Exendin-4 protects INS-1 cells against palmitate-induced apoptosis through the IRE1α-Xbp1 signaling pathway

DONGDONG JIANG[1] and FANG WAN[2]

[1]Department of Endocrinology and Metabolism, Huashan Hospital, Shanghai Medical College, Fudan University; [2]Department of Orthopedic Sports Medicine, Huashan Hospital, Fudan University, Shanghai 200040, P.R. China

Received July 19, 2017; Accepted May 24, 2018

DOI: 10.3892/etm.2018.6240

Abstract. The anti-apoptotic effect of the incretin analog, exendin-4 (EX-4) on pancreatic β cells is mediated via the activation of protein kinase B (Akt) signaling, and its effect is partly produced through the inhibition of endoplasmic reticulum (ER) stress. However, the molecular mechanisms that underlie the effect of EX-4 on the suppression of ER stress and the upregulation of Akt signaling are poorly understood. Inositol-requiring enzyme 1 (IRE1), a member of the ER-localized transmembrane protein family, activates its downstream transcription factor X-box binding protein 1 (XBP1) to mediate a key part of the cellular unfolded protein response in order to cope with ER stress. Using the clonal rat pancreatic β cell line INS-1, the present study produced an *in vitro* model of ER stress using palmitate (PA) in order to determine whether the beneficial effect of EX-4 under ER stress was regulated by the IRE1α-Xbp1 signaling pathway. The results demonstrated that the reduction in ER stress and the activation Akt by EX-4 may be associated with the upregulation of IRE1α phosphorylation and the splicing of Xbp1 mRNA, which improved PA-reduced cell viability. This effect was partially abrogated by the knockdown of IRE1α with small interfering RNA. Additionally, cellular IRE1α was phosphorylated by the protein kinase A (PKA) associated with EX-4 and the activation of IRE1α, as IRE1α phosphorylation was attenuated by the inhibition of PKA with its inhibitor. In conclusion, the data identified the IRE1α-Xbp1 signaling pathway as an essential mediator that associates EX-4 with the intracellular mechanism that inhibits ER stress and activates Akt in order to regulate β cell survival. This may provide important evidence for the use of EX-4 in treatments for type 2 diabetes.

Correspondence to: Dr Fang Wan, Department of Orthopedic Sports Medicine, Huashan Hospital, Fudan University, 12 Middle Urumqi Road, Shanghai 200040, P.R. China
E-mail: wolfwf01@gmail.com

Key words: exendine-4, endoplasmic reticulum stress, apoptosis, inositol-requiring enzyme 1α, X-box binding protein 1

Introduction

In type 2 diabetes, insulin resistance triggers the compensation response in β cells, including increased biosynthesis and secretion of insulin as well as proliferation of β cells (1). In eukaryotic cells, augmented protein folding demand perturb ER homeostasis and lead to a condition defined as endoplasmic reticulum (ER) stress (2). Likewise, increased demand for insulin secretion brings about a state of metabolic ER stress in β cells, and β cells are considered to be very sensitive to ER stress, which contributes to loss of β-cells that underlies the pathogenesis of type 2 diabetes (3).

The unfolded protein response (UPR), identified as expanding the protein-folding capacity of the ER, plays a pivotal role in the control of cell fate for survival under ER stress (4). If the UPR fails to restore homeostasis under excessive ER stress, cells will undergo apoptosis. Three mammalian ER-resident transmembrane proteins, inositol-requiring enzyme 1 (IRE1), PKR-like endoplasmic reticulum kinase (PERK), and activating transcription factor 6 (ATF6), mediate the canonical signaling branches of the UPR that orchestrate the adaptive response to resolve ER stress (5). IRE1α is the most highly conserved ER stress sensor which processes dual Ser/Thr kinase and endoribonuclease (RNase) activities in its cytoplasmic portion (6,7). In response to ER stress, IRE1α is activated through oligomerization and autophosphorylation (8,9). In mammals, activation of IRE1α's RNase activity leads to removal of a 26-nucleotide intron within the mRNA encoding the downstream transcription factor X-box binding protein 1 (XBP1). This non-conventional splicing event generates the spliced active form of XBP1 (XBP1s), which drives a major transcriptional program of the UPR (10-12). Proper activation of the IRE1a elicits the cytoprotective actions of the UPR and is essential to maintain cellular homeostasis and survival in response to ER stress (13). Notably, the IRE1α-Xbp1 pathway has been implicated in the homeostatic regulation of pancreatic islet β cells (14).

GLP-1 and its analogy exendin-4 (EX-4), has been shown to promote β cell replication and prevent β cell exhaustion under the diabetic conditions (15), and this effect was explained partly through the inhibition of ER stress (16). Therefore, in the present study, we tested the hypothesis that EX-4 could regulate β cell mass through IRE1α-Xbp1

signaling pathway. Our results demonstrate that the induction of IRE1α phosphorylation and the resultant splicing of Xbp1 mRNA evoked by EX-4 treatment could promote rapid phosphorylation of protein kinase B (Akt) and nuclear exclusion of FoxO1 in INS-1 cells, which improve cells survival under lipotoxic-induced ER stress condition.

Materials and methods

Cell culture and treatments. INS-1 832/13 (Cell Culture Centre, CAMS, Beijing, China) cells were maintained in RPMI-1640 containing 11.1 mM D-glucose, 10% fetal calf serum, penicillin (100 U/ml) and streptomycin (100 mg/ml), 10 mM Hepes, 2 mM L-glutamine, 1 mM sodium pyruvate, and 0.05 mM 2-bmercaptoethanol. After the cells reached 70% confluence, the medium was replaced with RPMI-1640 containing BSA-conjugated sodium palmitate (PA; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) at concentrations of 0.5 mM, with or without EX-4 (50 nM; Sigma-Aldrich, Merck KGaA), or 0.5% BSA as control for 24 h.

Cell transfection. To knock down IRE1α or RACK1 in INS-1 cells, cells were transfected with small interfering RNA (siRNA; Genepharma, Shanghai, China) targeting IRE1α (sequences: 5'-GGAATTACTGGCTTCTCATAG') or RACK1 (target sequences: 5'-GCTAAAGACCAACCACATTGG-3') at a concentration of 20 μM. Parallel cell cultures were transfected with control siRNA containing scrambled non-targeted sequence (5'-GTTCTCCGAACGTGTCACGTTT-3') at the equal concentrations (Genepharma). INS-1 cells were transfected with the plasmid containing human XBP1 s (Addgene Inc., Cambridge, MA, USA) for XBP1s overexpression. At 48 h after transfection, the medium was replaced with regular medium containing PA (0.5 mM), with or without EX-4 (50 nM), Forskolin (10 μM; Sigma-Aldrich; Merck KGaA) or H89 (10 μM; Sigma-Aldrich; Merck KGaA) for 24 h, INS-1 cells precultured for 30 min with PKA inhibitor H89 were treated with EX-4.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was isolated from INS-1 cells using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA). First-strand cDNA was synthesized with moloney murine leukemia virus (M-MLV) reverse transcriptase (Invitrogen) and random hexamer primers (Invitrogen; Thermo Fisher Scientific, Inc.). RT-qPCR was conducted using the SYBR Green PCR system, following the manufacturer's recommendations (Applied Biosystems; Thermo Fisher Scientific, Inc.). GAPDH was used as an internal control for normalization. The oligonucleotide primers used are shown in Table I.

Co-immunoprecipitation (Co-IP). For coimmunoprecipitation analysis, INS-1 cells were lysed with the lysis buffer [20 mM tris-HCl (pH 7.5), 100 mM KCl, 0.1% Nonidet P-40, 1 mM EDTA, and 10% glycerol containing 1 mM phenyl-methyl-sulfonyl-fluoride (PMSF), 1% Protease Inhibitor Cocktail (Sigma-Aldrich; Merck KGaA), and 1% Phosphatase Inhibitor Cocktails I/II (Sigma-Aldrich; Merck KGaA) for 0.5 h at 4˚C. After incubation with the desired primary antibody for 18 h at 4˚C via gentle rocking, immune complexes were captured by mixing with a final concentration of 2.5% protein G Sepharosebeads (Amersham; GE Healthcare, Chicago, IL, USA) for 2 h at 4˚C on a rotator. Anti-IRE1α antibody was used in the Co-IP assays. Beads were subsequently washed three times with the washing buffer [20 mM tris-HCl (pH 7.5), 150 mM KCl, 0.5% Nonidet P-40, 1 mM EDTA, and 10% glycerol supplemented with 1 mM PMSF, 1% Protease Inhibitor Cocktail, and 1% Phosphotase Inhibitor Cocktails I/II], followed by SDS-PAGE and immunoblotting analysis after elution by boiling in 2X SDS loading buffer.

Antibodies and immunoblotting. Antibodies against phospho-Akt (Ser473, no. 9271), phosphor-eIF2α (Ser 51, no. 3597), phospho-FoxO1 (Thr24, no. 9464), IRE1α (no. 3294), XBP1s (no. 12782), phosphor-CREB (Ser133, no. 9196), eIF2α (no. 9722), Akt (no. 4691), FoxO1 (no. 2880), CREB (no. 9197), RACK1 (no. 5432), PKA (no. 4782) were purchased from Cell Signaling Technologies, Inc. (Danvers, MA, USA). α-tubulin antibody (T6199) from Sigma-Aldrich and antibody against phospho-IRE1α (Ser724, ab124945) from Abcam (Cambridge, MA, USA). Tubulin antibody was diluted 1:10,000 and all other antibodies were diluted 1:1,000. For immune-blotting, cellular lysates were prepared by RIPA buffer. Protein extracts were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a polyvinylidene difluoride (PVDF) membrane filter (Merck KGaA, Darmstadt, Germany). After incubation with desired antibodies, the blots were developed with Thermo Scientific's SuperSignal West Pico Chemiluminescent substrate or Millipore's Immunobilon Western Chemiluminescent HRP substrate.

Cell viability and apoptosis assay. INS-1 cell viability was measured using WST-8 assay using Cell Counting Kit-8 (CCK-8; Dojindo, Gaithersburg, MD, USA) according to manufacturer's instruction. INS-1 β cells were seeded in 96-well culture plates at a density of 10^5/ml. The next day, the culture medium was replaced with RPMI-1640 containing BSA-conjugated PA at concentrations of 0.5 mM, with or without EX-4, or 0.5% BSA as control for 24 h. The CCK-8 assay reagent was added to the culture medium for the final 3 h. A microplate reader was used to measure the absorbance at 450 nm.

For apoptosis analysis, cells were washed twice with 1X binding buffer then labelled with Annexin V and propidium iodide (PI) following the manufacturer's instructions. The Apoptosis Analysis kit was ordered from Beyotime Institute of Biotechnology (Haimen, China). Cell apoptosis was analyzed by fluorescence-activated cell sorting (FACS) using a FACScan flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA).

Statistical analysis. All data are expressed as the mean ± standard error mean. Student's t-test was used to compare mean values between two groups using the Graph-Pad Prism 5 program (GraphPad Software, Inc., La Jolla, CA, USA). $P<0.05$ was considered to indicate a statistically significant difference.

Table I. Primer sequences used for reverse transcription-quantitative polymerase chain reaction.

Primer	Forward sequence (5'-3')	Reverse sequence (5'-3')
Bim	AGAGATACGGATCGCACAGG	GTCTTCCGCCTCTCGGTAAT
GAPDH	AGTTCAACGGCAGTCAAG	TACTCAGCACCAGCATCACC

Bim, B-cell lymphoma-2-like protein 11.

Results

EX-4 inhibits ER stress and stimulates IRE1α-Xbp1 signaling pathway. Exposure to free fatty acids such as PA has been shown to cause ER stress (17). In our study, 0.5 mM PA for 24 h did cause a significant increase in the protein level of eukaryotic initiation factor 2α (eIF2α), a ER stress marker, as well as the decrease in the phosphorylation of Akt (p-Akt) and its physiological target FoxO1 (p-FoxO1) (Fig. 1A), which leads to decreased cell viability (Fig. 1B) and increased Bim mRNA expression in INS-1 cells (Fig. 1C). The activation of Bim has been supposed to trigger cleavage of caspases and is critical for the apoptosis in β cells (18). In accord with this, PA induced significant cell apoptosis in INS-1 cells (Fig. 1D). However, EX-4 treatment induced a significant reverse in the protein level of p-Akt and p-FoxO1, displaying a protective role on the INS-1 cells, as demonstrated by the increased cell viability and decreased expression of Bim. IRE1α-Xbp1 signaling pathway has been implicated in the homeostatic regulation of pancreatic islet β cells, we then tested whether EX-4-induced protective roles on INS-1 cells was associated with this signaling pathway. We found that PA triggers the mild increase in the phosphorylation at the Ser 724 activation site in IRE1α as detected by a phosphorylation site-specific antibody, which triggered the increase of XBP1 s protein levels (Fig. 1A). Interestingly, the protein level of p-IRE1α was further increased in response to EX-4 treatment (Fig. 1A), accompanied by the enhancement in XBP1s, suggesting that EX-4 could stimulate IRE1α-Xbp1 signaling pathway.

EX-4-induced inhibition of ER stress and improvement of p-Akt is mediated by the stimulation of IRE1 α-Xbp1 signaling pathway. To determine whether IRE1α-Xbp1 signaling pathway acts as a critical component in mediating EX-4's beneficial effects in PA-induced INS-1 cells, we used siRNA to silence the IRE1α gene. INS-1 cells were transfected with siRNA specific for IRE1α and the efficiency of IRE1α knockdown was validated (Fig. 2A). In nontransfected cells treated with EX-4, a significant increase in p-Akt and a drastic decrease in p-eIF2α protein levels was observed (Fig. 1A), in contrast, transfection of INS-1 cells with siRNA targeting IRE1α, the expression of p-Akt was only partially improved by EX-4 treatment under PA-treated conditions (Fig. 2A). The decreased cell viability and increased mRNA level of Bim and cell apoptosis were also not ameliorated (Fig. 2B-D). As IRE1α associates with various signaling molecules, we then tested whether EX-4-induced upregulation of p-Akt is through the splicing of Xbp1 mRNA by IRE1α phosphorylation. In INS-1 cells with RNAi-mediated knockdown of IRE1α, the decreased protein levels of p-Akt and cell viability under EX-4 treatment was rescued by XBP1s overexpression (Fig. 2A and B), while the mRNA level of Bim and cell apoptosis markedly decreased (Fig. 2C and D) This data indicates that the beneficial roles of EX-4 treatment on PA-induced INS-1 cells might be associated with the IRE1α-Xbp1 signaling pathway.

EX-4-induced phosphorylation of IRE1α is dependent on PKA. Previous research has supported the idea that under ER stress conditions, IRE1α is activated through dimerization and transautophosphorylation (8,9). To test the idea that a protein kinase other than IRE1α itself may link EX-4 with the observed stimulation of IRE1α phosphorylation, we examined whether protein kinase A (PKA) is involved in the stimulation of IRE1α phosphorylation by EX-4. We found that EX-4 could promote the activation of PKA, as evidenced by the increased expression of cAMP response element-binding protein (CREB) phosphorylation (p-CREB), as well as the upregulation of IRE1α phosphorylation (Fig. 3). In accordance with this, forskolin, a chemical activator of PKA, also triggered the increase of IRE1α phosphorylation, while the increase in the IRE1α phosphorylation by EX-4 was suppressed by the inhibition of PKA using H89 (Fig. 3), a pharmacological PKA inhibitor, implying that EX-4-regulated IRE1α-phosphorylation is mediated through a PKA-dependent manner.

RACK1 is essential for PKA-dependent IRE1α phosphorylation in response to EX-4. RACK1, binding to membrane receptors and protein kinases, coordinates the interactions between signaling components in multiple cellular processes (19). We found that EX-4 treatment induced the association of IRE1α with PKA and RACK1 by Co-IP analysis (Fig. 4A). More importantly, INS-1 cells with knockdown of RACK1, although presenting increased p-CREB by EX-4, showed the absent increased IRE1α phosphorylation compared to cells transfected with a scrambled negative control siRNA (Fig. 4B). This confirms a possible function of RACK1 in the recruitment of PKA to phosphorylated IRE1α in response to EX-4 treatment.

Discussion

The suppression of ER stress and the activation of Akt in GLP-1-mediated β cell survival under lipotoxic conditions has previously been demonstrated (16,20), but the mechanisms are not well established. The present study provides evidence that induction of the IRE1α-Xbp1 axis, a signaling branch of the

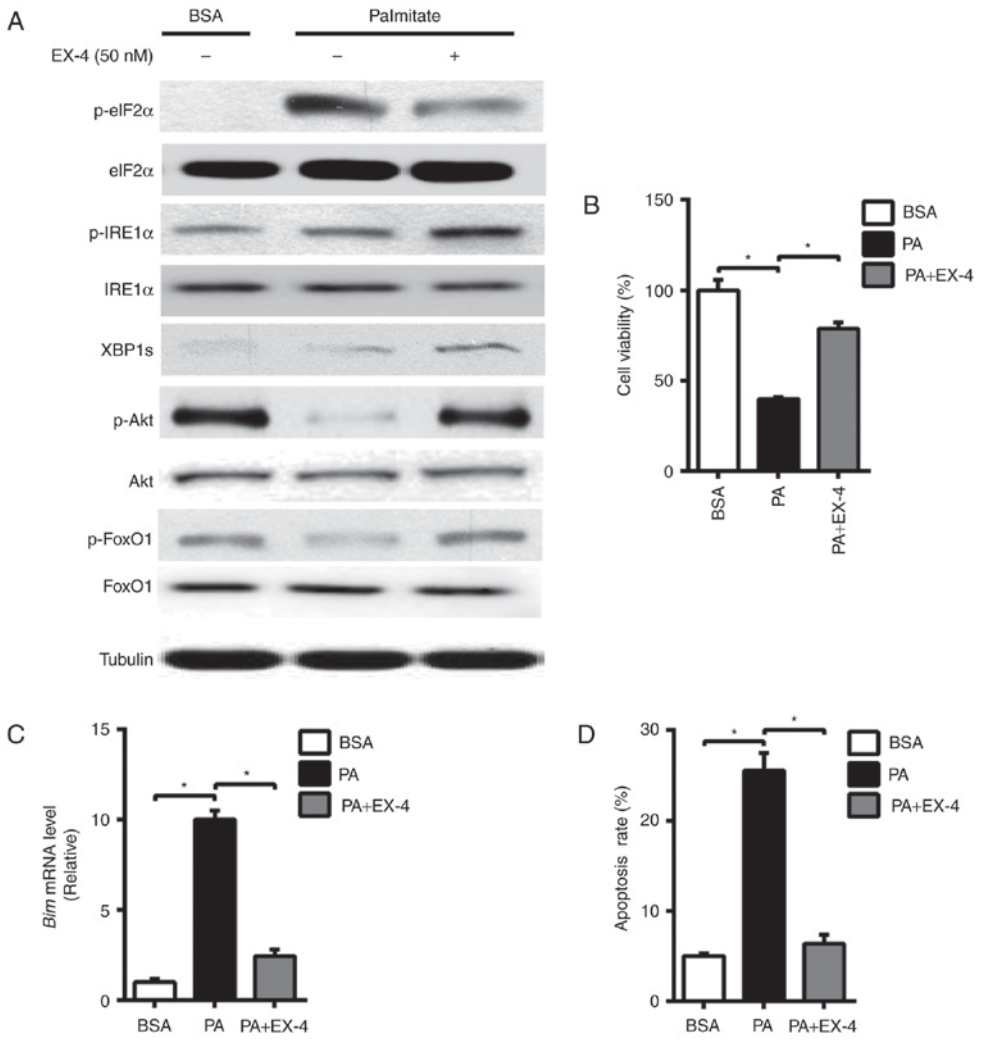

Figure 1. EX-4 exerts a protective role on palmitate-induced apoptosis and stimulates the IRE1α-Xbp1 signaling pathway in INS-1 cells. INS-1 cells were treated with PA (0.5 mM) and to which EX-4 (50 nM) was then added for 24 h. (A) Western blot analysis of p-eIF2α, eIF2α, p-Akt, Akt, p-FoxO1, FoxO1, p-IRE1α, IRE1α, XBP1s and Tubulin. (B) Cell viability. (C) Reverse transcription-quantitative polymerase chain reaction for Bim mRNA expression. (D) Cell apoptosis rate was determined by flow cytometry using Annexin V and propidium iodide. Tubulin was used as the internal control. Data are presented as the mean ± standard error mean of three independent experiments. *P<0.05, as indicated. IRE1α, inositol-requiring enzyme 1α; Xbp1, X-box binding protein 1; EX-4, exendin-4; p-, phosphorylated-; eIF2α, eukaryotic initiation factor 2α; Akt, protein kinase B; FoxO1, forkhead box protein O1; Bim, B-cell lymphoma-2-like protein 11; BSA, bovine serum albumin; PA, sodium palmitate.

UPR, by EX-4 treatment contributes to the alleviation of ER stress and the activation of Akt, which exerts anti-apoptotic roles on PA-treated INS-1 cells. These novel insights connect the EX-4 and the UPR response in a complex chain of events that inhibits ER stress and promotes β cell survival.

In mammals, IRE1α is recognized as a signaling branch to mediate UPR by splicing Xbp1 mRNA to generate an active form of this transcription factor that induces a major transcriptional program of the UPR (10,11,14). UPR can ameliorate ER stress by enhancing the ER's capacity to manage the workload of protein folding (5). In an *in vitro* 'ER stress' model produced by prolonged exposure of PA, EX-4-induced inhibition of ER stress and the improvement of cell viability is linked to the activation of IRE1α-Xbp1 signaling pathway, suggesting that IRE1α-Xbp1 may be involved in mediating the anti-apoptotic role of EX-4 on INS-1 cells by suppressing ER stress.

Activation of Akt results in the phosphorylation of various downstream protein targets that affect proliferation, cell cycle entry and intracellular apoptotic pathways (21,22). Akt signaling has been shown to protect β cells from ER stress-induced apoptosis and mediate part of the anti-apoptotic effects of GLP-1 agonists (23-25). FoxO1, a downstream target of Akt pathway, triggers cell death in different types of cells which involves transactivation of BH3-only molecule Bim (26-29). Bim is a member of pro-apoptotic BH3-only protein that plays an important role in mediating β cell apoptosis (18). Phosphorylation of FoxO1 by Akt results in the inhibition of FoxO1-dependent transcription that elicits the protective roles of pancreatic β cells against cell lipoapoptosis (30,31). Our data showed that IRE1α may be involved in the improvement of the phosphorylation of Akt, as the deficiency of IRE1α down-regulated its phosphorylation. Our global analysis of protein expression in IRE1α-deficient cells pointed to the suppression of ER stress and increase in the Akt signaling as possible mechanisms for the IRE1α-mediated β cell protection in response to EX-4 treatment. In agreement with anti-apoptotic role of IRE1α-Xbp1 signaling pathway, IRE1α has been previously shown to drive compensatory β cell proliferation

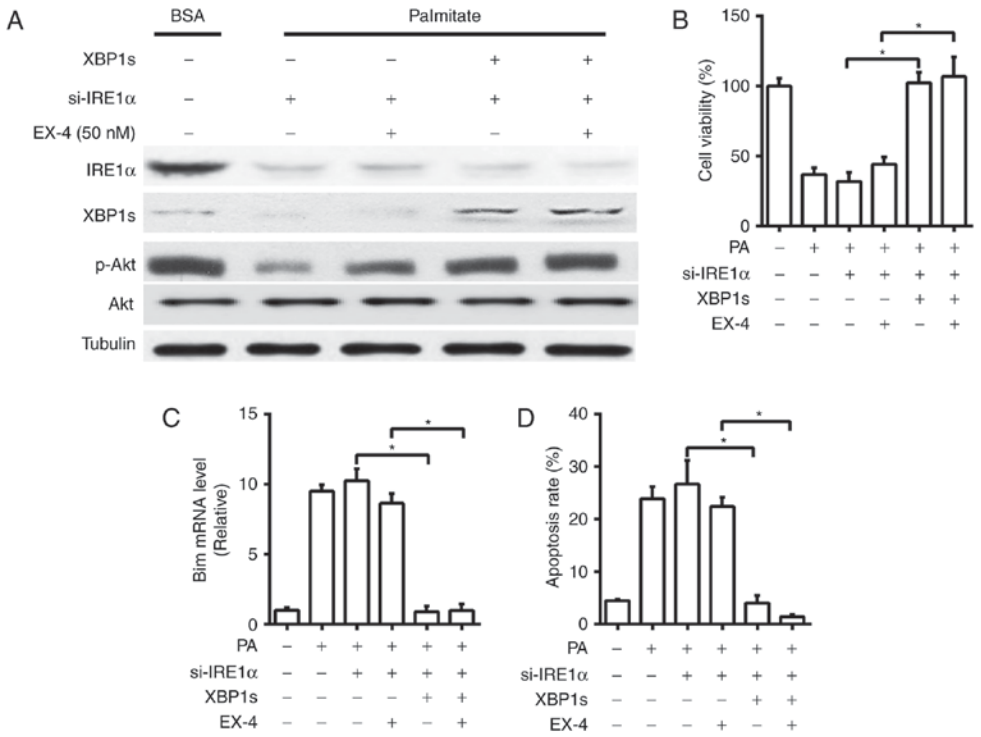

Figure 2. IRE1α-Xbp1 signaling pathway mediates the protective role of EX-4 on the palmitate-induced apoptosis in INS-1 cells. INS-1 cells with RNAi-mediated knockdown of IRE1α, coupled with or without the overexpression of XBP1s by transfection with a plasmid containing human XBP1s, were treated with PA (0.5 mM), with or without EX-4 (50 nM), for 24 h. (A) Western blot analysis of IRE1α, XBP1s, p-Akt, Akt and Tubulin. (B) Cell viability. (C) Reverse transcription-quantitative polymerase chain reaction for Bim mRNA expression. (D) Cell apoptosis rate was determined by flow cytometry using Annexin V and propidium iodide. Tubulin was used as the internal control. Data are presented as the mean ± standard error mean of three independent experiments. *P<0.05, as indicated. IRE1α, inositol-requiring enzyme 1α; Xbp1, X-box binding protein 1; EX-4, exendin-4; p-, phosphorylated-; si-, small interfering; Akt, protein kinase B; Bim, B-cell lymphoma-2-like protein 11; BSA, bovine serum albumin; PA, sodium palmitate.

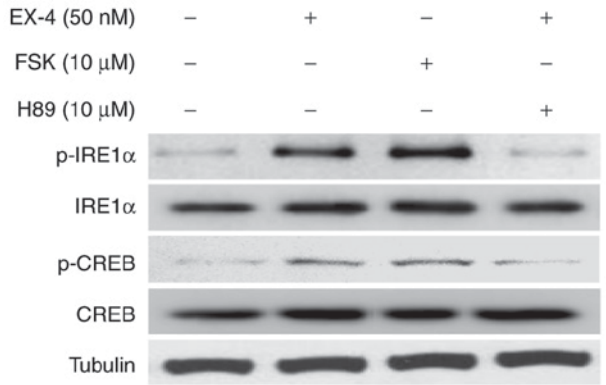

Figure 3. Protein kinase A is required for EX-4-induced phosphorylation of IRE1α. INS-1 cells were treated with Sodium palmitate (0.5 mM), to which FSK (10 μM), EX-4 (50 nM) or EX-4 with H89 (10 μM), was then added for 24 h. Western blot analysis was then performed for p-CREB, CREB, p-IRE1α, IRE1α and Tubulin. Tubulin was used as the internal control. Tubulin was used as the internal control. IRE1α, inositol-requiring enzyme 1α; EX-4, exendin-4; FSK, Forskolin; p-, phosphorylated-; CREB, cAMP response element-binding protein.

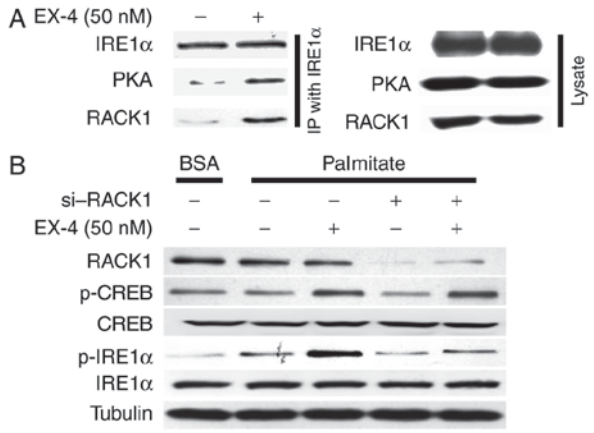

Figure 4. RACK1 is essential for PKA-dependent IRE1α phosphorylation in response to EX-4 treatment. (A) INS-1 cells were treated with Sodium palmitate (0.5 mM) and then with or without EX-4 (50 nM) for 24 h. Immunoprecipitation was performed with anti-IRE1α, followed by immunoblotting with RACK1, PKA and IRE1α antibodies. Immunoprecipitation reactions were replicated three times. (B) INS-1 cells with RNAi-mediated knockdown of RACK1, were then treated with Sodium palmitate (0.5 mM), with or without EX-4 (50 nM) for 24 h. Western blot analysis of RACK1, p-CREB, CREB, p-IRE1α, IRE1α and Tubulin was then performed. Tubulin was used as the internal control. RACK1, receptor for activated C kinase 1; PKA, protein kinase A; IRE1α, inositol-requiring enzyme 1α; EX-4, exendin-4; BSA, bovine serum albumin; p-, phosphorylated-; CREB, cAMP response element-binding protein.

during metabolic ER stress through XBP1s regulation of cell cycle machinery, as demonstrated by decreased islet mass and hypoinsulinemia in Ire1α$^{f/f}$: Cre mice when challenged with HFD feeding (14). Previous research also demonstrated the role of XBP1s on the protection of β cells against apoptosis through the induction of Akt (32). It is worth noting that our data showed that IRE1α may modulate Akt phosphorylation through an XBP1 dependent manner as well. In fact, the regulatory role of XBP1s on Akt activity has also been reported in

other cell types (33,34). Besides, an earlier study reported that in islets of db/db mice, increased activation of xbp1 mRNA was observed, presumably a compensatory mechanism to inhibit ER stress and β cell apoptosis induced by obesity (35). Our data raise the possibility that IRE1α-Xbp1 axis may serve as a component to regulate Akt phosphorylation in response to EX-4 treatment. Nonetheless, additional signaling regulators may also be involved in coordinating the anti-apoptotic actions of the IRE1α-Xbp1 branch. For instance, PI3K of the insulin/IGF-1 pathway can interact with XBP1s to modulate its functional behavior, implying that XBP1s may likely serve as a component of the insulin/IGF-1 signaling cascade in promoting β cell proliferation (36).

Notably, it was documented that IRE1α could also regulate insulin biosynthesis. For instance, increased phosphorylation of IRE1α is coupled to insulin production through mechanisms that do not involve Xbp1 splicing in response to acute glucose stimulation, whereas prolonged activation of IRE1α leads to suppression of insulin production after chronic exposure to high glucose (2). Interestingly, genetic deletion of Xbp1 specifically in pancreatic islet β cells of mice was reported to cause defective proinsulin processing and insulin secretion, as a result of the feedback hyperactivation of IRE1α that in turn degrades mRNAs encoding enzymes for proinsulin processing (37). Based on this evidence, it's likely that the the role of IRE1α in integrating ER stress could regulate both the cell mass and the function of β cells.

The activation of IRE1α is considered to be activated through autophosphorylation to initiate a key signaling arm of the mammalian UPR pathways (8). Previous research showed the anti-apoptotic effects of the GLP-1 analog in primary-cultured neonatal rat β cells could be reproduced by the activator of PKA (38), implying that PKA plays an important role in mediating the anti-apoptotic effect of GLP-1 analog. In our study, the early induction of IRE1α phosphorylation by PA may constitute an important component of UPR to cope with ER stress, while the further augment of IRE1α phosphorylation in response to EX-4 treatment was blunted by the pharmacological PKA inhibitor H89, suggesting that PKA, other than IRE1α itself, may link EX-4 with the observed stimulation of IRE1α phosphorylation. This finding is supported by a recent study showed that hepatic PKA can directly phosphorylate IRE1α at Ser 724 that modulates the metabolic activation of IRE1α in the mice with obesity (7). Although a direct activation of Akt by PKA cannot be completely excluded in the present study, our data favor the idea that PKA activates Akt through IRE1α-Xbp1 signaling pathway by EX-4. Furthermore, our study revealed a crucial role of RACK1, a multifaceted scaffolding protein, in the regulation of IRE1α phosphorylation by PKA in response to EX-4. RACK1 is a scaffold protein which contains seven Trp-Asp 40 (WD40) repeats, which binds to membrane receptors and protein kinases as well as coordinates the interactions between signaling components in the cellular processes (19,39,40). However, the precise pathophysiological effect of RACK1-dependent regulation of IRE1α by PKA in response to EX-4 remains to be further investigated.

In conclusion, we show that IRE1α-Xbp1 signaling pathway contributes to cope with ER stress and activate Akt signaling, thereby preventing PA-induced β cell apoptosis under EX-4 treatment conditions. A better understanding of the molecular components that link the EX-4 and UPR with the anti-apoptotic control of β cells may shed light on the new mechanisms of the EX-4 treatment for type 2 diabetes.

Acknowledgements

Not applicable.

Funding

The present study was supported by the National 863 Hi-Tech Project (grant no. 2015AA033703) and the National Key R&D Program of China (grant nos. 2016YFC1100300, 2016YFC1100304, 2017YFC0840100 and 2017YFC0840106).

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

FW and DJ conceived the initial idea of the work, supervised the study and wrote the manuscript. DJ contributed to the design of the study, and performed the experiments and data analysis. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Sachdeva MM and Stoffers DA: Minireview: Meeting the demand for insulin: Molecular mechanisms of adaptive postnatal beta-cell mass expansion. Mol Endocrinol 23: 747-758, 2009.
2. Lipson KL, Fonseca SG, Ishigaki S, Nguyen LX, Foss E, Bortell R, Rossini AA and Urano F: Regulation of insulin biosynthesis in pancreatic beta cells by an endoplasmic reticulum-resident protein kinase IRE1. Cell Metab 4: 245-254, 2006.
3. Wajchenberg BL: Beta-cell failure in diabetes and preservation by clinical treatment. Endocr Rev 28: 187-218, 2007.
4. Hetz C: The unfolded protein response: Controlling cell fate decisions under ER stress and beyond. Nat Rev Mol Cell Biol 13: 89-102, 2012.
5. Ron D and Walter P: Signal integration in the endoplasmic reticulum unfolded protein response. Nat Rev Mol Cell Biol 8: 519-529, 2007.
6. Bell GI and Polonsky KS: Diabetes mellitus and genetically programmed defects in beta-cell function. Nature 414: 788-791, 2001.
7. Mao T, Shao M, Qiu Y, Huang J, Zhang Y, Song B, Wang Q, Jiang L, Liu Y, Han JD, *et al*: PKA phosphorylation couples hepatic inositol-requiring enzyme 1alpha to glucagon signaling in glucose metabolism. Proc Natl Acad Sci USA 108: 15852-15857, 2011.

8. Shamu CE and Walter P: Oligomerization and phosphorylation of the Ire1p kinase during intracellular signaling from the endoplasmic reticulum to the nucleus. EMBO J 15: 3028-3039, 1996.
9. Welihinda AA and Kaufman RJ: The unfolded protein response pathway in Saccharomyces cerevisiae. Oligomerization and trans-phosphorylation of Ire1p (Ern1p) are required for kinase activation. J Biol Chem 271: 18181-18187, 1996.
10. Calfon M, Zeng H, Urano F, Till JH, Hubbard SR, Harding HP, Clark SG and Ron D: IRE1 couples endoplasmic reticulum load to secretory capacity by processing the XBP-1 mRNA. Nature 415: 92-96, 2002.
11. Lee K, Tirasophon W, Shen X, Michalak M, Prywes R, Okada T, Yoshida H, Mori K and Kaufman RJ: IRE1-mediated unconventional mRNA splicing and S2P-mediated ATF6 cleavage merge to regulate XBP1 in signaling the unfolded protein response. Genes Dev 16: 452-466, 2002.
12. Yoshida H, Matsui T, Yamamoto A, Okada T and Mori K: XBP1 mRNA is induced by ATF6 and spliced by IRE1 in response to ER stress to produce a highly active transcription factor. Cell 107: 881-891, 2001.
13. Lin JH, Li H, Yasumura D, Cohen HR, Zhang C, Panning B, Shokat KM, Lavail MM and Walter P: IRE1 signaling affects cell fate during the unfolded protein response. Science 318: 944-949, 2007.
14. Xu T, Yang L, Yan C, Wang X, Huang P, Zhao F, Zhao L, Zhang M, Jia W, Wang X and Liu Y: The IRE1α-XBP1 pathway regulates metabolic stress-induced compensatory proliferation of pancreatic β-cells. Cell Res 24: 1137-1140, 2014.
15. Drucker DJ: Glucagon-like peptide-1 and the islet beta-cell: Augmentation of cell proliferation and inhibition of apoptosis. Endocrinology 144: 5145-5148, 2003.
16. Tsunekawa S, Yamamoto N, Tsukamoto K, Itoh Y, Kaneko Y, Kimura T, Ariyoshi Y, Miura Y, Oiso Y and Niki I: Protection of pancreatic beta-cells by exendin-4 may involve the reduction of endoplasmic reticulum stress; in vivo and in vitro studies. J Endocrinol 193: 65-74, 2007.
17. Achard CS and Laybutt DR: Lipid-induced endoplasmic reticulum stress in liver cells results in two distinct outcomes: Adaptation with enhanced insulin signaling or insulin resistance. Endocrinology 153: 2164-2177, 2012.
18. Ren D, Sun J, Mao L, Ye H and Polonsky KS: BH3-only molecule Bim mediates β-cell death in IRS2 deficiency. Diabetes 63: 3378-3387, 2014.
19. McCahill A, Warwicker J, Bolger GB, Houslay MD and Yarwood SJ: The RACK1 scaffold protein: A dynamic cog in cell response mechanisms. Mol Pharmacol 62: 1261-1273, 2002.
20. Buteau J, El-Assaad W, Rhodes CJ, Rosenberg L, Joly E and Prentki M: Glucagon-like peptide-1 prevents beta cell glucolipotoxicity. Diabetologia 47: 806-815, 2004.
21. Scheid MP and Woodgett JR: PKB/AKT: Functional insights from genetic models. Nat Rev Mol Cell Biol 2: 760-768, 2001.
22. Hajduch E, Litherland GJ and Hundal HS: Protein kinase B (PKB/Akt)-a key regulator of glucose transport? FEBS Lett 492: 199-203, 2001.
23. Wrede CE, Dickson LM, Lingohr MK, Briaud I and Rhodes CJ: Fatty acid and phorbol ester-mediated interference of mitogenic signaling via novel protein kinase C isoforms in pancreatic beta-cells (INS-1). J Mol Endocrinol 30: 271-286, 2003.
24. Srinivasan S, Ohsugi M, Liu Z, Fatrai S, Bernal-Mizrachi E and Permutt MA: Endoplasmic reticulum stress-induced apoptosis is partly mediated by reduced insulin signaling through phosphatidylinositol 3-kinase/Akt and increased glycogen synthase kinase-3beta in mouse insulinoma cells. Diabetes 54: 968-975, 2005.
25. Cunha DA, Ladrière L, Ortis F, Igoillo-Esteve M, Gurzov EN, Lupi R, Marchetti P, Eizirik DL and Cnop M: Glucagon-like peptide-1 agonists protect pancreatic beta-cells from lipotoxic endoplasmic reticulum stress through upregulation of BiP and JunB. Diabetes 58: 2851-2862, 2009.
26. Gilley J, Coffer PJ and Ham J: FOXO transcription factors directly activate bim gene expression and promote apoptosis in sympathetic neurons. J Cell Biol 162: 613-622, 2003.
27. Stahl M, Dijkers PF, Kops GJ, Lens SM, Coffer PJ, Burgering BM and Medema RH: The forkhead transcription factor FoxO regulates transcription of p27Kip1 and Bim in response to IL-2. J Immunol 168: 5024-5031, 2002.
28. Dijkers PF, Birkenkamp KU, Lam EW, Thomas NS, Lammers JW, Koenderman L and Coffer PJ: FKHR-L1 can act as a critical effector of cell death induced by cytokine withdrawal: Protein kinase B-enhanced cell survival through maintenance of mitochondrial integrity. J Cell Biol 156: 531-542, 2002.
29. Dijkers PF, Medema RH, Lammers JW, Koenderman L and Coffer PJ: Expression of the pro-apoptotic Bcl-2 family member Bim is regulated by the forkhead transcription factor FKHR-L1. Curr Biol 10: 1201-1204, 2000.
30. Kitamura T, Nakae J, Kitamura Y, Kido Y, Biggs WH III, Wright CV, White MF, Arden KC and Accili D: The forkhead transcription factor Foxo1 links insulin signaling to Pdx1 regulation of pancreatic beta cell growth. J Clin Invest 110: 1839-1847, 2002.
31. Martinez SC, Tanabe K, Cras-Méneur C, Abumrad NA, Bernal-Mizrachi E and Permutt MA: Inhibition of Foxo1 protects pancreatic islet beta-cells against fatty acid and endoplasmic reticulum stress-induced apoptosis. Diabetes 57: 846-859, 2008.
32. Cunha DA, Gurzov EN, Naamane N, Ortis F, Cardozo AK, Bugliani M, Marchetti P, Eizirik DL and Cnop M: JunB protects β-cells from lipotoxicity via the XBP1-AKT pathway. Cell Death Differ 21: 1313-1324, 2014.
33. Hu MC, Gong HY, Lin GH, Hu SY, Chen MH, Huang SJ, Liao CF and Wu JL: XBP-1, a key regulator of unfolded protein response, activates transcription of IGF1 and Akt phosphorylation in zebrafish embryonic cell line. Biochem Biophys Res Commun 359: 778-783, 2007.
34. Akiyama M, Liew CW, Lu S, Hu J, Martinez R, Hambro B, Kennedy RT and Kulkarni RN: X-box binding protein 1 is essential for insulin regulation of pancreatic α-cell function. Diabetes 62: 2439-2449, 2013.
35. Laybutt DR, Preston AM, Akerfeldt MC, Kench JG, Busch AK, Biankin AV and Biden TJ: Endoplasmic reticulum stress contributes to beta cell apoptosis in type 2 diabetes. Diabetologia 50: 752-763, 2007.
36. Winnay JN, Boucher J, Mori MA, Ueki K and Kahn CR: A regulatory subunit of phosphoinositide 3-kinase increases the nuclear accumulation of X-box-binding protein-1 to modulate the unfolded protein response. Nat Med 16: 438-445, 2010.
37. Lee AH, Heidtman K, Hotamisligil GS and Glimcher LH: Dual and opposing roles of the unfolded protein response regulated by IRE1alpha and XBP1 in proinsulin processing and insulin secretion. Proc Natl Acad Sci USA 108: 8885-8890, 2011.
38. Bregenholt S, Møldrup A, Blume N, Karlsen AE, Nissen Friedrichsen B, Tornhave D, Knudsen LB and Petersen JS: The long-acting glucagon-like peptide-1 analogue, liraglutide, inhibits beta-cell apoptosis in vitro. Biochem Biophys Res Commun 330: 577-584, 2005.
39. Arimoto K, Fukuda H, Imajoh-Ohmi S, Saito H and Takekawa M: Formation of stress granules inhibits apoptosis by suppressing stress-responsive MAPK pathways. Nat Cell Biol 10: 1324-1332, 2008.
40. López-Bergami P, Habelhah H, Bhoumik A, Zhang W, Wang LH and Ronai Z: RACK1 mediates activation of JNK by protein kinase C [corrected]. Mol Cell 19: 309-320, 2005.