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Neuregulin-1 suppresses cardiomyocyte apoptosis by activating PI3K/Akt and inhibiting mitochondrial permeability transition pore

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Abstract Neuregulin-1 (NRG-1) has been shown to attenuate cardiomyocyte apoptosis but the underlying signaling mechanism remains elusive. In this study, we focused on mitochondrial permeability transition pore (mPTP) opening and PI3K/Akt pathway to investigate the effects of NRG-1 on oxidative stress-induced apoptosis of cardiomyocyte. Human cardiac myocytes and neonatal rat cardiac myocytes were exposed to hydrogen peroxide with or without pre-treatment with recombinant human neuregulin-1 (rhNRG-1). Cell apoptosis and mPTP opening were assayed by flow cytometry and confocal microscopy. The activation of Akt was detected by western blot analysis. The results showed that H_2O_2 induced cardiomyocyte apoptosis and activated mPTP. rhNRG-1 inhibited mPTP and activated Akt in the presence of H_2O_2, and further protected the cells from H_2O_2-induced apoptosis. However, rhNRG-1 failed to inhibit mPTP opening and cell apoptosis in the presence of PI3K inhibitor LY294002. Taken together, these findings suggest that NRG-1 activates PI3K/Akt signaling and inhibits mPTP opening, and downstream apoptotic events in cardiac myocytes subjected to oxidative stress.

Keywords Neuregulin · Apoptosis · Mitochondria · Cardiac myocyte

Introduction

Neuregulin-1 (NRG-1) is a peptide produced by endocardial and myocardial microvascular endothelial cells, and binds to receptor tyrosine kinases of the ErbB family on the cell surface of adjacent cardiomyocytes to activate phosphatidylinositol-3-kinase (PI3K)/Akt signaling [1]. NRG-1 has been shown to suppress the apoptosis of cardiac myocytes, but the underlying mechanism remains unclear [2]. Recent studies have suggested that many cardioprotective agents act through inhibiting mitochondrial permeability transition pore (mPTP) opening by PI3K/Akt-dependent pathways [3, 4]. Therefore, we hypothesized that NRG-1 could suppress cardiomyocyte apoptosis by inhibiting mPTP. In this study, we employed isolated neonatal rat cardiac myocytes (NRCM) and human cardiac myocytes (HCM) as the experimental model to examine the effects of recombinant human neuregulin (rhNRG-1) on the apoptosis of cardiac myocytes and investigate the underlying signaling mechanisms.

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Materials and methods

Cell culture and treatment

The hearts were excised from 1- to 3-day-old Sprague-Dawley rats provided by Vital River Laboratories (Beijing. China). Primary cultures of NRCM were isolated by multiple rounds of digestion using 0.4 % collagenase II and 0.05 % trypsin as previously described [5]. This protocol was approved by the Institutional Animal Care and Use Committee of Institute of Zoology accredited by AAALAC International. NRCM were enriched by 1 h of differential plating and viable cells were counted after trypan blue staining. The NRCM were then seeded at 500 or 1,000 cells/mm^2 in DMEM supplemented with 10 % newborn calf serum. One day after plating, the media were replaced with fresh cardiac myocyte medium (CMM) purchased from ScienCell Research Laboratories. This protocol yielded more than 98 % NRCM (see Video Files 1, 2 in Supplementary material, which showed beating NRCM) as defined by sarcomeric actin and Hoechst dye 33342 staining. HCM were purchased from ScienCell Research Laboratories and cultured in CMM. HCM and NRCM were maintained in tissue culture incubator at 37 °C under 5 % CO_2 atmosphere. The cells were treated by rhNRG-1 (Zensun Sci, Shanghai). To induce oxidative stress, cells were incubated with H_2O_2 for the indicated concentrations.

Cell viability assay

Cell viability was analyzed by the trypan blue exclusion method. After the cells were treated as indicated, they were washed with PBS, trypsinized, and then resuspended in PBS. After an incubation of 15 min with 0.4 % trypan blue stain, viable cells (unstained) and nonviable cells (stained) were counted using a standard hemocytometer under light microscopy. The results were presented as the percentage of the control values obtained using untreated cells.

Flow cytometric assay for apoptosis

Cells were grown in 6-well plates. The cells were collected and incubated with binding buffer (in mM: 10 HEPES, 140 NaCl, 5 $CaCl_2$, pH 7.4), then stained with Annexin V-FITC and propidium iodide (PI) for 20 min in the dark at room temperature. Flow cytometric analysis was performed to monitor the green fluorescence of the FITC (500–550 nm) and the red fluorescence of DNA-bound PI (565–615 nm). All data were analyzed with Cell Quest software (BD).

Detection of activation of caspase in situ

Cells were collected and washed with PBS. Caspase FITC-VAD-fmk in situ marker was added to the cells to a final concentration of 10 μM and incubated for 20 min in the dark. Cells were then washed three times with PBS, resuspended in 400 μL PBS and then analyzed with a FACScan.

Assessment of the $\Delta\Psi$m

Changes in the $\Delta\Psi$m were determined by flow cytometry using TMRE staining as described previously. In brief, aliquots of 10^6 cells were prepared as a single cell suspension in RPMI 1640 medium supplemented with 5 % heat-inactivated fetal bovine serum and stained for 20 min at 37 °C in the dark with 25 nM TMRE. Cells stained with TMRE were visualized by flow cytometry using the BD Biosciences FACscan flow cytometer (excitation/emission 549/574 nm).

Flow cytometric assay for mPTP

Calcein-AM is permeable to intact membranes but not to intact mitochondrial membranes. Therefore, mPTP opening leads to the exit of calcein in high conductance mode and the condition allows for monitoring of calcein fluorescence in mitochondria of intact cells [6]. The mPTP opening was measured in intact HCM and NRCM by monitoring calcein-AM fluorescence in the absence and presence of $CoCl_2$, which quenches cytosolic fluorescence, as described previously [6]. In brief, HCM and NRCM were washed twice with PBS and resuspended at 1×10^6 cells/mL in pre-warmed Hanks' balanced salt solution containing 2 mM Ca^{2+}. The cells were then loaded with 1 μM calcein-AM for 15 min at 37 °C in the absence and presence of 2 mM $CoCl_2$. After washing away excess stain and quenching reagent, the cell pellets were resuspended in 400 μL Hanks' balanced salt solution containing Ca^{2+} and analyzed for calcein-AM fluorescence by flow cytometry using a BD Biosciences FACscan flow cytometer in conjunction with WinMDI 2.8 software (excitation/emission, 494/517 nm).

Confocal imaging of mPTP in living cells

Cells were grown on 14-mm diameter glass-bottom microwell dishes. To evaluate mPTP opening, cells were loaded with 1 μM calcein-AM for 25 min at 37 °C in the absence and presence of 2 mM $CoCl_2$. This method allowed the selective loading of calcein in the mitochondria. After washing away excess stain and quenching reagent, the cells were imaged at 37 °C under 5 % CO_2 under a Zeiss LSM 510 confocal microscope. Calcein was excited at 488 nm, and emission was collected through a 505- to 550-nm bandpass filter. Real-time images were captured with a 60×/1.4 oil-immersion objective at a

sampling rate of 4.9 s/frame. At the end, pore-forming antibiotic alamethicin (10 μg/mL, Enzo Life Science) was applied to induce maximal calcein release from the mitochondrial matrix, and the minimum calcein fluorescence after alamethicin treatment was regarded as 0 % for the normalization of calcein fluorescence.

Immunofluorescence analysis

HCM and NRCM grown on glass coverslips were stained with MitoTracker RedCMXRos (Molecular Probes, Inc., Eugene, OR) according to the manufacturer's instructions. After staining, HCM and NRCM were washed in PBS, fixed in 3.7 % paraformaldehyde for 15 min at room temperature, and permeabilized in PBS containing 0.2 % Triton X-100 for 10 min at room temperature. After quenching in PBS containing 1 % bovine serum albumin for 1 h at room temperature, HCM and NRCM were incubated with antibody for cytochrome *c* or Bax (BD Biosciences, diluted in 0.1 % bovine serum albumin in PBS) for 1 h at room temperature. The cells were washed three times for 5 min and then were incubated with an antimouse IgG-AlexaFluor 488 conjugated for 1 h at room temperature. The cells were washed three times for 5 min and mounted on glass slides using the Prolong Antifade kit. Images were captured using a confocal fluorescence microscopy (Zeiss LSM 510 Meta).

Western blot analysis

Cells were lysed in lysis buffer (150 mmol/L NaCl, 25 mmol/L HEPES, 1 % NP40, 50 μmol/L phenylmethylsulfonyl fluoride, 50 μg/mL trypsin inhibitor, 3 mmol/L EDTA, 8 mmol/L EGTA, and 1 mmol/L DTT) on ice. Lysates were centrifuged at 12,000×*g* for 10 min to remove unbroken cells and nuclei. Proteins (20–80 μg) were loaded onto SDS-PAGE gels then transferred onto nitrocellulose membranes as described previously [7]. The membranes were blocked with 5 % nonfat dry milk and 0.1 % Tween 20 for 2 h at room temperature and then probed with primary antibodies against ErbB4, phospho-ErbB4, Akt and phospho-Akt (Ser473) (Cell Signaling Technology) at 4 °C overnight. Immune complexes were detected with horseradish peroxidase-conjugated secondary antibody (Amersham Life Science) and visualized by ECL.

Statistical analysis

All data were expressed as mean ± standard error of the mean (SEM). Significant differences between different groups were determined by two-tailed Student's *t* test or ANOVA with repeated measures and $P < 0.05$ was considered statistically significant.

Results

NRG-1 attenuates H_2O_2-induced apoptosis of cardiomyocytes

Because myocyte apoptosis is often a result of oxidative stress [8] and H_2O_2-induced oxidative stress has been well documented [9], we used HCM and NRCM to study the effect of rhNRG-1 on apoptosis induced by H_2O_2. HCM and NRCM were treated with increasing doses of H_2O_2 (100–400 μM) for 3 h, and cell death and apoptosis were detected by trypan blue exclusion method and flow cytometric analysis, respectively. We observed that H_2O_2 impaired cell viability in a concentration-dependent manner. At 200 μM, H_2O_2 induced approximately 40 % (42.37 ± 2.7 %) cell death and approximately 30 % (32.62 ± 2.5 %) cell apoptosis in HCM (Fig. 1a, c). We chose the concentration of H_2O_2 at 200 μM for subsequent experiments on HCM. Similarly, the concentration of H_2O_2 at 100 μM was selected for subsequent experiments on NRCM (data not shown). The concentration of NRG was selected on the basis of its physiological level and previous studies [10]. HCM and NRCM were treated with rhNRG-1 (0–400 ng/mL) for 3 h and we found that cell viability was not significantly affected (data not shown). However, when HCM and NRCM were pre-treated with 0–400 ng/mL rhNRG-1 for 0.5 h, and then treated by H_2O_2 for 3 h, we observed that 200 ng/mL rhNRG-1 exhibited a marked protection on HCM against H_2O_2-induced cell death and apoptosis (Fig. 1b, d). Similar results were observed in NRCM (data not shown). Thus, we chose 200 ng/mL rhNRG-1 for further experiments.

NRG-1 inhibits H_2O_2-induced mPTP opening in cardiomyocytes

It is known that irreversible mPTP opening leads to downstream proapoptosis events and triggers cell apoptosis. To evaluate whether H_2O_2 activates mPTP and rhNRG-1 inhibits H_2O_2-induced mPTP activation in HCM and NRCM, mPTP activation was examined in intact HCM and NRCM by flow cytometry assay based on monitoring the fluorescence of calcein-AM entrapped in the mitochondria through Co^{2+} quenching of cytosolic calcein fluorescence [11]. We observed that the fluorescence of calcein-AM was decreased in HCM treated by H_2O_2 but was partially recovered after rhNRG-1 treatment (Fig. 2a, b). Similar results were observed in NRCM (data not shown). These results suggest that oxidative stress leads to mPTP activation in cardiomyocytes which can be inhibited by NRG-1.

NRG-1 activates ErbB-PI3K/Akt signaling in cardiomyocytes

Previous studies have shown that H_2O_2 treatment activated PI3K/Akt pathway [12], suggesting the role of PI3KPI3K/

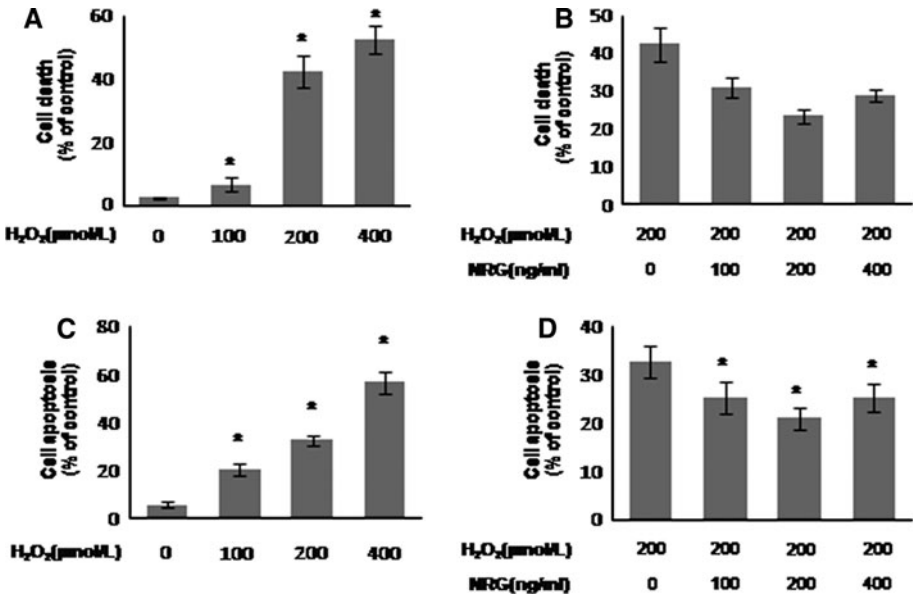

Fig. 1 rhNRG-1 attenuates H_2O_2-induced cell death or apoptosis in HCM. HCM were treated by H_2O_2 or rhNRG-1 as indicated and cell death or apoptosis was determined by trypan blue exclusion or flow cytometry 3 h after treatment. Each value represented the mean ± SEM of three independent experiments.*$P < 0.05$ compared with corresponding control

Akt in oxidative stress-induced injury. To examine whether ErbB-PI3K/Akt pathway is activated by NRG-1, HCM and NRCM were treated for 3 h with H_2O_2 in the absence or presence of rhNRG-1 or PI3K inhibitor LY294002. Western blot analysis showed that H_2O_2 increased the levels of phospho-ErbB4 and phospho-Akt in HCM. Furthermore, NRG-1 pre-treatment synergistically stimulated H_2O_2-induced activation of ErbB-PI3K signaling, which was completely abolished with LY294002 treatment (Fig. 3a, b). Similar results were observed in NRCM (data not shown).

NRG-1 inhibits mPTP opening in cardiomyocytes

It has been shown that PI3K/Akt signaling inhibits the opening of mPTP [3]. Next, we investigate whether PI3K/Akt pathway is involved in the inhibition of mPTP opening by NRG-1 in HCM. mPTP activation was analyzed by the detection of fluorescence of calcein-AM by flow cytometry assay or confocal microscopy. We observed that the fluorescence signal was greater in the cells pre-treated with rhNRG-1 than in the cells treated with H_2O_2 alone, indicating the inhibition of mPTP by NRG-1 in HCM. In addition, in the presence of LY294002, although the fluorescence signal was greater in the cells pre-treated with rhNRG-1 than in the cells untreated with rhNRG-1, the magnitude of the difference became smaller compared to the cells in the absence of LY294002 (Fig. 3c). These results demonstrate that the inhibition of PI3K/Akt pathway attenuated the inhibitory effects of rhNRG-1 on mPTP. mPTP opening analyzed by confocal microscopy showed similar results in HCM loaded with calcein-AM in the presence of $CoCl_2$ (Supplementary Fig. S1).

NRG-1 inhibits the apoptosis of cardiomyocytes

The irreversible opening of mPTP can lead to downstream proapoptosis events. To investigate the effects of NRG-1 on proapoptosis events, we analyzed $\Delta\Psi m$, caspase activity, Bax translocation, and cytochrome *c* release in cardiomyocytes. HCM were loaded with 100 nM TMRE or followed by incubation with FITC-VAD-fmk to assess $\Delta\Psi m$ or caspase activity using flow cytometry. Bax translocation or cytochrome *c* release was examined by immunofluorescence microscopy using anti-Bax or anti-cytochrome *c* mAb and mitochondrial marker MitoTracker CMXRos. Compared with control cells, H_2O_2 treatment resulted in the loss of $\Delta\Psi m$, enhanced caspase activation, significant subcellular BAX translocation, and significant cytochrome *c* release from the mitochondria (Fig. 4, Supplementary Figs. S2, S3). The combination of rhNRG-1 and H_2O_2 attenuated or reversed the changes. In addition, the inhibition of PI3K/Akt with LY294002 could block rhNRG-1-mediated protection from H_2O_2-induced proapoptosis events. Taken together, these data strongly support the idea that PI3K/Akt pathway is required for rhNRG-1-mediated inhibition of H_2O_2-induced mPTP opening.

Finally, we examined the effects of rhNRG-1 on the apoptosis of HCM. Flow cytometry analysis showed that combination treatment with both rhNRG-1 and H_2O_2 resulted in a reduction of cell apoptosis compared with corresponding cells treated with H_2O_2 alone. Moreover, LY294002 could block rhNRG-1-mediated protection from H_2O_2-induced apoptosis (Fig. 5). These data suggest that NRG-1 activates PI3K/Akt signaling to protect cardiomyocytes from oxidative stress-induced apoptosis.

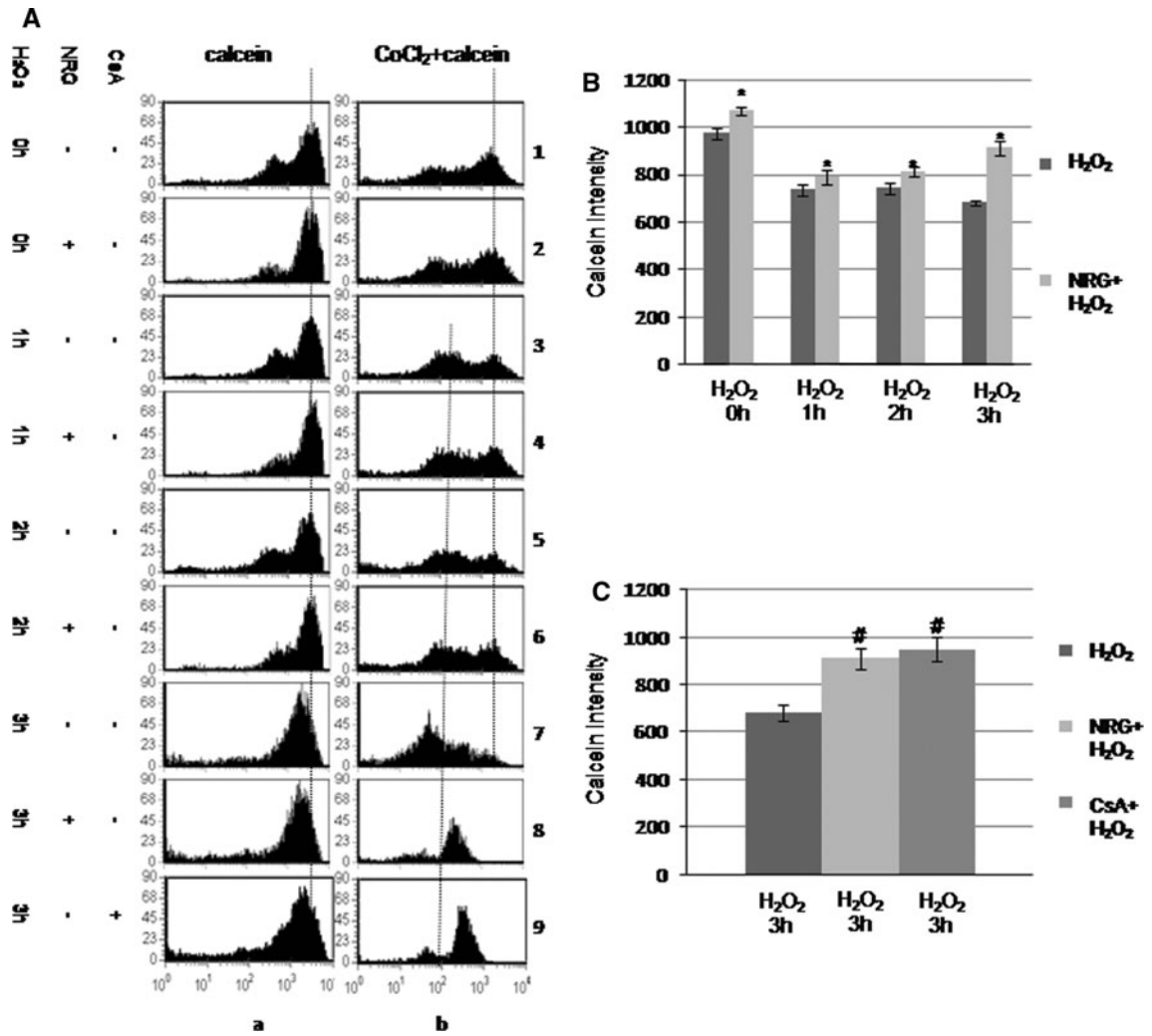

Fig. 2 rhNRG-1 inhibits H_2O_2-induced mPTP opening in HCM. **a** HCM were treated with 200 μM H_2O_2 for 0–3 h as indicated in the presence or absence of rhNRG-1, then incubated with calcein-AM alone (panels *a1–a8*) or with calcein-AM and quenching agent $CoCl_2$ (panels *b1–b8*) and analyzed by flow cytometry. As the positive control, HCM were treated with mPTP inhibitor CsA (0.2 μmol/L). **b**, **c** Quantitation of calcein-AM fluorescence in HCM loaded with calcein-AM in the presence of $CoCl_2$. Each value represented the mean ± SEM of three independent experiments.*$P < 0.05$ compared with corresponding controls without rhNRG-1 treatment, #$P < 0.05$ compared with corresponding controls without rhNRG-1 or CsA treatment

Discussion

In this study, we demonstrate that rhNRG-1 pre-treatment could inhibit mPTP opening and the apoptosis of HCM and NRCM during H_2O_2-induced oxidative stress. rhNRG-1 pre-treatment synergistically stimulated H_2O_2-induced activation of PI3K signaling. Inhibition of PI3K/Akt pathway with pharmacologic PI3K inhibitor, LY294002, significantly abolished rhNRG-1-mediated inhibition of mPTP opening induced by H_2O_2 in HCM and NRCM. These data suggest that NRG-1-mediated protection from H_2O_2-induced apoptosis is associated with PI3K/Akt-dependent mPTP inhibition in HCM and NRCM.

NRG-1 has been shown as a widely expressed signaling molecule involved in cell differentiation, proliferation, growth, survival, and apoptosis [13, 14]. The role of the NRG-1/ErbB signaling axis in heart development and function has been well investigated [2, 15]. NRG could protect cardiac myocytes against apoptosis in various physiological and etiological processes, acting through receptor tyrosine kinases of the ErbB family [2]. Cytoprotective NRG-1/ErbB4 signaling is activated at a cytotoxic concentration of H_2O_2, suggesting that this system may respond to and modulate myocardial stress [16]. Importantly, a recent study demonstrated that acute doxorubicin cardiotoxicity is associated with the inhibition of NRG-ErbB pathway [17]. In addition, recombinant NRG-1β could activate the growth and survival of isolated cardiac myocytes via the ErbB2 and ErbB4 receptors [18]. Consistent with these results, our present study

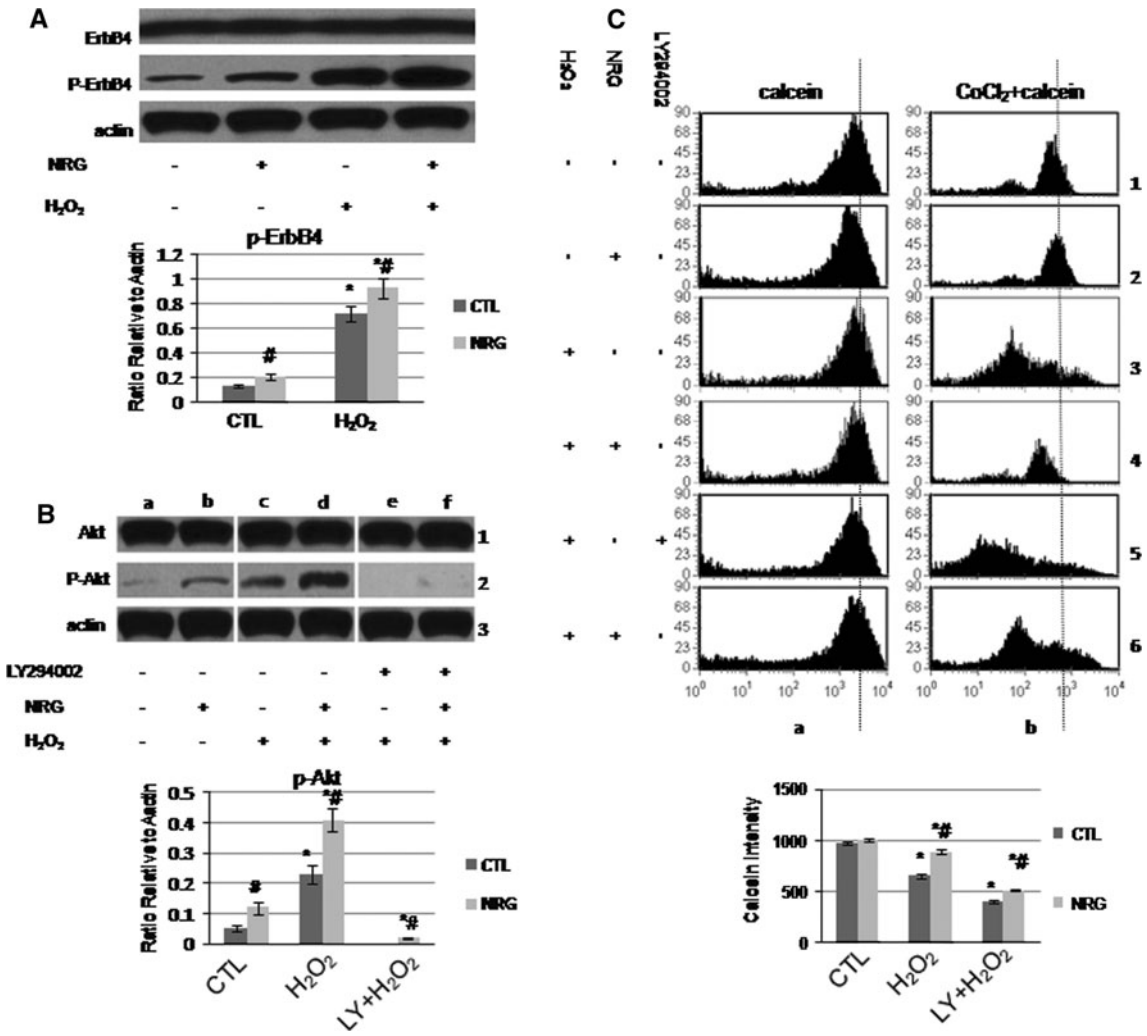

Fig. 3 rhNRG-1 activates ErbB4/Akt signaling to inhibit mPTP opening in HCM. **a** Western blot analysis of ErbB4/p-ErbB4 levels. Cells were treated with 200 μM H_2O_2 for 3 h in the presence or absence of 200 ng/mL rhNRG-1 as indicated. Actin was used as loading control. **b** Western blot analysis of Akt/p-Akt levels. Cells were treated with 200 μM H_2O_2 for 3 h in the presence or absence of 200 ng/mL rhNRG-1 or 50 μM LY294002 as indicated. Actin was used as loading control. **c** Cells were treated as indicated and then incubated with calcein-AM alone (panels *a1–a6*) or with calcein-AM and quenching agent $CoCl_2$ (panels *b1–b6*) and analyzed by flow cytometry. Quantitation of calcein-AM fluorescence in cells loaded with calcein-AM in the presence of $CoCl_2$ was showed at the bottom. Each value represented the mean ± SEM of three independent experiments. $^{*}P < 0.05$ compared with corresponding control, $^{\#}P < 0.05$ compared with corresponding controls without rhNRG-1 treatment. Blots were representative of three different experiments

demonstrated that rhNRG-1 at an optimal concentration, 200 ng/mL, significantly stimulated H_2O_2-induced activation of ErbB signaling to protect HCM and NRCM against H_2O_2-induced apoptosis.

The role of mitochondria, especially mPTP as a main integrator of apoptosis has been generally recognized. mPTP is a nonspecific pore in the inner mitochondrial membrane and its opening is responsible for subsequent necrosis and induction of apoptosis [19]. Once mPTP opening has occurred, the resulting downstream events such as the loss of mitochondrial membrane potential, translocation of Bax, release of cytochrome *c*, and activation of caspases lead to cell apoptosis. Our results showed that rhNRG-1 treatment markedly inhibited mPTP opening and reversed apoptotic events in the presence of H_2O_2. These findings suggest that anti-apoptotic effects of rhNRG-1 might be mediated, at least in part, by inhibiting apoptotic events downstream of mPTP inhibition.

Previous studies have confirmed that mPTP is regulated by PI3K/Akt signaling. PI3K/Akt pathway plays critical cardioprotection role during myocardial ischemia/reperfusion [20, 21]. In addition, PI3K/Akt signaling is the central regulator of NRG/ErbB signaling network [22]. Our results showed that rhNRG-1 stimulated the activation of PI3K signaling during H_2O_2-induced oxidative stress. rhNRG-1-mediated inhibition of mPTP was abolished by PI3K/Akt

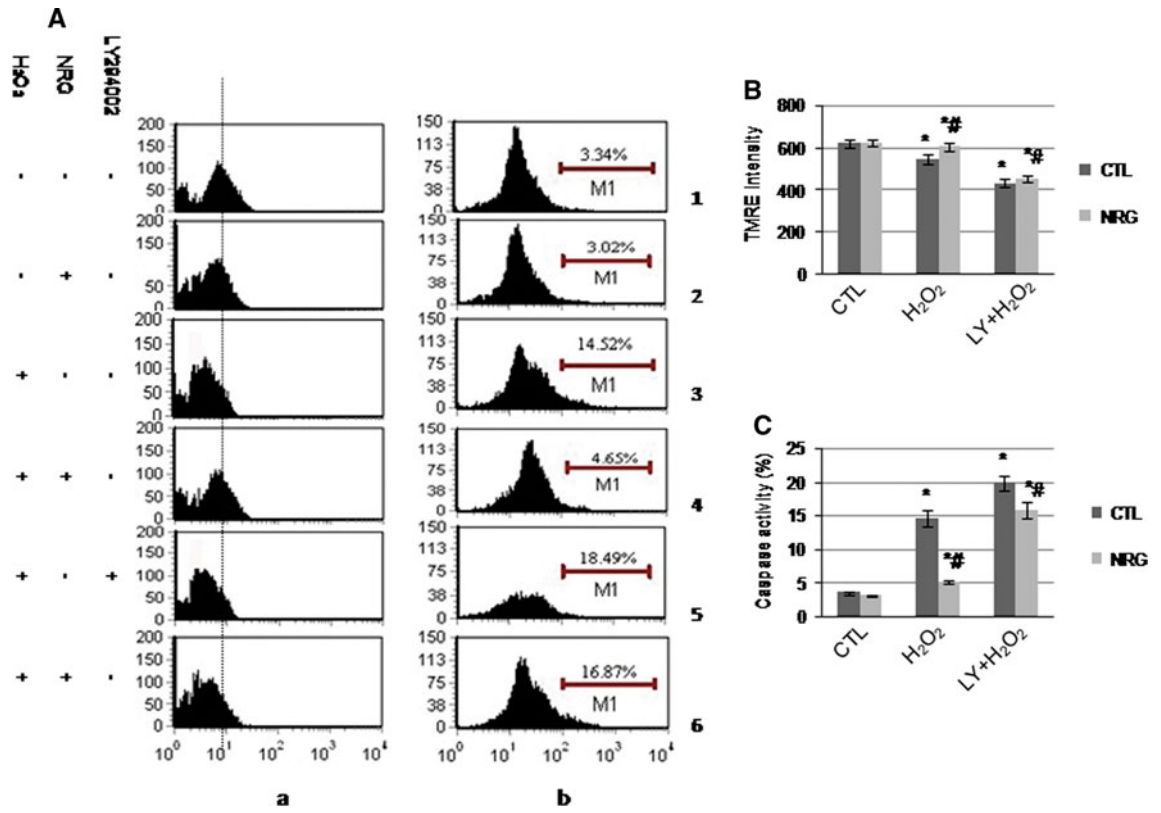

Fig. 4 rhNRG-1 inhibits mitochondrial membrane potential and caspase activity in HCM. **a** Cells were treated as described and then loaded with 100 nM TMRE or FITC-VAD-fmk to measure mitochondrial membrane potential or caspase activity by flow cytometry. **b**, **c** Quantitative analysis of mitochondrial membrane potential or caspase activity in HCM. Each value represented the mean ± SEM of three independent experiments. $^{*}P < 0.05$ compared with corresponding control, $^{\#}P < 0.05$ compared with corresponding controls without rhNRG-1 treatment

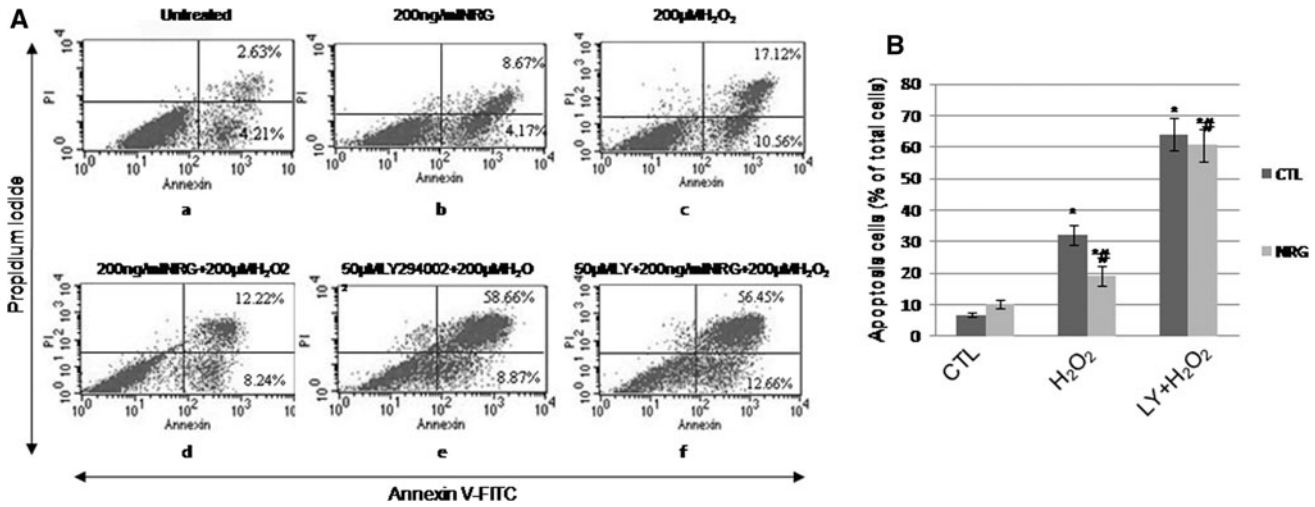

Fig. 5 rhNRG-1 inhibits apoptosis of HCM. **a** Cells were treated as indicated and apoptotic cells were defined as Annexin V positive and analyzed by flow cytometry. **b** Quantitative analysis of cell apoptosis in HCM. Each value represented the mean ± SEM of three independent experiments. $^{*}P < 0.05$ compared with corresponding control, $^{\#}P < 0.05$ compared with corresponding controls without rhNRG-1 treatment

inhibitor, LY294002, which effectively suppressed rhNRG-1-induced activation of Akt. These findings suggest that rhNRG-1 acts as a stabilizer of mPTP during H_2O_2-mediated oxidative stress and this effect is mediated by PI3K/Akt signaling. Furthermore, we demonstrated that rhNRG-1 exerted anti-apoptosis effects by inhibiting mPTP.

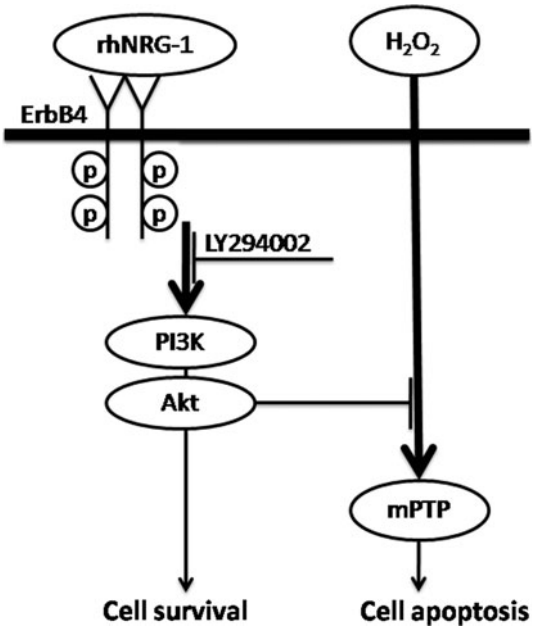

Fig. 6 A schematic model showing the signaling cascades by which rhNRG-1 protects cardiac myocytes from oxidative stress-induced apoptosis. → induction; ⊣ inhibition

Interestingly, although both NRG-1 and H_2O_2 activated Akt pathway, the mPTP opening were oppositely regulated, suggesting a possible bi-directional regulation of Akt signaling on mPTP status. The activation of Akt signaling by H_2O_2 may be a response of cardiomyocytes to oxidative stress [16]. Taken together, these data indicate that mPTP activation during oxidative stress is a key modulator of apoptotic cell death and NRG-1 inhibits H_2O_2-induced apoptosis by interfering with PI3K/Akt regulated mPTP opening (Fig. 6).

Recently, it was reported that the administration of rhNRG-1 to patients with stable chronic heart failure (CHF) resulted in improved cardiac function [23, 24]. Heart failure (HF) is the common clinical syndrome with high mortality that results from virtually all forms of cardiac disease. Cardiac remodeling is involved in the development and progression of HF. Among the three crucial components of remodeling, fibrosis, cardiomyocyte hypertrophy, and damage, cell death with deficient regeneration is considered one of the critical events for the cause of HF [25]. The inhibition of cardiac myocyte apoptotic death largely prevents the development of HF [26]. In light of the protective roles of rhNRG-1 shown in the present study, it is likely that rhNRG-1 induces improved cardiac function in patients with stable CHF by protecting cardiac myocytes from apoptosis via the inhibition of mPTP. Therefore, rhNRG-1 has a great potential to be applied as therapeutics for heart injury mediated by oxidative stress-induced apoptosis, including CHF.

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Conflict of interest The authors declared no conflict of interest.

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