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ORIGINAL PAPER

The altered expression of glucose-regulated proteins 78 in different phase of streptozotocin-affected pancreatic beta-cells

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Abstract Endoplasmic reticulum (ER) stress-mediated apoptosis plays an important role in the destruction of pancreatic beta-cells and contributes to the development of type 1 diabetes. The chaperone molecule, glucose-regulated proteins 78 (Grp78), is required to maintain ER function during toxic insults. In this study, we investigated the changes of Grp78 expression in different phases of streptozotocin (STZ)-affected beta-cells to explore the relationship between Grp78 and the response of beta-cells to ER stress. An insulinoma cell line (NIT-1) treated with STZ for different time periods and STZ-induced diabetic Balb/C mice at different time points were used as the model system. The level of Grp78 and C/EBP homologous protein (CHOP) mRNA were detected by real-time polymerase chain reaction and their protein by immunoblot. Apoptosis and necrosis was measured by flow cytometry. In addition, the changes of Grp78 protein in STZ-treated nondiabetic mice were also detected by immunoblot. Grp78 expression significantly increased in the early phase but decreased in the later phase of affected beta-cells, while CHOP was induced and apoptosis occurred along with the decrease of Grp78. Interestingly, the Grp78 protein of STZ-treated nondiabetic mice increased stably compared with that of the control. From the results, we can conclude that Grp78 may contribute to the response of beta-cells to ER stress, and more attention should be paid to Grp78 in the improvement of diabetes.

Keywords ER stress · Glucose-regulated proteins 78 · Streptozotocin · Diabetic mice · Beta-cells

Introduction

Type 1 diabetes results from autoimmune destruction of the insulin-producing pancreatic beta-cells and is characterized by hyperglycemia due to reduced insulin secretion. Apoptosis is the main mode of pancreatic beta-cell death in the development of diabetes (Maytin and Habener 1998; Mathis et al. 2001). Recent studies suggest that endoplasmic reticulum (ER) stress plays an important role in the loss of beta-cells (Oyadomari et al 2002a; Araki et al 2003; Laybutt et al 2007). Beta-cells have a highly developed and active ER, and early steps of insulin biosynthesis occur in the ER (Heller et al 1995; Eizirik and Mandrup 2001). Cells can regulate the capacity of their ER in protein folding and processing, and they can be tolerant to certain levels of imbalance between client protein loading and folding capacity. Disequilibrium between ER loading and folding capacity is referred to heuristically as ER stress, which triggers an evolutionarily conserved response-unfolded protein response (UPR). The initial intent of the UPR is to adapt to the changing environment and reestablish

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normal ER function (Kaufman 1999; Mori 2000). The ability to adapt to a physiological level of ER stress is important to cells, including professional secretory cells. This also holds true for the insulin-producing beta-cells, which process large amounts of ER client proteins (Heather and David 2002).

Glucose-regulated proteins 78 (Grp78), also referred to as immunoglobulin heavy chain binding protein, is one of the best-characterized ER chaperone proteins, and it has served as a classical marker for the UPR response. Grp78 functions as a master regulator of the UPR response by binding to and preventing the activation of all three proximal stress sensors: inositol requiring 1, PKR-like ER kinase, and activating transcription factor 6. Grp78 also binds transiently to the exposed hydrophobic residues of nascent folded proteins. These Grp78-mediated activities may lead to a reduction in the amount of newly synthesized protein translocated into the ER lumen, to reduce the load of client proteins the ER must process, to increase the translocation and degradation of ER-localized misfolded proteins, and to augment the protein folding capacity of the ER (Chunyan et al 2005; Gething 1999). When functions of the ER are severely impaired the UPR may lead to cell demise through the activation of programmed cell death signals (Ellgaard et al. 1999). In a word, the induction of Grp78 is required to alleviate ER stress, to maintain ER function, to facilitate protein folding, and to protect cells from apoptosis (Rao et al. 2004).

In the present experiment, we investigated Grp78 levels in different phases of affected beta-cells, about which limited information is available. We believe that to understand the relationship between Grp78 and the response of beta-cells to ER stress will guide rational therapeutic strategies for the improvement of diabetes.

Materials and methods

Streptozotocin induction of diabetes

Female Balb/c mice (8–10 weeks old, 20–22 g) obtained from the Institute of Organ Transplantation, Tongji Medical College, were used for the study. All of the studies were performed under the guidelines of Tongji Animal Use Regulations and with the approval of the Institutional Animal Care and Use Committee at Tongji Medical College. Diabetes was induced in Balb/c mice by multiple low doses of streptozotocin (STZ) treatment as reported (Holstad and Sandler 1999). Diabetes was induced by intraperitoneal injection of STZ (Sigma, St. Louis, MO, USA) with 40 mg/kg for five consecutive days and was monitored by measurement of blood glucose (BG) concentrations from the tail vein using a BG device (Roche Accu Check III). Mice with nonfasting BG >11.1 mmol/L for three consecutive days were considered to show onset of diabetes. The BG levels for diabetic mice were further monitored at least twice a week throughout the study. Fifteen of STZ-induced diabetic mice were divided into three test groups at different time points on the 1st, 7th, and 14th days respectively. Five citrate buffer-treated mice were used as controls.

Isolation of pancreatic islet

Pancreatic islets in mice of test, control, and STZ-treated nondiabetic groups were isolated by digestion with type V collagenase (2 mg/ml, Alexis Biochemicals, San Diego, CA, USA) and the Ficoll gradient centrifugation method.

NIT-1 cells treatment

NIT-1 cells (a kind gift from Dr. Thomas Kay, WEHI, Melbourne, Australia) belonging to an insulin-producing insulinoma cell line derived from nonobese diabetic mice prone to autoimmune diabetes (Hamaguchi et al. 1991) were used as a model cell system. These cells were expanded in 24-well tissue culture plates in Dulbecco's modified Eagle's medium (Sigma), containing STZ (8 mM) and 10% fetal calf serum (Gibco, Carlsbad, CA, USA). STZ-treated NIT-1 cells were harvested at different time points of 2, 6, and 24 h, respectively, for further studies. Citrate buffer-treated NIT-1 cells were used as control.

Real-time polymerase chain reaction

Total RNA of NIT-1 cells and islets were extracted with a QIAamp RNA kit (Qiagen, Valencia, CA, USA). Reverse transcription was carried out on 1 μg of RNA with RevertAidTM First Strand Synthesis Kit (Fermentas, Burlington, Canada). Equal amounts of cDNA were submitted to polymerase chain reaction (PCR) in the presence of SYBR green dye with the QuantiTect SYBR Green RT-PCR Kit (Qiagen) and the ABI PRISM 6700 real-time PCR detection machine (Fengling Biotechnology, Shanghai, China). The control groups described earlier were used as negative control. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control. PCR was performed by 40 cycles of 30 s at 94°C, 40 s at 55°C, and 30 s at 72°C. The specific primers used and their respective PCR fragment lengths were as follows: GAPDH forward, 5′-CTCCACTCACGGCA AAT TCAAC-3′, reverse, 5′-ACTCCACGACATACTCAGCACC-3′ (143 bp), C/EBP homologous protein (CHOP) forward, 5′-TCTTGACCCTGCGTCCCTAG-3′, reverse, 5′-TGGGCACTGACCACTCTGTTT-3′ (169 bp) and GRP78 forward, 5′-GAGGTGG GCAAACCAAGACATT - 3′, reverse, 5′-TCGCTGGGCATCATTGAAGTAAG-3′ (401 bp). The threshold cycle (Ct) reflects the point at which a sufficient

number of amplicons have accumulated to be statistically different from the baseline. ⊿Ct is the difference between the mean Ct values of the samples in the gene of interest wells and those of the internal standard gene. ⊿⊿Ct is the difference between the mean ⊿Ct values of the treated samples and the mean ⊿Ct values of the negative control sample. The mRNA levels of each sample were then compared using the equation $2^{-\Delta\Delta Ct}$. The expression level for samples from negative control group was arbitrarily assigned the value 1, and the final results were expressed as fold changes compared to samples from the control group.

Immunoblot

Total proteins of NIT-1 cells (1×10^6) and islets, described earlier, were extracted and electrophoresed in 10% SDS-

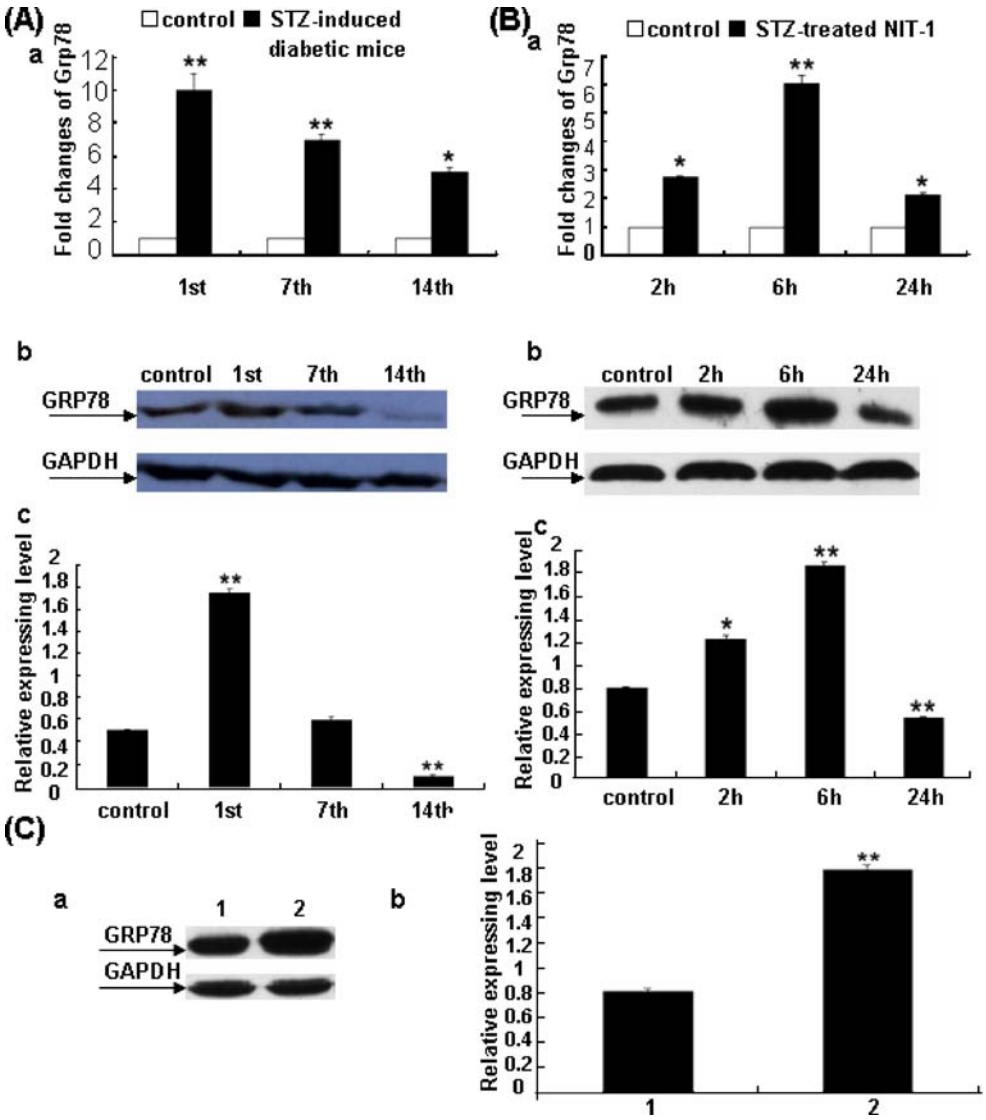

Fig. 1 Expression of Grp78 level in different phase of affected beta-cells STZ-treated NIT-1 cells were harvested at 2, 6, and 24 h, respectively. Islets of STZ-induced diabetic and nondiabetic mice were also isolated at different time points. NIT-1 cells or mice treated by citrate buffer were used as control. Total RNA and protein were extracted and used to study the Grp78 level. **A** Expression of Grp78 in different phase of STZ-induced diabetic mice. *a* Level of Grp78 mRNA detected by real-time PCR. *b* Expression of Grp78 protein detected by immunoblot. A representative blot is shown. *c* The *bar graph* represents the densitometric analysis of the bands. **B** Expression of Grp78 in different phase of STZ-treated NIT-1 cells. *a* Level of Grp78 mRNA detected by real-time PCR. *b* Expression of Grp78 protein detected by immunoblot. A representative blot is shown. *c* The *bar graph* represents the densitometric analysis of the bands. **C** Expression of Grp78 protein in STZ-treated nondiabetic mice. *a* Expression of Grp78 detected by immunoblot. A representative blot is shown. *b* The *bar graph* represents the densitometric analysis of the bands. *Lane 1* represents control and *lane 2* represents STZ-treated nondiabetic mice. $N=4$. $^*P<0.05$, $^{**}P<0.01$ vs control

PAGE and transferred to nitrocellulose membrane using semidry transfer (BioRad, Hercules, CA, USA). Membranes were blocked, incubated with antibody to GRP78 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), GAPDH (Santa Cruz Biotechnology), and CHOP (Santa Cruz Biotechnology). Membranes were then incubated to horseradish peroxidase-conjugated goat anti-rabbit IgG antibody (Amersham, Buckinghamshire, UK). The peroxidase reaction was visualized using an enhanced chemiluminescent substrate (Santa Cruz Biotechnology). Following activation of the chemiluminescent probe, membranes were exposed to film. The molecular sizes of bands were determined by comparison to prestained standards (BioRad).

Flow cytometry

STZ-treated NIT-1 cells were harvested at three time points of 2, 6, and 24 h, respectively, and citrate-buffer-treated NIT-1 cells were used as control. Apoptosis and necrosis were assessed by flow cytometry using Annexin-V-FITC and propidium iodide apoptosis detection kit (Baosai Bio, Beijing, China).

Data quantitation and statistical analysis

Analysis of variance was performed to compare means among the individual groups analyzed. Results were presented as mean ± standard error. P value<0.05 was considered statistically significant.

Results

Expression of Grp78 in different phase of affected beta-cells

As shown in Fig. 1, Grp78 mRNA significantly increased in all test groups compared with control (P<0.05, P<0.01), reaching its highest point first (P<0.01) in diabetic mice (Fig. 1A-a) and at 6 h (P<0.01) in STZ-treated NIT-1 cells (Fig. 1B-a), then decreased. The protein expression of Grp78

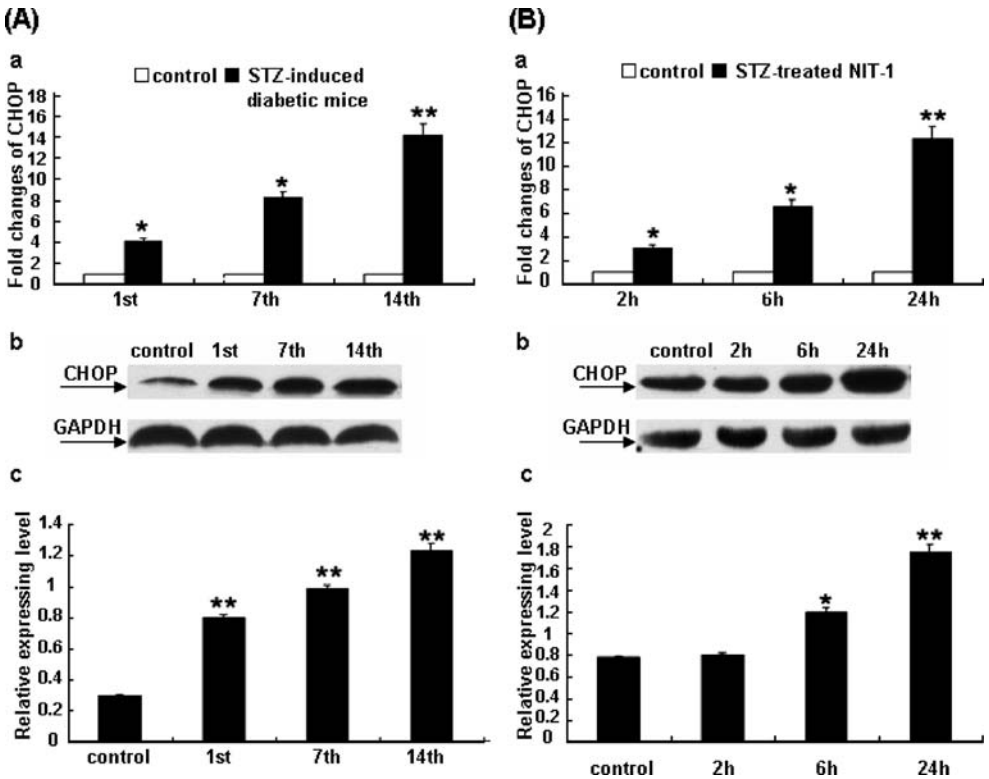

Fig. 2 Expression of CHOP level in different phase of affected beta-cells STZ-treated NIT-1 cells were harvested at 2, 6, and 24 h, respectively. Islets of STZ-induced diabetic and nondiabetic mice were also isolated at different time points. NIT-1 cells or mice treated by citrate buffer were used as control. Total RNA and protein were extracted and used to study the CHOP level. **A** Expression of CHOP in different phases of STZ-induced diabetic mice. *a* Level of CHOP mRNA detected by real-time PCR. *b* Expression of CHOP protein detected by immunoblot. A representative blot is shown. *c* The *bar graph* represents the densitometric analysis of the bands. **B** Expression of CHOP in different phases of STZ-treated NIT-1 cells. *a* Level of CHOP mRNA detected by real-time PCR. *b* Expression of CHOP protein detected by immunoblot. A representative blot is shown. *c* The *bar graph* represents the densitometric analysis of the bands. No significant changes of CHOP level were observed in STZ-treated but nondiabetic mice compared with control, data not shown. N=4. *P< 0.05, ** P<0.01 vs control

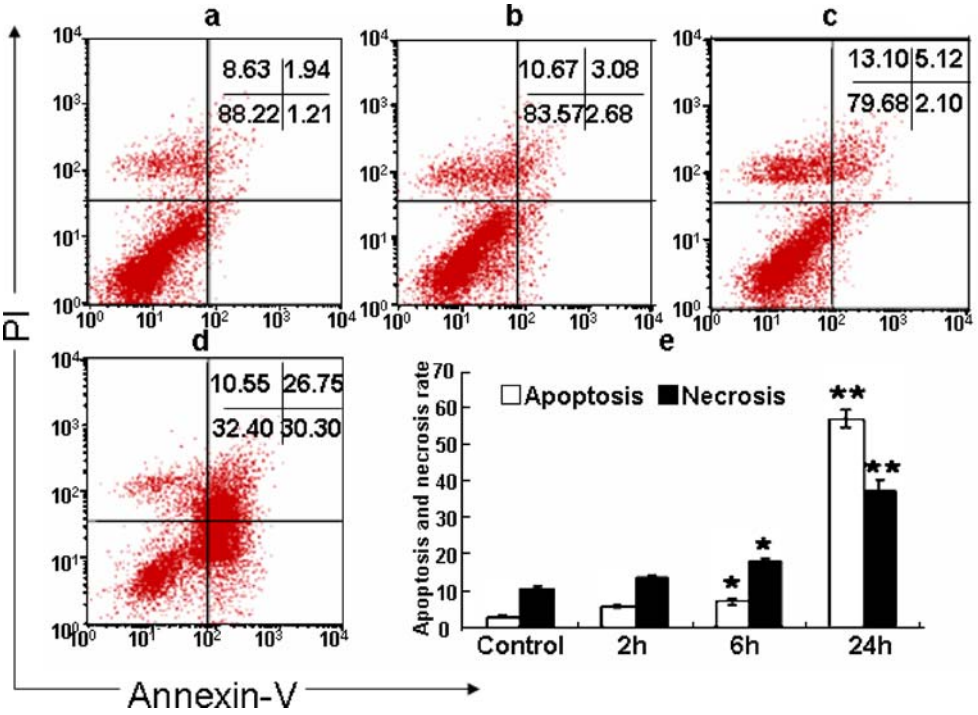

Fig. 3 Apoptosis and necrosis in NIT-1 cells treated by STZ at different time points STZ-treated NIT-1 cells were harvested at 2, 6, and 24 h, respectively; citrate-buffer treated NIT-1 cells were used as control. Apoptosis and necrosis were assessed by flow cytometry. **a** Control. **b** Two hours. **c** Six hours. **d** Twenty four hours. **e** The *bar graph* represents the statistic analysis. $N=6$. *$P<0.05$, ** $P<0.01$ vs control

did not cohere sufficiently with its mRNA level. Immunoblot results showed that Grp78 protein level significantly increased only on first-day groups ($P<0.01$) in diabetic mice (Fig. 1A-b,c) and on 2- and 6-h groups ($P<0.05$, $P<0.01$) in STZ-treated NIT-1 cells (Fig. 1B-b,c) compared with control, then sharply decreased. In addition, we found a stable increase of Grp78 level in STZ-treated but nondiabetic mice compared with control ($P<0.01$, Fig. 1C-a,b).

Expression of CHOP in different phases of affected beta-cell

Figure 2 shows that the CHOP mRNA levels of all test groups continually increased compared with control ($P<0.05$, $P<0.01$, Fig. 2A-a, B-a). The protein level of CHOP was in accordance with its mRNA level (Fig. 2A-b,c, B-b, c). No significant changes of CHOP level were observed in STZ-treated but nondiabetic mice compared with control (data not shown).

Apoptosis in NIT-1 cells treated by STZ at different time points

The results of flow cytometry showed that apoptosis and necrosis significantly increased in 6- and 24-h groups compared with control ($P<0.05$, $P<0.01$, Fig. 3).

Discussion

In our Introduction, we mentioned the relationship between ER stress and diabetes and the role of Grp78 in maintaining stable function of ER and in protecting cells from apoptosis. The expression level of Grp78 in diabetes has been incompletely reported earlier. Grp78 protein level significantly decreased in liver of Zucker rats (spontaneously diabetic rats), but no significant change was detected in its mRNA level compared with normal rats (Szanto et al. 1995). Parfett et al. reported an elevated expression of Grp78 mRNA in liver and brain only of nonobese diabetic mice, but the activation of Grp78 mRNA appeared to be transient (Parfett et al. 1990). We studied the changes of Grp78 expression in different phases of STZ-induced diabetic mice. The results revealed that the Grp78 level increased significantly in the earlier phase of diabetic mice, which indicated ER stress and UPR stimulation. Under conditions of prolonged ER stress, the level of Grp78 decreased. Grp78 protein decreased more than its mRNA, which implied the translational rate of Grp78 mRNA might be down-regulated. In addition, we also found a stable increase in Grp78 levels in STZ-treated nondiabetic mice compared with control. This intriguing result suggested that there exists a close relationship between Grp78 and diabetes, as has been reported by other groups. Ahmed et al. analyzed human pancreatic islets by two-dimensional gel electrophoresis and mass spectrometry and found that altered expression of Grp78 was associated with the development of diabetes (Ahmed et al. 2005). Could the decrease in Grp78 expression be a result of fewer beta cells in the diabetic mouse islets? For this purpose, an insulinoma cell line (NIT-1), which was treated with STZ at different time points, was used. These data confirm the results from diabetic mice. A comparable result was

reported by another group. Nozaki et al. reported that Grp78 was significantly increased in $Ins^{2+/Akita}$ cells (derived from the Akita diabetic mouse, which carries a C96Y mutation in the insulin genes) compared with wild-type ($Ins^{2+/+}$) cells (Nozaki et al. 2004). We found that Grp78 could protect NIT-1 cells from death induced by STZ, cytokines, or cytotoxic T lymphocytes. Additionally, Grp78 might exert an immunosuppressive and protective ability resulting in improvement of alloimmunity in beta-cells transplantation (Wang et al. 2007a,b). These combined results suggest that there was a close relationship between Grp78 and the response of beta-cells to ER stress.

One of the modulators of ER stress-induced cell death is CHOP, a transcription factor induced during ER stress (Harding et al. 2002). CHOP is a 29-kDa protein with 169 (human) or 168 (rodents) amino-acid residues and is first identified as a member of the CCAAT/enhancer binding proteins (C/EBPs). C/EBPs form a family of transcription factors that regulate a variety of genes involved in a broad range of physiological processes, including immune functions and cell differentiation and proliferation. CHOP has a dual role both as an inhibitor of C/EBPs function and as an activator of other genes (Oyadomari and Mori 2004). The CHOP level at different time points was measured by real-time PCR and immunoblot. With the decrease of Grp78, CHOP is continually induced and apoptosis occurs. Meanwhile, the identification of the downstream target genes that respond to CHOP is still unclear. CHOP−/− mice exhibit reduced apoptosis in response to ER stress. Over-expression of Grp78 attenuates the induction of CHOP in ER stress and reduces ER stress-induced apoptosis (Oyadomari et al. 2002b). Taken together, the results suggested that CHOP, ubiquitously expressed at a very low level, is robustly expressed by perturbations that induce stress in a wide variety of cells and plays an important role in ER stress-induced beta-cell apoptosis. In this current study, the results suggested that Grp78 was associated with the response of beta-cells to ER stress and may be important in the improvement of diabetes.

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