Ischemic postconditioning regulates cardiomyocyte autophagic activity following ischemia/reperfusion injury

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Abstract. Ischemic postconditioning (IPostC) is a promising protective mechanism for combating reperfusion injury. However, the role of autophagy in the protective effects of IPostC and the associated signaling pathways have remained to be elucidated. Male Sprague Dawley rats were subjected to 30 min ischemia and 1, 2, 3, 6, 12 and 24 h of reperfusion, with or without IPostC treatment. Autophagic flux was evaluated by detecting mRNA and protein expression levels of microtubule associated protein 1 light chain 3 and p62. Phosphorylated (p)-P70S6 kinase (P70S6K), p-adenosine monophosphate-activated kinase (AMPK) and Beclin 1 protein levels were measured by western blot analysis. Myocardial infarct size was measured using staining with Evans blue dye and myocardial apoptosis was evaluated by terminal deoxynucleotidyl transferase mediated dUTP nick end labeling staining. Autophagic activity was observed to be inhibited within the first hour of reperfusion, increased during 2-6 h and reduced from 12-24 h following IPostC compared with reperfusion without IPostC. Inhibition of autophagy by chloroquine significantly reversed the effects of IPostC on myocardial infarct size and the levels of apoptosis. IPostC significantly increased Beclin 1 levels, inhibited AMPK activation and increased P70S6K activation within the first hour compared with reperfusion without IPostC. IPostC attenuated the upregulation of Beclin 1 levels, increased AMPK activation and reduced P70S6K activation between 2 and 12 h, and subsequently exerted the opposite effects on these molecules between 12 and 24 h. IPostC was demonstrated to regulate autophagic activity in a time-dependent manner. The Beclin 1 and AMPK-mammalian target of rapamycin signaling pathways are suggested to be involved in the regulation of IPostC in autophagy.

Introduction

Myocardial ischemic postconditioning (IPostC) treatment, applied at the onset of reperfusion, has been reported to effectively ameliorate ischemia/reperfusion (I/R) injury of the heart (1). Previous studies have suggested that the protective effects of IPostC are predominantly established via activation of the reperfusion injury salvage kinase (RISK) pathway and the Janus kinase-signal transducer and activator of transcription pathway by inhibiting mitochondrial permeability transition pore opening (2,3). Previous studies have also indicated that autophagy is involved in the protective mechanisms of IPostC (4,5).

Autophagy is an intracellular degradation pathway, in which long-lived or aggregated proteins and damaged organelles are transported to lysosomes for destruction (6). In the heart, autophagy serves a homeostatic role under physiological conditions, but abnormal autophagy may result in cardiac dysfunction (7). A previous study demonstrated that autophagy was upregulated during myocardial I/R and suggested that it may be cardioprotective during ischemia (8). During myocardial ischemia and reperfusion, autophagy is upregulated through activation of an adenosine monophosphate-activated kinase (AMPK)- and Beclin 1-dependent mechanism (8). The AMPK-mammalian target of rapamycin (mTOR) pathway and Beclin 1 are core autophagy regulators, of which mTOR is additionally involved in the RISK pathway. However, the involvement of autophagy during the reperfusion period remains controversial (8-11). The current study aimed to investigate the role of autophagy in IPostC by comparing the myocardium autophagic flux and alterations in the key autophagy regulators within a 24 h interval between IPostC and I/R.

Materials and methods

Animals. A total of 192 male Sprague Dawley rats (SLAC Laboratory Animals Ltd., Hunan, China) weighing 200-300 g were used in the current study. The rats were housed in cages with ad libitum access to food pellets and water, were maintained at 22°C, and were exposed to a 12 h light/dark cycle. The animal protocol was approved by the Animal Care and Use Committee of Xiangya Medical College, Central South University (Changsha, China).
**I/R modeling.** Rats were anesthetized by intraperitoneal injection of pentobarbital (60 mg/kg; Sigma-Aldrich, St. Louis, MO, USA) and were placed on a heating pad (XR-YLS-20A; Xinruan Information Technology, Co., Ltd., Shanghai, China) maintained at 36-38°C. An electrocardiogram was recorded using a Power-Lab monitoring system (BL-410 system; Chengdu Tai Meng Science And Technology Co., Ltd., Chengdu, China). A left thoracotomy was subsequently performed to expose the heart at the fourth intercostal space. Ischemia was established by placing a snare on the top of the anterior descending branch of the left coronary artery (LAD) using a 6-0 nylon suture (Cheng-He Microsurgical Instruments Factory, Ningbo, China). Regional ischemia was confirmed by alterations in ST-T on the electrocardiogram.

**Western blot assay of autophagy.** Rats were randomly divided into I/R, IPostC and Sham-operation groups. Rats in the I/R group were subjected to 30 min ischemia to the heart followed by 1, 2, 3, 6, 12 and 24 h of reperfusion (n=6 per time-point). Rats in the IPostC group were subjected to 30 min ischemia to the heart prior to three cycles of 30-sec reperfusion/30-sec ischemia followed by 1, 2, 3, 6, 12 or 24 h reperfusion (n=6 per time-point). Rats in the Sham-operation group (n=6) underwent the same procedure without occlusion of the vessel. Rats were sacrificed by overdose of anesthesia (pentobarbital, 180 mg/kg).

Hearts were excised and left ventricular (LV) muscles were lysed in ice-cold radioimmunoprecipitation assay buffer containing protease and phosphatase inhibitor cocktails (EMD Millipore, Billerica, MA, USA). The concentration of the lysates was measured using a Bicinchoninic Acid Protein Assay kit (Beyotime Institute of Biotechnology, Beijing, China). Total protein (30 µg) was separated on 8-15% SDS polyacrylamide gels (Beyotime Institute of Biotechnology) and transferred onto polyvinylidene difluoride membranes (EMD Millipore). The membranes were blocked with blocking buffer [5% bovine serum albumin (Wuhan Boster Biological Technology, Ltd., Wuhan, China)]-buffered saline containing 0.05% Tween 20 (Sigma-Aldrich) for 1 h at room temperature. The blocked membranes were incubated with primary antibodies overnight at 4°C. Subsequent to washing (Western Washing Liquid; Beyotime Institute of Biotechnology), the membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit peroxidase-conjugated secondary antibody (1:1,000; cat. no. A0208; Beyotime Institute of Biotechnology) for 1 h at room temperature. Protein bands were visualized with the ECL Western Blotting Detection kit (EMD Millipore), and the chemiluminescence signal was detected using X-ray film (Kodak Digital Imaging Products GmbH, Nussloch, Germany). The LV sections were incubated with a 1% triphenyltetrazolium chloride solution (Sigma-Aldrich) at 37˚C for 15 min. The infarct area (pale), bated with a 1% triphenyltetrazolium chloride solution (Sigma-Aldrich) was injected through the caudal vein. The heart was excised and the LV tissues were cut into 1-mm cross-sections (Leica CM1950 cryostat; Leica Microsystems). Ischemia was established by placing a 1-µm-thick suture (Cheng-He Microsurgical Instruments Factory, Ningbo, China). Regional ischemia was confirmed by alterations in ST-T on the electrocardiogram.

**Determination of myocardial infarct size.** Rats were randomly divided into the I/R, IPostC and IPostC + CQ groups. Rats in the I/R group were subjected to 30 min ischemia to the heart followed by 3-h reperfusion. Rats in the IPostC group were subjected to 30 min ischemia to the heart prior to three cycles of 30-sec reperfusion followed by 30-sec ischemia, and finally 3 h of reperfusion. Rats in the IPostC + CQ group were pretreated with CQ (10 mg/kg, intraperitoneally) 1 h prior to the induction of ischemia (13), and were subsequently subjected to the same protocol as rats in the IPostC group (n=6-8 rats per group). Rats were sacrificed by overdose of anesthesia (pentobarbital, 180 mg/kg).

To measure the ischemic area at risk (AAR), the LAD was re-occluded with the same suture. Evans blue dye (3%; Sigma-Aldrich) was injected through the caudal vein. The heart was excised and the LV tissues were cut into 1-mm cross-sections (Leica CM1950 cryostat; Leica Microsystems GmbH, Nussloch, Germany). The LV sections were incubated with a 1% triphenyltetrazolium chloride solution (Sigma-Aldrich) at 37°C for 15 min. The infarct area (pale), the AAR (not blue) and the total LV area from both sides of each section were measured using Image-Pro Plus version 6.0 (Media Cybernetics, Inc., Rockville, MD, USA).

**Terminal deoxynucleotidyl transferase mediated dUTP nick end labeling (TUNEL) staining.** The hearts were rapidly excised following 3-h reperfusion and were fixed in 4% paraformaldehyde (Sinopharm Chemical Reagent Co., Ltd.,
Shanghai, China). The 10-µm transverse cryostat sections were cut from routinely Tissue-Tek OCT (Sakura Finetek, Inc., Torrance, CA, USA)-embedded heart tissues. The slices were processed for TUNEL staining using a commercially available in situ cell detection kit (Roche Diagnostics, Indianapolis, IN, USA). Nuclei were stained with DAPI (Beyotime Institute of Biotechnology). Apoptotic cell counts were expressed as a percentage of the total number of nuclei.

Statistical analysis. Data were presented as the mean ± standard error of the mean and were analyzed using SPSS version 17.0 (SPSS, Inc., Chicago, IL, USA). One-way analysis of variance was used for a comparison between multiple groups and the Bonferroni post hoc test was used for pairwise comparisons between groups. P<0.05 was considered to indicate a statistically significant difference.

Results

IPostC altered autophagic activity and Beclin 1, AMPK-mTOR expression in a time-dependent manner. In the myocardium of rats treated with IPostC compared with those of rats in the I/R group, the LC3-II/LC3-I ratios were significantly lower at 1, 12 and 24 h but significantly greater at 2, 3 and 6 h (P<0.05; Fig. 1A and B). The LC3-II protein levels were significantly upregulated at 1 h but significantly downregulated at the other time points (P<0.05; Fig. 1C). The p62 protein levels were significantly greater at 1, 12 and 24 h but significantly reduced at 2, 3 and 6 h (P<0.05; Fig. 1D and E). Furthermore, the LC3b mRNA levels were observed to be significantly lower at 1 and 24 h but significantly higher at 3 and 6 h (P<0.05; Fig. 2A), and the p62 mRNA levels were also significantly lower at 1 and 24 h but significantly higher at 3, 6 and 6 h (P<0.05; Fig. 2B). Overall, IPostC was observed to inhibit autophagic activity within the first hour, promote autophagic activity from 2 to 6 h and reduce autophagic activity from 12-24 h.

In the IPostC-treated myocardium, the Beclin 1 mRNA levels were significantly lower at 1 h and significantly higher at 3, 6, 12 and 24 h, as compared with the I/R group (P<0.05; Fig. 2C). The expression levels of P70S6K were significantly increased following 1 h, reduced at 2, 3 and 6 h and increased again at 12 and 24 h (P<0.05; Fig. 3A and B). AMPK activity
was significantly inhibited at 1 h, increased at 2, 3 and 6 h and reduced at 12 and 24 h (P<0.05; Fig. 3C and D). Beclin 1 protein levels were significantly increased at 1 h, reduced at 2, 3 and 6 h, followed by an increase again at 12 and 24 h (P<0.05; Fig. 3E and F).

Promotion of autophagic flux is significant in conferring IPostC-mediated cardioprotection. A previous study demonstrated that the reperfused myocardium exhibited impaired autophagosome clearance, which is associated with cardiomyocyte death (14). The current study also observed impaired autophagic flux in the reperfused myocardium following 2-6 h of reperfusion. Whether the relief of autophagy blocked by IPostC in this period serves a significant role in its myocardial protection was also investigated. The anti-malaria drug CQ is a well-known autophagy inhibitor, which inhibits lysosomal acidification and prevents autophagosome-lysosomal fusion (15). Rat hearts were pretreated with CQ 1 h prior to coronary artery occlusion. Following 3-h reperfusion, the ischemic AAR was measured. The infarct volume in the hearts subjected to I/R was 52% of the total LV area and this was reduced to 26% (P<0.05) in hearts subjected to IPostC. By contrast, the infarct volume in the IPostC + CQ rats was significantly increased to 53% of the total LV area (P<0.05 vs. IPostC group; Fig. 4A and B). Furthermore, the percentage of TUNEL-positive cells in the ischemic area was additionally increased in the CQ-treated myocardium (17%) compared with that of the IPostC-treated myocardium (7%) (Fig. 4C and D).

Discussion

Autophagy has been demonstrated to be upregulated during myocardial I/R and has been suggested to be cardioprotective during ischemia. IPostC has been previously observed to function as a protective mechanism against reperfusion injury (8). However, the role of autophagy in the protective effects of IPostC and the associated signaling pathways remain to be fully elucidated. The results of the current study suggested that IPostC regulates autophagy in a time-dependent manner through the regulation of Beclin 1 and AMPK-mTOR signaling pathways.

Autophagy is a dynamic process and its activity is predominantly reflected by autophagic flux, which is comprised of autophagosome formation and autolysosomal-lysosomal degradation. p62 is a protein adaptor, which is able to bind ubiquitinated cargo designated for autophagic breakdown. LC3-II, which is located on the inner autophagosomal membrane, is degraded when the autophagosome fuses with the lysosome (16). Detecting the LC3-II/LC3-I ratio and levels of p62 and LC3-II protein provides an improved representation of autophagic flux (6,15). p62 and LC3 are able to be transcriptionally regulated during autophagy, which may influence the efficacy of p62 and LC3 levels as indicators of autophagic substrates (15,17). Despite these potential limitations, p62 and LC3b gene expression levels were selected for detection in the myocardium during the reperfusion period by qPCR. The RISK pathway predominantly includes the phosphoinositide 3-kinase/protein kinase B (Akt)/mTOR pathway and p42/p44 extracellular signal-regulated kinase pathway. These two pathways converge at P70S6K. In mammalian cells, phosphorylation of mTOR inhibits cell autophagy (17). Numerous studies demonstrate that mTOR is a key factor for the regulation of autophagy in I/R (18-22). P70S6K is an effector of mTOR, and phosphorylated (p-)P70S6K levels are...
suggested to reflect the function of mTOR (23). AMPK is able to directly trigger autophagy or inhibit the suppressive effect of mTOR (24). Beclin 1 is a protein that controls the initiation of autophagy; however, greater Beclin 1 levels additionally impede lysosomal fusion by interacting with Rubicon or by directly downregulating transcriptional components of the autophagy-lysosome machinery (14,25). Therefore, Beclin 1 is a key regulator, which exerts dual-directional regulation on autophagic flux. For these reasons, the markers selected in the current study are appropriate for the identification of autophagy and the associated signaling.

During the first hour following treatment, reduced gene expression of LC3b and a lower LC3-II/LC3-I ratio were observed in the IPostC myocardium, which suggested a reduction in autophagosome formation. Meanwhile, high p62 protein levels with reduced p62 gene expression indicated that IPostC also inhibited autophagy clearance. Therefore, it was suggested that IPostC suppressed autophagic activity within the first hour following its application. Consistent with a previous study (26), P70S6K was observed to be significantly activated within 1 h following IPostC treatment, suggesting that mTOR was also activated. In addition, a significant reduction in AMPK activity was observed in the IPostC myocardium. Thus, IPostC may negatively regulate autophagy via the activation of mTOR. The expression of the Beclin 1 gene was reduced by IPostC in this first hour, but the Beclin 1 protein level was upregulated in the IPostC myocardium compared with the I/R group myocardium. Upregulation of Beclin 1 has been previously identified to be mediated by the high reactive oxygen species (ROS) content resulting in...
from reperfusion (14, 27) It is known that IPostC is able to reduce the production of ROS (26) therefore a reduction in the levels of ROS was hypothesized to contribute to the reduced Beclin 1 expression during the first hour following IPostC treatment A previous study observed that Akt may enhance the interaction among Beclin 1, 14-3-3 and vimentin, which inhibits the role of Beclin 1 in autophagy (28) Therefore activation of the RISK pathway by IPostC may be caused by
the accumulation of Beclin 1 in the cytoplasm. The higher concentration of Beclin 1 may then inhibit autophagic activity by impeding autophagy clearance or downregulating the transcription of autophagy-associated genes (14). Autophagy in the I/R myocardium at 1 h appears to be over-activated when compared with the myocardium in the IPostC group.

The excessive activation of autophagy may have detrimental effects due to a disproportionate degeneration of various key organelles and proteins (29). Thus, it is suggested that the temporary inhibition of autophagy induced by IPostC produced a protective effect on the myocardium.

The LC3-II/LC3-I ratio was observed to be higher in the IPostC myocardium during 2-6 h post-ischemia and the gene expression of LC3b was increased at 2 and 6 h, which suggested that autophagosome formation was increased. In addition, reduced levels of p62 protein expression together with significantly increased p62 gene expression levels indicates that IPostC also promotes autophagy clearance. The LC3-II levels were reduced in the IPostC-treated myocardium, which suggested that autophagy clearance was promoted by IPostC, rather than the blockage of autolysosome clearance induced by reperfusion. During this phase, P70S6K activation was reduced and AMPK was activated again, an effect which appeared to mediate autophagy activation in this period of time. The Beclin 1 protein level was reduced and Beclin 1 gene expression was increased in the IPostC myocardium compared with the reperfused myocardium. The reduced Beclin 1 protein level may have enhanced the autophagic flux during this time. Previous studies have demonstrated that autophagic flux was blocked during reperfusion (9,14). In the current study, it was reported that reperfusion impaired autophagic flux between 2 and 6 h in I/R, and IPostC enhanced autophagic activity during this period. It was further demonstrated that inhibition of autophagic flux by CQ significantly increased myocardial infarct volume and apoptosis, suggesting that relief of the impaired autophagic flux by reperfusion is important in IPostC-induced cardioprotection. During this period of time the reperfused myocardium exhibited impaired autophagic flux, which may be an endogenous adaptation to the overactivation of autophagy during the first hour; therefore, blocking the autophagic flux temporarily produced a protective effect on the myocardium. However, if this condition persists, it is damaging to the myocardium due to the fact that the heart must remove damaged proteins and organelles, particularly during the reperfusion phase (30). A previous study indicated that impaired autophagy was responsible for cardiomyocyte death (14). The enhanced autophagy induced by IPostC during this period produced a protective effect on the myocardium.

At the 12 and 24 h time-points, the reduced LC3-II/LC3-I ratio, LC3b and p62 gene expression levels together with increased p62 protein expression levels suggested that autophagic activity was reduced in the IPostC myocardium. Simultaneously, P70S6K was observed to be activated once again. Thus, mTOR may be involved in negative regulation of autophagy in the IPostC myocardium. The Beclin 1 protein and gene expression levels were all upregulated in the IPostC myocardium. In addition to participating in autophagy, Beclin 1 is also involved in various physiological processes, functioning as an important membrane binding protein (31,32). Considering the reduced autophagic activity, Beclin 1 may participate in additional physiological processes in the IPostC myocardium at these time-points. Autophagic activity was restored in the reperfused myocardium during the 12-24 h period, potentially as a result of the lower Beclin 1 levels improving autophagic flux or due to the fact that myocardial cells with autophagic dysfunction had already died. The autophagic activity was reduced in the IPostC myocardium due to the fact that the dysfunctional or damaged cellular components had been eliminated and mTOR was re-activated, thus promoting the production of proteins to repair the reperfused myocardium.

In conclusion, the current study highlighted the crucial role of autophagy in the protective mechanism of IPostC via regulation of the Beclin 1 and AMPK-mTOR signaling pathways. However, further studies are required to confirm an association between these signaling pathways and the regulation of autophagy. Regulating autophagy in a time-dependent manner may be an additional important end-effector of IPostC protection. Inhibition or activation of autophagy during reperfusion may be harmful to the myocardium, however regulation of autophagic activity in a time-dependent manner may be an effective novel cardioprotective treatment strategy.

References