0022-3565/07/3212-517–525
THE JOURNAL OF PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS
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JPET 321:517–525, 2007

Vol. 321, No. 2
119644/3202771
Printed in U.S.A.

Protective Role of Intracellular Zinc in Myocardial Ischemia/Reperfusion Is Associated with Preservation of Protein Kinase C Isoforms

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ABSTRACT

The recent discovery of zinc signals and their essential role in the redox signaling network implies that zinc homeostasis and the function of zinc-containing proteins are probably altered as a result of oxidative stress, suggesting new targets for pharmacological intervention. We hypothesized that the level of intracellular labile zinc is changed in hearts subjected to ischemia/reperfusion (I/R) and investigated whether the maintenance of myocardial zinc status protected heart functions. Using fluorescent imaging, we demonstrated decreased levels of labile zinc in the I/R hearts. Phorbol 12-myristate 13-acetate, a known trigger of zinc release, liberated zinc ions in control hearts but failed to produce any increase in zinc levels in the I/R rat hearts. Adding the zinc ionophore pyrithione at reperfusion improved myocardial recovery up to 100% and reduced the incidence of arrhythmias more than 2-fold. This effect was dose-dependent, and high concentrations of zinc were toxic. Adding membrane-impermeable zinc chloride was ineffective. Hearts from rats receiving zinc pyrithione supplements in their diet fully recovered from I/R. The recovery was associated with the prevention of degradation of the two protein kinase C isoforms, δ and ε, during I/R. In conclusion, our results suggest a protective role of intracellular zinc in myocardial recovery from oxidative stress imposed by I/R. The data support the potential clinical use of zinc ionophores in the settings of acute redox stress in the heart.

Zinc is important to the structure and function of a large number of macromolecules. Disruption of zinc homeostasis is associated with severe disorders, including injuries to cardiac tissues (Sandstead, 1995). The existence of regulatory mechanisms is evidenced by the large gradients of Zn^{2+}, free or loosely bound to proteins, so-called labile zinc, between the intracellular and extracellular milieu (nanomolar versus micromolar concentrations, respectively). Recent discoveries have revealed that the amount of intracellular free zinc is tightly controlled at the level of uptake, intracellular sequestration, redistribution, storage, and elimination, consequently creating a narrow window of optimal zinc concentration in the cells. The molecular players in zinc homeostasis have been identified. For example, membrane transport of zinc ions is mediated by zinc transporters encoded by two solute-linked carrier gene families, ZnT (SLC30), zinc extruders, and Zip (SLC39), zinc importers (Palmiter and Findley, 1995; Eide, 2004; Liuzzi and Cousins, 2004; Palmiter and Huang, 2004). Zinc storage sites, such as metallothioneins (MTs) (Fabisiak et al., 2002), mitochondria (Bossy-Wetzel et al., 2004), and zinc-rich vesicles "zincosomes" (Failla and Cousins, 1978) participate in sequestration of zinc ions as a way of cell protection from Zn^{2+} overload and provide zinc ions to structural and catalytic proteins under conditions of cellular zinc deficiency (Feng et al., 2005).

The central position of zinc in the redox signaling network

This study was supported by the National Institutes of Health (Grants RO1 HL77390-01 to I.K. and RO1 HL-077494 to M.B.) and by a Veterans Administration Merit grant (to M.B.).

This study was presented in abstract form at the *Third Annual Symposium of the American Heart Association Council on Basic Cardiovascular Sciences: Translation of Basic Insights into Clinical Practice*, 2006 Jul 31–Aug 3; Keystone, CO [Karagulova G, Yankun Yue, Boutjdr M, Moreyra AE, and Korichneva I (2006) Intracellular Zn protects isolated rat hearts from ischemia/reperfusion injury: involvement of protein kinase C. *Circ Res* **99:**136].

Article, publication date, and citation information can be found at http://jpet.aspetjournals.org.

doi:10.1124/jpet.107.119644.

ABBREVIATIONS: MT, metallothionein; PKC, protein kinase C; PMA, phorbol-12myristate-13acetate; I/R, ischemia/reperfusion; TSQ, *N*-(6-methoxy)-8-quinolyl-toluenesulfonamide; TPEN, *N*,*N*,*N*′,*N*′-tetrakis-(2-pyridylmethyl)ethylenediamine; LVDP, left ventricular developed pressure; HR, heart rate; dP/dT, maximal time rate of change of pressure; LVDP × HR, rate pressure product; VF, ventricular fibrillation; VT, ventricular tachycardia; OCT, optimal cutting temperature; RPP, rate pressure product.

is based on its unique chemical nature. Being itself redox inert, zinc creates a redox active environment when it binds to sulfur ligand (Maret, 2004). The chemical flexibility of transition metals allows zinc to impose conformational changes on the proteins it binds to, the required step in initiation of activation process of many signaling molecules (Clegg et al., 2005). The most important property of zinc-sulfur ligand interaction is the release of zinc under an oxidative environment (Barbirz et al., 2000). Protein kinase C (PKC) is one of the examples of redox-sensitive signaling molecules (Konishi et al., 1997; Imam et al., 2001). We have previously demonstrated that oxidative stress triggers zinc release from PKC (Korichneva et al., 2002). In addition, we showed that Zn^{2+} release from PKC is also triggered by a classic lipid activator, phorbol 12-myristate 13-acetate (PMA), pointing out convergence of the two signaling pathways. Cysteine-rich zinc binding regulatory domains were determined as sources of free zinc. These highly conserved structures are able to serve as "redox zinc switches," sensing the concentrations of both zinc and oxidants.

The ability of PKC to regulate many cardiovascular functions is supported by the facts that many cardiovasotropic growth factors target PKC (Feener et al., 1995; Xia et al., 199). Cardiac myocytes express multiple PKC isozymes, α, $\beta_{1/2}$, δ, ε, and ζ, which participate in the response of muscle cells to extracellular stimuli, modulate contractile properties, and promote cell growth and survival (Disatnik et al., 1994). The two novel PKC isoforms, δ and ε, are particularly important in myocardial responses to ischemia/reperfusion (I/R). The reports that PKCδ is activated by I/R, and PKCε is involved in ischemic preconditioning, link PKC function to redox control in vivo (Kawamura et al., 1998; Chen et al., 1999; Baines et al., 2003; Inagaki et al., 2003). Oxidative stress imposed on cardiac tissue under I/R conditions would probably trigger changes of the redox status and zinc content of PKC, as well as that of other cellular redox-sensitive proteins, thereby affecting myocardial zinc homeostasis as a whole. Although an understanding of the redox signaling mechanisms is of particular importance in the heart, very limited data exist regarding myocardial zinc distribution and metabolism. Studies by Powell et al. (1994) documented the protective effects of zinc ions in myocardial recovery from I/R. The authors used zinc-bis-histidinate to improve membrane permeability of the cation. Addition at preischemia conferred myocardial protection, whereas treatment starting at reperfusion worsened postischemic damage, thus limiting clinical applications of the drug. The protective effect was attributed to changes in redox metabolism, namely to decrease in ·OH formation and copper reactivity.

The present study investigated alterations in labile zinc in rat hearts subjected to I/R and attempted to improve myocardial recovery by maintaining intracellular zinc content using the zinc ionophore pyrithione. PKC isoforms, δ and ε, were assessed as possible zinc targets and as important regulators of cardiac function under the conditions of I/R. The protective role of postischemic intracellular zinc supplementation in myocardial recovery from ischemic stress was demonstrated, suggesting the potential of development of treatments based on zinc ionophores during acute redox stress in the heart.

Materials and Methods

Animals. All the procedures on the animals were carried out in accordance with the *Guide for Care and Use of Laboratory Animals* as adopted and promulgated by the United States National Institutes of Health and were approved by the Institutional Animal Care and Use Committee. Sprague-Dawley male rats, weighing 250 g (Taconic, Albany, NY), were used for heart isolation and preparation of cardiac myocytes. The animals were allowed 2 to 3 days of in-house acclimatization before experimental procedures.

Chemicals and Reagents. *N*-(6-Methoxy)-8-quinolyl-toluenesulfonamide (TSQ) was obtained from Molecular Probes/Invitrogen Labeling and Detection (Eugene, OR). Optimal cutting temperature (OCT) embedding medium for frozen tissue specimens was from Sakura Finetek U.S.A., Inc. (Torrance, CA). Cell-TAK cell and tissue adhesive was purchased from BD Bioscience (Franklin Lakes, NJ). Urethane, heparin ammonium salt, *N*,*N*,*N*′,*N*′-tetrakis-(2-pyridylmethyl)ethylenediamine (TPEN), 1-hydroxypyridine-2-thione zinc salt (mercaptopyridine *N*-oxide zinc salt pyrithione), and Cell-TAK cell and tissue adhesive were from BD Bioscience, and PMA was from Sigma Co. (St. Louis, MO).

Experimental Groups. Animals were assigned into experimental groups (six animals in each group) as follows: group 0 hearts were perfused for 1.5 h without I/R; group 1 did not receive any treatment before the I/R experiment and no treatment during heart perfusion and was used as a control; group 2 had no treatment prior I/R, but the perfused hearts were supplemented with zinc pyrithione at reperfusion; and groups 3, 4, and 5 were treated the same as group 2 except that membrane impermeable $ZnCl_2$ was used in place of zinc pyrithione in group 3, the Zn^{2+} chelator TPEN and zinc pyrithione in group 4, and PMA and zinc pyrithione in group 5. Animals in group 6 received zinc pyrithione in their diet for 1 week (35 mg/kg body weight), and the hearts from these animals had no subsequent treatment during I/R.

Ischemia/Reperfusion of Adult Rat Hearts. Rats were anesthetized with 1.6 to 2.2 g/kg urethane and 500 IU/kg heparin, administered i.p. The hearts were rapidly excised, cannulated, and perfused retrogradely with the Langendorff method with modified Krebs-Henseleit buffer containing 117 mM NaCl, 4.7 mM KCl, 2.7 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 21 mM $NaHCO_3$, 11 mM glucose, 2.0 pyruvate, and 0.2 mM EDTA, pH 7.4. The perfusion fluid was equilibrated with 95% O_2/5% CO_2 at 37°C. A latex balloon was inserted in the left ventricle and inflated to produce an end diastolic pressure of 8 to 12 mm Hg, and this volume was unchanged for the rest of the experiment. Coronary perfusion pressure was monitored at the point of cannulation of the aorta and adjusted to 60 to 70 mm Hg. The contractile and hemodynamic functions of the heart were continuously monitored with a computer-based data acquisition system (PowerLab; ADInstruments Inc., Colorado Springs, CO). All the hearts were subjected to a 30-min stabilization period, 15 or 30 min of global ischemia, followed by 30-min reperfusion. Hearts, which demonstrated contractile or other abnormalities during stabilization period, were discarded.

Preparation of Tissue Sections. At the end of perfusion, the hearts were quickly frozen in OCT using standard protocol to preserve tissue elements (Sheehan and Hrapchak, 1980). Cryosections of 45 μm were obtained at −14°C using a cryomicrotome and placed onto a dish with a glass bottom covered with Cell-TAK cell and tissue adhesive for confocal measurements. Control experiments revealed that OCT and isopentane did not alter subsequent loading of the tissues

with fluorescent probes, nor did these procedures affect tissue response to stimuli, PMA, or H_2O_2.

Labile Zn^{2+} Imaging by Confocal Microscopy. Fluorescent imaging of intracellular Zn^{2+} was performed with selective high-affinity Zn^{2+}-sensitive probe TSQ, as described previously (Frederickson et al., 2000; Korichneva et al., 2002). Thawed tissue sections were loaded with 5 μM TSQ for 20 min, washed intensively, and moved to the stage of confocal laser scanning microscope. Images were acquired with a Zeiss/LSM510 system (Carl Zeiss Microimaging Inc., Thornwood, NY) equipped with the Enterprise UV laser (Coherent Inc., Santa Clara, CA). The 351-nm line was used for the excitation of TSQ, and fluorescence between 420 and 480 nm was captured. To minimize the UV damage, laser intensity was kept at 5%. Fluorescence intensity was recorded on-line for several minutes to obtain the background steady-state fluorescence value, and after the addition of activator, the acquisition of images continued for 30 min to reach saturation. The PKC activator, PMA (50 nM), was used to assess Zn^{2+} release potential, as described previously (Korichneva et al., 2002). To ascertain the specificity of TSQ for Zn^{2+} and to address the factor of probe concentration and environment, the specific zinc chelator TPEN was used in competition experiments. Addition of PMA and TPEN to the tissue samples was performed directly on the microscope stage. Morphometric analysis was performed using the MetaMorph image analysis software.

Isolation and Treatment of Adult Rat Ventricular Myocytes and Cell Lysates. Isolated adult rat ventricular myocytes were prepared as described previously (Puceat et al., 1995). Hearts from anesthetized animals were quickly removed and perfused in a nominal Ca-free medium for 5 min and then with 1.2 mg/ml collagenase added with 30 μM $CaCl_2$. The cells were incubated for 15 min at 37°C. Meanwhile, Ca^{2+} concentration of the incubation medium was increased gradually up to 1 mM. The preparation provided at least 6×10^6 rod-shaped cells. Cells were kept until used at 37°C in HEPES-buffered medium adjusted to pH 7.4 and containing 117 mM NaCl, 5.7 mM KCl, 4.4 mM $NaHCO_3$, 1.5 mM KH_2PO_4, 1.7 mM $MgCl_2$, 1 mM $CaCl_2$, 21 mM HEPES, 11 mM glucose, 10 mM creatine, 20 mM taurine, and 0.5% bovine serum albumin. The cells were treated at indicated time intervals with PMA (50 nM) to initiate PKC activation followed by down-regulation in the presence or in the absence of TPEN (10 μM), pelleted, and resuspended in a 50 mM glycerophosphate buffer adjusted to pH 7.4 and supplemented with 1 mM EGTA, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 100 μM E64. The lysate was incubated at 4°C for 15 min and centrifuged at 15,000*g* for 15 min. Electrophoresis and Western blotting analysis were carried out as described previously (Korichneva and Hammerling, 1999).

Preparation of Whole-Heart Lysates and Western Blotting Analysis. At the end of each experiment, the hearts were cut into small pieces, extensively washed, and homogenized in a Tris-HCl buffer, pH 7.5, containing 50 mM Tris, 2 mM EDTA, 10 mM EGTA, 5 mM dithiothreitol, 250 mM sucrose, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 100 μM E64. The homogenates were centrifuged for 15 min at 15,000*g* to discard the myofilaments. Electrophoresis and Western blotting analysis were carried out as above.

Statistical Analysis. Data are presented as means ± S.D. The number of experiments (n) indicates the number of hearts used. The parameters obtained from experimental hearts were compared with the ones for control hearts, and the differences were determined with unpaired Student's t test. The results were considered significant at the $p < 0.05$ level.

Results

Effect of Ischemia/Reperfusion on Labile Zinc in Myocardial Tissue. To estimate relative levels of free/labile Zn^{2+} in cardiac tissue, we analyzed cryosections prepared from the Langendorff perfused hearts by confocal microscopy (Fig. 1). Experimental groups 0 to 2 were compared. Nomarski images reveal a regular pattern of rod-shaped cells with sarcomeric ultrastructure characteristic of cardiomyocytes in the tissue sections prepared from control hearts. In the sections prepared from the hearts subjected to I/R (group 1), in addition to rod-shaped structures, the areas with irregular morphology were identified, most probably represented by damaged cells. The cryosections prepared from the I/R hearts supplemented with zinc pyrithione at reperfusion (group 2) showed largely unchanged morphology.

Typical structures associated with TSQ-stainable Zn^{2+} in myocardial tissue were represented by sarcomeric units, filaments, perinuclear area, and intercalated disks (Fig. 1A). Nuclei were excluded from TSQ staining. In the I/R hearts, TSQ stained the zones of cell contacts, as well as patchy areas containing granulated vesicle-like structures reminiscent of the vesicle-associated zinc accumulation seen in neurons (Velazquez et al., 1999). The areas with vesicular zinc localized mainly to the zones of damaged cells, suggesting that zinc concentration had been elevated in these zones and sequestrated by zincosomes (Fig. 1B). The distribution of labile zinc in the I/R tissues supplemented with zinc pyrithione was similar to the control samples (Fig. 1C).

Comparison of the labile zinc levels in the tissue sections showed that overall fluorescence intensity was significantly lower in I/R-stressed hearts but not in those supplemented with zinc pyrithione (Fig. 1D). The latter tissues displayed the highest level of basal zinc. Treatment of the samples on the stage of the microscope with PMA significantly increased TSQ fluorescence of tissue sections obtained from the control hearts (90% increase) but not from the ones after I/R with or without zinc pyrithione (11 and 14% increase, respectively). The kinetic of Zn^{2+} release triggered by PMA in control hearts was similar to what we have previously reported (Korichneva et al., 2002); specifically, TSQ staining reached saturation within 10 min. Thus, our data suggest that heart tissue becomes zinc-depleted after I/R and that the capacity to liberate labile zinc by a classical trigger PMA is significantly diminished.

Myocardial Protection from I/R-Induced Damage by Intracellular Zinc. To examine whether replenishment of intracellular zinc improves heart function during reperfusion after a period of ischemia, we analyzed the recovery of hemodynamic parameters, left ventricular developed pressure (LVDP), heart rate (HR), maximal time rate of change of pressure (dP/dT), and rate pressure product (RPP) (LVDP × HR) as an index of cardiac performance and heart rate, as well as the incidence of arrhythmias in isolated adult rat

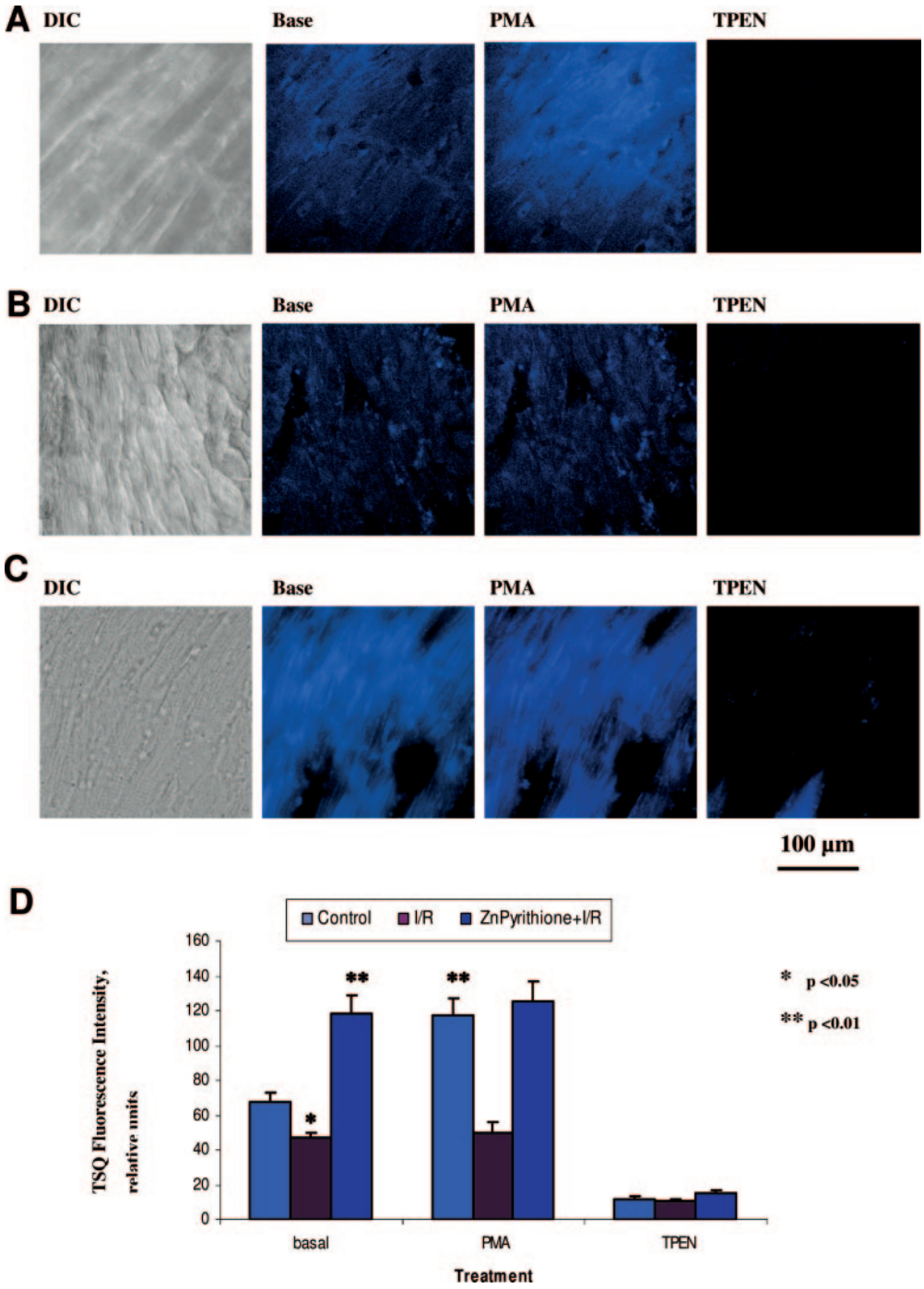


Fig. 1. Effect of I/R on functional zinc in cardiac tissue. Confocal images of labile Zn^{2+} in myocardial tissue (45-μm cryosections) were obtained from control (A) and I/R-stressed hearts in the absence (B) and presence (C) of zinc pyrithione in the reperfusion buffer. Images were acquired with a Zeiss/LSM510 system equipped with the Enterprise UV laser. The focal plane was set close to the nuclear center. The confocal projection images present one section at 1.6-μm optical resolution in the Z direction at 630-fold optical magnification. Cryosections were treated with PMA (50 nM) followed by TPEN (10 μM) for 10 min each on the stage of the confocal microscope. PMA triggers Zn^{2+} release in control but not in I/R tissue sections. A decrease in fluorescence intensity after TPEN treatment proved the specificity of TSQ as a reporter for zinc. D, quantitative analysis of TSQ fluorescence intensity in cryosections obtained from control and I/R-treated adult rat hearts in the presence or absence of zinc pyrithione. Bar graph, mean ± S.D., $n = 5$. *, $p < 0.05$; **, $p < 0.01$.

hearts subjected to both reversible (15 min) and irreversible (30 min) global ischemia followed by 30 min of reperfusion. The baseline LVDP of 101.37 ± 10.98 mm Hg recovered by 83.83% by the end of reperfusion after 15 min of global ischemia (Table 1). Zinc ionophore, pyrithione (10^{-5} M) applied at the time of reperfusion improved myocardial recovery up to 100%, at the same time reducing incidence of arrhythmias more than 2-fold, particularly obvious at late stages of reperfusion. The results summarizing the incidence of ventricular fibrillation (VF) and ventricular tachycardia (VT) are shown in Table 2. The zinc pyrithione effect was enhanced when the ischemia period was extended up to 30 min, yielding the improvement of LVDP recovery from 15.58% (experimental group 1) to 74.95% (experimental group 2) and diminishing VF (but not VT) 8-fold. The examples of the original recordings from these experiments are shown in Fig. 2A. The dose response of zinc pyrithione treatment was biphasic with the increase of protection at the concentrations up to 10 μM followed by a sharp decrease most likely due to Zn^{2+} toxicity. The recovery was attenuated by 10^{-5} M TPEN (experimental group 4). A significant finding was that zinc pyrithione in the diet (experimental group 6) could substitute for zinc pyrithione supplementation into the reperfusion buffer, showing similar recovery of LVDP (Fig. 3). Improved recovery was not observed with nonpermeable $ZnCl_2$ (experimental group 3). We found that the

TABLE 1
Effect of zinc pyrithione on myocardial recovery after 15- and 30-min ischemia

	Preischemia		Reperfusion	
	Control	10^{-5} M Zinc Pyrithione	Control	10^{-5} M Zinc Pyrithione
15-Min ischemia				
LVDP (mm Hg)	94.73 ± 4.59[a]	101.01 ± 5.83	77.98 ± 18.39	102.98 ± 13.79
Heart Rate (beats/min)	233.06 ± 36.66	262.10 ± 9.28	241.56 ± 31.79	281.26 ± 7.41
dP/dT max (mm Hg/s)	3844.45 ± 339.53	4222.83 ± 479.19	1861.30 ± 507.31	4496.80 ± 262.50[b]
RPP (mm Hg/beats/min)	22,087.41 ± 3665.29	26,404.04 ± 627.61	20,361.14 ± 6457.16	29,092.80 ± 4544.96
30-Min ischemia				
LVDP (mm Hg)	112.55 ± 2.12	103.01 ± 9.77	19.48 ± 6.92	82.65 ± 11.98[c]
Heart rate (beats/min)	235.44 ± 6.05	194.59 ± 24.67	101.79 ± 44.86	179.84 ± 32.73
dP/dT max (mm Hg/s)	3276.37 ± 301.03	3953.34 ± 591.76	160.05 ± 141.21	3259.24 ± 223.41[c]
RPP (mm Hg/beats/min)	26,506.88 ± 921.28	19,734.22 ± 2754.72	2892.86 ± 1784.34	15,268.77 ± 4185.71[c]

[a] The data are mean values ± S.D. taken from five to six hearts at 30-min reperfusion.
[b] $p < 0.05$.
[c] $p < 0.01$ in comparison with the values obtained after reperfusion in control conditions.

TABLE 2
Incidence of arrhythmias in adult rat hearts subjected to I/R

	Reperfusion					
	0–5 min	5–10 min	10–15 min	15–20 min	20–30 min	0–30 min
15-Min ischemia						
Control	43.0 ± 1.4[b]	36.0 ± 2.8	22.0 ± 3.1	18.0 ± 1.9	6.0 ± 0.7	125.0 ± 2.8[c]
VF	5.7 ± 0.4	4.0 ± 1.9	0	0	0	9.7 ± 0.1
VT[a]						
10^{-5} M zinc pyrithione	16.0 ± 1.9	12.0 ± 2.6	6.0 ± 1.9	4.0 ± 1.4	0	38.0 ± 7.4[c]
VF	4.0 ± 0.7	2.7 ± 1.1	0	0	0	6.7 ± 3.5
VT						
30-Min ischemia						
Control	169.6 ± 42.2	86.4 ± 30.2	8.6 ± 2.8	6.6 ± 2.4	7.0 ± 2.9	278.2 ± 78.9[c]
VF	0.8 ± 0.5	4.8 ± 1.0	1.6 ± 0.8	0.2 ± 0.2	0.2 ± 0.2	6.8 ± 1.9
VT						
10^{-5} M zinc pyrithione	21.0 ± 6.6	9.2 ± 3.3	1.6 ± 0.8	0.8 ± 0.4	0.2 ± 0.2	32.8 ± 11.0[c]
VF	2.6 ± 0.5	4.0 ± 1.0	1.3 ± 0.5	0	0	8.0 ± 1.0
VT						

[a] VT is defined as six or more rapid beats.
[b] The data are presented in seconds of VF or VT per the time period indicated and are mean ± S.D. of five independent experiments.
[c] $p < 0.05$.

effect of intracellular zinc supplementation on myocardial recovery depended on the activation status of PKC. The addition of the PKC trigger, PMA (50 nM, experimental group 5), after ischemia prior to zinc pyrithione, accelerated the recovery that was not sustained (Fig. 3).

PKC Involvement in Zinc-Mediated Cardioprotection. Because the two PKC isoforms, δ and ε, are among possible zinc controlled targets, we examined their alterations by Western blot analysis of the proteins prepared from rat hearts at the end of physiological experiments. Experimental groups 0 to 2 were compared. Although there were no visible changes in PKC levels after 15 min of ischemia followed by reperfusion, the results shown in Fig. 4A clearly demonstrate that PKCε level is diminished after 30 min of ischemia. Zinc pyrithione addition at reperfusion prevents PKCε reduction. Thus, prolonged stress leads to significant change in this important signaling kinase.

PKCδ activation during reperfusion has been linked to augmentation of myocardial injury (Inagaki et al., 2003). Upon proteolytic cleavage to 56- and 40-kDa fragments PKCδ can initiate aberrant signal transduction pathway leading to apoptosis (Chou et al., 2004). We determined that myocardial I/R is associated with PKCδ cleavage shifting the ratio in the levels of the 78- versus 56- and 40-kDa PKC bands toward lower molecular mass. The cleavage was diminished by supplementation of perfusion buffer with zinc pyrithione (Fig. 4B).

To determine whether zinc depletion plays a role in PKC changes, we performed the in vitro studies using the zinc chelator TPEN. To initiate PKC activation followed by down-regulation, cells were treated with PMA (50 nM). After 8 h of PMA treatment, PKCε level decreased by nearly 30% (Fig. 4C). The protein was barely detected after 16 h of PMA incubation. In the presence of TPEN, PKC degradation was markedly accelerated, reaching 30% decrease at 4 h of PMA incubation with subsequent elimination of the protein.

Discussion

Despite the vital role of zinc ions in physiology, zinc homeostasis and zinc-dependent redox regulatory mechanisms have not been extensively investigated in the heart. We present the data showing the importance of intracellular labile zinc in the myocardial response to oxidative stress imposed by I/R. Results obtained by quantitative confocal imaging technique demonstrate that cardiac tissue becomes Zn^{2+}-depleted under the conditions of I/R. We further used this approach to assess zinc functionality in vivo based on Zn^{2+} release in cryosections triggered by the known activator, PMA, and showed that in addition to decreased basal levels of labile zinc, stressed myocardial tissue loses the capacity to liberate zinc.

There is growing evidence that many proteins are regulated by a redox environment through reversible oxidation

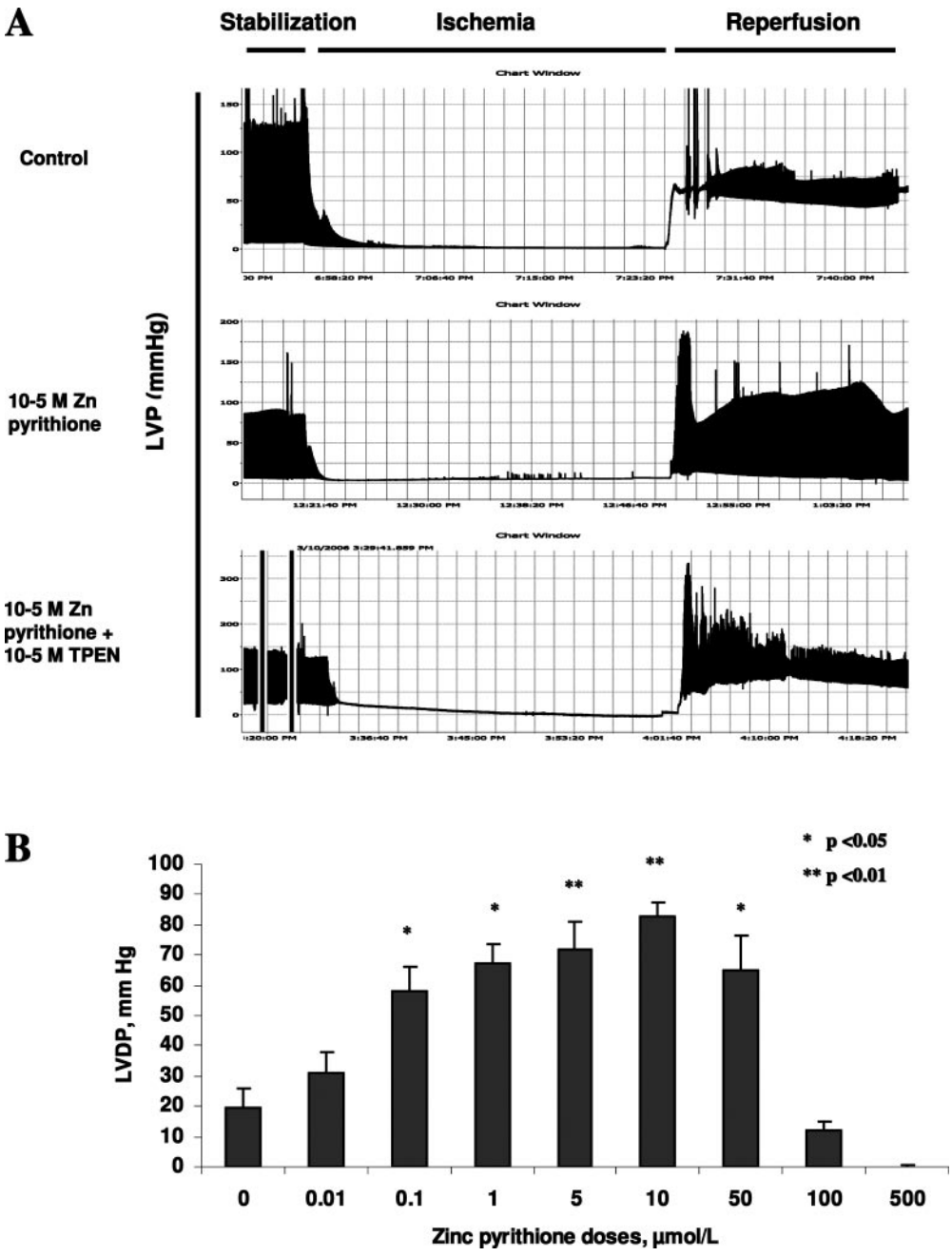


Fig. 2. Effect of zinc pyrithione supplementation at reperfusion on left ventricular pressure in Langendorff perfused adult rat hearts. The hearts were subjected to 30-min ischemia followed by reperfusion. Original recordings (A) demonstrate that addition of zinc pyrithione (10 μM) conferred the protection. The zinc-selective chelator TPEN (10 μM) added 2 min before zinc pyrithione supplementation abolished the protection. Dose dependence (B) was assessed in the hearts subjected to 30-min ischemia followed by 30-min reperfusion. Zinc pyrithione in the doses indicated was supplemented directly into the reperfusion buffer. LVDP was calculated as the difference between systolic and diastolic pressures of the left ventricular pressure trace, and the values obtained at 30-min reperfusion were used for dose dependence. The results on the bar graph are presented as percentage of recovery in relation to average LVDP during stabilization period. The data are mean ± S.D., $n = 5$. *, $p < 0.05$; **, $p < 0.01$ compared with the recovery in the absence of zinc pyrithione.

of their cysteine residues (Jakob et al., 2000). More refined redox zinc switches are in addition controlled by zinc availability (Maret, 2004; Korichneva, 2006). The underlying chemical changes of protein redox zinc switches seem to be among the very first responses to oxidative stress. Liberated zinc therefore would serve as a reliable indicator of the redox status of cellular proteins. Increase in free zinc has been observed in the cells treated by nitric oxide (Chang et al., 2004) or hydrogen peroxide (Korichneva, 2005).

Fluorescent probes designed to determine free zinc differ in their chemical properties and physical characteristics and provide different advantages depending on the experimental goals. The TSQ probe that features high sensitivity and selectivity for Zn^{2+} (Frederickson et al., 2000; Korichneva et al., 2002) was used as the Zn^{2+} reporter in this work. The specific requirement for such a reporter is the value of its affinity for Zn^{2+}, which is related to the potential to withdraw zinc ions from the numerous protein-binding sites. In other words, the probe should be sensitive enough to detect changes in intracellular Zn^{2+} concentration without competing with high-affinity protein binding sites. The submicromolar affinity of TSQ for Zn^{2+} meets these criteria exactly. The use of TSQ to determine PMA-triggered zinc release has been validated. We have reported previously that the probe does not bind to PKC upon stimulation of the cells with PMA (Korichneva et al., 2002). Likewise, we excluded the possible influence of PMA triggered alkalinization as a cause of TSQ fluorescence increase because the probe is known to be pH-independent (Frederickson et al., 2000). Fluorescent signal quenching by the selective Zn^{2+} chelator TPEN provides a "zero" point for calculations of Zn^{2+} increase and indicates

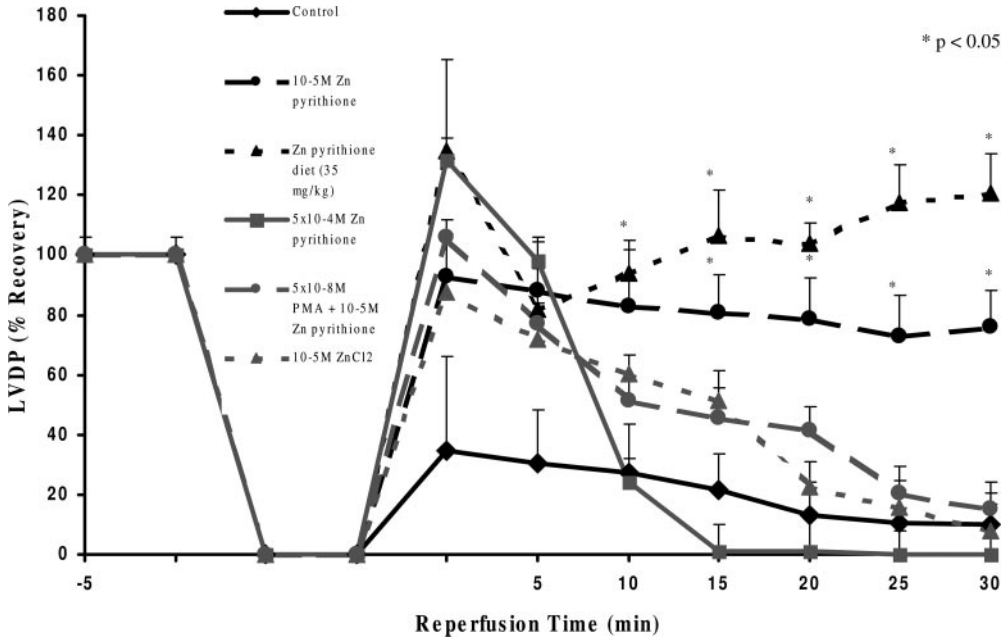
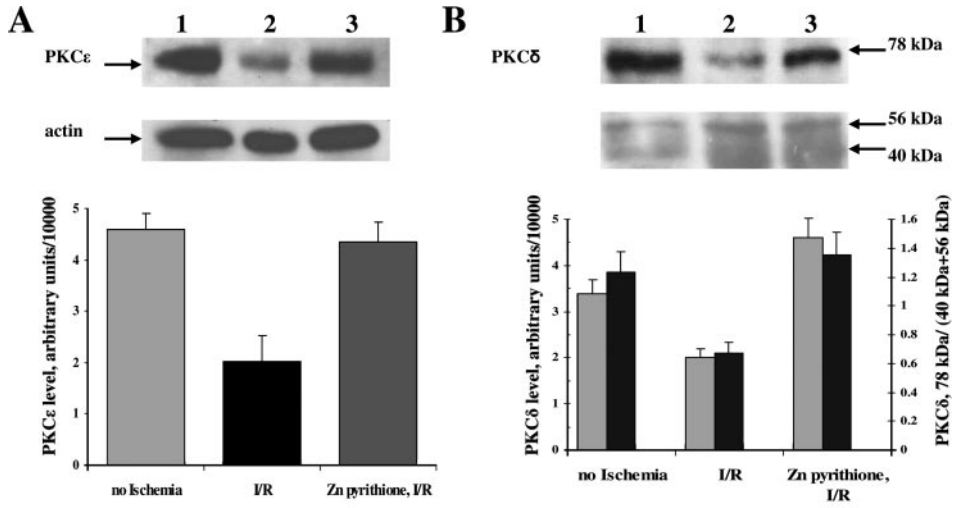


Fig. 3. Modulation of zinc-mediated effects on LVDP. The hearts were subjected to 30-min ischemia followed by reperfusion using the Langendorff technique. The pharmacological agents in the doses indicated were supplemented into the reperfusion buffer. One group of animals received a zinc diet (35 mg/kg) for 1 week before the experiment. LVDP was calculated as the difference between systolic and diastolic pressures of the left ventricular pressure trace. The results are presented as percentage of recovery in relation to average LVDP during the stabilization period. The data are mean ± S.D. compared with recovery in control conditions, $n = 5$. *, $p < 0.05$.



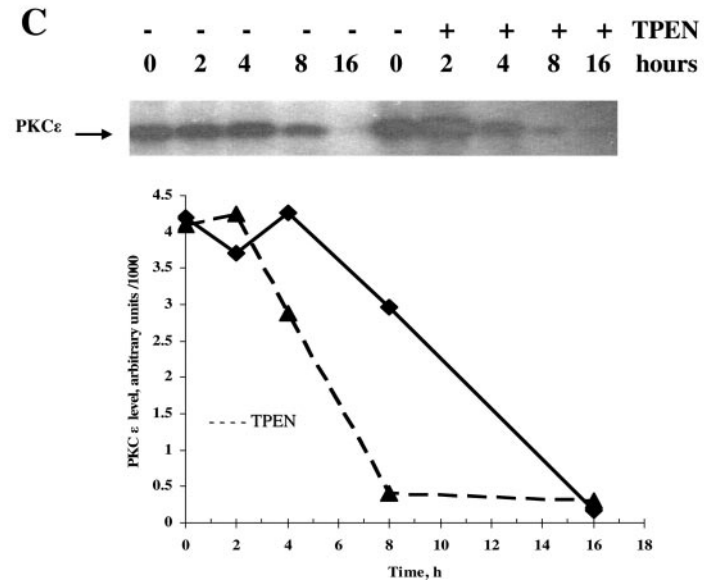


Fig. 4. Determination of PKC level by Western Blot analysis. Western blot of the proteins (20 μg per lane) from control perfused heart (lane 1), the heart subjected to I/R (lane 2), and the heart after reperfusion with 5 μM zinc pyrithione (lane 3) using the anti-PKCε and anti-actin antibodies (A) or anti-PKCδ antibodies (B). I/R triggers degradation of PKC isoforms. PKCδ degrades with formation of two products linked to apoptotic pathways. Zinc pyrithione shifts the ratio toward the 78-kDa PKCδ form. Bar graph, mean ± S.D. of three measurements. C, isolated cells were stimulated with PMA (50 nM) in the presence or absence of zinc chelator TPEN (10 μM). The reaction was stopped at the times indicated, and cell lysate was loaded on polyacrylamide gel electrophoresis for Western blotting with anti-PKCε antibodies. The PKC content (relative units) was analyzed with ImageJ software (National Institutes of Health). Molecular weights were estimated from prestained standards.

specificity. TSQ histofluorescence has been used in a number of tissues to ascertain the distribution of free zinc (Nitzan et al., 2004).

The areas of elevated labile zinc within the cells in myocardial cryosection include myofilaments, intercalated disks, and sarcomeres. The function of zinc ions in these zones is unknown. Possibly, increased TSQ fluorescence due to increased labile zinc is connected to localization of signaling modules in myocytes. Several PKC isoforms as well as their binding copartners, receptors for activated C kinase, have been shown to localize to the area of Z discs (Pyle and Solaro, 2004). Stress-induced change in sarcomeric pattern of Zn^{2+} staining may be related to destabilization of cytoskeletal elements similarly to those shown by us earlier on the model of anhydroretinol-induced apoptosis (Korichneva and Hammerling, 1999).

It is well known that PKCδ and PKCε have different functions in cardiac ischemia/reperfusion (Kawamura et al., 1998; Chen et al., 1999; Baines et al., 2003; Inagaki et al., 2003). However, they both are redox-sensitive, and both are subjected to degradation at prolonged ischemia (30 min). The degradation was not that obvious at shorter ischemic period (15 min), which is known as a reversible ischemia. Correspondingly, the effect of zinc ionophore on myocardial recovery was not that pronounced at 15-min ischemia, as compared with 30-min ischemia. Although it seems obvious that preservation of PKCε as an antiapoptotic PKC isoform should confer myocardial protection, the results obtained for PKCδ might be viewed as controversial. This isoform is activated at reperfusion, and its inhibition significantly diminishes the infarct zone (Inagaki et al., 2003). However, the timing of this inhibition seems to be important because this isoform can also mediate preconditioning (Hirotani and Sadoshima, 2005). Nonetheless, PKCδ is subject to processing to truncated catalytically active forms that mediate an aberrant signaling pathway leading to apoptosis (Chou et al., 2004), and our results demonstrated that zinc pyrithione treatment shifts the ratio of the truncated versus full-length PKCδ toward full-length protein, possibly explaining the protective role of intracellular zinc supplementation.

Activation of PKC by PMA prior reperfusion mediates postconditioning, but in combination with the zinc ionophore, the protection is not sustained. This suggests that if one of the mechanisms by which PMA confers the protection depends on zinc release, additional supplementation of intracellular Zn^{2+} may result in toxicity. Thus, the combination of protective agents should be carefully considered with regard to shared molecular mechanisms.

Based on our results, we suggest a hypothetical model of zinc role in cellular protein stress responses (Fig. 5). Reversible zinc release in response to redox changes is a signaling event that is associated with activation of zinc-containing molecules initiating a physiological adaptive response. Protein kinases possessing zinc fingers in the regulatory domains, like PKCε, are the primary examples. In case of irreversible activation similar to long-term PMA treatment or prolonged oxidative stress, the degradation pathway will be initiated. Thus, prolonged stress imposed by 30-min ischemia will lead to irreversible loss of zinc from the proteins triggering, or at least participating in, the initiation of protein degradation and cell death. Our in vitro experiments demonstrate that zinc removal by chelation accelerates down-regulation of PKCε. Although several mechanisms of this down-regulation, including transcriptional regulation, may be triggered by prolonged exposure to zinc chelator and PMA, protein degradation observed at 4 h of treatment is the likely mechanism shared in both in vivo and in vitro experimental models. As a consequence, elimination of Zn^{2+} renders sensitivity to damaging factors. As it has been reported, oxidative treatment of C6 glioma cells resulted in 50% of total zinc exported through the plasma membrane (Haase and Beyersmann, 2002).

In the situation of zinc loss, one would suggest that zinc replenishment could compensate at least in part for the damage associated with this loss. A potential mechanism utilizing zinc donation by MTs already has been proven in the model of myocardial I/R (Jiang et al., 2000; Kang et al., 2003). Studies using a cardiac-specific MT-overexpressing transgenic mouse model have demonstrated that MT inhibits myocardial injuries triggered by oxidative stress (Kang et al., 2003). We show here that supplementation of intracellular zinc using the zinc ionophore pyrithione confers a similar effect on stressed myocardium, significantly improving the mechanical properties of the heart and diminishing the incidence of fatal ventricular arrhythmias. Zinc pyrithione supplement in the diet, which had a similar effect in our experiments, probably confers protection through MT induction. We showed that PKC isoforms are among the potential tar-

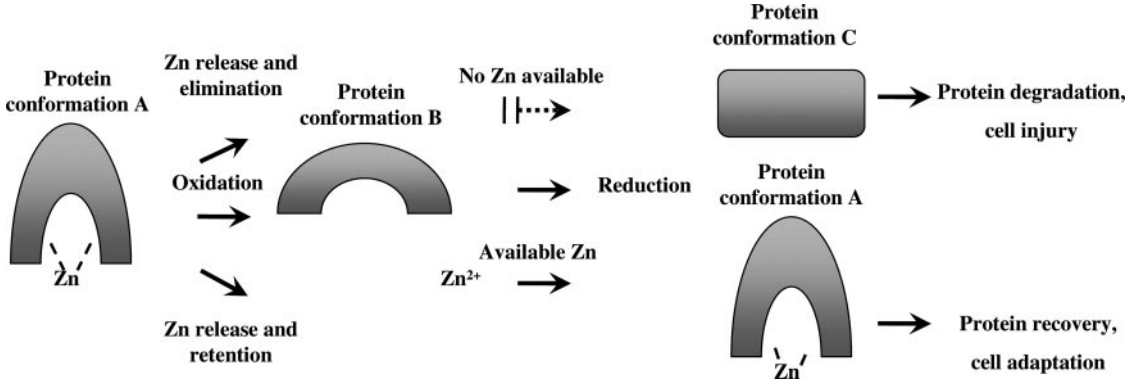


Fig. 5. Hypothetical model of zinc regulatory function in cellular stress responses. Under the condition of oxidative stress, zinc is released from the protein and either eliminated or retained by the cell. Protein conformation changes from A to B. Reduction in the presence of zinc leads to the reversal of the process, which takes place in physiological stress response resulting in adaptation. If zinc is not available, conformation A cannot be achieved, leaving the protein in conformation B or transforming it to C under reductive stress. The protein is not prepared to respond to the next message and is degraded as a result of pathophysiological stress response, leading to cell injury.

gets whose functionality is preserved by zinc, although we cannot exclude the effect of intracellular zinc supplementation on other mechanisms involved in injury following ischemia, including calcium entry by an L-type Ca channel (Turan, 2003). A better understanding of these mechanisms and of the physiological role of zinc in cellular redox control in myocardium may lead to the development of agents for zinc manipulation and promising new areas for future biomedical and pharmacological research.

Acknowledgments

We thank Alan Wilson for help in the preparation of the manuscript.

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