STAT5 activation by human GH protects insulin-producing cells against interleukin-1 β , interferon- γ and tumour necrosis factor- α -induced apoptosis independent of nitric oxide production

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Abstract

The proinflammatory cytokines interleukin-1 β (IL-1 β), interferon- γ (IFN- γ) and tumour necrosis factor- α (TNF- α) are toxic to pancreatic β -cells and are implicated in the pathogenesis of type 1 diabetes. We have previously found that GH and prolactin (PRL) stimulate both proliferation and insulin production in pancreatic β -cells and rat insulin-producing INS-1 cells. Here we report that human (h) GH can prevent the apoptotic effects of IL-1 β , IFN- γ and TNF- α in INS-1 and INS-1E cells. Using adenovirus-mediated gene transfer, we found that the anti-apoptotic effect of hGH is abrogated by expression of a dominant negative signal transducer and activator of transcription (STAT5) mutant in INS-1E cells. hGH and the cytotoxic cytokines was found to additively increase suppressor of cytokine signalling-3 mRNA expression after 4 h of exposure. In order to identify possible targets for the STAT5-mediated protection of INS-1E cells, we studied the effect of hGH on activation of the transcription factors STAT1 and nuclear factor- κ B (NF- κ B) by IFN- γ

and IL-1 β +TNF- α respectively. Gel retardation experiments showed that hGH affects neither IFN- γ +TNF- α -induced STAT1 DNA binding nor IL-1 β and IFN- γ +TNF- α -induced NF κ B DNA binding. The lack of influence of hGH on cytokine-mediated activation of STAT1 and NF κ B is in accordance with the finding that hGH had only a minor effect on cytokine-induced inducible nitric oxide synthase (iNOS) gene expression and in fact augmented the IL-1 β -stimulated nitric oxide production. As the anti-apoptotic Bcl-x_L gene has been shown to harbour a STAT5-binding element we measured the expression of Bcl-x_L as well as the pro-apoptotic Bax. We found that hGH increased the Bcl-x_L/Bax ratio both in the absence and in the presence of cytotoxic cytokines. In conclusion, these results suggested that GH and PRL protect β -cells against cytotoxic cytokines via STAT5-dependent mechanisms distal to iNOS activation possibly at the level of Bcl-x_L.

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Introduction

The proinflammatory cytokines interleukin-1 β (IL-1 β), interferon- γ (IFN- γ) and tumour necrosis factor- α (TNF- α) are toxic to pancreatic β -cells and have been implicated in the pathogenesis of type 1 diabetes (Eizirik & Mandrup-Poulsen 2001).

Growth hormone (GH) and the related hormones prolactin (PRL) and placental lactogen are potent growth factors as well as insulinotropic factors for pancreatic β -cells (Nielsen *et al.* 1989, 2001). In rodents, human (h) GH activates both GH receptor (GHR) and PRL receptor (PRLR) which are both expressed in rat islets and various insulinoma cell lines (Moldrup *et al.* 1990, 1993, Asfari *et al.* 1995). The GHR and PRLR activate the receptor-

associated Janus kinase (JAK2) leading to phosphorylation, dimerization and nuclear translocation of signal transducer and activator of transcription (STAT) proteins which bind specific consensus DNA sequences in the promoters of target genes and thereby regulate transcription (Carter-Su & Smit 1998).

In β -cells, GH and PRL primarily activate STAT5a and STAT5b and, to a lesser extent, STAT1 and STAT3 (Galsgaard *et al.* 1996, 1999). hGH-induced proliferation depends on activation of STAT5, as inhibition of STAT5 activity by expression of a dominant negative (DN) mutant (DN-STAT5) abolished the mitogenic effect of hGH in INS-1 cells and primary β -cells, whereas the constitutive active (CA) STAT5 mutant STAT5a/b1*6 (CA-STAT5b) induced proliferation in the absence of

hGH (Friedrichsen *et al.* 2001 2003). Furthermore, hGH-stimulated DNA binding of STAT5a and STAT5b to the promoters of rat insulin and PRLR genes, and this STAT5-DNA binding is required for hGH-induced transcriptional activation of these genes in the insulinoma cell lines RIN-5AH and INS-1 (Galsgaard *et al.* 1996, 1999).

In order to dissect the molecular mechanisms involved in the anti-apoptotic effect of hGH, the aim of the present study was to elucidate the role of STAT5 activation in INS-1E cells exposed to IL-1 β , IFN- γ and TNF- α . The involved signalling pathways share common mediators as GH, PRL and IFN- γ activate the JAK/STAT pathway, IL-1 β and TNF- α activate nuclear factor- κ B (NF κ B) and GH, PRL, IFN- γ and IL-1 β induce suppressor of cytokine signalling (SOCS) expression. We therefore studied possible interactions between hGH and IL-1 β , IFN- γ and TNF- α on the DNA-binding activity of STAT5, STAT1 and NF κ B, activation of SOCS-3 and induction of inducible nitric oxide synthase (iNOS) gene expression and nitric oxide (NO) production.

We also studied the influence of hGH on the expression pattern of the pro-apoptotic Bax and the anti-apoptotic Bcl-x_L in INS-1E cells upon hGH stimulation and cytokine treatment, as the susceptibility of β -cells to apoptosis seems to be regulated by the balance of pro- and anti-apoptotic members of the Bcl-2 protein family expressed in the cells.

Materials and Methods

Cells, hormones and viruses

The insulin-producing cell lines INS-1 and INS-1E were kindly provided by Dr C B Wollheim, University of Geneva, Switzerland. INS-1E is a more glucose-sensitive subclone of the INS-1 (Janjic *et al.* 1999). INS-1 and INS-1E cells were cultured at 37 °C in a humidified atmosphere containing 5% CO₂ in RPMI 1640 with Glutamax I (Gibco/Invitrogen) supplemented with 10% heat-inactivated fetal calf serum (FCS) (Biological Industries, Kibbutz Beit Haemek, Israel), 100 U/ml penicillin and 100 μ g/ml streptomycin (complete medium). The culture medium for the INS-1E cells was furthermore supplemented with 50 μ M β -mercaptoethanol.

The cytokines used were recombinant mouse IL-1 β (PharMingen/BD Bioscience, San Jose, CA, USA), recombinant rat IFN- γ (R&D Systems, Abingdon, Oxon, UK), recombinant rat TNF- α (R&D Systems) and recombinant human TNF- α (Genzyme, Cambridge, MA, USA). Recombinant human hGH was obtained from Novo Nordisk A/S (Gentofte, Denmark) and was used at a concentration of 500 ng/ml.

The recombinant adenoviruses encoding green fluorescent protein (GFP), DN-STAT5 or CA-STAT5b mutants were generated as previously described (Friedrichsen *et al.* 2003). The DN-STAT5 mutant is a truncated form

of the murine (m) STAT5a (STAT5 Δ 749) (Moriggl *et al.* 1996), whereas the CA-STAT5b mutant (mSTAT5b1*6) has two amino acid substitutions (H299R and S711F) (Onishi *et al.* 1998).

Annexin V-FITC apoptosis assay

INS-1 cells (2.5×10^5 cells/well) were seeded into 24-well tissue culture plates (Nunc, Roskilde, Denmark) in 1.5 ml complete medium. The cells were allowed to attach overnight and subsequently cultured in medium containing 0.5% FCS for an additional 24 h before addition of IFN- γ (100 U/ml), IL-1 β (30 U/ml), hTNF- α (100 U/ml) and/or hGH (500 ng/ml). After the indicated time points, apoptosis was measured using the TACS Annexin V-FITC apoptosis detection kit as described by the manufacturer (R&D Systems). Briefly, cells were harvested, washed and then incubated for 15 min with Annexin V-FITC and propidium iodide (PI). Subsequently, cells were analysed by flow cytometry using a FACScan (BD Biosciences).

DNA fragmentation

Cells (5×10^4 cells/well) were seeded in 48-multiwell plates (Falcon; BD Biosciences) and cultured for 2 days in complete medium. The adenoviruses were added in a concentration of 400 plaque forming units (PFU)/cell in the media and left for infection for 2.5 h before changing the media to media containing 0.5% FCS. IL-1 β (40 pg/ml), IFN- γ (50 U/ml), rat (r)TNF- α (0.5 ng/ml) and/or hGH (500 ng/ml) were added 24 h after infection. After an additional 24 h of culture, apoptotic cell death was measured using the Cell Death Detection Elisa plus kit (Roche) that detects cytoplasmic DNA-histone complexes generated during the apoptotic DNA fragmentation. In short, cells were lysed in lysis buffer and cell lysates were incubated with biotin-labelled anti-histone antibody and peroxidase-conjugated anti-DNA antibody in a streptavidin-coated microplate for 2 h. The ELISA was then developed with peroxidase substrate before measuring the absorbance at 405 nm according to the manufacturer's description.

Viability assay

INS-1 (10^4 cells/well) were set up in 96-well tissue culture plates (Costar, Bethesda, MD, USA) and cultured for 1 day before the addition of IFN- γ (200 U/ml), IL-1 β (150 pg/ml), hTNF- α (200 U/ml) and/or hGH (500 ng/ml). After 1–2 days of additional culture the proportion of viable cells in control vs cytokine-containing wells was determined based on the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Promega) measuring the conversion of an MTT tetrazolium salt to a coloured formazan product by the mitochondrial enzyme succinate dehydrogenase (Mosmann 1983).

Western blot analysis

INS-1E (5×10^5 cells/well) were seeded into six-well plates (Falcon) and cultured for 2 days in complete medium. Various titers of adenovirus (0–400 PFU/cell) were added to the media and left for infection for 2.5 h before changing the media to media containing 0.5% FCS. The cells were harvested 24 h after infection in RIPA buffer (1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM 4-(2-aminoethyl) benzene sulphonyl fluoridehydrochloride (AEBSF), 1 mM orthovanadate, 1 µg/ml aprotinin and 1 µg/ml leupeptin in phosphate-buffered saline (PBS)). Total protein concentrations were measured using the Bio-Rad protein assay (Bio-Rad Laboratories).

Cell lysates were normalized for protein concentration and 15 µg protein was separated by SDS-PAGE (7% Tris-Acetate NuPAGE; Invitrogen) and transferred by electroblotting to nitrocellulose membranes (Invitrogen). Membranes were blocked for 2 h in blocking buffer (5% non-fat dry milk and 5% FCS in Tris-buffered saline (TBS; 50 mM Tris-HCl, 27 mM KCl and 138 mM NaCl)), incubated overnight with primary antibody (monoclonal, mouse-anti-STAT5, no. 610191; Transduction Laboratories, BD Biosciences) diluted $250 \times$ in blocking buffer, washed four times in TBS containing 0.1% Tween (Sigma) and incubated for 2 h with secondary antibody (rabbit-anti-mouse, HRP-linked, P0260; DAKO, Glostrup, Denmark) diluted $1000 \times$ in blocking buffer. After several washing steps in TBS containing 0.1% Tween, proteins were visualized by an enhanced chemiluminescence detection system (Amersham Pharmacia Biotech).

Nuclear extracts and gel shift assays

INS-1E (4×10^6 cells) were seeded in 10 cm dishes (Falcon) and cultured for 2 days in complete medium. Cells were then serum starved in RPMI 1640 containing 0.5% FCS for 24 h and afterwards incubated in the presence or absence of IL-1β (80 pg/ml), IFN-γ (200 ng/ml), hTNF-α (50 ng/ml) and/or hGH (500 ng/ml) for 15 min. Alternatively, 2 days after seeding, the cells were infected with adenovirus (400 PFU/cell) for 24 h. Cells were washed twice in PBS and lysed for 15 min in buffer A (20 mM Hepes, pH 7.9, 1 mM EDTA, 1 mM MgCl₂, 10 mM KCl, 20% glycerol, 1 mM dithiothreitol, 0.5 mM AEBSF, 1 mM sodium orthovanadate, 1 µg/ml leupeptin and 1 µg/ml aprotinin) containing 0.5% Triton X-100. The lysates were centrifuged (5 min, 2500 g) and the nuclei were resuspended in buffer A containing 400 mM NaCl and incubated for 30 min on a rocking bench. The extracts were centrifuged (30 min, 20 000 g) and the supernatants were stored at -80°C . Total protein concentrations were measured using the Bio-Rad protein assay.

For detection of DNA-binding activity, the following double-stranded oligonucleotides were used: STAT1: the

optimized Sis-inducible element (SIE) from the c-fos gene (M67) (5'-AGCTTCATTTCCCGTAAATCCCTA-3') (Meyer *et al.* 1994); NFκB: the consensus NFκB-binding sequence (5'-AGCTTCAGAGGGGACTTTCCGAGAGG-3') (Zabel *et al.* 1991); STAT5: the sequence of the PRL-1A-promoter: 1A-GLE 5'-AGCTAGTTCTAGGAATAAGCT-3' (Galsgaard *et al.* 1999).

Nuclear extracts (10 µg) were incubated with 20 fmol ³²P-radiolabelled probe for 30 min at 30 °C and separated on a 5% polyacrylamide gel as described (Galsgaard *et al.* 1996). The gels were dried and exposed to PhosphorImager screen (Molecular Dynamics, Amersham Pharmacia Biotech).

Nitrite accumulation

INS-1 cells were treated with IL-1β (150 pg/ml), IFN-γ (200 U/ml) and hTNF-α (200 U/ml) either alone or in combination and IL-1β (150 pg/ml) in the presence or absence of hGH (500 ng/ml) for 24, 48 or 72 h. NO was measured as accumulated nitrite in the medium by mixing 100 µl with 100 µl Griess reagents (Green *et al.* 1982). The absorbance was measured at 540 nm and nitrite concentration calculated from the NaNO₂ standard curve.

Real-time PCR

INS-1E (1.8×10^6 cells) were seeded in 6 cm dishes (Falcon) and cultured for 2 days in complete medium. Cells were then serum starved in RPMI 1640 containing 0.5% FCS for 24 h and incubated for 1 or 4 h in the presence or absence of cytokines (40 pg/ml IL-1β, 50 ng/ml IFN-γ, 0.5 ng/ml rTNF-α and/or 500 ng/ml hGH). Total cellular RNA was isolated using RNeasy kit (Qiagen, Hilden, Germany) according to the manufacturer's recommended protocol. First-strand cDNA from the INS-1E total RNA (1 µg) was synthesized using the Promega reverse transcription system (Promega) with oligo d(T).

Quantitative real-time PCR of INS-1E cDNAs was performed using the LightCycler (Roche) with Quantitect SYBR Green PCR mix (Qiagen) according to the recommended protocol for the use of this reagent mix in the LightCycler. Briefly, all the quantitative PCRs with the various sets of primers were carried out using the same reaction conditions: initial activation, 15 min at 95 °C; three-step cycling, denaturation, 15s at 94 °C, annealing 20s at 55 °C, extension 25s at 72 °C. Melting curve analysis was performed at the end of each PCR run in addition to agarose gel electrophoresis of an aliquot of the LightCycler PCR run to verify the specificity and identity of the PCR products (data not shown). The real-time PCR data were from samples from four separate experiments done in duplicate in each run.

The forward and reverse primer sequences for each gene product are as the following: SOCS-3, 5'-GGG

CCC CTT CCT TTT CTT TAG-3' and 5'-GTC CAG GAA CTC CCG AAT G-3' (PCR fragment: 263 bp); Bcl-x_L, 5'-TGG AAA GCG TAG ACA AGG AGA T-3' and 5'-TCA CTT CCG ACT GAA GAG TGA G-3' (PCR fragment: 248 bp); Bax α , 5'-GAG ACA CCT GAG CTG ACC TTG-3' and 5'-CAA AGT AGA AGA GGG CAA CCA C-3' (PCR fragment: 229 bp); phosphoglycerate kinase (PGK), 5'-CTG GAA AAC CTC CGC TTT CA-3' and 5'-TGG CAG ATT CAC ACC CAC CA-3' (PCR fragment: 191 bp). The PCR fragments were analyzed by gel electrophoresis, cloned into pCR 2.1 vector by TOPO cloning kit (Invitrogen) and sequenced for PCR product confirmation.

To generate the reference standards for the quantitative real-time PCR experiments, PCR of the rat islets cDNA was performed for 35 cycles with PCR master mix (Roche) annealing at 55 °C (94 °C 2 min (94 °C 1 min, 55 °C 1.5 min, 72 °C, 2 min) 72 °C 10 min) using a gradient PCR cycler (Eppendorf, Hamburg, Germany) in a total reaction volume of 50 μ l. This PCR protocol was used throughout when different primer sets were used in the individual reaction to generate the corresponding primer-related DNA fragments. The PCR fragment containing TOPO plasmids were linearized by HindIII and subsequently acted as the DNA reference standards for the quantification of the gene expression studies.

Statistical analysis

Results are expressed as means $\pm$ S.E.M. Paired *t*-test was used for statistical analysis. Values of $P < 0.05$ were considered significant.

Results

Effect of hGH on cytokine-induced apoptosis

To study the effect of hGH on cytokine-induced apoptosis, INS-1 cells were treated with cytotoxic cytokines in the presence or absence of hGH. Apoptosis was measured using Annexin V-FITC conjugates for flow cytometry to detect phosphatidylserine exposed on the outer surface of apoptotic cells. Double staining with PI distinguished early apoptotic, i.e. Annexin V-positive/PI-negative cells (Fig. 1, upper panel) from late apoptotic/necrotic, i.e. Annexin V/PI double-positive cells (Fig. 1, middle panel). The percentage of early apoptotic INS-1 cells was not increased after exposure for 24 and 48 h to TNF- α or IL-1 β alone. However, exposure of INS-1 cells to IFN- γ significantly increased the percentage of early apoptotic cells to $15.2 \pm 2.6\%$ after 24 h and $21.9 \pm 3.4\%$ after 48 h. In comparison, the percentages in untreated control cells were $10.8 \pm 1.8\%$ and $9.7 \pm 1.4\%$ respectively. Apoptosis was further enhanced by the combination of all three cytokines (mix), especially after 48 h of exposure (24 h: $16.7 \pm 2.7\%$, $P < 0.05$ and 48 h: $32.8 \pm 4.7\%$, $P < 0.01$).

Co-treatment with hGH significantly inhibited the apoptotic effect of IFN- γ alone after both 24 h ($12.2 \pm 2.7\%$, $P < 0.05$) and 48 h ($16.5 \pm 2.2\%$, $P < 0.05$) and in combination with TNF- α and IL-1 β after 24 h ($9.5 \pm 1.5\%$, $P < 0.05$) but not after 48 h. The percentage of late apoptotic/necrotic cells markedly increased after 48 h of exposure of INS-1 cells to the combination of IFN- γ , TNF- α and IL-1 β from $2.7 \pm 0.5\%$ in untreated control cells to $20.6 \pm 3.8\%$ ($P < 0.05$) in cytokine-treated cells. Whereas co-treatment with hGH did not significantly decrease the toxic effect of the high cytokine concentrations (Fig. 1, middle panel) there was a significant protection when using a lower concentration of cytokines (1/2 mix), both after 24 and 48 h (Fig. 1, upper and middle panels).

Cell viability was determined using the MTT assay which measures mitochondrial succinate dehydrogenase activity (Fig. 1, lower panel). In accordance with the results obtained by Annexin V staining, exposure of INS-1 cells to IFN- γ alone significantly reduced MTT activity (24 h: $50.4 \pm 3.8\%$ and 48 h: $41.4 \pm 4.2\%$, data relative to control cells), whereas IL-1 β or TNF- α alone did not influence the MTT index. Combinations of IFN- γ with TNF- α and IL-1 β +TNF- α further reduced the MTT activity (e.g. IFN- γ +TNF- α , 24 h: $37.7 \pm 5.3\%$ and 48 h: $16.5 \pm 2.2\%$). Co-treatment with hGH significantly reduced the toxic effect of IFN- γ alone (24 h: $61.1 \pm 8.9\%$, $P < 0.05$ and 48 h: $56.8 \pm 6.4\%$, $P < 0.01$) or in combination with TNF- α (IFN- γ +TNF- α , 24 h: $44.5 \pm 6.6\%$, $P < 0.05$ and 48 h: $25.8 \pm 3.7\%$, $P < 0.005$). Together these results showed that hGH protects insulin-producing cells against cytokine-induced toxicity via inhibition of apoptosis.

Effects of DN- and CA-STAT5 mutants on cytokine-induced apoptosis

To address the role of STAT5 activation in hGH-induced protection against cytokine-induced apoptosis, we infected INS-1E cells with an adenovirus encoding either DN-STAT5 or CA-STAT5b mutants. The adenovirus encoding GFP infected up to 90% of the INS-1E cells as visualized by fluorescence microscopy (data not shown). Protein expression of the STAT5 mutants, DN-STAT5 and CA-STAT5b, was analyzed by Western blot analysis, which showed increased protein expression levels for both DN-STAT5 and CA-STAT5b with increasing virus titer (0–400 PFU/cell; Fig. 2A). As the inhibitory effect of DN-STAT5 is ascribed to its ability to dimerize with wild type (wt) STAT5 and bind DNA, thereby inhibiting wt STAT5 activity, DNA-binding activity of the DN-STAT5 mutant was measured by gel shift assay using a radiolabelled double-stranded oligonucleotide representing the STAT5-binding element of the PRLR 1A promoter (Galsgaard *et al.* 1999). hGH induced DNA binding of endogenous STAT5 in non-infected INS-1E cells

Figure 1 Protective effect of hGH on cytokine-induced apoptosis in INS-1 cells. INS-1 cells were treated with IL-1 β (150 pg/ml), IFN- γ (200 U/ml) and hTNF- α (200 U/ml) either alone or in combination (mix) in the presence or absence of hGH (500 ng/ml). After 24 and 48 h of treatment, cells were analysed by flow cytometry for Annexin V and IP staining. The upper panels show early apoptotic cells that are Annexin V positive only, whereas the middle panels show late apoptotic/necrotic cells that are Annexin V and PI double positive. Results (means $\pm$ S.E.M., $n=3-4$) are expressed as percentage of stained cells. Viability of the cells was determined by the MTT assay (lower panel). Results (means $\pm$ S.E.M., $n=5$) are expressed as percentage of untreated control cells. * $P<0.05$, ** $P<0.01$, *** $P<0.005$.

(Fig. 2B) and strongly potentiated DNA binding of DN-STAT5 which exhibited DNA-binding activity even under basal conditions, thereby showing successful expression of functional proteins.

Cytokine-induced apoptosis was studied in INS-1E cells infected with adenovirus (400 PFU/cell) using the Cell Death Detection Elisa assay to detect DNA fragmentation (Fig. 2C). Virus infection *per se* did not influence cytokine-induced apoptosis, since the mixture of IL-1 β , IFN- γ and TNF- α increased DNA fragmentation to a similar degree in the non-infected cells (3.4 ± 0.3 -fold), GFP-infected cells (2.7 ± 0.6 -fold) and cells infected with

DN-STAT5 (4.0 ± 0.4 -fold). Furthermore, in cells infected with GFP, hGH significantly inhibited cytokine-induced apoptosis to the same degree as in non-infected cells (1.7 ± 0.2 - and 1.9 ± 0.2 -fold respectively). However, hGH was not able to abolish cytokine-induced apoptosis in INS-1E cells infected with DN-STAT5 (3.7 ± 0.5 -fold in the DN-STAT5-infected cells compared with 1.9 ± 0.2 -fold in the non-infected cells, $P<0.05$), suggesting that activation of the STAT5 signalling pathway plays an important role in the anti-apoptotic effect of hGH. INS-1E cells infected with CA-STAT5b were more resistant to cytokine-induced apoptosis than

non-infected cells and DN-STAT5-infected cells (2.4 ± 0.2 -fold for CA-STAT5b-infected cells compared with 3.4 ± 0.3 -fold for non-infected cells and 4.0 ± 0.4 -fold for DN-STAT5-infected cells, $P < 0.05$), indicating an effect of the CA-STAT5b even without hGH-induced activation although the protection was enhanced by hGH. These results showed that STAT5 activation was necessary for the anti-apoptotic effect of hGH.

Figure 3 hGH- and cytokine-induced SOCS-3 mRNA expression. Real-time RT-PCR quantitative analysis of SOCS-3 and mRNA in INS-1E cells treated with a combination (mix) of IL-1 β (40 pg/ml), IFN- γ (50 U/ml) and rTNF- α (0.5 ng/ml) in the presence or absence of hGH (500 ng/ml) for 1 and 4 h as indicated. Results (means $\pm$ S.E.M., $n=4$) are expressed as the SOCS-3/PGK mRNA ratio normalized to untreated control cells. * $P < 0.05$ compared with control, † $P < 0.05$ compared with mix.

Effects of hGH and cytokines on SOCS-3 expression

The mRNA expression of SOCS-3 in INS-1E cells in response to hGH stimulation was measured by real-time PCR analysis (Fig. 3). SOCS-3 mRNA levels were normalized to mRNA levels of the internal control PGK. After 1 h of treatment with hGH alone SOCS-3 expression was increased $590 \pm 24\%$ compared with untreated control cells. The increase in SOCS-3 mRNA level induced by hGH alone was transient with a $276 \pm 13\%$ increase observed after 4 h. In contrast, treatment with a mixture of IL-1 β , IFN- γ and TNF- α (mix) increased SOCS-3 mRNA expression by $405 \pm 34\%$ after 1 h and $715 \pm 36\%$ after 4 h of treatment. The combined treatment with hGH and cytotoxic cytokines resulted in an enhancement of SOCS-3 mRNA induction when

Figure 2 Effect of DN-STAT5 expression on cytokine-induced DNA fragmentation. (A) Western blot analysis was performed on total protein extracts prepared from INS-1E cells that had been infected with the indicated amounts of adenovirus encoding either DN-STAT5 or CA-STAT5b. (B) INS-1E cells were infected with adenovirus encoding DN-STAT5 (400 PFU/cell) for 24 h and stimulated in the presence or absence of hGH for 15 min. Non-infected cells were included for comparison. Nuclear extracts were prepared and incubated with radiolabelled oligonucleotide containing the STAT5-binding site from the PRLR gene. Free and bound probe was separated by non-denaturing gel electrophoresis and visualized by exposure to a PhosphorImager screen. (C) INS-1E cells were infected with adenovirus (400 PFU/cell) containing the cDNA for GFP, DN-STAT5 or CA-STAT5b. Non-infected cells were included as a control. After 24 h of infection the cells were treated with a combination (mix) of IL-1 β (40 pg/ml), IFN- γ (50 U/ml) and rTNF- α (0.5 ng/ml) in the presence or absence of hGH (500 ng/ml) for an additional 24 h. Fragmented DNA was measured by ELISA. Results (means $\pm$ S.E.M., $n=3-10$) are normalized to untreated control cells in each experiment. The effect of hGH on cytokine-induced DNA fragmentation was significantly reduced ($P < 0.05$) in non-infected, GFP-infected cells and CA-STAT5-infected cells. The effect of cytokines was significantly reduced ($P < 0.05$) in CA-STAT5-infected cells compared with non-infected and DN-STAT5-infected cells.

Figure 4 Effect of hGH on cytokine-induced STAT1 and NFκB activation in INS-1E cells. (A) INS-1E cells were either untreated (lane a) or treated with IFN- γ (200 U/ml) (lanes c and d) or IFN- γ (200 U/ml) and hTNF- α (50 ng/ml) (lanes e and f) in the absence (lanes a, c and e) or presence (lanes b, d and f) of hGH (500 ng/ml). Nuclear extracts were prepared after 15 min and incubated with radiolabelled M67 oligonucleotide containing an optimized STAT1/3 binding site. (B) INS-1E cells were either untreated (lane a) or treated with IL-1 β (80 pg/ml) (lanes c and d), IFN- γ (200 U/ml) (lanes e and f) or IFN- γ (200 U/ml) and hTNF- α (50 ng/ml) (lanes g and h) in the absence (lanes a, c, e and g) or presence (lanes b, d, f and h) of hGH (500 ng/ml). Nuclear extracts were prepared after 15 min and incubated with radiolabelled oligonucleotide containing the consensus NFκB-binding sequence. Free and bound probe was separated by non-denaturing gel electrophoresis and visualized by exposure to a PhosphorImager screen. The arrows indicate migration of the (A) STAT1 and (B) NFκB protein–DNA complexes.

compared with either treatment alone after 4 h ($1201 \pm 54\%$). These results indicated that there was an additive effect of hGH and cytokines on the induction of SOCS-3 mRNA expression after extended exposure.

Effects of hGH and cytokines on activation of the transcription factors NFκB, STAT1 and STAT5

To determine whether activation of the two transcription factors NFκB and STAT1 by cytotoxic cytokines was inhibited by hGH stimulation, we performed gel shift assays. Activation of STAT1 DNA binding was detected by incubation of nuclear extracts with the radiolabelled double-stranded oligonucleotide representing the optimized SIE m67 from the *c-fos* gene (Fig. 4A). Treatment of INS-1E cells with IFN- γ either alone or in combination with TNF- α for 15 min strongly activated DNA binding of STAT1 (Fig. 4A, lanes c and e respectively). In comparison with the marked cytokine-induced STAT1 activation, STAT1 DNA binding was only weakly induced by hGH (Fig. 4A, lane b). Co-treatment with hGH for 15 min did not reduce cytokine-induced STAT1 DNA

binding (Fig. 4A, lanes d and f respectively). Furthermore, we did not observe any effect of hGH on cytokine-induced STAT1 DNA binding even after prolonged incubation time (1, 4 and 24 h; data not shown).

Nuclear translocation of NFκB was detected by incubation of nuclear extracts with an oligonucleotide representing the consensus NFκB-binding element (Fig. 4B). NFκB nuclear translocation was strongly induced within 15 min by IL-1 β (Fig. 4B, lane c) and by the combination of IFN- γ and TNF- α (Fig. 4B, lane g). In contrast, hGH alone did not induce NFκB translocation (Fig. 4B, lane b). The presence of hGH did not influence DNA binding of NFκB induced by IL-1 β or by the combination of IFN- γ and TNF- α after 15 min (Fig. 4B, lanes d and h respectively) or after 1, 4 and 24 h (data not shown). As previously described (Galsgaard *et al.* 1999), hGH strongly induced STAT5 DNA binding already after 15 min and this DNA-binding activity lasted for up to 24 h (data not shown). In contrast, IL-1 β , IFN- γ and IFN- γ +TNF- α did not induce STAT5 activation and, moreover, these cytokines did not interfere with hGH-induced STAT5 activation (data not shown).

Figure 5 Effect of hGH on cytokine-induced iNOS mRNA expression and NO accumulation. INS-1 cells were treated with (A) IL-1 β (150 pg/ml), IFN- γ (200 U/ml) and hTNF- α (200 U/ml) either alone or in combination and (B) IL-1 β (150 pg/ml) in the presence or absence of hGH (500 ng/ml) for 24, 48 and 72 h as indicated. NO accumulation was determined by the Griess assay. Results (means $\pm$ S.E.M., $n=5$) are expressed as percentage of 24 h IL-1-treated cells. * $P<0.05$ compared with IL-1 treated cells. (C) Real-time PCR quantitative analysis of iNOS mRNA in INS-1E cells treated with a combination (mix) of IL-1 β (40 pg/ml), IFN- γ (50 U/ml) and TNF- α (0.5 ng/ml) in the presence or absence of hGH (500 ng/ml) for 1 h and 4 h as indicated. Quantitative real-time PCR of iNOS and PGK mRNA expressed as the iNOS/PGK ratio normalized to untreated control cells (means $\pm$ S.E.M., $n=3$). * $P<0.05$ compared with control, † $P<0.05$ compared with mix.

Effects of hGH on cytokine-induced iNOS expression and NO production

To address whether the anti-apoptotic affect of hGH was mediated by inhibition of cytokine-induced NO production, we measured nitrite accumulation. As shown in Fig. 5A, IFN- γ alone did not induce NO production, whereas IL-1 β induced similar levels of NO as the combination of IFN- γ and TNF- α . The combination of IL-1 β , IFN- γ and TNF- α further enhanced NO production. As shown in Fig. 5B, hGH was found to have a significant potentiating effect on NO production induced by IL-1 β alone ($P<0.05$). A similar potentiating effect of hGH was seen on NO production induced by the combination of cytokines (data not shown). In order to see if this effect was due to changes in the expression of iNOS mRNA we performed quantitative RT-PCR of total RNA extracted from INS-1E cells exposed to combinations of hGH and cytokines. As shown in Fig. 5C, IL-1 β +IFN- γ +TNF- α (mix) induced a marked increase in iNOS expression after 4 h exposure (9.6 ± 1.2 -fold, $P<0.05$). However, addition

of hGH only slightly inhibited the cytokine-induced iNOS mRNA levels (8.4 ± 1.0 compared with 9.6 ± 1.2 , $P<0.05$). Thus, inhibition of iNOS transcription or enzymatic activity was not part of the anti-apoptotic effect of hGH.

Effects of hGH and cytokines on expression of Bcl-x_L and Bax

The protective effect of hGH on cytokine-induced apoptosis may involve changes in the balance between pro-apoptotic and anti-apoptotic proteins of the Bcl-2 family. Using real-time PCR, we studied mRNA levels of the STAT5-inducible anti-apoptotic Bcl-x_L and the pro-apoptotic Bax (Fig. 6A and 6B respectively). Stimulation of the INS-1E cells with hGH had a tendency to increase the Bcl-x_L mRNA expression although this was not statistically significant (1.5 ± 0.3) after 1 h, whereas 4 h of hGH stimulation resulted in a significant increase in the Bcl-x_L mRNA expression (1.6 ± 0.1 , $P<0.05$) relative to

Figure 6 hGH- and cytokine-induced Bcl-x_L and Bax mRNA expression. Real-time RT-PCR quantitative analysis of Bcl-x_L and Bax mRNA in INS-1E cells treated with a combination (mix) of IL-1 β (40 pg/ml), IFN- γ (50 U/ml) and rTNF- α (0.5 ng/ml) in the presence or absence of hGH (500 ng/ml) for 1 and 4 h as indicated. Results (means $\pm$ S.E.M., $n=4$) are expressed as the (A) Bcl-x_L/PGK and (B) Bax/PGK mRNA ratio normalized to untreated control cells. * $P<0.05$ compared with control, † $P<0.05$ compared with mix.

untreated control cells. Whereas treatment with the mixture of cytotoxic cytokines did not influence the expression of Bcl-x_L significantly ($117 \pm 11\%$ and $102 \pm 8\%$ after 1 h and 4 h respectively), the combination of hGH and cytotoxic cytokines further increased Bcl-x_L mRNA expression to $193 \pm 11\%$ ($P<0.05$) after 4 h of treatment. The Bax mRNA expression was unchanged during treatment with GH and/or cytotoxic cytokines (Fig. 6B), resulting in an overall increase in the Bcl-x_L/Bax ratio in cells treated with hGH alone or in combination with cytotoxic cytokines.

Discussion

Beside the stimulatory effect of GH and PRL on insulin gene transcription, biosynthesis and secretion, and β -cell proliferation, a role for GH and PRL in the regulation of cell death and survival has been observed in lymphoid

cells (LaVoie & Witorsch 1995, Jeay *et al.* 2000, 2002) and neurones (Shin *et al.* 2004). Furthermore, a recent study of primary β -cells showed that PRL reduces the expression of genes related to apoptosis and stimulates the transcription of genes associated with cell survival (Bordin *et al.* 2004). We therefore aimed to study whether hGH can protect β -cells against cytotoxic cytokines involved in the autoimmune destruction of β -cells in type 1 diabetes. We have included IL-1 β together with IFN- γ and TNF- α , as IL-1 β seems to be the most important cytotoxic cytokine in primary β -cells (Mandrup-Poulsen 1996).

We studied the effect of hGH on cytokine-induced apoptosis in the insulin-secreting rat insulinoma cell lines INS-1 and INS-1E. INS-1E is a more glucose-sensitive subclone of the INS-1 cell line (Janjic *et al.* 1999), which retains a number of differentiated features of the native β -cell and expresses functional GHR and PRLR (Asfari *et al.* 1992, Sekine *et al.* 1994, Asfari *et al.* 1995).

Our results have confirmed and extended previous findings that GH abolishes IFN- γ - and TNF- α -induced apoptosis in the insulin-producing cell line INS-1 (Sekine *et al.* 1999). However, our results differed in certain respects with regard to the molecular mechanisms.

Activation of STAT5 by GHR and PRLR is essential for GH and PRL signalling in most tissues, as indicated by the similar phenotypes of PRLR, GHR, STAT5a and STAT5b knockout mice (Bole-Feysot *et al.* 1998). Accordingly, in β -cells, GH and PRL activate STAT5 (Stout *et al.* 1997, Galsgaard *et al.* 1999, Brelje *et al.* 2002, 2004), which is the major mediator of the mitogenic effect of hGH on INS-1 cells (Friedrichsen *et al.* 2001). Furthermore, STAT5 has been associated with anti-apoptotic effects in cytokine-dependent erythroid progenitors (Socolovsky *et al.* 1999). In β -cells, GH and PRL stimulate the nuclear translocation of STAT5b more markedly than of STAT5a (Brelje *et al.* 2002, 2004).

Gel shift assays showed that hGH stimulation induced STAT5 DNA binding in the INS-1E cells, in accordance with what was found in INS-1 cells (Galsgaard *et al.* 1996), and lasted for at least 24 h. The role of STAT5 in the anti-apoptotic effect of hGH was studied by infecting the INS-1E cells with two mutant STAT5-variants, DN-STAT5, which is truncated at amino acid 749 making the protein unable to activate transcription but still able to dimerize with wt STAT5a and b, thereby inhibiting the transcriptional activity of both STAT5a and STAT5b (Moriggl *et al.* 1996), and CA-STAT5b which contains two point mutations that stabilize the activated form of the protein (Onishi *et al.* 1998).

We found that when STAT5 activity was inhibited by DN-STAT5, hGH was no longer able to inhibit the cytokine-induced apoptosis, showing that STAT5 is necessary for mediating the anti-apoptotic effect of hGH. CA-STAT5b had a small but significant anti-apoptotic effect compared with non-infected and DN-STAT5-infected cells. This effect was further augmented by hGH, supporting the essential role of STAT5 activation for the anti-apoptotic effect.

We followed STAT1 DNA-binding activity for 24 h, and found that hGH did not influence cytokine-induced STAT1 DNA binding within the time when hGH exerts its anti-apoptotic effect, showing that inhibition of STAT1 DNA binding is not involved in the anti-apoptotic effect of hGH under the conditions used in the present study.

Different mechanisms for the protective effect of hGH have been suggested. Luo & Yu-Lee (2000) suggested that STAT5 inhibits cytokine-induced activation of NF κ B. NF κ B is an important mediator of the cytokine signalling in β -cells (Eizirik *et al.* 2003). Our DNA-binding results showed that the cytokine-induced DNA-binding activity of NF κ B was not abrogated by hGH, as we did not see any changes in NF κ B DNA-binding activity in the INS-1E cells upon hGH treatment for up to 24 h. This is in

agreement with results reported in INS-1 cells by Sekine *et al.* (2001).

Cytokines (IL-1 β in particular) induce expression of iNOS and thus production of NO in β -cells (Eizirik *et al.* 1996, Rabinovitch & Suarez-Pinzon 1998). NO impairs β -cell function and may lead to β -cell death by causing DNA damage and inhibition of aconitase in the citric acid cycle resulting in impaired oxidative phosphorylation. However, the role of NO in cytokine-induced β -cell apoptosis is rather controversial, as studies in human islets suggest that NO may only be part of or not at all involved in cytokine-induced β -cell destruction (Eizirik *et al.* 1994, Rabinovitch *et al.* 1994).

Sekine *et al.* (2001) found a partial reduction in NO production when INS-1 cells were co-treated with GH in addition to IFN- γ and TNF- α , and therefore suggest that the protective effect of GH is partly explained by the inhibition of NO production. However, our data showed that the cytokine-induced increase in iNOS mRNA expression was not abolished by the co-treatment with hGH. The lack of correlation between NO production and β -cell death observed in our experiments rather suggested that GH and PRL may protect β -cells at a point beyond the level of NO production and thus, via STAT5, may protect against NO-induced β -cell death. Further studies using synthetic NO donors as well as longer exposure times may unravel the significance of this mechanism.

A newly described family of genes transiently induced by cytokines encodes SOCS proteins, which act as inhibitors of cytokine receptor signalling. In accordance with Sekine *et al.* (2001) we found that hGH treatment led to increases in both basal as well as cytokine-induced SOCS-3 mRNA levels in INS-1E cells. SOCS-3 overexpression prevents the toxic effect of IL-1 β and IFN- γ in INS-1 cells (Karlsen *et al.* 2001) and reduces IL-1 β -induced NF κ B activation, IFN- γ -induced STAT1 DNA-binding activity and decreases iNOS mRNA expression (Froboese *et al.* 2003). We did not see any of these changes in conjunction with hGH-induced SOCS-3 expression, neither NF κ B and STAT1 DNA binding nor NO production, suggesting that SOCS-3 expression levels induced by hGH are insufficient to inhibit these activities, and may therefore not be responsible for the anti-apoptotic effect of hGH observed in these cells.

The Bcl-2 family is one of the most prominent regulators of apoptosis. Gene transfer of the anti-apoptotic Bcl-2 has conferred *in vitro* protection from apoptosis in isolated human islets and in a mouse cell line exposed to pro-inflammatory cytokines (Iwahashi *et al.* 1996, Dupraz *et al.* 1999, Rabinovitch *et al.* 1999, Saldeen 2000). Additionally, the anti-apoptotic effect of STAT5 in erythroid progenitors has been explained by induction of the Bcl-x_L gene, which contains a STAT5 responsive element (Socolovsky *et al.* 1999), and reduction in Bcl-x_L expression has been associated with β -cell apoptosis (Pierucci *et al.* 2001).

We found that mRNA expression of the anti-apoptotic Bcl-x_L was increased by hGH, whereas the pro-apoptotic Bax was affected neither by hGH nor by the cytokines IL-1 β , IFN- γ and TNF- α . These results suggested that the anti-apoptotic effect of hGH is mediated by STAT5-induced increases in Bcl-x_L expression.

The biological significance of STAT5 activity in β -cells is emerging from a recent *in vivo* study in transgenic mice expressing either DN- or CA-STAT5 mutants under the insulin promoter. It was found that diabetes induced by multiple low-dose streptozotocin was more severe in DN-STAT5 mice than in CA-STAT5 mice (Jackerott *et al.* 2004). These results suggest that the level of STAT5 activity in β -cells contributes to the survival of β -cells *in vivo* and thus support our *in vitro* results.

In conclusion, we found that hGH can protect the insulin-producing INS-1E cells against cytokine-induced apoptosis and that STAT5 is necessary for mediating this effect. Although SOCS-3 expression was increased markedly by both cytokines and hGH alone and in combination, this probably does not explain the protective effect of hGH as no inhibition of STAT5, STAT1, NF κ B and NO production was observed. The anti-apoptotic effect of hGH on β -cells may therefore be beyond the level of NO production and the increased expression of Bcl-x_L may protect β -cells against reactive oxygen radicals induced by cytokines. Further studies focusing on the role of Bcl-x_L may provide evidence for its role in the STAT5-mediated anti-apoptotic effect of hGH.

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References

- Asfari M, Janjic D, Meda P, Li G, Halban PA & Wollheim CB 1992 Establishment of 2-mercaptoethanol-dependent differentiated insulin-secreting cell lines. *Endocrinology* **130** 167–178.
- Asfari M, De W, Postel-Vinay MC & Czernichow P 1995 Expression and regulation of growth hormone (GH) and prolactin (PRL) receptors in a rat insulin producing cell line (INS-1). *Molecular and Cellular Endocrinology* **107** 209–214.
- Bole-Feysot C, Goffin V, Edery M, Binart N & Kelly PA 1998 Prolactin (PRL) and its receptor: actions, signal transduction pathways and phenotypes observed in PRL receptor knockout mice. *Endocrine Reviews* **19** 225–268.
- Bordin S, Amaral ME, Anhe GF, Delghingaro-Augusto V, Cunha DA, Nicoletti-Carvalho JE & Boschero AC 2004 Prolactin-modulated gene expression profiles in pancreatic islets from adult female rats. *Molecular and Cellular Endocrinology* **220** 41–50.
- Brelje TC, Svensson AM, Stout LE, Bhargoo NV & Sorenson RL 2002 An immunohistochemical approach to monitor the prolactin-induced activation of the JAK2/STAT5 pathway in pancreatic islets of Langerhans. *Journal of Histochemistry and Cytochemistry* **50** 365–383.
- Brelje TC, Stout LE, Bhargoo NV & Sorenson RL 2004 Distinctive roles for prolactin and growth hormone in the activation of STAT5 in pancreatic islets of Langerhans. *Endocrinology* **145** 4162–4175.
- Carter-Su C & Smit LS 1998 Signaling via JAK tyrosine kinases: growth hormone receptor as a model system. *Recent Progress in Hormone Research* **53** 61–83.
- Dupraz P, Rinsch C, Pralong WF, Rolland E, Zufferey R, Trono D & Thorens B 1999 Lentivirus-mediated Bcl-2 expression in betaTC-tet cells improves resistance to hypoxia and cytokine-induced apoptosis while preserving *in vitro* and *in vivo* control of insulin secretion. *Gene Therapy* **6** 1160–1169.
- Eizirik DL & Mandrup-Poulsen T 2001 A choice of death – the signal-transduction of immune-mediated beta-cell apoptosis. *Diabetologia* **44** 2115–2133.
- Eizirik DL, Sandler S, Welsh N, Cetkovic-Cvrlje M, Nieman A, Geller DA, Pipeleers DG, Bendtzen K & Hellerstrom C 1994 Cytokines suppress human islet function irrespective of their effects on nitric oxide generation. *Journal of Clinical Investigation* **93** 1968–1974.
- Eizirik DL, Flodstrom M, Karlens AE & Welsh N 1996 The harmony of the spheres: inducible nitric oxide synthase and related genes in pancreatic beta cells. *Diabetologia* **39** 875–890.
- Eizirik DL, Kutlu B, Rasschaert J, Darville M & Cardozo AK 2003 Use of microarray analysis to unveil transcription factor and gene networks contributing to beta cell dysfunction and apoptosis. *Annals of the New York Academy of Science* **1005** 55–74.
- Friedrichsen BN, Galsgaard ED, Nielsen JH & Moldrup A 2001 Growth hormone- and prolactin-induced proliferation of insulinoma cells, INS-1, depends on activation of STAT5 (signal transducer and activator of transcription 5). *Molecular Endocrinology* **15** 136–148.
- Friedrichsen BN, Richter HE, Hansen JA, Rhodes CJ, Nielsen JH, Billestrup N & Moldrup A 2003 Signal transducer and activator of transcription 5 activation is sufficient to drive transcriptional induction of cyclin D2 gene and proliferation of rat pancreatic beta-cells. *Molecular Endocrinology* **17** 945–958.
- Froboese H, Heding P, Ronn, SG, Karlens AE, Mandrup-Poulsen T & Billestrup N 2003 Suppressor of cytokine signaling-3 inhibits IL-1 beta induced NF κ B signaling in beta-cells. *Diabetologia* **46** (Suppl 2) A109.
- Galsgaard ED, Gouilleux F, Groner B, Serup P, Nielsen JH & Billestrup N 1996 Identification of a growth hormone-responsive STAT5-binding element in the rat insulin 1 gene. *Molecular Endocrinology* **10** 652–660.
- Galsgaard ED, Nielsen JH & Moldrup A 1999 Regulation of prolactin receptor (PRLR) gene expression in insulin-producing cells. Prolactin and growth hormone activate one of the rat PRLR gene promoters via STAT5a and STAT5b. *Journal of Biological Chemistry* **274** 18686–18692.
- Green LC, Wagner DA, Glogowski J, Skipper PL, Wishnok JS & Tannenbaum SR 1982 Analysis of nitrate, nitrite, and [15N]nitrate in biological fluids. *Analytical Biochemistry* **126** 131–138.
- Iwahashi H, Hanafusa T, Eguchi Y, Nakajima H, Miyagawa J, Itoh N, Tomita K, Namba M, Kuwajima M, Noguchi T, Tsujimoto Y & Matsuzawa Y 1996 Cytokine-induced apoptotic cell death in a mouse pancreatic beta-cell line: inhibition by Bcl-2. *Diabetologia* **39** 530–536.
- Jackerott M, Tønnesen I, Moldrup A, Thams P & Nielsen JH 2004 STAT5 activity in beta cells affects the susceptibility to diabetes induced by multiple low-doses of streptozotocin. *Post-European Association for the Study of Diabetes (EASD) Symposium Conference*

- Proceedings, September 2004, Prien, Germany. Abstract p1. Ed S Lenzen. EASD Islet Study Group.
- Janjic D, Maechler P, Sekine N, Bartley C, Annen AS & Wolheim CB 1999 Free radical modulation of insulin release in INS-1 cells exposed to alloxan. *Biochemical Pharmacology* **57** 639–648.
- Jey S, Sonenshein GE, Postel-Vinay MC & Baixeras E 2000 Growth hormone prevents apoptosis through activation of nuclear factor-kappaB in interleukin-3-dependent Ba/F3 cell line. *Molecular Endocrinology* **14** 650–661.
- Jey S, Sonenshein GE, Postel-Vinay MC, Kelly PA & Baixeras E 2002 Growth hormone can act as a cytokine controlling survival and proliferation of immune cells: new insights into signaling pathways. *Molecular and Cellular Endocrinology* **188** 1–7.
- Karlsen AE, Romm SG, Lindberg K, Johannesen J, Galsgaard ED, Pociot F, Nielsen JH, Mandrup-Poulsen T, Nerup J & Billestrup N 2001 Suppressor of cytokine signalling 3 (SOCS-3) protects beta-cells against interleukin-1beta- and interferon-gamma-mediated toxicity. *PNAS* **98** 12191–12196.
- LaVoie HA & Witorsch RJ 1995 Investigation of intracellular signals mediating the anti-apoptotic action of prolactin in Nb2 lymphoma cells. *Proceedings of the Society for Experimental Biology and Medicine* **209** 257–269.
- Luo G & Yu-Lee L 2000 Stat5b inhibits NFkappaB-mediated signaling. *Molecular Endocrinology* **14** 114–123.
- Mandrup-Poulsen T 1996 The role of interleukin-1 in the pathogenesis of IDDM. *Diabetologia* **39** 1005–1029.
- Meyer DJ, Campbell GS, Cochran BH, Argetsinger LS, Lerner AC, Finbloom DS, Carter-Su C & Schwartz J 1994 Growth hormone induces a DNA binding factor related to the interferon-stimulated 91-kDa transcription factor. *Journal of Biological Chemistry* **269** 4701–4704.
- Moldrup A, Billestrup N & Nielsen JH 1990 Rat insulinoma cells express both a 115-kDa growth hormone receptor and a 95-kDa prolactin receptor structurally related to the hepatic receptors. *Journal of Biological Chemistry* **265** 8686–8690.
- Moldrup A, Petersen ED & Nielsen JH 1993 Effects of sex and pregnancy hormones on growth hormone and prolactin receptor gene expression in insulin-producing cells. *Endocrinology* **133** 1165–1172.
- Moriggl R, Gouilleux-Gruart V, Jahne R, Berchtold S, Gartmann C, Liu X, Hennighausen L, Sotiropoulos A, Groner B & Gouilleux F 1996 Deletion of the carboxyl-terminal transactivation domain of MGF-Stat5 results in sustained DNA binding and a dominant negative phenotype. *Molecular and Cellular Biology* **16** 5691–5700.
- Mosmann T 1983 Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *Journal of Immunological Methods* **65** 55–63.
- Nielsen JH, Linde S, Welinder BS, Billestrup N & Madsen OD 1989 Growth hormone is a growth factor for the differentiated pancreatic beta-cell. *Molecular Endocrinology* **3** 165–173.
- Nielsen JH, Galsgaard ED, Moldrup A, Friedrichsen BN, Billestrup N, Hansen JA, Lee YC & Carlsson C 2001 Regulation of beta-cell mass by hormones and growth factors. *Diabetes* **50** (Suppl 1) S25–S29.
- Onishi M, Nosaka T, Misawa K, Mui AL, Gorman D, McMahon M, Miyajima A & Kitamura T 1998 Identification and characterization of a constitutively active STAT5 mutant that promotes cell proliferation. *Molecular and Cellular Biology* **18** 3871–3879.
- Pierucci D, Cicconi S, Bonini P, Ferrelli F, Pastore D, Matteucci C, Marselli L, Marchetti P, Ris F, Halban P, Oberholzer J, Federici M, Cozzolino F, Lauro R, Borboni P & Marlier LN 2001 NGF-withdrawal induces apoptosis in pancreatic beta cells *in vitro*. *Diabetologia* **44** 1281–1295.
- Rabinovitch A & Suarez-Pinzon WL 1998 Cytokines and their roles in pancreatic islet beta-cell destruction and insulin-dependent diabetes mellitus. *Biochemical Pharmacology* **55** 1139–1149.
- Rabinovitch A, Suarez-Pinzon WL, Strynadka K, Schulz R, Lakey JR, Warnock GL & Rajotte RV 1994 Human pancreatic islet beta-cell destruction by cytokines is independent of nitric oxide production. *Journal of Clinical Endocrinology and Metabolism* **79** 1058–1062.
- Rabinovitch A, Suarez-Pinzon W, Strynadka K, Ju Q, Edelstein D, Brownlee M, Korbitt GS & Rajotte RV 1999 Transfection of human pancreatic islets with an anti-apoptotic gene (bcl-2) protects beta-cells from cytokine-induced destruction. *Diabetes* **48** 1223–1229.
- Saldeen J 2000 Cytokines induce both necrosis and apoptosis via a common Bcl-2-inhibitable pathway in rat insulin-producing cells. *Endocrinology* **141** 2003–2010.
- Sekine N, Cirulli V, Regazzi R, Brown LJ, Gine E, Tamarit-Rodriguez J, Girotti M, Marie S, MacDonald MJ, Wollheim CB *et al.* 1994 Low lactate dehydrogenase and high mitochondrial glycerol phosphate dehydrogenase in pancreatic beta-cells. Potential role in nutrient sensing. *Journal of Biological Chemistry* **269** 4895–4902.
- Sekine N, Takahashi T, Ishikawa T, Okazaki T, & Fujita T 1999 Effects of GH and PRL on apoptotic death of INS-1 cells. Conference Proceedings. *Diabetologia* **42** (Suppl 1) A158.
- Sekine N, Fukumoto S, Ishikawa T, Okazaki T & Fujita T 2001 GH inhibits interferon-gamma-induced signal transducer and activator of transcription-1 activation and expression of the inducible isoform of nitric oxide synthase in INS-1 cells. *Endocrinology* **142** 3909–3916.
- Shin DH, Lee E, Kim JW, Kwon BS, Jung MK, Jee YH, Kim J, Bae SR & Chang YP 2004 Protective effect of growth hormone on neuronal apoptosis after hypoxia-ischemia in the neonatal rat brain. *Neuroscience Letters* **354** 64–68.
- Socolovsky M, Fallon AE, Wang S, Brugnara C & Lodish HF 1999 Fetal anemia and apoptosis of red cell progenitors in Stat5a-/-5b-/- mice: a direct role for Stat5 in Bcl-X(L) induction. *Cell* **98** 181–191.
- Stout LE, Svensson AM & Sorenson RL 1997 Prolactin regulation of islet-derived INS-1 cells: characteristics and immunocytochemical analysis of STAT5 translocation. *Endocrinology* **138** 1592–1603.
- Zabel U, Schreck R & Baeuerle PA 1991 DNA binding of purified transcription factor NF-kappa B. Affinity, specificity, Zn2+ dependence, and differential half-site recognition. *Journal of Biological Chemistry* **266** 252–260.

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