Ciliary neurotrophic factor promotes survival of neonatal rat islets via the BCL-2 anti-apoptotic pathway

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Abstract

Ciliary neurotrophic factor (CNTF) belongs to the cytokine family and increases neuron differentiation and/or survival. Pancreatic islets are richly innervated and express receptors for nerve growth factors (NGFs) and may undergo neurotypic responses. CNTF is found in pancreatic islets and exerts paracrine effects in neighboring cells. The aim of this study was to investigate possible effects of CNTF on neonatal rat pancreatic islet differentiation and/or survival. For this purpose, we isolated pancreatic islets from neonatal rats (1–2 days old) by the collagenase method and cultured for 3 days in RPMI medium with (CNTF) or without (CTL) 1 nM CNTF. Thereafter, glucose-stimulated insulin secretion (RIA), general metabolism by (NAD(P)H production; MTS), glucose metabolism ($^{14}CO_2$ production), gene (RT-PCR), protein expression (western blotting), caspase-3 activity (Asp–Glu–Val–Asp (DEVD)), and apoptosis (DNA fragmentation) were analyzed. Our results showed that CNTF-treated islets demonstrated reduced glucose-induced insulin secretion. CNTF treatment did not affect glucose metabolism, as well as the expression of mRNAs and proteins that are crucial for the secretory process. Conversely, CNTF significantly increased mRNA and protein levels related to cell survival, such as Cx36, PAX4, and BCL-2, reduced caspase-3 activity, and islet cells apoptosis, suggesting that CNTF does not affect islet cell differentiation and, instead, acts as a survival factor reducing apoptosis by increasing the expression of the anti-apoptotic BCL-2 protein and decreasing caspase-3 activity.
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Introduction

Ciliary neurotrophic factor (CNTF) is a member of the IL-6 family of cytokines that includes IL-11, leukemia inhibitory factor, cardiotrophin-1, oncostatin-M, CNTF, and IL-6 itself, all using gp130 as a signal-transducing element in the functional receptor complexes and a specific receptor for each of them (CNTF-Rα for CNTF; Smith *et al.* 1993, Schulz-Key *et al.* 2002, Duff & Baile 2003).

CNTF is distributed all over the rat central nervous system in neurons and glial cells and also in high concentrations in Schwann cells in the peripheral nervous system. The peptide is known for its neurotrophic effects, being a survival factor for sympathetic, sensory, hippocampal and motor neurons *in vitro* and *in vivo*, and in type 2 astrocyte differentiation (Kirsch *et al.* 1997, Duff & Baile 2003).

Proper control of insulin secretion is crucial for the metabolism of mammals since it exerts a strict regulation of the plasma levels of nutrients, especially glucose. In pancreatic β-cells, a glucose-stimulated increase in the cytosolic ATP/ADP ratio, closes ATP-sensitive potassium (K_{ATP}) channels, which depolarizes the plasma membrane above a threshold, leading to Ca^{2+} entry into the cytosol through activation of voltage-dependent Ca^{2+} channels. The rise in cytosolic Ca^{2+} triggers exocytosis of insulin from secretory vesicles. Type 1 diabetes is characterized by a failure of the immune system that inappropriately recognizes β-cell peptides, leading to an islet infiltration by neutrophiles and a local increase in the concentration of many pro-apoptotic cytokines such as INF-γ, tumour necrosis factor-α and IL-1β, activating β-cell apoptotic pathways, suppression of protein expression, and membrane expression of apoptosis stimulating fragment (FAS) (Nagata *et al.* 1989, Jun *et al.* 1999, Park *et al.* 1999, Amrani *et al.* 2000) These effects culminate with an almost complete β-cell loss, leaving other islet cell types unharmed. The lack of circulating insulin alters the central nervous system's control of nutrient ingestion (Zhao & Alkon 2001, Zhao *et al.* 2004, Plum *et al.* 2005) and causes an inappropriate fuel metabolism, with plasma nutrient accumulation and impairment of its intracellular utilization, ultimately leading to organ and system degeneration (Gerbitz *et al.* 1995). These effects may be reversed or, at least, avoided by a proper activation of anti-apoptotic pathways, mainly via BCL-2, the major anti-apoptotic protein in islets (Polak *et al.* 1993, Mauricio & Mandrup-Poulsen 1998, Mizuno *et al.* 1998, Hanke 2001).

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CNTF impairs glucose-stimulated insulin secretion (GSIS) and potentiates the inhibitory effect of IL-1β on GSIS, in cultured islets (Wadt *et al.* 1998). In addition, it also exhibits many *in vivo* systemic effects, such as reduction of adiposity, body weight, hyperinsulinemia, and hyperglycemia in rats (Gloaguen *et al.* 1997, Lambert *et al.* 2001, Sakuma *et al.* 2002, Sleeman *et al.* 2003, Kelly *et al.* 2004, Ott *et al.* 2004, Ahima 2006, Graewin *et al.* 2006, Steinberg *et al.* 2006, Watt *et al.* 2006).

β-Cells express receptors for several neurotrophic factors and may undergo a neurotypic response to neuronal differentiation factors (Sundler & Böttcher 1991, Polak *et al.* 1993, Ahima 2006) CNTF is identified in pancreatic β-cells and in the islet-associated nervous system, exerting several actions on non-neuronal cells and may have a paracrine function inside the islets. Furthermore, CNTF expression has already been identified in β-cells (Wadt *et al.* 1998). For these reasons, we decided to study the possible effects of CNTF on rat pancreatic islets differentiation and/or survival.

Material and Methods

Chemicals

D-[U-^{14}C]glucose and ^{125}I-insulin were from G E. Health Care; aminotransferase inhibitor aminooxyacetate (carboxymethoxylamine) and Sybr-Green were from Sigma–Aldrich; MTS/phenazine methosulfate (PMS) preparation was from CellTitter96 aqueous assay (Promega), and all RT-PCR reagents were purchased from Invitrogen. Other reagents were from Sigma, whenever specified.

Islets isolation and culture

Neonatal (1–2 days old) Wistar rats came from the State University of Campinas animal facilities. After decapitation, the islets were isolated by collagenase (EC 3.4.24.3) digestion of pancreas in Hanks' balanced salt solution (137 mM NaCl, 5·5 mM KCl, 4·5 mM $NaHCO_3$, 0·4 mM KH_2PO_4, 0·4 mM Na_2HPO_4, 0·8 mM $MgSO_4$, and 1·5 mM $CaCl_2$, pH 7·4). Islets were extensively washed in sterile Hanks' solution and cultured in RPMI 1640 medium supplemented with 2 g/l $NaHCO_3$, 1% (v/v) penicillin/-streptomycin, 5·6 mM D-glucose and 2% fetal bovine serum (pH 7·4). Approximately, 1000 islets/dish were maintained at 37 °C in a humidified atmosphere with 3% CO_2 for 3 days in the presence or absence of 1 nM CNTF. The medium was renewed every 24 h. Islet experimental groups were assigned according to culture conditions: CTL (control group, islets cultured without CNTF) and CNTF (treated group, islets cultured with CNTF).

Insulin secretion

Batches of ten islets each were incubated in Krebs–Hepes-buffered saline (KHBS (mM): 115 NaCl, 10 $NaHCO_3$, 5 KCl, 1 $MgCl_2$, 2·5 $CaCl_2$, and 15 Hepes) containing 0·5 g/l BSA and 5·6 mM glucose (pH 7·4) and equilibrated with 95% O_2 and 5% CO_2 for 30 min at 37 °C. The medium was discarded and the islets incubated for a further period of 1 h in 1 ml KHBS containing 2·8 or 16·7 mM glucose. The supernatant was collected and insulin was measured by RIA.

Glucose metabolism

Batches of 50 islets each were incubated for 2 h at 37 °C in KHBS containing 2·8 or 16·7 mM glucose with trace amounts of either D-[U-^{14}C]glucose for $^{14}CO_2$ production. The batches were added with 1 M HCl to stop respiration and the $^{14}CO_2$ collected for 1 h at 4 °C 1 M NaOH.

RT-PCR

Groups of 1000 islets were homogenized in Trizol following phenol–chloroform RNA extraction, according to the manufacturer's instructions. RNA integrity was asserted through agarose gel. Reverse transcriptase reaction was performed using 3 μg total RNA. The reactions were incubated for 5 min at 65 °C before the addition of 150 ng random primers, for 10 min at 25 °C before the addition of 14·3 mM $MgCl_2$, 2·8 mM dithiothreitol, and 0·4 U/μl RNase-out, and at 42 °C for 2 min before the addition of 1·25 U/μl RNA Superscript II. Samples were incubated at 42 °C for 50 min, at 70 °C for 15 min, and then cooled to 4 °C. The cDNAs obtained were diluted in PCR buffer (60 mM Tris–HCl, 1·5 mM $MgCl_2$, and 15 mM NH_4SO_4, pH 10) with 50 mM $MgCl_2$, 0·3 mM each of dATP, dCTP, dGTP, and dTTP, 2·5 U/μl Taq DNA polymerase (Gibco/BRL), and 10 mM forward and reverse primers were then added. PCR amplification of cDNA was performed with a GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA, USA). The PCR program employed the following cycle profile: 32 cycles of denaturation for 1 min at 94 °C, annealing for 1 min, extension for 1·5 min at 72 °C, and maximization of strand completion for 7 min at 72 °C. The annealing temperatures and the number of cycles used in each amplification are shown in the legends. Following amplification, the cDNA fragments were analyzed on 1·6% agarose gels containing a 100 bp DNA molecular weight ladder (Gibco/BRL). PCR products were analyzed by ethidium bromide u.v. fluorescence in a Gel Doc EQ analyzer (Bio-Rad).

Internal controls for reactions were chosen among various controls used; of these, the RPS-29 subunit of the 40S small ribosomal unit showed the best homogeneity between treated and non-treated groups. Primers were designed and tested against *Rattus norvegicus* genome (Gene Bank) to ensure no amplification of other cDNAs. The sense (S) and anti-sense (AS) oligonucleotide primers used were as follows: RPS-29 (S) 5′-AGG CAA GAT GGG TCA CYCLE CCA GC-3′; RPS-29 (AS) 5′-AGT CGA ATC CAT TCA CYCLE GGT CG-3′; rat pro-insulin 2 (S) 5′-TTG CAG

TAG TTC TCC AGT T-3′; rat pro-insulin 2 (AS) 5′-ATT GTT CCA ACA TGG CCC TGT-3′; GLUT2 (A) 5′-CAT TGC TGG AAG CGT ATC AG-3′; GLUT2 (AS) 5′-GAG ACC TTC TGC TCA CYCLE GTC GAC G-3′; PKCα (S) 5′-CCT GCT CTA CGG ACT TAC T-3′; PKCα (AS) 5′-TGT AGT ATT CAC CCT CCT C-3′; NKX6.1 (S) 5′-AAA CAC ACC AGA CCC ACA TTC TC-3′; NKX6.1 (AS) 5′-TTC TCG TCG TCA GAG TTC GG-3′; glucokinase IV (S) 5′-ATG AAG ACC GCC AAT GTG AGG-3′; glucokinase IV 5′-TGT TGT GGA TCT GCT TTC GGT C-3′; CX36 (S) 5′-AGT GGT GGG AGC AAG CGA GAA G-3′; CX36 (AS) 5′-ACA ACC CTG GGA CAC TGA AGC C-3′; PAX4 (S) 5′-ACC AGC CAC AGG AAT CGG AC-3′; PAX4 (AS) 5′-AAG CCA CAG GAA GGA GGG AG-3′; BAD (S) 5′-CAG TGA TCT GCT CCA CAT TC-3′; BAD (AS) 5′-ATG ATA GGA CAG CAC CCA GT-3′; BAX (S) 5′-AAG AAG CTG AGC GAG TGT CT-3′; BAX (AS) 5′-CAA AGA TGG TCA CTG TCT GC-3′; AKT (S) 5′-CCT CAA GTA CTC ATT CCA GAC-3′; AKT (AS) 5-′CTC ATA CAC ATC TTG CCA CAC-3′; BCL2 (S) 5′-GTA TGA TAA CCG GGA GAT CG-3′; BCL2 (AS) 5′-AGC CAG GAG AAA TCA AAC AG-3′. All annealing temperatures and number of cycles were chosen to agree maximal sensibility to sample cDNA content.

Western blotting

After culture, groups of islets were pelleted by centrifugation and then resuspended in 50–100 μl homogenization buffer containing protease inhibitors, as described. The islets were sonicated (15 s) and the protein was determined by the Bradford method (Andersson *et al.* 2005) using BSA as standard. The samples volume was adjusted to provide the same amount of protein added to each lane. Samples containing 70 μg protein from each experimental group were separated by SDS-PAGE, transferred to nitrocellulose membranes, and stained with Ponceau S. No differences in the total amount of protein were observed as judged by densitometric analysis of the stained membranes (not shown). CX36 was detected in the membrane after 2-h incubation at room temperature with a rabbit polyclonal antibody against CX36 (Zymed, diluted 1:1500 in TTBS plus 30 g/l dry skimmed milk), and BCL-2 with a rabbit polyclonal antibody against BCL-2 (Santa Cruz, Biotechnologies, Santa Cruz, CA, USA, diluted 1:500 in TTBS plus 30 g/l dry skimmed milk). Detection was performed using enhanced chemiluminescence (SuperSignal West Pico, Pierce, Milwaukee, WI, USA) after incubation with a horseradish peroxidase-conjugated secondary antibody. Band intensities were quantified by optical densitometry (Scion Image, Frederick, MD, USA) of the developed autoradiogram.

NAD(P)H determination

NAD(P)H was measured by the coupled reduction of PMS and subsequent transfer of electrons to the tetrazolium salt, MTS, both of which are membrane permeable. Mixing of MTS and PMS solutions was carried out according to the manufacturer's instructions. Little change in 492 nm absorbance was seen with concentrations of NAD(P)H below 10 μM. Owing to the significant interference of proteins bound to NAD(P)H, standard curves were less accurate at low concentrations of NAD(P)H. However, spectroscopic analysis demonstrated an increase of $\sim 10^{-3}$/cm per islet light absorption at 650 nm when different numbers of islets were disrupted in a solution of MTS/PMS containing 2 mg/ml BSA. Heat-denatured islet homogenates were used as negative controls.

Static measurements Static measurements of NAD(P)H were performed by incubating groups of 200 islets in KHBS containing 2·8 or 16·7 mM glucose, reproducing the same conditions used in insulin secretion experiments. Islets were then washed in ice-cold Hanks' solution and immediately disrupted by sonication in 150 μl Hanks' solution. Homogenates were centrifuged at 10 000 ***g*** for 2 min to remove islet debris. Supernatants were then added to the MTS/PMS solution and incubated for 30 min at room temperature before recording absorbance at 650 and 405 nm (background). Samples with no islet were used as blanks.

Dynamic measurements Dynamic measurements of NAD(P)H were carried out by incubating groups of 20 islets in 200 μl KHBS containing 0 or 20 mM glucose and 5% (v/v) of MTS/PMS. This concentration of MTS/PMS was chosen after testing concentrations from 1 to 20% (v/v) and searching for the best sensitivity. As judged by a transient rate of MTS/PMS reduction, concentrations of PMS/MTS above 7% caused cell death due to NAD(P)H depletion. Samples were incubated for 3 h under 95% O_2 +5% CO_2 atmosphere (140 μM O_2 in KHBS). Samples with no islets were used as blanks and 492 nm absorbance values were recorded every 30 min. NADPH standard curves were used to calculate the reduced amounts of NAD(P)H in samples. The NAD(P)H reduction rate values were taken from the temporal increase of MTS/PMS absorbance in each sample.

Caspase-3 activity assay

The colorimetric method of cleavage of the DEVD was employed. Control and treated incubated neonatal rat islets were lysated in CHAPS-containing hypotonic buffer, centrifuged at 10 000 ***g***, and the supernatants were stored at −80 °C for further measurement. For the assay, up to 50 μl lysate (corresponding to 100 μg protein), 50 μl substrate, and enough volume of assay buffer for completing 100 μl total sample assay volume, were placed in clear flat bottom 96-well plates. The absorbance was measured at 405 nm, reading every 5 min for 2 h at 37 °C. Total protein content was determined by the Bradford assay. The specific activity of caspase-3 was calculated according to the manual, using a known standard pNA solution.

DNA fragmentation assay

DNA was isolated from neonatal rat islets, separated in fragmented and integral subunits by Trizol/Triton method. Both were quantified by Sybr-Green method, as ng/ml of DNA. Data are expressed as fragmented/total DNA.

Statistical analysis

Point-to-point comparisons were made by Student's *t*-test. Groups were compared by two-way ANOVA using the unpaired Tukey–Kramer method as post-test. Results were considered significantly different if $P<0{\cdot}05$. In RT-PCR experiments, results were considered different only if $P<0{\cdot}001$.

Results

The GSIS from islets cultured in the presence of 1 nM CNTF for 3 days, subsequently incubated for 1 h in Krebs-bicarbonate solution at 2·8 or 16·7 mM glucose, was significantly lower when compared with control islets ($P<0{\cdot}05$; Fig. 1). The total insulin (CTL 2·8, 291·38±60·99; CNTF 2·8, 293·20±31·42; CTL 16·7, 334·62±43·41; CNTF 16·7, 336·78±23·32 ng/islet, $n=6$); and DNA (CTL, 1·82±0·06; CNTF, 1·86±0·07 ng/ml, $n=4$) islet contents were similar between groups. In addition, a small reduction of the mitochondrial metabolism was observed in the CNTF-treated islets, as judged by the NADPH reduction rate ($P<0{\cdot}05$ versus CTL; Fig. 2), suggesting that CNTF treatment does not improve the maturation of the islet secretory process. To assess whether these inhibitory effects were due to an altered glucose metabolism, we measured the glucose utilization by CNTF and CTL islets. No differences in $^{14}CO_2$ production, at basal and stimulatory concentrations of glucose, were observed between groups (Fig. 3).

To further analyze the effects of CNTF on the maturation of neonatal islets, we investigated the transcription level of several proteins involved in this process, such as GLUT2 (Yasuda *et al.* 1992, Hathout *et al.* 1997, Wang & Gleichmann 1998), insulin, PKCα (Jones *et al.* 1992, Chen & Romsos 1997, Zawalich *et al.* 1998, Carpenter *et al.* 2004), PDX-1 (Beattie *et al.* 1999, Pedersen *et al.* 2002, Andersson *et al.* 2005) NKX6.1 (Schisler *et al.* 2005), and glucokinase IV (Matschinsky *et al.* 2006). None of the mRNA analyzed was significantly altered by CNTF treatment (Fig. 4A–F). The protein expression for some of these genes confirmed the results obtained by RT-PCR (Fig. 5A and B). Indicating that CNTF has no effect on islet cells differentiation.

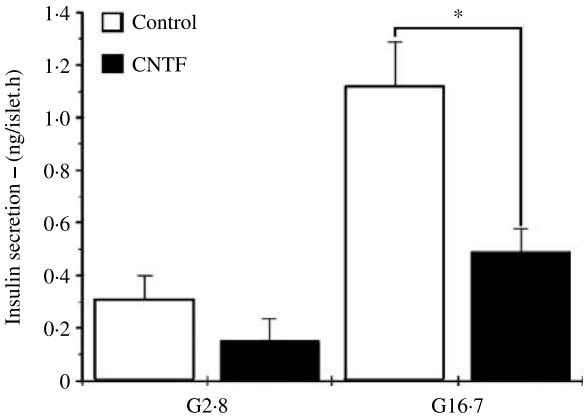

Figure 1 Insulin secretion in islets cultured for 3 days with 5·6 mM glucose, 2% FBS, and 1% penicillin, in the presence or absence of 1 nM CNTF (CNTF and control groups respectively). Islets were pre-incubated for 30 min in KHBS with 5·6 mM glucose, as described, and then incubated either with 2·8 or 16·7 mM glucose for 1 h. Bars are means±S.E.M. of eight independent experiments. *$P<0{\cdot}05$.

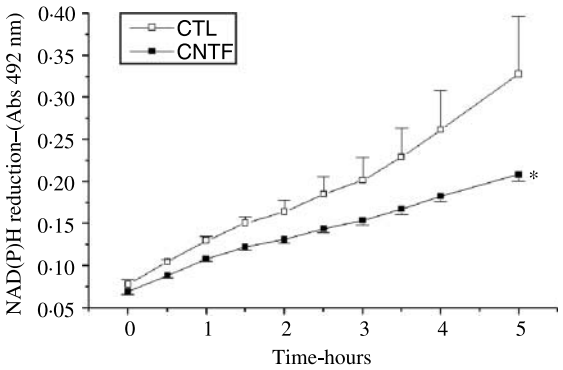

Figure 2 Islet metabolism, as evaluated by NAD(P)H reduction rate (NRR). Islets were incubated for 3 h in KHBS containing 5% (v/v) of MTS/PMS and 10 mM glucose. Incubations were performed in a 95% O_2 atmosphere that produced 140 μM O_2 in KHBS solution. NRR of each sample was calculated as the temporal change in NAD(P)H causing MTS/PMS reduction. Values are means±S.E.M. of ten experiments. *$P<0{\cdot}05$.

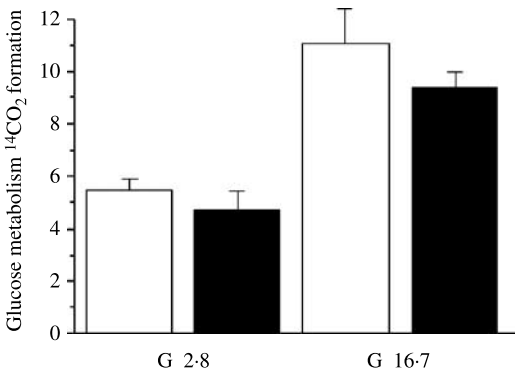

Figure 3 Glucose oxidation of islets cultured for 3 days with 5·6 mM glucose, 2% FBS, and 1% penicillin, in the presence or absence of 1 nM CNTF (CNTF and CTL groups respectively). Islets were incubated for 120 min in KHBS with 2·8 or 16·7 mM glucose containing equal amounts of D-[U-^{14}C]glucose to measure $^{14}CO_2$ production. Bars are means±S.E.M. of at least eight experiments. *$P<0{\cdot}05$ related to each respective control (2·8 mM glucose).

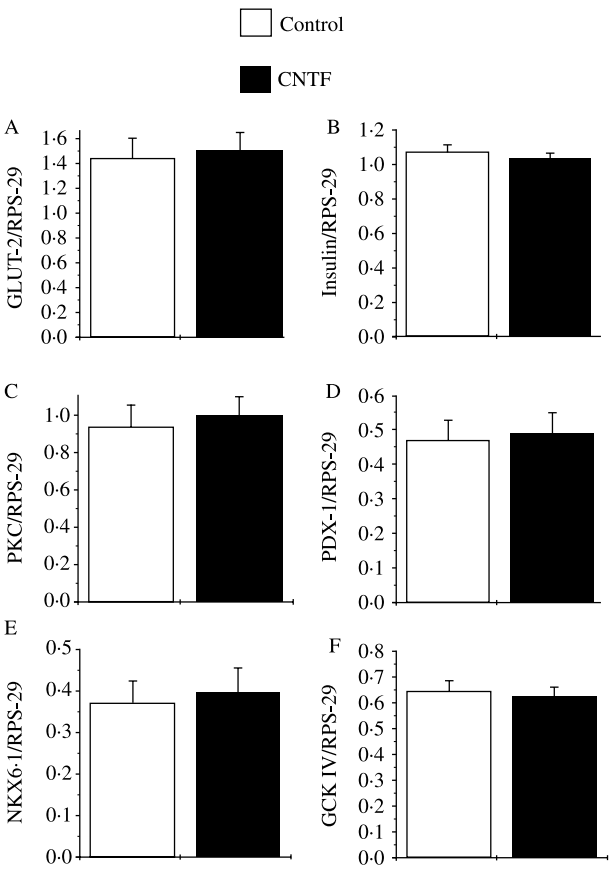

Figure 4 Effect of culture with 1 nM CNTF for 3 days on mRNA levels, as evaluated by RT-PCR, of GLUT-2 (A), insulin (B), PKC (C), PDX-1 (D) NKX6.1 (E), and glucokinase IV (GCK IV) (F). RT-PCRs annealing temperatures and cycle numbers used were as follows: 55 °C and 29 cycles for GLUT-2; 57 °C and 23 cycles for insulin; 57 °C and 31 cycles for PKC; 55 °C and 29 cycles for PDX-1; 60 °C and 30 cycles for NKX6.1; and 60 °C and 30 cycles for GCK IV. RPS-29 was used as an internal control (57 °C and 29 cycles), showing no variation among the conditions tested. Plotted columns are means ± S.E.M. of 12 experiments. *$P<0{\cdot}001$.

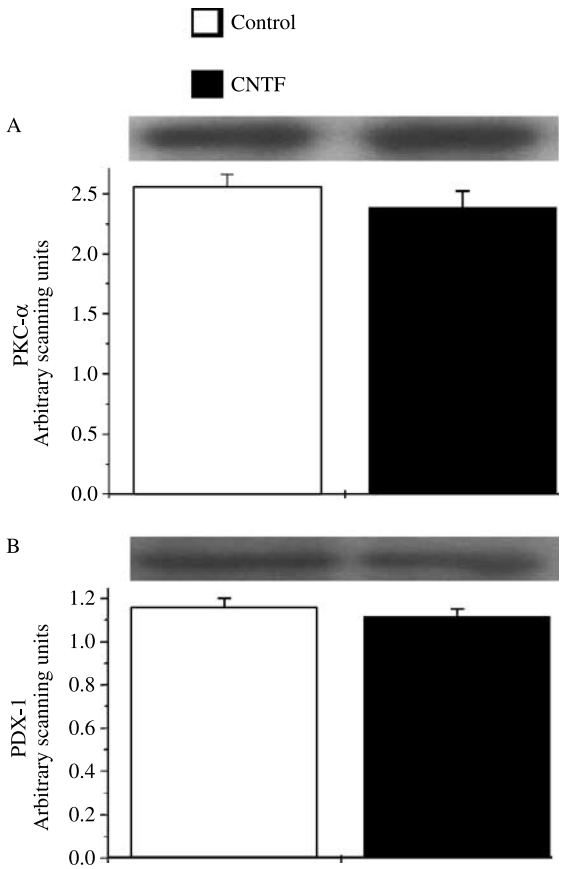

Figure 5 Protein expression, as measured by western blotting of PKC (A), PDX-1 (B) in neonate rat islets cultured for 3 days in the presence (CNTF ■) or absence (control □) of 1 nM CNTF. Values are means ± S.E.M. of six independent experiments. * $P<0{\cdot}05$.

In the next series of experiments, the expression of gene encoding proteins related to cells survival were analyzed, such as the pro-apoptotic BCL-2 antagonist of cell death (BAD; Hanke 2001) and BAX (Mizuno *et al.* 1998) and the anti-apoptotic Akt (Borner 2003), *Bcl-2* (Nunez & Clarke 1994, Gillardon *et al.* 1996, Reed *et al.* 1996, Chao & Korsmeyer 1998, Adams & Cory 2001, Kaufmann & Hengartner 2001, Borner 2003), *Cx36* (Calabrese *et al.* 2003, Le Gurun *et al.* 2003, Ravier *et al.* 2005, Striedinger *et al.* 2005), and *PAX4* (Brun *et al.* 2004, 2007) genes. No differences were observed between CNTF and CTL islets for *BAD*, *BAX*, and *Akt* (Fig. 6A–C) genes, whereas the *Bcl-2*, *Cx36*, and *PAX4* genes (Fig. 6D–F) were significantly higher in the CNTF-treated islets. The expression of two transcripts (Bcl-2 and Cx36) was confirmed by western blotting for the corresponding proteins (Fig. 7A and B), confirming that the peptide acts in neonatal rat islets as a survival factor.

Finally, the effect of CNTF on the final steps of cell death was assessed by evaluating the caspase-3 activity, an accurate marker for apoptosis (Medina *et al.* 1997, Juin *et al.* 1998, Yu *et al.* 1998, Jani *et al.* 2004), and islet cells DNA fragmentation (Gillardon *et al.* 1996). CNTF treatment significantly reduced the islet caspase-3 activity (Fig. 8A) and DNA fragmentation (Fig. 8B) compared with CTL islets. The unusual high levels of fragmented DNA can be explained by the lower fetal bovine serum (FBS; 2%) and glucose (5·6 mM) in culture medium compared with the usual 5–10% FBS and 11·2 mM glucose. These results clearly indicate a lower level of apoptosis and, therefore, increased islet survival promoted by CNTF.

Discussion

It has been suggested that CNTF, released from destroyed β-cells during the inflammatory process that occurs during

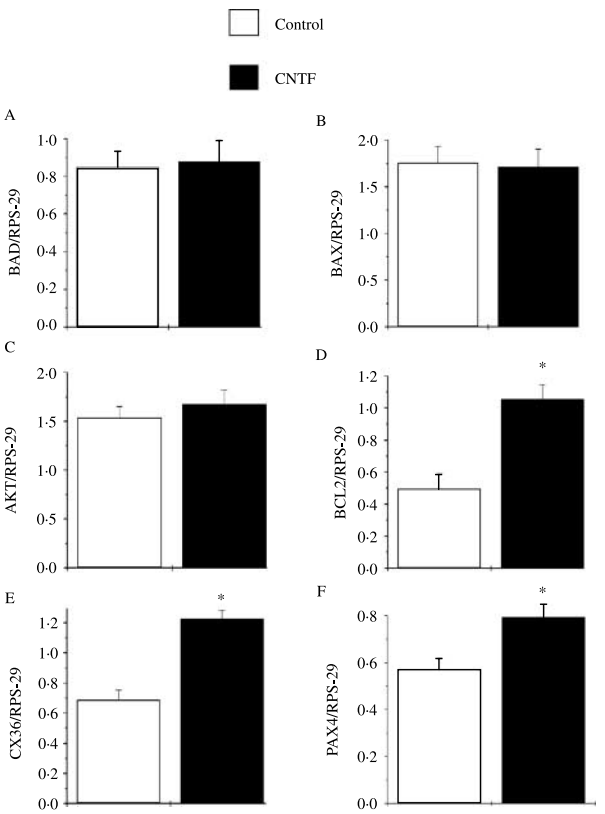

Figure 6 Effect of 3 days of culture with 1 nM CNTF on mRNA levels, as evaluated by RT-PCR, of BAD (A), BAX (B), AKT (C), BCL-2 (D), Cx36 (E), and PAX4 (F). RT-PCRs annealing temperatures and cycle numbers used were as follows: 60 °C and 29 cycles for BAD; 59 °C and 31 cycles for BAX; 59 °C and 30 cycles for AKT; 61 °C and 32 cycles for BCL-2; 57 °C and 31 cycles for CX36; and 62 °C and 31 cycles for PAX4. RPS-29 was used as an internal control (57 °C and 29 cycles), showing no variation among the conditions tested. Plotted columns are means±S.E.M. of 12 experiments. *$P<0{\cdot}001$.

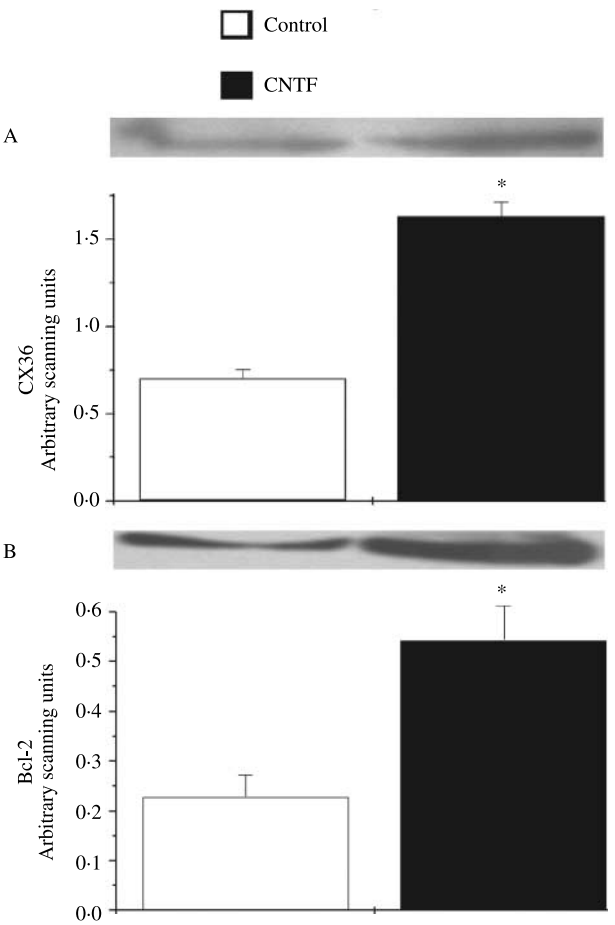

Figure 7 Protein expression, as measured by western blotting of Cx36 (A) and Bcl-2 (B) in neonate rat islets cultured for 3 days in the presence (CNTF ■) or absence (control □) of 1 nM CNTF. Values are means±S.E.M. of six independent experiments. *$P<0{\cdot}05$.

the onset of type 1 diabetes, may act as a proinflammatory cytokine by potentiating the action of IL-1β on β-cells (Wadt *et al.* 1998). However, to date, CNTF, in contrast to IL-6 (Ahima 2006) has not yet been tested as a differentiation and/or survival factor in pancreatic islets. The present results show that CNTF impairs GSIS in cultured islets, but promotes their survival by reducing apoptosis.

Basal and GSISs have been suggested to be accurate markers of differentiated and/or functionally mature pancreatic islets. Here, we show that mitochondrial islet metabolism was significantly reduced in CNTF-treated islets, whilst glucose metabolism remained unaltered; indicating that islets demonstrate a lower activity and responsiveness, but that they are integral, features typical of undifferentiated cells. Furthermore, the expressions of major proteins related to differentiated β-cells, such as insulin, Glut2, PKC, PDX-1, NKX6.1, and glucokinase IV were unaffected by CNTF treatment. The possible effects of CNTF on islets survival had yet to be investigated, thus, we first evaluated the mRNA levels of a number of apoptosis-related proteins, including BAD, BAX, AKT, Bcl-2, Cx36, and PAX4. No changes in BAD, BAX, and AKT expressions were observed; in contrast, Bcl-2, Cx36, and PAX4 mRNA levels were significantly higher in CNTF-treated islets, an effect subsequently confirmed by western blotting of two of the referred proteins. Owing to the anti-apoptotic function, attributed to these proteins, particularly Bcl-2, these results suggest a probable survival effect of CNTF in pancreatic islets.

The final steps of apoptosis involve the activation of caspase-3 in the cytosol and, depending on the degree of activation, the process is virtually irreversible. Thus, the reduced caspase-3 activity associated with the lower islet cells DNA fragmentation, observed in CNTF-treated islets, are markers of increased islets survival promoted by the peptide. The varying results observed following CNTF treatment in islets might be due to the increase in Bcl-2 expression; it has been suggested that in addition to its anti-apoptotic effects,

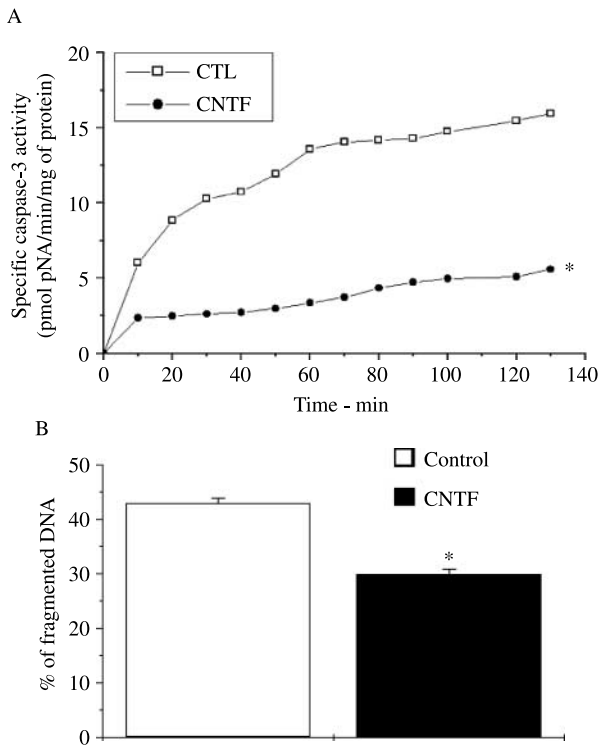

Figure 8 Caspase-3 activity (A) and percentage of DNA fragmentation (B) in neonate rat islets cultured for 3 days in the presence (CNTF ■) or absence (control □) of 1 nM CNTF. Values are means±S.E.M. of six (A) or four (B) independent experiments. *$P<0{\cdot}05$.

Bcl-2 may have a role in regulating metabolism, and many findings support this theory. Bcl-2, is involved in regulation or generation of ROS (Korsmeyer *et al*. 1995, Kowaltowski *et al*. 2004), can alter mitochondrial matrix volume or structure (Kowaltowski *et al*. 2002), permeability to or consumption of ATP (Imahashi *et al*. 2004), permeability of voltage-dependent anion channel (VDAC) (Tsujimoto & Shimizu 2006), and sensitivity of the MPT to Ca^{2+} (Murphy *et al*. 1996).

Given the strict relationship between these parameters and metabolic function, it may be proposed that Bcl-2 affects NADH reduction, an accurate indicative of metabolism, and this is exactly what we observed in the present study; a significantly lower NADH reduction rate that suggests a decrease in general metabolism, without affecting glucose metabolism.

We hypothesize that the observed CNTF effects could be explained by the increased Bcl-2 expression, leading on the one hand, to a lower generation of ROS and the inhibition of apoptotic pathways, a subsequent reduced caspase-3 activity and a lower apoptosis rate. On the other hand, Bcl-2 reduced the mitochondrial metabolism (as evaluated by NADH reduction rate). However, at the present time, we cannot state that the increase in Bcl-2 expression is responsible for the impaired GSIS observed in CNTF-treated islets.

Alternatively to previous proposals of Graewin *et al*. (2006), we suggest that the effect of CNTF in IL-1β action on pancreatic islets may be due to a parallel rather than a synergic pathway. Our findings support the idea that CNTF acts as an anti-apoptotic cytokine that protects islets against the inflammatory processes by increasing Bcl-2 expression and promoting its survival.

In conclusion, CNTF impairs GSIS, as well as the mitochondrial metabolism of pancreatic islets, and has no effect on glucose metabolism and the expression of genes and proteins related to pancreatic islet insulin secretion. Instead, the present data indicate that CNTF acts as an effective promoter of islet survival by enhancing the levels of survival proteins, especially Bcl-2. We hypothesize that during inflammatory processes, CNTF present in the islets or in the associated peripheral nervous system, acts as a survival factor for the neighboring islets during the early stages of lesion. CNTF has not been tested in an animal model for IDDM, mainly due to its harsh side effects, such as cachexia and anorexia, observed in other animal models. Thus, new methods of delivering CNTF to target cells to avoid its side effects may potentiate CNTF as an important therapeutic tool.

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