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Original Article

Qiliqiangxin protects cardiac myocytes against hypoxia-induced apoptosis via ErbB4/PI3K/Akt pathway

Jingmin Zhou[1*], Xueting Han[2*], Shijun Wang[1*], Mingqiang Fu[1], Zhiming Li[2], Xuefeng Ding[2], Jingjing Zhang[3], Chuhang Qing[2], Shuang Li[2], Li Chen[2], Kai Hu[1], Yunzeng Zou[1], Junbo Ge[1]

[1]Department of Cardiology, Shanghai Institute of Cardiovascular Diseases, Zhongshan Hospital, Fudan University, Shanghai, China; [2]Department of Cardiology, North Sichuan Medical College, The Affiliated Hospital of North Sichuan Medical College, Nanchong, Sichuan, China; [3]Shandong University School of Medicine, Ji'nan, Shandong, China. [*]Co-first authors.

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Abstract: Cardiac myocytes apoptosis played an important role in heart failure. Plenty of studies reported that Qiliqiangxin (QL), a traditional Chinese medicine, had therapeutic efficacy in chronic heart failure, but the underlying mechanism was not clear. Here we investigated whether QL prevented hypoxia-induced cardiac myocytes apoptosis and the possible mechanism. Isolated neonatal rat cardiac myocytes (NRCMs) were transfected by Lentiviral-ErbB4-siRNA, treated by a specific PI3K inhibitor (LY294002), and subjected to hypoxia with or without QL. Cell apoptosis was detected by terminal deoxynucleotide transferased UTP nick end labeling (TUNEL), flow cytometry and caspase-3 activity assay, respectively. ErbB4 mRNA level was detected by real-time PCR, protein expressions of ErbB4 and phosphorylated Akt were examined by Western blot. The results showed that hypoxia induced significant increase of apoptotic NRCMs, which was significantly attenuated by QL treatment. Further, QL induced the upregulation of ErbB4 and phosphorylation of Akt in hypoxic NRCMs. However, knocking down ErbB4 abolished these effects induced by QL. Furthermore, decrease of NRCMs apoptosis induced by QL was also abrogated by blocking PI3K/Akt signaling. Our data suggested that QL protected cardiac myocytes against hypoxia-induced apoptosis via regulating ErbB4-dependent PI3K/Akt pathway.

Keywords: Qiliqiangxin, hypoxia, cardiac myocytes, apoptosis, ErbB4

Introduction

Apoptosis is critically involved in the pathogenesis of cardiac dysfunction due to loss of normal functioned cardiac myocytes. One study suggests that both acute substantial loss of cardiac myocytes and chronic small amount of cell apoptosis can contribute to the development of cardiac dysfunction and heart failure [1]. Because cardiac myocytes have limited proliferative capacity, it is of crucial importance to preserve cardiac function by preventing cell loss.

Qiliqiangxin (QL), a type of traditional Chinese Medicine extracted from herbs, has a high value in the treatment of chronic heart failure (CHF). Recent clinical trial has revealed that QL treatment increases NT-pro-BNP level and improves cardiac function of CHF patients [2]. QL treatment also prevents cardiac remodeling through suppressing cardiac fibroblasts differentiation induced by AngII, decreasing the inflammatory response and promoting cardiac myocytes survival [3-5]. However, the role of QL in cardiac myocytes apoptosis has not been fully investigated.

The ErbB receptor is one of the receptor tyrosine kinases that belongs to the epidermal growth factor receptor (EGFR) family. ErbB receptors and their ligands play critical roles in the regulation of cell growth, differentiation and survival. Among them, ErbB2 and ErbB4 are highly expressed in cardiac myocytes. Activation of ErbB4 leads to ErbB2/ErbB4 heterodimerization or ErbB2 homodimerization, which stimulates the ErK1/2 cascades and PI3K/Akt pathway [6]. Recent studies indicate that ErbB receptors also play important roles in cardiac

myocytes apoptosis. Knocking down ErbB4 enhanced cleaved caspase-3 and weakened Bcl-2 expressions in cardiac myocytes [7]. Although QL has been proved to prevent rat cardiac myocytes apoptosis after acute myocardial infarction in vivo [8], the present study further investigates its effect on hypoxia-induced cardiac myocytes apoptosis and ErbB4-dependent PI3K/Akt signaling in vitro.

Materials and methods

Materials and reagents

QL was kindly provided by Yiling Pharmaceutical Corporation (Shijiazhuang, China). Low-glucose Dulbecco's modification of Eagle's medium Dulbecco (DMEM), fetal bovine serum (FBS) and Trypsin were purchased from life technology (New York, USA). In Situ Cell Death Detection Kit was purchased from Roche (USA). AnnexinV-FITC/PI Cell Apoptosis Assay Kit was purchased from KeyGENBioTECH (Nanjing, China). Caspase-3 Activity Assay Kit was obtained from Beyotime Institute of Biotechnology (Haimen, China). P-Akt antibody (Ser473), total-Akt antibody, LY294002 was obtained from Cell Signaling Technology (Boston, USA). ErbB4 was from Abcam (Cambridge, UK). ErbB4 siRNA lentivirus vector and ErbB4-siRNA were obtained from Shanghai GeneChem Corporation (Shanghai, China).

Cardiac myocytes isolation and treatment

Cardiac myocytes were isolated, as described previously [9]. Briefly, hearts from 1-2 day-old Sprague-Dawley rats were rinsed by ice-cold 1 × phosphate-buffered saline solution. After cutting off the atria, the remaining ventricles were minced with scissors. The well minced tissue was dispersed by digestion with 0.1% trypsin. NRCMs were cultured in Dulbecco's modified Eagle's Medium supplemented with 10% fetal bovine serum. Cells at a low density of 0.5 × 10^2 cells/mm^2 were planted on coverslips and placed into 24-well plates for TUNEL staining, and at a high density of 6 × 10^2 cells/mm^2 for flow cytometry. Hypoxia was achieved by placing NRCMs into a hypoxia incubator chamber (STEMCELL technologies, Vancouver, CAN) for 12 hours. Cells were treated with QL (0.5 mg/ml) or LY294002 (50 μM) at the onset of hypoxia exposure.

Knockdown of ErbB4 in NRCMs

The operation was carried out following the manufacture's instruction. Infections were performed at 70%-75% confluence in DMEM supplemented with 10% FBS. The optimal multiplicity of infection is 50. Transfected cells were then exposed to different treatment according to experiment procedures.

Western blot analysis

NRCMs were lysed by RIPA lysis buffer supplemented with 1 mmol/L PMSF and phosphates inhibitor on ice. Lysates were centrifuged at 12,000 g for 15 min to remove unbroken cells and nuclei. Equal amounts of proteins loaded onto SDS-PAGE gels were then transferred onto poly-vinylidene fluoride membrane (Thermo Scientific, USA). The membranes were blocked with 5% nonfat dry milk and 0.1% Tween 20 for 1 h at room temperature and then probed with primary antibodies against ErbB4, total-Akt and p-Akt (Ser473), at 4°C overnight. Immune complexes were detected with horseradish peroxidase-conjugated secondary antibody and visualized by ECL Plus (Thermo Scientific, USA).

Real-time quantitative RT-PCR

Total RNA was isolated from NRCMs after hypoxia using TRIzol reagent (Invitrogen, USA). cDNA was synthesized with PrimeScriptRT reagent Kit (TakaRa, JPN). Real-time quantitative PCR using SYBR Premix Ex TaqTM (TakaRa, JPN) was performed on an Applied Biosystems 7500 Real-Time PCR Detection System (Life Technologies, USA). The expression levels of examined transcripts were normalized to β-actin. The following were the sequences of primers: for ErbB4: 5'-AACCAGCACCATACCAGAGG-3' and 5'-TTCATCCAGTTCTGCTCGTG-3'; for β-actin: 5'-ACCACAGTCCATGCCATCAC-3' and 5'-TCCACCACCCTGTTGCTGTA-3'. PCR cycling conditions were: 95°C 60 sec., 95°C 15 sec., mPCR cycles (940 cycles): 95°C 15 sec., 65°C 25 sec. The relative expression level between treatments can be calculated using the following equation: relative gene expression = $2^{-(\Delta Ct\ sample-\Delta Ct\ control)}$.

Flow cytometry analysis

Cells were collected and made into single-cell suspensions with binding buffer. Then, cells

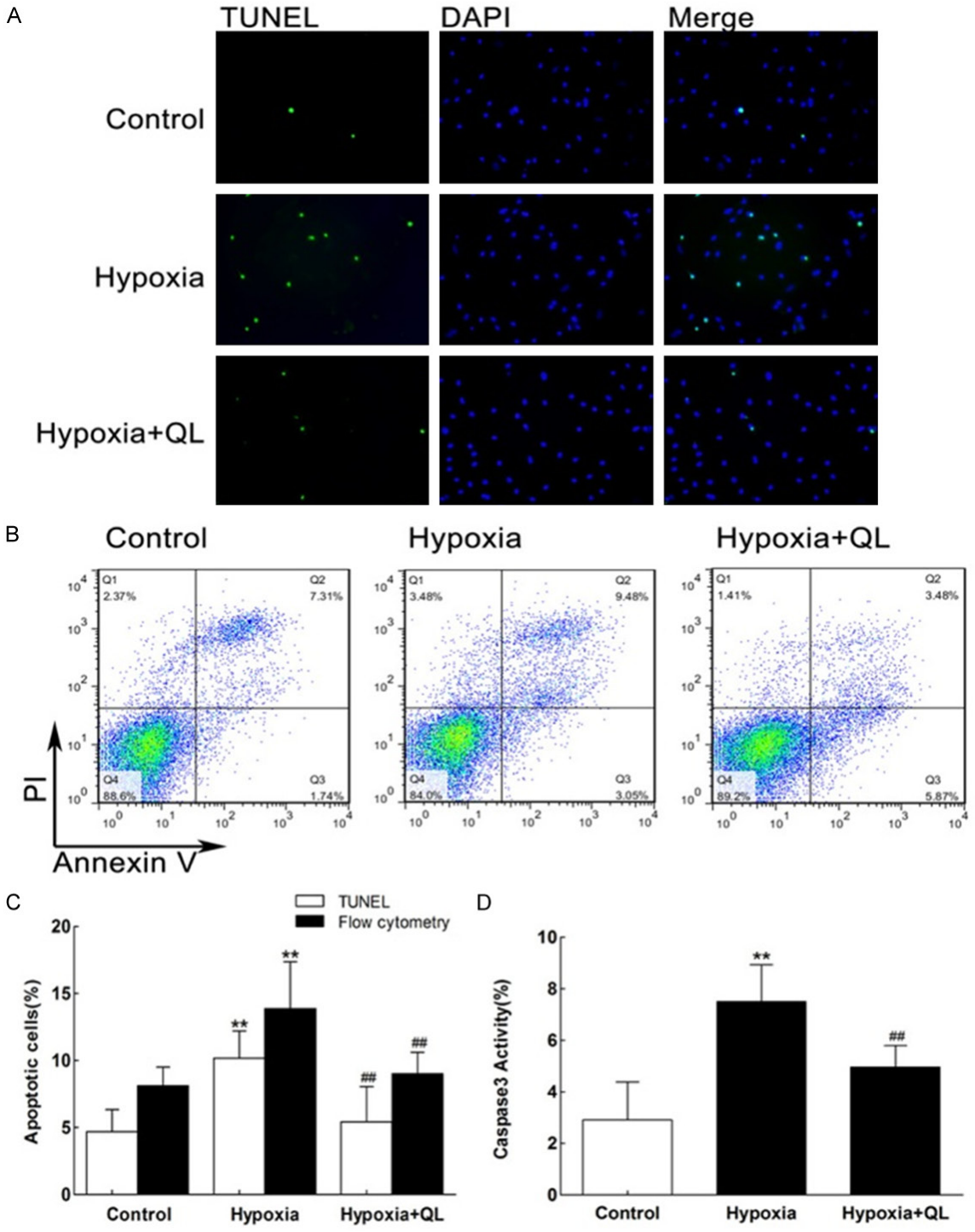


Figure 1. QL regulated NRCMs apoptosis in response to hypoxia. A. NRCMs placed on glass coverslips were stained for TUNEL or DAPI. The representative fields of cardiac myocytes stained with TUNEL or DAPI are shown. B. NRCMs apoptosis was measured by flow cytometry. C. Quantification of NRCMs apoptosis. D. NRCMs caspase-3 activity was assessed by Caspase-3 Activity Assay Kit (**P<0.01 versus control group, ##P<0.01 versus hypoxia group).

were stained with Annexin V-FITC/PI for 20 min in the dark at room temperature. Flow cytometry was performed to monitor the green fluorescence of the FITC and the red fluorescence of DNA-bound PI. All data were acquired using an LSRII flow cytometer (BD Biosciences) and were analyzed by FlowJo7 software (Tree Star, Inc.).

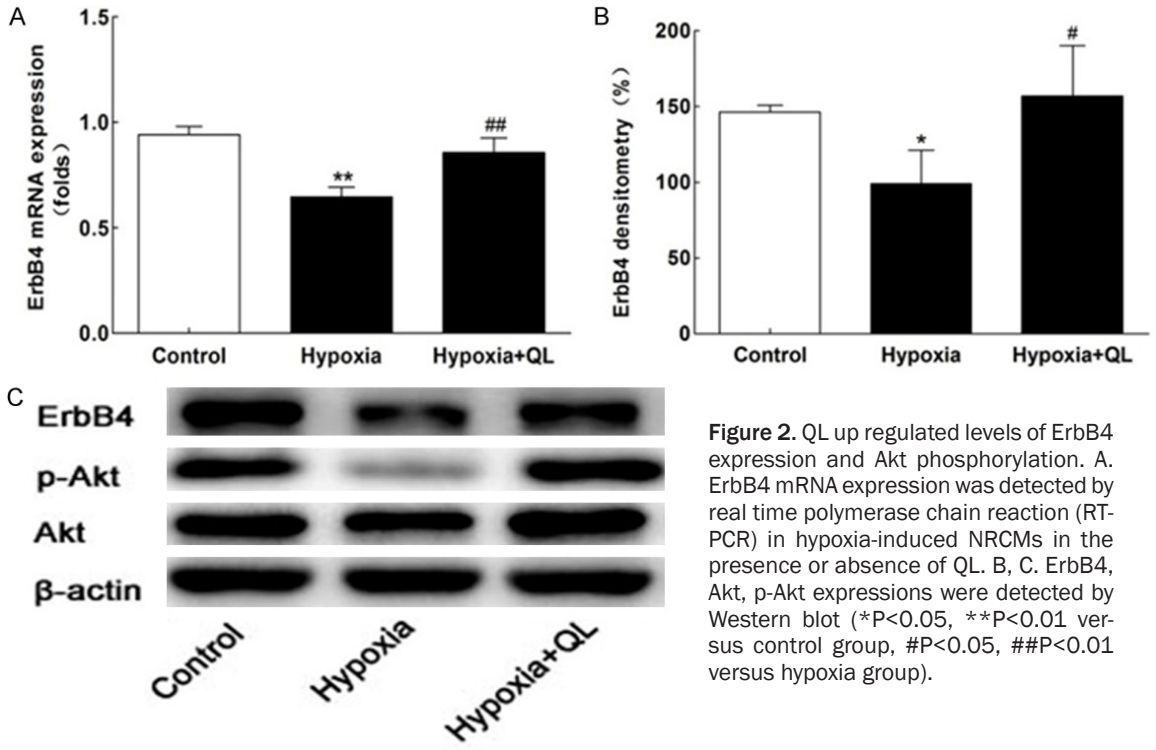


Figure 2. QL up regulated levels of ErbB4 expression and Akt phosphorylation. A. ErbB4 mRNA expression was detected by real time polymerase chain reaction (RT-PCR) in hypoxia-induced NRCMs in the presence or absence of QL. B, C. ErbB4, Akt, p-Akt expressions were detected by Western blot (*P<0.05, **P<0.01 versus control group, #P<0.05, ##P<0.01 versus hypoxia group).

TUNEL staining

NRCMs apoptosis was measured by TUNEL using the in situ cell death detection kit (Roche, USA) according to the manufacturer's protocol. In brief, NRCMs placed on coverslips were fixed with 4% paraformaldehyde and permeabilized by 0.1% Triton X-100. Cells were then incubated with fluorescein isothiocyanate-labeled TUNEL reaction mixture at 37°C for 1 h in the dark. The cells were counterstained with DAPI for 5 min in room temperature and rinsed with PBS for 3 times. TUNEL positive nuclei in 10 different fields per section were counted and normalized to the total number of cells based on DAPI positive nuclear staining. Data were expressed as a percentage of TUNEL-positive nuclei relative to total cell nuclei in each group.

Caspase-3 activity assay

Caspase-3 activity in NRCMs was determined by Caspase-3 Activity Assay Kit following manufacturer's instruction. In brief, NRCMs were collected and mixed with ice-cold lysis buffer for 15 min. Then the cell lysates were centrifuged at 20,000 × g for 20 min at 4°C and the supernatants were collected. The protein concentrations were measured by the Bradford Protein Assay Kit (Beyotime Institute of Biotechnology, Haimen, China). To each well of a 96-well plate, supernatant containing 200 μg of protein was loaded and incubated with 10 μL of the caspase-3 substrate N-acethyl-Asp-Glu-Val-Asp (DEVD)-p-nitroanilide at 37°C for 2 h in the dark. The absorbance was then measured at 405 nm.

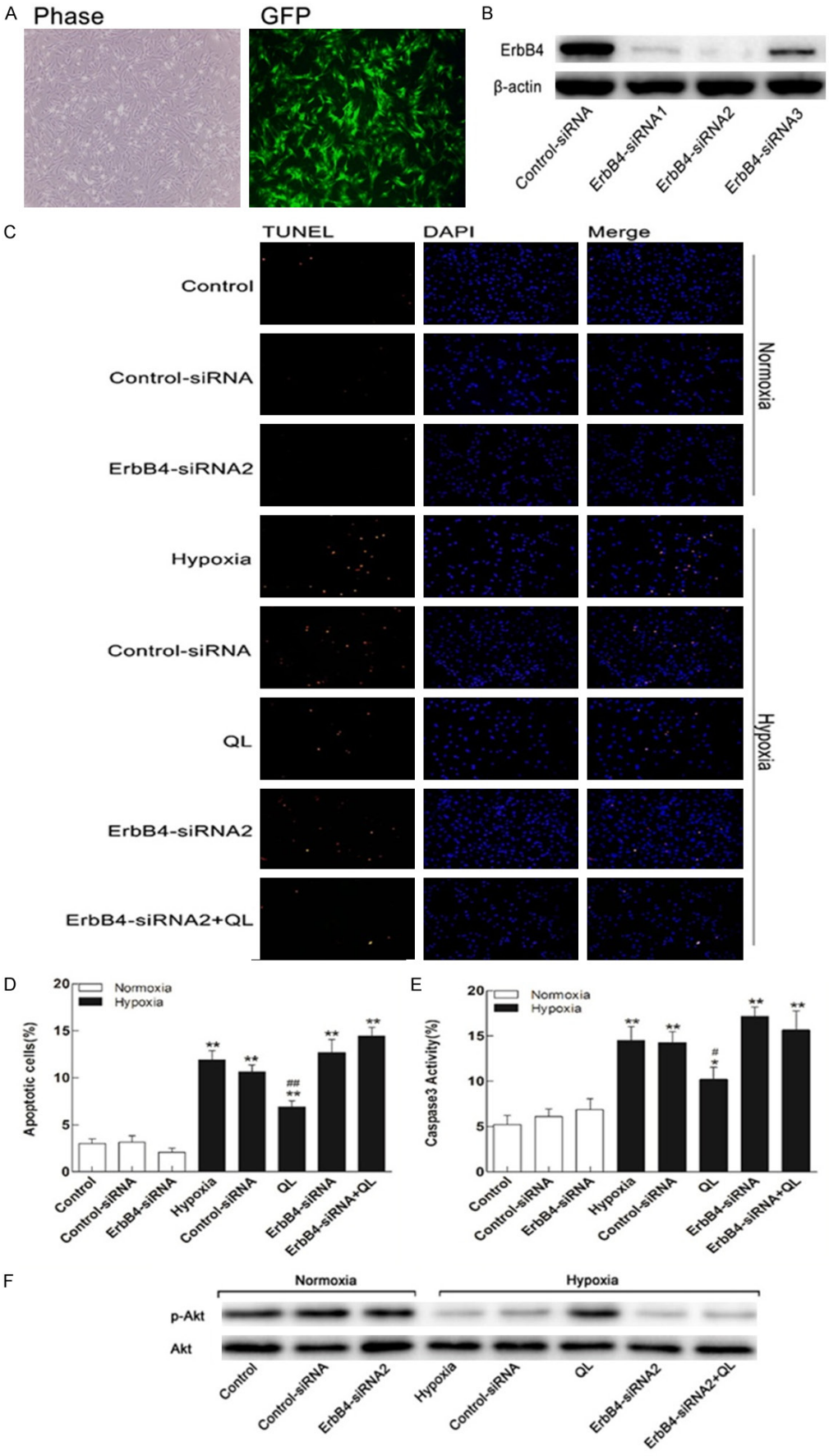
Statistics

All data were expressed as mean ± SD. For multiple-group comparisons, the statistical significance was assessed by using one-way ANOVA followed by an LSD test. Comparisons between two groups were carried out by a two-tailed Student's t test. All statistical analyses were conducted using SPSS17.0 (SPSS Inc., USA). A value of $P < 0.05$ was considered statistically significant.

Results

QL ameliorated hypoxia-induced apoptosis in NRCMs

To investigate the role of QL in cardiac myocytes in response to hypoxia, NRCMs were subjected to hypoxia for 12 hours with or without the treatment of QL. NRCMs apoptosis was evaluated by TUNEL staining, flow cytometry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DAPI
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Control
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Figure 3. ErbB4 mediated the anti-apoptotic effect of QL. A, B. Transfection efficiency and knockdown efficiency of ErbB4-siRNAs; C. Representative fields of NRCMs stained with DAPI or TUNEL; D. Quantification of apoptosis in response to hypoxia with the indicated treatments. E. NRCMs caspase-3 activity was assessed by Caspase-3 Activity Assay Kit. F. Akt, p-Akt protein expression were measured by Western blot. (*P<0.05, **P<0.01 versus control group, #P<0.05, ##P<0.01 versus hypoxia group).

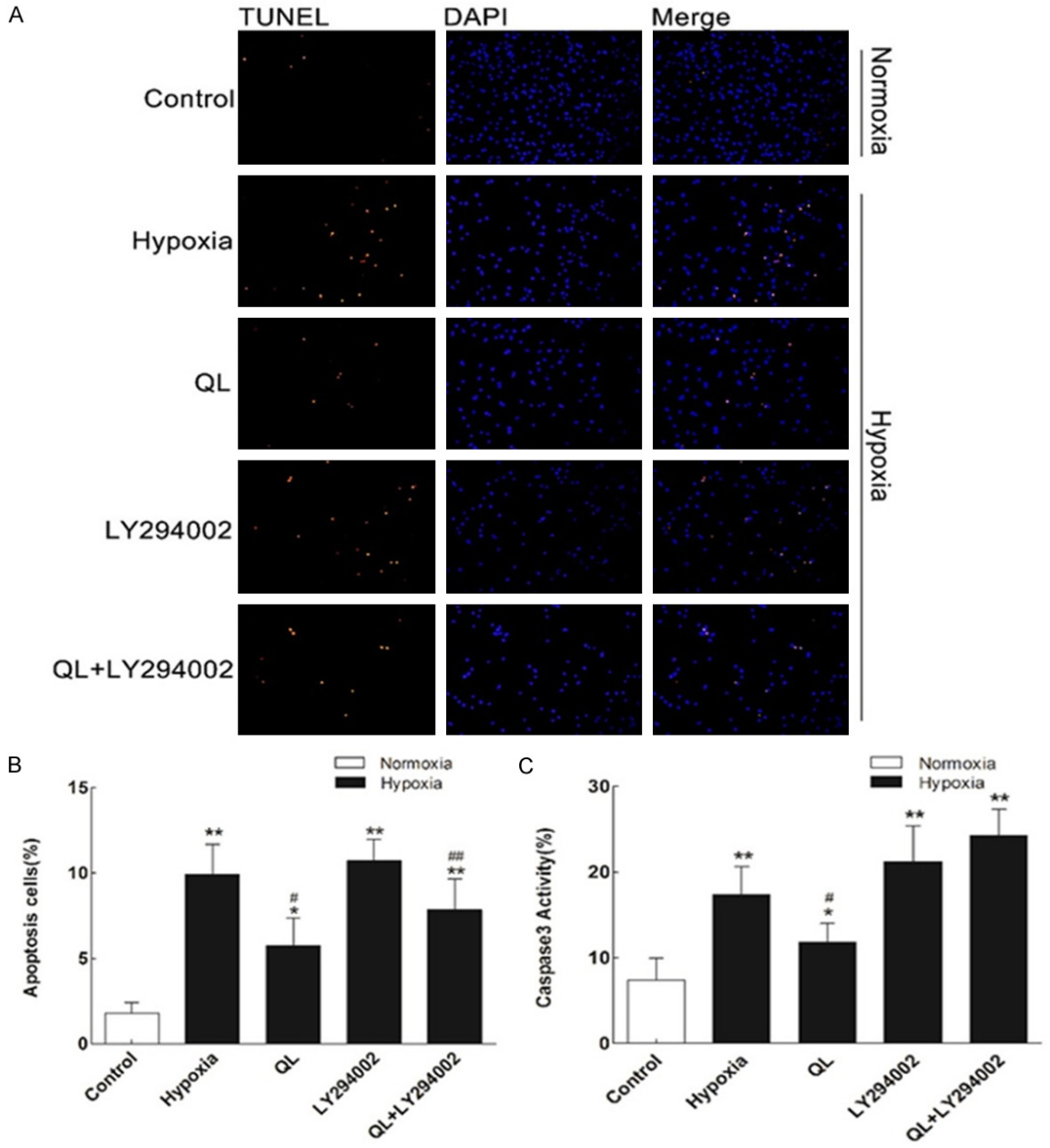

Figure 4. QL suppressed hypoxia induced NRCMs apoptosis via ErbB4 dependent PI3K/Akt pathway. A. Representative fields of NRCMs stained with DAPI or TUNEL; NRCMs were treated with LY294002 at the concentration of 50 μM in the presence or absence of QL under hypoxia. B. Quantification of cell apoptosis in response to hypoxia with the indicated treatments; C. NRCMs caspase-3 activity was assessed by Caspase-3 Activity Assay Kit (*P<0.05, **P<0.01 versus control group, #P<0.05, ##P<0.01 versus hypoxia group).

analysis and caspase-3 activity assay. Hypoxia induced a two-fold increase of apoptotic NRCMs when compared to control, which was decreased by the treatment of QL (**Figure 1**).

Similarly, hypoxia-induced upregulation of caspase-3 activity in NRCMs was also down-regulated by QL. These data suggested that QL treatment could reduce hypoxia-mediated NRCMs apoptosis.

QL stimulated ErbB4 expression and Akt phosphorylation in NRCMs in response to hypoxia

Previous studies highlighted the significance of ErbB4 in both cardiac development and cardiac myocytes survival of the failing heart [10]. Therefore, we determined whether QL protected NRCMs through activating ErbB4 signaling. mRNA and protein levels of ErbB4 were determined in hypoxia-induced NRCMs. As is shown in **Figure 2**, not only the mRNA and protein levels of ErbB4, but also ErbB4-dependent Akt phosphorylation was down regulated by hypoxia for 12 hours. QL treatment could reverse hypoxia-induced ErbB4 expression and Akt phosphorylation, indicated that QL was essential to regulate ErbB4 signaling pathway.

ErbB4 mediated the anti-apoptotic effect of QL in response to hypoxia

Next, we determined the role of ErbB4 activated by QL in NRCMs apoptosis. In vitro cultured NRCMs were transfected with lentiviral coding ErbB4 siRNAs. Western blot analysis showed that ErbB4 expression was markedly inhibited in ErbB4-siRNA2 transfected NRCMs compared to control-siRNA transfected NRCMs (**Figure 3**). NRCMs apoptosis was low and displayed no significant difference among groups treated with normoxic condition. However, NRCMs apoptosis was significantly increased by hypoxia and knocking down ErbB4 in NRCMs (**Figure 3**). Moreover, reduction of NRCMs apoptosis with QL was also reversed by knocking down ErbB4. Consistently, hypoxia-induced caspase-3 activity was enhanced by knocking down ErbB4, which could not be downregulated by QL treatment (**Figure 3**). Our results also demonstrated that Akt phosphorylation in response to hypoxia was enhanced by QL, and weakened by ErbB4 siRNAs (**Figure 3**). Collectively, these data indicated that ErbB4 might be the target of QL and hypoxia-induced NRCMs apoptosis could be avoid by QL through regulating ErbB4 signaling pathway.

QL activated ErbB4-dependent PI3K/Akt pathway

To confirm whether Akt phosphorylation benefits the survival of cardiac myocytes and ErbB4-dependent, we blocked PI3K/Akt signaling by treating NRCMs with LY294002. The results showed that LY294002 treatment increased NRCMs apoptosis, which was decreased by QL treatment (**Figure 4**). Interestingly, the co-treatment of QL and LY294002 led to a markedly increase in caspase-3 activity when compared to QL treatment alone (**Figure 4**). These results indicated that the protective effect of QL was partially blocked by LY294002, and QL stimulated the downstream of ErbB4, PI3K/Akt pathway, which was essential to cardiac myocytes survival in response to hypoxia.

Discussion

The effects of QL on protection of the failing heart against ischemia have been intensively studied for years. However, the underlying mechanism is still not clear. In this study, we demonstrate the findings that QL regulates the survival of cardiac myocytes in response to hypoxia. Concurrently, QL up regulated the expression of ErbB4 and the extent of Akt phosphorylation. Knockdown of ErbB4 impaired the anti-apoptotic effect of QL and reduced the level of Akt phosphorylation. Moreover, blocking PI3K/Akt signaling by LY294002 also hindered the protective effect of QL on cardiac myocytes.

Cardiac myocytes apoptosis plays important roles in the pathology of heart failure. In recent years, a growing number of studies have been devoted to shed light upon the regulatory role of QL on cell survival when exposed to a variety of cardiac insults. Tao L, et al [8] demonstrated by post myocardial infarction mice that QL couldimprove cardiac injury by reducing cell apoptosis, as evidenced by rebalanced Bax/Bcl-2, cleaved PARP/PARP and cleaved caspase-3/caspase-3 ratios. Yunzeng Z, et al [5] also found in chronic pressure overload mice that cardiac myocytes apoptosis was significantly reduced in QL treated mice, as compared to vehicle-treated ones. In the present study, we observed, in vivo, that QL is capable of attenuating hypoxia induced cardiac myocytes apoptosis through attenuation of TUNEL positive cells and inhibition of caspase-3 activation in hypoxia stimulated NRCMs.

ErbB receptors are indispensible for heart morphogenesis as well as the maintenance and function of the adult heart. Importance of ErbB receptors involved in the development of heart can be detected in a series of conditionally

knockout mice. Mice lacking ErbB4 and carrying ErbB2 null allele all died during mid-embryogenesis from the aborted development of myocardial trabeculae in the heart ventricles [11, 12]. Other studies reported that instead of overt cardiac phenotype following knockdown of ErbB receptors, those mice suffered a relatively high susceptibility towards severe dilated cardiomyopathy [13, 14]. On the other hand, ErbB receptors also critically involved in the protection of adult cardiac function. Recent study revealed that transplantation of MSC-ErbB4 (Mesenchymal stem cells overexpressed with ErbB4) into post myocardial infarcted hearts of mice showed better myocardial contractility, smaller fibrotic area and more effectively preserved left ventricular wall thickness compared to non-transplantation group [15]. Conversely, lacking of ErbB4 predispose cardiac myocytes to apoptotic death induced by chemical drugs or oxidative stress [7, 16]. ErbB receptor inactivation in the tissue samples of heart failure patients resulted in reversed Bcl-x/Bcl-Xs ratios, thereby contributing the human failing myocardium to enhance apoptosis susceptibility [17]. Accumulating evidence revealed that levels of ErbB receptor expressions are underlying biomarkers of cardiovascular disease. While ErbB2/ErbB4 mRNA expressions in hearts of CHF patients remain stable during the early stage of heart failure, it decreased by the onset of later stage when pump failure occurs [18]. In the present study, we also witnessed a decline in mRNA and protein levels of ErbB4 in NRCMs under hypoxic stimulation, which was effectively up-regulated following the treatment of QL, demonstrating a strong association between expression of ErbB4 and cell apoptosis in hypoxia induced NRCMs. Lacking of ErbB4 blunted the effect of QL in reducing the apoptotic cells, suggesting that ErbB4 mediates the anti-apoptotic effect of QL in the setting of hypoxia.

It has been proved that PI3K/Akt, Erk1/2 and JAK/STAT signaling cascades are the main downstream targets of ErbB4 [19], among which PI3K/Akt signaling pathway has a close link to cardiomyocyte survival and proliferation. Liang X, et al [15] reported that MSC-ErbB4 transplanted into post-MI heart suppressed hypoxia induced cardiac myocytes apoptosis via activating PI3K/Akt signaling pathway and enhancing Bcl-2 protein expression. Yukio Kuramochi, et al [16] also demonstrated that H_2O_2 induced cardiac myocytes apoptosis could be effectively suppressed by ErbB4 mediated PI3K/Akt signaling. In present study, we observed that QL activated PI3K/Akt signaling pathway in hypoxia induced NRCMs. However, Lentiviral-vector mediated conditionally knockdown of ErbB4 weakened PI3K/Akt signaling activity and QL-induced suppression of NRCMs apoptosis. Moreover, inhibiting PI3K/Akt signaling by LY294002 abrogated the protective effects of QL as well, indicating that QL exerts cardioprotective effects upon cardiac myocytes via, at least partially, ErbB4 mediated PI3K/Akt pathway.

Because cardiac myocytes apoptosis is intimately involved in the pathology of chronic heart failure and because hearts have restricted ability to regenerate, suppression of cardiac myocytes apoptosis may hold promise for heart failure therapy [20]. It has been suggested that stimulation of the Akt signaling or inhibition of caspases activation are effective in suppressing cardiac myocytes apoptosis and improving cardiac function subsequently [21]. In this study, we detected QL benefits hypoxia induced cardiac myocytes apoptosis by stimulating ErbB4 dependent PI3K/Akt signaling pathway. However, since QL is an herb compound, further investigations are required to probe the exact active pharmaceutical components that targeting this signaling pathway. Nevertheless, this study presented a novel perspective in the molecular mechanisms of QL in cardiac myocytes apoptosis in response to hypoxia.

Acknowledgements

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Disclosure of conflicts of interest

None.

Address correspondence to: Junbo Ge, Department of Cardiology, Shanghai Institute of Cardiovascular

Diseases, Zhongshan Hospital, Fudan University, Shanghai, China. E-mail: jbge@zs-hospital.sh.cn

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