Prohibitin inhibits high glucose-induced apoptosis via maintaining mitochondrial function in human retinal capillary endothelial cells

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Abstract. Mitochondrial dysfunction and excessive apoptosis of vascular endothelial cells play a critical role in the development of diabetic retinopathy (DR). Prohibitin (PHB), a significant regulator, maintains mitochondrial function and protects vascular endothelial cells against apoptosis. However, the mechanism underlying the protective effect of PHB on DR remains unclear. Since mitochondria are key regulators of vascular homeostasis, the present study aimed to investigate the molecular mechanism of PHB on maintaining mitochondrial function in human retinal capillary endothelial cells (HRCECs). To evaluate the role of PHB in cell apoptosis, HRCECs, transfected with or without PHB overexpression plasmid or small interfering RNA clones targeting PHB, were cultured in the presence of 5.5 mmol/l normal glucose (NG) or 30 mmol/l high glucose (HG). Subsequently, the apoptosis rate of HRCECs was determined using flow cytometry. The results showed that PHB was upregulated in HRCECs, while PHB knockdown promoted the generation of reactive oxygen species from mitochondria via inhibition of the activation of complex I. Additionally, the apoptosis rate of HRCECs in the HG group was notably enhanced compared with that in the NG group. Interestingly, PHB overexpression attenuated the increase in HG-mediated HRCEC apoptosis. Furthermore, treatment with HG upregulated expression of cleaved caspase-3 and cleaved poly(ADP-ribose) polymerase *in vitro*. The present study indicated that PHB could be a key modulator of mitochondrial homeostasis and could protect HRCECs against HG-induced apoptosis. Overall, the aforementioned findings provided experimental evidence supporting the potential protective effects of PHB on DR.

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Introduction

Diabetic retinopathy (DR) is considered as an important risk factor for ablepsia in developed countries. Mitochondrial dysfunction and hyperglycemia-mediated excessive apoptosis of microvascular cells are the most critical causes of diabetic microangiopathy. In addition, mitochondria play a key role in the vascular system via regulation of cellular energy production, and in the microvascular system via regulation of ATP formation and superoxide production (1). In particular, it has been reported that reactive oxygen species (ROS) are involved in cellular dysfunction such as apoptosis (2). It has been suggested that the hyperglycemia-induced production of mitochondrial ROS can be a key factor in initiating pathogenic signaling in DR (3). The aforementioned studies indicated that mitochondrial function and ROS could play a critical role in DR. Therefore, maintaining mitochondrial function and attenuating ROS production in retinal endothelial cells could be considered a significant approach for treating DR.

Prohibitin (PHB) is a negative regulator of mitochondrial ROS production (4). In addition, PHB, a conserved mitochondrial chaperone, plays a significant role in maintaining normal mitochondrial development and the subunits of mitochondrial proteins (5). A previous study showed that PHB could not only be translocated into the mitochondria, but also to other cellular areas, including the plasma and nucleus, and could therefore be involved in other cellular processes, including apoptosis and growth (6). Furthermore, previous studies demonstrated that PHB exerted a key role in cellular oxidative homeostasis and could protect intestinal epithelial cells and cardiomyocytes against oxidative stress, thus acting as potent antioxidant (7,8). However, the exact mechanism underlying the regulatory effect of PHB on retinal vascular cells in DR remains elusive.

Therefore, the present study aimed to investigate the expression levels of PHB in mitochondria of human retinal capillary endothelial cells (HRCECs) to further evaluate its effect on regulating mitochondrial function, ROS generation and high glucose (HG)-induced apoptosis, thus supporting its critical role in the development of DR.

Materials and methods

Reagents. CM-H_2-DCFDA, JC-1, rotenone, antimycin-A and both reduced and non-reduced Mito Tracker were obtained

from Invitrogen (Thermo Fisher Scientific, Inc.). The antibodies against PHB1 and cyclooxygenase (COX) IV were purchased from Abcam, while the Annexin V-FITC apoptosis kit was obtained from Becton, Dickinson and Company. The antibodies against β-actin, cleaved poly(ADP-ribose) polymerase (PARP) and cleaved caspase-3 were obtained from Cell Signaling Technology, Inc. and DAPI from MilliporeSigma.

HRCEC culture and treatment. HRCECs, obtained from the BeNa Culture Collection (Shanghai, China), were cultured in Endothelial Cell Medium (ECM; ScienCell Research Laboratories) supplemented with 5% FBS (Gibco; Thermo Fisher Scientific, Inc.) in 5% CO_2. The cells were used for subsequent experiments after 4-5 passages. When cells reached 50-60% confluence, they were treated with 5.5 mmol/l [normal glucose (NG)] or 30 mmol/l glucose (HG) for 4-6 days. Subsequently, the cells were divided into the following four groups: The NG group; the HG group; the HG + negative control (NC) group; and the HG + PHB-transfected group.

PHB overexpression. HRCECs were first cultured for 24 h and were then transfected with the pCMV6-XL5 plasmid (Clontech) encompassing the PHB sequence. When cells reached 50-60% confluency, they were transfected with the PHB overexpression plasmid (0.2 μg) using a X-tremeGENE HP DNA transfection reagent (Roche Applied Science) at 37˚C for 24 h according to the manufacturer's instructions. Following transfection for 24 h the medium was replaced with normal medium supplemented or not with HG (30 mmol/l). The transfection efficiency was ~70-80%.

PHB silencing. HRCECs were cultured in ECM supplemented with 5% FBS at 37˚C and 5% CO_2 for 24 h. When cells reached 50-60% confluency, they were transfected with small interfering RNA (siRNA, 50 nM) clones against PHB (Qiagen) using HiPerFect transfection reagent (Qiagen) for 10 h at 37˚C according to the manufacturer's instructions. Cells were collected at 48 h after transfection for various assays. The sequence of PHB-siRNA: 5'-AATGTGGATGCTGGGCAC AGA-3'; the sequence of negative control: 5'-AAGAGTGTC GGATGCAGGATC-3' (Qiagen).

Preparation of subcellular fractions of HRCECs. The subcellular fractions of HRCECs were separated as previously described (9). Briefly, HRCECs were rinsed in PBS and were then resuspended into Buffer A (20 mmol/l HEPES pH 7.5, 10 mmol/l KCl, 1.5 mmol/l MgCl2, 1 mmol/l EGTA, 1 mmol/l EDTA, 1 mmol/l DTT, 0.1 mmol/l PMSF, 250 mmol/l sucrose) supplemented with protease inhibitors. The cells were homogenized by 10 strokes in a Dounce homogenizer. Subsequently, cells were centrifuged at 1,000 x g for 4 min at 4˚C to isolate cell nuclei and DNA fragments. The supernatant was then collected and centrifuged at 8,000 x g for 20 min at 4˚C to isolate heavy cellular fragments rich in mitochondria. The resultant supernatant was centrifuged at 10,000 x g for 15 min at 4˚C to obtain the cytosolic fractions.

Western blot analysis. Western blot analysis was performed as previously described (10). Briefly, equal amounts of protein (20 μg) were separated by SDS-polyacrylamide gel electrophoresis and then transferred onto polyvinylidene difluoride membranes (Millipore, Sigma). The membranes were blocked with 5% bovine serum albumin (Sigma-Aldrich; Merck KGaA) in Tris-buffered saline Tween-20 (0.1%) for 2 h at room temperature and then incubated with primary antibodies at 4˚C overnight. The following primary antibodies were used: Anti-PHB (dilution, 1:500; product code ab75766; Abcam), anti-COX IV (dilution, 1:700; product code ab202554; Abcam), anti-cleaved-caspase 3 (dilution, 1:400; product no. 9661; Cell Signaling Technology, Inc.), anti-cleaved PARP (dilution, 1:500; product no. 5625; Cell Signaling Technology, Inc.) and anti-β-actin (dilution, 1:1,000; cat. no. sc-8432; Santa Cruz Biotechnology, Inc.). Immunoreactive proteins were detected with horseradish peroxidase-conjugated secondary antibodies (dilution, 1:5,000; cat. no. sc-2357; Santa Cruz Biotechnology, Inc.). Quantification was performed according to the NIH Image version-1.61 software (National Institutes of Health).

Immunofluorescence staining and live imaging. HRCECs were cultured on coverslips and were then fixed with 4% paraformaldehyde for 10 min at room temperature and 0.2% Triton X-100. Subsequently, cells were blocked with PBS for 1 h at room temperature supplemented with 0.2% Tween-20, 10% BSA and 10% serum (Gibco; Thermo Fisher Scientific, Inc.). Following blocking, the cells were first incubated with the indicated primary antibody (PHB; dilution, 1:100; product code ab75766; Abcam) and then with Alexa Fluor 488-conjugated secondary antibody (dilution, 1:1,000; product code ab150077; Abcam). The cell nuclei were stained for 5 min at room temperature with 2 μg/ml DAPI. For Mito Tracker staining, HRCECs were stained for 20 min at room temperature with 0.02 μM Mito Tracker (Invitrogen; Thermo Fisher Scientific, Inc.). For JC-1 staining, HRCECs were rinsed with PBS and incubated with JC-1 (Invitrogen) staining solution at 37˚C for 20 min. Pictures were obtained with an FV-1000 confocal laser scanning microscope (Olympus). For live cell imaging, images were captured under a phase contrast microscope (Carl Zeiss GmbH).

Analysis of mitochondrial complex I/III activity. Following HRCEC trypsinization, cells (1x10^6/ml) were resuspended and oxygen consumption was measured using an oxygen probe in the presence or absence of 10 μM rotenone (an inhibitor of complex I) 10 μM antimycin-A (an inhibitor of complex III) and 0.05% azide, an inhibitor of mitochondrial respiration. Finally, the activity of complex I/III was compared between PHB-knocked down and control cells.

FACS analysis of reactive oxygen species (ROS). Firstly, HRCECs (1x10^6/ml) cultured in DMEM supplemented with 0.2% Fetal Calf Serum (GIBCO) were incubated with 5 μM CM-H_2-DCDA and 5 μg/ml propidium iodide (PI) at room temperature for 20 min. HRCECs treated with 100 μM H_2O_2 for 20 min served as a positive control group. H_2O_2 levels were detected using CM-H_2-DCDA. Subsequently, PHB-depleted cells were treated with 10 μg/ml PEG-catalase for 2 h at room temperature. Living cells were analyzed using PI staining

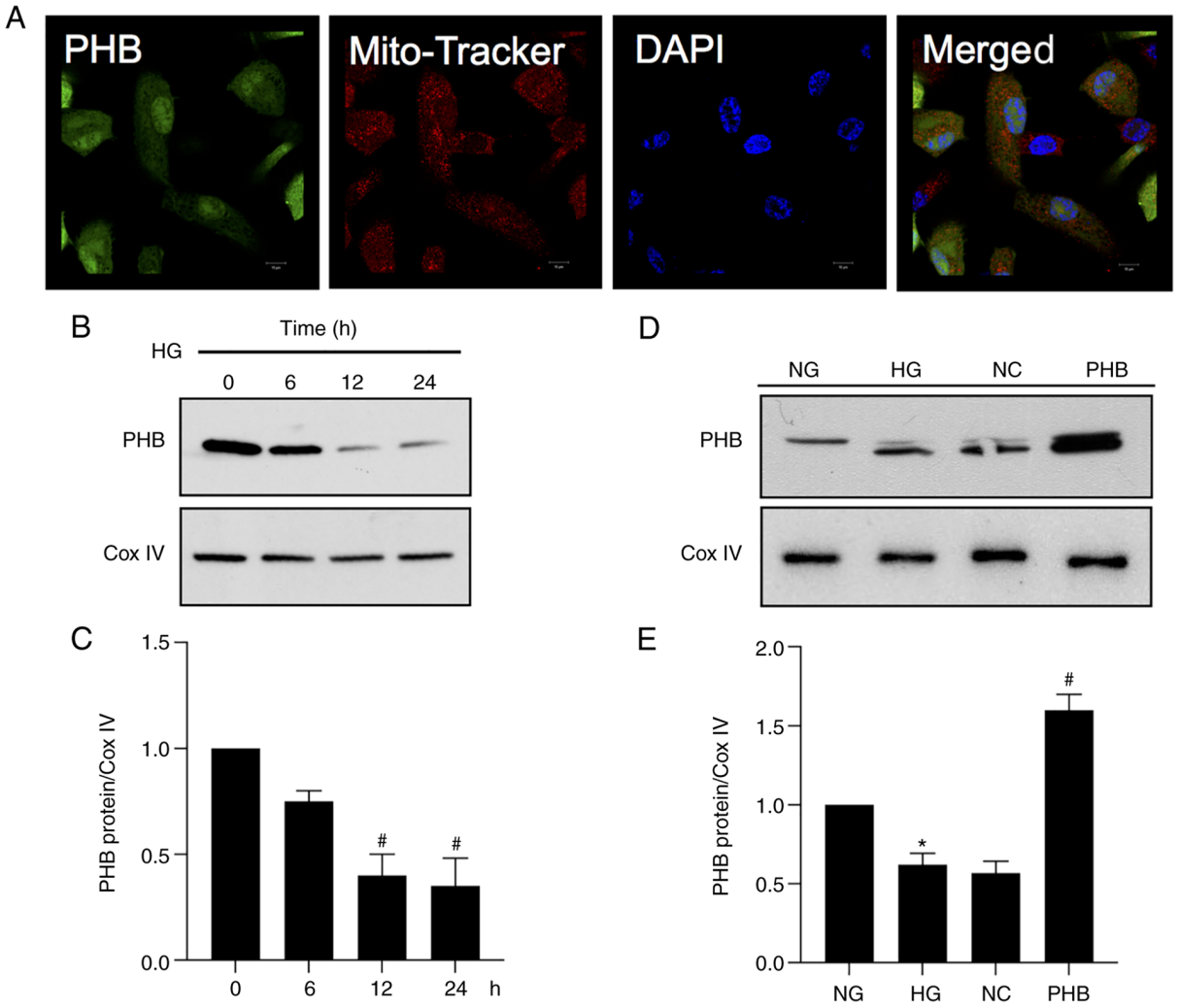

Figure 1. PHB localization in HRCECs. (A) PHB (green) and Mito Tracker (red) were detected in HRCECs using immunofluorescence staining. Co-localization is indicated by orange (scale bar, 10 μm). (B and C) Cell culture under high glucose conditions reduced PHB content in mitochondria. HRCECs were treated with 30 mmol/l glucose at different time-points. $^{\#}$P<0.01 vs. 0 h. (D and E) The protein expression levels of PHB were detected by western blot analysis. The experiments were repeated at least three times. *P<0.05 vs. NG; $^{\#}$P<0.01 vs. NC. PHB, prohibitin; HRCECs, human retinal capillary endothelial cells; HG, high glucose; NG, normal glucose; NC, negative control; COX, cyclooxygenase.

for 10 min at room temperature and the fluorescence intensity in the FL-2 and FL-1 channels was measured using a Becton-Dickinson FACSCalibur system (BD Biosciences) and FlowJo 7.6 software (Tree Star, Inc.).

Flow cytometric analysis. Flow cytometric analysis was performed as previously described (10). In brief, cells ($1x10^6$/ml) were used to assess cell apoptosis with an Annexin V/propidium iodide (PI) kit (Invitrogen, Thermo Fisher Scientific, Inc.) according the manufacturer's protocol. Flow cytometry was used determine the proportion of early apoptotic cells. Flow cytometry was performed using a FACSCalibur system (BD Biosciences).

Statistical analysis. Experiments were performed at least three times. All data are presented as the mean ± SD. Differences between multiple groups were assessed using a post hoc (Tukey's HSD) test used for one-way ANOVA in GraphPad Prism 6.0 (GraphPad Software, Inc.) and SPSS 17.0 (SPSS, Inc.). Comparison between non-parametric variables was performed using a χ^2 test. P<0.05 was considered to indicate a statistically significant difference.

Results

PHB localizes to the mitochondria and is downregulated under HG conditions. To detect the localization of PHB, immunofluorescence analysis was carried out in HRCECs. The analysis revealed that a small proportion of PHB was distributed in the nuclear compartment, while the majority of PHB was detected in the mitochondria, as shown using Mito Tracker (Fig. 1A). Furthermore, to determine the expression levels of PHB in the mitochondria under HG conditions, HRCECs were exposed to 30 mmol/l glucose (HG). The results revealed that PHB was downregulated in the mitochondria of HRCECs in the HG group (Fig. 1B and C). In addition, the expression levels of PHB were measured in all the different groups and the results demonstrated that PHB was successfully overexpressed in cells transfected with PHB overexpression plasmid (Fig. 1D and E). Collectively, these findings indicated

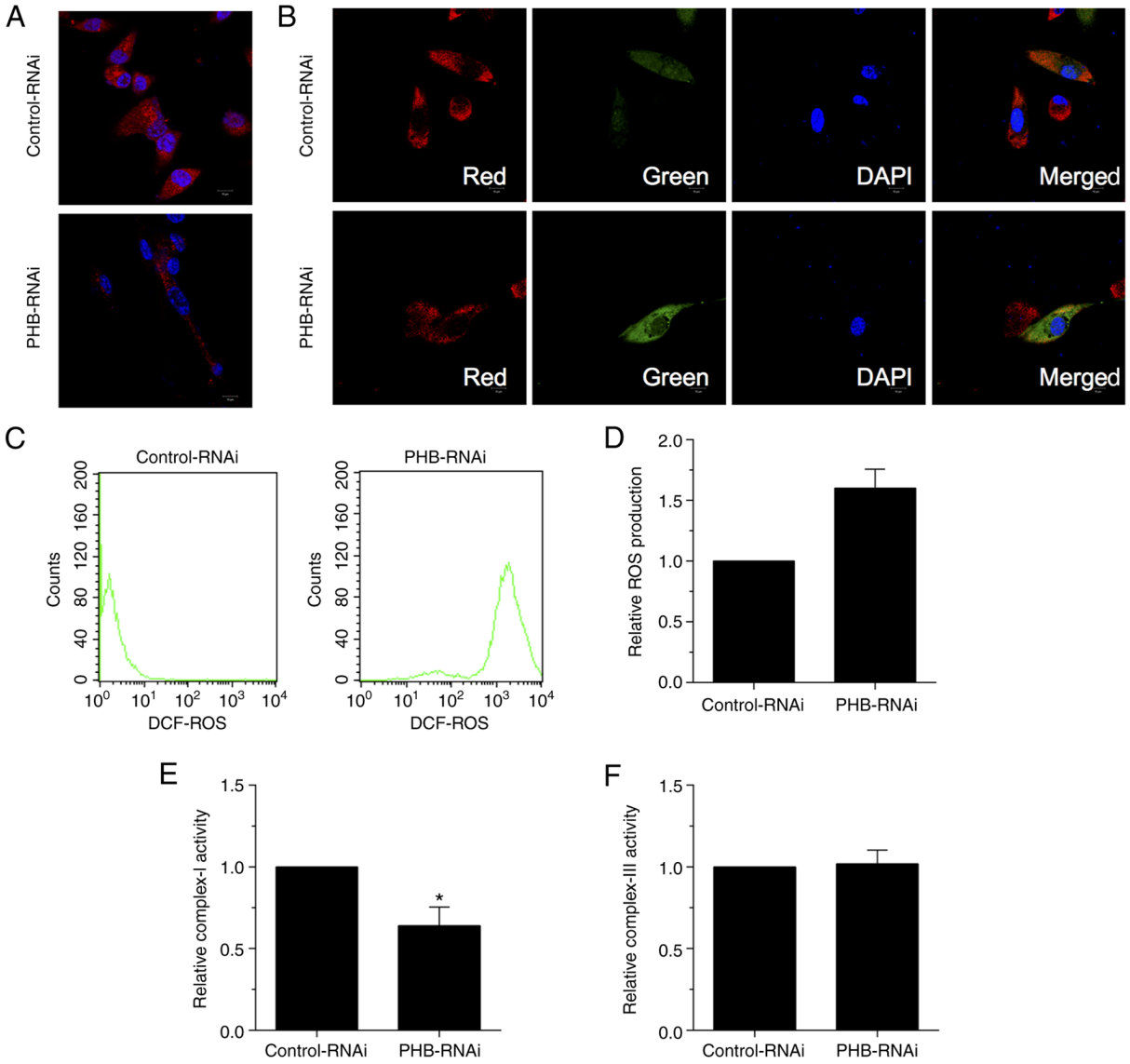

Figure 2. PHB regulates mitochondrial function in HRCECs. (A) Mitochondrial respiration in the si-NC (top) and si-PHB (bottom) groups was detected by immunofluorescence staining using Mito Tracker (red; scale bar, 10 μm). (B) PHB knockdown in HRCECs was determined by JC-1 and immunofluorescence staining (scale bar, 10 μm). (C and D) Reactive oxygen species generation was measured by fluorescence-activated cell sorting analysis. (E and F) The activity of complex I/III in PHB-depleted HRCECs was measured and normalized to the control group. The experiments were repeated at least three times. *P<0.05. PHB, prohibitin; HRCECs, human retinal capillary endothelial cells; si-NC, negative control small interfering RNA.

that PHB could be involved in the regulation of mitochondrial function in HRCECs.

PHB knockdown alters mitochondrial function in HRCECs. To investigate the role of PHB in mitochondria, PHB-depleted mitochondria were established in HRCECs and the levels of ROS were then measured. Immunofluorescence staining in HRCECs revealed reduced Mito Tracker positive staining, indicating that mitochondria were inactive with regard to respiration (Fig. 2A). This finding clearly suggested that the increased ROS production in PHB-depleted HRCECs was due to mitochondrial respiration. Furthermore, both immunofluorescence (Fig. 2B) and ROS production (Fig. 2C and D) analyses revealed reduced red and enhanced green fluorescence signals in PHB-depleted HRCECs, thus suggesting that mitochondrial membrane depolarization was promoted and ROS production was increased, respectively. In addition, the activity of complex I/III was assessed via measuring oxygen consumption. The results demonstrated that the activity of complex I was reduced in PHB-depleted HRCECs compared with the control group (Fig. 2E). However, the activity of complex III remained unchanged between both groups (Fig. 2F). Overall, the aforementioned results verified that mitochondria mediated ROS generation in PHB-depleted HRCECs.

PHB attenuates HG-induced apoptosis in HRCECs. To explore whether PHB could protect HRCECs, the effect of PHB on HG-induced cell apoptosis was assessed by evaluating the apoptosis-specific morphological changes in HRCECs. Therefore, HG markedly enhanced HRCEC apoptosis, which was restored by PHB (Fig. 3A). Additionally, HRCEC apoptosis was determined by Annexin V/PI staining. The apoptosis rate in the NG group was 9.96%, which was significantly different compared with that in the

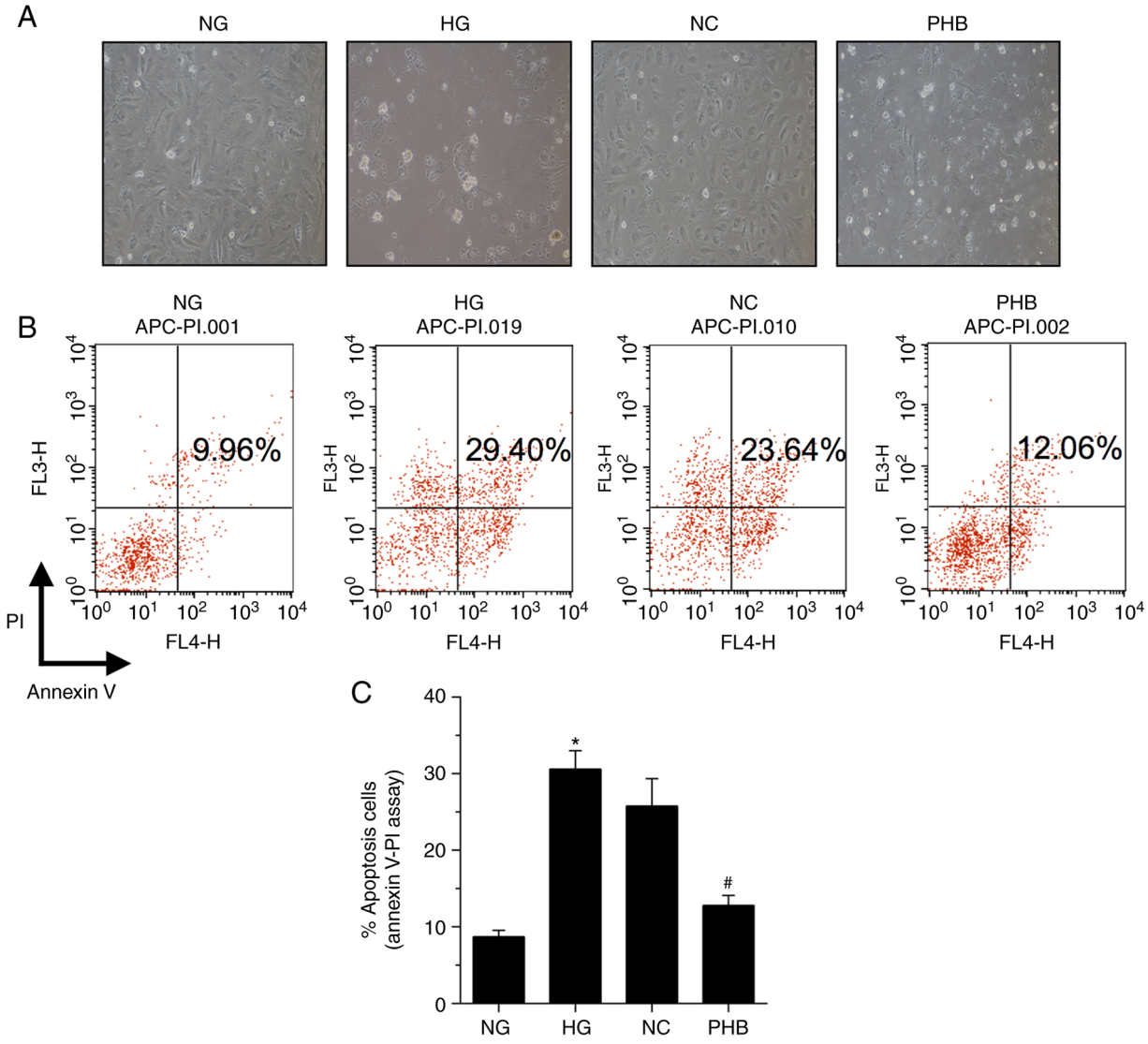

Figure 3. Effect of prohibitin on HG-induced HRCEC apoptosis. (A) Images of morphological changes in HRCECs from each group were captured under a phase contrast microscope (magnification, x400). (B and C) Apoptosis rate of HRCECs was measured using Annexin V/propidium iodide staining at 48 h. The experiments were repeated at least three times. *P<0.05 vs. NG; #P<0.01 vs. NC. HG, high glucose; HRCEC, human retinal capillary endothelial cell; NG, normal glucose; NC, negative control; PHB, prohibitin; PI, propidium iodide.

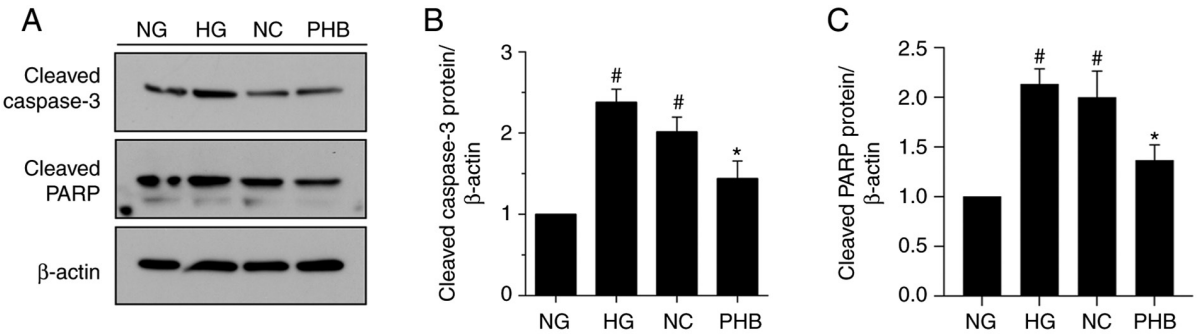

Figure 4. Effect of prohibitin on the expression levels of apoptosis-related proteins. (A-C) The protein expression levels of cleaved caspase-3 and poly(ADP-ribose) polymerase in the high glucose and negative vector control groups were notably enhanced compared with the normal glucose group. The experiments were repeated at least three times. *P<0.05 vs. NC. #P<0.01 vs. NG. NG, normal glucose; HG, high glucose; NC, negative control; PHB, prohibitin; PARP, poly(ADP-ribose) polymerase.

HG, NC or PHB groups, with apoptosis rates of 29.40, 23.64 and 12.06%, respectively (Fig. 3B and C). Collectively, the aforementioned findings suggested that PHB could attenuate HG-induced HRCEC apoptosis.

Effect of PHB on the expression of apoptosis-related proteins. Subsequently, the effect of PHB on the expression levels of the apoptosis-related proteins, PARP and caspase-3, in NG-, HG-, NC- and PHB-treated cells was evaluated by western blot analysis. Consistent with the Annexin V/PI staining results, western blot analysis revealed that the expression levels of both caspase-3 and PARP were significantly increased in the HG and NC groups compared with NG group. However, the expression levels of both proteins were not notably different between the HG and NC groups. Notably, caspase-3 and PARP were markedly downregulated in the PHB group (Fig. 4A-C). Collectively, the aforementioned findings suggested that PHB could protect HRCECs against HG-induced apoptosis via downregulating caspase-3 and PARP.

Discussion

The present study aimed to evaluate the role of PHB in HRCECs and demonstrated that HRCEC apoptosis was associated with mitochondrial function. The results showed that PHB was significantly upregulated in mitochondria, whereas PHB knockdown inhibited the activity of complex I and enhanced the production of mitochondrial ROS in HRCECs. In particular, PHB overexpression could protect HRCECs against HG-induced apoptosis. These results indicated that PHB could maintain mitochondrial function, inhibit HRCEC apoptosis and reduce mitochondrial ROS generation in DR.

PHB is a conserved and widely expressed protein that, is distributed in several cellular compartments, such as in the mitochondria, and lipid rafts in the plasma membrane and nucleus (11). The present study revealed that PHB was expressed in HRCECs. Furthermore, co-localization assays in HRCECs showed that PHB was mainly expressed in mitochondria. However, the mechanism underlying the effects of PHB under HG conditions remains elusive. To evaluate the function of PHB in HRCECs, cells were transfected with siRNA clones targeting PHB to silence its expression. Therefore, PHB knockdown affected mitochondrial function and promoted ROS production.

It has been reported that the production of ROS from mitochondria can be promoted by the depolarization of the mitochondrial membrane and blockade of electrons at complexes I and III, which are widely accepted as the source of ROS in mitochondria (12). The activity of complex III and the contribution of the mitochondrial connection observed in PHB-depleted cells suggested that a large number of electrons in complex I could be used for the production of ROS when the expression of PHB was knocked down. Furthermore, it was hypothesized that the function of cytochrome oxidase and ATP generation could be maintained through compensatory signaling pathways. These effects could be associated with the increase of electrons in the II/III complex. Additionally, ROS generation could be promoted in PHB-depleted HRCECs due to the lack of mitochondrial respiration in these cells.

Furthermore, PHB attenuated HG-induced apoptosis in HRCECs. A previous study revealed that treatment of endothelial cells with HG for three days enhanced cell apoptosis (13). Another study showed that the apoptosis rate of retinal pericytes, cultured under HG conditions for seven days, was higher compared with those cultured under NG conditions (14,15). Additionally, compared with endothelial cells cultured under NG conditions, cells exposed to 23 and 30 nM glucose for 25 and 45 days, respectively, exhibited enhanced apoptotic rates (16). Interestingly, the apoptosis rate in cells treated with 23 or 30 nM HG was not significantly different. The results of the present study were consistent with the aforementioned studies, as the apoptosis rate in the HG and NC groups was significantly increased compared with the NG group. However, the apoptosis rate was notably reduced in the PHB group. No statistically significant difference was observed in apoptosis between the HG and NC groups. Therefore, the present study indicated that PHB could attenuate HG-induced HRCEC apoptosis.

It has been reported that PHB exerts its anti-apoptotic effects through the caspase signaling pathway (17,18). Herein, to evaluate the anti-apoptotic effect of PHB on HRCECs, the protein expression levels of caspase-3 and PARP were determined by western blot analysis. The aforementioned proteins play a crucial role in regulating mitochondrial cell death (19). The results showed that caspase-3 and PARP were notably upregulated in the HG and NC groups compared with the NG group. However, the levels of both proteins were not statistically different between the HG and NC groups. Interestingly, the protein levels of PARP and caspase-3 were notably decreased in the PHB group compared with the NC group. Overall, PHB attenuated HG-induced apoptosis in HRCECs via downregulating caspase-3 and PARP.

PHB may be a promising approach for DR. However, the more detailed mechanism of PHB in DR need to be done *in vivo*. In summary, the results of the present study suggested that PHB could be necessary for maintaining mitochondrial function via regulating ROS generation and apoptosis in HRCECs. These findings highlighted the widely accepted notion that mitochondria are a significant cellular compartment and that ROS serves a crucial role in DR (20). Both apoptosis and mitochondrial function are associated with several pathological processes during microvascular damage, including DR (21). Overall, the results of the present study could provide an experimental basis for the protective effect of PHB in the management of DR. PHB could become an interesting candidate for some possible clinical implications in DR.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Author's contributions

LZ wrote the original draft, performed the experiments and collected the data. YH contributed to the analysis of data and

the design of this study. All authors read and approved the final manuscript. LZ and YH confirm the authenticity of all the raw data.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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