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ORIGINAL ARTICLE

Oleic acid protects insulin-secreting INS-1E cells against palmitic acid-induced lipotoxicity along with an amelioration of ER stress

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Abstract

Purpose It is demonstrated that unsaturated fatty acids can counteract saturated fatty acids-induced lipotoxicity, but the molecular mechanisms are unclear. In this study, we investigated the protective effects of monounsaturated oleic acid (OA) against saturated palmitic acid (PA)-induced cytotoxicity in rat β cells as well as islets, and mechanistically focused on its regulation on endoplasmic reticulum (ER) stress.

Methods Rat insulinoma cell line INS-1E cells and primary islets were treated with PA with or without OA for 24 h to determine the cell viability, apoptosis, and ER stress. SD rats were fed with high-fat diet (HFD) for 16 w, then, HFD was half replaced by olive oil to observe the protective effects of monounsaturated fatty acids rich diet.

Results We demonstrated that PA impaired cell viability and insulin secretion of INS-1E cells and rat islets, but OA robustly rescued cells from cell death. OA substantially alleviated either PA or chemical ER stressors (thapsigargin or tunicamycin)-induced ER stress. Importantly, OA attenuated the activity of PERK-eIF2α-ATF4-CHOP pathway and regulated the ER Ca^{2+} homeostasis. In vivo, only olive oil supplementation did not cause significant changes, while high-fat diet (HFD) for 32 w obviously induced islets ER stress and impaired insulin sensitivity in SD rats. Half replacement of HFD with olive oil (a mixed diet) has ameliorated this effect.

Conclusion OA alleviated PA-induced lipotoxicity in INS-1E cells and improved insulin sensitivity in HFD rats. The amelioration of PA triggered ER stress may be responsible for its beneficial effects in β cells.

Keywords Palmitic acid · Oleic acid · Apoptosis · ER stress · Pancreatic β cells

Introduction

Type 2 diabetes (T2DM) has emerged as a serious worldwide health problem. Notably, the population of prediabetes, a precursor stage before diabetes mellitus characterized as impaired fasting blood sugar and impaired glucose tolerance, has strikingly increased. It is reported that the prevalence of prediabetes reaches ~35% in the overall population of China, and higher than 40% in either the older or overweight population [1, 2]. Given that, it calls for more effective interventions targeting prediabetic patients to prevent the epidemiological explosion of diabetes.

Free fatty acids (FFA), especially saturated fatty acids (SFA) have been identified as one of the most important causal factors in the development of T2DM [3–5]. FFA are important to the physiological function of pancreatic β cells, however, chronic excess FFA supply can reduce insulin biosynthesis and secretion, resulting in β-cell apoptosis, a phenomenon called lipotoxicity. The most abundant SFA present in the diet and serum is palmitic acid (PA, 16:0) [6]. Piles of data demonstrated that PA is able to trigger lipotoxicity in β cells [7], hepatocytes [8], muscular cells [9], and so on. On the contrary, unsaturated fatty acids, including monounsaturated fatty acids (MUFA), appear healthier, and are able to counteract PA-induced lipotoxicity [10–13]. Moreover, obese patients given long-term MUFA rich diet have shown improved insulin sensitivity [14, 15] and lower risks of cardiovascular diseases [16, 17]. Oleic acid (OA, 18:1 $n = 9$) is a typical MUFA, and is the

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predominant component in olive oil (70–80%). The beneficial effects of olive oil as food supplement are widely investigated [18, 19], but the molecular mechanisms remain poor understood.

The mechanisms by which SFA exert their detrimental effects are still not clearly elucidated. SFA elicited stress in the endoplasmic reticulum (ER) plays an important role in lipotoxicity, especially in β-cell dysfunction. The imbalance between the ER protein load and ER folding capacity causes ER stress, leading to the hyperactivation of the unfolded protein response (UPR), which results in β-cell dysfunction and death [20, 21]. In the present study, we asked whether oleic acid was able to protect β cells against palmitic acid-induced lipotoxicity, and whether its protective effects were associated with the regulation on ER stress.

Materials and methods

In vivo experiments

Animals and diets

Male Sprague–Dawley (SD) rats (100–150 g body weight) were purchased from DASHUO animal company (SCXK (CHUAN) 2015-30, Chengdu, China). SD rats were housed in the animal care facilities under controlled temperature (21–25 °C) and humidity (40–70%) with 12-h light and dark cycle. After a 2-week adaptive period, rats were first fed with 1) chow diet (containing 4% fat, control group), 2) chow diet plus extra virgin olive oil (Baena, Spain, olive oil group) or 3) high fat diet (HFD, containing 10% fat and 2% cholesterol) for 16 w to induce obesity. The chow diet and HFD were purchased from Sichuan Academy of Agricultural Sciences (Chengdu, Sichuan, China). The olive oil was bought from supermarket, and the ingredients were indicated as 72% (weight ratio) oleic acid, 16% saturated fatty acids, and 12% polyunsaturated fatty acids. Thereafter, half of the HFD rats were continuously fed with HFD for a further 16 w (HFD group), and for another half of the HFD rats, the diet was replaced by a mixed diet with half HFD and half olive oil (calculated by calorie, HFD + Olive oil group) for a further 16 w. The food calorie in all the groups except for control group was equal as ~83 kcal/rat/day. All of the experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of Sichuan University.

Evaluation of islets function

Fasting blood glucose was measured with a One-Touch AccuChek Glucometer (Roche Diagnostics, Switzerland) and fasting serum insulin was detected using a Mercodia Ultrasensitive Rat Insulin ELISA Kit (Mercodia, Uppsala, Sweden). Glucose tolerance test (GTT) was carried out on 16 h fasted rats by intraperitoneally injection of 2 g kg^{-1} glucose (Kelun, Sichuan, China). Blood glucose was measured after 0, 15, 30, 60, and 120 min by venous tail bleeding. For insulin tolerance test (ITT), rats had been fasted for 6 h, followed by intraperitoneal injection with 1 U kg^{-1} insulin (Wanbang, Jiangsu, China), and blood glucose was measured at 0, 15, 30, 60, and 90 min, respectively. Immunohistochemical analysis of pancreas tissues was performed using anti-insulin antibody (LifeSpan BioSciences, USA) to identify the histological changes in islets.

Islet isolation and purification

After the treatment, pancreatic islets were isolated and purified from SD rats according to a previously described protocol [22]. Briefly, the pancreatic tissues were perfused with 15 ml collagenase P (1.5 mg/ml, Roche, Basel, Switzerland), and then digested at 37 °C for 10 min. The islets were then purified by centrifugation with human lymphocyte separation media (LSM; Sigma-Aldrich, St. Louis, MO). Single islet was manually picked up under microscope for the subsequent assessments.

In vitro experiments

Cell culture

Rat insulinoma cell line INS-1E cells were cultured in RPMI1640 supplemented with 10% fetal bovine serum (FBS), 1% antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin), 1 mM sodium pyruvate and 50 μM β-mercaptoethanol at 37 °C with 5% CO_2. When treated with PA or OA, the medium was changed to a fresh medium containing 2% FBS.

Preparation of fatty acids solutions

Fatty acids solutions were prepared by dissolving saturated fatty acid palmitic acid (Aladdin, China) and unsaturated fatty acids oleic acid, palmitoleic acid, or linoleic acid (Aladdin, China) in 100% ethyl alcohol to produce a high concentrated solution of 100 mM, then bound with 20% fatty acid-free BSA (Solarbio, China) by incubation at 50 °C for 1 h to yield a final stock solution of 10 mM.

Cell viability and apoptosis analysis

After the treatments, cells were washed three times with PBS and cell viability was measured using a Cell Counting Kit-8 (CCK8, DOJINDO, Japan). For apoptosis assay, both

attached and floating cells were harvested and centrifuged at 1000 rpm for 5 min. Annexin V-FITC and propidium iodide (PI) (BD, USA) staining were used to detect the apoptotic cells by a FACS Calibur instrument (BD Biosciences, USA).

Insulin secretion analysis

After 24 h treatments, the culture medium was collected to determine insulin level using a Mercodia Ultrasensitive Rat Insulin ELISA Kit (Mercodia, Uppsala, Sweden). Meanwhile, BCA Protein Assay Kit (Cwbio, China) was used to measure protein concentration. The chronic insulin secretion of INS-1E cells was presented as 24 h secretion of nanogram insulin per milligram of protein.

RNA isolation and quantitative real-time PCR

Total RNA was isolated using Trizol reagent (Ambion, USA), and RNA was reverse-transcribed into cDNA using a high capacity cDNA Synthesis Kit (Vazyme, China). Real-time PCR was performed to assess gene expression in a Bio-Rad QPCR Machine (CFX96, Bio-Rad, USA) using SYBR Green master mix (Vazyme, China).

Western blot analysis

Cells were lysed using RIPA lysis buffer, and the supernatant was collected after centrifugation at $14{,}000 \times g$ for 15 min at 4 °C. Protein extracts were separated by 10% SDS-PAGE gel and transferred to 0.2 μM PVDF membranes. After blocked for 2 h in 5% skimmed milk at room temperature, the membranes were subsequently probed by primary antibodies, including cleaved caspase-3 (CST, USA), BAX/BCL-2 (Abcam, USA), CHOP/phosphorylation of eIF2α (Santa, USA), and p-PERK/ ATF4/ ATF6 (Huaan, China). The immunoblots were visualized using a ChemiDoc™ imaging system (Bio-Rad, USA).

Electron microscopy

INS-1E cells were collected and centrifuged at 1000 rpm for 5 min. After washed one time with PBS, 500 μl of glutaraldehyde was added to the cells. The samples were stored at −20 °C before subjected to electron microscopy (it was conducted by the Department of Pathology of West China Hospital).

Measurements of cytosolic free Ca^{2+} by flow cytometry

After treatments, the INS-1E cells were collected and washed with HBSS. The cytosolic free calcium was labeled by 1 μM Fluo-4 AM (DOJINDO, Japan). Subsequently, the cells were washed with calcium-free HBSS, then incubated in HBSS to allow complete esterification of the probe. After basal measurements were done, calcium release from the ER was initiated using 5 μM thapsigargin, which inhibits Serca2b-mediated calcium reuptake. Calcium release into cytosol was monitored as time-dependent increase in Fluo-4 AM fluorescence intensity at excitation and emission wavelengths of 494 and 516 nm detected by flow cytometry (Beckman, USA).

Acridine orange and ethidium bromide (AO/EB) staining

Pancreatic islets were isolated from healthy SD rats, and cultured in 12-well plates for 24 h. Then, the islets were treated with BSA, PA or PA plus OA for a further 24 h and subjected to AO/EB staining (100 μg/ml of AO and 100 μg/ml of EB mixed in PBS, Aladdin, China). The fluorescence of cells was observed under fluorescent microscope.

Glucose stimulated insulin secretion

Pancreatic islets were isolated from healthy SD rats, and cultured in 6-well plates for 24 h. Then, manually pick-up appropriate number of islets into another 6-wells plate. The islets were treated with BSA, PA or PA plus OA for a further 24 h. After the treatments, these islets were washed with basal Krebs-Ringer bicarbonate (KRB) buffer (115 mM NaCl, 4.7 mM KCl, 10 mM $NaHCO_3$, 1.28 mM $CaCl_2{\bullet}2H_2O$, 1.2 mM $MgSO_4{\bullet}7H_2O$, 1.2 mM KH_2PO_4, 10 mM HEPES, 0.5% BSA, pH 7.4). Then pick them up again to new wells with basal KRB buffer to starve them for 40 min in incubator. Ten size matched islets (in triplicate samples) were placed in one well of 48-well plates, then stimulated with low glucose (1.1 mM) or high glucose (16.7 mM) KRB buffer for 1 h. In the end, the supernatant was collected and insulin secretion was determined by Rat Insulin ELISA Kit (Mercodia, Uppsala, Sweden). The stimulation index >1 (insulin secretion in high glucose divided by insulin secretion in low glucose) for each batch of isolated islets confirmed appropriately functioning islets.

Statistical analysis

Experiments were performed at least three times and all the quantitative data were presented as mean ± SD. GraphPad Prism 6 was used for statistical analyses. Data were evaluated with a two-tailed, unpaired Student's *t*-test or compared by one-way analysis of variance. A value of $P < 0.05$ was considered statistically significant.

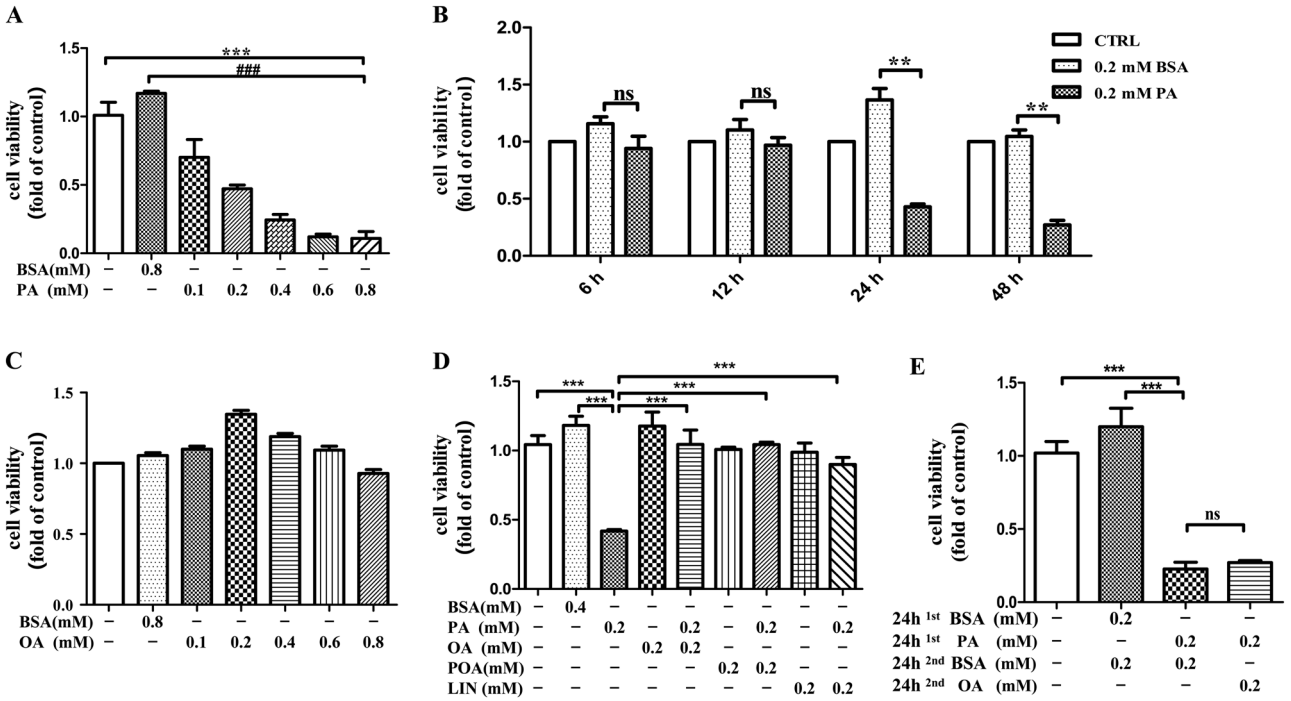

Fig. 1 Time- and dose-dependent effects of different fatty acids on INS-1E cells. Cell viability of INS-1E cells was assessed using CCK8 assay. **a** and **c** cells were exposed to various concentrations of PA or OA alone for 24 h. **b** Cells were treated with 0.2 mM PA for 6, 12, 24, and 48 h. **d** Cells were treated with 0.2 mM PA or combination of 0.2 mM PA plus 0.2 mM OA (PA + OA)/0.2 mM POA (PA + POA)/ 0.2 mM LIN (PA + LIN) for 24 h. **e** 0.2 mM BSA or OA was added after 0.2 mM PA withdraw (24 h PA followed by 24 h OA). The data are presented as means ± SD for $n > 4$ biological replicates; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. control; $^{\#\#\#}P < 0.001$ vs. BSA; ns, no significant differences between two connected groups

Results

Palmitic acid impaired the viability of INS-1E cells, and oleic acid robustly rescued cells from cell death

PA (C18:0) significantly deteriorated the cell viability of INS-1E cells in a dose and time-dependent manner (Fig. 1a, b). Conversely, monounsaturated OA (C18:1) was nontoxic to INS-1E cells, even at a high concentration (0.8 mM) after 24 h exposure (Fig. 1c). Interestingly, the combination of OA and PA at a mole ratio of 1:1 (0.2 mM PA + 0.2 mM OA) completely rescued the cells from cell damage (cell viability increased from 40.12% to 101.1%). Similarly, unsaturated palmitoleic acid (POA, C16:1) and linoleic acid (LIN, C18:2) at a concentration of 0.2 mM were able to completely block the cell death induced by 0.2 mM PA (Fig. 1d). However, when OA was added after PA withdrawal (24 h PA followed by 24 h OA only), OA showed bare protection on INS-1E cells. Likewise, replacement of PA containing media with normal media did not reverse the cell viability (Fig. 1e). These results suggested that the interaction between PA and OA probably has played an important role in the protective mechanism of OA in this scenario.

Oleic acid protected INS-1E cells from apoptosis and maintained the insulin secretion function

INS-1E cells were stimulated with 0.2 mM PA, 0.2 mM OA or the combination of both for 24 h. Annexin V/PI staining showed that the number of apoptotic cells was substantially increased after 24 h incubation with PA (37.3%), in comparison with that of BSA control cells (8.0%). OA did not trigger obvious apoptosis. But when concomitant added with PA, OA robustly suppressed the occurrence of β-cell apoptosis (Fig. 2a, b). Furthermore, we found consistent result in the protein expression of cleaved caspase-3, a typical biomarker of apoptosis. The combination of PA and OA significantly inhibited the expression of cleaved caspase-3, which convinced the protective role of OA in PA-induced apoptosis (Fig. 2c).

To assess the impact of PA or OA on chronic insulin secretion of INS-1E cells, we measured the insulin concentration in the culture medium after 24 h treatment. The results suggested that the basal insulin secretion was significantly inhibited after 24 h incubation with 0.2 mM PA. In contrast, cells stimulated with OA only or combination of PA and OA have maintained the insulin secretion capacity (Fig. 2d). Taken together, OA effectively prevented INS-1E

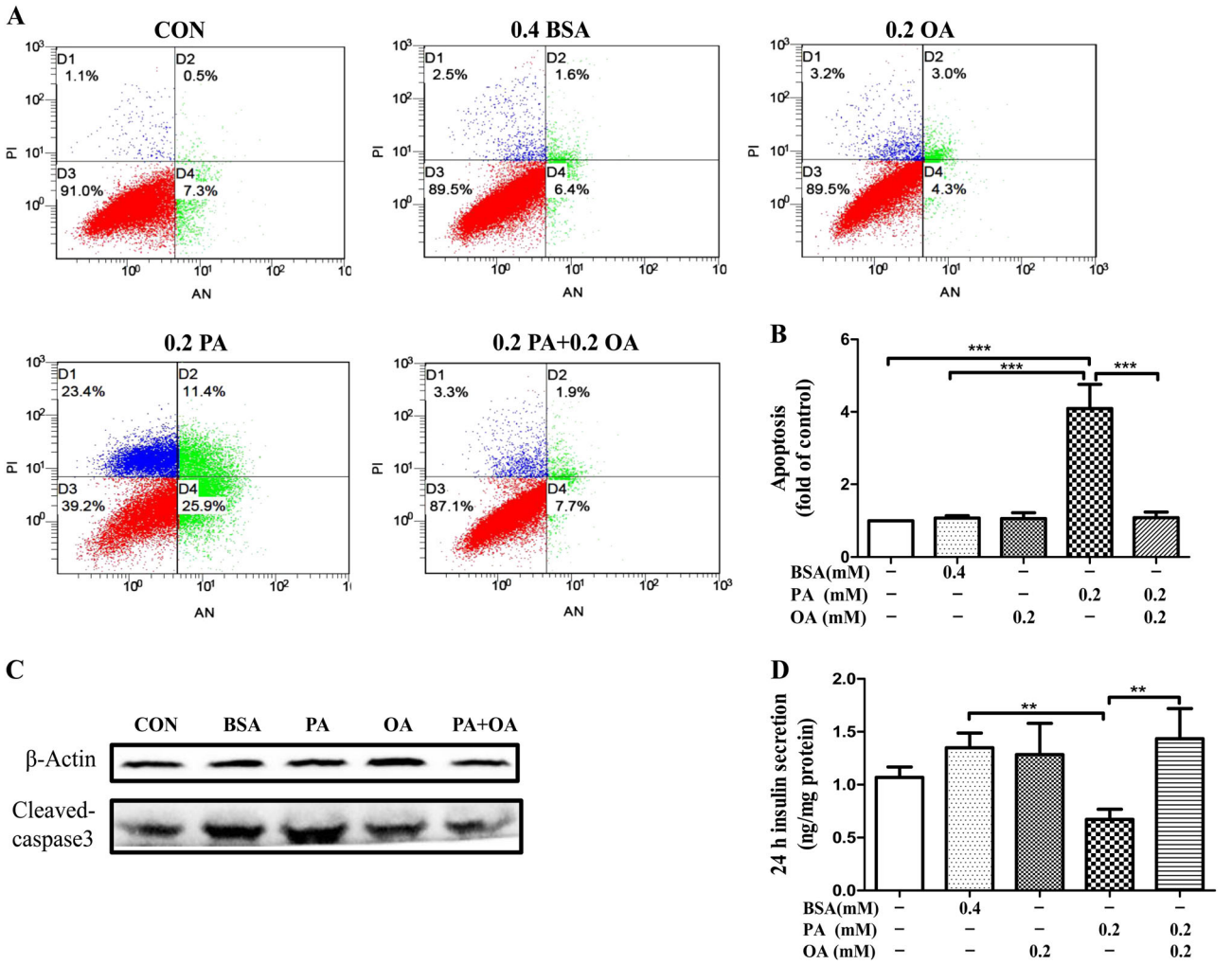

Fig. 2 OA suppressed PA-induced apoptosis in INS-1E cells. INS-1E cells were treated with 0.2 mM PA, 0.2 mM OA alone or combination of 0.2 mM PA plus 0.2 mM OA (PA + OA). **a** Apoptosis assay using FCM with AV/PI staining after 24 h treatment. The numbers at the lower or upper right indicated the percentages of early or late apoptotic cells, respectively. **b** Histogram analysis of FCM of apoptosis. **c** Representative western blots of Cleaved caspase-3 after 24 h treatment, and β-actin was used as a protein-loading control. **d** Chronic insulin secretion of INS-1E cells were detected after 24 h treatment using ELISA. The data are presented as means ± SD for $n > 4$ biological replicates; $^{***}P < 0.001$

cells from PA-induced lipoapoptosis, and maintained the basic insulin secretion function.

Oleic acid protected INS-1E cells from palmitic acid-induced ER stress

To answer the question that whether the protective effects of OA are related to the regulation of ER stress in the context of lipotoxicity, we first analyzed the expression of the ER stress-related molecular markers in INS-1E cells. We found that PA challenge obviously upregulated the protein expression of markers representing the three ER arms. The alterations included the PERK arm markers: phosphorylated protein kinase R (PKR) like endoplasmic reticulum kinase (P-PERK), phosphorylated eukaryotic initiation factor 2 alpha (P-eIF2α), activating transcription factor 4 (ATF4) and C/EBP homologous protein (CHOP); the Inositol- requiring enzyme 1(IRE-1) arm markers: sliced Xbp-1 (Xbp-1s), and activating transcription factor 6 (ATF6) representing the third ER arm. In addition, the ER chaperone protein BIP (GPR78) was slightly increased as well. Among them, the most remarkable alterations were observed in PERK arm (PERK-eIF2α-ATF4-CHOP). As shown in Fig. 3a, most of the upregulations occurred from 6 h after PA exposure, whereas the expressions of PERK, P-PERK and ATF4 dropped down at 24 h. Conversely, these upregulations were diminished at 6 h, 12 h, 18 h, or 24 h after concomitant addition of OA to counteract PA. Apart from the ER markers, we found that the protein expression of proapoptosis protein BAX was suppressed and antiapoptosis protein BCL-2 was increased by OA in the context of PA stimulation (Fig. 3a). Consistently, PA

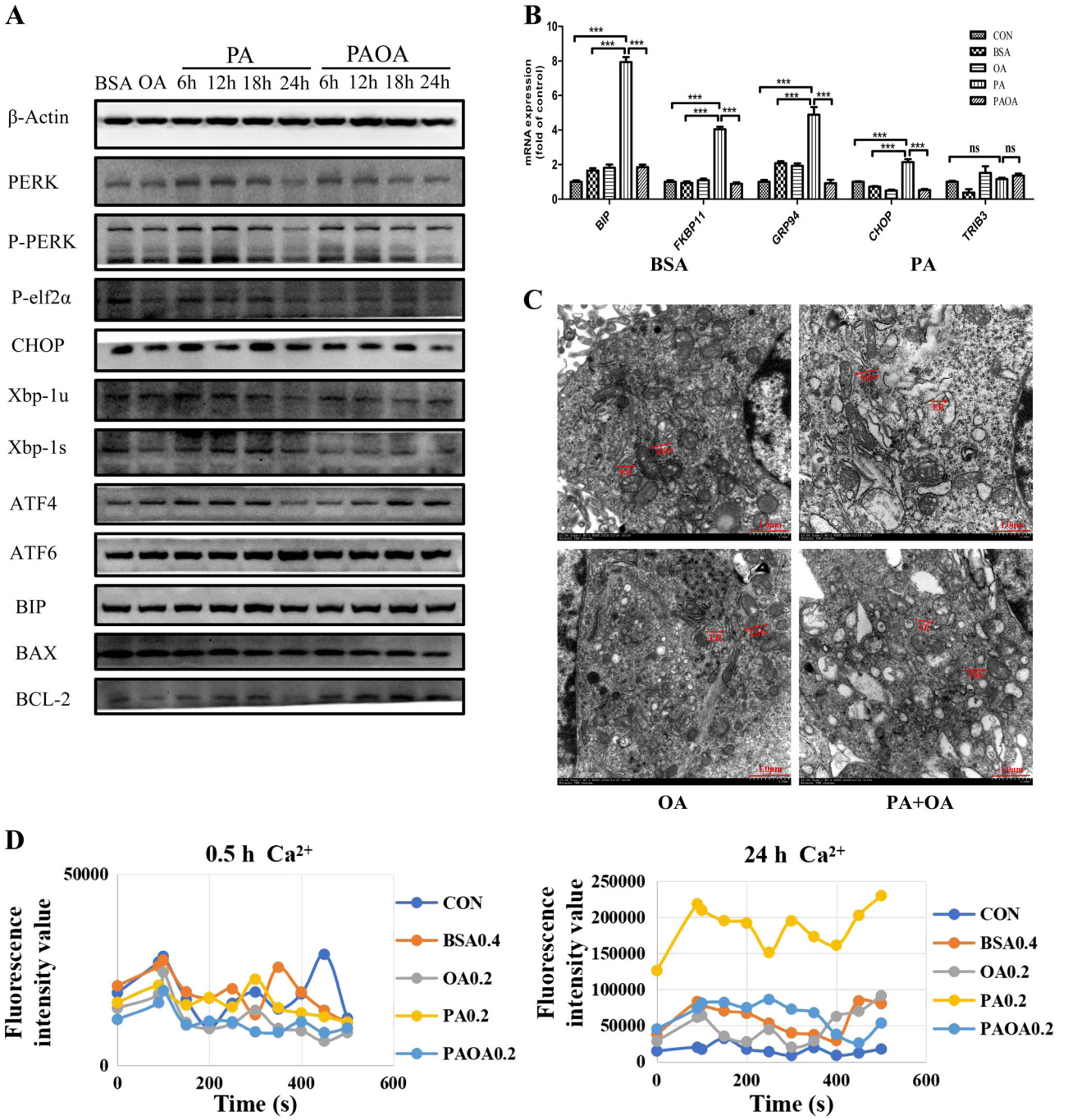

Fig. 3 Effects of OA on PA-induced ER stress. INS-1E cells were treated with 0.2 mM PA, 0.2 mM OA or combination 0.2 mM PA plus 0.2 mM OA (PA + OA) for 6, 12, 18, and 24 h. **a** Representative western blots of PERK, P-PERK, P-eIF2α, CHOP, Xbp-1u, Xbp-1s, ATF4, ATF6, BIP, BAX, and BCL-2 after 6, 12, 18, and 24 h treatment, respectively. β-actin was used as a protein-loading control. **b** The mRNA expression of a couple of UPR genes, including *BIP*, *FKBP11*, *GRP94*, *CHOP*, and *TRIB3* after 24 h treatment. **c** Ultrastructural alterations of ER were visualized by TEM. Shown are representative TEM images of INS-1E cells after 24 h incubation with BSA, PA, OA, or a combination of PA and OA. Arrows: the structure of ER. **d** Effects of PA and OA on intracellular Ca2+homeostasis. After basal measurements were made, 5 μM SERCA inhibiter thapsigargin (Thp) was added to deplete the ER Ca2+. The cytoplasmic Ca2 + was monitored by Fluo-4 AM fluorescence recorded by FCM. The data are presented as means ± SD for $n > 4$ biological replicates; ***$P < 0.001$

exposure increased the mRNA expression of a couple of unfolded protein response (UPR) genes, such as *BIP*, *FKBP11*, *GRP94*, and *CHOP*, while these upregulations have not been found in OA-treated cells. The combination of PA and OA inhibited the expression of UPR genes (Fig. 3b).

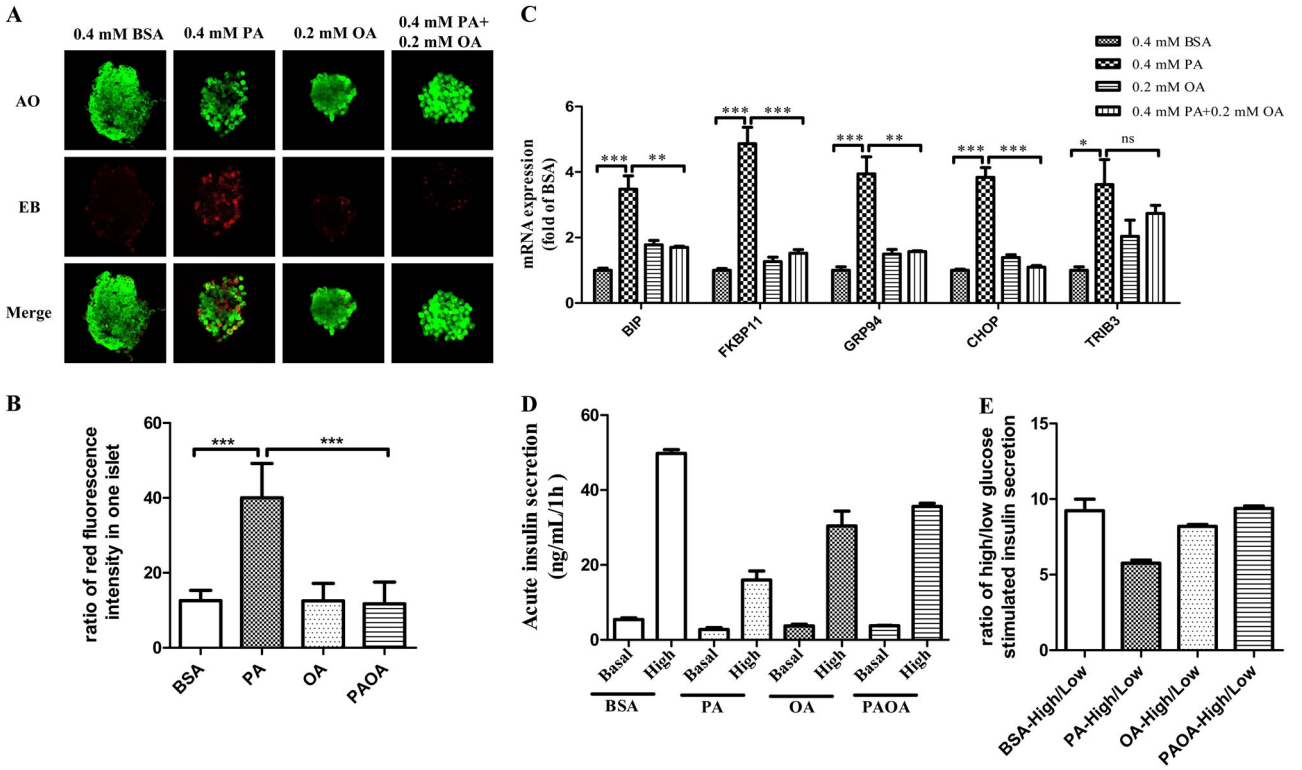

Fig. 4 Oleic acid protected islets from palmitic acid-induced ER stress. **a** The viability of islets was assessed by confocal microscopy after AO/EB staining. Viable cells exhibited green fluorescence by AO staining and dead cells exhibited red fluorescence by EB staining. **b** Quantification of results shown in **a** by calculating the ratio of red (EB) fluorescent intensity to the green (AO) fluorescent intensity in one islet ($n = 10$). **c** The mRNA expression of a couple of UPR genes in isolated rat islets, including *BIP, FKBP11, GRP94, CHOP*, and *TRIB3* after 24 h treatment. **d–e** GSIS with rat islets after the treatment with BSA, PA, or PA plus OA for 24 h and then cultured at low or high glucose KRB buffer for 1 h. The data are presented as means ± SD for >4 biological replicates; $^{***}P < 0.001$, except for **d** and **e**. The GSIS test was repeated twice, so the data are presented as the average value of two experiments

To obtain information upon the exact morphological changes of ER in the context of FFA exposure, an ultrastructural analysis by electron microscopy was performed. Incubation with PA for 24 h caused severe morphological changes in ER structure of INS-1E cells, displaying massive abnormal extension and swelling of ER lumens, but OA addition obviously improved this abnormality (Fig. 3c).

One of PA-mediated detrimental consequences is the perturbation of ER Ca^{2+} homeostasis. It is reported that PA inhibits the activity of SR Ca^{2+} ATPase (SERCA) pump that is responsible for retaking the cytoplasmic Ca^{2+} back into the ER Ca^{2+} pool [23–25]. Herein, we examined the effect of PA on cytoplasmic Ca^{2+} levels in INS-1E cells. The results showed that the cytoplasmic Ca^{2+} levels did not significantly change after 0.5 h treatment with 0.2 mM PA. But it was robustly increased after 24 h incubation with PA. This phenomenon may be explained by PA-induced deterioration of Ca^{2+} pumps in ER, leading to the depletion of ER Ca^{2+} storage and abnormal Ca^{2+} enrichment in cytoplasm. The combination of PA and OA abolished this effect, and brought the cytoplasmic Ca^{2+} to the control level (Fig. 3d). In summary, these results suggested that OA alleviated PA-induced ER stress in INS-1E cells.

Oleic acid protected primary islets from palmitic acid-induced ER stress

To validate the results observed in INS-1E cells, the analogous experiments were performed in primary islets isolated from healthy SD rat. AO/EB staining was used to evaluate the viability of islets. The results suggested that EB-positive dead cells were obviously increased in PA treated islets, compared to that in control or OA-treated islets. The combination of PA and OA significantly inhibited the islets death, which convinced the protective role of OA in primary rat islets (Fig. 4a, b). Similarly, PA exposure increased the mRNA expression of a couple of UPR genes, such as *BIP*, *FKBP11*, *GRP94*, *CHOP*, and *TRIB3* in rat islets, while these upregulations have not been found in OA-treated islets. Strikingly, the combination of PA and OA completely abolished these effects (Fig. 4c). In addition, glucose stimulated insulin secretion (GSIS) analysis is

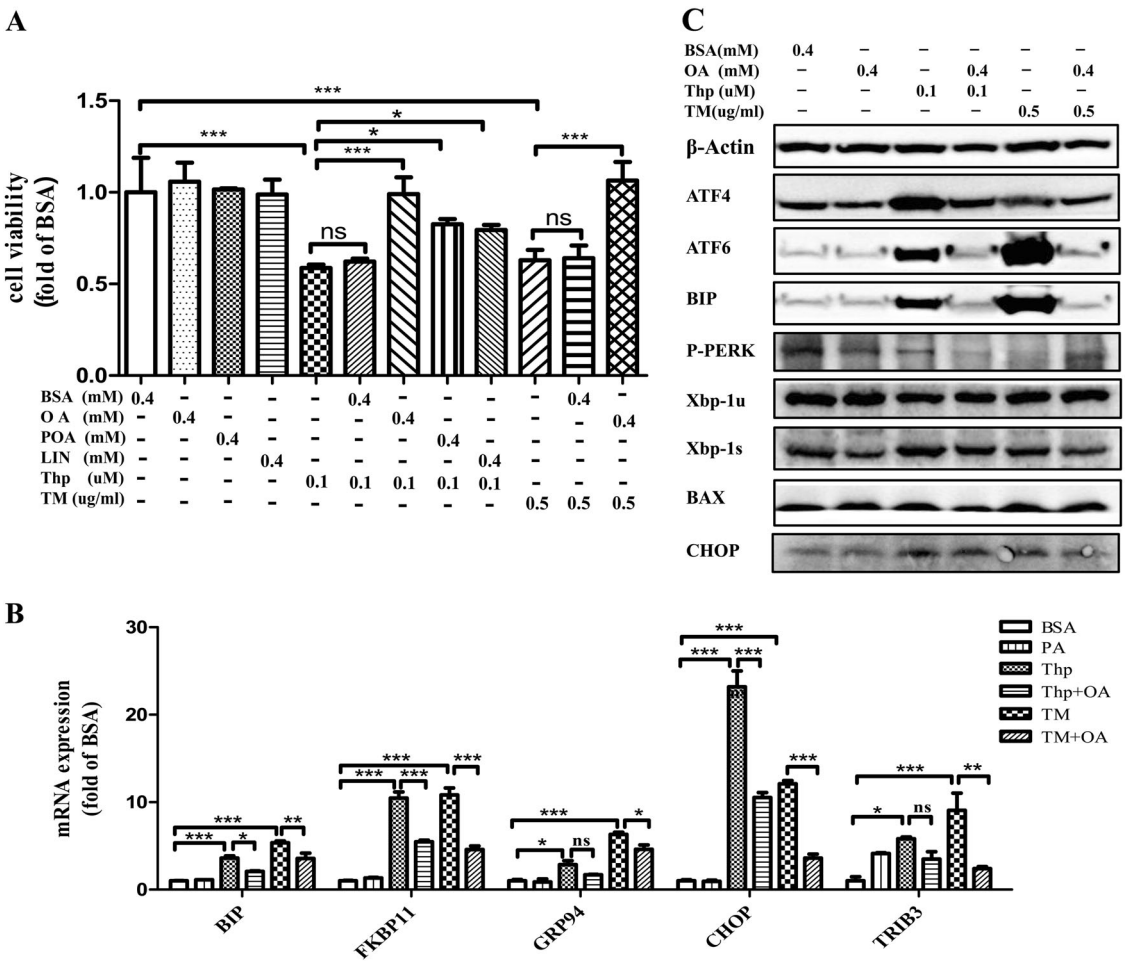

Fig. 5 Effects of OA on ER stressor-induced ER stress. The ER stress in INS-1E cells were elicited by chemical ER stressor thapsigargin (Thp) or tunicamycin (TM) for 24 h. **a** Cell viability of INS-1E cells was assessed using the CCK8 assay. **b** The relative mRNA expression of UPR genes, including *BIP*, *FKBP11*, *GRP94*, *CHOP*, and *TRIB3* after 24 h treatment. **c** Representative western blots of ATF4, ATF6, BIP, P-PERK, Xbp-1u, Xbp-1s, BAX, and CHOP after 24 h treatment, and β-actin was used as a protein-loading control. The data are presented as means ± SD for $n > 4$ biological replicates; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$; ns no significant differences between two connected groups

important to evaluate the physiological function of islets. The GSIS results suggested that PA tended to slightly inhibit basal insulin secretion after low-glucose stimulation, but remarkably decreased the high glucose stimulated insulin section. OA also mildly decreased the GSIS index, but the combination of PA and OA substantially restored the GSIS index in comparison with PA treatment, suggesting that OA was able to ameliorate PA-induced deterioration of islet function (Fig. 4d, e).

Oleic acid alleviated ER stressors-induced ER stress in INS-1E cells

ER stress can be chemically induced by some ER stressors, such as thapsigargin (Thp) and tunicamycin (TM). TM induces ER stress by inhibiting the glycosylation of newly synthesized proteins, and Thp-induced ER stress by inhibiting Ca^{2+}-ATPase, which destroyed Ca^{2+} homeostasis in the ER [26, 27]. Interestingly, either Thp or TM declined the viability of INS-1E cells, but the addition of OA completely restored the cell viability (Fig. 5a), suggesting OA was able to modulate general ER stress rather than specifically targeting PA. POA (C16:1) and LIN (C18:2) also showed protective effects under the simulation of ER stressors, but the protection was much less than OA did (Fig. 5a). As we expected, the mRNA levels of *UPR* genes were significantly increased after 24 h stimulation of Thp or TM, but these alterations were diminished when OA was added simultaneously (Fig. 5b). Additionally, Thp and TM treatments obviously increased the protein levels of ER stress markers. The most distinct upregulations presented on ATF4, ATF6, BIP, Xbp-1s, and CHOP. However, these alterations were substantially abolished by OA supplement (Fig. 5c).

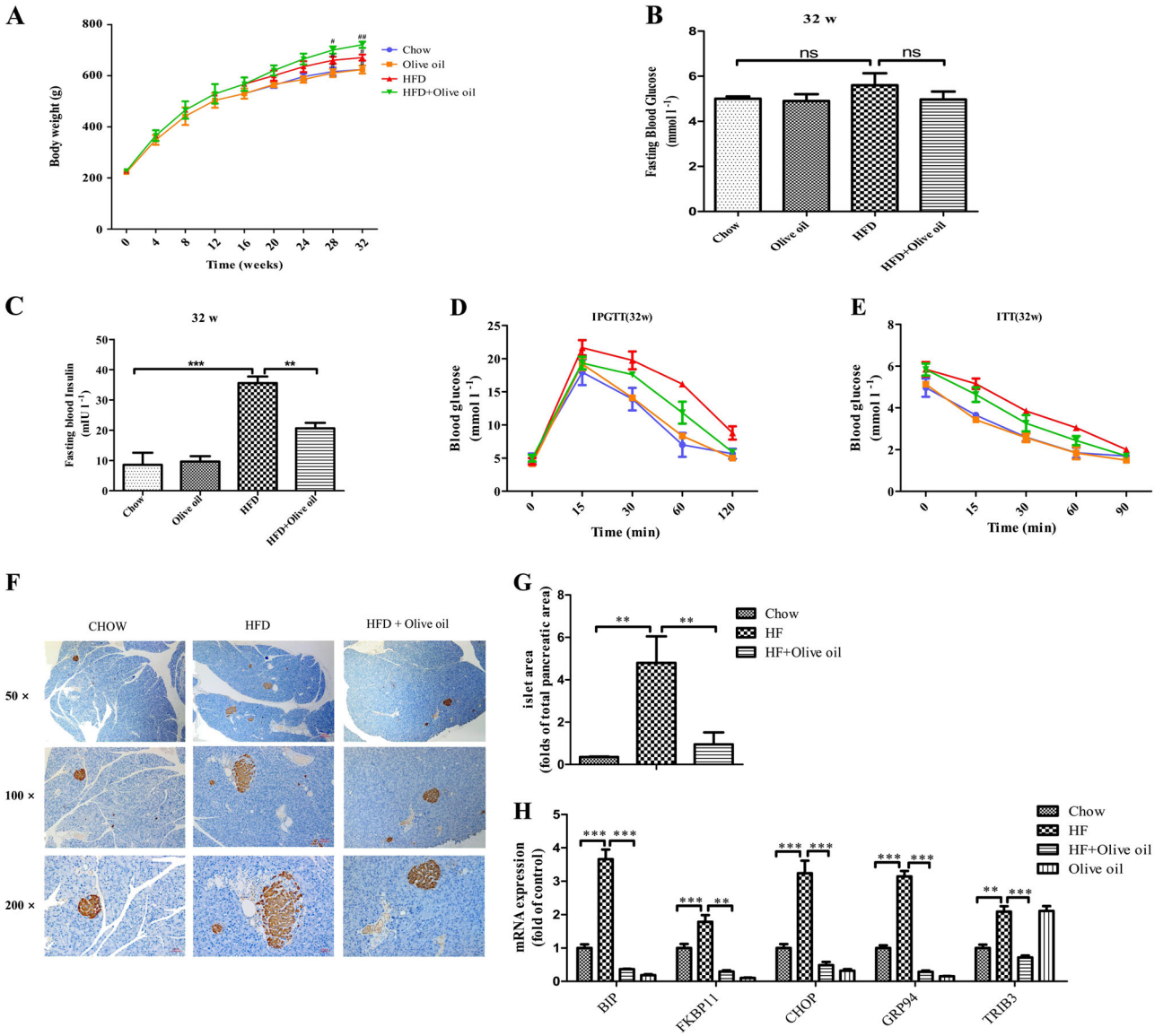

Fig. 6 Evaluation of pancreatic β-cell function in HFD fed SD rats. **a** Changes in body weights after the rats fed with chow diet, HFD, and olive oil for 32 w or HFD for 16 w then half replacement of HFD with olive oil for a further 16 w (HFD + olive oil). **b** and **c** Fasting levels of blood glucose and serum insulin. **d** Blood glucose levels in a glucose tolerance test (IPGTT) and **e** an insulin tolerance test (ITT) of rats after Chow diet, olive oil, HFD and HFD + olive oil administration for 32 w. $n > 4$ for each group and each time point. **f** Representative immunohistochemical images of pancreas sections from the indicated rats stained by anti-insulin antibody ($n > 4$ images per group for each staining). **g** Histogram analysis of islets mass calculated by the area of insulin positive cells in one visual field of microscope. **h** The relative mRNA expression of UPR genes in isolated islets from experimental rats after 32 w treatment, including BIP, FKBP11, GRP94, CHOP and TRIB3. β-actin was used as a protein-loading control. $N > 4$ for each group and each time point. The data are presented as means ± SD, $\#P < 0.05$, $\#\#P < 0.01$ vs. HFD; $^{**}P < 0.01$, $^{***}P < 0.001$; ns no significant differences between two connected groups

Long term of high-fat diet impaired insulin sensitivity and half replacement of HFD with olive oil ameliorated this effect in SD rats

To explore the in vivo effects of PA or OA, we fed SD rats with high fat diet for 16 w to induce obesity, then for the next 16 w, we half replaced the fat with olive oil in the diet, producing a mix diet with both saturated and unsaturated fatty acids to mimic the in vitro experimental setting in which PA and OA interacted together. The results showed that after 32 w HFD, the notable increase was observed in body weights of these animals (Fig. 6a). Particularly, after half replacement of HFD with olive oil, the animals showed even higher potential of body weight increase. In terms of insulin sensitivity, the HFD rats showed abnormal glucose dynamics in both GTT and ITT tests, suggesting the insulin sensitivity was compromised (Fig. 6d, e). Additionally, though the fasting blood glucose did not show significant

difference between groups, the elevated fasting serum insulin level in HFD rat indicated the development of hyperinsulinemia (Fig. 6b, c). Half replacement of HFD with olive oil (HFD + olive oil) ameliorated these abnormalities, indicated by decreased fasting insulin level as well as improved glucose tolerance and insulin tolerance (Fig. 6c–e). Interestingly, animals feeding the equal calories of olive from the beginning, did not show any detrimental impact with regards to insulin sensitivity. Additionally, the results of immunohistochemistry showed that the islets mass, indicated by insulin-positive staining cells, was obviously increased in HFD group. This was in consistence with hyperinsulinemia condition seen in HFD group, and implied that the islets functioned as abnormal adaptive enhancement of insulin synthesis and secretion in the compensation stage of prediabetes. Olive oil supplementation remarkably decreased the islets mass, suggesting the improvement of islets function as well as peripheral insulin resistance (Fig. 6f, g). Taken together, long term HFD impaired insulin sensitivity, whereas half replacement of HFD with olive oil was able to increase insulin sensitivity in SD rat.

Long term of high-fat diet-induced ER stress and olive oil ameliorated this effect in SD rats

To explore the in vivo effects of PA and OA on pancreatic β cell, primary pancreatic islets were isolated and purified from SD rats of different groups, and the mRNA expression of *UPR* genes were detected by qPCR. The results showed that long term HFD feeding induced ER stress, evidenced by the upregulated expression of UPR genes including *BIP, FKBP11, CHOP, Grp94*, and *TRIB3* (Fig. 6h). Olive oil treatment (HFD + olive oil) effectively abolished this effect, implying its potential to alleviate HFD induced ER stress.

Discussion

In this study, we used β-cell line INS-1E, primary rat pancreatic islets and high-fat diet-induced obese SD rats to determine the impacts of monounsaturated oleic acid on β-cell function in the context of saturated fatty acids-induced lipotoxicity. We found that concomitant supplementation of OA substantially alleviated the toxic effects of PA in vitro, and improved insulin sensitivity in vivo. Importantly, OA ameliorated either fatty acids or chemical ER stressors-induced ER stress in β cells, which contributed to OA-mediated protection against lipotoxicity.

The role of ER stress in the pathology of lipotoxicity is extensively discussed in a variety of diseases, including neurodegenerative diseases, diabetes and metabolic disorders, atherosclerosis, cancer, as well as renal and lung diseases [28–32]. Many cellular perturbations can lead to the accumulation of unfolded or misfolded proteins inside the ER. Initially, the unfolded protein response (UPR) acts as an important adaptive mechanism to restore protein-folding homeostasis. When the UPR is insufficient to handle the unfolded protein load, cells undergo apoptosis. So ER stress is recognized as a critical causal factor of cell death and cell dysfunction in the progression of many diseases [31]. Apparently, β cells are much more sensitive to disturbance of ER system than other cell types, since β cell has very elaborate ER networks responsible for protein folding of newly synthesized insulin as high as 1 million molecules per minute [33]. When the chaperone system becomes overloaded, the impaired ER function ultimately leads to β-cell apoptosis [34, 35]. The reported downstream molecular mechanisms include activation of CHOP, which is induced via PERK and ATF4. CHOP represses the antioxidant genes and induces growth arrest and DNA damage-inducible protein (GADD34) expression, which promotes dephosphorylation of eIF2α. The subsequent increased oxidative stress not only activates cell death but also further compromises ER capacity for protein folding. Alternatively, IRE1α can trigger apoptosis via activation of caspase 4 or 12, and the signaling pathway, including TNF receptor-associated factor 2 (TRAF2) and c-Jun N-terminal kinase (JNK) [36].

Our results agreed with previous reports [37–39] that palmitic acid impaired UPR balance and triggered ER stress in both INS-1E cells and rat islets. The results of INS-1E cells indicated that the most distinct alterations induced by PA stimulation were the activation of PERK arm, including the remarkably enhanced expressions of phosphorylated PERK, phosphorylated eIF2α and ATF4, which suggested the general protein translation was attenuated to relieve the ER workload after massive FFA influx. The consequence of the activation of ATF4 was its translocation from the cytosol to nucleus, and ultimately promoted the translation of CHOP and other proapoptosis, chaperone, and antioxidant response genes. In our results, both mRNA and protein level of CHOP have shown significant increase, and it was accompanied by the expression changes of proapoptotic BAX and antiapoptotic Bcl-2. Furthermore, these alterations occurred from 6 h after PA exposure, even earlier than the cell dysfunction (we found no significant impairment on cell viability before 12 h after PA exposure, Fig. 1b).

An interesting molecule in PA triggered ER stress is BIP (also named GRP78), a famous ER chaperone. It is well-known that ER stress is initiated by the dissociation of BIP from the ER transducers PERK, IRE1, and ATF6, resulting

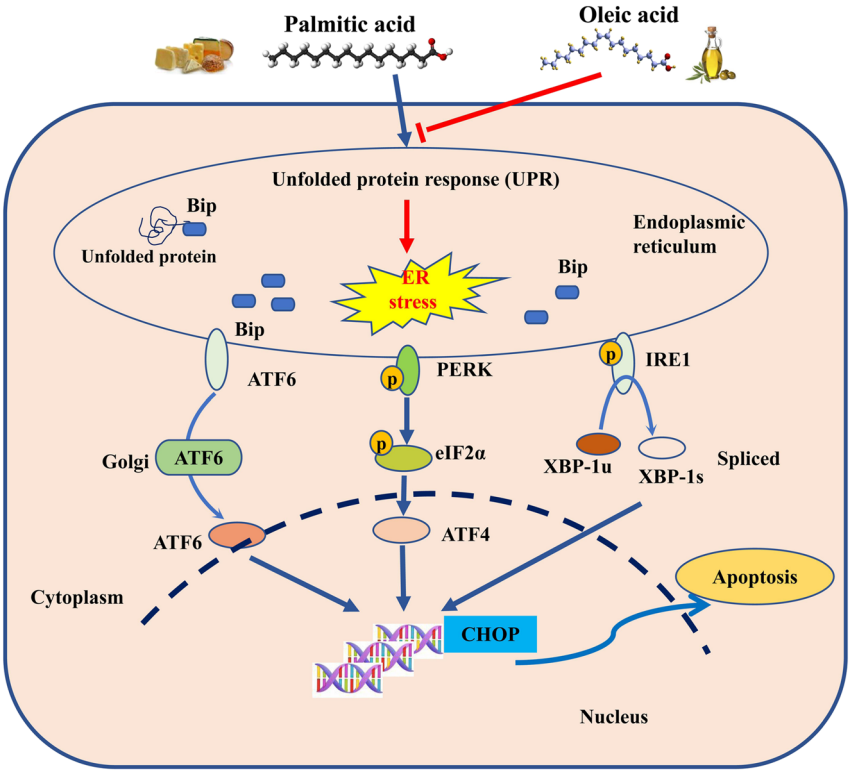

Fig. 7 Schematic diagram of the effects of palmitic acid and oleic acid on endoplasmic reticulum (ER) stress and apoptosis in β cell. Palmitc acid (C18:0, PA) is rich in fat food, and oleic acid (C18:1, OA) is rich in olive oil. When uptake excess PA, PA triggers unfolded protein response (UPR) in endoplasmic reticulum, and ER stress leads to cell apoptosis ultimately. Under normal conditions, the three critical transmembrane proteins PERK, IRE-1, and ATF6 are associated with the major ER chaperone Bip (GRP78) to maintain them in an inactive state. Under ER stress conditions, Bip is released and joins the increased unfolded or misfolded proteins in the ER lumen, participating in protein folding. Consequently, a complex signaling cascade, involving PERK, IRE-1, and ATF6, is activated. PERK dimerizes and autophosphorylates, leading to phosphorylation of eIF2a to decrease global translation. Phosphorylation of eIF2a then induces the selective translation of ATF4, which triggers the transcription of CHOP in nucleus. Autophosphorylation of IRE1 leads to splice of 26 nucleotides from the XBP1 mRNA, and the shorter spliced XBP1 (XBP-1s) promotes the transcription of CHOP as well. During ER stress, ATF6 is released from Bip and translocates to the Golgi where it is proteolytically activated. All the three signaling pathways trigger the expression of CHOP, and CHOP is positively connected to apoptosis. In the present study, we demonstrated that the activation of ER stress, particularly PERK-eIF2α-ATF4-CHOP pathway has played an essential role in PA triggered apoptosis, and OA-mediated attenuation of ER stress and regulation of UPR balance may be an important mechanism responsible for the protective role of OA in pancreatic β cells

in their activation [33]. However, there is no firm consensus about the role of BIP in lipotoxicity [40]. Some groups found that BIP was not significantly altered during exposure to palmitate in INS-1 cells [37, 38], assuming that palmitate activated the UPR in a manner of chaperone-independent pathway. In our experiments, we found mild alterations of protein expression of BIP in either PA or OA-treated INS-1E cells. Nevertheless, notable increase of the mRNA transcription of BIP gene was detected in both PA treated INS-1E cells and islets of HFD treated SD rats (Figs. 3b, 4c and 6h). Moreover, substantial increase of BIP protein was found in ER stressors thapsigargin and tunicamycin-treated INS-1E cells, indicating a successfully initiation of UPR (Fig. 5b). Consistent to the antagonistic role of OA on other ER markers as PERK, eIF2α and CHOP, the enhancement of BIP expression in all the scenarios was greatly diminished by OA addition. More detailed molecular (Fig. 7) mechanisms of OA need to be further intensively studied in the future.

The favorable effects of monounsaturated fatty acids are widely accepted in piles of studies including cardiovascular diseases, non-alcoholic fatty liver disease and hyperlipidemia [41, 42]. Many Nutrition Guidelines for Cardiovascular diseases or diabetes document a clear preference for MUFA consumption [43]. However, whether oleic acid benefits or impairs islets function is still controversial. Based on the literatures, a great number of experiments observed a similar phenomenon that OA is able to rescue β cells from palmitate-induced apoptosis [37, 44]. However, doubts have been developing during recent years regarding the validity

of the cardioprotective effect or antidiabetic effect of MUFAs. T Plötz et al. reported that PA and OA were both toxic to human Endoc-βH1 beta cells and pseudoislets, as well as to rat islets and human islets [45]. Using a proteomic approach, Michael Maris et al. demonstrated that exposure to oleate downregulated chaperones, hampered insulin processing and ubiquitin-related proteasomal degradation in INS1-E cells, leading to an opposite conclusion that oleate-induced beta cell dysfunction and apoptosis [46]. On the contrary, Maedler K et al. reported a protective role of OA on PA-induced apoptosis in human primary islets [47], and Wiebke Gehrmann et al. demonstrates that OA prevents PA-induced ROS generation in both rat RINm5F β-cell and rat primary β cells [39]. A possible explanation for this discrepancy is the differences of research subjects. T Plötz et al. concluded that rat insulin-secreting cell lines show no toxicity upon exposure to unsaturated fatty acids due to their inability to generate toxic hydrogen peroxide, as they apparently cannot metabolize unsaturated fatty acids in the peroxisomal beta-oxidation [45]. However, it seems this explanation could not fit all the paradoxes. In the present study, we validated the protective role of OA against SFA from three aspects, rat β cell line INS-1E, freshly isolated rat islets, as well as primary islets from fat rats. In the in vitro experiments, we observed that OA was able to protect β cells against PA-induced lipotoxicity in both INS-1E cells and primary rat islets. In the in vivo experiments, we reported the therapeutic effects of a mixed diet comprising half to half saturated and monounsaturated FFAs in the context of obesity. In comparison with other reported clinical or experimental nutrition studies [18], in which fat diet were stripped and replaced by MUFA rich diet after a long time HFD treatment, this mixed diet might be more acceptable and feasible for clinical patients, who feel extremely tough to strip fat food from their diet. On the other hand, this specific group setting was much closer to the in vitro treatment, the combination of PA and OA. The results convinced that olive oil was able to ameliorate HFD-induced insulin resistance and hyperinsulinemia. Additionally, in our previous study of OA-mediated protection against non-alcoholic steatohepatitis (NASH), we reported that half replacement of HFD with olive oil remarkably delayed the progression from steatosis to NASH in SD rats. The less expression of ER stress markers was also observed in olive oil or OA-treated hepatocytes [48]. Nevertheless, the fact is that we have not done any work on human islets, so we cannot tell whether it is true in human islets or β cells, or in clinical patients. If more dietitians, clinicians and lab researchers can work together to explore the therapeutic effects of monounsaturated fatty acids based on a more scientific and reliable experiment design, the argument will be clarified in the future.

Conclusion

In conclusion, we reported here the protective effects of oleic acids against saturated FFAs-induced lipotoxicity in INS-1E cells, rat islets as well as HFD-induced obese rats. The activation of PERK-eIF2α-ATF4-CHOP pathway has played an essential role in PA triggered cytotoxicity, and OA-mediated attenuation of ER stress and regulation of UPR balance may be an important mechanism responsible for the protective role of OA in pancreatic β cells.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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