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Cardioprotective time-window of Penehyclidine hydrochloride postconditioning: A rat study

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ABSTRACT

Pharmacological postconditioning offers a clinical perspective for all patients with ischemic heart disease. Penehyclidine hydrochloride (PHC) is a new type of anticholinergic drug. We previously reported that PHC preconditioning protects against I/R injury in rat hearts *in vivo*. Ischemic heart disease often occurs suddenly, so postconditioning is more significant than preconditioning. However, studies evaluating myocardial protective effects of PHC postconditioning are unavailable. We explored the effects and time-window of cardioprotection of PHC postconditioning in myocardial I/R injury. PHC was administered by intravenous at various times (t = −5, 0, 5, 10, 15, or 30 min) after the onset of reperfusion in addition to I/R rat. We observed five different indicators including infarct size, inflammatory response, myocardial enzyme, oxidative stress, and Ca^{2+} overload to quantify the effect of cardioprotection. Evans blue and TTC staining were used to measure myocardial infarct size. The expression of NF-κ B and IκB-α was analyzed using Western blot. ELISA was conducted to detect inflammatory and anti-inflammatory mediators. The Ca^{2+} level was determined using assay kit. PHC postconditioning (from −5 to 10 min after the onset of reperfusion) significantly reduced infarct size, downregulated NF-κ B expression, and decreased the release of inflammatory mediators, while significantly upregulating IκB-α expression and increasing the release of anti-inflammatory mediators. All PHC postconditioning groups significantly reduced Ca^{2+} level. PHC postconditioning is cardioprotective over a larger time-window (from −5 to 10 min after the onset of reperfusion). The probable mechanism is inhibition of NF-κB regulated inflammatory response pathway.

1. Introduction

Ischemic heart disease, which is one of the biggest healthcare challenges worldwide, is the leading cause of disability and death (Sacks et al., 2015). Rapid reperfusion of ischemic myocardium, such as primary percutaneous coronary intervention, thrombolysis, or coronary artery bypass graft, is the best treatment for attenuation of ischemic heart disease. However, reperfusion *per se* causes deleterious effects, such as lethal reperfusion injury, ventricular arrhythmias, myocardial stunning, and absence of reflow (Barrere-Lemaire et al., 2012). Therefore, attenuation of ischemia/ reperfusion (I/R) injury associated with the therapy of ischemic heart disease is very important. Development of novel drugs to improve clinical outcomes is an unmet medical need. Pharmacological postconditioning is a new alternative way, which can simulate the role of postconditioning (Cour et al., 2011). The concept of pharmacological postconditioning offers a clinical perspective for all patients with ischemic heart disease. Anticholinergic agents may play a helpful role in this respect.

Penehyclidine hydrochloride (PHC), which has been clinically used before surgical operations, is a new type of anticholinergic drug (Sun et al., 2013; Wang et al., 2013). It can selectively act on muscarinic 1 and 3 receptors (Ma et al., 2013; Wang et al., 2005; Xiao et al., 2009). Compared with other anticholinergic drugs, the specificity of PHC is that it has no muscarinic 2 receptor-associated cardiovascular side effects (Ma et al., 2013). So, PHC doesn't increase the heart rate. In recent years, with the exploration of organ-protective effects in I/R injury, PHC has attracted much attention. Animal studies have shown that PHC offers organ-protective effects, such as neuroprotective (Ma et al., 2013; Yu and Wang, 2013), lung protective, (Zhan et al., 2015) and renal protective (Wang et al., 2014). We previously reported that high dose (1 mg/kg) of PHC preconditioning protects against I/R injury in rat hearts *in vivo* (Lin et al., 2015). PHC provides organ protection effects against I/R injury by inhibiting apoptosis, attenuating oxidative stress, suppressing inflammatory response (Lin et al., 2015; Wang et al., 2014; Yu and Wang, 2013). Ischemic heart disease often occurs suddenly, so postconditioning is more significant than

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preconditioning. However, we looked up domestic and foreign literatures and found no related data about myocardial protective effects of PHC postconditioning.

We tested the hypothesis that PHC postconditioning may provide a cardioprotection in a rat model of myocardial I/R. First, we explored whether PHC postconditioning leads to myocardial protection in the rat model of myocardial I/R. Second, we identified the time-window of PHC postconditioning. This was the first study in the field. Third, we explored the possible myocardial protective mechanism of PHC postconditioning.

2. Material and methods

Ethical approval for this study (grant number 2014-X-6) was provided by the Ethics Committee of Animal Experiments and Experimental Animal Welfare Committee of Capital Medical University, Beijing, China (Chairperson Prof Wang) on 9 January 2014. The experiments adhered to the ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines. All rats received humane care according to National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 85-23, revised 1996).

2.1. Experimental model

Wistar rats (male, 220–250 g) aged 8 weeks were provided by Charles River Laboratories (Beijing, China). The rats were kept in standard cages: at 22 °C with a cycle of 12:12 light/dark in adjusted surroundings with free access to tap water and food. After intraperitoneal injection of 10% chloral hydrate (300 mg/kg), the rats were anesthetized, and then ventilated with a rodent ventilator (Jiangxi TELI Anaesthesia & Respiration Equipment Co., Ltd, Jiangxi, China) at a rate of 80 times/min with a volume of 6–8 ml/kg. After thoracotomy, the rat's heart was exposed. Surgical silk suture was applied to occlude the left anterior descending (LAD) coronary artery to establish I/R model. Coronary artery was subjected to 30 min ischemia followed by 3 h reperfusion. At the end of the experiments, the rats were euthanized by injection of potassium chloride.

2.2. Experimental design

PHC was provided by List Pharmaceutical Co., Ltd (Chengdu, China), and stored in a dry and cool place, a sealed preservation. PHC injection was prepared freshly as it was used in the experiments. PHC (1 mg/ml) was diluted in normal saline. Healthy male rats were evenly and randomly categorized into 9 experimental groups (n = 12 each group). Sham group, involving rats undergoing sham surgery; I/R group: rats were subjected to 30 min of ischemia followed by 3 h reperfusion. Sham+PHC group comprising rats administrated with PHC (1 mg/kg) intravenous at 25 min after sham operation; I/R+PHC postconditioning group: PHC (1 mg/kg) was administered by intravenous at various times (t = −5, 0, 5, 10, 15, or 30 min) after the onset of reperfusion in addition to I/R. I/R+PHC postconditioning group comprised 6 subgroups, including PHC-t-5 group (5 min before reperfusion), PHC-t0 group (0 min before reperfusion), PHC-t5 group (5 min after the onset of reperfusion), PHC-t10 group (10 min after the onset of reperfusion), PHC-t15 group (15 min after the onset of reperfusion), and PHC-t30 group (30 min after the onset of reperfusion). Experimental design was shown in Fig. 1.

2.3. Hemodynamic parameters

Heart rate and mean arterial pressure (MAP) were determined by the method of noninvasive pressure monitoring on the rat tail artery. The values of the heart rate and MAP were recorded at the 3 different time points: before sham surgery or the left anterior descending was occluded (baseline); at the 0 min before reperfusion (t0); and at the 3 h after the onset of reperfusion (t3h).

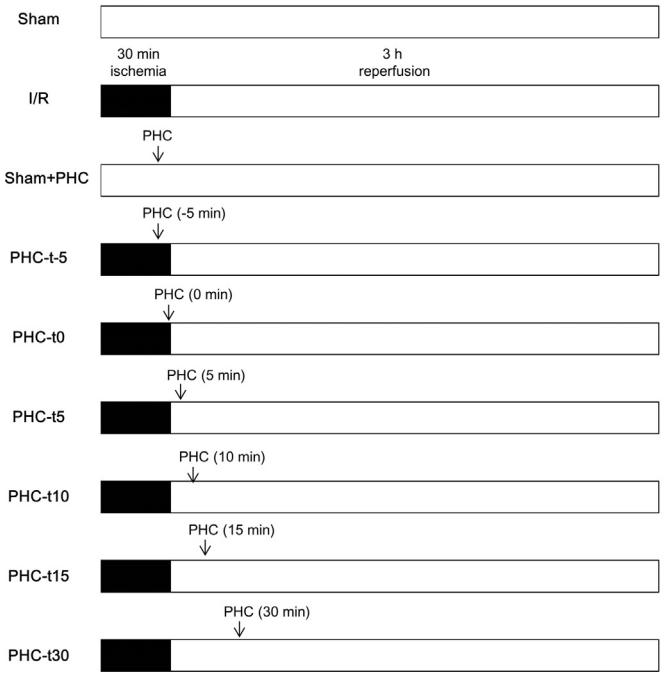

Fig. 1. Experimental design. Rats underwent myocardial Sham, I/R, Sham+PHC, and I/R+PHC postconditioning. I/R+PHC postconditioning group: PHC (1 mg/kg) was administered by intravenous at various times (t = −5, 0, 5, 10, 15, or 30 min) after the onset of reperfusion in addition to I/R (30 min of ischemia followed by 3 h reperfusion). The black box represents the period of ischemia (30 min). The blank box represents the period of perfusion or reperfusion.

2.4. Infarct size

Myocardial Evans blue-TTC staining was used to analysis the infarct size as previously described (Basalay et al., 2012; Lin et al., 2015; Roubille et al., 2011). After reperfusion for 3 h, the LAD was occluded again and 2% Evans blue dye (Beijing Solarbio Science & Technology Co., Ltd, Beijing, China) was injected into the jugular vein. The rat was then euthanized, and its heart was excised. The removed heart was frozen (−20 °C, 30 min), and cut into slices. The slices were incubated with 2% 2,3,5-triphenyltetrasolium chloride (TTC) (Beijing Solarbio Science & Technology Co., Ltd, Beijing, China) in the dark (37.5 °C, 20 min) room. A digital camera was used to take pictures. The infarct size was measured by a blind observer using Image software (Syngene, MD, USA).

2.5. Western blot

Rat heart was harvested and the protein extract was analyzed by Western blot. Nuclear factor-kappa B (NF-κB) and inhibitor of NF-κ B (IκB-α) concentrations were detected by protein assay kits (Beyotime, Beijing, China). Briefly, at the end of 3 h reperfusion, the rat was euthanized. The heart was excised and washed with normal saline. The removed heart was frozen in liquid nitrogen. The left ventricle was cut into slices. IκB-α protein was extracted by using a cytosol extraction kit (Wanleibio, Shenyang, China). NF-κB protein was extracted by using a nuclear protein extraction kit (Wanleibio, Shenyang, China). The sample protein concentration was quantified by protein quantification assay kit (Wanleibio, Shenyang, China). Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) was used to analyze the protein expression. The equal amount of protein (40 μg) was separated by 12% SDS-PAGE. The resolved proteins were transferred onto a polyvinylidene fluoride (PVDF) membrane. The membrane was washed for 5 min in Tris-buffered saline with 0.1% Tween 20 (TTBS), and then blocked for 1 h in 5% nonfat milk. The blocked PVDF membrane was incubated overnight at 4 °C with NF-κB antibody, or