European Journal of Pharmacology 780 (2016) 26–32

Contents lists available at ScienceDirect

European Journal of Pharmacology

journal homepage: www.elsevier.com/locate/ejphar

Cardiovascular pharmacology

Pinacidil-postconditioning is equivalent to ischemic postconditioning in defeating cardiac ischemia-reperfusion injury in rat

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ARTICLE INFO

Article history:
Received 22 December 2015
Received in revised form
11 March 2016
Accepted 15 March 2016
Available online 17 March 2016

Keywords:
Myocardial
Ischemia-reperfusion injury
Pinacidil
Mitochondria
Nrf2

ABSTRACT

Ischemic postconditioning (IPO) had been reported as a promising method against myocardial ischemia-reperfusion (I/R) injury, but IPO was later proved with poor clinical benefit. In this study, we compared the protective effects of pinacidil-postconditioning (PPO) and IPO against myocardial I/R injury. Langendorff rat hearts were randomly assigned to one of the following groups (n=8 each): Control group, I/R group (40 min ischemia and 60 min reperfusion), IPO group (6 successive cycles of 10 s reperfusion per 10 s occlusion before fully reperfusion), PPO group (perfused with 50 μM pinacidil for 5 min before reperfusion). Heart performance, infarct size and mitochondrial respiratory function were evaluated, and target genes/proteins of well-known Nuclear Factor-E2 Related Factor 2 (Nrf2) were assessed. Both IPO and PPO preserved heart function and myocardial ultrastructure at the end of reperfusion (all $P<0.05$ vs. I/R). The expression of Nrf2, NADH-quinone oxidoreductase-1 (NQO1), heme oxygenase 1 (HO-1) and superoxide dismutase 1 (SOD1) were similarly increased after IPO and PPO treatment (all $P<0.05$ vs. I/R). PPO exerted solid effect in defeating cardiac ischemia-reperfusion injury in rat.

1. Introduction

Early reports showed that ischemic postconditioning (IPO) was as effective as preconditioning in reducing infarct size, creatine kinase and preserving endothelial function of hearts which went through ischemia-reperfusion (I/R) injury (Zhao et al., 2003; Staat et al., 2005). To that end, a great number of clinical studies have investigated the effectiveness of IPO in human hearts, however, many of the these studies found IPO showed little effect in infarct size control. IPO brought little change when it was estimated by some more sensitive indicator such as troponin T (TnT), CK-MB, troponin I (TnI), magnetic resonance imaging (MRI) or cardiac magnetic resonance (CMR) (Freixa et al., 2012; Limalanathan et al., 2014; Sorensson et al., 2010).

IPO's myocardial protective effects may be associated with opioids, bradykinin, adenosine and other substances released during ischemia triggered cardioprotective signaling through their receptors, which ultimately prevents mitochondria from formation of lethal permeability transition pores (Cohen and Downey, 2015). In fact, these signaling can be pharmacologically activated. Our previous studies showed that artificially open adenosine triphosphate-sensitive potassium channel (Kir1.1), either the mitochondrial Kir1.1 (Cao et al., 2014) or sarcolemmal Kir1.1 (Yang and Yu, 2010b), can effectively reduce cardiac I/R injury. Pinacidil, a non-selective Kir1.1 opener, provides solid cardioprotective effect when it was added in the preservation solution. Pinacidil's cardioprotective effect was associated with ATP preservation resulted from the opening of mitochondrial Kir1.1. In addition, pinacidil postconditioning (PPO) has recently been proved to be cardioprotective in I/R heart.(Yang and Yu, 2010b).

The current study was therefore designed to compare the effects of PPO and IPO against myocardial I/R injury. Based on our previous data and the drawback of IPO, we hypothesize that the postconditioning with pinacidil would be an alternative strategy of IPO in reducing I/R injury in rat heart.

2. Material and methods

2.1. Animals

Male Sprague-Dawley rats, weighing 200–250 g, were supplied by the Center of Laboratory Animals in Third Military Medical

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http://dx.doi.org/10.1016/j.ejphar.2016.03.027
0014-2999/

University (Chongqing, China). Rats were housed in cages with *ad libitum* access to food and water and the room temperature was maintained at 24 ± 1 °C. All animals received humane care in compliance with the Guide for the Care and Use of Laboratory Animals in China (no. 14,924, 2001) and all experimental protocols were approved by the Zunyi Medical College Animal Care and Use Committee.

2.2. Langendorff reperfusion protocol

Reperfusion Protocol was prepared as we previously did (Yu et al., 2011; Yu et al., 2001). The rats were anesthetized with an intraperitoneal injection of 1% sodium pentobarbital (35 mg/kg) and heparin (250 U/kg). Sternotomy and rapid cardiectomy were performed and the isolated heart was put in precooled Krebs-Henseleit (K-H) buffer (4 °C). The aorta was rapidly connected to the Langendorff system by a perfusion column and perfused with K-H solution preheated at 37 °C (buffered with a gas mixture of 95% O_2 and 5% CO_2 for 60 s at a constant pressure of 7.5 mmHg). The perfusion pressure was maintained at 70–80 mmHg. After beating heart recovery, a microstomia cut was made at the left atrial appendage, and a fluid-filled latex balloon was inserted into the left ventricle through the mitral valve. The latex balloon was connected to a pressure transducer of Powerlab/8sp biological function test system. By adjusting the size and position of the latex balloon, the left ventricular end-diastolic pressure (LVEDP) was controlled between 7 and 9 mmHg. Above steps needed to be done within two mins after the microstomia cut was made. Criteria for successful perfusion models include: At the end of 20 min equilibrium perfusion, heart rate $\geq$250 beats/min, left ventricular developed pressure (LVDP) > 80 mmHg, ventricular premature beat < twice/min.

After a 20 min equilibration of K-H solution (37 °C), these isolated hearts were distributed into 4 groups: Control group (Control, n=8), continuous perfusion of K-H solution with 100 min; Ischemia-reperfusion group (I/R, n=8), undergoing 40 min ischemia and 60 min reperfusion; Ischemic postconditioning group (IPO, n=8), 40 min ischemia plus 6 cycles of 10 s reperfusion inserted by 6 cycles of 10 s ischemia were performed before 58 min reperfusion; Pinacidil-postconditioning group (PPO group, n=8), 40 min ischemia plus 5 min perfusion of K-H solution containing pinacidil (Sigma, Shanghai, China) 50 μM before 55 min reperfusion(Fig. 1). Heart function parameters including left ventricular end diastolic pressure (LVEDP), left ventricular developed pressure (LVDP), heart rate (HR), maximum rate of pressure change (dp/dtmax) and double product (DP=LVDP × HR) were recorded at the end of equilibration and reperfusion, and approximately 100 mg of myocardium of left ventricle was removed and quick frozen in liquid nitrogen for later ultrastructural and genes/proteins detection.

2.3. Measurement of myocardial infarct size

At the end of the reperfusion period, hearts were sectioned transaxially into five slices, and incubated in 1% TTC (Solarbio, Beijing, China) for 25 min at 37 °C. The tissue sections were then fixed in 10% formalin overnight at 8–12 °C and then photographed with a digital camera. The areas of normal (stained deep red) and infarcted ventricular myocardium (appeared yellow white) were measured with planimetry software (Image J for Windows version 1.4, National Institutes of Health, USA). The infarct size of the ventricle was calculated as the ratio (%) of cumulative infarct area to the entire ventricle area.

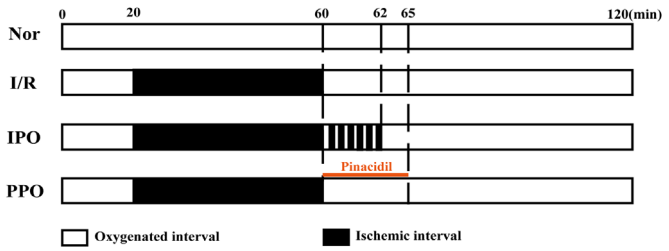

Fig. 1. Reperfusion Protocol. Rats were allocated into 4 groups: normal (Nor), I/R, IPO and PPO. Rat hearts were equilibrated for at least 20 min before the application of above protocols. Excluding Nor group, all hearts underwent ischemia for 40 min (indicated with black horizontal bar). I/R group were exposed to 40 min ischemia alone. Hearts in IPO group underwent IPO (palisade white and black bars) alone while pinacidil was administrated in the first 5 min of reperfusion in PPO group.

2.4. Morphologic evaluation of myocardium

Myocardiums in the non-infarcted area in the left ventricular from different groups were harvested immediately fixed in 3% paraformaldehyde and 0.25% glutaraldehyde solution, then washed in the buffer supplemented with 220 mM sucrose and post-fixed with 1% osmic acid. Dehydration in acetone and embedding in ethoxyline 618 were done by standard procedures. Ultrathin sections were cut from each sample and examined with electron microscope (H7500, HITACHI Japan). The extent of ultrastructural damage was scored by Flameng grading detailed elsewhere (Han et al., 2010): grade 0 showed integrated granules but the structure of mitochondria was normal; grade 1, the granules were lost but the structure of mitochondria was normal; grade 2, the granules were lost plus swelling mitochondria and limpid matrix; grade 3, the granules were lost plus limpid matrix and ruptured cristae; grade 4, the granules were lost plus ruptured inner and external membranes.

2.5. Myocardial mitochondria extraction

The mitochondria were prepared as Kim et al. (2006) and Jiang et al. (2005) reported but with some necessary modifications. Briefly, hearts were cut and placed in ice-cold mitochondrion-separating medium containing 225 mM mannitol, 75 mM sucrose, 10 mM Tris-HCl, and 1 mM ethylenediamine tetraacetic acid at pH 7.4. Tissue was then dissociated by a high-speed disperser. The homogenate was first centrifuged at 600 g for 7 min to eliminate nuclei and cell debris, and the supernatant was then centrifuged at 10,000 g (4 °C) for 11 min to get precipitated mitochondria. The mitochondria were washed by separating medium and isolated by dispersing medium containing 150 mM KCl, 5 mM Tris–HCl, and 1 mM ethylenediamine tetraacetic acid at pH 7.4. The mitochondrial protein quantization was tested by Bradford method and kept between 1.0 and 2.0 mg/ml. The isolated mitochondria were stored at −80 °C.

2.6. Mitochondrial respiratory function test

The mitochondrial respiratory function was detected with a computer-controlled Clark-type O_2 electrode unit (Hansatech Instruments, Pentney, UK) according to the protocol described previously (Yang and Yu, 2010a). Briefly, 0.5 ml mitochondrial suspension was added to 2.5 ml of assay buffer at 30 °C. The assay buffer contained 2 mM hydroxyethylpiperazine ethanesulfonic acid, 70 mM sucrose, 220 mM D-mannitol, and KH_2PO_4 with pH 7.0. Oxygen consumption curves were recorded and measured in the absence (state 4 respiration) and presence of ADP (50 μl of 100 mM ADP was add for state 3 respiration) and succinate (50 μl of 250 mM succinate was add). The respiratory control ratio (RCR)