ERp46 mitigates lipotoxic ER stress to preserve GLUT2 expression and insulin secretion in β-cells

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Abstract. Lipotoxicity-induced β-cell dysfunction is a critical contributor to the pathogenesis of type 2 diabetes mellitus. The aim of the present study was to investigate the role of endoplasmic reticulum-resident protein 46 (ERp46) in regulating glucose transporter 2 (GLUT2) expression and insulin secretion in β-cells under palmitic acid (PA)-induced lipotoxic stress. β-TC6 cells were treated with PA to induce lipotoxicity, and ERp46 expression was silenced using specific small interfering RNA. GLUT2 expression and insulin secretion were assessed, and the involvement of protein kinase B (AKT) signaling was evaluated. The results demonstrated that PA significantly decreased GLUT2 expression and insulin secretion, while ERp46 expression was upregulated as a potential compensatory response. ERp46 knockdown exacerbated the reduction of GLUT2 expression and insulin secretion. Furthermore, PA treatment reduced phosphorylated AKT (p-AKT) levels without altering total AKT expression, and ERp46 knockdown further decreased p-AKT levels. The activation of AKT using AKT activator compound SC79 restored GLUT2 expression and insulin secretion in ERp46-depleted cells. These findings indicated that ERp46 helps preserve β-cell function under lipotoxic stress, potentially by stabilizing ER proteostasis and supporting AKT phosphorylation.

Introduction

Diabetes is one of the most common and serious global health problems, as well as one of fastest growing chronic diseases (1). For the 10th edition of the IDF Diabetes Atlas, by 2045, the global prevalence of diabetes among adults aged 20-79 years is projected to increase from 9% (463 million adults) in 2019 to 12.2% (783.2 million) (2). Among all cases of diabetes, type 2 diabetes mellitus (T2DM) accounts for ~90% of all cases (3). Obesity is an important risk factor for T2D (4). The increase in free fatty acids (FFAs) caused by obesity plays a crucial role in the occurrence and development of T2D (4).

T2D is characterized by insufficient insulin secretion and chronic hyperglycemia caused by pancreatic β-cell dysfunction (5). Regulating glucose transporter 2 (GLUT2) is a key protein for β-cell function and a transmembrane protein expressed in pancreatic β-cells, liver, kidneys and intestines, playing a key role in glucose sensing and insulin secretion (6). GLUT2 has a low affinity but high transport capacity for glucose, allowing β-cells to rapidly take up glucose when blood levels rise. This triggers metabolic pathways that increase intracellular ATP, leading to insulin secretion (7). The dysregulation of GLUT2 expression or function impairs glucose sensing and insulin release, which has been reported to contribute to β-cell dysfunction and the pathogenesis of T2D (8,9).

Among the various factors implicated in β-cell dysfunction, chronic exposure to high levels of FFAs is a major factor (10), with evidence suggesting that elevated FFAs have deleterious effects on β-cell function and survival, a phenomenon often referred to as lipotoxicity (5,11). Research has shown that metabolic stressors, such as elevated FFAs, can alter GLUT2 expression, further exacerbating β-cell dysfunction (12). A saturated fatty acid, palmitic acid (PA), has been shown to impair glucose-stimulated insulin release (13-17), and can induce lipotoxicity, leading to β-cell dysfunction and apoptosis (often referred to as lipotoxic β-cell apoptosis) (10,15,18,19).

PA can exert these detrimental effects on β-cells through various mechanisms, including the induction of endoplasmic reticulum (ER) stress, oxidative stress and inflammatory response (20). Given their critical role in insulin synthesis, β-cells have a highly developed ER network to synthesize insulin. Consequently, ER stress is particularly important in β-cell dysfunction (21). Under stress conditions, it leads to the accumulation of misfolded proteins in the ER and the activation of the unfolded protein response (UPR) (22). Long-term

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or excessive ER stress eventually triggers β-cell apoptosis, leading to the gradual loss of functional β-cells in patients with T2D (23).

A recent study highlighted the role of specific ER proteins in regulating ER stress and β-cell survival (24). Endoplasmic reticulum-resident protein 46 (ERp46), a thiol-disulfide oxidoreductase that is highly expressed in endothelial cells, pancreatic β-cells, hepatocytes and hypoxic tissues (25-27), plays a crucial role in protein folding and redox regulation (24,28). ERp46 has been identified as a key regulator of cellular homeostasis, and its activity is particularly important under conditions of ER stress, including maintenance of β-cell function and involvement in insulin secretion (29). In addition, GLUT2 plays a role in the intracellular signaling pathways involved in glucose uptake and metabolism in ER stress and apoptosis (30), suggesting that ERp46 may also be linked to these processes, although this has not been directly demonstrated. Despite the potential importance of ERp46 in β-cell function and the fact that its expression is reduced in β-cells in diabetic mouse models and under high glucose stimulation (31), the mechanisms through which ERp46 regulates β-cells in diabetic mouse models and, particularly how it may link ER stress to GLUT2 expression, remain unclear. The aim of the present study was to explore the role of ERp46 in pancreatic β-cells, particularly in the regulation of insulin secretion under PA-induced lipotoxic stress. By elucidating the molecular mechanism through which ERp46 affects β-cell function, this study may help develop novel therapeutic strategies targeting ER stress to preserve β-cell function in patients with T2D.

Materials and methods

Cell culture and PA treatment. The β-TC6 mouse insulinoma cell line was purchased from the National Collection of Authenticated Cell Cultures. Cells were maintained in DMEM (Sigma-Aldrich; Merck KGaA) supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.) at 37˚C and 5% CO_2. A stock solution of PA (Sigma-Aldrich; Merck KGaA) was prepared in 100% ethanol at 55˚C until it was completely dissolved at a concentration of 100 mM. The final ethanol concentration in culture did not exceed 0.1% (v/v), and vehicle controls were included accordingly. Prior to PA treatment, the stock solution was added to DMEM with 10% FBS at the indicated concentrations.

AKT activator compound SC79 (SC79) treatment. SC79 (Selleck Chemicals) was dissolved in DMSO to prepare a 50-mM stock solution stored at -20˚C. For experiments, the stock was diluted in DMEM to a final concentration of 5 or 10 μM, ensuring the DMSO concentration did not exceed 0.1%. β-TC6 cells at 70-80% confluency were treated with SC79 for 4 h at 37˚C, 5% CO_2. DMSO-treated cells were used as controls. Following treatment, cells were harvested for protein extraction or insulin secretion assays.

Differential expression analysis and functional enrichment. RNA-seq data (GSE53949) was downloaded from the GEO database (32) and analyzed in RStudio (version 1.4.1717; Posit PBC) using the DESeq2 package (version 1.30.1; https://bioconductor.org/packages/release/bioc/html/DESeq2.html). GSE53949 included five control and five PA-treated human pancreatic islets samples; all ten samples were used for differential and correlation analyses. Following initial quality control, normalization was performed to adjust for library size variations. Differential expression analysis identified genes with $|\log2FC|>1$ and $P<0.05$ as significant, and P-values were adjusted using the Benjamini-Hochberg method to control for false discovery rate. Volcano plots were generated with ggplot2 (version 3.3.5; http://ggplot2.tidyverse.org), where upregulated genes were highlighted in red and downregulated genes in green. Functional enrichment, including Gene Ontology (http://geneontology.org) and Kyoto Encyclopedia of genes and genomes (KEGG; https://www.genome.jp/kegg/) pathway analysis, was conducted using the clusterProfiler package (version 4.0.5; http://bioconductor.org/packages/release/bioc/html/clusterProfiler.html), and results were visualized through bar plots. To assess the relationship between thioredoxin domain-containing protein 5 [*TXNDC5* (ERp46)] and solute carrier family 2 member 2 [*SLC2A2* (GLUT2)], Pearson's correlation analysis was conducted using normalized RNA-seq expression data. A linear regression model was applied, and the coefficient of determination (R^2) was calculated to quantify the strength of the correlation. Scatter plots with fitted regression lines were generated to visualize the results.

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was extracted from β-TC6 cells using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.). First-strand complementary DNA was synthesized using the Hifair® II 1st Strand cDNA Synthesis Kit (Shanghai Yeasen Biotechnology Co., Ltd.), according to the manufacturer's instructions. Subsequently, RT-qPCR was performed using the Hieff® qPCR SYBR Green Master Mix (Shanghai Yeasen Biotechnology Co., Ltd.) according to the standard method. The thermocycling conditions were as follows: Initial denaturation at 95˚C for 2 min, followed by 40 cycles of denaturation at 95˚C for 10 sec and annealing/extension at 60˚C for 30 sec. A melting curve analysis was performed to verify amplification specificity. The relative expression (fold) was calculated using the comparative method $2^{-\Delta\Delta Cq}$ method (33). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used for normalization. The primer sequences are shown in Table I.

Cell protein extraction. The culture dish in which β-TC6 cells were seeded was washed twice with cold PBS to completely remove the culture medium, and the cells were lysed using RIPA lysis buffer (Beijing Solarbio Science & Technology Co., Ltd.) supplemented with protease inhibitors. After gently scraping the cells with a cell scraper, they were transferred to a 1.5 ml Eppendorf tube and incubated on ice for 30 min for lysis. The cells were vortexed every 5 min to enhance protein extraction. The lysate was centrifuged at 12,000 x g for 15 min at 4˚C to precipitate cell debris. The supernatant containing the extracted protein was carefully transferred to a new tube for subsequent analysis.

Protein quantification. Protein concentration was determined using the bicinchoninic acid (BCA) assay (Thermo

Table I. Primer sequences used for quantitative PCR analysis of target genes in β-cells.

Primer	Sequence (5'-3')
ERp46-F	GGATGCCAAGGTCTACGTG
ERp46-R	CGGAGCGAAGAACTTGATA
PDX1-F	GGCGGCCGCAGGAACCAC
PDX1-R	GAGGGCCCCAATACTACAAAAACC
GLUT2-F	CCTGCTTGGCCTGTCTGGTGT
GLUT2-R	TGTCGGTAGCTGGAATTGGTGAAG
GAPDH-F	CGGGGCTCTCCAGAACATCATCC
GAPDH-R	CCAGCCCCAGCGTCAAAGGTG

ERp46, endoplasmic reticulum-resident protein 46; PDX1, pancreatic and duodenal homeobox' GLUT2, glucose transporter 2; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; F, forward; R, reverse.

Fisher Scientific, Inc.). A standard curve was prepared using bovine serum albumin (Thermo Fisher Scientific, Inc.) as a standard. Protein samples were added to different wells of a 96-well plate. Subsequently, 200 μl of BCA reagent mixture (comprised of reagent A and reagent B mixed at a ratio of 50:1) was added to each well. The plate was incubated at 37˚C for 30 min, and absorbance was measured at 562 nm using a microplate reader. Protein concentration was calculated by comparing the absorbance of the sample with the standard curve. Depending on the concentration, protein samples were diluted to 1 μg/μl using RIPA and then added to protein loading buffer, boiled at 100˚C for 5 min, aliquoted and stored in a freezer at -80˚C.

Western blotting. Protein samples (50 μg) were separated by 10% SDS-PAGE and transferred to PVDF membranes (Wuhan Servicebio Technology Co., Ltd.). Following blocking with 5% skim milk in TBST for 1 h at room temperature, the membranes were incubated with primary antibodies overnight at 4˚C: Anti-ERp46 (1:1,000; cat. no. sc-271667; Santa Cruz Biotechnology, Inc.), anti-GLUT2 (1:1,000; cat. no. K006592P; Beijing Solarbio Science & Technology Co., Ltd.), anti-p-Akt (1:1,000; cat. no. GB150002), anti-Akt (1:1,000; cat. no. GB111114), anti-pancreatic and duodenal homeobox 1 (PDX1; 1:1,000; cat. no. GB11917), and anti-tubulin (1:2,000; cat. no. GB11017; all from Wuhan Servicebio Technology Co., Ltd.), anti-binding immunoglobulin protein (BiP; 1:1,000; cat. no. 3177T), C/EBP homologous protein (CHOP; 1:1,000; cat. no. 2895T), and anti-Na^+/K^+-ATPase [1:1,000; cat. no. 3010S; all from Cell Signaling Technology, Inc.]. Following washing three times with TBST (TBS containing 0.1% Tween-20) for 10 min each time, the membranes were incubated with secondary antibodies: Anti-rabbit IgG, HRP-linked antibody (cat. no. 7074) and anti-mouse IgG, HRP-linked antibody (cat. no. 7076) (both 1:5,000; Cell Signaling Technology, Inc.), for 1 h at room temperature. Protein bands were visualized using enhanced chemiluminescence (Thermo Fisher Scientific, Inc.), and band intensities were quantified using ImageJ software (version 1.53t; National Institutes of Health).

Insulin secretion assay. Supernatants of β-TC6 cells exposed to various concentrations (0, 0.1, 0.25 and 0.5 mM) of PA for 24 h at 37˚C were collected, and insulin levels were measured using a mouse insulin ELISA kit [cat. no. 634-01481 (formerly AKRIN-011T) FUJIFILM Wako Pure Chemical Corporation], according to the manufacturer's instructions. Absorbance was measured at 450 nm, and insulin concentrations were calculated using a standard curve.

Transfection. For the knockdown of ERp46, $1x10^6$ cells were seeded into a 6-well plate. A total of 50 pmol ERp46 small interfering (siRNA) (sense, 5'-GUACUCGGUACGAGG UUAUTT-3' and antisense, 5'-AUAACCUCGUACCGAGUA CTT-3') or negative control siRNA (sense, 5'-UUCUCCGAA CGUGUCACGUTT-3' and antisense, 5'-ACGUGACACGUU CGGAGAATT-3') were transfected using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's instructions (at 3˚C for 6 h; typically 5 μl reagent per well in Opti-MEM). The transfection medium was then replaced with DMEM containing 12% FBS, and cells were cultured for 24 h at 37˚C. The expression of ERp46 was detected using western blotting and RT-qPCR.

Statistical analysis. Statistical analysis was performed using GraphPad Prism 8.0 (GraphPad Software, Inc.). Data are presented as the mean ± standard error of the mean from three to five independent experiments, with each experiment including n=6 biological replicates per group. Statistical comparisons between two groups were performed using an unpaired two-tailed Student's t-test, and multiple-group comparisons were analyzed using ANOVA followed by Sidak's multiple-comparisons test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

PA activates ER stress-related pathways and impairs β-cell function. As aforementioned, FFAs can cause lipotoxicity in pancreatic β-cells (10,15,19), and it was found that following PA stimulation, pancreatic β-cells secreted less insulin, which was proportionate to the dose of PA (Fig. 1A), consistent with previous studies (16,17). When stimulated with 0.5 μM PA, insulin secretion decreased over time; however, there was no significant difference between 24 and 48 h, suggesting that the effect reached saturation (Fig. 1A). To further investigate the cause of the reduced insulin secretion, RNA-seq analysis of a public dataset (GSE53949) was performed. By analyzing the RNA-seq results of PA-stimulated β-cells compared with the control group, *TXNDC5* (gene name of ERp46) was significantly upregulated (Fig. 1B). This upregulation was supported by enrichment analysis, which highlighted the activation of ER stress-related pathways in β-cells following PA stimulation (Fig. 1C). These findings were consistent with previous reports, in which the upregulation of TXNDC5 was associated with its role in promoting correct protein folding and maintaining redox homeostasis under ER stress conditions (25-27).

By contrast, the key glucose transporter *SLC2A2* (gene name of GLUT2) in β-cells was significantly downregulated following PA stimulation. Enrichment analysis identified the ‘negative regulation of transport’

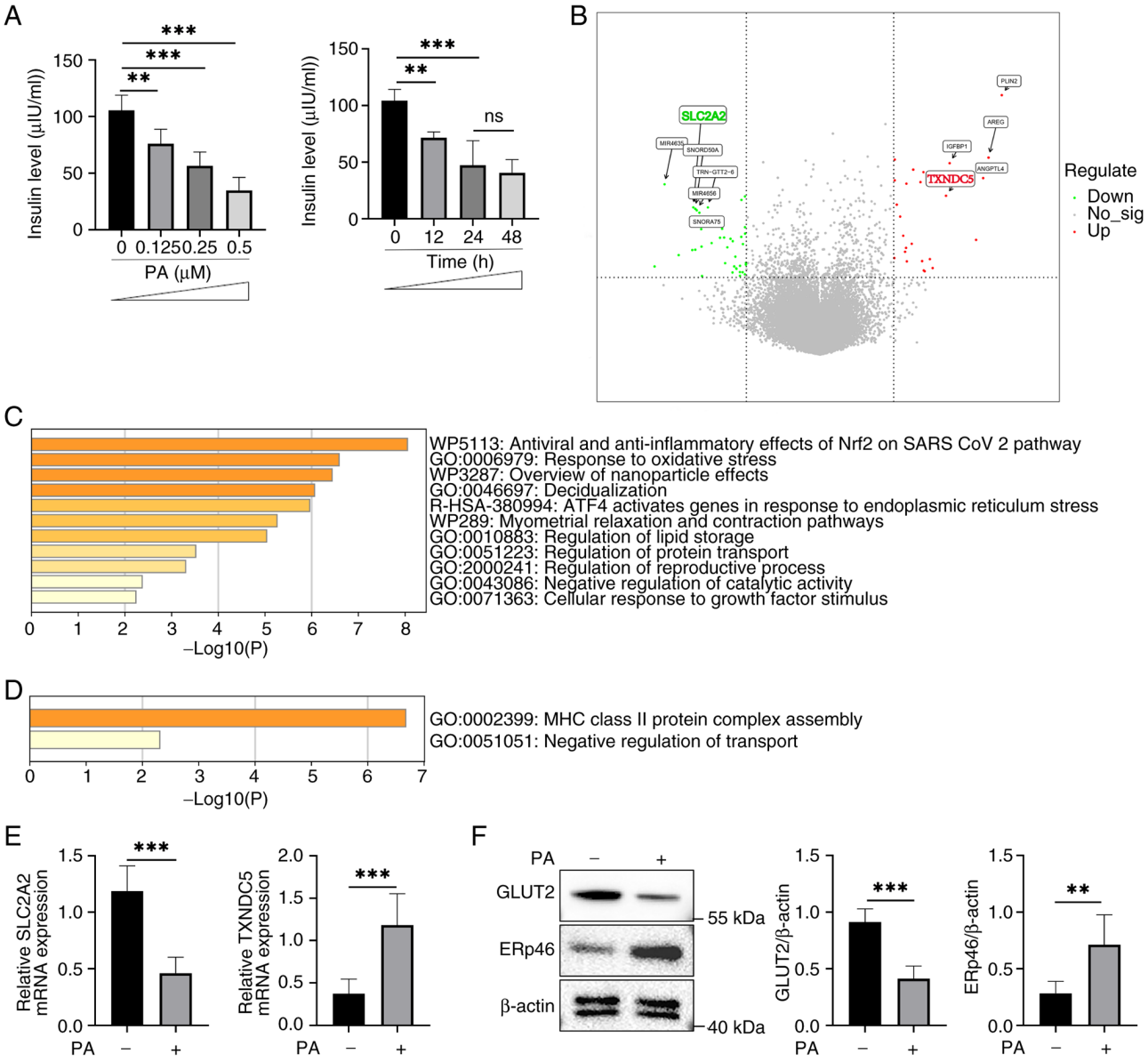

Figure 1. PA-induced ER stress reduces GLUT2 expression and impairs insulin secretion in β-cells. (A) Insulin secretion levels under varying concentrations of PA (0, 0.125, 0.25 and 0.5 μM) and at different time points (0, 12, 24 and 48 h). Insulin secretion decreased in a dose- and time-dependent manner. Data are presented as the mean ± SEM (n=6). Statistical significance was determined using one-way ANOVA. (B) Volcano plot showing the DEGs in PA-stimulated β-cells compared with controls. *TXNDC5* (ERp46) was significantly upregulated, whereas *SLC2A2* (GLUT2) was downregulated. (C) GO and KEGG pathway enrichment of upregulated DEGs. Key pathways included oxidative stress response, UPR and ER stress-related pathways. (D) Enrichment analysis of downregulated DEGs, highlighting impaired transport regulation and immune-related processes. (E) RT-qPCR validation of *TXNDC5* and *SLC2A2* expression in PA-stimulated β-cells. (F) Western blotting showing increased ERp46 and decreased GLUT2 protein levels following PA treatment. **P<0.01 and ***P<0.001. PA, palmitic acid; ER, endoplasmic reticulum; GLUT2, glucose transporter 2; SEM, standard error of the mean; DEGs, differentially expressed genes; *TXNDC5*, thioredoxin domain-containing protein 5; ERp46, endoplasmic reticulum-resident protein 46; *SLC2A2*, solute carrier family 2 member 2; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; UPR, unfolded protein response; RT-qPCR, reverse transcription-quantitative PCR; ns, not significant.

pathway (Fig. 1D), highlighting the importance of GLUT2 in β-cell function. The downregulation of GLUT2 suggested a reduced glucose-transport capacity and was compatible with the decreased insulin secretion observed in Fig. 1A. Previous studies have demonstrated that GLUT2 plays a critical role in glucose sensing and insulin secretion in β-cells (8,9), although the present data did not directly establish causality. Consistently, RT-qPCR confirmed that *SLC2A2* mRNA was decreased while *TXNDC5* mRNA was increased in PA-treated cells (Fig. 1E). Western blotting further demonstrated decreased GLUT2 protein and increased ERp46 protein levels following PA treatment (Fig. 1F). In conclusion, PA stimulation was associated with the activation of ER stress-related pathways and reduced GLUT2 expression and insulin secretion in β-cells.

ERp46 depletion exacerbates PA-induced ER stress, reducing GLUT2 expression and insulin secretion in β-cells. ERp46 knockdown efficiency was first verified. Western blotting confirmed a robust reduction of ERp46 protein following siERp46 transfection (Fig. 2A). This result validated the effectiveness of the knockdown system for subsequent experiments.

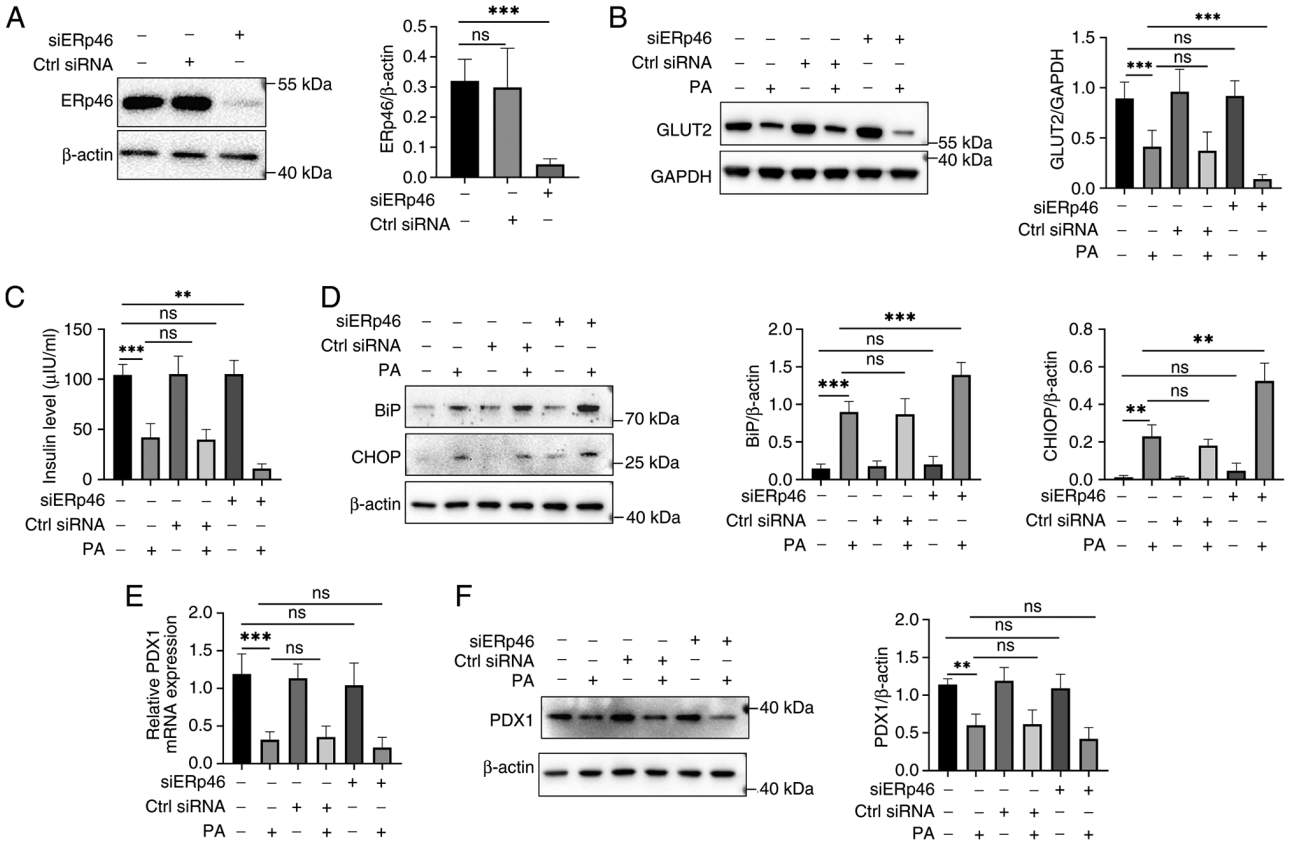

Figure 2. ERp46 regulates GLUT2 expression and insulin secretion in β-cells under PA-induced stress. (A) Western blotting confirming ERp46 knockdown efficiency by siRNA transfection. (B) Western blotting showing that GLUT2 expression was further decreased in PA-treated cells following ERp46 knockdown. (C) Insulin secretion was significantly reduced in ERp46-depleted β-cells exposed to PA, as determined by ELISA. (D) Western blotting of ER stress markers BiP and CHOP revealed that ERp46 knockdown aggravated PA-induced ER stress. (E) RT-qPCR analysis of *PDX1* mRNA expression showed no significant changes with ERp46 knockdown under PA stimulation. (F) Western blotting confirmed that PDX1 protein levels were not significantly altered by ERp46 knockdown. Data are presented as the mean ± SEM (n=6). Statistical significance was determined using one-way ANOVA. **P<0.01 and ***P<0.001. ERp46, endoplasmic reticulum-resident protein 46; GLUT2, glucose transporter 2; PA, palmitic acid; siRNA, small interfering RNA; ER, endoplasmic reticulum; BiP, binding immunoglobulin protein; CHOP, C/EBP homologous protein; RT-qPCR, reverse transcription-quantitative PCR; *PDX1*, pancreatic and duodenal homeobox 1; SEM, standard error of the mean; ns, not significant.

Next, GLUT2 protein was examined across the four conditions (Control, PA, siERp46 and PA + siERp46). As shown in Fig. 2B, PA alone decreased GLUT2, whereas siERp46 alone had no detectable effect. Of note, the combination of siERp46 with PA further reduced GLUT2 compared with PA alone. These findings indicated that ERp46 is not essential for basal GLUT2 expression, but contributes to the maintenance of GLUT2 under lipotoxic stress.

Functionally, insulin secretion followed the same pattern (Fig. 2C): PA reduced insulin release; siERp46 alone did not alter secretion; and siERp46 plus PA led to a further decrease relative to PA alone. This indicates that the protective role of ERp46 becomes evident only in the presence of PA-induced stress. Since ERp46 is an ER oxidoreductase, ER stress markers were assessed. PA increased BiP and CHOP, and siERp46 further increased their levels under PA, whereas siERp46 alone showed no significant change (Fig. 2D). These results are consistent with the interpretation that ERp46 depletion aggravates ER stress in β-cells exposed to PA. PDX1, a β-cell identity/functional factor (12,30), was also evaluated. PA decreased PDX1 mRNA and protein levels, while siERp46 did not produce an additional significant reduction beyond PA in our conditions (Fig. 2E and F). This suggested that ERp46 depletion primarily impacted GLUT2 and insulin secretion rather than β-cell identity markers under PA stress.

Collectively, these data indicated that ERp46 depletion exacerbates PA-induced ER stress and is associated with a greater loss of GLUT2 and insulin secretion, whereas ERp46 knockdown alone has minimal effects at baseline. Thus, ERp46 appears to play a stress-dependent protective role in sustaining GLUT2 expression and β-cell functionality.

AKT signaling contributes to GLUT2 maintenance under PA-induced stress; ERp46 depletion reduces AKT phosphorylation. The relationship between *TXNDC5* (ERp46) and *SLC2A2* (GLUT2) was first examined using the public RNA-seq dataset (GSE53949). In the correlation plot, each dot represents one RNA-seq sample from GSE53949 (PA-stimulated or control β-cell samples). A modest positive correlation was observed (R^2=0.4001, P=0.0497; Fig. 3A), supporting an association between ERp46 and GLUT2 under lipotoxic conditions. Next, AKT activation was assessed. Total AKT (t-AKT) remained unchanged across groups, whereas PA decreased phosphorylated AKT (p-AKT). Of note, siERp46 alone had little effect, but siERp46 in the presence of PA further reduced p-AKT (Fig. 3B). These findings indicated

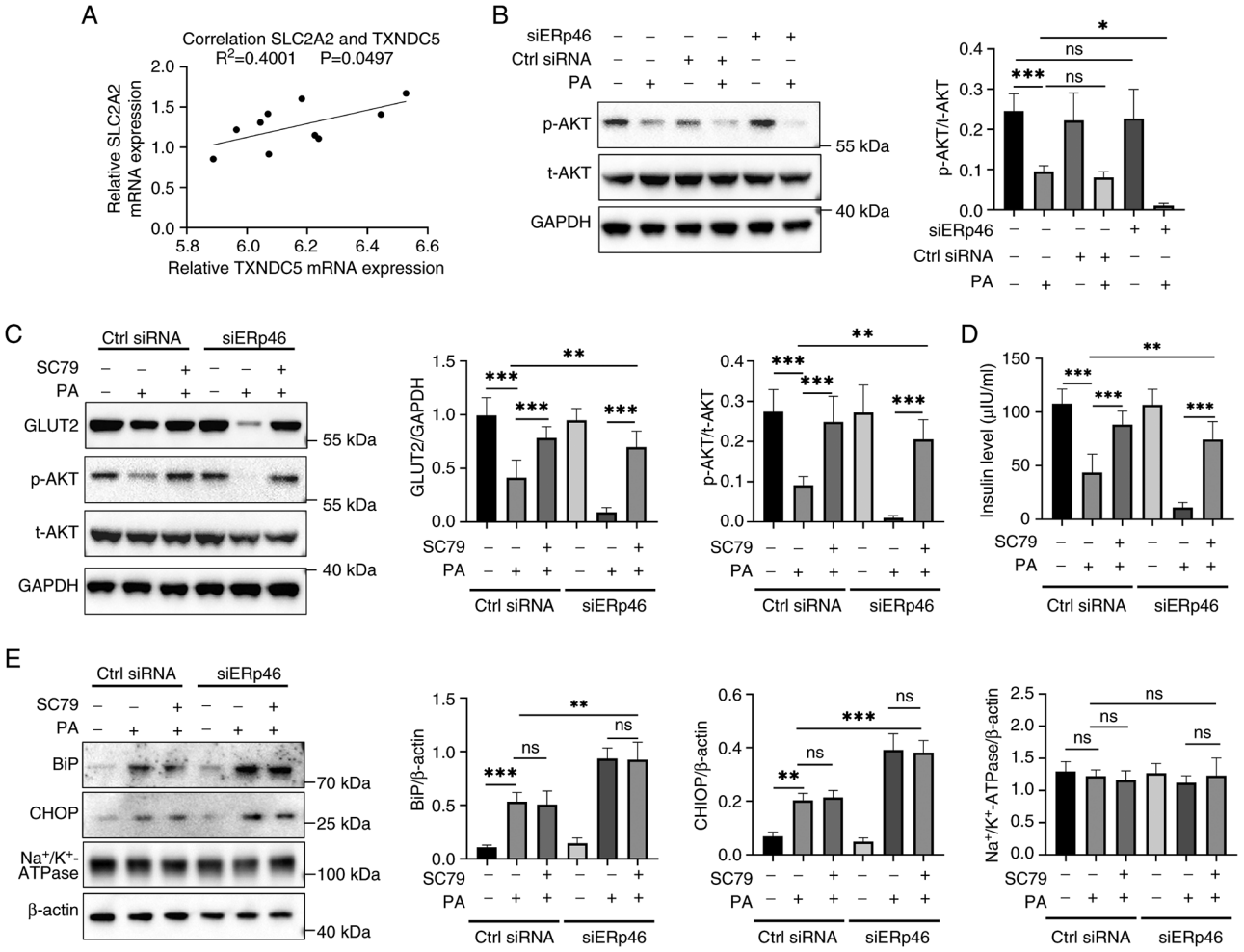

Figure 3. ERp46 regulates GLUT2 expression through AKT activation under PA-induced stress. (A) Correlation analysis of RNA-seq dataset (GSE53949) showing a positive correlation between *TXNDC5* (ERp46) and *SLC2A2* (GLUT2) expression in PA-stimulated and control β-cell samples. (B) Western blotting showing that p-AKT levels were decreased by PA and further reduced by ERp46 knockdown, while t-AKT remained unchanged. (C) Western blotting demonstrating that treatment with the AKT activator SC79 restored GLUT2 expression and p-AKT levels in ERp46-depleted cells under PA stimulation. (D) ELISA assay showing that SC79 treatment rescued insulin secretion impaired by ERp46 knockdown in PA-treated β-cells. (E) Western blotting of ER stress markers BiP and CHOP revealed that SC79 treatment did not significantly reverse PA-induced ER stress, indicating that AKT activation specifically rescued GLUT2 and insulin secretion without directly modulating ER stress. Na^+/K^+-ATPase served as a membrane-protein specificity control. Data are presented as the mean ± SEM (n=6). Statistical significance was determined using one-way ANOVA. $^*P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$. ERp46, endoplasmic reticulum-resident protein 46; GLUT2, glucose transporter 2; AKT, protein kinase B; PA, palmitic acid; *TXNDC5*, thioredoxin domain-containing protein 5; *SLC2A2*, solute carrier family 2 member 2; p-AKT, phosphorylated AKT; t-AKT, total AKT; SC79, AKT activator compound SC79; ER, endoplasmic reticulum; BiP, binding immunoglobulin protein; CHOP, C/EBP homologous protein; SEM, standard error of the mean; ns, not significant.

that ERp46 depletion exacerbated the PA-induced suppression of AKT phosphorylation, while having a minimal impact under basal conditions.

To determine whether AKT activation is sufficient to counteract the PA/siERp46 effects, cells were treated with the AKT activator SC79. SC79 increased p-AKT and partially restored GLUT2 protein in PA-exposed cells under both control siRNA and siERp46 conditions (Fig. 3C). Functionally, SC79 also improved insulin secretion that was reduced by PA and further diminished by siERp46 (Fig. 3D). To evaluate specificity, an additional membrane protein and ER-stress markers were probed. Na^+/K^+-ATPase was selected as a representative plasma membrane housekeeping protein whose expression remains stable under metabolic or ER stress conditions, serving as a control for nonspecific changes in membrane protein abundance. Na^+/K^+-ATPase levels were unchanged across treatments, and SC79 did not reduce PA-induced increases of BiP or CHOP (Fig. 3E), indicating that AKT activation does not broadly elevate membrane proteins or alleviate ER stress but can still restore GLUT2 and insulin secretion.

In conclusion, ERp46 depletion was demonstrated to enhance the PA-induced loss of p-AKT, GLUT2 and insulin secretion, while pharmacological AKT activation rescued GLUT2 and insulin without reducing ER-stress markers. These findings supported a model in which AKT acts downstream or in parallel to ER-stress pathways, with ERp46 helping to preserve AKT activity and β-cell function specifically under lipotoxic stress.

Discussion

The present study revealed the critical role of ERp46 in regulating GLUT2 expression and insulin secretion in pancreatic β-cells under PA-induced lipotoxic stress. These

findings demonstrated that ERp46 exerted a protective effect on β-cell function, primarily by alleviating PA-induced ER stress, which in turn helps sustain AKT phosphorylation and maintain GLUT2 expression. These observations suggested that AKT may act downstream or in parallel to ER stress, rather than being directly regulated by ERp46 under basal conditions. However, the possibility that ERp46 may influence AKT signaling more directly through effects on the folding or stability of upstream signaling components cannot be excluded and should be further investigated. The findings of the present study provide novel insights into the molecular mechanisms underlying β-cell dysfunction in T2DM.

PA-induced ER stress is a well-known contributor to β-cell dysfunction, leading to impaired insulin secretion and increased cell death through mechanisms such as protein misfolding and oxidative stress (23,30). Consistent with these observations, the present study showed that PA stimulation significantly decreased GLUT2 expression and insulin secretion in β-cells. Previous studies have shown that ER stress disrupts glucose sensing by reducing GLUT2 expression and impairing insulin granule exocytosis (22,23). Furthermore, prolonged ER stress was shown to contribute to β-cell apoptosis, further limiting insulin secretion capacity (10,18). The chronic activation of the UPR has been demonstrated to further promote β-cell apoptosis and functional decline under lipotoxicity (10,34). Of note, ERp46 expression was upregulated in response to PA, suggesting a compensatory mechanism to counteract ER stress. However, the knockdown of ERp46 further reduced GLUT2 expression and exacerbated the decline in insulin secretion, highlighting its protective role in maintaining β-cell functionality. As a member of the protein disulfide isomerase (PDI) family, ERp46 is essential for protein folding and redox homeostasis in the ER (6,12). This is consistent with its reported ability to mitigate cellular stress by ensuring proteostasis under adverse conditions. Concordantly, multiple PDI paralogs (such as PDI family A member 1 (PDIA1)/pyruvate dehydrogenase E1 component subunit beta and ERp57/PDIA3 modulate β-cell stress adaptation and insulin biosynthesis, underscoring a conserved ER proteostasis axis in the endocrine pancreas (24,35). Previous studies have further emphasized the role of ERp46 in alleviating ER stress and apoptosis in β-cells under diabetic conditions (22,31), suggesting its broader function in metabolic regulation. A recent comprehensive review of TXNDC5/ERp46 across diseases also highlighted its metabolic relevance and therapeutic tractability (29).

In addition to its role in maintaining ER homeostasis, ERp46 may influence GLUT2 expression, at least partly through the modulation of the AKT signaling pathway. AKT activation has been widely reported as a crucial regulator of glucose uptake and insulin secretion in β-cells (30,36). In the present study, PA stimulation significantly reduced p-AKT, and this effect was further exacerbated by ERp46 knockdown, suggesting that ERp46 supports AKT activation under lipotoxic conditions, which in turn may help preserve GLUT2 expression. Although total AKT levels remained unchanged across experimental groups, PA stimulation significantly reduced p-AKT levels, and this reduction was further exacerbated by ERp46 knockdown. This suggested that ERp46 primarily sustains AKT phosphorylation indirectly by alleviating ER stress, as siERp46 had no effect under baseline conditions but markedly suppressed AKT activity in the presence of PA. Thus, AKT is more likely to act downstream or in parallel to ER stress rather than being directly controlled by ERp46. Of note, treatment with SC79, a direct AKT activator, successfully rescued GLUT2 expression and restored insulin secretion in ERp46-depleted cells. This is consistent with broader evidence that phosphoinositide 3-kinase (PI3K)/AKT signaling is a central survival and metabolic pathway in β-cells and a candidate lever to counter lipotoxic dysfunction (37,38). These findings provided direct evidence that ERp46 supports GLUT2 expression and β-cell functionality by modulating AKT activation.

UPR signaling has been reported to intersect with AKT activity in other cellular contexts. For example, PERK-eIF2α signaling can suppress insulin biosynthesis, whereas adaptive inositol-requiring enzyme 1-X-box binding protein 1 and activating transcription factor 6 branches promote β-cell survival and proteostasis (39,40). Additional studies have suggested that maladaptive ER stress can impair AKT phosphorylation, while protective UPR responses may sustain it (41,42). Although these findings indicated potential crosstalk between UPR and AKT, whether this mechanism operates in β-cells under lipotoxic stress remains uncertain. The present data that ERp46 depletion reduces p-AKT under PA stress raise the possibility that ERp46 may act by stabilizing ER proteostasis, thereby preventing maladaptive UPR activation and indirectly supporting AKT activity. Nevertheless, the alternative hypothesis that ERp46 may also regulate AKT through more direct mechanisms, cannot be excluded.

The positive correlation between ERp46 and GLUT2 highlights the importance of maintaining ER homeostasis in preserving β-cell function under lipotoxic stress. Furthermore, the ability of AKT activation to mitigate the negative effects of ERp46 depletion suggests that targeting the ERp46-AKT-GLUT2 axis could offer a therapeutic strategy for improving β-cell survival and insulin secretion in T2DM. In parallel, interventions that lower ER stress, such as chemical chaperones or UPR modulators, have demonstrated the protection of β-cell mass and function in preclinical models and early translational studies (43-46), supporting an 'ER-proteostasis-first' approach that aligned with the present model. These findings supported the rationale that enhancing ERp46 activity may represent a novel strategy to stabilize β-cell proteostasis and insulin secretory function.

Beyond diabetes, ER stress is a convergent mechanism in multiple chronic diseases, including cardiovascular disease, neurodegeneration and cancer (40,47-50). Placing these results in this broader context underscores the translational significance of modulating ER proteostasis; targeting ERp46 or allied PDI nodes could complement metabolic therapies and potentially benefit co-morbid conditions characterized by secretory stress. Of note, the spleen has recently been recognized as an active immunometabolic hub that communicates with the gut and liver to shape lipid and glucose metabolism, as well as systemic inflammation, forming distinct spleen-organ axes (51). In addition, in a high-fat/streptozotocin rat model with splenectomy, adipose tissue-derived stem cells protected against T2D through the induction of spleen-derived IL-10; this benefit was blunted by splenectomy, highlighting a spleen-IL-10 anti-inflammatory circuit

with a metabolic impact (52). An independent study further showed that spleen-derived or exogenous IL-10 can dampen obesity-associated inflammation and insulin resistance in liver and adipose tissues (53). Of note, IL-10 has also been demonstrated to alleviate ER stress and apoptosis in non-islet tissues, such as cardiomyocytes under doxorubicin challenge (54) and skeletal muscle of aged mice (55). These findings suggested that splenic IL-10-driven anti-inflammatory circuits could, in principle, buffer β-cell ER stress and indirectly preserve AKT phosphorylation and GLUT2 expression, consistent with our ERp46-proteostasis model.

Despite these insights, the precise mechanisms through which ERp46 regulates AKT activation remain to be fully elucidated. It is possible that ERp46 interacts directly with components of the PI3K/AKT pathway, but it is also plausible that the effects are mediated indirectly through ER stress and UPR signaling. Further studies are warranted to dissect these molecular interactions and to explore the *in vivo* role of ERp46, particularly in diabetic animal models. In addition, complementary approaches such as conditional β-cell-specific ERp46-knockout models or the pharmacological modulation of ER stress may provide more definitive evidence. In addition, the involvement of other ER stress-related pathways in regulating GLUT2 expression and insulin secretion deserves further investigation. Collectively, these findings not only enhanced our understanding of β-cell dysfunction under lipotoxic stress but also underscored the potential of ERp46 as a promising therapeutic target for diabetes and possibly other ER stress-related diseases.

In conclusion, the present study demonstrated that ERp46 is a key regulator of GLUT2 expression and insulin secretion in β-cells under lipotoxic stress. Rather than directly activating AKT, ERp46 primarily alleviated PA-induced ER stress, which in turn supported AKT phosphorylation and helped preserve GLUT2 expression. These findings provided a mechanistic basis for the development of therapeutic strategies targeting ER stress modulators in combination with ERp46- and AKT-related pathways to combat β-cell dysfunction in T2DM.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

DC acquired the funding and contributed to the conceptualization, data curation, formal analysis, investigation, methodology, and drafting and revision of the manuscript. CH contributed to the conceptualization, investigation, methodology, project administration, supervision and manuscript revision. XC contributed to the investigation, data curation and formal analysis. YT and KW contributed to data curation and formal analysis. DC and CH confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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