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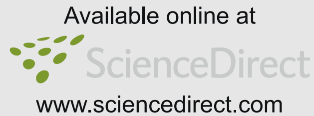

Dossier

RNAi targeting ryanodine receptor 2 protects rat cardiomyocytes from injury caused by simulated ischemia-reperfusion

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ABSTRACT

The effects of a small interfering RNA targeting ryanodine receptor 2 (si-Ryr2) on cardiomyocytes injury following a simulated ischemia-reperfusion (I/R) were investigated. Pretreated with si-Ryr2 or ryanodine, primary cultures of neonatal rat cardiomyocytes were subjected to a protocol of simulated I/R. Compared with control, the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) and the generation of reactive oxygen species (ROS) was significantly augmented after I/R. Concomitant with these, cell injury assessed by Annexin V/PI staing, mitochondria membrane potential ($\Delta\Psi m$) and the leakage of lactic dehydrogenase (LDH) and creatine phosphokinase (CPK) were aggravated. Si-Ryr2 treatment reduced $[Ca^{2+}]_i$ and ROS generation and protected the cardiomyocytes from subsequent I/R injury, as evidenced by stable $\Delta\Psi m$ and decreased Annexin V^+ PI^- staing and enzymes release. Moreover, si-Ryr2 exerted more effective protection on I/R injury compared to ryanodine. The present study demonstrated for the first time that in neonatal cardiomyocytes, si-Ryr2 reduces cell death associated with attenuating $[Ca^{2+}]_i$ and ROS production. Furthermore, we attempt to speculate that si-Ryr2 excel ryanodine in Ryr2 function research of cardioprotection.

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1. Introduction

Among the molecular mechanisms leading to cardiomyocyte injury caused by I/R, changes in $[Ca^{2+}]_i$ are thought to be of major importance [1]. The most remarkable event leading to $[Ca^{2+}]_i$ alteration appears to be the Ca^{2+}-induced Ca^{2+} release, i.e., increase in cytosolic Ca^{2+} due to Ca^{2+} entry through the sarcolemma induces further Ca^{2+} release from the SR. Ca^{2+} release by SR is mediated by the SR Ca^{2+} release channel, known as ryanodine receptor (Ryr, Ryr2 in cardiomyocytes). Ischemia or reperfusion exerts profound effects on the function of Ryr2, resulting in increased $[Ca^{2+}]_i$ [2]. So many modulators which disturb Ryr2 function could protect myocardium from injury caused by I/R.

Ryanodine, a vegetable alkaloid derived from *Ryania speciosa*, is an ester of pyrrole-α-carboxylic acid with ryanodol. It has been shown to interact specifically with Ryr. Several groups of investigators have observed beneficial effects when ryanodine is administrated before ischemia or I/R [3]. However, ryanodine may produce complex effects, both beneficial and deleterious, on the physiological function of SR channel, and the protective one was usually preceded by a negative inotropic response [4,5].

Here, we presented the results of our preliminary in vitro studies performed in rat cardiomyocytes that aimed to explore the protective effect of gene silencing on cardiomyocyte injury caused by I/R using the RNA interference technique that specifically suppresses the expression of Ryr2 gene. Since many previous reports [6–8] have shown that $[Ca^{2+}]_i$ alteration during reperfusion is associated with generation of ROS, and the latter plays crucial role in the induction of cell death following I/R, we also examined the ROS production in the present study. And by comparing the protection efficacy of si-Ryr2 or ryanodine on cardiomyocytes following I/R, reflected by Annexin V/PI staing, $\Delta\Psi m$ and the leakage of LDH and CPK, we explored if RNA interference technique has any superiority over ryanodine.

2. Materials and methods

2.1. Animals

Animals used in this study were maintained in accordance with the Guide for the Care and Use of Laboratory Animals published by the US NIH (publication No. 96-01), and all procedures were approved by the Institutional Review Board of the Institute of

Abbreviations: siRNA, a small interfering RNA; Ryr 2, ryanodine receptor 2; si-Ryr2, a small interfering RNA targeting ryanodine receptor 2; I/R, ischemia-reperfusion; $[Ca^{2+}]_i$, cytosolic Ca^{2+} concentration; ROS, reactive oxygen species; $\Delta\Psi m$, mitochondria membrane potential; LDH, lactic dehydrogenase; CPK, creatine phosphokinase; SR, sarcoplasmic reticulum.

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2.2. Neonatal rat cardiomyocyte preparation

Cardiomyocytes from 1–3 day old Sprague-Dawley (SD) rats were prepared as described previously [9]. Briefly, hearts were harvested and placed in Hanks' balanced salt solution lacking magnesium and calcium. Ventricles were minced, and myocytes were dissociated with the use of 17–18 repeats of trypsin (0.1%, Lot 0458, Amresco) degradation at 37 °C with gentle agitation. Isolated cells were then transferred to modified Eagle medium (MEM, Cat 41500, Lot 1267191, GIBCO) containing 15% fetal bovine serum (Cat CCS00101, Lot 402007, MD genics Inc.) for inhibiting trypsin, centrifuged for 5 min at 1,500 rpm at 4 °C, and finally resuspended in the medium mentioned above. Resuspended cells were placed in a Petri dish in a humidified incubator (5% CO_2-95% O_2 at 37 °C) for 2 h to promote early adherence of fibroblasts, then filtered through a 100 μm mesh. Nonadherent cells were counted with a hemocytometer, and viability reached 90% as estimated by staining with trypan blue (0.4%). Cells were transferred to a medium containing 0.1 mM 5-BrdU for 48 h and then cultured in a medium without 5-BrdU.

2.3. Simulated I/R

On the third or fourth day after plating, the cells which formed interconnected confluent networks that exhibited rhythmic spontaneous contractions were used. Myocytes were plated in HEPES buffered solution on 35 mm cell culture dishes. "Ischemia" (6 h) was reproduced by a buffer exchange to ischemia-mimetic solution (in mM: 98.5 NaCl, 20.0 HEPES, 0.9 NaH_2PO_4, 6.0 $NaHCO_3$, 10.0 KCl, 1.2 $MgSO_4$, 1.8 $CaCl_2$ and 40.0 Na-lactate, gassed with 95% N_2 and 5% CO_2, and pH adjusted to 6.8 with NaOH). A 95% N_2 and 5% CO_2 gas phase was layered over the experimental chamber during simulated ischemia. "Reperfusion" (2 h) was initiated by returning to the reperfusion-mimetic solution (in mM: 129.5 NaCl, 20.0 HEPES, 0.9 NaH_2PO_4, 20.0 $NaHCO_3$, 5.0 KCl, 1.2 $MgSO_4$, 1.8 $CaCl_2$ and 55.0 D-glucose, gassed with 95% O_2 and 5% CO_2, and pH adjusted to 7.4). During simulated reperfusion, N_2 was replaced by O_2.

2.4. RNA interference

In order to inhibit Ryr2 expression, siRNA targeting Ryr2 (si-Ryr2) was used (Shanghai GeneChem Company, Shanghai, China). The cardiomyocytes were grown in MEM supplemented with 15% fetal bovine serum and beaten at equal pace. Transfection of si-Ryr2 was carried out using HiperFect (Qiagen) with 3'-FITC-labeled control siRNA (AUAUAAUUGUGGCCGUUGCdTdT) or 3'- FITC-labeled si-Ryr2 (GCGAUGCAAGAGAGAGAAGAAdTdT) according to the manufacturer's protocol. Transfection efficiencies were determined 36 h later by fluorescence microscopy (model IX 50, Olympus).

The cells were selected according to the following strategy: first, reduced expression of Ryr2 mRNA was determined by RT-PCR; secondly, reduced expression of Ryr2 was checked by flow cytometry.

2.5. Ryanodine treatment

On the third or fourth day after plating, the cells which formed interconnected confluent networks and exhibited rhythmic spontaneous contractions were used. Ryanodine (0.1 μM, Lot P8226n, BIOMOL) was administrated and the cells preceded incubation for 30 min.

2.6. Grouping

A known number of cultured neonatal rat cardiomyocytes were randomly and homogeneously distributed into different experimental groups as follows: (1) Control group: cardiomyocytes were incubated in cell incubator for 45 h. (2) siRNA-C group: the protocol was the same as that in control group except that RNA interference was applied. (3) Rya-C group: the protocol was the same as that in control group except that ryanodine exhausting was applied. (4) I/R group: cardiomyocytes were directly exposed to ischemia (for 6 h) followed by reperfusion (for 2 h) as described above. (5) siRNA-I/R group: the protocol was the same as that in I/R group except that RNA interference was applied. (6) Rya-I/R group: the protocol was the same as that in I/R group except that ryanodine exhausting was applied. The experimental protocol for each group is given in Fig. 1.

2.7. Measurement of intracellular calcium

Fluo-3 AM (Cat 50016, Lot 6F0221, Biotium) was dissolved in dimethyl sulfoxide (Sigma) at a concentration of 10 mM. The cardiomyocytes were resuspended at 10^6/ml in HBSS buffer (Hank's balanced salt solution without calcium chloride, magnesium sulfate, and phenol red). The cells were adjusted to 1 μM fluo-3 AM and incubated at 37 °C for 30 min in the dark. Finally, the cells were washed two times in Hank's solution. Analysis was performed on the Beckman Coulter EPICS-XL flow cytometer.

2.8. Flow cytometric determination of ROS

ROS formation was assessed in cardiomyocytes using ROS assay kit (S0033, Beyotime Institute of Biotechnology, China). Cardiomyocytes were dislodged with trypsin and were then loaded (30 min at 37 °C) with 10 μM DCFH-DA, which becomes fluorescent on oxidation to DCF by hydrogen peroxide (H_2O_2) within the cell. Levels of ROS were measured by flow cytometry (EPICS-XL, Beckman Coulter) as the fluorescence of DCF. Excitation and emission wavelengths were 488 and 525 nm, respectively. Tracings were obtained by displaying the log fluorescence of the samples generated against the background staining of cells.

2.9. Flow cytometry analysis of cell apoptosis

Cells were labeled with annexin V and propidium iodide (PI) (PF032-IEA D38501, Merck, USA) according to the manufacturer's instructions. Briefly, 1×10^5 cells were washed with cold PBS, and resuspended in the binding buffer. The cells were stained with Annexin V and PI in the dark for 15 min before analyzed by Beckman Coulter EPICS-XL flow cytometer.

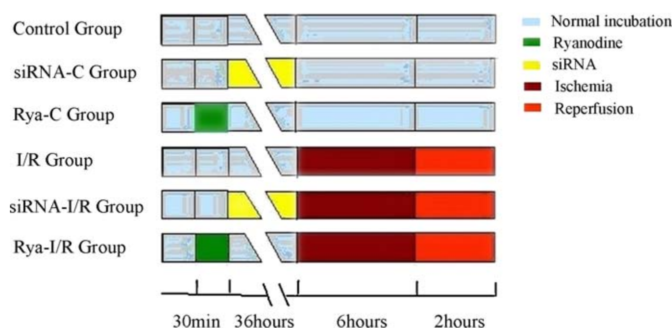

Fig. 1. Schematic representation of the experimental design. See materials and methods for details.

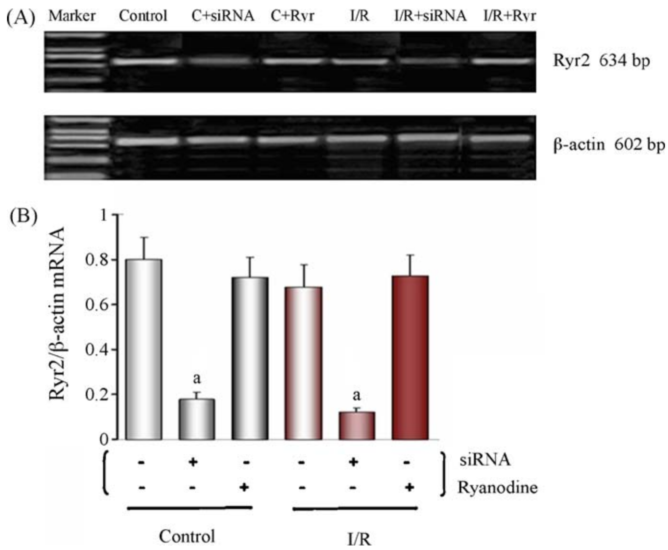

Fig. 2. Effect of siRNA on Ryr2 mRNA expression in cardiomyocytes as evidenced by RT-PCR (A) and expression of the data by histogram (B) ($n = 8$, means ± S.D.). [a]$P < 0.01$ *vs* corresponding nontransfected cells.

2.10. Flow cytometry analysis of $\Delta\Psi m$

Cell suspensions were stained with Rh123 (5 mg/L, DFRH-001, Beijing Dingguo Genetech Co., Ltd) for 10 min at room temperature in the dark, and were washed twice with PBS (pH 7.4). FCM analysis was performed on a Beckman Coulter EPICS-XL flow cytometer.

2.11. Measurement of LDH and CPK release

The amount of LDH and CPK released into the culture medium from the cardiomyocytes of each group was assayed using an automatic biochemical analyzer (model LX20, Beckman Coulter) according to the manufacturer's instructions.

2.12. Statistical analysis

Data are presented as means ± S.D. Significant differences were evaluated using ANOVA and an LSD test as appropriate. $P < 0.05$ was regarded as statistically significant.

3. Results

3.1. Effect of siRNA or ryanodine on Ryr2 expression

First, to verify the specificity and effectiveness of si-Ryr2, RT-PCR was performed on the si-Ryr2-transfected primary cardiomyocytes. As shown in Fig. 2, Ryr2 expression was significantly inhibited by si-Ryr2 ($P < 0.01$), but not by ryanodine ($P > 0.05$). In control and I/R group, Ryr2 mRNA levels were reduced by 27.4 and 20.2% respectively, compared with nontransfected cells.

Similarly, transfection of si-Ryr2 resulted in a 34.2 and 26.1% reduction in Ryr2 protein compared with nontransfected cells. These results confirmed the effectiveness of this siRNA in silencing Ryr2 expression in cardiomyocytes. SR Ca^{2+} depletion by ryanodine did not influence significantly Ryr2 protein expression in all groups (Fig. 3).

3.2. Effect of siRNA or ryanodine on [Ca2+]i of cardiomyocytes following I/R

As Ca^{2+} overload is a determinant of the irreversible cardiomyocyte injury. $[Ca^{2+}]_i$ in cardiomyocytes from different groups was measured by flow cytometry (Fig. 4). The $[Ca^{2+}]_i$ in I/R group was increased compared to that in the control group ($P < 0.01$), whereas in the cardiomyocytes treated with si-Ryr2 or ryanodine $[Ca^{2+}]_i$ maintained at a low level ($P < 0.01$), with si-Ryr2 being more effective than ryanodine ($P < 0.05$).

3.3. Effect of siRNA or ryanodine on ROS during reperfusion

To check that si-Ryr2 or ryanodine induces ROS in cardiomyocyte, the cells were incubated with DCFH-DA, a sensitive oxidant

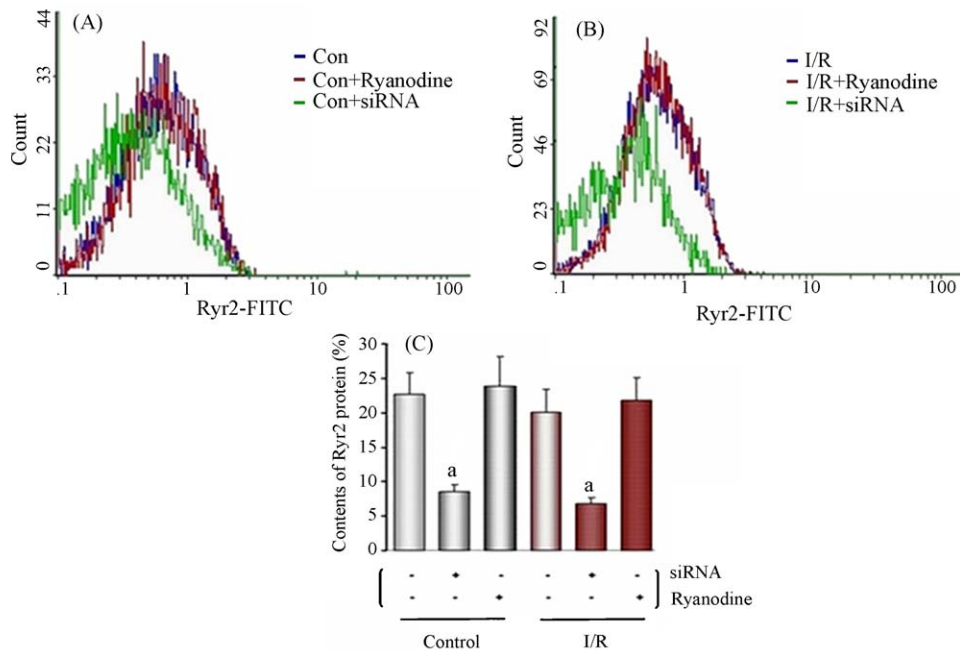

Fig. 3. Effect of siRNA on the expression of Ryr2 protein in cardiomyocytes estimated by flow cytometry (A and B) and expression of the data by histogram (C) ($n = 8$, means ± S.D.). [a]$P < 0.01$ *vs* corresponding nontransfected cells.

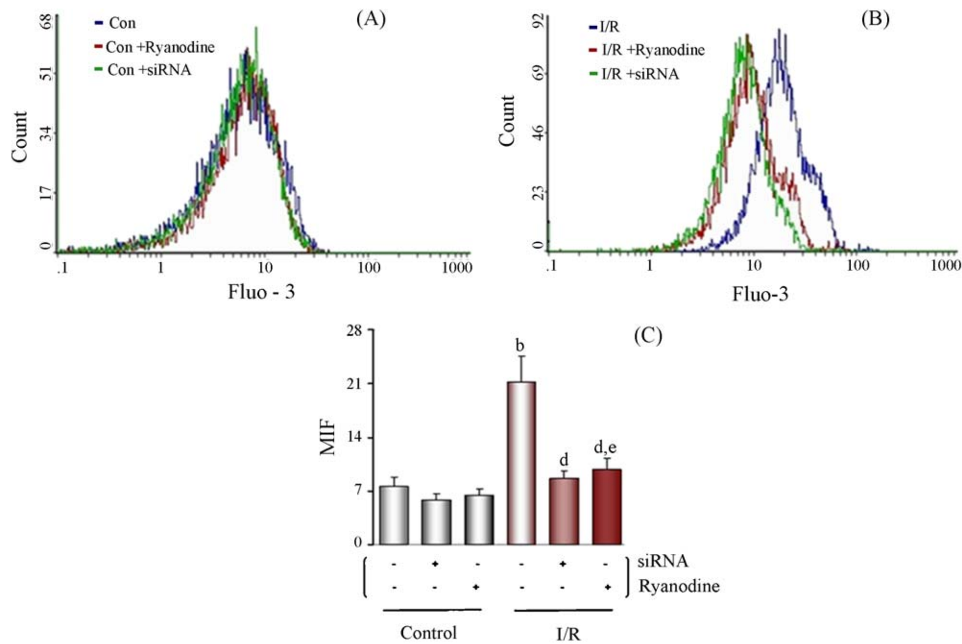

Fig. 4. Flow cytometric analysis of $[Ca^{2+}]_i$ in cardiomyocytes from different groups showing effects of si-Ryr2 and ryanodine on $[Ca^{2+}]_i$ in the absence (A) and presence (B) of I/R. Histogram transition of data from A and B (C) ($n = 8$, means ± S.D.). $^{b}P < 0.01$ *vs* control group; $^{d}P < 0.01$ *vs* I/R group; $^{e}P < 0.05$ *vs* I/R + siRNA group.

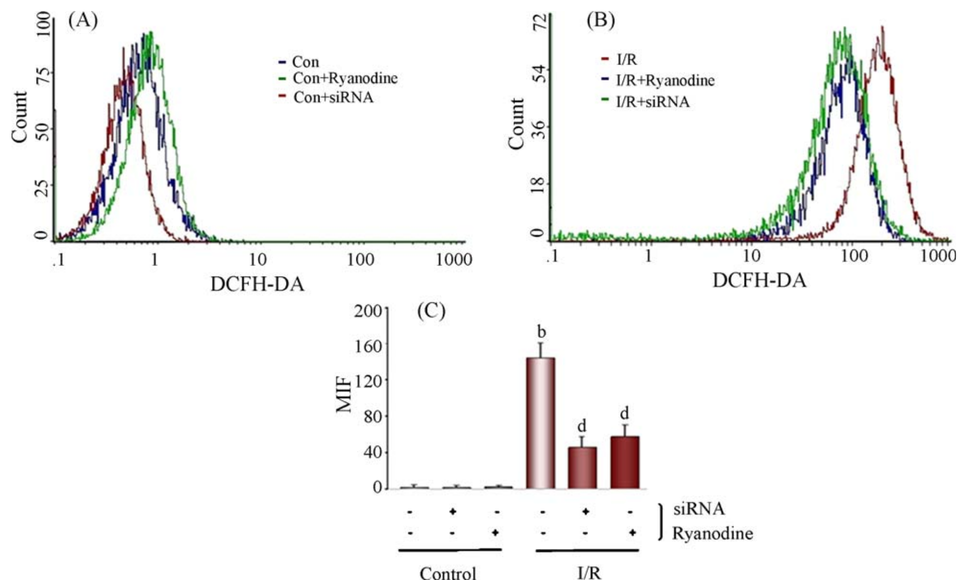

Fig. 5. The effect of si-Ryr2 or ryanodine on the generation of ROS in cardiomyocyte in the absence (A) and presence (B) of I/R. Histogram transition of data from A and B (C) ($n = 8$, means ± S.D.). The cardiomyocytes were dislodged with trypsin and incubated with DCFH-CA for 30 min, and were analyzed to estimate fluorescence. The experiment was repeated three times. A and B: The mean fluorescence intensity of DCF of all groups. C: Results are expressed as means ± S.D. $^{b}P < 0.01$ *vs* control group; $^{d}P < 0.01$ *vs* I/R group.

indicator dye. ROS levels, as detected by DCF fluorescence, were increased significantly at the end of reperfusion compared with control. The mean fluorescence intensity of DCF signal was decreased by ryanodine added to give a final concentration of 0.1 μM at 30 min before the onset of I/R. And si-Ryr2 further reduced the DCF signal compared with ryanodine (Fig. 5).

3.4. siRNA or ryanodine reduced apoptotic cardiomyocytes following I/R

To determine whether si-Ryr2 or ryanodine had anti-apoptotic effects, the cardiomyocytes were labelled with annexin V and PI, and cell apoptosis was detected by flow cytometry (Fig. 6). Consistent with the $[Ca^{2+}]_i$ results, I/R promoted cell apoptosis ($P < 0.01$ vs. control group). Treatment with si-Ryr2 or ryanodine before I/R both exerted significant anti-apoptotic effects ($P < 0.01$ vs. I/R group), and si-Ryr2 was more powerful than ryanodine ($P < 0.05$).

3.5. siRNA or ryanodines stabilized $\Delta\Psi$m of cardiomyocytes following I/R

$\Delta\Psi$m is considered one of the earliest events in apoptosis. In the present study, we analyzed $\Delta\Psi$m by flow cytometry using Rh123 staining (Fig. 7). The Rh123 staining is intense and strictly located at the mitochondria and a reduced Rh123 staining

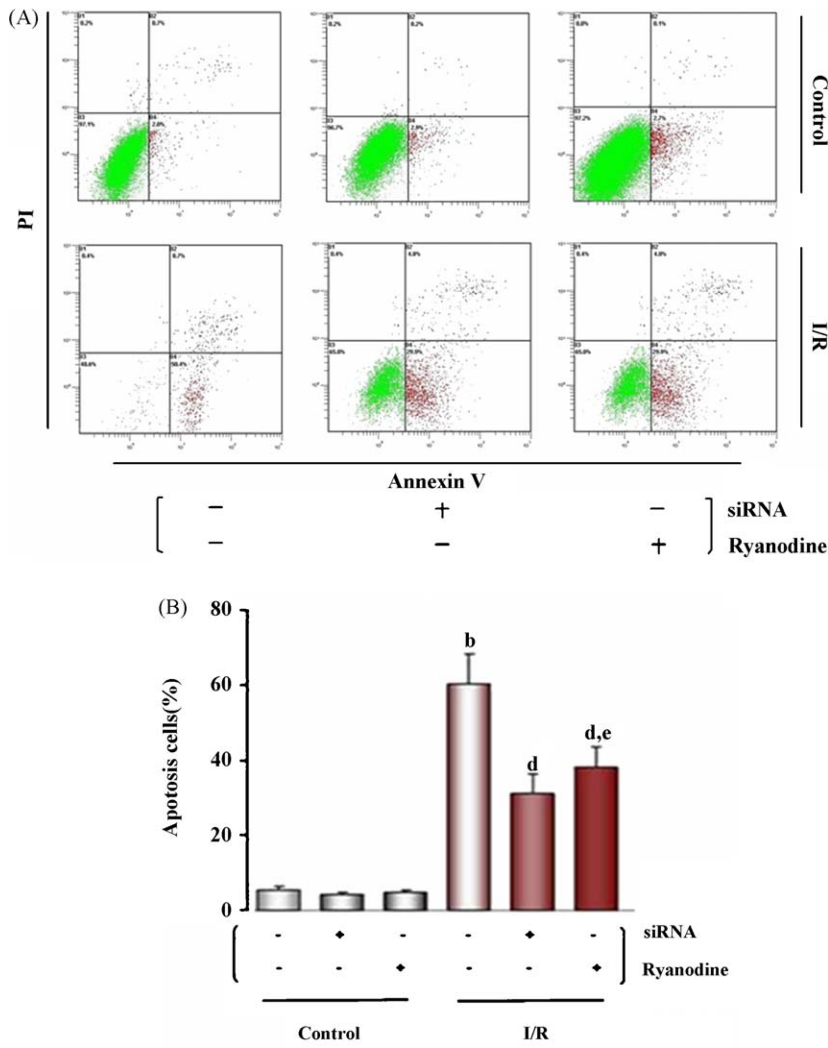

Fig. 6. Anti-apoptotic effect of siRNA and ryanodine shown by flow cytometry (A) and histogram transition of data from A (B) (n = 8, means ± S.D.). $^{b}P < 0.01$ *vs* control group; $^{d}P < 0.01$ *vs* I/R group; $^{e}P < 0.05$ *vs* I/R + siRNA group.

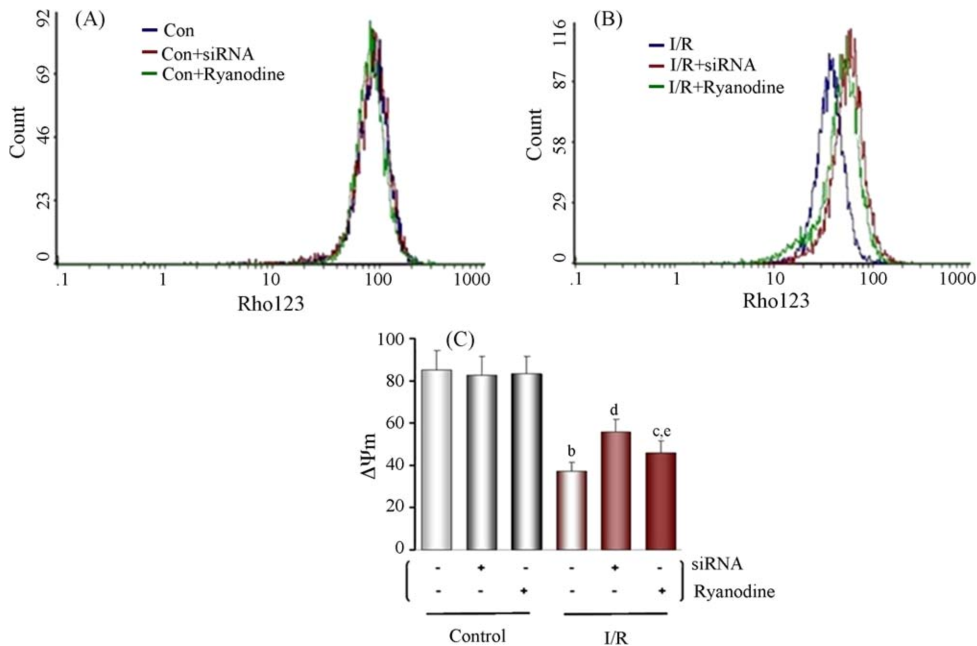

Fig. 7. Effect of si-Ryr2 or ryanodine on mitochondria $\Delta\Psi$m of cardiomyocytes in the control group (A) and in the I/R group (B) as evidenced by flow cytometry. Histogram transition data from A and B (C) (n = 8, means ± S.D.). $^{b}P < 0.01$ *vs* control group; $^{c}P < 0.05$, $^{d}P < 0.01$ *vs* I/R group; $^{e}P < 0.05$ *vs* I/R + siRNA group.

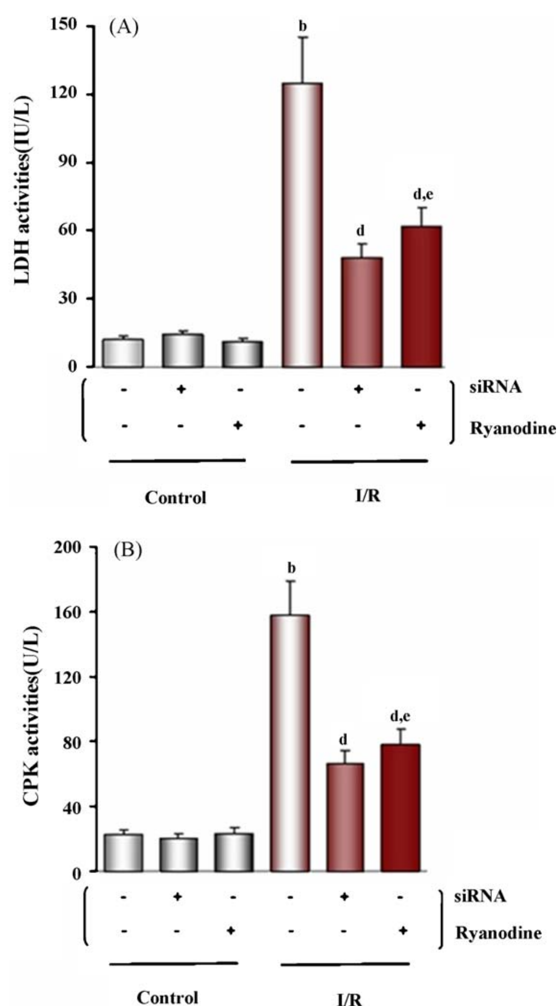

Fig. 8. SiRNA and Ryanodine were only protective for cardiomyocytes that had undergone I/R in terms of membrane integrity, as evidenced by reduced enzymatic leakage in the I/R, but not in the control group (n = 8, means ± S.D.). (A) LDH leakage and (B) CPK leakage. $^{b}P < 0.01$ *vs* control group; $^{d}P < 0.01$ *vs* I/R group; $^{e}P < 0.05$ *vs* I/R + siRNA group.

indicates $\Delta\Psi$m reduction. The results showed that si-Ryr2 or ryanodine treatment could prevent I/R-induced $\Delta\Psi$m depress, and that si-Ryr2 was more effective than ryanodine ($P < 0.05$).

3.6. Leakage of myocardial enzymes

LDH and CPK leakage detected in the culture medium of cardiomyocytes was increased in the I/R group compared with that in the control group ($P < 0.01$). Treatment of cardiomyocytes with si-Ryr2 or ryanodine did not show any effects on the LDH and CPK leakage of normal cells in the control group, but significantly reduced enzyme leakage in cells which had undergone I/R ($P < 0.01$). In addition, si-Ryr2 had a stronger effect compared with ryanodine (Fig. 8).

4. Discussion and conclusion

Reperfusion of the ischemic myocardium caused reperfusion injury, which is associated with and possibly mediated by Ca^{2+} overload. The increase in cytosolic Ca^{2+} is largely due to Ca^{2+} entry through Ryr2 from the SR. Many modulators including ryanodine which disturb Ryr2 function could protect myocardium from injury caused by I/R. However, ryanodine may produce concentration-dependent complex effects on the physiological function of Ryr2, and the protective one was usually preceded by a negative inotropic response. RNA interference is a powerful targeting technique that specifically suppresses the expression of the targeted gene at the post-transcriptional, and sometimes, transcriptional level. This study was designed to investigate the protective effect of gene silencing on cardiomyocyte injury caused by I/R using the RNAi technique that specifically suppresses the expression of Ryr2 gene. And by comparing the efficacy of this technique with that of ryanodine, we explored if RNAi technique could have more effective protection on cardiomyocytes following I/R than ryanodine.

The present study shows that siRNA has a powerful and specialized inhibitory effect on Ryr2 (Figs. 2 and 3). In the isolated cardiomyocytes that were subjected to I/R, inhibition of Ryr2 by si-Ryr2 resulted in decreased apoptosis level as examined by annexin V/PI staining and $\Delta\Psi$m detection, and reduced cell death by measurement of LDH and CPK release. Moreover, although statistically significant differences are not obvious between si-Ryr2 and ryanodine, the stronger trend of si-Ryr2 seems to exist. Compared with ryanodine, si-Ryr2 appeared more effective in the protection of cardiomyocyte from damage by I/R. Considering that ryanodine would affect basal contractility or Ca^{2+} dynamics in cardiomyocyte, si-Ryr2 would be a much more sensitive and specific tool in Ryr2 function research.

In the present study, we also demonstrate for the first time that si-Ryr2 prevents cell injury associated with attenuating cytosolic Ca^{2+} and ROS production in cardiomyocytes. Both intracellular Ca^{2+} overload and generation of ROS appear crucial in the induction of cell death during reperfusion and reoxygenation.

On one hand, si-Ryr2 exerted powerful protection on cardiomyocyte mediated by $[Ca^{2+}]_i$ reduction by means of interfering Ryr2. SR is the major intracellular Ca^{2+} store, and SR Ca^{2+} release occurs through Ryr2 in cardiomyocyte. The increase in $[Ca^{2+}]$i following I/R is largely due to Ca^{2+} release through Ryr2. So interventions able to affect Ryr2 function like si-Ryr2 have a deep influence on the development of I/R injury.

On the other hand, the effect of si-Ryr2 on ROS generation drew our attention. Si-Ryr2 reduced the generation of ROS evidenced by a decrease in oxidation of DCFH. A significant burst of ROS formation occurs during reperfusion of the ischemic heart [10]. Si-Ryr2 possibly reduces ROS production indirectly by decreasing $[Ca^{2+}]_i$. Mitochondria are a significant source of ROS. Ca^{2+} could enhance mitochondrial ROS output by improving metabolic rate, blocking the respiratory chain, diminishing glutathione level, and so on [11]. However, the exact source and mechanism of ROS formation during reoxygenation are unknown. Furthermore, some experimental observations support the idea that the generation of ROS occurs first and then induces intracellular Ca^{2+} overload [12]. Additional studies are required to clarify the mechanisms in attenuating ROS production by si-Ryr2.

In conclusion, the present study demonstrates for the first time that si-Ryr2 reduces cell death associated with attenuating cytosolic Ca^{2+} and ROS production in isolated neonatal rat cardiomyocytes following I/R. Furthermore, as far as its effect against I/R injury are concerned, it is tempting to speculate that si-Ryr2 excel ryanodine in the research of Ryr2 function.

Acknowledgements

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