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Original Article

Role of prohibitin overexpression in proliferation of vascular smooth muscle cells

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Abstract: We aim at studying the effect of transfection of prohibitin (PHB) overexpression on biological characteristics of rat vascular smooth muscle cells. 12, 24 and 48 h after transfection, the expression of PHB protein expression was detected by RT-PCR and western-blot blot. Cell proliferation, apoptosis and mitochondrial membrane potential were detected by MTT and flow cytometry. Western blot was used for identifying AIF, cytc, caspase 8 and 9. The amount of the relative expression of PHB protein of experimental group was significantly higher than that of control group ($P < 0.05$); 12, 24 and 48 h after transfection, PHB cells proliferation in experimental group was significantly lower than that of control group ($P < 0.05$). 48 h after transfection, the apoptosis rate of PHB cells of the experimental group was obviously higher than that of control group ($P < 0.05$). MMP was blocked by PHB overexpression, and mitochondria-dependent apoptosis proteins were regulated by PHB overexpression. After PHB gene overexpression, the ability of smooth muscle cell proliferation was suppressed and the apoptosis rate was increased. Prohibitin can inhibit the proliferation of vascular smooth muscle cells by mitochondria-dependent apoptosis *in vitro*.

Keywords: Vascular smooth muscle cell, Prohibitin, proliferation, apoptosis

Introduction

At present, cardiovascular disease has become one kind of serious diseases that is harmful to human health. Abnormal proliferation of vascular smooth muscle cells generally exists in various cardiovascular diseases, which is the basic pathological change of the coronary atherosclerotic heart disease [1], vein bridge stenosis after coronary artery bypass grafting, coronary restenosis after stent implantation and other diseases. Thus inhibiting the proliferation of VSMC cells is an important target for the prevention and treatment of cardiovascular diseases.

Prohibitin (PHB) is a chaperone protein that is highly conserved evolutionarily and present in different cellular compartments [2]. PHB is a highly conserved protein with diverse functions including regulation of cell cycle progression, apoptosis, and transcription depending on its subcellular localization.

In the present study, we aimed at explore the effect of PHB on cell proliferation, apoptosis and MMP of VSMC and clarify the possible mechanisms involved in.

Materials and methods

Cell culture

Male SD rats (150 g-200 g), using tissue explant method to cultivate aortic smooth muscle cells in primary cultured rat, and the primary culture medium contained 20% FBS, 1 × 10 U/L penicillin and DMEM with 1 g/L streptomycin. After the first passage in about 2 weeks, the culture medium was changed to 10% FBS DMEM. And the cells can be used for experiment after subcultured to 3-7 generation.

Reagents

Protein electrophoresis and transmembrane device were provided by the Bio-Rad Company. PI staining solution was provided by the Jingmei Biotechnology Company; first antibody of Prohibitin was provided by Abcam Company; second antibody of Prohibitin was provided by Santa Cruz Company. Methylthiazolyldiphenyl-

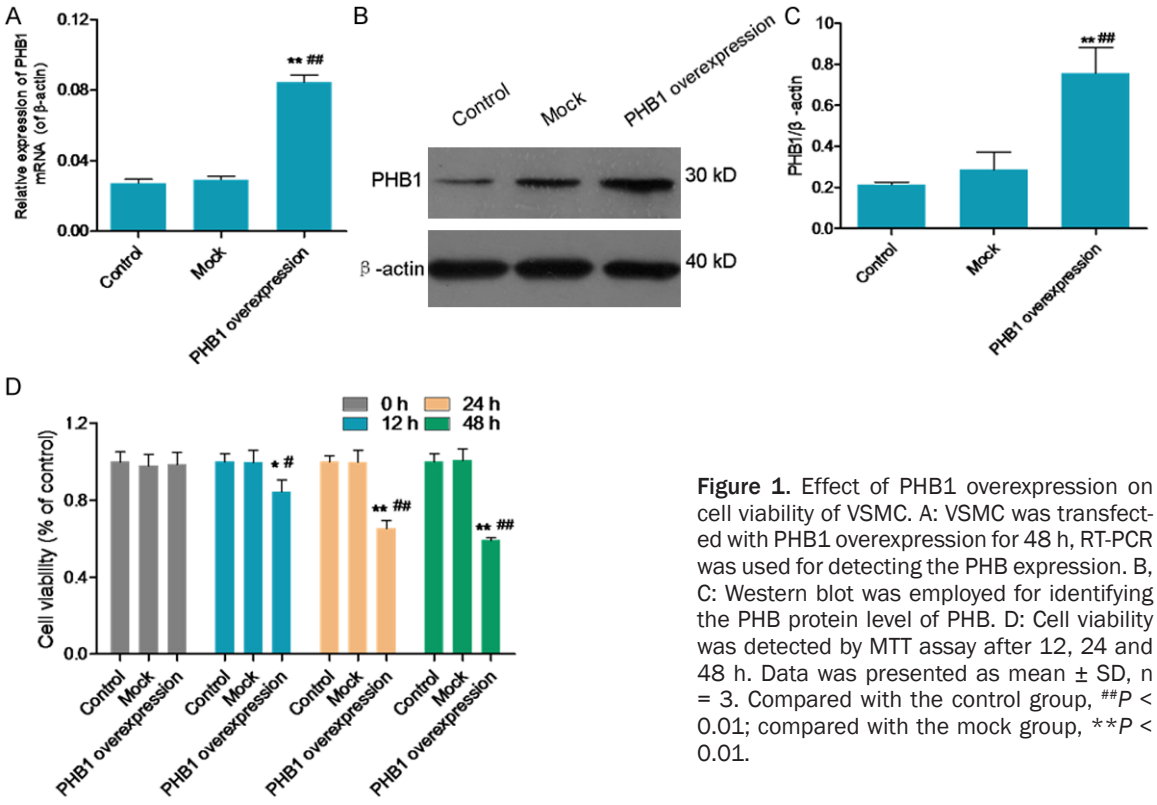

Figure 1. Effect of PHB1 overexpression on cell viability of VSMC. A: VSMC was transfected with PHB1 overexpression for 48 h, RT-PCR was used for detecting the PHB expression. B, C: Western blot was employed for identifying the PHB protein level of PHB. D: Cell viability was detected by MTT assay after 12, 24 and 48 h. Data was presented as mean ± SD, n = 3. Compared with the control group, $^{\#\#}P < 0.01$; compared with the mock group, $^{**}P < 0.01$.

tetrazolium bromide (MTT) was purchased from Sigma Company in USA.

Transfection

The plasmid pCMV-PHB; provided by Shenggong Biotechnology (Shanghai) Co. Ltd. Plasmid PHB (3 mg) or mock-vehicle plasmid were transfected into VSMC lines in 6-well plates (1 mg/ml) using the lentiviral vector, according to the manufacturer's instructions.

Western blot

Cells in each group were collected respectively 72 h after transfection, and the total cell proteins were extracted, and electrotransferred to a nitrocellulose membrane in 12% polyacrylamide gel electrophoresis, Then the proteins were sealed 1 h in TBST (containing 5% skim milk powder); 1 to 1000 diluent monoclonal Prohibitin antibody and beta-actin antibody were added in to react the whole night under 4°C. After washing with TBST, the reaction system was reacted with 1:1000 dilute fluorescent second antibody marked with HRP at room temperature for 1 h. Finally, the TBST membrane was washed, colored, exposed, developed, fixed, scanned and imaged using the ImageScanner scanner. The result was analyzed using Photoshop CS2 software.

Cell proliferation

The cells were, made into single cell suspension, which was then adjusted to the cell concentration of 1×10^4/ml after counting and cultivated in a 96 orifice plate. These cell suspension in each orifice was cultured in DMEM liquid culture with 10% FBS for 48 h, and added with 5 mg/ml MTT for 20 μL to culture in the incubator for 4 h. The supernatant was discarded and each orifice was added with DMSO for 150 uL and shaked in an oscillator for 10 min. The value of [OD (570 nm)] was measured in the absorption wavelength of 570 nm. A set of samples of 4 orifices were collected every 24 h and the experiment was repeated 3 times.

Cell apoptosis

The apoptosis cells of blank group, empty vector group and experimental group were detected after transfection through Annexin-V FITC/PI flow-type double staining experiments. After transfection of 48 h, the collected cells in 6-ori-

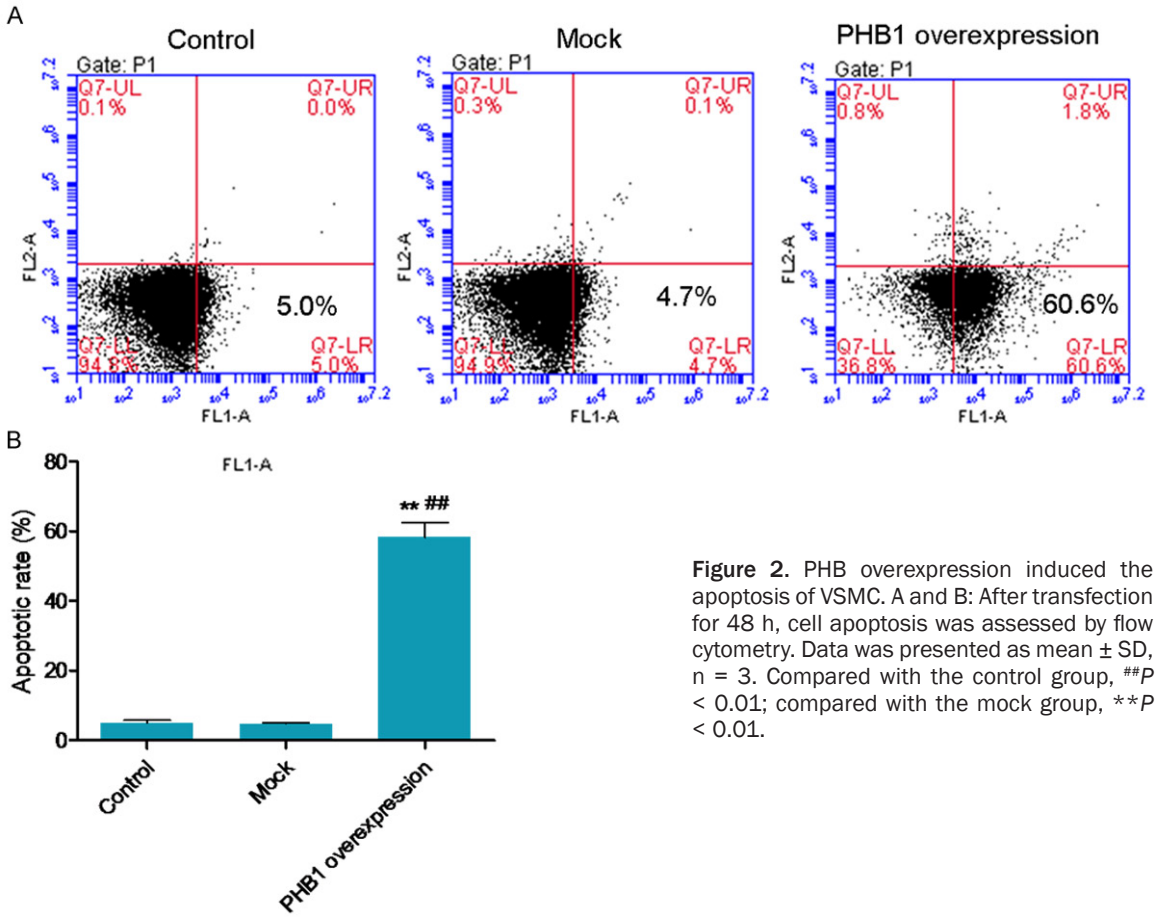

Figure 2. PHB overexpression induced the apoptosis of VSMC. A and B: After transfection for 48 h, cell apoptosis was assessed by flow cytometry. Data was presented as mean ± SD, n = 3. Compared with the control group, $^{\#\#}P < 0.01$; compared with the mock group, $^{**}P < 0.01$.

fice plate were fixed with 70% pre-cold ethanol and preserved at 4°C for at least 18 h, which were then send to Shanghai Genechem Co. Ltd for the flow-type double staining detection and analysis of apoptosis by using FACS Calibur flow cytometer (Beckman) and Summit 5.0, respectively.

Mitochondrial membrane potential

Rhodamine-123 (Rho-123) dye (Sigma) was used to detect the changes in MMP. Cells (5×10^4 cells/well) were cultured in 24-well plate. After a period of transfection (24 h), cells were washed with PBS, incubated with Rho-123 (10 mg/ml) and subsequently subjected to flow cytometry.

Statistical analysis

The experimental results of the statistical treatment were analyzed by SPSS17 statistics software and expressed by mean ± SD. Multiple group comparison was performed by using single-factor variance analysis and two-sample Q test, and $P < 0.05$ the statistical significance of the difference.

Results

Effect of PHB overexpression on cell viability

After PHB overexpression transfection for 48 h, PHB expression and protein level of VSMC was identified by RT-PCR and western blot. The relative expression in the PHB overexpression group was notably lower than the control group and mock group (**Figure 1A**). In **Figure 1B** and **1C**, PHB protein level in PHB overexpression group was significantly decreased compare with the control and mock group.

After the transfection for 12, 48 and 72 h, cell viability was detected by MTT assay. Compared with the control group, cell viability in PHB overexpression group was decreased by 84.5 ± 6.53%, 65.30 ± 4.08% and 59.2 ± 6.32% at 12, 24 and 48 h respectively (**Figure 1D**).

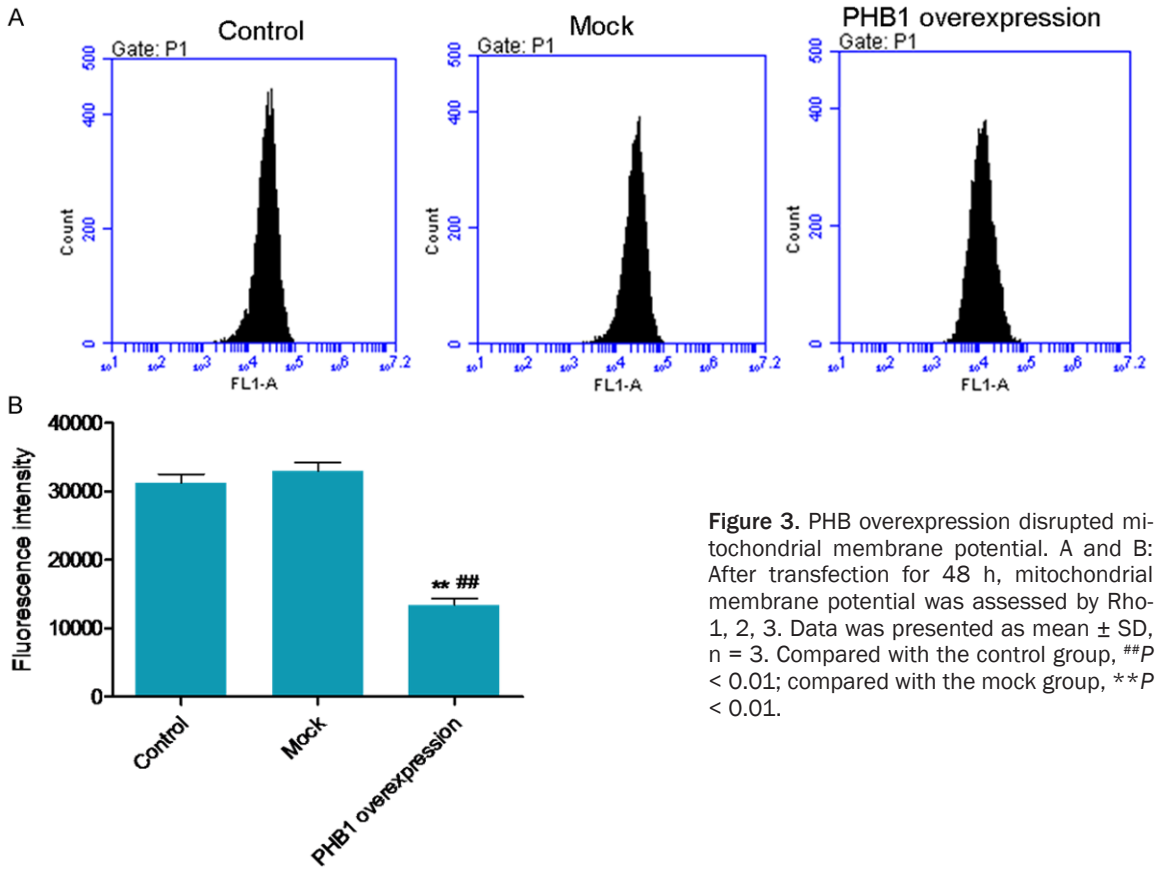

Figure 3. PHB overexpression disrupted mitochondrial membrane potential. A and B: After transfection for 48 h, mitochondrial membrane potential was assessed by Rho-1, 2, 3. Data was presented as mean ± SD, n = 3. Compared with the control group, $^{\#\#}P < 0.01$; compared with the mock group, $^{**}P < 0.01$.

Effect of PHB overexpression on cell apoptosis

As PHB overexpression effectively suppressed cell viability of VSMC, flow cytometry was used to identify whether it can cause cell apoptosis. After the transfection for 48 h, the cell apoptosis rate of PHB overexpression group was significantly decreased, compared with the control and mock control (**Figure 2A** and **2B**). Cell apoptotic rate in PHB overexpression group was increased by 60.6 ± 5.45%.

Effect of PHB overexpression on mitochondrial membrane potential (MMP)

The collapse of mitochondrial membrane potential is the essential step of mitochondrial apoptosis. MMP was detected by Rho-123. As shown in **Figure 4**, MMP in PHB overexpression group was dramatically descended compared with that of control and mock group.

Effect of PHB overexpression on AIF, CytoC, caspase 3 and 9 expression

Protein expression of AIF, CytoC, caspase 3 and 9 expression was identified by western blot analysis. As shown in **Figure 4A-C**, protein level of AIF and CytoC in mitochondria was significantly decreased by PHB overexpression, while protein level of AIF and CytoC in cytoplasm was increased in PHB overexpression group. In addition, caspase 3 and 9 expression in PHB overexpression group were increased in comparison with that of control and mock group (**Figure 4D** and **4E**).

Discussion

The proliferation of vascular smooth muscle cells is one of important causes of atherosclerosis, hypertension, restenosis after stent implantation and other diseases [3]. The mechanical properties of vessel wall are mainly determined by the membrane, and the only cellular component of membrane is VSMC. So VSMC plays an important role in the vascular activity [4-7].

Anti-proliferative protein, as a highly conservative protein, is widely distributed in a variety of biological cells, which is named because of its obviously anti-proliferation effect [8, 9]. Anti-

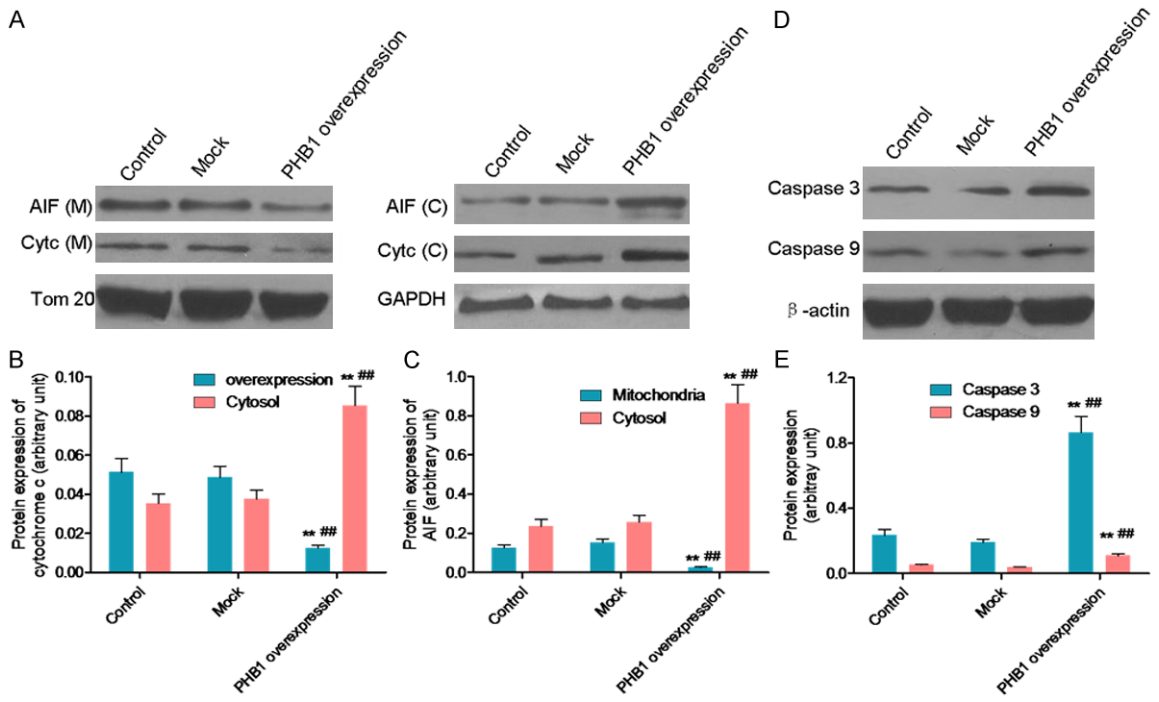

Figure 4. PHB regulated protein expression of AIF, cytc, caspase 8 and caspase 9. A-C: After transfection for 48 h, AIF and cytc in mitochondria and cytoplasm was detected by western blot. D, E: Caspase 8 and caspase 9 was detected by western blot. Data was presented as mean ± SD, n = 3. Compared with the control group, $^{\#\#}P < 0.01$; compared with the mock group, $^{**}P < 0.01$.

proliferation proteins can inhibit the cell cycle transitions and DNA synthesis in normal cells [10]. So far, the expression of the protein have been found in a variety of tumor cells, and the anti-proliferative proteins are known to be relevant to cell proliferation, cell differentiation, cell apoptosis, the regulation of cell growth and so on [11, 12].

In the present study, we found that PHB overexpression effectively suppressed the cell viability, induced cell apoptosis and damaged the MMP. The break of MMP is considered as the initial process of mitochondria-dependent apoptosis. The loss of MMP causes an increase in the permeability of the mitochondrial membrane, followed by the release of pro-apoptotic molecules such as cytochrome c. Cytochrome c releasing from mitochondrial interacts with AIF, ATP, Apaf-1 and caspase 9, and subsequently activates caspase 3, which consequently elicits caspase-dependent apoptotic cell death [13, 14]. The western blot analysis results suggest that the protein expression levels of AIF, cytochrome c, caspase 3 and 9 were increased after treatment with PHB overexpression transfection.

The preliminary experimental results demonstrate that PHB is able to inhibit proliferation of *in vitro* cultured rat vascular smooth muscle cells. The incidence of the many diseases including vein bridge stenosis after coronary artery bypass graft, atherosclerosis, coronary restenosis after stent implantation and can be reduced in theory, which provide a new breakthrough point for the treatment of further experimental animal studies need to be carried out for *in vivo* environment test on the proliferation effect of vascular smooth muscle because of the difference between *in vitro* proliferative smooth muscle cells and *in vivo* proliferative smooth muscle cells and the limitation of many other conditions.

Disclosure of conflict of interest

None.

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