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

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Abstract

The calcium-regulated transcription coactivator, Ca$^{2+}$-responsive transactivator (CREST) was expressed in pancreatic $\beta$-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 $\beta$-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 $\beta$-cell function, we established a stable $\beta$-cell line with inducible CREST expression. Hence, CREST overexpression mimicked the glucotoxic effects on insulin secretion and cell growth in $\beta$-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 $\beta$-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 $\beta$-cells, and these effects were specifically offset by the siRNA knockdown of CREST. These results indicated that CREST is implicated in $\beta$-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

Introduction

Insulin resistance and $\beta$-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 $\beta$-cells and impairing cellular functioning (Elsner et al. 2011). However, the mechanisms underlying $\beta$-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, Junitti-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:v) 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-1r9 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 μg/ml streptomycin, 1 mM sodium pyruvate, and 50 μM 2-mercaptoethanol. Cells were incubated in 5% CO₂ 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-CAACCACCTGA and GGCTGGAACCGCTCTGACTG (Crest, 215 bp); GACATCCGTAAGACCTCTATGCC and AATAGAGCCACCACAATCCACAGAG (β-actin, 173 bp).
The expression levels of Crest were normalized to those of β-actin. Data were analyzed by the 2^−ΔΔCt method.

For immunohistochemistry, excised pancreas were fixed in 4% paraformaldehyde and paraffin embedded. Pancreatic tissue sections (5 μm) 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 antibinin (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 referred 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, Burlingame, CA, USA) 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: TGCCCAAGCTTTGTCAAAACAGCCACCTTT and CTCCAGTGCCAAGGTCTGAAG (Insulin, 187 bp); GACATCCGTAAGACCTCTATGCCG and AATAGGCGCAATCCACACAGAG (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^−ΔΔCt 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 μl Annexin V-FITC and 5 μl (50 μg/ml) propidium iodide (PI) on ice and then analyzed by FACSscan (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 GAAGAGTTCCTCCACCAC (Bcl2, 350 bp); GCAGAGGATGATTGCTGATG and CTCAGCCCATCTTCTTCCAG (Bax, 353 bp); GACA-TCCGTAAAGACCTCTATGCC and AATAGGCCAC-CAATCCACACAG (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−ΔΔCt 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 5′ 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 ± 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 Ca2+-dependent mechanism

As demonstrated by quantitative real-time PCR, the levels of Crest mRNA in INS-1E cells were increased in a 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 (Efano et al. 1998), we speculated that high glucose may increase Crest expression via a cytoplasmic Ca^{2+} increase. Therefore, we investigated the role of the increased 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 impairs glucose-stimulated insulin secretion. As shown in Fig. 4, the overexpression of Crest impaired the glucose-stimulated secretion of insulin (Fig. 4A) and

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 ± 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 ± 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 ± S.E.M. of three independent experiments. **P < 0.01, high glucose vs low glucose. *P < 0.01, low glucose + tolbutamide vs low glucose. *P < 0.05, high glucose + diazoxide vs high glucose.
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...
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 Ca²⁺ 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 Ca²⁺ 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 Ca²⁺-dependent manner (Efano et al. 1998).
Furthermore, in cultured human pancreatic islets, over-stimulation 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 \(\beta\)-cell function (Grill & Bjorklund 2001). However, while the intracellular Ca\(^{2+}\) concentrations may play an important role in high glucose-induced \(\beta\)-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 \(\beta\)-cell apoptosis was ill-defined.

In this study, we demonstrated for the first time that CREST is indeed expressed in pancreatic \(\beta\)-cells, hence providing an avenue for deciphering its role in the processes leading to cell death.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure6.png}
\caption{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 ± 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 ± S.E.M. from three independent experiments. *P<0.05, **P<0.01.}
\end{figure}
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 7](http://joe.endocrinology-journals.org/C209/2013/Society-for-Endocrinology/DOI:10.1530/JOE-12-0286/Printed-in-Great-Britain)

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 ± S.E.M. from three independent experiments. **P < 0.01.
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 ± 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

![Graph A](image1)

![Graph B](image2)

![Graph C](image3)

**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 μM TG or 0.5 mM PA for 24 h. Values represent the mean ± 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 Ca2+ was also involved in lipotoxicity- and ER stress-induced βcell 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.

Funding

This study was supported by National Basic Research Program of China (No. 2012CB966402 to Lou), National Nature Science Foundation of China (No. 30971410 to Men and No. 84200616 to Peng), and Beijing Natural Science Foundation of China (No. 7122160 to Peng), National High Technology Research and Development Program of China (863 Project, No. 2011AA020107 and National Science and Technology Major Project (No.2011ZX09102-010-03).

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Received in final form 24 October 2012
Accepted 16 November 2012
Accepted Preprint published online 16 November 2012