# Notch1 signaling activation protected myocardium against hypoxia injury via reducing programmed cell death

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**Abstract**: Programmed cell death plays an important role in cardio protection, and Notch1 was an important factor related to programmed cell death. The role of Notch1 on ischemia myocardium remains unclear.H9C2 myocardial cells were cultured with routine medium, transfected with Notch1 over expression plasmid, Notch1-siRNA-overexpression plasmid and vehicle plasmid for further hypoxic experiment. Condition of hypoxic experiment was 1% oxygen centration and culturing for 12hours, then the cell proliferation activity and apoptosis rate was assessed by MTS kit and flow cytometry, respectively. The expressions of Caspase-3, Caspase-9 and Bcl-2 were determined by RT-qPCR and Western Blot, respectively. Compared with normoxia treatment, hypoxia could decrease H9C2 cell proliferation activity as well as Bcl-2 mRNA expression, and increase cell apoptosis rate as well as Caspase-3 and Caspase-9 mRNA expression. Notch1 activation could increase proliferation activity as well as Bcl-2 mRNA expression, while decrease apoptosis rate as well as Caspase-3 and Caspase-9 mRNA expression. Compared with Notch1 activation H9C2 cells, the opposite effect on programmed cell death was observed in cells with Notch1-siRNA-overexpression plasmid. Targeted activation of Notch1 gene to reduce hypoxia-induced programmed cell death in myocardial cells via up-regulating the expression of Caspase-3 and Caspase-9 and inhibiting the expression of Bcl-2.

**Keywords**: Notch1, myocardial, hypoxia, programmed cell death.

## INTRODUCTION

Ischemia is the main cause of myocardial injury, and results in multiple cardiovascular diseases (Kallinen *et al*., 2012). Complex mechanisms were involved in progress of myocardial ischemia injury, in which hypoxia played a pivotal role (Rocha *et al*., 2015; Horton *et al*., 1995). Although myocardium adapt at physiological low oxygen enrichment condition, aberrant hypoxia accompanied with ischemia always enhanced myocardial death, which worsens prognosis of cardiovascular diseases (Zhang *et al*., 2013; Jiang *et al*., 2013). Recent studies indicated that, under adverse stimulus, programmed cell death was promoted in ischemia myocardium, and hypoxia was proved with enhancing effect on programmed cell death (Jiang *et al*., 2013; Liu *et al*., 2015; Zhang *et al*., 2012). Accordingly, it is a potential that hypoxia injury was alleviated via programmed cell death pathways.

Notch1 and its related pathways are important parts of programmed cell death (Geisler and Strazzabosco 2015). Moreover, animal experiments suggested Notch1 was associated with myocardial injury, such as ischemia injury and ischemia-reperfusion injury (Gude *et al*., 2015; Quillard *et al*., 2013). Despite of promising clinical significance, detailed mechanisms were warranted to understand effect of Notch1. No report was about effect of Notch1 on hypoxia-induced programmed cell death. On the other hand, the overall data linking Notch1 and myocardial hypoxia injury is controversial (Felician *et al*., 2014; Nemir *et al*., 2014). Some researchers suggested that too much and too less Notch1 were both detrimental to myocardium in hypoxia condition (Pei *et al*., 2013). Collectively, it is essential to fig. out whether Notch1 was associated with programmed cell death in hypoxia myocardium, and to explore specific effects of different Notch1 expressions on myocardium.

In this work, we hypothesized that the Notch1 signaling pathway is a positive regulator of programmed cell death in H9C2 cell line under hypoxia condition. Complementary studies with different Notch1 expressions were performed to prove relationship between Notch1 and myocardial hypoxia injury.

## MATERIALS AND METHODS

### *Experimental materials*

H9C2 cell line was from the ATCC cell bank in the United States, Notch1-siRNAoverexpression plasmid and blank plasmid were synthesized and provided by Shanghai Innovation Company, Lipofectamine™ 2000 liposomes (from Invitrogen Company), MTS kits (from Promega Company), Annexin V kits (from eBioscience Company) as well as RNA extraction kits, reverse transcription kits and fluorescence quantitative PCR kits (from Takara Company).

### *Cell culture and hypoxia treatment methods*

H9C2 cells were cultured in DMEM (dulbecco's modified eagle medium) medium containing 10% calf serum,

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digested and sub-cultured with trypsin, inoculated in culture plate and treated when the cell density reached about 90%.

Lipofectamine™ 2000 liposomes were used to transfect plasmids, and the transfected plasmids included Notch1 over expression plasmid, Notch1-siRNA-overexpression plasmid and blank plasmid. All cells were randomly assigned into two groups, including control group (normoxia) and experimental group (hypoxia). Condition of hypoxia treatment was low oxygen concentration (1%) for 12 hours.

***Cell proliferation activity detection methods***

The cells digested and sub-cultured by trypsin were inoculated in 96-well cell plates (inoculation density 5 × 103/well), different plasmids were used to transfect the cells after 12h for control group and experimental group, 20mL of testing liquid from MTS kit was added in each cell well after 24h, the cells continued to be cultured in incubator for 4h, the cell culture plates were taken out and shaken for 10min, then the absorbance value (OD value) at 570nm wavelength was read from the micro plate reader, and the OD value was used as the cell proliferation activity. Three parallel wells were made for each bath of cells, and 4 batches were repeated.

***Cell apoptosis detection methods***

The cells digested and sub-cultured by trypsin were inoculated in 6-well cell plates (inoculation density 1 × 106/well) and transfected with different concentrations of paclitaxel and two different plasmids after the cell density reached 80%, the supernatant was abandoned after 24 h, the cells were kept, digested with trypsin and centrifuged, the obtained cells were added in 5mL AnnexinV and incubated for 15 min away from light, and the percentage of apoptotic cells was detected in flow cytometer.

***Fluorescence quantitative PCR***

The cells digested and sub-cultured by trypsin were inoculated in 12-well cell plates (inoculation density 0.5 × 105/well) and transfected with different concentrations of oxygen and two different plasmids after the cell density reached 80%, the supernatant was abandoned after 24 h, the cells were kept, RNA extraction kits were used to extract the total RNA in the cells, reverse transcription kits were used to reverse transcribe the total RNA into cDNA, finally fluorescence quantitative PCR kits were used to amplity Caspase-3, Caspase-9, Bcl-2 and b-actin, and bactin was used as reference to calculate Caspase-3, Caspase-9 and Bcl-2 mRNA expression. Caspase-3, Caspase-9 and Bcl-2 primer sequences and annealing temperature were shown in table 1.

***Western blot***

Proteins were extracted from transfected cells for Western blot with routine protocol. Detailed processes were as follows:

Protein suspension (15μg protein) was prepared for electrophoresis. Block proteins for 1.5 hours after transferring. Incubate with primary antibody (1: 800) at 4°C for 12 hours. React with second antibody (1:1500) at 37°C for 2 hours. Gel imaging system was used for analysis of specific protein bands.

## STATISTICAL ANALYSIS

SPSS17.0 software was used to input and analyze data, the measurement data analysis between two groups was by t test (Independent-Samples Test), measurement data analysis among groups was by variance analysis and P< 0.05 indicated statistical significance in differences.

## RESULTS

***Hypoxia increased cell apoptosis and decreased cell proliferation activity in H9C2 cell line***

Compared with normoxia-treated H9C2 cells, cells under hypoxia indeed showed significant higher rate of apoptosis and lower rate of proliferation activity (fig. 1), consistent with previous report.

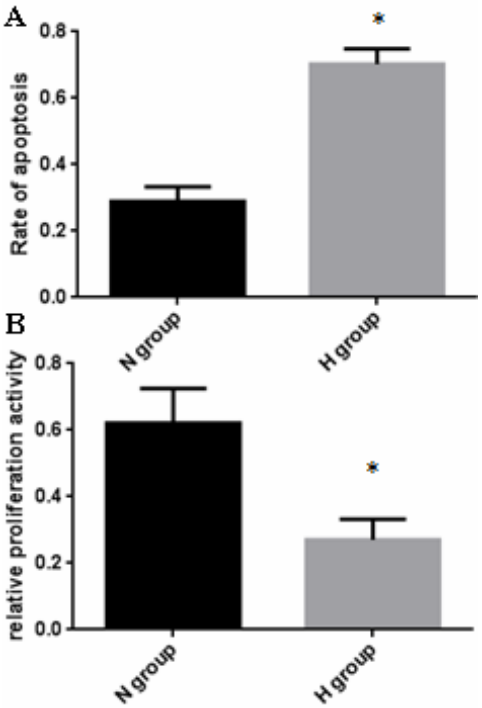


**Fig. 1**: (A) Analysis of apoptosis rate in N group and H group (B) Analysis of proliferation activity in N group and H group.*P<0.05, versus N group.

***Hypoxia enhanced programmed cell death of H9C2 cells***

Compared with normoxia, hypoxia significantly increased expression of Caspase-3 and Caspase-9, but decreased expression of Bcl-2, verified by rt-qPCR and Western blot (fig. 2). These suggested hypoxia enhanced programmed cell death of H9C2 cells.

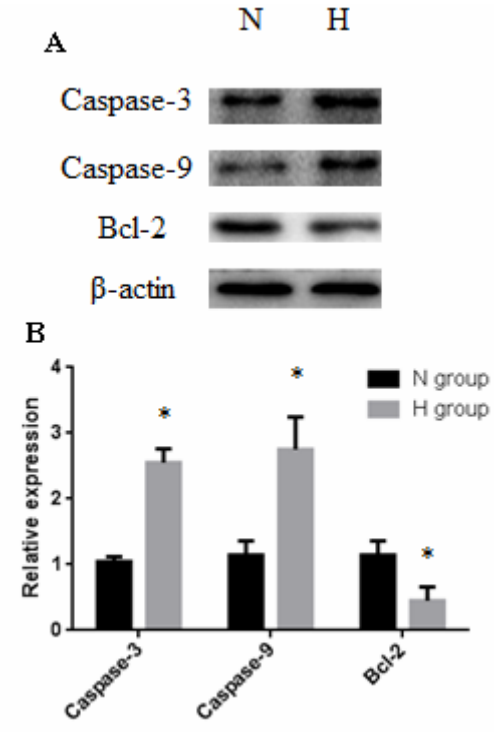


**Fig. 2**: (A) Protein bands of Western Blot for Caspase-3, Caspase-9 and Bcl-2. (B) Analysis of expressions of Caspase-3, Caspase-9 and Bcl-2. *P<0.05, versus N group.

***Over expression of Notch1 reduced hypoxia-induced programmed cell death of H9C2 cells***

In hypoxia-treated H9C2 cells, over expression of Notch1 significantly decreased cell apoptosis and increased cell proliferation activity, verified by MTS kit experiment and flow cytometry (fig. 3A). Moreover, over expression of Notch1 increased expression of Bcl-2 and decreased expression of Caspase-3 and Caspase-9 (figs. 3B&C)

***Inhibition of Notch1enhanced hypoxia-induced programmed cell death of H9C2 cells***

In hypoxia-treated H9C2 cells, inhibition of Notch1 significantly increased cell apoptosis and decreased cell proliferation activity, verified by MTS kit experiment and flow cytometry (fig. 4A). Moreover, inhibition of Notch1 decreased expression of Bcl-2 and increased expression of Caspase-3 and Caspase-9 (fig. 4B&C).

## DISCUSSION

Our study elucidated that hypoxia damaged myocardium (H9C2 cells) via inducing programmed cell death. Moreover, we proved expression of Notch1 was negatively associated with degree of programmed cell death. Over expression of Notch1 alleviated hypoxia-induced myocardial programmed cell death via decreasing Caspase-3, Caspase-9 and increasing Bcl-2.

It is no doubt that hypoxia damaged myocardium in the progress of myocardial ischemia, but the mechanisms still

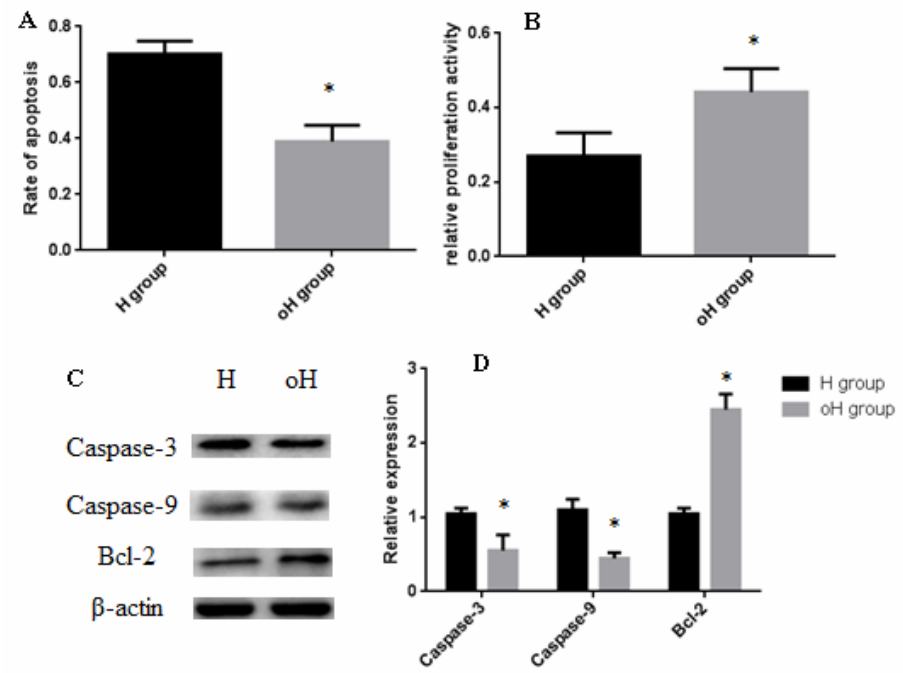


**Fig. 3**: (A)Analysis of apoptosis rate in H group and oH group (B)Analysis of proliferation activity in H group and oH group .(C) Protein bands of Western Blot for Caspase-3, Caspase-9 and Bcl-2. (D) Analysis of expressions of Caspase-3, Caspase-9 and Bcl-2. *P<0.05, versus H group, Caspase-9 and Bcl-2. (D) Analysis of expressions of Caspase-3, Caspase-9 and Bcl-2. *P<0.05, versus H group

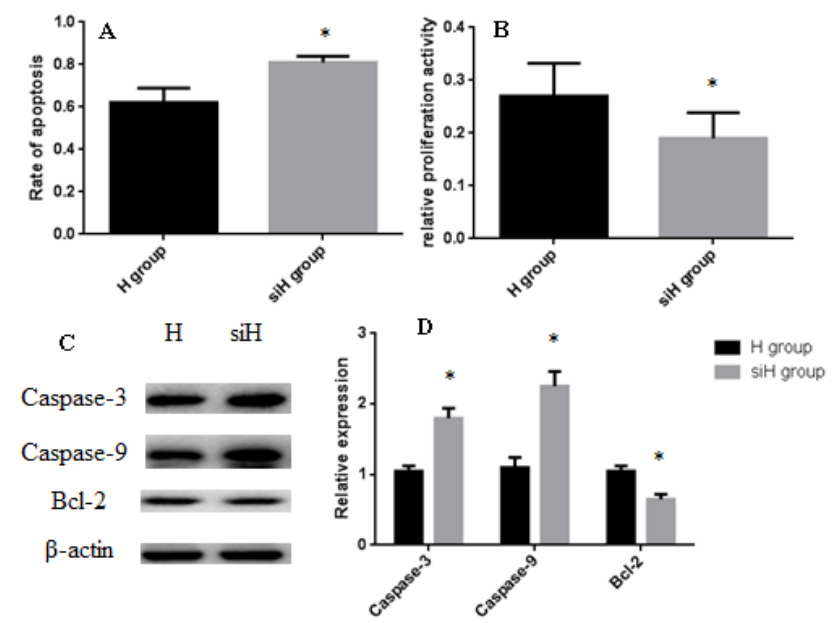


**Fig. 4**: (A) Analysis of apoptosis rate in H group and siH group (B) Analysis of proliferation activity in H group and siH group. (C) Protein bands of Western Blot for Caspase-3, Caspase-9 and Bcl-2. (D) Analysis of expressions of Caspase-3, Caspase-9 and Bcl-2. *P<0.05, versus H group

need to be further unveiled (Yu *et al*., 2011). We found that hypoxia induced programmed cell death of H9C2 cell, verified by increased Caspase-3, Caspase-9 and increased Bcl-2. Intriguingly, Bcl-2 was also a marker for autophagy (Wang *et al*., 2014), and our result indicated more pathways were involved in myocardial hypoxia injury except programmed cell death.

Notch related pathways were proved with clinical significance in many kinds of tissue injury, especially hypoxia-caused injury (Bai *et al*., 2015; Zhang *et al*., 2011). In the Notch family, Notch1 is a star molecule due to various effects in multiple diseases (Krzyzaniak *et al*., 2011). For example, Notch1 is aberrantly over-expressed in tumors, including prostate cancer, lung cancer, breast cancer and glioma Liu *et al*., 2014; Xiao *et al*., 2012; Yao *et al*., 2014), moreover, targeting inhibition of Notch1 not only itself increasing apoptosis of tumor, but also enhances the killing effects of chemotherapy (Cai *et al*., 2014). In the field of heart, Notch1 is highly expressed in heart at embryonic development stage, and its expression gradually decreased with the maturing of heart (Caliceti *et al*., 2014). Previous animal studies showed that Notch1 increased in myocardium after myocardial infarction, suggesting over expression of Notch1 was detrimental to heart (Yu *et al*., 2013; Kornfeld *et al*., 2015). However, experiment *in vitro* showed over expression of Notch1 enhanced proliferation of cardiac fibroblasts and reduced apoptosis of myocardium (Ha *et al*., 2004).

To explore whether over expression of Notch1 was detrimental to heart, we performed this study. In our study, over expression of Notch1 decreased programmed cell death of H9C2 cells, while inhibiting Notch1 promoted programmed cell death of H9C2 cells. Taken together, we believed Notch1 was a protecting factor for hypoxia myocardium, and its increase could protect myocardium against hypoxia injury via reducing programmed cell death. On the other hand, it should be also pointed out that our study focused on cell experiment, and the effect of Notch1 on hypoxia myocardial could be more complex *in vivo*, which need further exploration.

## CONCLUSION

Targeted over expression of Notch1 protects myocardial cells against hypoxia injury via up-regulating the expression of Caspase-3 and Caspase-9 and inhibiting the expression of Bcl-2 to reduce programmed cell death.

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