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# Knockdown of p66ShcA activates Nrf2 pathway to protect cardiomyocytes from oxidative stress and inflammation induced by $H_2O_2$

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**Abstract. – OBJECTIVE: Oxidative stress and inflammation are the most common causes of myocardial ischemia and hypoxia. This article focuses on the effect of p66ShcA on $H_2O_2$-induced cardiomyocytes.**

**MATERIALS AND METHODS: The p66ShcA knockdown model of H9c2 cells was constructed by plasmid transfection. After treatment of different groups with $H_2O_2$, oxidative stress-related factors and inflammatory factors were detected.**

**RESULTS: The expressions of SOD1, SOD2, GPX1, and GPX3 in $H_2O_2$ cells were significantly decreased, IL-1β and IL-6 expression were significantly increased, while p66ShcA siRNA negative group could promote the expression of SOD1, SOD2, GPX1, and GPX3, inhibit the expression of IL-1β and IL-6 significantly, and activates the Keap1/Nrf2 pathways.**

**CONCLUSIONS: Knockdown of p66ShcA can activate Keap1/Nrf2 pathway, which inhibits $H_2O_2$-induced oxidative stress and inflammation in H9c2 cells.**

*Key Words:*
p66ShcA, H9c2, Oxidative stress, Inflammation, Keap1/Nrf2.

## Introduction

A large number of oxygen free radicals during myocardial ischemia (MI) and ischemia-post reperfusion are the main pathogenesis of MI and reperfusion injury after ischemia[1]. Numerous studies have shown that reactive oxygen species (ROS) plays an important role in mediating cardiomyocyte apoptosis during ischemia-reperfusion injury. Whether oxidative stress induces apoptosis depends on the concentration of intracellular ROS[2]. To protect cardiomyocytes and prevent their apoptosis is of positive clinical significance for the prevention and treatment of cardiovascular diseases, such as heart failure and MI, and the development of antioxidant drugs and targets is effective in the prevention and treatment of cardiomyocyte inflammation caused by oxidative stress, thus, protecting the heart has become one of the research hotspots[3].

The incidence and development of ischemic heart disease (IHD) are closely related to inflammatory factors. Inflammatory factors are produced by lymphocytes and are cytokines that reflect the severity of inflammation in the body[4]. The level of inflammatory factors is accompanied by the development and development of IHD, and is related to apoptosis of cardiomyocytes and immune activation signaling pathways[5]. Oxidative stress and inflammation have mutual effects[6]. Therefore, we will explore the inflammatory damage associated with $H_2O_2$-induced H9c2 production.

The adaptor protein (SHC) family containing Src homology domain 2 includes SHCA, SHCB, SHCC, SHCD, which have specific prototype signaling cells in mammals[7]. ShcA (also known as Shc1) is a scaffolding protein of TKs that increases the complexity and specificity of the tyrosine kinase (TK) signal. The mammalian ShcA gene encodes three cytoplasmic protein isomers (66, 52, and 46 kDa) that bind to motifs containing phosphotyrosine (pTyr) on activated TKs *via* the n-terminal PTB binding domain (PTB) and the c-terminal Src homologous domain (SH). Once recruited into activated TKs by such a pTyr recognition domain, ShcA itself can tyrosine phosphorylation in the central region of CH1 (collagen homology 1), thereby stimulating activation of specific cytoplasmic signaling pathways[8]. Miyazawa and Tsuji[9] have reported that p66Shc/IQGAP1/nuclear factor NF-E2 re-



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lated factor (Nrf) 2 and AKT/Nrf2/glutathione (GSH) pathways are involved in the regulation of oxidative stress.

Kelch-like epichlorohydrin-related protein-1 (Keap1)-Nrf2 signaling pathway is particularly important for the regulation of cellular oxidative stress, and its regulation of the downstream metabolic enzymes and antioxidant proteins/enzymes in cellular defense plays an important role in protection and is also a hot spot in the field of antioxidant research in recent years. Bellezza et al[10] have shown that Nrf2 is a member of the basic leucine zipper (bZIP) protein family in the oxidative stress response of the body and is an important nuclear transcription factor regulating antioxidant stress. Nrf2 is an important transcription factor that plays a role in cell self-protection and has physiological activities, such as anti-oxidation, maintenance of homeostasis, and immune surveillance[11]. Mizunoe et al[12] have observed that Keap1/Nrf2 signaling pathway is involved in oxidative stress response. Therefore, we have investigated the knockdown of p66ShcA to activate Keap1/Nrf2 pathway to protect $H_2O_2$-induced oxidative stress and inflammation in cardiomyocytes.

## Materials and Methods

### Material

Lipofectamine™ 2000 transfection reagent (Shanghai Jikai Gene Chemical Technology, Shanghai, China), small interfering RNA (siRNA) sequence (Shanghai Jikai Gene Chemical Technology, Shanghai, China). Horseradish peroxidase (HRP)-labeled goat anti-rabbit Ig G and anti-mouse Ig G (Beijing Zhongshan Jinqiao Biotechnology, Beijing, China). Dulbecco's Modified Eagle's Medium (DMEM; Life Technology, Wuhan, China) and fetal bovine serum (FBS; Life Technology, Wuhan, China).

### Cell Culture

The culture conditions of cardiomyocytes H9c2 (Cell Culture Center, Shanghai, China) were: 37°C, volume fraction 95% air, 5% $CO_2$ incubator, Dulbecco's Modified Eagle's Medium (DMEM) containing $1\times10^5$ $UL^{-1}$ penicillin, 100 $\mu g/mL^{-1}$ streptomycin, and in DMEM. We, then, added 10% FBS. Cell passage was performed at a cell density of 90%, and 0.25% trypsin digest was passaged.

### Transfection

H9c2 cells in logarithmic growth phase were seeded in 6-well plates. When the cell confluence rate reached 70%, the recombinant plasmid and the plasmid transfected with unrelated sequence were transferred into H9c2 cells by liposome Lipofectamine™ 2000 (Shanghai Jikai Gene Chemical Technology, Shanghai, China). Plasmid transfection group cells transfected with unrelated sequences were set as negative controls.

### Cell Processing

The stably transfected H9c2 cells were treated with $H_2O_2$ and divided into p66ShcA siRNA negative group and siRNA NC group. H9c2 cells treated with $H_2O_2$ but without transfection with plasmid were set as $H_2O_2$ group. H9c2 cells without any treatment were recorded as a control group.

### Detection of Biochemical Indicators

According to the instructions of the commercial kit (Jiancheng, Nanjing, China), the supernatants of each group of cells were collected, and after the working solution was configured using the kit, the supernatants of the cells were measured for lactate dehydrogenase (LDH), malondialdehyde (MDA) levels, and catalase (CAT) activity.

### Flow Cytometry

H9c2 cells in each group were collected and screened to adjust the cell concentration. The prepared single cell suspension was incubated with 2',7'-diacetate dichlorofluorescein, and the supernatant was centrifuged and incubated with 10% FBS. After centrifugation at 4°C, a H9c2 single cell suspension was prepared again. The average fluorescence intensity of the intracellular marker fluorescent probe was measured by flow cytometry (BD FACSCalibur type, Becton-Dickinson (BD), Franklin Lakes, NJ, USA).

### Immunofluorescence

The cells were fixed with 4% paraformaldehyde, and the goat serum was added dropwise for 1 h. The diluted antibody (SOD2, 1:500, Abcam, Cambridge, MA, USA; IL-1β, 1:500, Abcam, Cambridge, MA, USA; Nrf2, 1:500, Abcam, Cambridge, MA, USA) was added dropwise and placed in a refrigerator at 4°C overnight. The second antibody was added to the next day in the dark, and after 1 h of incu-

bation, 4',6-diamidino-2-phenylindole (DAPI) was stained and incubated in the dark. After 15 minutes, the images were observed under a fluorescence microscope.

### Real Time-PCR

Total RNA was used in advance using TRIzol (Thermo Fisher Scientific, Waltham, MA, USA). Reverse transcription synthesis of complementary deoxyribose nucleic acid (cDNA). Real Time-PCR amplification was performed on iCycler using SYBR Premix Ex Taq (Thermo Fisher Scientific, Waltham, MA, USA). RT-PCR primers: GAPDH, Forward 5'-ACAACTTTGGTATCGTGGAAGG-3', Reverse 5'-GCCATCACGCCACAGTTTC-3', SOD1, Forward 5'-GGTGAACCAGTTGTGTTGTC-3', Reverse 5'-CCGTCCTTTCCAGCAGTC-3', SOD2, Forward 5'-CAGACCTGCCTTACGACTATGG-3', Reverse 5'-CTCGGTGGCGTTGAGATTGTT-3', GPX1, Forward 5'-ATCATATGTGTGCTGCTCGGCTAGC-3', Reverse 5'-TACTCGAGGGCACAGCTGGGCCCTTGAG-3', GPX3, Forward 5'-AGAGCCGGGGACAAGAGAA-3', Reverse 5'-ATTTGCCAGCATACTGCTTGA-3', IL-1β, Forward 5'-GCAACTGTTCCTGAACTCAACT-3', Reverse 5'-ATCTTTTGGGGTCCGTCAACT-3', IL-6, Forward 5'-TAGTCCTTCCTACCCCAATTTCC-3', Reverse 5'-TTGGTCCTTAGCCACTCCTTC-3', Keap1, Forward 5'-TGCCCCTGTGGTCAAAGTG-3', Reverse 5'-GGTTCGGTTACCGTCCTGC-3', Nrf2, Forward 5'-TCTTGGAGTAAGTCGAGAAGTGT-3', Reverse 5'-GTTGAAACTGAGCGAAAAAGGC-3', HO-1, Forward 5'-GTGACAGAAGAGGCTAAGACCG-3', Reverse 5'-CAACAGGAAGCTGAGAGTCAGG-3', p66ShcA, Forward 5'-AAGTACAACCCACTTCGGAATG-3', Reverse 5'-GAAAGAAGGAACACAGGGTAGTC-3'.

### Western Blot

Whole cell proteins were extracted using radioimmunoprecipitation assay (RIPA) lysate (Beyotime, Shanghai, China). After the protein was separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), the target protein was transferred to the polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). After 5% of the skim milk powder was blocked for 2 h, it was incubated with a specific primary antibody at 4°C overnight. The next day, after washing with Tris-Buffered Saline-Tween (TBST), the horse radish peroxidase (HRP)-labeled secondary antibody was added and incubated for 1 hour on a shaker. Then, the membrane was observed by an enhanced chemiluminescence (ECL) system. Specific primary antibody: SOD1, Abcam, Cambridge, MA, USA, Rabbit, 1:3000; SOD2, Abcam, Cambridge, MA, USA, Rabbit, 1:3000; IL-1β, Abcam, Cambridge, MA, USA, Rabbit, 1:5000, IL-6, Abcam, Cambridge, MA, USA, Rabbit, 1:5000; Keap1, Abcam, Cambridge, MA, USA, Mouse, 1:1000; Nrf2, Abcam, Cambridge, MA, USA, Mouse, 1:1000; HO-1, Abcam, Cambridge, MA, USA, Mouse, 1:2000; p66ShcA, Abcam, Cambridge, MA, USA, Mouse, 1:1000; GAPDH, ProteinTech, Rosemont, IL, USA, 1:5000.

### Enzyme-Linked Immunosorbent Assay (ELISA)

The supernatants of each group of cells were collected and centrifuged. After setting the standard wells, we added different concentrations of the standard, dropped the corresponding antibodies (Jianglai, Shanghai, China) into the standard wells and the sample wells, incubated for 1 hour, and then incubated with the working solution for 15 min. After termination, the optical density (OD) value of each well was measured at a wavelength of 450 nm.

### Statistical Analysis

Data were expressed as mean ± standard deviation. Data analysis was performed using Statistical Product and Service Solutions (SPSS) 13.0 statistical software package (SPSS Inc., Chicago, IL, USA). The *t*-test was used for comparison between the two groups. $p<0.05$ was considered statistically significant.

## Results

### Knockdown of p66ShcA can Inhibit $H_2O_2$-Induced H9c2 Injury

First, we confirmed the successful transfection by Western blot and Real Time-PCR (Figure 1A and 1B). Again, we collected the supernatants of each group and found that compared with the control group, the LDH and MDA contents in the $H_2O_2$ group and the siRNA NC group were significantly increased, while the CAT content was significantly decreased. Conversely,

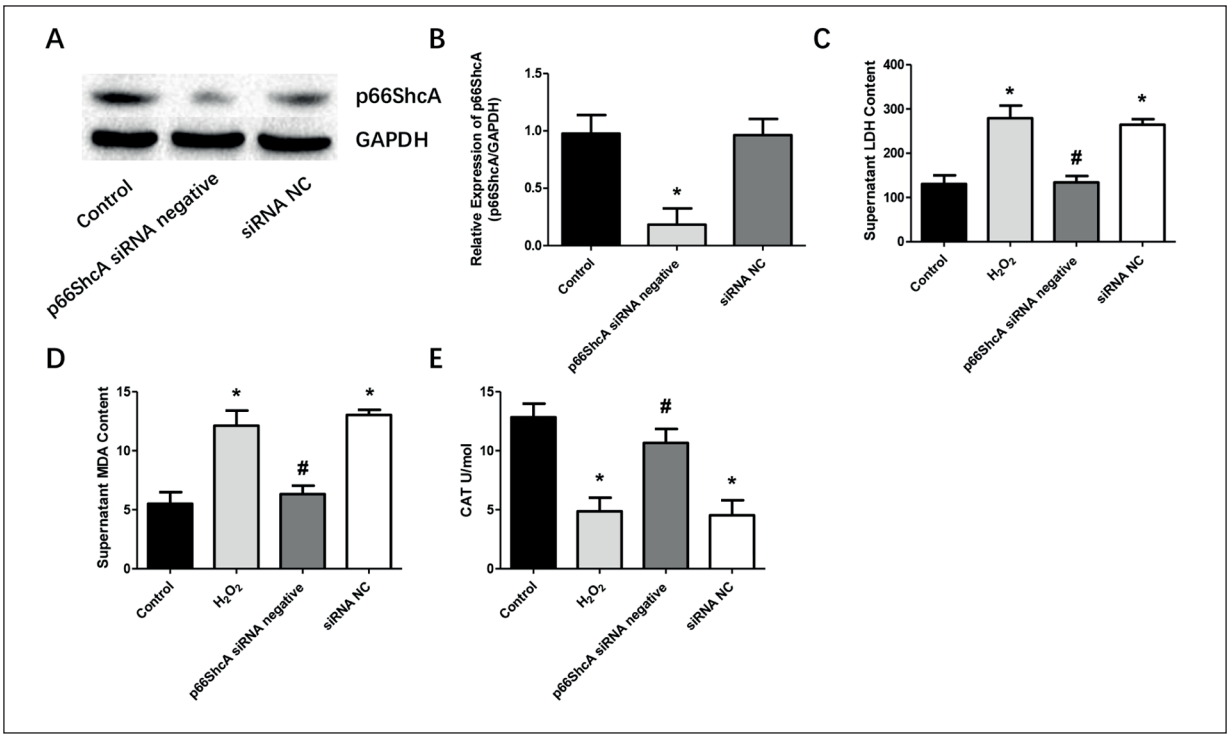


**Figure 1.** Knockdown of p66ShcA can inhibit $H_2O_2$-induced H9c2 injury. **A, B,** p66ShcA transfection assay by Western blot and Real Time-PCR, GAPDH was used as an internal control. **C,** LDH content detection. **D,** MDA content detection. **E,** CAT activity detection. ("*" indicates statistical difference from the control group $p<0.05$, "#" indicates statistical difference from the contrast group $p<0.05$).

in p66ShcA siRNA negative group, LDH and MDA levels were significantly reduced, and CAT content was significantly relieved (Figure 1C-1E).

### Knockdown of p66ShcA Inhibits $H_2O_2$-Induced H9c2 Oxidative Stress

Western blot (Figure 2A) and Real Time-PCR (Figure 2B and 2C) showed that compared with the control group, the expression of SOD1 and SOD2 in $H_2O_2$ group and siRNA NC group was significantly inhibited, and the expression of GPX1 and GPX3 mRNA was also significantly decreased (Figure 2D and 2E). ELISA detection also obtained similar results (Figure 2F). The knockdown of p66ShcA can effectively alleviate the decreased expression of SOD1, SOD2, GPX1, and GPX3. Flow cytometry showed that $H_2O_2$ treatment could significantly increase the ROS levels, while the knockdown of p66ShcA could effectively inhibit ROS elevation (Figure 2G). In addition, immunofluorescence assay showed that SOD2 expression was effectively inhibited in $H_2O_2$ group and siRNA NC groups, but in p66ShcA siRNA negative group, the expression of SOD2 was significantly higher than that in the former (Figure 2H).

### Knockdown of p66ShcA Inhibits $H_2O_2$-Induced H9c2 Inflammatory Response

First, we detected IL-1β and IL-6, and the expression of IL-1β and IL-6 protein in $H_2O_2$ group and siRNA NC group was significantly increased. The knockdown of p66ShcA could effectively inhibit the expression of IL-1β and IL-6 (Figure 3A and 3B). At the same time, mRNA levels (Figure 3C and 3D) and ELISA results (Figure 3E and 3F) were the same as before. Immunofluorescence results confirmed that $H_2O_2$ treatment can stimulate the expression of IL-1β, and its expression in siRNA NC group was significantly also increased, but the knockdown of p66ShcA can significantly inhibit IL-1β expression (Figure 3G).

### Downregulation of p66ShcA Expression Activates the Keap1/Nrf2 Pathway

The Keap1/Nrf2 pathway plays an important role in the regulation of ischemic and hypoxic car-

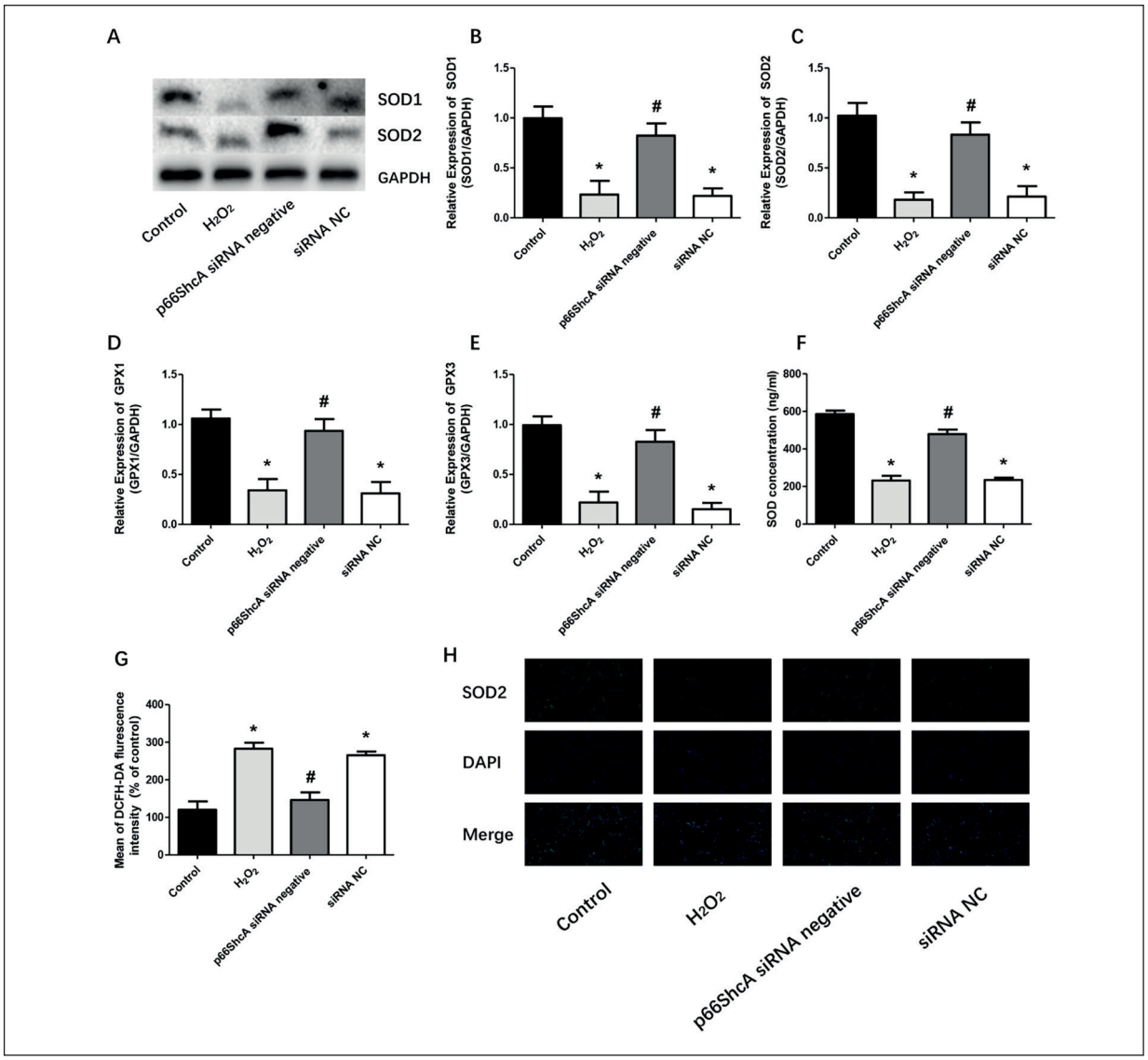


**Figure 2.** Knockdown of p66ShcA inhibits $H_2O_2$-induced H9c2 oxidative stress. **A,** Western blot was used to detect the expression of SOD1 and SOD2, and GAPDH was used as an internal control. **B-E,** Real Time-PCR was used to detect SOD1, SOD2, GPX1, and GPX3 levels. **F,** The ELISA method detects the SOD content. **G,** Flow cytometry was used to detect ROS levels. **H,** Immunofluorescence was used to detect SOD2 expression (magnification: 400×). ("*" indicates statistical difference from the control group $p<0.05$, "#" indicates statistical difference from the contrast group $p<0.05$).

diomyopathy. Western blot (Figure 4A) showed that $H_2O_2$ treatment can significantly increase Keap1 expression, but can inhibit the expression of Nrf2, and decreased expression of downstream factor HO-1, while the knockdown of p66ShcA can stimulate the expression of Nrf2 and HO-1 and inhibit the expression of Keap1. At the same time, similar results were obtained for mRNA levels (Figure 4B-4D). By immunofluorescence, we found that Nrf2 expression was significantly increased in the p66ShcA siRNA negative group, while Nrf2 expression was significantly inhibited in the $H_2O_2$ group and the siRNA NC group (Figure 4E). This indicates that knocking down p66ShcA activates the Keap1/Nrf2 pathway.

## Discussion

In the metabolic process, the body can generate superoxide anion, hydrogen peroxide molecules, hydroxyl radicals, and other oxygen-con-

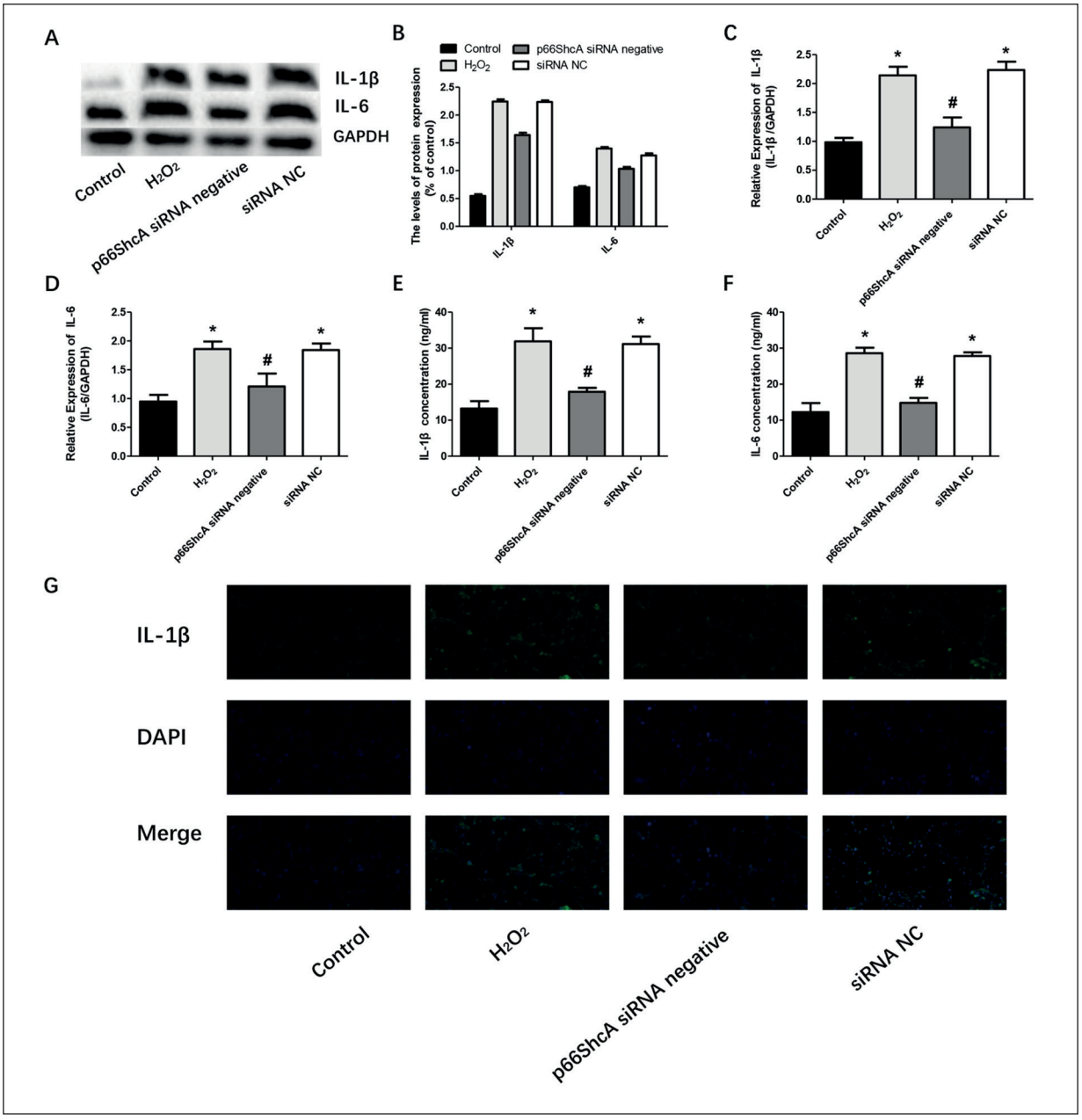


**Figure 3.** Knockdown of p66ShcA inhibits $H_2O_2$-induced H9c2 inflammatory response. **A, B,** Western blots detected IL-1β and IL-6 expression, GAPDH was used as an internal control. **C, D,** Real Time-PCR was used to detect IL-1β and IL-6 levels. **E, F,** The ELISA method was used to detect IL-1β and IL-6 levels. **G,** Immunofluorescence was used to detect IL-1β expression (magnification: 400×). ("*" indicates statistical difference from the control group $p<0.05$, "#" indicates statistical difference from the contrast group $p<0.05$).

taining free radicals called oxygen radicals[13]. Under physiological conditions, the production and elimination of oxygen free radicals are in a physiologically low level of equilibrium, which is beneficial to the human body, but under certain pathological conditions, due to the excess of these reactive oxygen species metabolites, the organism's biological macromolecules, such as proteins, lipids, nucleic acids, etc., cause damage[14]. As a member of the ROS, ROS can directly act on membrane lipids to form lipid peroxides, leading to damage to cell membranes[15]. We found that $H_2O_2$ treatment significantly promoted H9c2 secretion of LDH and MDA, and

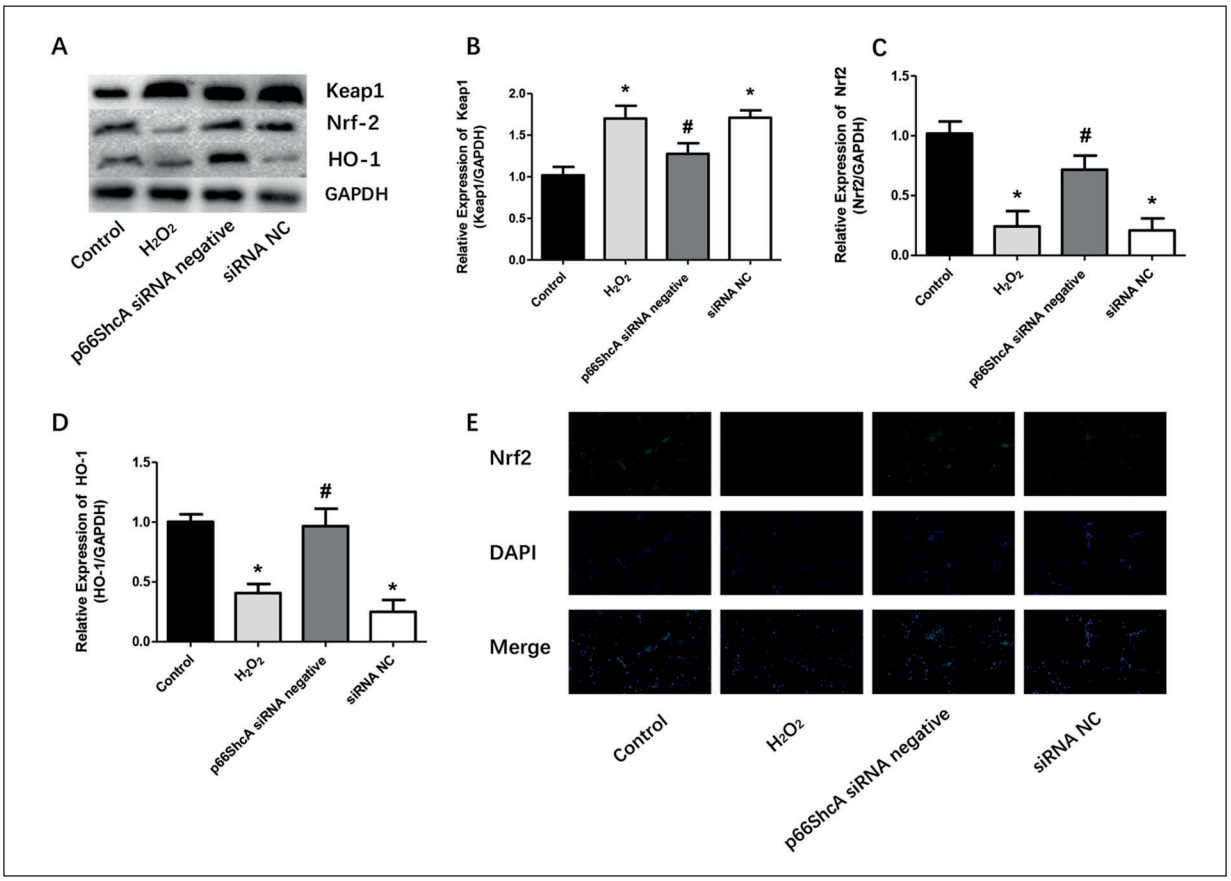


**Figure 4.** Downregulation of p66ShcA expression activates the Keap1/Nrf2 pathway. **A,** Western blot was used to detect Keap1.Nrf2 and HO-1 expression, GAPDH was used as an internal control. **B-D** Real Time-PCR was used to detect Keap1, Nrf2, and HO-1 levels. **E,** Immunofluorescence was used to detect Nrf2 expression (magnification: 400×). ("*" indicates statistical difference from the control group $p<0.05$, "#" indicates statistical difference from the contrast group $p<0.05$).

inhibited CAT secretion, while promoting intracellular ROS accumulation. The knockdown of p66ShcA can effectively inhibit the increase of LHD and MDA, promote the expression of SOD1 and SOD2, and reduce the level of ROS. Thereby, the $H_2O_2$-induced redox imbalance is restored, thereby inhibiting cell senescence and apoptosis.

Oxidative stress often leads to activation of the inflammatory pathway, and Karam et al[16] found that oxidative stress and inflammation are the key causes of atrial fibrillation. We found that $H_2O_2$ treatment resulted in increased expression of IL-1β and IL-6, while the knockdown of p66ShcA effectively inhibited the expression of IL-1β and IL-6. The above results indicate that p66ShcA has a regulatory effect on the regulation of inflammatory factor expression. Bosutti et al[17] found that plasma cholesterol levels in patients implanted with pacemakers were associated with inflammatory factors and p66ShcA levels. However, this study did not investigate on how p66ShcA affects inflammatory factor expression.

Nrf2 is a key regulator of maintenance of redox balance, especially under conditions that continuously activate the PI3K-AKT signal[18]. The activated PI3K-AKT pathway enhances the accumulation of Nrf2 in the nucleus, allowing Nrf2 to promote the expression of antioxidant, anti-inflammatory, and anti-apoptotic genes. The mechanism is to activate the AKT/Nrf2 pathway and increase the anti-oxidation of Nrf2-induced HO-1 and Cu/Zn SOD expression[19]. This study aimed to investigate the effect of Keap1/Nrf2 pathway on $H_2O_2$-induced H9c2 injury. The results showed that the knockdown of p66ShcA can activate the Keap1/Nrf2 pathway, thereby regulating the expression of anti-oxidative stress and anti-inflammatory related

factors in H9c2 cells. However, the mechanism of knockdown of p66ShcA to activate Keap1/Nrf2 pathway has not been studied in detail, so it needs further study.

## Conclusions

This work showed that downregulation of p66ShcA expression activates Keap1/Nrf2 pathway, thereby inhibiting $H_2O_2$-induced H9c2 cell redox imbalance and inflammatory response. Therefore, this provides a potential intervention target for the treatment of ischemic heart disease.

### Conflict of Interest

The Authors declare that they have no conflict of interests.

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