Biometals (2011) 24:171–180
DOI 10.1007/s10534-010-9371-8

Cardioprotective effect of zinc requires ErbB2 and Akt during hypoxia/reoxygenation

Kasi Viswanath · Sreedhar Bodiga · Victor Balogun · Anita Zhang · Vijaya Lakshmi Bodiga

Received: 23 May 2010 / Accepted: 20 August 2010 / Published online: 31 August 2010
© Springer Science+Business Media, LLC. 2010

Abstract Recent literature suggests that exogenous zinc can prevent ischemia reperfusion injury by activating phosphoinositide-3 kinase (PI3K)/Akt and by targeting the mitochondrial permeability transition pore (mPTP). It is known that ErbB2 expression promotes association and activation of PI3-kinase/Akt, resulting in growth and survival of cardiac myocytes. In this study, we found that zinc-induced ErbB2 protein expression and Akt activation are required for preventing reperfusion injury. Neonatal rat cardiac myocytes subjected to 8 h of hypoxia, followed by 16 h of reoxygenation induced cardiomyocyte apoptosis, as assessed by increased caspase-3 activity, annexin V staining and lowered MTT reduction and ATP levels. However, addition of zinc-pyrithione (ZPT) before onset of reoxygenation effectively lowered the apoptotic indices and restored the ATP levels. ZPT induced a significant increase in ErbB2 protein expression and Akt activation. Pre-treatment with Hsp 90 inhibitor, geldanamycin or PI3-kinase inhibitor, wortmannin prevented the increase in ATP levels and abrogated the protective effect of zinc-pyrithione. Taken together, these data suggest that zinc prevents reperfusion injury by modulating the ErbB2 protein expression and Akt activation.

Keywords Zinc · Cardiomyocytes · Hypoxia · Reoxygenation · ErbB2 · Akt · Hsp90

K. Viswanath · S. Bodiga
Cardiovascular Center, Medical College of Wisconsin, Milwaukee, WI, USA

V. Balogun · A. Zhang · V. L. Bodiga (✉)
Blood Research Institute, 8701, Watertown Plank Road, Milwaukee, WI, USA
e-mail: bodigavijayasri@gmail.com

Introduction

Multiple factors contribute to ischemia-mediated cardiomyocyte cell death. Hypoxia diminishes oxidative phosphorylation resulting in decreased levels of ATP, increased lactic acid production, and lower intracellular pH (Buja et al. 1993). Metabolic stress, often coupled with oxidative stress as seen during reoxygenation can affect intracellular zinc homeostasis. Zinc, an essential micronutrient, plays a critical role in cellular biology affecting many key cellular processes. It can alter cellular signals transduction, DNA synthesis, gene transcription, post translational modifications, protein–protein interactions, and protein function (Beyersmann and Hasse 2001). The cellular homeostasis of zinc is at least in part controlled by metallothionein (MT), which is a highly conserved, low molecular weight, cysteine-rich protein. Increases or decreases in intracellular zinc have been associated with apoptotic cell death. As the essential role of zinc is being recognized, there have been attempts to manipulate intracellular zinc levels, using chelators or sequesters and ionophores. Most of the intracellular

zinc is tightly bound to metallothionein and thus the level of intracellular free zinc is very low (Krezel et al. 2007). Therefore, release of intracellular zinc or supply of extracellular zinc is required to increase cytosolic zinc. The elevated intracellular zinc can act upon multiple targets to elicit the end effect. In this direction, various studies have produced strong evidence to support the concept that MT functions as a metal chaperone for the regulation of gene expression and for synthesis and functional activity of proteins, such as metalloproteins and metal-dependent transcription factors (Maret 1995; Zeng et al. 1991; Jacob et al. 1998). Also, MT functions as a free radical scavenger and antioxidant (Thornalley and Vasak 1985). Thus, zinc might play a major role in several pathophysiological derangements, including cardiotoxicity induced by environmental chemicals, therapeutic drugs, ischemia-reperfusion injury, and heart failure resulting from multifactorial manifestations. A Cardiac-specific, MT-overexpressing transgenic mouse model provided strong evidence in favour of the protective ability of MT against ischemia-reperfusion injury and significantly improved postischemic recovery of the contractile force (Kang et al. 1999).

The role of zinc in preconditioning and toxicity in various tissues and underlying mechanisms are beginning to be understood in more detail. Anoxia-hypoglycemia in organotypic hippocampal cultures demonstrated that zinc can inhibit glutamate release via activation of pre-synaptic K_{ATP} channels and reduce ischaemic damage in rat hippocampus (Bancila et al. 2004). Interestingly, the level of zinc and metallothionein was found to decrease during ischemia-reperfusion injury, implying a possible role for enhanced apoptosis and significant disturbance in redox balance in hepatic ischemia (Vali et al. 2008). Thus, evidence obtained so far suggests that zinc preconditioning is a complex process and involves a spectrum of mechanisms involving intracellular and intercellular transients of zinc, ability to induce metallothionein, a potential scavenger of hydroxyl radicals and the ability of zinc per se to act as a major signaling molecule, triggering a cascade of events that are both redox-dependent and independent. It is highly improbable that a single mechanism mediates zinc-induced cytoprotection and toxicity and the search for probable targets continues.

Pyrithione (1-hydroxypyridine-2-thionine, omadine) had long been recognized to have ionophore properties. Zinc salt of pyrithione is highly lipophilic and therefore penetrates membranes easily. This permits transport of zinc across cell membranes. The anti-apoptotic effect of zinc pyrithione was first observed in vitro by Zalewski and coworkers, who showed that micromolar concentrations of this compound protected lymphocytic leukemia cells against colchicine-induced apoptosis (Giannakis et al. 1991). Zalewski's group has since published a number of other studies, confirming the ability of micromolar concentrations of zinc-pyrithione to rapidly transport Zn^{2+} into cells. Further, zinc is known to affect cardiac differentiation (Apostolova et al. 1999), regeneration of cardiac muscle (Coudray et al. 1993), lipid peroxidation and the outcome of surgical procedures in open heart (Powell et al. 1995). On the other hand, existing evidence implicates zinc in the pathogenesis of cerebral ischemia. Many in vitro and in vivo studies have examined the role of zinc during both global and focal cerebral ischemia. These studies, direct (using zinc chloride or zinc protoporphyrin) and indirect (using chelators of zinc) have reported zinc to possess either neurotoxic or neuroprotective capabilities in a temporal manner in experimentally induced ischemia (see review, Galasso and Dyck 2007). This inconsistency may have arisen due to employment of different model systems and much more needs to be learnt about the critical role of zinc in brain ischemia. It was argued that calcium may act as an accomplice to zinc and their cellular transients can be influenced by each other in potentially mediating ischemic injury in the brain. Translocation of zinc from presynaptic neurons to specific postsynaptic neurons was shown to be responsible for cerebral ischemic injury (Galasso and Dyck 2007).

ErbB2 is a transmembrane receptor tyrosine kinase that heterodimerizes with other members of the ErbB family (Muthuswamy et al. 1999; Gordon et al. 2009). The stability of ErbB2 requires its association with Hsp90 (Xu et al. 2001). Inhibitors of Hsp90 induce the release of ErbB2 resulting in subsequent ErbB2 proteosomal degradation (Neckers 2002; Zaarur et al. 2006). The chaperone activity of Hsp90 requires ATP, which binds with approximately 10-fold lower affinity than ADP. Lowering ATP concentration in isolated myocytes triggers rapid dissociation of Hsp90 from ErbB2 and degradation of ErbB2 along with other client proteins (Peng et al. 2005). ErbB2 plays an essential role in cardiomyocyte survival and the

expression levels of ErbB2 receptor are significantly decreased in an animal model of ischemic heart disease and in human ischemic cardiomyopathy (Gordon et al. 2009). One of the downstream growth signaling effectors of ErbB2 is PI3 kinase (Yarden and Sliwkowski 2001). Recent experimental studies in vitro have demonstrated the cardioprotective effect of zinc to depend on the ability to activate PI3-kinase/Akt by targeting the mitochondrial permeability transition pore (mPTP) via glycogen synthase kinase 3β (GSK-3β) (Chanoit et al. 2008; Lee et al. 2009; Karagulova et al. 2007; Mocanu and Yellon 2009; Chanoit et al. 2009). Also, zinc sulphate supplementation effectively reduced infarct size in dog model (Lal 1991). In the present study, we have examined the following: (1) Whether the protective effect of zinc derives from an altered expression of ErbB2; (2) Whether this is mediated by Hsp90 stabilization of ErbB2; and (3) if it involves changes in intracellular ATP levels.

Materials and methods

Chemicals and reagents

$ZnCl_2$ and pyrithione (ZPT) were procured from Sigma (St. Louis, MO). Wortmannin (WT) and geldanamycin (GA) were obtained from LC laboratories (Woburn, MA). Phospho-Akt rabbit polyclonal antibody Ser-473, total Akt rabbit polyclonal antibodies were obtained from Cell signaling Technology. ErbB2 rabbit polyclonal antibody and Hsp90 antibody was obtained from Santa Cruz Biotechnology. Anti-ErbB2 (clone B10, antibody 9) was purchased from Neomarker, Fremont, CA, USA.

Primary culture of neonatal rat ventricular myocytes

Cardiac ventricular myocytes were prepared from 1 to 2 day old Sprague–Dawley rats and cultured as described elsewhere (Bodiga et al. 2009). Hearts were digested with trypsin and the dissociated cells were preplated for 1 h to select the non-adherent myocytes. These cells were cultured in the presence of bromodeoxyuridine (0.1 mM) for 3 days to inhibit fibroblast growth.

Treatment of cells

Rat neonatal myocytes were cultured to 70% confluency and then serum starved in basal medium (DMEM) for 24 h. NRCMs were maintained at 37°C under an atmosphere of 95% N_2 and 5% CO_2 (hypoxia) in an air tight chamber or 95% air and 5% CO_2 (normoxia) for 8 h followed by reoxygenation for 16 h in serum- and glucose-free DMEM, but containing 10 mM 2-deoxyglucose. The oxygen content (FiO_2) during hypoxia was continuously monitored to be below 1% (Pro-Ox 110, Biospherix Ltd, Redfield, NY). Myocytes not exposed to hypoxia/reoxygenation served as normoxic controls. Various groups of myocytes were treated with 10 μM $ZnCl_2$ and 4 μM pyrithione, or 1 μM geldanamycin (GA) (Peng et al. 2005), 5.0 μg/ml B10 (Pugatsch et al. 2006), or 200 nM wortmannin (Bodiga et al. 2009) at the onset of reoxygenation. At the end of the hypoxia/reoxygenation, myocytes were examined for viability and apoptosis by MTT, caspase-3, and annexin V staining. The rationale for the use of zinc pyrithione was to facilitate the transport of zinc into the target cells. This was necessitated by the fact that all eukaryotic cells strictly regulate membrane transport of zinc making it very difficult to modulate the intracellular concentration and distribution of zinc.

3-(4,5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide assay

Neonatal myocytes were cultured in 60-mm dishes (plated at a density approximately half million cells) to 80% confluency and then maintained in either complete medium or switched to basal medium after 42 h. The samples were maintained under normoxia or subjected to H/R as described in "Treatment of cells" section. At the end of the incubation period, the survival of the cells was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as described in the manufacturer's protocol (Molecular Probes, Eugene, OR).

Caspase-3 assay

The NRCMs were processed for the caspase-3 colorimetric assay as described by the manufacturer (R&D Systems) and results were normalized for protein content.

Annexin V binding

Annexin V binds to phosphatidylserine, which appears in the outer leaflet of the plasma membrane in early apoptotic cells. Neonatal myocytes were cultured in 60-mm dishes to 70% confluency and subjected to normoxia or H/R (8/2 h) with or without pretreatment with ZPT as described in "Treatment of cells" section. The cells were washed with PBS and treated with FITC-labeled annexin V (0.2 μg/ml) for 20 min at room temperature. Labeling with FITC-coupled annexin V was performed according to the manufacturer's protocol (BD Biosciences, San Diego, CA). The labeled cells (10,000/sample) were analyzed by measuring fluorescence intensity using a FACScan flow cytometer (Becton Dickinson) in conjunction with CellQuest software (BD Biosciences).

Cellular ATP levels

Bioluminescent determination of ATP was performed as per the manufacturer's instructions, using a kit (Sigma). Briefly, cellular ATP was determined on a Biotek Synergy HT luminescence plate reader (Biotek) and normalized to cell number using a coulter counter (Coulter Electronics, Hialeah, FL). Results are reported as a percentage of normoxic values.

Western blot analysis

Neonatal rat cardiac myocytes of various groups were exposed or not exposed to hypoxia (8 h) and treated with zinc pyrithione at the onset of reoxygenation. Where indicated, pharmacological inhibitors were applied 30 min prior to zinc pyrithione treatment. For these studies, reoxygenation was done for 1 h in order to assess the immediate effect of reoxygenation and the ability of zinc pyrithione. Cells were solubilized with a lysis buffer containing 1% Triton X-100, 20 mM Tris, pH 7.4, 100 mM NaCl, 1 mM EDTA, 1% sodium deoxycholate, 0.1% SDS, and protease inhibitor cocktail (Pierce). Insoluble proteins were precipitated by centrifugation at 13,000 rpm for 10 min at 4°C, and the supernatants were boiled in SDS sample buffer, and subjected to SDS-PAGE on 4–12% polyacrylamide gels and transferred to a nitrocellulose membrane (Bio-Rad). Equal amounts of protein (50 μg) from each group were probed by western blotting. Reactive bands were visualized using the SuperSignal West Femto Maximum Sensitivity Substrate Kit (Pierce, Rockford, IL) and Kodak 440CF image station (Kodak, New Haven, CT). The intensity of the reactive bands was quantified using the Kodak 1D software.

Statistical analysis

All values are expressed as means ± SE from at least three or more samples in each experiment. Comparisons between controls and treatments were analyzed by ANOVA, followed by Tukey's test when permitted. $P \leq 0.05$ were considered significant.

Results and discussion

Cardiomyocyte death by H/R and the protective effect of zinc pyrithione treatment

To evaluate the functional impact of zinc pyrithione (ZPT) treatment on hypoxia/reoxygenation induced apoptosis, neonatal rat cardiac myocytes were subjected to 8 h of hypoxia followed by 16 h of reoxygenation or left in normoxia for 24 h. 10 μM Zn^{2+} and 4 μM pyrithione was added to cells immediately after hypoxia and just before reoxygenation. Cell viability decreased by 46% following H/R (8/16 h) as compared to aerobically incubated myocytes (normoxia). However, addition of ZPT before reoxygenation significantly increased cell survival to 85% (Fig. 1a). We also assessed caspase-3 activity as a measure of apoptosis. Caspase-3 activity increased over twofold after H/R, while treatment with ZPT significantly decreased caspase-3 activity (Fig. 1b). Both MTT and caspase-3 assays demonstrated that ZPT is effective in reducing cell death after H/R. We also tested for apoptosis using a specific marker, annexin V staining at an early time point, i.e., after 2 h of reoxygenation. Cardiomyocytes subjected to hypoxia (8 h), followed by reoxygenation (2 h) showed significantly higher intensity of annexin V staining as assessed by FACS analysis (Fig. 2). Similar to caspase-3 activity, ZPT treatment effectively lowered the annexin V staining in myocytes subjected to H/R. These data clearly demonstrate that zinc pyrithione treatment at the onset of reoxygenation can effectively lower cell death/apoptosis. These results are in line with previous observations that exogenous zinc supplementation

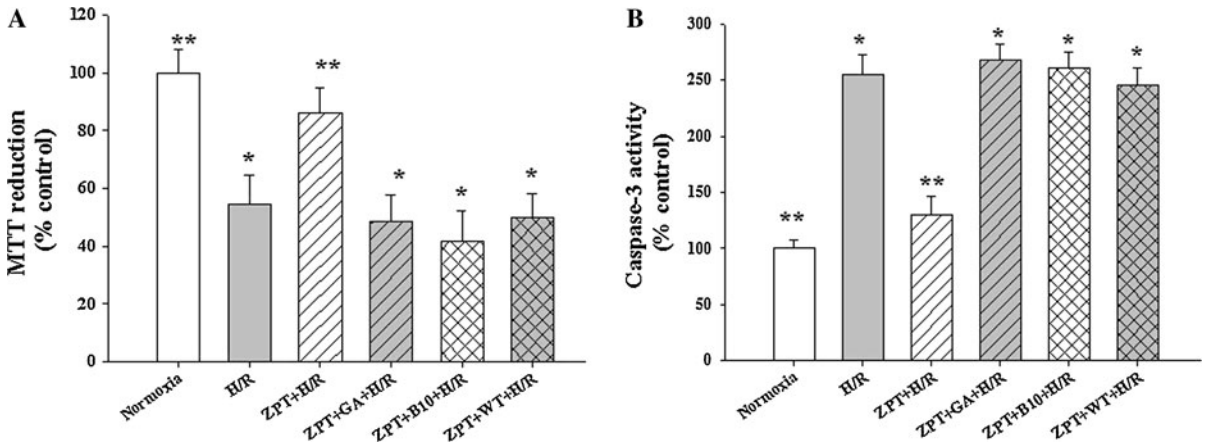

Fig. 1 Cardioprotective effect of zinc pyrithione in NRCM during ischemic injury is blocked by treatment with geldanamycin, ErbB2 antibody and PI3-kinase inhibitor. NRCM were subjected to 8 h hypoxia and treated with 10 μM zinc chloride and 4 μM pyrithione at the onset of reoxygenation for 16 h. Cells were treated with GA (geldanamycin, 1 μM) or B10 (anti-ErbB2 antibody, 5.0 μg/ml) or WT (wortmannin, 200 nM) for 30 min before zinc pyrithione application, and cell survival was measured via **a** MTT reduction **b** caspase-3 activity and normalized to percent control (* $P < 0.028$ compared with normoxia, ** $P < 0.074$ compared with normoxia, $n = 5$). Data are presented as mean ± SE

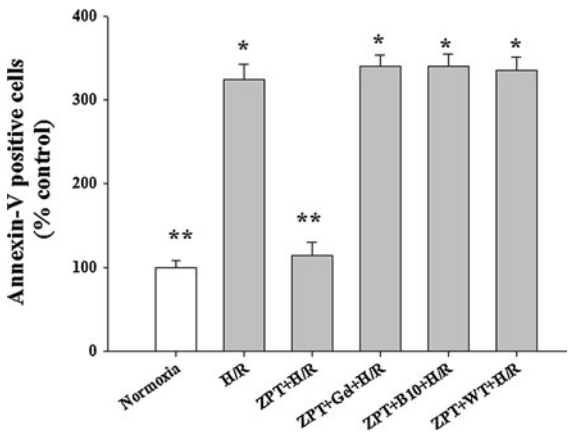

Fig. 2 ZPT decreases annexin V binding after H/R. Neonatal myocytes were subjected to H/R in the presence of ZPT. Inhibitors were applied 30 min before ZPT treatment, where indicated. Cells were incubated with binding buffer containing annexin V, as described in "Materials and methods" section. Bar graph showing mean ± SE of percent increase of annexin binding over control (100%). * $P \leq 0.05$ compared with control; ** $P \leq 0.05$ compared with H/R-treated cells

can ameliorate ischemic injury and prevent apoptosis in cardiac myocytes (Chanoit et al. 2008; Lee et al. 2009; Karagulova et al. 2007).

Targeted deletion of cardiac ErbB2 results in cardiac myopathy in adult animals with increased DNA fragmentation (Crone et al. 2002). Also blockade of ErbB2 receptor results in mitochondrial dysfunction and apoptosis (Grazette et al. 2004). A key mediator of ErbB2 stability is the molecular chaperone Hsp90, which also functions as physiological ATP sensor (Chavany et al. 1996). 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 PI3-kinase (Hellyer et al. 2001). Activation of Akt through the PI3-kinase is well known to couple to receptor tyrosine kinases, including those in the ErbB family, and this pathway has been shown to inhibit apoptosis through inactivation of caspases (Baliga et al. 1999). We therefore examined whether the ErbB2 blockade or Hsp90 inhibition and PI3-kinase inhibition interfere with the cytoprotective effect of zinc.

To address the specific role of ErbB2 and PI3-kinase/Akt in zinc pyrithione mediated protection against H/R, we tested three different agents: (1) geldanamycin, Hsp90 inhibitor; (2) anti-ErbB2 antibody (B10); and (3) wortmannin, PI3-kinase inhibitor. These inhibitors were applied 30 min before addition of zinc pyrithione to myocytes during reoxygenation. Geldanamycin induces the release of ErbB2 from Hsp90, resulting in subsequent proteasomal degradation (Zaarur et al. 2006; Peng et al. 2005). Pretreatment with Hsp90 inhibitor, geldanamycin did not further lower the viability or increase caspase-3 activity after H/R, but eliminated the protective effect of zinc pyrithione (Fig. 1a, b). Similarly, incubation of myocytes with an anti-ErbB2 antibody (B10) or wortmannin

abrogated the protective effect of zinc (Fig. 1a, b). All the pharmacological inhibitors tested here significantly decreased the ability of zinc in lowering the annexin V positive cells after H/R. These results provide the first line of evidence that zinc pyrithione may be acting by modulating the expression and interaction of ErbB2, Hsp90, and PI3-kinase/Akt. Thus, loss of ErbB2 function, whether through antibody blockade or enhanced degradation due to Hsp90 inhibitor, dramatically decreased the cytoprotective effect of zinc. Our results indicate that the activity of Hsp90 is essential for maintaining the function of ErbB2, Akt in neonatal rat cardiac myocytes.

To determine further whether zinc/pyrithione mediated reperfusion salvage is mediated by the altered expression of ErbB2, we measured the expression of ErbB2 by western blotting. Also to verify that Hsp90-mediated changes in ErbB2 expression or stability account for the observed anti-apoptotic effect of zinc, the expression of Hsp90 and Akt levels were monitored. Levels of ErbB2 decreased significantly upon hypoxia/reoxygenation compared to normoxia (Fig. 3a). Also there was a modest phosphorylation of Akt at Ser473 (Fig. 3b). In contrast zinc/pyrithione treatment resulted in a significant increase of ErbB2 and robust activation of Akt, without any changes in the levels of Hsp90 and Akt (Fig. 3a, b). These results suggest a direct relationship between the levels of ErbB2 and the phosphorylation of Akt at ser473. Pretreatment with geldanamycin before zinc/pyrithione during reperfusion dramatically lowered the levels of ErbB2 and phospho-Akt, without altering the levels

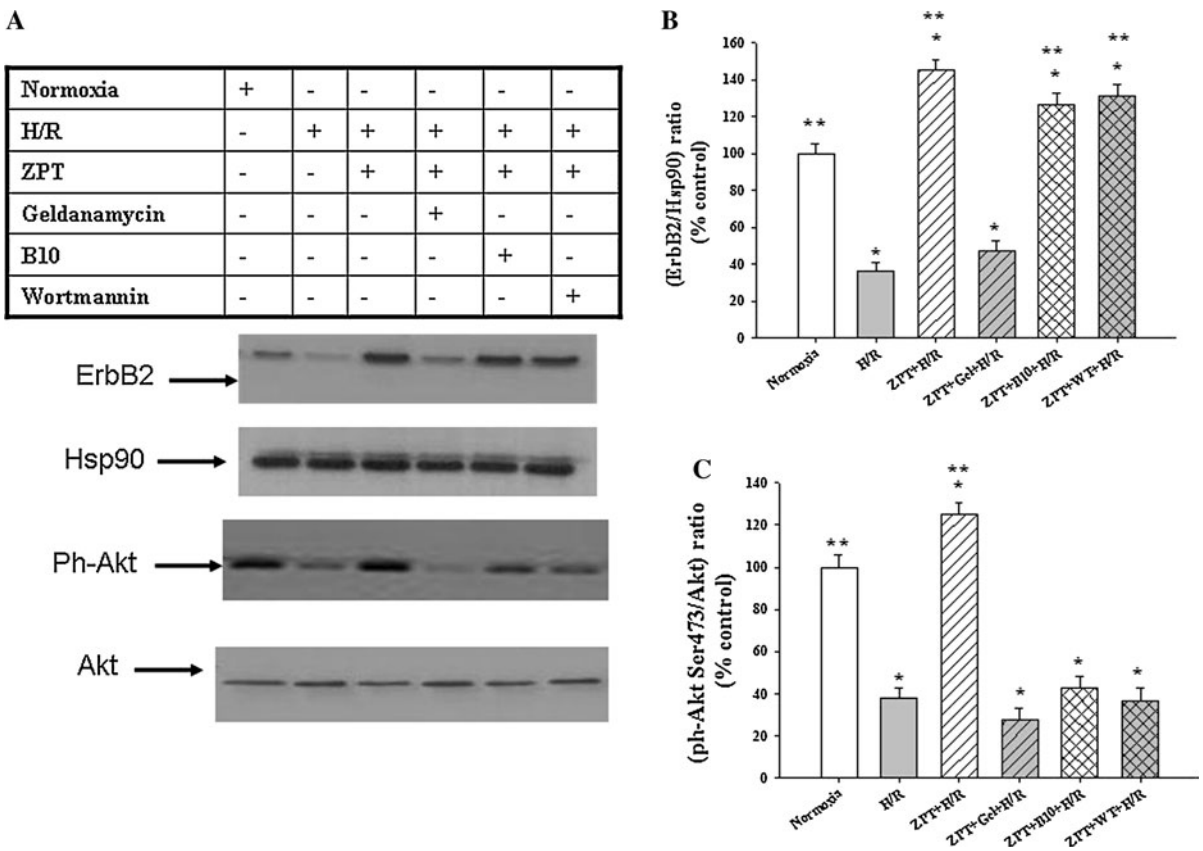

Fig. 3 Effect of zinc pyrithione on ErbB2 and phospho-Akt during ischemic hypoxia and reoxygenation. NRCM were subjected to 8 h hypoxia and treated with 10 μM zinc chloride and 4 μM pyrithione at the onset of reoxygenation for 16 h. Cells were treated with GA (geldanamycin, 1 μM) or B10 (anti-ErbB2 antibody, 5.0 μg/ml) or WT (wortmannin, 200 nM) for 30 min before zinc pyrithione application. Cell lysate supernatants were immunoblotted with anti-ErbB2 antibody and anti-Hsp90 antibody (**a**) and anti-phospho-Akt (Ser473) and anti-Akt antibody (**b**). The figures show representative immunoblots obtained from five independent experiments. **c** Protein levels were determined from the immunoblots by densitometric analysis (mean ± SE), $n = 5$; * $P < 0.05$ versus normoxia control; ** $P < 0.05$ versus H/R

of Hsp90 and Akt. Similarly anti-ErbB2 antibody reduced phospho-Akt levels, without any change in the expression of ErbB2, although a recent study reported that after 24 h treatment with B10, ErbB receptor levels were downregulated (Pugatsch et al. 2006). We have not observed any change in the expression of ErbB2 after blockade with B10 within 12 h. Inhibition of PI3-kinase with wortmannin did not affect ErbB2 levels, but significantly lowered Akt activation. These results indicate that Hsp90 inhibition and ErbB2 receptor blockade negatively affect Akt activation and that Akt activation is required for cytoprotective effect of zinc as assessed by caspase-3 activity and annexin V staining (Fig. 1, 2). Our results are consistent with previous literature that blockade or loss of ErbB2 mediates cell death during ischemic injury (Gordon et al. 2009; Grazette et al. 2004). Of note, there has been a significant loss in the levels of ErbB2 with no change in Hsp90 levels after H/R, suggesting that ErbB2 signaling is important for normal cardiomyocytes function. On the other hand, zinc supplementation effectively prevented the decrease in ErbB2 and enhanced phospho-Akt levels after H/R resulting in increased cell viability. As shown in Fig. 3a, geldanamycin potently decreased ErbB2 levels, consistent with the role of Hsp90 in stabilization of ErbB2. Inhibition of Hsp90 function has been demonstrated to block the function of Hsp90-client proteins, either through inhibition of maturation or through their destabilization and subsequent degradation (Kupatt et al. 2004). Together, this data suggest a functional relationship between Hsp90, ErbB2 and phospho-Akt in zinc-pyrithione mediated cardioprotection. Although our study has not directly tested the above hypotheses, our data imply that ErbB2 alone is not cytoprotective, but requires Akt activation as seen in the presence of wortmannin.

Previous studies with various cell types have shown dependence of apoptosis on ATP levels (Shiraishi et al. 2001; Nicotera et al. 1998; Reed and Paternostro 1999; Taimor et al. 1999). In contrast, there is increasing evidence that, even under glucose deprivation and presumed ATP depletion, cell death occurs primarily by apoptosis (Malhotra and Brosius 1999; Bialik et al. 1999). Although the ability of hypoxia/reoxygenation to deplete ATP and induce apoptosis in isolated cardiac myocytes has been demonstrated previously (Mizukami et al. 2004), the causal link between Hsp90–ErbB2 expression and ATP levels were

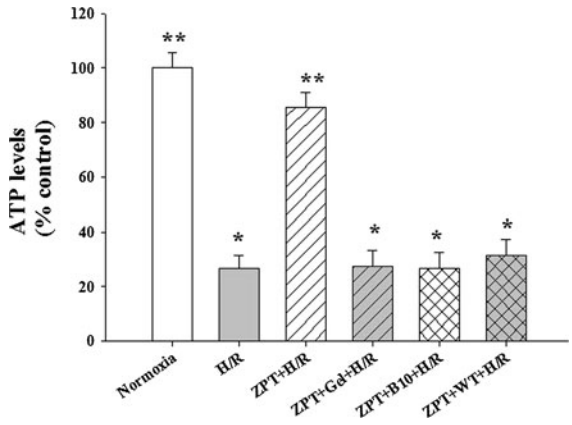

Fig. 4 ATP levels during ischemic hypoxia and reoxygenation in NRCM treated with zinc pyrithione. NRCM were subjected to hypoxia and treated with 10 μM zinc chloride and 4 μM pyrithione at the onset of reoxygenation. Cells were treated with GA (geldanamycin, 1 μM) or B10 (anti-ErbB2 antibody, 5.0 μg/ml) or WT (wortmannin, 200 nM) for 30 min before zinc pyrithione application. ATP levels were determined by the luciferin-luciferase method at the end of reoxygenation (mean ± SE, $n = 6$; * $P < 0.05$ versus normoxia, ** $P < 0.05$ versus H/R)

evaluated in the present study. Exposure of NRCM to hypoxia for 8 h followed by reoxygenation for 16 h resulted in a severe depletion of ATP levels (Fig. 4). Low levels of ErbB2 after H/R can be explained on the basis that ATP-depletion can induce disruption of Hsp90 chaperone function and result in ErbB2 degradation (Fig. 3a). Zinc treatment significantly elevated intracellular ATP levels after H/R. In contrast, application of pharmacological inhibitors targeting Hsp90, ErbB2 and PI3-kinase resulted in depletion of ATP, despite zinc pyrithione treatment. Loss of intracellular ATP is probably due to H/R and not due to the actions of the pharmacological agents used, although ErbB2 blockade using B10 and disruption of Hsp90–ErbB2 interaction using geldanamycin are known to deplete ATP levels and inhibit mitochondrial function (Grazette et al. 2004; Griffin et al. 2004). Hypoxia/reoxygenation is known to result in loss of mitochondrial membrane potential, reactive oxygen species generation, and the uncoupling of oxidative phosphorylation so that ATP is hydrolyzed rather than synthesized (White and Wittenberg 2000; Stanley and Chandler 2002; Plas et al. 2001). None of the inhibitors tested here showed a further decrease in ATP levels after H/R. However, geldanamycin and anti-ErbB2 blockade interfered with the ability of zinc to activate

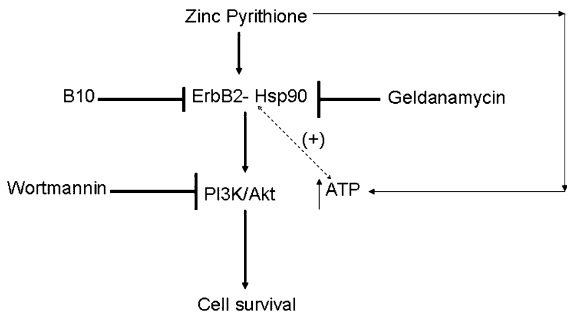

Fig. 5 A schematic depicting the probable mechanism(s) involved in zinc pyrithione mediated protection of neonatal rat cardiac myocytes against hypoxia-reoxygenation injury. ZPT induced a significant increase in ErbB2 protein expression and Akt activation. Pretreatment with B10, anti-ErbB2 antibody negatively affected Akt activation. Pretreatment with Hsp 90 inhibitor, geldanamycin or PI3-kinase inhibitor, wortmannin prevented the increase in ATP levels and abrogated the protective effect of zinc-pyrithione

Akt and replete ATP levels upon hypoxia/reoxygenation. Wortmannin did not affect ErbB2 and Hsp90 levels, but being an inhibitor of PI3-kinase, antagonized the effect of zinc in restoring ATP levels and in preventing apoptosis. This data suggests that both ErbB2 and PI3-kinase/Akt are required for zinc to salvage reperfusion injury while maintaining ATP levels (Fig. 5). It is interesting to note that neither hypoxia/reoxygenation nor zinc-pyrithione induced any changes in Hsp90 expression in NRCM despite a significant change in ATP levels. These data also suggest that zinc pyrithione can preserve and maintain mitochondrial function.

This study identified ErbB2 as a potential target in zinc mediated cardioprotection. Zinc/pyrithione not only enhanced the expression of ErbB2, but also resulted in significant increase of Akt phosphorylation. These new findings warrant further investigations to establish if there is a direct role for zinc in induction of ErbB2, stabilization of Hsp90, ErbB2 complex and phospho Akt activation. ErbB2 is an orphan receptor, but can heterodimerize with ErbB4 or ErbB3. Neuregulins 1 and 2 can stimulate heterodimerization with ErbB4 or ErbB3 and capacitate to interact with distinct complements of intracellular signaling proteins, giving rise to a broad range of cellular responses (Fuller et al. 2008). It is of great interest to further understand if zinc can elicit the expression of peptide growth factors like Neu 1 after H/R and to pinpoint if they play a major role in the observed cardioprotective effects. These investigations also provide a clue in the direction that ErbB2 may act upstream of Akt activation, which is essential in preventing myocardial injury. These data have significance with regard to prevention of reperfusion injury and myocardial apoptosis. Although zinc pyrithione protects the cardiomyocytes against hypoxia/reoxygenation-induced apoptosis, it should be noted that results obtained in cell cultures cannot be directly compared and extended to pathological states of living organisms.

References

Apostolova MD, Ivanova IA, Cherian MG (1999) Metallothionein and apoptosis during differentiation of myoblasts to myotubes: protection against free radical toxicity. Toxicol Appl Pharmacol 159:175–184

Baliga RR, Pimental DR, Zhao YY, Simmons WW, Marchionni MA, Sawyer DB, Kelly RA (1999) NRG-1-induced cardiomyocyte hypertrophy. Role of PI-3-kinase, p70(S6K), and MEK-MAPK-RSK. Am J Physiol 277:H2026–H2037

Bancila V, Nikonenko I, Dunant Y, Bloc A (2004) Zinc inhibits glutamate release via activation of pre-synaptic K_{ATP} channels and reduces ischaemic damage in rat hippocampus. J Neurochem 90:1243–1250

Beyersmann D, Hasse H (2001) Functions of zinc in signaling, proliferation and differentiation of mammalian cells. Biometals 14:331–341

Bialik S, Cryns VL, Drincic A, Miyata S, Wollowick AL, Srinivasan A, Kitsis RN (1999) The mitochondrial apoptotic pathway is activated by serum and glucose deprivation in cardiac myocytes. Circ Res 85:403–414

Bodiga S, Zhang R, Jacobs DE, Larsen BT, Tampo A, Manthati VL, Kwok WM, Zeldin DC, Falck JR, Gutterman DD, Jacobs ER, Medhora MM (2009) Protective actions of epoxyeicosatrienoic acid: dual targeting of cardiovascular PI3K and KATP channels. J Mol Cell Cardiol 46:978–988

Buja LM, Eigenbrodt ML, Eigenbrodt EH (1993) Apoptosis and necrosis. Basic types and mechanisms of cell death. Arch Pathol Lab Med 117:1208–1214

Chanoit G, Lee S, Xi J, Zhu M, McIntosh RA, Mueller RA, Norfleet EA, Xu Z (2008) Exogenous zinc protects cardiac cells from reperfusion injury by targeting mitochondrial permeability transition pore through inactivation of glycogen synthase kinase-3beta. Am J Physiol Heart Circ Physiol 295:H1227–H1233

Chanoit G, Lee S, Xu Z (2009) Reply to "letter to the editor: 'zinc and cardioprotection: the missing link' ". Am J Physiol 296:H235

Chavany C, Mimnaugh E, Miller P, Bitton R, Nguyen P, Trepel J, Whitesell L, Schnur R, Moyer J, Neckers L (1996) p185erbB2 binds to GRP94 in vivo. Dissociation of the p185erbB2/GRP94 heterocomplex by benzoquinone

ansamycins precedes depletion of p185erbB2. J Biol Chem 271:4974–4977

Coudray C, Charlon V, de Leiris J, Favier A (1993) Effect of zinc deficiency on lipid peroxidation status and infarct size in rat hearts. Int J Cardiol 41:109–113

Crone SA, Zhao YY, Fan L, Gu Y, Minamisawa S, Liu Y, Peterson KL, Chen J, Kahn R, Condorelli G, Ross J Jr, Chien KR, Lee KF (2002) ErbB2 is essential in the prevention of dilated cardiomyopathy. Nat Med 8:459–465

Fuller SJ, Sivarajah K, Sugden PH (2008) ErbB receptors, their ligands, and the consequences of their activation and inhibition in the myocardium. J Mol Cell Cardiol 44:831–854

Galasso SL, Dyck RH (2007) The role of zinc in cerebral ischemia. Mol Med 13:380–387

Giannakis C, Forbes IJ, Zalewski PD (1991) Ca^{2+}/Mg^{2+}-dependent nuclease: tissue distribution, relationship to inter-nucleosomal DNA fragmentation and inhibition by Zn^{2+}. Biochem Biophys Res Commun 181:915–920

Gordon LI, Burke MA, Singh AT, Prachand S, Lieberman ED, Sun L, Naik TJ, Prasad SV, Ardehali H (2009) Blockade of the erbB2 receptor induces cardiomyocyte death through mitochondrial and reactive oxygen species-dependent pathways. J Biol Chem 284:2080–2087

Grazette LP, Boecker W, Matsui T, Semigran M, Force TL, Hajjar RJ, Rosenzweig A (2004) Inhibition of ErbB2 causes mitochondrial dysfunction in cardiomyocytes: implications for herceptin-induced cardiomyopathy. J Am Coll Cardiol 44:2231–2238

Griffin TM, Valdez TV, Mestril R (2004) Radicicol activates heat shock protein expression and cardioprotection in neonatal rat cardiomyocytes. Am J Physiol Heart Circ Physiol 287:H1081–H1088

Hellyer NJ, Kim MS, Koland JG (2001) Heregulin-dependent activation of phosphoinositide 3-kinase and Akt via the ErbB2/ErbB3 co-receptor. J Biol Chem 276:42153–42161

Jacob C, Maret W, Vallee BL (1998) Control of zinc transfer between thionein, metallothionein, and zinc proteins. Proc Natl Acad Sci USA 95:3489–3494

Kang YJ, Li G, Saari T (1999) Metallothionein inhibits ischemia-reperfusion injury in mouse heart. Am J Physiol 276:H993–H997

Karagulova G, Yue Y, Moreyra A, Boutjdir M, Korichneva I (2007) Protective role of intracellular zinc in myocardial ischemia/reperfusion is associated with preservation of protein kinase C isoforms. J Pharmacol Exp Ther 321: 517–525

Krezel A, Hao Q, Maret W (2007) The zinc/thiolate redox biochemistry of metallothionein and the control of zinc ion fluctuations in cell signaling. Arch Biochem Biophys 463:188–200

Kupatt C, Dessy C, Hinkel R, Raake P, Daneau G, Bouzin C, Boekstegers P, Feron O (2004) Heat shock protein 90 transfection reduces ischemia-reperfusion-induced myocardial dysfunction via reciprocal endothelial NO synthase serine 1177 phosphorylation and threonine 495 dephosphorylation. Arterioscler Thromb Vasc Biol 24:1435–1441

Lal A (1991) Effect of zinc sulphate on infarct size in experimental myocardial infarction in dogs. Indian J Med Res 94:316–319

Lee S, Chanoit G, McIntosh R, Zvara DA, Xu Z (2009) Molecular mechanism underlying Akt activation in zinc-induced cardioprotection. Am J Physiol Heart Circ Physiol 297:H569–H575

Malhotra R, Brosius FC III (1999) Glucose uptake and glycolysis reduce hypoxia-induced apoptosis in cultured neonatal rat cardiac myocytes. J Biol Chem 274:12567–12575

Maret W (1995) Metallothionein/disulfide interactions, oxidative stress, and the mobilization of cellular zinc. Neurochem Int 27:111–117

Mizukami Y, Iwamatsu A, Aki T, Kimura M, Nakamura K, Nao T, Okusa T, Matsuzaki M, Yoshida K, Kobayashi S (2004) ERK1/2 regulates intracellular ATP levels through alpha-enolase expression in cardiomyocytes exposed to ischemic hypoxia and reoxygenation. J Biol Chem 279: 50120–50131

Mocanu MM, Yellon DM (2009) Letter to the editor: "zinc and cardioprotection: the missing link". Am J Physiol 296: H233–H234

Muthuswamy SK, Gilman M, Brugge JS (1999) Controlled dimerization of ErbB receptors provides evidence for differential signaling by homo- and heterodimers. Mol Cell Biol 19:6845–6857

Neckers L (2002) Hsp90 inhibitors as novel cancer chemotherapeutic agents. Trends Mol Med 8:S55–S61

Nicotera P, Leist M, Ferrando-May E (1998) Intracellular ATP, a switch in the decision between apoptosis and necrosis. Toxicol Lett 102–103:139–142

Peng X, Guo X, Borkan SC, Bharti A, Kuramochi Y, Calderwood S, Sawyer DB (2005) Heat shock protein 90 stabilization of ErbB2 expression is disrupted by ATP depletion in myocytes. J Biol Chem 280:13148–13152

Plas DR, Talapatra S, Edinger AL, Rathmell JC, Thompson CB (2001) Akt and Bcl-xL promote growth factor-independent survival through distinct effects on mitochondrial physiology. J Biol Chem 276:12041–12048

Powell SR, Aiuto L, Hall D, Tortolani AJ (1995) Zinc supplementation enhances the effectiveness of St. Thomas' Hospital No. 2 cardioplegic solution in an in vitro model of hypothermic cardiac arrest. J Thorac Cardiovasc Surg 110:1642–1648

Pugatsch T, Abedat S, Lotan C, Beeri R (2006) Anti-erbB2 treatment induces cardiotoxicity by interfering with cell survival pathways. Breast Cancer Res 8:R35

Reed JC, Paternostro G (1999) Postmitochondrial regulation of apoptosis during heart failure. Proc Natl Acad Sci U A 96:7614–7616

Shiraishi J, Tatsumi T, Keira N, Akashi K, Mano A, Yamanaka S, Matoba S, Asayama J, Yaoi T, Fushiki S, Fliss H, Nakagawa M (2001) Important role of energy-dependent mitochondrial pathways in cultured rat cardiac myocyte apoptosis. Am J Physiol Heart Circ Physiol 281:H1637–H1647

Stanley WC, Chandler MP (2002) Energy metabolism in the normal and failing heart: potential for therapeutic interventions. Heart Fail Rev 7:115–130

Taimor G, Lorenz H, Hofstaetter B, Schluter KD, Piper HM (1999) Induction of necrosis but not apoptosis after anoxia and reoxygenation in isolated adult cardiomyocytes of rat. Cardiovasc Res 41:147–156

Thornalley PJ, Vasak M (1985) Possible role for metallothionein in protection against radiation-induced oxidative stress: Kinetics and mechanism of its reaction with

superoxide and hydroxyl radicals. Biochim Biophys Acta 827:36–44

Vali L, Stefanovits-Banyai E, Szentmihalyi K, Drahos A, Sardy M, Febel H, Feher E, Bokori E, Kocsis I, Blazovics A (2008) Alterations in the content of metal elements and fatty acids in hepatic ischemia-reperfusion: induction of apoptotic and necrotic cell death. Dig Dis Sci 53: 1325–1333

White RL, Wittenberg BA (2000) Mitochondrial NAD(P)H, ADP, oxidative phosphorylation, and contraction in isolated heart cells. Am J Physiol Heart Circ Physiol 279: H1849–H1857

Xu W, Mimnaugh E, Rosser MF, Nicchitta C, Marcu M, Yarden Y, Neckers L (2001) Sensitivity of mature Erbb2 to geldanamycin is conferred by its kinase domain and is mediated by the chaperone protein Hsp90. J Biol Chem 276:3702–3708

Yarden Y, Sliwkowski MX (2001) Untangling the ErbB signalling network. Nat Rev Mol Cell Biol 2:127–137

Zaarur N, Gabai VL, Porco JA Jr, Calderwood S, Sherman MY (2006) Targeting heat shock response to sensitize cancer cells to proteasome and Hsp90 inhibitors. Cancer Res 66: 1783–1791

Zeng J, Heuchel R, Scahffner W, Kagi JH (1991) Thionein (apometallothionein) can modulate DNA binding and transcription activation by zinc finger containing factor Sp1. FEBS Lett 279:310–312