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ORIGINAL ARTICLE

Oxidative stress suppresses PHB2-mediated mitophagy in β-cells *via* the Nrf2/PHB2 pathway

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ABSTRACT

Aims/Introduction: Mitochondrial damage caused by oxidative stress is a main driver of pancreatic β-cell dysfunction in the pathogenesis of type 2 diabetes mellitus. Prohibitin2 (PHB2) is a vital inner mitochondrial membrane protein that participates in mitophagy to remove the damaged mitochondria. This study aimed to investigate the role and mechanisms of PHB2-mediated mitophagy in oxidative stress-induced pancreatic β-cell dysfunction.

Materials and Methods: PHB2 and mitophagy-related protein expression were analyzed by real-time polymerase chain reaction and western blotting in RINm5F cells treated with H_2O_2 and islets of diabetic rats. Mitophagy was observed by mitochondrial and lysosome colocalization. RINm5F cells were transfected by *phb2* siRNA or overexpression plasmid to explore the role of PHB2 in mitophagy of RINm5F cells. The mechanism of Nrf2 regulating PHB2 was explored by Nrf2 inhibitor and agonist.

Results: The expression of PHB2, mitophagy related protein PINK1, and Parkin were decreased in RINm5F cells incubated with H_2O_2 and in islets of diabetic rats. Overexpression of PHB2 protected β-cells from oxidative stress by promoting mitophagy and inhibiting cell apoptosis, whereas transfection with PHB2 siRNA suppressed mitophagy. Furthermore, PHB2-mediated mitophagy induced by oxidative stress was through the Nrf2/PHB2 pathway in β-cells. Antioxidant NAC alleviated oxidative stress injury by promoting PHB2-mediated mitophagy.

Conclusion: Our study suggested that PHB2-mediated mitophagy can protect β-cells from apoptosis *via* the Nrf2/PHB2 pathway under oxidative stress. Antioxidants may protect β-cell from oxidative stress by prompting PHB2-mediated mitophagy. PHB2-mediated mitophagy as a potential mechanism takes part in the oxidative stress induced β-cell injury.

INTRODUCTION

Type 2 diabetes mellitus is a metabolic disease with multiple etiologies characterized by chronic hyperglycemia. The prevalence of diabetes is increasing rapidly all over the world, placing a heavy burden on many countries. It is increasingly important to understand its pathogenesis[1]. The crucial pathophysiological disorder of diabetes is pancreatic β-cell dysfunction leading to reduced insulin secretion. Mitochondria are essential for preventing pancreatic β-cells from being impaired[2]. Mitochondria trigger insulin exocytosis by metabolizing pyruvate to produce ATP in β-cells[3]. Changes in mitochondrial morphology have been observed in type 2 diabetes mellitus model mouse β-cells[4]. Impaired mitochondrial morphology and function have been

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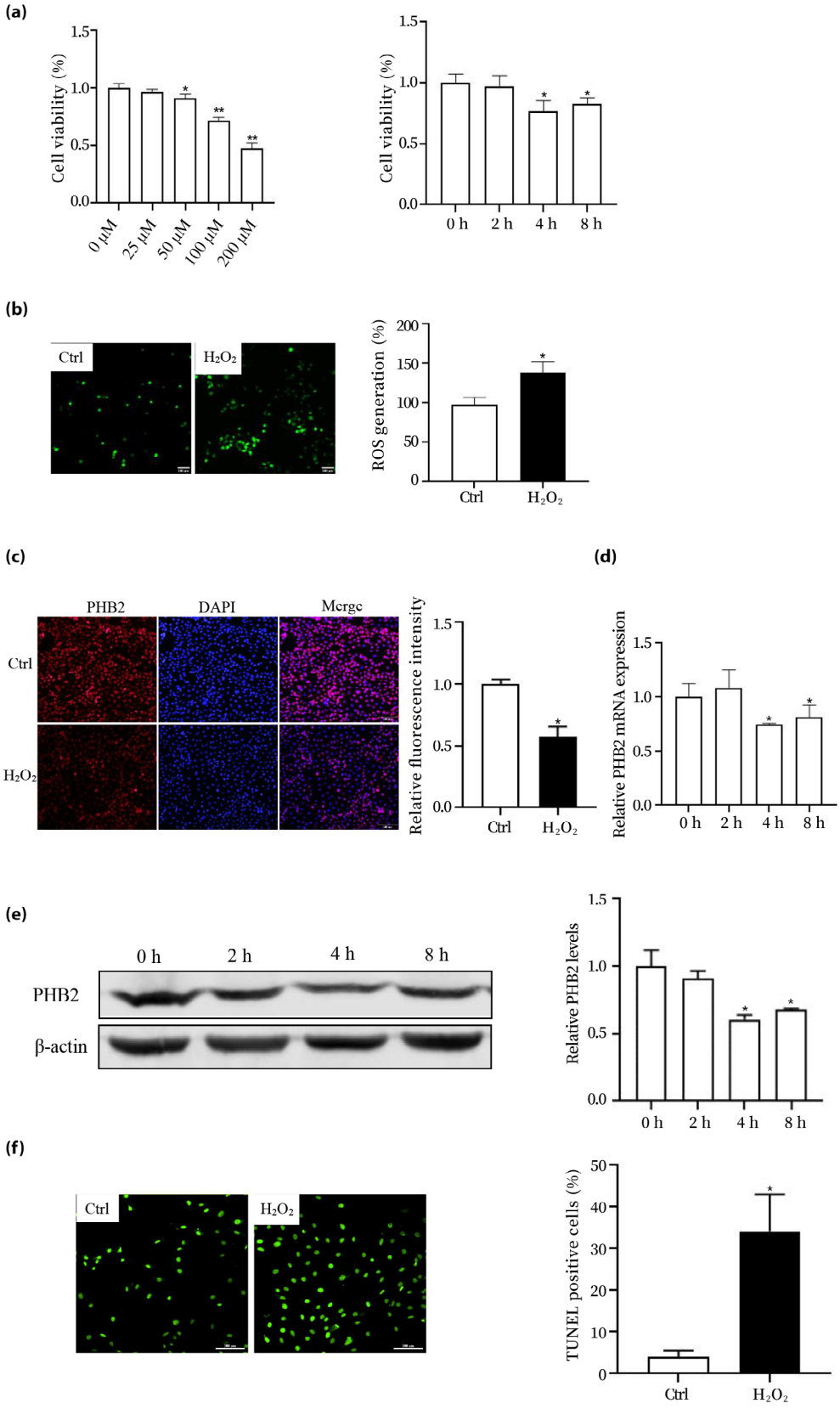


associated with β-cell deficiency in insulin secretion in type 2 diabetes mellitus patients[5]. Therefore, the stable function of mitochondria is indispensable to β-cell insulin secretion. However, the mechanism of β-cell injury by mitochondria is still unclear.

β-Cells are particularly vulnerable to oxidative stress due to their lack of antioxidant enzymes. Reactive oxygen species (ROS) are significant factors that participate in β-cell signal transduction and regulate glucose-stimulated insulin secretion (GSIS). Oxidative stress is considered to be a contributor to the onset and development of diabetes[6].

The mitochondrial respiratory chain is the primary source of ROS in β-cells[7]. However, excessive ROS leads to mitochondrial oxidative stress which causes mitochondrial dysfunction and cellular injury[8,9]. The removal of damaged mitochondria can repress cell apoptosis and preserve cell function. Oxidative stress can be generated by numerous sources, such as superfluous nutrition, smoking and sleep-deprivation. Previous studies have mostly focused on the effect of oxidative stress induced by high glucose or elevated free fatty acids on β-cells. In fact, β-cell dysfunction predates the occurrence of type 2 diabetes mellitus. Hence, we hope to explore the impact of oxidative stress-induced mitochondrial injury in β-cells on the onset of diabetes before the occurrence of hyperglycemia.

Mitophagy is crucial to protect cells against the lesions from damaged mitochondria. A deficiency of mitophagy may lead to neurodegenerative disease, carcinoma, inflammation, and metabolic disease. Mitophagy deficiency in β-cells causes damaged mitochondria and insulin secretion dysfunction[10].

PHB includes two homologous subunits PHB1 and PHB2. PHB2 is a pleiotropic inner mitochondrial membrane protein that is crucial for maintaining the homeostasis of mitochondrial morphology and function[11]. Wei *et al.*[12] reported that PHB2 is a key mitophagy receptor that participates in targeting mitochondria for degradation.

Lee *et al.*[13] found that the expression of PHB increased with oxidative stress induced by ethanol in pancreatic β-cells. Nuclear factor erythroid-2 related factor 2 (Nrf2) is a transcription factor which plays an important role in antioxidant stress and the homeostasis of mitochondrial function. Previous studies showed that geraniol, fucoxanthin, and kirenol inhibit oxidative stress and inflammation *via* activating Nrf2 signaling pathways in vascular endothelial cells[14–17]. Recent research showed that mitophagy reduces oxidative stress injury in subarachnoid hemorrhage rats *via* the Keap1/Nrf2/PHB2 pathway[18].

It is unknown whether oxidative stress could lead to β-cell dysfunction by regulating PHB2 and its mechanism. In this research, we will investigate the effects of oxidative stress on PHB2-mediated mitophagy and explore the role of PHB2 in the pathogenesis of diabetes.

MATERIALS AND METHODS

Cell culture and treatment

RINm5F cell lines (RINm5F cell lines purchased from Cell Resource Center, IBMS, CAMS/PUMC) were cultured in RPMI 1640 medium containing 10% fetal bovine serum and 1% penicillin and streptomycin in a 37°C humidified incubator with 5% CO_2. RINm5F cells were cultured for 24 h until 70% confluence and then the cells were incubated with or without 100 μM hydrogen peroxide (H_2O_2) for 4 h. The H_2O_2 dosage was determined by a dose–response curve in RINm5F cells.

Experimental animals and induction of type 2 diabetes

A total of 20 male Sprague–Dawley (SD) rats aged 8 weeks and weighing 150–200 g were obtained from the Experimental Animal Center of Hebei Medical University [SCXK 2018-004]. Animal experiments were approved by the Animal Ethical Experimental Committee of The First Hospital of Hebei Medical University. Animals were kept at a constant temperature (22–25°C) for 12 h in light–dark alternating cycles with adequate food and water. The rats were randomly divided into two groups: control (n = 10) and diabetic model group (n = 10). The control group rats were fed an ordinary diet. Diabetic model rats were fed a high-fat diet for 4 weeks and then administered a single intraperitoneal injection of 30 mg/kg STZ (Sigma Chemical Co., Saint Louis, MO, USA). Tail vein blood samples were measured with a glucometer to check blood glucose (Accu-Check; Roche Diagnostics, Rotkreuz, Switzerland). Hyperglycemia and diabetes were defined as a random blood glucose ≥16.7 mmol/L according to the criteria published by the American Diabetes Association[19].

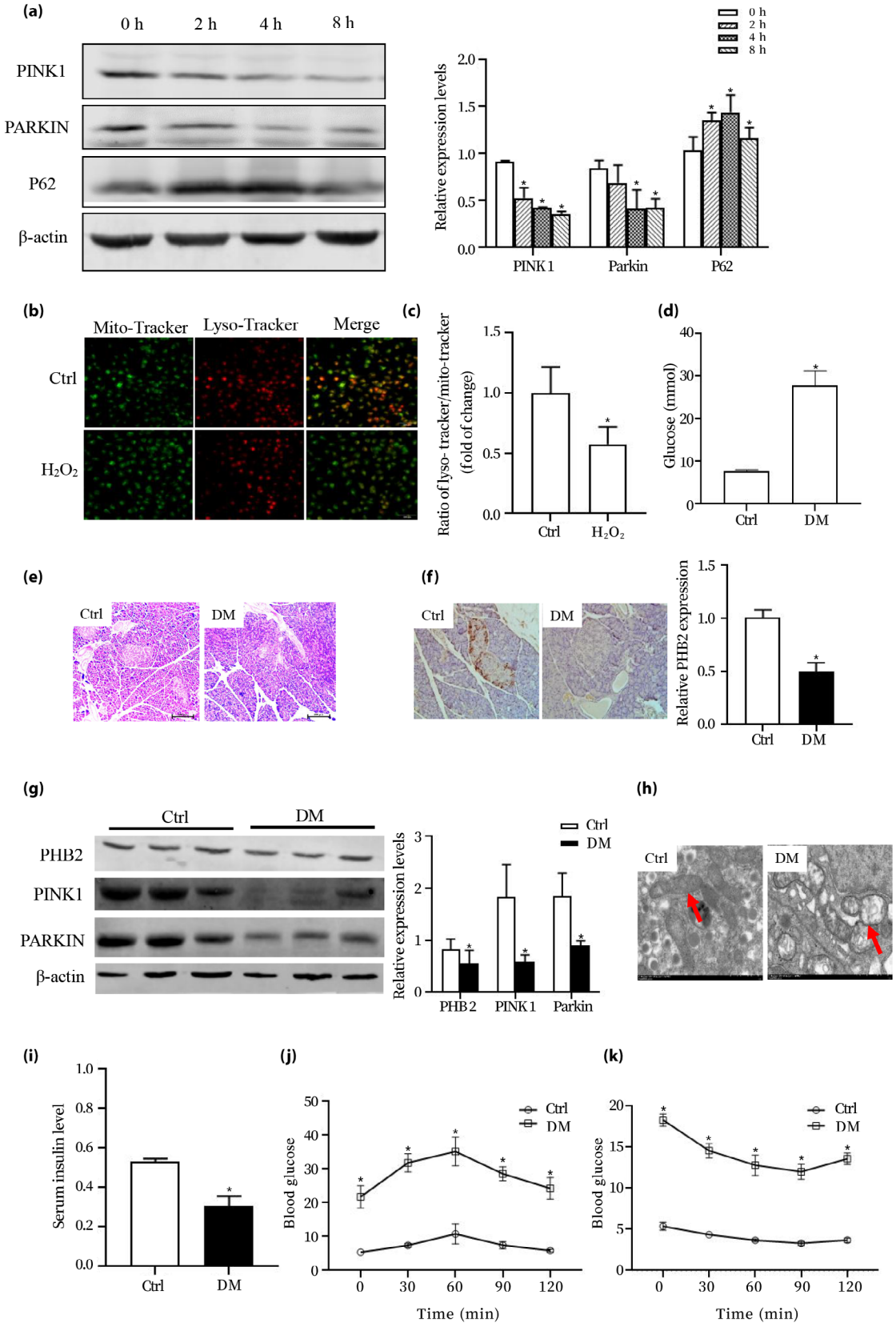
Cell viability assay

The proliferation of RINm5F cells was evaluated by a commercial kit (Cell Counting Kit-8 (CCK-8); Soribio, Inc., Beijing, China). The cells were inoculated into 96-well plates at a density of 5×10^4 cells per well and incubated with 0, 25, 50, 100, and 200 μM H_2O_2 for 4 h at 37°C. The operation was carried out according to the instructions.

EdU click chemistry assay and fluorescence imaging

RINm5F cells were cultured with 10 μM EdU solution for 2 h. After washing, fixing solution was added. Then the cells were permeabilized using Triton. The cells were washed and CLICK

Figure 1 | Effect of H_2O_2 on the PHB2 expression in RINm5F cells. (a) Effect of H_2O_2 on the relative cell viability in RINm5F cells. The relative viability was reduced significantly after treatment with 100 μM H_2O_2 for 4 h. RINm5F cells were incubated in 100 μM H_2O_2 for different times. N = 3 experiments. (b) ROS level of RINm5F cells incubated with or without 100 μM H_2O_2 for 4 h (scale bar 100 μm). (c) Expression of PHB2 in RINm5F cells incubated with or without H_2O_2 by immunostaining (scale bar 100 μm). (d) qRT-PCR analysis of PHB2 mRNA levels. (e) Western blot analysis of PHB2 in RINm5F cells with or without H_2O_2. (f) Representative images and quantification of TUNEL staining of RINm5F cells (scale bar 100 μm). *Compared with the control group, $P < 0.05$.



reaction solution added. DAPI seal and pictures taken (EdU assay, C0078S; Beyotime, Inc., Shanghai, China). Images were obtained by an inverted fluorescence microscope (Nikon, Eclipse Ts2R, Tokyo, Japan).

ROS assay

After cell culture and treatment, DCFDA was diluted to 10 μmol/L in serum-free medium and incubated in a carbon dioxide incubator for 30 min[20]. A fluorescence microscope (Nikon, Eclipse Ts2R) was used to detect ROS levels in cells and statistically analyzed.

Western blot analysis

RINm5F cell extracts and pancreatic islet specimens were obtained for Western blot analysis. The membrane was blocked for 1 h at room temperature with 5% nonfat milk. Western blot were carried out with PHB2 (#14085, 1:2000; Cell Signaling Technology, Boston, MA, USA), PINK1 (DF7742, 1:1000; Affinity, Changzhou, Jiangsu, China), Parkin (#4211, 1:1000; Cell Signaling Technology), P62 (ab56416, 1:1000; Abcom, Cambrige, UK), and Nrf2 (NBP1-32822, 1:1000; Novus Biologicals, Littleton, CO, USA). The membranes were incubated with primary antibody at 4°C overnight. After washing three times, the membrane was incubated with a fluorescent secondary antibody at room temperature for 1 h. Quantitative image analysis was performed using NIH Image J software to determine the intensity of the individual proteins.

Quantitative real-time polymerase chain reaction

Total RNA from cell cultures and rat pancreatic islets was extracted by TRIzol reagent (Thermo, Waltham, MA, USA). Reverse transcription of the isolated RNA into cDNA was performed using PrimeScript Master Mix (Takara, Osaka, Japan). Real-time PCR was performed with SYBR Select Master Mix (Thermo) on Bio-Rad CFX96 quantitative PCR system (Bio-Rad, Hercules, CA, USA). The primer sequences used PHB2: 5′-GGCGTCCGTGAGTCCGTAT-3′ (forward), 5′-GGTCT-TTGGAGCCT GTGGG-3′ (reverse); Nrf2: 5′-CAGCACATC-CAGACAGACACC-3′ (forward), 5′-CTCCTGCCA AACTTGCTCCAT-3′ (reverse); β-actin as an internal control were quantified in parallel with target genes. Normalization and fold changes were calculated by the comparative $\Delta\Delta C_T$ method.

PHB2 siRNA transfection

On the day before transfection, the cells were plated to the desired cell density (at least 50% confluency). Small interfering RNAs (NC and PHB2) were separately diluted in Opti-MEM (Life Technologies, Carlsbad, CA, USA) and incubated at room temperature for 5 min. The diluted siRNAs were added to diluted Lipofectamine 2000 (11668; Invitrogen, Waltham, MA, USA) and further incubated for 20 min. Small interfering RNAs (siRNAs) were transferred into cells according to the manufacturer's protocol. The above sequence used for siRNA cell transfection was 5′-GCTATATCAAGCTCCGAAA-3′.

PHB2 overexpression

The RINm5F cells were cultured for 24 h with a (pcDNA3.1 (+)-mCherry) vector containing PHB2 clone before transfection. The transfection was conducted with a FuGENE HD transfection reagent (Roche Applied Science, Laval, QC, Canada) according to the protocol supplied by the manufacturer. Then the cells were washed at 24 h and replaced in medium with or without 100 μM H_2O_2 for 4 h.

Immunocytochemistry staining

The RINm5F cells were cultured on chamber slides (Sorlabio, Beijing, China) and incubated for 4 h with or without H_2O_2 (100 μM). The cells were incubated with paraformaldehyde at room temperature for 10 min and washed in PBS three times. Then, the cell membrane was permeated with 0.1% Triton X-100 at room temperature for 5 min. The cells were incubated in PHB2 (1:200; Cell Signaling Technology, Hercules, MA, USA) overnight. After washing, the cells were blocked with 5% nonfat milk for 1 h and then incubated with secondary antibody (FITC-conjugated goat anti-rabbit IgG) for 1 h at 37°C. DAPI was used to label nuclei. Images were obtained by an inverted fluorescence microscope (Nikon, Eclipse Ts2R). For quantification, the semi-quantitative immunofluorescence score was obtained by examining at least five random fields per section under ×400 magnification and with digital image analysis by Image J software.

Histological and immunohistochemical staining

The pancreas samples were fixed in 10% formaldehyde for 48 h at room temperature and embedded in paraffin. The sections were stained with histology and immunohistochemistry using a commercial kit [UltraSensitive™ SP (mouse/rabbit) IHC kit;

Figure 2 | PHB2 mediated mitophagy decreased in β-cells incubated in H_2O_2 and diabetic rats. (a) Western blot analysis of PINK1 and Parkin in RINm5F cells with or without H_2O_2. *Compared with the 0 h control group, $P < 0.05$. (b) The colocalization of mitochondria (MitoTracker green) and lysosomes (LysoTracker deep red) was observed in RINm5F cells with or without H_2O_2. Images were captured with a confocal laser scanning microscope (scale bar 100 μm). (c) Analysis of the levels of colocalization of mitochondria and lysosomes. (d) The blood glucose of SD rats in the control and diabetic models. (e) HE staining of pancreatic tissue in SD rats (scale bar 100 μm). (f) Immunochemical staining for PHB2 in pancreatic islets in SD rats (scale bar 100 μm). (g) Western blot analysis of PHB2, PINK1, and Parkin in control and diabetic rats. (h) Representative TEM images of pancreatic β cell mitochondria in control and diabetic rats (scale bar 2 μm). Red arrows indicate mitochondria. In image of DM rats, red arrows indicate disruption of the mitochondria with derangement of the mitochondrial crests. (i) The serum insulin level of SD rats in the control and diabetic models. (j) Blood glucose values under intraperitoneal glucose tolerance test of SD rats in the control and diabetic models (IPGTT). (k) Blood glucose values during intraperitoneal insulin tolerance test of SD rats in the control and diabetic models (IPITT). *Compared with control group, $P < 0.05$.

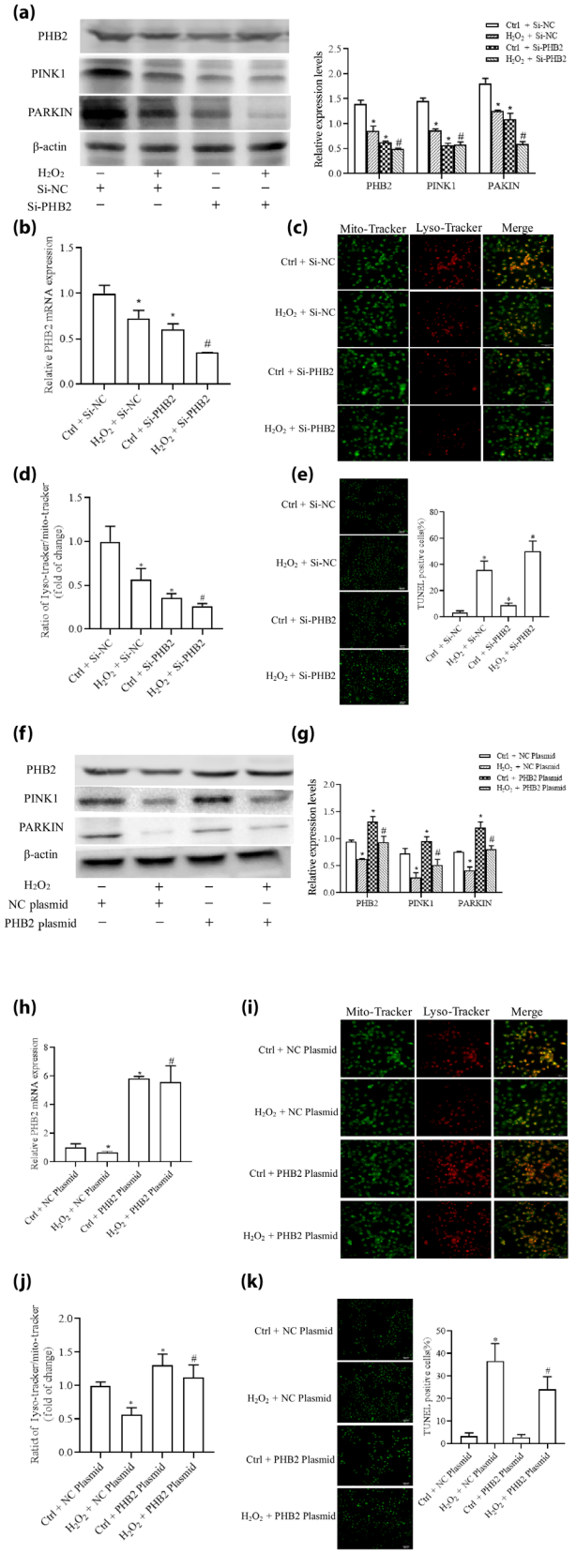


Figure 3 | PHB2 plays a key role in mitophagy of β-cells under oxidative stress. (a) Effect of PHB2 siRNA on PINK1 and Parkin in RINm5F cells. Si-NC represents the corresponding scrambled siRNA (+). Si-PHB2 represents small interfering PHB2 (+). Ctrl +Si-NC group represents Si-NC(+)H_2O_2 (–). H_2O_2+ Si-NC group represents Si-NC(+)H_2O_2 (+). Ctrl +Si-PHB2 group represents Si-PHB2(+)H_2O_2 (–). H_2O_2+ Si-PHB2 group represents Si-PHB2(+)H_2O_2 (+). *Compared with the Ctrl +Si-NC group, $P < 0.05$. #Compared with the H_2O_2+ Si-PHB2 group, $P < 0.05$. (b) qRT-PCR determination of the mRNA expression of PHB2 in RINm5F cells transfected with PHB2 siRNA. (c) The colocalization of mitochondria and lysosomes were observed in RINm5F cells transfected with PHB2 siRNA. Images were captured with a confocal laser scanning microscope (scale bar 100 μm). (d) Analysis of the levels of colocalization of mitochondria and lysosomes in RINm5f cells. (e) Effect of PHB2 siRNA on H_2O_2 induced apoptosis in RINm5F cells by TUNEL staining (scale bar 100 μm). (f) Western blot of effect of PHB2 overexpression on PINK1 and Parkin in RINm5F cells. (g) Western blot analysis of effect of PHB2 overexpression on PINK1 and Parkin in RINm5F cells. (h) qRT-PCR determination of the mRNA expression of PHB2 in RINm5F cells overexpressing PHB2. (i) The colocalization of mitochondria and lysosomes were observed in RINm5F cells transfected with PHB2 plasmid. Images were captured with a confocal laser scanning microscope (scale bar 100 μm). (j) Analysis of the levels of colocalization of mitochondria and lysosomes transfected with PHB2 plasmid. (k) Effect of PHB2 overexpression on H_2O_2-induced apoptosis in RINm5F cells by TUNEL staining (scale bar 100 μm). *Compared with the Ctrl +NC plasmid group, $P < 0.05$. #Compared with the H_2O_2+ NC plasmid group, $P < 0.05$.

MXB Biotech, Inc., Fuzhou, Fujian, China]. Pancreas sections were incubated with anti-Nrf2 (1:200) or anti-PHB2 (1:300) overnight at 4°C. Positive staining was observed with 3, 3′-diaminobenzidine (DAB). After staining, the pancreas sections were observed under a microscope. For quantification, the semi-quantitative immunohistochemical score was obtained by examining at least five random pictures per section under ×400 magnification and with digital image analysis by Image J software.

Analysis of apoptosis

The TUNEL assay was performed on RINm5F cells by the one-step TUNEL apoptosis assay kit (Beyotime, Shanghai, China) following the manufacturer's instructions. The cells were incubated with TUNEL reaction mixture for 1 h in the dark at 37°C and then washed twice in PBS. Condensed or fragmented nuclei in apoptotic cells were observed under a fluorescence microscope at 200× magnification.

Isolation of pancreatic islets

Rat islets were separated by collagenase V digestion and filtration[21]. Islets were collected by hand under a microscope[22]. Thirty milliliters of cold Hanks' buffer/type XI collagenase solution was injected into the pancreatic duct of rats. The expanded pancreas was removed into a 37°C shaker water bath, chopped up and digested. According to Ishikawa's method, the number

of isolated islets was counted by two persons in a double-blinded manner[23].

Mitochondria and lysosome colocalization imaging

The cells were cultured on chamber slides (Sorlabio) until 70–80% confluence. According to the instructions, the cells were coincubated with MitoTracker green (Beyotime) and LysoTracker deep red (Beyotime). The cells were stored in the dark at 37°C for 20 min, then washed three times with primary medium. The images were observed using an inverted fluorescence microscope (Nikon, Eclipse Ts2R). Image J was used to analyze the mean gray value of immunofluorescence.

Transmission electron microscopy imaging

The 1 mm^3 tissue blocks were transferred into an EP tube with fresh TEM fixative for further fixation, which was fixed at 4°C for preservation and transportation. Then the tissues were washed using 0.1 M PB (pH 7.4) three times for 15 min each. Then 2% uranium acetate saturated alcohol solution was added to avoid light staining for 8 min. Then 2.6% lead citrate was added to avoid CO_2 staining for 8 min, and then rinsed with ultra pure water three times. The samples were dried overnight at room temperature. The images were analyzed by transmission electron microscopy (HITACHI, HT7800/HT7700).

IPGTT and IPITT

For glucose tolerance tests, the rats were fasted overnight for 16 h followed by an intraperitoneal glucose injection (1 g/kg BW). Blood glucose measurements from the tail veins were performed at 0, 30, 60, 90, and 120 min after the injection. For insulin tolerance tests, the rats were injected intraperitoneally with regular human insulin (at 0.75 U/kg body weight) after a 6 h fast, and the blood glucose was measured 0, 30, 60, 90, and 120 min later. Serum insulin levels from blood samples were detected by a rat insulin enzyme-linked immunosorbent assay (Linco Research, St Charles, MO, USA).

Statistical analysis

In the present study, all the data were analyzed using SPSS 22.0. One-way ANOVA or Student's *t*-test was used to evaluate the statistical significance of differences among mean values in different experimental groups. The SNK-q test was used post-hoc after the one-way ANOVA when compared between three or more groups. A value of $P < 0.05$ indicated that the differences were statistically significant.

RESULTS

PHB2 is expressed in β-cells and decreased by hydrogen peroxide

To observe the effect of oxidative stress on PHB2 in RINm5F cells, we first generated a dose-dependent curve of hydrogen peroxide toxicity in RINm5F cells incubated with H_2O_2 for 4 h. A significant decline in cell activity was observed at 100 μM compared with untreated cells. At this concentration, a time-dependent curve of H_2O_2 was generated. There was an obvious decline in cell activity at 4 h (Figure 1a). The EdU incorporation assay showed that a significant inhibition of proliferation was observed in RINm5F cells incubated with 100 μM H_2O_2 for 4 h (Figure S1A,B). Then, the RINm5F cells were divided into two groups, one was the control group, and the other was the group of cells incubated with 100 μM H_2O_2 for 4 h. It was found that cells incubated in H_2O_2 had markedly more ROS generation than the control group (Figure 1b).

The levels of PHB2 mRNA and protein were determined by a time course. The expression of PHB2 in the H_2O_2 group significantly decreased compared with the control group by immunofluorescence staining (Figure 1c). The levels of PHB2 mRNA and protein were determined by a time course in 100 μM H_2O_2. The PHB2 mRNA and protein levels decreased significantly in the cells with 100 μM H_2O_2 for 4 h (Figure 1d, e). Furthermore, apoptosis increased significantly in the H_2O_2 group (Figure 1f).

Decreased PHB2 leads to reduced mitophagy in β-cells incubated with H_2O_2

We observed a decline in PHB2 in H_2O_2-incubated β-cells as described above. As PHB2 is a critical mitochondrial inner protein for mitophagy and required for Parkin-mediated mitophagy. We were interested in whether PHB2 mediated mitophagy was reduced in H_2O_2-incubated β-cells. We measured the expression of the mitophagy-related proteins PINK1, Parkin and P62 in RINm5F cells. PINK1 and Parkin decreased and P62 increased significantly in the H_2O_2 group cells (Figure 2a). Mitophagy analysis by colocalization showed that mitophagy was significantly reduced in the H_2O_2 group compared with the control group (Figure 2b,c). All of the above suggests that H_2O_2 leads to mitophagy suppression in β-cells.

To investigate the expression of PHB2 *in vivo*, type 2 diabetes mellitus model rats were used. The random blood glucose of diabetic model rats was above 16.7 mmol/L (Figure 2d). The H&E staining showed morphological shrinkage of pancreatic islets, nuclear arrangement disorder, and vacuolation of cytoplasm in diabetic rats (Figure 2e). Western blot analysis showed that the expression of PHB2 obviously decreased in diabetic rat β-cells compared with the control group (Figure 2g). A significant decline in PHB2 expression was observed in the pancreatic islets of diabetic rats by immunohistochemical staining (Figure 2f). Electron microscopy showed mitochondrial swelling, cristae loss, decreased secretory granules, decreased core density, nuclear deformation, and endoplasmic reticulum expansion in β-cells of diabetic rats (Figure 2h). The concentration of serum insulin decreased significantly in the diabetic rats compared with the control which suggested the function of the β-cells was impaired (Figure 2i). The DM rats displayed impairments in glucose (Figure 2j) and insulin responses (Figure 2k).

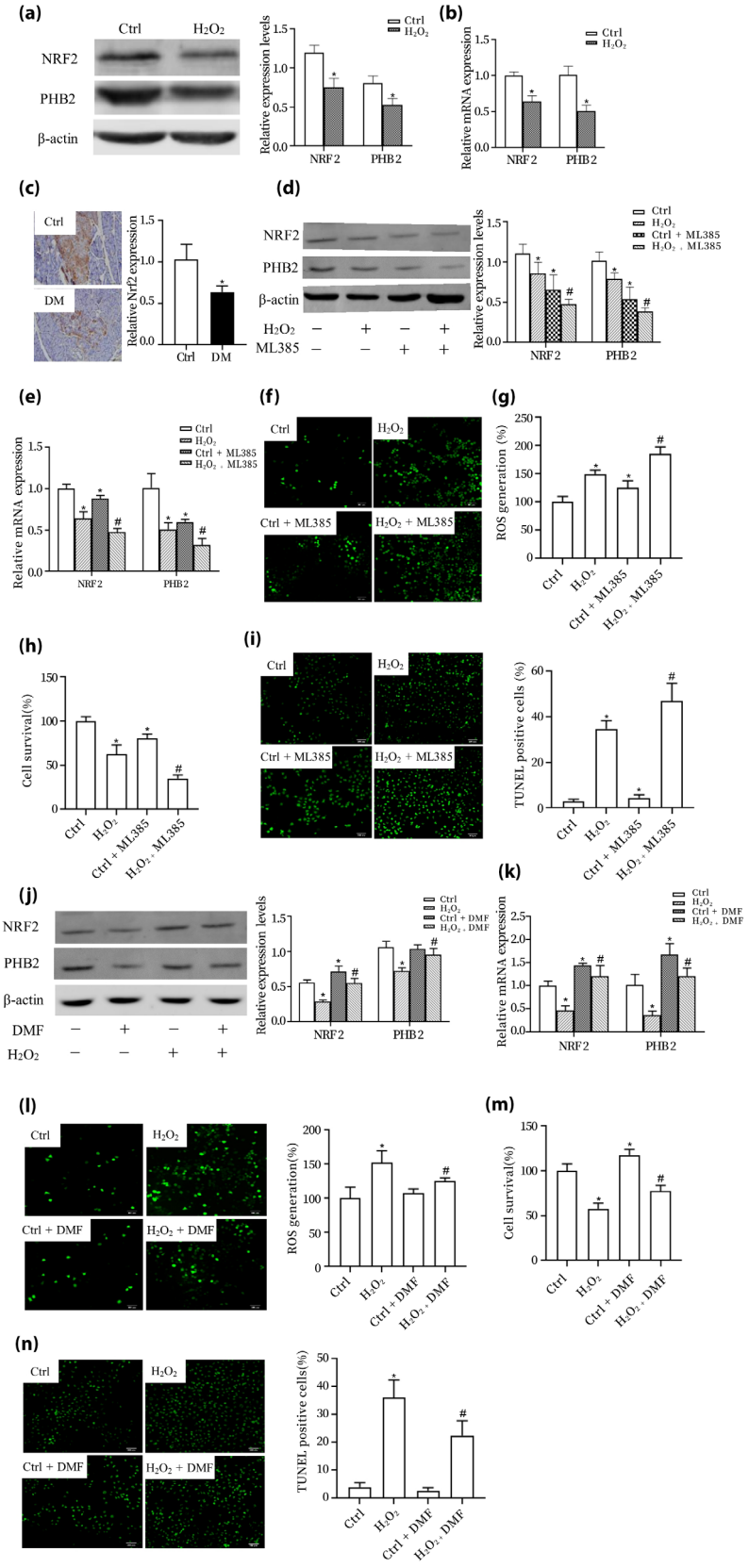


Figure 4 | Oxidative stress reduces mitophagy through the Nrf2/PHB2 pathway in β-cells. (a) Western blot analysis of NRF2 in RINm5F cells incubated with and without H_2O_2. (b) qRT-PCR determination of the mRNA expression of NRF2 and PHB2 in RINm5F cells with and without H_2O_2. (c) Immunochemical staining for NRF2 in pancreatic tissue in SD rats (scale bar 100 μm). (d) Western blot analysis of PHB2 in RINm5F cells pretreated with the NRF2 inhibitor ML385. (e) qRT-PCR determination of the mRNA expression of NRF2 and PHB2 in RINm5F cells treated with the NRF2 inhibitor ML385. (f) ROS level of RINm5F cells incubated with or without ML385 (scale bar 100 μm). (g) ROS level of RINm5F cells incubated with or without ML385 (scale bar 100 μm). (h) Cell survival of RINm5F cells incubated with or without ML385. (i) Effect of ML385 on H_2O_2 induced apoptosis in RINm5F cells by TUNEL staining (scale bar 100 μm). (j) Western blot analysis of PHB2 in RINm5F cells pretreated with NRF2 agonist DMF. (k) qRT-PCR determination of the mRNA expression of NRF2 and PHB2 in RINm5F cells treated with the NRF2 agonist DMF. (l) ROS level of RINm5F cells incubated with or without DMF (scale bar 100 μm). (m) Cell survival of RINm5F cells incubated with or without DMF. (n) Effect of DMF on H_2O_2-induced apoptosis in RINm5F cells by TUNEL staining (scale bar 100 μm). *Compared with the control group, $P < 0.05$. #Compared with the H_2O_2 group, $P < 0.05$.

PHB2 plays a key role in mitophagy in β-cell under oxidative stress

To elucidate the pivotal function of PHB2 in β-cell mitophagy, the β-cells were transfected with PHB2 siRNA. PINK1 and Parkin were significantly reduced in the H_2O_2+ si-PHB2 group compared with the H_2O_2+ si-NC group (Figure 3a,b). Mitophagy analysis showed that mitophagy was significantly reduced in the H_2O_2+ si-PHB2 group compared with the H_2O_2+ si-NC group (Figure 3c,d). β-cell apoptosis was significantly increased in the H_2O_2+ si-PHB2 group compared with the H_2O_2+ si-NC group (Figure 3e). These results suggested that inhibition of PHB2 led to mitophagy reduction and apoptosis augmentation in β-cells under oxidative stress. In contrast, the expression of PINK1 and Parkin was significantly increased in the H_2O_2+ PHB2 plasmid group compared with the H_2O_2 group (Figure 3f,g,h). Mitophagy analysis showed that mitophagy obviously increased in the H_2O_2+ PHB2 plasmid group compared with the H_2O_2+ NC plasmid group (Figure 3i,j). Meanwhile, β-cell apoptosis was significantly decreased in the H_2O_2+ PHB2 plasmid group compared with the H_2O_2+ NC plasmid (Figure 3k). These results suggested that overexpression of PHB2 may protect β-cells by augmenting mitophagy and inhibiting apoptosis under oxidative stress. Transfection experiments verified that PHB2 plays a key role in β-cell mitophagy.

Oxidative stress reduced mitophagy through the Nrf2/PHB2 pathway in β-cells

To investigate whether oxidative stress reduces mitophagy through the Nrf2/PHB2 pathway in β-cells, Western blotting revealed that Nrf2 and PHB2 were obviously decreased significantly in the H_2O_2 group compared with the control group (Figure 4a). A similar result was observed in mRNA expression (Figure 4b). Immunocytochemistry also showed that Nrf2 was decreased in the β-cells of diabetic rats compared with the control group (Figure 4c).

To confirm whether Nrf2 is the upstream regulatory protein of PHB2, β-cells were pretreated with Nrf2 inhibitor and agonist to observe the expression of PHB2. After pretreatment with the Nrf2 inhibitor ML385 (4 μM), PHB2 was significantly decreased in the H_2O_2+ ML385 cells compared with H_2O_2 incubated alone at the protein and mRNA levels, followed by a decline in Nrf2 (Figure 4d,e). There was more ROS generation in the H_2O_2+ ML385 group compared with the H_2O_2 group (Figure 4f,g). Cell survival was decreased in the H_2O_2+ ML385 group compared with the H_2O_2 group (Figure 4h). Meanwhile, β-cell apoptosis was significantly increased in the H_2O_2+ ML385 group compared with the H_2O_2 group (Figure 4i). Conversely, after being pretreated with the Nrf2 agonist dimethyl fumarate (DMF, 10 μM), PHB2 was significantly increased in the H_2O_2+ DMF group compared with the H_2O_2 group at the protein and mRNA levels, followed by an increase in Nrf2 (Figure 4j,k). There was less ROS generation in the H_2O_2+ DMF group compared with the H_2O_2 group (Figure 4l). Cell survival was increased in the H_2O_2+ DMF group compared with the H_2O_2 group (Figure 4m). Meanwhile, β-cell apoptosis was significantly decreased in the H_2O_2+ DMF group compared with the H_2O_2 group (Figure 4n). Nrf2 may be the upstream regulatory factor of PHB2 and oxidative stress reduced mitophagy through the Nrf2/PHB2 pathway.

N-acetylcysteine alleviated oxidative stress leading to an increase in mitophagy in β-cells

N-acetylcysteine (NAC) is a commonly used antioxidant. To further examine whether oxidative stress restrains mitophagy in β-cells through the Nrf2/PHB2 pathway, RINm5F cells were pretreated with NAC. A significant decline in cell activity was observed after 4 mM NAC treatment (Figure 5a). We detected that ROS generation was significantly reduced in the H_2O_2+ NAC group compared with the H_2O_2 group (Figure 5b). In determining whether alleviating oxidative stress could increase the expression of PHB2, Western blotting and qPCR showed that the levels of PHB2 and Nrf2 were both significantly increased in the H_2O_2+ NAC group compared with the H_2O_2 group (Figure 5c,d). Mitophagy analysis showed that mitophagy in the H_2O_2+ NAC group was obviously greater than that in the H_2O_2 group (Figure 5e,f). Meanwhile, β-cell apoptosis was significantly decreased in the H_2O_2+ NAC group (Figure 5g). Considering the regulatory relationship between Nrf2 and PHB2 found above, these data indicated that NAC treatment may attenuate oxidative stress leading to increased PHB2-mediated mitophagy *via* the Nrf2/PHB2 pathway.

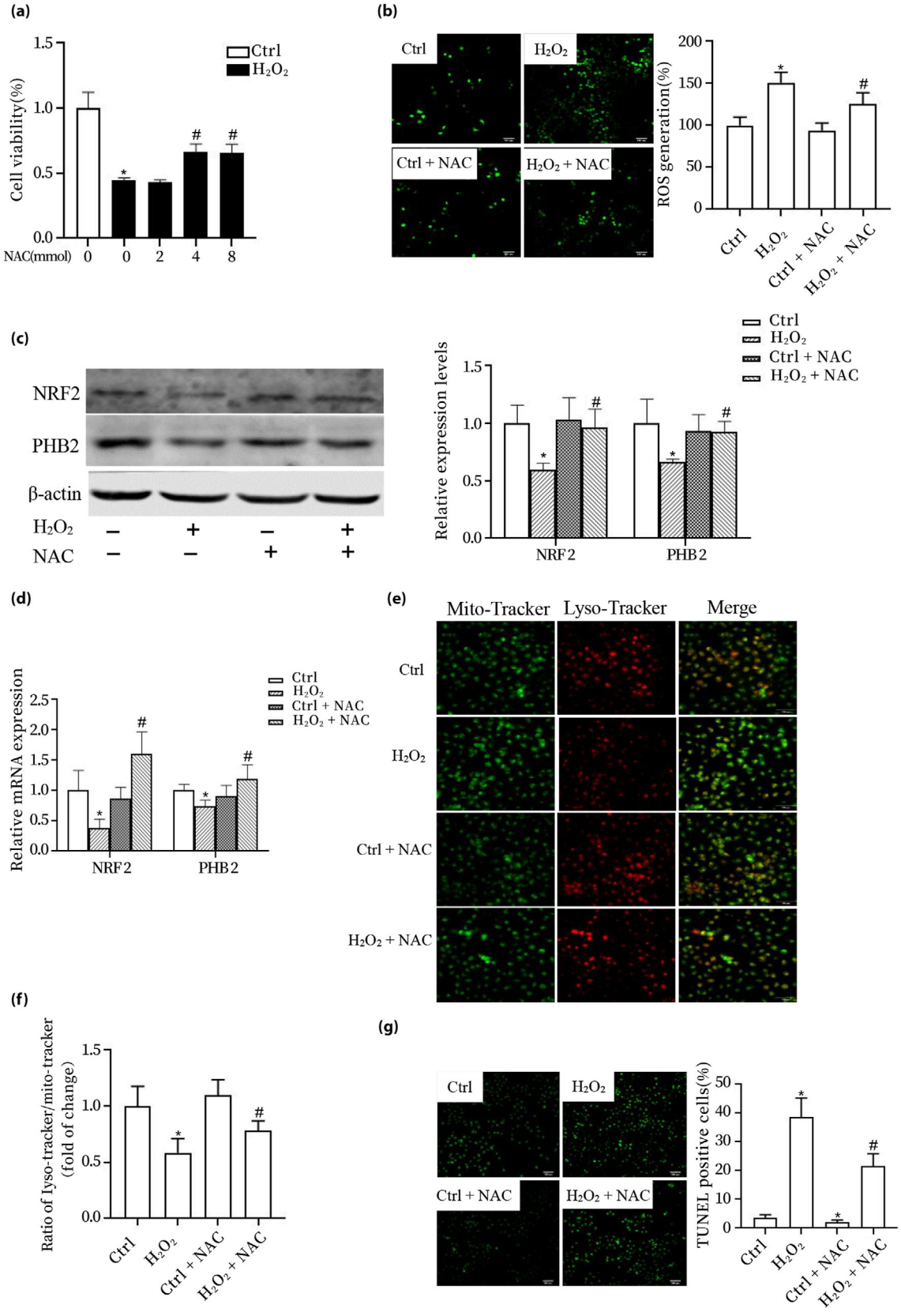


Figure 5 | N-acetylcysteine alleviated oxidative stress leading to an increase in mitophagy in β-cells. (a) Effect of NAC on the relative cell viability in RINm5F cells. The relative viability was increased significantly in 4 mM NAC incubated with 100 μM H_2O_2 for 4 h. (b) ROS level of RINm5F cells pretreated with NAC (scale bar 100 μm). (c) Western blot analysis of PHB2 and NRF2 in RINm5F cells pretreated with NAC. (d) qRT-PCR determination of the mRNA expression of NRF2 and PHB2 in RINm5F cells treated with NAC. (e) The colocalization of mitochondria and lysosomes were observed in RINm5F cells pretreated with or without NAC before incubation with H_2O_2. Images were captured with a confocal laser scanning microscope (scale bar 100 μm). (f) Analysis of the levels of colocalization of mitochondria and lysosomes. (g) Effect of NAC on H_2O_2 induced apoptosis in RINm5F cells by TUNEL staining (scale bar 100 μm). *Compared with the control group, $P < 0.05$. #Compared with the H_2O_2 group, $P < 0.05$.

DISCUSSION

Oxidative stress is part of the pathogenesis of type 2 diabetes mellitus due to the excessive accumulation of ROS that causes cellular dysfunction and induces various diseases. In rodent models of diabetes, ROS have been demonstrated to participate in β-cell dysfunction and apoptosis[24–27]. ROS-induced mitochondrial injury may be a vital activator of mitophagy. ROS generation induced by high glucose inhibits mitophagy of retinal pigment epithelium cells[28]. However, the effects and mechanism of ROS on β-cell mitophagy under oxidative stress conditions are still unclear.

β-cells are especially sensitive to oxidative stress due to a lack of antioxidant enzymes[29]. Excessive ROS have complex connections to obesity and related pathophysiological disorders, particularly type 2 diabetes mellitus[30]. Nutritional stress promotes oxidative stress as evidenced by increased lipid peroxidation products, protein carbonylation, and decreased antioxidant status[8]. Previous studies have mostly focused on the effect of oxidative stress induced by high glucose on β-cells. However, oxidative stress can be generated by numerous sources, such as superfluous nutrition, smoking and sleep-deprivation. Therefore, we investigated whether oxidative stress could damage β-cell function by inhibiting mitophagy before the occurrence of hyperglycemia. Therefore, we used H_2O_2 rather than high glucose to stimulate the oxidative stress model.

In our study, PHB2-mediated mitophagy was decreased in the oxidative stress model of RINm5F cells. Similar results were obtained in a type 2 diabetes mellitus rat model. Subsequently, insulin secretion was reduced and β-cell apoptosis was increased following mitophagy reduction in H_2O_2-cultured cells. Oxidative stress induced inhibition of mitophagy in β-cells through downregulation of PHB2, contributing to the pathogenesis of type 2 diabetes mellitus.

PHB is a dimer structure containing PHB1 and PHB2[31]. PHB2 is an inner mitochondrial membrane protein that is required for Parkin induced mitophagy[12]. A previous study found that the expression of PHB increased after alcohol-induced oxidative stress in RINm5F cells[13]. Nevertheless, this study is about the PHB complex, but not single PHB2. In our study, PHB2 was decreased in the H_2O_2-induced oxidative stress cell model.

Another study showed that PHB2 gene knockout in mice induced mitochondrial injury, which caused β-cell dysfunction. However, the relationship between PHB2 deficiency and mitophagy disorder in β-cells has not been elucidated. Our study revealed that PHB2 expression and mitophagy decreased accordingly in RINm5F cells under oxidative stress. Increased β-cell apoptosis and insulin secretion disorder were observed. Overexpression of PHB2 could protect β-cells by increasing mitophagy from oxidative stress. *In vivo*, we also observed that PHB2 expression decreased and mitochondria swelled in pancreatic β-cells of type 2 diabetes mellitus rat model. These results suggested that PHB2 plays a vital role in mitophagy in β-cells. Then, we further explored the mechanism of PHB2 regulation.

Nrf2 is an antioxidative transcription factor which could be activated by mtROS and influence mitochondrial function[32,33]. Yagishita *et al.*[34] found that Nrf2 inhibited the accumulation of ROS in β-cells. This suggests that Nrf2 protects β-cells through antioxidant effects. Zhang *et al.*[18] reported that Nrf2 is the regulator of PHB2 in a subarachnoid hemorrhage rat model. Another study showed PHB prevented inflammation-related oxidative stress by continuous activation of Nrf2 in intestinal epithelial cells[35]. Both studies found a relationship between PHB and Nrf2, but the upstream and downstream relationship is not clear.

We hypothesized that Nrf2 could augment mitophagy by regulating PHB2 to protect β-cells. *In vitro*, we observed that both Nrf2 and PHB2 were decreased in the H_2O_2 group cells. *In vivo*, Nrf2 and PHB2 were decreased in the β-cells of type 2 diabetes mellitus rats. To test the upstream and downstream relationship, we used the Nrf2 agonist DMF and the inhibitor ML-385. The expression of PHB2 in β-cells under oxidative stress increased after DMF treatment. Concurrently, apoptosis of β-cells decreased in the DMF group. In contrast, the expression of PHB2 decreased and apoptosis increased in the ML385 group. These results suggest that Nrf2 may be one of the upstream regulators of PHB2 in β-cells.

Our study proposed that oxidative stress suppressed PHB2-mediated mitophagy in β-cells through the Nrf2/PHB2 pathway. To further demonstrate the effect of oxidative stress on mitophagy, we used the antioxidant NAC to pretreat β-cells before H_2O_2 incubation. NAC has antioxidant effects by scavenging oxygen free radicals[36].

A previous study found that NAC protected cells by preserving mitochondrial function. Some studies have shown that NAC can ameliorate mitochondrial function through mitophagy or remove ROS[37,38]. In our study, we observed that ROS

production was reduced and PHB2-mediated mitophagy was increased in the NAC group. We observed a reduction in apoptosis and an increase of Nrf2 in the NAC group. All of the above results confirmed that oxidative stress suppressed PHB2-mediated mitophagy in β-cells. NAC may increase mitophagy through the Nrf2/PHB2 pathway.

In this study, we investigated the role of PHB2-mediated mitophagy in β-cells under oxidative stress. However, the *in vivo* experiments are limited to animal studies. PHB2-mediated mitophagy will be detected in the peripheral blood and pancreatic islets of diabetic patients in the future. The mitochondrial protective effects of NAC in β-cells are limited to *in vitro* experiments. An NAC intervention study could be conducted in animals to observe the therapeutic effects.

In conclusion, this study found that oxidative stress reduced PHB2-mitophagy *via* the Nrf2/PHB2 pathway in β-cells, which may be one of the pathogenesis of type 2 diabetes mellitus. The antioxidant NAC may protect the mitochondrial function of β-cells by upregulating the Nrf2/PHB2 pathway. In the future, agents targeting PHB2 to protect mitochondria of β-cells could be further explored to prevent and treat type 2 diabetes.

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DISCLOSURE

The authors declare no conflict of interest.

Approval of the research protocol: This study passed the review of the Clinical Medical Ethics Committee of The First Hospital of Hebei Medical University.

Informed consent: N/A.

Registry and the registration no. of the study/trial: Approval date of Registry of study is March 6, 2020 and the Registration No. is 20200324.

Animal studies: Animal experiments were approved by the Animal Ethical Experimental Committee of The First Hospital of Hebei Medical University.

DATA AVAILABILITY STATEMENT

The data are available from the corresponding author on reasonable request.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1 | (A) Effect of H_2O_2 on the relative cell proliferation in RINm5F cells. The proliferation was reduced significantly after treatment with 100 μM H_2O_2 for 4 h. (B) RINm5F cells were incubated in 100 μM H_2O_2 for different times. The proliferation was reduced significantly at 4 h. (C) Western blot analysis of PHB2 in RINm5F cells pretreated with the NRF2 siRNA.