**Panax Notoginseng** Saponins Protect H9c2 Cells From Hypoxia-reoxygenation Injury Through the Forkhead Box O3a Hypoxia-inducible Factor-1 Alpha Cell Signaling Pathway

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**Abstract:** *Panax notoginseng* saponins (PNS) are commonly used in the treatment of cardiovascular diseases. Whether PNS can protect myocardial ischemia-reperfusion injury by regulating the forkhead box O3a hypoxia-inducible factor-1 alpha (FOXO3a/HIF-1α) cell signaling pathway remains unclear. The purpose of this study was to investigate the protective effect of PNS on H9c2 cardiomyocytes through the FOXO3a/HIF-1α cell signaling pathway. Hypoxia and reoxygenation of H9c2 cells were used to mimic MIRI in vitro, and the cells were treated with PNS, 2-methoxyestradiol (2ME2), and LY294002. PNS could reduce H9c2 hypoxia-reoxygenation injury by promoting autophagy and inhibiting apoptosis through the HIF-1α/FOXO3a cell signaling pathway. Furthermore, the protective effects of PNS were abolished by HIF-1α inhibitor 2ME2 and PI3K/Akt inhibitor LY294002. PNS could reduce H9c2 hypoxia-reoxygenation injury by promoting autophagy and inhibiting apoptosis through the HIF-1α/FOXO3a cell signaling pathway.

**Key Words:** PNS, HIF-1α, FOXO3a, autophagy, apoptosis

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INTRODUCTION

Ischemic cardiomyopathy is the main cause of death in cardiovascular diseases. With the acceleration of aging, the prevalence of cardiovascular disease is also increasing. Excessive reactive oxygen species (ROS) production is induced by ischemia and hypoxia, which leads to cardiomyocyte necrosis. Excessive oxidative stress can cause ROS to regulate the PI3K/Akt cell signaling pathway, which give rise to activate death receptor-dependent and mitochondrial-dependent apoptosis pathways. Forkhead box O3a (FOXO3a) transcription factor is a fundamental regulator of apoptosis and it is also a crucial downstream target of PI3K/Akt signaling pathway. FOXO3a plays an important pathophysiological role in the occurrence and development of ischemic heart disease. Elucidating the mechanism of cardiomyocyte apoptosis after oxidative stress is helpful to clarify the mechanism of cardiomyocyte apoptosis after oxidative stress. It provides a new target for the study of cardiomyocyte apoptosis in ischemic heart disease.

Previous studies have shown that myocardial ischemia and hypoxia can induce autophagy, but the role of autophagy in myocardial hypoxia-reoxygenation has not been fully illuminated. Moderate autophagy may play a protective role as an emergency mode of myocardial cells, whereas excessive autophagy may aggravate cell damage and eventually lead to apoptosis. At the same time, myocardial ischemia and hypoxia can induce the production of hypoxia-inducible factor-1α (HIF-1α), and the expression of HIF-1α is enhanced under hypoxia conditions. However, HIF-1α is degraded under normoxic conditions, and the expression is very low. It has been reported that HIF-1α transcription factor expression is enhanced during hypoxia, which leads to the increase of BNIP3 protein expression and induces myocardial autophagy. Similarly, some studies have found that blocking FOXO3a or BNIP3 can significantly reduce hypoxia-induced mitochondrial-dependent apoptosis, inhibit FOXO3a, and play a protective role in the process of cardiomyocyte apoptosis. The role of HIF-1 and FOXO3a in regulating autophagy and apoptosis during myocardial ischemia and hypoxia needs further clarification.

*Panax notoginseng* saponins (PNS) are effective medicinal components extracted from a high quality of *P. notoginseng* [(Burk.) F.H. Chen] (Sanqi or Tianqi in Chinese). PNS are commonly used in the treatment of cardiovascular diseases,
which can dilate coronary artery, can resist vasospasm, reduce myocardial oxygen consumption, and improve microcirculation. Modern studies have shown that PNS have anti-inflammatory, antioxidant, and antiapoptotic effects. Our previous study found that PNS attenuate myocardial ischemia-reperfusion injury through the HIF-1α/BNIP3 pathway of autophagy. However, the involvement of PNS in the FOXO3a/HIF-1α signaling pathway to protect H9c2 cardiomyocytes from H/R injury and the relationship between apoptosis and autophagy remain uncertain. In this study, we established an H9c2 cardiomyocyte hypoxia-reoxygenation model and explored the possible molecular mechanism of PNS alleviating hypoxia-reoxygenation injury by autophagy and apoptosis mediated by the FOXO3a/HIF-1α signaling pathway so as to provide a theoretical basis for PNS in clinical treatment of myocardial ischemic diseases.

MATERIALS AND METHODS

Cell Culture and H/R Injury Model

Rat H9c2 myocardial cell line was obtained from Nanjing Cobioer Co, Ltd, China. H9c2 cells were cultured in DMEM high glucose medium supplemented with 10% FBS (Gibco, Carlsbad, CA), 100 μg/mL streptomycin (Gibco), and 1% penicillin-streptomycin (Gibco) in a humidified 5% CO2 atmosphere at 37°C. The hypoxia group was placed into a normoxic incubator, the control group was placed into a triple gas incubator, the control group was filled with 95% N2/5% CO2 for 18 hours under 8C. The hypoxia group was placed into a triple gas incubator at 37°C filled with 95% N2/5% CO2 for 18 hours under anoxia and then put into an incubator at 37°C filled with 95% air and 5% CO2 for 6 of the 18 hours under reoxygenation.

Drug Treatment

In this study, powder injection of PNS (batch number: 19AA210) was provided by the KPC Pharmaceuticals, Inc (Yunnan, China). PNS was added 24 hours before hypoxia treatment. The HIF-1α inhibitor 2-methoxyestradiol (2ME2) and the PI3K/Akt inhibitor LY294002 were added 1 h before hypoxia treatment.

Determination of Cell Viability

Cell Counting Kit-8 (CCK-8) was used to determine the cell viability. The cell suspension (100 μL/well) was inoculated into 96-well plates, and the culture plate was placed in the incubator for preculture. Each well was added with 10 μL of CCK-8 solution; after incubation for 1 hour, the absorbance at 450 nm was determined by enzyme-labeled instrument.

Determination of Lactate Dehydrogenase and Malonaldehyde Content

Lactate dehydrogenase (LDH) assay kit (cat. no. A020-2-2) and malonaldehyde (MDA) assay kit (cat. no. A003-1-2) were used to assay cell injury, which were purchased from Nanjing Jiancheng Bioengineering Institute Co, Ltd. LDH was detected by the microplate method, and MDA by the thiobarbituric acid method. After treatment, the cells of each group were collected, operated, and tested according to the instructions of each kit.

Determination of ROS

ROS assay kit (Cat. No. S0033; Beyotime Institute of Biotechnology, China) was used to evaluate ROS production in H9c2 cells. H9c2 cells were incubated with 10 μM 2′,7′-dichlorofluorescein diacetate for 20 minutes and washed 3 times with serum-free medium. The photographs of culture dishes were taken immediately by fluorescence microscopy (Olympus) using a suitable analysis software system.

Measurement of Apoptosis by Flow Cytometry

The apoptotic rates of H9c2 cells were detected by Annexin V/FITC Apoptosis Detection kit (Sigma, St. Louis, MO). In brief, after the different treatments, the cells were collected, resuspended in 1 mL of binding buffer, and centrifuged at 300g for 10 minutes. After centrifugation, the supernatant was discarded and 5 μL of annexin V-FITC was added to the cells at room temperature and kept away from light, and then 5 μL of PI was added. To make the total volume to 500 μL, 490 μL of PBS was added and mixed gently. Flow cytometry was performed within 1 hour.

Western Blot

The H9c2 cells in each group were collected after treatment according to the experimental method. Protein was extracted by RIPA lysis buffer, the protein concentration was detected by the BCA Kit (Shanghai Beyotime Biotechnology Co Ltd, China), and 5x sample loading buffer was added and boiled for 10 minutes. Proteins were separated by SDS-PAGE (12% Tris-Gly per gel) and transferred onto PVDF by electrophoresis. The protein content of each sample was 4 g/L, the voltage of electrophoresis was 80V, the electrophoresis time was 80 minutes, the membrane transfer current was 100mA, and the membrane transfer time was 90 minutes. The membranes were blocked with sealing solution for 1 hour, incubated with primary antibodies against HIF-1α (Cell Signaling, cat. no. 12791s, 1:1000), LC3 (Cell Signaling, cat. no. 12741s, 1:1000), Bnip3 (Cell Signaling, cat. no. 3769s, 1:1000), FOXO3a (Cell Signaling, cat. no. 12829s, 1:1000), p-FOXO3a (Cell Signaling, cat. no. 2933s, 1:1000), Akt (Cell Signaling, cat. no. 4685s, 1:1000), p-Akt (Cell Signaling, cat. no. 4060s, 1:2000), Bim (Cell Signaling, cat. no. 2933s, 1:1000), and β-actin (Santa Cruz Biotechnology, cat. no. 2933s, 1:1000) overnight on 4°C shaker. After 12 hours, the PVDF membranes were washed and then incubated with appropriate secondary antibody (anti-rabbit IgG, cat. no. 926-32211, 1:15,000 in tPBS; LI-COR) for 2 hours. Odyssey infrared fluorescence scanning imaging system (Olympus) using a suitable analysis software system.

Statistical Analysis

The SPSS 22.0 software was used to analyze the experimental data. All data were expressed in the form of
RESULTS

Effect of PNS on the Proliferation of H9c2 Cells

Effect of PNS on the proliferation of H9c2 cells at different reperfusion time is shown in Fig. 1A. H9c2 cells were subjected to hypoxia for 18 hours, and cell viability was detected after reperfusion for 0, 3, 6, 12, and 24 hours. We found that the viability of H9c2 cells decreased with the prolongation of reperfusion time but increased slightly after reperfusion 24 hours.

H9c2 cells were pretreated with different concentrations of PNS (50, 100, 200, 400, 800, and 1600 µg/mL) for 24 hours. The cell proliferation activity was measured by the CCK-8 test kit. It was found that the concentration of PNS ≥800 µg/mL had no effect on the cell proliferation, and the cell proliferation activity was declined to a great extent when the concentration was ≥1600 µg/mL (P < 0.01) (Fig. 1B).

The effects of different concentrations of PNS on cell viability of H9c2 cells induced by H/R injury. Compared with the control group, the proliferation activity of cells in the H/R model group was decreased significantly (P < 0.01). Compared with the H/R model group, the proliferation activity of cells in the PNS group with the concentration of 200 and 400 µg/mL was increased significantly (P < 0.01) (Fig. 1C). We found that PNS could protect H9c2 cells from hypoxia-reoxygenation injury in a dose-dependent manner, and the optimal concentration of PNS was 400 µg/mL. Therefore, we chose 400 µg/mL PNS as the dose of this experiment.

Effect of PNS on the Changes of LDH and MDA in H9c2 Cells

The LDH and MDA assay showed that compared with the control group, the LDH and MDA content in the H/R model group were increased significantly (P < 0.01). Compared with the H/R model group, the LDH and MDA content in the PNS group decreased significantly (P < 0.01). Compared with the PNS group, the cell activity of the PNS + LY294002 group was increased significantly (P < 0.01) (Figs. 2A, B).

Effect of PNS on Apoptosis in H9c2 Cells Against H/R Injury

We verified the effect of PNS on apoptosis by regulating the PI3K/Akt signaling pathway. Compared with the control group, the apoptotic rate of the H/R group was significantly increased (P < 0.01), whereas the apoptosis rate of the PNS group was significantly lower than that of the H/R group (P < 0.01). The results showed that PNS could reduce the apoptosis rate of hypoxia-reoxygenation injury cells and protect the cells. Compared with the PNS group, the cell activity of the PNS + LY294002 group was increased significantly (P < 0.01). These results indicate that the antiapoptotic effect of PNS could be blocked by LY294002, and PNS could reduce apoptosis by regulating the PI3K/Akt cell signaling pathway (Fig. 2C).

In addition, we also verified the effect of PNS on apoptosis by regulating the HIF-1α/BNIP3 signaling pathway. Compared with the control group, the apoptotic rate of the H/R group was significantly increased (P < 0.01), whereas the apoptosis rate of the PNS group was significantly lower than that of the H/R group (P < 0.01). Compared with the PNS group, the apoptotic rate of the PNS + 2ME2 group was increased significantly (P < 0.01). These results indicated that the antiapoptotic effect of PNS could be blocked by 2ME2, and PNS could reduce apoptosis by regulating the HIF-1α/BNIP3 cell signaling pathway (Fig. 3A).

Effect of PNS in H9c2 Cells Against H/R Injury by Reducing ROS Accumulation

The increase of ROS production is the basis of oxidative stress. The results showed that the production of cellular ROS was increased under H/R conditions compared with the control group and further increased in the PNS +
FIGURE 2. PNS protects H9c2 cells against H/R injury through the PI3K/Akt signaling pathway. A, The levels of LDH. B, The levels of MDA. C, Apoptosis rate detected by Annexin V/PI flow cytometry. D, The ROS levels of 4 groups. The results are presented as the mean ± SD, n = 3, data derived from 3 individual experiments. *P < 0.01 versus the control group, #P < 0.01 versus the H/R group, &P < 0.01 versus the PNS group.
LY294002 group ($P < 0.01$), whereas compared with the H/R group, the production of cellular ROS in the PNS group was significantly decreased (Fig. 2D). At the same time, compared with the H/R group, ROS in the PNS + 2ME2 group increased significantly (Fig. 3B). These results indicate that PNS could protect against H/R injury by reducing ROS; ROS may affect the protection of PNS by regulating downstream PI3K/Akt and HIF-1α/BNIP3 cell signaling pathways.

**PNS Protect H9c2 Cardiomyocytes Against H/R Injury Through Autophagy Involving the HIF-1α/BNIP3 Pathway**

Western blot analysis showed that compared with the control group, the expression of HIF-1α and BNIP3 protein in H9c2 cells of the H/R group was significantly increased ($P < 0.01$). The expression of HIF-1α and BNIP3 protein in H9c2 of the PNS group was significantly higher than that in the H/R group ($P < 0.01$), indicating that PNS can increase the expression of HIF-1α and BNIP3 protein in H9c2. Interestingly, compared with the PNS group, the expression of HIF-1α and BNIP3 in the PNS + 2ME2 group was decreased significantly ($P < 0.01$), indicating that 2ME2 can block the effect of PNS on the regulation of HIF-1α and BNIP3 protein expression (Figs. 4A–C).

We observed autophagy by detecting the expression of LC3-I and LC3-II protein (Fig. 4A). The western blotting analysis showed that hypoxia-reoxygenation induced the increase of autophagy protein LC3 and the ratio of LC3II/LC3I ($P < 0.01$). Interestingly, after PNS intervention, autophagy protein LC3 and the ratio of LC3II/LC3I increased significantly ($P < 0.01$) (Fig. 4B). After 2ME2 intervention, the autophagy protein LC3 and the ratio of LC3II/LC3I decreased significantly ($P < 0.01$), indicating that 2ME2...
blocked the expression of autophagy protein regulated by PNS (Fig. 4D).

**PNS Protect H9c2 Cardiomyocytes Against H\R-Induced Apoptosis Involving the PI3K/Akt Pathway**

To confirm whether PNS can protect H9c2 cells through the PI3K/Akt/FOXO3a pathway, H9c2 cells were pretreated with PNS and PI3K/Akt inhibitor LY294002. The PI3K/Akt/FOXO3a pathway could be blocked by LY294002. The results showed that the expression of FOXO3a and Akt in the H/R group was significantly lower than that in the control group, whereas the expression of FOXO3a and Akt in the PNS group was significantly higher than that in the H/R group (P < 0.01). Compared with the PNS group, the expression of Bim in the PNS + LY294002 group was significantly higher than that in the PNS group (P < 0.01) (Fig. 5D).

**DISCUSSION**

PNS are the main component of traditional Chinese medicine *P. notoginseng*. PNS have a variety of pharmacological effects, such as antiapoptotic, antioxidant, antitumor, and antidiabetic activities. Most studies have reported that PNS have a good therapeutic effect on myocardial ischemic diseases. Myocardial ischemia-reperfusion injury (MIRI) is an important cause of death in clinical myocardial ischemic diseases. At present, the pathogenesis of MIRI has not been clarified, and there is no effective treatment. This study confirmed the potential therapeutic effect of PNS on MIRI and found that PNS decimated hypoxia-reoxygenation injury in cardiomyocytes. These effects may be correlated to the induction of HIF-1α/FOXO3a-mediated autophagy and apoptosis.
Autophagy plays a leading part in myocardial ischemia-reperfusion. Autophagy refers to the process in which organelles and proteins that need to be degraded in cells are encapsulated in the membrane to form autophagosome, which is finally marred by lysosomes. However, the autophagy mechanism of MIRI remains controversial: Whether autophagy is the stress mechanism of cell survival, a pathological mechanism of cell death or disease, or both, is still unclear. In the early stage of anoxia reoxygenation, autophagy is essential for cell survival by removing damaged organelles timely and selectively, reducing ROS production and providing nutrients.

Myocardial ischemia-reperfusion injury is of great significance to mitochondrial homeostasis. Acute and chronic ischemia and hypoxia can cause mitochondrial damage to myocardial cells, producing excessive ROS, and further activating autophagy. Excessive ROS can damage mitochondrial protein and DNA, lead to mitochondrial dysfunction, and eventually result in cell apoptosis. ROS can induce the production of HIF-1. HIF-1 is inducible with 3 subunits, and HIF-1α is the main functional subunit. Our previous study found that HIF-1α was sharply increased in myocardial ischemia-reperfusion rats, reflecting the degree of cell damage to a certain extent. HIF-1α is a critical regulator of mitochondrial autophagy. HIF-1α can directly regulate BNIP3, which results in the increase of BNIP3 expression. Mitochondria are abundant in cardiomyocytes. The mitochondrial dysfunction caused by ischemia-reperfusion injury increases ROS and BNIP3 expression.

**FIGURE 5.** Effects of PNS on apoptosis protein expression in H9c2 cells. A, The protein levels of Akt, p-Akt, FOXO3a, p-FOXO3a, and Bim detected by western blot. a, The control group; b, the H/R group; c, the PNS group; d, the PNS + LY294002 group. B, The p-FOXO3a/FOXO3a ratio. C, The p-Akt/Akt ratio. D, The protein level of Bim expression in H9c2 cells. The results are presented as the mean ± SD, n = 3, data derived from 3 individual experiments. *P < 0.01 versus the control group, #P < 0.01 versus the H/R group, &P < 0.01 versus the PNS group.

**FIGURE 6.** Schematic of the mechanism of PNS protecting cardiomyocytes from hypoxia-induced injury through the FOXO3a/HIF-1α pathway. PNS can protect cardiomyocytes by regulating HIF-1α-mediated autophagy and FOXO3a-mediated apoptosis. The effect of PNS was blocked by HIF-1α inhibitor 2ME2 and PI3K/Akt inhibitor LY294002.
which can activate autophagy to degrade damaged mitochondria and promote mitochondrial renewal in myocardial cells. In our study, HIF-1α-related and autophagy-related proteins LC3 and BNIIP3 were significantly increased after hypoxia-reoxygenation injury. When PNS was used, HIF-1α was significantly increased in hypoxia-reoxygenation cardiomyocytes. At the same time, PNS could greatly reduce LDH, MDA, and ROS production. When 2ME2 was used, HIF-1α and autophagy levels were cut down enormously. These results suggest that PNS can protect cardiomyocytes from hypoxia-reoxygenation injury by regulating HIF-1α and increasing autophagy.

With the prolongation of hypoxia-reoxygenation time, excessive autophagy leads to increased cell apoptosis. Transcription factors FOXO family is a decisive regulator of apoptosis. Nonphosphorylated FOXO3α is an active transcription factor. After phosphorylation, FOXO3α loses its transcriptional activity. Studies have found that FOXO3α plays an important role in inhibiting cardiomyocyte hypertrophy and antioxidant stress. Activation of the PI3K/Akt signaling pathway can phosphorylate downstream target FOXO3α. The downstream target gene Bim of FOXO3α is an important proapoptotic protein containing only BH3 domain in Bcl-2 family. In this study, to confirm the role of the FOXO3α/Akt pathway in the protection of PNS, H9c2 cells were pretreated with LY294002, a PI3K/Akt signaling pathway inhibitor. LY294002 inhibited the phosphorylation of Akt and FOXO3α and blocked the Akt/FOXO3α signal pathway. In this experiment, we found that PNS could increase the expression of p-FOXO3α and p-Akt, decrease the expression of Bim, and decrease the apoptosis rate in H/R injury of H9c2 cells. The expressions of FOXO3α and Akt in the PNS + LY294002 group were significantly lower than those in the PNS group, whereas Bim was significantly higher, and the apoptosis rate was on the contrary. It indicated that PNS could regulate the apoptosis through the PI3K/Akt signaling pathway mediated by FOXO3α.

Under the induction of ischemia and hypoxia, autophagy occurs in the prophase to protect the cells from excessive injury. When the cell is damaged excessively, the excessive autophagy of proapoptotic protein promotes apoptosis. The proapoptotic protein Bim is the binding point of autophagy and apoptosis. Excessive autophagy of cardiomyocytes leads to increased apoptosis and upregulation of Bim expression. Bim may be a bridge between autophagy and apoptosis. Some studies have shown that HIF-1α and FOXO3α could increase the expression of BNIIP3 and induce cardiomyocyte apoptosis. Simplified overview of the above signaling pathways is as illustrated in Figure 6. PNS intervention on the HIF-1α/FOXO3α target may be an effective strategy to treat myocardial diseases, but the mechanism of PNS regulating HIF-1α/FOXO3α autophagy and apoptosis during myocardial ischemia needs further study.

CONCLUSION

Our study provides new evidence for the protective effect of PNS on H9c2 cardiomyocytes through the FOXO3α/HIF-1α signaling pathway. PNS can promote autophagy through HIF-1α/BNIIP3 and protect cardiomyocytes from hypoxia-reoxygenation injury by inhibiting apoptosis by FOXO3α/Akt. This result provides not only a solid basis for further study of the protective effect of PNS on myocardial tissue but also a new target for early clinical treatment of the myocardial ischemia-reperfusion injury.

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