Involvement of the Ca^{2+} -responsive transactivator in high glucose-induced β -cell apoptosis

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Abstract

The calcium-regulated transcription coactivator, Ca^{2+} -responsive transactivator (CREST) was expressed in pancreatic β -cells. Moreover, CREST expression became significantly increased in pancreatic islets isolated from hyperglycemic Goto-Kakizaki rats compared with normoglycemic Wistar controls. In addition, culture of β -cells in the presence of high glucose concentrations also increased CREST expression *in vitro*. To further investigate the role of this transactivator in the regulation of β -cell function, we established a stable β -cell line with inducible CREST expression. Hence, CREST overexpression mimicked the glucotoxic effects on insulin secretion and cell growth in β -cells. Moreover, high glucose-induced apoptosis was aggravated by upregulation of the transactivator but inhibited when CREST expression was partially silenced by siRNA technology. Further investigation found that upregulation of *Bax* and downregulation of *Bcl2* was indeed induced by its expression, especially under high glucose conditions. In addition, as two causing factors leading to β -cell apoptosis under diabetic conditions, endoplasmic reticulum stress and high free fatty acid, mimicked the high glucose effects on CREST upregulation and generation of apoptosis in β -cells, and these effects were specifically offset by the siRNA knockdown of CREST. These results indicated that CREST is implicated in β -cell apoptosis induced by culture in high glucose and hence that CREST may become a potential pharmacological target for the prevention and treatment of type 2 diabetes mellitus.

Key Words

- CREST
- glucotoxicity
- lipotoxicity
- beta-cell apoptosis
- type 2 diabetes mellitus

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Introduction

Insulin resistance and β -cell dysfunction are main characteristics of type 2 diabetes mellitus (T2DM). Chronic high glucose, free fatty acids, and endoplasmic reticulum (ER) stress have been found to be toxic to pancreatic β -cells and impairing cellular functioning (Elsner *et al.* 2011). However, the mechanisms underlying β -cell dysfunction

and the resulting apoptosis via those factors have not been fully characterized (Donath *et al.* 1999, Kim *et al.* 2005, Lablanche *et al.* 2011). It is well known that Ca^{2+} plays an important role in the regulation of apoptosis in multiple experimental models (Orrenius *et al.* 1989, Juntti-Berggren *et al.* 1993, He *et al.* 1997). In this regard, high

glucose induced apoptosis in pancreatic β -cells via a Ca^{2+} -dependent process (Efanova *et al.* 1998), although the specific molecular players directing this process were not investigated.

As a calcium-regulated transcription coactivator, Ca^{2+} -responsive transactivator (CREST) has been recently isolated by a transactivator trap strategy (Aizawa *et al.* 2004). Thus, CREST is composed of three major functional domains: an N-terminal region with an auto-regulatory role, an internal methionine-rich domain with unknown functions, and a large C-terminal glutamine-rich domain responsible for transactivation. Its expression has been reported in brain, heart, liver, kidney, and testis as well as in various other cell types (Aizawa *et al.* 2004, Pradhan & Liu 2005). However, there are no reports regarding CREST expression in pancreas or its involvement in high glucose-induced pancreatic β -cell apoptosis. Notably, some reports have shown that culturing pancreatic islets under high glucose concentrations might lead to a persistent elevation of cytoplasmic Ca^{2+} so as to trigger apoptosis and that this could lead to the long-term irreversible deterioration of β -cell function (Efanova *et al.* 1998, Bjorklund *et al.* 2000). Given the aforementioned premises, this study was designed to examine the potential involvement of CREST in glucotoxicity and apoptosis in pancreatic β -cells.

For this purpose, we established an INS-1E stable cell line permitting inducible expression of CREST. The effects of CREST overexpression on insulin secretion and apoptosis were examined when the cell line was cultured under high glucose conditions. In addition, the mechanisms of CREST-mediated apoptosis upon such cultured cells were analyzed. Moreover, given that some recent studies found that Ca^{2+} was also involved in lipotoxicity- and ER stress-induced apoptosis in pancreatic β -cells (Choi *et al.* 2007, Sano *et al.* 2009, Wang *et al.* 2011), the role of CREST in mediating these processes was also investigated.

Materials and methods

Reagents and materials

INS-1E cells (between passages 54 and 94) were kindly provided by Dr Haiyan Wang (University of Geneva, Geneva, Switzerland). All general reagents for cell culture were purchased from GIBCO, unless otherwise stated. Palmitate (PA), doxycycline (Dox), tolbutamide, diazoxide, thapsigargin (TG), FCS, and BSA were purchased from Sigma-Aldrich Company. TG was dissolved in DMSO.

Dox was dissolved in RPMI 1640 medium. Tolbutamide and diazoxide were added to the medium from stock solutions in NaOH. All chemicals were handled in accordance with the supplier's recommendations.

Preparation of PA

PA/BSA conjugates were made by soaping PA in NaOH and mixing with BSA (Lee *et al.* 2011). In brief, a 20 mM solution of PA in 0.01 M NaOH was incubated for 30 min at 70 °C. Then, 5% (w:vol) fatty acid-free BSA in PBS was added to the fatty acid soaps at a 3:1 volume ratio. The resulting conjugates contained 5 mM PA and 3.75% BSA. The PA/BSA conjugates were diluted in RPMI 1640 supplemented with 10% FCS.

Cell culture

The INS-1E and INS-r9 cell lines (also referred to as r9, which carry the reverse tetracycline/Dox-dependent transactivator) were cultured in complete medium composed of RPMI 1640 supplemented with 10 mM HEPES, 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin, 1 mM sodium pyruvate, and 50 μM 2-mercaptoethanol. Cells were incubated in 5% CO_2 incubator at 37 °C and the medium was changed every 3 days.

Quantitative real-time PCR and immunohistochemistry

Sixteen-week-old, male diabetic Goto-Kakizaki (GK) rats and age- and sex-matched nondiabetic male Wistar rats were purchased from Shanghai SLAC Laboratory Animal Co. Ltd (Shanghai, China). Mean nonfasting blood glucose concentrations were determined to be 16.45 and 4.32 mmol/l for the GK and Wistar rats respectively. Pancreatic tissues and islets were isolated respectively from the two groups of rats for immunohistochemistry, protein extraction, and RNA extraction. The procedures of animal experiments were in agreement with the institutional guidelines of the animal ethics committee. All surgery was performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering.

Quantitative real-time PCR was performed as described previously (Qian *et al.* 2008). The following forward and reverse primers were used: GGATGAGAAC-CACCACCTGA and GGCTGGAACCGCTCTGACTG (Crest, 215 bp); GACATCCGTAAAGACCTCTATGCC and AATAGAGCCACCAATCCACACAGAG (β -actin, 173 bp).

The expression levels of *Crest* were normalized to those of β -actin. Data were analyzed by the $2^{-\Delta\Delta C_t}$ method.

For immunohistochemistry, excised pancreas were fixed in 4% paraformaldehyde and paraffin embedded. Pancreatic tissue sections (5 μ m) were then incubated with goat anti-CREST primary antibodies (Santa Cruz, 1:100 dilution) followed by biotinylated secondary antibodies. Immunoreactivity was visualized using a HRP-conjugated antibiotin (ABC-Peroxidase kit; Vector Laboratories, Burlingame, CA, USA) according to the manufacturer's protocol.

Establishment of INS-1E stable cell lines allowing inducible CREST expression

The plasmids were constructed by subcloning the cDNA-encoding *Crest* into plasmids PUHD10-3. The stable INS-r9 cells (also refer red to as r9), carrying the reverse tetracycline/Dox-dependent transactivator (Gossen *et al.* 1995, Wang & Iynedjian 1997), were used for the secondary stable transfection following the procedures described previously (Wang & Iynedjian 1997, Wang *et al.* 2001). Then, the CREST cells were induced with 500 ng/ml Dox for 72 h and the CREST expression was analyzed by western blot and immunofluorescence as described previously (Men *et al.* 2009).

Cell viability

The CREST cells were cultured in medium containing either 2.8 mM glucose or 30 mM glucose for 0, 1, 3, 5, and 7 days, in the presence or absence of 500 ng/ml Dox. After the corresponding incubation times, cells were counted using a cell counting kit-8 (CCK-8; Dojindo Laboratories, Kumamoto, Japan) according to the manufacturer's instructions, with OD measurements performed at 450 nm. Duplicate measurements were performed for each of three different experiments.

Measurements of insulin secretion and the expression of insulin mRNA expression

For determination of insulin secretion levels, CREST-expressing cells were seeded in 24-well plates and cultured in medium containing either 2.8 or 30 mM glucose, with or without 500 ng/ml Dox, for 96 h at 37 °C under a 5% CO₂ atmosphere. After this incubation, cells were equilibrated in medium containing 2.8 mM glucose for an additional 6 h. Cells were then stimulated with Krebs–Ringer bicarbonate HEPES buffer (129 mM NaCl,

5 mM NaHCO₃, 4.7 mM KCl, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄, 10 mM HEPES, and 2 mM CaCl₂, pH 7.4) containing either 2.8 or 30 mM glucose. Following a 1-h stimulation period, cells were treated in lysis buffer (1% Triton-X, 20 mM HEPES, pH 7.4, 100 mM KCl, 2 mM EDTA, and 1.0 mM PMSF) containing protease inhibitors (Roche Diagnostics). Protein concentrations were determined using the BCA assay (Pierce, Rockford, IL, USA). Insulin concentration was determined using an ELISA kit (Linco, St Charles, MO, USA).

For insulin mRNA expression analysis, the CREST cells were cultured in medium containing either 2.8 or 30 mM glucose, in the presence or absence of 500 ng/ml Dox for 96 h. Quantitative real-time PCR was performed as described previously (Qian *et al.* 2008) using the following forward and reverse primers: TGCCAGGCTTTTGTCAAACAGCACCTT and CTCCAGTGCCAAGGTCTGAA (*Insulin*, 187 bp); GACATCCGTAAAGACCTCTATGCC and AATAGAGCCACCAATCCACACAGAG (β -actin, 173 bp). The expression levels of insulin gene were normalized to those of the housekeeping gene β -actin, and data were analyzed by the $2^{-\Delta\Delta C_t}$ method.

Assessment of apoptotic rates

To measure the rates of apoptosis, the CREST cells were cultured in medium containing either 2.8 mM glucose or 30 mM glucose, in the presence or absence of 500 ng/ml Dox, for 96 h as for the previous experiments. The TUNEL assay (Li *et al.* 2012) was performed using an *in situ* cell death detection kit (Roche). The DNA fragmentation was assessed by the quick apoptosis DNA ladder detection kit (LabForce/MBL, Nunningen, Switzerland). For Annexin V staining, cells were incubated with 10 μ l Annexin V-FITC and 5 μ l (50 μ g/ml) propidium iodide (PI) on ice and then analyzed by FACScan (Beckman Counter Epics XL, Miami, FL, USA). This allowed the discrimination among intact cells (FITC–/PI–), early apoptotic cells (FITC+/PI–), and late apoptotic or necrotic cells (FITC+/PI+ or PI+).

Quantitative real-time PCR for detection of *Bcl2* and *Bax* gene expression

After culturing in six-well plates with medium containing 2.8 or 30 mM glucose in the presence or absence of 500 ng/ml Dox for 4 days, the CREST cells were harvested and washed thrice in PBS. Quantitative real-time PCR was performed as described previously (Qian *et al.* 2008). The following forward and reverse primers were used:

TCCATTATAAGCTGTCACAG and GAAGAGTTCCTC-CACCAC (*Bcl2*, 350 bp); GCAGAGGATGATTGCTGATG and CTCAGCCCATCTTCTCCAG (*Bax*, 353 bp); GACA-TCCGTAAAGACCTCTATGCC and AATAGAGCCAC-CAATCCACACAGAG (β -actin, 173 bp). The expression levels of *Bcl2* and *Bax* gene were normalized to that of the housekeeping gene β -actin, and data were analyzed by the $2^{-\Delta\Delta C_t}$ method.

Silencing of CREST expression with siRNAs

A siRNA duplex corresponding to the 603–623 nucleotide of the rat *Crest* (AGG CGG CAC GTC CCA CTA CAA or GCC CAT GAG TCA ACA GTA CAT) was generated (Qiagen). A BLAST search revealed that this region displayed no significant homology to any other known gene. These siRNA duplexes consisted of a 21-nucleotide sense strand (5'-GCG GCA CGU CCC ACU ACA AdTdT-3') (No. 303473) and a 21-nucleotide antisense strand (5'-UUG UAG UGG GAC GUG CCG C dCdT-3') (No. 303474) paired in a manner that yielded a 19-nucleotide duplex region with a two-nucleotide dithymidine overhang at each 3' terminus. A scrambled siRNA (sense strand, 5'-CGUGAUUGCGAGACUCUGAdTdT-3'; antisense strand, 5'-UCAGAGUCUCGCAAUCACGdTdT-3'), without any significant homology to any known protein (as determined by BLAST search), was used as a negative control.

The siRNAs were introduced into CREST cells by Lipofectamine 2000 (Invitrogen). To measure high glucose-induced apoptosis in cells transfected with control (scrambled) or *Crest* siRNAs, the cells were cultured in medium containing 30 mM glucose for 96 h following siRNA transfection. Apoptosis was detected by Annexin V-PI and TUNEL staining, as explained earlier.

Statistical analysis

The results were expressed as mean $\pm$ S.E.M. Comparisons were made using unpaired Student's *t*-tests or one-way ANOVA, as appropriate. The *P* value <0.05 indicated statistical significance while *P* value <0.01 denoted that the difference was highly significant.

Results

Upregulated CREST expression in diabetic GK rat islets

The pancreatic islets from nondiabetic Wistar rats (glycemia 4.32 mmol/l) were oval or round with a smooth circumference, whereas those from GK rats (glycemia

16.45 mmol/l) became fibrotic with irregular contours (Fig. 1B). Moreover, the CREST staining was stronger in islets from diabetic GK rats than in those from nondiabetic Wistar rats (Fig. 1B). Additionally, quantitative real-time PCR and western blot results showed that CREST was drastically upregulated in islets from diabetic GK rats compared with that from the islets of nondiabetic Wistar rats (Fig. 1A and C). Altogether, these data suggest that CREST upregulation may play a role in T2DM.

High glucose increases CREST expression via a cytoplasmic Ca^{2+} -dependent mechanism

As demonstrated by quantitative real-time PCR, the levels of *Crest* mRNA in INS-1E cells were increased in a glucose

Figure 1

CREST expression in islet cells from Wistar and GK rats. (A) Quantitative real-time PCR revealed that *Crest* expression was higher in isolated pancreatic islets from hyperglycemic GK rats than in those from age- and sex-matched normoglycemic Wistar rats. Data represent mean $\pm$ S.E.M. of three independent experiments; **P* <0.05 . (B) Representative images of immunohistochemical analysis showing islet structure as well as distribution of CREST-positive cells (brown) in Wistar (left) and GK (right) rats. (C) Representative western blot corroborating the change in CREST expression detected by immunohistochemistry in pancreatic islets from diabetic GK rats.

Figure 2

High glucose increased *Crest* expression via a cytoplasmic Ca^{2+} -dependent mechanism. (A) Glucose dose-response on *Crest* expression in INS-1E cells. Cells were cultured for 5 days in medium containing 2.8, 10, 20, or 30 mM glucose. Data represent mean $\pm$ S.E.M. of three independent experiments. $^{**}P < 0.01$. (B) Time-course of the effects of high glucose on the expression of *Crest* in INS-1 cells. Cells were cultured in 30 mM glucose medium for 0, 1, 3, or 5 days. Data represent mean $\pm$ S.E.M. of three independent experiments. $^{**}P < 0.01$. (C) The effect of glucose on *Crest* expression is dependent on cytoplasmic Ca^{2+} . INS-1E cells were cultured in low glucose (2.8 mM), low glucose + tolbutamide (100 μM), high glucose (30 mM), or high glucose + diazoxide (50 μM) for 3 days. Quantitative real-time PCR was used to detect *Crest* expression. Data represent mean $\pm$ S.E.M. of three independent experiments. $^{**}P < 0.01$, high glucose vs low glucose. $^{\dagger}P < 0.01$, low glucose + tolbutamide vs low glucose. $^{*}P < 0.05$, high glucose + diazoxide vs high glucose.

concentration-dependent manner (Fig. 2A). Thus, at high glucose concentrations (30 mM), *Crest* expression increased sixfold over that of the basal glucose concentrations (2.8 mM glucose) (Fig. 2A). Moreover, treatment

of INS-1E cells with high glucose also increased the levels of *Crest* transcript in a time-dependent manner (Fig. 2B). Indeed, over a fivefold increase in *Crest* expression was observed after a 5-day culture in high glucose concentrations (Fig. 2B).

As CREST is a Ca^{2+} -responsive transactivator, and some reports had found that high glucose led to a persistent elevation of cytoplasmic Ca^{2+} in β -cells (Efanova *et al.* 1998), we speculated that high glucose may increase CREST expression via a cytoplasmic Ca^{2+} increase. Therefore, we investigated the role of cytoplasmic Ca^{2+} in high glucose-induced CREST expression. As shown in Fig. 2C, we observed that the high glucose effect on *Crest* expression could be reproduced by raising cytoplasmic Ca^{2+} with tolbutamide under the low glucose condition. Conversely, we also observed that the high glucose effect on *Crest* expression could be inhibited by decreasing cytoplasmic Ca^{2+} with diazoxide. Therefore, our experiments suggested that the high glucose-driven CREST induction was cytoplasmic Ca^{2+} dependent.

Characterization of CREST-inducible cell lines

The INS-r9 cells, which carry the reverse tetracycline/Dox-dependent transactivator, were cotransfected with plasmids PUHD10-3, carrying the *Crest* and a plasmid pTKhygro containing the hygromycin-resistance marker. Hygromycin-resistant clones were screened by Northern blotting for clones positively expressing *Crest* after Dox induction. One clone, termed CREST, showing a high induction level and the lowest background levels, was selected for this study. Hence, these cells were then induced with different concentrations of Dox for specified time periods and the transgene-encoded protein levels were analyzed by immunofluorescence and quantitative real-time PCR. As shown in Fig. 3A and B, CREST proteins were induced in a Dox-dependent manner. The time-course and dose-response of Dox induction in CREST cells are shown in Fig. 3C. Dox alone did not affect the endogenous expression of CREST in the control INS-r9 cells (data not shown).

Effects of CREST overexpression on insulin secretion and insulin mRNA expression in INS-1E cells

We then investigated the possible involvement of CREST in impairing glucose-stimulated insulin secretion. As shown in Fig. 4, the overexpression of CREST impaired the glucose-stimulated secretion of insulin (Fig. 4A) and

Figure 3

Characterization of the CREST-inducible INS-1E cell lines. (A) Phase-contrast images (top) and immunofluorescence staining (bottom) using a goat anti-CREST antibody showed that CREST protein was induced by Dox. Cells were cultured with (Dox+) or without (Dox-) 500 ng/ml Dox for 72 h. (B) Quantitative real-time PCR analysis of CREST expression in CREST cells cultured with or without 500 ng/ml Dox for 72 h. Values represent the mean $\pm$ S.E.M. of three independent experiments. ** $P < 0.01$. (C) Time-course and dose-response of the Dox effect on the expression of CREST in CREST cells. To study the time-course of Dox-dependent CREST expression, cells were cultured in medium containing 500 ng/ml Dox and harvested for cytosolic protein at the indicated times. To study the dose-response to Dox treatment, cells were cultured under different concentrations of Dox for 96 h. Then, 20 μ g cytosolic proteins were resolved by 10% SDS-PAGE, transferred to a PVDF membrane, and immunoblotted with an antibody against CREST.

downregulated *insulin* mRNA levels (Fig. 4B). This result supports the hypothesis that CREST is implicated in β -cell glucotoxicity.

Effects of CREST overexpression on cell viability

To examine the effects of CREST overexpression on the growth/survival of pancreatic β -cells, a cell growth curve was plotted by the CCK-8 assay. After treatment with 500 ng/ml Dox, the number of viable cells decreased in a

time-dependent manner. Moreover, CREST induction combined with chronic high glucose concentrations resulted in a synergistic right shift of growth retardation in CREST cells (Fig. 5).

Overexpression of CREST induced apoptosis and aggravated glucotoxicity

To further substantiate our hypothesis, we analyzed apoptotic rates in CREST cells using Annexin V-PI double staining and TUNEL staining. As shown by the sensitive Annexin V-PI double staining, exposure of CREST cells to

Figure 4

Overexpression of CREST inhibited glucose-stimulated insulin secretion and *insulin* mRNA expression. (A) CREST cells were cultured in medium with or without 500 ng/ml Dox, containing 2.8 mM glucose or 30 mM glucose, for 96 h. Cells were then equilibrated in 2.8 mM glucose-containing medium for another 6 h followed by stimulation with Krebs-Ringer bicarbonate HEPES buffer containing 2.8 or 30 mM glucose for 1 h. Insulin release was normalized by total protein and time of stimulation. Control: cells pre-incubated with medium containing 2.8 mM glucose for 96 h. High glucose: cells pre-incubated with medium containing 30 mM glucose for 96 h. Basal: cells stimulated with 2.8 mM glucose for 1 h. Glucose: cells stimulated with 30 mM glucose for 1 h. Values represent mean $\pm$ S.E.M. from three independent experiments. * $P < 0.05$, ** $P < 0.01$. (B) Effects of CREST overexpression on *insulin* mRNA expression in INS-1E cells. CREST cells were cultured in medium containing either 2.8 or 30 mM glucose for 96 h, in the presence or absence of 500 ng/ml Dox. Values represent the mean $\pm$ S.E.M. from three independent experiments. * $P < 0.05$, ** $P < 0.01$.

Figure 5

Effects of CREST expression on INS-1E cell growth. CREST overexpression and chronic high glucose had a synergistic inhibitory effect on INS-1E cell growth. CREST cells were cultured with medium containing basal (2.8 mM, left panel) or high (30 mM, right panel) glucose, in the presence or absence of 500 ng/ml Dox for 0, 1, 3, 5, and 7 days. Data represent four separate experiments. * $P < 0.05$, ** $P < 0.01$.

30 mM glucose for 96 h caused moderate apoptosis. However, concomitant overexpression of CREST aggravated the glucotoxicity-triggered β -cell apoptosis (Fig. 6A). This synergistic effect was further confirmed by TUNEL staining (Fig. 6B). By contrast, neither CREST overexpression nor high glucose alone could cause such deleterious consequences on INS-1E cell apoptosis. These results suggest that CREST is involved in high glucose-induced β -cell apoptosis.

Expression of *Bcl2* and *Bax* in INS-1E cell following CREST overexpression

We next wanted to examine the pathway by which CREST overexpression may induce β -cell apoptosis. Real-time PCR analysis indicated a 138.3% increase in *Bax* expression and a 39.1% reduction in *Bcl2* expression when cells were treated with 500 ng/ml Dox under 2.8 mM glucose for 4 days. Moreover, treatment with Dox under high glucose concentrations resulted in a 217.6% upregulation and a 49.6% downregulation in *Bax* and *Bcl2* expression levels respectively (Fig. 7). Altogether, these results suggest that CREST overexpression promoted *Bax* upregulation and *Bcl2* downregulation, hence contributing to glucose-induced apoptosis in pancreatic β -cells.

CREST knockdown effects on high glucose-induced apoptosis, *insulin* gene expression, and glucose-stimulated insulin secretion

As demonstrated in Fig. 8B and C, CREST silencing largely offset the synergistic effects of CREST and high glucose in pancreatic β -cell apoptosis, thus suggesting a predominant

role for CREST in high glucose-induced apoptosis. The efficiency of siRNA CREST knockdown is summarized in Fig. 8A. In addition, siRNA against CREST improved *insulin* gene expression and glucose-stimulated insulin secretion (Fig. 8D).

ER stress- and lipotoxicity-induced CREST expression in INS-1E cells

Given that recent studies have confirmed the involvement of Ca^{2+} in lipotoxicity- and ER stress-induced apoptosis, the potential implication of CREST in these events was further explored. As shown in Fig. 9A, TG-induced ER stress as well as treatment with the saturated free fatty acid PA increased the expression of *Crest* in INS-1E cells. Moreover, such effects were specifically suppressed by *Crest* silencing.

Crest knockdown effects on lipotoxicity- and ER stress-induced apoptosis

Further supporting the above results, *Crest* knockdown significantly inhibited TG- or PA-induced apoptosis, as demonstrated by both DNA fragmentation (Fig. 9B) and TUNEL assays (Fig. 9C). Altogether, these data suggest that CREST is also implicated in lipotoxicity- and ER stress-induced β -cell apoptosis.

Discussion

Pancreatic islet β -cell apoptosis has been implicated in the pathogenesis of T2DM by causing absolute or relative insulin deficiencies (Elsner *et al.* 2011). During the progression of T2DM, glucotoxicity is an important contributing factor for the progression of β -cell failure and the development of diabetic complications (Rhodes 2005). It has been demonstrated that chronic exposure of β -cells to high glucose resulted in the apoptosis of β -cells (Donath *et al.* 1999, Kim *et al.* 2005, Lablanche *et al.* 2011). In addition, lipotoxicity and ER stress were also the important factors leading to β -cell apoptosis (Choi *et al.* 2007, Sano *et al.* 2009, Wang *et al.* 2011). However, the mechanisms for this factor-associated apoptosis of β -cells have remained elusive.

It is well known that Ca^{2+} plays an important role in the regulation of apoptotic process in multiple experimental models (Orrenius *et al.* 1989, Juntti-Berggren *et al.* 1993, He *et al.* 1997). For instance, high glucose concentrations induced pancreatic β -cell apoptosis in a Ca^{2+} -dependent manner (Efanova *et al.* 1998).

Figure 6

Effect of CREST on apoptosis in INS-1E cells. (A) Flow cytometric analysis for phosphatidylserine exposure in CREST cells cultured in 2.8 mM glucose or 30 mM glucose with (Dox+) or without (Dox-) 500 ng/ml Dox. Intact cells (FITC-/PI-), early apoptotic cells (FITC+/PI-), and late apoptotic or necrotic cells (FITC+/PI+ or PI+) were discriminated. Values represent the mean $\pm$ s.e.m. of early and late apoptotic cells from three independent

experiments. * $P < 0.05$, ** $P < 0.01$. (B) CREST cells were cultured in medium containing 2.8 or 30 mM glucose, with (Dox+) or without (Dox-) 500 ng/ml Dox, for 4 days, and then processed for TUNEL assay. Values represent the mean $\pm$ s.e.m. from three independent experiments. * $P < 0.05$, ** $P < 0.01$.

Furthermore, in cultured human pancreatic islets, overstimulation by high glucose concentrations led to a rise in cytoplasmic Ca^{2+} levels, which persisted after normalization of glucose levels. Thus, a sustained cytoplasmic Ca^{2+} increase might trigger the onset of apoptosis and lead to the long-term irreversible deterioration of β -cell function (Grill & Bjorklund 2001). However, while the intracellular Ca^{2+} concentrations may play an important role in high glucose-induced β -cell apoptosis, the exact mechanisms underlying these effects remain unknown.

CREST is composed of three major functional domains: an N-terminal region with an auto-regulatory role, an internal methionine-rich domain with unknown functions, and a large C-terminal glutamine-rich domain

responsible for transactivation. Immunohistochemical localization showed that it was a nuclear protein (Aizawa *et al.* 2004) encoding a protein of 402 amino acids (55 kDa) and there was a striking homology (54% amino acid identical) with the SYT proto-oncogene. The level of *Crest* mRNAs was shown to be high in brain and moderate in kidney, liver, and heart from normal adult rats. However, its expression was absent in normal adult rat pancreatic tissues and hence the potential role of this protein in high glucose-induced β -cell apoptosis was ill-defined.

In this study, we demonstrated for the first time that CREST is indeed expressed in pancreatic β -cells, hence providing an avenue for deciphering its role in the

Figure 7

Effects of CREST on *Bcl2* and *Bax* mRNA expression in INS-1E cells. (A) Effect of CREST overexpression on *Bcl2* mRNA expression. (B) Effect of CREST overexpression on *Bax* mRNA expression. INS-r9 and CREST cells were cultured in medium containing 2.8 or 30 mM glucose, with (Dox+) or

without (Dox-) 500 ng/ml Dox, for 96 h. Effects of CREST overexpression on *Bcl2* and *Bax* mRNA expression were assessed by quantitative real-time PCR. Values represent the mean $\pm$ S.E.M. from three independent experiments. ** $P < 0.01$.

pathogenesis of diabetes. The mRNA level of *Crest* becomes significantly elevated in *ex vivo* islets and its protein expression is also upregulated in pancreatic tissues from hyperglycemic GK rats vs normoglycemic Wistar rats. In this regard, as an animal model for T2DM, the GK rat is characterized by a progressive loss of β -cell mass and deteriorating function of pancreatic islets. Glucotoxicity (sustained hyperglycemia) has been shown to accelerate the apoptosis and ensuing loss of β -cells in GK rats (Koyama *et al.* 1998). Therefore, we hypothesized that high glucose-induced β -cell dysfunction would be correlated with CREST expression levels.

Indeed, herein, we observed that high glucose could upregulate the expression of *Crest* as well as increase intracellular Ca^{2+} concentrations in β -cells, consistent with findings in previous reports (Efanova *et al.* 1998, Grill & Bjorklund 2001). Additionally, our experiments supported the notion that high glucose-driven CREST upregulation was cytoplasmic Ca^{2+} dependent. Moreover, because we had previously reported that high glucose could induce β -cell apoptosis (Wang *et al.* 2001, Qian *et al.* 2008), it could be inferred that high glucose-induced β -cell apoptosis may be correlated with intracellular Ca^{2+} concentrations and *Crest* expression levels.

Thus, in order to analyze the effect of CREST expression on pancreatic dysfunction, we established a β -cell stable line allowing an inducible expression of CREST. We then demonstrated the Dox-dependent cellular induction of CREST protein by immunofluorescence, as well as confirming the dose and time dependence of Dox induction by western blotting.

In our experiments, the cultural medium used for β -cells was RPMI 1640 containing 11.2 mM glucose, which has insulin content similar to normal islets and exhibits glucose-stimulated insulin secretion (Hohmeier *et al.* 2000). It has been reported that 30 mM glucose could lower the levels of *insulin* mRNA (Ubeda *et al.* 2006) and induce ER stress in INS-1 cells through glucotoxicity (Wang *et al.* 2005).

Our experimental results showed that an induction of CREST alone was sufficient to evoke apoptosis in INS-1E cells. In addition, the overexpression of CREST aggravated high glucose-elicited apoptosis in INS-1E cells while CREST silencing largely overcame the synergistic effects of CREST and glucotoxicity. Moreover, our results showed not only that CREST was involved in high glucose-induced apoptosis but also that CREST expression could inhibit insulin gene expression. That is, under the basal

Figure 8

Effects of CREST knockdown on apoptosis, insulin gene expression, and glucose-stimulated insulin secretion in CREST cells. (A) Western blot confirming CREST silencing in CREST cells. After transfection with CREST siRNA for 2 days, these CREST cells were cultured for 4 days in medium containing 30 mM glucose plus 500 ng/ml Dox. (B, C) High glucose-induced apoptosis was partially inhibited by CREST knockdown in CREST cells. After transfection with CREST siRNA for 2 days, CREST cells were cultured for 4 days in medium containing 30 mM glucose plus 500 ng/ml Dox. Apoptosis was assessed by (B) Annexin V-PI double staining and (C) TUNEL staining. (D) CREST knockdown improved glucose-stimulated insulin secretion (left) and *insulin* gene expression (right) in CREST cells. Left: after transfection with CREST siRNA for 2 days, these CREST cells were cultured in medium

containing 2.8 or 30 mM glucose, in the presence of 500 ng/ml Dox, for 96 h. The cells equilibrated in 2.8 mM glucose medium for another 6 h and then were stimulated with Krebs-Ringer bicarbonate HEPES buffer containing 2.8 or 30 mM glucose for 1 h. Insulin release was normalized for total protein and time of stimulation. Control: cells pre-incubated with medium containing 2.8 mM glucose for 96 h. High glucose: cells pre-incubated with medium containing 30 mM glucose for 96 h. Basal: cells stimulated with 2.8 mM glucose for 1 h. Glucose: cells stimulated with 30 mM glucose for 1 h. Right: after transfection with CREST siRNA for 2 days, these CREST cells were cultured in medium containing 2.8 or 30 mM glucose, in the presence of 500 ng/ml Dox for 96 h. Values represent the mean $\pm$ S.E.M. from three independent experiments. * $P < 0.05$, ** $P < 0.01$.

glucose condition, overexpression of CREST resulted only in ~10% cell apoptosis. However, this led to a ~50% decrease in *insulin* gene expression and glucose-stimulated insulin secretion. Furthermore, siRNA directed against *Crest* improved *insulin* gene expression and glucose-stimulated insulin secretion.

In this study, we also observed that overexpression of CREST in β -cells increased apoptotic rates in these cells as

assessed by different methods. When CREST was induced by Dox, and in particular under high glucose concentrations, there was not only an increase in DNA fragmentation but also changes in *Bax* and *Bcl2* expression levels, thus tilting the balance toward cell death. Notably, Bcl2 family proteins localize within mitochondria and belong to one of the most biologically relevant classes of apoptosis-regulatory gene products acting at the effector

Figure 9

Effect of CREST knockdown on ER stress- and lipotoxicity-induced apoptosis. (A) Quantitative real-time PCR showed that both the ER stress inducer thapsigargin (TG) and the saturated free fatty acid palmitate (PA) markedly upregulated *Crest* expression in INS-1E cells. Two days after siRNA transfection, INS-1E cells were treated with 0.1 μ M TG or 0.5 mM PA for

24 h. Values represent the mean $\pm$ s.e.m. from three independent experiments. * P < 0.05, ** P < 0.01. (B) ER stress or lipotoxicity evoked DNA fragmentation that was offset by CREST silencing. (C) TUNEL staining also corroborated that CREST knockdown diminished ER stress- and lipotoxicity-induced apoptosis.

stage of the programmed cell death event (Mizuno *et al.* 1998). It has been postulated that these proteins act at the level of controlling the permeability of the outer mitochondrial membranes by forming autonomous pores or by opening a multiprotein complex, known as the mitochondrial permeability transition core complex (Martinou & Green 2001). Overall, the ratio of death antagonists (Bcl2) to agonists (Bax) within the Bcl2 superfamily determines whether a cell will ultimately respond to an apoptotic stimulus. Under the present experimental conditions, this ratio was clearly altered.

Finally, as recent studies have revealed that Ca^{2+} was also involved in lipotoxicity- and ER stress-induced β -cell apoptosis (Choi *et al.* 2007, Sano *et al.* 2009, Wang *et al.* 2011), the potential role of CREST in these processes was also investigated. Interestingly, ER stress and FFA mimicked the high glucose effects in regard to CREST upregulation and apoptosis induction in cultured β -cells. These effects were specifically offset by siRNA-driven knockdown of *Crest*.

In conclusion, the upregulation of CREST, a calcium-regulated transcription coactivator, is implicated in glucotoxicity-, lipotoxicity-, and ER stress-induced β -cell apoptosis. Therefore, we speculate that CREST could be a potential pharmacological target for the prevention and treatment of T2DM.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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