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Original article

Loss of ErbB2-PI3K/Akt signaling prevents zinc pyrithione-induced cardioprotection during ischemia/reperfusion

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ABSTRACT

Objectives: The purpose of this study was to determine if zinc homeostasis is affected during ischemia/reperfusion, if so, whether zinc pyrithione limits myocardial cell death and improves hemodynamics when administered as an adjunct to reperfusion and if ErbB receptor tyrosine kinases that are important for the long-term structural integrity of the heart are indispensable for reperfusion salvage.
Methods: Isolated perfused rat hearts were subjected to 35 min of global ischemia and reperfused for 120 min to determine the relative intracellular zinc levels by TSQ staining. The hearts were reperfused in the presence of incremental concentrations of zinc pyrithione for the first 10 min during reperfusion. Silencing or blockade of ErbB2 using a monoclonal antibody, ErbB2 kinase inhibition and PI3kinase inhibition was used to study their critical role in zinc pyrithione-induced cardioprotection.
Results: We found that there was a profound decrease in intracellular zinc after ischemia/reperfusion resulting in increased apoptosis, caspase-3 activation, and infarct size. A dose-dependent reduction of infarct size with zinc pyrithione in the range of 5–20 μmol/l (optimal protection was seen at 10 μmol/l with infarct size of $16 \pm 2\%$ vs. I/R vehicle, $33 \pm 2\%$, $p < 0.01$). Increased TUNEL staining and caspase-3 activity observed after ischemia/reperfusion were attenuated by zinc pyrithione administration during the reperfusion. Moreover, this protection was sensitive to silencing and blockade of ErbB2, inhibition of ErbB2 kinase activity or PI3-kinase activity. Western blot analysis revealed that zinc pyrithione resulted in decreased caspase-3 activation, rapid stabilization of ErbB2/ErbB1 heterodimers, and increased activation of PI3K/Akt signaling cascade.
Conclusions: Zinc pyrithione attenuates lethal perfusion-induced injury in a manner that is reliant on ErbB2/PI3K/Akt activity.

1. Introduction

Heart disease is currently the leading cause of mortality in industralized countries. Therapies aimed at reducing the extent of myocardial reperfusion injury through lowering the risk posed by various injurious triggers and potentiating various aspects of cardioprotection relating to ischemic duration [1], oxygen free radicals [2,3], profinflammatory cytokines [4,5] and preconditioning [6–10] have been reported. Ischemic preconditioning as a cardioprotective strategy has not fulfilled its clinical potential,

Abbreviations: ATP, adenosine triphosphate; AAR, area at risk; AG825, (*E*)-3-[3-[2-benzothiazolythio)methyl]-4-hydroxy-5-methoxyphenyl]-2-cyano-2-propenamide; B10, anti-ErbB2 blocking antibody; BSA, bovine serum albumin; CK, creatine kinase; DAF, diaminofluorescein; DEVD-pNA, *N*-acetyl-Asp-Glu-Val-Asp *p*-nitroanilide; DMSO, dimethyl sulphoxide; ErbB, avian erythroblastosis oncogene B; ERK1/2, extracellular signal regulated protein kinases 1 and 2; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IP, immunoprecipitation; IS, infarct size; I/R, ischemia/reperfusion; JNK, c-Jun N-terminal kinase; kDa, kilo Dalton; K-H, Krebs-Henseleit; LVDP, left ventricular developed pressure; LVEDP, left ventricular end diastolic pressure; CF, coronary flow; LVESP, left ventricular end systolic pressure; MTT, 2-(4,5-dimethyltriazol-2-yl)-2,5-diphenyl tetrazolium bromide); NO, nitric oxide; ph-ErbB, phosphorylated ErbB protein; p38MAPK, p38 mitogen activated protein kinase; RT-PCR, reverse transcriptase-polymerase chain reaction; siRNA, small interference ribonucleic acid; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TUNEL, terminal deoxynucleotidyl transferase mediated nick end labeling; TSQ, *N*-(6-metoxy)-8-quinolyl-toluenesulfonamide; TTC, triphenyl tetrazolium chloride; WT, wortmannin; ZPT, zinc pyrithione.

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primarily because of the need to intervene before the index ischaemic event, which is impossible to predict in patients presenting with an acute myocardial infarction. Thus, there is a need for identification of the molecular alterations after ischemia and prior to reperfusion and choosing the signaling pathways that can be recruited and activated at the time of reperfusion. There is large body of evidence implicating the deregulation of protein–protein interactions and protein degradation mechanisms with the pathogensis of multiple forms of the heart disease [11]. Development of novel therapeutic strategies based on moduating protein degradation in cardiac tissue is warranted.

Hypoxic incidence significantly influences cardiomyocyte structure and function, due to oxygen deficiency and associated loss of metabolic function. Subsequent reoxygenation-associated events have also been shown to be significant contributors to a pathological outcome. Zinc homeostasis is adversely affected by myocardial ischemia/reperfusion injury [12–15]. TSQ staining for the presence of Zn^{2+} revealed a significant decline in TSQ fluorescence in the hearts subjected to global ischemia and reperfusion compared to normoxic perfused hearts [15]. The zinc levels of cardiomyocytes and myocardial tissue after ischemia and reperfusion are lower than the normal myocardial tissue. Thus it appears that impaired zinc accumulation in the myocardium critically relates to ischemia reperfusion injury and if such is the case, one should expect that the zinc-accumulating process is absent or defective in the myocardium during I/R and necessitates the use of ionophores to transport zinc across the lipid membrane.

Zinc, in addition to being an antioxidant through induction of metallothionein [16], can directly or indirectly influence the stability of various proteins [17–19]. It can also reduce the caspase activation and prevent the target proteins from degradation and thereby influence the pathological outcome [20–22]. Cellular metabolic and oxidative stress during ischemia/reperfusion is known to destabilize and degrade some proteins within the cell [23]. The Her-2/neu oncogene, also known as ErbB2 is a transmembrane receptor tyrosine kinase that belongs to EGFR [24,25]. The importance of ErbB2 in normal cardiac development and physiology was demonstrated in mice by cardiac-specific knock-out of ErbB2 [26]. The mice were initially normal, but developed cardiomyopathy as adults and showed increased DNA fragmentation [26,27]. Recently, Grazette et al. [28] studied the effects of ErbB2 blockade on cardiomyocyte survival, and showed that ErbB2 antibody caused a loss of mitochondrial membrane potential and an increase in cell death. Primary cultures of neonatal rat cardiac myocytes exposed to anti-ErbB2 antibody (anti-ErbB2 Ab) for up to 24 h decreases cell viability, affects mitochondrial function. The mechanism for the deleterious effects of ErbB2 blockade remains unclear, but a recent report showed that activation of ErbB2 reduces doxorubicin-induced oxidative stress in cardiomyocytes [29]. Yet another study showed that ErbB2 levels decreased in an animal model of myocardial ischemia and in patients with ischemic cardiomyopathy [30]. Previous reports also suggested that PI3K and Akt have a role in ErbB2-mediated signal transduction [30,31]. Further evidence accumulated from recent studies suggests that exposure to exogenous zinc ions may trigger EGFR signaling in cardiomyocytes through a trans-activation mechanism involving c-Src kinase activity [32,33]. In vitro studies have shown that ErbB2 does not directly mediate anti-apoptotic effects, although there are reports suggesting that ErbB2 can directly couple to PI3K [34]. Activation of Akt through PI3K is well known to couple to receptor tyrosine kinases, including those in ErbB family, and this pathway has been shown to inhibit apoptosis through inactivation of caspases [35].

The present study was therefore undertaken to probe the effect of acute dose of zinc pyrithione given during reperfusion in the isolated rat heart on functional and metabolic damage induced by ischemia/reperfusion. The study also probed the role of ErbB2/PI3K/Akt signaling in zinc pyrithione-induced cardioprotection during I/R.

2. Materials and methods

2.1. Materials

Wortmannin, dimethyl sulphoxide (DMSO) were purchased from Sigma (Bangalore, India). Dimethyl sulfoxide was used as the solvent for wortmannin and used at a final concentration in the perfusion buffer at 0.001%. 1-hydroxypyridine-2-thione zinc salt (mercaptopyridine N-oxide zinc salt pyrithione) was supplied by Sigma (St. Louis, MO). AG825 was obtained from Biomol (Plymouth Meeting, PA). Anti-ErbB2 antibody (Ab-9, clone B10) was purchased from Neomarkers. N-(6-Metoxy)-8-quinolyl-toluene-sulfonamide (TSQ) was obtained from Molecular Probes/Invitrogen (Eugene, OR). Optimal cutting temperature (OCT) embedding medium for frozen tissue specimens was from Sakura Finetek U.S. A., Inc. (Torrance, CA).

2.2. Animals

Male Sprague-Dawley rats weighing 250–280 g were procured from the National Center for Laboratory Animal Sciences, NIN, Hyderabad, and were housed in a temperature-controlled room with a 12: 12-h light-dark cycle. A total of 75 animals were used. No hearts were excluded after the onset of the ischemia/reperfusion protocol. The animals were provided free access to standard laboratory chow and water. This investigation was approved by the Institutional Animal Ethics Committee, Kakatiya University (Registration #885/ac/05/CPCSEA), registered under "Committee for the Purpose of Control and Supervision of Experiments on Laboratory Animals," Ministry of Environment and Forests, Government of India and conforms with the *Guide for the Care and Use of Laboratory Animals* published by the United States National Institutes of Health Publication 85–23, revised 1996.

2.3. Perfusion of the rat hearts

Rats were sacrificed by cervical dislocation. Hearts were immediately excised and the ascending aorta was cannulated and immediately retrogradely perfused on a Langendorff apparatus with Krebs-Henseleit (K-H) solution (in mM: 119 NaCl, 4.7 KCl, 1.25 $CaCl_2$, 1.24 $MgSO_4$, 20.1 $NaHCO_3$, 1.24 KH_2PO_4, and 11.2 glucose) at a constant pressure of 80 mmHg. Hearts were perfused for a total of 175 min, consisting of a 20-min pre-ischemia period followed by 35 min of global ischemia and 120 min of reperfusion at 37 °C, as illustrated in Fig. 1. The hearts were divided into various experimental groups. Sham group hearts were continuously perfused with K-H buffer without ischemia. I/R group hearts were perfused with K-H buffer for a 20 min pre-ischemia period followed by 35 min of global ischemia and 120 min reperfusion at 37 °C. I/R + ZPT group hearts were perfused in the same manner as I/R, except for the first 10 min which included different concentrations of zinc pyrithione. Further reperfusion continued with KHB. PI3K inhibitor, Wortmannin (100 nmol/mL) or ErbB2 blocking antibody, B10 (1 mg/mL) or ErbB2 kinase inhibitor (AG825, 100 μM) were included in the perfusate during the last 10 min of ischemia. Perfusate was bubbled with 95% O_2 and 5% CO_2 and kept at 37 °C. In order to assess contractile function, a latex balloon connected to a pressure transducer (ML-820, AD Instruments Pvt. Ltd.) was inserted into the left ventricular cavity via the left atrium. Left ventricular end diastolic pressure (LVEDP) was set at 5–10 mmHg by inflating the balloon with physiological saline, and the left ventricular pressure was then continuously recorded. Coronary flow was measured by collecting the effluent. Pacing