European Review for Medical and Pharmacological Sciences 2018; 22: 6475-6484

Remote ischemic postconditioning alleviates myocardial ischemia/reperfusion injury by up-regulating ALDH2

Z.-X. ZHANG[1], H. LI[1], J.-S. HE[2], H.-J. CHU[3], X.-T. ZHANG[2], L. YIN[1]

[1]Emergency Department, The Affiliated Hospital of Qingdao University, Qingdao, Shandong, China
[2]Department of Anesthesiology, The Affiliated Hospital of Qingdao University, Qingdao, Shandong, China
[3]Department of Gynecology, The Affiliated Hospital of Qingdao University, Qingdao, Shandong, China

Abstract. – OBJECTIVE: The myocardial ischemia/reperfusion (I/R) injury is a significant challenge, and the clinical significance of remote ischemic postconditioning (RIPostC) in cardioprotection has been confirmed. However, the molecular mechanism remains unclear. We aimed to explore the regulatory mechanism of RIPostC in myocardial I/R.

MATERIALS AND METHODS: A mouse model of myocardial I/R injury and cell model of oxygen-glucose deprivation (OGD)/re-oxygenation (OGD/R) injury were constructed. Infarct size was measured by Evans blue dye staining and TTC staining. mRNA and protein expression levels of aldehyde dehydrogenase 2 (ALDH2) were determined by RT-qPCR and Western blot analysis, respectively. Cell viability, p53 expression, apoptotic cells, expression of proteins related to apoptosis, and reactive oxygen species (ROS) generation were evaluated by CCK-8 assay, Western blot analysis, flow cytometry assay, Western blot analysis, and DCFH-DA staining, respectively. ALDH2 in H9c2 cells was knocked down, and its effects on cells treated with OGD/R and RIPostC were tested. How RIPostC affected ALDH2 expression was finally studied.

RESULTS: RIPostC reduced infarct size in mice and attenuated OGD/R-induced H9c2 cell injury. Myocardial I/R-induced down-regulation of ALDH2 was abrogated by RIPostC. Moreover, the effects of RIPostC on OGD/R-treated H9c2 cells were significantly reversed by ALDH2 silence. Finally, we found RIPostC-induced up-regulation of ALDH2 in OGD/R-treated cells could be bated by activation of PI3K and/or mTOR.

CONCLUSIONS: RIPostC exerted cardioprotective role against myocardial I/R both *in vivo* and *in vitro*. Up-regulation of ALDH2 might be a reason for the cardioprotection, and RIPostC might regulate ALDH2 expression via the PI3K/mTOR pathway.

Key Words:
Myocardial ischemia/reperfusion, RIPostC, H9c2 cells, ALDH2, PI3K/mTOR.

Introduction

Ischemic heart disease is the leading cause of mortality in the industrialized societies, causing a huge burden of morbidity[1]. Prolonged ischemia resulting from occlusion caused by atherosclerotic plaque-induced clotting cascade may lead to ischemic cardiomyopathy, myocardial cell loss and fibrosis in the shape of interstitial collagen deposition[2-4]. Although rapid reperfusion is the current standard treatment for myocardial ischemia, reperfusion itself has the potential for additional lethal injury[5,6]. Myocardial ischemia/reperfusion (I/R) injury is a significant challenge which needs more effective measures to improve the outcome.

Remote ischemic preconditioning (RIPC) is a non-invasive, feasible and low-cost strategy to protect organs against irreversible I/R injury by means of repetitive and short induction of I/R in a distant organ prior to ischemia[7,8]. Many experimental studies[9-11] have proved the protective role of RIPC against myocardial I/R injury. However, the clinical application of RIPC is limited since RIPC should be conducted before ischemia. Different from RIPC, remote ischemic postconditioning (RIPostC) refers to repeated I/R following a prior I/R injury in another organs[12]. A previous study[13] has provided support for the protection of RIPostC against myocardial I/R injury following the primary percutaneous coronary intervention. Meanwhile, limb RIPostC is reported as a power-

Corresponding Author: Lei Yin, MD; e-mail: leiyin0122@sina.com

ful non-drug cardioprotective strategy for prolonged myocardial ischemia[14]. Since RIPostC can be carried out after ischemia, which is the case in most patients with coronary events, RIPostC is more applicable in clinic than RIPC[15]. The clinical significance of RIPostC in cardioprotection has been confirmed in 2003[16]. However, the molecular mechanism underlying RIPostC in cardioprotection remains poorly understood.

Aldehyde dehydrogenase 2 (ALDH2) is a mitochondrial enzyme, acting a key metabolic function in the oxidation and detoxification of reactive aldehydes in diverse cell types[17]. Mitochondria are an important source of reactive oxygen species (ROS) implicated in I/R injury[18]. What's more, ALDH2 is critical in the ethanol detoxification pathway, and ALDH2 deficiency is related to elevated ROS level[19]. Considering that increased generation of ROS initiates perturbation of the cellular redox balance leading to cell apoptosis, ALDH2 is critical in myocardial I/R-induced cellular and tissue damages[20].

In this study, we focused on the correlation between RIPostC and ALDH2 expression, aiming to figure out the molecular mechanism of RIPostC in cardioprotection. Accordingly, the protective roles of RIPostC in mice with myocardial I/R and H9c2 cells with oxygen-glucose deprivation (OGD)/re-oxygenation (OGD/R) were verified. Furthermore, we also preliminarily explored the possible regulatory mechanism of RIPostC in ALDH2 expression.

Materials and Methods

Animals

A total of 30 male C57BL/6 mice (ages 7-8 weeks) were obtained from Beijing Vital River Laboratory Animal Technology Corporation (Beijing, China). Mice were maintained under a 12 h light/dark cycle (lights on at 7:00 am) in cages with water ad libitum. Experimental protocols were approved by the Ethics Committee of the Affiliated Hospital of Qingdao University. We tried our best to minimize the number and the pain of mice.

In Vivo Experiments

Mice were randomly assigned into three groups (n = 10 per group) including the Sham group, I/R group, and I/R + RIPostC group. Mice in the I/R group were intraperitoneally injected with pentobarbital sodium (70 mg/kg), and the anesthesia was maintained via supplemental injection of pentobarbital sodium (30 mg/kg) as needed. Then, a tracheotomy was performed, and mice were ventilated with a mixed atmosphere consisting of air and oxygen. After a left thoracotomy, an 8-0 silk suture with a slipknot was ligated around the left anterior descending coronary artery (LCA). Successful ischemia which lasted for 45 min was confirmed by the pale color of the ligated area, and reperfusion (2 h) was established through releasing the slipknot. The RIPostC was induced by three cycles of left femoral artery occlusion (5 min) with a microvascular clamp under an operating microscope and reperfusion (5 min), during the reperfusion period. Mice in the Sham group received the same surgical procedures without I/R and RIPostC.

Determination of Infarct Size

After reperfusion, the LCA was re-occluded at the same site, and 2% Evans blue dye solution (0.3 mL) was injected into the abdominal vein to identify the area at risk (AAR; unstained). Non-ischemic part of the myocardium was stained blue. Then, the heart was cut transversely into 1-mm-thick sections after storing at -20°C for 20 min. Subsequently, heart slices were stained in 2,3,5-triphenyltetrazolium chloride solution (TTC; 2%, Sigma-Aldrich, St. Louis, MO, USA) for 20 min at 37°C, and fixed in 4% paraformaldehyde solution overnight. The viable myocardium was stained red whereas the infarcted tissues remained pale. The extent of the area was quantified by computerized planimetry (ImageJ software, National Institutes of Health, Bethesda, MA, USA).

Cell Culture and Treatments

H9c2 cells (American Type Culture Collection, ATCC® CRL-1446™, Manassas, VA, USA) were grown in Dulbecco's Modified Eagle's Medium (DMEM; Gibco-BRL, Grand Island, NY, USA) containing 10 % (v/v) fetal bovine serum (FBS; Gibco-BRL, Grand Island, NY, USA), and a mixture of 1% penicillin and streptomycin at 37°C. Normally, cells were cultured in a humidified incubator containing 95% air and 5% CO_2. However, to stimulate OGD, the culture medium was replaced by DMEM without FBS and glucose, and cells were incubated in a hypoxic incubator containing 94% N_2, 5% CO_2, and 1% O_2 for 3 h. At the end of hypoxia, the culture medium was replaced by complete medium, and cells were cultured under normoxia for 2 h to induce re-oxygenation. For inhibition of PI3K and mTOR, cells were incubated in DMEM containing LY294002 (PI3K

inhibitor, 50 μM, Sigma-Aldrich, St. Louis, MO, USA) or rapamycin (mTOR inhibitor, 50 nM, Sigma-Aldrich, St. Louis, MO, USA) for 0-24 h prior to ischemia.

In Vitro Experiments

Cells were randomly divided into three groups including the control group (cells were kept in normoxic culture for 5 h), the OGD/R group (cells received OGD for 3 h followed by re-oxygenation for 2 h), and the OGD/R + RIPostC group (OGD for 3 h, followed by three cycles of 5 min OGD and 5 min re-oxygenation, then re-oxygenation for 90 min).

Lentiviral Infection

Short-hairpin RNA directed against ALDH2 or a non-targeting sequence was inserted into the pGPU6 plasmid (GenePharma, Shanghai, China), and the reconstructed plasmids were referred to as sh-ALDH2 and sh-NC, respectively. Cells were infected with lentivirus carrying sh-NC or sh-ALDH2, followed by selection with puromycin (Sigma-Aldrich, St. Louis, MO, USA) for 2 days. Then, single colonies were isolated, and the medium was replaced with fresh medium containing puromycin every two days. After one-month selection, cells were isolated for measurements of the ALDH2 expression.

Cell Viability Assay

A Cell Counting Kit-8 (CCK-8; Dojindo Molecular Technologies, Gaithersburg, MD, USA) was used for determination of cell viability. In brief, cells were seeded in 96-well plates at a density of 5.0×10^3 cells/well, and were incubated at 37°C for attachment. After treatments, 10 μL of CCK-8 solution was added into the culture medium and cells were incubated at 37°C. An hour later, the absorbance at 450 nm was measured using a Microplate Reader (Bio-Rad, Hercules, CA, USA).

Apoptosis Assay

The FITC Annexin V/Dead Cell Apoptosis Kit with FITC Annexin V and PI, for flow cytometry (Invitrogen, Carlsbad, CA, USA) was used for identification and quantification of apoptotic cells. In brief, treated cells were harvested and washed by phosphate-buffered saline (PBS). Then, cells suspended in binding buffer were stained by fluorescein isothiocyanate (FITC)-Annexin V and PI according to the manufacturer's instructions. Subsequently, stained cells were subjected into a FACS scan (Beckman Coulter, Fullerton, CA, USA) for flow cytometry analysis. Data were analyzed by using FlowJo software (Tree Star, San Carlos, CA, USA).

ROS Assay

Intracellular ROS level was measured by staining with 2,7-dichlorofluorescein diacetate (DCFH-DA; Nanjing Jiancheng, Nanjing, China). In brief, after treatments, cells were washed twice with PBS and incubated in DMEM containing 10 μM DCFH-DA for 20 min at 37°C in the dark. Thereafter, cells were washed with PBS again, followed by sample collection using trypsin treatment (0.25% trypsin-EDTA). After centrifugation and washing with PBS, cells resuspended in 500 μL PBS were subjected into a FACS can (488 nm excitation, 521 nm emission) for measurement of fluorescent intensity.

Reverse Transcription-Quantitative PCR (RT-qPCR)

Tissues of ventriculus sinister from mice were homogenized in TRIzol reagent (Invitrogen, Carlsbad, CA, USA), and total RNA was isolated according to the supplier's instructions. Isolated RNAs were dissolved in RNase-free water, and the concentration and purity of RNA were determined by spectrophotometric analysis with a NanoDrop™ (Thermo Scientific, Waltham, MA, USA). cDNA was synthesized by using a PrimeScript™ 1st Strand cDNA Synthesis kit (TaKaRa, Dalian, China) following the supplier's protocol. Real Time-PCR reactions were performed according to the instructions of the SYBR® Advantage® qPCR Premix (TaKaRa, Dalian, China). Relative expression fold of ALDH2 mRNA was analyzed according to the $2^{-\Delta\Delta Ct}$ method[21], and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was acted as the internal control. Primer sequences were as follows: mouse ALDH2 (F: 5'-CGTAG ACAAG GCAGT GAA-3', R: 5'-GCGTA ATAGC GGAGA CAT-3'), mouse GADPH (F: 5'-TCTGA CGTGC CGCCT GGAG-3', R: 5'-TCGCA GGAGA CAACC TGGTC-3'), rat ALDH2 (F: 5'-GTAGA CAAGG CAGTG AAGG-3', R: 5'-CAGGT AGGAG ATGAC ATAAG G-3'), rat GAPDH (R: 5'-CTCAA GATTG TCAGC AATGC-3', R: 5'-TTCCA CGATG CCAAA GTTGT-3').

Western Blot Analysis

Tissues of ventriculus sinister from mice and treated H9c2 cells were respectively lysed in RIPA lysis buffer (Beyotime Biotechnology, Shanghai, China) supplemented with 1 mM

PMSF (Beyotime Biotechnology, Shanghai, China). Proteins in the supernatant were quantified using the BCA™ Protein Assay Kit (Pierce, Appleton, WI, USA), and protein samples were electrophoresed by SDS-PAGE. Proteins were then transferred to nitrocellulose membranes, and those membranes were blocked by 5% bovine serum albumin (Solarbio Science and Technology Co., Ltd., Beijing, China). Afterwards, membranes were incubated with primary antibody against ALDH2 (ab108306), p53 (ab131442), B cell lymphoma-2 (Bcl-2; ab196495), Bcl-2-associated X protein (Bax; ab182733), procaspase-3 (ab90437), cleaved caspase-3 (ab49822), phosphatidylinositol-3-kinase (PI3K; ab133595), phospho (p)-PI3K (ab182651), β-actin (ab8227), all from Abcam, Cambridge, MA, USA), mechanistic target of rapamycin (mTOR; #2983), or p-mTOR (#5536, both from Cell Signaling Technology, Danvers, MA, USA), followed by incubation with a goat anti-rabbit horseradish peroxidase-conjugated secondary antibody (ab205718, Abcam). Finally, proteins in the membranes were visualized by using the ECL Western blotting detection reagent (GE Healthcare, Braunschweig, Germany). The intensity of the bands was quantified by ImageJ software.

Statistical Analysis

Experiments were performed in triplicate with three repeats. All data were shown as the mean ± standard deviation (SD). Statistical analysis was performed using GraphPad Prism 5 software (GraphPad, San Diego, CA, USA). The p-values were calculated using the one-way analysis of variance (ANOVA) with Tukey correction or unpaired two-tailed t-test. The differences were considered statistically significant at $p < 0.05$.

Results

RIPostC Reduced Myocardial Infarct Size and Up-Regulated ALDH2 Expression in Mice

After Evans blue staining and TTC staining, infarct size/AAR was calculated. In Figure 1A, RIPostC significantly reduced the infarct size caused by I/R (RIPostC group, 27.9% ± 4.1%, vs. IR group, 56.9% ± 2.9%; $p < 0.01$). RT-qPCR results showed ALDH2 mRNA levels in the I/R group were markedly lower than that in the Sham group ($p < 0.01$), and RIPostC notably up-regulated the ALDH2 mRNA expression relative to the I/R group ($p < 0.05$, Figure 1B). The effects of I/R and RIPostC on ALDH2 protein expression were consistent with that on ALDH2 mRNA expression ($p < 0.05$ or $p < 0.01$, Figure 1C-1D). These results suggested that I/R-induced myocardial infarct size could be reduced by RIPostC, and the ALDH2 expression was up-regulated in mice, simultaneously.

RIPostC Attenuated OGD/R-Induced H9c2 Cell Injury

Effects of RIPostC on OGD/R-induced cell injury were subsequently explored. Compared with the control group, OGD/R induced significant reduction of cell viability ($p < 0.01$, Figure 2A), up-regulation of p53 protein expression ($p < 0.01$, Figure 2B-2C), increase of apoptotic cells ($p < 0.001$, Figure 2D), up-regulation of pro-apoptotic Bax and cleaved caspase-3 as well as down-regulation of anti-apoptotic Bcl-2 (Figure 2E), and enhancement of ROS generation ($p < 0.01$, Figure 2F). In the meantime, we found that those effects of OGD/R on H9c2 cells were all significantly mitigated by RIPostC ($p < 0.05$ or $p < 0.01$). Those results collectively indicated that RIPostC could alleviate OGD/R-induced H9c2 cell injury.

RIPostC Up-Regulated ALDH2 Expression in H9c2 Cells

Expression of ALDH2 in H9c2 cells with OGD/R or OGD/R plus RIPostC was measured. As evidenced in Figure 3, OGD/R prominently down-regulated ALDH2 protein expression relative to the control group ($p < 0.01$), and the down-regulation was bated by RIPostC relative to the OGD/R group ($p < 0.05$). Results suggested the possible involvements of ALDH2 in the protective role of RIPostC against OGD/R injury.

RIPostC Functioned Through Up-Regulating ALDH2 Expression in OGD/R-Treated H9c2 Cells

After lentiviral infection, ALDH2 protein levels in cells transfected with sh-ALDH2 were markedly lower than that in sh-NC-transfected cells ($p < 0.001$, Figure 4), presenting that ALDH2 was successfully silenced after lentiviral infection. Then, transfected and untransfected cells received OGD/R with or without RIPostC, and cell viability, p53 expression, cell apoptosis and ROS generation were all testified. Results showed that effects of RIPostC on OGD/R-treated cells were all remarkably reversed by ALDH2 knockdown, as evidenced by decreased cell viabi-

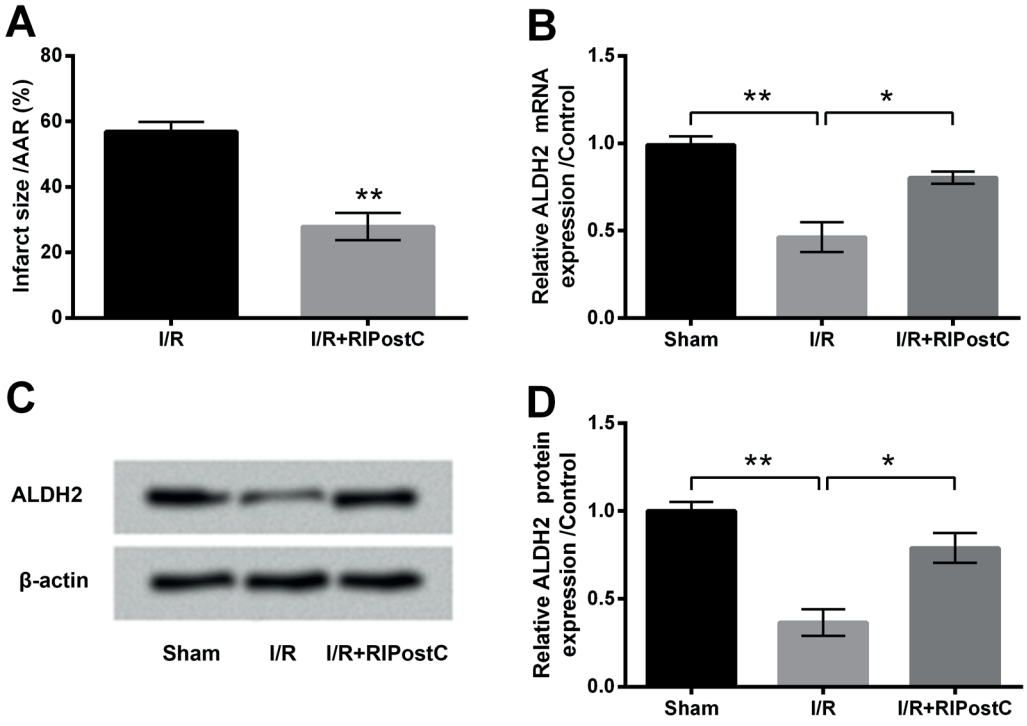

Figure 1. RIPostC reduced infarct size and up-regulated ALDH2 expression in mice with myocardial I/R injury. Mice were assigned into three groups, including the Sham, I/R and I/R + RIPostC groups. ***(A)*** Infarct sized by Evans blue dye staining and TTC staining. ***(B)*** mRNA expression of ALDH2 by RT-qPCR. ***(C-D)*** Protein expression of ALDH2 by Western blot analysis. Data are shown as the mean ± SD of three independent experiments. *, $p < 0.05$; **, $p < 0.01$.

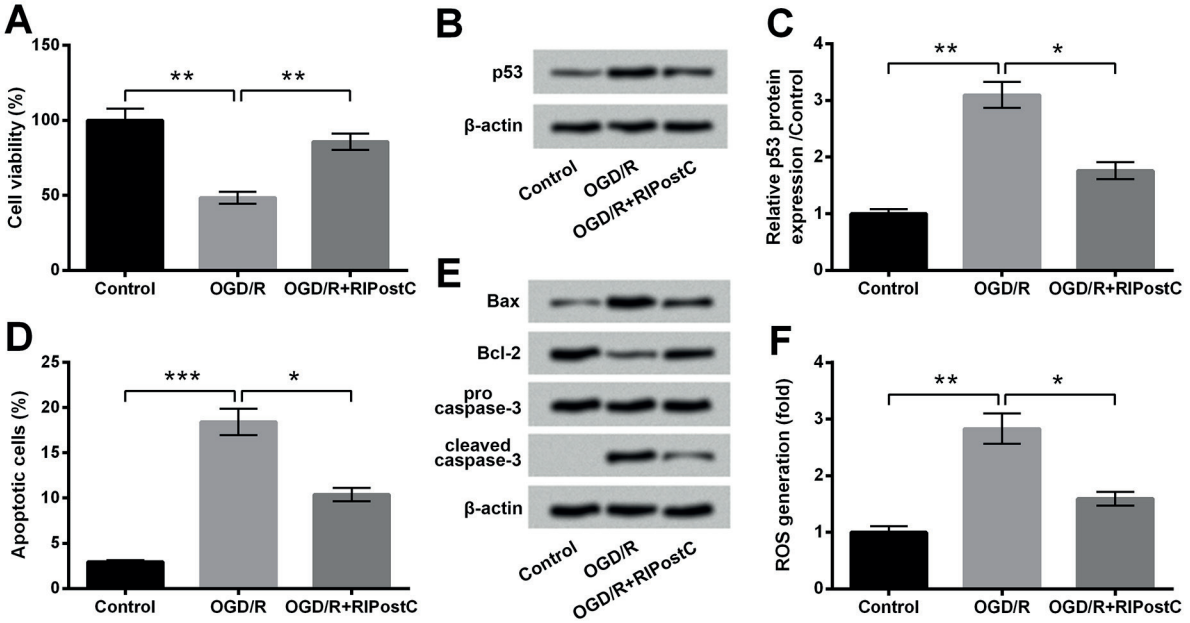

Figure 2. RIPostC alleviated OGD/R-induced H9c2 cell injury. H9c2 cells received OGD/R alone or with RIPostC, and non-treated cells were acted as control. ***(A)*** Cell viability by the CCK-8 assay. ***(B-C)*** Protein expression of p53 by Western blot analysis. ***(D)*** Percentage of apoptotic cells by flow cytometry assay. ***(E)*** Expression of proteins associated with apoptosis by Western blot analysis. ***(F)*** ROS generation by DCFH-DA staining. Data are shown as the mean ± SD of three independent experiments. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

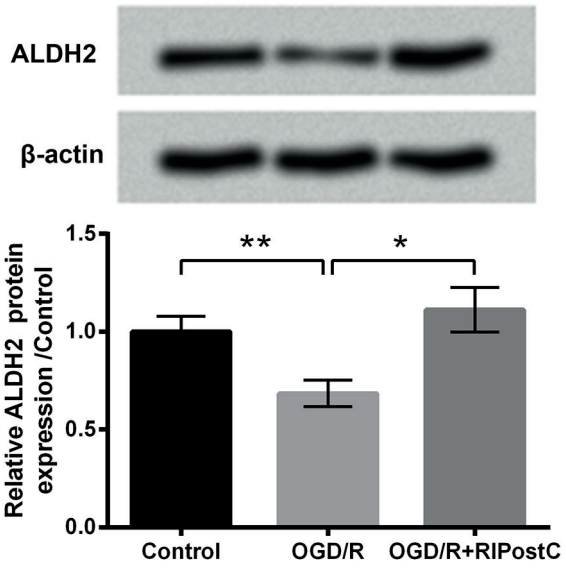

Figure 3. RIPostC up-regulated ALDH2 expression in OGD/R-treated H9c2 cells. H9c2 cells received OGD/R alone or with RIPostC, and non-treated cells were acted as control. Protein expression of ALDH2 was measured by Western blot analysis. Data are shown as the mean ± SD of three independent experiments. *, $p < 0.05$; **, $p < 0.01$.

lity ($p < 0.01$, Figure 5A), elevated p53 protein level ($p < 0.01$, Figure 5B-5C), enhanced apoptotic cells ($p < 0.001$, Figure 5D), up-regulation of Bax and cleaved caspase-3 as well as down-regulation of Bcl-2 (Figure 5E), and increased ROS level ($p < 0.01$, Figure 5F). Results indicated that RIPostC might protect H9c2 cells against OGD/R injury through up-regulating ALDH2 expression.

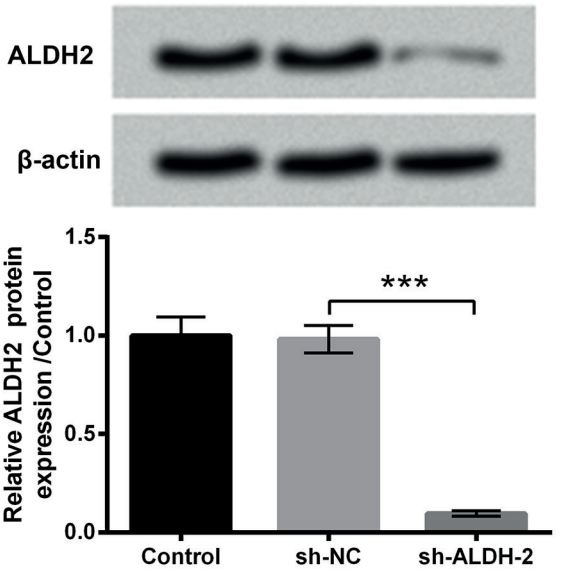

Figure 4. Expression of ALDH2 was silenced successfully in H9c2 cells. H9c2 cells were transfected with sh-NC or sh-ALDH2, and non-treated cells were acted as control. Protein expression of ALDH2 was measured by Western blot analysis. Data are shown as the mean ± SD of three independent experiments. ***, $p < 0.001$.

RIPostC Up-Regulated ALDH2 Via the PI3K/mTOR Pathway in H9c2 Cells

How RIPostC affected the expression of ALDH2 was preliminarily studied. After stimulation with PI3K inhibitor (LY294002), phosphorylated levels of PI3K and mTOR were observably decreased with time (Figure 6A). Under stimulation with mTOR inhibitor (rapamycin), phosphorylated level of mTOR was observably decreased with time while the phosphorylated level of PI3K remained unchangeable (Figure 6B). Afterwards, we found effects of RIPostC on expression of ALDH2 in OGD/R-treated H9c2 cells were significantly abrogated by either LY294002 ($p < 0.01$, Figure 6C) or rapamycin ($p < 0.01$, Figure 6D), when compared to the OGD/R + RIPostC group. Those results suggested that RIPostC might up-regulate ALDH2 expression via the PI3K/mTOR pathway in H9c2 cells.

Discussion

The possible cardioprotective mechanisms of RIPostC have been reported to relate to mitochondrial permeability transition pore, protein kinase C, autophagy in animal I/R models[15]. However, the molecular mechanism remains unclear. In this study, we consolidated the cardioprotective role of RIPostC both *in vivo* and *in vitro*. Interestingly, ALDH2 expression, which was down-regulated in myocardial I/R injury, was markedly up-regulated after RIPostC in mice and H9c2 cells. Subsequently, we found that the ALDH2 silence could reverse the protective effects of RIPostC, indicating that RIPostC might affect H9c2 cells under OGD/R through up-regulating ALDH2. Finally, we identified that RIPostC up-regulated ALDH2 expression via the PI3K/mTOR pathway.

The effects of RIPostC on myocardial I/R injury were explored in mice and H9c2 cells. In mice, we found RIPostC significantly reduced the infarct size. In H9c2 cells which retain many cardiomyocyte phenotypes, OGD/R was utilized to mimic myocardial I/R. Cardiomyocyte apoptosis occurring during I/R injury lead to irreversible damage to cardiac function[22,23]. p53 is a tumor sup-

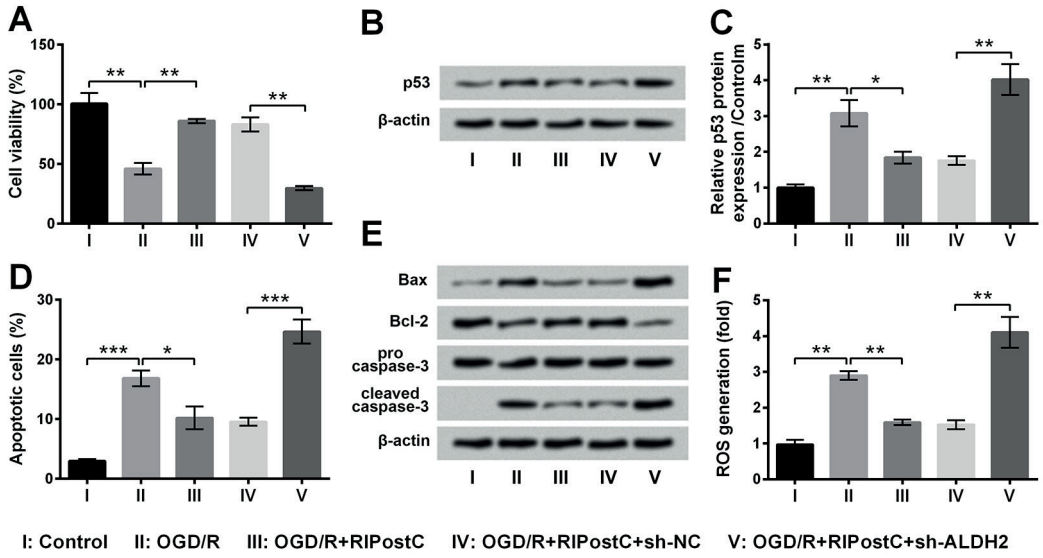

Figure 5. RIPostC affected OGD/R-treated H9c2 cells through up-regulating ALDH2 expression. Transfected or untransfected H9c2 cells received OGD/R alone or with RIPostC, and non-treated cells were acted as control. ***(A)*** Cell viability by the CCK-8 assay. ***(B-C)*** Protein expression of p53 by Western blot analysis. ***(D)*** Percentage of apoptotic cells by flow cytometry assay. ***(E)*** Expression of proteins associated with apoptosis by Western blot analysis. ***(F)*** ROS generation by DCFH-DA staining. Data are shown as the mean ± SD of three independent experiments. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

pressor which may induce apoptosis to eliminate damaged cells[24]. During myocardial I/R, ROS is excessively produced and causes oxidative stress, resulting in severe cellular and tissue damages[25]. A previous literature[26] has elaborated that p53 can promote ROS production due to the positive feedback loop between p53 and ROS level. Therefore, cell viability, apoptosis, p53 expression, and ROS generation were measured to analyze the effects of RIPostC on H9c2 cells with OGD/R injury. Results showed that OGD/R-induced alterations were all attenuated by RIPostC, proving the cardioprotective role of RIPostC *in vitro*.

Despite the cardioprotective role of RIPostC both *in vivo* and *in vitro*, we also discovered for the first time that ALDH2 level, which was down-regulated in mice with myocardial I/R injury and H9c2 cells with OGD/R injury, was elevated by RIPostC. The effects of RIPostC on ALDH2 expression made us hypothesize that there might be a correlation between ALDH2 expression and the cardioprotective role of RIPostC. To verify this conjecture, we constructed a stable cell line, in which ALDH2 was stably silenced. Then, the difference of cell viability, apoptosis, p53 expression, and ROS generation between the cells transfected with sh-NC and the cells transfected with sh-ALDH2 after OGD/R and RIPostC was testified. In the present study, results illustrated that ALDH2 silence could reverse the effects of RIPostC on OGD/R-treated H9c2 cells. In other words, RIPostC-induced up-regulation of ALDH2 might be an explanation for the cardioprotective role of RIPostC. Results of our work were partially consistent with several previous studies. Sun et al[27] have demonstrated that increased ALDH2 expression might reduce cardiomyocyte apoptosis through inhibition of p53. In the report of Leo et al[28], the increasing ALDH2 expression is accompanied by a reduction of ROS level in mice.

The PI3K/AKT pathway acts a critical role in proliferation, migration, adhesion, protein synthesis, survival, etc.[29,30]. The cardioprotective effects of Bauhinia championii flavone against myocardial I/R injury were associated with the activation of the PI3K in rats[31]. Both *in vivo* and *in vitro* protective roles of basic fibroblast growth factor in myocardial I/R injury were attributed to activation of the PI3K and mTOR[32]. Activation of PI3K and mTOR was also identified to be an explanation for the protective role of Transhinone IIA against myocardial I/R injury[33]. Therefore, we explored the association between activation of PI3K/mTOR and ALDH2 expression. Results showed that the RIPostC-induced up-regulation of ALDH2 was abrogated when the PI3K and/or mTOR was inhibited. Hence, we concluded that RIPostC might modulate the ALDH2 expression

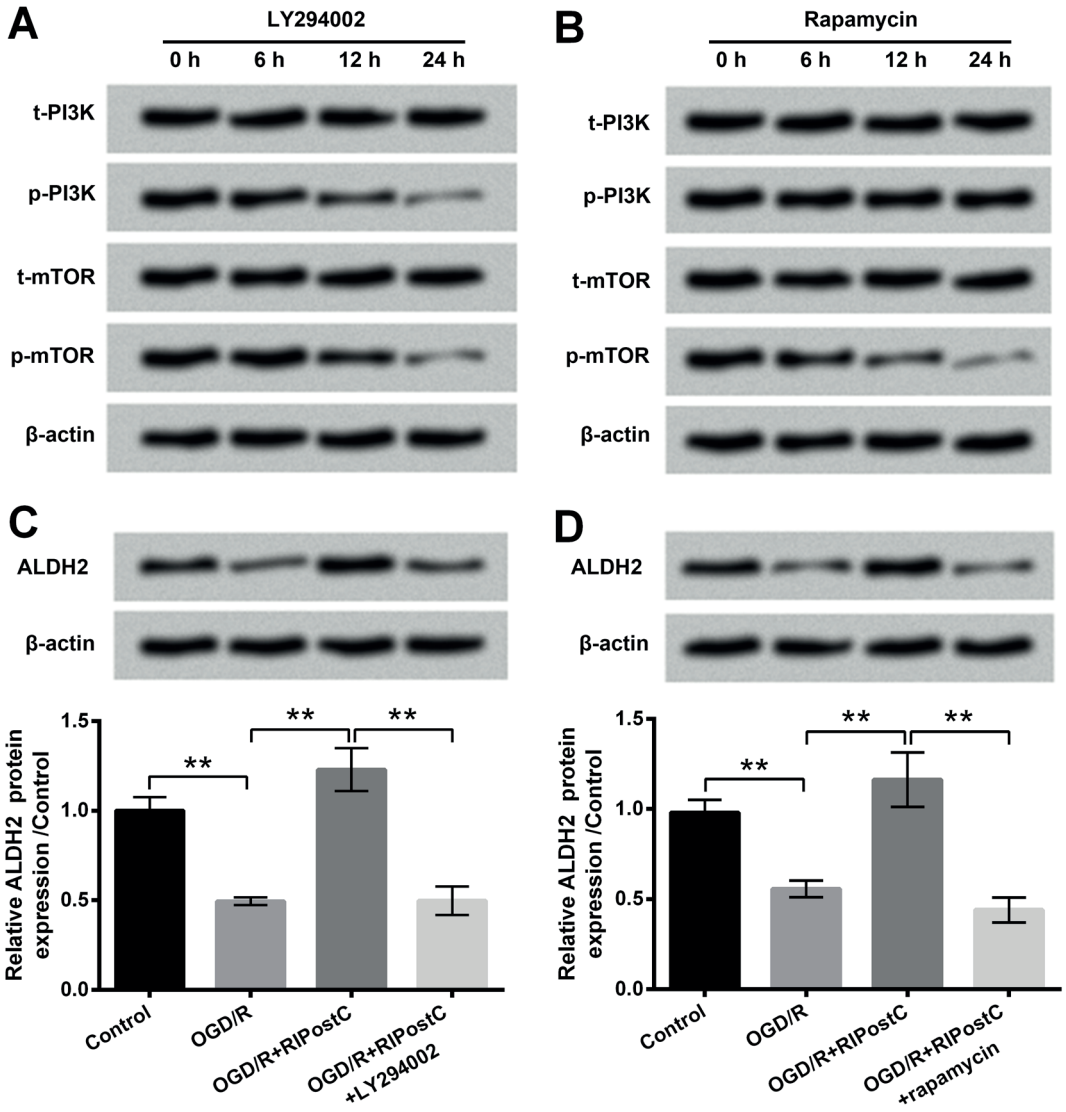

Figure 6. RIPostC up-regulated ALDH2 expression via the PI3K/mTOR pathway. H9c2 cells were stimulated with 50 μM PI3K inhibitor LY294002 ***(A)*** or 50 nM mTOR inhibitor rapamycin ***(B)*** for 0 h, 6 h, 12 h and 24 h. Expression of key kinases in the PI3K/mTOR pathway was tested by Western blot analysis. H9c2 cells were stimulated with LY294002 ***(C)*** or rapamycin ***(D)*** for 24 h, followed by OGD/R and RIPostC. Non-treated cells were acted as control. Expression of ALDH2 was determined by Western blot analysis. Data are shown as the mean ± SD of three independent experiments. **, $p < 0.01$.

through the PI3K/mTOR pathway. More details should be investigated in the future to verify this conclusion.

Conclusions

We found that the cardioprotective role of RIPostC against myocardial I/R injury was consolidated both *in vivo* and *in vitro*. RIPostC induced up-regulation of ALDH2 expression, which might be the reason for the cardioprotection of RIPostC. Moreover, RIPostC might up-regulate ALDH2 expression through activation of the PI3K/mTOR pathway. This investigation not only verified the cardioprotection function of RIPostC, but also provided an innovative regulatory mechanism for RIPostC in myocardial I/R. This study might enrich the theoretical basis for the RIPostC.

Conflict of Interest

The Authors declare that they have no conflict of interest.

References

1) Clark JE, Dudler T, Marber MS, Schwaeble W. Cardioprotection by an anti-MASP-2 antibody in a murine model of myocardial infarction. Open Heart 2018; 5: e000652.
2) Thygesen K, Alpert JS, Jaffe AS, Simoons ML, Chaitman BR, White HD; Writing Group on behalf of the Joint ESC/ACCF/AHA/WHF Task Force for the Universal Definition of Myocardial Infarction. Third universal definition of myocardial infarction. Glob Heart 2012; 7: 275-295.
3) Tsujita K, Kaikita K, Soejima H, Sugiyama S, Ogawa H. [Acute coronary syndrome-initiating factors]. Nihon Rinsho 2010; 68: 607-614.
4) Andalib S, Divani AA, Michel TM, Hoilund-Carlsen PF, Vafaee MS, Gjedde A. Pandora's Box: mitochondrial defects in ischaemic heart disease and stroke. Expert Rev Mol Med 2017; 19: e5.
5) Heusch G. Cardioprotection: chances and challenges of its translation to the clinic. Lancet 2013; 381: 166-175.
6) Tullio F, Angotti C, Perrelli MG, Penna C, Pagliaro P. Redox balance and cardioprotection. Basic Res Cardiol 2013; 108: 392.
7) Siedek F, Persigehl T, Mueller RU, Burst V, Benzing T, Maintz D, Haneder S. Assessing renal changes after remote ischemic preconditioning (RIPC) of the upper extremity using BOLD imaging at 3T. MAGMA 2018; 31: 367-374.
8) Gedik N, Kottenberg E, Thielmann M, Frey UH, Jakob H, Peters J, Heusch G, Kleinbongard P. Potential humoral mediators of remote ischemic preconditioning in patients undergoing surgical coronary revascularization. Sci Rep 2017; 7: 12660.
9) Yellon DM, Ackbarkhan AK, Balgobin V, Bulluck H, Deelchand A, Dhuny MR, Domah N, Gaoneadry D, Jagessur RK, Joonas N, Kowlessur S, Lutchoo J, Nicholas JM, Pauvaday K, Shamloll O, Walker JM, Hausenloy DJ. Remote Ischemic Conditioning Reduces Myocardial Infarct Size in STEMI Patients Treated by Thrombolysis. J Am Coll Cardiol 2015; 65: 2764-2765.
10) Liu Z, Zhao L, Hong D, Gao J. Remote ischaemic preconditioning reduces myocardial ischaemic reperfusion injury in patients with ST-elevation myocardial infarction undergoing primary percutaneous coronary intervention. Acta Cardiol 2016; 71: 596-603.
11) Zhou FZ, Song W, Yin LH, Song ZF, Yang S, Yang FB, Liu JF, Song YG, Zhang HY, Zhang ZM. Effects of remote ischemic preconditioning on myocardial injury and endothelial function and prognosis after percutaneous coronary intervention in patients with acute coronary syndrome. Eur Rev Med Pharmacol Sci 2017; 21: 4642-4648.
12) Chen GZ, Shan XY, Li XS, Tao HM. Remote ischemic postconditioning protects the brain from focal ischemia/reperfusion injury by inhibiting autophagy through the mTOR/p70S6K pathway. Neurol Res 2018; 40: 182-188.
13) Cao B, Wang H, Zhang C, Xia M, Yang X. Remote ischemic postconditioning (RIPC) of the upper arm results in protection from cardiac ischemia-reperfusion injury following primary percutaneous coronary intervention (PCI) for acute ST-segment elevation myocardial infarction (STEMI). Med Sci Monit 2018; 24: 1017-1026.
14) Ovize M, Baxter GF, Di Lisa F, Ferdinandy P, Garcia-Dorado D, Hausenloy DJ, Heusch G, Vinten-Johansen J, Yellon DM, Schulz R; Working Group of Cellular Biology of Heart of European Society of Cardiology. Postconditioning and protection from reperfusion injury: where do we stand? Position paper from the Working Group of Cellular Biology of the Heart of the European Society of Cardiology. Cardiovasc Res 2010; 87: 406-423.
15) Gao Y, Song J, Chen H, Cao C, Lee C. TRPV1 activation is involved in the cardioprotection of remote limb ischemic postconditioning in ischemia-reperfusion injury rats. Biochem Biophys Res Commun 2015; 463: 1034-1039.
16) Zhao ZQ, Corvera JS, Halkos ME, Kerendi F, Wang NP, Guyton RA, Vinten-Johansen J. Inhibition of myocardial injury by ischemic postconditioning during reperfusion: comparison with ischemic preconditioning. Am J Physiol Heart Circ Physiol 2003; 285: H579-588.
17) Liu X-z, Sun X, Shen K-p, Jin W-j, Fu Z-y, Tao H-r, Xu Z-x. Aldehyde dehydrogenase 2 overexpression inhibits neuronal apoptosis after spinal cord ischemia/reperfusion injury. Neural Regen Res 2017; 12: 1166-1171.
18) Korge P, Ping P, Weiss JN. Reactive oxygen species production in energized cardiac mitochondria during hypoxia/reoxygenation: modulation by nitric oxide. Circ Res 2008; 103: 873-880.
19) Sun X, Zhu H, Dong Z, Liu X, Ma X, Han S, Lu F, Wang P, Qian S, Wang C, Shen C, Zhao X, Zou Y, Ge J, Sun A. Mitochondrial aldehyde dehydrogenase-2 deficiency compromises therapeutic effect of ALDH bright cell on peripheral ischemia. Redox Biol 2017; 13: 196-206.
20) Wang Z, Wang Y, Ye J, Lu X, Cheng Y, Xiang L, Chen L, Feng W, Shi H, Yu X, Lin L, Zhang H, Xiao J, Li X. bFGF attenuates endoplasmic reticulum stress and mitochondrial injury on myocardial ischaemia/reperfusion via activation of PI3K/Akt/ERK1/2 pathway. J Cell Mol Med 2015; 19: 595-607.
21) Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 2001; 25: 402-408.
22) Liu X, Zhang C, Qian L, Zhang C, Wu K, Yang C, Yan D, Wu X, Shi J. NF45 inhibits cardiomyocyte apoptosis following myocardial ischemia-reperfusion injury. Pathol Res Pract 2015; 211: 955-962.
23) Huang LH, Li J, Gu JP, Qu MX, Yu J, Wang ZY. Butorphanol attenuates myocardial ischemia reperfusion injury through inhibiting mitochondria-mediated apoptosis in mice. Eur Rev Med Pharmacol Sci 2018; 22: 1819-1824.

24) Taira N, Yamaguchi T, Kimura J, Lu Z-G, Fukuda S, Higashiyama S, Ono M, Yoshida K. Induction of amphiregulin by p53 promotes apoptosis via control of microRNA biogenesis in response to DNA damage. Proc Natl Acad Sci U S A 2014; 111: 717-722.

25) Bae S, Park M, Kang C, Dilmen S, Kang TH, Kang DG, Ke Q, Lee SU, Lee D, Kang PM. Hydrogen peroxide-responsive nanoparticle reduces myocardial ischemia/reperfusion injury. J Am Heart Assoc 2016; 5. pii: e003697.

26) Chen Y, Liu K, Shi Y, Shao C. The tango of ROS and p53 in tissue stem cells. Cell Death Differ 2018; 25: 637-639.

27) Sun A, Zou Y, Wang P, Xu D, Gong H, Wang S, Qin Y, Zhang P, Chen Y, Harada M, Isse T, Kawamoto T, Fan H, Yang P, Akazawa H, Nagai T, Takano H, Ping P, Komuro I, Ge J. Mitochondrial aldehyde dehydrogenase 2 plays protective roles in heart failure after myocardial infarction via suppression of the cytosolic JNK/p53 pathway in mice. J Am Heart Assoc 2014; 3: e000779.

28) Leo CH, Fernando DT, Tran L, Ng HH, Marshall SA, Parry LJ. Serelaxin treatment reduces oxidative stress and increases aldehyde dehydrogenase-2 to attenuate nitrate tolerance. Frontiers Pharmacol 2017; 8: 141.

29) Sussman MA, Volkers M, Fischer K, Bailey B, Cottage CT, Din S, Gude N, Avitabile D, Alvarez R, Sundararaman B, Quijada P, Mason M, Konstandin MH, Malhowski A, Cheng Z, Khan M, McGregor M. Myocardial AKT: the omnipresent nexus. Physiol Rev 2011; 91: 1023-1070.

30) Xu XY, Zhang J, Qi YH, Kong M, Liu SA, Hu JJ. LincROR promotes endometrial cell proliferation by activating the PI3K-Akt pathway. Eur Rev Med Pharmacol Sci 2018; 22: 2218-2225.

31) Jian J, Xuan F, Qin F, Huang R. Bauhinia championii flavone inhibits apoptosis and autophagy via the PI3K/Akt pathway in myocardial ischemia/reperfusion injury in rats. Drug Design, Dev Ther 2015; 9: 5933-5945.

32) Wang ZG, Wang Y, Huang Y, Lu Q, Zheng L, Hu D, Feng WK, Liu YL, Ji KT, Zhang HY, Fu XB, Li XK, Chu MP, Xiao J. bFGF regulates autophagy and ubiquitinated protein accumulation induced by myocardial ischemia/reperfusion via the activation of the PI3K/Akt/mTOR pathway. Sci Rep 2015; 5: 9287.

33) Li Q, Shen L, Wang Z, Jiang H-P, Liu L-X. Tanshinone IIA protects against myocardial ischemia reperfusion injury by activating the PI3K/Akt/mTOR signaling pathway. Biomed Pharmacother 2016; 84: 106-114.