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

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Abstract

The proinflammatory cytokines interleukin-1β (IL-1β), 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-1β, 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 cytokotoxic 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-κB (NF-κB) by IFN-γ and IL-1β+TNF-α respectively. Gel retardation experiments showed that hGH affects neither IFN-γ+TNF-α-induced STAT1 DNA binding nor IL-1β and IFN-γ+TNF-α-induced NFκB DNA binding. The lack of influence of hGH on cytokine-mediated activation of STAT1 and NFκB 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-1β-stimulated nitric oxide production. As the anti-apoptotic Bcl-xL gene has been shown to harbour a STAT5-binding element we measured the expression of Bcl-xL as well as the pro-apoptotic Bax. We found that hGH increased the Bcl-xL/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 cytokotoxic cytokines via STAT5-dependent mechanisms distal to iNOS activation possibly at the level of Bcl-xL.


Introduction

The proinflammatory cytokines interleukin-1β (IL-1β), 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-5A 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-1β, IFN-γ and TNF-α. The involved signalling pathways share common mediators as GH, PRL and IFN-γ activate the JAK/STAT pathway, IL-1β and TNF-α activate nuclear factor-κB (NFκB) and GH, PRL, IFN-γ and IL-1β induce suppressor of cytokine signalling (SOCS) expression. We therefore studied possible interactions between hGH and IL-1β, IFN-γ and TNF-α on the DNA-binding activity of STAT5, STAT1 and NFκB, 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-xL 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 µg/ml streptomycin (complete medium). The culture medium for the INS-1E cells was furthermore supplemented with 50 µM β-mercaptoethanol.

The cytokines used were recombinant mouse IL-1β (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) STAT5α (STAT5A749) (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 × 10⁵ 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-1β (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 FACSScan (BD Biosciences).

DNA fragmentation

Cells (5 × 10⁴ 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-1β (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 (10⁴ 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-1β (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 titer 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-amoethox) benzene sulphonyl fluoridehydrochloride (AEBSF), 1 mM ortovanadate, 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-fold 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, and incubated for 2 h). Western blot analysis was performed at the end of each PCR run in a 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 × 10^5 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.

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′-AGCTTCATTTCCCCGTAAATCCCTA-3′) (Meyer et al. 1994); NFκB: the consensus NFκB-binding sequence (5′-AGCTCCAGGGGACTTTCCGAG AGG-3′) (Zabel et al. 1991); STAT5: the sequence of the PRL-1A-promoter: 1A-GLE 5′-AGCTAGTTTCTAGGA ATAAGCT-3′ (Galsgaard et al. 1999).

Nuclear extracts (10 μg) were incubated with 20 finol 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.
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-1β alone. However, exposure of INS-1 cells to IFN-γ significantly increased the percentage of early apoptotic cells to 15·2 ± 2·6% after 24 h and 21·9 ± 3·4% after 48 h. In comparison, the percentages in untreated control cells were 10·8 ± 1·8% and 9·7 ± 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 ± 2·7%, P<0·05 and 48 h: 32·8 ± 4·7%, P<0·01).

Co-treatment with hGH significantly inhibited the apoptotic effect of IFN-γ alone after both 24 h (12·2 ± 2·7%, P<0·05) and 48 h (16·5 ± 2·2%, P<0·05) and in combination with TNF-α and IL-1β after 24 h (9·5 ± 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-1β from 2·7 ± 0·5% in untreated control cells to 20·6 ± 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 ± 3·8% and 48 h: 41·4 ± 4·2%, data relative to control cells), whereas IL-1β or TNF-α alone did not influence the MTT index. Combinations of IFN-γ with TNF-α and IL-1β+TNF-α further reduced the MTT activity (e.g. IFN-γ+TNF-α, 24 h: 37·7 ± 5·3% and 48 h: 16·5 ± 2·2%). Co-treatment with hGH significantly reduced the toxic effect of IFN-γ alone (24 h: 61·1 ± 8·9%, P<0·05 and 48 h: 56·8 ± 6·4%, P<0·01) or in combination with TNF-α (IFN-γ+TNF-α, 24 h: 44·5 ± 6·6%, P<0·05 and 48 h: 25·8 ± 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
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-1β, 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) 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

(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-1β, 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) 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 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 (500 ng/ml) for 1 and 4 h as indicated. Results (means ± 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 ± 24% compared with untreated control cells. The increase in SOCS-3 mRNA level induced by hGH alone was transient with a 276 ± 13% increase observed after 4 h. In contrast, treatment with a mixture of IL-1β, IFN-γ and TNF-α (mix) increased SOCS-3 mRNA expression by 405 ± 34% after 1 h and 715 ± 36% after 4 h of treatment. The combined treatment with hGH and cytotoxic cytokines resulted in an enhancement of SOCS-3 mRNA induction when
compared with either treatment alone after 4 h (1201 ± 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 NFkB, STAT1 and STAT5**

To determine whether activation of the two transcription factors NFkB 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 containing an optimized STAT1 binding site (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 d). 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 NFkB was detected by incubation of nuclear extracts with an oligonucleotide representing the consensus NFkB-binding sequence (Fig. 4B). NFkB nuclear translocation was strongly induced within 15 min by IL-1β (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 NFkB translocation (Fig. 4B, lane b). The presence of hGH did not influence DNA binding of NFkB induced by IL-1β 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-1β, 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 4** Effect of hGH on cytokine-induced STAT1 and NFkB 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 binding site. (B) INS-1E cells were either untreated (lane a) or treated with IL-1β (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 NFkB-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) NFkB protein–DNA complexes.
Effects of hGH on cytokine-induced iNOS expression and NO production

To address whether the anti-apoptotic effect 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-1β induced similar levels of NO as the combination of IFN-γ and TNF-α. The combination of IL-1β, 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-1β 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-1β+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-xL 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-xL 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-xL 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-xL mRNA expression (1.6 ± 0.1, P<0.05) relative to control.
untreated control cells. Whereas treatment with the mixture of cytotoxic cytokines did not influence the expression of Bcl-xL significantly (117 ± 11% and 102 ± 8% after 1 h and 4 h respectively), the combination of hGH and cytotoxic cytokines further increased Bcl-xL mRNA expression to 193 ± 11% (P < 0.05) after 4 h of treatment. The Bax mRNA expression was unchanged during treatment with GH and/or cytokine (Fig. 6B), resulting in an overall increase in the Bcl-xL/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-1β together with IFN-γ and TNF-α, as IL-1β 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-Feyset et al. 1998). Accordingly, in β-cells, GH and PRL activate STAT5 (Stout et al. 1997, Galsgaard et al. 1999, Brejl 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 (Brejl 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 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 GH. 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 NFKB. NFKB 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 NFKB was not abrogated by hGH, as we did not see any changes in NFKB 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-1β 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 over-expression prevents the toxic effect of IL-1β and IFN-γ in INS-1 cells (Karlsen et al. 2001) and reduces IL-1β-induced NFKB 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 NFKB 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-xL gene, which contains a STAT5 responsive element (Socolovsky et al. 1999), and reduction in Bcl-xL expression has been associated with β-cell apoptosis (Pierucci et al. 2001).
We found that mRNA expression of the anti-apoptotic Bcl-xL was increased by hGH, whereas the pro-apoptotic Bax was affected neither by hGH nor by the cytokines IL-1β, IFN-γ and TNF-α. These results suggested that this anti-apoptotic effect of hGH is mediated by STAT5-induced increases in Bcl-xL 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, NFkB 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-xL may protect β-cells against reactive oxygen radicals induced by cytokines. Further studies focusing on the role of Bcl-xL may provide evidence for its role in the STAT5-mediated anti-apoptotic effect of hGH.

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