European Journal of Pharmacology 765 (2015) 472–481

Contents lists available at ScienceDirect

European Journal of Pharmacology

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

Cardiovascular pharmacology

Inhibition of the nitric oxide/cyclic guanosine monophosphate pathway limited the cardioprotective effect of post-conditioning in hearts with apical myocardial infarction

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ARTICLE INFO

Article history:
Received 15 April 2015
Received in revised form
9 September 2015
Accepted 14 September 2015
Available online 18 September 2015

Keywords:
Post-conditioning
Isoproterenol
Reperfusion injury
cGMP

ABSTRACT

Reperfusion damage involves opening of the mitochondrial permeability transition pore (mPTP) and loss of ATP synthesis. Several cardioprotective pathways are activated by ischemic or pharmacological post-conditioning (PC). The mechanisms that are activated by PC in no co-morbidity murine models include: activation of rescue kinases, oxidative stress reduction, glycolytic flux regulation and preservation of ATP synthesis. However, relatively scarce efforts have been made to define whether the efficacy of PC signaling is blunted by risk factors or systemic diseases associated with ischemic heart pathology. Experimental evidence has shown that the nitric oxide (NO)/cyclic guanosine monophosphate (cGMP) signaling is a main mechanism activated by PC in hearts without pathological history. In this work we evaluated the participation of the NO pathway, through downstream kinase activation and inhibition of mPTP in hearts with previous infarct.

Myocardial infarction was induced with a single dose of isoproterenol (85 mg/kg i.p.) to male Wistar rats. After 24 h, the hearts were mounted into the Langendorff system and subjected to 30 min of ischemia and 60 min of reperfusion. PC consisted of 5 cycles of 30 s of reperfusion/30 s of ischemia, then the hearts were reperfused with or without inhibitors of the NO/cGMP pathway.

PC activates the NO/cGMP pathway, as increased cGMP and NO levels were detected in isoproterenol-treated hearts. The cardioprotective effect of PC was abolished with both L-NAME (inhibitor of constitutive NO synthase) and ODQ (inhibitor of soluble guanylate cyclase), whereas the NO donor (DETA-NO) restored cardioprotection even in the presence of L-NAME or ODQ. We also found that mitochondrial structure and function was preserved in PC hearts.

We conclude that PC exerts cardioprotection in hearts with previous infarct by maintaining mitochondrial structure and function through NO-dependent pathway.

1. Introduction

Cardiovascular ischemic diseases affect a high proportion of world population, being the leading cause of mortality and disability in adults. Timely reperfusion promotes cardiomyocyte survival, decreasing morbidity and mortality. Paradoxically, reperfusion of ischemic tissue by thrombolysis, percutaneous coronary intervention, coronary artery bypass grafting or cardiac transplantation, may result in additional cardiomyocyte dysfunction, a phenomenon termed “reperfusion injury”. In this sense, post-conditioning (PC) has emerged as a powerful strategy to contend against reperfusion injury in animals models, as well as in

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http://dx.doi.org/10.1016/j.ejphar.2015.09.018
0014-2999/

some clinical studies (Zhao et al., 2003). It consists of the application of brief cycles of ischemia–reperfusion, after ischemia and before prolonged reperfusion. PC has re-attracted scientific and clinical interest on reperfusion injury, particularly due to the acquired knowledge of the cellular and molecular events activated during the early phase of reperfusion (Costa et al., 2008; Zhao et al., 2003). However, most of PC studies had been performed in health and young animals. The application of experimental knowledge on the clinical practice requires demonstration of PC effectiveness in heart diseases resulting from co-morbidities.

In this sense, it has been described that patients with non ST-elevation acute coronary syndrome are at high risk for recurrent ischemic events due to complex coronary artery disease (CAD), and had lesser possibilities to be completely revascularized. Also, a percentage of patients ≥ 70 years of age with unstable angina discharged after myocardial infarction (PREVESE Study) and followed during three months, presented a second ischemic event (2.2%); whereas 11.2% developed angina (Kumbhani et al., 2013; de Velasco et al., 1997).

Isoproterenol (ISO) hydrochloride, a synthetic catecholamine and β-adrenergic agonist causes severe stress in the myocardium resulting in infarct-like necrosis damage in animal's models (Díaz-Muñoz et al., 2006). The pathophysiological changes produced by ISO in rat hearts are comparable to those observed in human myocardial infarction. Particularly cardiac apoptosis and/or necrosis, increase the incidence of cardiac arrhythmias (Tappia et al., 2001; Stelzner et al., 1987). ISO generates free radicals leading to lipid peroxidation and membrane permeability alterations (Sushamakumari et al., 1989; Tood et al., 1980). Augmented oxidative stress depresses sarcolemmal Ca^{2+} transport, promoting intracellular Ca^{2+} overload and ventricular dysfunction (Prabhu et al., 2006). It has also been reported that ISO decreases the antioxidant defense mechanisms (Chien et al., 1978) and that β-adrenergic receptors stimulates apoptosis in myocytes through reactive oxygen species (ROS) dependent activation of the mitochondrial pathway (Remondino et al., 2003).

As it is known that the nitric oxide/cyclic guanosine monophosphate (ON/cGMP) pathway is involved in PC protection in isolated rat hearts (without co-morbidity) (Penna et al., 2006) and, that ROS attenuation depends on the antioxidant action of NO (Todd et al., 1980), the aim of the present investigation was to demonstrate the effectiveness of PC in a heart model with previous infarct induced by ISO, to evaluate the robustness of the NO/cGMP pathway to confer cardioprotection and its impact on mitochondrial structure and function.

2. Materials and methods

This investigation was performed in accordance with the Guide for the Care and Use of Laboratory Animals, published by the United States National Institutes of Health (US-NIH).

2.1. Cardiac function in post-conditioned hearts from isoproterenol-treated rats

Male Wistar rats, weighing 250–300 g and provided with food and water *ad libitum*, were injected subcutaneously with a single dose of ISO (85 mg/kg/day) between 08:00 and 09:00 a.m.. Control animals received a subcutaneous injection of saline solution.

After 24 h the animals were anesthesized with sodium pentobarbital (60 mg/kg) and sodium heparine (100 U/kg). Five min after heparin injection, a midsternal thoracotomy was performed and the heart was rapidly excised and placed in ice-cold Krebs–Henseleit buffer solution of pH 7.4, consisting of 118 mM NaCl, 4.75 mM KCl, 1.18 mM KH_2PO_4, 1.18 mM $MgSO_4 \cdot 7H_2O$, 5 mM $CaCl_2$, 25 mM $NaHCO_3$, 5 mM glucose, and 100 μM sodium octanoate. Within 45 s, the heart was mounted onto a Langendorff heart perfusion system. The hearts were perfused retrogradely via the aorta at a constant flux of 12 ml/min with Krebs–Henseleit solution, which was continuously bubbled with 95% O_2 and 5% CO_2 at 37 °C.

Cardiac performance was measured at left ventricular end-diastolic pressure (LVEDP) of 10 mm Hg using a latex balloon inserted into the left ventricle and connected to a pressure transducer (Correa et al., 2008). Throughout the experiment, left ventricular developed pressure (LVDP) was continuously recorded using a computer acquisition data system designed by the Instrumentation and Technical Development Department of the National Institute of Cardiology (México, D.F., México). Heart rate (HR) expresses beat number/min. Cardiac contractile function was calculated by subtracting LVEDP from left ventricular peak systolic pressure, yielding LVDP. The double product (DP) was calculated by multiplying HR by LVDP. All variables were recorded using a computer acquisition data system designed by the Instrumentation and Technical Development Department of the National Institute of Cardiology (México, D.F., México).

The heart was perfused till stabilization with Krebs buffer for 20 min. The ISO hearts were subjected to global ischemia for 30 min by turning off the pumping system and then to 60 min of reperfusion (ISO+I/R). The post-conditioning maneuver consisted of five cycles of ischemia-reperfusion (30 s reperfusion and 30 s ischemia per cycle), followed by 60 min of reperfusion (ISO+I/R+PC). Hearts from the control group were also evaluated to compare functional and biochemical changes induced by isoproterenol, but were not subjected neither to I/R or PC.

2.2. Measurement of infarct size

At the end of the experiments the hearts to be used for infarct size calculations were frozen at −20 °C. Heart slices of ~3 mm were obtained and immersed in 1% triphenyltetrazolium chloride solution in phosphate buffer (8.8 mM Na_2HPO_4, 1.8 mM NaH_2PO_4, pH 7.4) for 10 min at 37 °C; the slices were incubated in a solution of formalin for 5 min, and digitalized on a Hewlett-Packard Scanjet 3800 scanner (Hewlett-Packard, Palo Alto, CA, USA). In each image the risk and the infarct zones were traced and the respective areas were calculated in terms of pixels using the software Image J®. Other group of hearts was assigned for histological analysis with the haematoxylin/eosin technique and electron microscopy. To study the mitochondrial ultrastructural morphology, small cardiac tissue fragments were obtained and immersed into 10% glutaraldehyde dissolved in cacodylate buffer pH 7.2. Then, the fragments were deposited into glass tubes and fixed by immersion in the same solution during 24 h at 4 °C. After extensive washing with cacodylate buffer, tissue fragments were post-fixed with osmium tetraoxide, dehydrated in graded ethyl alcohol solutions and embedded in Epon resin (London Resin Company, London, UK). Thin sections from 70 to 90 nm were placed on cooper grids, contrasted with lead and uranium salts and examined with a FEI Technei electron microscope.

2.3. Echocardiography analysis

Echocardiographic images were obtained using a Sonos 5500 echocardiographer (Koninlijke Philips Electronics, Eindhoven, NL) with a 12 MHz transducer. Parasternal long and short axis views were analyzed in the anesthetized rats. Two-dimensional-guided (2D) M-mode echocardiography was performed and determinations were made from at least 3 beats in each rat. Left ventricular (LV) cavity and wall thickness were measured to calculate the ejection fraction (EF) as follows: $\%EF = [(EDV - ESV/EDV) \times 100]$