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Original Article

Inhibiting KDELC1 may prevent palmitate-induced apoptosis in INS-1 cells by reducing ER stress

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Abstract: Several endoplasmic reticulum resident proteins contain a unique C-terminal sequence Lys-Asp-Glu-Leu-KDEL, which is required for the retention of these proteins in the endoplasmic reticulum. Recently, the induction of endoplasmic reticulum (ER) stress is one mechanism proposed to contribute to the detrimental effects of FFA on β-cells. In this study, INS-1 cells were exposed to PA and BSA for 24 hours. Cell viability was assessed using the Cell Counting Kit-8 (CCK8) viability assay. We use siRNA transfection method to inhibit the expression of KDELC1. The ER stress-related effectors were measured by western blotting. The results showed that the expression of ER stress-related chaperones GRP78, IRE1α, ATF4 and Caspase-3 were reduced markedly after KDELC1 siRNA transfected in PA-induced INS-1 cells. And after silencing the expression of KDELC1, the cell viability was increased significantly. These finding provide that, inhibiting KDELC1 could reduce the expression of some ER stress-related chaperones and show a novel role for reduction of KDELC1 in preventing or treating with PA-treated insulinoma cell line.

Keywords: Free fatty acid, KDELC1, endoplasmic reticulum stress, INS-1, palmitate, apoptosis

Introduction

Increasing apoptosis of pancreatic beta cells mass is recognized as one of the importantcontributing factors to the pathogenesis of type 2 diabetes mellitus. It has been documented in numerous in vitro experimental systems that exposure to chronically elevated free fatty acid (FFA) causes β-cell dysfunction and apoptosis, a phenomenon what we often call lipotoxicity [1]. Endoplasmic reticulum (ER) stresses and oxidative both lead to beta cell loss, increase pancreatic dysfunction and worsen type 2 diabetes [2-4]. In the insulin-resistant (IR) state, misfolded proteins accumulation also activates apoptotic pathway via ER stress [6]. Numbers of evidence showed that ER stress is a pivotal factor in type 2 diabetes [7, 8].

Endoplasmic reticulum (ER) isan organelle responsible for lipid and protein biosynthesis and Ca^{2+} storage [9]. Disruptions of the ER homeostasis was one crucial features of type 2 diabetes. When the ER becomes stressed due to the accumulation of synthesized unfolded proteins, the ER stress activated [10]. However, if the adaptive unfolded protein response (UPR) fails to restore, the ER stress would initiate apoptosis pathways [11]. Pancreatic beta cells, due to have the function of synthesize and secrete large amounts of insulin, have been found to be very sensitive to perturbation in ER homeostasis [12, 13].

KDELC1, also known as EP58, is a novel KDEL motif-bearing protein [15, 16]. KDELC1 is a small gene, whichcontains 10 exonsspanning ~15 kb and encodes a protein product localized to the lumen of the ER. KDELC1 is a new type of ER-resident protein, based on its lack of homology to any of the known proteins resident in the ER [17, 18]. Our previous study had been indicated that KDELC1 participate in ER stress [16]. Unfortunately, less information is available about the functional characters of KDELC1 in type 2 diabetes. In the present study, we investigated the effects of KDELC1 on FFA (palmitate)-induced cytotoxicity in rat INS-1 cells and whether inhibiting KDELC1 could prevent palmitate-induced apoptosis in INS-1 cells by reducing ER stress.

Materials and methods

Main reagents

Palmitate was obtained from Sigma-Aldrich (St. Louis, MO). Cell Counting Kit-8 was purchased from DOJINDO (Japan). Antibodies were obtained as follows: KDEL and IRE1α antibodies were obtained from Novus Biotechnology (Colorado, U.S.); anti-GRP78 Bip antibody from abcam (Abcam Biotechnology, CA); antibody for ATF4 and Caspase-3 were obtained from CST (Cell Signaling Technology, Beverly, MA); anti-actin antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

FFA preparation

And accumulating evidence suggests that saturated free fatty acids (FFAs), such as palmitate, have been shown to trigger ER stress that may contribute to promoting β-cells apoptosis [14]. In this study, FFA were used as inducer and FFA solutions were prepared as described previously [19]. 100 m palmitate was prepared in 0.1 m NaOH at 70°C and filtered. Five percent (w/v) FFA-free bovine serum albumin (BSA) solution was prepared indouble-distilled H_2O and filtered. A 5 mm FFA/5% BSA solution was prepared by complexing an appropriate amount of FFA to 5% BSA ina 55°C water bath. The above solution was then cooled to room temperatureand diluted in RPMI 1640 without fetal bovine serum (FBS) to desired concentration.

CCK8 viability assay

Cell viability was measured with Cell Counting Kit-8 (CCK8) according to themanufacture's instructions. CCK8 is more sensitive than the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay [4]. INS-1 cells were treated with cck8 at 37°C for 1 h. Absorbency was measured at 450 nm using a microplate reader. The percentage of cell viability was calculated as follows: cell viability (%) = (OD of treatment - OD of blank control)/(OD of control - OD of blank control) × 100.

Cell culture

The rat insulin-secreting INS-1 pancreatic β-cells were routinely maintained in RPMI 1640 medium supplementing with 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 mg/ml streptomycin, 1 mM sodium pyruvate, 2 mM L-glutamine, and 50 μM 2-mercaptoethanol. Cells were incubated at 37°C in a humidified atmosphere of 5% CO_2. All experiments were performed between the 15th and 25th passages to minimize the effects of phenotypic variation in continuous culture. The cells were split four days and the medium changed two days.

INS-1 cells were divided into four groups: (1) control (RPMI 1640); (2) normal (RPMI 1640 medium supplementing with 10% FBS); (3) BSA; (4) PA. To investigate the effects of FFA on the expression of KDELC1, GRP78, ATF4 and IRE1α, cells were stimulated with BSA or PA for 24 h. At the end of 24 h, total protein of the cells was extracted for the target protein's expression.

To examine the effect of KDELC1 on some ER stress-related chaperones expression by PA, KDELC1 small interfering RNA (siRNA) was transfected 6 h before stimulation and cultured for 24 h in PA or BSA medium; then, total protein was extracted from cells for analysis of GRP78, ATF4, IRE1α and Caspase-3 expression.

siRNA transfection

Cells were replated and transfected in 6-well plates with 125 μl Opti-MEM (Invitrogen, CA) and 7.5 μl/well Lipofectamine 3000 (Invitrogen, CA) with 100 pmol siRNA and its controls. The sense and antisense sequences of the primers were 5'-GGUUAAACACCAUGGGCAATT-3' and 5'-UUGCCCAUGGUGUUUAACCTT-3'. As a negative control, we used randomly mixed sequences of KDELC1 siRNA, 5'-UUCUCCGAACGUGUCACGUTT-3', and 5'-ACGUGACACGUUCGGAGAATT-3'. The effect of siRNA knockdown of KDELC1 on the expression of the ER stress-related effectors were evaluated by western blot analysis at 24 h. All RNAi experiments were repeated at least three times.

Western blot analysis

Cell proteins were extracted as described previously [2]. The protein extracts were separated on 10% SDS-polyacrylamide gels, and then transferred to 0.22 μm PVDF membranes, and blocked in TBST with 5% no fat milk at room temperature for 2 h. The blots were incubated

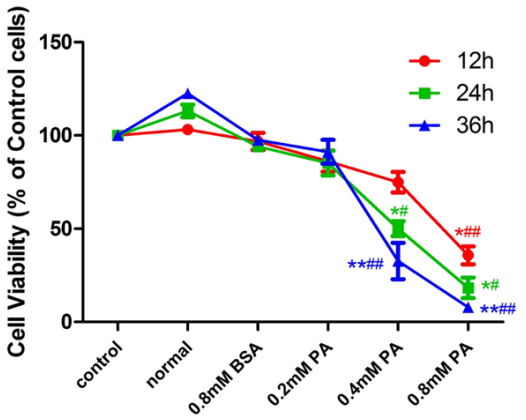

Figure 1. The viability of INS-1 cells was inhibited by palmitate (PA) stimulation at different times. INS-1 cells were incubated with different concentrations of PA (0.2 mM, 0.4 mM, and 0.8 mM) or BSA (0.8 mM) for 12 h, 24 h and 36 h. Cells cultured with RPMI 1640 medium was used as control. The viability was determined by CCK8 analysis. Results were mean ± SD for three individual experiments. *P < 0.05; **P < 0.01 vs. control; #P < 0.05; ##P < 0.01 vs. BSA (0.8 mM).

with antibodies for KDELC1 (1:1000), GRP78 (1:1000), ATF4 (1:1000), IRE1α (1:1000) and Caspase-3 (1:1000) overnight at 4°C, washed, and then incubated with horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG (1:5000) at room temperature for 1 h. The signals were detected with the ECL system. To control equal lane loading, the other membrane was probed with anti-β-actin (1:1000) antibody. Protein bands were analyzed by use of AlphaView SA software. All experiments were repeated at least three times.

Statistical analyses

All values were expressed as means ± SD. Groups were compared by one-way ANOVA analysis method. Statistical analysis involved SPSS v17.0 for Windows (SPSS Inc., Chicago, IL). $P < 0.05$ was considered statistically significant.

Results

Palmitate induces cell death

INS-1 cells were incubated with 0.2, 0.4, and 0.8 mM PA or 0.8 mM BSA for 12, 24, and 36 h. The cells maintained in RPMI 1640 mediumwere used as control. CCK8 analysis showed that after 12 h incubation, 0.8 mM PA reduced possible cell numbers by approximately 30%, whereas incubation of cells with 0.2 and 0.4 mM PA for 12 h did not totally impair cell viability as compared with control and 0.8 mM BSA (**Figure 1**). When the incubation time extended to 24 h, both 0.4 and 0.8 mM PA induced apoptosis significantly, by about 50% and 18%, respectively, as compared with control and 0.8 mM BSA (**Figure 1**). As shown in **Figure 1**, when the incubation time was extended to 36 h, apoptosis was increased in 0.4 and 0.8 mM concentrations by about 32% and 7%, respectively. Whereas 0.2 mM PA decreased cell viability, but the differences did not reach any statistical significance. Seeing that 0.4 mM PA was large enough to induce biological events, it was used in the subsequent experiments.

Palmitate induces ER stress in INS-1 cells

To see whether FFAs would induce ER stress in INS-1 cells, the ER stress-related effectors and chaperones were examined by western blotting. As compared with control or BSA, PA increased the protein expression of GRP78, ATF4 and IRE1α (**Figure 2A-C**) at 24 h. However, Caspase-3 expression did not change under any conditions in 24 h (data not shown). Therefore, PA increased GRP78, ATF4 and IRE1-α secreted from cultured INS-1 cells.

Effect of PA on the expression of KDELC1 in INS-1 cells

To confirm the effect of PA on the expression of KDELC1, INS-1 cells were stimulated with PA or BSA for 24 h. KDELC1 protein level was increasedunder PA at 24 h (**Figure 2A**). However, leveldid not increase significantly under BSA at the same time. Thus, PA can upregulate the expression of KDELC1 in INS-1 cells.

Effect of KDELC1 on the activation of ER stress and apoptosis

To verify the effect of KDELC1 on the ER stress and apoptosis in INS-1 cells, we transfected KDELC1 siRNA into INS-1 cells exposed to PA medium for 24 h and evaluated GRP78, ATF4, IRE1α and Caspase-3 levels. Transient transfection of siRNA into INS-1 cells induced FAM expression, which indicated successful transfection. Expression of FAM peaked at 6 h (**Figure 3A**). To test the efficacy of the selected siRNA sequence, we measured the protein level of KDELC1 after 24 h transfection with KDEL-

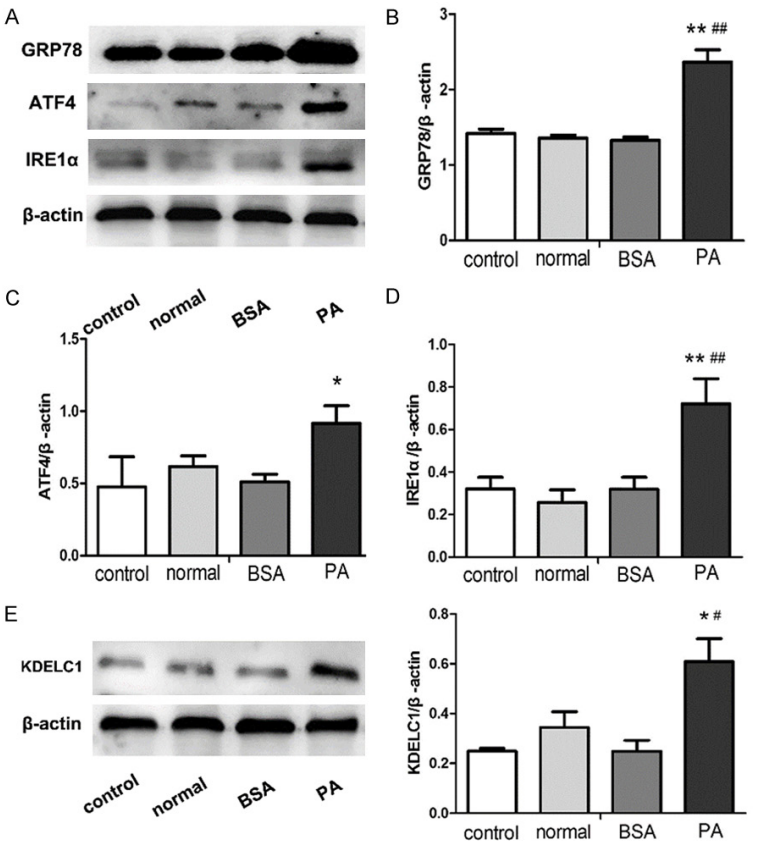

Figure 2. Effect of PA on protein levels of ER stress-related effectors and KDELC1 in INS-1 cells at 24 h. INS-1 cells were cultured in 1640 media and then stimulated with 0.4 mM PA or 0.4 mM BSA for 24 h. Western blot analysis of protein level of GRP78 (A, B), ATF4 (A, C), IRE1α (A, D) and KDELC1 (E). Data are mean ± SD. *P < 0.05; **P < 0.01 vs. control; #P < 0.05; ##P < 0.01 vs. BSA (0.4 mM).

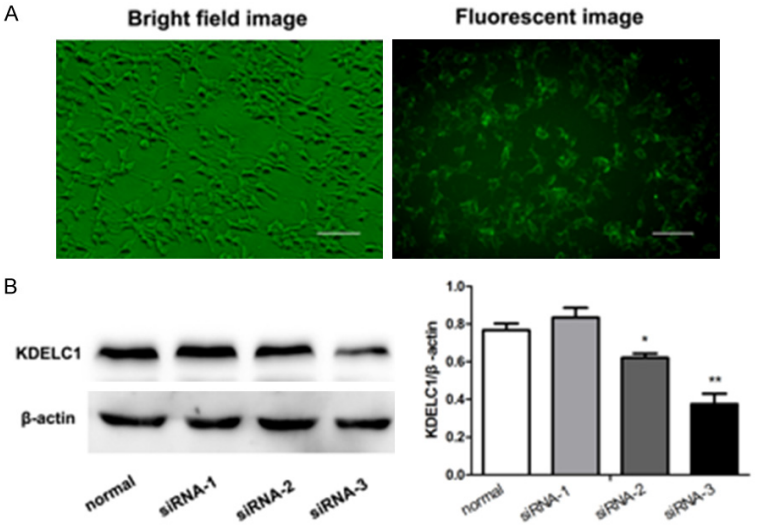

Figure 3. Effect of siRNA on the expression of FAM and KDELC1 in cells. Transient transfection of cells with KDELC1 siRNA induced FAM expression, indicating successful transfection. FAM expression was observed at 6 h after transfection. Cells were transfected with three different KDELC1 siRNA and then incubated with normal culture media for 24 h. A. FAM expression of KDELC1 was observed byfluorescent imaging (400×). B. Western blot analysis of the protein expression of KDELC1 at 24 h after KDELC1 siRNA transfection. Data are mean ± SD. *P < 0.05 vs. normal; **P < 0.01 vs. normal. Scale bars, 50 μm.

C1 siRNA. KDELC1 protein expression was lower in INS-1 cells with KDELC1 siRNA-3 than in cells with siRNA-1 and siRNA-2 ($p < 0.01$, versus normal) (**Figure 3B**). The ER stress-related effectors GRP78, ATF4 and IRE1α expression were decreased in INS-1 cells transfected with KDELC1 siRNA-3 ($p < 0.01$, versus normal; **Figure 4A-C**). Interestingly, the level of apoptotic factor Caspase-3 was decreased significantly ($p < 0.05$, versus normal; **Figure 4D**). And after transient transfection of siRNA of KDELC1, the cell viability was increased significantly ($p < 0.01$, versus PA; **Figure 5**). KDELC1 may activate the ER stress and apoptosis pathway in INS-1 cells.

Discussion

Type 2 diabetes is characterized by progressive dysfunction and apoptosis of pancreatic beta cell [20, 21]. Increasing results of numbers studies show that ER stress is necessary for promoting beta cell apoptosis both in humans and animal models with type 2 diabetes [22]. When more unfolded protein accumulates under stress, universal protein synthesis will be inhibited and lead to cell apoptosis [23].

Results of many studies showed that lipotoxicity, saturated free acids (FFA) exposure indudce pancreatic beta cell dysfunction and death, plays

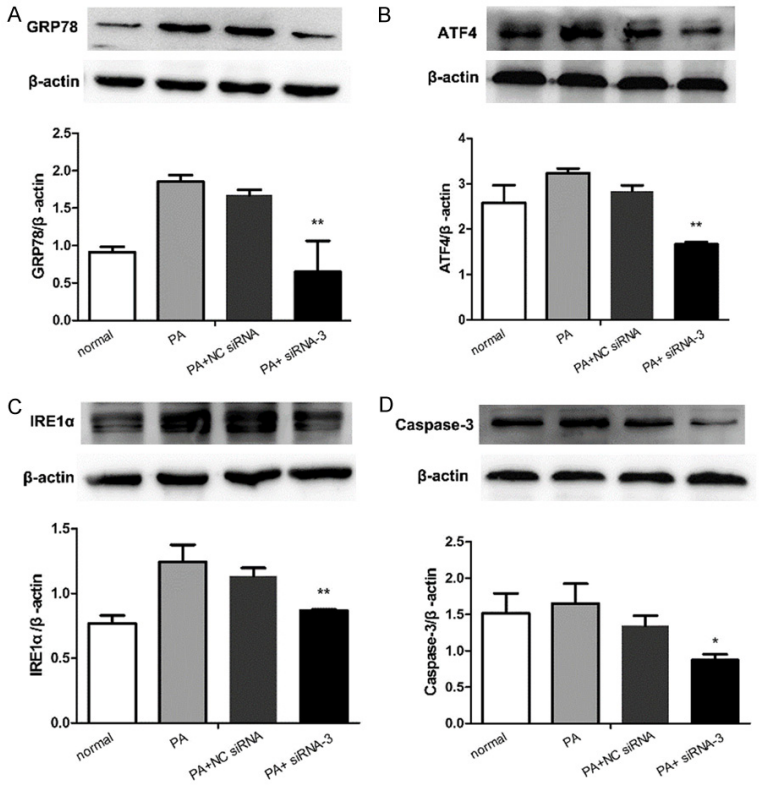

Figure 4. Effect of silencing KDELC1 protein on PA induction of GRP78 (A), ATF4 (B), IRE1α (C) and Caspase-3 (D) level in INS-1 cells. INS-1 cells were stimulated with PA after blockade of KDELC1 with KDELC1 siRNA-3 or its corresponding negative control siRNA. Data are mean ± SD. *P < 0.05, **P < 0.01 versus PA.

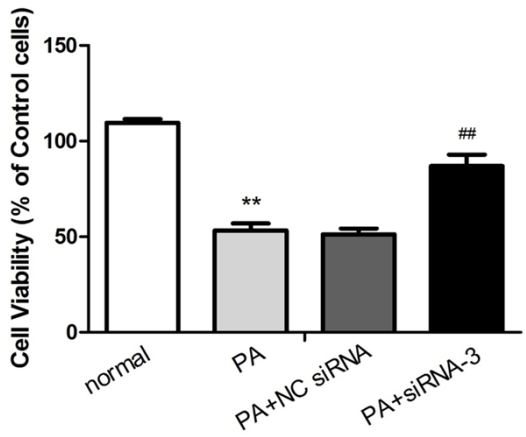

Figure 5. Effect of silencing KDELC1 protein on viability of INS-1 cellsinhibited by 0.4 mM palmitate (PA) stimulation at 24 h. The viability was determined by CCK8 analysis. Results were mean ± SD for three individual experiments. **P < 0.01 vs. normal; ##P < 0.01 vs. PA.

an important role in pathogenesis of type 2 diabetes [24, 25]. It has been reported that prolonged pancreatic β-cells exposed to fatty acid environment can cause cell apoptosis, and lead to β-cells loss via the induction of ER stress in type 2 diabetes [26]. In our study, we researched PA-induced apoptosis in INS-1 cells in order to investigate the effects of FFA on β-cell function and survival in the lipotoxic environment. Our results showed that the INS-1 cell survival rate was reduced to about 50% after incubated in 0.4 mM palmitate at 24 h. What's more, the results of our study showed that exposure of INS-1 cells to lethal doses of palmitate leads to an increase in the expression or activity of factors involved in the ER stress response, including GRP78, IRE1α and ATF4, which have been used as biomarkers of ER stress [27].

Several ER resident proteins contain a unique C-terminal sequence (KDEL) and it is required for the retention of these proteins in the ER. The ER resident proteins act as molecular chaperones assisting in protein folding and assembly [28]. KDELC1 is a novel KDEL motif- bearing ER resident glycoprotein but its functions in type 2 diabetes are not well understood. Here our results confirmed that paltimate upregulated the expression of KDELC1. In this study, we further assess the role of KDELC1 in palmitate-exposed ER stress and cytotoxicity in beta cell line INS-1. Our results showed thatafter inhibiting KDELC1 with KDELC1 siRNA, the palmitate-induced level of ER stress was attenuated and palmitate-induced expression of GRP78, IRE1α and ATF4 decreased. Our results showed that KDELC1 may be involved in the pathogenesis of type 2 diabetes. The results of our previous experiment showed that KDELC1 participates inthe response of ER stress, regulates the HepG2 cellproliferation and might have relationship with the hepatic dysfunction [16].

Previous studies showed that in multiple cell types, palmitate via activation of caspase-3 in

microvascular endothelial cells [29]. Growing evidences have shown that ER stress causes apoptosis mainly through activation of four pathways, IRE1α-JNK pathway, PKR-CHOP pathway, the family of Bcl2 and caspases-3 [30]. Ning Lin and colleaguesreported that INS-1 cells cultured for 24 h in the presence of 0.25 mM PA elevated the caspase-3 activity by 4.21 folds, while OL did not cause caspase-3 activation [31]. But our results have not found significant changes about protein level of caspase-3 in INS-1 cells cultured for 24 h in the absence or presence of 0.4 mM PA. Interestingly, we found that caspase-3 was significantly reduced in INS-1 cells stimulated with PA after blockade of KDELC1 with KDELC1 siRNA-3, however there was no change with the corresponding negative control siRNA. And after silencing of KDELC1, we measured the cell viability with CCK8 Viability Assay. The cell viability was increased significantly versus PA. KDELC1 may activate the ER stress and apoptosis pathway in INS-1 cells.

ER stress is a double-edged sword. When homeostasis is disturbed, ER stress has three molecular components which serve to ameliorate stress: inositol requiring enzyme (IRE)-1/X-box binding protein (XBP)-1, activating transcription factor (ATF)-6, and pancreatic ER kinase (PERK) [32, 33]. In PERK branch, phosphorylated eukaryotic translation initiation factor 2 alpha (eIF2α) promotes the translation of ATF4, which induces the UPR effector C/EBPα-homologous protein (CHOP). And we all know that prolonged CHOP expression triggers apoptosis through a number of mechanisms. Huang et al showed that ER stress was involved in palmitate-induced caspase-3 dependent apoptosis in INS-1 cells [34]. Studies by Dietlind considered palmitate activated the PERK and IRE1 pathwway of the UPR as shown by phosphorylation of eIF2α and splicing of Xbp1 [35].

In summary, we proved that palmitate was able to elicit pancreatic β-cells dysfunction through the induction of ER stress response. And inhibiting KDELC1 could attenuate palmitate-mediated β-cells apoptosis, which was due to amelioration the ER stress. A decrease in KDELC1 protein or activity in beta-cells may contribute to enhance beta cell survival and stop type 2 diabetes development. Targeting KDELC1 may be useful for therapeutic development in treatment of type 2 diabetes.

Conclusions

Inhibiting KDELC1 could reduce the expression of some ER stress-related chaperones and show a novel role for reduction of KDELC1 in preventing or treating with PA-treated insulinoma cell line.

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Disclosure of conflict of interest

None.

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