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Original Article

Upregulation of polycystin 2 and activation of autophagy contribute to imparting cardioprotection during late phase of remote preconditioning in rats

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Abstract: Purpose: The present study was aimed to explore the role of mechanosensitive polycystin proteins and autophagy in remote preconditioning (RP)-induced cardioprotection in rats. Methods: The hind limb was subjected to transient episodes of ischemia-reperfusion using a neonatal blood pressure cuff to induce RP. In early phase of RP, hearts were isolated immediately; while in late phase, hearts were isolated after 24 hours. Results: There was a significant decrease in ischemia-reperfusion-induced cardiac injury parameters including CK-MB, cTnT, myocardial infarction and apoptosis in both phases of RP. There was a selective increase in the expression of polycystin 2 (not polycystin 1) and LC3-II/LC3-I ratio (marker of autophagosome) in the heart homogenates during late phase of RP. There were no changes in polycystin and LC3-II/LC3-I during early phase of RP, suggesting that the mechanisms involved in early and late phases of RP are different. Pretreatment with autophagy inhibitor, 3-methyladenine abolished the late cardioprotective effects of RP and attenuated LC3-II/LC3-I ratio without any significant effect on the expression of polycystin 2, suggesting that polycystin 2 is upstream mediator of autophagy. Conclusion: Upregulation of polycystin 2 with subsequent activation of autophagy may be critical in inducing cardioprotection during the late phase of RP.

Keywords: Heart, ischemia, reperfusion, remote preconditioning, mechanosensitive

Introduction

Remote ischemic preconditioning is the novel intervention in which repeated episodes of transient, non-lethal ischemia-reperfusion to a remote organ (other than the target organ) protect the target organ from sustained, lethal ischemia-reperfusion injury [1]. This sort of tissue protection has been documented for a number of organs including brain, liver, kidney and heart [2, 3]. There have been a number of preclinical as well as clinical studies documenting the usefulness of remote preconditioning in imparting protection to the heart against sustained ischemic injury [4-6]. Indeed, it has been reported that remote preconditioning exerts tissue protection in two windows, first window of protection (also termed as early phase of preconditioning) and second window of protection (also termed as late phase of preconditioning). The effects of late preconditioning appear after 24 hours and persist for relatively longer time. On the other hand, the cardioprotective effects of early preconditioning are observed and persist for relatively shorter time [7, 8].

Polycystins are the mechanosensitive proteins and act as key regulators of mechanosensitivity and mechanotransduction. Indeed, these are G protein-coupled receptor-like proteins and function as mechanosensor in a variety of cell types [9, 10]. The important biological role of these proteins was identified from the studies showing that the mutations in the genes encoding polycystin proteins-1 and -2 are associated with the development of autosomal dominant polycystic kidney disease (ADPKD). Due to their close relationship with polycystic kidney disease, the genes were named as PKD1 and PKD2 and these genes encode for the proteins polycystin 1 and polycystin 2, respectively [11]. Subsequent studies have identified the diverse

functions of these mechanosensitive polycystin proteins including regulation of mitochondrial function and cellular metabolism [12], calcium signaling [13, 14], and regulating autophagy [15] etc. Moreover, studies have identified the important roles of these proteins related to the cardiovascular system including maintenance of contractile function of heart [16]. The deficiency of these proteins is associated with cardiac dysfunction [17-19] and heart failure due to cardiomyopathy [13]. Considering the role of mechanosensitive polycystin proteins in regulating heart functions and role of mechanosensitive channels in remote preconditioning [20], the present study was designed to explore the role of mechanosensitive polycystin proteins in the early and late phases of remote preconditioning-induced cardioprotection. Moreover, due to the studies showing the close association of polycystin and autophagy [15, 21] along with the role of autophagy in different types of remote conditioning [22, 23], the present study was also aimed to explore the role of autophagy in early and late phases of remote preconditioning.

Material and methods

Animals, chemicals and drugs

Wistar albino male rats were used for this study. The Institutional Animal Ethics Committee of The Second Affiliated Hospital of Xi'an Jiaotong University approved the experimental protocol (Number: 2018061). All experiments were performed as per ethical guidelines. The assay kit for CK-MB was procured from Elabscience, Wuhan, China; ELISA kit for the assay of cTnT was procured from Cloud-Clone Corp, Texas, USA. The ELISA assay kits for caspase 3 activity and polycystin protein estimation were procured from LifeSpan BioSciences, USA.

Induction of early and late phases of remote preconditioning

In this study, remote preconditioning was induced by alternate occlusion and deocclusion of left hind limb by a neonatal blood pressure cuff. The cuff inflated up to 150 mm of Hg was used to induce hind limb ischemia for 5 minutes, which was followed by deflation of cuff to restore the blood supply in the hind limb for 5 minutes. Four such episodes of transient limb ischemia and reperfusion (five minutes each) were employed for remote preconditioning protocol. For the early phase of remote preconditioning, hearts were isolated immediately after last episode of transient limb ischemia-reperfusion and mounted on Langendorff apparatus. On the other hand, in late phase of remote preconditioning, hearts were isolated 24 hours after the last episode of ischemia-reperfusion [7, 24].

Induction of ischemia-reperfusion injury using Langendorff system

The hearts were isolated immediately after last episode of remote preconditioning protocol (for early phase) or 24 hours later after the last episode of remote preconditioning (for late phase). The isolated hearts were mounted on the Langendorff apparatus and perfused with Kreb's Henseleit (KH) physiological solution. The inflow of KH solution to the heart was stopped for 30 minutes to induce global ischemia and inflow of KH solution was restored for 120 minutes to induce reperfusion injury [25, 26]. The KH solution after passing through the coronary arteries of heart, called coronary effluent, was collected immediately before ischemia (basal) and immediately after reinstitution of reperfusion to quantify the release of heart-specific biomarkers from the heart into the coronary effluent.

Assessment of myocardial injury in terms of release of CK-MB and cardiac troponins (cTnT)

The extent of ischemia-reperfusion-induced myocardial injury was assessed by quantifying the release of heart-specific biochemical markers in the coronary flow. Indeed, the levels of specific isoform of creatine kinase (CK), i.e. CK-MB and cardiac troponins (cTnT), were measured in the coronary effluent using the commercially available assay kits.

Assessment of myocardial injury in terms of necrosis and apoptosis

After subjecting the hearts to ischemia-reperfusion injury, the hearts were divided into two halves. One of the halves was employed to assess the myocardial infarction, while the other half was used to make a homogenate solution. The extent of necrotic cell death was assessed by quantifying the infarction area after staining with triphenyltetrazolium chloride (TTC).

Table 1. Representation of experimental protocol

S. No	Groups	Names	Interventions
1.	Group I	Normal control	No intervention
2.	Group II	Ischemia reperfusion injury	Thirty minutes of ischemia and 120 minutes of reperfusion to the isolated heart
3.	Group III	Early phase of remote preconditioning	Thirty minutes of ischemia and 120 minutes of reperfusion in isolated hearts immediately after remote preconditioning stimulus
4.	Group IV	Late phase of remote preconditioning	Thirty minutes of ischemia and 120 minutes of reperfusion in isolated hearts twenty four hours after remote preconditioning stimulus
5.	Group V	3-methyadenine (15 mg/kg *i.p.*) in late phase of remote preconditioning	Administration of 3-methyadenine (15 mg/kg) thirty minutes prior to remote preconditioning stimulus, followed by isolation of heart after 24 hours to induce ischemia reperfusion injury
6.	Group VI	3-methyadenine (30 mg/kg *i.p.*) in late phase of remote preconditioning	Administration of 3-methyadenine (30 mg/kg) thirty minutes prior to remote preconditioning stimulus, followed by isolation of heart after 24 hours to induce ischemia reperfusion injury
7.	Group VII	3-methyadenine (30 mg/kg *i.p.*) in ischemia reperfusion injury	Administration of 3-methyadenine (30 mg/kg) prior to ischemia reperfusion injury to isolated hearts

This stains the viable heart portions as red, while the infarcted portions remained unstained and thus, the percentage of dead/infarcted area was calculated to assess the extent of myocardial injury [27, 28]. Apoptosis is another form of cell death induced in hearts after ischemia-reperfusion injury [29]. The extent of activation of apoptosis was assessed by quantifying the caspase 3 activity in the heart homogenates using commercially available ELISA kits. The caspase 3 activity was standardized with respect to total protein content, which was assessed quantitatively by Lowery method [30, 31].

Quantification of polycystin 1, 2 and LC3-II/LC3-I ratio

The levels of polycystin 1, polycystin 2 along with the LC3-II/LC3-I ratio, a marker of autophagosome formation, were assessed in the heart homogenate solution using commercially available ELISA kits.

Experimental groups

To achieve the aim and objective of this study, seven experimental groups were employed and each group comprised of six animals (**Table 1**). In normal control (group I), no intervention was done and heart was isolated for biochemical estimations. For ischemia reperfusion injury (group II), hearts were subjected to 30 minutes of ischemia and 120 minutes of reperfusion. For early phase of remote preconditioning (group III), animals were subjected to remote preconditioning and hearts were isolated immediately to induce ischemia-reperfusion injury. For late phase of remote preconditioning (group IV), animals were subjected to remote preconditioning and 24 hours later, hearts were isolated to induce ischemia-reperfusion injury. For late phase of remote preconditioning (group V), animals were administered 3-methyadenine (15 mg/kg *i.p.*) thirty minutes before subjecting to remote preconditioning and 24 hours later, hearts were isolated to induce ischemia-reperfusion injury. For late phase of remote preconditioning with high dose of 3-methyadenine (group VI), animals were administered 3-methyadenine (30 mg/kg *i.p.*) thirty minutes before subjecting to remote preconditioning and 24 hours later, hearts were isolated to induce ischemia-reperfusion injury. For ischemia reperfusion injury (group VII), animals were administered 3-methyadenine (30 mg/kg *i.p.*) before subjecting to ischemia reperfusion injury.

Statistical analysis

The data were presented in the form of mean ± standard deviation (S.D.). The data of CK-MB and cTnT were analyzed by two-way repeated measure ANOVA, while the data of infarct size, caspase 3 activity, polycystin proteins and LC3-II/LC-3I ratio were analyzed by one-way ANOVA. Tukey's multiple comparison test was employed for *post hoc* analysis. The statistical significance was defined with $P < 0.05$.

Results

Effect of early and late phases of remote preconditioning on ischemia reperfusion injury

Ischemia for thirty minutes and twenty minutes of reperfusion markedly produced myocardial

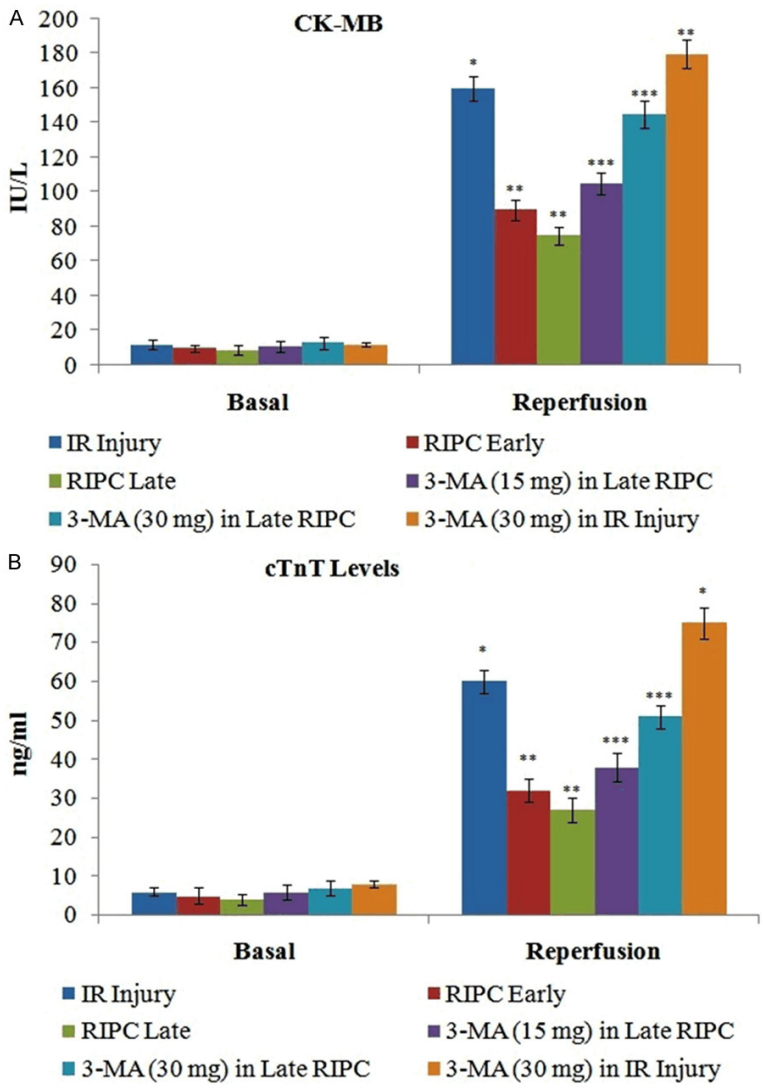

Figure 1. Influence of early and late phases of remote preconditioning (RIPC) along with 3-methyladenine (3-MA) on ischemia-reperfusion (IR)-induced (A) CK-MB levels (B) cTnT levels. Results were expressed in mean ± S.D. *: $P < 0.05$ *vs*. basal; **: $P < 0.05$ *vs*. I/R injury during reperfusion; ***: $P < 0.05$ *vs*. RIPC late.

injury. Indeed, there was a marked increase in the levels of CK-MB (**Figure 1A**) and cTnT (**Figure 1B**) in the coronary effluent measured during reperfusion phase in comparison to basal levels (before instituting ischemia). Moreover, there was an increase in percentage of heart area undergoing myocardial infarction in comparison to normal hearts, which were not subjected to ischemia-reperfusion injury (**Figure 2A**). Apart from this, there was also a marked increase in the caspase 3 activity in the heart homogenates subjected to ischemia-reperfusion injury in comparison to normal hearts (**Figure 2B**). Transient, short episodes of ischemia-reperfusion to the hind limb in the form of remote preconditioning decreased the release of CK-MB and cTnT along with reduction in infarct size and caspase 3 activity, suggesting the attenuation of myocardial injury. The cardioprotection was observed for both early as well as late phases of remote preconditioning. However, the effects of late remote preconditioning were relatively more pronounced (though not significantly) in comparison to early phase of preconditioning.

Effect of early and late phases of remote preconditioning on polycystin proteins and autophagy

Ischemia-reperfusion did not modulate the expression of polycystin 1 and 2 in the heart homogenates in comparison to normal hearts, not subjected to ischemia-reperfusion (**Figure 3A** and **3B**). However, the levels of polycystin 2 proteins were significantly increased in the heart homogenates during late phase of remote preconditioning. Interestingly, early phase of remote preconditioning did not modulate the expression of polycystin 2 in ischemia-reperfusion-subjected rat hearts. The levels of polycystin 1 were not modulated either during early or late phases of remote preconditioning.

The ratio of LC3-II/LC-I is employed as a maker of autophagosome formation and an increase in its ratio indicates the activation of autophagy. In the present study, there was no significant change in the LC3-II/LC-I ratio in hearts subjected to ischemia-reperfusion injury in comparison to normal hearts. Late phase of remote preconditioning led to significant increase in the LC3-II/LC-I ratio, indicating the

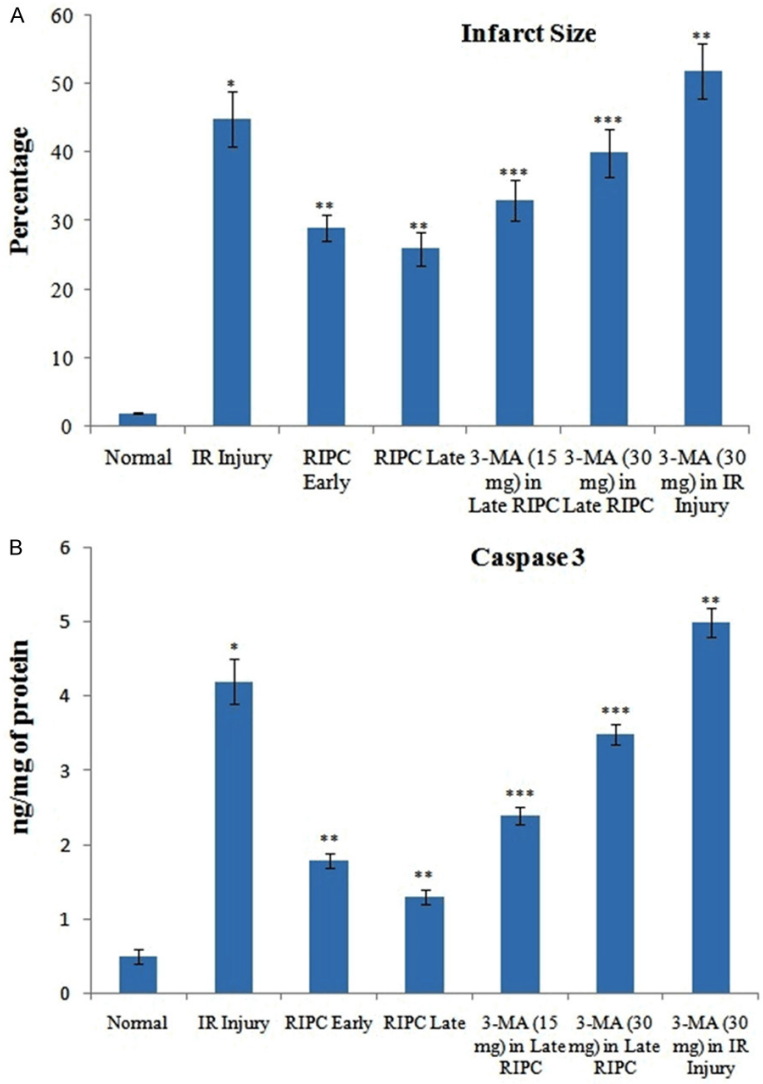

Figure 2. Influence of early and late phases of remote preconditioning (RIPC) along with 3-methyladenine (3-MA) on ischemia-reperfusion (IR)-induced (A) myocardial infarction (B) caspase 3 activity. Results were expressed in mean ± S.D. *: $P < 0.05$ *vs*. normal; **: $P < 0.05$ *vs*. I/R injury; ***: $P < 0.05$ *vs*. RIPC late.

activation of autophagy. However, early phase of remote preconditioning did not modulate the LC3-II/LC-I ratio in ischemia-reperfusion-subjected rat hearts (**Figure 4**).

Effect of autophagy modulator 3-methyladenine on myocardial injury, polycystin proteins and LC3-II/LC3-I ratio

Considering the role of autophagy in ischemia-reperfusion injury and late phase of remote preconditioning, the effects of pharmacological inhibitor of autophagy, i.e. 3-methyladenine, on remote preconditioning and ischemia-reperfusion were investigated. Administration of 3-methyladenine (15 mg and 30 mg/kg *i.p.*) attenuated the cardioprotective effects of late phase of remote preconditioning. It also significantly attenuated late phase of remote preconditioning-induced increase in the LC3-II/LC-I ratio. Interestingly, it did not modulate the levels of polycystin proteins in remote preconditioning-subjected rats. Furthermore, administration of 3-methyladenine aggravated ischemia-reperfusion-induced myocardial injury and decreased the LC3-II/LC-I ratio. However, it did not modulate the polycystin levels in ischemia-reperfusion-subjected rat hearts (**Figures 1**-**4**).

Discussion

The results of present study depict the cardioprotective effects of early as well as late phases of remote preconditioning in ischemia-reperfusion-subjected hearts. In both phases of remote preconditioning, a significant reduction in ischemia-reperfusion-induced increase in the levels of cardiac injury biomarkers, i.e. CK-MB and cTnT, in the coronary effluent was observed. Moreover, a significant decrease in the myocardial infarction (necrosis) and caspase 3 activity (marker of apoptosis) was also observed in both phases of remote preconditioning. The cardioprotective effects of late phase of remote preconditioning were comparatively more (although not significantly) in comparison to early phase. Remote preconditioning exerts protection in two phases, early and late phases. The effects of early phase appear immediately after remote preconditioning stimulus and remain for shorter period. However, the effects of late phase of remote preconditioning appear after 24 hours and remain for comparatively longer period [8]. It is also reported that the

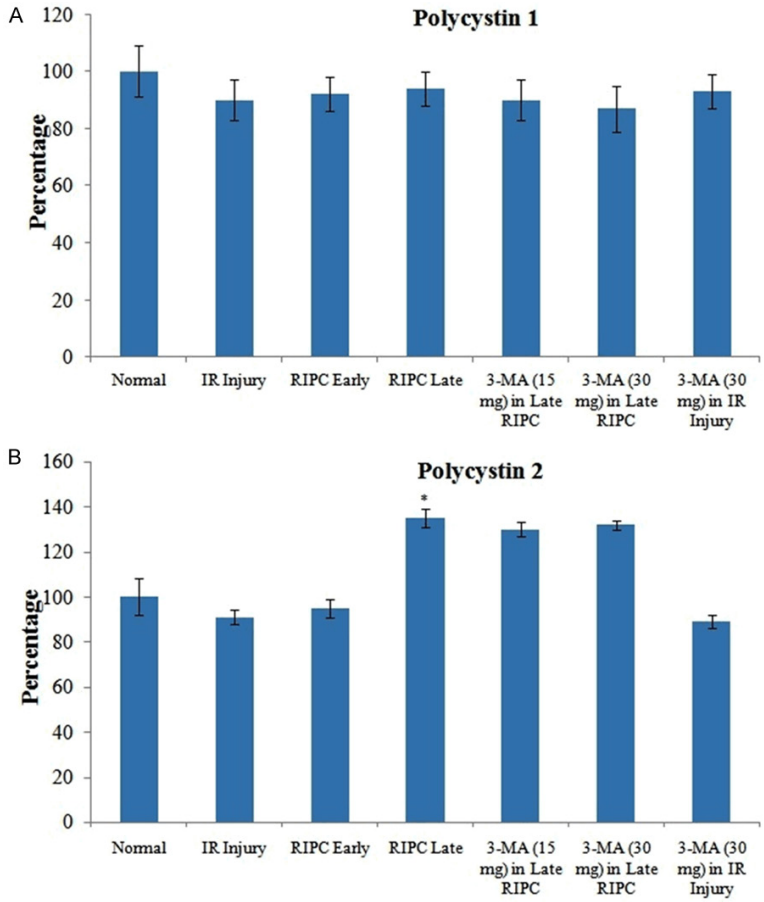

Figure 3. Influence of early and late phases of remote preconditioning (RIPC) along with 3-methyladenine (3-MA) on ischemia-reperfusion (IR)-induced changes in (A) polycystin 1 (B) polycystin 2. Results were expressed in mean ± S.D. *: $P < 0.05$ *vs*. normal.

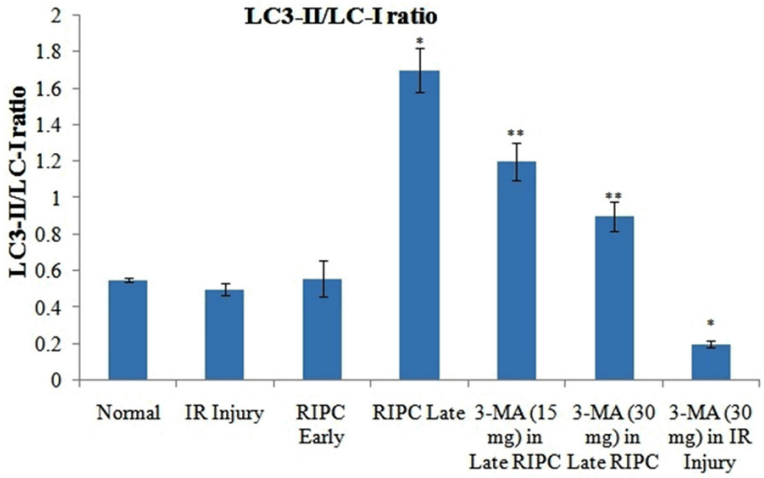

Figure 4. Influence of early and late phases of remote preconditioning (RIPC) along with 3-methyladenine (3-MA) on ischemia-reperfusion (IR)-induced changes in LC3-II/LC-1 ratio. Results were expressed in mean ± S.D. *: $P < 0.05$ *vs*. normal, **: $P < 0.05$ *vs*. RIPC late.

cardioprotective effects of late remote preconditioning are comparatively more than the early phase of remote preconditioning [7].

Remote preconditioning is a unique phenomenon in which short episodes of ischemia to a remote organ send signals to the heart to induce cardioprotection. The precise signaling pathways triggered during remote preconditioning are not identified. There have been studies suggesting that the backflow of the blood from the occluded region towards the heart may activate the mechanosensitive channels on the heart to trigger cardioprotection [20]. Polycystin 1 and 2 are membrane localized, G-protein coupled receptor-like proteins and these serve as mechanosensors in variety of cells including heart cells [9, 15, 16]. To explore their possible involvement in remote preconditioning-induced cardioprotection, the expression of polycystin 1 and 2 were analyzed in the heart homogenates. The present study revealed that there was a significant upregulation of polycystin 2 protein in the heart homogenates in ischemia-reperfusion-subjected hearts, without any significant alteration in the expression of polycystin 1 protein. However, there was no significant alteration in the expression of either of polycystin during the early phase of remote preconditioning. It suggests that the upregulation of mechanosensitive protein i.e. polycystin 2 may be up-regulated during the late phase of remote preconditioning to trigger cardioprotection. It also suggests

that the mechanisms involved in inducing cardioprotection during the early and late phases of remote preconditioning are different. Nevertheless, it is the first report depicting the role of mechanosensitive polycystin 2 protein in cardioprotection offered during the late phase of remote preconditioning.

There have been studies describing the relationship between polycystin 2 and autophagy. Indeed, it has been shown that polycystin 2 may lead to activation of autophagy in cardiomyocytes [15, 21]. To explore the relationship between polycystin 2 and autophagy in remote preconditioning, the ratio of LC3-II/LC3-I was assessed in the present study. The ratio of LC3-II/LC3-I is considered as a marker of autophagosome formation and accordingly, it is used to indicate the activation/inhibition of autophagy [32, 33]. In the present study, there was a marked increase in the LC3-II/LC3-I ratio in the heart homogenate during the late phase of remote preconditioning. It possibly suggests that the activation of autophagy during the late phase of remote preconditioning may contribute to cardioprotection. However, there was no significant alteration in LC3-II/LC3-I ratio in early phase of remote preconditioning again suggesting that the mechanisms involved in cardioprotection during the early and late phases of remote preconditioning are different. The role of autophagy in late phase of remote preconditioning was further verified by administering 3-methyladenine, a pharmacological inhibitor of autophagy [34]. Administration of 3-methyladenine significantly abolished the cardioprotective effects observed during the late phase of remote preconditioning along with the reduction in LC3-II/LC3-I ratio. It further signifies the importance of autophagy activation in inducing cardioprotection during the late phase of remote preconditioning. There have been studies showing that the activation of autophagy contributes in imparting tissue protection in different forms of ischemic and remote conditioning [22, 23]. However, to the best of our knowledge, it is the first study showing that the activation of autophagy helps in imparting cardioprotection during the late phase, but not in the early phase, of remote preconditioning.

Another important finding of this study was that administration of 3-methyladenine abolished the effects of remote preconditioning without altering the levels of polycystin 2. In other words, autophagy inhibitor abolished the effects of remote preconditioning despite the elevated levels of polycystin 2. It signifies that autophagy is a downstream target of polycystin 2 and the latter activates the process of autophagy to impart protection from ischemia-reperfusion injury. Accordingly, it may be proposed that the upregulation of polycystin 2 protein activates the process of autophagy to impart cardioprotection from ischemia-reperfusion injury during the late phase of remote preconditioning. Nevertheless, future studies shall be designed to fully elucidate this signaling pathway using polycystin 2 knockout rats/mice.

Conclusion

The upregulation of mechanosensitive polycystin 2 protein along with activation of autophagy may be critical in inducing cardioprotection during the late phase of remote preconditioning in rats.

Disclosure of conflict of interest

None.

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