

Stimulation of pancreatic β -cell proliferation by growth hormone is glucose-dependent: signal transduction via Janus kinase 2 (JAK2)/signal transducer and activator of transcription 5 (STAT5) with no crosstalk to insulin receptor substrate-mediated mitogenic signalling

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Mitogenic signal-transduction pathways have not been well defined in pancreatic β -cells. In the glucose-sensitive rat β -cell line, INS-1, glucose (6–18 mM) increased INS-1 cell proliferation (> 20-fold at 15 mM glucose). Rat growth hormone (rGH) also induced INS-1 cell proliferation, but this was glucose-dependent in the physiologically relevant concentration range (6–18 mM glucose). The combination of rGH (10 nM) and glucose (15 mM) was synergistic, maximally increasing INS-1 cell proliferation by > 50-fold. Moreover, glucose-dependent rGH-induced INS-1 cell proliferation was increased further by addition of insulin-like growth factor 1 (IGF-1; 10 nM) to > 90-fold at 12 mM glucose. Glucose metabolism and phosphatidylinositol-3'-kinase (PI3'K) activation were necessary for both glucose- and rGH-stimulated INS-1 cell proliferation. Glucose (> 3 mM) independently increased tyrosine-phosphorylation-mediated recruitment of growth-factor-bound protein 2 (Grb2)/murine sons of sevenless-1 protein (mSOS) and PI3'K to insulin receptor substrate (IRS)-1 and IRS-2, as well as SH2-containing protein (Shc) association with Grb2/mSOS and downstream activation of mitogen-

activated protein kinase and 70 kDa S6 kinase. Glucose-induced IRS- and Shc-mediated signal transduction was enhanced further by the addition of IGF-1, but not rGH. In contrast, rGH was able to activate Janus kinase 2 (JAK2)/signal transducer and activator of transcription 5 (STAT5) signal transduction at glucose concentrations above 3 mM, but neither glucose independently, nor glucose with added IGF-1, were able to activate the JAK2/STAT5 signalling pathway. Thus rGH-mediated proliferation of β -cells is directly via the JAK2/STAT5 pathway without engaging the Shc or IRS signal-transduction pathways, although activation of PI3'K may play an important permissive role in the glucose-dependent aspect of rGH-induced β -cell mitogenesis. The additive effect of rGH and IGF-1 on glucose-dependent β -cell proliferation is therefore reflective of rGH and IGF-1 activating distinctly different mitogenic signalling pathways in β -cells with minimal crosstalk between them.

Key words: MAP kinase, nutrient stimulus-response coupling, phosphatidylinositol-3'-kinase, tyrosine phosphorylation.

INTRODUCTION

Pancreatic β -cell proliferation can be increased by nutrients, certain pharmacological agents and several growth factors [1,2]. In particular, somatotrophic hormones (prolactin and growth hormone, GH) and insulin-like growth factor 1 (IGF-1) have been shown to increase the number of replicating β -cells in rodent islets from a resting 0.5% up to 6% of the islet cell population [1,3]. GH is one of the most potent of peptide growth factors to induce proliferation of differentiated β -cells [4]. It is likely that GH instigates a mitogenic signal in β -cells predominantly via activation of the Janus kinase 2 (JAK2)/signal transducer and activator of transcription 5 (STAT5) signal-transduction pathway [5–7]. However, it has been suggested that GH can also mediate increases in β -cell proliferation via local IGF-1 production [8]. Previous studies have shown that IGF-1

can instigate a marked stimulation of β -cell proliferation and is involved in endocrine pancreatic regeneration [9]. IGF-1 stimulation of pancreatic β -cell (INS-1) mitogenesis was instigated via tyrosine autophosphorylation activation of the IGF-1-receptor tyrosine kinase activity, resulting in downstream tyrosine phosphorylation of insulin receptor substrate (IRS) and SH2-containing protein (Shc) and subsequent activation of signal-transduction pathways for activation of mitogen-activated protein kinase (MAPK; Erk-1 and Erk-2 isoforms), phosphatidylinositol-3'-kinase (PI3'K) and 70 kDa S6 kinase (p70^{S6K}) [10,11]. Since IRS-2 is more abundant than IRS-1 in pancreatic β -cells, it was presumed that most IRS-mediated mitogenic signal transduction was via IRS-2 [12], complementary to a lack of β -cell growth in the IRS-2 knock-out mouse [13]. Notwithstanding, it was apparent that activation of the PI3'K branch of IRS-mediated signal-transduction pathways was more important than

Abbreviations used: Grb2, growth-factor-bound protein 2; MAPK, mitogen-activated protein kinase; MEK, MAPK kinase; GH, growth hormone; rGH, rat GH; IGF-1, insulin-like growth factor 1; IRS-1–4, insulin receptor substrate 1–4; PI3'K, phosphatidylinositol-3'-kinase; p85 PI3'K, 85 kDa regulatory subunit of PI3'K; PKC, protein kinase C; p70^{S6K}, 70 kDa S6 kinase; Shc, SH2-containing protein; mSOS, murine sons of sevenless-1 protein; JAK, Janus kinase; STAT, signal transducer and activator of transcription; PKA, protein kinase A.

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that leading to MAPK activation in terms of increased β -cell proliferation in response to IGF-1 [10]. Moreover, unlike the mitogenic effect of IGF-1 in other eukaryotic cells [11], IGF-1-induced β -cell proliferation was dependent on glucose being present in the physiologically appropriate range (3–15 mM) and readily metabolized [10]. It is not yet clear if GH's effect on stimulating β -cell proliferation is likewise glucose-dependent, and, if so, whether this might be mediated by GH secondary effects inducing IGF-1 signal transduction [8]. In this respect, it has been found in a variety of eukaryotic cell types that GH is capable of activating IRS-mediated signal-transduction pathways as well as JAK2/STAT5 signalling, probably via JAK2 activation and subsequent tyrosine phosphorylation of IRS and/or insulin receptor/IGF-1-receptor tyrosine kinase activity [6,7,14]. Likewise, IGF-1 can induce activation of JAK1 and JAK2 [15]. However, it has not been clear whether such crosstalk to IRS-mediated signal-transduction pathways occurs in GH-stimulated β -cells. In this study we have examined whether GH-induced β -cell proliferation is glucose-dependent, and requires IGF-1 signalling via local IGF-1 production and/or crosstalk to IRS-mediated signal-transduction pathways.

EXPERIMENTAL

Materials

[methyl- 3 H]Thymidine (20 Ci/mmol) was from NEN (Boston, MA, U.S.A.). Anti-'active-MAPK' antiserum was purchased from Promega (Madison, WI, U.S.A.), the 'total MAPK' antiserum (Erk1/Erk2) was a gift from Dr. M. Cobb (University of Texas Southwestern Medical Center, Dallas, TX, U.S.A.), and the IRS-1 and IRS-2 antisera were generated as described previously [16]. The p70^{S6K} antisera were generated and used as described in [17]. Anti-JAK2 antibodies were rabbit antisera generated against a keyhole limpet haemocyanin-coupled synthetic peptide corresponding to the N-terminal 12 amino acids of murine JAK2. All other antisera were purchased from Upstate Biotechnology (Lake Placid, NY, U.S.A.). Recombinant rat GH (rGH) was provided by Eli Lilly (Indianapolis, IN, U.S.A.; N-terminal leucine) or purchased from Diagnostic System Laboratories (Webster, TX, U.S.A.; N-terminal methionine). Both rGH preparations had equivalent biological activity. Transblot nitrocellulose membranes (0.2 μ m pore size) were from Bio-Rad (Hercules, CA, U.S.A.) and immunoblot chemiluminescence detection kit was from NEN. IGF-1 and protein kinase/phosphatase inhibitors were purchased from Calbiochem-Novabiochem (La Jolla, CA, U.S.A.). The Rp-2'-O-monobutyl-AMP and Sp-2'-O-monobutyl-AMP were from Biolog Life Science Institute (La Jolla, CA, U.S.A.). All the other biochemicals were purchased from either Sigma (St. Louis, MO, U.S.A.) or Fisher Scientific (Pittsburgh, PA, U.S.A.) and were of the highest purity available.

Cell culture

The glucose-sensitive pancreatic β -cell line, INS-1 [18], was used in the experiments. INS-1 cells were maintained in RPMI 1640 medium, containing 2 mM L-glutamine, 1 mM sodium pyruvate, 50 μ M β -mercaptoethanol, 100 units/ml penicillin, 100 μ g/ml streptomycin, 10% fetal calf serum and 11.2 mM glucose, and incubated at 37 °C and under 5% CO₂ as described in [18]. Cells were subcultured at 80% confluence.

[3 H]Thymidine incorporation

Incorporation of [3 H]thymidine was used as an indicator of DNA synthesis and INS-1 cell proliferation [19]. A 96-well-plate

[3 H]thymidine assay was established as described previously [10]. Essentially, INS-1 cells were cultured on 96-well plates and incubated for 2 days at 37 °C in INS-1 medium to reach a maximum density of approximately 10⁵ cells per well. The medium was removed and the cells made quiescent by serum and glucose deprivation for 24 h in RPMI 1640 containing 0.1% BSA instead of serum and no glucose. The INS-1 cells were then incubated for a further 24 h in RPMI 1640/0.1% BSA at different glucose concentrations (0–24 mM glucose) with or without IGF-1 and/or rGH (0.1–100 nM), \pm various inhibitors. The last 4 h of this latter incubation period was carried out in the additional presence of 5 μ Ci [3 H]thymidine/ml to monitor the degree of DNA synthesis and gain an assessment of the β -cell proliferation rate. After this final incubation period, the cells were centrifuged (3000 g, 10 min at 4 °C; Sorvall RT7 centrifuge and RT750 rotor and 96-well-plate holders), washed and lysed using a semi-automatic cell harvester (Packard Instrumentation, Meriden, CT, U.S.A.), and the cell lysates were transferred to Whatman glass-fibre micropore filters. The [3 H]thymidine specifically incorporated into the INS-1 cell DNA trapped on glass-fibre filters was counted by liquid scintillation counting.

Protein immunoblot and co-immunoprecipitation analysis

INS-1 cells were subcultured on 10-cm plates to about 70% confluence as described previously [18]. The cells were then subjected to a 24-h period of quiescence by serum and glucose deprivation in RPMI 1640 medium containing 0.1% BSA instead of serum and no glucose. After the quiescent period, INS-1 cells were then incubated in fresh RPMI 1640 medium containing 0, 3 or 15 mM glucose \pm 10 nM IGF-1 or 10 nM rGH for between 5 and 60 min, as indicated. The cells were then lysed in 0.5 ml of ice-cold lysis buffer consisting of 50 mM Hepes (pH 7.5), 1% (v/v) Nonidet P-40, 2 mM sodium vanadate, 50 mM NaF, 10 mM sodium pyrophosphate, 4 mM EDTA, 10 μ M leupeptin, 10 μ g/ml aprotinin and 100 μ M PMSF.

Immunoblot analysis of mitogenic signal-transduction protein expression and protein-tyrosine phosphorylation was as described previously, using horseradish peroxidase-based chemiluminescence reaction as a secondary detection method [10]. Examination of stimulated protein-protein interactions between mitogenic signal-transduction-pathway proteins was by co-immunoprecipitation analysis, with subsequent densitometric scanning of immunoblots for quantification, as described previously [10]. For immunoblot analysis, 50–75 μ g of INS-1 cell total protein lysate was used, and for immunoprecipitation 750–1000 μ g of INS-1 cell total protein.

Other procedures

Protein assay was by the bicinchoninic acid method (Pierce, Rockford, IL, U.S.A.). Data are presented as means \pm S.E. with the number of individual observations indicated (*n*). Differences between groups were analysed for statistical significance using Student's *t* test, where *P* < 0.05 was considered statistically significant.

RESULTS

rGH only stimulates INS-1 cell proliferation in the presence of physiological glucose concentrations

The effect of glucose (0.1–24 mM) \pm rGH (10 nM) on INS-1 cell DNA synthesis/proliferation was determined by [3 H]thymidine incorporation. INS-1 cells were chosen as a model to examine pancreatic β -cell proliferation as they respond to glucose, at least

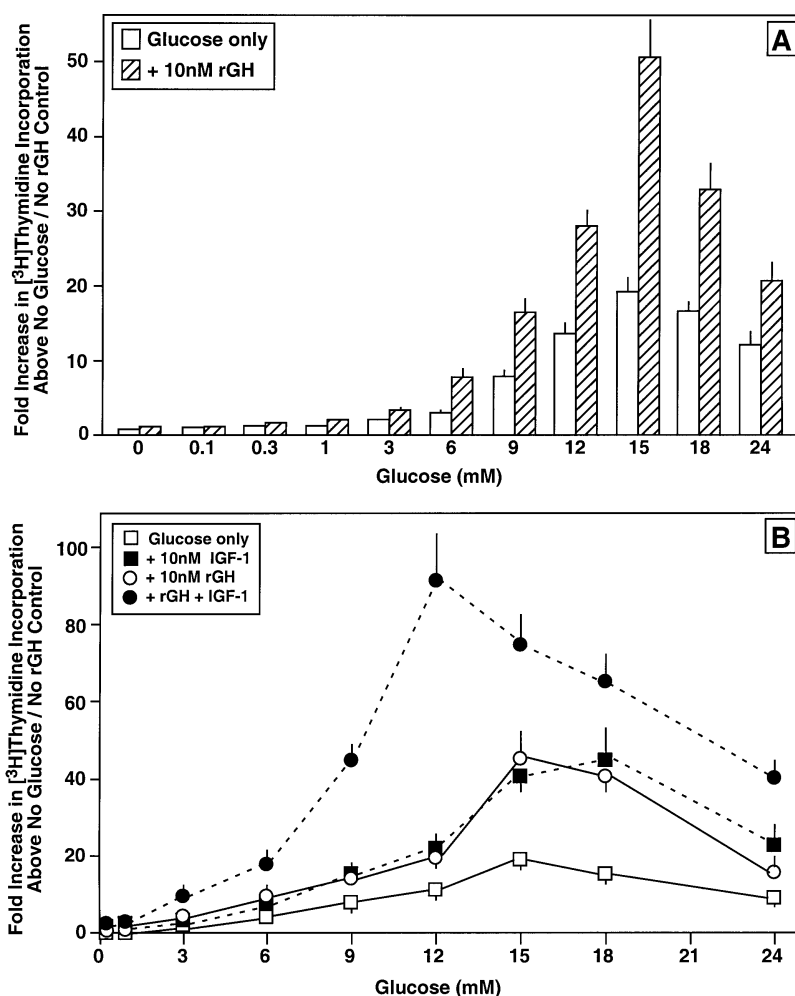


Figure 1 rGH-induced [^3H]thymidine incorporation in INS-1 cells at different glucose concentrations

Approximately 10^5 quiescent INS-1 cells/well were incubated for 24 h in RPMI 1640 medium containing 0.1% BSA, 0–24 mM glucose \pm 10 nM rGH (A) or 10 nM IGF-1 and/or 10 nM rGH (B), then assessed for proliferation rate by [^3H]thymidine incorporation as outlined in the Experimental section. All experiments were done in triplicate on at least six independent occasions. The data are expressed as fold increase above the control observation in the absence of glucose, rGH and IGF-1 (i.e. 500–1200 c.p.m./ 10^5 cells), and are shown as means \pm S.E. ($n \geq 6$).

in terms of insulin secretion, in the physiologically relevant range (6–18 mM glucose) [18]. This is unlike the majority of other pancreatic cell lines, such as RIN and βTC3 cells, which respond to glucose at non-physiological micromolar concentrations. Furthermore, INS-1 cells grow at a convenient rate so that DNA synthesis can be measured sensitively by [^3H]thymidine incorporation, unlike other slower-growing glucose-responsive β -cell lines such as MIN6. However, it should be understood that INS-1 cells are an experimental model, and as insulinoma cells they will have a degree of aberrant growth control. As such, the results should be interpreted with a degree of reservation, and only considered as a guide as to what might potentially occur in differentiated β -cells. In INS-1 cells glucose independently increased [^3H]thymidine incorporation between 3 and 24 mM glucose (Figure 1), as observed previously [10]. In the absence of rGH, no significant difference in [^3H]thymidine incorporation in INS-1 cells was observed between 0 and 3 mM glucose (Figure 1A). However, the most effective glucose-concentration range for INS-1 cell proliferation occurred between 6 and 18 mM glucose (4–20-fold above that in the absence of glucose, $P < 0.001$; Figure 1). Maximum [^3H]thymidine incorporation into

INS-1 cells, compared with that in the absence of glucose, occurred at 15 mM glucose (20-fold increase, $P < 0.0001$; Figure 1A). This was equivalent to a 2-fold increase in INS-1 cell number as determined by cell counting. Above 15 mM glucose the rate of [^3H]thymidine incorporation into INS-1 cells declined; nonetheless, at 24 mM glucose, this was still 12-fold higher than in the absence of glucose ($P < 0.001$; Figure 1).

At 1 mM glucose, rGH modestly increased [^3H]thymidine incorporation into INS-1 cells (1.9-fold increase above that in the absence of glucose and rGH, $P < 0.001$; Figure 1A), which further increased to a 3.8-fold rise in INS-1 [^3H]thymidine incorporation at a basal 3 mM glucose + 10 nM rGH ($P < 0.005$; Figure 1A). However, this effect was rather small compared with the synergistic effects of nGH and glucose in the physiologically relevant range (6–18 mM; Figure 1A), similar to that of IGF-1 (Figure 1B) [10]. At 6 mM glucose, rGH instigated an 8-fold increase in [^3H]thymidine incorporation above that in the absence of glucose and rGH ($P < 0.001$; Figure 1A), which was 2.5-fold higher than the rate of INS-1 cell proliferation at 6 mM glucose alone ($P < 0.01$; Figure 1A). rGH gave a maximum increase in INS-1 cell [^3H]thymidine incorporation at

15 mM glucose (51-fold above that in the absence of glucose and rGH, $P < 0.001$; Figure 1A), which was 2.5-fold higher than that at 15 mM glucose alone ($P < 0.01$; Figure 1A). This was equivalent to a 3–4-fold increase in INS-1 cell number as determined by cell counting. Above 18 mM glucose the synergistic effect of rGH and glucose on [^3H]thymidine incorporation into INS-1 cells was diminished ($P < 0.001$ above 15 mM glucose + 10 nM rGH; Figure 1A), so that at 24 mM glucose addition of rGH only surpassed the glucose effect by 40% (Figure 1A). Notwithstanding, this was 22-fold higher than the proliferation rate in the absence of glucose and rGH ($P < 0.001$; Figure 1A). In dose-response studies performed at 15 mM glucose it was found that 10 nM rGH gave a maximum stimulation of INS-1 cell proliferation, with an ED_{50} of 3.4 ± 0.5 nM rGH. It was also found that human GH did not provoke an as effective response as rGH on rat INS-1 cell proliferation. This was probably due to human GH exerting its effect via prolactin rather than GH receptors [20,21]. Unlike rGH, human GH did not further increase a prolactin-induced INS-1 cell proliferative response at 15 mM glucose (results not shown).

The glucose-dependent effect of rGH increasing INS-1 cell proliferation was not significantly different from that instigated by IGF-1 (Figure 1B) [10]. However, the combination of rGH and IGF-1 gave a synergistic increase in INS-1 cell [^3H]thymidine incorporation (Figure 1B). Moreover, the requirement for glucose was when both rGH and IGF-1 were present, giving an apparent 'left-shift' in the glucose dose-response curve (Figure 1B). This shift indicated that the maximal INS-1 cell proliferation response was observed at 12 mM glucose in the presence of both rGH and IGF-1, compared with between 15 and 18 mM glucose with rGH or IGF-1 separately (Figure 1B). At 3 mM glucose, the combination of rGH and IGF-1 instigated a 9-fold increase in [^3H]thymidine incorporation above that in the absence of glucose and rGH/IGF-1 ($P < 0.001$; Figure 1B), which was > 3-fold higher than the rate of INS-1 cell proliferation at 3 mM glucose alone or in the presence of either 10 nM rGH or 10 nM IGF-1 ($P < 0.01$; Figure 1B). The rGH and IGF-1 combination instigated a maximum increase in INS-1 cell [^3H]thymidine incorporation at 12 mM glucose (92-fold above no glucose, $P < 0.001$; Figure 1B), which was > 4-fold higher than that at 12 mM glucose plus rGH or IGF-1 ($P < 0.001$; Figure 1B) and > 9-fold higher than 12 mM glucose alone ($P < 0.001$; Figure 1B). This was equivalent to a 5–6-fold increase in INS-1 cell number, as determined by cell counting. Above 12 mM glucose the synergistic effect of rGH, IGF-1 and glucose on [^3H]thymidine incorporation into INS-1 cells was diminished (Figure 1B). Nonetheless, at 24 mM glucose the combination of rGH and IGF-1 still gave a 40-fold increase in INS-1 cell proliferation above that in the absence of glucose and rGH/IGF-1 ($P < 0.001$; Figure 1B), 2-fold higher than that at 24 mM glucose plus rGH or IGF-1 ($P < 0.01$; Figure 1B) and 4-fold higher than 24 mM glucose alone ($P < 0.001$; Figure 1B). INS-1 cells have been used in other studies of glucose- and growth-factor-induced β -cell proliferation [19,22], but the fold response was less than that observed in this and our other studies [10]. However, it should be noted that the [^3H]thymidine-incorporation assay conditions used were quite different between these studies; most notably, growth factors including IGF-1 supplemented the serum-deprived medium, and the incubation times after serum deprivation and for [^3H]thymidine incorporation were much longer in the previous studies [19,22].

Both rGH- and glucose-stimulated INS-1 cell proliferation required glucose metabolism. Mannoheptulose (at 3 or 15 mM), a competitive inhibitor of glycolysis [23], completely inhibited 15 mM glucose-stimulated INS-1 cell [^3H]thymidine incorpor-

ation in the presence or absence of rGH ($P < 0.001$). Likewise, the non-metabolizable glucose analogues, 2-deoxyglucose or 3-*O*-methylglucose (at concentrations of 3 or 15 mM), had no effect on [^3H]thymidine incorporation into INS-1 cells and could not provide a suitable platform for rGH-instigated INS-1 cell proliferation. Fructose (15 mM), which is not efficiently metabolized in pancreatic β -cells [23], did not significantly increase INS-1 cell proliferation in the presence or absence of rGH. These observations further emphasize the requirement of physiologically relevant concentrations of glucose for rGH to stimulate INS-1 cell proliferation.

The effects of various protein-phosphorylation inhibitors on glucose- and rGH-stimulated INS-1 cell proliferation

The effect of specific protein kinase and phosphatase inhibitors on stimulation of [^3H]thymidine incorporation in INS-1 cells by 15 mM glucose \pm 10 nM GH was examined (Table 1). In this series of experiments 15 mM glucose instigated a 19-fold increase in [^3H]thymidine incorporation above that in the absence of glucose ($P < 0.001$), and the combination of 15 mM glucose and 10 nM rGH gave a 48-fold increase above that in the absence of glucose and rGH ($P < 0.001$), similar to that observed previously (Figure 1A). Addition of Sp-2'-*O*-monobutryryl-cAMP (5 μM), a cell-permeant protein kinase A (PKA) agonist [24], did not show any significant changes in the rate of INS-1 cell [^3H]thymidine incorporation at either 15 mM glucose alone or in the additional presence of rGH (Table 1). However, in the presence of Rp-2'-*O*-monobutryryl-cAMP (5 μM), a cell-permeant antagonist of PKA activity [24], 15 mM glucose-induced [^3H]thymidine incorporation in INS-1 cells was inhibited by 76% ($P < 0.005$; Table 1), whereas it was not significantly affected in the additional presence of rGH (Table 1). This supported a putative role for PKA in glucose-induced INS-1 cell proliferation [25], but this was overcome by addition of rGH, or IGF-1 [10].

In the presence of sphingosine (10 μM), a selective inhibitor of protein kinase C (PKC) [26], both glucose-stimulated and glucose-dependent rGH-stimulated [^3H]thymidine incorporation in INS-1 cells were inhibited significantly (45–50% inhibition, $P < 0.02$; Table 1). This suggested that certain PKC isoform(s) could be involved in regulating glucose-stimulated and glucose-dependent rGH-mediated INS-1 cell proliferation [25]. Staurosporine (20 nM), an inhibitor of PKC, PKA and protein kinase G [26], inhibited glucose-stimulated [^3H]thymidine incorporation into INS-1 cells (50% inhibition, $P < 0.001$; Table 1), but not in the additional presence of rGH (Table 1). This was similar to Rp-2'-*O*-monobutryryl-cAMP inhibition of INS-1 cell proliferation (Table 1), suggesting staurosporine may be mediating its effect via inhibition of PKA.

Intracellular [Ca^{2+}] could be involved in signalling pathways for glucose-induced β -cell mitogenesis [19,27] that may be partly mediated by activation of Ca^{2+} /calmodulin-dependent proteins. Calmidazolium (50 nM), a calmodulin antagonist, significantly reduced glucose-stimulated [^3H]thymidine incorporation into INS-1 cells by 65% ($P < 0.01$; Table 1), but did not inhibit rGH-stimulated INS-1 cell proliferation at 15 mM glucose (Table 1). KN-93 (1 μM), an inhibitor of calmodulin kinase II activity in β -cells [28], showed no significant decrease in glucose-stimulated INS-1 cell proliferation whether rGH was present or not (Table 1). These data suggested a role for Ca^{2+} /calmodulin (but not necessarily calmodulin kinase II), for glucose-induced INS-1 cell proliferation [25], which was overcome by addition of rGH.

The possible role of phosphoprotein phosphatase activities on glucose-/rGH-induced INS-1 cell proliferation was investigated. Okadaic acid (50 nM), an inhibitor of phosphoprotein phosphatase

Table 1 The effect of phosphorylation inhibitors on glucose-/rGH-stimulated [3 H]thymidine incorporation in INS-1 cells

Approximately 10^5 quiescent INS-1 cells/well were incubated for 24 h in RPMI 1640 medium containing 0.1% BSA, 15 mM glucose \pm 10 nM rGH, with or without various inhibitors of protein kinases, phosphoprotein phosphatases or tyrosine kinase signalling cascades, as indicated, then assessed for proliferation rate by [3 H]thymidine incorporation as outlined in the Experimental section. The data are presented as percentages of either the control [3 H]thymidine incorporation at 15 mM glucose in the absence of rGH, or control [3 H]thymidine incorporation at 15 mM glucose + 10 nM rGH, as appropriate. Means \pm S.E. of at least three experiments done in triplicate are given. *, Statistically significant difference from equivalent control at $P < 0.05$.

| Inhibitor | [3 H]Thymidine incorporation (%) | |
|---|--|--|
| | Incorporation of 15 mM glucose control | Incorporation of 15 mM glucose + 10 nM rGH control |
| Protein kinase A | | |
| Rp-2'- <i>O</i> -monobuturyl-cAMP (5 μ M) | 24.2 \pm 3.6* | 88.5 \pm 12.1 |
| Sp-2'- <i>O</i> -monobuturyl-cAMP (5 μ M) | 86.8 \pm 11.4 | 96.8 \pm 8.4 |
| Protein kinase C | | |
| Sphingosine (10 μ M) | 54.6 \pm 8.6* | 48.7 \pm 4.3* |
| Staurosporine (20 nM) | 48.4 \pm 5.1* | 106.3 \pm 13.2 |
| Ca $^{2+}$ /calmodulin kinase | | |
| Calmidazolium (50 nM) | 34.9 \pm 4.7* | 94.8 \pm 3.7 |
| KN-93 (1 μ M) | 96.3 \pm 4.8 | 86.8 \pm 7.8 |
| Phosphoprotein phosphatase | | |
| Okadaic acid (50 nM) | 81.8 \pm 9.3 | 95.5 \pm 3.3 |
| Cyclosporin A (5 μ M) | 98.6 \pm 4.6 | 105.1 \pm 2.7 |
| Orthovanadate (0.5 mM) | 2.5 \pm 0.2* | 1.8 \pm 0.3* |
| Tyrosine kinase cascades | | |
| Genistein (25 μ M) | 2.2 \pm 0.3* | 3.6 \pm 0.3* |
| Wortmannin (10 nM) | 0.7 \pm 0.1* | 1.1 \pm 0.1* |
| LY294002 (5 μ M) | 1.5 \pm 0.1* | 1.6 \pm 0.1* |
| PD98059 (50 μ M) | 72.8 \pm 8.6 | 87.4 \pm 7.2 |
| Rapamycin (10 nM) | 52.5 \pm 6.4* | 46.8 \pm 4.4* |

tases 1 and 2A, and cyclosporin A (5 μ M), an inhibitor of phosphoprotein phosphatase 2B, had no significant effect on glucose-stimulated or glucose-dependent rGH-stimulated INS-1 cell proliferation (Table 1). However, the protein-tyrosine-phosphatase inhibitor, orthovanadate (0.5 mM), markedly inhibited glucose-stimulated and glucose-dependent rGH-stimulated [3 H]thymidine incorporation into INS-1 cells by $> 95\%$ ($P < 0.001$; Table 1). Protein-tyrosine phosphorylation was thus an important aspect of glucose/rGH mitogenic signalling in INS-1 cells.

Protein-tyrosine-phosphorylation cascades have been implicated strongly in mitogenic signal-transduction pathways [16,29]. Genistein (25 μ M), a generic inhibitor of protein-tyrosine kinase activity, markedly inhibited both glucose-stimulated and glucose-dependent rGH-stimulated [3 H]thymidine incorporation into INS-1 cells by $> 95\%$ ($P < 0.001$; Table 1). Specific inhibitors of PI3'K activity (wortmannin, 10 nM, and LY294002, 5 μ M) also markedly inhibited both glucose-stimulated and glucose-dependent rGH-induced INS-1 cell proliferation by $> 95\%$ ($P < 0.001$; Table 1). Activation of p70 S6K lies downstream of PI3'K activation in mitogenic signalling pathways and, correspondingly, specific inhibition of p70 S6K by rapamycin (10 nM) resulted in a significant 50% inhibition of both 15 mM glucose- and rGH-induced [3 H]thymidine incorporation into INS-1 cells ($P < 0.02$ and $P < 0.005$, respectively; Table 1). In contrast, the MAPK kinase (MEK) inhibitor PD98059 (which in turn inhibits MAPK activation [30]), had no significant effect on [3 H]thymidine incorporation into INS-1 cells incubated with 15 mM glucose in the absence or presence of rGH (Table 1). These data imply that the PI3'K branch of mitogenic signal-transduction pathways was more important than the MAPK branch for glucose and the glucose-dependent aspect of rGH-stimulated INS-1 cell proliferation.

Such inhibitor experiments can be useful, but not conclusive, as indicators of signalling elements required for a mitogenic response. Certain reservations must be made concerning the specificity and potential cytotoxicity of these pharmaceutical agents. In this study, inhibitor studies have been used to support biochemical analysis of mitogenic signal-transduction pathways in β -cells. Cytotoxic effects were not evident. At the concentration of each inhibitor used in a 24-h period, no effect on INS-1 cells was observed by phase-contrast light microscopy, Neutral Red staining or Trypan Blue exclusion compared with control cells. Moreover, there was negligible effect of any of the chosen inhibitors (outlined in Table 1) on total protein biosynthesis, as determined by specific radioactivity of [35 S]methionine incorporation into trichloroacetic acid-precipitable material over a 30-min incubation at 15 mM glucose \pm 10 nM rGH or IGF-1 [31] (results not shown). As such, it was thought unlikely that inhibition of [3 H]thymidine incorporation into INS-1 cells was due to non-specific cytotoxic effects of these inhibitors.

Glucose and IGF-1, but not rGH, activate IRS-mediated mitogenic signal-transduction pathways in INS-1 cells

Protein-phosphorylation activation of mitogenic signal-transduction pathways by 3 or 15 mM glucose \pm 10 nM IGF-1 or 10 nM rGH in INS-1 cells was investigated using co-immunoprecipitation and immunoblot analysis. Immunoprecipitation of the 85 kDa regulatory subunit of PI3'K (p85 PI3'K) followed by immunoblot analysis with antiserum to recognize IRS-1 (Figure 2A) or IRS-2 (Figure 2B) revealed a specific increased association of PI3'K with IRS-1 and IRS-2 instigated by both 15 mM glucose and IGF-1, but not rGH (Figures 2A and 2B). At a basal 3 mM glucose, 10 nM IGF-1 increased the amounts of IRS-1 (2.8 \pm 0.4-fold, $n = 4$; $P < 0.05$) and IRS-2 (7.4 \pm 0.9-fold, $n = 4$;

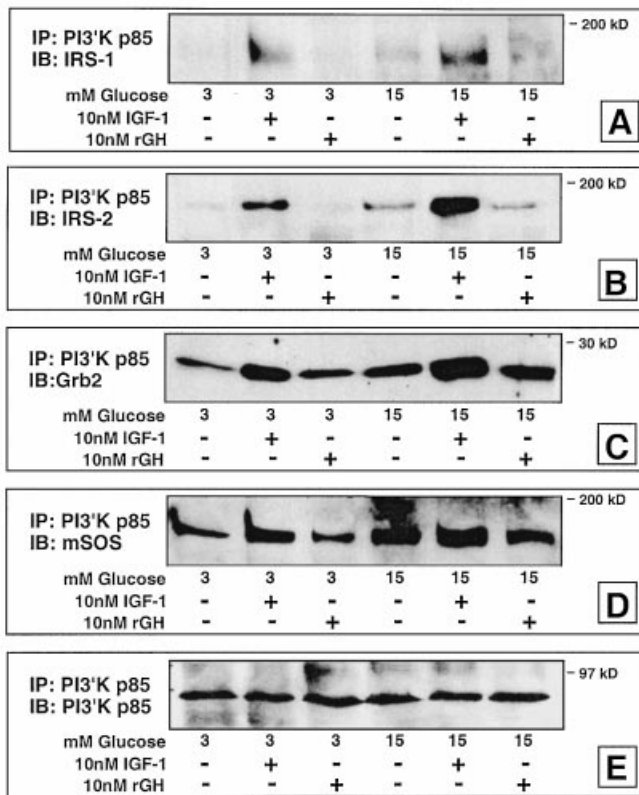


Figure 2 IGF-1- and glucose-mediated, but not rGH-mediated, association of IRS-1, IRS-2, PI3'K, Grb2 and mSOS

INS-1 cells (70% confluent on a 10-cm diameter dish) were stimulated with 3 or 15 mM glucose \pm 10 nM IGF-1 or 10 nM rGH for 10 min, and cell lysates generated as outlined in the Experimental section. INS-1 cell lysates were then subjected to immunoprecipitation (IP) with antiserum against p85 PI3'K. Immunoprecipitates were then immunoblotted (IB) analysed with IRS-1 (A), IRS-2 (B), Grb2 (C), mSOS (D) and p85 PI3'K (E) antibodies, as described in the Experimental section. An example blot for such co-immunoprecipitation analysis is shown from at least four independent experiments.

$P < 0.02$) associated with PI3'K within 10 min (Figures 2A and 2B). At a stimulatory 15 mM glucose, IRS-1 association with PI3'K only slightly increased above that at basal 3 mM glucose (1.4 ± 0.2 -fold, $n = 4$), but this was not statistically significant (Figure 2A). However, in the additional presence of IGF-1 at 15 mM glucose, IRS-1/PI3'K association significantly increased above that at basal 3 mM glucose (5.2 ± 0.7 -fold, $n = 4$; $P < 0.02$; Figure 2A). In the additional presence of rGH at 15 mM glucose, IRS-1/PI3'K association did not change above that at 3 or 15 mM glucose alone (Figure 2A). The specific association of IRS-2 and PI3'K increased at 15 mM glucose compared with that at basal 3 mM glucose (3.3 ± 0.4 -fold, $n = 4$; $P < 0.01$), and more so in the additional presence of IGF-1 (12.7 ± 0.8 -fold, $n = 4$; $P < 0.001$; Figure 2B). In contrast, in the additional presence of rGH at 15 mM glucose, IRS-2/PI3'K association did not significantly change from that at 15 mM glucose alone (Figure 2B). Immunoblotting of the PI3'K immunoprecipitates with antiserum recognizing growth-factor-bound protein 2 (Grb2; Figure 2C) or murine sons of sevenless-1 protein (mSOS; Figure 2D) indicated an increased association of Grb2 (3.9 ± 0.5 -fold, $n = 4$; $P < 0.02$) and mSOS (2.7 ± 0.2 -fold, $n = 4$; $P < 0.01$) with a PI3'K/IRS signalling complex [16,29] within 10 min in INS-1 cells stimulated with IGF-1 at basal

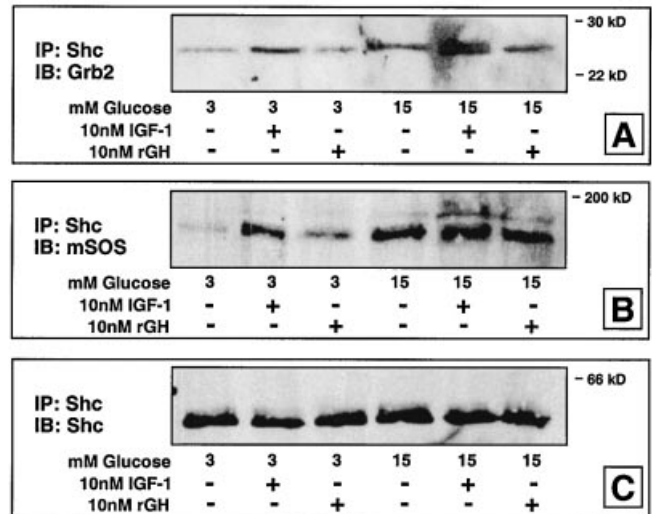


Figure 3 IGF-1 and glucose, but not rGH, increase the association of Grb2 and mSOS with Shc

INS-1 cells (70% confluent on a 10-cm diameter dish) were stimulated with 3 or 15 mM glucose \pm 10 nM IGF-1 or 10 nM rGH for 10 min, and cell lysates generated as outlined in the Experimental section. INS-1 cell lysates were then subjected to immunoprecipitation (IP) with antiserum against Shc. The Shc immunoprecipitates were then subjected to immunoblot (IB) analysis with Grb2 (A), mSOS (B), and Shc (C) antibodies, as described in the Experimental section. An example blot for such co-immunoprecipitation analysis is shown from at least four independent experiments.

3 mM glucose, but not rGH (Figures 2C and 2D). The association of PI3'K with Grb2/mSOS in INS-1 cells was increased further by 15 mM glucose alone (2.8 ± 0.4 -fold, $n = 4$, $P < 0.05$ for Grb2; and 3.6 ± 0.3 -fold, $n = 4$, $P < 0.01$ for mSOS), and more so by the additional presence of IGF-1 at 15 mM glucose (5.7 ± 0.7 -fold, $n = 4$, $P < 0.02$ for Grb2; and 4.7 ± 0.5 -fold, $n = 4$, $P < 0.02$ for mSOS), above a basal 3 mM glucose (Figures 2C and 2D). In contrast to IGF-1, rGH had no additional effect on recruitment of Grb2/mSOS to a PI3'K/IRS signalling complex at 15 mM glucose (Figures 2C and 2D). The specific nature of this glucose/IGF-1-induced Grb2/mSOS, IRS-1 and IRS-2 interaction with PI3'K was indicated in that p85 PI3'K immunoblot analysis of PI3'K immunoprecipitates revealed that an equivalent amount of PI3'K was present in each sample (Figure 2E).

IGF-1, but not rGH, was also able to activate the Ras/Raf/MEK/MAPK mitogenic signal-transduction pathway independent of IRS, by IGF-1-receptor tyrosine-mediated phosphorylation of Shc [16,29,32]. Immunoprecipitation of Shc followed by immunoblot analysis of Grb2 (Figure 3A) and mSOS (Figure 3B) from INS-1 cells incubated for 10 min at basal 3 mM glucose \pm IGF-1 indicated that IGF-1 could promote the association of Grb2 (3.3 ± 0.4 -fold, $n = 4$; $P < 0.02$), and mSOS (4.9 ± 0.6 -fold, $n = 4$; $P < 0.02$) with Shc compared with 3 mM glucose alone (Figures 3A and 3B). In four individual parallel analyses it was found that rGH had no significant effect on promoting Grb2/mSOS association with Shc at a basal 3 mM glucose. A stimulatory 15 mM glucose alone was found to promote the association of Grb2 (4.2 ± 0.3 -fold, $n = 4$; $P < 0.01$), and mSOS (6.6 ± 0.8 -fold, $n = 4$; $P < 0.02$), with Shc above that at basal 3 mM glucose (Figures 3A and 3B). In the additional presence of IGF-1 at 15 mM glucose, Shc association with Grb2 (8.0 ± 0.7 -fold, $n = 4$; $P < 0.01$) and mSOS (7.5 ± 0.8 -

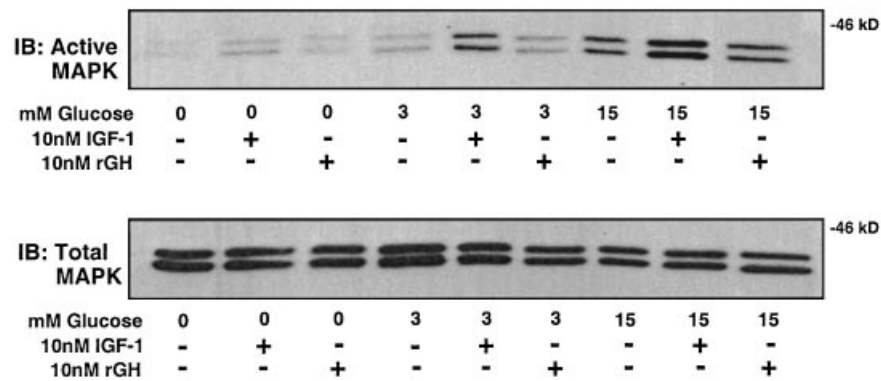


Figure 4 IGF-1 and glucose, but not rGH, stimulated phosphorylation activation of MAPK (Erk-1/Erk-2 isoforms) in INS-1 cells

INS-1 cells (70% confluent on a 10-cm diameter dish) were stimulated with 0, 3 or 15 mM glucose \pm 10 nM IGF-1 or 10 nM rGH for 10 min, and cell lysates generated as outlined in the Experimental section. Specific immunoblot (IB) phosphorylation-activated MAPK (active MAPK) and total MAPK was examined in the INS-1 cell lysates as described in the Experimental section. Representative immunoblot analyses of activated MAPK and total MAPK are shown from at least five independent experiments.

fold, $n = 4$; $P < 0.01$) was further increased above basal 3 mM glucose (Figures 3A and 3B). In contrast to IGF-1, rGH did not exert any significant change in Grb2/mSOS association with Shc compared with 15 mM glucose alone (Figures 3A and 3B). The specific nature of glucose/IGF-1-induced Grb2/mSOS interaction with Shc in INS-1 cells was indicated, in that an equivalent amount of Shc was detected in Shc immunoprecipitates (Figure 3C).

The activation of mSOS by its Grb2-mediated association with tyrosine-phosphorylated IRS and/or Shc results in downstream activation of Ras, which in turn stimulates Raf-1 serine kinase activity [16,29,32]. Raf-1 then activates MEK, which in turn activates MAPK (Erk-1 and Erk-2 isoforms) by serine phosphorylation [16,19,27,29,32]. Activated MAPK can be detected with specific antiserum that only recognizes the phosphorylation-activated forms of Erk-1 and Erk-2 [19,27]. Immunoblot analysis of IGF-1- or rGH-stimulated INS-1 cells with 'activated phospho-MAPK' antiserum showed little activated MAPK in the absence of glucose or IGF-1/rGH (Figure 4). However, in the presence of 10 nM IGF-1 but the absence of glucose, activated phospho-MAPK could be detected (2.6 ± 0.4 -fold, $n = 5$; $P < 0.05$; Figure 4) after a 10-min exposure, as observed previously [10]. In contrast, rGH did not significantly activate MAPK in the absence of glucose (1.3 ± 0.2 -fold, $n = 5$; not significant; Figure 4). At a basal 3 mM glucose, significant active phospho-MAPK was observed above that in the absence of glucose (2.5 ± 0.4 -fold, $n = 5$; $P < 0.05$; Figure 4), and was further increased at 15 mM glucose (6.2 ± 0.8 -fold, $n = 5$) above that in the absence of glucose ($P < 0.01$; Figure 4), independently of IGF-1 or rGH

(Figure 4). At a basal 3 mM glucose, IGF-1 instigated a marked increase in activation of MAPK in INS-1 cells above that in the absence of glucose (6.8 ± 0.6 -fold, $n = 5$; $P < 0.005$; Figure 4) and that at 3 mM glucose alone (2.6 ± 0.4 -fold, $n = 5$; $P < 0.05$; Figure 4). At a stimulatory 15 mM glucose, IGF-1 only modestly further increased MAPK activation (1.8 ± 0.2 -fold, $n = 5$) above that at 15 mM glucose alone (not significant; Figure 4), but this was increased markedly above that in the absence of glucose or IGF-1/rGH (12.5 ± 1.7 -fold, $n = 5$; $P < 0.001$; Figure 4).

Addition of rGH in the absence of glucose, at basal 3 mM or stimulatory 15 mM glucose concentrations, failed to induce any significant activation of MAPK above that of a glucose effect (Figure 4). The amount of total MAPK in INS-1 cells, as ascertained by immunoblot analysis with antisera recognizing both active and inactive forms of MAPK, was not significantly altered by glucose, IGF-1 or rGH treatment (Figure 4, lower panel).

The $p70^{S6K}$ is activated downstream of PI3'K activation [32]. Phosphorylation activation of $p70^{S6K}$ occurs on multiple sites, so that $p70^{S6K}$ phosphorylation can be detected on immunoblot analysis by an apparent electrophoresis mobility retardation [33]. INS-1 cells were incubated for 5–60 min at 15 mM glucose \pm 10 nM IGF-1. Maximal $p70^{S6K}$ phosphorylation was observed at 30 min (results not shown), and subsequent experiments examining $p70^{S6K}$ activation were done over a 30-min time course. Immunoblot analysis with $p70^{S6K}$ -specific antiserum indicated phosphorylation activation of $p70^{S6K}$ in response to 15 mM glucose, which was further increased in the presence of IGF-1 but not rGH (Figure 5). Phosphorylation activation of

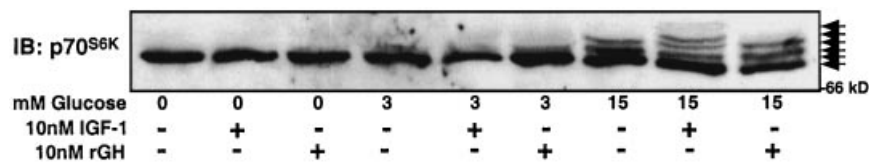


Figure 5 IGF-1 and glucose, but not rGH, stimulated phosphorylation activation of $p70^{S6K}$ in INS-1 cells

INS-1 cells (70% confluent on a 15-cm diameter dish) were stimulated with 0, 3 or 15 mM glucose \pm 10 nM IGF-1 or 10 nM rGH for 30 min, and cell lysates generated as outlined in the Experimental section. Specific phosphorylation activation of $p70^{S6K}$ was examined in the INS-1 cell lysates by immunoblot (IB) analysis as described in the Experimental section. A representative immunoblot for $p70^{S6K}$ is shown from at least five independent experiments. Phosphorylated forms of $p70^{S6K}$ become retarded on SDS/PAGE analysis, and these multi-phosphorylated $p70^{S6K}$ forms are indicated by the arrows.

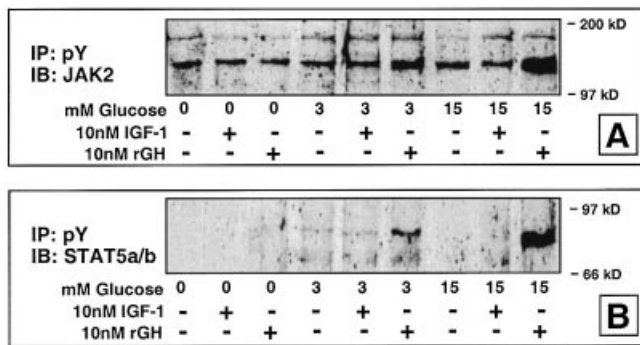


Figure 6 rGH, but not IGF-1 or glucose alone, activate JAK2 and STAT5a/b signal-transduction pathway in a glucose-dependent manner

INS-1 cells (70% confluent on a 15-cm diameter dish) were stimulated with 0, 3 or 15 mM glucose \pm 10 nM IGF-1 or 10 nM rGH for 10 min, and cell lysates generated as outlined in the Experimental section. INS-1 cell lysates were then subjected to immunoprecipitation (IP) with anti-phosphotyrosine (pY) antiserum. The anti-phosphotyrosine immunoprecipitates were then subjected to immunoblot (IB) analysis with specific antisera recognizing either JAK2 (A) or both STAT5a/b isoforms (B), as described in the Experimental section. An example blot for such co-immunoprecipitation analysis is shown from at least three independent experiments.

p70^{S6K} in β -cells was not detected in the absence of or at a basal 3 mM glucose, either with glucose alone or in the additional presence of IGF-1/rGH (Figure 5), similar to that observed previously for IGF-1 [10].

Glucose-dependent rGH-induced, but not IGF-1-induced, activation of JAK2/STAT5 signal transduction in INS-1 cells

GH mediates a mitogenic effect downstream of the GH receptor via protein-tyrosine-phosphorylation activation of JAK2, which recruits the transcription factor STAT5 for subsequent phosphorylation activation [5,7]. Activation of the JAK2/STAT5 signal-transduction pathway in INS-1 cells was assessed by anti-phosphotyrosine immunoprecipitation and subsequent immunoblot analysis of JAK2 and STAT5a/b (using antisera that recognized both STAT5 isoforms; Figure 6). INS-1 cells were incubated for 10 min in the absence of glucose, or at a basal 3 mM or stimulatory 15 mM glucose \pm 10 nM IGF-1 or 10 nM rGH. Analysis of phosphotyrosine immunoprecipitates revealed a basal tyrosine phosphorylation of JAK2 independent of glucose concentration (Figure 6A). The tyrosine-phosphorylation state of JAK2 was observed to be increased modestly at basal 3 mM glucose in the presence of rGH (2.3 ± 0.4 -fold, $n = 3$) above that in the absence of glucose or IGF-1/rGH, but this was not statistically significant (Figure 6A). However, a more substantial tyrosine phosphorylation of JAK2 by rGH was observed at a stimulatory 15 mM glucose (5.9 ± 0.7 -fold, $n = 3$) above that in the absence of glucose or IGF-1/rGH ($P < 0.02$; Figure 6A). JAK2 tyrosine phosphorylation was not affected independently by glucose or by IGF-1 (Figure 6). Likewise, STAT5a/b phosphorylation activation by JAK2 was unaffected by IGF-1 or glucose independently (Figure 6B). In the presence of rGH, STAT5a/b phosphorylation activation was not detectable in the absence of glucose. However, rGH was able to induce STAT5a/b phosphorylation activation at a basal 3 mM glucose (4.2 ± 0.6 -fold, $n = 3$) above that in the absence of glucose or IGF-1/rGH, and more so at a stimulatory 15 mM glucose (9.1 ± 1.5 -fold, $n = 3$) above that in the absence of glucose or IGF-1/rGH (both $P < 0.05$; Figure 6B). As such, glucose-dependent rGH-induced

activation of JAK2 correlated with activation of STAT5a/b (Figure 6).

DISCUSSION

GH has been shown previously to increase pancreatic β -cell proliferation in a variety of β -cell preparations [4,5,21]. However, it has not hitherto been realized that GH action upon pancreatic β -cells is glucose-dependent. In this study, it has been outlined that rGH would not exert any effect on INS-1 cell proliferation unless glucose was present in the appropriate physiological concentration range (6–18 mM). Above 3 mM glucose, 10 nM GH was found to specifically increase the tyrosine-phosphorylation state of JAK2 in pancreatic β -cells, as observed previously [22], which was further enhanced as the glucose concentration increased to 15 mM. Normally, GH-induced JAK2 tyrosine autophosphorylation results in association and predominately downstream tyrosine phosphorylation of STAT5a/b isoforms to form a transient GH receptor/JAK2/STAT5 signalling complex [6,7]. In most eukaryotic cells, tyrosine-phosphorylated STAT5a/b then translocates to the nucleus to activate transcription of a group of genes that will subsequently lead to increased mitogenesis [6,7]. In pancreatic β -cells incubated in the absence of glucose, GH-induced STAT5a/b phosphorylation was not observed, which correlated with a failure of JAK2 phosphorylation activation and no significant GH-induced increase in β -cell proliferation. At basal 3 mM glucose, modest GH-induced STAT5a/b was found in anti-phosphotyrosine immunoprecipitates correlating with a small degree of JAK2 autophosphorylation, but at higher stimulatory glucose concentrations a marked activation of STAT5a/b was observed that correlated with significant JAK2 activation and a distinct increase in GH-induced β -cell mitogenesis. Nonetheless, it should be noted that, at a basal 3 mM glucose, although there was significant rGH-induced tyrosine-phosphorylation activation of STAT5a/b, there was only a modest increase in INS-1 cell [³H]thymidine incorporation. Thus at least at sub-stimulatory glucose concentrations, activation of the JAK2/STAT5 pathway alone was not sufficient to evoke a robust response of rGH-induced proliferation of β -cells. It was likely that input from activation of other mitogenic signal-transduction pathways in the β -cell, probably as a consequence of increased glucose metabolism in the β -cell, were required to evoke a full rGH-induced mitogenic response. This would be in support of the notion that activation of more than one signal-transduction pathway is required to commit a eukaryotic cell to a mitogenic response [34].

Glucose appeared to have no independent effect on activating a JAK2/STAT5 signal-transduction pathway. Nonetheless, glucose metabolism was essential for GH to provoke an INS-1 cell proliferative response. This suggests that the regulation of mitogenesis in pancreatic β -cells is likely to be more complicated than in other eukaryotic cells, due to the unique characteristic of the β -cell's stimulus-coupling mechanisms being tightly linked to its metabolic state [35,36]. It has become apparent that β -cell glucose metabolism within the physiologically relevant concentration range (6–18 mM) provides a permissive platform for growth factors, such as GH, to elicit a mitogenic effect. A similar glucose-dependency has been observed previously for IGF-1-induced pancreatic β -cell proliferation [10]. However, it remains unclear what secondary signals emanating from glucose metabolism in the β -cell are pertinent for allowing an IGF-1 and/or GH proliferative response. Previous studies have suggested that glucose-induced increases in cytosolic [Ca^{2+}] [35] may in part provide a signalling basis for GH [5] and IGF-1 [10] to induce a

proliferative response in β -cells. This may be associated with a potential role of calmodulin, as indicated by inhibition of glucose-induced β -cell proliferation by trifluoperazines, but not via activation of calmodulin kinase II, since inhibitors of calmodulin kinase II have no effect on β -cell proliferation (Table 1) [10]. Also based on inhibitor studies (Table 1) [5,10], it is possible that glucose-induced activation of a PKC isoform [25] and/or transient increases in cytosolic [cAMP] for subsequent PKA activation [5,19] could also be involved in stimulus-coupling signalling mechanisms for glucose-induced β -cell proliferation [1,2], and provides a permissive platform for GH and IGF-1 to provoke a proliferative response in β -cells. In addition, this study (Figures 2–5) and previous observations [10,19,27] have indicated that, at glucose concentrations of > 6 mM, glucose can independently activate IRS- and Shc-mediated signal-transduction pathways resulting in downstream activation of MAPK (Erk1 and Erk-2 isoforms), PI3'K and p70^{S6K}. It is possible that glucose-induced activation of MAPK in β -cells might be mediated by a rise in cytosolic [Ca²⁺], since removal of extracellular [Ca²⁺] inhibits this response [27]. However, although MAPK activation may be partly required for IGF-1-induced β -cell proliferation [10], it appears that it does not play an essential role for glucose-stimulated or glucose-dependent GH-induced β -cell mitogenesis. Inhibition of MAPK activation by the MEK inhibitor PD98059 had no effect on glucose/GH-induced β -cell proliferation (Table 1; [10]), and neither does glucose-dependent IGF-1-induced activation of MAPK correlate with glucose-dependent IGF-1-stimulated β -cell growth [10]. In contrast, inhibition of PI3'K activity abolished glucose-stimulated and glucose-dependent GH-induced β -cell proliferation, similar to glucose-dependent IGF-1-induced β -cell growth [10]. GH was unable to independently increase association of IRS-1 or IRS-2 with PI3'K, and was thus unlikely to elicit PI3'K activity. As such, the requirement of PI3'K activity for GH-induced β -cell proliferation probably resides in the glucose-dependent nature of the GH response, in particular a need for glucose-induced activation of PI3'K in β -cells. It is not entirely clear what the appropriate signal(s) downstream of PI3'K might be. It should be noted that rapamycin-induced inhibition of p70^{S6K} activation only inhibited glucose-stimulated and glucose-dependent GH-induced β -cell proliferation by 50%. This suggested that other signalling molecules acting downstream of PI3'K and/or protein kinase B (Akt) [11,29,37] might be involved in the glucose-dependent aspect of GH- and IGF-1-induced β -cell proliferation [10]. Notwithstanding, it should be noted that whereas inhibitor studies can be useful for an initial indication of which signalling elements are important for a mitogenic response in β -cells, they should be interpreted with reservation, due to the limited specificity of some of these pharmaceutical tools. It is important to complement inhibitor data (Table 1) with independent biochemical evidence (Figures 2–6), as in this study, but even so further biochemical experiments will be required to pinpoint the appropriate signals emanating from glucose metabolism that lead to increased β -cell mitogenesis.

It has been suggested previously, mostly from experiments *in vitro*, that GH-induced activation of JAK2 might also result in activation of IRS-mediated signal-transduction pathways, as well as STAT5 activation [6,7,14]. In addition, it has been postulated that GH might exert some of its mitogenic effects on β -cells by inducing local production of IGF-1 [8], that would then signal via a Shc- and/or IRS-mediated signal-transduction pathway [11]. However, in this study, it was found in pancreatic β -cells that GH could only activate the JAK2/STAT5 signal-transduction pathway as long as the glucose concentration was > 3 mM (Figure 6). GH was unable to independently, or in a

glucose-dependent manner, activate Shc- or IRS-mediated signal-transduction pathways for downstream MAPK or p70^{S6K} activation in a manner instigated by IGF-1 in parallel experiments (Figures 2–5). As such, it is unlikely that there was any significant GH-induced crosstalk of JAK2 to Shc- and IRS-mediated signal-transduction pathways in pancreatic β -cells. Moreover, these observations reaffirm the notion that GH does not stimulate mitogenesis via induction of local production of IGF-1 in β -cells, rather directly via a predominant JAK2/STAT5 signal-transduction pathway [6,7]. This supports previous observations *in vivo* where there appeared to be no GH-mediated activation of Shc- or IRS-mediated signal transduction and exclusive signalling via a JAK2/STAT5 pathway [38].

In summary, this study establishes that a GH-induced mitogenic signal-transduction pathway via JAK2/STAT5 activation is present in pancreatic β -cells, and can be stimulated by GH in a glucose-dependent manner. GH does not appear to signal via Shc- or IRS-mediated signal-transduction pathways in β -cells, although activation of PI3'K may play an important role in the glucose-dependent aspect of GH-induced β -cell proliferation. However, only a limited number of mitogenic signal-transduction elements have been examined in this study, and other factors should not yet be ruled out. In addition, it will be important in future studies to identify the appropriate transcription factors relevant to β -cell mitogenesis that are activated downstream of JAK2/STAT5 signal transduction (e.g. c-Jun and c-Fos [6] and the insulin gene [39]), and the 'signalling factor' that renders GH signal transduction in β -cells dependent on glucose metabolism. Notwithstanding, the characteristics of the signal-transduction pathways activated by GH and IGF-1 for induction of β -cell proliferation unveiled in this study suggested negligible interaction between different mitogenic signalling pathways in β -cells. As such, IGF-1 and GH gave a synergistic induction of β -cell growth.

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