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Original article

Mechanisms involved in postconditioning protection of cardiomyocytes against acute reperfusion injury

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ABSTRACT

Experimental and clinical studies demonstrated that postconditioning confers protection against myocardial ischemia/reperfusion injury. However the underlying cellular mechanisms responsible for the beneficial effect of postconditioning are still poorly understood. The aim of the present study was to examine the role of cytosolic and mitochondrial Ca^{2+}-handling. For this purpose adult rat cardiomyocytes were subjected to simulated *in vitro* ischemia (glucose-free hypoxia at pH 6.4) followed by simulated reperfusion with a normoxic buffer (pH 7.4; 2.5 mmol/L glucose). Postconditioning, i.e., 2 repetitive cycles of normoxic (5 s) and hypoxic (2.5 min) superfusion, was applied during the first 5 min of reoxygenation. Mitochondrial membrane potential ($\Delta\Psi_m$), cytosolic and mitochondrial Ca^{2+} concentrations, cytosolic pH and necrosis were analysed applying JC-1, fura-2, fura-2/manganese, BCECF and propidium iodide, respectively. Mitochondrial permeability transition pore (MPTP) opening was detected by calcein release. Hypoxic treatment led to a reduction of $\Delta\Psi_m$, an increase in cytosolic and mitochondrial Ca^{2+} concentration, and acidification of cardiomyocytes. During the first minutes of reoxygenation, $\Delta\Psi_m$ transiently recovered, but irreversibly collapsed after 7 min of reoxygenation, which was accompanied by MPTP opening. Simultaneously, mitochondrial Ca^{2+} increased during reperfusion and cardiomyocytes developed spontaneous cytosolic Ca^{2+} oscillations and severe contracture followed by necrosis after 25 min of reoxygenation. In postconditioned cells, the collapse in $\Delta\Psi_m$ as well as the leak of calcein, the increase in mitochondrial Ca^{2+}, cytosolic Ca^{2+} oscillations, contracture and necrosis were significantly reduced. Furthermore postconditioning delayed cardiomyocyte pH recovery. Postconditioning by hypoxia/reoxygenation was as protective as treatment with cyclosporine A. Combining cyclosporine A and postconditioning had no additive effect. The data of the present study demonstrate that postconditioning by hypoxia/reoxygenation prevents reperfusion injury by limiting mitochondrial Ca^{2+} load and thus opening of the MPTP in isolated cardiomyocytes. These effects seem to be supported by postconditioning-induced delay in pH recovery and suppression of Ca^{2+} oscillations. This article is part of a Special Issue entitled "Calcium Signaling in Heart".

1. Introduction

Reperfusion of the myocardium, after a prolonged ischemic period, is associated with ultrastructural changes, which can finally lead to irreversible injury. Prevention of the reperfusion injury is still a challenging therapeutic goal for cardiologists. Postconditioning is a promising strategy that protects human hearts, i.e. it improves cardiac function and reduces infarct size in reperfused hearts following ischemia/reperfusion [1–3].

The pioneer study of postconditioning was performed in dogs [4], in which the alternating cycles of 30 s reperfusion and 30 s occlusion, i.e. postconditioning, were applied at the onset of reperfusion. Postconditioning decreased infarct size, tissue edema, and neutrophil accumulation in the myocardial area at risk and improved post-ischemic endothelial function. The follow-up studies applying various experimental models, including isolated cardiomyocytes, confirm the protective potential of ischemic postconditioning [5,6].

Mitochondrial permeability transition pores (MPTP) play an important role in the cardiomyocyte injury during initial phase of

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reoxygenation/reperfusion. It has been reported that MPTP are a key player in the cardioprotection afforded by postconditioning [7,8] and the pharmacological inhibition of MPTP with cyclosporine A leads to protection of the reperfused heart in acute myocardial infarction [9], including the human heart [10]. Opening of MPTP is associated with the collapse of the mitochondrial membrane potential and disruption of the permeability barrier of the inner mitochondrial membrane [11,12]. However, during ischemia the MPTP remains inhibited due to low pH. With the start of reperfusion the mitochondria resume their respiration and certain factors, like Ca^{2+} overload and oxygen radicals, favor the opening of the MPTP [11]. The only restraining factor at this stage is the acidic pH, but this returns back to normal within the early minutes of reperfusion [11]. The cell death resulting from the sudden recovery of intracellular pH during the early minutes of reperfusion has been described as the "pH paradox", and, indeed, prolongation of acidosis acts protective during early reperfusion and contributes to postconditioning's protection. [13–15].

Aside of pH modulation, the role of spontaneous Ca^{2+} oscillations during initial phase of reoxygenation for the reoxygenation-induced injury was suggested [16]. These Ca^{2+} oscillations are due to a repetitive uptake and release of Ca^{2+} by the sarcoplasmic reticulum (SR). Interventions directed to reduce the spontaneous Ca^{2+}-oscillations by interfering with the Ca^{2+}-uptake or -release can prevent the reperfusion-induced injury of cardiac myocytes [16,17].

It has been also shown that under physiological conditions mitochondria actively reply to SR-mediated Ca^{2+} release by Ca^{2+} uptake due to anatomical proximity to SR organized in functional intracellular microdomains [18,19]. These spontaneous Ca^{2+} release from the SR may contribute to mitochondrial Ca^{2+}-load during reperfusion, which thus may promote the MPTP opening resulting in a generation of mitochondrial ROS. Indeed, the interplay between the Ca^{2+} cycling and MPTP has been recently found to be an important cause of the loss of mitochondrial membrane potential and death of cardiac myocytes during reoxygenation [16]. Whether postconditioning may affect this Ca^{2+} handling was unknown. Therefore, we aimed to investigate the effects of postconditioning on the cytosolic and mitochondrial Ca^{2+} handling in relation to MPTP opening and cell death.

2. Material and methods

2.1. Isolation of cardiomyocytes

The investigation conforms to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1996). Cardiac myocytes were isolated from 200 to 250 g male Wistar rats as previously described [20].

2.2. Experimental protocol

Experiments were performed with a HEPES buffer (composition in mmol/L: 140.0 NaCl, 2.6 KCl, 1.2 KH_2PO_4, 1.2 $MgSO_4$, 1.3 $CaCl_2$, 2.5 glucose and 25.0 HEPES). To simulate ischemia the pH of the buffer was adjusted to 6.4 at 37 °C, glucose was omitted, and the buffer was equilibrated with 100% N_2 before and during experiments. To simulate reperfusion buffer pH was adjusted to 7.4 at 37 °C, glucose was added, and the buffer was equilibrated with air as previously described [21]. In the postconditioning group 2 repetitive cycles of normoxic (5 s) followed by hypoxic (2.5 min) superfusion were applied during initial phase of reoxygenation. This protocol of postconditioning has been chosen from several protocols tested, i.e. variation in time of normoxic superfusion (5–120 s) and in time of hypoxic period (1.5–5 min) as it provides the maximal protection against reperfusion-induced injury in our model.

2.3. Hypoxia-reoxygenation experiments

Five hours after isolation, cardiomyocytes attached to the glass cover slip were loaded with the appropriate dye, introduced into a gas tight and temperature controlled (37 °C) perfusion chamber (0.5 ml filling volume) and superfused at a flow rate of 0.5 ml/min. The buffers were transferred into the perfusion chamber through gas tight steel capillaries. After 10 min of normoxic superfusion, cardiomyocytes were superfused with the hypoxic buffer for 75 min to simulate ischemia, followed by 30 min of normoxic superfusion to simulate reperfusion.

This protocol of simulated ischemia/reperfusion was established in our laboratory and reported previously [16], as it provides the marked, but not complete injury of cardiomyocytes, observed as hypercontracture, MPTP opening and necrosis. During the entire experiments, cardiac myocytes were quiescent and not stimulated.

The pO_2 of ischemic buffer at the chamber outlet was less than 1 mmHg as determined by a polarographic oxygen sensor. A field of 4–6 rod shaped cardiomyocytes was chosen for each experiment and condition. The temperature controlled perfusion chamber (37 °C) was mounted to a Microscope (Olympus IX-70, Germany) adapted to a Video-Imaging-System (Till Photonics, Germany), containing a monochromator excitation side and a CCD-Camera (Retiga 2000-RV) for fluorescence detection. Fluorescence data were analysed using Till-Vision Software (Till Photonics, Germany). The fluorescent signal was recorded continuously every 6 seconds throughout the experiment excepting measurement during reperfusion, which was performed every 0.25 seconds to determine the oscillation frequency of cytosolic Ca^{2+}. The inhibitor of MPTP, cyclosporine A (0.5 μmol/L) was administrated 10 min before and during reperfusion.

2.4. Determination of cytosolic and mitochondrial Ca^{2+}

To measure cytosolic Ca^{2+}, cardiomyocytes were loaded at 37 °C with acetoxymethyl ester of fura-2 (5 μmol/L) for 30 min and then washed for further 20 min in a dye-free buffer as previously described [22]. After the loading, cells were washed twice with medium 199. This was followed by incubation in medium 199 for 30 min to allow hydrolysis of the acetoxymethyl esters within the cell. The fluorescence from dye-loaded cells was 20 to 30 times higher than background fluorescence from unloaded cells.

To measure mitochondrial Ca^{2+}, the fura-2 loaded cardiomyocytes were washed for further 20 min in the presence of 50 μM $MnCl_2$ to quench the cytosolic fura-2 compartment [23]. The fura-2 loaded cells were excited at 340, 360, and 380 nm and the fluorescence emission was collected at 510 nm. Mitochondrial Ca^{2+} was monitored as the ratio of the wavelength at 340 and 380 nm. Emission fluorescence of 360 nm excitation wave length was used to monitor mitochondrial fura-2 content.

2.5. Determination of mitochondrial inner membrane potential ($\Delta\Psi_m$)

$\Delta\Psi_m$ was monitored by applying the fluorescence dye JC-1. For this purpose, cardiac myocytes were loaded with JC-1 (5 μmol/L) at 37 °C for 20 min and then washed twice for 20 min. The loaded cells were excited at 490 nm and the emitted fluorescence was collected at 530 nm and 590 nm. $\Delta\Psi_m$ was expressed as the emitted fluorescence ratio (590 nm/530 nm) in percent to the initial level, whereas 0% level was found after complete mitochondrial depolarization with carbonyl cyanide-p-trifluoromethoxyphenylhydrazone.

2.6. Determination of MPTP opening

MPTP opening was determined by analyzing the mitochondrial calcein leak as previously described [24]. For this purpose, cardiac myocytes were incubated for 20 min with acetoxymethyl ester of calcein