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Nuclear factor-Y mediates pancreatic β-cell compensation by repressing reactive oxygen species-induced apoptosis under metabolic stress

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Abstract

Background: Pancreatic β-cells elevate insulin production and secretion through a compensatory mechanism to override insulin resistance under metabolic stress conditions. Deficits in β-cell compensatory capacity result in hyperglycemia and type 2 diabetes (T2D). However, the mechanism in the regulation of β-cell compensative capacity remains elusive. Nuclear factor-Y (NF-Y) is critical for pancreatic islets' homeostasis under physiological conditions, but its role in β-cell compensatory response to insulin resistance in obesity is unclear.

Methods: In this study, using obese (*ob/ob*) mice with an absence of NF-Y subunit A (NF-YA) in β-cells (*ob*, *Nf-ya* βKO) as well as rat insulinoma cell line (INS1)-based models, we determined whether NF-Y-mediated apoptosis makes an essential contribution to β-cell compensation upon metabolic stress.

Results: Obese animals had markedly augmented NF-Y expression in pancreatic islets. Deletion of β-cell *Nf-ya* in obese mice worsened glucose intolerance and resulted in β-cell dysfunction, which was attributable to augmented β-cell apoptosis and reactive oxygen species (ROS). Furthermore, primary pancreatic islets from *Nf-ya* βKO mice were sensitive to palmitate-induced β-cell apoptosis due to mitochondrial impairment and the attenuated antioxidant response, which resulted in the aggravation of phosphorylated c-Jun N-terminal kinase (JNK) and cleaved caspase-3. These detrimental effects were completely relieved by ROS scavenger. Ultimately, forced overexpression of NF-Y in INS1 β-cell line could rescue palmitate-induced β-cell apoptosis, dysfunction, and mitochondrial impairment.

Conclusion: Pancreatic NF-Y might be an essential regulator of β-cell compensation under metabolic stress.

Keywords: Nuclear factor-Y; β-cell compensation; Apoptosis; Mitochondria; Oxidative stress; Obesity; Lipotoxicity

Introduction

Pancreatic islet β-cells secrete insulin in response to an increased level of blood glucose. Insulin effectively maintains normoglycemia through the stimulation of glucose uptake by peripheral tissues and suppression of endogenous glucose production. In the face of insulin resistance or increased circular nutrients imposed by obesity, pancreatic β cells adaptively augment insulin production and secretion through compensatory expansion of β-cell mass.[1] In the case of an individual whose insulin demand exceeds the β-cell functional capacity, hyperglycemia, a hallmark of type 2 diabetes (T2D), develops as a result. Thus, a failure of β-cell compensatory response is a principal risk factor at the onset and during the progression of T2D.[2] However, the mechanism underlying the β-cell compensation and failure in the course of T2D remains incompletely understood.

Although β-cell proliferation or neogenesis is considered to be the primary determinant of β-cell mass,[3] increasing evidence suggests that β-cell apoptosis is implicated in the deficit of β-cell compensatory expansion in T2D.[4] Mice with a deficiency of B-cell lymphoma-2 (BCL-2) associated agonist of cell death (BAD) protein, one of the proapoptotic BCL-2 family members, show an increased β-cell mass and are resistant to high-fat diet-induced glucose intolerance.[5] Indeed, autopsy sections of T2D patients have indicated that apoptosis is one of the major contribu-

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tors to the loss of β-cell mass in T2D islets.[6] Mechanistically, β-cell apoptosis during the progression of T2D involves oxidative damage, mitochondrial dysfunction, unfold proteins overload and endoplasmic reticulum stress, disruption of autophagy pathway, amyloid deposits, and inflammatory stimulation.[7] Given the critical role of apoptosis in pancreatic β-cell failure during the course of T2D, finding strategies to maintain β-cell survival is of particular interest in preventing the occurrence of diabetes.

Nuclear factor-Y (NF-Y) consisting of three subunits (i.e., NF-YA, NF-YB, and NF-YC) is ubiquitously expressed in various cell types. NF-Y regulates expression of multiple genes involved in cell replication, survival, and endoplasmic reticulum stress.[8,9] NF-Y is essential for metabolic homeostasis. Liver-specific NF-Y deficient mice exhibited reduced blood glucose levels due to the transcriptional inhibition of the gluconeogenic program.[10] NF-Y interacting with sterol regulatory element-binding protein-1c (SREBP-1c) enhances the transcription of genes involved in fatty acid and cholesterol metabolism, such as patatin-like phospholipase domain containing 3 (PNPLA3) and 3-hydroxy-3-methylglutaryl coenzyme A synthase (HMG-S).[11] Moreover, NF-Y is critical for the maintenance of pre-adipocyte and adipocyte by regulating their specific gene expression, such as adiponectin and leptin.[12–14] In the pancreas, NF-Y is reported to regulate β-cell proliferation and glucose uptake into β-cells, and is therefore critical for pancreatic islet homeostasis.[15] Nevertheless, the physiological role of NF-Y in adaptive increase of β-cell mass and its regulatory mechanism have been little studied.

In this study, we extended previous reports on the NF-Y function in β-cell mass maintenance. We determined whether NF-Y is required for the adaptive response of β-cell to metabolic stress in *ob/ob* mice with an absence of *Nf-ya* specific in β-cells as well as insulinoma cell line (INS1) cell-based models.

Methods

Animals

All animal use protocols were approved by the Committee on the Ethics of Animal Experimentation at West China Hospital (No. 20220302006). Mice were maintained under a 12-h light–dark cycle with an ambient temperature and fed regular rodent chow (Trophic Animal Feed High-Tech Co., Nantong, China) *ad libitum*. C57BL/6J-*Lep*$^{ob/+}$ (*ob*/+) mice (catalog 000632) and *Rip-Cre* mice (catalog 003573) were bought from The Jackson Laboratory (Bar Harbor, Maine, USA). *Nf-ya*$^{fl/fl}$ mice were kindly provided by Dr. N. Nobuyuki (Juntendo University, Tokyo, Japan). *Nf-ya*$^{fl/fl}$*; Rip-Cre* mice (referred to as *Nf-ya* βKO) were created by crossing *Nf-ya*$^{fl/fl}$ mice with *Rip-Cre* mice as previously described.[15] *Nf-ya*$^{fl/fl}$ βKO mice were then bred with *ob*/+ mice to create *ob*/+; *Nf-ya*$^{fl/+}$; *Cre* mice, which were then intercrossed to produce *ob*, *Nf-ya* βKO mice. Littermate mice with the genotypes of *ob*; *Rip-Cre*, *Rip-Cre*, and *Nf-ya* βKO were used for controls. Mice were confirmed by genotyping [Supplementary Figure 1, http://links.lww.com/CM9/B493] with the primers [Supplementary Table 1, http://links.lww.com/CM9/B493] and NF-YA Western blotting [Supplementary Figure 1, http://links.lww.com/CM9/B493].

Metabolic analysis

Blood samples were obtained by retro-orbital bleedings when mice were at the age of 12 weeks. The supernatants of plasma were separated after centrifugation at 3500 rounds per min (r = 17.5 cm) for 5 min. Total plasma concentrations of insulin, total cholesterol (TC), triglyceride (TG), and free fatty acid (FFA) for each individual sample were determined using commercial assay kits (Jiancheng Bioengineering, Nanjing, China) as previously described.[16]

Overnight-fasted mice were injected intraperitoneally with glucose (2 g/kg body weight). The concentrations of blood glucose and plasma insulin were determined at 0 min, 15 min, 30 min, 60 min, and 120 min after glucose injection.

Immunostaining and measurement of β-cell mass, apoptosis, and proliferation

Freshly dissected mouse pancreas tissues were fixed in 4% paraformaldehyde overnight and then embedded in paraffin. The β-cell proliferation and apoptosis were examined on paraffin sections by immunofluorescence staining of insulin (1:2000, EM80714, HuaBio, Hangzhou, China) with Ki-67 (1:800, ab15580, Abcam, Cambridge, UK) or with terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) (Yeasen Biotech, Shanghai, China) as described previously.[15] The percentage of double-stained cells was quantified from the digitized images. About 3000 insulin positive cells of 50 islets per animal were counted. Apoptotic cell death was also examined in primary islets as previously described,[17] in which the 4′,6-diamidino-2-phenylindole (DAPI) staining was used to visualize total cells. Relative β-cell mass was estimated by point counting after insulin staining.[18] An average of 7000 points/mouse was counted.

Mouse islets' isolation

Mice islets were isolated following the protocol described previously.[19] Briefly, the dissected pancreas was digested with collagenase at 37°C for 30 min. Thereafter, pancreatic islets were sequentially pelleted using 29%, 24%, and 15% of Ficoll gradient solutions (GE Healthcare, Uppsala, Sweden) and then were manually picked under a stereoscopic microscope. Finally, selected islets were maintained in the Roswell Park Memorial Institute 1640 (RPMI-1640) medium containing 10% fetal bovine serum and 100 U/mL penicillin-streptomycin at 37°C.

Plasmid, virus, and stable cell line establishment

Complementary DNAs (cDNAs) encoding mouse *Nf-y* (ya, yb, and yc) amplified using gene-specific primers [Supplementary Table 2, http://links.lww.com/CM9/B493] were cloned into the lentiviral expression vector pLVX-Puro to generate a recombinant *Nf-y* overexpression lentiviral vector, which was then co-transfected with viral pack-

aging vectors (psPAX2 and pMD2G) into HEK293 (American type culture collection, Manassas, VA, USA) cells using lipofectamine 2000 for virus production and amplification. Recombined lentivirus then infected INS1 cells for 48 h, and cell lines stably expressing Nf-y were screened by 10 μg/μL puromycin (Sigma-Aldrich, St. Louis, MO, USA). The blank pLVX-Puro vector was used as negative control.

Cell viability analysis

Stably overexpressing *Nf-y* INS-1 cells were placed in a 96-well plate for 24 h and treated with palmitic acid at various concentrations for an additional 24 h. Cell viability was measured using a CCK-8 kit (4A Biotech, Beijing, China) in accordance with the manufacturer's instructions.

Glucose-stimulated insulin secretion (GSIS)

INS-1 cells with a stable *Nf-y* overexpression were treated with 200 μmol/L palmitate for 24 h. Thereafter, cells were incubated in Krebs-Ringer HEPES (KRH; Sigma-Aldrich) buffer at 37°C for 60 min, and then incubated for 60 min in KRH buffer containing 2.8 mmol/L or 16.7 mmol/L of glucose. Secreted insulin in the buffer was measured with a mouse insulin immunoassay kit (Millipore, Billerica, MA, USA).

Reactive oxygen species (ROS) assay

Intracellular ROS and mitochondrial ROS production were estimated using 2′7′-dichlorodihydrofluorescein diacetate (DCFDA)-cellular ROS assay kit (Yeasen Biotech, Shanghai, China) and MitoSOX™ Red superoxide indicator (Yeasen Biotech), respectively. In short, INS-1 cells or mouse islets were maintained in black 96-well plates with RPMI 1460 medium. After a 12-h incubation of palmitic acid (200 μmol/L), the cells were incubated with DCFDA (20 μmol/L) for 30 min or MitoSOX™ Red (5 μmol/L) for 10 min at 37°C. Fluorescence was detected using a laser confocal microscope (Carl Zeiss (Shanghai) Co. Ltd., China), and the fluorescence intensity was analyzed using the ImageJ software (National Institutes of Health, Bethesda, MA, USA).

Measurement of antioxidant enzymes

Protein of INS-1 cells was collected for 50 μg per sample. Total superoxide dismutase-2 (SOD2) activity was determined by Total Superoxide Dismutase Activity Assay Kit (Yeasen Biotech). Total glutathione peroxidase 1 (GPX1) activity was determined by Total Glutathione Peroxidase Assay Kit (Beyotime Biotech, Shanghai, China) in accordance with the manufacturer's protocol.

Mitochondrial membrane potential (MMP) measurement

Mitochondrial potential was determined with the JC-1 fluorescent probe (Yeasen Biotech) as previously described.[15] JC-1 forming a monomeric form in low-potential mitochondria produces green fluorescence, while red fluorescence is emitted by the aggregated JC-1 in high-potential mitochondria. After being treated with 200 μmol/L palmitate for 24 h, primary islets or INS-1 cells were incubated in culture media containing JC-1 (10 μmol/L) at 37°C for 30 min. The fluorescence was observed under a laser confocal microscope (Carl Zeiss).

Mitochondrial specific fluorescence staining

Mitochondria structure were stained using MitoTracker® Red CM-H2XRos (Yeasen Biotech), which is a mitochondrial potential-independent mitochondrial staining reagent. The mitochondrial shape was observed with the fluorescence microscope.

Quantitative real-time polymerase chain reaction

RNA isolation from primary islets or INS-1 cells was performed using RNeasy Mini Kit following the manufacturer's protocol (Vazyme Biotech, Nanjing, China) and converted into complementary DNA (cDNA) with HiScript III 1st Strand cDNA Synthesis Kit (+genomic DNA wiper) (Vazyme Biotech). Real-time polymerase chain reaction (PCR) analysis was carried out using the AceQ qPCR SYBR Green Master Mix (Vazyme Biotech) and the indicated primers [Supplementary Table 3, http://links.lww.com/CM9/B493]; glyceraldehyde-3-phosphate dehydrogenase (GAPDH) messenger RNA (mRNA) was used for the endogenous control and gene expression was calculated by the ΔΔCt method.

Western blotting analysis

Protein extraction from primary mouse islets or INS-1 β-cells, protein concentration measurement, and Western blotting processes were performed as described before.[20] Antibodies against α-tubulin (1: 3000, M1501), BCL-2 associated X protein (BAX) (1:1000, ET1603-34), B-cell lymphoma 2 (BCL-2) (1:800, ET1603-11), p-Jun N-terminal kinase (JNK) (1:1500, ET1609-42), SOD2 (1:1200, ET1701-54), and GPX1 (1:800, ET1605-38) were purchased from HuaBio. Antibodies against NF-YA (1:1500, sc17753), NF-YB (1:800, sc-376546), and NF-YC (1:1200, sc-390985) were from Santa Cruz Biotechnology (Shanghai, China). Hybridized primary antibodies were detected with horseradish peroxidase (HRP)-labeled secondary antibodies. Protein signals were detected using the enhanced chemiluminescence reagents (Thermo Fisher Scientific, Waltham, MA, USA).

Statistical analysis

The data representing at least three independent experiments were presented as the mean ± standard deviation (SD). Statistical analysis was conducted using GraphPad Prism (GraphPad Software Inc., La Jolla, CA, USA). A P value <0.05 was defined as statistically significant.

Results

Islet NF-Y expression correlates with β-cell compensation in obese mice

Genetically obese *ob/ob* mouse is generally accepted as a model of successful β-cell compensation.[21] Although

ob/ob mice had striking increase in weight gain [Supplementary Figure 2A, http://links.lww.com/CM9/B493] and hyperlipidemia [Supplementary Figure 2B, http://links.lww.com/CM9/B493], they can maintain normal blood glucose levels [Supplementary Figure 2C, http://links.lww.com/CM9/B493] owing to the adaptively compensatory β-cell hyperplasia [Supplementary Figure 2D, http://links.lww.com/CM9/B493] and sustained insulin secretion [Supplementary Figure 2E, http://links.lww.com/CM9/B493], underpinning the notion that insulin resistance drives β-cell compensative response. To determine whether NF-Y contributes to β-cell compensation in obesity, we analyzed islets' NF-Y abundance. Obese *ob/ob* mouse showed remarkably increased islets' NF-Y expression of both mRNA [Supplementary Figure 2F, http://links.lww.com/CM9/B493] and protein [Supplementary Figure 2G, http://links.lww.com/CM9/B493] compared with lean mice. Together, these observations suggest that islets' NF-Y might play a role in β-cell compensation at states of insulin resistance.

NF-Y depletion in β-cell of obese mice impairs β-cell compensation and glucose homeostasis

Because the absence of NF-Y in β cells causes β-cell failure,[15] and islets' NF-Y abundance is increased in obese mice, we raised the question of whether NF-Y expression is essential to islets' β-cell compensatory response to insulin resistance under conditions of obesity. We generated *ob/ob* (*ob, Nf-ya* βKO) and lean (*Nf-ya* βKO) mice with a β-cell-specific deletion of *Nf-ya* gene, and *ob/ob* (*ob, RIP-Cre*) and lean (*RIP-Cre*) control mice by crossing *Nf-ya*$^{flox/flox}$ *RIP-Cre* mice and *Lep*$^{ob/+}$ mice. The *Nf-ya* knockout in the islets was confirmed by NF-YA Western blotting [Supplementary Figure 1C, http://links.lww.com/CM9/B493]. As *RIP-Cre* is also expressed at a low level in a subset of neurons in the hypothalamus,[22] we examined *Nf-ya* expression in this tissue. The unchanged NF-YA protein in the whole brain of the *Nf-ya* βKO mice demonstrated that the *Nf-ya* knockout was pancreatic β-cell-specific [Supplementary Figure 1, http://links.lww.com/CM9/B493]. Since *RIP-Cre* transgenic mice has been suggested to develop some glucose intolerance,[23] this *Cre* line was used as controls in the present study.

Ob, Nf-ya βKO mice showed a significant increase in body weight compared with lean mice (*RIP-Cre* and *Nf-ya* βKO), but the weight gain was lower than that in *ob, RIP*-Cre mice [Figure 1A]. *ob, Nf-ya* βKO mice displayed remarkably higher random blood glucose levels compared to other-group animals having comparable blood glucose levels [Figure 1B], demonstrating that *Nf-ya* ablation in β cells results in dysregulation of glucose homeostasis under obese conditions. Compared to lean *RIP-Cre* mice, obese *ob, RIP-Cre* mice displayed the expected glucose intolerance [Figure 1C] and a significant increase in insulin secretion [Figure 1D] in response to glucose challenge, providing evidence of an upregulated islets' β-cell function to maintain insulin hypersecretion in obesity. In contrast, *Nf-ya* deleted obese (*ob, Nf-ya* βKO) mice showed a significant reduction in insulin excretion [Figure 1D] and were even more glucose intolerant than *ob, RIP-Cre* mice [Figure 1C].

To account for the reduced insulin secretion in *ob, Nf-ya* βKO islets, we examined β-cell mass in the pancreas. Obese *ob*, *RIP-Cre* mice displayed significant increase in islets' size and β-cell mass in comparison with lean *RIP-Cre* mice [Figures 1E–G]. However, *Nf-ya* deletion significantly diminished the β-cell compensatory ability, and β-cell mass was dramatically reduced in *ob*, *Nf-ya* βKO mice compared with *ob*, *RIP-Cre* mice [Figures 1E–G]. In addition, phenotypic analysis of female experimental mice was also tested. Similar to the male animals, female *ob*, *Nf-ya* βKO mice also exhibited higher random blood glucose levels and severer glucose intolerance, accompanied with significant reductions in insulin secretion and β-cell mass compared to *ob, RIP-Cre* mice [Supplementary Figure 3, http://links.lww. com/CM9/B493]. Collectively, these findings support the requirement of NF-Y in β-cell compensation under conditions of obesity.

NF-Y depletion induces β-cell apoptosis in ob/ob mice

The decline of β-mass in *ob*, *Nf-ya* βKO mice could be due to an alteration of β-cell proliferation or cell apoptosis. Proliferating cell nuclear antigen (PCNA), cyclin D1, and cyclin D2 are cell-cycle regulators required for β-cell proliferation, and their mRNA levels were markedly reduced in pancreatic islets from *ob, Nf-ya* βKO and *Nf-ya* βKO mice relative to respective wild-type littermates [Figure 2A], which was in parallel with declined β-cell proliferation as indicated by the lower number of Ki-67-positive β cells [Figures 2B, C]. Apart from the deterioration of β-cell proliferation, we were curious about whether NF-Y deletion can lead to β-cell apoptosis that exaggerates the β-cell failure under obese conditions. Obese *ob, RIP-Cre* mice and control lean *RIP-Cre* mice had a similarly undetectable occurrence of TUNEL-positive β cells [Figures 2D, E]. Thus, β-cell apoptosis seems therefore to be unaltered in diabetes-resistant *ob/ob* mice. In contrast, TUNEL-positive β cells, although nearly undetected in β-cell *Nf-ya*-deficient (*Nf-ya* βKO) lean animals, were present abundantly in obese *ob, Nf-ya* βKO mice [Figures 2D, E]. These results suggest that NF-Y depletion augments β-cell apoptosis under obese condition.

Given that hyperlipidemia is a key pathological feature of obesity and that lipotoxicity can ultimately result in β cell dysfunction and death,[24] the pro-apoptotic effect of NF-Y deficiency on β-cell was further recapitulated in an *ex vivo* lipotoxic model using mouse primary islets subjected to saturated palmitic acid. As expected, palmitate exposure significantly increased the number of TUNEL-positive apoptotic cells and the cleavage of caspase-3, an inducer of apoptosis relative to the bovine serum albumin (BSA) control [Supplementary Figures 4A,B, http://links.lww.com/CM9/B493]. Intriguingly, palmitate treatment decreased NF-YA protein levels in a time- and dose-dependent manner [Figure 2F], but without effect on the levels of NF-YB and NF-YC [Supplementary Figure 4C, http://links.lww.com/CM9/B493]. Thus, lipotoxicity-induced β-cell apoptosis is concurrent with a reduction of NF-YA expression. Moreover, the palmitate-induced apoptosis was more intense

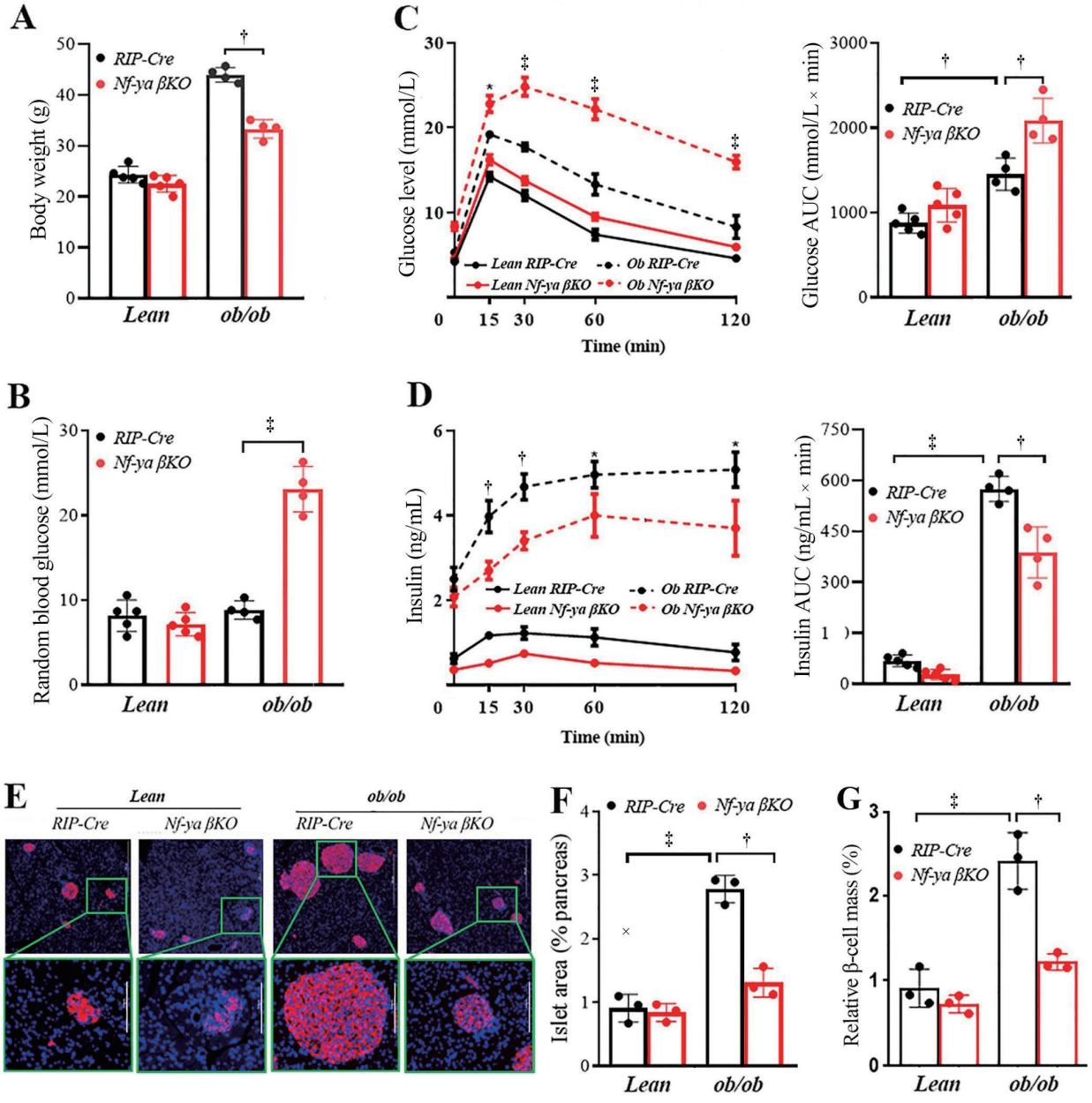

Figure 1: NF-YA specifically deleted in pancreatic β-cells in obese *ob/ob* mice leads to diabetes due to failure of β-cell compensation. (A) Body weight and (B) random blood glucose levels were examined in 12-week-old mice ($n = 5–8$ for each group). Blood glucose levels and AUC for glucose (C), and plasma insulin levels and AUC for insulin (D) during intraperitoneal GTT in mice given glucose after 12 h fasting (2 g/kg body weight) ($n = 6–8$ for each group). (E) Example of immunofluorescent staining for insulin (red) on pancreatic cryosections from 12-week-old mice with the indicated genotypes. The lower panels (scale bars = 50 μm) show high-magnification (original magnification ×10) images of the green-line boxed areas in the upper panels. (F) Quantitative assessment of the proportion of insulin-positive area/islet area in mice pancreatic islets. A total of 20–30 islets were analyzed for each group ($n = 4$ mice/group). (G) Relative β-cell mass. $^{*}P<0.05$, $^{\dagger}P<0.01$, $^{\ddagger}P<0.001$. AUC: area under the curve; GTT: Glucose tolerance test; NF-YA: NF-Y subunit A.

in islets from *Nf-ya* βKO mice [Figures 2G,H]. Collectively, these findings demonstrated that NF-Y had a protective function in regulating apoptosis of β-cell response to metabolic stress.

NF-Y depletion augments β-cell apoptosis by inducing oxidative injury during metabolic stress

Obesity-associated hyperlipidemia significantly induces ROS production through disrupting intracellular redox homeostasis.[25] The ROS increase has been found to trigger pancreatic β-cell apoptosis and consequent cellular dysfunction in the development of T2D.[26,27] To determine whether the change of lipotoxic apoptosis in *Nf-ya* βKO islets is due to the alteration of redox status, we have measured the intracellular ROS production by incubating mouse islets with ROS-sensitive fluorescence dye DCFDA in presence or absence of palmitic acid.[28] As expected, ROS accumulated more in *Nf-ya* βKO islets than in *RIP-Cre* islets. Notably, palmitate treatment enhanced the ROS to a much greater extent in *Nf-ya* βKO

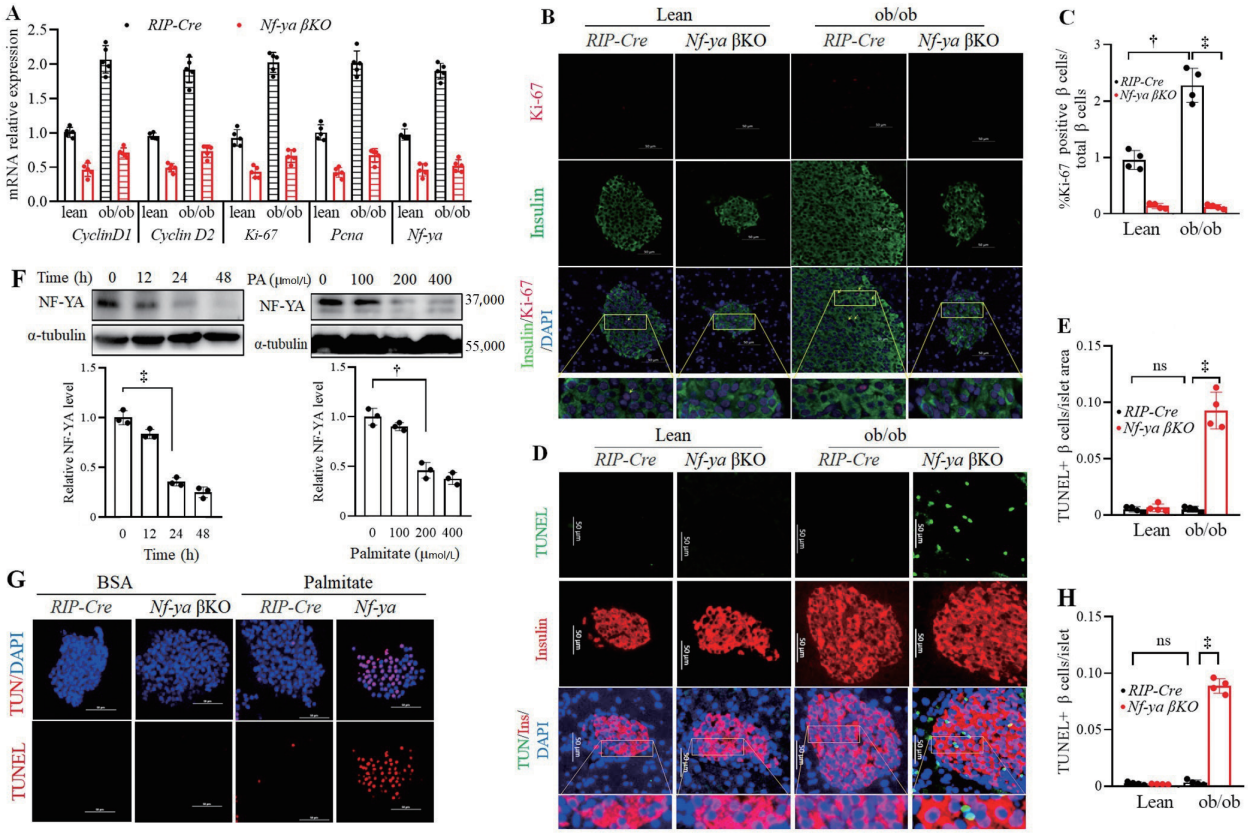

Figure 2: NF-Y depletion in β-cells leads to reduced proliferation and increased apoptosis of β-cells under metabolic stress conditions. (A) Real-time qPCR showing the mRNA expressions of cell cycle regulators in pancreatic islets from 12-week-old lean and *ob/ob* mice with or without a *Nf-ya* deletion (200 islets each from four individual mice/group). (B) Representative images of immunofluorescence staining of Ki-67 (red), insulin (green), and DAPI (blue) on pancreatic sections from 12-week-old lean and *ob/ob* mice with or without a *Nf-ya* deletion (scale bar = 50 μm). (C) Proliferation of β-cells was quantified as the number of nuclei from both Ki-67- and insulin-positive cells divided by the number of nuclei from only insulin-positive cells (about 5000 β cells per animal from 4–5 mice per genotype were counted). (D) Representative images of immunofluorescence staining of TUNEL (green), insulin (red), and DAPI (blue) on pancreatic sections of *ob,Nf-ya* βKO and *Nf-ya* βKO mice and their control mice (scale bar = 50 μm). (E) β-cell apoptosis was quantified as the number of nuclei from both TUNEL and insulin positive cells divided by the number of nuclei from only insulin positive cells (about 5000 β-cells/animal from four mice per genotype were counted). (F) Time course of NF-YA protein levels in INS1 cells in response to 200 μmol/L palmitate (upper), and NF-YA protein levels of INS1 cells in response to various c concentrations of palmitate for 24 h (lower). (G,H) TUNEL assay analysis of β-cell apoptosis in the islets from 12-week-old mice with the indicated genotypes (Scale bar = 50 μm). $^{*}P$ <0.05, $^{\dagger}P$ <0.01, $^{\ddagger}P$ <0.001. DAPI: 4′,6-diamidino-2-phenylindole; mRNA: Messenger ribonucleic acid; ns: not significant; NF-YA: NF-Y subunit A; PA: Palmitic acid; PCR: Polymerase chain reaction;ns: non-significant; TUN: Tunel; Ins: Insulin; mRNA: message RNA; BSA: Bovine serum albumin.

islets than in *RIP-Cre* islets [Figure 3A]. Increasing evidence indicates that ROS-induced activation of JNK is crucial for the initiation of apoptosis.[29] Palmitate treatment increased JNK phosphorylation in *RIP-Cre* islets, and *Nf-ya* depletion further exaggerated the upregulation of phosphorylated JNK [Figure 3B]. Intracellular ROS is efficiently detoxified by the endogenous enzymatic antioxidants like SOD2 and GPX1.[30] We next examined whether expression levels of these antioxidant enzymes were altered in *Nf-ya* βKO islets. Palmitate treatment reduced their expression in *RIP-Cre* islets and this reduction was further accentuated in *Nf-ya* βKO islets [Figure 3B]. Consistently, the palmitate-attenuated enzymic activities were aggravated by the *Nf-ya* ablation [Figures 3C,D]. These results suggested that NF-Y depletion suppressed the antioxidant response to metabolic stress. Subsequently, we subjected primary islets to palmitate together with or without the ROS scavengers *N*-acetyl-cysteine (NAC). The palmitate-enhanced phosphorylation of JNK and cleavage of caspase-3 in *Nf-ya* βKO islets were suppressed with NAC treatment [Figure 3E]. Moreover, NAC co-treatment remarkably rescued *Nf-ya* βKO islets from the palmitate-induced apoptosis [Figure 3F]. Together, these observations indicated that the protection of NF-Y against metabolic stress-stimulated β-cell apoptosis could arise from the maintenance of cellular redox status.

NF-Y absence induces β-cell mitochondrial ROS production

Because ROS is mainly derived from mitochondria, and the mitochondrial ROS can contribute to mitochondrial stress response initiating downstream apoptosis pathways,[31] we then determined the level of mitochondrial ROS production in mouse islets by using mitochondrial specific superoxide indicator MitoSOX Red. Similar to the change in intracellular ROS level, *Nf-ya* βKO islets displayed a higher mitochondrial ROS distribution than *RIP-Cre* islets [Figure 4A]. Furthermore, palmitate treatment remarkably increased ROS production in mitochondria and this increment was further potentiated in *Nf-ya* βKO islets [Figure 4A]. Since MMP reflects the mitochondrial function, and the potential drop coinciding with the activation of caspase can lead to apoptosis,[32] we subsequently examined the impact of NF-Y

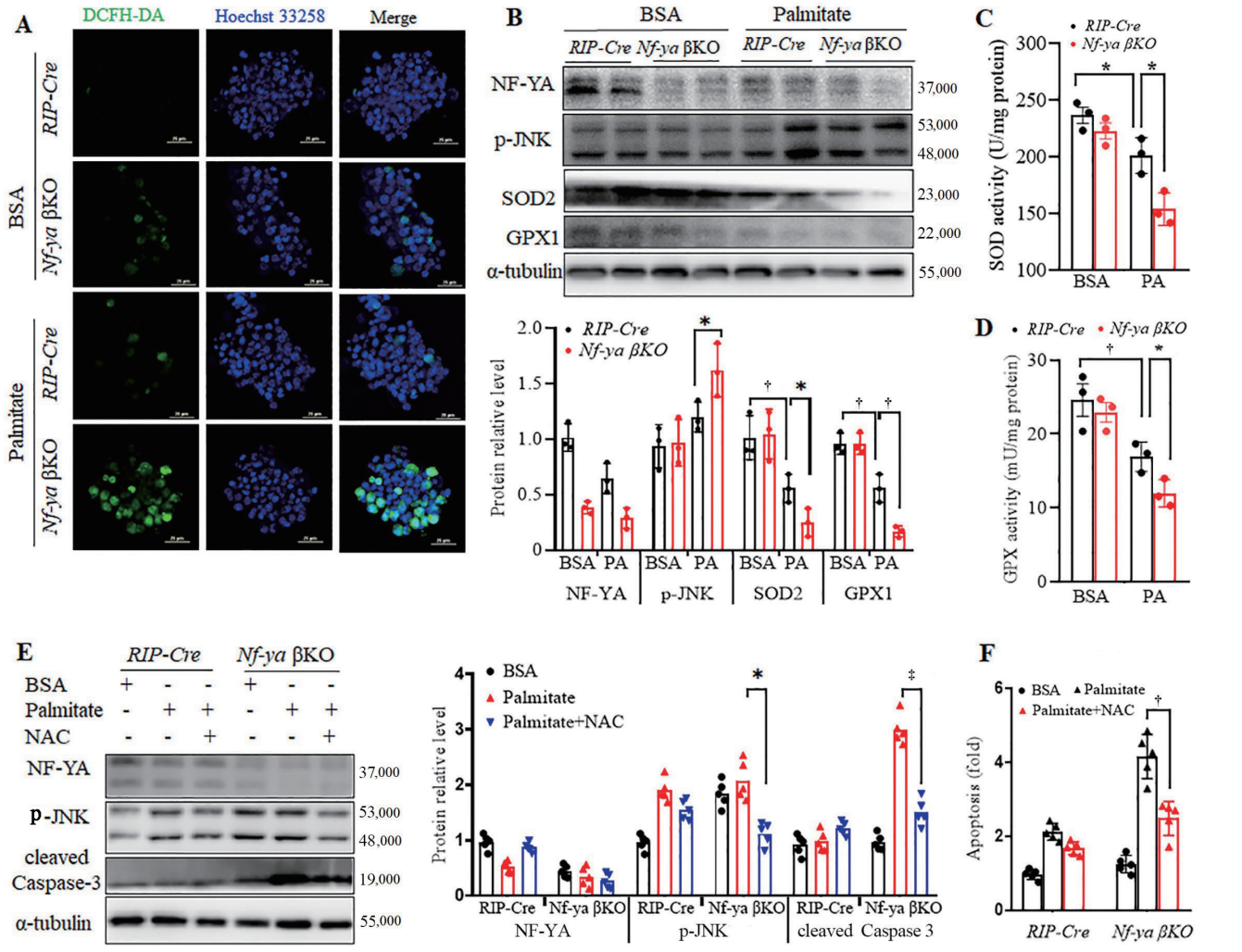

Figure 3: NF-Y deficiency accelerates palmitate-induced oxidative stress and JNK activation in mouse islets. Primary islets isolated from *RIP-Cre* or *Nf-ya* βKO mice were incubated with 200 μmol/L palmitic acid or 18% (w/v) BSA for 24 h ($n = 3$ mice in each group). (A) ROS was measured with a DCFH-DA ROS assay kit. The fluorescence signal was monitored with a 448-nm excitation filter (Scale bar = 25 μm). (B) Western blotting detected the JNK phosphorylation levels and protein levels of SOD2 and GPX1. The activities of antioxidant enzymes SOD (C) and GPX (D) were determined by commercial assay kits. (E) Western blot analysis of JNK phosphorylation and cleaved caspase-3 expression in islets co-treated in the absence or presence of the antioxidant NAC and palmitate for 24 h, and (F) apoptosis rate in islets determined by cell death detection ELISA. All data are represented as mean ± standard deviation. $^{*}P$ <0.05, $^{\dagger}P$ <0.01, $^{\ddagger}P$ <0.001. BSA: Bovine serum albumin; DCFH-DA: Dichlorofluorescein-diacetate; ELISA: Enzyme-linked immunosorbent assay; GPX1: Glutathione peroxidase 1; JNK: c-Jun N-terminal kinase; NAC: *N*-acetyl-cysteine; NF-Y: Nuclear factor-Y; NF-YA: NF-Y subunit A; PA: Palmitic acid; ROS: Reactive oxygen species; SOD2: Superoxide dismutase-2.

on the mitochondrial potential of β-cells under metabolic stress by using potential-sensitive indicator JC-1 dye. NF-Y deletion markedly potentiated the palmitate-induced mitochondrial potential collapse in isolated mouse islets as reflected by the remarkable decreased ratio of red/green fluorescence intensity [Figures 4B, C]. Given that the dynamic processes of mitochondrial fusion and fission are critical for maintaining mitochondrial optimal function and potential,[33] we also examined the potential change of mitochondrial morphology with Mito-Tracker red staining. Our results showed that palmitate stimulated a partial mitochondrial fission process as shown by the production of dramatic mitochondrial fragmentation, which was further exaggerated in NF-Y deficient β-cells [Figure 4D]. Additionally, the BCL-2 family members (such as BCL-2 and BAX) regulate mitochondrial fission-fusion balance,[34] and the ratio of BCL-2 to BAX is an index determining apoptotic status.[35] Hence, we further evaluated the levels of BCL-2 and BAX in mouse islets. In comparison with control *RIP-Cre* islets, *Nf-ya* βKO islets exhibited a marked reduction in BCL-2 expression at both mRNA and protein levels while a negligible alteration of BAX, and palmitate exposure to *Nf-ya* βKO islets further potentiated the BCL-2 reduction but with a minor effect on BAX [Figures 4E,F], leading to a marked decrease in the ratio of BCL-2/BAX in *Nf-ya* βKO islets [Figure 4G]. In sum, these results revealed that the absence of NF-Y promoted mitochondrial ROS accumulation and mitochondrial fission, which might be partially mediated through the regulation of BCL-2 and BAX abundance.

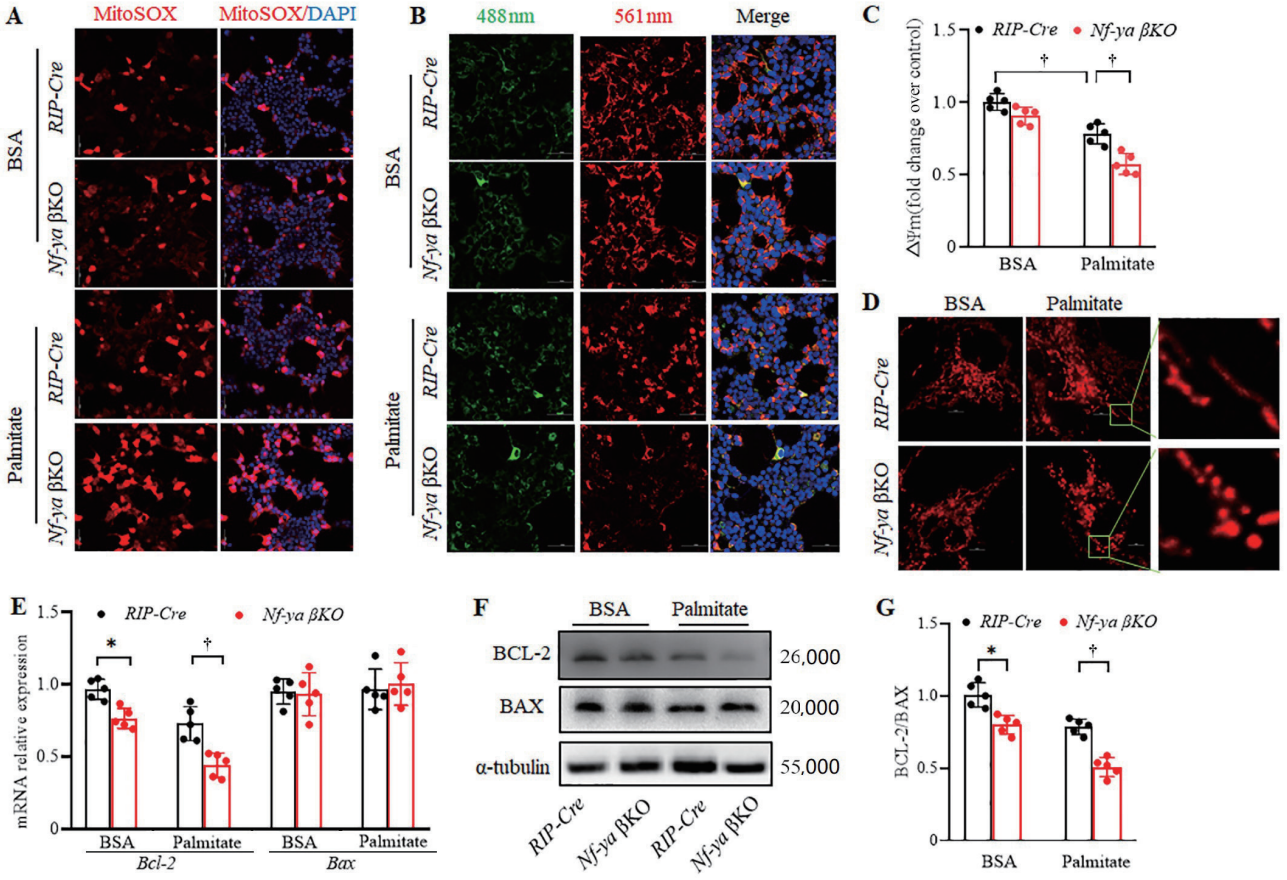

Figure 4: NF-Y depletion accelerates palmitate-induced mitochondrial dysfunction. Mouse islets from *Nf-ya* βKO and control (*RIP-Cre*) mice were incubated with palmitate (100 μmol/L) or BSA (18% w/v) for 24 h ($n = 3$ for each group). (A) The content of mitochondrial superoxide was measured by an immunofluorescence staining of MitoSOX (Scale bar = 50 μm). (B,C) MMP was assayed in JC-1 sending red or green fluorescence when MMP was high in the normal mitochondria or low in the unhealthy mitochondria, respectively. Representative images of immunofluorescence staining (Scale bar = 50 μm) (B) were taken at emission wavelenght of 488 nm (green-indicating JC-1 monomers in the cytoplasm) and at 561 nm (red-indicating JC-1 polymers in the mitochondrial matrix), and the fluorescence intensity analysis (C). (D) Mitochondria shapes were monitored using MitoTracker Red (red) (Scale bar = 5 μm). (E) Real-time PCR analysis of mRNA expressions of *Bcl-2* and *Bax* genes. Individual measurement was normalized to 18s rRNA expression, and the BSA-treated control average was set to 1. (F) Western blotting detected the protein levels of BCL-2 and BAX, and (G) the ratio of BCL-2 and BAX was compared. $^{*}P < 0.05$, $^{\dagger}P < 0.01$, $^{\ddagger}P < 0.001$. BAX: BCL-2 associated X protein; BCL-2: B cell lyphoma; BSA: Bovine serum albumin; DAPI: 4,6-diamidino-2-phenylindole; mRNA: messenger RNA; NF-Y: Nuclear factor-Y; MMP: Mitochondrial membrane potential.

NF-Y overexpression protects b- cells from palmitate-induced impairment

Given that NF-Y deletion exaggerates the β-cell failure during hyperlipidemic stress, we subsequently determined whether NF-Y could be an efficient therapeutic target in palmitate-treated INS1 cells. NF-YA was overexpressed in INS1 β-cell lines by lentivirus vectors. The cell line stably overexpressing NF-YA was subjected to puromycin screening and forced overexpression of NF-YA was confirmed by immunoblotting [Supplementary Figure 5A, http://links.lww.com/CM9/B493]. Although there was no effect on cell survival under regular culture conditions [Supplementary Figure 5B, http://links.lww.com/CM9/B493], NF-Y overexpression significantly alleviated the palmitate-mediated suppression of cell viability [Figure 5A]. We next determined the β-cell function, revealing that overexpression of NF-Y rescued the reduction of palmitate-induced GSIS in INS1 cells [Figure 5B]. In addition, the palmitate-stimulated mitochondrial ROS generation was also ameliorated by NF-Y overexpression [Figure 5C]. Furthermore, both the MMP collapse [Figure 5D] and excessive mitochondrial fission [Figure 5E] caused by palmitate were improved after NF-Y overexpression. Besides, overexpression of NF-Y also significantly suppressed palmitate-stimulated JNK phosphorylation and caspase-3 cleavage [Figure 5F]. Expectedly, the reduction of BCL-2 expression in INS1 cells exposed to palmitate was significantly improved by NF-YA overexpression [Figures 5G, H]. Altogether, these findings evidently suggested that NF-Y could maintain β-cell function and protect β-cell from damage under metabolic stress condition.

Discussion

To maintain normoglycemia in the face of insulin resistance in peripheral tissues, islets' β-cells adaptively augment insulin production and secretion through β-cell compensation. Thus, the ability of β-cell to undergo compensatory hyperplasia is critical for the maintenance of adequate β-cell function and glucose homeostasis.[2] Nonetheless, the regulatory mechanisms by which β-

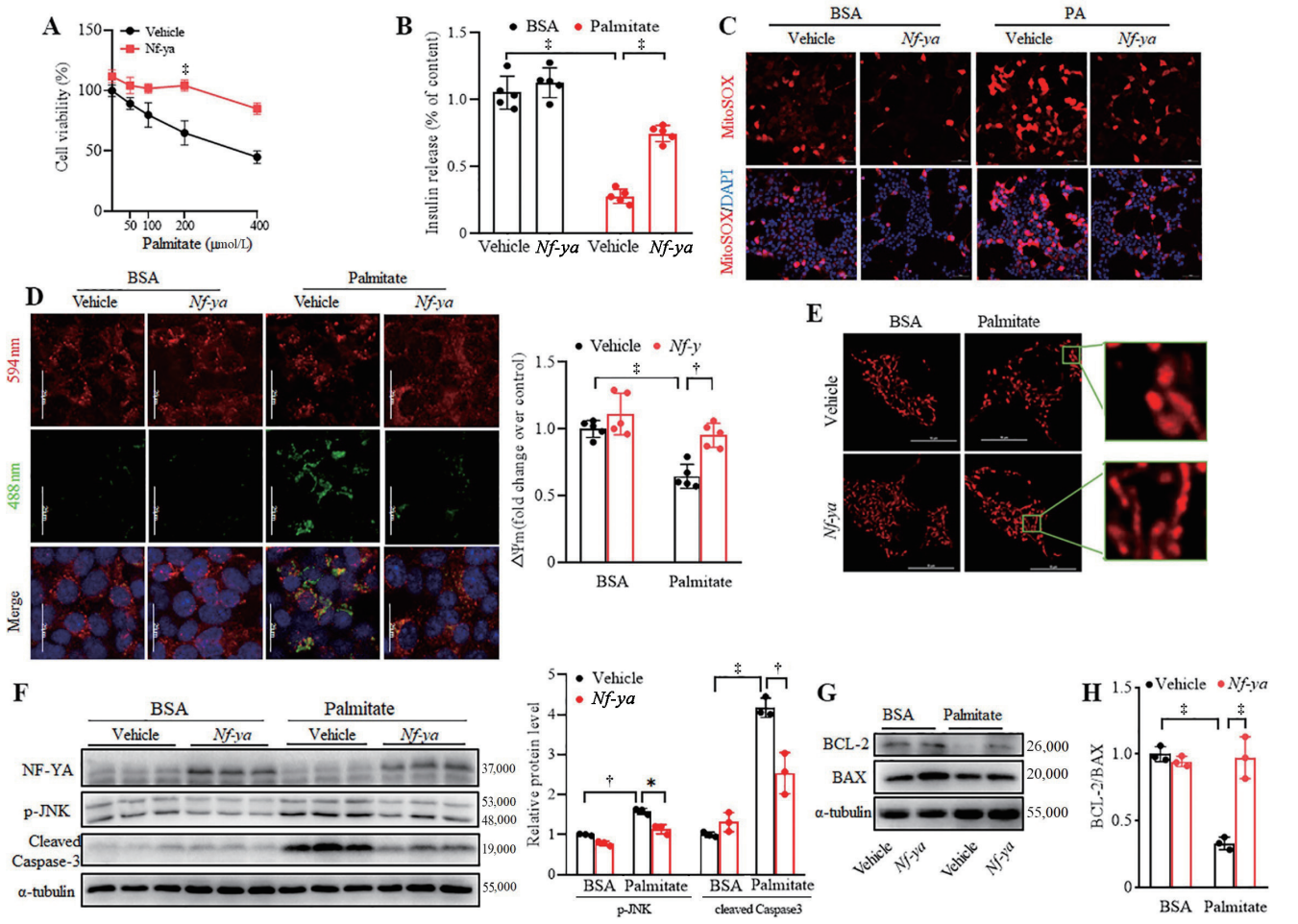

Figure 5: NF-YA overexpression partially rescues palmitate-induced impairment on beta cells. *Nf-ya* overexpression cells or empty vector (vehicle) transfected control cells were treated with palmitate at the indicated dosage or 18% (w/v) BSA for 24 h. (A) Cell viability, (B) GSIS, (C) MitoSOX staining (Scale bar = 50 μm), (D) MMP (Scale bar = 25 μm), (E) MitoTracker Red staining (Scale bar = 10 μm), (F) JNK phosphorylation and caspase-3 cleavage, (G) protein levels of BCL-2 and BAX, and (H) the ratio of BCL-2 and BAX were compared after different treatments. Data are presented as mean ± standard deviation. $^{*}P < 0.05$, $^{\dagger}P < 0.01$, $^{\ddagger}P < 0.001$. BAX: BCL-2 associated X protein; BCL-2: B cell lymphoma 2; BSA: Bovine serum albumin; DAPI: 4,6-diamidino-2-phenylindole; GSIS: Glucose-stimulated insulin secretion; JNK: c-Jun N-terminal kinase; MMP: Mitochondrial membrane potential; NF-Y: Nuclear factor-Y; NF-YA: NF-Y subunit A; PA: Palmitic acid.

cells compensate for insulin resistance remain elusive. Here, we demonstrated that NF-Y plays a fundamental role in the regulation of β-cell compensatory responses. NF-Y is abundantly expressed in pancreatic islets of obese animals,[15] and an absence of NF-Y in pancreatic β-cells resulted in metabolic decompensation as shown by the exaggerated glucose intolerance and decreased GSIS. Using primary islets and the INS1 β-cells subjected to palmitic acid, we demonstrated that the NF-Y ablation in pancreatic β-cells under metabolic stress conditions leads to an increased apoptosis and an impaired handling of oxidative stress, whereas NF-Y forced overexpression could protect β-cells from ROS-induced apoptosis and improve the capacity of insulin secretion in response to metabolic stress. Thus, our findings proposed that NF-Y played a key role in β-cell compensation, which in part was mediated by ameliorating oxidative stress.

Our previous work showed that NF-Y ablation in pancreatic β-cells leads to diminished islets' volume due to impaired β-cell replication.[15] Instead, the current study demonstrated that β-cell loss induced by NF-Y deletion under metabolic stress conditions was attributable to increased apoptosis. Furthermore, our findings revealed that palmitate-induced lipotoxicity stimulated a small increase in oxidative stress levels in control islets, but this was markedly exaggerated in NF-Y deficient animals. Excessive ROS induces oxidative stress, which is implicated as one of the major factors in palmitate-induced β-cell dysfunction and apoptosis.[36] Mitochondria are the primary ROS source and also the major target of their action. Mitochondrial failure has been associated with β-cell dysfunction in both T2D patients and mouse model.[37,38] Our findings demonstrated that the absence of NF-Y caused an imbalance of mitochondrial dynamics and an excessive mitochondrial ROS production in β-cells, which might contribute to the elevated apoptosis of the NF-Y deficient β-cells under metabolic stress conditions. In addition to the oxidative stress, the function of NF-Y in cell apoptosis is often connected to tumor inhibitor p53, and the inactivation

of NF-YA triggers the activation of p53-mediated apoptosis.[39,40] Further investigations are necessary to clarify the interaction between NF-Y and p53 in the regulation of β-cell apoptosis.

The accumulation of ROS will increase the phosphorylation of JNK, which subsequently induces the programed cell death.[41] NF-Y ablation led to increased JNK phosphorylation as well as reduced activities of SOD2 and GPX1 in primary islets and INS 1 cells after palmitate treatment, while they were negligibly affected by the NF-Y deficiency under physiological conditions. Thus, NF-Y might be important for compensatory pathways maintaining normal cellular oxidative stress level and β-cell function under metabolic stress conditions. The exact mechanism underlying the complexity of NF-Y in compensatory pathways is worthy of further investigation.

On the whole, we present the evidence that NF-Y is a key mediator of adaptive β-cell compensation to override insulin resistance. Inactivation of NF-Y resulted in β-cell apoptosis due to the deficiencies of antioxidant defense and mitochondrial functions. These detrimental changes were partially restored through NF-Y gain-of function in pancreatic β-cells. Our data reinforced the idea that targeting NF-Y might help to preserve and restore functional β-cell mass in at-risk subjects with obesity. However, the clinical relevance of these findings is needed to be confirmed in human β-cells.

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Conflicts of interest

None.

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