Int J Clin Exp Pathol 2016;9(12):12543-12550
www.ijcep.com /ISSN:1936-2625/IJCEP0039747

Original Article

Silencing Rac1 protects H9c2 cells against hypoxia-reoxygenation injury by inactivation of notch pathway

Huimin Liu, Maoen Xu, Lijia Ma, Huai Yu, Jingbo Hou, Bo Yu

Department of Cardiology, The 2nd Affiliated Hospital of Harbin Medical University, Key Laboratory of Myocardial Ischemia Mechanism and Treatment Ministry of Education, Harbin 150086, China

Received September 9, 2016; Accepted October 28, 2016; Epub December 1, 2016; Published December 15, 2016

Abstract: This study was aimed to determine the regulatory landscape of ectopic Ras-related C3 botulinum toxin substrate 1 (Rac1) in cardiomyocytes with hypoxia-reoxygenation (H/R) injury. We constructed a cell model with H/R injury in H9c2 cardiomyocytes to simulate myocardial Ischemia reperfusion injury (MIRI). When Rac1 was overexpressed or silenced, 3-(4,5-dimethylthiazol -2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay and flow cytometry were used to detect cell viability, reactive oxygen species (ROS) content and apoptosis in transformed cells. Key proteins Notch 1, Notch 2 and Jagged 1 (JAG1) of the Notch signaling pathway and apoptosis mediators B-cell lymphoma-2 (Bcl-2) and Caspase-3 were observed. Reoxygenation treatment provoked decline in the H9c2 cell viability in a time-dependent manner and about 54.67% viability was identified for 12 h. Thereby, 12 h reoxygenation duration was determined in further studies. Silencing Rac1 could remarkably raise cell viability ($P < 0.05$), suppress apoptosis ($P < 0.05$), scavenge ROS ($P < 0.01$) and inhibit expressions of key proteins of Notch signaling pathway, while overexpressing Rac1 had opposite effects on H/R-treated H9c2 cardiomyocytes ($P < 0.05$). Furthermore, silencing Rac1 suppressed H9c2 cell apoptosis by increasing Bcl-2 and decreasing cleaved Caspase-3. Collectively, our findings suggest that Rac1 acts as a key mediator of cardiomyocytes survival and responding oxidant stress after H/R injury. This study provides novel insight into the MIRI suppressive capacity of silencing Rac1 by inactivation of Notch signaling pathway, which holds great clinical potential in the cardioprotection against MIRI.

Keywords: Rac1, hypoxia-reoxygenation injury, notch signaling pathway, ischemic-reperfusion injury

Introduction

Ischemic-reperfusion injury (IRI) is severe structural damages with cell metabolic disorders when blood returns to organs following a period of ischemia [1, 2]. Reintroduction of blood flow contributes to the increase of reactive oxygen species (ROS), which brings about tissue functional damage and initiates a cellular cascade resulting in inflammations and cell apoptosis [3, 4]. Myocardial IRI (MIRI) has been reported to inflict irreversible necrosis of cardiomyocytes and poor prognosis in acute myocardial infarction (AMI) patients [5]. Currently, MIRI has been the major barrier of ischemia myocardium achieving the optimal efficacy from cardioprotective therapy [6]. In the present study, we established a model with hypoxia-reoxygenation (H/R)-treated H9c2 cardiomyocytes to simulate MIRI [7].

Ras-related C3 botulinum toxin substrate 1 (Rac1) is a key member of Ras superfamily of small guanosine triphosphatases (GTPases), which regulates cell cycle, apoptosis, protease activation and membrane trafficking [8]. Evidence points to a pivotal role for Rac1 acting as a molecular switch to conduct cell growth and cytoskeletal rearrangements. Raz *et al.* found that Rac1 GTPase mediated IRI-induced ROS generation and oxidative stress in the rat following cerebral ischemia [9]. However, little is known concerning the role of Rac1 in pathological process of MIRI.

In this study, we aimed to determine the regulating landscape of ectopic Rac1 in cardiomyocytes with H/R injury, which is expected to contribute to the therapy of MIRI. We constructed a cell model with H/R injury in H9c2 cardiomyo-

cytes to simulate MIRI. Subsequently, we studied whether overexpression and knockdown of Rac1 play a role in the cell viability, ROS content and apoptosis of H/R-induced H9c2 cells, as well as its underlying mechanism. This study was expected to offer a novel Rac1-based therapy for protection against MIRI.

Materials and methods

Cell culture and H/R treatment

The rat embryonic heart-derived H9c2 cardiomyocytes were obtained from the American Type Culture Collection (ATCC; Manassas, USA). The cell line was maintained in high glucose Dulbecco's Modified Eagle's Medium (DMEM, Lonza, USA) supplemented with 10% fetal bovine serum (FBS; Hyclone Laboratories, Logan, USA), 100 μg/mL streptomycin, 100 IU/mL penicillin (both from Gibco-Invitrogen Corp., UK) and 2 mM L-glutamine (Sigma-Aldrich, Saint Louis, USA). All cells were cultured at 37°C in a humidified atmosphere containing 5% CO_2 for 2-3 d. Then for the H/R treatment, the medium was replaced with none glucose DMEM to simulate myocardial ischemia. The hypoxic condition was founded by incubating H9c2 cells in an anaerobic chamber equilibrated with 5% H_2, 5% CO_2 and 90% N_2 at 37°C for 6 h. Subsequently, the medium was replaced with high glucose DMEM and H9c2 cells were reoxygenated under normoxic conditions in a humidified atmosphere (5% CO_2) at 37°C for the indicated time (2, 4, 8, 12 and 24 h). In addition, the control H9c2 cardiomyocytes were incubated in high glucose DMEM under normoxic conditions for equivalent durations. For following analyses, H9c2 cells grew to 70%-80% confluence as judged under a phase contrast microscopy (Olympus, Tokyo, Japan) for 1 d before experimentations.

Rac1 overexpression and silence plasmids transfection

H9c2 cells were incubated at 1×10^5 cells per well of six-well plates overnight. The plasmid pcDNA3.1-Rac1 or small interfering RNA (siRNA)-Rac1 (GenePharma Co, Shanghai, China) was constructed and transfected to H/R-treated H9c2 cells on the next day, respectively. The empty vector pcDNA3.1 and siRNA were considered to be negative control (NC). Cell transfections were carried out using Lipofectamine 3000 reagent (Invitrogen, CA, USA) based on manufacturer' s introductions.

Cell viability assay

The cell viability was determined with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay [10]. After transformation for 2 d, the control and H/R-treated H9c2 cells were cultured in DMEM (with 10% FBS) containing 0.5 mg/mL MTT (Sigma, USA) for 4 h. Then 100 μL dimethylsulfoxide (DMSO; Lonza, USA) was added to dissolve the blue formazan (Sigma, USA) product. The cell viability was measured by absorbance at a wavelength of 550 nm. After the reoxygenation duration was determined, cell viability of H/R-treated negative control and transfected H9c2 cells were identified at 1 d after reoxygenation with the same method.

Apoptosis assay

Apoptotic H9c2 cells were identified and quantified by flow cytometry with Annexin V-FITC/propidium iodide (PI) apoptosis detection kit (Beijing Biosea Biotechnology, Beijing, China). After transfecting pc-Rac1/pcDNA3.1 or siRac1/siRNA for 48 h, H/R-treated H9c2 cells (1×10^5 cells/well) were seeded in 6-well culture plates respectively and washed twice with cold phosphate buffer saline (PBS). Then they were co-incubated with serum-free culture medium containing 10 μM dichlorofluorescein diacetate (DCFH-DA; Jiancheng, Nanjing, China) for 20 min at room temperature in the dark. Subsequently, samples were collected by a trypsin digestion approach and centrifuged. Then they were resuspended in 100 μl annexin-binding buffer and apoptotic H9c2 cells measured with flow cytometer according to the manufacturer's protocol.

ROS content

ROS was measured by flow cytometry using DCFH-DA. Briefly, after treated by H/R, the negative control and transfected H9c2 cells (for 2 d) were conducted in the same way as apoptosis assay. Then they were centrifuged and resuspended to 500 μl PBS again. The fluorescent intensities were measured using a flow cytometer (Becton Dickinson, Mountain View, USA) with 488 nm excitation and 521 nm emission.

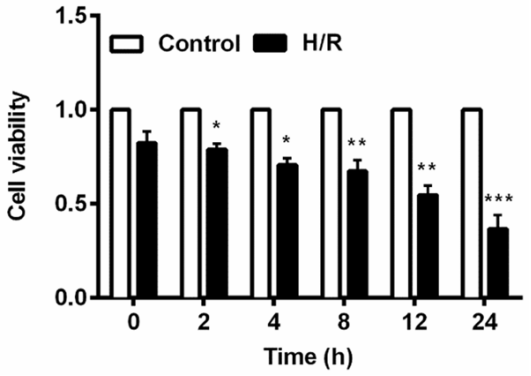

Figure 1. The H9c2 cell viability after different durations of H/R treatment. H/R, Hypoxia-Reoxygenation. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

Quantitative reverse-transcription polymerase chain reaction (qRT-PCR)

Total RNA of transfected H9c2 and control cells was isolated respectively using Trizol reagent (Invitrogen, CA, USA) and treated with DNaseI (Promega, Madison, USA). A total of 2 μg RNA was used to synthesize poly-oligo (dT) primed complementary DNA (cDNA) with the RevertAid H Minus First strand Cdna Synthesis Kit (Thermo Fischer Scientific Inc., MA, USA). QRT-PCR reactions for Rac1, Notch 1, Notch 2, Jagged 1 (JAG1), B-cell lymphoma-2 (Bcl-2), Caspase-3 were performed using RiboMAX Large Scale RNA Production System T7 (Promega, Karlsruhe, Germany). The primer sequences were: Rac1 forward primer: 5'ATGCAGGCCATCAAGTATGTGGTG'3, reverse primer: 5'TTACAACAGCAGG CATTTTCTCTTCC'3 [8]; Notch 1 forward primer: 5'GATGACCTGGGGCA AGTCCG'3, reverse primer: 5'TGCGCTCCTGTGCGATGT'3; Notch 2 forward primer: 5'TGACTGT TCCCTCACTATGG'3, reverse primer: 5'CACGTCTTGC TATTCCTCTG'3; JAG1 forward primer: 5'ACGACCCCCTGTGAAGTGAT'3, reverse primer: 5'TCCCGACTGACTCTTGCACT'3 [11]; Bcl-2 forward primer: 5'ATCGCCCTGTGGATGACTGAG'3, reverse primer: 5'CAGCCAGGAGAAATC AAACAGAGG'3 [12]; Caspase-3 forward primer: 5'ATGGACAACAACGAAA CCTC'3, reverse primer: 5'TTAGTGATAAAAGTACAGTTCTT'3 [13]; GAPDH forward primer: 5'GCACCGTCAAGGCTGAGAAC'3, reverse primer: 5'TGGTG AAGACGCCAGTGGA'3.

Western blot analysis

The protein used for western blotting was extracted from H9c2 cells using RIPA lysis buffer (Beyotime Biotechnology, Shanghai, China) with protease inhibitors (Applygen Technologies Inc., Beijing, China). The total amount of proteins was quantified by Bicinchoninic Acid (BCA) Protein Assay Kit (Pierce, Appleton, USA). A Bio-Rad Bis-Tris Gel system was employed to establish the western blot system, in which GAPDH (Abcam, Cambridge, United Kingdom) was regarded as an internal control. Primary antibodies Rac1 (ab33186), Notch 1 (ab52-301), Notch 2 (ab201616), JAG 1 (ab89663), Bcl-2 (ab59348), Pro-Caspase-3 (ab90437) and Cleaved-Caspase-3 (ab32042) were obtained from Abcam (Cambridge, United Kingdom). After incubation with the membrane at 4°C overnight, secondary antibodies were marked by horseradish peroxidase for 1 h at 37°C. Then the polyvinylidenefluoride (PVDF) membrane carrying blots and antibodies was transferred into the Bio-Rad ChemiDoc™ XRS system. Images were developed and photographed using Image Lab statistical software (Bio-Rad, Richmond, USA).

Statistical analysis

The results were presented as mean ± standard deviation (SD). Values were performed by one-way analysis of variance (ANOVA) with SPSS 19.0 software (SPSS, IL, USA). A P value of < 0.05 was considered significant.

Results

Determination of H/R treatment duration

The anoxic H9c2 cells were reoxygenated under normoxic conditions for 2, 4, 8, 12 and 24 h, respectively. We sought to determine the H/R treatment duration via measuring H9c2 cell viability after different durations of reoxygenation. The H9c2 cell viability varied with different H/R treatment durations as compared to cells without H/R treatment (**Figure 1**). The cell viability was strikingly reduced by H/R treatment after 2, 4 h ($P < 0.05$), 8, 12 h ($P < 0.01$) and 24 h ($P < 0.001$), respectively. Reoxygenation treatment provoked decline in the H9c2 cell viability in a time-dependent manner and about 54.67% viability was identified for 12 h. Thereby, 12 h reoxygenation duration was determined among H/R-treated H9c2 cardiomyocytes in further studies [14].

Transfection efficiency of Rac1 overexpression and knockdown in H9c2 cells

The pc-Rac1/pcDNA3.1 or siRac1/siRNA were transformed to H/R-treated H9c2 cells respec-

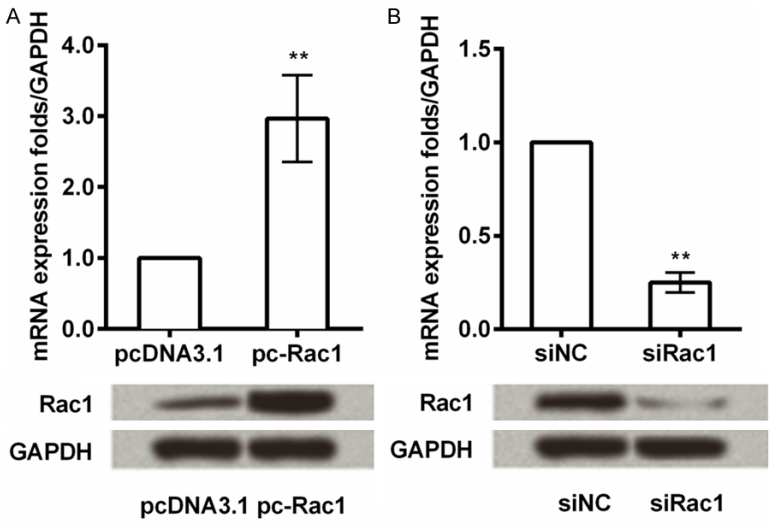

Figure 2. The expressions of ectopic Rac1 in H/R-treated H9c2 cardiomyocytes. A. The expressions of Rac1 overexpression in H/R-treated H9c2 cardiomyocytes; B. The expressions of Rac1 knockdown in H/R-treated H9c2 cardiomyocytes. GAPDH, glyceraldehyde-3-phosphate dehydrogenase; si, small interfering; NC, negative control; Rac1, Ras-related C3 botulinum toxin substrate 1. $^{**}P < 0.01$.

tively in order to examine the effects of ectopic Rac1 on H9c2 cardiomyocytes. **Figure 2A** and **2B** reflected that relative expression of overexpressing Rac1 was significantly higher than the NC ($P < 0.01$) and silencing Rac1 was statistically lower than the NC ($P < 0.01$). Accordingly, it was concluded that overexpression and silence of Rac1 were effective and these transferred H9c2 cells could be used in the further study.

The effects of Rac1 overexpression and knockdown on cell viability, ROS content and apoptosis in H9c2 cells subjected to H/R

To functionally characterize the impact of aberrant Rac1 on H/R-treated H9c2 cells, the effects of Rac1 overexpression and knockdown on cell viability, ROS content and apoptosis were measured in transformed cells. Overexpressing Rac1 observably diminished cell viability and silencing Rac1 accelerated it compared to the NC ($P < 0.05$, **Figure 3A**). Additionally, ROS content in H9c2 cardiomyocytes were increased by overexpressing Rac1 ($P < 0.05$) and decreased by silencing Rac1 ($P < 0.01$) as shown in **Figure 3B**. The flow cytometry assay revealed that Rac1 overexpression raised percentage of apoptosis cells from 26.2% (NC) to 34.9% ($P < 0.05$) and Rac1 suppression dropped it from 27.0% (NC) to 12.7% ($P < 0.05$) in H9c2 cells (**Figure 3C** and **3D**). Thus, Rac1 overexpression inhibited H9c2 cell viability, promoted ROS content and apoptosis, while Rac1 suppression possessed opposite effects on H9c2 cells.

The effects of Rac1 overexpression and knockdown on Notch signaling pathway

The Notch signaling pathway is responsible for cell fate determination and development in mature tissue, which attracts a host of interests in human diseases [15, 16]. We investigated the effects of Rac1 overexpression and knockdown on the expressions of Notch family in H/R-treated H9c2 cells. The expressions of Notch 1, Notch 2 and JAG1 mRNA and proteins were higher by Rac1 overexpression and lower by Rac1 suppression as compared with control cells ($P < 0.05$ or $P < 0.01$; **Figure 4A** and **4B**). It indicated that silencing Rac1 inhibited the Notch signaling pathway in H/R-treated H9c2 cells. Moreover, emerging evidence has revealed that the best recognized biochemical hallmark of apoptosis is the anti-apoptotic Bcl-2 and activation of cysteine proteases (caspases) [17]. We examined the anti-apoptotic protein Bcl-2 was down-regulated by overexpressing Rac1 ($P < 0.01$) and up-regulated by silencing Rac1 ($P < 0.05$). Further, the activated Caspase-3 was increased by overexpressing Rac1 ($P < 0.01$) and decreased by silencing Rac1 ($P < 0.05$). These findings claimed that silencing Rac1 inhibited H9c2 cell apoptosis in H/R injury condition.

Discussion

The current study makes a primary research on the regulating role and mechanism of Rac1 in MIRI. H9c2 cardiomyocytes were exposed to hypoxia for 6 h and reoxygenation for 12 h to establish the MIRI process model. Then we observed the effects of overexpressing and silencing Rac1 on cell growth, apoptosis and ROS content of H/R-induced H9c2 cells. Silencing Rac1 could remarkably raise cell via-

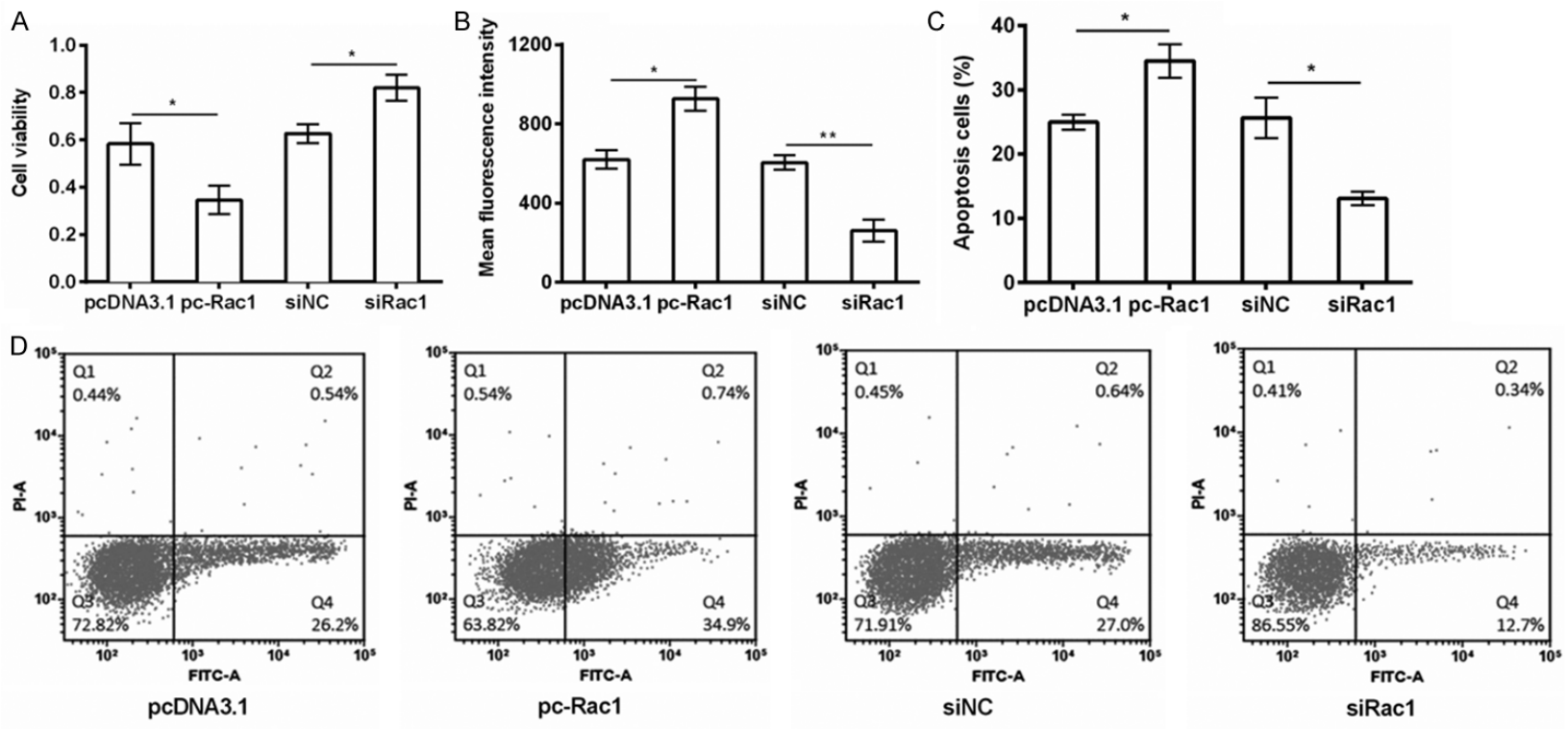

Figure 3. The effects of Rac1 overexpression and knockdown on cell viability, ROS content and apoptosis in H/R-treated H9c2 cardiomyocytes. A. The effects of Rac1 overexpression and knockdown on cell viability in H9c2 cells; B. The effects of Rac1 overexpression and knockdown on ROS content in H9c2 cells; C. The effects of Rac1 overexpression and knockdown on apoptosis in H9c2 cells; D. Apoptotic H9c2 cells were identified and quantified by flow cytometry. si, small interfering; NC, negative control; Rac1, Ras-related C3 botulinum toxin substrate 1. *$P < 0.05$; **$P < 0.01$.

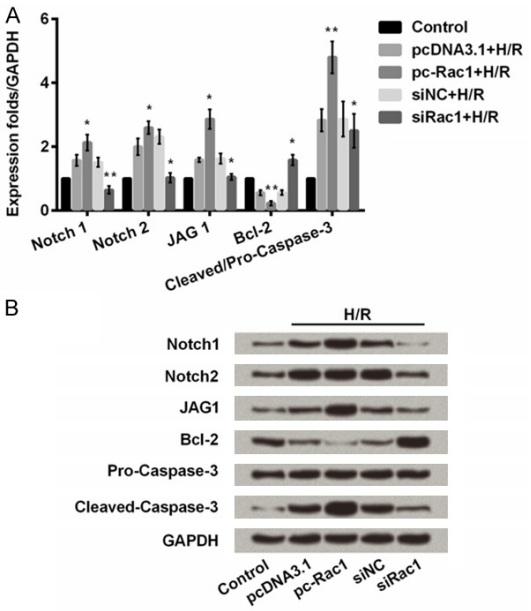

Figure 4. The effects of Rac1 overexpression and knockdown on the Notch family and apoptosis indicators. A. The effects of aberrant Rac1 on the expressions of Notch family and apoptosis indicators mRNAs; B. The effects of aberrant Rac1 on the expressions of Notch family and apoptosis indicators proteins; JAG1, Jagged 1; Bcl-2, B-cell lymphoma-2; si, small interfering; NC, negative control; Rac1, Ras-related C3 botulinum toxin substrate 1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; H/R, Hypoxia-Reoxygenation. $*P < 0.05$; $**P < 0.01$.

bility, suppress apoptosis and scavenge ROS by inhibiting Notch signaling pathway, while overexpressing Rac1 had opposite effects on H/R-treated H9c2 cardiomyocytes. Therefore, these findings hinted the importance of Rac1 in regulating tissue damage and responding oxidant stress resulting from IRI and made the suppression of Rac1 an attractive candidate for MIRI therapeutic target.

Appreciable literatures on cardioprotection are undertaken to construct cell culture models, in which H/R is applied to simulate IRI, leading to developments of preclinical results to clinical therapy [7, 18, 19]. H/R is considered to expedite oxidant products and cause cell apoptosis as well as activation of inflammatory responses [20], providing a clue for our MIRI model. Our data revealed that Rac1 suppression promoted H9c2 cell viability and inhibited apoptosis, indicating that silencing Rac1 protected H9c2 cardiomyocyte growth against H/R injury.

There is extensive evidence confirming that following reperfusion of the cardiac muscle, there is a rapid augment of ROS content, inflicting increased lipid peroxidation, protein nitrosylation and DNA damage [9, 21]. ROS, comprising H_2O_2, O^{2-} and OH-, is considered to be the main indicator of oxidative stress [22]. Moreover, superfluous ROS restrains the action of endogenous antioxidant enzymes and aggravates lipid peroxidation as well as causes cell damage [23-25]. Our data revealed that silencing Rac1 could strikingly scavenge ROS in H/R-treated H9c2 cells, which was corroborated by an anterior report that the administration of a Rac GTPase inhibitor attenuated superoxide production in rats at 3 h after cerebral stroke [9]. Thus, suppression of Rac1 might possess a protective effect in scavenging ROS by preventing the cardiac muscle from lipid peroxidation in H/R-induced cardiomyocytes.

The past decades have seen a multitude of developments in the Notch signaling pathway. Notch signaling, including four receptors (Notch 1-4) and five ligands (Deltal, Deltal 3-4 and JAG 1-2), is an evolutionarily conserved cell fate regulator which serves as a key driver regulating homeostasis, proliferation, survival, apoptosis and neurogenesis in diverse embryonic and tissue systems [11, 26, 27]. Nakhai *et al.* claimed that the inactivation of Notch 1 and Notch 2 repressed epithelial cells growth in the early pancreatic embryonic development stage [28]. Besides, the suppression of Notch1 moderately inhibits invasion of prostate cancer (PC) cells, which was suggestive of a therapeutic approach against PC [29]. In IRI research, emerging studies point out IRI could induce the activation of Notch signaling pathway. Notch signaling protected non-parenchymal cells from IRI by decreasing ROS content [30] and the inhibitor of the Notch-activating enzyme lessened the proliferation of reactive astrocyte [31]. Based upon the previous facts, we investigated the effects of aberrant Rac1 on the expressions of Notch family to characterize endogenous Notch signaling activation in H9c2 cardiomyocytes induced by H/R injury. The results gave strength to that H/R treatment leaded to the activation of Notch signaling pathway and Rac1 effects might be by regulating downstream Notch signaling pathway. Consequently, silencing Rac1 alleviated H/R injury by inactivation of Notch pathway in H9c2 cells.

Dysfunction of apoptosis is involved in numerous pathological processes in human, including

MIRI. In previous MIRI model, the apoptotic events were evidenced in cardiomyocytes in response to hypoxia and reoxygenation injury [14, 32]. It is well known that the execution of cell apoptosis processes is adjusted by the Bcl-2 and caspase families of proteins. The curcumin improved apoptosis in human cervical cancer cells by increasing cleavage of Caspase-3 [33]. Our data validated that H/R-treated H9c2 cell damage was related to apoptosis via the restraint of Bcl-2 and activation of Caspase-3. Hence, silencing Rac1 suppressed cell apoptosis as evidenced by the increase in Bcl-2 and decrease in cleaved Caspase-3. These results indicated that silencing Rac1 protected H9c2 cardiomyocytes from H/R-induced cell apoptosis.

Collectively, our findings suggest that Rac1 acts as a key mediator of cardiomyocytes survival and responding oxidant stress after H/R injury. This study provides novel insight into the injury suppressive capacity of silencing Rac1 by inactivation of Notch signaling pathway, which holds great clinical potential in the cardioprotection against MIRI.

Disclosure of conflict of interest

None.

Address correspondence to: Bo Yu, Department of Cardiology, The 2nd Affiliated Hospital of Harbin Medical University, Key Laboratory of Myocardial Ischemia Mechanism and Treatment Ministry of Education, No. 246, Xuefu Street, Nangang District, Harbin 150086, China. E-mail: yubodr@126.com

References

[1] Gao S, Zhu Y, Li H, Xia Z, Wu Q, Yao S, Wang T and Yuan S. Remote ischemic postconditioning protects against renal ischemia/reperfusion injury by activation of T-LAK-cell-originated protein kinase (TOPK)/PTEN/Akt signaling pathway mediated anti-oxidation and anti-inflammation. Int Immunopharmacol 2016; 38: 395-401.

[2] Turkmen S, Cekic Gonenc O, Karaca Y, Mentese A, Demir S, Beyhun E, Sahin A, Gunduz A, Yulug E and Turedi S. The effect of ethyl pyruvate and N-acetylcysteine on ischemia-reperfusion injury in an experimental model of ischemic stroke. Am J Emerg Med 2016; 34: 1804-1807.

[3] Tang B, Wang Z, Qi G, Yuan S, Yu S, Li B, Wei Y, Huang Q, Zhai R and He S. MicroRNA-155 deficiency attenuates ischemia-reperfusion injury after liver transplantation in mice. Transpl Int 2015; 28: 751-760.

[4] Varga ZV, Zvara A, Farago N, Kocsis GF, Pipicz M, Gaspar R, Bencsik P, Gorbe A, Csonka C, Puskas LG, Thum T, Csont T and Ferdinandy P. MicroRNAs associated with ischemia-reperfusion injury and cardioprotection by ischemic pre- and postconditioning: protectomiRs. Am J Physiol Heart Circ Physiol 2014; 307: H216-227.

[5] Eltzschig HK and Eckle T. Ischemia and reperfusion--from mechanism to translation. Nat Med 2011; 17: 1391-1401.

[6] Lum JJ, DeBerardinis RJ and Thompson CB. Autophagy in metazoans: cell survival in the land of plenty. Nat Rev Mol Cell Biol 2005; 6: 439-448.

[7] Cao X, Chen A, Yang P, Song X, Liu Y, Li Z, Wang X, Wang L and Li Y. Alpha-lipoic acid protects cardiomyocytes against hypoxia/reoxygenation injury by inhibiting autophagy. Biochem Biophys Res Commun 2013; 441: 935-940.

[8] Schnelzer A, Prechtel D, Knaus U, Dehne K, Gerhard M, Graeff H, Harbeck N, Schmitt M and Lengyel E. Rac1 in human breast cancer: overexpression, mutation analysis, and characterization of a new isoform, Rac1b. Oncogene 2000; 19: 3013-3020.

[9] Raz L, Zhang QG, Zhou CF, Han D, Gulati P, Yang LC, Yang F, Wang RM and Brann DW. Role of Rac1 GTPase in NADPH oxidase activation and cognitive impairment following cerebral ischemia in the rat. PLoS One 2010; 5: e12606.

[10] Xu Z, Dong D, Chen X, Huang H and Wen S. MicroRNA-381 Negatively Regulates TLR4 Signaling in A549 Cells in Response to LPS Stimulation. Biomed Res Int 2015; 2015: 849475.

[11] Liu W, Jin G, Long C, Zhou X, Tang Y, Huang S, Kuang X, Wu L, Zhang Q and Shen H. Blockage of Notch signaling inhibits the migration and proliferation of retinal pigment epithelial cells. ScientificWorldJournal 2013; 2013: 178708.

[12] Porichi O, Nikolaidou ME, Apostolaki A, Tserkezoglou A, Arnogiannaki N, Kassanos D, Margaritis L and Panotopoulou E. BCL-2, BAX and P53 expression profiles in endometrial carcinoma as studied by real-time PCR and immunohistochemistry. Anticancer Res 2009; 29: 3977-3982.

[13] Zhu ZH, Wan HT and Li JH. Chuanxiongzine-astragaloside IV decreases IL-1beta and Caspase-3 gene expressions in rat brain damaged by cerebral ischemia/reperfusion: a study of real-time quantitative PCR assay. Sheng Li Xue Bao 2011; 63: 272-280.

[14] Sun J, Sun G, Meng X, Wang H, Wang M, Qin M, Ma B, Luo Y, Yu Y, Chen R, Ai Q and Sun X. Ginsenoside RK3 Prevents Hypoxia-Reoxygenation Induced Apoptosis in H9c2 Cardiomyocytes via AKT and MAPK Pathway. Evid Based Complement Alternat Med 2013; 2013: 690190.

[15] Takebe N, Nguyen D and Yang SX. Targeting notch signaling pathway in cancer: clinical development advances and challenges. Pharmacol Ther 2014; 141: 140-149.

[16] Yabuuchi S, Pai SG, Campbell NR, de Wilde RF, De Oliveira E, Korangath P, Streppel MM, Rasheed ZA, Hidalgo M, Maitra A and Rajeshkumar NV. Notch signaling pathway targeted therapy suppresses tumor progression and metastatic spread in pancreatic cancer. Cancer Lett 2013; 335: 41-51.

[17] Choudhary GS, Al-Harbi S and Almasan A. Caspase-3 activation is a critical determinant of genotoxic stress-induced apoptosis. Methods Mol Biol 2015; 1219: 1-9.

[18] Heusch G, Kleinbongard P and Skyschally A. Myocardial infarction and coronary microvascular obstruction: an intimate, but complicated relationship. Basic Res Cardiol 2013; 108: 380.

[19] Matsui Y, Takagi H, Qu X, Abdellatif M, Sakoda H, Asano T, Levine B and Sadoshima J. Distinct roles of autophagy in the heart during ischemia and reperfusion: roles of AMP-activated protein kinase and Beclin 1 in mediating autophagy. Circ Res 2007; 100: 914-922.

[20] Gustafsson AB and Gottlieb RA. Eat your heart out: Role of autophagy in myocardial ischemia/reperfusion. Autophagy 2008; 4: 416-421.

[21] Sun M, Li M, Huang Q, Han F, Gu JH, Xie J, Han R, Qin ZH and Zhou Z. Ischemia/reperfusion-induced upregulation of TIGAR in brain is mediated by SP1 and modulated by ROS and hormones involved in glucose metabolism. Neurochem Int 2015; 80: 99-109.

[22] Zhang X, Sun CY, Zhang YB, Guo HZ, Feng XX, Peng SZ, Yuan J, Zheng RB, Chen WP, Su ZR and Huang XD. Kegan Liyan oral liquid ameliorates lipopolysaccharide-induced acute lung injury through inhibition of TLR4-mediated NF-kappaB signaling pathway and MMP-9 expression. J Ethnopharmacol 2016; 186: 91-102.

[23] Comhair SA and Erzurum SC. Antioxidant responses to oxidant-mediated lung diseases. Am J Physiol Lung Cell Mol Physiol 2002; 283: L246-255.

[24] Li J, Wang F, Xia Y, Dai W, Chen K, Li S, Liu T, Zheng Y, Wang J, Lu W, Zhou Y, Yin Q, Lu J, Zhou Y and Guo C. Astaxanthin Pretreatment Attenuates Hepatic Ischemia Reperfusion-Induced Apoptosis and Autophagy via the ROS/MAPK Pathway in Mice. Mar Drugs 2015; 13: 3368-3387.

[25] Fatemikia H, Ketabchi F, Karimi Z and Moosavi SM. Distant effects of unilateral renal ischemia/reperfusion on contralateral kidney but not lung in rats: the roles of ROS and iNOS. Can J Physiol Pharmacol 2016; 94: 477-487.

[26] Gonsalves FC and Weisblat DA. MAPK regulation of maternal and zygotic Notch transcript stability in early development. Proc Natl Acad Sci U S A 2007; 104: 531-536.

[27] Leong KG and Karsan A. Recent insights into the role of Notch signaling in tumorigenesis. Blood 2006; 107: 2223-2233.

[28] Nakhai H, Siveke JT, Klein B, Mendoza-Torres L, Mazur PK, Algul H, Radtke F, Strobl L, Zimber-Strobl U and Schmid RM. Conditional ablation of Notch signaling in pancreatic development. Development 2008; 135: 2757-2765.

[29] Bin Hafeez B, Adhami VM, Asim M, Siddiqui IA, Bhat KM, Zhong W, Saleem M, Din M, Setaluri V and Mukhtar H. Targeted knockdown of Notch 1 inhibits invasion of human prostate cancer cells concomitant with inhibition of matrix metalloproteinase-9 and urokinase plasminogen activator. Clin Cancer Res 2009; 15: 452-459.

[30] Yu HC, Bai L, Yue SQ, Wang DS, Wang L, Han H and Dou KF. Notch signal protects non-parenchymal cells from ischemia/reperfusion injury in vitro by repressing ROS. Ann Hepatol 2013; 12: 815-821.

[31] Marumo T, Takagi Y, Muraki K, Hashimoto N, Miyamoto S and Tanigaki K. Notch signaling regulates nucleocytoplasmic Olig2 translocation in reactive astrocytes differentiation after ischemic stroke. Neurosci Res 2013; 75: 204-209.

[32] Namura S, Zhu J, Fink K, Endres M, Srinivasan A, Tomaselli KJ, Yuan J and Moskowitz MA. Activation and cleavage of caspase-3 in apoptosis induced by experimental cerebral ischemia. J Neurosci 1998; 18: 3659-3668.

[33] Dang YP, Yuan XY, Tian R, Li DG and Liu W. Curcumin improves the paclitaxel-induced apoptosis of HPV-positive human cervical cancer cells via the NF-kappaB-p53-caspase-3 pathway. Exp Ther Med 2015; 9: 1470-1476.