Involvement of DJ-1 in ischemic preconditioning-induced delayed cardioprotection in vivo

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Abstract. DJ-1 protein, as a multifunctional intracellular protein, has been demonstrated to serve a critical role in regulating cell survival and oxidative stress. To provide in vivo evidence that DJ-1 is involved in the delayed cardioprotection induced by ischemic preconditioning (IPC) against oxidative stress caused by ischemia/reperfusion (I/R), the present study subjected male Sprague-Dawley rats to IPC (3 cycles of 5-min coronary occlusion/5-min reperfusion) 24 h prior to I/R (30-min coronary occlusion/120-min reperfusion). A lentiviral vector containing short hairpin RNA was injected into the left ventricle three weeks prior to IPC, to knockdown DJ-1 in situ. Lactate dehydrogenase (LDH) and creatine kinase-MB (CK-MB) release, infarct size, cardiac function, superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) activities, malondialdehyde (MDA), intracellular reactive oxygen species (ROS), and DJ-1 protein expression levels were assessed. IPC caused a significant increase in the expression levels of DJ-1 protein. In addition, IPC reduced LDH and CK-MB release, attenuated myocardial infarct size, improved cardiac function following I/R, and inhibited the elevation of ROS and MDA and the decrease in activities of the antioxidant enzymes SOD, CAT and GPx. However, in situ knockdown of DJ-1 attenuated the IPC-induced delayed cardioprotection, and reversed the inhibitory effect of IPC on I/R-induced oxidative stress. The present study therefore provided novel evidence that DJ-1 is involved in the delayed cardioprotection of IPC against I/R injury in vivo. Notably, DJ-1 is required for IPC to inhibit I/R-induced oxidative stress.

Introduction

Ischemic preconditioning (IPC) is an important endogenous adaptive phenomenon first described by Murry et al (1) in 1986. IPC involves single or multiple brief periods of sublethal ischemia, which increase myocardial resistance to a greater subsequent insult. It is now well established that IPC confers two separate phases of cardioprotection; an early phase occurring instantly and continuing for 3-4 h and a late (or delayed) phase occurring ~12 h after the preconditioning stimulus that may persist to 72 h (2,3). The late phase is clinically relevant due to its persistent and effective cardioprotection against myocardial stunning and infarction (4); therefore, extensive investigations have been performed to elucidate its underlying mechanisms.

Previous studies have revealed that the delayed cardioprotective mechanisms underlying IPC are complex and involve upregulation of endogenous cardioprotective proteins and oxidative stress inhibition (5-7). Genetic and pharmacological studies have identified aldose reductase, heme oxygenase-1 (HO-1), and Mn-superoxide dismutase (MnSOD) as critical mediators of the antioxidative stress effects of late preconditioning (8-10). However, accumulating evidence indicates that IPC is a complex polygenic adaptation (3); therefore, as yet unidentified antioxidant proteins may additionally be involved.

DJ-1, a novel oncogene product identified in 1997, is a ubiquitously expressed and highly conserved intracellular protein (11). Subsequent research suggested that DJ-1 has various potential functions, including transcriptional regulation, oxidative stress inhibition, acting as a chaperone or protease and mitochondrial regulation (12). DJ-1 may act as an antioxidant and be important for cellular defense in response to oxidative stress (13-16). Our previous study revealed that hypoxia preconditioning of H9c2 cardiomyocytes significantly increased the de novo synthesis of DJ-1 and induced cardioprotection against prolonged hypoxic injury 24 h later (17). However, whether the increase in DJ-1 expression mediates protection against ischemia/reperfusion (I/R) injury in vivo during the late phase of IPC remains to be determined.

The present study therefore used an in vivo rat model of IPC and I/R to determine whether DJ-1 was upregulated 24 h after IPC, which has previously been demonstrated to induce delayed cardioprotection against oxidant stress caused by I/R. It was subsequently investigated whether in situ knockdown
of DJ-1 interfered with the delayed cardioprotective effects mediated by IPC and blocked the inhibition of oxidative stress generated by I/R. The results of the present study demonstrated that IPC upregulates DJ-1 protein expression levels in the heart and that DJ-1 is essential for the antioxidative stress effects of late phase IPC in vivo, thereby identifying DJ-1 as an endogenous cardioprotective protein.

Materials and methods

Chemicals and reagents. Anti-DJ-1 (N-20; catalog no. sc-27004) and anti-β-actin (I-19; catalog no. sc-1616) goat polyclonal primary antibodies, and the horseradish peroxidase-conjugated rabbit anti-goat secondary antibody (catalog no. sc-2768) were purchased from Santa Cruz Biototechnology, Inc. (Dallas, TX, USA). Dihydroethidium (DHE) was obtained from Molecular Probes; Thermo Fisher Scientific, Inc. (Waltham, MA, USA). Malondialdehyde (MDA), glutathione peroxidase (GPx), catalase (CAT) and superoxide dismutase (SOD) assay kits were purchased from Beijing Solarbio Science & Technology Co., Ltd. (Beijing, China). All other chemicals were purchased from Sigma-Aldrich; Merck Millipore (Darmstadt, Germany) unless otherwise stated.

Animals. A total of 75 adult, healthy, male Sprague-Dawley rats (weight, 210-240 g) were purchased from the Animal Center of Nanchang University (Nanchang, China). Rats were housed at a temperature of 23±1°C and a relative humidity of 55±10%, under a 12-h light/dark cycle and were allowed free access to water and a standard diet. All procedures performed in the present study were in accordance with the Guidelines on the Use of Laboratory Animals (National Institutes of Health, Bethesda, MD, USA) and were approved by the Ethics Committee for the Use of Experimental Animals at Nanchang University.

In situ knockdown of DJ-1. A lentiviral vector containing DJ-1 short hairpin (sh)RNA (lenti-shDJ-1; GeneChem Co., Ltd., Shanghai, China) was used to selectively knockdown DJ-1 in situ according to the method of Das et al (18). In brief, rats were anesthetized with an intraperitoneal injection of 50 mg/kg sodium pentobarbitone (Sigma-Aldrich; Merck Millipore) and orotracheally intubated; a positive-pressure ventilator was used to maintain breathing. A left thoracotomy was performed at the fourth intercostal space, and the heart was exposed by stripping the pericardium. A 7/0 silk suture was placed around the LCA 3-4 mm distal to the LCA origin, and an occlusive snare was placed around it. Artery occlusion was achieved by tightening the snare and verified by epicardial cyanosis. Successful reperfusion of the heart was achieved by releasing the snare, and confirmed by visualizing a clear epicardial hyperemic response.

For IPC, a sequence of three cycles of 5-min coronary occlusion/5-min reperfusion was performed. I/R was induced 24 h following IPC, in the late phase of delayed preconditioning, and achieved by 30 min of coronary occlusion followed by 120 min of reperfusion.

Experimental groups. The present study consisted of two successive phases. The objective of the first phase was to determine the effect of IPC on the expression of DJ-1 protein in rat myocardium. Male Sprague-Dawley rats were assigned to six groups (n=5/group). Group I (control) did not undergo coronary occlusion. Groups II, III, IV, V, and VI underwent IPC with no treatment and were sacrificed by cervical dislocation 0 (group II), 12 (group III), 24 (group IV), 48 (group V) or 72 h (group VI) following the final reperfusion. Myocardial samples were rapidly removed, frozen in liquid nitrogen, and stored at -140°C until analysis of DJ-1 protein expression levels by western blotting.

The aim of the second phase was to determine whether in situ knockdown of DJ-1 interferes with delayed cardioprotection induced by IPC against oxidative stress caused by I/R. Male Sprague-Dawley rats were randomly assigned to one of three experimental groups (n=15/group). Rats in group VII (WT) were untreated. Rats in group VIII (lenti-shC) received left intramyocardial injection of control virus lenti-shC. Rats in group IX (lenti-shDJ-1) received left intramyocardial injection of lenti-shDJ-1. A total of three weeks later, rats in each of these three experimental groups were randomly divided into three subgroups (n=5/group): i) Sham, in which rats underwent the surgical procedure without coronary occlusion; ii) I/R, in which rats were subjected to 30 min coronary occlusion followed by 120 min reperfusion; and iii) IPC + I/R, in which rats were preconditioned with a sequence of three cycles of 5-min coronary occlusion/5-min reperfusion 24 h prior to coronary I/R. Lactate dehydrogenase (LDH) and creatine kinase-MB (CK-MB) release, infarct size, cardiac function, CAT, SOD and GPx activities, MDA, and intracellular reactive oxygen species (ROS) were assessed following I/R. Rats were sacrificed immediately following I/R.

Determination of cardiac function. I/R-induced cardiac dysfunction was evaluated by invasive hemodynamic evaluation methods. A microcatheter was inserted into the left ventricle via the right carotid artery to measure the left ventricular pressure (LVP). LVP was tracked on a RM-6200C polygraph. Computer algorithms measured left ventricular...
end-diastolic pressure (LVEDP), left ventricular systolic pressure (LVSP), and first derivative of LVP (±dP/dt\text{max}) 120 min following reperfusion.

**LDH and CK-MB release evaluation.** Myocardial cellular damage was evaluated by measuring LDH and CK-MB activities. Blood samples were collected from the carotid artery after 120 min reperfusion and placed in heparinized tubes. The blood was centrifuged at 3,000 g for 10 min at 4°C. The plasma was recovered and used for measuring the activities of LDH and CK-MB using commercially available assay kits (cat nos. BC0681 and 1855105; Beijing Solarbio Science & Technology Co., Ltd.).

**Determination of infarct size.** Myocardial infarct size was evaluated by Evans blue/2,3,5-triphenyl-2H-tetrazolium chloride (TTC) staining as previously described (20). Following 120 min coronary artery reperfusion, the snare around the LCA was retightened and 1 ml 2% Evans blue was perfused into the aorta and coronary arteries to delineate the area at risk (AAR). AAR was defined as the area of myocardium that was not stained with Evans blue. The heart was excised and cut transversely into 1-mm thick slices from the apex to the base. The slices were incubated with 1% TTC in 0.2 M Tris buffer (pH 8.0) at 37°C for 10 min. Following TTC staining, the infarcted area appears white, whereas the healthy tissue appears red. Each slice was imaged and analyzed using Image-Pro Plus (version, 6.0; Media Cybernetics, Inc., Rockville, MD, USA). The myocardial infarct size was expressed as the percentage of the infarct area/AAR.

**Measurement of the activities of the antioxidant enzymes CAT, SOD and GPx, and MDA content.** The border zone was identified as ‘Evans blue unstained’ and ‘TTC stained’. A 2 mm section of myocardium tissue from the border zone was obtained for CAT, SOD, GPx and MDA assays. Following 120 min reperfusion, ventricular tissues were removed and stored in radioimmunoprecipitation assay buffer (Beijing Solarbio Science & Technology Co., Ltd.) containing 1 mM ethylene glycol-bis (β-aminooxyethyl)ether)-N,N,N′,N′-tetraacetic acid, 5 mM sodium fluoride, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 220 mM mannitol, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 70 mM sucrose and 1 mM Na\text{3} β-glycerol phosphate (Sigma-Aldrich; Merck Millipore), supplemented with 5 µl/ml protease inhibitor mixture, pH 7.4 at 4°C. The tissues were cut into pieces and homogenized with a Teflon Potter homogenizer. Following centrifugation at 13,000 x g, 4°C for 20 min, the supernatants were used to determine the activities of cellular CAT, SOD and GPx, and MDA content, using commercial assay kits (cat nos. BC0200, BC0170, BC1190, BC0020). The activities of CAT, SOD and GPx, and MDA content, were expressed relative to the protein levels in the supernatant determined by the Lowry method using the Detergent-Compatible Protein assay kit II (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

**Statistical analysis.** Data are presented as the mean ± standard error. Statistical comparisons between groups were performed by one-way analysis of variance followed by a least significant difference post hoc test. Statistical analyses were performed in SPSS software version 11.0 (SPSS, Inc., Chicago, IL, USA). P<0.05 was considered to indicate a statistically significant difference.

**Results**

**Effect of IPC on DJ-1 protein expression levels in rat myocardium.** To provide in vivo evidence that DJ-1 is involved in the delayed protection of IPC, DJ-1 protein expression levels were analyzed in rat myocardium at 0, 12, 24, 48 and 72 h after IPC. DJ-1 protein expression levels were increased at 12 h (P=0.0112), peaked at 24 h (P=0.0002), and persisted at 48 h (P=0.0016) following IPC compared with control group (Fig. 1). The time points of DJ-1 upregulation were consistent with the established time window of delayed cardioprotection induced by IPC in vivo (24,25). These results demonstrated that IPC increased DJ-1 protein expression levels in the late phase.

**Effect of DJ-1 knockdown on the IPC-induced improvement of cardiac function following I/R.** To determine the in vivo under a fluorescence microscope (Olympus IX-81; Olympus Corporation, Tokyo, Japan). DHE staining was performed on 10-µm thick frozen myocardial sections as previously described (21-23). In brief, following 120 min reperfusion, hearts were snap-frozen in Tissue-Tek embedding medium (Sakura Finetek USA, Inc., Torrance, CA, USA), and 10-µm thick sections were cut using a cryostat. Sections were air-dried, hydrated with PBS and incubated with 10 µM DHE at 37°C for 30 min in a dark humidified chamber. The sections were washed briefly and the relative DHE fluorescence was quantified by calculating the mean value of fluorescence intensity within three identical circles using Image-Pro Plus software version 6.0. Three to five sections from each rat were analyzed.

**Western blot analysis.** A total of three weeks following left intramyocardial injection of lenti-shDJ-1 or control lenti-shC, or at various time points following IPC, total proteins were extracted with RIPA buffer (cat. no. R0010; Beijing Solarbio Science & Technology Co., Ltd.) from cardiac left ventricular samples and quantified by the Lowry method using the Detergent-Compatible Protein assay kit II. Total proteins (50 µg) were subjected to 12% SDS-PAGE and transferred onto a polyvinylidine fluoride membrane. The membrane was blocked for 2 h at 4°C with 5% nonfat milk and subsequently probed with primary antibodies directed against DJ-1 (1:1,000) at 4°C overnight, followed by incubation with a horseradish peroxidase-conjugated secondary antibody (1:5,000) at 4°C for 2 h. The signals were visualized with an Enhanced Chemiluminescence reagent (Pierce; Thermo Fisher Scientific, Inc.). To normalize lane loading, the same membranes were reprobed with anti-β-actin (1:1,000). The levels of DJ-1 protein were standardized to the loading control and were quantified using Quantity One® software version 4.62 (Bio-Rad Laboratories, Inc.).
contribution of DJ-1 to IPC-induced delayed cardioprotection, cardiac function was measured in rats subjected to IPC 24 h prior to I/R. As presented in Fig. 2, a 61% decrease in DJ-1 protein expression levels was observed in the heart of sham rats three weeks after left intramyocardial injection of lenti-shDJ-1 compared with injection with control lenti-shC (P=0.0003). Notably, in WT and lenti-shC-infected rats, IPC significantly attenuated the reduction of LVSP (P=0.0056 and P=0.0082, respectively) and $\pm dP/dt_{\text{max}}$ (P=0.0003 and 0.0011 for $dP/dt_{\text{max}}$; P=0.0020 and 0.0005 for $-dP/dt_{\text{max}}$, respectively) induced by I/R injury (Fig. 3). Additionally, IPC significantly inhibited the I/R-induced increase of LVEDP in WT (P=0.0025) and lenti-shC-infected rats (P=0.0039). However, IPC did not attenuate these effects in lenti-shDJ-1-infected rats. These results indicated that DJ-1 knockdown abrogates the recovery effect of IPC on rat cardiac function following I/R.

**Effect of DJ-1 knockdown on the delayed cytoprotection of IPC against rat myocardial I/R injury.** The effect of DJ-1 knockdown on the delayed cytoprotection of IPC *in vivo* was determined by measuring myocardial infarct size and plasma CK-MB and LDH levels post I/R. As presented in Fig. 4, no myocardial infarction (Fig. 4A) was observed in sham-operated hearts. I/R resulted in significant infarction in the I/R compared with sham group rats (P<0.0001). However, IPC pretreatment significantly decreased I/R-induced myocardial infarction in WT (P=0.0002) and lenti-shC-infected rats (P=0.0002). Notably, the infarct-decreasing effect of IPC was attenuated following knockdown of DJ-1 by lenti-shDJ-1 infection.

The serum levels of the necrotic cell death markers, LDH and CK-MB, were evaluated. I/R significantly increased CK-MB (all P<0.0001; Fig. 4B) and LDH (all P<0.0001; Fig. 4C) release in all groups. IPC significantly attenuated the increases in CK-MB (P=0.0005 and P=0.0033, respectively) and LDH (P=0.0002 and P=0.0049, respectively) levels caused by I/R injury in WT and lenti-shC-infected rats (P<0.01); however, no significant effect was observed following lenti-shDJ-1-infection. These results provided direct evidence that DJ-1 knockdown abrogated the delayed cardioprotective effect of IPC against rat myocardial I/R injury.

**Effect of DJ-1 knockdown on the inhibitory action of IPC on oxidative stress caused by I/R.** Oxidative stress is one of the primary causes of I/R injury; IPC-induced delayed cardioprotection is associated with the attenuation of oxidative stress. As DJ-1 serves an important role in regulating cell survival and oxidative stress, the present study determined whether DJ-1 knockdown abrogates the inhibitory effect of IPC on I/R-induced oxidative stress. To evaluate oxidative stress, MDA content, ROS levels and the activities of SOD, CAT and GPx were measured. As presented in Fig. 5, in WT or lenti-shC-infected rats, IPC attenuated the I/R-induced accumulation of ROS (all P<0.0001; Fig. 5A) and MDA (all P<0.0001; Fig. 5B) and partially reversed I/R-induced effects on the activities of the cellular antioxidant enzymes SOD (P=0.0096 and 0.0032, respectively), CAT (P=0.0037 and P=0.0096, respectively) and GPx (all P<0.0001; Fig. 5C), again indicating that IPC attenuates I/R-induced oxidative stress *in vivo*. However, when DJ-1 expression was specifically knocked down in lenti-shDJ-1-infected rats, the inhibitory effect of IPC on oxidative stress was abrogated. These data demonstrated that DJ-1 is required for the delayed protective effect of IPC against oxidative stress induced by I/R.

**Discussion**

The present study used a well-characterized rat model of I/R and IPC to demonstrate that DJ-1 is involved in the delayed...
Cardioprotection of IPC in vivo. DJ-1 protein expression levels were increased following IPC, peaking at 24 h and being maintained for up to 72 h. This time course was consistent with delayed preconditioning. Targeted in vivo knockdown of DJ-1 using lenti-shRNA abrogated the antioxidative stress effects of IPC, and significantly inhibited the delayed cardioprotection provided by IPC. These findings improve understanding of delayed preconditioning by identifying DJ-1 as an essential molecular effector of this cardioprotective phenomenon in vivo.

It is widely accepted that oxidative stress is a primary cause of I/R injury (26-28), whereas delayed cardioprotection is associated with limited intracellular oxidative stress following I/R (29,30). In the present study, using a well-characterized in vivo model of IPC and I/R, IPC efficiently reduced LDH and CK-MB release, attenuated myocardial infarct size and improved cardiac function when performed 24 h prior to I/R, suggesting that IPC may exert delayed cardioprotection against I/R in vivo. Furthermore, IPC significantly inhibited I/R-induced increases in intracellular ROS production and MDA content, and decreases in the activities of the antioxidant enzymes SOD, CAT and GPx. Therefore, these results additionally demonstrated that the delayed cardioprotection induced by IPC is associated with the attenuation of oxidative stress caused by I/R.

Although the mechanism underlying IPC-induced delayed cardioprotection remains to be fully elucidated, it is clear that the delayed cardioprotection of IPC is dependent upon de novo protein synthesis (5-7). Recent genetic and pharmacological studies have identified aldose reductase, MnSOD and HO-1 as essential antioxidative stress mediators in late preconditioning (8-10). However, as myocardial preconditioning is a complex polygenic adaptation (3), other endogenous antioxidant proteins may be involved.

DJ-1 is a highly conserved and ubiquitously expressed intracellular protein with multiple functions. The inhibition
of oxidative stress is a primary function of DJ-1. To exert this effect, DJ-1 eliminates ROS by self-oxidation (15) and modulates the expression of genes including glutamate cysteine ligase, extracellular SOD (SOD3) and MnSOD by activating Nrf2, a master transcription factor in the redox system (16,31,32). Therefore, DJ-1 may serve as a general survival factor by enhancing cellular antioxidant capacity whilst suppressing ROS production. Consistent with this, Yokota et al (33) reported that cell death induced by hydrogen peroxide exposure was markedly inhibited by overexpression of WT DJ-1, while Taira et al (15) reported that DJ-1 knockdown rendered neuroblastoma cells more susceptible to hydrogen peroxide-induced cell death. These reports further suggest that DJ-1 is a stress responder and may serve a potentially cytoprotective role due to its antioxidant activity. Our previous study demonstrated that hypoxia preconditioning may induce delayed cardioprotection against hypoxia/reoxygenation-induced oxidative stress in an H9c2 cellular model. This was accompanied by enhanced expression of DJ-1, and DJ-1 knockdown abrogated the delayed cardioprotection, indicating that DJ-1 may be involved in the delayed cardioprotection induced by hypoxia preconditioning against oxidative stress caused by hypoxia/reoxygenation (17). However, there was no in vivo evidence that the induction of DJ-1 was responsible for the acquisition of tolerance to I/R in the late phase of IPC. Therefore, the present study used an in vivo model of IPC and I/R to investigate the role of DJ-1. DJ-1 protein expression levels were increased at 12 h, peaked at 24 h and persisted for up to 72 h following IPC. This was in accordance with the time course of the attenuation of cell injury induced by subsequent prolonged ischemia. To further verify the in vivo contribution of DJ-1 to IPC-induced delayed cardioprotection, the effect of DJ-1 knockdown in situ was investigated. IPC efficiently reduced LDH and CK-MB release, attenuated myocardial infarct size and improved cardiac function following I/R injury in WT rats, but not in lenti-shDJ-1-infected rats, suggesting that DJ-1 is required for the delayed cardioprotective effect induced by IPC.

DJ-1 protein serves a critical role in the regulation of cell viability and oxidative stress. Oxidative stress is a primary cause of I/R injury, and delayed cardioprotection of IPC is associated with the attenuation of oxidative stress. Therefore, it was hypothesized that DJ-1 may be essential for the antioxidant stress effect of IPC. To test this hypothesis, the effect of DJ-1 knockdown on oxidative stress during the late phase of IPC was investigated. IPC attenuated the I/R-induced production of ROS and MDA and maintained the activities of the cellular antioxidant enzymes SOD, CAT and GPx. However, following DJ-1 knockdown by lenti-shRNA, the antioxidative stress effects of IPC were abrogated. Taken together, these data provide, to the best of our knowledge, the first in vivo evidence to suggest that DJ-1 has a crucial role in the antioxidative stress effects of late phase IPC, and further demonstrated that DJ-1 contributes to the delayed protection induced by IPC via its antioxidant action.

In conclusion, the present study identified DJ-1 as an essential mediator responsible for the beneficial effects of the late phase of IPC in vivo. In addition, the results of the present study suggested that IPC exerts delayed cardioprotective effects by a hitherto unrecognized underlying mechanism; the attenuation of I/R-induced oxidative stress via the upregulation of DJ-1. These findings provide a basis for the further investigations that are required to elucidate the detailed molecular mechanism underlying the effect of DJ-1 in the delayed protection of myocardial preconditioning in vivo.
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