Anti-proliferative effect of pro-inflammatory cytokines in cultured β cells is associated with extracellular signal-regulated kinase 1/2 pathway inhibition: protective role of glucagon-like peptide -1

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Abstract

Pancreatic β-cell homeostasis is a balance between programmed cell death (apoptosis) and regeneration. Although autoimmune diabetes mellitus type 1 (DM1) is the most-studied cause of β-cell mass loss by pro-inflammatory cytokine-induced apoptosis, influences of a pro-inflammatory environment on β-cell regenerative response have been poorly studied. In this study, we assess the anti-proliferative effect of pro-inflammatory cytokines and glucose concentration on rat pancreatic β cells and the potential protective role of glucagon-like peptide (GLP-1). Apoptotic and proliferating islet cells were stained using the DeadEnd Fluorimetric TUNEL System and 5-bromo-2′-deoxyuridine label respectively, in the presence–absence of varying concentrations of glucose, pro-inflammatory cytokines, and GLP-1. The potential signaling pathways involved were evaluated by western blot. Considerable anti-proliferative effects of pro-inflammatory cytokines interleukin (IL)-1β, interferon (IFN)-γ, and tumour necrosis factor-α (TNF-α) were observed. The effects were synergistic and independent of glucose concentration, and appeared to be mediated by the inhibition of extracellular signal-regulated kinase 1/2 (ERK1/2) activation, the signaling pathway involved in β-cell replication. GLP-1 completely reversed the cytokine-induced inhibition of ERK phosphorylation and increased β-cell proliferation threefold in cytokine-treated cultures. While pro-inflammatory cytokines reduced islet cell ERK1/2 activation and β-cell proliferation in pancreatic islet culture, GLP-1 was capable of reversing this effect. These data suggest a possible pharmacological application of GLP-1 in the treatment of early stage DM1, to prevent the loss of pancreatic β cells as well as to delay the development of overt diabetes.

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Introduction

Autoimmune diabetes mellitus type 1 (DM1) is due to a selective death of pancreatic islet β cells, mainly by apoptosis (Castano & Eisenbarth 1990). Pro-inflammatory cytokines such as IL-1β, alone or in combination with IFN-γ and tumour necrosis factor-α (TNF-α), inhibit the glucose-induced insulin secretion and play an important role in β-cell damage (Andersen *et al.* 1994, Maedler *et al.* 2002).

β-cell homeostasis appears to be a balance between death and regeneration such that pancreatic β-cell mass remains constant. This is in agreement with the earlier studies that reported a basal replication rate in murine and human systems (Finegood *et al.* 1995, Butler *et al.* 2003, Teta *et al.* 2005). In addition, in some situations such as pregnancy or weight gain, β-cell mass must rise to adapt to a greater insulin demand (Sorenson & Brelje 1997, Montanya *et al.* 2000). Replication and neogenesis processes appear to participate in this homeostasis. Recent studies have provided support for a significant neogenesis in humans (Bonner-Weir *et al.* 2000, Butler *et al.* 2003, Xu *et al.* 2008), and β-cell self-duplication has been described as the main form for β-cell mass increase in adult mice (Dor *et al.* 2004). There are several growth factors that play important roles in the regulation of the β-cell cycle. These include hepatocyte growth factor, insulin-like growth factors, and glucagon-like peptide-1 (GLP-1). All of these are capable of triggering proliferation signals, such as extracellular signal-regulated kinase 1/2 (ERK1/2), phosphoinositide-3 kinase (PI3-K), and protein kinase C (PKC) (Vasavada *et al.* 2006) as well as acting upon basic machinery controlling the cell cycle in β cells. This machinery, which acts upon G2/M and more importantly on G1/S checkpoints, includes elements such as pRb (protein defective in human retinoblastoma), some cyclins, and cyclin-dependent kinases (For an extensive review see Cozar-Castellano *et al.* 2006).

Some pathological situations produce a loss of β cells and result in an induction of compensatory responses via proliferation and/or neogenesis; partial pancreatectomy

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in rats results in a loss of β-cell differentiation (Jonas *et al.* 1999, Laybutt *et al.* 2001) and diet-inducing hyperglycemia has been shown to be associated with a transient proliferative response (Donath *et al.* 1999). However, no increased β-cell proliferation has been observed associated with the recent onset of DM1 in humans (Butler *et al.* 2007).

One of the common characteristics of pancreatic injury with inflammatory infiltration is the production of pro-inflammatory cytokines such as IL-1β, IFN-γ, and TNF-α, which have been shown to inhibit proliferation in certain experimental models (Dai *et al.* 2003, Song *et al.* 2005, Tellez *et al.* 2005).

GLP-1 and its analogs have beneficial effects on β-cell proliferation. GLP-1 induces islet cell regeneration following partial pancreatectomy (Xu *et al.* 1999, De Leon *et al.* 2003) and prevents diabetes by apoptosis inhibition (Wang & Brubaker 2002). *In vitro* and *ex vivo* studies using GLP-1, or its receptor agonist exendin-4, have demonstrated that several signaling pathways are implicated in the proliferative action on β cells. GLP-1 activates PI3K-Akt (Buteau *et al.* 2003, Wang *et al.* 2004) and protein kinase A (PKA) via increased cAMP levels (Briaud *et al.* 2003, Park *et al.* 2006). PKA, in addition to exerting an inhibitory action on the ERK pathway, can activate ERK by the alternative Rap-1 > B-Raf > MEK1 pathway (Pearson *et al.* 2001). An activation of the ERK1/2 pathway in response to glucose and GLP-1 has been described in human islet cells (Trumper *et al.* 2005).

Since β-cell mass is greatly reduced at the onset of DM1, it is crucial to know the early events occurring just before the disease is established and the influence of the pancreatic *milieu* on the capacity of islet cells to maintain a balance between apoptotic death and regeneration. In the present study, we evaluated the anti-proliferative effect of pro-inflammatory cytokines on β cells and provide initial evidence of ERK1/2 signaling inhibition as one of the mechanisms underlying this effect. The capacity of GLP-1 to reverse this process could extend therapeutic perspectives of analogs of this compound for use in the preventive treatment of DM1.

Materials and methods

Isolation and culture of rat islets

All animal procedures were performed with the approval of the Animal Ethical Use and Care Committee of the Cádiz University School of Medicine (Cádiz, Spain). Pancreatic islets were isolated from adult male Wistar rats as described previously (McDaniel *et al.* 1983). Isolated islets were cultured in RPMI medium (Sigma–Aldrich) supplemented with 2 mM L-glutamine (Gibco Invitrogen), 100 U/ml penicillin, and 100 μg/ml streptomycin (Pen-Strep; Bio-Whittaker Europe, Verviers, Belgium). Fetal bovine serum (10%) (FBS, Gibco Invitrogen) or albumin from bovine serum (5%) (BSA, Sigma–Aldrich) were used for supplementing the cultures. Glucose concentration used in proliferation time-course assays was 5·5 mM and, in the dose–response experiments, 5·5, 7, 11, 17, and 24·4 mM glucose concentrations were used. Pro-inflammatory cytokines (PeproTech EC Ltd, London, UK) used in the experiments were recombinant human IL-1β (50 U/ml), recombinant rat IFN-γ (1000 U/ml), and recombinant rat TNF-α (1000 U/ml). These concentrations were selected as being appropriate, based on the results of previous published studies (Eizirik *et al.* 1994, Delaney *et al.* 1997, Hoorens & Pipeleers 1999). In assays for phosphorylated ERK quantification, the islets were maintained for 12 h in the RPMI culture medium described above, and with 5·5 mM glucose for stability following isolation. Subsequently, the islets were treated with cytokines or GLP-1 (30 nmol/l; a kind gift from Novo Nordisk A/S, Bagsværd, Denmark). After 15-min, 30-min, 60-min, and 48-h incubation, the islets were harvested, washed in cold PBS, pelleted, and the pellet resuspended in sample buffer.

Proliferation assays

Proliferating cells were detected using 5-bromo-2′-deoxyuridine 5 μmol/l (BrdU) label (Sigma–Aldrich), which was added to the cultures from the start of the assay together with, when appropriate, the cytokines being tested. Following the appropriate culture duration, the islets were recovered and incubated for 15 min with trypsin–EDTA (0·25% trypsin, 1 mM EDTA) in Hanks' balanced salt solution without Ca^{2+} and Mg^{2+} (Gibco Invitrogen) at 37 °C, and the islets were gently dispersed. After washing with PBS, the cells were cytospun onto poly-L-lysine-coated slides and fixed in 4% methanol-free formaldehyde. The slides were immunostained using monoclonal mouse anti-bromodeoxyuridine (Dako Cytomation, Denmark) and polyclonal guinea pig anti-insulin (Sigma–Aldrich) antibodies, according to the manufacturer's instructions. The cells were permeabilized by incubation for 30 min with 0·1% Triton-X100 in PBS and washed twice with 100 mM glycine buffer containing 0·1% Triton-X100 and 3% BSA. The cells were then treated with HCl (2 M) in PBS for 30 min, neutralized with borax/borate buffer (0·1 M, pH 8·9) for 30 min, washed, and incubated overnight at 4 °C with anti-bromodeoxyuridine and anti-insulin antibodies. The stained cells were revealed using anti-mouse IgG (alexa-546 conjugated) and anti-guinea pig IgG (alexa-488 conjugated) antibodies (Molecular Probes Inc.; Eugene, OR, USA). The cell nuclei were stained with 4′-6-diamidino-2-phenylindole (DAPI). To determine the proliferating fraction, total and insulin-positive/BrdU-positive cells were analyzed using a fluorescence microscope in

randomized conditions by a single investigator (M B-R) and, for statistical purposes, the percentage of positive cells was calculated for each incubation condition.

Apoptosis and proliferation double staining

The pancreatic islets were cultured for 48 h and BrdU was added to the cultures from the start of the assay together with the cytokines, when appropriate. Then, islet cells were dispersed, cytospun, and fixed, as described above. Apoptotic and proliferating islet cells were stained simultaneously using the DeadEnd Fluorimetric TUNEL System (Promega) according to the manufacturer's instructions and then treated with HCl (2 M) in PBS for 30 min, neutralized with borax/borate buffer (0·1 M, pH 8·9) for 30 min, washed, and incubated overnight at 4 °C with anti-bromodeoxyuridine. BrdU stain uptake was revealed using anti-mouse IgG antibody (alexa-546 conjugated). The cell nuclei were stained with DAPI. Total, TUNEL-positive (green), BrdU-positive (red), and double-stained cells were analyzed using a fluorescence microscope in randomized conditions by a single investigator (M B-R) and, for statistical purposes, the percentage of positive cells was calculated for each incubation condition.

Western blot

Equivalent numbers of islets under the different experimental conditions described above were lysed in 60 mM Tris–HCl buffer (pH 6–8), containing 2% SDS, 10% glycerol, 0·1% bromophenol blue, and 5% β-mercaptoethanol. Lysed islets were boiled for 5 min and then loaded and electrophoresed on 10–12% SDS-PAGE. The proteins were transfer-blotted on to polyvinylidene fluoride membrane. The blot was then incubated in blocking buffer (5% non-fat milk in 10 mM Tris–HCl, 1·15 M NaCl, and 0·1% Tween-20) for 1 h at room temperature. The blots were then incubated with rabbit polyclonal antibody against phospho-ERK1/2 (Cell Signaling, Beverly, MA, USA) overnight according to the manufacturer's instructions, followed by incubation with peroxidase-conjugated anti-rabbit IgG for 1 h at room temperature. Phosphorylated ERK1/2 bands were revealed using LiteA-blot (EuroClone, Italy) and quantified by Quantity One software Version Upgrade (Bio-Rad). The membranes were then stripped in Restore western blot stripping buffer (Pierce, Rockford, IL, USA), washed with Tween–TBS, and immunoblotted using rabbit polyclonal antibody against ERK1/2 (Cell Signaling). ERK1/2 bands were quantified and the ratios between total and phosphorylated ERK1/2 were calculated.

Statistical analyses

Results are presented as means ± S.E.M. of at least three independent and separate experiments. Statistical comparisons were performed by either Mann–Whitney test or ANOVA. $P<0·05$ were considered statistically significant.

Results

Pro-inflammatory cytokines act synergistically, and in a glucose-independent manner, in suppressing cultured β-cell proliferation

Islets were cultured for 24, 48, and 72 h with 5·5 mM glucose and BrdU-positive β cells were quantified. At 72 h, the percentage of proliferating β cells reached a tenfold increase with respect to the 24-h value, and the addition of IL-1β + TNF-α + IFN-γ to the culture reduced the number of positive BrdU-positive β cells (Fig. 1A). Cytokine-induced apoptosis and the proliferating cells undergoing apoptosis were studied at 48-h culture using double-staining TUNEL/BrdU. The cytokines induced apoptosis in islet cells, but there was no apoptosis increase in proliferating islet cells; the double-staining fraction remaining near-undetectable in control cultures and in cytokine-supplemented cultures (Fig. 1B).

The effect of pro-inflammatory cytokines, alone or in combination, was tested following 48-h culture. Individual cytokines had no significant effect on β-cell proliferation when incubated alone. When incubated in pairs, IL-1β + TNF-α and IL-1β + IFN-γ had significant intermediate effects. Only TNF-α + IFN-γ acted similarly to that of all cytokines in combination (Fig. 2).

To investigate whether the anti-proliferative effect of cytokines depended on glucose concentration, pancreatic islets were cultured at 5·5, 7, 11, 17, and 24·4 mM glucose with and without IL-1β + TNF-α + IFN-γ. Although in the absence of cytokines BrdU-positive β-cell number appeared to increase at 7·7 mM glucose, the difference did not reach statistical significance at 48-h culture. The percentage of β-cell proliferation in the presence of cytokines was the same at all the different glucose concentrations, indicating that this effect was independent of glucose levels (Fig. 3).

ERK1/2 activation is inhibited by pro-inflammatory cytokines

Phospho-ERK1/2 was quantified in the islet cells following exposure to cytokines for 15, 30, and 60 min. The values were compared with control cultures without cytokines. Phospho-ERK1/2 expression in the islet cells was reduced along the time course of exposure to cytokines, reaching a maximum level of inhibition at

30 min, which was maintained up to 60 min (Fig. 4). No changes were observed in the ERK expression over the time course of culture prior to the assay, probably due to isolation procedures used (data not shown).

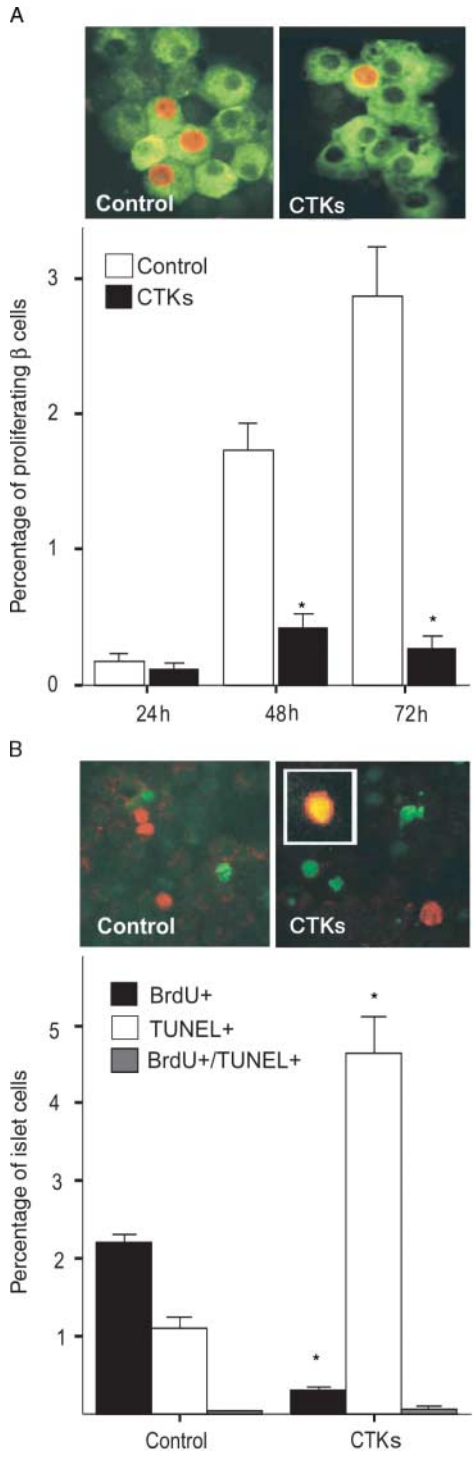

Figure 1 Effect of pro-inflammatory cytokines on cultured β-cell proliferation. (A) Rat islets were cultured for 24, 48, and 72 h in 5·5 mM glucose medium alone (control, white bars) or in combination with IL-1β (50 U/ml) + IFN-γ (1000 U/ml) + TNF-α (1000 U/ml) (CTKs, black bars) and stained with BrdU during the whole course of the culture period. Upper panels show a representative image of insulin- (green) and BrdU (red)-stained islet cells after 48-h culture. Bar diagram expresses results as means ± S.E.M. of percentage of accumulated BrdU-positive/insulin-positive cells over the culture period relative to insulin-positive cells, in a minimum of five experiments. (B) Rat islets were cultured for 48 h in 5·5 mM glucose medium, alone or with cytokines and stained with BrdU during the whole course of the culture period. BrdU-positive (black bars), TUNEL-positive (white bars), and double-stained BrdU-positive/TUNEL-positive (grey bars) cells were quantified. Upper panels show representative images of TUNEL (green), BrdU-(red), and BrdU-positive/TUNEL-positive (yellow, in square) stained islet cells. Graph expresses results as means ± S.E.M. of the percentages of three populations of cells in five experiments and refers to total cell number. *$P < 0{\cdot}05$ treated versus control without cytokines.

ERK1/2 inhibition has parallel effects on β-cell proliferation as pro-inflammatory cytokines

MEK1 inhibitor PD98059 was used to test the role of ERK1/2 in cultured β cells, and to explore the effect of cytokines on the ERK1/2 expression and β-cell proliferation. PD98059 alone as well as PD98059 plus cytokines induced between 2·6- and 5-fold decreases in β-cell proliferation. The rate was similar to that induced by cytokines (4·2-fold relative to control) in 48-h islet cultures (Fig. 5).

GLP-1 has a protective role against anti-proliferative effects of cytokines

To test the effect of a recognized β-cell ERK activator, as GLP-1, in the anti-proliferative action of cytokines, β-cell cultures were performed with both, cytokines and GLP-1 (30 nmol/l). Overall effects of GLP-1 and cytokines were evaluated in terms of ERK activation and β-cell proliferation. For the control of GLP-1 action in the absence of growth factors, a culture supplemented with 5% BSA instead of FBS was performed. In BSA-supplemented cultures, ERK activation was induced by GLP-1 thus confirming the capacity to activate ERK pathway in β cells (Fig. 6A). However, in FBS-supplemented cultures, high level of ERK activation without any effect of GLP-1 was observed. When cytokines were added to the culture, an ERK phosphorylation inhibition was observed after 30 min, which was completely reversed by GLP-1 (Fig. 6B). Nevertheless, although the addition of GLP-1 increased β-cell proliferation by threefold compared with cytokine-treated 48-h cultures (Fig. 7), this effect did not imply a total recovery to previous proliferation rates. A concentration of 60 nmol/l did not increase GLP-1 effect on β-cell proliferation under these conditions (data not shown).

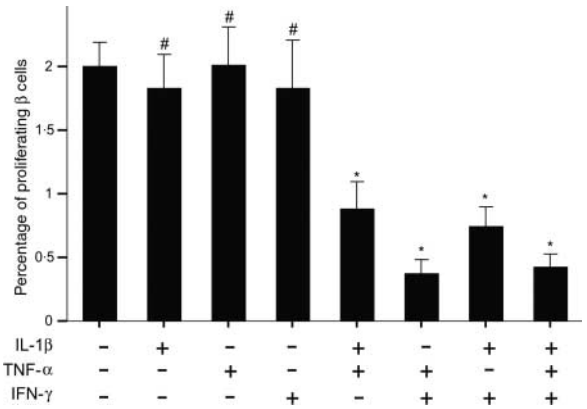

Figure 2 Effect of pro-inflammatory cytokines alone or in combination. Rat islets were cultured for 48 h in 5·5 mM glucose medium, alone or in combination with IL-1β (50 U/ml), IFN-γ (1000 U/ml), TNF-α (1000 U/ml), IL-1β+ IFN-γ, IFN-γ+ TNF-α, IL-1β+TNF-α, and IL-1β+IFN-γ+TNF-α and stained with BrdU during the whole course of the culture period. Results are expressed as means±S.E.M. of the percentage of combined BrdU/insulin-positive cells relative to insulin-positive cells in a minimum of five experiments. *$P<0·05$ treated versus islets control without cytokines; #$P<0·05$ treated versus islets exposed to IL-1β+IFN-γ+TNF-α.

Signaling pathway effects of GLP-1 were tested using H89 (10 μmol/l) and PD98059 (50 μmol/l), inhibitors of PKA and ERK1/2 pathways respectively. The addition of H89 barely alters the recovery of the proliferation induced by GLP-1 in cultures treated with cytokines. On the other hand, PD98059 abolishes GLP-1-mediated recovery of proliferation in cytokine-treated islet cultures. This effect is clear, albeit proliferation values in PD98059-treated cultures are low due to the important role of the ERK1/2 signaling in β-cell replication (Fig. 8).

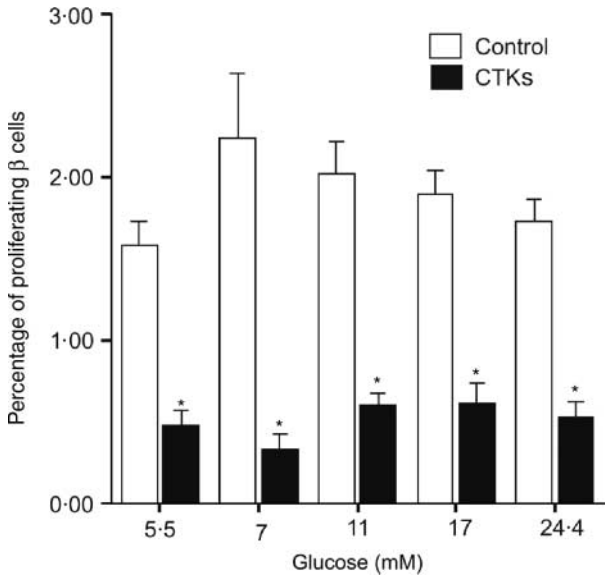

Figure 3 Role of glucose concentration in the anti-proliferative effect of cytokines. Rat islets were cultured for 48 h in 5·5, 7, 11, and 24·4 mM glucose medium, alone (control, white bars) or in combination with IL-1β (50 U/ml) +IFN-γ (1000 U/ml) +TNF-α (1000 U/ml) (CTKs, black bars) and stained with BrdU during the whole course of the culture period. Results are expressed as means±S.E.M. of the percentage of combined BrdU/insulin-positive cells relative to insulin-positive cells in a minimum of five experiments. *$P<0·05$ treated versus control without cytokines.

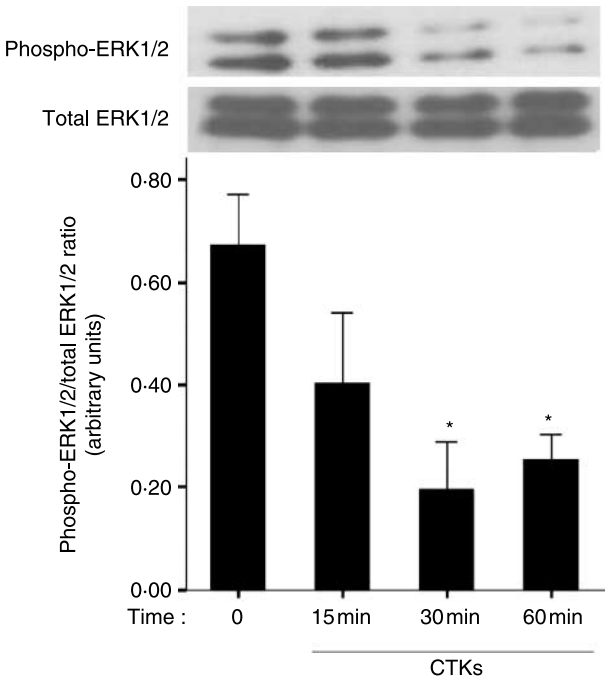

Figure 4 Effect of pro-inflammatory cytokines on ERK1/2 activation. Rat islets were cultured for 12 h and had additional IL-1β (50 U/ml) +IFN-γ (1000 U/ml) +TNF-α (1000 U/ml) (CTKs) for 15 min, 30 min, 60 min, and 48 h, and had levels of ERK1/2 phosphorylation assayed. Upper panel is a representative image of phospho-ERK1/2 and total ERK1/2 immunoblotting. Results are expressed as means±S.E.M. of phospho-ERK1/2 to total-ERK1/2 ratio measured by densitometry in a minimum of five experiments. Units on the *y*-axis are arbitrary. *$P<0·05$ treated versus control without cytokines.

Discussion

Pro-inflammatory cytokines such as IL-1β, IFN-γ, and TNF-α play essential roles in the pathogenesis of DM1. Many studies have focused on the apoptotic effects of cytokines, such as in the destruction of pancreatic β cells. Cell culture assays have demonstrated significant anti-proliferative action in immortalized cell lines (Li *et al.* 2005) and in primary cultures, suggesting that pro-inflammatory cytokines may be involved in the loss of regenerative response when a damaging circumstance such as insulitis is present. In the present study, the rat pancreatic islets were cultured in 5·5 mM glucose and 10% FCS-supplemented medium. Due, probably, to the

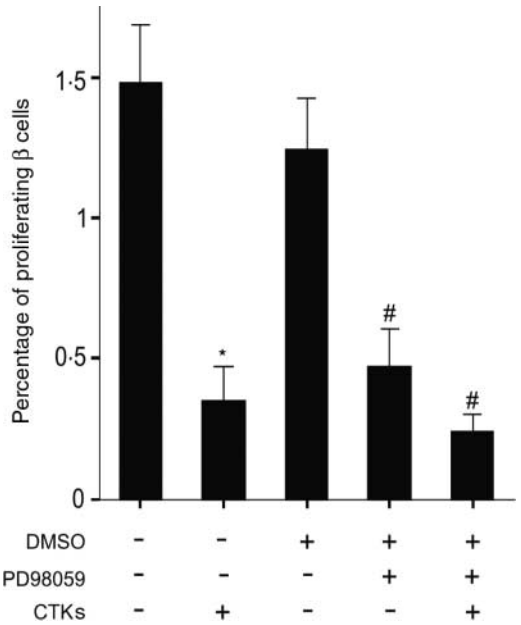

Figure 5 Effect of ERK1/2 signal pathway inhibition in islet β-cell proliferation. Rat islets were cultured for 48 h in 5·5 mM glucose medium, alone or in combination with IL-1β (50 U/ml) + IFN-γ (1000 U/ml) + TNF-α (1000 U/ml) (CTKs), PD98059, and PD98059 + CTKs, and stained with BrdU during the whole course of the culture period. A control with DMSO was performed for cultures containing PD98059 (50 μM). Results are presented as means ± S.E.M. of the percentage of combined BrdU/insulin-positive cells relative to insulin-positive cells in a minimum of five experiments. *$P<0·05$ treated versus control; #$P<0·05$ treated versus control with DMSO.

impact of the isolation procedure, β cells showed a low proliferation rate over the first 24 h and, subsequently, proliferative activity remained at a constant rate. Under these conditions, when cytokines were added to the culture, an anti-proliferative effect was observed. This effect is higher when the three-set cytokines (IL-1β, IFN-γ, and TNF-α) are added in combination, or when the combination was IFN-γ + TNF-α. These data are in contrast to a previous report in which the addition of IL-1β alone appeared to have a significant anti-proliferative effect (Tellez *et al.* 2005). This difference in outcome could have been related to the culture conditions and cytokine concentrations used (Mellado–Gil & Aguilar-Diosdado 2005). We had used the set of three pro-inflammatory cytokines (IL-1β, IFN-γ, and TNF-α) to mimic the pancreatic *milieu* during the pre-diabetic stage. Observed loss of proliferative response could be due to cytokine-mediated apoptosis affecting, mainly, the replicating β cells. To assess this possibility, we quantified the double TUNEL- and BrdU-positive cells. The double-stained cells were almost non-existent, and their numbers were not increased by the presence of cytokines. This suggests that replicating β cells are not more susceptible to cytokine-mediated apoptosis, and

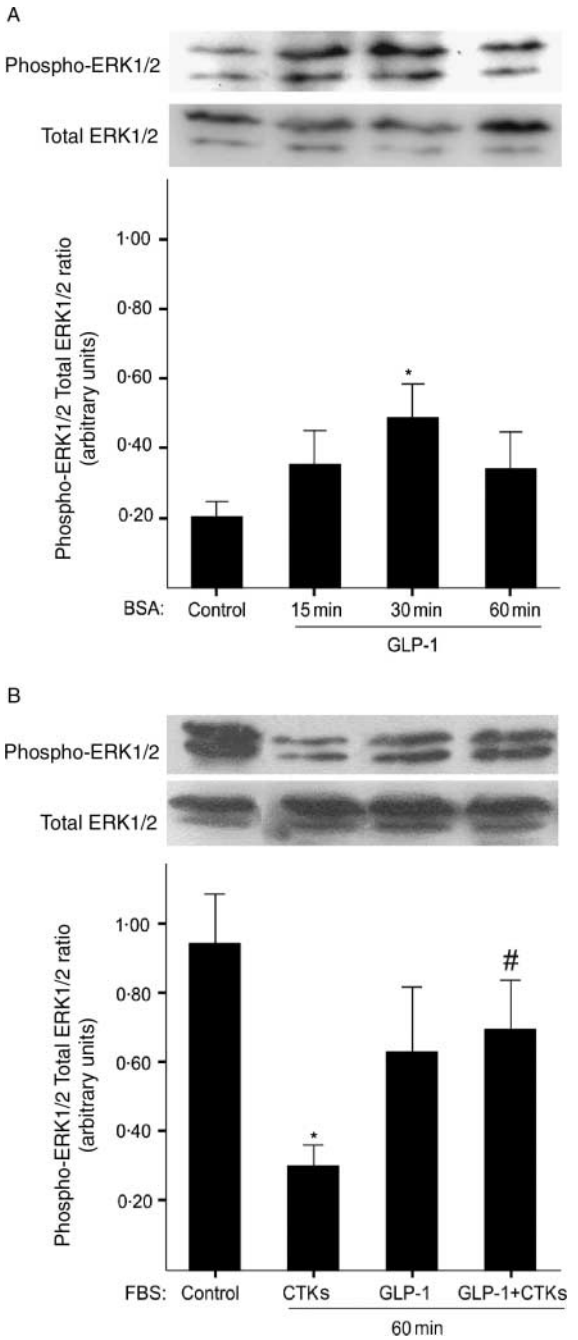

Figure 6 Effect of GLP-1 in cytokine-induced ERK1/2 inactivation. Rat islet cells pre-cultured for 12 h in culture medium supplemented with (A) 5% BSA or (B) 10% FBS were treated with GLP-1 (30 nmol/l) in the presence or absence of IL-1β (50 U/ml) + IFN-γ (1000 U/ml) + TNF-α (1000 U/ml) (CTKs) for the indicated times. The levels of ERK1/2 phosphorylation were measured and compared with untreated control. Upper panel is a representative image of phospho-ERK1/2 and total ERK1/2 immunoblotting. Results are expressed as means ± S.E.M. of phospho-ERK1/2 to total-ERK1/2 ratio measured by densitometry in a minimum of five experiments. Units on the *y*-axis are arbitrary. *$P<0·05$ treated versus control culture; #$P<0·05$ treated versus CTKs.

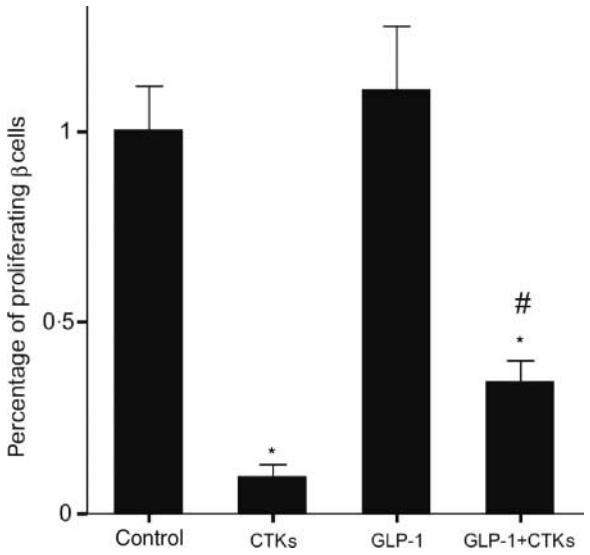

Figure 7 Effect of GLP-1 on β-cell anti-proliferative action of pro-inflammatory cytokines. Rat islets were cultured for 48 h in 5·5 mM glucose medium, alone or in combination with IL-1β (50 U/ml) + IFN-γ (1000 U/ml) + TNF-α (1000 U/ml) (CTKs), GLP-1, and GLP-1 (15, 30, and 60 nmol/l) + CTKs, and stained with BrdU during the whole course of the culture period. Proliferation was quantified in insulin-positive cells and expressed as means ± S.E.M. of the percentage of combined BrdU/insulin-positive cells relative to insulin-positive cells in a minimum of five experiments. $^{*}P<0{\cdot}05$ treated versus untreated control; $^{\#}P<0{\cdot}05$ treated versus CTKs.

that the anti-proliferative effect of cytokines on β cells is due to an active process.

We found no changes in the anti-proliferative effect of cytokines when β cells were cultured at different glucose concentrations in the culture medium. This independence of glucose concentration indicates that the cytokine anti-proliferative effect would be a relevant phenomenon from the earliest stages in the clinical evolution of the disease; especially when glycemia is within the normal range in the stages of subclinical diabetes. Conversely, the effect of cytokines in inducing apoptosis is increased at 24·4 mM, as previously reported by our group (Mellado-Gil & Aguilar-Diosdado 2004). These results suggest that the cytokine-mediated anti-proliferative events could be responsible for the β-cell mass loss from the beginning of insulitis, and that the apoptotic events would become more important at the same time that glucose levels increase.

ERK1/2, which belongs to the MAPK family, is a key factor in the control of survival, growth, differentiation, and transformation in many cell systems. In β cells, ERK1/2 is activated in response to extracellular stimuli such as the glucose concentration, or other growth factors implicated in pancreatic tissue homeostasis (Vasavada *et al.* 2006). As well, ERK1/2 induction is mediated by β-cell integrins and extracellular matrix interaction (Hammar *et al.* 2004). We observed that, in islet culture, the pharmacological inhibition of ERK1/2 by PD98059 considerably reduces β-cell proliferation, demonstrating that ERK1/2 is a major pathway involved in β-cell proliferation. Previous studies in immortalized cell lines have reported an induction of the ERK1/2 pathway in response to pro-inflammatory cytokines, mainly IL-1β (Larsen *et al.* 1998, Maedler *et al.* 2004). By contrast, we found in β cells from *ex vivo* cultured islets in the presence of optimal glucose and 10% FCS, a basal level of ERK1/2 activation that was drastically inhibited by the IL-1β+ IFN-γ + TNF-α combination. The mechanisms by which pro-inflammatory cytokines inhibit ERK1/2 activation in β cells are as yet unclear although inhibition has been shown to occur within 30 min and, as such, would indicate that non-transcriptional regulation could be involved. Loss of activated ERK1/2 in a short time period suggests that the cytokines' anti-proliferative effect is due to an active process and not due to consequences of apoptosis. Several routes of ERK1/2 phosphorylation–mediation could crosswalk with ERK pathways triggering its negative regulation in response to cytokines. Most of these trigger Raf-1, which in turn activates mitogen-activated protein kinase and ERK kinase 1 (MEK1), which will phosphorylate ERK1/2. Raf-1 may be phosphorylated in multiple sites and, depending on the site phosphorylated, will be positively or negatively regulated. Little is known regarding the molecular pathways involved in the negative regulation of Raf-1, and ERK1/2 has been suggested as being operative via a negative feed-back loop (Dougherty *et al.* 2005). Thus, p38 belonging to MAPK family is activated by pro-inflammatory cytokines and studies in immortalized cell lines have shown that an inhibitor of p38, SB203580, significantly enhances epidermal growth factor-induced ERK activation demonstrating a crosstalk between ERK and p38. The mechanism of this inhibition may be the p38-mediated activation of protein phosphatase 2A (pp2A) that inhibits the level of MEK phosphorylation. The cAMP/PKA/CREB pathway is activated by IFN-γ and TNF-α and may be also involved in the negative regulation of ERK pathway. The proposed mechanism for PKA-inducing ERK inhibition involves direct phosphorylation of c-Raf by PKA, which blocks c-Raf interaction with Ras (Dumaz & Marais 2005). PKB/Akt is another candidate implicated in negative regulation of the ERK pathway by either phosphorylating or forming a complex with Raf. (Rommel *et al.* 1999, Zimmermann & Moelling 1999, Chaudhary *et al.* 2000). Although PKB/Akt pathway can be potentially activated by pro-inflammatory cytokines, there is evidence from immortalized β-cell lines that PKB/Akt activation is also abrogated in a pro-inflammatory cytokine-dependent manner (Li *et al.* 2005). Moreover, AMP-activated protein kinase, a sensor of cellular energy charge that has been shown recently to be involved in pro-inflammatory cytokines β-cell damage (Richards *et al.* 2005, Riboulet-Chavey *et al.* 2008)), may have also an anti-proliferative effect (Jones *et al.* 2005, Motoshima *et al.* 2006).

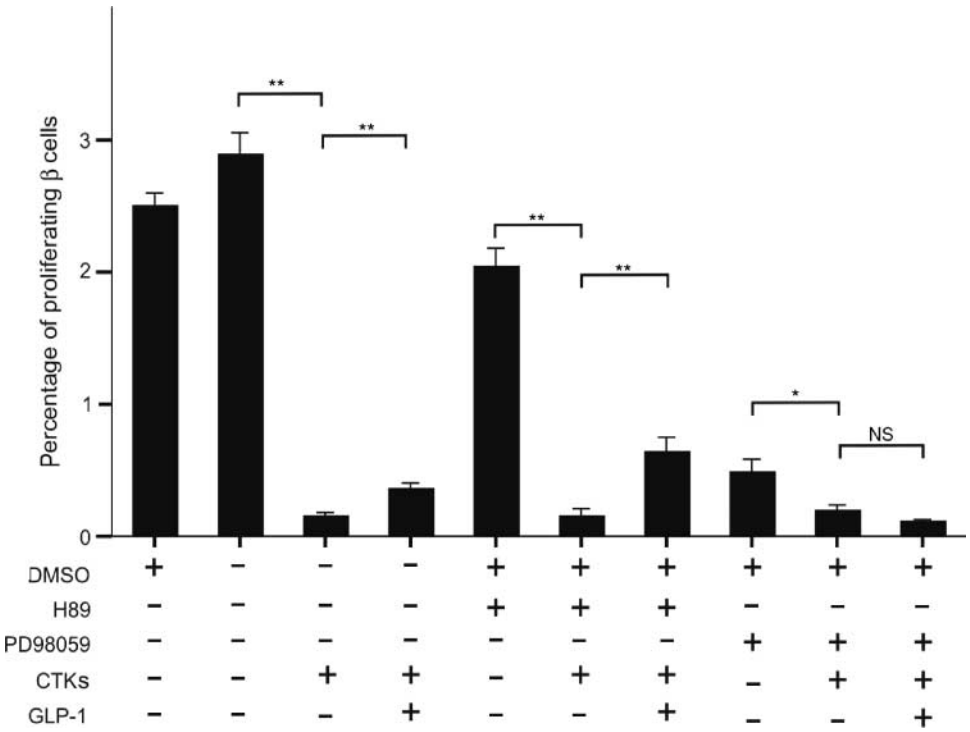

Figure 8 Effect of signaling pathway inhibitors on GLP-1 action. Rat islets were cultured for 48 h in 5·5 mM glucose medium, alone or in combination with IL-1β (50 U/ml) + IFN-γ (1000 U/ml) + TNF-α (1000 U/ml) (CTKs), H89 (10 nmol/l), H89+ CTKs, H89+ CTKs + GLP-1 (30 nmol/l), PD98059 (50 μmol/l), PD98059+ CTKs, and PD98059+ CTKs + GLP-1, and stained with BrdU during the whole course of the culture period. A control with DMSO was performed for cultures containing H89 and PD98059. Results are represented as means±S.E.M. of the percentage of combined BrdU/insulin-positive cells relative to insulin positive cells in a minimum of five experiments. *$P \leq 0{\cdot}05$, **$P < 0{\cdot}01$, NS: not significant.

Since GLP-1 has, among its effects, the ability to increase β-cell proliferative response in most systems studied, we tested whether GLP-1 could modify cytokine-mediated inhibition of ERK1/2 phosphorylation or the effect on β-cell proliferation. In the absence of cytokines, we observed no significant effect of GLP-1 on ERK activation. In addition, GLP-1 did not modify β-cell proliferation under these conditions. However, in β-cell cultures in which FCS is replaced by BSA, a positive effect of GLP-1 on ERK activation was observed. The explanation for this finding could be that fetal calf serum and glucose in the culture medium are able, of themselves, to trigger the highest signals for proliferation.

Nevertheless, the GLP-1 effect was quite different in the presence of cytokines. ERK1/2 activation was totally recovered and β-cell proliferation rate increased by threefold, albeit still remaining lower than the control value. This lack of a complete recovery of proliferation, despite complete activation of ERK1/2, could be due to multiple signaling pathways triggered by GLP-1, which may act negatively on β-cell proliferation (Zimmermann & Moelling 1999, Dhillon *et al.* 2002). Although GLP-1 and glucose activate the ERK1/2 by a cAMP–PKA-dependent pathway in immortalized β cells (Briaud *et al.* 2003), we found that the GLP-1 had a protective effect through reversed cytokine-induced ERK1/2 phosphorylation inhibition. The PKA inhibitor, H89, did not have an effect on GLP-1 protective action due, probably, to a different system (*ex vivo* culture), that used a culture medium with several growth factors contained in the fetal calf serum, and the possibility of cAMP induced ERK1/2 activation in a PKA-independent manner. In this sense, the role of cAMP-regulated guanine nucleotide exchange factor (Epac) in GLP-1 receptor-mediated signal transduction in β cells, and Epac-dependent, PKA-independent ERK1/2 activation in response to cAMP has been described (Busca *et al.* 2000, Lin *et al.* 2003, Holz 2004, Johnson-Farley *et al.* 2005, Fang & Olah 2007). On the other hand, treatment with PD98059, which blocks ERK1/2 pathway by inhibiting MAP kinase (MEK), abrogates the effect of GLP-1 on cytokine-treated cultures. This feature confirms that restoration of ERK activation is important in the mechanism of GLP-1-mediated proliferation recovery and that a more detailed knowledge of these pathways would be of considerable interest in enabling the pharmacological modulation of β-cell response to GLP-1, in the presence of cytokines.

The β-cell proliferation recovery mediated by GLP-1 in the presence of cytokines is noteworthy because, in maintaining cell proliferation rate during the subclinical diabetes phase, it may be possible to avoid or delay the loss of pancreatic β cells leading to overt DM1. In this sense, earlier reports showed that an analog of GLP-1 synergistically augments the remission-inducing effect of a polyclonal anti-T-cell antibody on overtly diabetic NOD mice (Ogawa *et al.* 2004) and may enhance β-cell mass in a streptozotocin-induced diabetes model (Soltani *et al.* 2007). Further details of potential signaling pathways implicated in β-cell proliferation, which are inactivated by pro-inflammatory cytokines and regulated by GLP-1, will enable the selection of more specific drugs which, administered alone or in combination with GLP-1, would restore β-cell proliferation in the presence of cytokines without causing ablation of the immune system.

In conclusion, our data suggest that, in autoimmune cytokine-mediated diabetes, the inhibition of β-cell proliferation causes loss of β-cell mass which, together with the apoptosis phenomenon, leads to a massive destruction and a non-regeneration of pancreatic β cells. We describe an important non-glucose-dependent anti-proliferative effect of pro-inflammatory cytokines due, especially, to synergistic action of IFN-γ and TNF-α and have shown that an inhibition of the ERK1/2 pathway mediated by cytokines could, at least in part, be the mechanism involved. The capacity of GLP-1 to reverse the anti-proliferative effect of cytokines in β cells suggests that its use in early stages of autoimmune diabetes would protect β-cell proliferation, and could be potentially effective in preventing β-cell mass loss and clinical diabetes.

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