Retraction

Retraction Notice: "Jinshui He, Xu Zhang, Chaowei Lian, Jinzhi Wu, Yanling Fang, Xiaoling Ye. Exendin-4 prevented pancreatic beta cells from apoptosis in (Type I) diabetic mouse via keap1-Nrf2 signaling"

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Jinshui He, Xu Zhang, Chaowei Lian, Jinzhi Wu, Yanling Fang, Xiaoling Ye. Exendin-4 prevented pancreatic beta cells from apoptosis in (Type I) diabetic mouse via keap1-Nrf2 signaling. Exp Biol Med (Maywood). 2019; 244(1): 28–36. DOI: 10.1177/1535370218823549.

This manuscript is being retracted at the request of the authors because it was determined that experiment(s) could not be repeated.

Original Research

RETRACTED: Exendin-4 prevented pancreatic beta cells from apoptosis in (Type I) diabetic mouse via keap1-Nrf2 signaling

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Impact statement

Nrf2 is an essential part of the defense mechanism of vertebrates and protects them from surrounding stress via participation in stimulated expression of detoxification as well as antioxidant enzymes. It also exerts a role in defending hosts from different stress in the environment, including reactive oxygen species. Our study investigates the role of exendin-4 on Nrf2 pathway as well as cell death in pancreatic β-cell and in non-obese diabetic mice. Result of study indicates exendin-4 mediates activation of Keap1-Nrf2-ARE pathway and may serve as a potential agent to treat type I diabetes mellitus. In our research, we observed excessive reactive oxygen species production, low level of cell death, and PKC phosphorylation on exendine-4 treatment. Nrf2 knockdown led to suppression of reactive oxygen species generation as well as increasing apoptosis. Moreover, siRNA-mediated Nrf2 down-regulation attenuated the suppressive effect of exendin-4 in pancreatic β-cell viability, via modulating apoptosis promoting- and counteracting-proteins, Bax, and Bcl-2.

Abstract

Type I diabetes mellitus (TIDM) serves as a large contributor to morbidity as well as mortality globally with persistently elevating prevalence. It is known that oxidative damage by free radicals participates in etiology, complications, as well as progression of TIDM. In our research, we demonstrate that Keap1-Nrf2-ARE pathway was stimulated by exendin-4 treatment during the development of type I diabetes in murine TIDM model as well as in pancreatic β-cells. We also observed excessive reactive oxygen species (ROS) production, low level of cell death, and PKC phosphorylation on exendine-4 treatment. Nrf2 knockdown led to suppression of ROS generation as well as increasing apoptosis. Moreover, siRNA-mediated Nrf2 down-regulation attenuated the suppressive effect of exendin-4 in pancreatic β-cell viability, through modulating apoptosis promoting- and counteracting-proteins, Bax, and Bcl-2. Thus, the present study indicates exendin-4 mediates activation of Keap1-Nrf2-ARE pathway and may serve as a promising agent to treat TIDM.

Keywords: IDM, Keap1-Nrf2-ARE, exendin-4, ROS, apoptosis, PKC

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Introduction

Featured as chronic hyperglycemia, type I diabetes mellitus (TIDM) is caused by insufficient insulin generation. Persistent hyperglycemia brings about malfunction of endothelium as well as complications in kidneys, retina, and blood vessels.[1]

Exendin-4 contains 39 amino acids and can decrease blood glucose concentration not only during satiation but also during starvation. Exendin-4 is tolerant to dipeptidyl peptidase-IV and serves as the only agent able to postpone and turn over diabetes development in theory.[2] Previous study has demonstrated the ability of exendin-4 to shorten the time interval required to restore from hyperglycemia to normoglycemia as compared to untreated group. Additionally, it expanded grafted β-cell number in streptozotocin-induced diabetic mice, which were transplanted with marginal number of fresh islets.[3] However, understanding of its role in TIDM mice and β-cell is lacking.

As a common pathological reaction, chronic oxidative stress (OS) participates in generation as well as development of some amyloid generating disorders including TIDM, Alzheimer's and Parkinson's disease.[4–6] Enhanced OS harms the majority of cells as well as tissues including pancreatic islets, which triggers the stimulation of multiple apoptosis promoting factors such as caspases, ASK1, p38MAPK, as well as JNK, leading to TIIDM generation.[7–11] Recent studies demonstrated that NF-E2-related factor 2 (Nrf2)/Kelch-like ECH-associated protein 1 (Keap1)/ARE antioxidant pathway serves as a potential regulator in apoptosis research. Nrf2 is an essential part of the defense mechanism of vertebrates and protects them from surrounding stress via participation in stimulated expression of detoxification as well as antioxidant enzymes.[12] Besides the aforementioned functions, it also plays a role in defending hosts from different stress in the environment, including reactive oxygen species (ROS) and electrophiles.[13,14] Consequently, this research investigates the role of exendin-4 on Nrf2 pathway as well as cell death in pancreatic β-cell and in non-obese diabetic (NOD) mice.

Material and method

Cell culture

RIN-m5F, a rat pancreatic β-cell line (ATCC, CRL-11605), was cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), streptomycin (100 μg/mL), and penicillin (100 U/mL). Cells were grown in humidified incubator containing 5% CO_2 at 37°C. Cells were subcultured at 80% confluency.

Material

NOD/Lt mice[15] (weighing 17–25 g) were purchased from Institute of Experimental Animals of Chinese Academy of Medical Sciences. Every procedure was approved by the Animal Care and Use Committee of the Affiliated Hospital of Zhangzhou, Fujian Medical University and was in conformity with the guidelines of National Institute of Health (No81004).

Nrf2 knockdown (KD) was achieved with the help of short hairpin RNA (shRNA). Nrf2 shRNAs were produced in order to silence Nrf2 in order to understand the role of Nrf2 in exendin-4-supplemented TIDM mice. Recombinant lentivirus was prepared via transient transfection with the help of packaging cell line. Briefly, the packaging human cell line 293T HEK was co-transfected with packaging plasmid vector and lentivirus expression plasmid containing Nrf2-targeted shRNA, by Lipofectamine 2000. Infectious recombinant lentiviruses were obtained 72 h post transfection and the medium was collected, centrifuged, and filtered through 0.45 μm syringe filter to obtain a debris-free supernatant. Murine infection was carried out with the help of recombinant lentiviruses. Cultivating media was renewed subsequent to infection. KD of Nrf2 was confirmed using Western blot (WB) assay

Forty healthy mice were divided into four groups randomly ($n = 10$): low-dose (2 μg/kg/d) exendin-4 group A, medium-dose (4 μg/kg/d) exendin-4 group B, high-dose (8 μg/kg/d) exendin-4 group C, and blank control group D treated with saline. Treatment lasted for eight weeks and mice were killed subsequently; 1 mL of blood was obtained from inner canthus and centrifuged at 5000 × *g* for 10 min at 4°C.

Exendin-4 treatment in cells and animals

RiN-m5F cells were treated with 1 μM of exendin-4 for 48 h and then harvested for further experiments. For animal experiments *in vivo*, mice received six-week exendin-4 supplement (3 mg/kg subcutaneously twice a day).[3] Mice without exendin-4 supplement acted as the control group.

RNA extraction from pancreatic tissue and cell line

Total RNA isolation from pancreatic tissue and cell line was conducted using TRIZOL reagent. Purity of RNA was checked by estimating the A260/A280 ratio using the NanoDrop 2000 (Beijing Koled Traffic Co., Ltd, Beijing, China). RNA on the other hand was quantitated using 2100 biological analyzer (Agilent Technology Co., Ltd, New York, USA).

Quantitative real-time PCR

mRNA levels of Keap1, Nrf2, ARE, HO-1, caspases 3, Bax, and Bcl-2 were quantitated using gene specific primers by qRT-PCR. Briefly, cDNA was generated using RT2 Easy First Strand Kit according to the manufacturer's instructions. The qRT-PCR mix contained 100 μL of cDNA template, 125 μL of 2X RT2 SYBR Green qPCR Master Mix, gene specific primers and was suspended in dd H_2O to obtain a final volume of 250 μL. qPCR was performed on ABI7500 real-time fluorescence quantitative PCR instrument (Life Tech applied Biosystems, New York, USA) with the following PCR conditions: denaturation at 95°C for 10 min, 40 cycles of 95°C for 15 s, 60°C for 1 min, and 72°C for 30 s.

The sequences of these genes were listed as follows:

Rat Keap1 F: 5′-GGA CGG CAA CAC TGA TTC-3′;
Rat Keap1 R: 5′-TCG TCT CGA TCT GGC TCA TA-3′;
Rat Nrf2 F: 5′-CAC ATC CAG ACA GAC ACC AGT-3′;
Rat Nrf2 R: 5′-CTA CAA ATG GGA ATG TCT CTG C-3′;
Rat HO-1 F: 5′-ACA GGG TGA CAG AAG AGG CTA A-3′;
Rat HO-1 R: 5′-CTG TGA GGG ACT CTG GTC TTT G-3′;
Rat ARE F: 5′-CGG TAC TTG CCT GCC TTT G-3′;
Rat ARE R: 5′-ATT TGT TTT GCA TCC ACG GG-3′;
Rat Bax F: 5′-AAG AAG CTG AGC GAG TGT CT-3′;
Rat Bax R: 5′-CAA AGA TGG TCA CTG TCT GC-3′;
Rat Bcl-2 F: 5′-GTA TGA TAA CCG GGA GAT CG-3′;
Rat Bcl-2 R: 5′-AGC CAG GAG AAA TCA AAC AG-3′;
Rat PKC F: 5′-CAA GCA GTG CGT GAT CAA TGT-3′;
Rat PKC R: 5′-GGT GAC GTG CAG CTT TTC ATC-3′;
Rat GAPDH F: 5′-CGG AGT CAA CGG ATT TGG TCG TAT-3′;
Rat GAPDH R: 5′-AGC CTT CTC CAT GGT GGT GAA GAC-3′;
Mouse Keap1 F: 5′-ATG ACG GAG TGT AAG GCG G-3′;
Mouse Keap1 R: 5′-CAG GCC GTT GGT GAA CAT G-3′;

Mouse NRF2 F: 5′-GAG AGC CCA GTC TTC ATT GC-3′;
Mouse NRF2 R: 5′-TGC TCA ATG TCC TGT TGC AT-3′;
Mouse HO-1 F: 5′-GCA GAG AAT GCT GAG TTC ATG-3′;
Mouse HO-1 R: 5′-CAC ATC TAT GTG GCC CTG GAG GAG G-3′;
Mouse ARE F: 5′-CCC TGT CCT CAA AGG AAC-3′;
Mouse ARE R: 5′-AGC AGC CGG AGG AGG AAG G-3′;
Mouse GAPDH F: 5′-GCC TCA AGA TCA TCA GCA ATG C-3′;
Mouse GAPDH R: 5′-CCT TCC ACG ATA CCA AAG TTG TCA T-3′.

ROS detection

Cells (2×10^5 cells) were seeded in each well of 24-plates. They were then treated with exendin-4 (0.2 to 1 μM) for 1 h. Subsequently, cells were incubated with 20 μM DCFH-DA for 15 min at 37°C. Flow cytometer was applied to quantified ROS. Furthermore, about one hundred islets from five independent isolations from each group were used to quantify ROS.

Immunofluorescence assay

RIN-m5F cells were seeded on coverslips for 36 h in 12-plates. Standard immunofluorescence assay (IFA) was carried out as previously described.[13] Control and treated cells were washed with PBS for 10 min. The cells were then incubated with anti-Nrf2 primary antibody (1:200, Abcam) at 37°C for 1 h. Cells were subsequently washed five times with PBS (8 min/wash) followed by incubation with anti-rabbit IgG TRITC (1:100, CWBIO) secondary antibody. Fluorescence in the samples was observed under a Leica SP8 fluorescence microscope.

WB analysis

Total proteins from cell lysates of pancreatic tissue and cell line were extracted and were quantitated using a bicinchoninic acid assay kit. Proteins were resolved by 10% SDS-PAGE and transferred to PVDF membranes. The membranes were incubated with specific primary antibodies overnight at 4°C after blocking. Membranes were then incubated with appropriate secondary antibody. Immunoreactivity was detected by enhanced chemiluminescence (ECL kit, Amersham), and quantitated using C-DiGit Blot Scanner.

Evaluation of cell apoptosis

β-cell death was assessed using annexin V-FITC/PI apoptosis detection kit following manufacturer's protocol. In brief, transfected and control cells were resuspended in 20 μl of binding buffer. Annexin V-FITC (10 μl) and PI (5 μl) were added to the suspension and incubated in dark for 20 min. Apoptosis was analyzed using the flow cytometry (FC).

Statistical analysis

Data analysis was performed using the GraphPad 5.0 software (San Diego, California, USA). Difference between two groups was compared using *t*-test, and difference between more than two groups was compared with one-way ANOVA method. $P < 0.05$ was considered statistically significant.

Results

Keap1/Nrf2 signaling was activated after exendin-4 administration

To explore the effect of exendin-4 on the Keap1/Nrf2 signaling in pancreatic β-cell and TIDM mouse model, expression of key proteins in this pathway was examined. The WB and qPCR data indicated that exendin-4 treatment caused a significant reduction of Keap-1 and upregulation of Nrf-2, its downstream targets, ARE and HO-1 at both protein and mRNA levels (Figure 1(a) and (b)). Additionally, immunofluorescence staining of Nrf2 in presence of exendin-4 resulted in nuclear accumulation of Nrf-2, partially indicating activation of Keap1/Nrf2 pathway (Figure 1(c)). Similarly, *in vivo* data of exendin-4 administration in TIDM mouse model led to decrease in Keap1 and an increase in Nrf2, ARE, and HO-1 expressions as determined by WB and qPCR analysis (Figure 1(d) and (e)). These results demonstrated that exendin-4 administration leads to activation of the Keap1/Nrf2 signaling both *in vitro* and *in vivo*.

Exendin-4 treatment modulated ROS production, cell death, and PKC activation in pancreatic *β*-cells

To further understand the mechanism of exendin-4 on pancreatic β-cells, ROS production, apoptosis, and PKC activation were estimated in exendin-4-treated β-cells. We found that the treatment of pancreatic β cells with different concentrations of exendin-4 led to an obvious augment in ROS production in dose-dependent (Figure 2). Apoptosis measured by FC, indicated that exendin-4 treatment decreased the cell number of apoptoticβ-cells (7.8%), compared with untreated group (19.8%) (Figure 3(a)). Decrease in apoptotic cell number was also supported by decreased caspase-3 and Bax, as well as increased Bcl-2 protein and mRNA levels (Figure 3(b) and (c)). Additionally, quantitation of phospho-PKC on exendin-4 treatment showed an increase in protein levels. Similarly, PKC mRNA expression was also significantly upregulated (Figure 4(a) and (b)).

Nrf2 silencing restored ROS levels, apoptotic cells, and PKC activation in exendin-4-treated pancreatic *β*-cells

To further explore the role of Nrf2 in exendin-4-mediated ROS generation, apoptosis, and PKC phosphorylation in pancreatic β-cells, Nrf2 was KD in RIN-m5F cells with Nrf2 siRNA. We found that increased ROS levels were significantly reduced after Nrf2 silencing (Figure 5(a)). Apoptotic cell number in exendin-4-treated β-cells was increased in Nrf2 KD cells (Figure 5(b)). Meanwhile, the silencing of Nrf2 was confirmed by measuring Nrf2 protein as well as mRNA levels in transfected cells (Figure 5(c)). Expressions of caspase-3, Bax, and Bcl-2 at protein and mRNA levels were partially restored in Nrf2 knockout cells (Figure 5(c) and (d)). Additionally, PKC expression

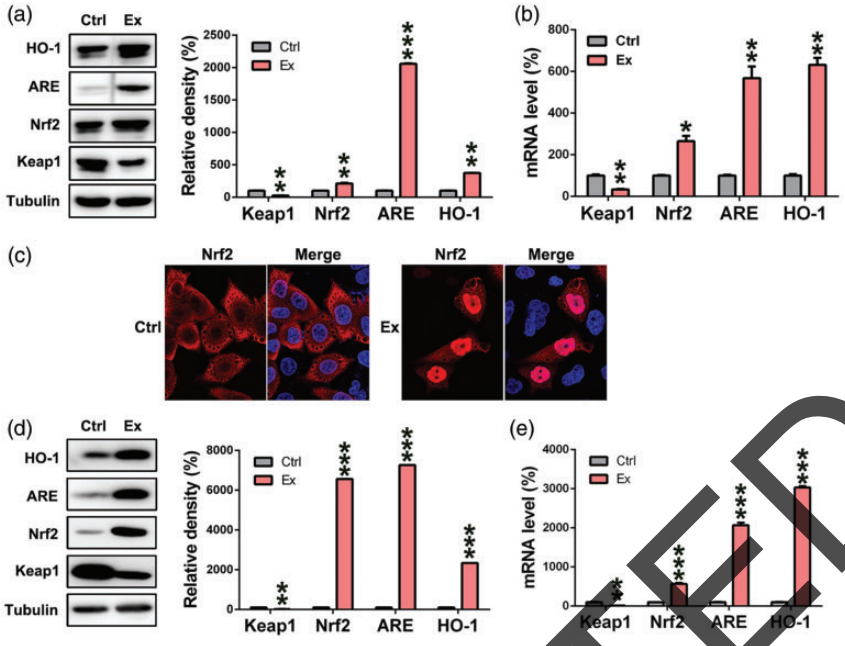

Figure 1. Exendin-4 treatment activated Keap1/Nrf2 pathway in both pancreatic β-cell and TIDM mouse model. (a) Western blot analysis of control and 1 μM exendin-4-treated cells using Keap1, Nrf2, ARE, and HO-1 specific antibodies. (b) qPCR was carried out to examine the expression of Keap1, Nrf2, ARE, and HO-1 in cells at 48 h post exendin-4 treatment. (c) IFA was performed to examine the nuclear and cytoplasmic localization of Nrf2 (TRITC, red). Nuclear DNA was stained with DAPI (blue). (d) Subsequent to exendin-4 administration in mice, islet tissue was isolated and subjected to Western blot analysis of Keap1, Nrf2, ARE, and HO-1 in treated and non-treated group. (e) qPCR was carried out to observe transcriptional levels of Keap1, Nrf2, ARE, and HO-1. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. indicated group. (A color version of this figure is available in the online journal.)

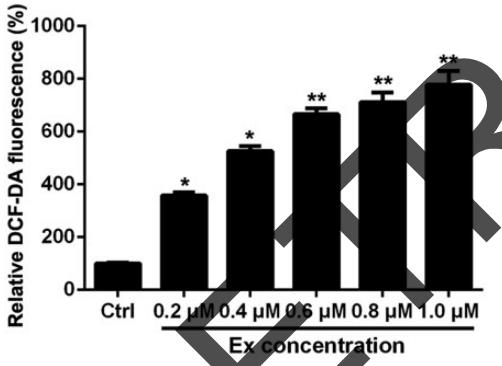

Figure 2. Exendin-4 treatment induced ROS production in a dose-dependent manner. Pancreatic β-cells were treated with different doses of exendin-4 and DCFH-DA fluorescence was measured using flow cytometry to examine the ROS levels in cell. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. indicated group.

and phosphorylation induced by exendin-4 treatment were recovered by Nrf2 silencing (Figure 5(e) and (f)). These data suggested that the Keap1/Nrf2 signaling is responsible for exendin-4-mediated regulation of ROS levels, apoptosis, and PKC activation.

We also utilized the $Nrf2^{-/-}$ and WT mouse to confirm the effect of Nrf2 in exendin-4-treated TIDM mice. The pancreatic islet tissues of mice were isolated, homogenized, and analyzed for ROS levels with DCFH fluorescence assay. The results showed that exendin-4 administration caused robust ROS production in WT mice (Figure 6(a)), while Nrf2 depletion ablated the effect of exendin-4 on ROS production (Figure 6(b)). FC data also showed that exendin-4 administration resulted in decreased apoptosis of islet cells isolated from WT mice (Figure 6(c)), whereas no significant difference was showed in apoptotic cell number between mice (Figure 6(d)). *In vivo* observations for expression levels of caspase-3, Bax, and Bcl-2 were consistent with the in vitro data: caspase-3 and Bax levels augmented, while Bcl-2 level decreased subsequent to exendin-4 administration (Figure 6(e)). In $Nrf2^{-/-}$ mice, KD of Nrf2 was confirmed by decreased levels of Nrf2 using WB analysis. However, the expression of caspase-3, Bax, and Bcl-2 remained unaltered on exendin-4 administration (Figure 6(f)). Furthermore, we also found that exendin-4 increased the expression and activation of PKC in TIDM WT mice (Figure 6(g)), but PKC signal was unchanged in $Nrf2^{-/-}$ mice (Figure 6(h)). Collectively, these *in vivo* and *in vitro* data demonstrated that exendin-4-mediated regulation of ROS production, apoptosis, and PKC activation was Nrf2-dependent.

Discussion

Nrf2 stimulation is essential to counteract harmful effects of oxidation as well as inflammation. However, ROS is generated during oxidation inside the cells as well as in response to environmental stress. OS is triggered upon excessive ROS.[16] Previous research demonstrated the role of ROS in TIIDM etiology.[17] The influence of exendin-4 supplement on pancreatic β-cell apoptosis as well as its mechanism was investigated in this study. It was observed that exposure to exendin-4 could remarkably suppress proliferation as well as cell death of RIN-m5F cells. Exendin-4 treatment brought about apoptotic down-regulation, enhanced ROS generation, as well as PKC phosphorylation not only in

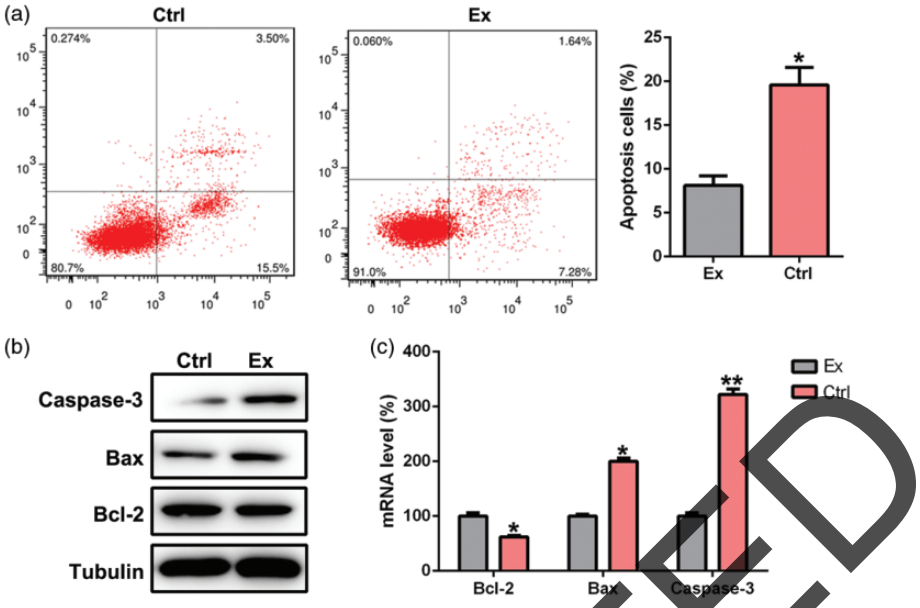

Figure 3. Exendin-4 treatment impaired cell viability of pancreatic β-cell. (a) Annexin V-FITC/PI staining was performed to detect induction of apoptosis in exendin-4-treated β-cells and control cells using flow cytometer. (b) WB analysis and (c) qPCR showed that exendin-4 treatment upregulated the expression of Bax and Caspase-3, and downregulated Bcl-2 in treated pancreatic β-cell. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. indicated group. (A color version of this figure is available in the online journal.)

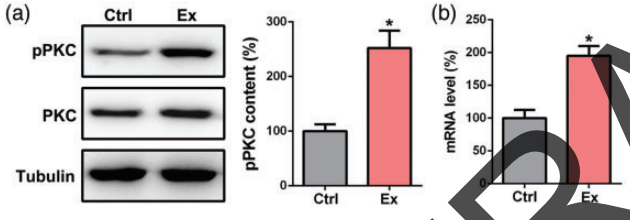

Figure 4. Exendin-4 treatment induced PKC activation. (a) Western blot for phosphorylated PKC in cells treated with 1 μM exendin-4 and control cells at 48 h post treatment. (b) qPCR of PKC mRNA levels in Rin-5mF cells. $^{*}P < 0.05$ vs. indicated group. (A color version of this figure is available in the online journal.)

RIN-m5F cells but also in TIDM mice. Moreover, it was revealed that Nrf2 silencing promoted apoptosis, which consequently suppressed Bcl-2 level and elevated Bax as well as caspase-3 expression in both protein and mRNA levels. Nrf2 silencing in mice led to decreased ROS expression and increased cell death, which consequently attenuated the effect of exendin-4 in TIDM mice. Briefly, this study indicated that exendin-4 led to ROS generation, cell death suppression, as well as PKC stimulation in pancreatic β-cells via activation of the Nrf2/Keap1/ARE pathway, which was further confirmed by Nrf2 silencing or KD studies.

Increasing evidence has revealed that ROS generation as well as exposure is closely associated with injury, death, as well as necrosis of multiple cell lines.[18–20] Cell death pathway is triggered via interaction of death domain-containing adaptor proteins with C-terminal death domain of several death receptors. It is indicated by recent evidences that ROS mediates cell death via ROS-triggered receptor clustering as well as generation of lipid raft-derived signaling platforms.[21,22] Pathophysiological reactions that serve as a primary mechanism of cell death require deeper understanding. In terms of cell death, stimulated caspase-8 triggers caspase-3 directly (type 1 apoptosis), while low caspase-8 stimulation modulates caspase-3 activation via an amplification loop associated with mitochondria (type 2 apoptosis).[23] During type 2 apoptosis, cleaved caspase-8 stimulates apoptosis promoting Bid which triggers permeabilization of outer mitochondrial membrane by interaction of truncated Bid (tBid) and Bax/Bak, leading to release of cytochrome C, AIF, as well as second mitochondria-derived activator of caspases/direct IAP binding protein with low pI from the mitochondria. Essentially, apoptosis counteracting (Bcl-X_L, Bcl-2 as well as Bcl-w) and promoting (Bak, Bim, Bid, Bad as well as Bax) proteins serve as primary contributors to permeabilization of mitochondrial outer membrane as well as vulnerability to cell death.[24] Growing evidence has demonstrated that the Nrf2/Keap1 pathway can modulate apoptosis as well as generation of malignancy in order to defend against toxic levels of chemical compounds or disorders which change redox state inside the cells.[25] This development attracts the majority of attention to cytotoxicity brought about via xenobiotics, malignancy counteracting drugs, as well as high ROS levels.[26–29] It has been reported by Taguchi *et al.*[30] that murine Keap1 gene impairment in liver via Atg7 triggers damage of hepatic cells and is related to Nrf2 accumulation. Moreover, caspase-3 stimulation participates in apoptosis and was found in VSMCs subsequent to Keap1 KD. Synchronous Nrf2 exhaustion via siRNA transfection prevented from cell death.[30] Ashino *et al.*[31] have proved that Keap1 exhaustion stimulates the characteristics of VSMC death, including positive TUNEL staining as well as Annexin-V binding. These alterations are related to enhanced nuclear Nrf2 expression. Nrf2 exhaustion additionally prohibits cell death triggered via Keap1

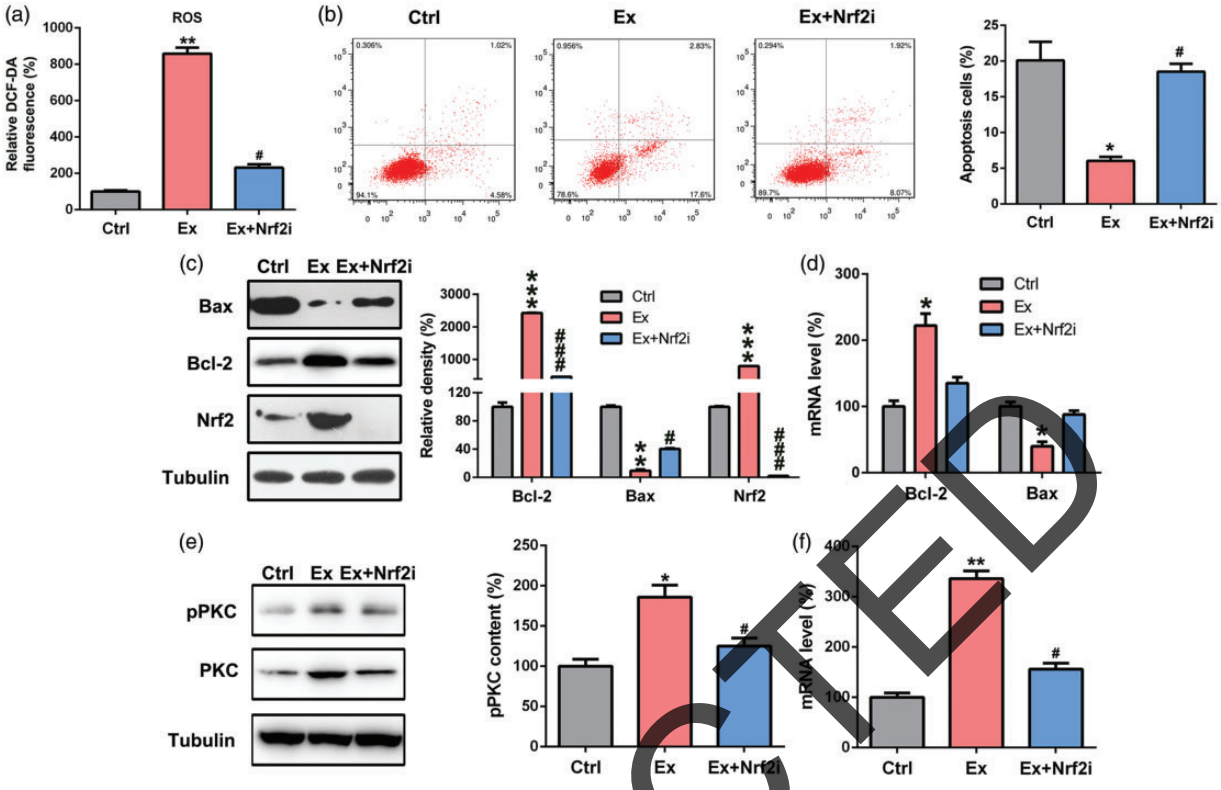

Figure 5. Nrf2 silencing ameliorated the effect of exendin-4 on β-cells. Cells were transfected with Nrf2 siRNA followed by treatment with 1 μM exendin-4. (a) DCFH-DA fluorescence was measured using flow cytometer to examine the ROS levels in the cells. (b) PI/Annexin V-FITC flow cytometry analysis was performed to display the extent of apoptosis in the treated and control β-cells. (c) WB analysis and (d) qPCR were performed to quantitate expression levels of Nrf2, caspase-3, Bax, and Bcl-2 in different groups. (e) WB analysis of phosphorylated PKC in Nrf2 siRNA transfected cells and exendin-4 treatment. (f) qPCR was carried out to measure the expression level of PKC in different groups. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. Ctrl group; $^{\#}P < 0.05$ vs. Ex group. (A color version of this figure is available in the online journal.)

depletion. For instance, depleting NRF2 after the transfection of KEAP1 siRNA protected these cells against apoptosis.[30] Ashino *et al.*[31] showed that features of apoptosis could be induced by KEAP1 depletion in vascular smooth muscle cells, including positive terminal deoxynucleotidyl transferase dUTP nick end labeling and annexin-V binding. The above phenomena were found to be related to an increase in the nuclear localization of NRF2. Furthermore, the depletion of NRF2 was found to inhibit the induction of apoptosis by KEAP1 depletion. Despite more and more studies investigating Nrf2/Keap1 modulated death of multiple cell lines,[32–34] the understanding of whether Nrf2/Keap1 pathway protects pancreatic β-cell via modulating cell death on ROS exposure is insufficient. In this study, it was displayed that exendin-4 treatment to pancreatic β-cells as well as in TIDM mice could suppress β-cell death, as demonstrated by decrease in apoptotic cell number, as well as changed expressions of Bax and Bcl-2 in transcriptional and translational pattern, which was consistent with previous reports. Moreover, Nrf2 silencing as well as KD could remarkably restore cell death and Bax and caspase-3 protein and mRNA levels while suppressing the expression of Bcl-2. The reason why Nrf2 silencing only partially recovered the expression of Bax protein may be attributed to some post-translational regulation. Consequently, the above findings confirm the role of Nrf2 in cell death regulation.

Some other mechanisms associated with Nrf2 stimulation have been reported, including acetylation, phosphorylation, and cysteine post-translational modification. These modifications regulate activities of glycogen synthase kinase-3 beta (GSK3β), phosphatidylinositol 3-kinase pathway (PI3K/AKT), PKC, MAPK, as well as extracellular regulated protein kinases (ERKs). Specifically, PKC-mediated Ser-40 phosphorylation of Nrf2 brings about disassociation of Keap1 and Nrf2. It has been previously demonstrated that PKC regulates Nrf2 level and consequently has an impact on transcription from AREs.[35] Recent studies have revealed that mechanisms relying on cytosolic kinase as well as Keap1 act in coordination.[36] PKC has various isoenzymes, which differ remarkably in terms of specificity, functional regulation, as well as location. It has been proved that PKCδ participates in translocation of Nrf2.[37] It is illustrated in our study that exendin-4 stimulates Nrf2 pathway as well as nuclear localization, along with increased PKC phosphorylation as well as enhanced mRNA expression, which is in accordance with previous research. Moreover, Nrf2 silencing and KD suppresses PKC phosphorylation as well as its transcription and translation, suggesting that Nrf2 serves as a downstream sensor and exerts feedback influence on PKC stimulation.

Thus, our research proves that exendin-4 treatment can suppress β-cell death, generate more ROS, and stimulate PKC pathway. These functions rely on Keap1/Nrf2/ARE axis.

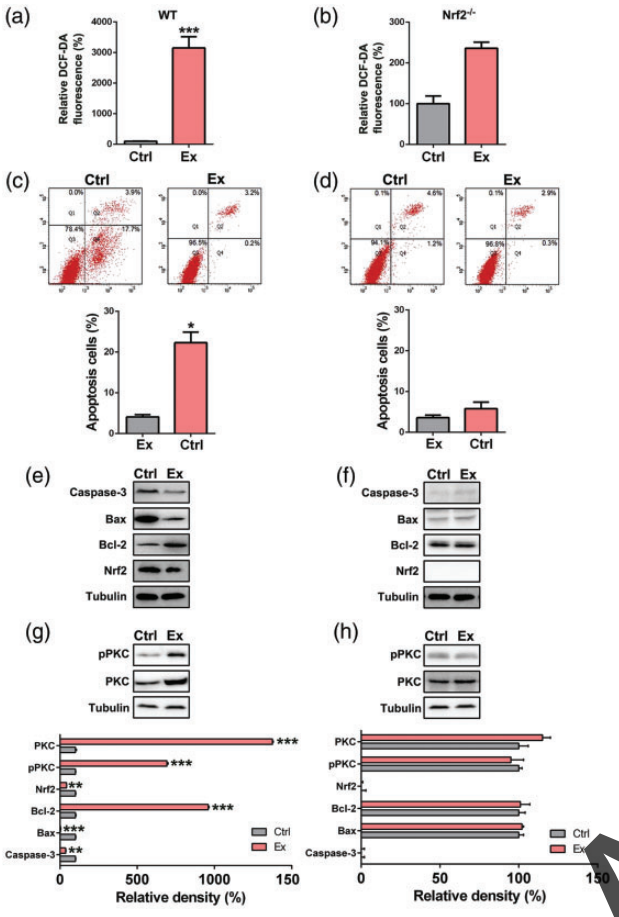

Figure 6. Nrf2 knockout nullifies the effect of exendin-4 in TIDM mouse model. Both WT TIDM and Nrf2$^{-/-}$ mouse were treated with exendin-4. Islet tissue was isolated, homogenized, and used for further assays. DCFH-DA fluorescence was measured to check the ROS levels in (a) WT and (b) Nrf2$^{-/-}$ islet tissue using a flow cytometer. PI/Annexin V-FITC flow cytometry analysis was performed to exhibit the extent of apoptotic cell content in (c) WT and D) Nrf2$^{-/-}$ islet cells. Protein levels of caspase-3, Bax, Bcl-2, and Nrf2 in (e) WT and (f) Nrf2$^{-/-}$ mice were quantitated using WB. Phosphorylation of PKC in (g) WT and H) Nrf2$^{-/-}$ islet cells were quantitated using Western blot. $^{*}P < 0.05$; $^{**}P < 0.001$ vs. indicated group. (A color version of this figure is available in the online journal.)

Findings above demonstrate that exendin-4 counteracts apoptosis in not only pancreatic β-cells but also in TIDM mice, at least in part through Keap1/Nrf2 axis.

Authors' contributions: JH, XZ, CL, JW, YF and XY conducted the experiments, JH supplied critical reagents, XY wrote the manuscript.

DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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