

Dominant negative mutant forms of the cAMP response element binding protein induce apoptosis and decrease the anti-apoptotic action of growth factors in human islets

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Abstract

Aims/hypothesis Transplantation of islets is a viable option for the treatment of diabetes. A significant proportion of islets is lost during isolation, storage and after transplantation as a result of apoptosis. cAMP response element binding protein (CREB) is an important cell survival factor. The aim of the present study was to determine whether preservation of CREB function is needed for survival of human islets.

Materials and methods To determine the effects of down-regulation of CREB activity on beta cell apoptosis in a transplantation setting, adenoviral vectors were used to express two dominant negative mutant forms of CREB in human islets isolated from cadaveric donors. Markers of apoptosis were determined in these transduced islets under basal conditions and following treatment with growth factor.

Results Expression of CREB mutants in human islets resulted in significant ($p < 0.001$) activation of caspase-9, a

key regulatory enzyme in the mitochondrial pathway of apoptosis, when compared with islets transduced with adenoviral beta galactosidase. Immunocytochemical analysis showed the activation of caspase-9 to be predominantly in beta cells. Other definitive markers of apoptosis such as parallel activation of caspase-3, accumulation of cleaved poly-(ADP-ribose) polymerase and nuclear condensation were also observed. Furthermore, the anti-apoptotic action of growth factors exendin-4 and betacellulin in human islets exposed to cytokines was partially lost when CREB function was impaired.

Conclusions/interpretation Our findings suggest that impairment of CREB-mediated transcription could lead to loss of islets by apoptosis with potential implications in islet transplantation as well as in the mechanism of beta cell loss leading to diabetes.

Keywords Apoptosis · Betacellulin · Caspases · cAMP response element binding protein · CREB · Cytokines · Exendin-4 · Human islets · Transplantation

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Abbreviations

BTC	betacellulin
CRE	cAMP response element
CREB	cAMP response element binding protein
DAPI	4',6-diamidino-2-phenylindole
GFP	green fluorescent protein
HEK	human embryonic kidney
KCREB	dominant negative mutant form of CREB
IEQ	islet equivalents
MCREB	dominant negative mutant form of CREB
MnSOD	manganese superoxide dismutase
MOI	multiplicity of infection
OCT	optimal cutting temperature compound
PARP	poly-(ADP-ribose) polymerase

TBST tris-buffered saline with tween-20
TUNEL terminal deoxynucleotidyl transferase-mediated
dUTP-biotin nick-end labelling

Introduction

Islet transplantation is a viable option for the treatment of type 1 diabetes. Advances in transplantation techniques from several laboratories culminated in 2000 in the development of the Edmonton protocol for clinically successful islet transplantation [1]. The major limitation of this therapeutic approach is that islets from two to four cadaveric pancreases are required for insulin independence in one diabetic patient. A significant portion of the islet mass is lost during isolation, storage and after transplantation as islets are subjected to stress from multiple sources. Apoptosis is considered to be the major cause of islet loss as shown by the presence of several apoptotic markers [2, 3].

The mechanism of beta cell apoptosis in human islets isolated from cadaveric donors is not clearly understood. Apoptosis takes place during the isolation process when extracellular matrix surrounding islets is disrupted by exogenous isolation enzymes [4]. In addition, nonspecific inflammatory reaction at the site of transplantation could lead to release of proinflammatory cytokines and free radicals, potential inducers of apoptosis [2]. Intra-islet cytokine production [5] can further aggravate the apoptotic pathway. Cytokines contribute to oxidative stress through inducible nitric oxide synthase-mediated generation of nitric oxide [6]. Pancreatic beta cells are vulnerable to injury under conditions of oxidative stress since they are characterised by a significantly low-level production of antioxidant enzymes, including manganese superoxide dismutase (MnSOD), catalase and glutathione peroxidase [7].

Previous *in vitro* and *in vivo* studies have used several approaches to improve islet survival and transplantation outcomes. For example, Bertera et al. [8] showed that production of the antioxidant enzyme, MnSOD, leads to improved survival of mouse islet grafts. Overexpression of the B cell CLL/lymphoma 2 gene (*Bcl2*), an anti-apoptotic gene, resulted in significant protection against apoptosis *in vivo* [9]. Similar results have been obtained by transferring the insulin gene [10], anti-apoptotic gene *Bcl-XL* [11] and *XIAP* (now known as baculoviral IAP repeat-containing 4 gene [*BIRC4*]), an endogenous inhibitor of caspases [12]. These studies suggested that manipulation of isolated islets is a potential strategy to improve islet survival after transplantation.

It is also important to understand the beta cell survival pathways that preserve islet function. One such pathway is growth factor-mediated activation of the transcription factor cAMP response element (CRE) binding protein (CREB),

which leads to the expression of genes needed for beta cell function and survival. In a recent study [13], we made two significant observations in the context of beta cell apoptosis. First, we demonstrated that cytokines decrease CREB-mediated expression of the anti-apoptotic gene *Bcl2* in MIN6 cells, a mouse beta cell line and in mouse islets. Second, we observed that overexpression of mutant forms of CREB led to the activation caspase-9, a marker for the mitochondrial pathway of apoptosis. CREB-mediated transcription is likely to be impaired after transplantation of human islets. Factors contributing to CREB downregulation in islets could be cytokines and oxidative stress. In addition, the immunosuppressive drug tacrolimus (FK506), used in patients receiving islets, has been shown to inhibit CREB-mediated transcription [14]. Under conditions of CREB downregulation, growth factors are likely to be less effective, since many of them target CREB to induce cytoprotective genes [15, 16].

Although recent reports [13, 17, 18] have suggested that CREB plays a role in beta cell survival, no previous study has examined its role in the survival of human islets. The objectives of the present study were to determine the effects CREB downregulation in human islets and to examine the anti-apoptotic actions of the beta cell-specific growth factors in islets exposed to cytokines. By transduction of islets with adenoviral vectors expressing dominant negative mutant forms of CREB, we demonstrate that CREB downregulation could play a role in beta cell apoptosis in human islets in conditions leading to beta cell loss in diabetes and during islet transplantation.

Materials and methods

Materials CMRL 1066 medium (Mediatech, Hendon, VA, USA) was supplemented with human serum albumin (0.5%), nicotinamide (10 mmol/l), and is referred to below as Miami medium. Exendin-4, betacellulin (BTC) and 4',6-diamidino-2-phenylindole (DAPI) were purchased from Sigma Chemical (St Louis, Missouri, USA). Human cytokines IL-1 β (5 U/ng), TNF- α (100 U/ng) and IFN- γ (50 U/ng) were obtained from Roche Applied Science (Indianapolis, IN, USA). Antibodies specific for CREB, phospho CREB, AKT, phospho AKT, BCL2, cleaved poly-(ADP-ribose) polymerase (PARP), beta galactosidase, active cleaved forms of caspase-3, -7, -8 and -9 and beta actin were from Cell Signaling (Beverly, MA, USA). Cy3-conjugated anti rabbit IgG and FITC-conjugated anti-guinea pig IgG were obtained from Jackson Immuno Research Laboratories (West Grove, PA, USA). The caspase-3 assay kit was purchased from Sigma Chemical and the caspase-9 assay kit was from Chemicon International (Temecula, CA, USA). Terminal deoxynucleotidyl

transferase-mediated dUTP-biotin nick-end labelling (TUNEL) In Situ Cell Death Detection Kit was purchased from Roche Diagnostics (Mannheim, Germany).

Preparation of recombinant adenovirus For generation of recombinant adenoviruses by homologous recombination, cDNAs encoding dominant negative mutant forms of CREB (KCREB, MCREB) were first subcloned into the plasmid pACCMVpLpA, which encodes the left end of the adenovirus chromosome containing the *E1A* gene and the 5' half of the *E1B* gene replaced with cytomegalovirus major immediate early promoter [19]. Plasmids containing the appropriate constructs in pACCMVpLpA were co-transfected with BstBI-digested Ad5dl327_{Bst}β-gal-TP complex in human embryonic kidney (HEK)-293 cells by the LipofectAMINE Plus (Invitrogen, Carlsbad, CA, USA) method. After complete cytopathic effect was observed (7–10 days), cells were harvested, freeze-thawed to release virus and used for plaque purification as described previously [20]. Virus was propagated in HEK-293 cells and purified by CsCl gradient purification [21].

Isolation and adenoviral transduction of human islets Human islets were isolated by collagenase digestion (cold ischaemia time 4–9 h) by the Islet Cell Resource Center at the University of Colorado at Denver and Health Sciences Center using the Edmonton protocol [1]. All donors were brain-dead, heart-beating individuals from the state of Colorado who had died in motor vehicle accidents. Written informed consent was obtained from next of kin of donors. None had a previous history of diabetes or inflammatory diseases. Islet purity (65–90%) and viability (65–85%) were determined by dithizone and Syto13/ethidium bromide staining, respectively, using standard operation procedures defined by the Clinical Islet Laboratory (SMRI, Edmonton, AB, Canada). Islet preparations (2,000 islet equivalents [IEQ]) when transplanted under the kidney capsule of streptozotocin-diabetic *Rag2*^{-/-} B6 mice normalised hyperglycaemia, indicating long-term survival and functionality. Islets were precultured for 12 to 18 h in Miami medium in 5% CO₂ and 37°C. For adenoviral transduction, islets in a small volume of 200 μl medium were mixed with recombinant adenoviruses at room temperature for 1 h and later the islets were mixed with medium (3 ml/1,000 IEQ) and incubated at 37°C in non-treated culture dishes.

Assay of caspase-3 and caspase-9 The activities of caspase-3 and caspase-9 were determined using assay kits from Sigma Chemical and Chemicon International, respectively. After appropriate adenoviral transduction and treatment, islets were lysed with the lysis buffer provided in the kit and 10,600 g supernatant fractions were used for the

assay. The substrates for the caspase-3 and caspase-9 assays were *p*-nitroanilide-labelled peptides, DEVD and IETD, respectively. The released chromophore was read at 405 nm in a microplate reader. In addition, activation of caspases was also determined by immunoblot analysis of the cleaved active fragments of the respective enzymes.

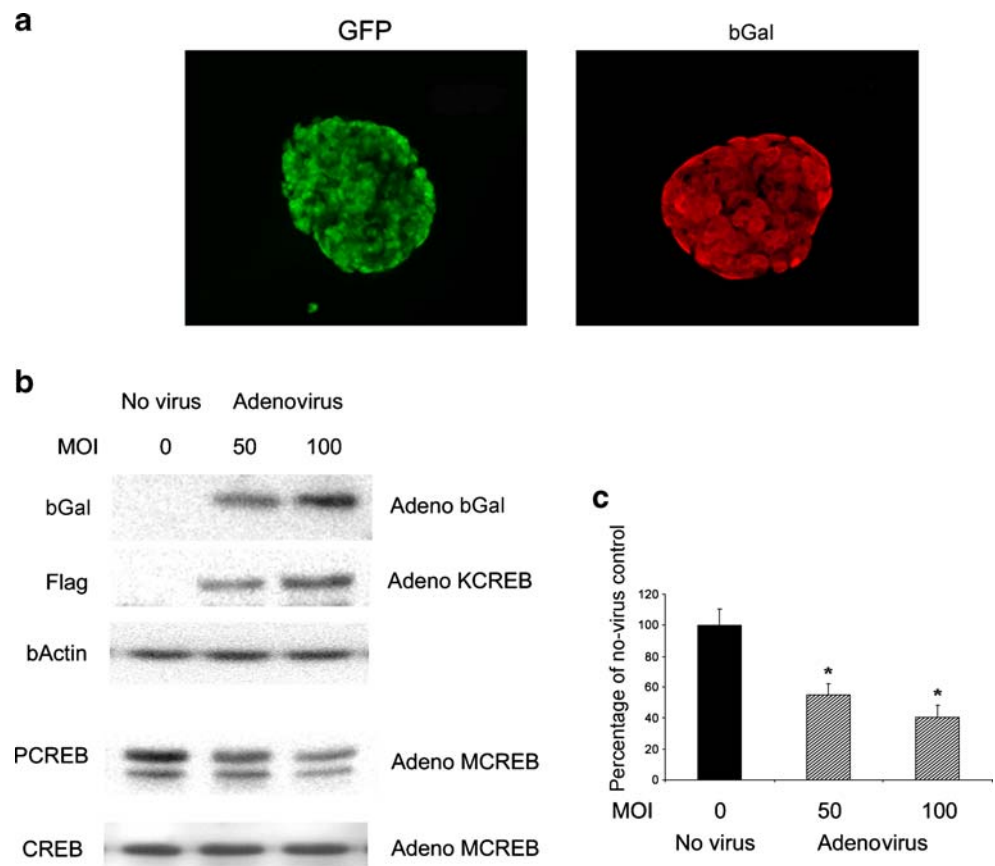
TUNEL staining In situ detection and quantification of apoptosis was done by labelling DNA strand breaks by terminal-deoxynucleotidyl transferase enzyme, which catalyses the polymerisation of labelled nucleotides to the free 3' hydroxyl end of DNA by using a TUNEL cell death detection kit according to the manufacturer's protocol. Briefly, frozen sections (7 μm) were hydrated in PBS for 30 min and treated with freshly prepared permeabilisation solution containing 0.1% (vol./vol.) Triton X-100 in 0.1% (wt/vol.) sodium citrate (pH 6.0) for 2 min on ice and rinsed twice in PBS. The slides were then incubated in labelling and enzyme solution for 60 min at 37°C in a humid chamber in the dark. The samples were then washed twice in PBS and analysed by fluorescence microscopy. Appropriate positive and negative controls were also included in the assay as suggested in the protocol.

Immunocytochemical analysis of islets After transducing the islets with adenoviral vectors and appropriate treatments, the islets were fixed in 4% (wt/vol.) paraformaldehyde solution for 15 min, suspended in 30% (wt/vol.) sucrose solution for another 30 min, embedded in optimal cutting temperature compound (OCT) from Tissue-Tek (Sakura Finetek USA, Torrance, CA, USA) and frozen. Immunocytochemistry was carried out with 7 μm thick sections. The sections, circled with PAP pen, were incubated at 37°C for 10 min and soaked in PBS for 10 min. The slides were heated for 5 min in 10 mmol/l citrate buffer (pH 6.0) in a steamer and cooled for 15 min. After washing in PBS, the slides were incubated in blocking solution (5% normal goat serum and 0.2% [vol./vol.] Triton in PBS) for 1 h in humidified chamber. The slides were exposed to primary antibodies in 3% BSA at 4°C overnight in a humidified chamber, washed in PBS and exposed to secondary antibodies linked to Cy3 or FITC in 3% BSA for 1 h in the dark. After PBS wash, the slides were incubated with DAPI (2 μg/ml) for 10 min, washed in PBS and mounted with glycerol mounting medium. The sections were examined by fluorescent microscopy using a Zeiss Axioplan 2 microscope (Carl Zeiss MicroImaging, Thornwood, NY, USA) fitted with a high-performance charged-coupled device (Cooke SensiCam; The Cooke Corporation, Romulus, MI, USA). For quantitation, the mean integrated fluorescence intensity of the images was calculated using Slide Book Application software (Intelligent Imaging Innovations, Denver, CO, USA).

Immunoblotting Islets incubated under appropriate conditions were washed with ice-cold PBS and cell lysates were prepared. The protein content of the lysates was measured [22]. Diluted samples containing equal amounts of protein were mixed with 2× Laemmli sample buffer. The proteins were resolved on 12% SDS-polyacrylamide gels and transferred to polyvinylidene difluoride (PVDF) membranes. The blots were blocked with Tris-buffered saline with Tween-20 (TBST; 20 mmol/l Tris-HCl [pH 7.9], 8.5% NaCl [wt/vol.] and 0.1% Tween-20 [vol./vol.]) containing 5% non-fat dry milk at room temperature for 1 h and exposed to primary antibody in TBST containing 5.0% BSA at 4°C overnight. After washing with TBST, anti-rabbit IgG conjugated to alkaline phosphatase was added for 1 h at room temperature. Blots were then rinsed with washing buffer (10 mmol/l Tris-HCl [pH 9.5], 10 mmol/l NaCl and 1 mmol/l MgCl₂), developed with CDP-Star reagent (New England Biolabs, Beverly, MA, USA) and exposed to X-ray film. The intensity of bands was measured using Fluor-S MultiImager and Quantity One software from Bio-Rad (Hercules, CA, USA).

Statistical analysis This was performed by one-way ANOVA with Dunnett's multiple comparison test. Data are presented as means±SEM unless stated otherwise.

Fig. 1 Adenoviral transduction of human islets. **a** Human islets were transduced with adenoviral GFP or beta galactosidase (*bGal*; MOI 50) for 48 h. The later set of islets was immunostained with Cy3 for *bGal* and examined by fluorescent microscopy along with GFP-expressing cells. **b** Human islets were transduced with recombinant adenoviruses encoding *bGal*, KCREB or MCREB at an MOI of 50 or 100 for 48 h. Lysates were prepared and immunoblotted for *bGal*, Flag tag, PCREB, CREB or beta (b) actin respectively. A representative blot from three batches of islets is provided. **c** The bands were quantitated by scanning densitometry and the ratio of PCREB: CREB was determined. The results are means±SEM of three batches of islets. **p*<0.001 vs no-virus control



Results

Adenoviral transduction of human islets We were able to introduce genes of interest in human islets by transducing with recombinant adenoviruses at a multiplicity of infection (MOI) of 50 and 100. Immunocytochemical analysis of human islets transduced with adenoviral green fluorescent protein (GFP) showed a 30 to 55% efficiency of gene transfer (Fig. 1a). Production of beta galactosidase was demonstrated by immunofluorescent labelling and immunoblot analysis. We also verified the transfer of mutant forms of CREB, namely KCREB and MCREB. Both mutants were transduced with similar efficiency at an MOI of 50 to 100 as evaluated by western blot analysis of flag tag production for KCREB and of decreased CREB phosphorylation for MCREB, which interferes with phosphorylation/activation of endogenous CREB (Fig. 1b). The decreases were 46 and 60% at an MOI of 50 and 100 respectively (Fig. 1c).

Dominant negative mutant forms of CREB induce the mitochondrial pathway of apoptosis in human islets To determine if CREB is essential for islet survival we examined a panel of markers of apoptosis in human islets transduced with KCREB or MCREB. KCREB is mutated at

the DNA binding domain. It will heterodimerise with endogenous CREB and reduce its binding to CRE by sequestering it away from the promoter. MCREB is mutated at the phosphorylation site (S133A). Although MCREB binds to the CRE site, it cannot bind to the coactivator, CREB binding protein. Thus both mutants interfere with CREB-mediated transcription. Adenoviral beta galactosidase was used as control. We carried out immunoblot analysis of cleaved active fragment of different caspases (Fig. 2a). Some basal activation of caspases was seen in islets not exposed to adenovirus. There are mainly two pathways of apoptosis, the extrinsic death receptor pathway, which activates caspase-8, and the intrinsic mitochondrial pathway, which activates caspase-9. Apoptosis in isolated human islets is inevitable as they are exposed to stress from multiple sources. Apoptosis in islets

transduced with adenoviral beta galactosidase was comparable to that in no-virus control. Both mutant forms of CREB increased activation of caspase-9, a marker for the mitochondrial pathway of apoptosis by two to fourfold (Fig. 2a). The active form of caspase-8 was not detected, suggesting that the extrinsic pathway of apoptosis is not likely to play a significant role under these conditions. The active form of caspase-3, which is downstream of caspase-9, was also elevated after transduction of islets with adenoviral KCREB and MCREB. Quantitation of the bands in immunoblot analysis showed 2.4-fold and fourfold increases in active caspase-3 with KCREB and MCREB (MOI 100) respectively (Fig. 2b). We also detected markers of executive and terminal phases of apoptosis. For example, activation of caspase-7, which is downstream of caspase-3, was observed. Accumulation of caspase-cleaved PARP was also detected. PARP, a nuclear enzyme that is involved in the recovery of cells after DNA damage, is cleaved by caspase-9 and caspase-3. Next we examined the activities of the caspases as another measure of caspase activation. Substrates specific for caspase-9 (DEVD) and -3 (IETD) linked to *p*-nitroaniline were used. Both KCREB and MCREB increased the enzymic activities of caspase-9 and -3 with the latter being more apoptotic than the former (Fig. 3a,b), a result that paralleled the changes in immunoblot analysis. Nuclear condensation as detected by DAPI staining was significantly ($p < 0.001$) greater in human islets transduced with adenoviral KCREB and MCREB when compared with beta galactosidase control (Fig. 3c). Taken together, these findings suggest that induction of the mitochondrial pathway of apoptosis in human islets occurs when CREB function is downregulated. Generally, we observed 10 to 15% of cell death by necrosis in our islet preparations. Further increases in necrosis were observed when islets were cultured for longer time periods, e.g. 4 to 7 days. The experiments described in this study were completed within 3 to 4 days after islet isolation. We observed the mechanism of cell death to be mainly apoptotic under conditions of CREB downregulation.

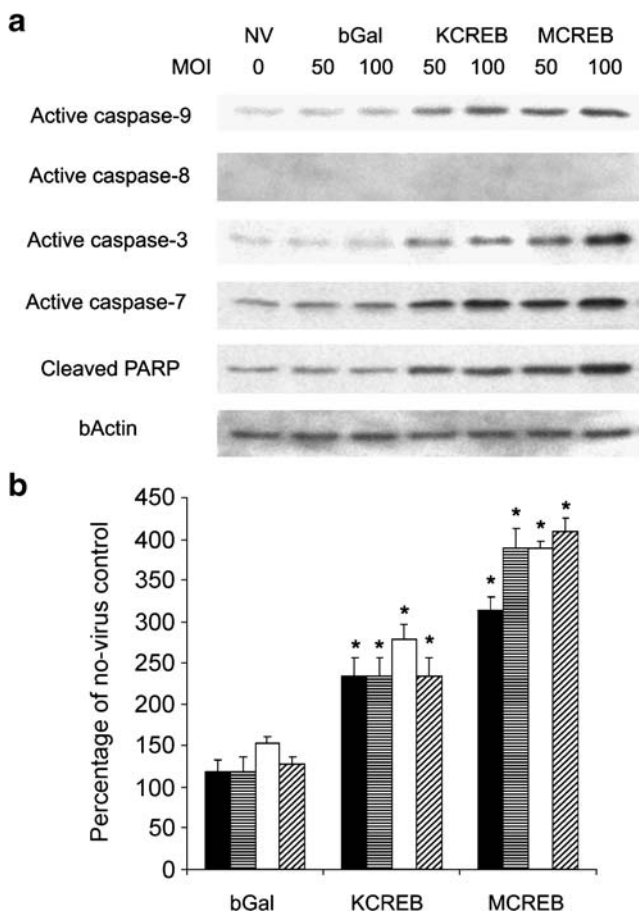
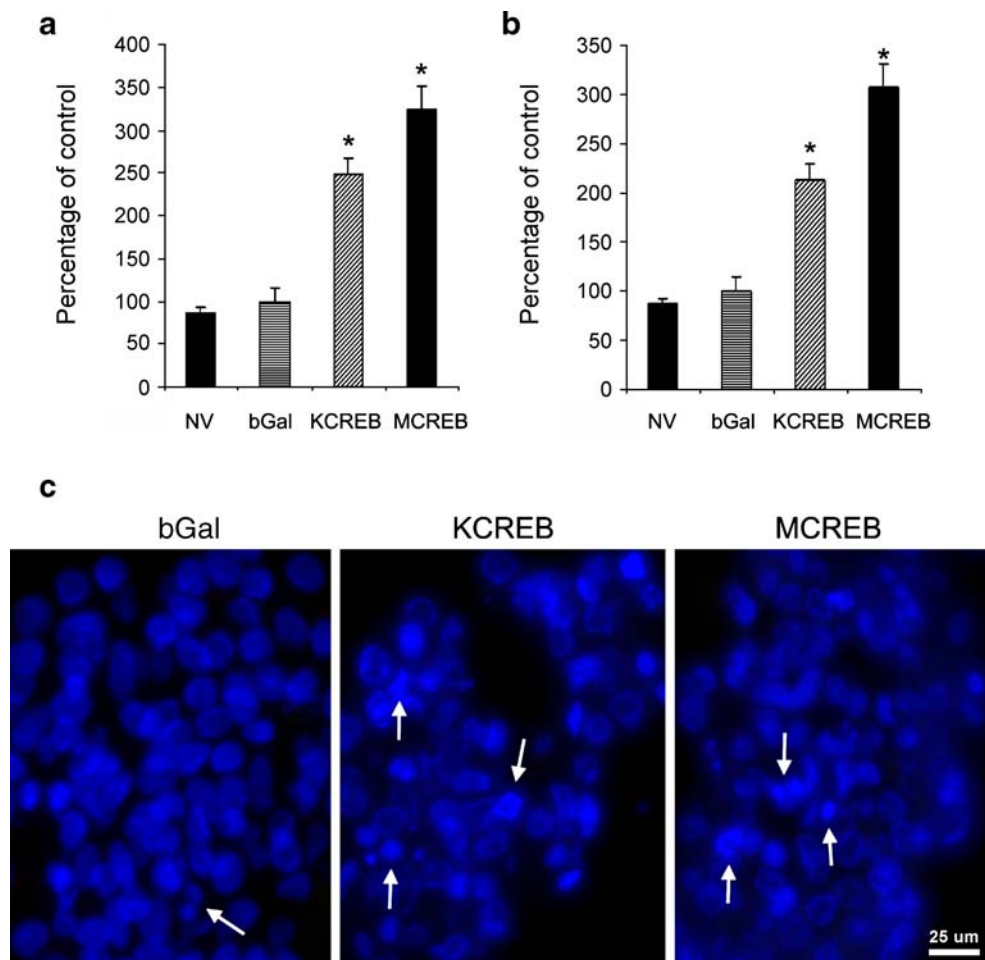


Fig. 2 Mutant forms of CREB activate caspase-9 and caspase-3 in human islets. Human islets were transduced with adenoviral beta galactosidase (*bGal*), KCREB and MCREB at an MOI of 50 and 100 for 48 h. Transduced islets along with no-virus control (*NV*) were processed for immunoblot analysis (**a**) of active forms of caspase-9, -8, -3 and -7 and cleaved PARP. The blots were reprobbed for beta (**b**) actin. **b** The bands were quantitated by scanning densitometry and corrected for beta actin. The results are means \pm SEM of three batches of islets. * $p < 0.001$ vs *bGal* control. *Black bars*, active caspase-9; *horizontal-hatched bars*, active caspase-3; *white bars*, active caspase-7; *diagonal-hatched bars*, cleaved PARP

Dominant negative mutant forms of CREB activate caspase-9 and caspase-3 in beta cells Immunocytochemical analysis of islets using antibodies to active caspase-9 and -3 demonstrated that the major changes induced by KCREB or MCREB occurred predominantly within beta cells (Figs. 4 and 5). The Cy3 (red) signal from active caspase-9 and -3 as quantitated by mean integrated fluorescence intensity of the images using Slide Book Application software showed a four to sixfold increase in beta cells by mutant forms of CREB. This is significantly more than the two to fourfold increase observed in immunoblot analysis of whole islets, probably due to the increased susceptibility of beta cells to injury. Another interesting aspect of the observation in

Fig. 3 Mutant forms of CREB induce apoptosis in human islets. **a, b** Human islets were transduced with adenoviral beta galactosidase (*bGal*), KCREB and MCREB at an MOI of 100 for 48 h. The activities of caspase-9 (**a**) and caspase-3 (**b**) were determined using kits. The results are means±SEM of three batches of islets. * $p<0.001$ vs *bGal* control. *NV* no-virus. **c** A portion of the treated islets was embedded in OCT and 7 μm sections were made. The sections were stained for nuclei with DAPI and condensed nuclei were counted



these experiments is that the majority of insulin-positive cells showed activation of caspases although we had indicated that transduction efficiency was at a maximum of 55%. A possible explanation is that apoptosis induced in transduced beta cells could have an overall impact on the beta cell population of islets.

CREB downregulation potentiates cytokine-induced apoptosis in human islets Activation of caspase-9 and caspase-3 by cytokines (4 ng/ml of IL-1 β +20 ng/ml of TNF- α +20 ng/ml of IFN- γ) in human islets was further potentiated ($p<0.001$) by twofold when transduced with MCREB (MOI 50) as shown by the immunoblot analysis ([Electronic supplementary material \[ESM\] text, supplementary Fig. 1](#)).

Exendin-4 and betacellulin decrease cytokine-induced downregulation of CREB function and apoptosis To determine if activators of CREB could reduce cytokine-induced apoptosis, we used two beta cell-specific growth factors, exendin-4 and BTC. Our preliminary studies showed that these two growth factors are less effective when used alone and therefore we used a combination of these two. They

activated CREB through multiple and largely independent signalling pathways and showed additive effects in raising the active phosphorylated form of CREB (results not shown). When human islets were preincubated for 24 h with a combination of exendin-4 (100 ng/ml) and BTC (10 nmol/l) prior to exposure to cytokines, the number of TUNEL-positive beta cells in islets decreased significantly ($p<0.001$; Fig. 6a). TUNEL-positive cells among cells stained for insulin were counted in multiple fields from nonadjacent sections of embedded islets. The percentage of TUNEL-positive cells was 11.4±1.8 and 3.6±0.8 in cells incubated in the absence or presence of growth factors, respectively. When the islets were exposed to a mixture of cytokines, the percentage of TUNEL-positive cells increased to 32.7±4.8, which was reduced by 60% ($p<0.001$) after preincubation with exendin-4 and BTC. These observations suggest that apoptosis in beta cells of isolated islets is significantly reduced under basal as well as cytokine-treated conditions by a combination of growth factors that activate CREB. Next, we also measured anti-apoptotic markers in these islets. Decreases in phosphorylated active forms of AKT (Fig. 6b), one of the upstream

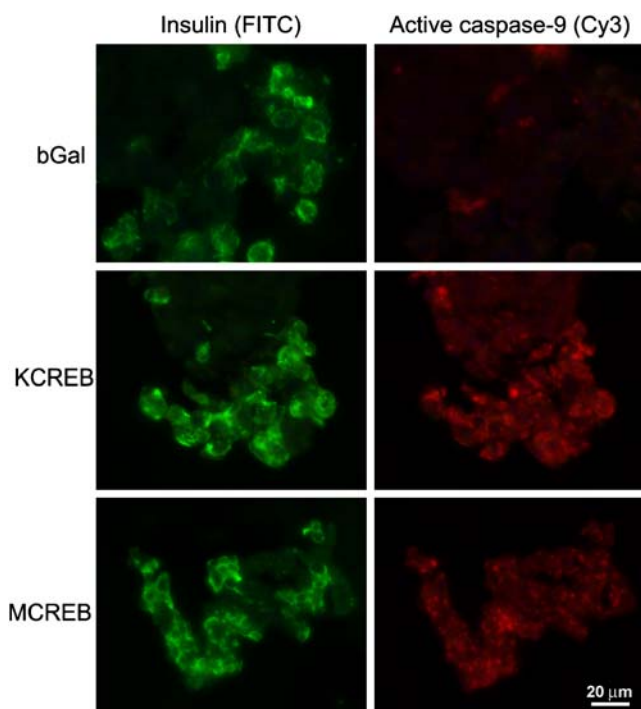


Fig. 4 Dominant negative mutant forms of CREB activate caspase-9 in beta cells of human islets. Human islets from three different batches were transduced (MOI 100) with recombinant adenoviruses encoding control beta galactosidase (*bGal*) or mutant forms of CREB (KCREB or MCREB) for 48 h. The treated islets were embedded in OCT and 7 μ m sections were made, on which immunocytochemical analysis of islets was carried out. After fixing and permeabilisation, sections were exposed to guinea pig anti-insulin antibody and rabbit active caspase-9 antibody. After washing, islets were stained with appropriate secondary antibodies linked to FITC (insulin) and Cy3 (caspase-9). Sections were mounted with mounting medium for fluorescent microscopy. A representative image is provided

kinases of CREB and phosphorylation of CREB (Fig. 6c), were restored to the levels in control islets not exposed to cytokines. It should be mentioned here that the increases in phosphorylated forms of AKT and CREB in growth factor-treated islets above the basal condition were moderate (42–50%; $p < 0.05$), since treatment with exendin-4 and BTC was for 24 h. Activation of signalling by growth factors in general reaches a maximum at 10 to 15 min. The decreases in these signals induced by cytokines over 24 h were significantly restored by growth factors (two to threefold; $p < 0.001$). Next, the production of anti-apoptotic protein BCL2, which is positively regulated by CREB, was also maintained in cytokine-treated islets at control levels (Fig. 6d). These actions of exendin-4 and BTC are likely to play a role in their cytoprotective action.

Anti-apoptotic effects of exendin-4 and BTC in human islets are partially blocked by a mutant form of CREB To determine if CREB plays a role in mediating the effects

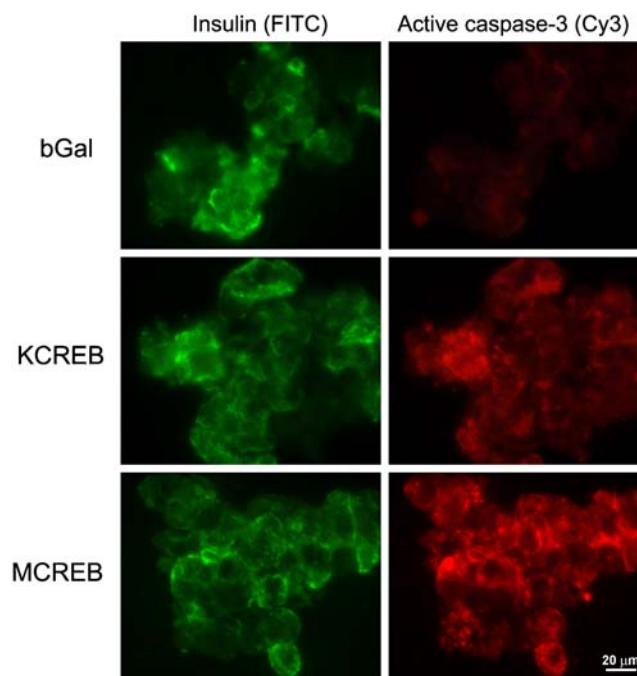


Fig. 5 Dominant negative mutant forms of CREB activate caspase-3 in beta cells of human islets. Human islets transduced with adenoviral beta galactosidase (*bGal*) or KCREB or MCREB (MOI 100) for 48 h were embedded in OCT and frozen. Sections (7 μ m thickness) were permeabilised and incubated in the presence of guinea pig anti insulin antibody and rabbit active caspase-3 antibody. After washing, islets were stained with secondary antibodies linked to FITC (insulin) and Cy3 (caspase-3). Sections were mounted with mounting medium for fluorescent microscopy. Representative images from immunofluorescent analysis of three batches of islets are provided

of exendin-4 and BTC, we examined the effects of growth factors on activation of caspase-3 in human islets transduced with MCREB. First we examined the effect of transduction with MCREB on growth factor-mediated CREB phosphorylation, an important step needed for its activation at the peak time point of 10 min (Fig. 7a). This mutant decreased the level of phosphorylated CREB in islets exposed to growth factors by 75% ($p < 0.001$; Fig. 7b). Next, we observed that growth factors exendin-4 and BTC decreased ($p < 0.001$) cytokine-mediated activation of caspase-3 in islets as shown by the assay of enzyme activities (Fig. 7c). However, the anti-apoptotic effect of growth factors was partially and significantly lost when the CREB function was downregulated by transduction with MCREB. For example, caspase-3 activity in islets producing MCREB and treated with cytokines and growth factors was 55% ($p < 0.01$) more than in the corresponding beta galactosidase control but 21% ($p < 0.05$) less than in cytokine-treated islets. The probable reason could be that adenoviral transduction of MCREB was achieved only in ~50% of islets, whereas the growth factors exert their action in all islets.

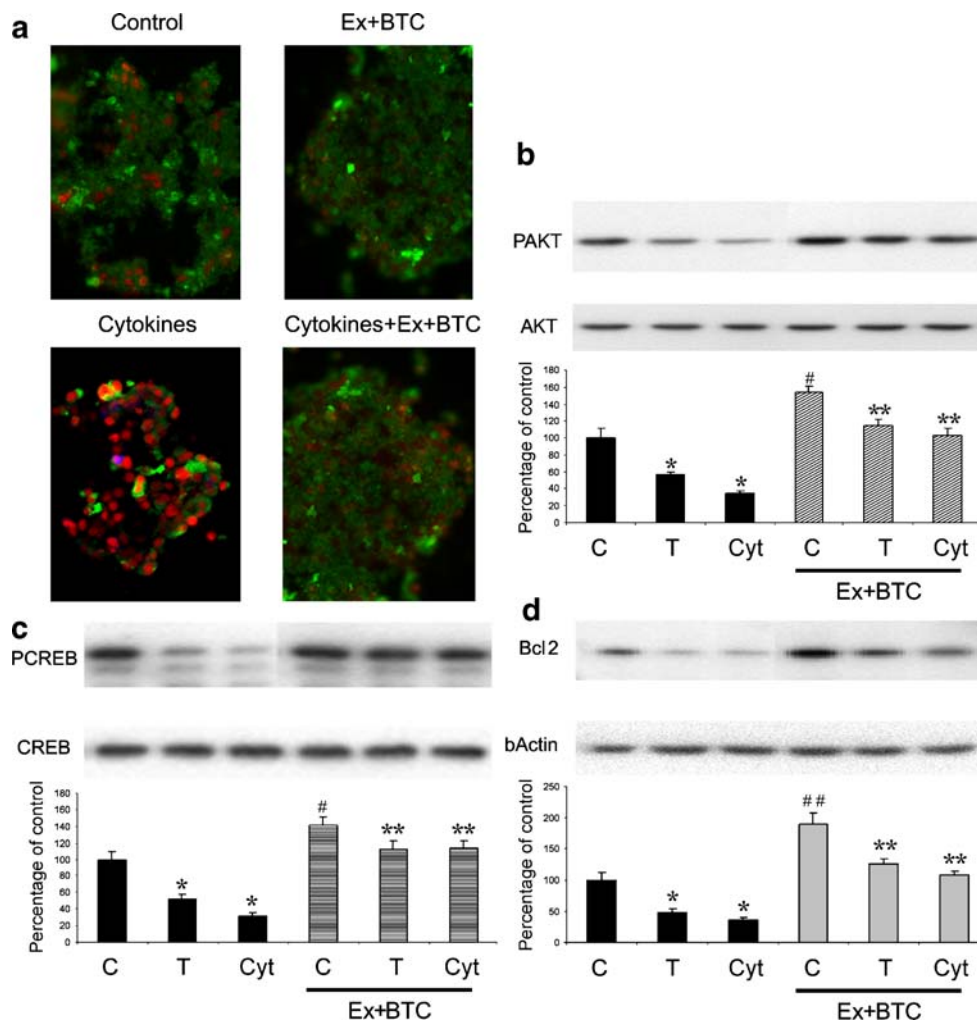


Fig. 6 Activators of CREB exert anti-apoptotic effects in human islets. **a** Human islets were exposed to a mixture of cytokines (4 ng/ml of IL-1 β , 20 ng/ml of TNF- α and 20 ng/ml of IFN- γ) and/or a combination of exendin-4 (*Ex*; 100 ng/ml) and BTC (10 nmol/l) for 24 h. The treated islets were embedded in OCT and frozen. Sections (7 μ m thickness) were TUNEL-stained (*red*) using a cell death detection kit, stained for insulin with FITC (*green*) and analysed by fluorescence microscopy. A representative image for each treatment is provided. TUNEL-positive beta cells were counted in multiple fields of four nonadjacent sections. The results are means \pm SEM of three batches of islets. TUNEL-positive cells: control, 11.4 \pm 1.8; Ex+BTC, 3.6 \pm 0.8 (*p*<0.001 vs control); cytokines, 32.7 \pm 4.8 (*p*<0.001 vs control); cytokines+Ex+BTC, 13.1 \pm 1.4 (*p*<0.001 when compared with cytokine-treated islets). **b–d** Human

islets from three different batches were exposed to TNF- α (*T*; 50 ng/ml) or a mixture of cytokines (*Cyt*; 4 ng/ml of IL-1 β , 20 ng/ml of TNF- α and 20 ng/ml of IFN- γ) in the absence and presence of a combination of exendin-4 (100 ng/ml) and BTC (10 nmol/l) for 24 h. After washing the islets in cold PBS, they were lysed with cell lysis buffer and the protein content was determined. Samples with equal protein content were immunoblotted for phospho (P) AKT, AKT, phospho (P) CREB, CREB, BCL2 and beta actin (bActin). Representative blots are presented. The band intensities were quantitated for PAKT/AKT (**b**), PCREB/CREB (**c**) and BCL2/bActin (**d**) by scanning densitometry. The results are means \pm SEM of three different batches of islets. **p*<0.001, #*p*<0.05 and ##*p*<0.01 vs untreated islets; ***p*<0.001 when compared with corresponding cytokine treatment without growth factors

Discussion

Reducing apoptosis in human islets during storage and after transplantation is potentially an important strategy to improve transplantation outcomes. CREB is a transcription factor with critical survival gene targets and loss of CREB function leads to mouse beta cell apoptosis in vitro and in vivo [13, 17, 18]. In this study, we demonstrate that when CREB-mediated transcription is downregulated by domi-

nant negative mutant forms of CREB (MCREB and KCREB), the apoptotic profile of human islets in the transplantation setting is modified. Furthermore, we observed that the anti-apoptotic effects of the growth factors exendin-4 (an analogue of glucagon-like peptide-1) and BTC are reduced in human islets when CREB function is compromised. Taken together, these observations suggest that maintenance of CREB-mediated transcription could be cytoprotective to human islets.

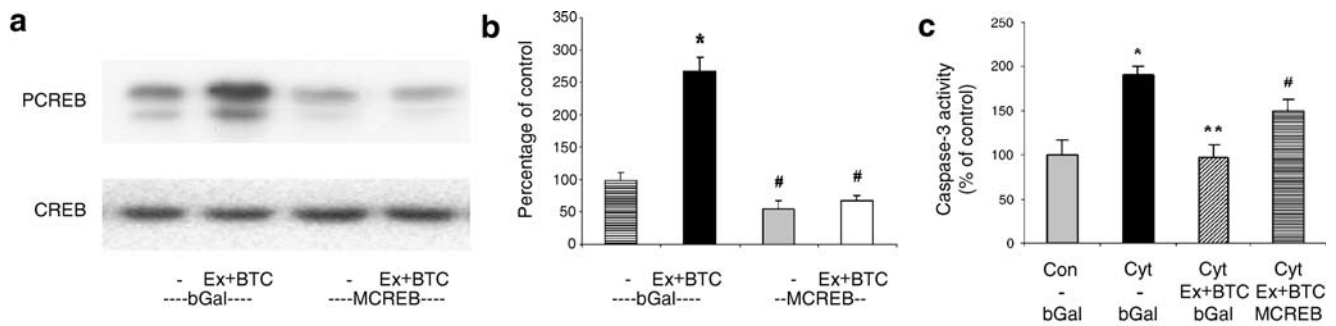


Fig. 7 Dominant negative MCREB partially blocks growth factor-mediated anti-apoptotic effects in human islets **a** Human islets were transduced with adenoviral beta galactosidase (*bGal*) or MCREB. After 24 h, they were exposed to exendin-4 (*Ex*; 100 ng/ml) and BTC (10 nmol/l) for 15 min. The treated islets were processed for immunoblot analysis of PCREB and CREB. A representative blot is presented. **b** The intensity of bands was measured (see “Materials and methods”). The results shown are means \pm SEM of three different batches of islets. * p <0.001 vs bGal control; # p <0.001 vs corresponding bGal control in the

absence and presence of growth factors. **c** Human islets were transduced with adenoviral bGal or MCREB. After 24 h, they were exposed to a mixture of cytokines (*Cyt*; 4 ng/ml of IL-1 β , 20 ng/ml of TNF- α and 20 ng/ml of IFN- γ) and/or a combination of exendin-4 (100 ng/ml) and BTC (10 nmol/l) as indicated for another 24 h. The treated islets were processed for caspase-3 assay. The results are means \pm SEM of three independent batches of islets. * p <0.001 vs bGal control; ** p <0.001 vs bGal+cytokines; # p <0.01 vs bGal+Cytoines+Ex+BTC and p <0.05 vs bGal+cytokines

To understand the pathway of apoptosis induced by CREB downregulation, we examined a panel of apoptotic markers (Fig. 2). First, we saw activation of caspase-9, a marker for the activation of the intrinsic mitochondrial pathway, but not of caspase-8, a marker for the extrinsic pathway. The mitochondrial pathway of apoptosis is regulated by the BCL2 family of proteins, which includes anti-apoptotic BCL2, BCL-XL and the pro-apoptotic proteins BAD and BAX. The integrity of the mitochondrial membrane is dependent on the balance between these two groups of proteins [23]. In the present study, there was also evidence for the later committed stages of apoptosis as shown by an increase in active forms of caspase-3 and caspase-7. Caspase-7 plays an important role in cell death, causing cleavage of important cellular regulatory proteins. Furthermore, we detected significant amounts of cleaved PARP, a substrate for caspase-3 and caspase-7. Cells with condensed nuclei, another marker of apoptosis, were significantly more frequent in islets transduced with adenoviral KCREB and MCREB when compared with control (Fig. 3). Thus observations from this detailed examination of apoptotic pathways provide new information regarding beta cell death under conditions of CREB downregulation.

CREB regulates the expression of several genes involved in cell growth, function and survival [24, 25]. Although CREB-mediated gene expression has been characterised in neurons, limited information is available regarding its role in beta cell survival [26–28]. The promoter region of the *c-IAP2* gene, which belongs to the family of inhibitors of apoptosis, contains CRE sites [29]. *IRS2* an important mediator of growth factor action is also a CREB-dependent gene [17]. Inada et al. [18] demonstrated that transgenic mice with beta cell-targeted expression of inducible cyclic

AMP early repressor, which interferes with CREB function, is characterised by early diabetes due to impaired beta cell proliferation. Transgenic mice expressing ACREB, a dominant negative form of CREB, develop diabetes as a result of beta cell apoptosis [17]. We and others have shown that CREB plays a critical role in inducing the anti-apoptotic gene *Bcl2* [15, 20, 30]. Therefore, downregulation of CREB could be expected to induce apoptosis by the mitochondrial pathway as observed in this study. The role of CREB in cell survival has been demonstrated in other cell types as well by in vivo and in vitro studies [31–34].

Next we examined the role of CREB in mediating the anti-apoptotic action of growth factors. Treatment of isolated human islets with growth factors for improving beta cell survival has been previously suggested as an important strategy for improving islet survival [35]. We suggest that CREB needs to be an important component of this strategy. Even after transplantation, when CREB function is reduced, growth promoting effects of endogenous growth factors are likely to be reduced significantly and it can lead to graft failure. In this study, exendin-4 and BTC together increased CREB phosphorylation/activation, restored BCL2 production and decreased apoptosis in cytokine-treated islets (Fig. 6). Activation of CREB seems to be needed for the anti-apoptotic effects of exendin-4 and BTC, since overexpression of the gene encoding MCREB, which interferes with CREB phosphorylation, reduced significantly the cytoprotective effects of these two growth factors (Fig. 7). We have previously demonstrated that IGF-I induces the expression of *Bcl2*, by activating CREB through multiple signalling pathways in PC12 cells, a neuronal cell line [15, 20]. Therefore, treating human islets in vitro with growth factors for improving islet survival needs to be done in conjunction with other agents such as

antioxidants that preserve CREB function [36]. Bottino et al. [37] have demonstrated the beneficial effects of a novel antioxidant compound in improving islet survival. We have previously demonstrated that antioxidants restore CREB-mediated transcription [36, 38].

Although islet transplantation is a promising therapy, the potential demand for such a treatment greatly outstrips the supply of human islets from cadaveric donors. In addition to multiple approaches taken to increase the supply of islets, there is a need to limit islet loss during isolation and following transplantation, ensuring that this precious resource is efficiently utilised. Findings from our current study suggest that preservation of CREB function could lead to improvement in beta cell survival in transplanted islets.

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