

Cite this: *RSC Adv.*, 2018, 8, 13871

Notoginsenoside R₁ prevents H9c2 cardiomyocytes apoptosis against hypoxia/reoxygenation *via* the ERs/PI3K/Akt pathway

Guang Li,^{abc} Xiaoyan Xing,^{*ac} Yun Luo,^{ac} Xuehong Deng,^{ac} Shan Lu,^{ac} Shimin Tang,^d Guibo Sun^{ac} and Xiaobo Sun^{*ac}

Notoginsenoside R₁ (NGR₁) is separate from Panax notoginsenosides (PNS), and plays a role similar to phytoestrogen in preventing and treating cardiovascular diseases. However, the protective mechanism of NGR₁ in the myocardial ischemia/reperfusion injury *via* the estrogen receptor (ER) pathway remains unclear, which hinder its application. This study aimed to study the preventive mechanisms of NGR₁ in the apoptosis of H9c2 cardiomyocytes after hypoxia/reoxygenation (H/R). NGR₁ did not affect the expression of ER α and ER β proteins in normal H9c2 cardiomyocytes. However, NGR₁ could upregulate the ER α and G protein-coupled receptor 30 (GPR30) proteins in H9c2 cardiomyocytes after H/R without affecting ER β levels. Moreover, it significantly affected the expression levels of PI3K and its downstream apoptosis proteins such as Bcl-2 Associated X Protein (Bax), B cell lymphoma/leukemia-2 (Bcl-2), caspase-3, and so forth. Whereas, after adding the PI3K protein antagonist, the modulatory expression levels of PI3K and its downstream apoptosis proteins were remarkably abolished. After adding ER α and GPR30 antagonists, NGR₁ had no significant effect on the expression of PI3K and its downstream Akt protein in the model group. The data of flow cytometry showed that after adding the ER α , GPR30 and PI3K antagonists, the apoptotic rate of cardiomyocytes had no significant changes compared with the model group. This study demonstrated that NGR₁ protected H9c2 cardiomyocytes from the injury after H/R by affecting ER α and GPR30 to regulate the expression levels of PI3K and its downstream apoptosis proteins.

Received 7th July 2017
Accepted 29th March 2018

DOI: 10.1039/c8ra02554a

rsc.li/rsc-advances

1. Introduction

Coronary heart disease is one of the main causes of death in humans.¹ In most cases, reperfusion therapy after myocardial ischemia could restore heart function and repair damaged structures. However, sometimes, reperfusion after ischemia not only is unable to restore organ functions, but also causes myocardial energy metabolism disorder, ultrastructural changes, and no vascular reflow, aggravating functional disorder and structural damage. This phenomenon is termed as myocardial ischemia-reperfusion injury (MIRI).² At present, MIRI is the major obstacle in obtaining the optimal therapeutic effect from reperfusion of ischemic myocardium. Prevention

and mitigation of a reperfusion injury have become the major focus for researchers nowadays.

Clinical studies have shown that estrogen plays a role in preventing ischemic heart disease.³ In 1997, Kolodgie *et al.* proved that 17 β -estradiol could effectively reduce the myocardial infarct size after ischemia-reperfusion in a female rat model with bilateral ovarian resection. Kang *et al.* found that estrogen (E₂) significantly reduced the myocardial oxidative stress injury and dilated the coronary artery.^{4,5} Li *et al.* found that estrogen could significantly reduce heart rate and blood pressure in normal and propyleneephine-treated hearts and myocardial energy consumption and prevent Ca²⁺ overload.⁶ However, the underlying mechanisms of these effects remain unclear. Moreover, estrogen could also protect myocardial cells through anti-inflammatory and anti-apoptotic responses.⁷

It is expensive and has obvious adverse effects for treating menopausal syndrome and cardiovascular disease using estrogen replacement therapy (ERT). The long-term use of ERT could increase the incidence of a series of diseases, such as uterine and breast cancers,⁸ endometrial hyperplasia and hypercoagulable state,^{9–13} venous thrombosis,¹⁴ gallbladder disease and stroke.^{15,16} Drug withdrawal after ERT could easily result in repeated illness.¹⁷ On the contrary, oral estrogen had

^aThe Institute of Medicinal Plant Development, Chinese Academy of Medical Sciences, Beijing 100093, China. E-mail: sun_xiaobo163@163.com; lilyclxy@163.com; Fax: +86-10-57833013; Tel: +86-10-57833013

^bYunnan Branch, Institute of Medicinal Plant Development, Chinese Academy of Medical Sciences, Jing Hong 666100, China

^cBeijing Key Laboratory of Innovative Drug Discovery of Traditional Chinese Medicine (Natural Medicine) and Translational Medicine, Institute of Medicinal Plant Development, Peking Union Medical College and Chinese Academy of Medical Sciences, Beijing 100193, China

^dChangchun University of Chinese Medicine, 130117, Changchun, China



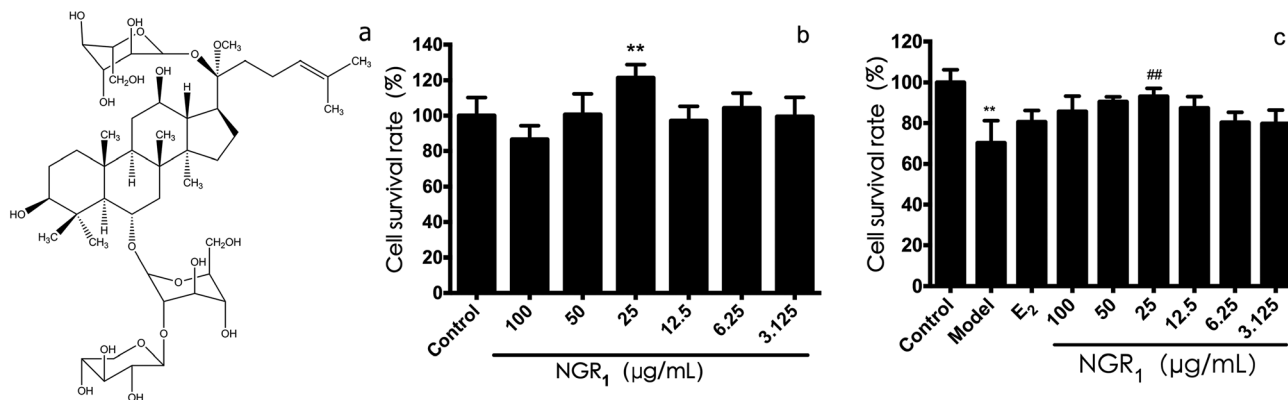


Fig. 1 Protective effects of NGR₁ against H/R-induced H9c2 cell injury. (a) Structural formula of NGR₁. (b) Effects of NGR₁ on H9c2 cell viability. Cells were incubated with different doses of NGR₁ for 24 h. Cell viability was measured by MTT assay (c) effects of NGR₁ on H/R-induced H9c2 cell viability. H9c2 cells were pretreated with indicated doses of NGR₁ and E₂ for 24 h before exposure to H/R for 12 h. Cell viability was measured by MTT assay. (B and C, expressed as the percentage of control). All results are expressed as the mean \pm SD of three independent experiments. ** $P < 0.01$ vs. control group; ## $P < 0.01$ vs. H/R-treated cells.

a severe first-pass effect on liver,¹⁸ but subcutaneous injection or changing the dosage form virtually increases costs and difficulty in treatment. Considering the aforementioned disadvantages of ERT, estrogen replacements are urgently needed. It was hoped that these substitutes could preserve the protective effects of estrogen on the cardiovascular system while avoiding the adverse effects. Phytoestrogen is one of them.¹⁹

Pseudo-ginseng is the subterranean root of *Panax notoginseng* (Burk) F. H. Chen (Araliaceae, Panax), which was used for promoting blood circulation and removing blood stasis. It has obvious controlling effects on cardio-cerebrovascular diseases,²⁰ and has attracted much attention for an effective MIRI control drug in the worldwide.²¹ Notoginsenoside R₁ (NGR₁, Fig. 1a), one of the main components, accounts for about 5% of the total saponin. Wang *et al.* proved that NGR₁ exerted phytoestrogen-like effects by activating the phosphorylation of phosphorylated estrogen response element (pERE).²² Our group found that NGR₁ could protect cardiomyocytes from H/R-induced injuries by regulating the expression of endoplasmic reticulum stress-related proteins, thereby promoting the expression of apoptosis proteins.²³ Moreover, we proved that NGR₁ could inhibit SH-SY5Y cell apoptosis *via* the mitogen-activated protein kinase 1 and 2-extracellular signal-regulated kinases 1 and 2 (MEK1/2-ERK1/2) pathway.²⁴

However, the mechanisms underlying its estrogen-like effects and its role in preventing MIRI remain unclear. Using ER and PI3K pathway antagonists, this study proved that NGR₁ could antagonize the apoptosis of cardiomyocytes by regulating PI3K and its downstream pathways *via* the ER pathway, highlighting the mechanism of MIRI prevention and treatment by NGR₁.

2. Materials and methods

2.1 Drug and reagents

NGR₁ (molecular weight: 933.15, purity \geq 98%) was obtained from the Shanghai Winherb Medical S & T Development Co. Ltd (Shanghai, China). ER α , ER β , and GPR30 receptor agonists and

antagonists were procured from Cayman Chemical Inc (Michigan, USA). ER β , GPR30, and Bax antibodies, as well as secondary antibodies, including goat anti-rabbit immunoglobulin G (IgG) and goat anti-mouse IgG, were purchased from Abcam (Cambridge, England). PI3K, Akt, caspase-3, Bcl-2, ER α , and β -actin antibodies were purchased from Millipore Corporation (CA, USA). Dulbecco's modified Eagle's medium (DMEM) was purchased from Hyclone (Utah, USA). Foetal bovine serum (FBS) was purchased from Gibco (NY, USA). Double antibiotics were purchased from Gibco (NY, USA). The Alexa Fluor 488 Annexin V/propidium iodide (PI) Apoptosis Kit was procured from Life Technologies (CA, USA). The protein extraction and separation reagent were obtained from CW Biotech (Beijing, China). All other reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2 Cell culture and treatment

H9c2 cells were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China), cultured in Dulbecco's modified Eagle's medium with high glucose, supplemented with 10% fetal bovine serum (FBS), 100 U mL⁻¹ of penicillin, 100 mg mL⁻¹ of streptomycin, and maintained in a humidified incubator at 37 °C with 5% CO₂. The H/R model was established according to previous study²⁵ The anaerobic incubator (Coy Laboratory, Michigan, USA) was used to establish the model at 37 °C. First, the complete medium was replaced with the FBS-free and sugar-free medium to culture cells at 37 °C with 5% CO₂, 5% H₂, and 90% N₂ for 6 h. The medium was replaced with the FBS-free high-glucose medium to continue cell culture with 5% CO₂ for another 12 h. The control group was cultured under the same conditions in the carbon dioxide incubator with 5% CO₂.

2.3 Cell viability

The cell viability was measured using the MTT assay. The H9c2 cells were cultured in a 96-well plate (10 000 cells per well), and NGR₁ was added at doses of 100, 50, 25, 12.5, 6.25, and 3.125 µg mL⁻¹ for 24 h. MTT solution (1 mg mL⁻¹) was added after administrating NGR₁, and the cells were cultured continuously



for another 4 h at 37 °C. By the end of cell incubation, dimethyl sulfoxide (100 μ L per well) was added for dissolution. Optical density (OD) values were measured at 570 nm using the automatic multifunctional microplate reader (Model i3, Molecular Devices, CA, USA) for calculating cell survival rates.

2.4 Flow cytometry

Cell apoptosis was detected by flow cytometry using the Annexin V/PI kit. The cell culture medium in each group was collected after treatment. The cells were trypsinized, and culture solutions were collected and combined with centrifugation at 1000 rpm for 10 min. The cells were then washed with cold phosphate-buffered saline and centrifuged at 1000 rpm for 10 min. The cells in each group were treated following the manufacturer's instructions for detection. The flow cytometer was BD Accuri C6 (Accuri Cytometers, Inc. Michigan, USA).

2.5 Detection of protein expression using western blot

The cells were cultured in 25 cm² flasks. Molding was performed 24 h after drug administration, and then the proteins were extracted and purified using the mammalian protein extraction kit (CW Biotech, Beijing, China). Then, the proteins were

quantified by the BCA protein quantification kit (CW Biotech, Beijing, China). Protein extracts were diluted to the same concentration and added into the loading buffer (CW Biotech, Beijing, China) at a ratio of 1 : 4 for 10 min in a boiling water bath. The protein samples were separated from the sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred onto the polyvinylidene difluoride (PVDF) membrane using a semidry blotter. The resulting PVDF membrane was blocked for 2 h using the blocking buffer [5% milk in Tris-buffered saline with Tween 20 (TBST)]. Then, primary antibodies were prepared and incubated overnight at 4 °C. The membrane was washed three times using TBST. The membrane was incubated with secondary antibodies in the blocking buffer for 2 h and then washed three times using TBST. After adding the enhanced chemiluminescence, the membrane was incubated in the dark for 5 min. The band intensities were quantified using the Photo-Image System (Molecular Dynamics, Sweden).

2.6 Statistical analysis of data

All data were repeated three times. Three to five wells for each group were placed on the 96-well plate. Data were presented as mean \pm SD. The statistical analysis was performed by one-way ANOVA using the SPSS version 13.0 (SPSS, IL, USA).

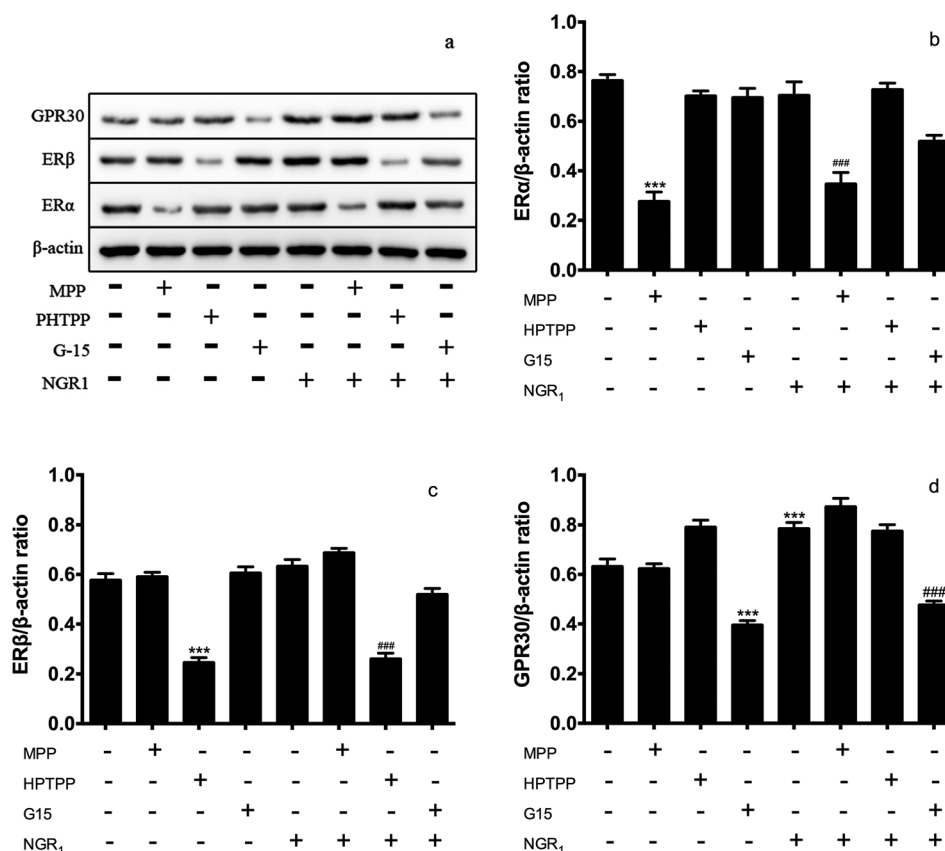


Fig. 2 Effects of ER antagonists on the expression of ER α/β in normal H9c2 cardiomyocytes. H9c2 cells were pretreated with ER α antagonist MPP (100 nM) for 30 h, ER β antagonist PHTPP (1 μ M) for 24 h and GPR30 receptor antagonist G-15 (1 μ M) for 24 h, then cells were exposed to NGR₁ (25 μ g mL⁻¹) for 24 h. Expression levels of GRP30, ER β , ER α , and β -actin were investigated by western blot. (a) Representative images of GRP30, ER β , ER α , and β -actin (b–d) statistical results of GRP30, ER β , ER α relative to control group. *** p < 0.001 vs. control; ### p < 0.001 vs. H/R-treated cells.



Comparisons were tested by one-way ANOVA and least significant difference. A *P* value less than 0.05 was considered to be statistically significant.

3. Results

3.1 Effects of NGR₁ on the survival rate of H9c2 cells after normal and H/R treatment

First of all, we evaluated the cell viability effects of various concentration of NGR₁. As shown in Fig. 1b, the survival rate of H9c2 cells at the dose of 25 $\mu\text{g mL}^{-1}$ was the highest ($121.4 \pm 7.5\%$), which was significantly different from that in the control group. Next, the protective effects of NGR₁ on H/R induced H9c2 cell injury were investigated. As shown in Fig. 1c, after 6 h of hypoxia and 12 h of reoxygenation, the cell survival rate ($70 \pm 10\%$) decreased obviously compared with the control group. However, 25 $\mu\text{g mL}^{-1}$ of NGR₁ significantly increased the cell survival rate ($86.53 \pm 7.77\%$) compared with the model group, suggesting that 25 $\mu\text{g mL}^{-1}$ of NGR₁ could obviously inhibit cell apoptosis induced by H/R. Therefore, 25 $\mu\text{g mL}^{-1}$ was considered to be the concentration of drug administration for NGR₁.

3.2 Effects of NGR₁ on the expression of ER proteins

ER protein plays an important role in ischemia reperfusion injury.^{26–28} Then, we accessed the effects of NGR₁ on the expression of ER proteins in H/R treated H9c2 cells. Different doses of ER antagonists were added for different times. Fig. 2a and b showed that a single administration of ER α antagonist MPP significantly decreased the expression of ER α . If NGR₁ was added, the expression of ER α did not change significantly. A single administration of NGR₁ had no effect on the expression of ER α and ER β in normal H9c2 cardiomyocytes. Fig. 2a and c indicated that a single administration of ER β antagonist HPTPP significantly decreased the expression of ER β . When NGR₁ was added, the expression of ER β did not change significantly. A single administration of GPR30 receptor antagonist G-15 significantly decreased the expression of GPR30. If NGR₁ was added, the expression of GPR30 increased. A single administration of NGR₁ also increased the expression of GPR30 (Fig. 2a and d). Thus, when ER antagonists were added, they directly inhibited the expression of ER α and ER β . NGR₁ upregulated the expression of GPR30 in normal H9c2 cardiomyocytes

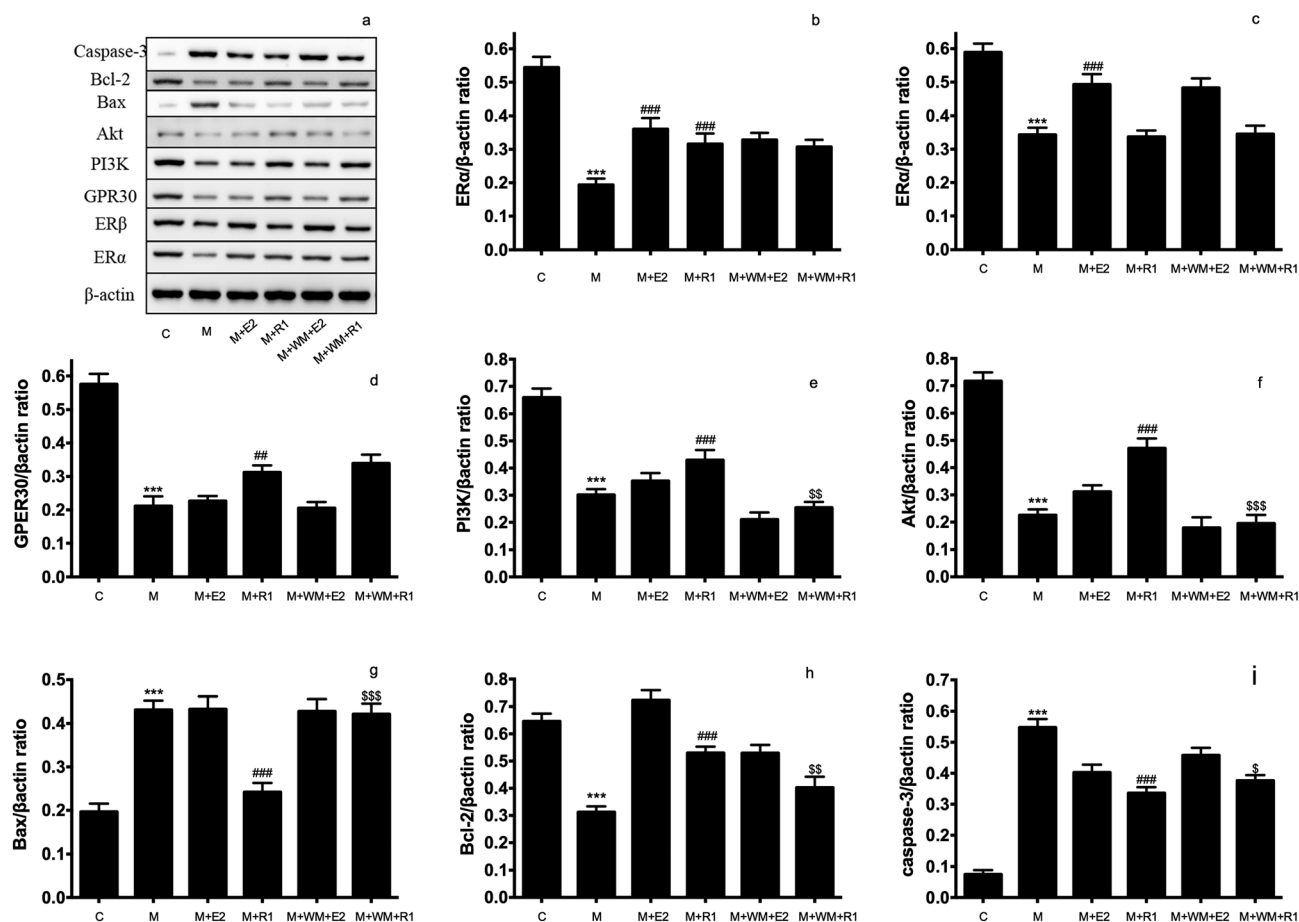


Fig. 3 Effects of PI3K inhibitor on the protein expression in H/R-induced H9c2 cardiomyocytes. H9c2 cells were pretreated with PI3K inhibitor WM (100 nM) for 24 h, then cells were exposed to NGR₁ (25 $\mu\text{g mL}^{-1}$) for 24 h. Expression levels of Caspase-3, Bcl-2, Bax, Akt, PI3K, GPR30, ER β , ER α and β -actin were investigated by western blot. (a) Representative images of Caspase-3, Bcl-2, Bax, Akt, PI3K, GPR30, ER β , ER α and β -actin (b–i) Statistical results of ER β , ER α , GPR30, PI3K, Akt, Bax, Bcl-2, Caspase-3 relative to control group. ****p* < 0.001 vs. control; ###*p* < 0.001 vs. H/R-treated cells; §*p* < 0.05, §§*p* < 0.01 vs. model + NGR₁ group.



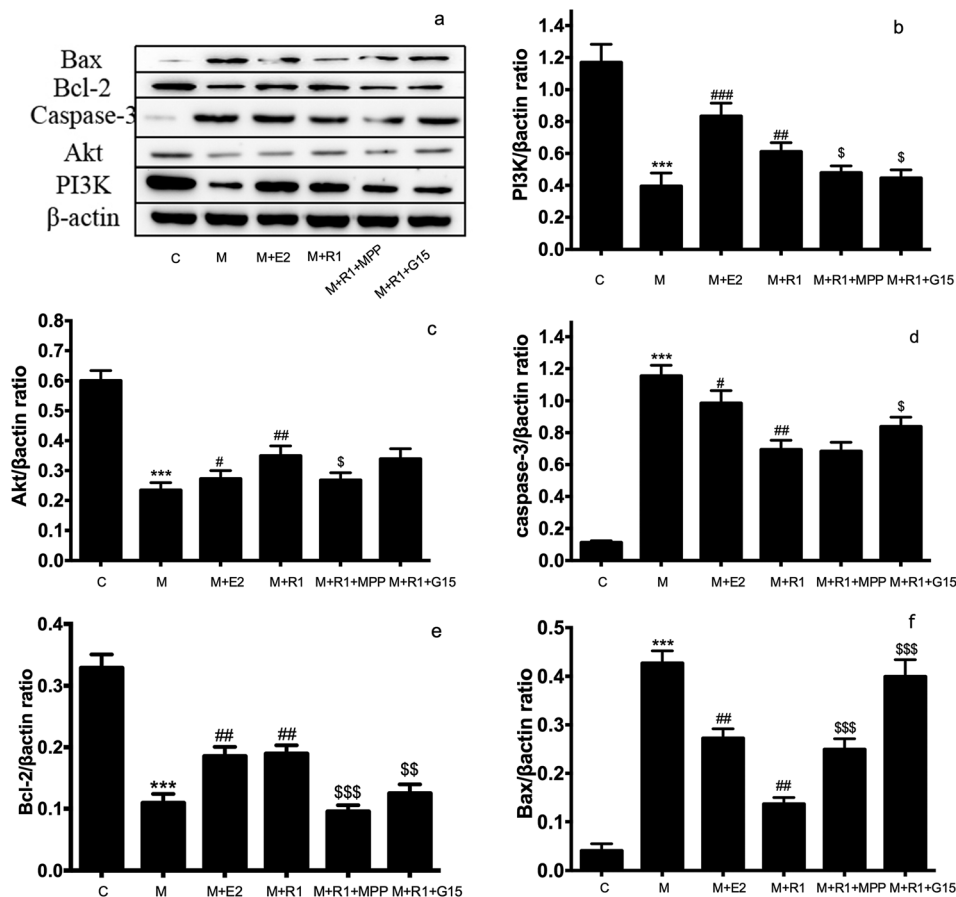


Fig. 4 Effects of ER inhibitor on the expression of PI3K/Akt pathway and apoptosis-related proteins in H/R-induced H9c2 cardiomyocytes. H9c2 cells were pretreated with ER α antagonist MPP (100 nM) for 30 h and GPR30 receptor antagonist G-15 (1 μ M) for 24 h, then cells were exposed to NGR₁ (25 μ g mL⁻¹) for 24 h. Expression levels of Bax, Bcl-2, Caspase-3, Akt PI3K and β -actin were investigated by western blot. (a) Representative images of Bax, Bcl-2, Caspase-3, Akt PI3K and β -actin. (b–f) Statistical results of Bax, Bcl-2, Caspase-3, Akt PI3K relative to control group. *** p < 0.001 vs. control; # p < 0.05, ## p < 0.01, ### p < 0.001 vs. H/R-treated cells; \$ p < 0.05, \$\$ p < 0.01, \$\$\$ p < 0.001 vs. model + NGR₁ group.

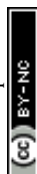
irrespective of the addition of G-15. These data together suggested that the mechanism underlying the regulatory effect of NGR₁ on the expression of ER α and ER β was different from that in the case of GPR30. The aforementioned findings also showed that NGR₁ had no effect on the expression of ER α and ER β protein in normal H9c2 cardiomyocytes, but had a significant stimulatory effect on the expression of GPR30.

3.3 Effects of NGR₁ on the PI3K/Akt pathway and the expression level of downstream proteins

3.3.1 Effects of PI3K antagonist on the PI3K pathway and the expression of its downstream proteins. The PI3K inhibitor was added to H9c2 cardiomyocytes according to the previous report.²⁹ It was observed that the expression of PI3K protein decreased significantly in the model group compared with the control group. However, it increased significantly after treatment with NGR₁ (Fig. 3). After the addition of PI3K inhibitor wortmannin (WM), the expression of PI3K in the model group decreased significantly compared with the model group + NGR₁, suggesting that NGR₁ could increase the expression of PI3K protein in H9c2 cardiomyocytes after H/R treatment. The

expression level of PI3K downstream protein Akt changed with the expression of PI3K protein (Fig. 3a, e and f). After H/R treatment, the expression of ER in H9c2 cardiomyocytes decreased significantly. Estradiol could significantly increase the expression of ER α and ER β in the model group, but it had no effect on the expression of GPR30. NGR₁ could significantly increase the expression of ER α and GPR30 in the model group, but had no effect on the expression of ER β , suggesting that NGR₁ played a role in preventing H9c2 cardiomyocytes injury from H/R treatment by affecting ER α and GPR30, without any effect on ER β (Fig. 3a–d). Apoptosis proteins, such as caspase-3, Bax, and Bcl-2 in the model group had no significant changes, suggesting that NGR₁ affected the apoptosis of cardiomyocytes by regulating the expression of PI3K protein and therefore the expression of its downstream apoptosis proteins through ER (Fig. 3a and g–i).

3.3.2 Effects of ER antagonists on the expression of PI3K and its downstream proteins. We furthermore investigated the modulative effects of MPP and G-15 on the PI3K pathway. As shown in Fig. 4, after adding MPP and G-15, the expression of PI3K decreased compared with the model group with NGR₁,



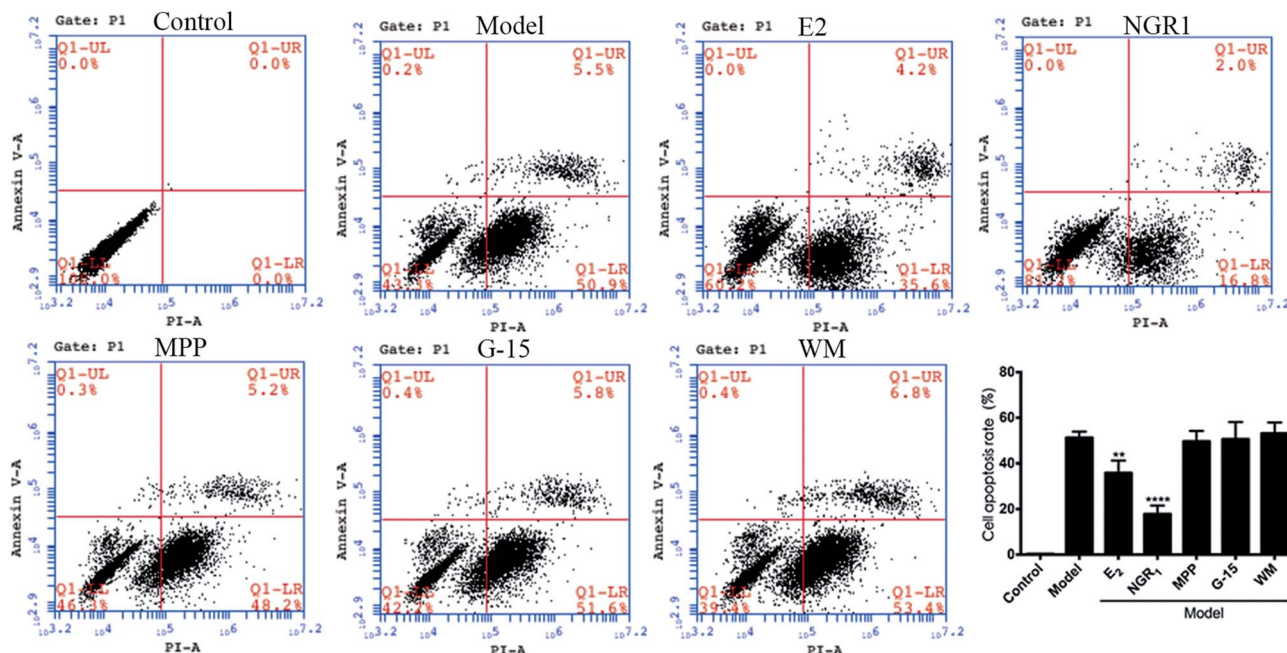


Fig. 5 Effects of NGR₁ on H/R-induced apoptosis in H9c2 cardiomyocytes. H9c2 cells were pretreatment with ER α antagonist MPP (100 nM) for 30 h, PI3K inhibitor WM (100 nM) for 24 h and GPR30 receptor antagonist G-15 (1 μ M) for 24 h, then cells were exposed to NGR₁ (25 μ g mL⁻¹) for 24 h. Annexin V-FITC/PI staining in H9c2 cells was detected by flow cytometry. ^{##} $p < 0.01$, ^{###} $p < 0.001$ vs. H/R-treated cells.

suggesting that NGR₁ could regulate the expression of PI3K through the ER pathway. The addition of G-15 exerts no obvious effects on the expression of Akt compared with the model group with NGR₁, suggesting that the PI3K protein was not the only upstream pathway for Akt protein regulation. NGR₁ may regulate the expression of Akt through bidirectional regulation.

3.4 Effects of ER and PI3K inhibitors on the apoptosis of H9c2 cardiomyocytes

Cell apoptosis and necrosis were identified by the Annexin V-FITC/PI assay. The cell apoptosis rate increased significantly after H/R treatment compared with the normal control group. The addition of E₂ and NGR₁ significantly decreased cell apoptosis. After adding the antagonists, the cell apoptosis rate increased significantly, suggesting that NGR₁ could inhibit the apoptosis of H9c2 cardiomyocytes *via* the ER α , GPR30, and PI3K pathways (Fig. 5).

4. Discussion

NGR₁ is one of the major components of PNS. The therapeutic effects of NGR₁ on organ ischemia-reperfusion injury has gained the attention of researchers in recent years. NGR₁ plays a therapeutic role through anti-inflammatory, anti-oxidative stress, and anti-apoptosis pathways. It has been proved that NGR₁ could be used for treating a neonatal cerebral hypoxic-ischemic injury by affecting endoplasmic reticulum stress through ERs.³⁰ NGR₁ could decrease the inflammatory damage induced by oxidized low-density lipoprotein in vascular endothelial cells by activating the nuclear transcription factor peroxisome proliferator-activated receptor γ .³¹ At the same

time, NGR₁ could improve the intestinal metabolism, and thereby exerted a protective effect on the intestinal ischemia-reperfusion injury. Moreover, NGR₁ played a protective role in lung functions through the transforming growth factor β 1/TGF β activated kinase 1 (TGF- β 1/TAK1) signaling pathway in the pulmonary ischemic model.³² NGR₁ affected cardiac function by inhibiting a myocardial ischemia-reperfusion injury through the tumor suppressor protein vitamin D3 upregulated protein 1/Nuclear factor- κ B (NF- κ B) pathway.³³ For H9c2 myocardial cell apoptosis induced by endotoxins, NGR₁ played an anti-oxygenation and anti-apoptosis role through ER α .³⁴ Chen proved that PNS upregulated the expression of PI3K and Akt proteins in heart and H9c2 cardiomyocytes after hypoxia stimulation.³⁵ It was found in a recent study that NGR₁ could inhibit the apoptosis of H9c2 cardiomyocytes by affecting the expression of endoplasmic reticulum stress-related proteins such as GRP78, p-PERK, ATF6, and IRE.²³

H9c2 cardiomyocytes treated with H/R is the major *in vitro* model of myocardial ischemia-reperfusion injury.^{36–39} This study focused on the roles of NGR₁ in the ER and PI3K pathways during the myocardial H/R injury. We found that NGR₁ could not directly stimulate the expression of ER α and ER β proteins in normal H9c2 cardiomyocytes. However, NGR₁ could increase the expression of GPR30 in normal H9c2 cells, and increase the expression of ER α and GPR30 in H9c2 cardiomyocytes after H/R treatment, suggesting that although NGR₁ could be used as a phytoestrogen, its mechanism of preventing the myocardial ischemia-reperfusion injury was not completely consistent with that of estradiol.⁴⁰ NGR₁ could regulate the expression of PI3K and its downstream protein in the model, thereby controlling the expression of apoptosis proteins such as caspase-3 and Bax to inhibit the



apoptosis of cardiomyocytes. The inhibition of apoptosis of cardiomyocytes disappeared on adding PI3K inhibitors, suggesting that NGR₁ protects cells from the ischemic-reperfusion injury through the PI3K pathway. ER α inhibitor and MPP and GPR30 inhibitor G-15 were used to evaluate the expression of PI3K and its downstream proteins to further explore effects of ER α and GPR30 on the PI3K pathway. Interestingly, although NGR₁ upregulated the expression of GPR30, the addition of GPR30 inhibitor G-15 could also increase the expression of PI3K and its downstream protein Akt, thereby promoting the apoptosis of cardiomyocytes. This is likely to be one of the mechanisms of action of estrogen with bidirectional regulation.

The role of ER in preventing and treating cardiovascular diseases has long been the interest of many researchers. Simoncini *et al.* found that estradiol could reduce the ischemia-reperfusion injury by increasing endothelial nitric oxide synthase and PI3K activities.²⁹ The role of the nonnuclear ER signaling pathway between ER α and PI3K was elucidated. GPR30 agonist could also regulate the expression of PI3K and its downstream proteins to prevent the myocardial ischemia-reperfusion injury.⁴¹ We have proved that NGR₁ could protect the myocardium of mice *via* the ER pathway,⁴² suggesting an estrogen-like effect of NGR₁. The present study found that NGR₁ had no effect on the expression of ER in normal H9c2 cardiomyocytes, probably because NRG₁ could not activate the estrogen response elements in normal H9c2 cardiomyocytes but activated the estrogen response elements after H/R treatment.²²

This study proved that one of the mechanisms of NGR₁ in preventing the myocardial H/R injury is by controlling the expression of PI3K and its downstream apoptosis proteins. However, some mechanisms remain unclear: first, the mechanism how NGR₁ increases the expression of proteins in the model group, although it has no effect on the expression of ER α in normal H9c2 cardiomyocytes; second, the mechanism for GPR30 to regulate the expression of Akt and the downstream apoptosis proteins through non-PI3K pathways. These would be the focus of the in-depth studies in the future.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

The National Natural Science Foundation of China (Grant No supported this study. 81503289, 81303257), the Central Public-interest Scientific Research Found of China, Peking Union Medical College (Grant No. 2016ZX350064), the Applied basic Research Foundation of Yunnan Province, China (Grant No. 2016FB143), Beijing Natural Science Foundation (Grant No. 7172138).

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