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Original Article

Paeonol protects H9C2 cardiomyocytes from ischemia/reperfusion injury by activating Notch1 signaling pathway in vitro

Xianli Li, Rui Yang, Xiaohong Yang, Xiaohui Zheng

Department of Cardiovascular, Anyang District Hospital, Henan Province, People's Republic of China

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Abstract: Objective: To investigated the protective effects of paeonol on simulated ischemia/reperfusion treated cardiomyocytes in vitro. Methods: In this research, the rat cardiomyocyte line, H9c2, was treated with hypoxia/reoxygenation as an I/R model in vitro. Cell viability was assayed by CCK-8 kit; cell apoptosis was detected by flow cytometry and protein expression was assayed by western blot. Results: SIR injury can decrease the viability of the H9c2 cells and significant increase the apoptosis-related protein bax, caspase-3 and down regulation of the anti-apoptosis-related protein bcl-2. Paeonol can down regulated the expression of apoptosis-related protein following SIR injury and restored the viability of the H9c2 cells. A signifiant increase in Notch1 protein levels were observed in the H9c2 cells treated with both paeonol and SIR injury. However, the anti-apoptotic effect conferred by paeonol was blocked by DAPT (the specific inhibitor of Notch1 signaling) in the cultured H9c2 cells. Conclusion: The findings of our study suggest that paeonol protects the H9c2 cells against SIR injury-induced injury through the activation of the Notch1 signaling. These results provide new evidence for the potential protective effects of paeonol.

Keywords: Paeonol, ischemia/reperfusion injury, simulated ischemia/reperfusion, Notch1 signaling pathway

Introduction

Cardiovascular diseases are the major contributor to mortality both in developed and developing country today and Myocardial ischemia is one of the main results of cardiovascular diseases. Because of lacking effective therapies, myocardial ischaemia/reperfusion (I/R) injury remains a unsolved medical problem [1]. Timely reperfusion is essential for recovery of the ischemia myocardium; however, reperfusion itself can result in major cardiac injury [2]. Although the underlying mechanism regulating myocardial injury induced by I/R remains to be fully elucidated, numerous evidences indicated that cellular apoptosis is an essential form in I/R injury [3].

The Notch1 signaling pathway involves in multiple cellular processes, including cell fate determination, development, differentiation, proliferation, apoptosis, and regeneration [4-7]. In heart, Notch1 signaling not only relates to embryonic cardiac development and differentiation, but also stimulates proliferation of immature cardiomyocytes and promotes quiescent cardiomyocytes to reenter the cell cycle [8-11]. All these effects promote regeneration and repair of the injured myocardium. Expression levels for Notch1, decline in cardiomyocytes during cardiac postnatal development, are activated and restored in the adult injured myocardium.

Paeonol, 2'-hydroxy-4'-methoxyacetophenone is a major active component extracted from Moutan Cortex, a Chinese medicine prepared from the root bark of Paeonia suffruticosa Andrews and Cynanchum paniculatum (Bunge) Kitagawa [12]. Traditional Chinese medicine literature has documented that paeonol possesses abundant therapeutic effects including anti-oxidation, anti-tumor, anti-inflammation, anti-atherosclerosis and anti-proliferation of vascular smooth muscle cells [13-17]. Despite these pharmacological findings, whether paeonol protects cardiomyocytes against I/R-induced injury through a Notch1-dependent mechanism is not yet known. To examine this hypothesis, in this study, we investigated the effects of paeonol on Notch1 activity as a means of modulating apoptosis in vitro. Our data demonstrated

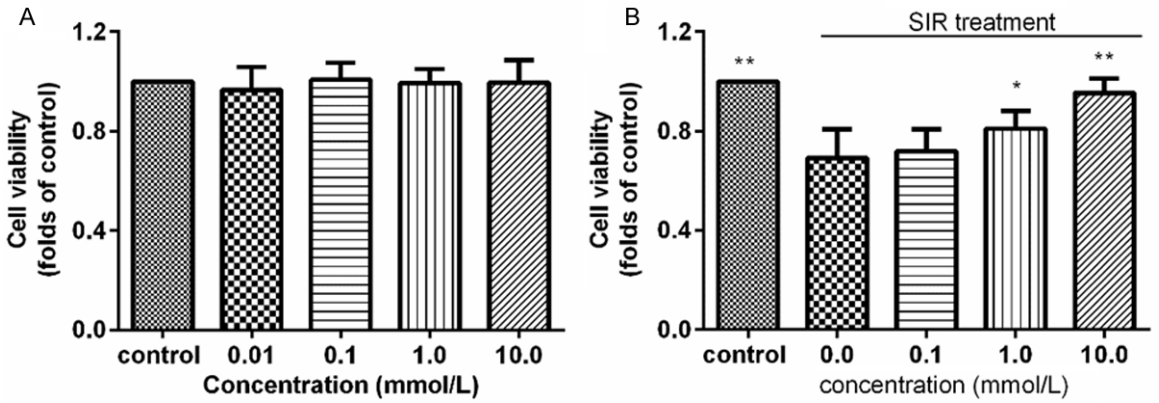

Figure 1. The effect of paeonol treatment on cell viability in SIR-treated cardiomyocyte cells. A: The cells were incubated with the different concentrations of paeonol for 12 h; B: Cells were subjected to SIR treatment in the absence or presence of paeonol (0.1, 1.0 or 10.0 mmol/L). Then, the cell viability was assayed by the MTT method. Results were expressed as mean ± S.E.M. from six independent experiments, *P < 0.05 or **P < 0.01 vs. SIR treatment.

that the treatment of H9c2 cells with paeonol attenuated SIR-induced cell apoptosis, upregulated Notch expression.

Materials and methods

Regents

Paeonol was purchased from the National Food and Drug Testing Institute (Beijing, China). The DAPT were purchased from Sigma-Aldrich (St. Louis, MO, USA); Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco Thermo Fisher Scientific, Inc. (Waltham, MA, USA). Penicillin and streptomycin were purchased from Beijing Solarbio Science & Technology Co., Ltd. (Beijing, China). The Cell Counting kit-8 (CCK-8) was purchased from Boster biotech Co., Ltd. (Wuhan, China). Protein quantification kit was purchased from Bio-Rad (Hercules, CA, USA). Primary antibodies against Notch1 were obtained from Abcam (Cambridge, MA, USA); cleved-caspase-3, bax, bcl-2, and GAPDH were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Secondary antibodies were purchased from the Zhongshan Company (Beijing, China).

Cell culture

H9c2 embryonic rat myocardium-derived cells, a well-characterized and widely used cell line to study myocardial cell ischemia, were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic-antimycotic. The H9c2 cells were maintained at 37°C with 5% CO_2.

Simulated ischemia/reperfusion treatment

The SIR treatment was performed by physiological concentrations of potassium, hydrogen, and lactate. The procedure was performed as described previously [18]. Briefly, the H9c2 cells were exposed to an ischemic buffer containing (in mmol/L) 137 NaCl, 12 KCl, 0.49 $MgCl_2$, 0.9 $CaCl_2$, and 4.0 HEPES. This buffer was also supplemented with (in mmol/L) 10.0 deoxyglucose, 0.75 sodium dithionate, and 20.0 lactate. The buffer pH was 6.5, and the cells were incubated for 50 min in a humidified cell culture incubator (21% oxygen, 5% CO_2, 37°C). Reperfusion was initiated by returning the cells to normal culture medium for 4 h in a humidified cell culture incubator (21% oxygen, 5% CO_2, 37°C).

Cell viability assay

After the H9c2 cells were seeded in 96-well culture plates and received different treatments, 10 μl CCK-8 solution was added to each well at a 1/10 dilution. After 3 h of incubation, the absorbance was measured using a microtiter plate reader Bio-Rad (Hercules, CA, USA) at a wavelength of 450 nm. The cell viability was calculated by dividing the optical density of samples with the optical density of control group.

Determination of cell apoptosis

Apoptotic cells were identified by an Annexin V/PI apoptosis kit (Keygen Biotech, Nanjing, China) and flow cytometry. After 12 h of culture

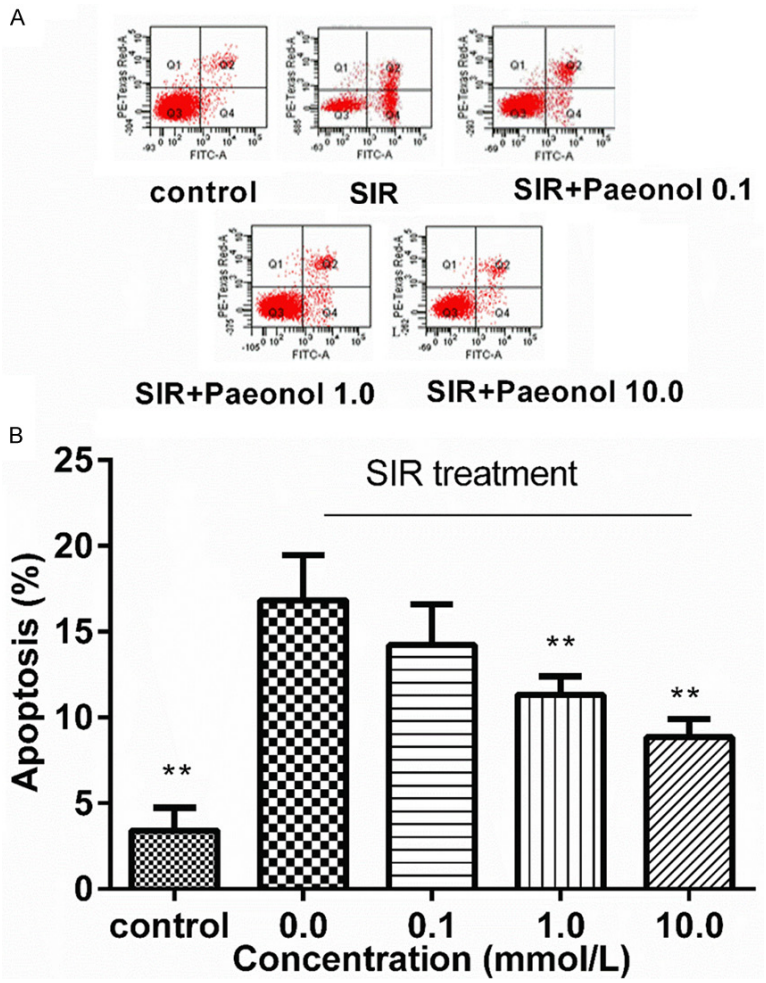

Figure 2. Impacts of paeonol on SIR-induced cardiomyocytes apoptosis by annexin V/PI staining. Cardiomyocytes were pre-treated with paeonol (0.1, 1.0 or 10.0 mmol/L) for 1 h and then were stimulated with SIR for 12 h. At the end of the incubation period, the myocytes were then prepared for annexin V/PI staining. The data expressed as % apoptotic cardiac myocytes. Results were expressed as mean ± S.E.M. from 3 independent experiments, **P < 0.01 compared with SIR treatment.

under the different treatments, (1-2) × 10^6 cells were harvested and re-suspended in 500 μl binding buffer. Next, the cells were incubated with 5 μl Annexin V-fluorescein isothiocyanate (FITC) and 5 μl PI (50 mg/ml) for 25 min in the dark and immediately analyzed by flow cytometry. Data from at least 2 × 10^5 cells of each sample were acquired and analyzed using Cell Quest software, version 7.5.3 (Becton Dickinson). In the PI vs. FITC scatter plot, the percentage of cells in the lower right quadrant of (early apoptotic cells), upper right quadrant (late apoptotic cells), upper left quadrant (necrotic cells) and lower left quadrant (live cells) was calculated for comparison.

Western blot analysis

The H9c2 cells were harvested and lysed after the indicated treatments. The lysates were clarified by centrifugation at 4°C for 15 min at 12,000 rpm. After quantitation of protein concentration, 40 μg of total protein was separated by SDS-PAGE and then transferred to a poly vinylidene difluoride membrane Bio-Rad (Hercules, CA, USA). The membrane was blocked with 8% free-fat milk in TBST for 4 h at room temperature, then incubated with primary antibodies specific to Notch1, Caspase-3, Bcl-2, Bax and β-actin, at least 12 h at 4°C. After washing three times with TBST for 15 min, the membrane was incubated with the secondary antibodies for 4 h at room temperature, and then washed as above. The positive protein bands were developed using a chemiluminescent system, and the bands were scanned and quantified by densitometric analysis using an image analyzer Quantity One System (Bio-Rad, Richmond, CA, USA).

Data analysis

All values are presented as mean ± S.E.M. Differences were compared by ANOVA followed by Bonferroni correction for post hoc t test, where appropriate. P < 0.05 were considered to be statistically significant. All of the statistical tests were performed with the GraphPad Prism software version 6.0 (GraphPad Software, Inc., San Diego, CA).

Results

The effect of paeonol treatment on cell viability in SIR-treated H9C2 cells

To explore whether paeonol was able to protect against cardiac injury induced by SIR in vitro.

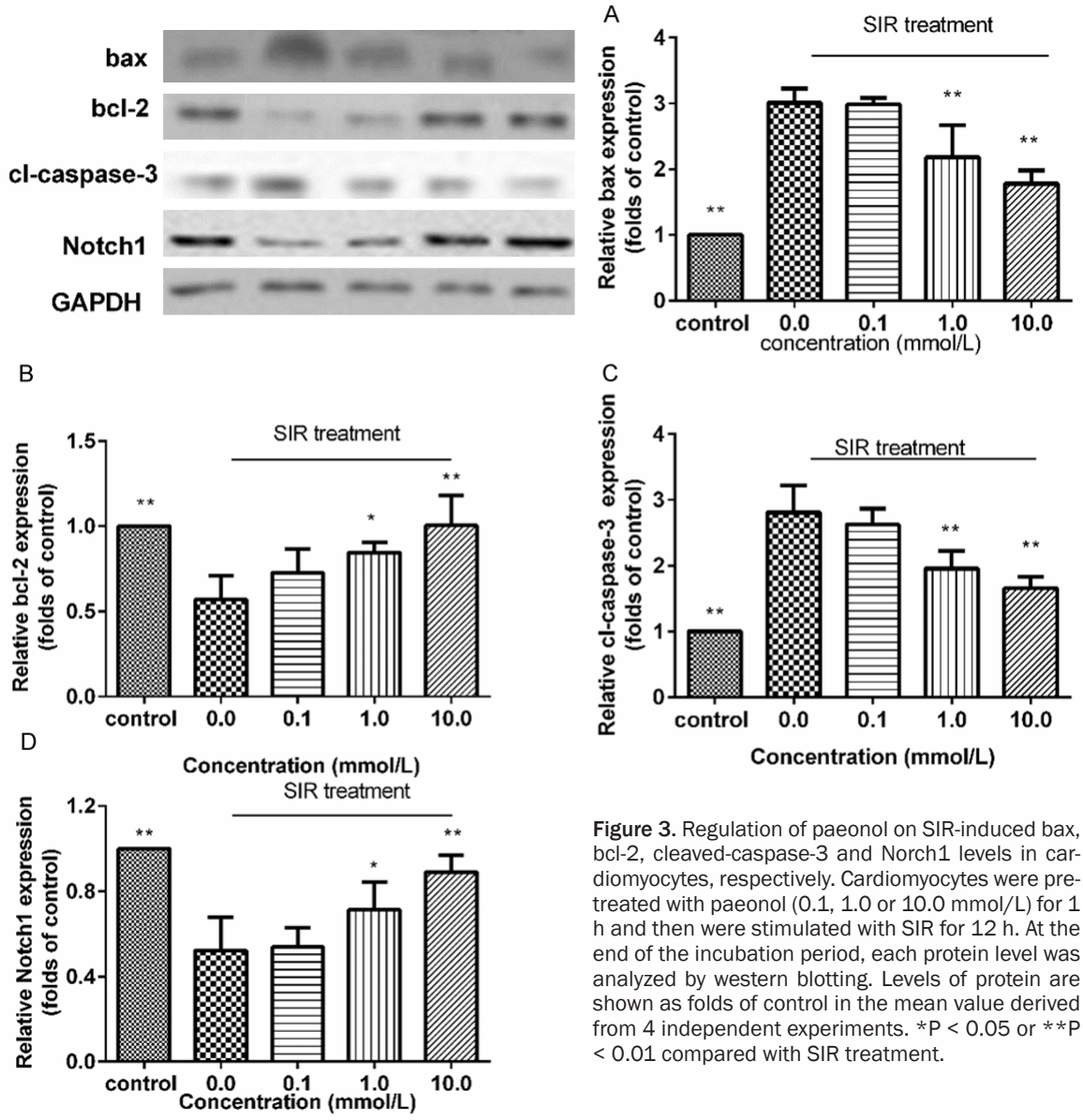

Figure 3. Regulation of paeonol on SIR-induced bax, bcl-2, cleaved-caspase-3 and Norch1 levels in cardiomyocytes, respectively. Cardiomyocytes were pretreated with paeonol (0.1, 1.0 or 10.0 mmol/L) for 1 h and then were stimulated with SIR for 12 h. At the end of the incubation period, each protein level was analyzed by western blotting. Levels of protein are shown as folds of control in the mean value derived from 4 independent experiments. *P < 0.05 or **P < 0.01 compared with SIR treatment.

Firstly, determined the effects of paeonol on the viability of H9c2 cells. The H9c2 cells were exposed to paeonol (0.01, 0.1, 1.0 and 10.0 mmol/L) for 8 h. Then, cell viability was determined by CCK-8. As shown in **Figure 1A**, no significant cell viability changes were found compared with the control group (P > 0.05). Next, the cells were subjected to SIR treatment in the absence or presence of paeonol (0.1, 1.0 and 10.0 mmol/L) treatment. SIR treatment significantly decreased cell viability (**Figure 1B**, P < 0.05, compared with the control group), while paeonol (1.0 and 10.0 mmol/L) treatment for 12 h resulted in a significant increase in cell viability, in a dose-dependent manner (**Figure 1B**, P < 0.05, compared with the SIR group). However, 0.1 mmol/L paeono had almost no effect on improving the cell viability (vs. IR group, P > 0.05).

Paeonol prevented the cell apoptosis induced by SIR treatment

To examine whether paeonol has any effect on SIR-induced apoptosis in H9c2 cells, the present study performed by flow cytometry. Results showed that SIR treatment increased the percentage of apoptotic cells (P < 0.05, compared with control group, **Figure 2**). Compared with the SIR group, paeonol (1.0 and 10.0 mmol/L) treatment attenuated the H9c2 cells apoptosis, in a dose-dependent manner (P < 0.05, **Figure 2**), this result is identical with the CCK-8

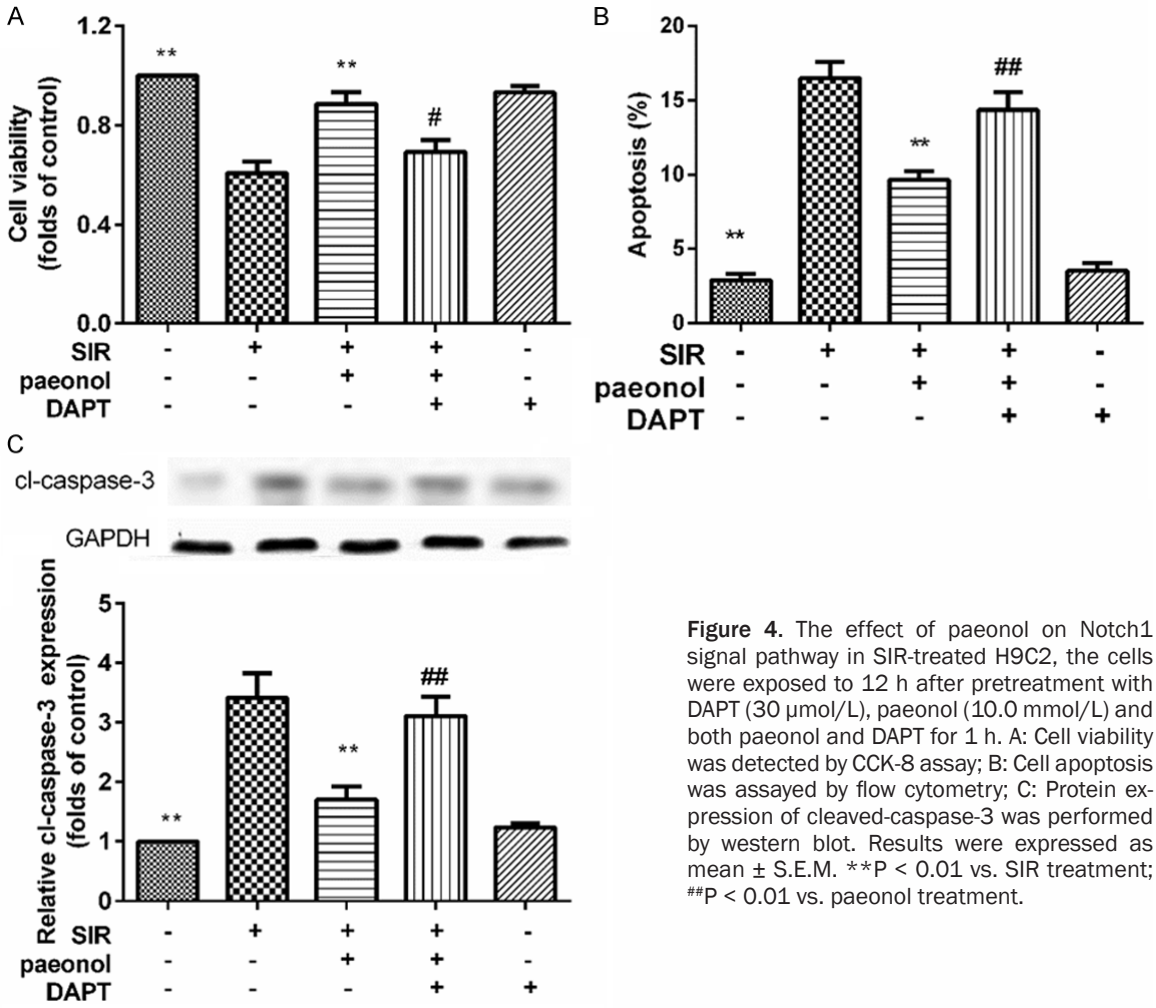

Figure 4. The effect of paeonol on Notch1 signal pathway in SIR-treated H9C2, the cells were exposed to 12 h after pretreatment with DAPT (30 μmol/L), paeonol (10.0 mmol/L) and both paeonol and DAPT for 1 h. A: Cell viability was detected by CCK-8 assay; B: Cell apoptosis was assayed by flow cytometry; C: Protein expression of cleaved-caspase-3 was performed by western blot. Results were expressed as mean ± S.E.M. **P < 0.01 vs. SIR treatment; ##P < 0.01 vs. paeonol treatment.

results. In conclusion, these data confirmed that exposure of the H9c2 cells to SIR induced apoptotic cell death, however, pretreatment with paeonol reversed this effect and protected the H9c2 cells.

The effect of paeonol treatment on caspase-3, bax, bcl-2 and notch1 proteins expression in SIR-treated H9C2 cells

To investigate whether the protective effect of paeonol is associated with apoptosis related protein, the protein levels of pro-apoptotic cleaved-caspase-3, bax, and anti-apoptotic Bcl-2 were measured using Western blot analysis. As shown in **Figure 3**, the protein expression of cleaved-Caspase-3, and bax in the SIR group was significantly upregulated, compared with that in the control group ($P < 0.05$). However, paeonol attenuated the SIR-induced upregulation in the expression of cleaved-caspase-3 and bax, compared with the SIR group ($P < 0.05$). In addition, conversely, SIR produced a significant decrease in the expression of Bcl-2 ($P < 0.05$, compared with control group). While, paeonol treatment up-regulated the ratio of Bcl-2 ($P < 0.05$, compared with the SIR group). The results suggested that the protective effects of paeonol were associated with the down regulation of cleaved-caspase-3 and bax, and up regulation the expression of bcl-2. As shown in **Figure 3D**, SIR treatment can significantly down regulate the level of Noch1 in compared with control group. However, paeonol (1.0 and 10.0 mmol/L) can up regulate the expression of Notch 1 protein.

The effect of paeonol on Notch1 signal pathway in SIR-treated H9C2 cells

The above experiments indicated that paeonol protected the H9c2 cells from SIR-induced cell death and this mechanism may be related to Notch1 signaling pathway. The following experi-

ment was to explore the role of the Notch1 signaling pathway in paeonol's protective effect on SIR-treated H9c2 cells. We determined whether treatment with DAPT, a specific inhibitor of the Notch1 signaling pathway, affected cell viability in SIR-treated H9c2 cells. The cells were subjected to SIR treatment in the absence or presence of paeonol (1.0 mmol/L). SIR treatment significantly decreased cell viability, similar to our previous experiments ($P < 0.05$, compared with the control group), while paeonol treatment for 12 h resulted in a significant increase in cell viability ($P < 0.05$, compared with the SIR group). However, paeonol-induced cardioprotective effect was significantly blunted by co-administration of DAPT (**Figure 4A**, $P < 0.05$, compared with the SIR+ paeonol group). As assayed by flow cytometry, paeonol (1.0 mmol/L) treatment significantly attenuated SIR-induced cell apoptosis. However, this protective effect was partially blunted by co-administration of DAPT (**Figure 4B**). As shown in **Figure 4C**, paeonol (1.0 mmol/L) treatment also reduced the apoptotic protein cleave-caspase-3, while DAPT co-administration significantly blocked this protective effect ($P < 0.05$, compared with the SIR+ paeonol group). These findings indicated that paeonol had direct anti-apoptotic effect against SIR injury and Notch1 signaling might play a key role in this protective action.

Discussion

Recent years, increasing attentions have been focused on the potential various pharmacological properties of paeonol, including sedation, antipyresis, analgesic, anti-tumor, anti-oxidation, anti-inflammation and immunoregulation and so on [19-23]. The present study was carried out to further elucidate the underlying mechanisms of paeonol's cardioprotective action. In this study, the vitro model was used to investigate the protective effect of paeonol against SIR-induced cardiac damage. We found that paeonol markedly ameliorated the SIR injury by activating Notch1 signal pathway.

Notch1 signaling has indispensable functional to control tissue formation in cardiogenesis [24, 25]. Intriguingly, the modulation effect of Notch1 signaling on cardioprotection attracted increasing attention recently. Notch1 signaling played a cardio protective role following cardiac injury and the activation of Notch1 signaling in the H9C2 cells contributed to the cardio protection provided by ischemic preconditioning and postconditioning [9, 26, 27]. However, the relationship between paeonol and Notch1 signaling in myocardium has not been elucidated. In this study, we found that the expression of Notch1 was significantly up-regulated by paeonol treatment in SIR-treated H9c2 cells, which indicated the critical role of Notch1 signaling in paeonol's protective action.

To further discover the role of Notch1 signaling in paeonol's cardio protective action, DAPT was added in the H9C2 cells. DAPT, a potent and specific inhibitor of γ-secretase, has been widely used to specifically block the Notch1 signal pathway. In the present study, DAPT significantly attenuated paeonol's protective effect against SIR treatment on the H9c2 cells. This finding indicated that the potential relevance of Notch1 signaling. In this study, we provided direct in vitro evidence that Notch1 signaling activation by paeonol reduced the SIR-treated H9c2 cells and that blocking this signaling significantly reverse this effect.

In summary, our findings suggested that paeonol exerted a profound cardio protective effect against apoptosis of the H9c2 cells induced by SIR treatment. This protection appeared to be largely due to the modulation of Notch1 signaling pathway. These results indicated that paeonol may be a promising candidate for the treatment of ischemic heart diseases.

Disclosure of conflict of interest

None.

Address correspondence to: Xiaohui Zheng, Department of Cardiovascular, Anyang District Hospital, Puyang, Henan Province, People's Republic of China. Tel: +86 13460946833; Fax: +86 29 82644367; E-mail: zhengxiaohui41@163.com

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