIN6 cells, consistent with recent findings in other β -cell lines [19], insulin-independent signalling mechanisms are able to transduce the effects of glucose to this gene when insulin secretion is suppressed. Thus a complex interplay exists between insulin-dependent and -independent signalling pathways in the control of β -cell genes by glucose, the relative contributions of which are gene-specific and dependent on the presence of both the IR and IGF-1R.

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