(Pro)renin receptor contributes to hypoxia/reoxygenation-induced apoptosis and autophagy in myocardial cells via the β-catenin signaling pathway

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Running title: PRR induces apoptosis and autophagy via β-catenin

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Summary

(Pro)renin receptor (PRR) contributes to regulating many physiological and pathological processes; however, the role of PRR-mediated signaling pathways in myocardial ischemia/reperfusion injury (IRI) remains unclear. In this study, we used an *in vitro* model of hypoxia/reoxygenation (H/R) to mimic IRI and carried out PRR knockdown by siRNA and PRR overexpression using cDNA in H9c2 cells. Cell proliferation activity was examined by MTT and Cell Counting Kit-8 (CCK-8) assays. Apoptosis-related factors, autophagy markers and β-catenin pathway activity were assessed by real-time PCR and western blotting. After 24 h of hypoxia followed by 2 h of reoxygenation, the expression levels of PRR, LC3B I/II, Beclin1, cleaved caspase-3, cleaved caspase-9 and Bax were upregulated, suggesting that apoptosis and autophagy were increased in H9c2 cells. Contrary to the effects of PRR downregulation, the overexpression of PRR inhibited proliferation, induced apoptosis, increased the expression of pro-apoptotic factors and autophagy markers, and promoted activation of the β-catenin pathway. Furthermore, all these effects were reversed by treatment with the β-catenin antagonist DKK-1. Thus, we concluded that PRR activation can trigger H/R-induced apoptosis and autophagy in H9c2 cells through the β-catenin signaling pathway, which may provide new therapeutic targets for the prevention and treatment of myocardial IRI.

Key words:

PRR, hypoxia/reoxygenation, apoptosis, autophagy, β-catenin signaling pathway

Introduction:

Myocardial infarction (MI) leads to deaths worldwide and places a tremendous burden on individuals and society (Benjamin *et al.* 2017). Blockade of the coronary artery completely or partially deprives the downstream tissue of oxygen and nutrients. Timely and effective reperfusion therapy (emergency percutaneous coronary intervention, thrombolysis or coronary artery bypass surgery) can reduce acute myocardial injury and limit the MI area. However, the resumption of oxygen delivery triggers a second wave of insult, termed reperfusion injury (Yellon and Hausenloy 2007). Myocardial ischemia/reperfusion injury (IRI) has been reported to attenuate the benefit of reperfusion and lead to cardiac failure and arrhythmia. Thus, there is great interest in identifying the mechanisms of IRI in the

hopes of mitigating this damage. However, the pathophysiological mechanism of IRI has not been elucidated in detail. Many factors, especially cell apoptosis and autophagy, are involved in the pathogenesis of IRI.

The renin-angiotensin (Ang) system (RAS) is a hormonal system that regulates blood pressure by affecting vasomotor tone and salt and fluid retention. The RAS is also involved in IRI, and Ang II levels are elevated in the damaged myocardium (Zhu *et al.* 2000, Yahiro *et al.* 2003, Dogan *et al.* 2001). (Pro)renin receptor (PRR), a new member of the RAS, was discovered and cloned from the mesangium of glomeruli by Nguyen in 2002 (Nguyen *et al.* 2002). Both renin and PR bind PRR, which not only catalyzes the production of active Ang II but also activates an intracellular signal pathway independent of Ang II formation (Zhou *et al.* 2010, Kouchi *et al.* 2017, Krop *et al.* 2013). Accumulating evidence has shown that PRR is activated in hypertension and cardiovascular and renal diseases. Furthermore, data have shown that PRR is expressed at high levels in neurons for the central control of blood pressure, the heart in patients with dilated cardiomyopathy, and chronic kidney disease patients (Nguyen 2011, Mahmud *et al.* 2012, Hamada *et al.* 2013, Ramkumar *et al.* 2018).

β-Catenin signaling, which plays a vital role in the progression of several diseases, is involved in regulating cell survival/apoptosis in different cell types, including cardiomyocytes (Ding *et al.* 2000, Fukumoto *et al.* 2001). Moreover, the β-catenin signaling pathway contributes to cell autophagy in adipogenesis (Romero *et al.* 2018) and human tumor cell lines (Hou *et al.* 2018, Hu *et al.* 2019). In addition, PRR, acting as a specific adaptor, is involved in Wnt/β-catenin signaling (Cruciat *et al.* 2010). Therefore, we hypothesized that PRR can affect cell autophagy and apoptosis in IRI in cardiomyocytes via the activation of β-catenin signaling.

Despite the previous finding that PRR activation could trigger the H/R-induced apoptosis of H9c2 cells through the p38 MAPK/caspase-3 signaling pathway (Liu *et al.* 2015), the association between PRR and β-catenin signaling in IRI is unclear. H9c2 is an original clonal cell line derived from embryonic BD1X rat heart tissue by B. Kimes and B. Brandt. We illustrated the molecular mechanism underlying the regulatory effects of PRR associated with β-catenin signaling on H/R-induced apoptosis and autophagy in H9c2 cells. Additionally, *in vitro* studies were beneficial in excluding the effects of nerves, body fluids and the interactions between cardiomyocytes. In the present study, we established an *in vitro* model of H/R using H9c2 cells and transfected specific siRNAs or a recombinant plasmid encoding PRR into the cells to knock down or overexpress PRR, respectively. In addition, we examined key genes

related to the β-catenin pathway. Finally, we treated H9c2 cells with the β-catenin antagonist DKK-1 to investigate the role of PRR in β-catenin signaling in H9c2 cells after H/R.

Materials and Methods

Cell culture and transfection

H9c2 cells were obtained from Zhong Qiao Xin Zhou Biotechnology Co., Ltd. (Shanghai, China) and cultured in DMEM (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS) (HyClone, Logan, UT, USA) in 5% CO_2 in air at 37°C. H9c2 cells were prepared by treatment with 10 μmol/L valsartan (MCE, USA) (Angiotensin II receptor 1 blocker) and 20 μmol/L PD123319 (Selleck, USA) (Angiotensin II receptor 2 blocker). The siRNA sequences were as follows: siRNA-1, 5'- GCGAAUGAAUUUAGCAUAU -3'; siRNA-2: 5'- GCUGCAUGAUAUUUCAAGU -3'; and siRNA-3: 5'- CCUUGCGUAUAAGUAUAAU -3'. The PRR coding sequence was inserted into the pcDNA3.1 plasmid (Clontech, Palo Alto, CA, USA) as previously reported(Peng *et al.* 2018). Cell transfection was performed with Lipofectamine 2000 (Invitrogen, CA, USA) according to the manufacturer's instructions. For DKK-1 treatment, cells were treated with 20 ng/ml DKK-1 (Sino Biological, Beijing, China) for 2 h before transfection. For H/R treatment, cells were treated with 24 h hypoxia (1% O_2, 5% CO_2, and 94% N_2), followed by 2 h of reoxygenation (5% CO_2, 37°C) at 48 h after transfection.

MTT assay

Cells were seeded in 96-well plates at a density of 3×10^3/well, and after incubation under normoxia or H/R treatment, a 5 mg/ml MTT solution (Wanleibio, Shenyang, China) was added, and cells were incubated for 4 h. The supernatant was then removed and replaced with 200 μl of dimethylsulfoxide (DMSO, Sigma, St. Louis, MO, USA) to dissolve the blue-purple formazan crystals. The absorbance at 490 nm was then measured using a microplate reader (ELx800, BioTek Instruments, VT, USA).

Real-time PCR

mRNA expression was detected by real-time PCR. Total RNA was extracted with an RNA pure total RNA kit (BioTeke, Beijing, China) according to the manufacturer's instructions. RNA was quantified, and then 1 μg of total RNA from each sample was used for cDNA synthesis with Super M-MLV reverse transcriptase (BioTeke). The cDNA product was subsequently subjected to real-time PCR with a SYBR Green kit (Solarbio, Beijing, China). β-Actin was used as a reference gene for the normalization of mRNA expression levels. RNA expression was calculated with the $2^{-\Delta\Delta Ct}$ method. Sequences of the

primers used in this study are presented in Table 1.

Western blot analysis

Total protein was extracted from cells with RIPA buffer (Beyotime, Haimen, China), and nuclear proteins were extracted with a Nuclear and Cytoplasmic Protein Extraction Kit (Beyotime). Then, the concentrations of the extracted nuclear and total proteins were measured by using a BCA kit (Beyotime). Histone H3 and β-actin were used as internal controls for nuclear and total proteins, respectively. Subsequently, 40 micrograms of protein samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes (Thermo Fisher Scientific, Waltham, MA, USA). After blocking with 5% (M/V) BSA for 1 h, the membranes were incubated with anti-PRR antibody (diluted 1:1000, Proteintech, Wuhan, China), anti-LC3B-I/II antibody (diluted 1:500, CST, Danvers, MA, USA), anti-Beclin1 antibody (diluted 1:500, CST), anti-cleaved caspase-3 antibody (diluted 1:500, CST), anti-cleaved caspase-9 antibody (diluted 1:500, CST), anti-Bax antibody (diluted 1:500, Proteintech), anti-Bcl-2 antibody (diluted 1:500, Abcam, Cambridge, MA, USA), anti-Dvl-1 antibody (diluted 1:500, Santa Cruz, CA, USA), anti-β-catenin antibody (diluted 1:500, Proteintech), anti-Histone H3 antibody (diluted 1:500, Proteintech) or anti-β-actin antibody (diluted 1:500, Santa Cruz) overnight at 4°C. Then, the membranes were incubated with goat anti-rabbit IgG-HRP (diluted 1:10000, Proteintech) or goat anti-mouse IgG-HRP (diluted 1:10000, Proteintech) for 40 min at room temperature. Protein bands were visualized using an ECL kit (7 Sea Biotech, Shanghai, China).

Flow cytometry

Flow cytometry was performed to detect the cell apoptosis status. Cells were collected by centrifugation (309 ×g) for 5 min. Thereafter, the cells were washed twice with phosphate-buffered saline (PBS). Next, 5 μl of Annexin V-FITC and 10 μl of propidium iodide were added to each sample and mixed well. Finally, the cells were incubated at room temperature for 15 min and then analyzed by flow cytometry.

CCK-8 assay

Cells were seeded in 96-well plates at a density of 3×10^3/well, and 10 μl of CCK-8 reagent (Wanleibio) was added to each well and incubated in 5% CO_2 at 37°C for 1 h. Then, the OD value at 450 nm was determined with a microplate reader (ELx800, BioTek, VT, USA).

Statistical analysis

Data are expressed as the mean ± standard deviation (SD) and were analyzed using the unpaired Student's t-test or one-way analysis of variance (ANOVA) with GraphPad Prism 5.0 software (GraphPad Software, Inc., San Diego, CA, USA). $p < 0.05$ indicated significant differences.

Results

H/R induced apoptosis and autophagy in H9c2 cells

To investigate H/R-induced apoptosis in H9c2 cells, we first examined cell viability by the MTT assay. The cell viability after H/R was decreased by 23.89% compared to control cells (Fig. 1A). Furthermore, cell apoptosis was detected by flow cytometry, the results of which suggested that the apoptosis rate of cells treated with H/R was remarkably higher than that of the control cells (14.31% vs. 7.58%, respectively) (Fig. 1B). Moreover, the mRNA levels of PRR, Beclin1, Bax and Bcl-2 were assessed by real-time PCR, which showed that the mRNA levels of PRR, Beclin1 and Bax were significantly increased, while the Bcl-2 mRNA level was decreased by H/R treatment in H9c2 cells compared to control cells (Fig. 1C). Finally, the expression levels of PRR, LC3B-I/II, Beclin1, cleaved caspase-3, cleaved caspase-9, Bax and Bcl-2 were measured by western blotting, the results of which suggested that the expression levels of PRR, LC3B-Ⅰ/Ⅱ, Beclin1, cleaved caspase-3, cleaved caspase-9 and Bax were increased in cells after H/R, while the Bcl-2 expression level was decreased compared to that in control cells (Fig. 1D), similar to the real-time PCR results.

The expression of PRR in H9c2 cells was reduced by siRNA

To explore the effect of PRR in H9c2 cells after H/R, endogenous PRR was knocked down in H9c2 cells with three siRNAs: siRNA-1, siRNA-2 and siRNA-3. As shown in Fig. 2A and B, the mRNA and protein levels of PRR were significantly decreased in siRNA-transfected cells compared to standard negative control (NC)-transfected cells, suggesting that PRR was efficiently silenced. Moreover, the mRNA and protein levels of PRR in siRNA-1-transfected cells were the lowest, suggesting that siRNA-1 was the most efficient in silencing PRR among the three siRNAs. Therefore, siRNA-1, which inhibited the PRR mRNA level by 72% and the PRR protein level by 69%, was chosen for further experiments. **Overexpression of PRR aggravated H/R-induced cell autophagy and apoptosis**

To investigate the function of PRR in H/R-treated H9c2 cells, an expression plasmid encoding PRR was transfected into H9c2 cells to overexpress PRR before H/R treatment, and cell proliferation and the apoptosis status were detected by CCK-8 and flow cytometry assays, respectively. According to the results, PRR downregulation significantly promoted cell proliferation and suppressed apoptosis in H9c2

cells, whereas the overexpression of PRR inhibited proliferation and induced apoptosis in H9c2 cells (Fig. 3A and B). The expression levels of apoptosis and autophagy-related factors were also examined by real-time PCR and western blotting. As shown in Fig. 3C and D, expression levels of the proapoptotic factors cleaved caspase-3, cleaved caspase-9 and Bax as well as the autophagy markers LC3B- I /II and Beclin-1 were markedly reduced by the knockdown of PRR and increased by the overexpression of PRR. Moreover, Bcl-2, an antiapoptotic factor, was increased by PRR knockdown and inhibited by PRR overexpression.

Furthermore, to investigate whether the expression of PRR affects the β-catenin pathway, we examined the expression of Dvl-1 and β-catenin by western blotting. As shown in Fig. 3E, the expression levels of Dvl-1 and nuclear β-catenin were increased in H9c2 cells overexpressing PRR and decreased in cells in which PRR had been silenced. The expression levels of Dvl-1 and nuclear β-catenin were increased by 160% and 67% in cells overexpressing PRR, respectively, and decreased by 47% and 60% in cells in which PRR had been silenced, respectively, compared to the control cells. These results suggested that PRR contributes to H/R-induced apoptosis and autophagy in H9c2 cells and that these effects of PRR are related to the β-catenin pathway.

Deactivation of the β-catenin pathway depressed H/R-induced cell apoptosis and autophagy mediated by PRR

To confirm the association between the β-catenin pathway and PRR-induced cell apoptosis as well as autophagy, we added 20 ng/ml DKK-1, a β-catenin antagonist, to H9c2 cells before their transfection and H/R treatment. As expected, the PRR-mediated inhibition of proliferation was reversed by treatment with DKK-1 (Fig. 4A), and proliferation was increased by 44.53% in PRR+DKK-1 cells compared to PRR cells. PRR-induced cell apoptosis was abolished by treatment with DKK-1 (Fig. 4B), and the apoptosis rate was decreased by 22.44% in PRR+DKK-1 cells compared to PRR-overexpressing cells. This effect was also confirmed by changes in the expression of apoptosis-related factors (Fig. 4C). Furthermore, treatment with the β-catenin antagonist blocked the PRR-induced upregulation of the autophagy markers LC3BI/II and Beclin-1 (Fig. 4D). All the results provide evidence to confirm that deactivation of the β-catenin pathway can depress PRR expression and then inhibit H/R-induced cell autophagy and apoptosis.

Discussion

In this study, we found that H/R increased apoptosis and autophagy in H9c2 cells. To the best of our

knowledge, this study is the first to demonstrate that PRR mediates the apoptosis and autophagy of cardiomyocytes induced by H/R via activating the β-catenin signaling pathway.

Acute IRI results in cardiomyocyte death, cardiac failure, and arrhythmias and increases 30-day patient mortality rates (Zhou *et al.* 2017). The mechanism of myocardial IRI is complex and has not been fully elucidated. Additionally, cell apoptosis and autophagy may play pivotal roles in the pathogenesis of IRI. Autophagy-related gene expression was found to be activated in the human myocardium after acute IRI (Singh *et al.* 2014). In addition, cardiomyocyte cell death in IRI is critical to the progression of IRI and probably related to specific signal transduction mechanisms (Ibanez *et al.* 2015). Furthermore, PRR overexpression has been associated with hypertension, diabetes, heart failure, proteinuria, and nephropathy. In addition, PRR has been reported to promote kidney fibrosis and injury through amplifying Wnt/ β-catenin signaling (Li *et al.* 2017). Moreover, PRR silencing attenuated activation of the β-catenin signaling pathway, reduced proliferation and induced apoptosis in pancreatic ductal adenocarcinoma cells *in vitro* (Shibayama *et al.* 2015). However, the role of Wnt/β-catenin signaling mediated by PRR in IRI has not been well studied thus far. Therefore, we used an *in vitro* H/R model to mimic IRI *in vivo* and demonstrated the effects of PRR and Wnt/β-catenin signaling on apoptosis and autophagy under H/R *in vitro*. We first added Ang II receptor blockers to avoid the influence of Ang II.

Cardiomyocyte apoptosis plays vital roles in the progression of myocardial disorders, including IRI. Furthermore, high glucose was found to decrease autophagy and increases apoptosis in mouse podocytes through the PRR signaling pathway (Li and Siragy 2015). Detection of apoptosis-related proteins by flow cytometric analysis showed that the overexpression of PRR downregulated the expression of the antiapoptotic protein Bcl-2 and promoted the activation of cleaved caspase-3 and cleaved caspase-9, thereby inducing apoptosis. These findings suggest that cell apoptosis induced by H/R can be enhanced by PRR overexpression and depressed by PRR silencing in H9c2 cells. Consistent with the results of flow cytometric and western blot analyses, the effect of PRR overexpression in promoting cardiomyocyte autophagy was also demonstrated in H/R-treated H9c2 cells. Autophagy is a bulk protein and organelle degradation process (Matsui *et al.* 2007). PRR has been reported to play a vital role in regulating the maintenance of cellular homeostasis via autophagy-related signaling pathways (Binger and Muller 2013). In addition, PRR mediates the function and survival of podocytes by maintaining autophagy (Riediger *et al.* 2011). In the heart, ischemia/reperfusion stimulates autophagy via a Beclin 1-dependent mechanism (Matsui *et al.* 2007). In this study, H/R induced an increase in the expression of the key autophagy-related

genes LC3B-Ⅰ/Ⅱ and Beclin1, resulting in autophagy flux. Our results demonstrated the inhibitory effect of PRR knockdown on myocardial apoptosis and autophagy.

PRR has been identified as a crucial player in the β-catenin signaling pathway (Cruciat *et al.* 2010). Several studies have confirmed that the β-catenin signaling pathway contributes to a variety of biological processes, including cell apoptosis and autophagy (MacDonald *et al.* 2009). In addition, PRR is involved in the RAS and an auxiliary subunit of the V-ATP enzyme and plays an important role in maintaining the intracellular pH. Notably, the increased expression of PRR enhanced β-catenin activation and augmented the expression of its downstream targets. Conversely, the knockdown of PRR abolished β-catenin activation(Li *et al.* 2017). In a mouse model of IRI, transfection with PRR expression vectors promoted β-catenin activation. PRR overexpression significantly activated the β-catenin signaling pathway, and PRR silencing deactivated β-catenin signaling in human kidney proximal tubular cells (HKC-8 cells) (Li *et al.* 2017). In this study, we found that β-catenin was activated by PRR overexpression and deactivated by PRR silencing, which is consistent with the results of a previous study in the kidney (Li *et al.* 2017). Dvl positively regulates β-catenin signaling. Activated Dvl inhibits the degradation of β-catenin and induces the cytoplasmic accumulation of β-catenin, which then enters the nucleus to regulate the expression of many downstream genes (Kockeritz *et al.* 2006, Forde and Dale 2007). Several studies documented potential positive role of β-catenin in adaptive responses to ischemic (hypoxic) stress, which regulated via GSK3β, and GSK3β phosphorylation played a protective role in ischemic preconditioning by increasing the threshold for the oxidative stress-induced transition in mitochondrial permeability (Juhaszova *et al.* 2004, Thirunavukkarasu *et al.* 2015, Potz *et al.* 2017). However, the results were not always concordant. Another study showed that preventing GSK3β phosphorylation is unlikely to be the key determinant of the cardioprotective effects of ischemic preconditioning (Nishino *et al.* 2008). In addition, The study of Barandon et al. showed the antagonist for the wnt/frizzled pathway decreased the cytosolic accumulation of β-catenin and reduced infarct size (Barandon *et al.* 2003). These conflicting results are probably due to the different experimental models, duration of ischemia or infarction, and the modulating complexity of upstream signals of β-catenin signaling. The present results were obtained on embryonic cardiac muscle cells. We examined the effect of PRR on the expression levels of Dvl-1 and β-catenin. Increased PRR was found to upregulate the expression of Dvl-1 and β-catenin and induce cell apoptosis and autophagy caused by H/R in H9c2 cells.

Notably, the relationship between PRR and the β-catenin pathway was demonstrated in our study

by the treatment of H9c2 cells with an inhibitor of the β-catenin pathway—DKK-1. DKK-1, a natural antagonist of the Wnt signaling pathway, interrupts the Wnt signaling pathway by directly or indirectly competitively binding a receptor common to Wnt-related proteins. We found that DKK-1 downregulated PRR expression after H/R treatment and thereby partially antagonized apoptosis and autophagy induced by PRR. Hence, PRR regulates H/R-induced apoptosis and autophagy via the β-catenin pathway. Some experiments on PRR have been conducted *in vivo*, but the mechanism of PRR remains unknown. Reperfusion strategies for MI may result in paradoxical cardiomyocyte dysfunction, also known as IRI. In a MI rat model, the mRNA level of PRR was remarkably increased(Ribeiro *et al.* 2018). In an IRI rat model, PRR blockade ameliorated MI and IRI(Ellmers *et al.* 2016). Moreover, the increased expression of PRR induced cell apoptosis in an IRI rat model(Granado *et al.* 2014). In PRR knockout mice, the loss of PRR disturbed autophagic flux(Binger and Muller 2013, Kinouchi *et al.* 2010). Given these experimental results in animal model, we predict that results collected *in vivo* will be similar to those of previous studies, which provide evidence for the regulatory effects of PRR on cell apoptosis and autophagy in cardiac myocytes. Overall, further research is still required to better understand the function of PRR and its underlying mechanism in cardiomyocytes *in vivo*.

Conclusions

In conclusion, PRR regulates H/R-induced apoptosis and autophagy in cardiomyocytes through activation of the β-catenin signaling pathway. This study is expected to provide a new therapeutic target for the prevention and treatment of myocardial IRI.

Acknowledgment

This study was supported by a grant from the National Natural Science Foundation of China (No. 81500304).

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Table

Table 1 Sequences of primers used for real-time PCR

Primer name	Sequence (5'-3')
PRR F	CGAGTGCTTTAGCGAATGAA
PRR R	GAGCCAGTTTATCTACCCCC
beclin1 F	TGCGTCAGCTCTCGTCAAG
beclin1 R	ACCTCCAGAGTTCCCATCGC
Bax F	GGCGAATTGGAGATGAACTGGAC
Bax R	GCAAAGTAGAAGAGGGCAACCAC
Bcl-2 F	ATGCGACCTCTGTTTGATTTCTC
Bcl-2 R	AACTTTGTTTCATGGTCCATCCT
β-actin F	CACTGTGCCCATCTACGAGG
β-actin R	TAATGTCACGCACGATTTCC

FIGURE LEGENDS

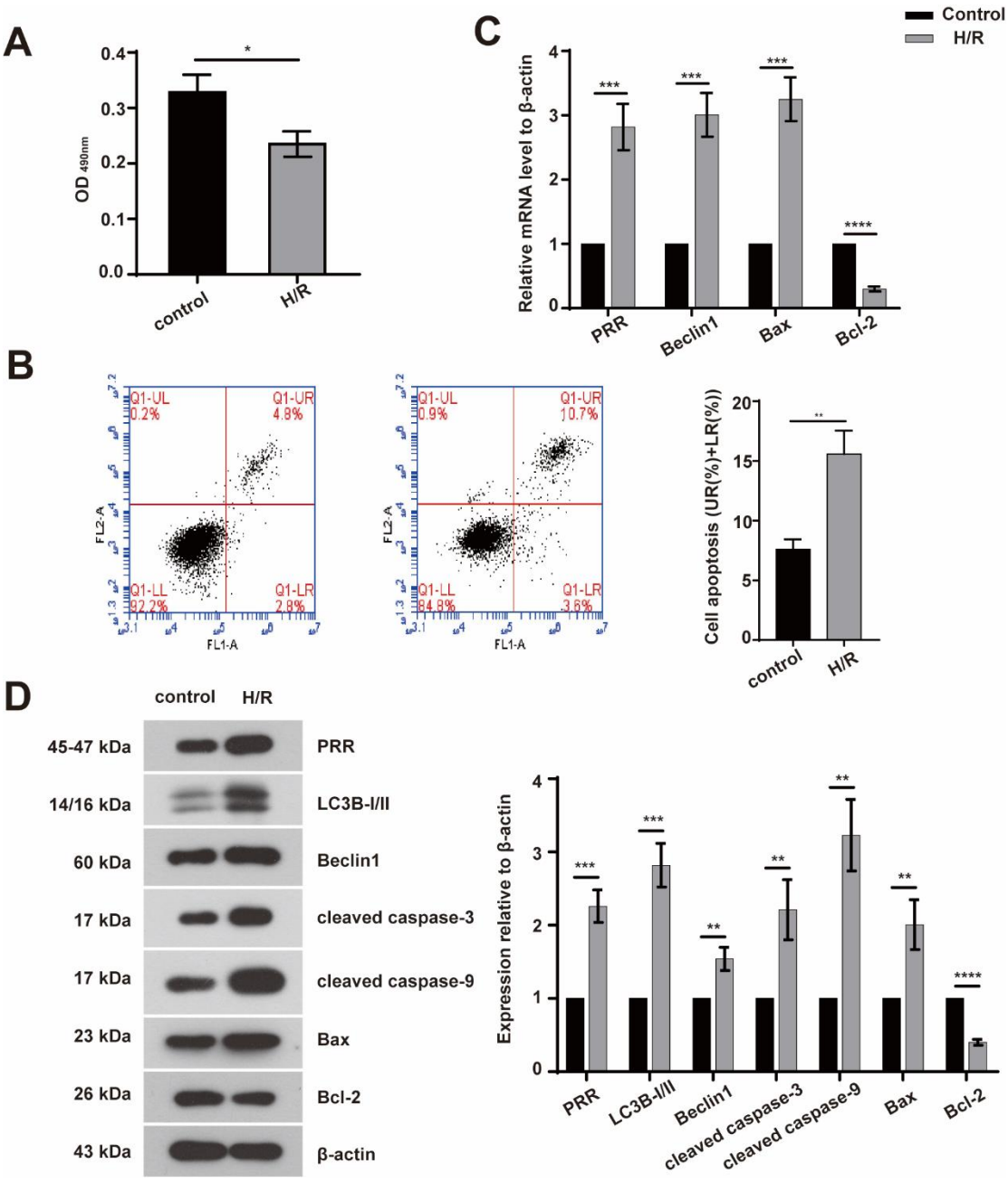

Fig. 1. H/R induced autophagic activity and apoptosis in H9c2 cells. (A) H9c2 cells were treated with (called H/R) or without (called control) hypoxia (1% O_2, 5% CO_2, and 94% N_2) for 24 h, followed by reoxygenation (5% CO_2, 37℃) for 2 h. H9c2 cell viability was measured at 490 nm using the MTT method. (B) Apoptosis of control and H/R cells (UR (%) + LR (%)) was measured with an apoptosis

assay kit. (C) The mRNA levels of PRR, Beclin1, Bax and Bcl2 were determined by real-time PCR. (D) The protein levels of PRR, LC3BI/II, Beclin1, cleaved caspase-3, cleaved caspase-9, Bax and Bcl-2 were analyzed using western blotting. Data are expressed as the mean ± standard deviation (SD) and were analyzed using the unpaired Student's t-test or one-way ANOVA (n =3). *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

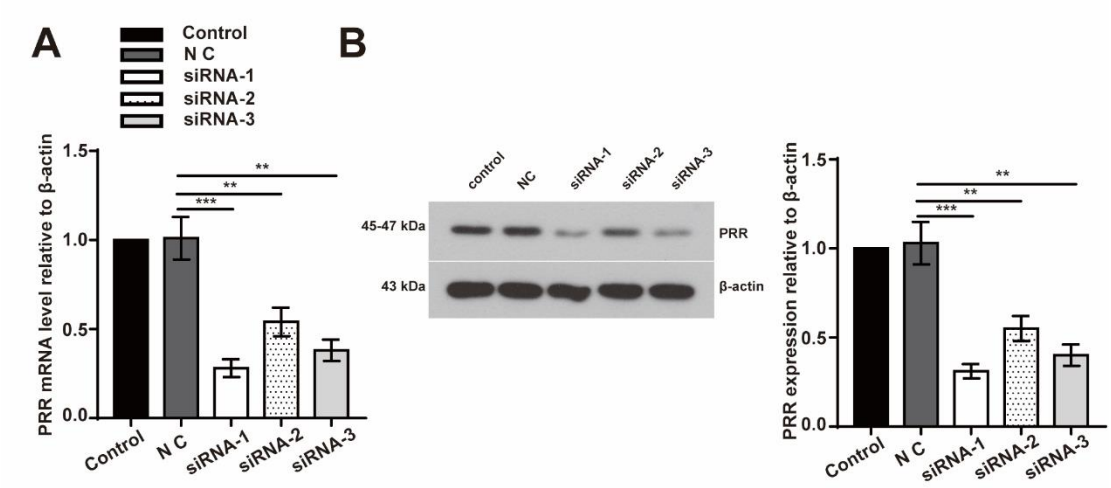

Fig. 2. PRR expression was most effectively silenced in H9c2 cells transfected with siRNA-1. (A) After transfection with siRNA-1, siRNA-2 or siRNA-3, the mRNA levels of PRR in H9c2 cells were determined by real-time PCR. (B) The expression level of PRR was assessed by western blotting. Data are expressed as the mean ± standard deviation (SD) and were analyzed using one-way ANOVA (n =3). **p<0.01, ***p<0.001.

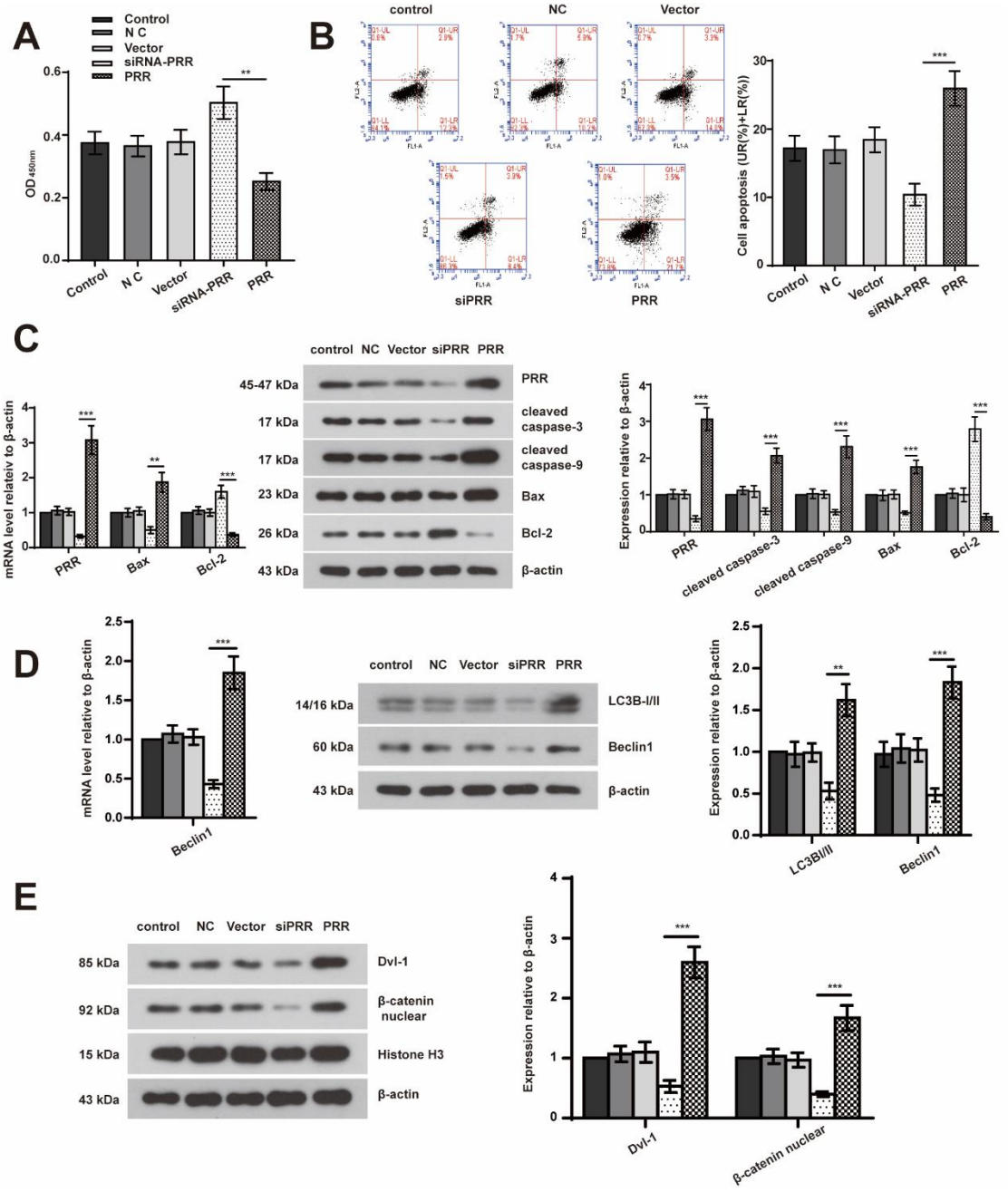

Fig. 3. Overexpression of PRR induced cellular autophagy and apoptosis after H/R. H9c2 cells were treated with hypoxia (1% O_2, 5% CO_2, and 94% N_2) for 24 h, followed by reoxygenation (5% CO_2, 37℃) for 2 h. Cell proliferation (A) and apoptosis (B) were detected by CCK-8 and flow cytometry assays, respectively. Cell proliferation (A) was decreased, and apoptosis (B) was increased by PRR overexpression in H9c2 cells compared to siRNA-mediated PRR knockdown cells. (C) Detection of cell apoptosis-related genes. The mRNA levels of PRR, Bax and Bcl-2 were determined by real-time PCR,

and the expression levels of PRR, cleaved caspase-3, cleaved caspase-9, Bax and Bcl-2 were assessed by western blotting. (D) The mRNA level of Beclin1 and the expression levels of LC3BI/II and Beclin1 were detected by real-time PCR and western blotting, respectively. (E) The expression levels of Dvl-1 and nuclear β-catenin were determined by western blotting. Data are expressed as the mean ± standard deviation (SD) and were analyzed using one-way ANOVA (n =3). $^{**}p<0.01$, $^{***}p<0.001$.

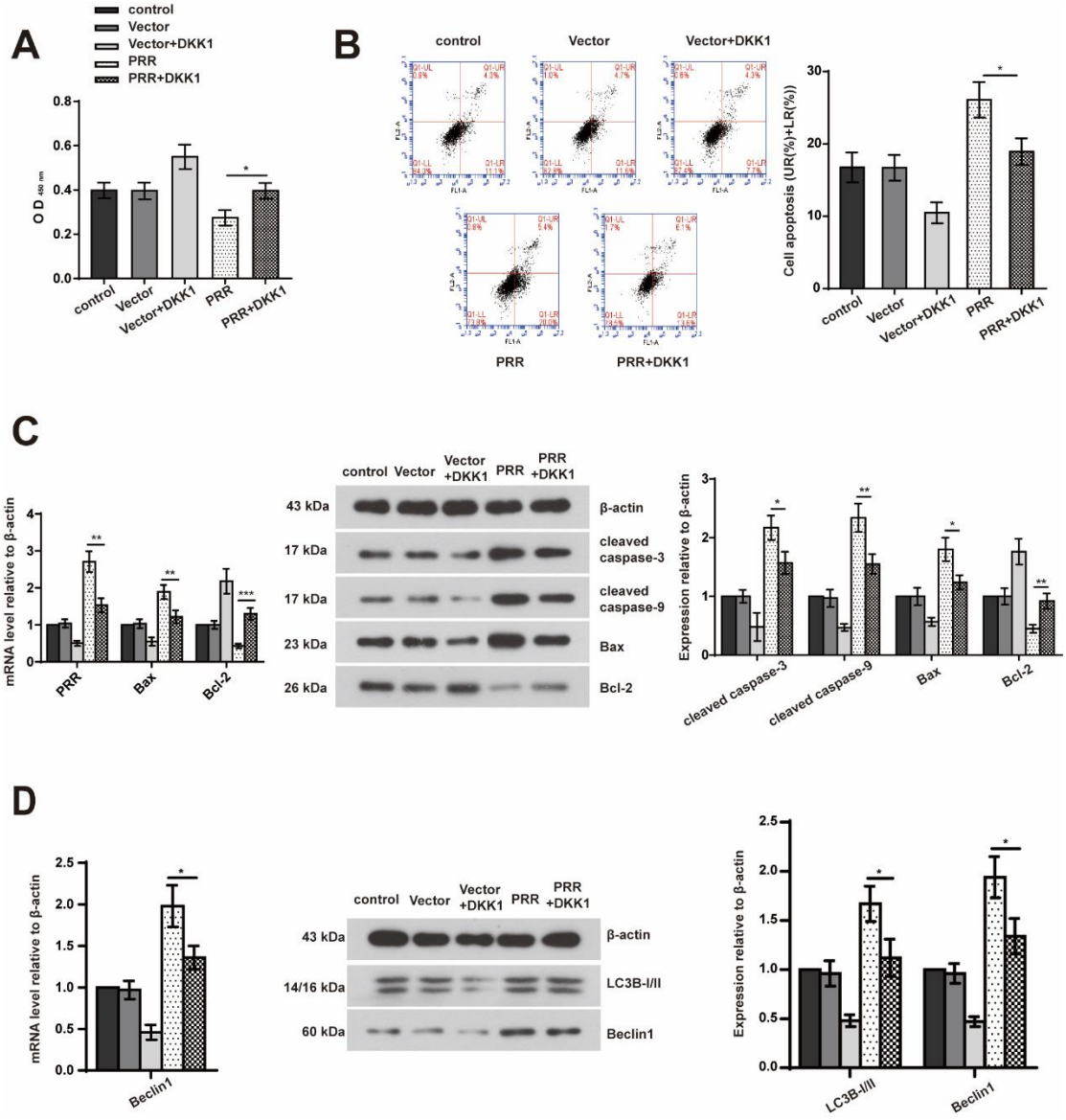

Fig. 4. Deactivation of the β-catenin pathway depressed PRR-induced cell autophagy and apoptosis after H/R. H9c2 cells were prepared by treatment with 10 μmol/L valsartan and 20 μmol/L PD123319. Then, the cells were treated with or without 20 ng/ml DKK-1 before their transfection. After transfection, the cells were subjected to H/R treatment. Cell proliferation (A) and apoptosis (B) were detected by CCK-8 and flow cytometry assays, respectively. Cell proliferation (A) was increased, and apoptosis (B) was decreased in H9c2 cells treated with DKK-1 compared with cells without DKK-1 treatment. (C) The mRNA levels of PRR, Bax and Bcl-2 were determined by real-time PCR. Moreover, the expression levels of cleaved caspase-3, cleaved caspase-9, Bax and Bcl-2 were assessed by western blotting. (D) The mRNA level of Beclin1 and the expression levels of LC3BI/II and Beclin1 were detected by real-time PCR and western blotting, respectively. Data are expressed as the mean ± standard deviation (SD) and were analyzed using one-way ANOVA (n =3). *p<0.05, **p<0.01, ***p<0.001.