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Role of ERp46 in β-cell lipoapoptosis through endoplasmic reticulum stress pathway as well as the protective effect of exendin-4

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ABSTRACT

Endoplasmic reticulum (ER) stress is considered as a key factor in free fatty acid (FFA)-induced apoptosis. ERp46, a new member of the thioredoxin family, is highly expressed in pancreatic β-cells and plays an important role in glucose toxicity. In this study we examined the potential role of ERp46 in palmitic acid (PA)-induced cell apoptosis and the protective role of exendin-4, a long-acting agonist of the hormone glucagon-like peptide-1 (GLP-1) receptor. The glucose-sensitive mouse β-pancreatic cell line, βTC6, was used to investigate the mechanisms of PA-induced apoptosis. Our results showed that ERp46 expression was reduced in a dose- and time-dependent manner after PA treatment. Furthermore, inhibition of ERp46 expression by small interfering (si)RNA-mediated silencing enhanced the ER stress response via three separate pathways and increased βTC6 cell apoptosis rates. Moreover, exendin-4 reduced the ER stress response and levels of apoptosis in NC transfected cells after PA treatment, but not in cells transfected with ERp46siRNA. In conclusion, ERp46 plays a protective role in PA-induced cell apoptosis by decreasing the ER stress response and might be a novel target for anti-diabetic drugs. Exendin-4 might protect against βTC6 cell lipoapoptosis in part by activating ERp46 signaling pathway.

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1. Introduction

Accumulating evidence indicates that chronic exposure to a high concentration of free fatty acids (FFAs) is a principal cause of β-cell apoptosis through a process referred to as lipotoxicity [1–3]. The saturated fatty acid, such as palmitic acid (PA) has been shown to impair glucose-stimulated insulin release [3–5]. There are a number of mechanisms that contribute to the effect of FFAs on β-cells, including endoplasmic reticulum (ER) stress, which leads to insulin resistance and β-cell failure and apoptosis [6–10]. However, the exact molecular events in this process are not well understood.

Destruction of ER homeostasis can lead to an accumulation of unfolded and misfolded proteins, termed ER stress, which activates the unfolded protein response (UPR) [11]. Persistent activation of the UPR may trigger the pro-apoptosis pathway. There are three main proapoptotic proteins: C/EBP homologous protein transcription factor (CHOP), c-Jun NH2-terminal kinase (JNK), and Caspase12 [12–14].

Endoplasmic reticulum protein 46 (ERp46), also called endothelial protein-disulfide isomerase (EndoPDI), thioredoxin domain containing protein 5 precursor (TXNDC5) and plasma cell thioredoxin-related protein (PC-TRP), was first identified via three independent proteomic approaches [15–17]. ERp46 is expressed in various tissues and is highly expressed in liver, endothelial tissues and plasma. Under hypoxic conditions, ERp46 expression was up-regulated and could have a protective effect on endothelial cell survival by upregulating the antiapoptosis factor CD105 [16]. Recent work has found that ERp46 was highly expressed in pancreatic islets compared with other exocrine tissue and it was associated with pancreatic β-cell glucose toxicity [18]. However, the role of ERp46 in pancreatic β-cell lipoapoptosis has not yet been described.

Increasing evidence suggests that the incretin hormone glucagon-like peptide-1 (GLP-1) not only acts directly on β-cells to upregulate insulin biosynthesis, but also enhances β-cell resistance to apoptosis [19–21]. Exendin-4, a GLP-1 receptor agonist, is a new anti-diabetes drug. To explore the possible mechanism of lipotoxicity in β-cells, in this study we sought to determine whether ERp46 is involved in PA-mediated β-cell lipoapoptosis and to clarify the protective role of exendin-4.

2. Methods

2.1. Cell culture

The mouse insulinoma β-cell line βTC6 (American Type Culture Collection) was used in all experiments. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) medium containing

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5.6 mM glucose, 4.0 mM L-glutamine and supplemented with 10% fetal bovine serum (Gibco) at 37 °C and 5% CO_2, and then were subcultured at 70–80% confluence.

2.2. PAor exendin-4 treatment

A stock solution of 50 mM PA (Sigma) was prepared in 90% ethanol by heating to 70 °C. The fluid was bound to 10% bovine serum albumin by incubating at 55 °C until it was completely dissolved. The mixture was added to DMEM with 1% fetal bovine serum at the indicated concentrations. The culture medium contained 0.5% bovine serum albumin and 1% fetal bovine serum and was changed the day before. Exendin-4 (Sigma) was dissolved in 1 ml ddH_2O to a concentration of 1 g/l. The mixture was diluted in DMEM medium with 10% fetal bovine serum before treating the cells. The medium was changed the day before.

2.3. Real-time PCR

Total RNA was isolated from βTC6 cells using TRIZOL Reagent (Invitrogen) and reverse transcribed to cDNA after DNase treatment. The Real-time PCR amplification reaction system (TAKARA) was performed in a final volume of 25 μl containing 0.5 μM forward and reverse primers, 12.5 μl of SYBR Green PCR master mix, 0.4 μl Taq DNA polymerase and 2 μl cDNA template. The expression level of the gene of interest was normalized to β-actin expression levels. The specific primers (Invitrogen) used and their respective PCR fragment lengths were as follows: ERp46 (forward GGATGCCAAGGTCTACGTG, reverse CGGAGCGAAGAACTTGATA, 316 bp); β-actin (forward GCCTTCCTTCTTGGGTATG, reverse CAGCAATGCCTGGGTACAT, 143 bp). A PRISM7500 Real-Time PCR instrument (ABI, USA) was used to detect gene expression.

2.4. Western blot

For Western blot experiments, cells were washed twice with PBS and lysed in radioimmune precipitation assay buffer containing 50 mM Tris (pH 7.4), 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, and 0.1% SDS with protease inhibitors on ice for 30 min. Protein concentrations were determined via bicinchoninic acid (BCA) assay. 25 μg protein samples were separated via SDS–polyacrylamide gel and transferred onto polyvinylidene difluoride (PVDF) membranes (Sigma). Membranes were blocked with 5% fat-free milk in Tris-buffered saline solution (TBS) containing 0.1% Tween-20 and incubated overnight at 4 °C with anti-ERp46 (sc-134829, santacruz), anti-CHOP (sc-575, santacruz), anti-Cleaved caspase12 (NO. 2202, Cell Signaling), anti-P-JNK (sc-6254, santacruz) or anti-β-actin (sc-47778, santacruz) primary antibodies diluted 1:200–500. Membranes were incubated with Horseradish peroxidase-labeled secondary antibodies (1:500) for 1 h at room temperature. Protein signals were visualized using an enhanced chemiluminescence detection system. Protein expression levels were normalized to β-actin.

2.5. Knockdown of ERp46 by small interfering (si)RNA

ERp46 expression was suppressed using the small interfering (si)RNA. 2×10^6 cells were seeded into a 6 cm cell culture dish and cultured for 24 h prior to transfection. 10 μl Lipofectamine 2000 and 10 μl 20 nM ERp46siRNA were mixed to transfect βTC6 cells. Cells were then cultured for 4–6 h, and the medium was replaced with DMEM containing 12% fetal bovine serum. After 48 h, Western blot and Real-time PCR analysis were performed to detect the expression of ERp46. The target sequence of the ERp46siRNA duplexes was GUACUCGGUACGAGGUUAUTT. Non-Targeting siRNA was used as negative control siRNA (NC). The cells treated with transfection reagent were used as blank control (BC).

2.6. Detection of β-cell apoptosis by flow cytometric analysis

βTC6 cells were seeded in 6-well plates and treated as indicated. The cells were collected after digestion by 0.25% EDTA-free trypsin and centrifuged at 10,000 rpm for 5 min. The cell pellet was then washed twice with cold PBS, and an AnnexinV-FITC/PI Apoptosis Detection Kit (BD Biosciences) was used to assess cell death. 10,000 cells were measured by FACScan. Annexin V-FITC(+)/PI(−) cells were scored as apoptotic cells.

2.7. Statistical analysis

Statistical analysis was performed using the SPSS statistical analysis program. The data were presented as means ± SEM of three to five independent experiments. The statistical significance of quantitative results was evaluated using an analysis of variance (ANOVA) test. A P value < 0.05 was considered statistically significant.

3. Results

3.1. Effects of PA on ERp46 expression in βTC6 cells

To determine ERp46 expression under PA-induced conditions by Real-time PCR and Western blot analysis, βTC6 cells were cultured in increasing concentrations of PA respectively for 12–48 h. Our results indicated that ERp46 mRNA and protein levels were decreased in a dose- and time-dependent manner after PA treatment (Fig. 1A and B).

3.2. Effect of ERp46 on ER stress response in βTC6 cells

The functional role of ERp46 in βTC6 cells was studied under high lipid conditions. Gene silencing methods was used to knockdown ERp46 gene expression and monitored the changes of ER stress response. Transfection of βTC6 cells with NCsiRNA had no significant effect on the expression of ERp46 compared to cells transfected with BC. The expression of ERp46 mRNA was decreased by 76%, while protein levels were reduced by 82% compared to cells transfected with NCsiRNA (Fig. 2A).

After transfection with siRNA directed against ERp46, βTC6 cells were cultured in 0.5 mM PA for 24 h. The results showed that CHOP, Cleaved Caspase12, and P-JNK expression enhanced in cells transfected with NCsiRNA and cultured with 0.5 mMPA compared to that of cells transfected with NC alone. The expression of CHOP, Cleaved Caspase12, and P-JNK in NC and ERp46siRNA sample had no significant changed. Cells transfected with ERp46siRNA showed increased ER stress markers expression after PA treatment (Fig. 2B).

3.3. Role of ERp46 in βTC6 cells lipoapoptosis

Using ERp46siRNA to down-regulate ERp46, we detected the extent of βTC6 cell apoptosis after ERp46 knockdown under PA-induced conditions via Annexin V-PI analysis. Transfection with NC plus 0.5 mM PA and ERp46siRNA plus 0.5 mM PA caused higher rates of apoptosis, about 35.6% ± 4.4% and 49% ± 3.5% respectively, compared to a 1.2% ± 0.5% rate for cells transfected with NC alone. The cell apoptosis rate of ERp46 siRNA alone was 2% ± 0.9% (Fig. 2C).

3.3.1. Effect of exendin-4 on ERp46 expression in TC6 cells exposed to PA

To determine whether exendin-4 affects the expression of ERp46 under PA-induced conditions, cells were pre-treated with

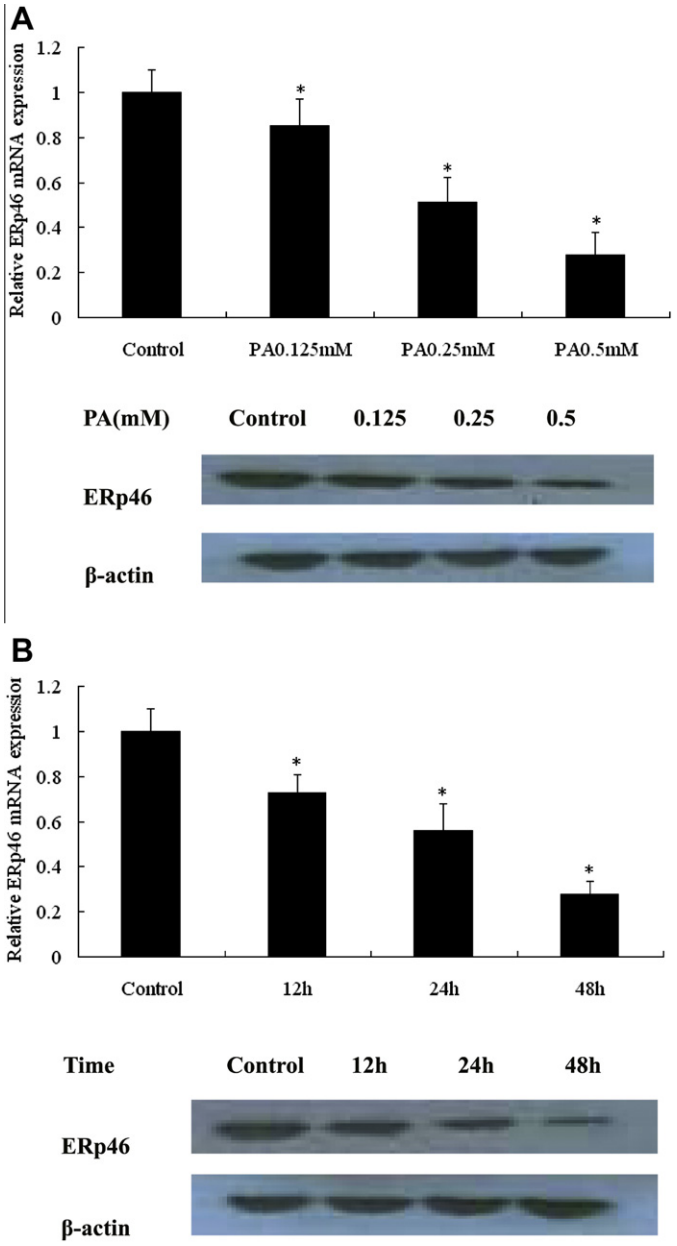

Fig. 1. Chronic PA treatment results in reduced ERp46 gene expression. (A) βTC6 cells were treated with 0.125 mM, 0.25 mM or 0.5 mM PA for 48 h. (B) βTC6 cells were cultured in 0.5 mM PA for 12, 24 or 48 h. ERp46 expression was measured by Real-time PCR and Western blot. Data represent means ± SEM of three to five independent experiments. $^{*}P < 0.05$ versus control.

10–50 nM of exendin-4 for 24 h, then exposed to 0.5 mMPA plus 10–50nM of exendin-4 for 24 h. The expression of ERp46 decreased when treated with 0.5 mM PA alone compare to control (without PA or exendin-4). ERp46 levels increased gradually as the concentration of exendin-4 was raised from 10 nM to 50 nM (Fig. 3).

3.3.2. Effect of exendin-4 on ER stress in ERp46siRNA transfected TC6 cells treated with PA

To investigate whether the effects of exendin-4 on changes in the stress response in βTC6 cells were associated with ERp46, the expression levels of CHOP, Cleaved Caspase12 and P-JNK were analyzed by Western blot in NC and ERp46siRNA transfected βTC6 cells. Cells were pre-treated with 50 nM exendin-4 for 24 h, then exposed to 0.5 mM PA plus 50 nM exendin-4 for 24 h. Exendin-4

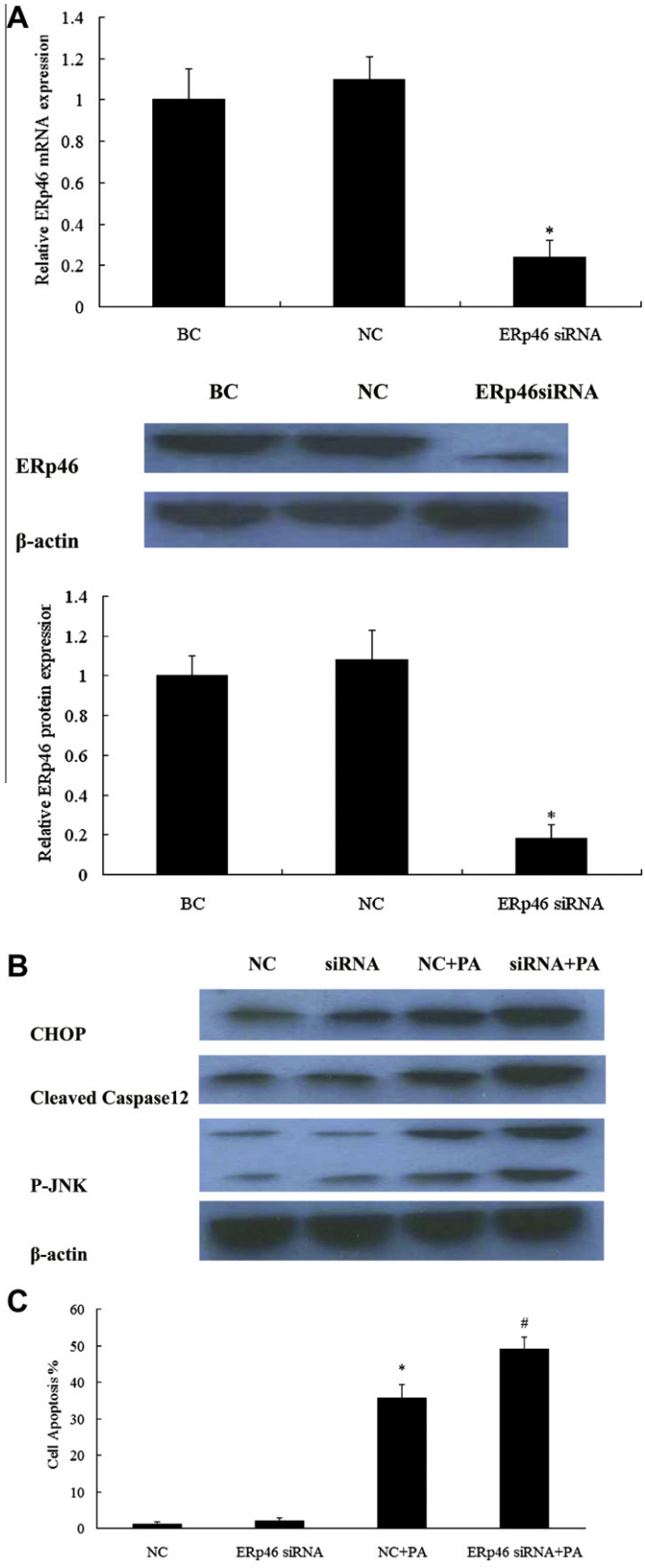

Fig. 2. Suppression of ERp46 by siRNA increases PA-induced ER stress and cell apoptosis. (A) ERp46 expression was measured by Real-time PCR and Western blot after transfection with BC, NC and ERp46siRNA. ERp46 protein levels reflect the ratio of ERp46 to β-actin expression. Western blot (B) analysis was used to measure the protein levels of ER stress markers CHOP, P-JNK, and Cleaved Caspase12 in negative control (NC), ERp46 siRNA, NC plus 0.5 mM PA and ERp46 siRNA plus 0.5 mM PA samples. (C) AnnexinV-PI analysis was used to detect βTC6 cell apoptosis. Data represent means ± SEM from three independent experiments. A representative Western blot is shown. $^{*}P < 0.05$ versus NC, $^{\#}P < 0.05$ versus NC + PA.

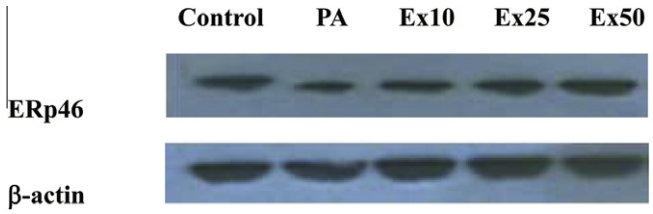

Fig. 3. Exendin-4 increases the expression of ERp46 induced by chronic PA exposure. βTC6 cells were cultured for 24 h in the presence of 0.5 mM PA alone or in combination with various concentrations of exendin-4 (Ex) (10 nM, 25 nM or 50 nM). ERp46 expression was measured by Western blot analysis of three independent experiments. β-actin was detected in Western blot as the loading control.

treatment had no effect on CHOP or Cleaved Caspase12 protein levels in NC and ERp46siRNA transfected βTC6 cells cultured in absence of PA. However, exendin-4 reduced the protein levels of CHOP, Cleaved Caspase12 and P-JNK in NC transfected cells after PA treatment, but not in cells transfected with ERp46siRNA (Fig. 4A).

3.3.3. Effect of exendin-4 on cell apoptosis in ERp46siRNA transfected TC6 cells exposed to PA

Flow cytometric analysis was used to detect whether exendin-4 was able to exert its anti-apoptotic effects on PA-induced apoptosis via ERp46. NC and ERp46siRNA transfected βTC6 cells were treated with PA, exendin-4 or a combination of PA and exendin-4. Results showed that exendin-4 exposure induced a significant decrease in apoptosis levels in NC transfected βTC6 cells with PA treatment, but not in cells transfected with ERp46siRNA. The apoptosis rate was not significantly altered in NC and ERp46siRNA transfected βTC6 cells exposed to exendin-4 without PA treatment (Fig. 4B).

4. Discussion

Thioredoxin family proteins are the largest group of chaperones resident in the ER. Moreover, members of the thioredoxin family play an important role in antihypoxia-induced apoptosis, proliferation, differentiation, and angiogenesis [22]. ERp46 is a newly identified member of the thioredoxin group of ER chaperones and has been shown to contain an active protein disulfide isomerase (PDI) domain. However, few studies regarding the biological function of ERp46 have been completed. Recent studies have indicated that ERp46 is highly expressed in non-small cell lung carcinoma [23], and that reducing its expression restrains the growth and proliferation of gastric cancer cells [24]. ERp46 was shown to be the first AdipoR1-specific interacting protein with a suggested function in modulating adiponectin signaling [25]. In the fructose-fed hamster liver, ERp46 was shown to be 7-fold down-regulated compared to controls [26]. More recently, ERp46 was shown to be highly expressed in pancreatic islet cells, and its expression was correlated with pancreatic β-cell glucose toxicity. These findings indicate a possible role for ERp46 in diabetes and metabolic syndromes. Our results showed that ERp46 expression was reduced after PA treatment in a dose- and time-dependent manner. Previously, expression of the ER chaperone protein BiP was shown to be upregulated upon cellular stress under PA-induced conditions to increase folding capacity [27,28]. We inferred that this cellular defense mechanism might be impaired in lipotoxic conditions, leading to a reduction in ERp46 expression. However,

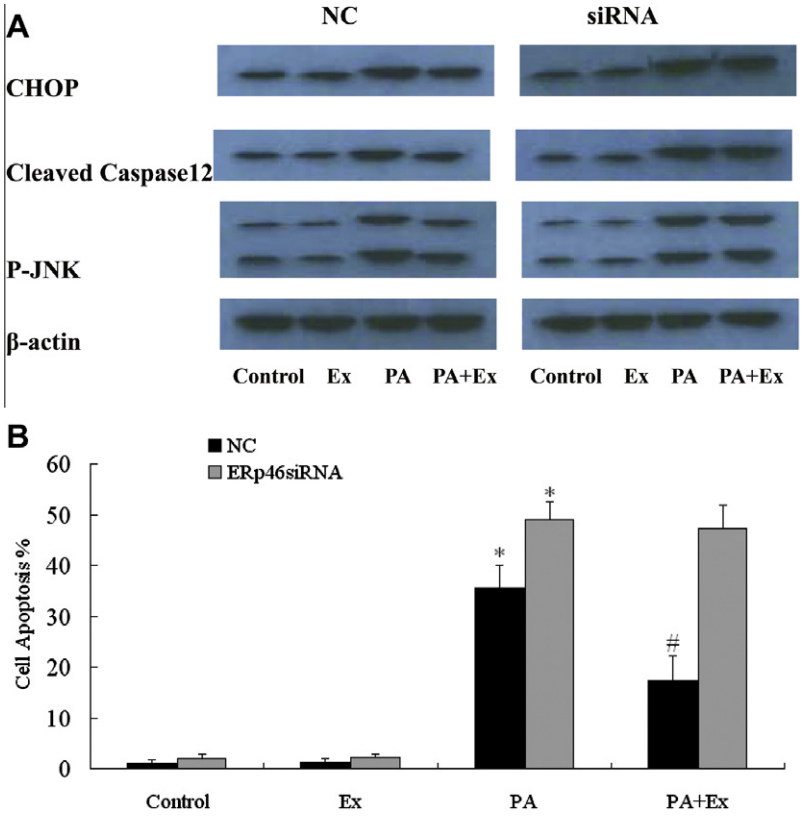

Fig. 4. Effects of exendin-4 on PA-induced ER stress response and cell apoptosis in βTC6 cells. The effect on CHOP, P-JNK, and Cleaved Caspase12 levels and apoptosis after exposure to 0.5 mMPA or 50 nM exendin-4 or a mixture of both was measured in NC and ERp46siRNA transfected βTC6 cells. Western blot analysis was performed using anti-CHOP, anti-P-JNK, and anti-Cleaved Caspase12 antibodies (A). βTC6 cell apoptosis was measured by Annexin V – PI analysis (B). Data represent means ± SEM of three independent experiments. Representative Western blot analysis is shown. $^{*}P < 0.05$ versus control. $^{\#}P < 0.05$ versus PA.

further studies will be needed to determine the mechanisms at play during this process.

Many harmful factors can trigger an ER stress response in β-cells. The saturated fatty acid PA can activate the ER stress response via three pathways [10,28]. Our present work supports these findings. Furthermore, knockdown of ERp46 potentiated the expression of the ER stress markers CHOP, Cleaved Caspase12 and P-JNK in our study. This indicates that ERp46 may have protective effects on the ER stress response through three pathways after PA exposure. ER stress was considered as a key link between obesity, insulin resistance and T2DM. This demonstrates the possibility of ameliorating ER stress as a therapeutic target for T2DM, and ERp46 could act as an intervention point in reducing ER stress.

Apoptosis occurs in the presence of persistent ER stress associated with a detrimental factor and might be the initiating factor in many ER stress-related disorders, including diabetes. In fact, a growing body of evidence has shown that the loss of β-cell mass due to increased β-cell apoptosis is an important factor in the development of diabetes mellitus [29,30]. ER stress was proposed as a key mechanism leading to increased β-cell apoptosis. Because of high protein production levels, including insulin production, which accounts for about 50% of total protein synthesis [31], β-cells are particularly sensitive to ER stress-induced dysfunction and death [32]. The mechanisms of apoptosis include a highly complex molecular cascade. So far, the ER stress markers CHOP, JNK and Cleaved Caspase12 have been shown to be the key ER stress-induced proapoptotic mediators. However, the mechanisms linking ER stress to apoptosis require further clarification. βTC6 cell apoptosis increased after PA treatment, and knockdown of ERp46 increased apoptosis rates in our study. This led us to the hypothesis that the increased apoptosis rate might be due to the activation of CHOP, P-JNK and Cleaved Caspase12 after ERp46 inhibition. Thus, ERp46 might play an anti-apoptotic role in the process of PA-induced cell apoptosis in βTC6 cells. So far, no studies have implicated a role for thioredoxin/PDI proteins in PA-induced cell apoptosis. Our studies emphasize the crucial role played by ERp46, a thioredoxin/PDI family member, in the process of lipoapoptosis.

Many mechanisms of β-cell protection by exendin-4 have been previously reported. The mechanisms for its action include activation of protein kinase B/Akt through cAMP-dependent phosphorylation of cAMP-responsive element-binding protein [33] and decreased caspase 3 expression [34]. In rodent islet cells, exendin-4 therapy reduced the expression of CHOP and other ER stress markers [35–37]. However, there have been few reports regarding the effect of exendin-4 on the ER stress response in βTC6 cells exposed to PA. Our study indicates that the ER stress markers CHOP, P-JNK, and Cleaved Caspase 12 decreased with chronic exendin-4 treatment under PA-induced conditions. In addition, exendin-4 protected cells from lipotoxic ER stress through up-regulating the expression of ER chaperone BiP and the antiapoptotic protein JunB [38]. In our observation, exendin-4 increased ERp46 expression in a dose-dependent manner between 10 nM and 50 nM. Furthermore, the increased ER stress response and apoptosis rate induced by PA were not altered with exendin-4 administration in cells transfected with ERp46siRNA. These findings led us to hypothesize that exendin-4 activates ERp46 expression, leading to down-regulation of CHOP, P-JNK, and Cleaved Caspase 12, and by blocking lipoapoptosis-promoting signaling events. This favors the view that ERp46 induction is one of the mechanisms by which exendin-4 protects against lipoapoptosis by enhancing cellular defense mechanisms. However, the mechanism of exendin-4 in this process is still not fully understood.

In conclusion, the resident ER protein ERp46 may be an anti-apoptotic factor involved in the process of PA-induced apoptosis through alleviating the ER stress response. The mechanism by which ERp46 achieves this has not been conclusively demonstrated. We suggest that ERp46 might be a novel target in attempts to identify anti-diabetes drugs. Furthermore, the protective effects of exendin-4 on lipoapoptosis may involve activation of the ERp46 signaling pathway.

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