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Original article

Zinc plays a critical role in the cardioprotective effect of postconditioning by enhancing the activation of the RISK pathway in rat hearts

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ABSTRACT

This study investigated if zinc plays a role in postconditioning-induced cardioprotection in rat hearts. Isolated rat hearts were subjected to 30 min regional ischemia followed by 2 h of reperfusion. Postconditioning was elicited by 6 cycles of 10 s reperfusion and 10 s ischemia. Cytosolic zinc concentrations were measured with inductively coupled plasma optical emission spectroscopy (ICPOES). Infarct size was assessed by triphenyltetrazolium chloride staining. Cytosolic zinc concentrations were decreased dramatically upon reperfusion in the control hearts. In contrast, postconditioning increased cytosolic zinc levels at reperfusion. The anti-infarct effect of postconditioning was inhibited by the selective zinc chelator N,N,N′,N′-tetrakis-(2-pyridylmethyl) ethylenediamine (TPEN). Postconditioning significantly increased phosphorylation levels of the reperfusion injury salvage kinases (RISK) including Akt (Ser^{473}), extracellular signal-regulated kinase1/2 (ERK1/2) (Thr^{202}/Tyr^{204}), and glycogen synthase kinase-3β (GSK-3β) (Ser^{9}) at reperfusion, which were nullified by TPEN. Postconditioning decreased the activity of protein phosphatase 2A (PP2A) in a zinc-dependent manner. Knockdown of the zinc transporter Zip2 inhibited the protective effect of postconditioning on hypoxia/reoxygenation injury in H9c2 cells. These results suggest that zinc plays an important role in the cardioprotective effect of postconditioning presumably by enhancing the activation of the RISK pathway. Zip2 and inactivation of PP2A by zinc may, at least in part, account for the activation of the RISK pathway.

1. Introduction

Brief cycles of ischemia and reperfusion during the early phase of reperfusion protect the heart from infarction, a phenomenon termed postconditioning [1]. Activation of cell surface G-protein coupled receptors (GPCRs) has been reported to trigger postconditioning [2–5]. Several pro-survival kinases including extracellular signal regulated kinase (ERK) [6], PI3K/Akt [7], glycogen synthase kinase 3β (GSK-3β) [8], PKC [9], and PKG [2,5] are proposed to be involved in the transduction of the protective signals. Among these kinases, ERK, PI3K/Akt, and GSK-3β have been suggested to be the major components of the reperfusion injury salvage kinase (RISK) pathway [10]. Inhibition of the mitochondrial permeability transition pore (mPTP) through the RISK pathways may serve as an important mechanism by which postconditioning protects the heart from reperfusion injury [11–13]. Although the establishment of these signaling events has propelled investigators to mimic postconditioning experimentally with chemicals that activate some signaling elements, a thorough understanding of the underlying mechanism through further studies will help us to develop fundamental therapeutic strategies to be applied in the clinical settings of acute myocardial infarction [13].

Zinc plays an important role in cellular signaling by regulating activities of several important protein kinases. The kinases of the RISK pathway including PI3K/Akt [14,15], ERK [15], and GSK-3β [16] have been demonstrated to be phosphorylated by zinc. It has also been reported that the maintenance of myocardial zinc status is cardioprotective at reperfusion in rat hearts [17]. Our recent study has demonstrated that cellular zinc is lost upon reperfusion and adenosine A_2 receptor activation protects the heart from reperfusion injury by preventing the loss of zinc at reperfusion [18]. Therefore, it is possible that postconditioning enhances the activation of the RISK pathway by preventing zinc loss at reperfusion.

In this study, we tested the hypothesis that postconditioning protects the heart from reperfusion injury by regulating intracellular zinc levels at reperfusion. Our study demonstrated that compared to the control hearts in which cytosolic zinc levels were rapidly decreased upon reperfusion, postconditioning increased cytosolic zinc levels and reduced infarct size in a zinc-dependent manner. We further demonstrated that postconditioning enhanced phosphorylation of Akt, ERK, and GSK-3β, which was reversed by the chelation of free zinc. Moreover, postconditioning could inhibit the Ser/Thr protein phosphatase PP2A via zinc.

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2. Methods

This study conforms to the NIH Guide for the Care and Use of Laboratory Animals (NIH publication No. 85-23, revised 1996).

2.1. Chemicals and antibodies

N,N,N′,N′-tetrakis-(2-pyridylmethyl) ethylenediamine (TPEN) was purchased from Sigma. All antibodies were purchased from Cell Signaling (Danvers, MA). PP2A activity assay kit was purchased from Promega (Madison, WI).

2.2. Perfusion of isolated rat hearts

Male Wistar rats (250–350 g) were anesthetized with thiobutabarbital sodium (100 mg/kg i.p.). The hearts were removed rapidly and mounted on a Langendorff apparatus. The hearts were perfused with Krebs–Henseleit buffer containing (in mM) 118.5 NaCl, 4.7 KCl, 1.2 $MgSO_4$, 1.8 $CaCl_2$, 24.8 $NaHCO_3$, 1.2 KH_2PO_4, and 10 glucose, which was heated to 37 °C and gassed with 95% O_2/5% CO_2. A latex balloon connected to a pressure transducer was inserted into the left ventricle through the left atrium. The left ventricular pressure and heart rate were continuously recorded with a PowerLab system (ADInstruments, Mountain View, CA). A 4–0 silk suture was placed around the left coronary artery, and the ends of the suture were passed through a small piece of soft vinyl tubing to form a snare. All hearts were allowed to stabilize for at least 20 min. Ischemia was induced by pulling the snare and then fixing it by clamping the tubing with a small hemostat. Total coronary artery flow was measured by timed collection of the perfusate dripping from the heart into a graduated cylinder.

2.3. Measurement of infarct size

At the end of the experiments, the coronary artery was reoccluded, and fluorescent polymer microspheres (2–9 μM diameter, Duke Scientific Corp) were infused to demarcate the risk zone as the tissue without fluorescence. The hearts were weighed, frozen and cut into 1 mm slices. The slices were incubated in 1% triphenyltetrazolium chloride (TTC) in sodium phosphate buffer at 37 °C for 20 min. The slices were immersed in 10% formalin to enhance the contrast between stained (viable) and unstained (necrotic) tissue and then squeezed between glass plates spaced exactly 1 mm apart. The myocardium at risk was identified by illuminating the slices with U.V. light. The infarcted and risk zone regions were traced on a clear acetate sheet and quantified with ImageTool. The areas were converted into volumes by multiplying the areas by slice thickness. Infarct size is expressed as a percentage of the risk zone.

2.4. Measurement of cytosolic zinc concentrations in cardiac tissue

Myocardial samples taken from risk zones were homogenized in a buffer containing (in mM) 250 sucrose, 10 Tris–HCl, 1 Na_3CO_4, 1 NaF and protease inhibitor cocktail. The homogenate was centrifuged at 1000 *g* for 10 min to pellet the nuclei. The supernatant was then centrifuged at 10,000 *g* for 30 min. The resultant supernatant was subsequently centrifuged at 10,000 *g* for 1 h to yield the cytosolic fraction (supernatant). The cytosolic samples were added into 125 μl RIPA buffer and 1.0 ml of 3 N HCl/10% trichloroacetic acid (TCA) and hydrolyzed at 70 °C for 24 h. The concentration of zinc was quantified using inductively coupled plasma optical emission spectroscopy (ICPOES, Model Optima 4300D, Perkin Elmer, Norwalk, CT) at a wavelength of 206.2 nm. A multi-element standard (Spex Certiprep, Metuchen, NJ) was used to calibrate the instrument. The limits of detection approximated 1 ppb [19].

2.5. Western blotting analysis

Myocardial samples taken from risk zones were homogenized in ice-cold lysis buffer. Equal amounts of cytosolic protein were loaded and electrophoresed on SDS-polyacrylamide gel and transferred to a PVDF membrane. Membranes were blocked with nonfat milk, and then incubated with the primary antibodies (1:1000) at 4 °C overnight. The primary antibody bindings were detected with a secondary anti-rabbit antibody (1:2000) and visualized by the ECL method.

2.6. PP2A activity assay

PP2A activity was determined using Serine/Threonine phosphatase assay kit (Promega) according to the manufacturer's instruction. Briefly, tissue lysates (25 μl) were centrifuged at 15,000 ×*g* for 1 h after a short sonication. Endogenous free phosphate was removed from the lysate supernatant with a Sephadex G-25 resin spin column. Serine/Threonine phosphate activity was measured colorimetrically by the capacity to dephosphorylate a synthetic −754 Da phosphopeptide through formation of molybdate. The total Serine/Threonine phosphatase activity for each sample was expressed as a percentage of the value measured in cell lysate without PP2A substrates.

2.7. Hypoxia/reoxygenation (H/R) in H9c2 cells

Rat heart tissue-derived H9c2 cardiac myoblast cell line was purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 100 U penicillin/streptomycin at 37 °C in a humidified 5% CO_2–95% air atmosphere. To induce H/R injury, cells cultured in a 24-well plate filled with DMEM deficient in glucose and FBS were exposed to hypoxia (<1% O_2) by placing the plate in a hypoxia chamber for 20 h. Then the hypoxic medium was replaced by the normal DMEM and cells were cultured in an incubator under normoxic conditions (room air with 5% CO_2) for 4 h. At the end of experiment, cell viability was measured propidium iodide.

2.8. siRNA transfection

H9c2 cells with >80% subconfluency were transfected with Zip2 siRNA (Santa Cruz) using transfection reagents (Santa Cruz). All experiments were done 48 h after transfection.

2.9. Cell viability assay

The cell viability was assessed by propidium iodide fluorometry using a fluorescence reader at the excitation and emission wavelengths of 540 and 590 nm, respectively. The background fluorescence intensity (B) was measured 20 min after addition of propidium iodide (30 μM) before hypoxia. The fluorescence intensity (R) was measured again. The final fluorescence intensity (F) was measured 20 min after addition of digitonin (300 μM). The cell viability was calculated by the following formula: $100(F - R) / (F - B)$ (%).

3. Experimental protocols

All hearts were subjected to 30 min regional ischemia followed by 2 h of reperfusion. Postconditioning was induced by 6 cycles of 10 s reperfusion and 10 s occlusion started immediately after the release of the index ischemia. Cardiac samples were collected from the risk zone −5, 5, 10, and 30 min after the onset of reperfusion. Infusion of TPEN (10 μM) was started 5 min before the onset of reperfusion and continued for 35 min. Infarct size was measured 2 h after the start of reperfusion. Hypoxic postconditioning was induced by three cycles of 5 min reoxygenation and 5 min hypoxia as previously described [20].