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Original Article

Na^+/Ca^{2+} exchanger 1 (NCX-1) mediates the anti-apoptotic effect of Akt1 in neonatal rat cardiomyocytes during ischemia/reperfusion

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Abstract: The purpose of this study was to investigate the anti-apoptotic role of Akt1 gene in neonatal rat cardiomyocytes and the relationship with Na^+/Ca^{2+} exchanger 1 (NCX1) during ischemia/reperfusion (IR). The cultured original rat cardiomyocytes were randomly divided into five groups: normal control group (C group), hypoxia/reoxygenation group (HR group), the control vector pLVX-EGFP-3FLAG group (CV group), the gene pLVX-EGFP-3FLAG-Akt1 transfection group (A group), and Akt1 inhibitor LY294002 group (LY group). Cardiomyocyte vitality was determined using MTT, and the apoptosis was determined by TUNEL to verify the anti-apoptotic role of Akt1. The mRNA levels of Akt1 and NCX1 were determined by RT-PCR, the protein expression of Akt1, p-Akt1, NCX1 and the apoptotic proteins of mitochondrial pathway cytochrome C (Cyto C) and caspase-9 were measured by Western blot. As a result, transfected Akt1 (A group) showed increased myocardial cell viability and reduced apoptosis, with increase in Akt1 expression and decrease in NCX1 expression. The levels of apoptotic proteins Cyto C and caspase-9 also declined. This study demonstrated that lentivirus-mediated transfection of *Akt1* played an anti-apoptotic role during IR of rat cardiomyocytes, via inhibition of NCX1 and other mitochondrial proteins.

Keywords: Akt1, cardiomyocytes, ischemia/reperfusion, NCX1, apoptosis

Introduction

Myocardial ischemia/reperfusion injury (IRI) is a clinical complication associated with procedures such as angioplasty, thrombolysis and coronary bypass, which are common revascularization procedures. Apoptosis or programmed cell death is triggered by ischemia/reperfusion (IR). A number of studies have shown that myocardial IRI is associated with increased apoptosis [1-3]. However, the mechanisms of apoptosis in myocardial IRI are not completely understood, although intracellular Ca^{2+} overload is widely recognized [4-6]. The PI-3 kinase/Akt pathway is a cell survival mechanism, which is one of the main reperfusion injury salvage kinase pathways (RISK) [7, 8]. Akt is a key component of the PI3K/Akt signaling pathway. Akt is a serine/threonine kinase that mediates several functions via phosphorylation and inactivation of substrates such as GSK-3β, mTOR, Bcl-2, Bad, Bax, FOXO and eNOS. Studies suggest that Akt expression inhibited myocardial cell apoptosis mediated via GSK-3β, Bcl-2 and eNOS [9-14]. The three subtypes of Akt include: PKB alpha/Akt1, PKB beta/Akt2 and PKB gamma/Akt3. The three subtypes are distributed spatially, and the Akt1 is highly expressed in myocardium.

The Na^+/Ca^{2+} exchanger 1 (NCX1) is a protein localized to the myocardial cell membrane, and is used to balance the Na^+, Ca^{2+} exchange across the cell membrane. NCX1 operates via two modes: forward and reverse. The forward mode pumps Ca^{2+} out, and the reverse mode promotes Ca^{2+} entry into the cells [15, 16]. Generally, Na^+ and Ca^{2+} are exchanged in a 3:1 ratio by NCX1. However, there are other studies suggesting a 2:1 or 4:1 exchange. In brief, NCX1 plays a key role in regulating intracellular Ca^{2+} concentration.

Under physiological conditions, the levels of myocardial intracellular Ca^{2+} ions are regulated by sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA)

and the forward mode of NCX1 (92% to 7% ratio) to maintain an equilibrium [17]. In IR, excessive Ca^{2+} flow into myocardial cells under the action of NCX1 reverses the transport mechanism, resulting in Ca^{2+} overload during reperfusion [18-21]. However, Ca^{2+} overload triggers the opening of mitochondrial permeability transition pore (MPTP) [22-24], leading to the release of mitochondrial cytochrome C (Cyto C) and other apoptotic proteins into the cytoplasm. Cyto C combines with the caspase-9/Apaf1 complex, to activate caspase-9, and other caspases downstream resulting in myocardiocyte apoptosis [25].

We investigated the close relationship between NCX1, Ca^{2+} overload and MPTP by transfecting rat cardiomyocytes undergoing hypoxia reoxygenation (HR, simulated IR model) with *Akt* using a lentiviral vector pLVX-EGFP-3FLAG pLVX-EGFP-3FLAG-Akt1 (LV-Akt1). We determined the anti-apoptotic role of Akt1 in neonatal rat myocardiocytes, and further elucidated the role of NCX1 in anti-apoptotic mechanisms.

Materials and methods

Animals

Sprague-Dawley neonatal rats (n = 300, 1-3 d), were purchased from Experimental Animal Centre of Xuzhou Medical University. The study protocol was approved by the Animal Ethics Committee of the Medical College of Xuzhou (permit number: xz11-12540). All animals were treated in accordance with the Guide for the Care and Use of Laboratory Animals, published by the National Institutes of Health (NIH publication No. 85-23, revised 1996). The control vector pLVX-EGFP-3FLAG (LV) and the LV-Akt1 were purchased from Shang Hai Sibo company.

Cell culture and treatment

The method of Franke [26] was used to isolate and culture neonatal rat cardiomyocytes. Briefly, the cardiac tissue was cut into 1 mm^3 blocks, and digested with equal volumes of 0.08% pancreatic enzymes and 0.08% type I collagenase. Myocardioctes suspended in DMEM after centrifugation were used for *in vitro* studies. The myocardial cells were inoculated into different culture plates, as needed. BrdU was added to inhibit the growth of fibroblasts. Finally, the cardiomyocytes were incubated for 48 h at 37°C and 5% CO_2.

The cultured cardiomyocytes were randomly divided into five groups.

1. Normal control group (C group): The cells were cultured under normal experimental conditions, without HR process.

2. Hypoxia reoxygenation (HR group): The cardiomyocytes were cultured under hypoxia for 2 h followed by reoxygenation for 24 h.

3. Control vector group (CV group): LV was transfected for 48 h, followed by HR.

4. Gene transfection group (A group): LV-Akt1 was transfected for 48 h, followed by HR.

5. Akt1 inhibitor LY294002 group (LY group): Following cardiomyocyte culture for 2 h under hypoxia, LY294002 cells (50 μmol/L) were added before reoxygenation for 24 h. Each group experiment was performed three times.

Identification of cardiomyocytes

The cTroponin I (cTn I) was only expressed in myocardiocytes, and serves as an experimental marker [27]. Immunofluorescence using Cy3-labeled secondary antibody combined with the cTn I antibody enables identification of myocardial cells, followed by DAPI staining of all nuclei.

Tested the optimal MOI value

Detection of enhanced green fluorescent protein (eGFP) 48 h after LV-Akt1 transfection indicated stable expression. The first generation of myocardial cells were vaccinated in 96-well plate, using a concentration of 1×10^5 cells per hole, to which the LV-Akt1 was added according to the multiplicity of infection (MOI) 0, 5, 10, 15, 20 25, 30, 35, 40 and 45. After culturing for 48 h, the myocardiocytes were observed under a fluorescent microscope.

Simulated ischemia/reperfusion

The hypoxia reoxygenation model was improved based on the method described by Rakhit et al [28]. The culture medium containing 20% fetal bovine serum was depleted of fetal bovine serum and cultured for 2 h, and then substituted with D-Hank's buffer saturated with 95% N_2-5% CO_2 for 30 min. The culture plate was

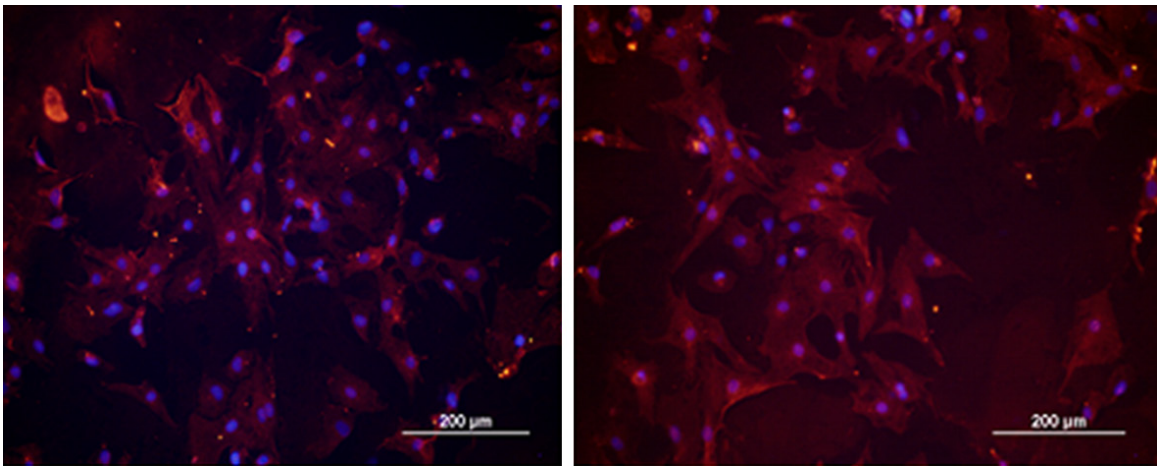

Figure 1. Identification of myocardial cells. Myocardial cells showed the red fluorescence, the nuclei of all cells showed the blue fluorescence.

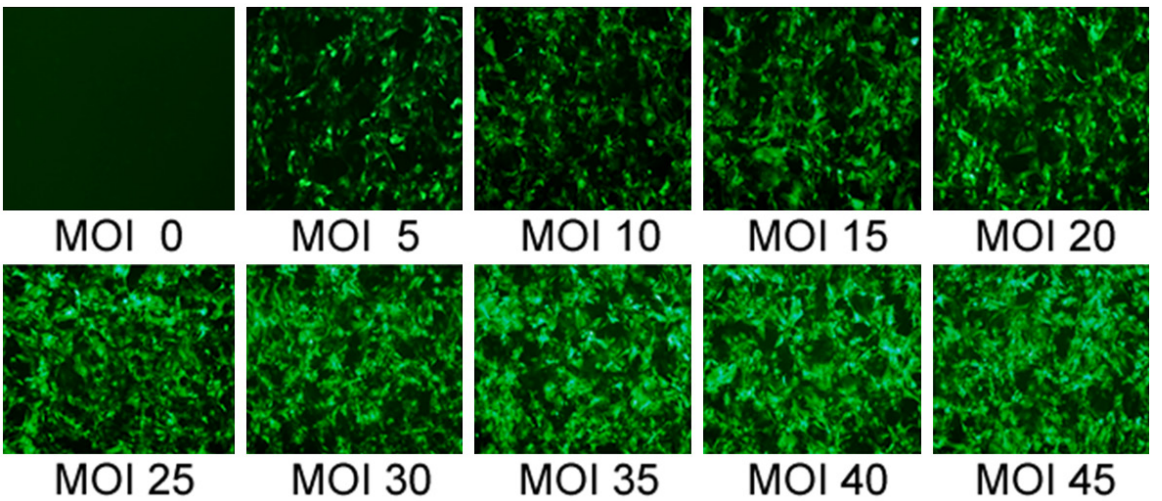

Figure 2. Tested the optimal MOI value. Because of the eGFP, the myocardial cells showed the green fluorescence. When the MOI was 25, the transfection rate reached 83%, as the MOI was 30, the transfection rate reached 88%, when the MOI was 35, 40 and 45, there was no obvious difference for the transfection rate.

transferred into anoxic incubator to simulate hypoxia for 2 h, under the following conditions: 5% CO_2 and O_2 < 1%. Finally, the D-Hank's medium was replaced by normal culture medium containing 20% fetal bovine serum, and incubated at 37°C and 95% air plus 95% CO_2 for 24 h. The C group was incubated directly in the CO_2 incubator, while the gene transfected groups were subjected to lentiviral transfection before HR.

Cell vitality assay

The original generation of myocardiocytes was vaccinated in 96-well plate at a concentration of 1 ×10^5 cells per well. The C group was vaccinated in a 96-well plate, and each group was assigned three wells. Myocardial cell vitality was detected using the MTT method after HR. Under dark, MTT 20 μL (5 mg/mL) was added to each well and incubated at 37°C and 5% CO_2 for 4 h. The culture medium was replaced with 150 μL DMSO, and 10 min later, the OD value was determined using standard enzyme tests (BIO-TEK company, USA).

TUNEL for cardiomyocytes apoptosis

The original generation of myocardial cells was used to inoculate in 24-well plate at a con-

Table 1. The changes in neonate rat myocardial cells vitality

Groups	*Cell vitality (% fold increase)*
C	100.000±0.000
HR	47.130±1.888*
CV	46.360±1.944*
A	77.730±3.790*,#,$
LY	36.460±4.799*,&

Date are presented as mean ± SEM. *p value < 0.05 as compared with C group; #p value < 0.05 as compared with HR group; $$p$ value < 0.05 as compared with CV group; &p value < 0.05 ascompared with A group.

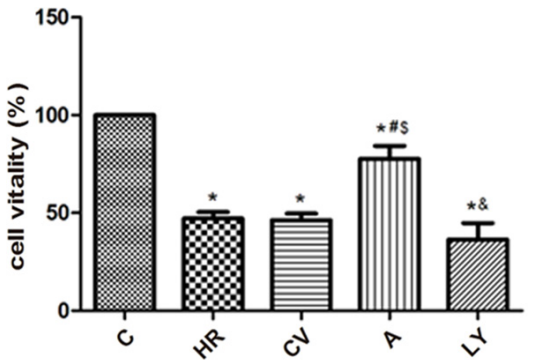

Figure 3. The changes in neonate rat myocardial cells vitality. Data are presented as mean ± SEM. C normal control group, HR hypoxia-reoxygenation group, CV the control vector group, A the gene transfection group, LY Akt inhibitor LY294002 group.*P value < 0.05 as compared with C group; #P value < 0.05 as compared with HR group; $$P$ value < 0.05 as compared with CV group; &P value < 0.05 as compared with A group. Compared with C group, the other groups of cell vitality decreased significantly (P < 0.05), compared with HR group, the myocardial cells of the A group vitality increased significantly (P < 0.05).

centration of 3×10^5 cells per well. Following treatment of different groups, TUNEL (Roche Company, Germany) assay was used to detect cardiomyocyte apoptosis. To each well, a 4% paraformaldehyde fixed solution was added at room temperature for 30 min, and washed for 5 min with PBS, three times. A 0.3% methanol solution of H_2O_2 was used to incubate for 30 min at room temperature. After PBS wash, the transparent liquid (0.1% Triton X–100 soluble in 0.1% sodium citrate solution) was used to incubate for 2 min in an ice bath. After washing twice with PBS, the sample was dried, followed by addition of 50 μL TUNEL reaction mixture and incubation for 60 min at 37°C under dark. After washing with PBS three times, the sample was dried, and supplemented with 50 μL converting agent -POD followed by incubation for 30 min in the wet carton at 37°C under dark. After another PBS wash three times, 50-100 μL DAB substrate solution was added and the sample was incubated for 10-30 min at room temperature under dark. After the final PBS wash three times, the results were analyzed using a microscope. Three visual fields were selected for each group. The apoptotic cell number and the total cell number were calculated, respectively.

RT-PCR

The original generation of myocardial cells was used for inoculation in the 12-well plates at a concentration of 5×10^5 cells per well. The mRNA was extracted and measured using a spectrophotometer and amplified using RT-PCR according to the manufacturer's nstructions (TaKaRa company, Japan).

The RT-PCR primer sequences were as follows: Akt1: Sense: 5'-ATGGACTTCCGGTCAGGTTCA-3', antisense: 5'-GCCCTTGCCCAGTAGCTTCA-3', expected fragments amplified: 126 bp. NCX1: Sense: 5'-GTTCTGTTTGGAGGGCCAAG-3', antisense: 5'-GTTTGGAGGAGGTGAGGAAGAGA-3', expected fragments amplified: 120 bp. GAPDH: Sense: 5'-TGACTTCAACAGCGACACCCA-3', antisense: 5'-CACCCTGTTGCTGTAGCCAAA-3', expected fragments amplified: 121 bp.

Due to short fragment size, a 3% agarose gel was used for electrophoresis followed by imaging. The images were analyzed using the ImageJ software (National Institutes of Health, USA).

Western blot analysis

The original generation of myocardial cells was added to a 6-well plate at a concentration of 1×10^6 cells per well, and the proteins were extracted by RIPA strong lysis buffer (with SDS, cholate and NP-40) after appropriate treatment of the different groups. The BCA method was used to determine the protein concentrations. Based on the different molecular weights of the proteins, the concentration of enrichment gel was 5%. After loading 20 μL proteins into each well, SDS-PAGE was performed followed by electrotransfer to PVDF membrane. The transfer time was adjusted by the protein molecular

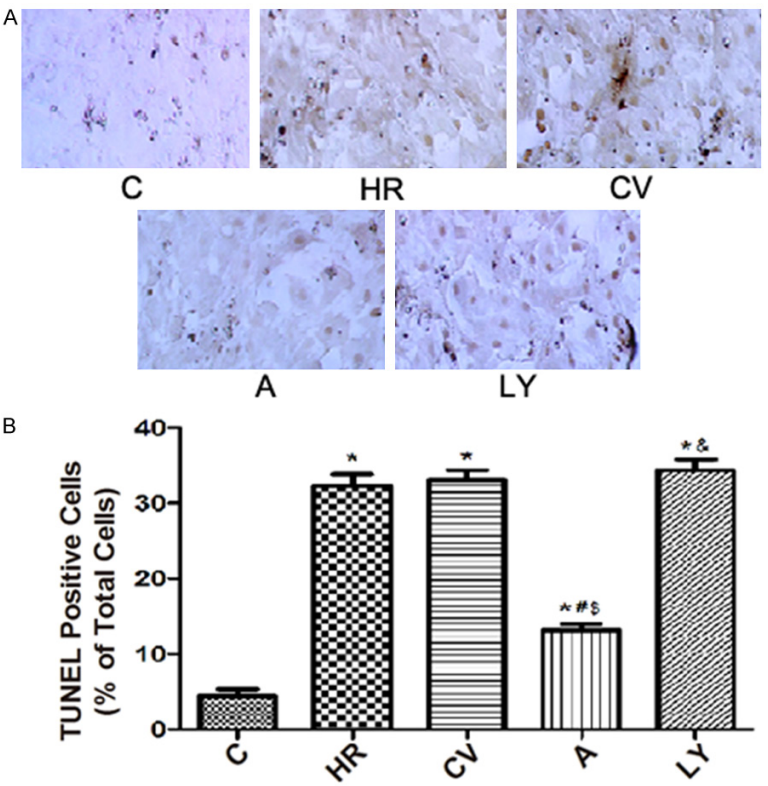

Figure 4. TUNEL detected apoptosis of cardiomyocytes. The nuclei of apoptotic myocardial cells were dyed brown. Data are presented as mean ± SEM. C normal control group, HR hypoxia-reoxygenation group, CV the control vector group; A the gene transfection group, LY Akt inhibitor LY294002 group. *P value < 0.05 as compared with C group; #P value < 0.05 as compared with HR group, \$$P$ value < 0.05 as compared with CV group; &P value < 0.05 as compared with A group. Statistical results showed that compared with C group, the other groups of myocardial cell apoptosis rate increased significantly (P < 0.05). Compared with the HR group, A group apoptosis rate decreased (P < 0.05), LY group and CV group all had no significant difference Compared with the HR group (P > 0.05).

weight. The PVDF membrane was blocked for 2 h using 5% skimmed milk powder. After the membrane was washed three times in 5 min, the membrane was incubated with NCX1 antibody (Swant company, Switzerland) and antibodies to Akt1, p-Akt1 (Ser473, Thr308), caspase-9, and Cyto C (Cell Signaling Technology, USA), in a 4°C refrigerator overnight. Following incubation with the first antibody, a secondary antibody was added for 3 h, followed by the AP method. The color stripe was analyzed using ImageJ software after scanning.

Statistical analysis

All data were obtained from at least three independent experiments, which were replicated three times under each condition. Data are expressed as mean ± SEM. Comparison of multiple groups was performed using one-way ANOVA. Values of P < 0.05 were considered statistically significant.

Results

Identification of myocardial cells and the purity

As shown in **Figure 1**, myocardial cells showed red fluorescence due to Cy3, and the nuclei of all cells showed blue fluorescence under DAPI staining. The purity of cardiomyocytes exceeded 95%.

MOI testing

After culturing for 48 h, the myocardial cells were observed under a fluorescent microscope. As shown in **Figure 2**, when the MOI was 25, the transfection rate reached 83%. At an MOI of 30, the transfection rate reached 88%. At MOI of 35, 40 and 45, no obvious differences were detected in the transfection rate. The optimal MOI value was 25.

MTT staining for cardiomyocyte vitality

MTT staining is shown in **Table 1** and **Figure 3**. Compared with the C group, the vitality of the other groups of cells decreased significantly (P < 0.05). Compared with HR, CV and LY groups, the vitality of cardiomyocytes in the A group increased significantly (P < 0.05). No significant differences were detected among HR, CV and LY groups (P > 0.05). The LY group cell vitality was lower than that of the HR group, but not statistically significant (P > 0.05).

TUNEL staining for apoptosis

TUNEL staining is illustrated in **Figure 4**. The nuclei of apoptotic cardiomyocytes were dyed brown. Compared with the C group, the other

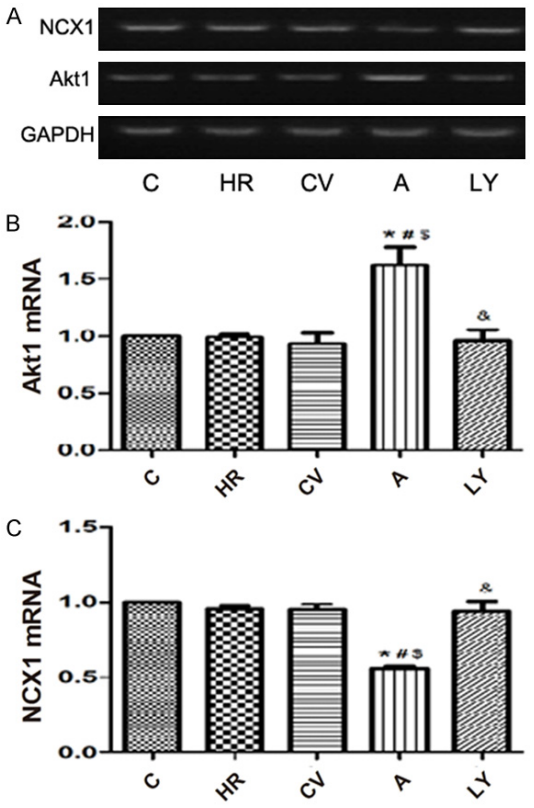

Figure 5. RT-PCR detected the mRNA level of Akt1 and NCX1. Data are presented as mean ± SEM. C normal control group, HR hypoxia-reoxygenation group, CV the control vector group, A the gene transfection group, LY Akt inhibitor LY294002 group. **P* value < 0.05 as compared with C group; #*P* value < 0.05 as compared with HR group, $*P* value < 0.05 as compared with CV group, &*P* value < 0.05 as compared with A group. Compared with C group, Akt1 and NCX1 mRNA levels were no significant change in HR, CV and LY groups (*P* < 0.05); compared with HR group, A group Akt1 mRNA levels increased significantly (*P* < 0.05), however, the level of NCX1 mRNA decreased (*P* < 0.05).

groups of cardiomyocytes showed significantly increased apoptosis (*P* < 0.05). Compared with the HR, CV and LY groups, the rate of apoptosis in the A group decreased (*P* < 0.05). The differences between LY and CV groups were not significant compared with the HR group (*P* > 0.05).

RT-PCR of Akt1 and NCX1

As shown in **Figure 5**, compared with C group, Akt1 and NCX1 mRNA levels were not significantly different in HR, CV and LY groups (*P* > 0.05), but the A group Akt1 mRNA level increased significantly (*P* < 0.05). However, the level of NCX1 mRNA decreased (*P* < 0.05).

Western blot of Akt1, p-Akt1, NCX1, caspase-9 and Cyto C

Western blot (**Figures 6**-**8**) results showed that compared with C group, the HR group Akt1 and NCX1 protein expression was not significantly changed (*P* > 0.05). However, the background p-Akt1 expression increased (*P* < 0.05), and the apoptotic protein levels of caspase-9, and Cyto C increased (*P* < 0.05); compared with HR group. In the A group, along with increased Akt1 and p-Akt1 protein expression, the expression of NCX1 increased (*P* < 0.05), and the levels of caspase-9, and Cyto C decreased significantly (*P* < 0.05).

Discussion

Advances in gene therapy, and transgenic technology facilitated the transfection of several genes such as NF-κB [29, 30], Bcl-2 [31], Bax [32], PI3K/Akt [33, 34], SOD [35] and eNOS [36] associated with IR to target cells via appropriate carriers after gene recombination. Gene transfection vectors include recombinant viruses and physical and chemical carriers. Viral vectors used for gene therapy include recombinant adenovirus, adeno-associated virus, retrovirus, vaccinia virus, herpes simplex virus and lentivirus. Compared with other viral vectors, lentivirus enables easy transfection, with minimal immune response, and stable expression.

Akt-mediated inhibition of cardiomyocyte apoptosis has been demonstrated in many experiments [9-14]. Our findings are consistent with previous studies. This study adopted MTT assay to detect myocardial cell vitality, and adopted TUNEL assay to determine the rate of apoptosis in cardiomyocytes. The results showed that HR in the cardiomyocytes diminished the vitality and increased the apoptotic rate. However, after transfection of LV-Akt1, the myocardial cell vitality increased, and the apoptotic rate declined. The combined results of MTT and TUNEL suggest successful creation of HR model. The *Akt1* expression in rat cardiomyocytes was protective. It also suggests that the control vector had no apparent effect on the cells, and Akt1 in IR had no effect on the vitality of cardiomyocytes.

The relationship between Akt and NCX1 during heart IR has not been confirmed. We first stud-

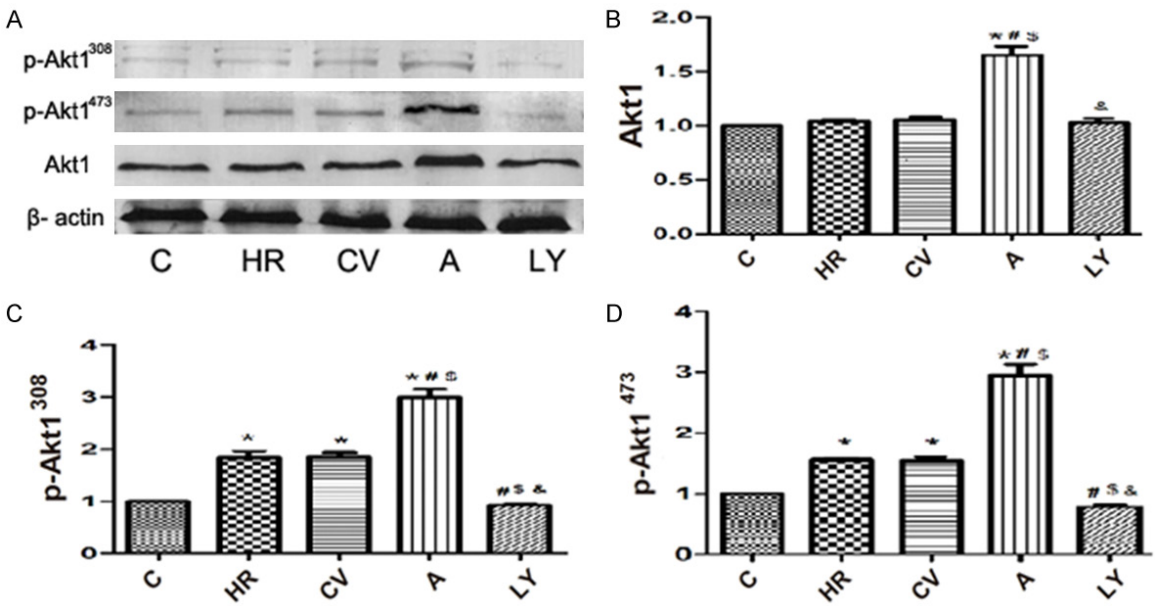

Figure 6. Expression of Akt1 and p-Akt1 determined by Western blotting. Data are presented as mean ± SEM. C normal control group, HR hypoxia-reoxygenation group, CV the control vector group, A the gene transfection group, LY Akt inhibitor LY294002 group. **P* value < 0.05 as compared with C group, #*P* value < 0.05 as compared with HR group, $*P* value < 0.05 as compared with CV group, &*P* value < 0.05 as compared with A group. Compared with C group, HR group Akt1 protein expression had no significant change (*P* > 0.05), but the background of p-Akt1 expression increased (*P* < 0.05), Compared with HR group, A group, Akt1 and p-Akt1 protein expression increased significantly (*P* < 0.05).

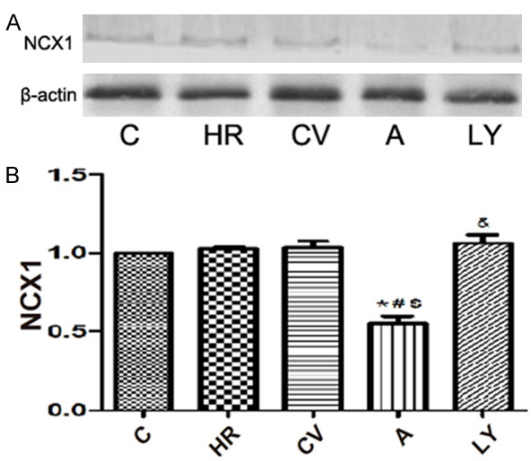

Figure 7. Expression of NCX1 determined by Western blotting. Data are presented as mean ± SEM. C normal control group; HR hypoxia-reoxygenation group, CV the control vector group; A the gene transfection group, LY Akt inhibitor LY294002 group. **P* value < 0.05 as compared with C group; #*P* value < 0.05 as compared with HR group, $*P* value < 0.05 as compared with CV group; &*P* value < 0.05 as compared with A group. Compared with C group, hypoxia 2 h and reoxygenation 24 h could not make NCX1 protein take place obvious change (*P* > 0.05), but when transfected Akt1 gene, the NCX1 protein level was upregulated (*P* < 0.05).

ied the relationship between Akt and NCX1 in IR neonatal rat cardiomyocytes. We further analyzed whether Akt1 induced NCX1 in IR to inhibit apoptosis. We therefore, transfected LV-Akt1. RT-PCR and Western blot were used to detect mRNA levels and the protein expression of Akt1 and NCX1. The results showed that compared with the C group, the Akt1 and NCX1 expression in HR group was not altered. Other experiments [37] showed such results. *In vitro* studies used rat hearts in IR models. Rat hearts were randomly divided into three groups: ischemia for 45 min and without reperfusion, ischemia for 45 min and reperfusion for 24 h, and ischemia for 45 min and reperfusion for 7 d. Compared with hearts subjected to ischemia for 45 min and without reperfusion, rat hearts subjected to 24 h reperfusion showed slightly increased NCX1 expression. Reperfusion 7d later reduced the NCX1 protein expression compared with the control group. Compared with the HR group, transfection with *Akt1* increased the Akt1 and p-Akt1 protein expression, and reduced NCX1 protein levels. Akt1 and NCX1 showed different results in other experimental conditions. Annunziato. L et al.

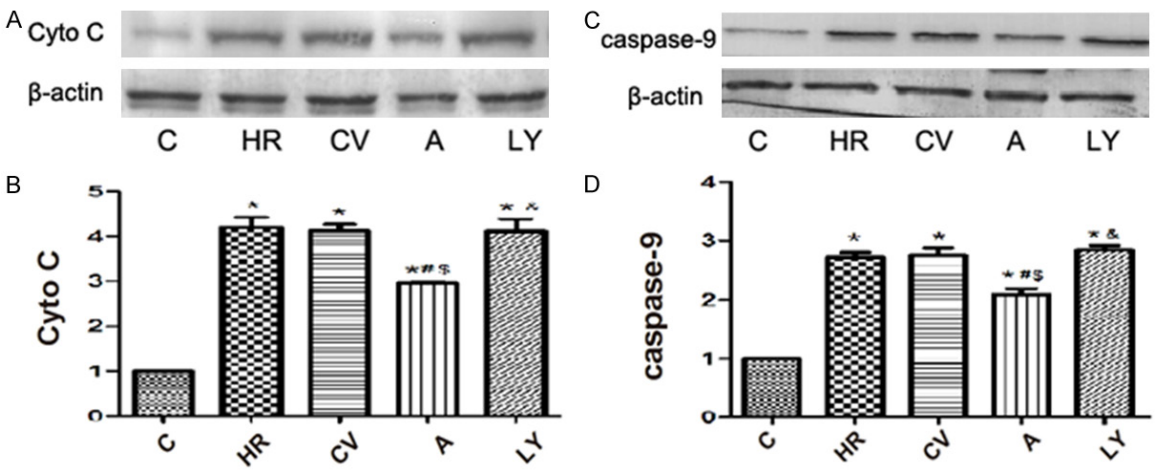

Figure 8. Expression of Cyto C and caspase-9 determined by Western blotting. Data are presented as mean ± SEM. C normal control group; HR hypoxia-reoxygenation group; CV the control vector group, A the gene transfection group, LY Akt inhibitor LY294002 group. **P* value < 0.05 as compared with C group; #*P* value < 0.05 as compared with HR group, $*P* value < 0.05 as compared with CV group, &*P* value < 0.05 as compared with A group. Compared with C group, hypoxia 2 h and reoxygenation 24 h could make the apoptotic related proteins of caspase-9, Cyto C increased obviously ($P < 0.05$), but transfected Akt1 gene could downregulate the Cyto C and caspase-9 protein levels ($P < 0.05$).

[38] established a brain ischemia model via permanent occlusion of middle cerebral artery (pMCAO). Nerve growth factor (NGF) was used to stimulate the brain. Akt expression was increased in response to NGF. The NCX1 mRNA levels were increased as well, but the cerebral infarct area was reduced. Other experiments [39] arrived at similar conclusions suggesting that ischemic preconditioning protected against cerebral ischemia, following increased NCX1 and NCX3 expression. After addition of Akt inhibitors LY-294002, the protective effect disappeared, and the corresponding NCX1 and NCX3 expression was reduced.

Cyto C is a transmembrane protein in the mitochondria. Mitochondrial dysfunction induced by IR triggers the release of Cyto C into the cytoplasm, followed by the activation of caspase-9. Caspase-9 induces apoptosis, and is the most directly activated caspase kinase in mitochondrial apoptosis [40, 41]. Caspase-9 further activates downstream apoptotic protein caspase-3. Caspase-3 acts on endonuclease, damaging the nuclear DNA, resulting in cardiomyocyte apoptosis. Caspase-8 and caspase-12 also activate caspase-3. We, therefore, selected caspase-9 in the mitochondrial dysfunction-induced apoptosis mediated by IR. In our experiment, the cardiomyocytes in HR, were transfected with *Akt1*. Increased Akt1 levels and reduced NCX1 led to diminished cytoplasmic levels of Cyto C and caspase-9.

Transfection of lentivirus-mediated Akt1 played an anti-apoptotic role during IR in rat cardiomyocytes. The underlying mechanism includes inhibition of NCX1 expression, which further reduces the release of proteins in mitochondrial apoptosis, and results in anti-apoptotic effect.

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Disclosure of conflict of interest

None.

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References

[1] McCully JD, Wakiyama H, Hsieh YJ, Jones M and Levitsky S. Differential contribution of necrosis and apoptosis in myocardial ischemia-reperfusion injury. Am J Physiol Heart Circ Physiol 2004; 286: 1923-1935.

[2] Armstrong SC. Protein kinase activation and myocardial ischemia/reperfusion injury. Cardiovasc Res 2004; 61: 427-436.

[3] Du Y, Zhu H, Li D, Wang L, Zhang L, Luo Y, Pan D and Huang M. Lentiviral-mediated overexpression of Akt1 reduces anoxia-reoxygenation injury in cardiomyocytes. Cell Biology International 2014; 38: 488-496.

[4] Zhang WH, Fu SB, Lu FH, Wu B, Gong DM, Pan ZW, Lv YJ, Zhao YJ, Li QF, Wang R, Yang BF and Xu CQ. Involvement of calcium-sensing receptor in ischemia/reperfusion-induced apoptosis in rat cardiomyocytes. Biochem Biophys Res Commun 2006; 347: 872-881.

[5] Kalogeris T, Baines CP, Krenz M and Korthuis RJ. Cell biology of ischemia/reperfusion injury. Int Rev Cell Mol Biol 2012; 298: 229-317.

[6] Shintani-Ishida K, Inui M and Yoshida K. Ischemia-reperfusion induces myocardial infarction through mitochondrial Ca2+ overload. J Mol Cell Cardiol 2012; 53: 233-239.

[7] Hu Y, Chen X, Pan TT, Neo KL, Lee SW, Khin ES, Moore PK and Bian JS. Cardioprotection induced by hydrogen sulfide preconditioning involves activation of ERK and PI3K/Akt pathways. Pflugers Arch 2008; 455: 607-616.

[8] Lee S, Chanoit G, McIntosh R, Zvara DA and Xu Z. Molecular mechanism underlying Akt activation in zinc-induced cardioprotection. Am J Physiol Heart Circ Physiol 2009; 297: 569-575.

[9] Viswanath K, Bodiga S, Balogun V, Zhang A and Bodiga VL. Cardioprotective effect of zinc requires ErbB and Akt during hypoxia/reoxygenation. Biometals 2011; 24: 171-180.

[10] Routhu KV, Tsopanoglou NE and Strande JL. Parstatin (1-26): the putative signal peptide of protease-activated receptor 1 confers potent protection from myocardial ischemia- reperfusion injury. J Pharmacol Exp Ther 2010; 332: 898-905.

[11] Zhu J, Rebecchi MJ, Glass PS, Brink PR and Liu L. Cardioprotection of the aged rat heart by GSK-3 inhibitor is attenuated: age-related changes in mitochondrial permeability transition pore modulation. Am J Physiol Heart Circ Physiol 2011; 300: 922-930.

[12] Rakhit RD, Edwards RJ, Mockridge JW, Baydoun AR, Wyatt AW, Mann GE and Marber MS. Nitric oxide-induced cardioprotection in cultured rat ventricular myocytes. Am J Physiol Heart Circ Physiol 2000; 278: 1211-1217.

[13] Bharti S, Golechha M, Kumari S, Siddiqui KM and Arya DS. Akt/GSK-3beta/eNOS phosphorylation arbitrates safranal-induced myocardial protection against ischemia-reperfusion injury in rats. Eur J Nutr 2012; 51: 719-727.

[14] Yu LN, Yu J, Zhang FJ, Yang MJ, Ding TT, Wang JK, He W, Fang T, Chen G and Yan M. Sevoflurane postconditioning reduces myocardial reperfusion injury in rat isolated hearts via activation of PI3K/Akt signaling and modulation of Bcl-2 family proteins. J Zhejiang Univ Sci B 2010; 11: 661-672.

[15] Sher AA, Noble PJ, Hinch R, Gavaghan DJ and Noble D. The role of the Na+/Ca2+ exchangers in Ca2+ dynamics in ventricular myocytes. Prog Biophys Mol Biol 2008; 96: 377-398.

[16] Imahashi K, Pott C, Goldhaber JI, Steenbergen C, Philipson KD and Murphy E. Cardiac-Specific Ablation of the Na^+/Ca^{2+} Exchanger Confers Protection Against Ischemia/Reperfusion Injury. Circ Res 2005; 97: 916-921.

[17] Zhang X, Klein AL, Alberle NS, Norby FL, Ren BH, Duan J and Ren J. Cardiac-specific overexpression of catalase rescues ventricular myocytes from ethanol-induced cardiac contractile defect. J Mol Cell Cardiol 2003; 35: 645-652.

[18] Eigel BN and Hadley RW. Antisense inhibition of Na^+/Ca^{2+} exchange during anoxia/reoxygenation in ventricular myocytes. Am J Physiol Heart Circ Physiol 2001; 281: 2184-2190.

[19] Satoh H, MukaiM, Urushida T, Katoh H, Terada H and Hayashi H. Importance of Ca^{2+} influx by Na^+/Ca^{2+} exch ange under normal and sodium-loaded conditions in mammalian ventricular. Mol Cell Biochem 2003; 242: 11-17.

[20] Komuro I, Ohtsuka M. Forefront of Na^+/Ca^{2+} exchanger studies: role of Na^+/Ca^{2+} exchanger-lessons from knockout mice. J Pham acol Sci 2004; 96: 23-26.

[21] Iwamoto T. Sodium-calcium exchange inhibitors: therapeutic potential in cardiovascular diseases. Future Cardiol 2005; 1: 519-529.

[22] Halestrap AP. What is the mitochondrial permeability transition pore? J Mol Cell Cardiol 2009; 46: 821-831.

[23] Gomez L, Li B, Mewton N, Sanchez I, Piot C, Elbaz M and Ovize M. Inhibition of mitochond rialpermeability transition pore opening: translation to patients. Cardiovasc Res 2009; 83: 226-233.

[24] Li M, Xia T, Jiang CS, Li LJ, Fu JL and Zhou ZC. Cadmium drectly unduced the opening of membrane permeability pore of mitochondria which possibly involved in cadmium-triggered apoptosis. Toxicology 2003; 194: 19-33.

[25] El-Assal ON and Besner GE. HB-EGF enhances restitution after intestinal ischemia/reperfusion via PI3K/Akt and MEK/ERK1/2 activation. Gastroenterology 2005; 129: 609-625.

[26] Franke WW, Schumacher H, Borrmann CM, Grund C, Winter-Simanowski S, Schlechter T, Pieperhoff S and Hofmann I. The area compositа of adhering junctions connecting heart muscle cells of vertebrates-III: Assembly and sintegration of intercalated disks in rat cardiomyocytes growing in culture. Eur J Cell Biol 2007; 86: 127-142.

[27] Bergmann O, Zdunek S, Alkass K, Druid H, Bernard S and Frisén J. Identification of cardiomyocyte nuclei and assessment of ploidy for the analysis of cell turnover. Exp Cell Res 2011; 317: 188-194.

[28] Han L, Xu C, Jiang C, Li H, Zhang W, Zhao Y, Zhang L, Zhang Y, Zhao W and Yang B. Effects of polyamines on apoptosis induced by simulated ischemia/reperfusion injury in cultured neonatal rat cardiomyocytes. Cell Bio Int 2007; 31: 1345-1352.

[29] Van der Heiden K, Cuhlmann S, Luong le A, Zakkar M and Evans PC. Role of nuclear factor kappaB in cardiovascular health and disease. Clin Sci 2010; 118: 593-605.

[30] Wilhide ME, Tranter M, Ren X, Chen J, Sartor MA, Medvedovic M and Jones WK. Identification of a NF-κB cardioprotective gene program: NF-κB regulation of Hsp70.1 contributes to cardioprotection after permanent coronary occlusion. J Mol Cell Cardiol 2011; 51: 82-89.

[31] Isaka Y, Suzuki C, Abe T, Okumi M, Ichimaru N, Imamura R, Kakuta Y, Matsui I, Takabatake Y, Rakugi H, Shimizu S and Takahara S. Bcl-2 protects tubular epithelial cells from ischemia/reperfusion injury by dual mechanisms. Transplant Proc 2009; 41: 52-54.

[32] Honda K, Tohyama T, Kotegawa H, Kojima Y, Kushihata F, Watanabe J and Kobayashi N. Protective effect of adeno-mediated human Bcl-xL gene transfer to the mouse liver in a partial ischemia/reperfusion model. J Surg Res 2009; 157: 107-116.

[33] Ruan H, Li J, Ren S, Gao J, Li G, Kim R, Wu H and Wang Y. Inducible and cardiac specific PTEN inactivation protects ischemia/reperfusion injury. J Mol Cell Cardiol 2009; 46: 193-200.

[34] Nagoshi T, Matsui T, Aoyama T, Leri A, Anversa P, Li L, Ogawa W, del Monte F, Gwathmey JK, Grazette L, Hemmings BA, Kass DA, Champion HC and Rosenzweig A. PI3K rescues the detrimental effects of chronic Akt activation in the heart during ischemia/reperfusion injury. J Clin Invest 2005; 115: 2128-2138.

[35] Ye N, Liu S, Lin Y and Rao P. Protective effects of intraperitoneal injection of TAT-SOD against focal cerebral ischemia/reperfusion injury in rats. Life Sci 2011; 89: 868-874.

[36] O'Connor DM and O'Brien T. Nitric oxide synthase gene therapy: progress and prospects. Expert Opin Biol Ther 2009; 9: 867-878.

[37] Aurora AB, Mahmoud AI, Luo X, Johnson BA, van Rooij E, Matsuzaki S, Humphries KM, Hill JA, Bassel-Duby R, Sadek HA and Olson EN. MicroRNA-214 protects the mouse heart from ischemic injury by controlling Ca2+ overload and cell death. J Clin Invest 2012; 122: 1222-1232.

[38] Annunziato L, Pignataro G, Boscia F, Sirabella R, Formisano L, Saggese M, Cuomo O, Gala R, Secondo A, Viggiano D, Molinaro P, Valsecchi V, Tortiglione A, Adornetto A, Scorziello A, Cataldi M and Di Renzo GF. ncx1, ncx2, and ncx3 gene product expression and function in neuronal anoxia and brain ischemia. Ann N Y Acad Sci 2007; 1099: 413-426.

[39] Pignataro G, Boscia F, Esposito E, Sirabella R, Cuomo O, Vinciguerra A, Di Renzo G, and Annunziato L. NCX1 and NCX3: Two new effectors of delayed preconditioning in brain ischemia. Neurobio Dis 2012; 45: 616-623.

[40] Zhao ZQ and Vinten-Johansen J. Myocardial apoptosis and ischemic preconditioning. Cardiovasc Res 2002; 55: 438-455.

[41] Qin Y, Vanden Hoek TL, Wojcik K, Anderson T, Li CQ, Shao ZH, Becker LB and Hamann KJ. Caspase-dependent cytochrome c release and cell death in chick cardiomyocytes after simulated ischemia-reperfusion. Am J Physiol Heart Circ Physiol 2004; 286: 2280-2286.