Endocrine (2012) 42:107–117
DOI 10.1007/s12020-012-9633-z

ORIGINAL ARTICLE

Mitochondrial reactive oxygen species (ROS) inhibition ameliorates palmitate-induced INS-1 beta cell death

Ning Lin · Hanbei Chen · Hongmei Zhang · Xiaoyu Wan · Qing Su

Received: 5 October 2011 / Accepted: 8 February 2012 / Published online: 20 February 2012

Abstract The purpose of this study is to explore the possible link between oxidative stress and endoplasmic reticulum (ER) stress in palmitate (PA) induced apoptosis of INS-1 cells, and to figure out the main source of reactive oxygen species (ROS) and the effect of ROS inhibition on the level of ER stress. In this study, INS-1 cells were exposed to PA and oleate for the indicated times. Cell viability and apoptosis were measured by MTT and ELISA; ROS was detected by the probe DCFH-DA and MitoSOX Red using flow cytometer; and the ER stress-related chaperones were measured by western blotting and real time PCR. The level of JNK phosphorylation was also measured by western blotting. The results showed that, in PA-treated cells, apoptosis increased in a dose-dependent way. ROS generation was mainly increased through mitochondrion, and ROS inhibition reduced the expression of some ER chaperones and transcription factors levels. Also, inhibition of JNK phosphorylation ameliorated PA-induced apoptosis. It is concluded that, ROS inhibition, especially inhibiting the ROS from mitochondria, may reduce the expression of some ER stress-related effectors and show a protective role in PA-induced pancreatic beta-cell apoptosis.

Keywords Reactive oxygen species · Endoplasmic reticulum stress · Free fatty acids · INS-1 cell

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Introduction

Free fatty acids (FFAs) have been implicated as an important causative link among obesity, insulin resistance, and type 2 diabetes mellitus (T2DM) [1]. Elevation of circulating FFAs contributes to the pathogenesis of T2DM, and high concentrations of FFAs may impair insulin action and β-cell dysfunction [2]. Two processes that are becoming recognized as inducers of pathological cell death are oxidative stress and endoplasmic reticulum (ER) stress [3, 4].

Oxidative stress is provoked in various tissues under diabetic conditions, and involved in the development of diabetic complications [5]. Patients with T2DM have significantly higher glucose excursions, which could trigger the activation of oxidative stress [6]. Mitochondria and the Nox family of NADPH oxidase are the two major sources of reactive oxygen species (ROS) that are induced by external stimuli, and the mitochondria respiratory chain is considered as an important site of ROS production within most cells [7–9]. There are no exceptions in pancreatic β-cells, which are vulnerable to oxidative stress due to relatively low expression of antioxidant enzymes such as catalase and glutathione peroxidase [10]. Nakamura et al. [11] demonstrated that palmitate increased the production of intracellular ROS, thus activating c-jun-N-terminal kinase (JNK), one of the most important pathways about apoptosis.

Ample evidence shows that palmitate induces β-cell dysfunction and apoptosis via activation of ER stress [12, 13]. The accumulation of unfolded or misfolded proteins in the lumen of the ER is pursued by translation attenuation, degradation of misfolded proteins, and increased protein folding capacity through augmented transcription of ER chaperones. When unfolded, protein response (UPR) fails to restore adequate ER function, it turns on signaling pathways leading to apoptosis [14].

Many diseases that feature ER and oxidative stress are associated with aging and obesity, including diabetes, atherosclerosis, nephropathy, and neurodegenerative disease, all of which are becoming epidemic in modern society [15]. However, progress in translating this understanding into useful therapeutic strategies has been disappointing. The reason is that ER stress involves many signaling pathways with differing pathological and physiological functions, and, likewise, there exist many sources of cellular oxidative stress [16, 17]. An important approach to this problem is to gain a more in-depth understanding about molecular and cellular mechanisms and/or clarify the link between ER stress and oxidative stress, particularly in the process of cell death. It is known that "glucose toxicity" could provoke oxidative stress and ER stress and activate the JNK pathway in various tissues [18, 19].

The present research was designed to study the effect of mitochondrial ROS on ER stress and clarify the possible link between them by incubating the rat pancreatic β-cell strain, INS-1 cells with palmitate.

Research design and methods

Cell culture

INS-1 cells (obtained from the Shanghai Institute for Endocrine and Metabolic Diseases) were cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 10 mmol/L HEPES, 2 mmol/L L-glutamine, 1 mmol/L sodium pyruvate, 50 μmol/L β-mercaptoethanol, 100 U penicillin/mL, and 100 μg streptomycin/mL at 37°C in a humidified 5% CO_2 atmosphere. RPMI 1640, FBS, HEPES, L-glutamine, sodium pyruvate, penicillin, and streptomycin were all from Invitrogen (USA). After removing the medium, cells were then incubated in RPMI medium supplemented as above with 1% FBS, in the presence of BSA alone (control) or with various FFAs. In some experiments, following culture establishment, cells were pretreated with thenoyltrifluoroacetone (TTFA, a specific inhibitor of mitochondrial electron transport complex II), diphenylene iodonium (DPI, inhibitor of NAD(P)H oxidase), N-acetylcysteine (NAC), and SP600125 (inhibitor of JNK) for 30 min in complete RPMI containing 10% FBS, followed by FFAs treatment for certain time in the presence of the inhibitor. All the reagents mentioned above were obtained from Sigma (USA).

Preparation of BSA-bound fatty acids

FFAs' solutions were prepared as described previously [20]. Briefly, 100 mM palmitate (PA) and 100 mM oleate (OL) stocks (Sigma, USA) were prepared in 0.1 m NaOH at 70°C and filtered. Five percent (wt/vol) FFAs-free BSA (Roche, USA) solution was prepared in double-distilled H_2O and filtered. A 5 mM FFAs/5% BSA solution was prepared by complexing an appropriate amount of FFAs to 5% BSA in a 60°C water bath. The above solution was then cooled to room temperature and diluted in RPMI 1640 to final concentrations.

Determination of cell viability

MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide, Sigma) was added to the cells in a 96-well plate, 24 h after the activation of FFAs. After 4 h incubation at 37°C, the stain was diluted with 150 μL DMSO. The absorbance of each well was then measured using a FluoStar-Optima microplate reader at 492 nm, and the viability of cells was presented as percentage of the control. Each treatment was replicated at least five times.

Detection of cytosolic and mitochondrial ROS

Cytosolic ROS was measured using 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA) (Sigma, USA), which can readily enter cells and be cleaved by esterase to yield DCFH, a polar, nonfluorescent product. ROS in cells promotes the oxidation of DCFH to yield the fluorescent product, dichlorofluorescein. After treatment for the indicated time periods, cells were collected and then incubated in PBS containing the reagent DCFH-DA (10 μmol/L) for 40 min at 37°C. Blank controls were set, in which DCFH-DA incubation was omitted. After incubation, cells were washed with PBS twice, trypsinized, re-suspended, and immediately submitted to flow cytometric analysis using a FACScan flow cytometer. The data based on the FL1 channel were analyzed with the CellQuest program.

Mitochondrial ROS was measured using MitoSOX Red (Invitrogen, USA), which is a live-cell permeant and is rapidly and selectively targeted to mitochondria. Once in the mitochondria, MitoSOX™ Red reagent is oxidized by superoxide and exhibits red fluorescence (with excitation at 510 nm and emission at 580 nm). After drug treatment for the indicated time periods, cells were collected and then incubated in Hank's balanced salt solution (HBSS) containing 5 μM MitoSOX Red for 30 min at 37°C. After incubation, cells were washed with PBS twice, then trypsinized, re-suspended, and immediately submitted to flow cytometric analysis. Data based on the FL2 channel were analyzed by the CellQuest program.

Measurement of NADPH oxidase activity

NADPH oxidase activity was measured using lucigenin chemiluminescence. Cells were trypsinized, pelleted by

centrifugation, and resuspended at 1×10^6 cells/mL with cold Krebs-Hepes buffer containing NaCl 119 mmol/L, Hepes 20 mmol/L, KCl 4.6 mmol/L, $CaCl_2$ 1.2 mmol/L, Na_2HPO_4 0.15 mmol/L, KH_2PO_4 0.4 mmol/L, $MgSO_4$ 1.0 mmol/L, $NaHCO_3$ 25.0 mmol/L, and glucose 5.5 mmol/L (pH 7.4). 300 μL cellular suspensions was put into a 96-well white plate in a luminescence reader and added with 10 μmol/L dark-adapted lucigenin (Sigma, USA) to start the reaction. Chemiluminescence was recorded every 15 s for 10 min. The lucigenin chemiluminescence was expressed as counts per minute per 10^6 cells. After measurement of background lucigenin chemiluminescence, NADPH (final concentration 100 μmol/L, Sigma) was added, and the measurements were continued for another 10 min. Fluorescence was analyzed using a FluoStar-Optima microplate reader in a fluorescence mode with an excitation filter of 440-nm wavelength. The difference between the values obtained before and after addition NADPH was calculated as the activity of NADPH oxidase.

Small interfering RNA transfection

Annealed double-stranded small interfering RNAs (siRNAs) for IRE1α were purchased from Takara. The sequences for rat IRE1α were designed and synthesized: AAGGCGATGATCTCAGACTTT. The scrambled siRNA sequences were also obtained from Takara. INS-1 Cells were seeded in 6-well plates and allowed to reach 60% confluence on the day of transfection. Cells were then transfected with siRNA by Lipofectamine reagent (Invitrogen) according to the manufacturer's transfection protocol. The transfected cells were incubated at 37°C for 24 h and then stimulated by PA.

Apoptosis ELISA

Cells were plated in 96-well plates and grown in RPMI 1640, exposed for certain concentration of FFAs-BSA in indicated times, and lysed. Oligonucleosomes in cytosol indicative of apoptosis-induced DNA degradation was quantified by the Cell Death ELISA^{PLUS} assay (Roche, USA) according to the manufacturer's instructions.

Caspase-3 activity assay

Caspase-3 activity (Sigma, USA) was measured using the substrate Ac-DEVD-AFC, and assayed according to the manufacturer's protocol. Briefly, INS-1 cells established in culture were incubated in RPMI containing 0.5% BSA with or without various fatty acids as indicated in the figure legends. Both adherent and detached cells were then harvested and combined. After sedimentation at 500 g for 10 min, the cells were washed twice with ice-cold PBS, lysed for 10 min on ice with a cell lysis buffer, and centrifuged (10 min, 15,000 g, 4°C) to remove debris. 50 mg proteins determined by the BCA protein quantification kit (Beyotime, China) was incubated with 50 μM Ac-DEVD-AFC at 30°C for 30 min. Fluorescence was analyzed using a FluoStar-Optima microplate reader in a fluorescence mode using an excitation filter of 380-nm wavelength and an emission filter of 505-nm wavelength. The reaction was allowed to proceed for 30 min with a reading every minute. Caspase-3 activity was obtained by calculating the slope of the reaction over 30 min and reporting the slope for each condition.

Real-time PCR analysis of mRNA expression

Total RNA was isolated with Trizol (Invitrogen, USA) and then subjected to cleanup using RNase-Free Water. The resultant DNA-free RNA was quantitated by UV spectroscopy at 260 nm. Then, total RNA from each sample was reverse transcribed to cDNA using PrimeScript™ RT reagent Kit (Takara, Japan). The SYBR Premix Ex Taq™ kit (Takara, Japan) was used for quantitative real-time RT-PCR analysis. The primers were designed using Primer Express software (Applied Biosystems) and synthesized by Takara. The specific primers used were as follows: GADD153/CHOP, forward 5′-CCAGCAGAGGTCACAAGCAC-3′, reverse 5′-CGCACTGACCACTCTGTTTC-3′; ATF-4, forward 5′-GTTGGTCAGTGCCTCAGACA-3′, reverse 5′-CATTCGAAACAGAGCATCGA-3′; BiP, forward 5′-CCACCAGGATGCAGACATTG-3′, reverse 5′-AGGGCCTCCACTTCCATAGA-3′; spliced XBP1, forward 5′-GAGTCCGCAGCAGGTG-3′, reverse 5′-GCGTCAGAATCCATGGGA-3′; Ire1α, forward 5′-TGTCCCACTTTG TGTCCAATGG-3′, reverse 5′-TTGCTCTTGGCCTCTGTCTCCTT-3′; and GAPDH, forward 5′-GCAAGTTCAACGGCACAG-3′, reverse 5′-GCCAGTAGACTCCACGACAT-3′. Real time fluorescence detection was carried out using an ABI 7500 Sequence Detector (Applied Biosystems). The results were analyzed using the relative standard curve method of analysis/ΔCt (threshold cycle) method of analysis.

Western blotting analysis of protein expression

Total protein extraction was done using RIPA lysis buffer (Sigma, USA), plus 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 mM NaF, 2 mM Na_3VO_4, and 1× complete protease inhibitor mixture (Sigma, USA). The protein concentration was determined using a BCA Protein Assay Kit (Beyotime, China). Equal protein concentrations were separated by 10% SDS-PAGE gel by a standard Western blot protocol. In brief, protein was transferred to Immuno-Blot PVDF Membrane and blocked in 5% nonfat milk in TBST. The membrane was incubated with corresponding

antibodies. P-IRE1α, IRE1α, p-PERK, PERK, p-JNK, and JNK were purchased from Cell Signal, and β-actin was from Sigma. Secondary peroxidase-labeled anti-rabbit or anti-mouse IgG (H + L) antibody (Abcam, USA) was applied. The signals were developed with the addition of an enhanced chemiluminescence solution (Invitrogen, USA). The gray value of each strap was detected and analyzed using Bio-Rad ChemiDoc XRS + (Bio-Rad).

Statistical analyses

Each experiment was carried out in duplicate or triplicate and three independent experiments were performed. Data are expressed as mean + standard deviation (SD) and analyzed using SPSS 16.0 software. For comparisons between groups, a Student's t test was performed. A one-way ANOVA followed by a Tukey's or Dunnett's test was performed to compare all groups or selected groups to control. A level of $P < 0.05$ was considered as statistically significant.

Results

Chronic FFAs treatment induces INS-1 apoptosis

INS-1 cells were incubated with 0.1, 0.25, and 0.5 mM PA for 2, 12, and 24 h. The same concentrations of OL and BSA were used as control. MTT analysis showed that incubation of cells with 0.1 and 0.25 mM PA for 2 h did not totally impair cell viability as compared with control and OL (Fig. 1a). When the incubation time extended to 12 h, both 0.25 and 0.5 mM PA induced apoptosis significantly, by about 21 and 43%, respectively, as compared with control (Fig. 1b). As shown in Fig. 1c, when the incubation time was extended to 24 h, apoptosis was increased in all concentrations by about 18, 52, and 90%, respectively. Seeing that 0.25 mM PA was large enough to induce biological events, it was used in the subsequent experiments.

The effect of mitochondria is earlier and more important as the source of ROS induction in INS-1 cells

In this study, INS-1 cells were used to evaluate the effect of FFAs on oxidative stress. ROS generation was tested in FFAs-treated INS-1 cells. As shown in Fig. 2a, the intracellular fluorescence, which represents the ROS in cytosol, was increased significantly at different times as compared with the BSA control and oleate group. The MitoSOX Red probe was used to measure mitochondrial ROS. It was found that the mitochondrial ROS was increased by 42 and 51% at 2 and 12 h, respectively (Fig. 2b).

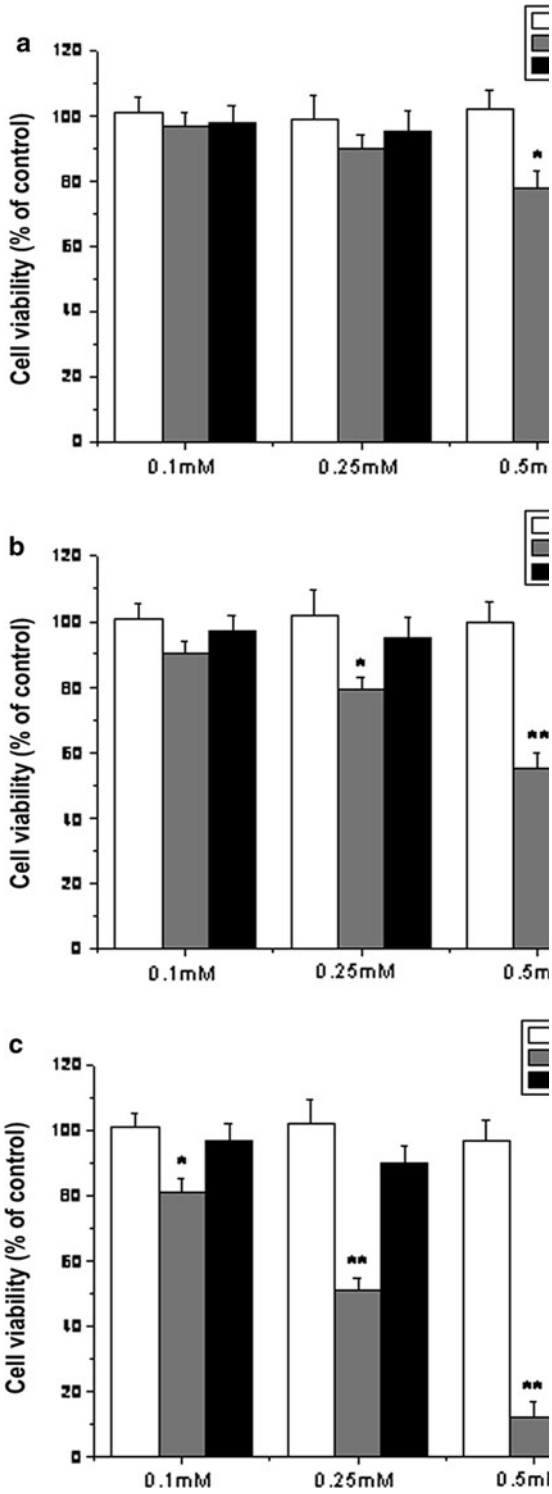

Fig. 1 The viability of INS-1 cells was inhibited by palmitate (PA) stimulation at different times. INS-1 cells were incubated with different concentrations of PA or OL (oleate) (0.1 mM, 0.25 mM, and 0.5 mM) for 2 h (**a**), 12 h (**b**) and 24 h (**c**). Common culture with 1% FBS was used as the total control (not shown). The viability was determined by MTT assay. Results were mean ± SD for five individual experiments where, for each condition, were performed in duplicate. * $P < 0.05$; ** $P < 0.01$ vs. control

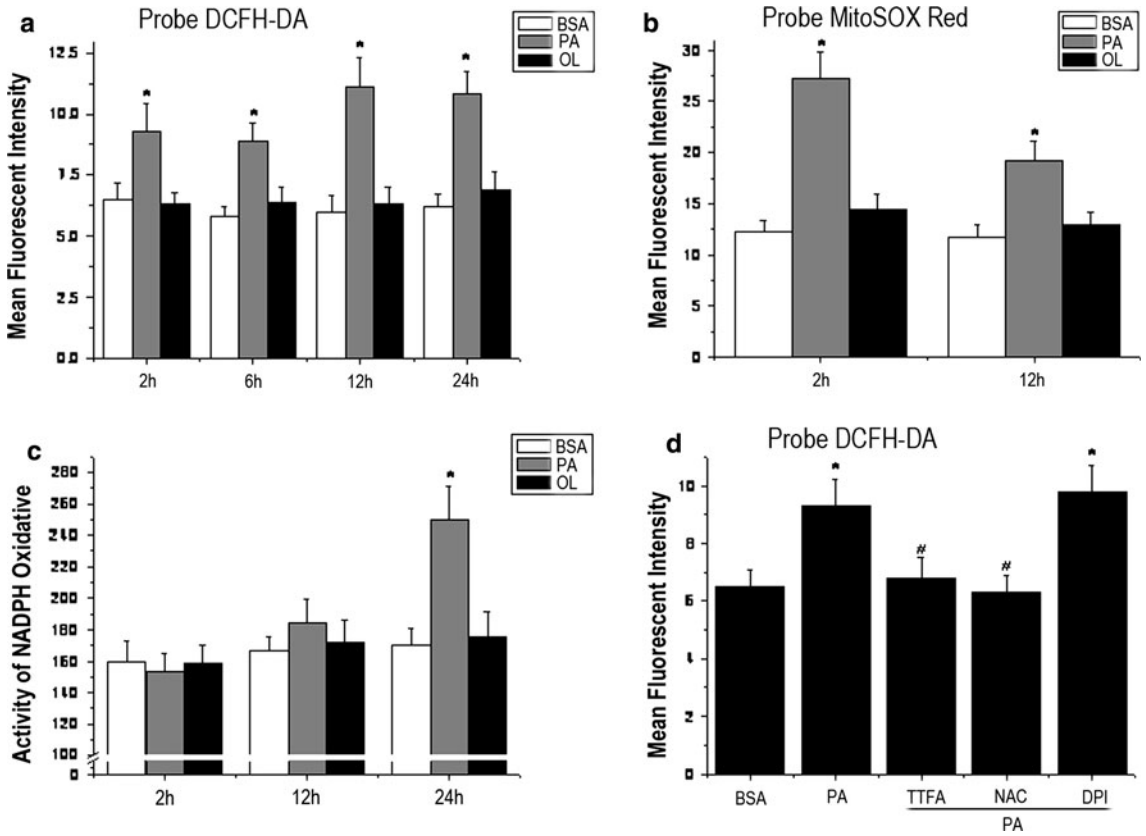

Fig. 2 The mitochondria respiratory chain is the major source in the early phase of PA-induced ROS generation. **a** INS-1 cells were cultured for 2, 6, 12, and 24 h in the presence of PA or OL (0.25 mM), and BSA with the same concentration as control. ROS was measured with DCFH-DA by flow cytometry. **b** INS-1 cells in the same condition incubated for 2 and 12 h. ROS from the mitochondrial pathway was detected with probe MitoSOX Red also by flow cytometry. **c** INS-1 cells were incubated with PA or OL (0.25 mM) for 2,12, and 24 h, chemiluminescence method was used to measure the activity of NADPH oxidase. **d** INS-1 cells cultured for 2 h with FFAs were pretreated for 30 min with different inhibitors (TTFA 0.5 μM, NAC 10 mM, or DPI 2 μM),and DCFH-DA was used to detect the ROS generation. Results from five separate experiments were pooled and the mean ± SD calculated. * $P < 0.05$ significantly different from the control; # $P < 0.05$, significantly different from PA group

Next, chemiluminescence method was used to measure the activity of NADPH oxidase, it was found that the activity of NADPH oxidase was raised by 47.1% in the present of PA at 24 h ($P < 0.05$). However, little change in the activity of NADPH oxidase was observed both at 2 and 12 h (Fig. 2c).

To study the source of ROS, TTFA (a specific inhibitor of mitochondrial electron transport complex II), DPI (inhibitors of NAD(P)H oxidase), and NAC were used in culture. At 2 h of incubation, cytosolic ROS generation in PA group with TTFA and NAC was inhibited ($P < 0.05$), while no inhibition was observed in cells treated with DPI (Fig. 2d).

Palmitate induces caspase-3 activation

To determine whether these changes would induce an apoptotic process, the effect of various FFAs on caspase-3 activation as a hallmark of apoptosis was evaluated (Fig. 3). In INS-1 cells, PA elevated the caspase-3 activity by 4.21 folds, while OL did not cause caspase-3 activation. Pretreatment of cells with TTFA and NAC reduced the activity of caspase-3 by 42.9 and 50.1%, respectively ($P < 0.05$).

Inhibition of ROS decreases FFAs-induced 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 real time PCR and western blotting. Each mRNA expression was examined in INS-1 cells treated with PA, OL, or BSA control with or without the presence of TTFA and NAC. CHOP, ATF-4, BIP, and spliced XBP1 mRNA expressions were elevated by 52, 63%, 1.2 folds, and 2.0 folds, respectively, after the treatment with PA for 12 h (Fig. 4a–d), which was consistent with other studies [21]. Pretreatment with TTFA or NAC reduced each

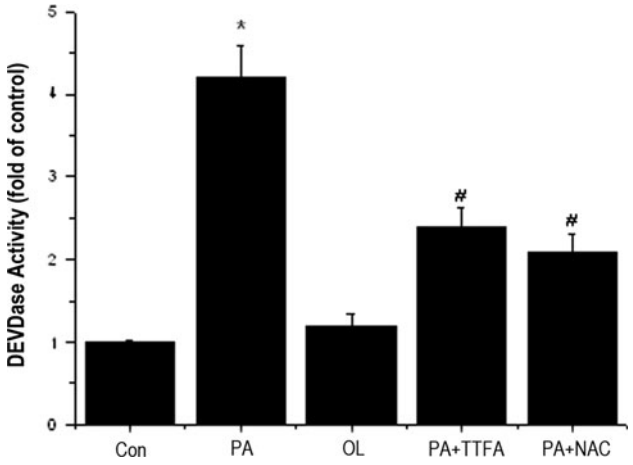

Fig. 3 The action of FFA on caspase-3 activation could be ameliorated by ROS inhibition. INS-1 cells were cultured for 24 h in the absence (control, Con) or presence of 0.25 mM PA, OL. Some groups were pretreated with TTFA (0.5 μM) or NAC (10 mM) for 30 min. Cell lysates were incubated with the caspase-3 substrate Ac-DEVDAFC. Fluorescence of released AFC was measured as described. The results were presented as fold increase over control and expressed as means ± SD of five separate experiments. * $P < 0.05$ significantly different from control; # $P < 0.05$, significantly different from PA group

mRNA expression ($P < 0.05$). The mRNA level of IRE1α changed little in the presence of PA (Fig. 4e), but the protein level of p-IRE1α increased by 5.6 folds compared with the control when cells were cultured for 24 h (Fig. 4f, h). Pretreated with TTFA or NAC decreased the protein level of p-IRE1α by 54.3 and 45.4%, respectively. PERK phosphorylation level, which was increased by 2.17 folds in PA-treated group compared with the control, was also down-regulated by ROS inhibitors (TTFA by 60.4% and NAC by 76%, Fig. 4f, g). In addition, ROS inhibition can reduce the phosphorylation of IRE1α and PERK in OL-treated groups although the ER stress was not increased compared with the control.

Inhibition of ROS decreases the phosphorylation of JNK

We next studied the JNK pathway, which could be activated by both ER stress and oxidative stress. INS-1 cells were incubated with FFAs for 24 h, in the pretreatment with TTFA, NAC, or SP600125. As shown in Fig. 5a, PA elevated the phosphorylation of JNK significantly by 3.76-fold as compared with the BSA control, and OL increased the phosphorylation of JNK by 1.7-fold, which was consistent with the previous study [11]. This effect was totally inhibited by SP600125, an inhibitor of JNK. ROS inhibition also reduced the phosphorylation level of JNK. TTFA inhibited the phosphorylation of JNK by 29.7% and NAC by 25.6% ($P < 0.05$).

Given that both ER stress and oxidative stress can activate JNK phosphorylation, we further examined the inhibition of IRE1α by siRNA to analyze the source of JNK activation induced by PA. As shown in Fig. 5b, JNK phosphorylation was significantly reduced by IRE1α siRNA both in PA and BSA group, and little difference was observed between them. These data indicate that activation of JNK by PA is mainly through ER stress.

Inhibition of ROS and JNK has a protective effect on apoptosis

Knowing that ROS inhibition decreased ER stress and phosphorylation of JNK, we next observed the effect of JNK blockade on apoptosis of INS-1 cell. Apoptosis was quantified by an ELISA assay for cytosolic oligonucleosome content indicative of apoptosis-induced DNA degradation. INS-1 cells were incubated with FFAs for different times, in the presence/absence of pretreatment (30 min) with TTFA, NAC, and SP600125 (Fig. 6a). After 2 h incubation, PA did not affect β-cell apoptosis, and the tendency for apoptosis was 1.35-fold higher than that in untreated cells incubated at 0.25% BSA (w/v). A similar result was obtained with OL, where little changes were observed after intervention with the same inhibitors. Incubation of INS-1 cells for 24 h sensitized the β-cells to a toxic effect of PA. The apoptotic rate of INS-1 cells treated with OL and PA was 1.5 and 6.1-fold higher, respectively, than that of the untreated control. However, co-culture with inhibitors for ROS generation induced less apoptosis as compared with cells treated with FFAs only. As shown in the Figure, TTFA decreased apoptosis by 47.5%, and NAC by 49.1%. Meanwhile, SP600125 decreased β-cells apoptosis of INS-1 cells with a 36% reduction of apoptosis in cells treated with PA, which was consistent with other studies [11, 22]. IRE1α knockout also demonstrated a protective role in INS-1 death caused by PA. As shown in Fig. 6b, the blockade of IRE1α decreased apoptosis by 49.8% compared with PA control.

Discussion

Palmitate is one of the most abundant saturated fatty acids in human diets and is the major fatty acid synthesized de novo in liver. In addition, plasma PA level is elevated in T2DM patients [1]. Exposure of pancreatic β-cells to PA could lead to the reduction in β-cell mass [23]. As shown by previous studies [24, 25], treatment of pancreatic β-cells with PA induces considerable β-cells apoptosis. Although oxidative stress and ER stress has been implicated as a pro-apoptotic process in ER stress-induced cell death, there are

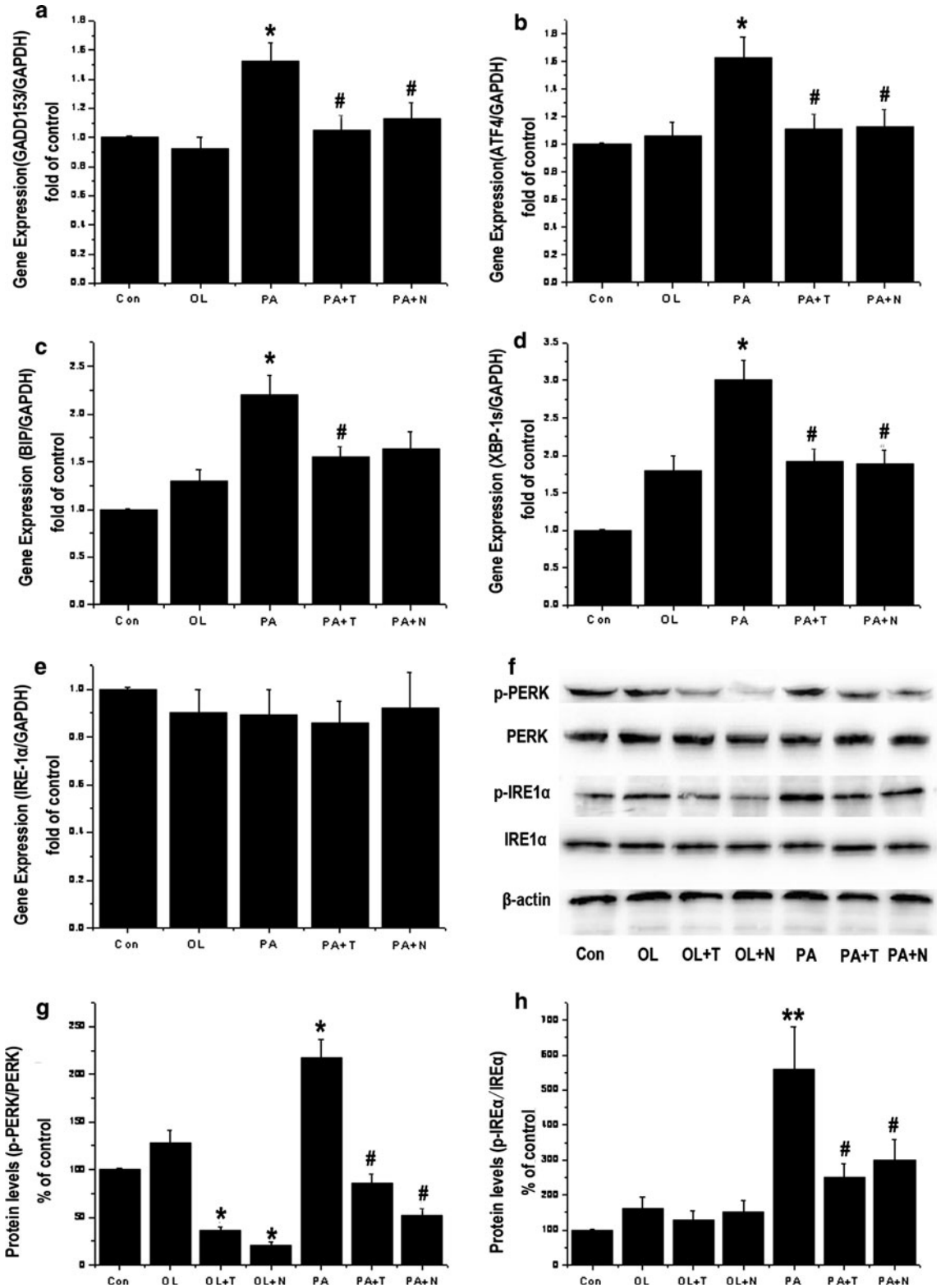

Fig. 4 ROS inhibition reduced PA-induced ER stress in INS-1 cells. INS-1 cells were incubated with and without 0.25 mM OL or PA for 12 h. Some groups were pretreated with TTFA (T, 0.5 μM) and NAC (N, 10 mM) for 30 min. Quantification of GADD153/CHOP (**a**), ATF4 (**b**), BIP (**c**), XBP-1s (**d**) and IRE1α (**e**) mRNA level. **f–h** The effects of FFAs on p-PERK and p-IRE1α protein level with or without ROS inhibition. INS-1 cells were incubated with and without 0.25 mM PA or OL for 24 h. Quantification of phosphorylation protein level. Results are presented as fold increase over control and expressed as means ± SD of five separate experiments. * $P < 0.05$, ** $P < 0.01$ significantly different from control; # $P < 0.05$, significantly different from PA group

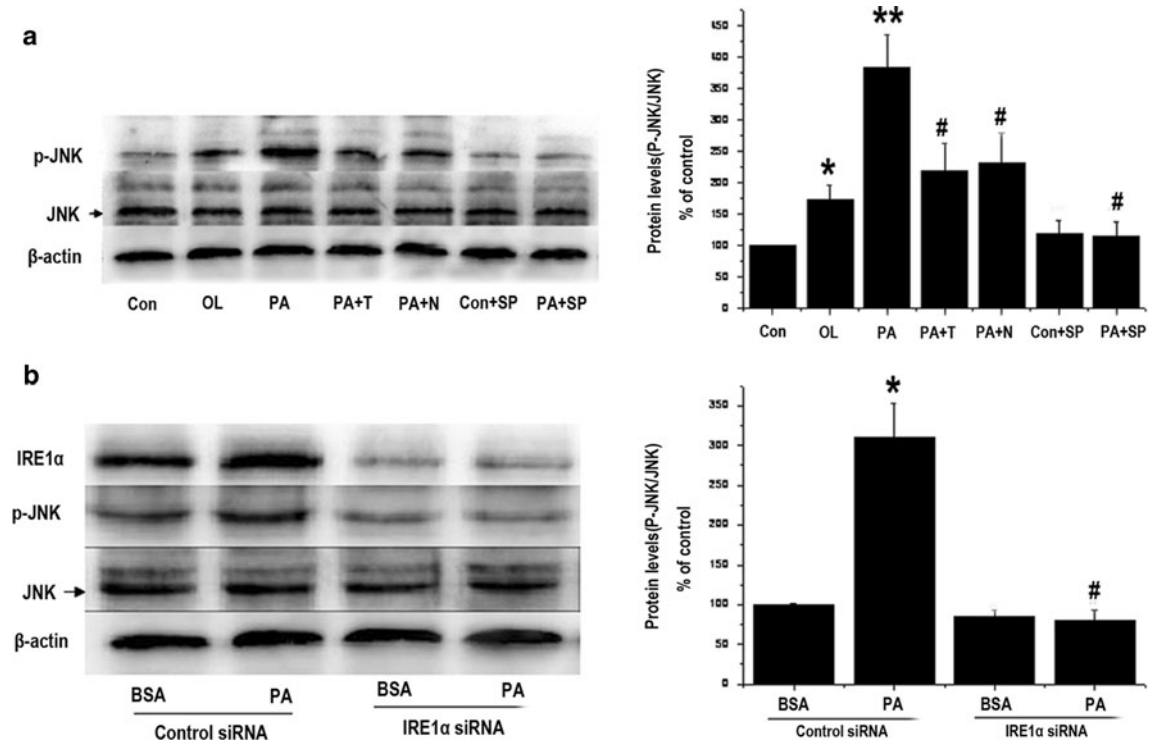

Fig. 5 PA increased the phosphorylation of JNK and this effect can be partly eliminated by ROS inhibition and IRE1α blockade. **a** INS-1 cells were incubated for 24 h with PA, OL (0.25 mM), or BSA (2.5 g/L). Cells were pre-incubated with TTFA (T, 0.5 μM), NAC (N, 10 mM), and specific inhibitors of JNK (SP, SP600125, 10 μM) for 30 min. **b** INS-1 cells were transfected with siRNA specific for IRE1α (20 nM) or negative control siRNA as a control. After transfection, the cells were treated with PA (0.25 mM) or BSA for 24 h. Quantification of p-JNK/JNK protein level. Results were presented as fold increase over control and are means ± SD of five separate experiments. * $P < 0.05$, ** $P < 0.01$ significantly different from control; # $P < 0.05$ significantly different from PA group

few studies describing the source of oxidative stress and its exact molecular connections to ER stress.

Mitochondria respiratory chain is an important site of ROS production within many cells [9]. Our study showed that in the early phase (at 2 h) of PA-induced ROS generation, the mitochondria pathway was the major source, when the Nox family of NADPH oxidase showed little influence. However, co-cultured for longer time (48 h), NOX2-derived ROS might play a critical role in the dysfunction and apoptosis of β-cells induced by FFA. Li [26] reported that NOX amplified CHOP induction through activation of the double-stranded RNA-dependent protein kinase (PKR), and apoptosis can be blocked by both genetic deletion of the NOX subunit Nox2. Some researchers pointed out that ROS from the mitochondria pathway increased the expression of nox1 and enhanced its activity in tumor cells and human 293T cells [27, 28]. Other studies showed that it took at least 12–72 h to generate ER stress treated with FFAs [12, 13, 29], which was consistent with our study in INS-1. In this research, we found that TTFA, a specific inhibitor of complex II in mitochondrial electron transport, inhibited ROS generation in mitochondria, also significantly reduced ROS generation in cytosol. All these results suggest that the mitochondrium is the major source of ROS generation and plays a vital role in apoptosis in the early phase.

Endoplasmic reticulum is known to be the major site in cells where protein is synthesized. Because of high rate protein synthesis, pancreatic β-cells are susceptible to changes in ER homeostasis [10, 30]. When abnormal oxidation reaction occurs in ER, disulfide-bond formation is increased and the unfolded proteins are accumulated in the ER lumen, which in turn causes accumulation of excess ROS, thus forming a vicious circle and ultimately leading to cell death [31]. Synthetic analog of preimplantation factor (sPIF), the analog of a novel embryosecreted immunomodulatory peptide, could preserve the function of islets by controlling protein processing, preventing protein misfolding and aggregation, and reducing associated oxidative stress [32]. The data in our study showed that oxidative stress, especially the ROS from mitochondria, enhanced ER stress, thus induced β-cells apoptosis. These effects were mediated by ROS, which was suggested by the suppression of ROS production in the presence of TTFA,

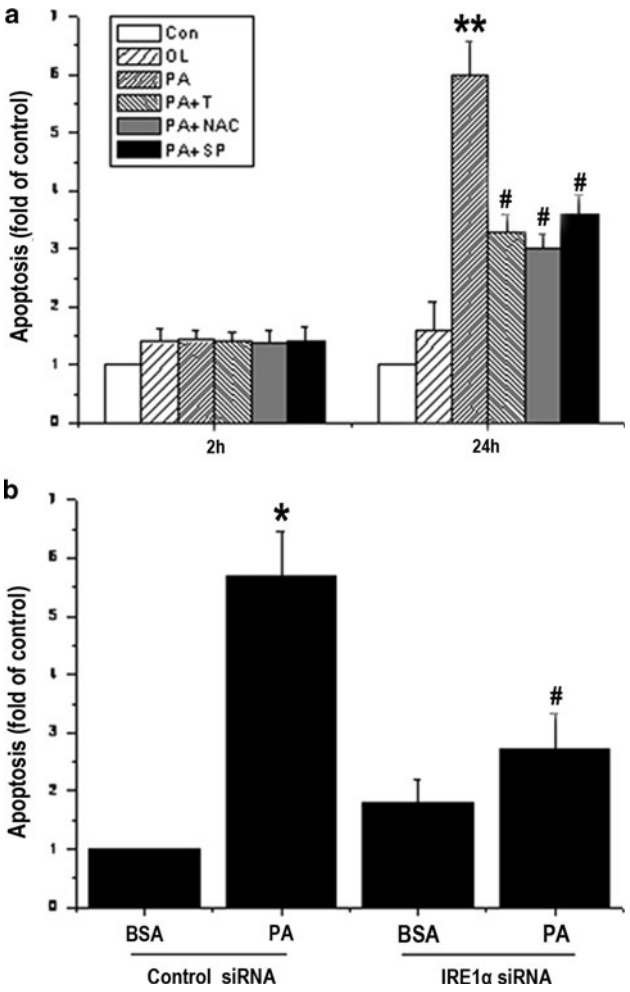

Fig. 6 JNK inhibition protects INS-1 against apoptosis. **a** INS-1 cells were incubated with FFAs, in the presence or absence of TTFA (0.5 μM), NAC (10 mM), and SP600125 (10 μM). **b** INS-1 cells were transfected with siRNA specific for IRE1α and then treated with PA (0.25 mM) for 24 h. Apoptosis was assessed using the cell death apoptosis kit that measures the amount of cytoplasmic DNA-associated histone complexes. Results were presented as fold increase over control and are means ± SD of five separate experiments. * $P < 0.05$, ** $P < 0.01$ significantly different from control; # $P < 0.05$ significantly different from PA group

NAC. More importantly, the levels of ER stress-related effectors were also decreased when pretreated with ROS inhibitors. These data indicate that ROS, especially from the source of mitochondrial pathway, may also participate in the signal of ER stress. However, the concrete mechanisms of the regulation need to be further studied.

ER stress causes apoptosis mainly through activation of the PKR-CHOP pathway, IRE1α-JNK pathway, caspases-3 and the family of bcl2 [33]. It was found in our study that ER stress-related factors CHOP, ATF4, spliced XBP1, and BiP were elevated with PA for 12 h. The protein expression of p-IRE1α and p-PERK were also up-regulated in PA group, while the total IRE1α and PERK changed little, which indicate that PA induces up-regulation of p-IRE1α and p-PERK at the post-transcriptional level. Based on detailed analysis of IRE1α expression, JNK, and phosphorylation, our findings suggest the IRE1α-JNK pathway is involved in lipotoxicity induced by PA in INS-1 cells, which is consistent with other studies [22, 34].

Next, we focused on JNK signal pathway, activated both by ER stress and oxidative stress. Activation of JNK signal is involved in the oxidative stress-induced reduction of insulin gene expression. Suppression of JNK signal can protect β-cells from oxidative stress [35]. JNK phosphorylation also can be increased by ER stress. Urano et al. [34] showed that ER stress suppressed insulin receptor signaling via IRE-1α-dependent activation of the JNK pathway. PA-induced ER stress can activate the JNK pathway through activation of Foxo1 [36]. Our research showed that ROS inhibition could reduce the level of JNK phosphorylation by TTFA and NAC. More importantly, IRE1α knockout can mostly inhibit JNK activation induced by PA. This means, although oxidative stress induced by PA can directly activate JNK phosphorylation, the main source of JNK activation probably comes from IRE1α-JNK pathway by ER stress. However, ROS can regulate JNK pathway by affecting p-IRE1α level.

Inhibition of JNK phosphorylation by the synthetic inhibitor SP600125 ameliorated PA-induced apoptosis. Similar results were also observed by ROS inhibition and IRE1α knockout. Considering ROS inhibition can reduce IRE1α phosphorylation, we believe ROS inhibition protects INS-1 cells from apoptosis by inhibiting both oxidative stress and ER stress. However, we found that JNK inhibition cannot totally reduce apoptosis. So, other mechanisms may also participate in PA-induced death, such as activation of protein kinase C, inhibition of protein kinase B activity, activation of calpain-10, and ceramide formation [37–40].

In this research, ROS inhibition decreased caspase-3 activity, another mechanism mediated apoptosis in ER stress. Given that caspases are also factors belonging to the mitochondrial apoptotic pathway [41], mitochondria may play a pivotal role in inducing apoptosis by ER stress and oxidative stress.

Above all, this study identified mitochondrial ROS as an important inducer to ER stress, also as a critical factor in PA-induced apoptosis. ROS production exerts amplification and regulation on JNK activity, and there may be some other transduction mechanisms that in turn contribute to cell death. Although the concrete mechanisms need to be further explored, ROS inhibition could potentially be a therapeutic target in protecting pancreatic β-cells.

Acknowledgment This study was supported by the National Natural Science Foundation of China (No. 30872727 and No. 81170757).

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