Ischemic preconditioning/Ischemic postconditioning alleviates anoxia/reoxygenation injury via the Notch1/Hes1/VDAC1 axis

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Abstract

Background

It has been demonstrated that ischemic preconditioning (IPC), and ischemic postconditioning (IPost) have a significant protective effect on myocardial ischemia/reperfusion (MI/R) injury by alleviating oxidative stress and mitochondrial disturbances, although the underlying molecular mechanisms are not clear. To demonstrate that cardioprotection against anoxia/reoxygenation (A/R) injury is transduced via the Notch1/Hes1/VDAC1 signaling pathway.

Methods

Using proteomics techniques, liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS) and tandem affinity purification (TAP), to screen for differentially expressed proteins associated with Hes1, followed by standard bioinformatics analysis. The co-immunoprecipitation (Co-IP) assay confirmed an interaction between Hes1 and VDAC1 proteins. H9c2 cells were transfected with Hes1 adenoviral N-terminal TAP vector (AD-NTAP/Hes1) and Hes1-short hairpin RNA adenoviral vector (AD-Hes1-shRNA) to establish A/R injury, ischemic preconditioning (IPC), and ischemic postconditioning (IPost) models, respectively. The expression of Hes1 and VDAC1 proteins were measured by western blotting, while the levels of intracellular reactive oxygen species (ROS), mitochondrial membrane potential (ΔΨm), and apoptosis were evaluated by flow cytometry.

Results

AD-NTAP/Hes1 can activate the exogenous protein expression of Hes1, thus decreasing creatine phosphokinase (CPK) and lactate dehydrogenase (LDH) activity and promoting cell viability. The study found that VDAC1 was a potential target protein for Hes1 and the overexpression of Hes1 protein expression downregulated protein expression levels of VDAC1, reduced ROS production, stabilized ΔΨm, and inhibited apoptosis in H9c2 cells. Additionally, downregulation of Hes1 protein expression also upregulated VDAC1 protein expression, increased ROS production, imbalanced ΔΨm, promoted cell apoptosis, and attenuated the cardioprotection afforded by IPC and IPost.

Conclusions

The Notch1/Hes1 signaling pathway activated by IPC/IPost can directly downregulate the protein expression of VDAC1 and consequently relieve A/R injury.

Background

Ischemic heart disease has serious global consequences resulting in high levels of morbidity and mortality [1]. The most effective current treatment is myocardial reperfusion using thrombolytic therapy, percutaneous coronary intervention, or coronary artery bypass grafting [2,3]. However, reperfusion itself can induce additional impairment, known as MI/R injury [4]. Target-oriented therapy of MI/R injury is of great importance but remains challenging due to the ambiguity of its underlying mechanisms [5], the pathophysiology of which mainly involves calcium overload, oxidative stress, and mitochondrial dysfunction which induce cardiomyocyte apoptosis [6]. Evidence suggests that MI/R leads to mitochondrial dysfunction combined with excessive production of ROS and opening of mitochondrial permeability transition pores (mPTPs) which can, in turn, exacerbate mitochondrial dysfunction, thereby resulting in a self-amplifying vicious cycle [6,7]. Therefore, maintenance of mitochondrial function has been identified as an essential therapeutic target to prevent cardiomyocyte apoptosis during MI/R injury.

The well-conserved Notch1 signaling pathway can regulate a variety of intracellular processes, including cell cycle regulation, cell differentiation, and apoptosis [8]. Several studies have demonstrated convincingly that activation of the Notch1 signaling pathway plays a key role in myocardial protection, and conversely, its dysregulation promotes a variety of cardiac diseases, such as pulmonary hypertension, ventricular septal defect, and perinatal cardiomyopathy [9–12]. Recently, several pieces of evidence showed that Notch signaling may play a pivotal role in the estrogen-mediated cardiac effects [13]. For instance, estrogen receptor β activation exerts its cardioprotective function through the Notch1/PI3K/Akt signaling cascade [14]. Notch1 mediates preconditioning protection induced by protein-coupled oestrogen receptor in normotensive and hypertensive female rat hearts [15]. In a previous study, we revealed that Notch1 signaling was activated during myocardial IPC/IPost, thus enhancing cell viability and inhibiting apoptosis [12,16,17]. Moreover, activation of Notch1 signaling can stabilize ΔΨm and reduce ROS during MI/R injury [18]. Additionally, activation of the Notch1 signaling pathway can regulate mitochondrial fusion and function, thereby preventing MI/R injury [19]. It has been suggested that there is a close connection between Notch1 signaling and mitochondrial function, but the precise molecular mechanism remains unclear. Several studies have reported that the mitochondrial proteome could be modified by activation of the Notch1 signaling pathway [20]. However, there is no evidence as to whether the pathway can preserve mitochondrial function in MI/R injury via VDAC1. Hes1, which is a classical downstream target gene of the Notch1 signaling pathway, encodes a repressive basic Helix-Loop-Helix that functions as a transcriptional repressor [21,22]. Hes1 is associated with the PTEN/Akt signaling pathway that produces antioxidant/anti-nitrification effects [23]. It can also participate in the formation of the Hes1-Stat3 complex which promotes Stat3 phosphorylation and HIF-1α activation, in turn preventing MI/R injury [24,25]. It has been suggested that Hes1 may be a pivotal molecule between the Notch1 signaling pathway and other endogenous factors or signaling pathways. In recent years, cardiovascular proteomics has flourished, becoming a key technique in modern biotechnology. Through the use of proteomics, differentially expressed proteins in cardiovascular diseases can be studied and screened for functional studies and in-depth validation [26]. By combining multiple proteomics techniques, the function of target proteins can be more accurately studied by combining the respective advantages of the different techniques [27].

Here, we hypothesize that Notch1 may interact with the mitochondrial proteome through the downstream target Hes1, thus preventing MI/R injury. Proteomics methods (LC-ESI-MS/MS and TAP) were utilized to screen for differentially expressed ligand proteins of Hes1, and bioinformatics analysis was performed of a particular differentially expressed ligand-protein that was potentially relevant to myocardial protection. Interestingly, we found that the activated Notch1/Hes1 signaling pathway induced by myocardial IPC/IPost was able to protect H9c2 cells against A/R induced injury via its interaction with VDAC1.

Materials And Methods

Reagents

Hes1 antibody was purchased from Abcam (Cambridge, UK), and VDAC1 and β-actin antibodies were obtained from Santa Cruz (Dallas, Texas, USA). AD-Hes1-shRNA and AD-NTAP/Hes1 were provided by Hanbio Biotechnology (Shanghai, China), and the NTAP tag was a tandem combination of streptavidin binding peptide (SBP) tag and calmodulin binding peptide (CBP) tag, which loaded onto the pNTAP vector through the InterPlay Adenoviral TAP system. ROS assay kits and mitochondrial staining kits (5,5',6,6'-Tetrachloro-1,1',3,3'-tetraethyl-imidacarbocyanine, JC-1) were purchased from Invitrogen. CPK and LDH assay kits were supplied by Jiancheng Bioengineering Institute (Nanjing, China).

Cell culture and model establishment

H9c2 cardiomyocytes, obtained from the Cell Bank of Shanghai Institutes for Biological Sciences, were cultured in Dulbecco's modified Eagle's medium (DMEM; Hyclone) containing 4.5 g/L glucose and supplemented with 10% fetal bovine serum (FBS; Hyclone, Logan, UT, USA), 100 U/ml penicillin and 100 mg/ml streptomycin (New Cell Molecular Biotech, Suzhou, China) in a humidified incubator (37 °C, 95% air, 5% CO_2).

The experimental design was as follows: (1) Control group: H9c2 cardiomyocytes routinely cultured as described previously. (2) A/R group: The A/R model was established as previously described [28]. Briefly, H9c2 cells underwent anoxia using a simulated anoxia buffer (6.0 mM NaHCO3, 98.5 mM NaCl, 0.9 mM NaH2PO4, 10.0 mM KCl, 1.8 mM CaCl2, 1.2 mM MgSO4, 20 mM HEPES, and 40.0 mM Sodium lactate, pH = 6.8) in a tri-gas incubator with 95% N2 and 5% CO2 for 3 h at 37 °C.Then, anoxia buffer was changed to reoxygenation buffer (20 mM NaHCO3, 129.5 mM NaCl, 0.9 mM NaH2PO4, 5.0 mM KCl, 1.8 mM CaCl2, 1.2 mM MgSO4, 20 mM HEPES, 5.5 mM Glucose, pH = 7.4) incubated with an 95% O2 and 5% CO2 for 2 h at 37 °C. (3) IPC group: The IPC model was established following the published protocols with a minor modification [12, 29, 30]. Briefly, under sterile conditions, H9c2 cardiomyocytes were preincubated for 15 min in the simulated anoxia buffer in a hypoxic incubator (95% N2 - 5% CO2) as described above and then incubated in simulated reoxygenation buffer in a high oxygen incubator (95% O2-5% CO2) for another 15 min. This process was repeated 3 times to induce anoxic preconditioning and then followed by the A/R treatment as previously described in the A/R group. (4) IPost group: According to our previously published

protocols with a minor modification [12, 31-33]. The IPost model was established as follows: At the end of 3 h of hypoxia, subsequently, H9c2 cardiomyocytes were exposed to 3 cycles of 15 min of reoxygenation and then 15 min of hypoxia, and at last, followed by 2 h of reoxygenation. (5) A/R+AD-NTAP/Hes1 group: H9c2 cardiomyocytes were treated as the cells in the A/R group then subsequently transfected with AD-NTAP/Hes1. (6) IPC+AD-Hes1-shRNA group: H9c2 cardiomyocytes were transfected with AD-Hes1-shRNA for 48 h then subjected to the same treatment as cells in the IPC group. (7) IPost+AD-Hes1-shRNA group: H9c2 cardiomyocytes were transfected with AD-Hes1-shRNA for 48 h then treated the same way as cells in the IPost group.

CPK, LDH release, and Cell viability assay

CPK and LDH release and the viability of cells in each experimental group were measured using an LDH assay kit (Jiancheng Bioengineering Institute, Nanjing, China), CPK assay kit (Jiancheng Bioengineering Institute, Nanjing, China), and a commercial CellTiter 96® Aqueous One solution cell proliferation assay (Promega), respectively, in accordance, in each case, with the manufacturer's instructions.

Extraction and purification of Hes1 ligand-protein by TAP

Total proteins were extracted from AD-NTAP/Hes1 infected H9c2 cardiomyocytes after 48-72 h, in accordance with the InterPlay TAP purification kit (Agilent Technologies,USA) instructions. The total number of infected H9c2 cardiomyocytes was approximately 3×10^8, while the quantity of reagents for this experimental step was calculated based on 10^7 cells.

LC-ESI-MS/MS analysis

The peptides were separated using an LC-20AD nanoliter liquid chromatograph in accordance with the manufacturer's instructions. The peptides were separated by liquid phase separation into a Q-EXACTIVE mass spectrometer (primary mass spectrometry resolution: 70,000, secondary resolution: 17,500). Fifteen parent ions with charges of 2+~5+ and peak intensities greater than 20000 were selected for secondary analysis. The peptides were fragmented in HCD mode using a collision energy of 27, after which the fragments were detected in Orbi software. The following parameters were used: Dynamic exclusion time: chromatographic half-peak width duration; ion source voltage: 1.6 kV. Automatic gain control was achieved using Orbi, by setting the mass-to-charge ratio range of the scan (primary: 350-2000; secondary: 100-1800).

Standard bioinformatics analysis

The original mass spectrometry files were converted into mass spectrometry peak files and then searched against sequences in protein databases, in this case the ipi_rat database (39925 sequences), using protein identification software Mascot 2.3.02. The search results were filtered and quality controlled in order to identify the proteins. The results of the identified proteins were analyzed by functional annotation, including gene ontology (GO) and Clusters of Orthologous Groups (COG) databases.

Co-IP assay

Proteins were collected from H9c2 cardiomyocytes, lysed in pre-cooled RIPA buffer then incubated with an anti-VDAC1 or anti-GST-tag antibody (as negative control) overnight at 4 °C. The proteins were then incubated for 4-5 h at 4 °C with protein A/G agarose suspension (Merck Millipore, Germany) which was pre-washed with cold PBS. The mixture was then centrifuged for 5 min at 4,000 g at 4 °C, from which the supernatant was collected for subsequent analysis and the pellet washed 3 times in pre-cooled PBS followed by recentrifugation for 5 min at 4,000 g at 4 °C to obtain the immunoprecipitation complex. The supernatant and immunoprecipitation complex were boiled in 1× SDS-PAGE sample buffer and then prepared for Western blot analysis.

Western Blotting Assay

Proteins were isolated from H9c2 cardiomyocytes in accordance using a method described previously [34]. Protein concentration was measured using a Bradford assay (Beyotime). Protein samples (40 μg) were separated using 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to polyvinylidene difluoride membranes. Following blocking with 5% skimmed milk for 2 h at 37 °C, the membranes were then incubated with primary antibodies overnight against Hes1 (Abcam, Cambridge, MA, USA), VDAC1 (Abcam), and β-actin (Cell Signaling Technology). The molecular weight of exogenous NTAP/Hes1 and endogenous Hes1 is about 50 kDa and 35 kDa, respectively. The membranes were then washed with 1×TBST 3 times then incubated with a secondary antibody (Cell Signaling Technology) for 2 h at room temperature. Finally, protein bands were visualized using enhanced chemiluminescence (GE Healthcare, USA) after incubation with an enhanced chemiluminescence substrate and quantified using a Quantity One System image analyzer (Bio-Rad Laboratories, Hercules, CA, US).

ROS production assay

The production of ROS was measured using a ROS assay kit (Invitrogen, USA) in accordance with the manufacturer's instructions, as we have described in our previous study [34]. Briefly, treated

H9c2 cardiomyocytes were mixed with DMEM supplemented with 10 µM DCFH-DA (Invitrogen, USA) and then incubated for 20 min at 37 °C in the dark. Fluorescence was monitored at excitation and emission wavelengths (ex/em) of 485 and 528 nm by flow cytometry (Becton Dickinson, USA).

Mitochondrial Membrane Potential (ΔΨm) assay

A mitochondrial staining kit (JC-1) (Invitrogen, USA) was used to measure ΔΨm concentration, following the manufacturer's instructions, as we have described in a previous study [34]. JC-1 was added to a final concentration of 200 µM in H9c2 cardiomyocytes and then incubated in the dark for 20 min at 37 °C from which fluorescence was measured using flow cytometry (Becton Dickinson, USA) at ex/em of 530/580 nm (red) and then at 480/530 nm (green), respectively. MMP was calculated from the ratio of red to green fluorescence.

Flow cytometric analysis of apoptosis

An Annexin V-FITC/PI apoptosis detection kit (BD Biosciences, San Jose, CA, USA) was utilized to detect the apoptosis of H9c2 cardiomyocytes. Briefly, the H9c2 cardiomyocytes cells were trypsinized, resuspended in 100 µL 1x binding buffer, and then incubated with 5 µL annexin V-FITC and 5 µL PI in the dark for 15 min. Apoptosis was identified in cells using a FACScan flow cytometer using CellQuest[TM] software (BD Biosciences, San Jose, CA, USA) by virtue of their green but not red fluorescence.

Statistical analysis

All values were presented as mean ± SEM. One-way ANOVA was used to compare the biochemical indices of different groups. SPSS 18.0 was used for statistical analysis. A p-value less than 0.05 was considered statistically significant.

Results

AD-NTAP/Hes1 upregulated the expression of Hes1, decreased CPK and LDH activity, and increased cell viability in H9c2 cells suffering A/R injury.

Firstly, AD-NTAP/Hes1 constructed in a previous study was transfected into H9c2 cells, in order that an A/R injury model was established, allowing cell viability, and LDH and CPK activity to be measured in different groups. The results demonstrated that Hes1 protein expression in the A/R group was slightly higher than that of the Control group, although not statistically significantly so, while there was greater Hes1 protein expression in the A/R+AD-NTAP/Hes1 group than in the A/R group (p<0.01) (**Figure 1A**),

indicating that AD-NTAP/Hes1 upregulates the expression of Hes1 protein after A/R injury. LDH, CPK, and cell viability were measured to confirm that AD-NTAP/Hes1 exhibited biological and myocardial protective functionality (**Figures 1B, 1C, and 1D**). Compared with the Control group, CPK and LDH activity was significantly higher in the A/R group (p<0.01) but lower in the A/R+AD-NTAP/Hes1 group (p<0.01). Cell viability of the A/R+AD-NTAP/Hes1 group was lower than that of the Control group but significantly higher than that of the A/R group (p<0.01). Therefore, AD-NTAP/Hes1 was able to upregulate the expression of Hes1 protein following A/R injury, further reducing the CPK and LDH activity and enhancing cell viability, thus exerting a cardioprotective effect.

Figure 1: Hes1 decreases CPK and LDH activity and increases cell viability in H9c2 cells suffering A/R injury. (A) Western blotting was used to confirm the exogenous NTAP/Hes1 and endogenous Hes1 upregulation in A/R+AD-NTAP/Hes1 cells. The molecular weight of exogenous NTAP/Hes1 and endogenous Hes1 is 50 kDa and 35 kDa, respectively; **(B-D)** CPK and LDH activity and cell viability in each group. **p <0.01 vs. Control group; ▲▲p < 0.01 vs. A/R group. Data are expressed as the mean ± SEM, *n* = 5.

A/R: anoxia/reoxygenation; CPK: creatine phosphokinase; LDH: lacate dehydrogenase; NTAP: N-terminal tandem affinity purification.

Extraction and screening of ligand-proteins (VDAC1) binding to Hes1 in H9c2 cells.

To explore the molecular mechanism of the cardioprotective effect of Hes1 protein, ligand-protein complexes that potentially interact with Hes1 protein were screened using proteomics methods (TAP and LC-ESI-MS/MS). Firstly, total protein extracted from H9c2 cells transfected with AD-NTAP/Hes1 was purified with an InterPlay TAP purification kit then separated by SDS-PAGE then silver-stained. As show in **Figure 2A**, Hes1 ligand protein was purified by TAP Successfully. Line 1 is the protein supernatant after binding streptavidin resin, Line 2 is the protein solution combined with streptavidin resin, Line 3 is the protein supernatant after binding calmodulin resin, and Line 4 is the final eluate of Hes1 ligand protein after purification by TAP. The heterobands in Line 4 were obviously reduced, the molecular weight of exogenous NTAP/Hes1 was about 50 kDa.

Subsequently, a total of 88 proteins containing 25 non-specific, 21 unknown, and 42 possibly interacting proteins (Table 1) were characterized by LC-ESI-MS/MS. The functional annotations of the proteins were conducted using GO and COG database (**Figures 2B-E**). Following the bioinformatics analysis and a search of the literature, VDAC1 was finally selected as the ligand-protein binding to Hes1 in follow-up research. Co-IP experiments were performed to confirm that Hes1 interacts with VDAC1, using anti-GST-tag antibody as a negative control. As expected, Hes1 successfully co-immunoprecipitated with VDAC1 but failed to precipitate with GST-Tag, confirming that Hes1 can interact with VDAC1 (**Figure 2F**).

Figure 2: Identification and analysis of Hes1 ligand-protein. (A) SDS-PAGE colloidal silver staining verifies purification by TAP, Line 1 is the protein supernatant after binding streptavidin resin, Line 2 is the protein solution combined with streptavidin resin, Line 3 is the protein supernatant after binding calmodulin resin,

and Line 4 is the final eluate of Hes1 ligand protein after purification by TAP. The molecular weight of exogenous NTAP/Hes1 and endogenous Hes1 is 50 kDa and 35 kDa, respectively. **(B-D)** GO classification chart (pie chart represents the percentage of items in total protein). **(E)** COG classification chart. **(F)** Western blotting identification of Hes1 protein expression following Co-IP.

COG: cluster of orthologous groups; Co-IP: co-immunoprecipitation; GO: gene ontology; SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis.

Table 1
Identification of proteins

Sample	Total number of spectra	Number of identification spectra	Identify the number of peptides	Identify the number of proteins
TAP purified protein	7239	360	145	88
TAP: tandem affinity purification.				

Relationship between Hes1 and VDAC1 in H9c2 cells.

Although Hes1 interacts with VDAC1, as demonstrated by the results above, the mechanism by which Hes1 affects the expression of VDAC1 remains unclear. To further explore the influence of Hes1 on VDAC1, we established A/R, IPC, and IPost models to analyze the expression of Hes1 and VDAC1 proteins by western blotting after transfecting H9c2 cells with AD-NTAP/Hes1 and AD-Hes1-shRNA, respectively **(Figure 3)**. Interestingly, compared with the Control group, Hes1 expression in the A/R group was slightly higher, but not significantly so, while VDAC1 expression was significantly upregulated ($p<0.01$). Compared with the A/R group, Hes1 expression in the IPC, IPost, and A/R+AD-NTAP/Hes1 groups was significantly upregulated, while VDAC1 was correspondingly significantly downregulated ($p<0.01$). We can conclude that IPC, IPost, and AD-NTAP/Hes1 activate Hes1 and downregulate VDAC1 expression. In contrast, in the IPC+AD-Hes1-shRNA and IPost+AD-Hes1-shRNA groups, Hes1 protein expression was downregulated and the expression of VDAC1 protein upregulated ($p<0.01$, IPC+AD-Hes1-shRNA group vs. IPC group, IPost+AD-Hes1-shRNA group vs. IPost group). This, therefore, confirmed that AD-Hes1-shRNA is able to inhibit the activation of Hes1 in IPC and IPost and upregulate the expression of VDAC1. In summary, we can speculate that the Notch1/Hes1 signaling pathway directly downregulates the expression of VDAC1, thereby exhibiting cardioprotective functionality.

Figure 3: Relationship between Hes1 and VDAC1 in H9c2 cells. Western blotting was used to measure the expression of Hes1 and VDAC1 in each experimental group. **$p <0.01$ vs. Control group; ▲▲$p < 0.01$ vs. A/R group; ##$p<0.01$ vs. IPC group; ■■$p <0.01$ vs. IPostC group. Data are expressed as the mean ± SEM, n = 3.

IPC: ischemic preconditioning; IPostC: ischemic postconditioning; VDAC1: voltage dependent anion channel 1.

Hes1 inhibits ROS generation and plays a protective role in IPC and IPost.

To verify the relationship between myocardial protection and Hes1/VDAC1, we further examined the change in ROS generation. As shown in **Figure 4**, the generation of ROS in the A/R group was significantly higher than that in the Control group (p<0.01), while ROS generation in the IPC, IPost, and A/R+AD-NTAP/Hes1 groups was significantly lower than that in the A/R group (p<0.01). In the AD-Hes1-shRNA treatment group, ROS generation was significantly higher (p<0.01, IPC+AD-Hes1-shRNA group vs. IPC group, IPost+AD-Hes1-shRNA group vs. IPost group). The results demonstrate that Hes1 can inhibit the generation of ROS and plays a protective role in IPC and IPost, possibly related to the direct downregulation of VDAC1 expression mediated by the Notch1/Hes1 signaling pathway.

Figure 4: Effect of Hes1 on the generation of ROS in ischemic myocardial cells. (A) ROS flow cytometry; (B) histogram of ROS generation. **p <0.01 vs. Control group; ▲▲p < 0.01 vs. A/R group; ##p<0.01 vs. IPC group; ■■p <0.01 vs. IPostC group. Data are expressed as the mean ± SEM, n = 3.

ROS: reactive oxygen species.

Hes1 stabilizes ΔΨm of A/R-injured H9c2 cells and plays a role in the myocardial protection in IPC and IPost.

ΔΨm was calculated using the ratio of the number of cells in the upper right quadrant to the lower right quadrant in a graph of red and green fluorescence. As displayed in **Figure 5**, the red/green fluorescence ratio of the A/R group was lower than that of the control group (p<0.01). The red/green fluorescence ratio of the IPC, IPost, A/R+AD-NTAP/Hes1 groups was higher than that of the A/R Group (p<0.01). In the AD-Hes1-shRNA treatment group, the red/green fluorescence ratio decreased (p<0.01, IPC+AD-Hes1-shRNA group vs. IPC group, IPost+AD-Hes1-shRNA group vs. IPost group). These results suggest that Hes1 stabilizes the ΔΨm in ischemic cardiomyocytes and plays a role in the protection in IPC and IPost. This effect may be related to the direct downregulation of VDAC1 expression by the Notch1/Hes1 signaling pathway.

Figure 5: Effect of Hes1 on the ΔΨm of ischemic myocardial cells. (A) Flow cytometric analysis of ΔΨm; (B) ΔΨm bar graph. **p <0.01 vs. Control group; ▲▲p < 0.01 vs. A/R group; ##p<0.01 vs. IPC group; ■■p <0.01 vs. IPostC group. Data are expressed as the mean ± SEM, n = 3.

ΔΨm: mitochondrial membrane potential.

Hes1 can reduce apoptosis in A/R injured H9c2 cells and participate in protection in IPC and IPost.

The effect of Hes1 on the apoptosis of A/R injured H9c2 cells (**Figure 6**) was tested. Compared with the Control group, apoptosis was significantly increased (p<0.01) in the A/R, IPC+AD-Hes1-shRNA, and IPost+AD-Hes1-shRNA treatment groups but reduced in the IPC, IPost, and A/R+AD-NTAP/Hes1 treatment groups p<0.01). The results suggest that Hes1 reduces apoptosis in A/R injured H9c2 cells and

participates in protection in IPC and IPost. Thus, Hes1 may directly downregulate VDAC1 expression via the Notch1/Hes1 signaling pathway.

Figure 6: Effect of Hes1 on apoptosis in ischemic cardiomyocytes. (A) Flow cytometric analysis of apoptosis; (B) Histogram of apoptosis. **p <0.01 vs. Control group; ▲▲p < 0.01 vs. A/R group; ##p<0.01 vs. IPC group; ■■p <0.01 vs. IPostC group. Data are expressed as the mean ± SEM, n = 3.

Discussion

As morbidity due to myocardial infarction has increased combined with the widespread use of revascularization therapy, MI/R injury has now become a common pathophysiological phenomenon in clinical practice, resulting in the presentation of arrhythmias, myocardial electrical shocks, and heart failure [35]. Therefore, mitigation of MI/R injury has now become an urgent clinical issue.

Mitochondria, that have been recognized as key triggers of MI/R injury, are abundant in cardiomyocytes and provide more than 90% of the energy supply. Mitochondria not only promote cardiomyocyte death by inducing apoptosis or necroptosis in MI/R injury, but also trigger and regulate many pathological processes, such as calcium overload, oxidative stress, and immune response [36-38]. Recent studies show that the mitochondrial quality control system plays an important role in preserving mitochondrial structure and function, improving mitochondrial biochemical processes and signal transduction, and regulating mitochondria-dependent programmed cell death [39-41]. They are therefore a potential target for cardioprotective intervention [18, 42].

Since the concept of IPC and IPost was proposed 30 years ago, it has been demonstrated that both of them have a significant protective effect on MI/R injury by activating multiple signaling pathways [43]. Interestingly, these signaling pathways have a common endpoint, which is the mitochondria[7]. Vélez *et al.* [44] demonstrated that IPC can act on mitochondria through the PI3K signaling pathway, thereby promoting ATP synthesis and cellular autophagy and reducing mitochondrial damage, thus exerting a protective effect on cardiomyocytes. González *et al.* [45] found that IPC and IPost exert myocardial protection by acting on mitochondria through PKC-ε/VDAC, reducing mitochondrial permeability. IPC activates downstream protective pathways against I/R injury by triggering the opening of mitochondrial Cx43 HCs [46]. In contrast, a reduction in mitochondrial Cx43 content was shown to abolish IPC-mediated cardioprotection [47]. These reports suggest that the endogenous protection mechanisms of IPC and IPost are closely related to mitochondrial regulatory mechanisms.

Hes1 is a transcription factor regulated by the Notch1 signaling pathway and acts as a crosstalk hub between Notch1 signaling and a variety of other signaling pathways [21, 22, 48]. Cancer studies have demonstrated that the Notch1/Hes1 signaling pathway is involved in cancer genesis and progression through crosstalk with other oncogenic signaling pathways such as PI3K-Akt [49] and NF-κb [50], and the WNT signaling pathway [51]. Studies in neuroprotection have demonstrated that the Notch1/Hes1

signaling pathway has cross-talked with the NF-κb pathway against cerebral ischemia-reperfusion injury [52]. The Notch1/Hes1 signaling pathway can activate the PI3K/Akt signaling pathway and regulates the PTEN/Akt signaling pathway through Hes1 to reduce ROS generation, stabilizing ΔΨm, which ultimately reduces apoptosis, thus exerting a myocardial protective effect [12, 17]. Therefore, we speculate that Hes1 may be a bridge between the Notch1 signaling pathway and other endogenous factors or signaling pathways. Based on our previous findings that activation of the Notch1 signaling pathway regulates mitochondrial fusion and function to prevent myocardial ischemia-reperfusion injury [19], we further speculate that Notch1/Hes1 may mediate mitochondrial function via linking with particular mitochondrial proteins. Interestingly, we identified the expected outer mitochondrial membrane protein VDAC1 using a Co-IP assay and MS analysis. No studies have so far demonstrated a connection between Hes1 and VDAC1 and thus, we have identified for the first time a direct molecular target of the Notch1/Hes1 signaling pathway located in mitochondria.

Rigaut *et al.* [53] first proposed the TAP technique to obtain specific protein complexes close to their natural state after two consecutive affinity purifications using a special protein tag. After years of improvement and optimization by other researchers, it is now widely used for the study of protein-protein interactions in proteomics. Currently, TAP technology has superior specificity, sensitivity, and reliability and so obtains more closely interacting proteins, which are more closely related to physiological protein interactions. TAP-MS coupling technology has become a common means to identify protein interactions, characterize protein complexes, and complete protein networks [54]. It has been used to validate mixed lineage kinase domain-like kinases, such as mixed lineage kinase domain-like protein and heat shock protein 90 [55]. We identified a total of 88 proteins of which 25 were non-specific proteins, 21 that were unknown in the database, and 42 possibly-interacting proteins, including VDAC1, using TAP and LC-ESI-MS/MS proteomic techniques.

VDAC is located in the outer mitochondrial membrane and has three isoforms (VDAC1-3) in mammalian cells. VDAC1 is the most widely expressed protein, followed by VDAC2, then VDAC3 [56]. VDAC1 plays many important functions in cells, including maintaining mPTP stability, regulating mitochondrial morphological structure, and participating in mitochondrial-mediated apoptosis [57, 58]. Huang *et al.* [59] found that cyathin-R mediates apoptosis through VDAC1 in Bax/Bak-deficient cells, a process that is dependent on VDAC1. Martel *et al.* [60] demonstrated that VDAC1 interacts with multiple molecules, such as nicotinamide adenine dinucleotide, lipids, GSK3, Bax, Bcl-2, and adenine nucleotide translocase. Their interactions also demonstrate the decisive role of VDAC1 in cell fate. Liao *et al.* [61] found that VDAC1 protein expression was upregulated during myocardial injury, and resveratrol, an active monomer in traditional Chinese medicine, can also exert myocardial protective effects via the downregulation of VDAC1 protein expression, decreasing CPK and LDH activity, inhibiting caspase-3 activity, reducing apoptosis, and ultimately reducing the area of myocardial infarcts.

We then demonstrated that IPC and IPost can activate Notch/Hes1 and regulate VDAC1 expression levels, thereby attenuating A/R injury in H9c2 cardiomyocytes. Because previous studies have demonstrated

that VDAC1 is a critical protein in mitochondrial-mediated apoptosis [62], we hypothesized that Notch/Hes1 may inhibit apoptosis via regulation of VDAC1. We further demonstrated that overexpression or silencing of Hes1 could correspondingly ameliorate or exacerbate apoptosis in cardiomyocytes in A/R injury, which was accompanied by a decrease or increase in VDAC1 expression. We further demonstrated that Hes1 overexpression corresponded to reduced apoptosis in cardiomyocytes in A/R injury, accompanied by decreased VDAC1 expression, reduced ROS production, and improved ΔΨm stability, whereas silencing Hes1 reversed these effects.

Conclusions

In summary, the study demonstrated that IPC and IPost can activate the Notch/Hes1 signaling pathway in hypoxia/reoxygenation-induced injury of H9c2 cardiomyocytes. The molecular mechanism of this process may be the direct binding of Hes1 to, and the negative regulation of the mitochondrial protein VDAC1, reducing ROS production, stabilizing ΔΨm, and ultimately reducing H9c2 cardiomyocyte apoptosis.

Abbreviations

AD-Hes1-shRNA   Hes1-short hairpin RNA adenoviral vector

AD-NTAP/Hes1   Hes1 adenoviral N-terminal TAP vector

A/R: anoxia/reoxygenation

COG: cluster of orthologous groups

Co-IP: co-immunoprecipitation

CPK: creatine phosphokinase

GO: gene ontology

IPC: ischemic preconditioning

IPost: ischemic postconditioning

JC-1:   5,5',6,6'-Tetrachloro-1,1',3,3'-tetraethyl-imidacarbocyanine

LC-ESI-MS/MS: liquid chromatography-electrospray ionization-tandem mass spectrometry

LDH: lactate dehydrogenase

MI/R: myocardial ischemia/reperfusion

mPTPs: mitochondrial permeability transition pores

ROS   reactive oxygen species

SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis

TAP: tandem affinity purification

ΔΨm   mitochondrial membrane potential

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

The data used to support the findings of this study are available from the corresponding author upon request.

Competing interests

The authors declare no conflicts of interest.

Funding

Authors' contributions

Lijun Wang, Huang Huang, and Jichun Liu provided substantial contributions to the conception and design of this study and drafted the article or critically revised it for important intellectual content. Lijun

Wang, Songqing Lai, Xueliang Zhou, Qing Wan, Yong Luo, Huaxi Zou, Qicai Wu, and Li Wan performed data acquisition, data analysis, and interpretation. All authors gave final approval of the version to be published and agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of the work are appropriately investigated and resolved.

Acknowledgements

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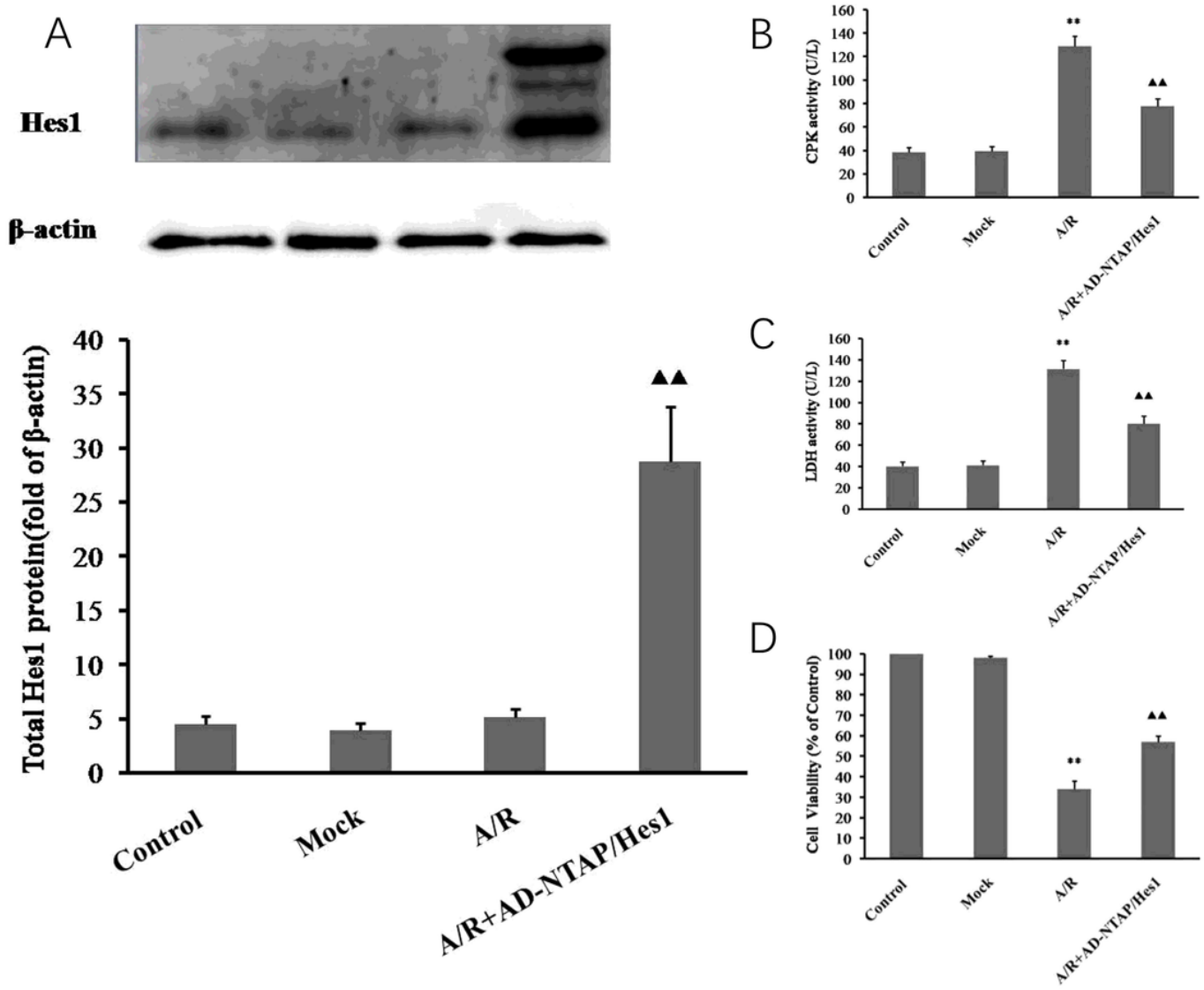
Figures

Figure 1

Hes1 decreases CPK and LDH activity and increases cell viability in H9c2 cells suffering A/R injury. (A) Western blotting was used to confirm the exogenous NTAP/Hes1 and endogenous Hes1 upregulation in A/R+AD-NTAP/Hes1 cells. The molecular weight of exogenous NTAP/Hes1 and endogenous Hes1 is 50 kDa and 35 kDa, respectively; (B-D) CPK and LDH activity and cell viability in each group. $**p <0.01$ vs. Control group; ▲▲$p < 0.01$ vs. A/R group. Data are expressed as the mean ± SEM, n = 5. A/R: anoxia/reoxygenation; CPK: creatine phosphokinase; LDH: lacate dehydrogenase; NTAP: N-terminal tandem affinity purification.

Figure 2

Identification and analysis of Hes1 ligand-protein. (A) SDS-PAGE colloidal silver staining verifies purification by TAP, Line 1 is the protein supernatant after binding streptavidin resin, Line 2 is the protein solution combined with streptavidin resin, Line 3 is the protein supernatant after binding calmodulin resin, and Line 4 is the final eluate of Hes1 ligand protein after purification by TAP. The molecular weight of exogenous NTAP/Hes1 and endogenous Hes1 is 50 kDa and 35 kDa, respectively. (B-D) GO classification chart (pie chart represents the percentage of items in total protein). (E) COG classification chart. (F) Western blotting identification of Hes1 protein expression following Co-IP. COG: cluster of orthologous groups; Co-IP: co-immunoprecipitation; GO: gene ontology; SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis.

Figure 3

Relationship between Hes1 and VDAC1 in H9c2 cells. Western blotting was used to measure the expression of Hes1 and VDAC1 in each experimental group. **p <0.01 vs. Control group; ▲▲p < 0.01 vs. A/R group; ##p<0.01 vs. IPC group; ■■p <0.01 vs. IPostC group. Data are expressed as the mean ± SEM, n = 3. IPC: ischemic preconditioning; IPostC: ischemic postconditioning; VDAC1: voltage dependent anion channel 1.
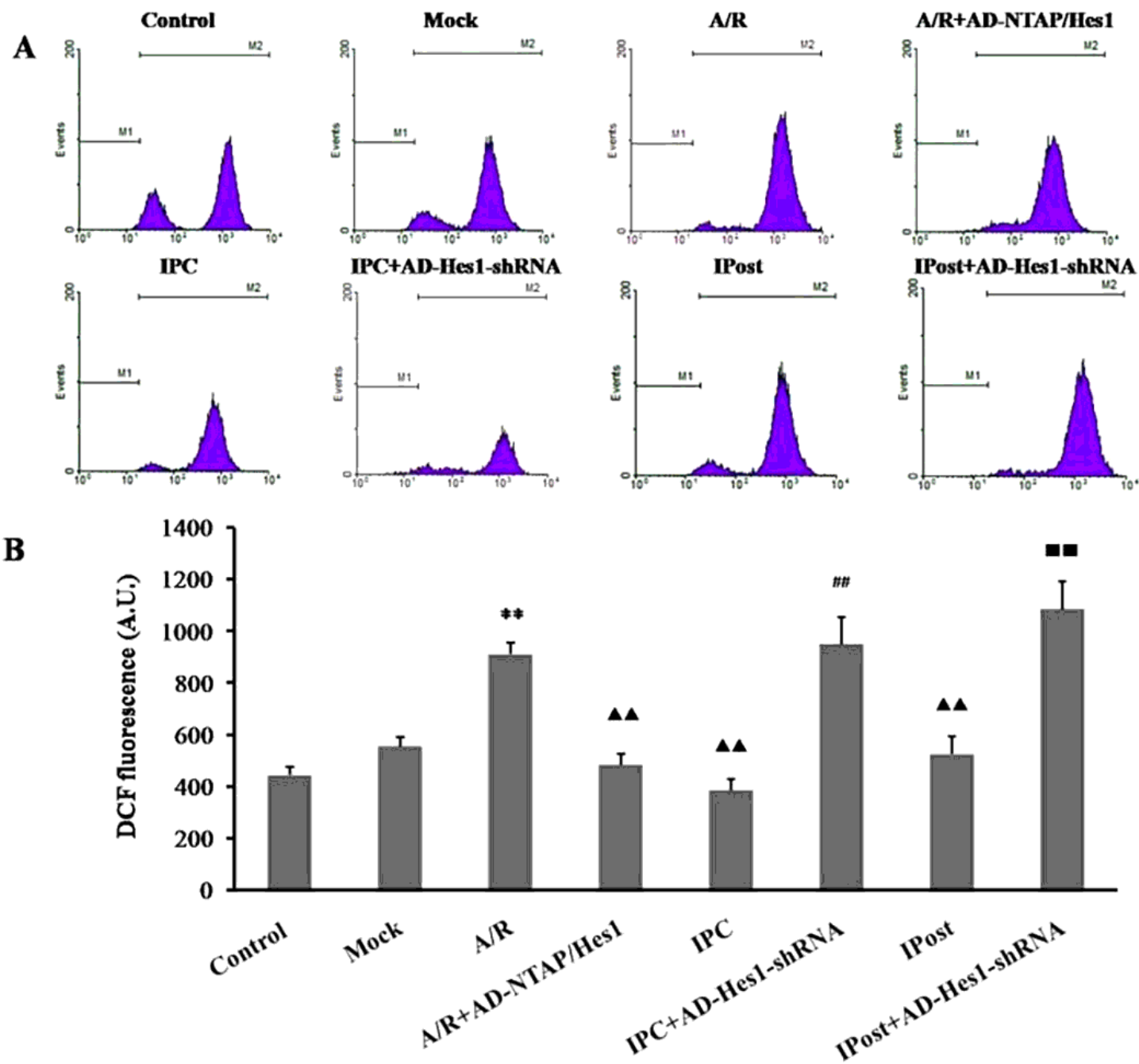
Figure 4

Effect of Hes1 on the generation of ROS in ischemic myocardial cells. (A) ROS flow cytometry; (B) histogram of ROS generation. ✳✳p <0.01 vs. Control group; ▲▲p < 0.01 vs. A/R group; ##p<0.01 vs. IPC group; ■■p <0.01 vs. IPostC group. Data are expressed as the mean ± SEM, n = 3. ROS: reactive oxygen species.
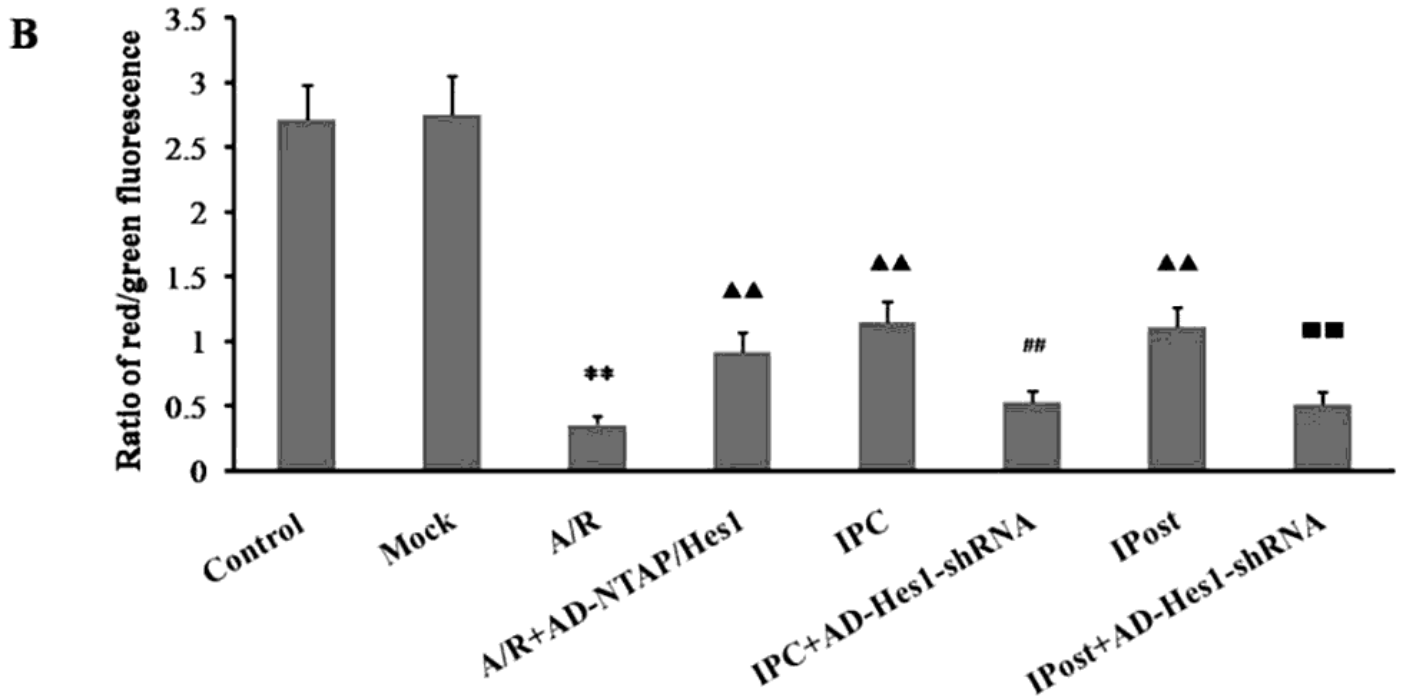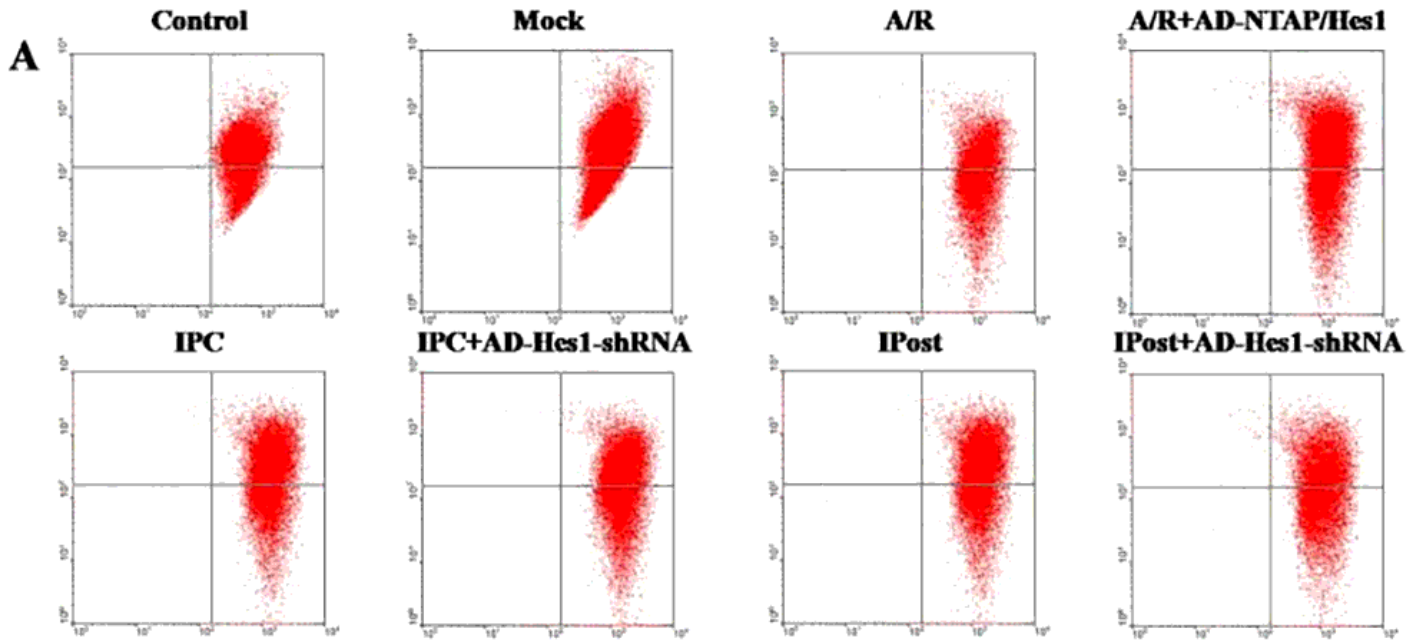
Figure 5

Effect of Hes1 on the ΔΨm of ischemic myocardial cells. (A) Flow cytometric analysis of ΔΨm; (B) ΔΨm bar graph. ＊＊$p < 0.01$ vs. Control group; ▲▲$p < 0.01$ vs. A/R group; ##$p < 0.01$ vs. IPC group; ■■$p < 0.01$ vs. IPostC group. Data are expressed as the mean ± SEM, n = 3. ΔΨm: mitochondrial membrane potential.
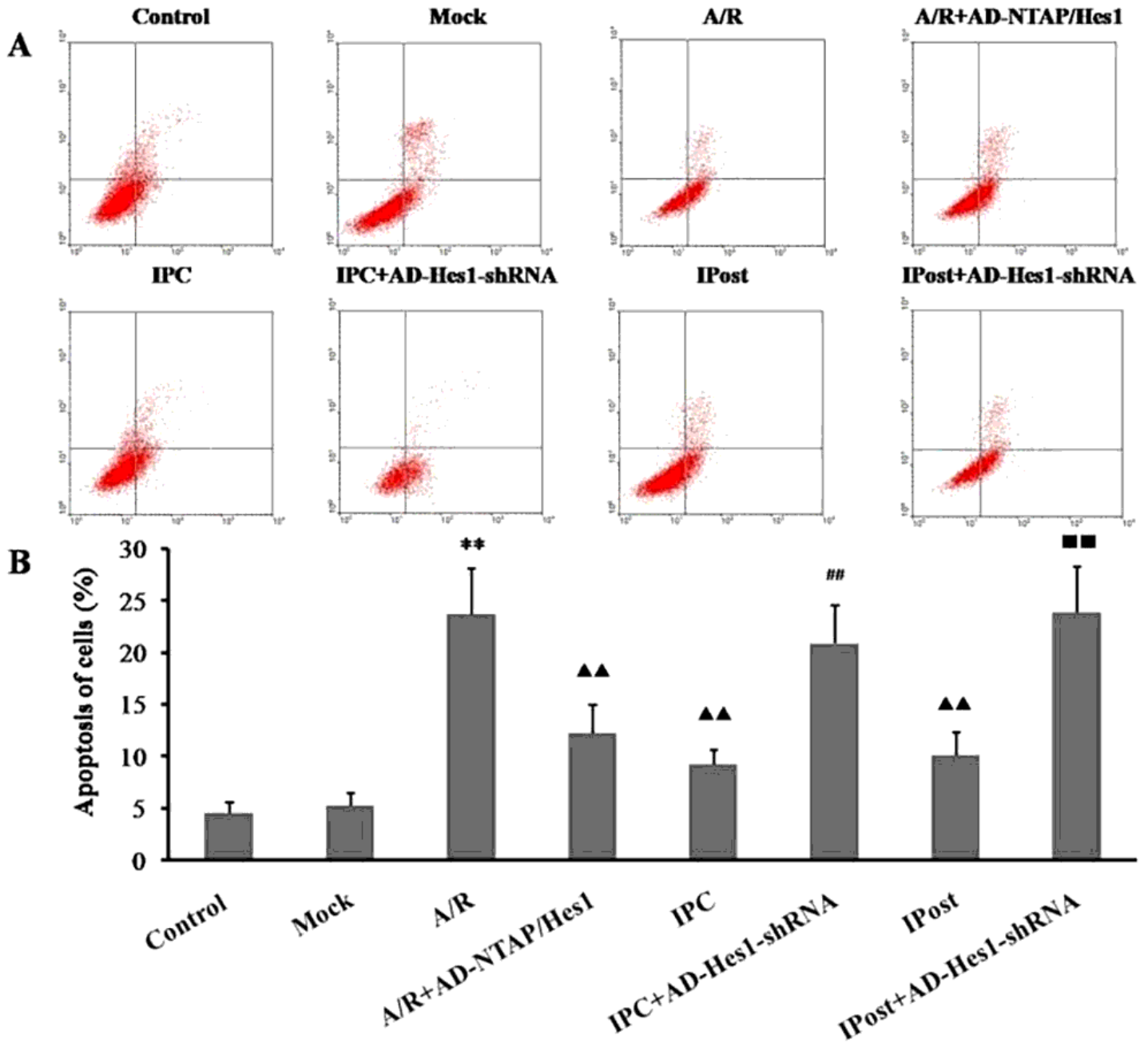
Figure 6

Effect of Hes1 on apoptosis in ischemic cardiomyocytes. (A) Flow cytometric analysis of apoptosis; (B) Histogram of apoptosis. ✶✶p <0.01 vs. Control group; ▲▲p < 0.01 vs. A/R group; ##p<0.01 vs. IPC group; ■■p <0.01 vs. IPostC group. Data are expressed as the mean ± SEM, n = 3.