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Glucose regulated proteins 78 protects insulinoma cells (NIT-1) from death induced by streptozotocin, cytokines or cytotoxic T lymphocytes

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Abstract

Endoplasmic reticulum stress-mediated apoptosis plays an important role in the destruction of pancreatic beta-cell, 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 effect of GRP78 on the beta-cell apoptosis. We first measured GRP78 protein expression in different phase of streptozotocin-affected beta-cell by immunoblotting analysis. An insulinoma cell line, NIT-1, transfected with GRP78 was established, named NIT-GRP78, and used to study apoptosis, which was induced by streptozotocin or inflammatory cytokines. Apoptosis of NIT-1 or NIT-GRP78 cells was detected by flow cytometry, the transcription of C/EBP homologous protein (CHOP) was monitored by real-time PCR, the concentration of nitric oxide and the activity of superoxide dismutase were measured by colorimetric method. We found that, in comparison to NIT-1 cells, NIT-GRP78 cells responded to the streptozotocin or cytokines treatments with decreased concentration of nitric oxide, but increased activity of superoxide dismutase. In addition, the level of CHOP was also decreased in the NIT-GRP78 cells, which may mediate the resistance of the GRP78 overexpressed NIT-1 cells from apoptosis. Finally, we found that NIT-GRP78 cells were also more resistant than NIT-1 cells to cytotoxic T lymphocyte (CTL) specific killing detected by flow cytometry through target cells expressing green fluorescent protein cultured with effector cells and finally stained with propidium iodide. The data suggest that modulating GRP78 expression could be useful in preventing pancreatic beta-cell from the immunological destruction in type 1 diabetes individuals.
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Keywords: Beta-cell apoptosis; Glucose regulated proteins 78; Streptozotocin; Cytokine; Cytotoxic T lymphocyte

1. Introduction

Type 1 diabetes (T1D) results from autoimmune destruction of the insulin-producing pancreatic beta-cell, and is characterized by hyperglycemia due to reduced insulin secretion. Apoptosis is the main mode

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of pancreatic beta-cell death in the development of diabetes (Maytin & Habener, 1998; Mathis, Vence, & Benoist, 2001). Recent studies suggest that endoplasmic reticulum stress (ER stress) plays an important role in the loss of beta-cells (Araki, Oyadomari, & Mori, 2003; Laybutt et al., 2007; Oyadomari, Araki, & Mori, 2002a). Beta-cells have a highly developed and active ER, and early steps of insulin biosynthesis occur in the ER (Eizirik & Mandrup, 2001; Heller et al., 1995). 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 re-establish 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 & David, 2002).

Glucose regulated proteins 78 (GRP78), also referred to as immunoglobulin heavy chain binding protein (BIP), is one of the best-characterized ER chaperone proteins, and it has served as a classical marker for the UPR response (Gething, 1999). 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 (IRE1), PKR-like ER kinase (PERK) and activating transcription factor 6 (ATF6). 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, so that to reduce the load of client proteins the ER must process, to increase the translocation and degradation of ER-localized misfolded proteins, as well as to augment the protein folding capacity of the ER (Chunyan, Beatrice, & John, 2005). When functions of the ER are severely impaired the UPR may lead to cell demise through the activation of programmed cell death signals (Ellgaard, Molinari, & Helenius, 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, Ellerby, & Bredesen, 2004).

In this present experiments, we investigated the cytoprotective activity of GRP78 in pancreatic beta-cell, of which limited information is available. We believe that to understand the ER stress-induced beta-cell apoptosis will guide rational therapeutic strategies for the improvement of diabetes.

2. Materials and methods

2.1. Plasmids

The pIRES2-GRP78 plasmid expressing the green fluorescent protein (GFP) was kindly provided by Dr. Congyi Wang (Department of Pathology, Medical College of Georgia, USA). DNA fragment for GRP78 were amplified from mouse cDNA and then subcloned into the pIRES2 vector in frame with GFP using the BamH1 cutting sites.

2.2. Establishment of GRP78-GFP stable transfectants in NIT-1 cells

NIT-1 cells (a kind gift from Dr. Thomas Kay, WEHI, Melbourne, Australia) belonging to an insulin-producing insulinoma cell line derived from non-obese diabetic (NOD) mice prone to autoimmune diabetes (Hamaguchi, Gaskins, & Leiter, 1991) were used as a cell model system. These cells were expanded in 24-well tissue culture plates in dulbecco's modified eagale's medium (DMEM, Sigma) supplemented with 10% fetal calf serum (Gibco). pGRP78-GFP and pGFP (empty vector) were transfected into NIT-1 cells by the Lipofectamine™ 2000 transfection reagent (Invitrogen), respectively. After 72 h transfection, G418(600 μg/ml, Sigma) was added into the cultures for the establishment of stable transfectants. Stable individual clones were selected by limited dilute and maintained with a low dose of G418 (300 μg/ml), then were mixed together to expand to stable multiple clones. Stable transfectants were confirmed by fluorescence microscopy and flow cytometry of GFP expression. Stable transfectants with GFP only (pGFP) were used as a control.

2.3. Immunoblotting analysis

To investigate how the expression of GRP78 varies in different phase of affected beta-cells, NIT-1 cells (1×10^6) were passaged on 6-well tissue culture plates full of DMEM (2 ml per well), supplemented with 10% fetal calf serum, containing STZ (8 mM). STZ-treated NIT-1 cells were harvested on 2 h, 6 h, 24 h for detection of GRP78 protein expression; citrate buffer-treated NIT-1 cells were used as control group simultaneously. Total cellular proteins were electrophoresed in 10% SDS-PAGE and transferred to nitrocellulose membrane using semi-dry transfer (BioRad). Membranes were

blocked, incubated with antibody to GRP78 (Santa Cruz Biotechnology) and glyceraldehyde3-phosphate dehydrogenase (GAPDH, Santa Cruz Biotechnology). Then membranes were incubated to horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG antibody (Amersham). 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).

2.4. Flow cytometry

The percentages of apoptotic and necrotic cells were determined by flow cytometry combined application of Annexin V-PE.Cy5 (MBL international) and propidium iodide (PI, Jingmei Biotech Co., Ltd.). For this purpose, the NIT-1, NIT-GFP and NIT-GRP78 cells were treated with combination of recombinant mouse IFN-gamma (100 U/ml, Beyotime Company, China) and IL-1beta (50 U/ml, Beyotime Company, China) for 36 h or STZ (8 mM) for 24 h, then were suspended in the 500 μl of 1× binding buffer at a concentration of approximately 1×10^6 cells/ml. Samples were incubated with 5 μl Annexin V-PE.Cy5 and 5 μl PI for 5 min at room temperature, then were measured by FACScan flow cytometer (Becton Dickinson). Data were analyzed by CellQuest software (Becton Dickinson).

2.5. Real-time PCR

Total RNA of the NIT-1, NIT-GFP and NIT-GRP78 cells, treated with STZ or cytokines, were extracted with a QIAamp RNA kit (Qiagen). Reverse transcription was carried out on 1 μg of RNA with RevertAidTM First Strand Synthesis Kit (Fermentas). Equal amounts of cDNA were submitted to 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 Inc.). The GAPDH was used as an internal control. Citrate buffer-treated (in STZ-treated samples) or non-treated (in cytokines-treated samples) cells were used as negative 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′-CTCCACTCACGGCAAATTCAAC-3′; reverse, 5′-ACTCCACGACATACTCAGCACC-3′ (143 bp) and C/EBP homologous protein (CHOP) forward, 5′-TCTTGACCCTGCGTCCCTAG-3′; reverse, 5′-TGGGCACTGACCACTCTGTTT-3′ (169 bp). The threshold cycle (Ct) reflects the point at which a sufficient number of amplicons have accumulated to be statistically different from the baseline. ΔCt is the difference between the mean Ct values of the samples in the gene of interest wells and those of the internal standard gene. ΔΔCt is the difference between the mean ΔCt values of the treated samples and the mean ΔCt 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 sample from negative control group was arbitrarily assigned value 1 and the final results were expressed as fold changes compared to sample from control group.

2.6. Measurement of nitric oxide (NO) concentration and superoxide dismutase (SOD) activity

The supernatant fluid of NIT-1, NIT-GFP and NIT-GRP78 cells was harvested and treated by STZ (24 h) or cytokines (36 h), then the concentrations of NO were measured with the use of the Griess reagent using total nitric oxide assay kit (Beyotime Company, China), and the SOD activities were measured by colorimetric mothod using SOD assay kit (Jiancheng Bio. Co. Ltd. China).

2.7. In vitro cytotoxic T lymphocyte (CTL) assay

After Balb/C mice were immunized twice with NIT-1 cells (5×10^6 cells per mouse) by abdominal cavity injection, spleen cells were collected and stimulated with 10% NIT-1 cells pre-treated with mitomycin C (30 μg/ml, Alexis Biochemicals) for 7 days, used as effector cells. NIT-1, NIT-GFP and NIT-GRP78 cells were used as target cells. Assessment of viability of target cells and effector cells were performed using trypan blue (0.2%). The in vitro CTL assay was performed as described earlier, with some modifications (Betts et al., 2003; Karina et al., 2005; Lecoeur, Fevrier, Garcia, Rivere, & Gougeon, 2001). Target cells (1×10^5 ml in 100 μl) expressing GFP were cultured with effector cells (1×10^6 ml in 100 μl) for 4 h, and finally stained with PI. Determination of cytolysis is based on the numeration of viable target cells (VTC), which are characterized by GFP-positivity and PI-negativity (GFP^+PI^-), using the following equation: cytolysis (%) = $[(VTC_{control\ sample} - VTC_{test\ sample})/VTC_{control\ sample}]$. CTLs-mediated cell lysis was analyzed using a Becton-Dickinson FACScan flow cytometer. Target cells

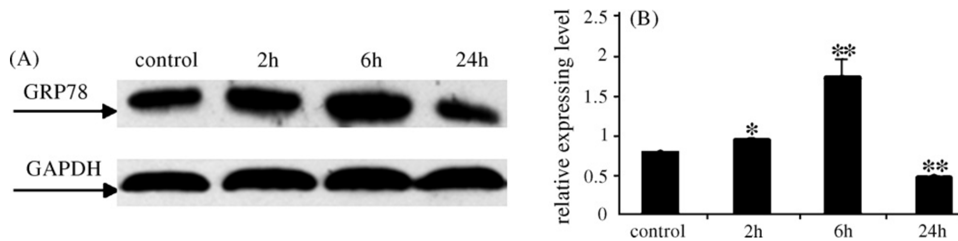


Fig. 1. The GRP78 protein expression in different phase of STZ-treated NIT-1 cells. STZ-treated NIT-1 cells were harvested in 2 h, 6 h and 24 h for detection of GRP78 protein expression by immunoblotting assay; citrate buffer-treated NIT-1 cells were used as control group. The GAPDH was used to ensure equal loading of the sample. A representative blot is shown. The bar graph represents the densitometric analysis of the bands. $N = 6$. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. control.

cultured with non-treated spleen cells were used as control sample.

2.8. Data analysis

All of the data were analyzed by Student's *t*-test for unpaired or paired data as appropriate. Results were present as mean ± standard error (SE). *P*-value < 0.05 was considered to have statistical significant differences.

3. Results

3.1. Expression of GRP78 in STZ-affected NIT-1 cells

The results of Western blot displayed that the expression of GRP78 protein significantly increased in 2 h and 6 h groups ($P < 0.05$, $P < 0.01$), reaching its highest point in 6 h in NIT-1 cells after STZ treatment, then sharply decreased in 24 h group ($P < 0.01$) compared with control (Fig. 1).

3.2. GRP78 prevents NIT-1 cells from STZ or cytokines induced apoptosis

NIT-1, NIT-GFP and NIT-GRP78 cells were treated by STZ or cytokines, then apoptosis, necrosis and CHOP mRNA were detected. As shown in Fig. 2A, NIT-GRP78 cells show lower level of CHOP mRNA in comparison to NIT-1 and NIT-GFP cells ($P < 0.05$), while there was no significant difference between NIT-1 and NIT-GFP cells ($P > 0.05$). Consistent with the observation of CHOP mRNA, NIT-1 cells and NIT-GFP cells showed similar levels of apoptosis and necrosis (data not shown). While NIT-GRP78 cells showed significantly lower apoptosis ($P < 0.01$) and necrosis ($P < 0.05$) as compared to NIT-GFP cells (Fig. 2B and C). Taken together, GRP78 might prevent apoptosis of NIT-1 cells induced by STZ or cytokines through down regulating CHOP mRNA level.

3.3. Secretions of NO and activities of SOD in NIT-1, NIT-GFP and NIT-GRP78 cells treated with STZ or cytokines

After treatment with STZ or cytokines, NIT-GRP78 cells showed the lowest concentration of NO ($P < 0.05$, Fig. 3A) compared with NIT-1 and NIT-GFP cells. The highest activity of SOD ($P < 0.05$, Fig. 3B) was shown in NIT-GRP78 cells treated by STZ. While no significant difference of SOD activity was observed in samples treated by cytokines (data not shown).

3.4. GRP78 attenuates CTLs-mediated NIT-1 cells lysis

As shown in Fig. 4, NIT-GRP78 cells displayed the lowest susceptibility (25%, $P < 0.05$) to T cell-mediated cytotoxicity as compared to the NIT-GFP cells (41%). We failed to detect the significant difference between NIT-1 and NIT-GFP groups (data not shown).

4. Discussion

NIT-1 cells are a NOD-derived beta-cell line that possesses the characteristics of primary beta-cell such as the capability for secretion of insulin (Robbins, Maksumova, Pocock, & Chantler, 2003). Therefore, NIT-1 cells are a well validated cell model for the study of T1D. We have mentioned the relationship between ER stress and diabetes, and the role of GRP78 in maintaining stable function of ER, in protecting cell from apoptosis in Section 1 earlier. Nozaki et al. reported that GRP78 was significantly increased in $Ins^{2+/Akita}$ cells (derived from Akita diabetic mouse, carries a C96Y mutation in the insulin genes) compared with wild-type ($Ins^{2+/+}$) cells (Nozaki et al., 2004). We investigated the expression of GRP78 in different phase of STZ-treated NIT-1 cells. The results (Fig. 1) displayed that the expression of GRP78 protein significantly increased,

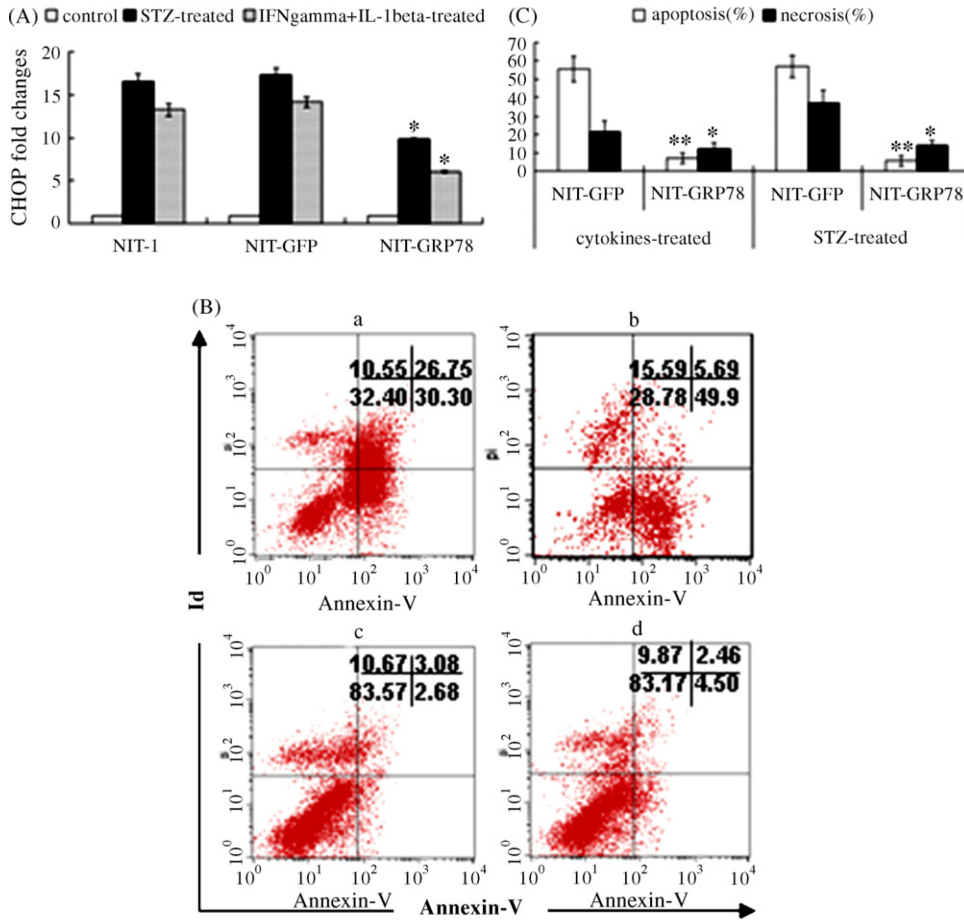


Fig. 2. Effects of GRP78 on apoptosis of NIT-1 cells induced by STZ or cytokines. NIT-1, NIT-GFP and NIT-GRP78 cells were treated with combination of recombinant mouse IFN-gamma (100 U/ml) and IL-1beta (50 U/ml) for 36 h or STZ (8 mM) for 24 h. Panel (A): after treatment, total RNA were extracted and used to investigate the level of CHOP mRNA by real-time PCR. Panel (B): apoptosis and necrosis were detected using Annexin V-PE.Cy5 and PI double staining method by flow cytometer. (a) NIT-GFP cells + STZ; (b) NIT-GFP cells + combination of IL-1 beta and IFN-gamma; (c) NIT-GRP78 cells + STZ; (d) NIT-GRP78 cells + combination of IL-1beta and IFN-gamma. Panel (C): The bar graph represents the statistic analysis of panel (B). $^{*}P < 0.05$, $^{**}P < 0.01$ NIT-GRP78 vs. NIT-GFP cells. There has no significant difference between NIT-1 and NIT-GFP cells, data not shown. $N = 6$.

in the early phase of affected NIT-1 cells, which indicated ER stress and UPR happening. Under conditions of prolonged ER stress, the level of GRP78 protein decreased. Taken together we consider that GRP78 has a close relationship with the response of beta-cell to ER stress.

Macrophages and $CD4^{+}$ T cells can secrete soluble mediators such as oxygen free radicals, NO, and

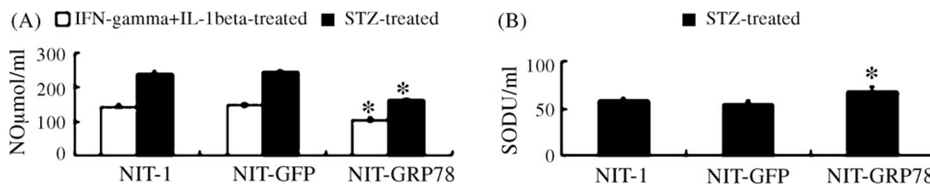
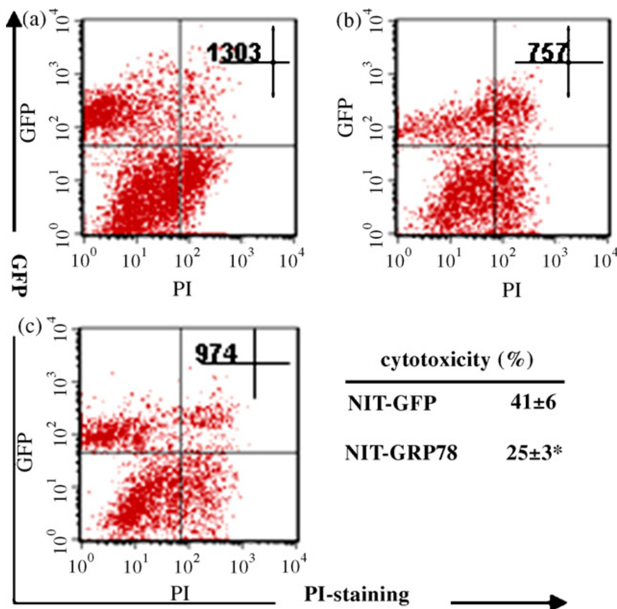


Fig. 3. Comparison of the secretions of NO and the activities of SOD in NIT-1, NIT-GFP and NIT-GRP78 cells after treatment with STZ or cytokine. NIT-1, NIT-GFP and NIT-GRP78 cells were treated with combination of recombinant mouse IFN-gamma (100 U/ml) and IL-1beta (50 U/ml) for 36 h or STZ (8 mM) for 24 h. Panel (A): after treatment with STZ or cytokines, concentrations of NO in NIT-1, NIT-GFP and NIT-GRP78 cells were measured. Panel (B): after treatment with STZ, activities of SOD in NIT-1, NIT-GFP and NIT-GRP78 cells were measured. $^{*}P < 0.05$ NIT-GRP78 vs. NIT-1 and NIT-GFP cells. $N = 8$.



cytotoxicity (%)	
NIT-GFP	41±6
NIT-GRP78	25±3*

Fig. 4. Cytolysis mediated by CTL in NIT-1, NIT-GFP and NIT-GRP78 cells. Target cells expressing GFP were cultured with effector cells for 4 h at ratio 10:1, and finally stained with PI. Determination of cytolysis is based on the numeration of viable target cells (VTC), which characterized by GFP-positivity and PI-negativity ($GFP^{+}PI^{-}$), using the following equation: cytolysis (%) = $[(VTC_{control\ sample} - VTC_{test\ sample})/VTC_{control\ sample}]$. Target cell cultures with non-treated spleen cells were used as control. (a) Control sample; (b) NIT-GFP + allo-reactive CTL. The representative sample shown here demonstrates $[(1303 - 757)/1303] \times 100\% = 41.9\%$ specific cytotoxicity. (c) NIT-GRP78 + allo-reactive CTL. The representative sample shown here demonstrates $[(1303 - 974)/1303] \times 100\% = 25.2\%$ specific cytotoxicity. The table shows the statistic analysis of cytolysis mediated by CTL in NIT-GFP and NIT-GRP78 cells. There is no significant difference between NIT-GFP vs. NIT-1 cells (data not shown). $N = 6$. $^{*}P < 0.05$ NIT-GRP78 vs. NIT-GFP cells.

cytokines. Apoptosis induced by these mediators is the main mode of beta-cell death in the development of T1D (Ilham et al., 2004). CTL is also involved in apoptosis of beta-cell through perforin/granzyme or Fas/FasL pathway. In this study, we demonstrated that GRP78 can attenuate CTL-mediated NIT-1 cell lysis and prevent NIT-1 cell from STZ, cytokines mediated apoptosis and necrosis. CHOP is a 29 kDa protein with 169 (human) or 168 (rodents) amino-acid residues, and is first identified to be a member of the CCAAT/enhancer binding proteins (C/EBPs). C/EBPs form a family of transcription factors that regulates a variety of genes involved in a broad range of physiological processes, including immune functions as well as 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 & Mori, 2004). Our results suggested that overexpression of CHOP was induced by STZ or cytokines (IL-1beta plus IFN-gamma) in NIT-1 cells, and GRP78 could down-regulate the CHOP mRNA level (Fig. 2A), prevent NIT-1 cells from apoptosis (Fig. 2B). CHOP−/− mice exhibit reduced apoptosis in response to ER stress. Overexpression of GRP78 attenuates the induction of CHOP in ER stress and reduces ER stress-induced apoptosis (Oyadomari, Koizumi & Takeda, 2002b). Taken together, the results suggested that CHOP, ubiquitously expressed at 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 apoptosis. GRP78 could prevent beta-cell from apoptosis through down-regulating CHOP level.

STZ and inflammatory cytokines, such as IL-1beta, TNF alfa, and IFN-gamma can induce the production of NO, which is an important mediator of beta-cell failure in T1D. Oxygen free radical, also a mediator of beta-cell death, can be catalyzed by SOD. So SOD can protect oxygen-metabolizing cells against harmful effects of superoxide free-radicals, and exogenous SOD might have an anti-inflammatory ability. In this experiment, after cells were treated with STZ or cytokines, NIT-GRP78 group showed decreased secretions of NO, while increased activities of SOD compared with NIT-1 and NIT-GFP group (Fig. 3). The results suggest that GRP78 might reduce the production of NO to improve the destruction mediated by it, but increase the activities of SOD, which can clear superoxide free-radicals, to increase NIT-1 cells viability. However, the mechanism underlying this phenomenon is unclear. Bodman-Smith et al. (Bodman-Smith, Corrigall, Kemeny, & Panayi, 2003) reported that antibodies to GRP78 could be found in the serum of RA patients and of mice with collagen- or pristane-induced arthritis. And they cloned T cells from individuals whose T cells proliferated in response to GRP78 and to investigate their function, they found that GRP78 was an antigen that might specifically stimulate CD8 cells with the ability to produce large amounts of IL-10. This IL-10 production may be part of a mechanism to down-modulate an immune response. These intriguing reports give us a direction to explore the mechanism of GRP78 protective ability on beta-cell further.

In the current studies, we demonstrated the capability of GRP78 in prevention of STZ- or cytokines-induced beta-cell apoptosis and CTLs-mediated lysis. Several groups have also reported that GRP78 was associated with diabetes. 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, Forsberg, & Bergsten, 2005). Laybutt et al.

found that attenuation of ER stress by overproduction of GRP78 in a beta-cell line, MIN6 cells, significantly protected the cells from lipid-induced apoptosis (Laybutt et al., 2007). In addition, other studies suggested that GRP78 may also protect the host cells from cell death by suppressing oxyradical accumulation so as to stablize mitochondrial function (Gething, 1999). The results together suggested that modulating GRP78 expression could be useful in preventing pancreatic beta-cell from the immunological destruction in type 1 diabetes individuals.

Acknowledgements

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