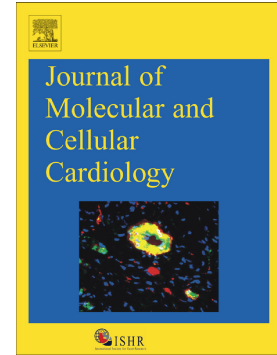


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**The critical role of the zinc transporter Zip2 (SLC39A2) in ischemia/reperfusion injury in mouse hearts**

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Running title: Zip2 and cardioprotection

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**Abstract**

Although zinc homeostasis has been demonstrated to play a role in myocardial ischemia/reperfusion (I/R) injury, the roles of zinc transporters that are critical for zinc homeostasis in I/R injury are poorly understood. The purpose of this study was to test if Zip2, an important zinc importer, plays a role in I/R injury in mouse hearts and explore the mechanism by which Zip2 expression is regulated. Zip2 expression was increased at reperfusion in *in vivo* mouse hearts, an effect that was abolished by ZnCl<sub>2</sub>, indicating Zip2's attempt to compensate for zinc loss at reperfusion. Further studies showed that upregulation of Zip2 expression was reversed by either pharmacological or genetic inhibition of signal transducers and activators of transcription 3 (STAT3), whereas STAT3 overexpression increased Zip2 expression, indicating that STAT3 accounts for Zip2 upregulation. In support, reperfusion enhanced STAT3 phosphorylation (Tyr<sup>705</sup>), which was blocked by ZnCl<sub>2</sub>, implying that STAT3 is activated in response to zinc loss. To determine the role of Zip2 in I/R injury, we assessed I/R injury by genetically disrupting Zip2 expression. Knockout of Zip2 genes (Zip2<sup>+/-</sup> and Zip2<sup>-/-</sup>) exacerbated I/R injury by increasing infarct size as well as the serum LDH, troponin I (cTnI), and CK-MB activities. In contrast, delivery of Zip2 genes reduced I/R injury. Delivery of STAT3 genes increased STAT3 phosphorylation and reduced I/R injury. However, delivery of the dominant negative STAT3 mutant did not reduce I/R injury. Moreover, delivery of STAT3 genes failed to reduce I/R injury in Zip2<sup>-/-</sup> mice. Zip2 upregulated upon reperfusion via STAT3 is cardioprotective and this upregulation may serve as an important intrinsic protective mechanism by which the heart is resistant to I/R injury. The factors involved in the zinc homeostasis (zinc and Zip2) are responsible for STAT3 activation and its subsequent cardioprotective action.

**Key words:** zinc; zinc transporter; zinc homeostasis; STAT3; Zip2

## 1. Introduction

Zinc is essential for the structure and function of many proteins [1] and has been demonstrated to be cytoprotective [2, 3]. An early study documented that treatment of isolated perfused rat hearts with zinc reduced cardiac dysfunction and LDH release at reperfusion, suggesting that zinc may play a role in prevention of myocardial ischemia/reperfusion injury [4]. Recently, we found that exogenous zinc protects cardiac cells from ischemia/reperfusion injury by targeting the mitochondrial permeability transition pore (mPTP) via Akt and glycogen synthase kinase  $\beta$  (GSK-3 $\beta$ ) [5, 6]. Exogenous zinc was also shown to protect cardiac cells from reoxygenation injury by inducing ErbB2 expression and Akt activation [7]. We further demonstrated that activation of adenosine A<sub>2</sub> receptors protects rat hearts from ischemia/reperfusion injury by preventing reperfusion-induced zinc loss and by relocating zinc to mitochondria, indicating that the maintenance of cardiac zinc homeostasis is critical for cardioprotection against ischemia/reperfusion injury [8]. In support, a recent report addressed that intracellular free zinc levels were decreased in rat hearts subjected to ischemia/reperfusion and the treatment of hearts with zinc ionophore pyrithione at reperfusion induced cardioprotection [9]. Thus, it is reasonable to propose that the impairment of zinc homeostasis characterized by the loss of zinc may serve as an important mechanism responsible for ischemia/reperfusion injury.

Recent studies indicate that zinc transporters play a critical role in the maintenance of intracellular zinc homeostasis. Zinc transporters are encoded by two gene families: Znt (SLC30) and Zip (SLC39) [10, 11]. They have opposite roles in zinc homeostasis. Znt transporters lower cytoplasmic zinc levels by promoting zinc efflux from cells or influx into intracellular vesicles, whereas Zip transporters increase cytoplasmic zinc by promoting zinc transport from the

extracellular fluid or from intracellular vesicles into cytoplasm [10]. Expression of zinc transporters is regulated by cellular zinc levels to maintain zinc homeostasis [11]. Accordingly, it is imaginable that the loss of zinc at reperfusion may lead to alterations in expression levels of the zinc transporters. In this regard, Zip2 (SLC39A2) may be the most noticeable zinc transporter, since depletion of intracellular free zinc greatly increased Zip2 expression [12, 13] and Zip2 is demonstrated to be involved in cellular zinc uptake [14]. Thus, it is tenable to hypothesize that Zip2 expression is upregulated in the setting of ischemia/reperfusion to compensate for zinc loss, which may serve as an important innate adaptive mechanism by which the heart attempts to reduce cardiac injury.

Although it is well known that the expression of the Zip family zinc transporters is regulated by zinc availability, the molecular mechanism underlying the regulation is still largely unknown. Zinc can inhibit STAT3 (signal transducer and activator of transcription 3) signaling in T<sub>H</sub>17 cells [15] and Zip6 serves as a downstream target of STAT3 during zebrafish gastrulation [16]. STAT3 is activated during ischemia/reperfusion (I/R) [17, 18]. Overexpression of STAT3 showed reduced infarct size compared to wild-type mice after I/R [19]. In contrast, STAT3 knockout mice revealed larger infarcts after I/R [20]. These observations suggest that STAT3 activation during I/R is protective and can modulate cardiac injury. In addition, STAT3 is also critical for cardioprotective effects of ischemic preconditioning and postconditioning [21, 22]. Thus, it is tenable to hypothesize that STAT3 may play a role in the regulation of Zip2 expression in the setting of I/R. If this is true, this study will not only disclose the role of Zip2 in I/R injury but also provide useful knowledge on the mechanism by which STAT3 exerts its cardioprotection in the setting of I/R.

The purpose of this study was to identify the role of Zip2 during I/R injury and to explore the mechanism by which Zip2 expression is regulated. First, we tested if Zip2 expression is upregulated upon reperfusion in mouse hearts. We then explored the mechanism by which Zip2 expression is regulated, focusing on the role of STAT3. Lastly, we determined the role of Zip2 in I/R injury by genetically altering Zip2 expression levels.

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## 2. Materials and Methods

### 2.1 Chemicals

ZnCl<sub>2</sub> and N,N,N',N'-Tetrakis (2-pyridylmethyl) ethylenediamine (TPEN) were purchased from Sigma (St.Louis,MO,USA). Stattic (Selective STAT3 inhibitor) was purchased from TOCRIS (Bio- techne, UK). Antibodies including anti-phospho-STAT3,-tubulin, -GAPDH, and the secondary antibody were purchased from Cell Signaling Technology (Beverly, MA, USA). Anti-Zip2 was purchased from Sigma-Aldrich. Zinc fluorescent probe ZinPyr-1 was purchased from Santa Cruz Biotechnology.

### 2.2 Animals

Male C57BL/6 mice (8-10 weeks old) were purchased from the Institute of Laboratory Animal Science, Chinese Academy of Medical Sciences (Beijing, China).The source of Slc39a2 (Zip2) knockout mice is described in this article. All the animal treatments and subsequent analysis were performed in a blind fashion for all groups. All of the animal experiment procedures were approved by the Tianjin Medical University Animal Care and Use Committee. All animal experiments described were carried out in accordance with the NIH Guide for the Care and Use of Laboratory Animals (Eighth Edition).

### 2.3 *In vivo* mouse heart study

Male mice (8-10 weeks) anesthetized with sodium pentobarbital (80 mg/kg, i.p.) were intubated through a tracheotomy, and ventilated with 3 cm H<sub>2</sub>O positive-end expiratory pressure.

Adequacy of anesthesia was monitored using the corneal and withdrawal reflexes. Ventilation frequency was kept at 110 breaths per minute with a tidal volume between 135 - 150  $\mu$ L. After opening the chest, the left anterior descending coronary artery (LAD) was surrounded by a 7-0 Prolene suture that was then passed through a small plastic tube. Ischemia was induced by

tightening the tubing against the heart surface. Regional ischemia was confirmed by visual inspection of pale color of the myocardium and the elevation of ST segment on the electrocardiogram. All hearts underwent 30 min of LAD occlusion followed by 2 h of reperfusion, and the sham-operation group was also placed with the suture under the LAD but without occlusion.

#### **2.4 Measurement of infarct size**

Evans Blue and triphenyltetrazolium chloride (TTC) double staining methods were used to determine myocardial infarct size. Infarct size was assessed after the end reperfusion. Hearts were excised and sliced. The slices were incubated in 1% TTC in sodium phosphate buffer at 37 °C for 20 min and fixed with 10% formalin at room temperature. Infarct size was measured with ImageTool by Dr. Thomas Krieg's lab at the University of Cambridge in a single-blind mode and was expressed as a percentage of the risk zone.

#### **2.5 Measurement of zinc concentrations in cardiac tissue**

Cardiac tissue was weighed and digested in 0.7–1.5 ml concentrated HNO<sub>3</sub> at 120°C by using a Hot Plate (LabTech, USA) for 1-2 h. Each sample was diluted with 13% HNO<sub>3</sub>. The concentration of zinc was quantified by using inductively coupled plasma optical emission spectroscopy (ICPOES, Optima™ 8000, Perkin Elmer, CT, USA) at a wavelength of 206.200. A multi-element standard (Spex Certiprep, Metuchen, NJ) was used to calibrate the instrument. The limits of detection approximated 1 ppb. Tissue concentrations are expressed as µg/g wet weight.

#### **2.6 *In vivo* gene transfer**

Recombinant adeno-associated virus (serotype 9) (AAV9) vector has been reported as an excellent candidate for intravascular gene delivery. The AAV9 vectors carrying mouse STAT3, STAT3 Y705F or Zip2 with a c-TNT promoter (AAV9-cTNT-STAT3, AAV9- cTNT-



STAT3Y705F, AAV9-cTNT-Zip2) were generated by Hanbio Biotechnology Co. Ltd (Shanghai, China). The dominant negative mutant (STAT3Y705F) was obtained by substituting phenylalanine for tyrosine at 705 site. AAV9-cTNT was used as a negative control. AAV9-cTNT-STAT3, AAV9-cTNT-STAT3Y705F, AAV9-cTNT-Zip2 or AAV9-cTNT [3×10<sup>11</sup> vector genomes (vg) per mouse] were injected into mice respectively in a total volume of 0.1 mL via the tail vein. Experiments were done 4 weeks after the injection.

## 2.7 Generation of Slc39a2 (Zip2) knockout mice

A construct was engineered for disruption of the Slc39a2 (Zip2) gene in which a 3.7 kb fragment spanning the Zip2 gene was flanked by two loxP sites. The 3.7 kb fragment, the 5.1 kb left homology arm, and the 3.1 kb right homology arm were amplified from C57BL/6 BAC DNA and cloned into the pBasicLFNeoFL vector sequentially by in-fusion cloning and confirmed by sequencing. In addition to the knockout region and homology arms, the targeting construct also contains Frt sites flanking the neomycin cassette for positive selection and a DTA cassette for negative selection. The targeting construct linearized with NotI was electroporated into C57BL/6 ES cells. Six-positive clones were identified from 96 G418-resistant clones by PCR screening. The positive clones were expanded and further confirmed by Southern blot. The random integration of extra copies of targeting construct was excluded by hybridization with a neomycin probe. Chimeric mice were generated by microinjection of two independent ES cell clones into blastocysts, and the blastocysts were implanted into pseudo-pregnant foster mice by Cyagen Biosciences Inc (Guangzhou, China). Chimeric males were bred with Flp deleter mice from Jackson Laboratories to generate neomycin-free floxed mice. The correct integration of loxP sites and the successful removal of the neomycin cassette were confirmed by PCR analysis with the primers listed below.

mSlc39a2\_F1: ATTCACACAGAAGAGTAGCAACTCCACC

mSlc39a2\_R1: CTGTCTGGGTTTACACAGCTCT

mSlc39a2\_F2: CTTCTICCTTGTGCTTCTCTTCCA

mSlc39a2\_R2: GGCTGGTCCTTCTGGTTTCTACTA

Heterozygous Zip2-knockout mice (Zip2<sup>+/-</sup>) were generated by crossing Ella-cre mice (strain name: B6.FVB-TgN C5379 Lmgd from JAX.org) which express Cre in all tissues with Zip2<sup>flox/flox</sup> mice. Heterozygous Zip2-knockout mice were inbred to generate homozygous knockout mice and wild-type mice.

## 2.8 Cell culture

Rat heart tissue-derived H9c2 cardiac myoblast cell line was purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were cultured in culture dishes with Dulbecco's modified Eagle's medium (DMEM) (Gibco), supplemented with 10% fetal bovine serum (FBS) (Gibco) and 100 U penicillin/streptomycin at 37 °C in a humidified 5% CO<sub>2</sub>-95% air atmosphere.

## 2.9 Hypoxia/reoxygenation (H/R) of H9c2 cells

To induce H/R injury, cells cultured in the culture plates filled with DMEM deficient in glucose and FBS were exposed to hypoxia (1% O<sub>2</sub>) 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<sub>2</sub>) for 4 h.

## 2.10 siRNA and plasmid transfection

H9c2 cells with >80% subconfluency were transfected with Zip2 siRNA (Santa Cruz) using Lipofectamine RNAiMAX Reagent (Invitrogen) and Zip2 plasmid (GenScript) using

Lipofectamine 3000 Reagent (Invitrogen) according to the manufacturer's instruction. All experiments were done 48 h after transfection.

### **2.11 Cell viability assay**

H9c2 cells were seeded in 96-well plates ( $1 \times 10^4$  cells/ml). Following hypoxia/reoxygenation, cells viability was measured using the CCK-8 kit (DOJINDO) according to the manufacturer's protocol. Absorbance was measured at 450 nm with a microplate reader (VersaMax, Molecular Devices, USA).

### **2.12 Real-time quantitative RT-PCR**

Total RNA was extracted using Trizol reagent (Invitrogen). For RT-PCR, the cDNA Reverse Transcription Kit (Promega, Madison, USA) was used according to the manufacturer's protocol. Relative quantification of mRNA was performed by real-time quantitative SYBR Green MasterMix using the CFX96 Real-Time System (BIO-RAD, CA, USA). The primers used were as follows.

Mouse Zip2 primer

Forward: AGCTACAGGTCATCACCACAGG

Reverse: TGCATCAACCCTGCTCCCAA;

Mouse GAPDH primer

Forward: CGTGCCGCCTGGAGAAACCTG,

Reverse: AGAGTGGGAGTTGCTGTTGAAGTCG.

### **2.13 Measurements of cardiac marker enzymes**

The activities of cardiac troponin I (cTnI), creatine kinase-MB (CK-MB), and the release of lactate dehydrogenase (LDH) in serum were analyzed using a standard kit by enzyme-linked immunosorbent assay (Bio-Swamp, China).

#### **2.14 Hematoxylin and eosin (HE) staining**

After fixed with 4% paraformaldehyde, cardiac tissues were paraffin-embedded and cut into 6- $\mu$ m sections, The sections were then stained with hematoxylin and eosin according to the standard protocol including deparaffinization and hydration.

#### **2.15 Measurement of intracellular free zinc with flow cytometry**

Free zinc concentrations in H9c2 Cells were detected with ZinPyr-1, a cell-permeable zinc fluorescence probe, according to the manufacturer's instructions. Briefly, 72 h after transfection of Zip2 plasmid or negative control (pcDNA 3.1), cells were stained with ZinPyr-1 (5 $\mu$ M) for 30 min at 37°C. Then cells were filtered with nylon mesh. Fluorescence was determined with the FACSVERSE Flow Cytometer (BD Biosciences, CA, USA). The Flow J software was used to determine the fluorescence value.

#### **2.16 Western blotting analysis**

Equal amounts of protein lysates were loaded and electrophoresed on a SDS-polyacrylamide gel and transferred to a PVDF membrane. Membranes were probed with primary antibodies overnight at 4 °C. Each primary antibody binding was detected with a secondary antibody and visualized by the enhanced chemiluminescence (ECL) method. The ECL-image was captured with Biospectrum Imaging System (UVP, Upland, CA).

#### **2.17 Measurement of total zinc concentrations in cardiac tissue**

Tissue samples weighed 100 mg and digested by 2 mL 65% nitric acid (HNO<sub>3</sub>) at 120 °C for 30min. After cooling for 15 min at room temperature, the samples were diluted by mineral-free water. The concentration of Zn<sup>2+</sup> was quantified using inductively coupled plasma optical emission spectroscopy (ICPOES, Perkin Elmer, USA) at a wavelength of 206.200 nm. A multi-

element standard solution (Perkin Elmer, USA) was used to set up standard curve of  $Zn^{2+}$  concentration. The limits of detection approximated 1 ppb.

### 2.18 Experimental protocols

All hearts were subjected to 30 min of regional ischemia followed by 30, 60, or 120 min of reperfusion.  $ZnCl_2$  (300  $\mu$ g/kg) was infused starting 5 min before the onset of reperfusion and continuing for 30 min via the tail vein. Statin (3 mg/kg) was given starting 20 min before ischemia and continuing for 20 min. Cardiac samples were collected 30, 60, or 120 min after the onset of reperfusion from the risk zone. In studies with H9c2 cells, TPEN (10  $\mu$ M) was given 5 min before the onset of reperfusion for 4 h.  $ZnCl_2$  (1  $\mu$ M) together with pyrithione was given 5 min before the onset of reperfusion for 30 min.

### 2.19 Statistical analysis

Investigators measuring mouse heart infarct size and Western blotting intensity were blind with respect to the experimental protocols. All data were presented as mean  $\pm$  SD. Data were test for the normality distribution with Shapiro-Wilk test. Statistical significance was determined by using the Student's t-test or one-way ANOVA followed by Tukey's test. A value of  $p < 0.05$  was considered as statistically significant.

### 3. Results

Since depletion of intracellular  $Zn^{2+}$  induces Zip2 expression [12], we tested if I/R could alter Zip2 mRNA expression levels in mouse hearts. Zip2 mRNA expression was significantly increased after reperfusion (Fig. 1A), which was prevented by  $ZnCl_2$  (Fig. 1B), pointing to that zinc loss leads to the increase of Zip2 expression. Western blotting analysis also showed that increased Zip2 protein expression at reperfusion was inhibited by  $ZnCl_2$  (Fig. 1C). Moreover, Zip2 overexpression increased  $Zn^{2+}$  levels in cardiac H9c2 cells, as indicated by an enhancement of Zinpyr-1 fluorescence (Fig. 1D). To test if Zip2 overexpression can alter cardiac zinc levels *in vivo*, we measured cardiac  $Zn^{2+}$  levels in *in vivo* mouse hearts with ICPOES. As shown in Fig. 1E, delivery of adeno-associated virus (AAR) carrying STAT3-overexpressing plasmid increased cardiac  $Zn^{2+}$  levels.

To investigate the mechanism by which I/R induces Zip2 expression, we examined if static, a selective inhibitor of STAT3, could alter I/R induced Zip2 expression. Fig. 2A and B show that Zip2 mRNA and protein expressions at reperfusion were inhibited by static, indicating that STAT3 as an upstream signal regulates Zip2 expression at reperfusion. In support, delivery of AAR carrying STAT3-overexpressing plasmid enhanced Zip2 protein expression, whereas a dominant negative STAT3 mutant (Y705F) in which tyrosine 705 (Y705F) was replaced by phenylalanine failed to upregulate Zip2 expression (Fig. 2C). To confirm this role of STAT3, we measured STAT3 phosphorylation at Tyr<sup>705</sup> after reperfusion. STAT3 phosphorylation was increased at reperfusion (Fig. 3A) and this was prevented by  $ZnCl_2$  (Fig. 3B), indicating that STAT3 is activated in response to zinc loss upon reperfusion.

To determine the exact role of Zip2 in I/R injury, we created heterozygous ( $Zip2^{+/-}$ ) and homozygous ( $Zip2^{-/-}$ ) Zip2 knockout mice and evaluated cardiac injury in the setting of I/R. As

shown in Fig. 4A and B, Zip2<sup>+/-</sup> mice revealed a significant decrease in Zip2 mRNA and protein expression levels in the heart and skin. Fig 4C shows that Zip2<sup>+/-</sup> mice showed a significant increase in infarct size after I/R compared to the wild type mice, suggesting that Zip2 is protective against I/R injury. Fig. 5A shows that Zip2 expression was completely suppressed in the heart and skin of the Zip2 homozygous mice (Zip2<sup>-/-</sup>). After I/R, the serum LDH, cTnl, and CK-MB activities (Fig.5B-D) were increased in Zip2<sup>-/-</sup> mice compared to those in the WT mice. In contrast, injection of AAV carrying Zip2 over-expressing plasmid reduced the LDH, cTnl, and CK-MB activities (Fig.5B-D). In support, infarct size was dramatically increased by the homozygous knockout of Zip2 genes (Fig. 5E). Similar to the above observation, injection of AAR carrying Zip2 plasmid reduced infarct size compared to the wild type (Fig. 5E). To further confirm the role of Zip2, we measured cell viability in H9c2 cells subjected to hypoxia/reoxygenation (H/R). Zip2 siRNA or Zip2 plasmids were transfected to cells to assess the role of Zip2. As shown in Fig. 6A, H/R significantly reduced cell viability. Transfection of Zip2 siRNA further reduced cell viability, an effect that was reversed by the treatment with ZnCl<sub>2</sub>. Fig. 6B shows that Zip2 overexpression increased cell viability compared to the vector, whereas the zinc chelator TPEN abolished the effect of Zip2 plasmid.

To determine the role of STAT3 in the protective effect of Zip2, we evaluated cardiac injury in mouse hearts subjected to I/R by altering STAT3 levels through injection of AAV carrying the wild-type STAT3-overexpression plasmid or Y705F. Both the plasmids increased the total STAT3 expression in cardiac tissue (Fig. 7A). However, the wild-type STAT3-overexpression plasmid but not Y705F upregulated STAT3 phosphorylation (Fig. 7B). Overexpression of STAT3 but not Y705F reduced the serum cTnl (Fig. 7C) as well as LDH and CK-MB (data not shown) activities, and infarct size (Fig. 7D). Further experiments showed that

STAT3-overexpression failed to alleviate cardiac injury in Zip2<sup>+/-</sup> mice (Fig. 7C-F), implying that activated STAT3 protects the heart from I/R injury through Zip2 and STAT3 serves as an upstream signal upregulating Zip2 expression.

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#### 4. Discussion

This study demonstrates that Zip2 is an endogenous cardioprotective molecule in the setting of I/R in mouse hearts. I/R-induced zinc loss triggers STAT3 activation, which accounts for Zip2 upregulation.

Zinc is an essential micronutrient with catalytic, structural, and regulatory roles in humans [23]. Cellular zinc homeostasis is maintained by the two distinct families of zinc transporters: ZnT (SLC30) and Zip (SLC39) [10, 11]. ZnT transporters reduce intracellular free zinc by promoting zinc efflux from cells or into intracellular vesicles, whereas Zip transporters increase intracellular free zinc by promoting extracellular zinc uptake and vesicular zinc release into the cytosol [10]. In a previous study, Cousins et al demonstrated that zinc transporter gene expression levels were markedly altered in response to cellular zinc depletion and supplementation in THP-1 cells [13]. They further found that Zip2 was most sensitive to zinc depletion and proposed that this may indicate a cellular attempt to homeostatically compensate for zinc loss through increased cellular uptake, since Zip2 was shown to play a role in cellular zinc uptake [14]. This may suggest that Zip2 could play a role in myocardial ischemia/reperfusion injury because we and others have demonstrated that zinc loss contributes to ischemia/reperfusion injury in rat hearts [8, 9]. In the present study, I/R induced Zip2 upregulation at reperfusion. We further found that the I/R-induced increase in Zip2 expression was prevented by the treatment of mice with  $Zn^{2+}$ , whereas Zip2 overexpression led to an increase in intracellular  $Zn^{2+}$ . These results indicate that zinc loss during I/R stimulates Zip2 expression to attempt maintenance of cellular zinc homeostasis. This finding supports the previous observations by others that Zip2 plays a critical role in adapting zinc deficiency or depletion [12, 24].

Although, as mentioned above, Zip2 has been proposed to play a critical role in the maintenance of cellular zinc homeostasis in the setting of zinc depletion or deficiency, its role under pathophysiological conditions especially in myocardial I/R injury remains unknown. Zinc depletion with TPEN leads to apoptosis [12, 25], indicating that cellular zinc levels are critical for cell survival. Cellular zinc levels may also be associated with various cardiovascular diseases [26, 27]. In isolated perfused rat hearts, I/R decreased levels of intracellular labile zinc and the treatment of hearts with zinc attenuated myocardial dysfunction, indicating a critical role of cellular zinc homeostasis in cardiac cell survival in the setting of myocardial I/R [9]. Our group has also demonstrated that reperfusion after 30 min of regional ischemia triggered zinc loss in isolated rat hearts and activation of adenosine A<sub>2</sub> receptors led to cardioprotection against reperfusion injury by preventing zinc loss, suggesting that zinc homeostasis is closely related to cardiac reperfusion injury [8]. Zinc plays a critical role in ischemic postconditioning by enhancing the activation of the RISK pathway in isolated rat hearts [28]. In this study, Zip2 gene knockout exacerbated myocardial I/R injury, whereas Zip2 gene transfer reduced myocardial infarction. These observations suggest that the upregulation of Zip2 expression in response to I/R might be an important intrinsic adaptive mechanism by which cardiac cells attempt to protect themselves from I/R injury. Prevention of the decrease in cellular zinc levels through increased cellular zinc uptake may account for the protection by Zip2 upregulation, since cellular zinc levels are associated with cardiac survival/death during I/R. However, it cannot be excluded that Zip2 exerts its cardioprotection through a different mechanism other than the regulation of zinc homeostasis, since ZnT-1, an important zinc exporter, protects HL-1 cells from simulated I/R by activating the Ras-ERK signaling pathway [29]. Furthermore, it has been reported that inhibition of protein synthesis did not block cardioprotection induced by ischemic

preconditioning [30]. Obviously, more studies are required to examine if Zip2 protects the heart through an unknown mechanism that is independent of regulation of zinc homeostasis.

STAT3 is crucial for cell proliferation, differentiation, and survival, and is activated through its tyrosine residue phosphorylation (Tyr<sup>705</sup>). Phosphorylation of STAT3 leads to its dimerization and subsequent translocation into the nucleus to interact with regulatory elements for gene expression [31]. Zinc has been reported to inhibit STAT3 signaling in T<sub>H</sub>17 cells [15] and Zip6 serves as a downstream target of STAT3 [16]. Thus, it is possible that zinc loss during I/R triggers Zip2 mRNA expression by activating STAT3. STAT3 has been demonstrated to be activated during ischemia and reperfusion in rat hearts [17, 18]. Transgenic mice with overexpression of a constitutively active STAT3 showed reduced infarct size compared to wild-type mice after ischemia/reperfusion [19]. In contrast, STAT3 knockout mice revealed larger infarcts after 1 h ischemia followed by 24 h reperfusion [20]. Thus, STAT3 activation during I/R is protective and can modulate cardiac injury. In the present study, I/R-induced upregulation of Zip2 expression was reversed by the inhibition of STAT3 with statin and the dominant negative mutation of STAT3, and I/R significantly increased STAT3 phosphorylation at Tyr<sup>705</sup> in response to cellular zinc loss. Moreover, delivery of STAT3 genes reduced myocardial I/R injury in mouse hearts, which was nullified by the genetic disruption of Zip2 expression. These data suggest that zinc loss resulted from I/R activates STAT3, which in turn leads to Zip2 upregulation. Since STAT3 is activated in response to zinc loss (zinc deficiency) and activated STAT3 upregulates leading to cardioprotection against I/R injury, STAT3 may serve as an important zinc sensor to protect the heart by maintaining zinc homeostasis. Thus, this finding provides a new insight into the molecular mechanism underlying STAT3's cardioprotection. A number of factors that activate STAT3 and several downstream targets (effectors) of STAT3

have been proposed [32], we are the first to report that the factors related to zinc homeostasis (zinc loss and zinc transporter) serve as STAT3's activator and effector in the setting of I/R. Accordingly, our finding will add new knowledge on STAT3's cardioprotection, although more studies are needed to determine how zinc loss leads to STAT3 activation and how Zip2 is activated upon STAT3 activation.

It has been proposed that STAT3 is unlikely to exert the cardioprotective effects of classic (early) ischemic preconditioning or postconditioning through regulation of target gene transcription [33], because the transcriptional function of STAT3 may be too slow to account for the immediate rescue of cardiac cell death during the early minutes of reperfusion [22]. In the present study STAT3 activated upon reperfusion led to cardioprotection against I/R injury through upregulation of Zip2 protein expression. Moreover, we found that while STAT3 phosphorylation was prominent 10 min after the onset of reperfusion, Zip2 expression was increased starting 60 min after the onset of reperfusion. These observations suggest that STAT3 is cardioprotective against the late-stage reperfusion injury by upregulating Zip2 gene transcription. Thus, it should be mentioned here that Zip2 may not mediate cardioprotection by inhibiting the mPTP opening, since inhibition of the pore opening at very early stage of reperfusion leads to cardioprotection [34].

In summary, Zip2 expression is upregulated at reperfusion as an intrinsic protective mechanism by which the heart is resistant to I/R injury. STAT3 activation triggered by cellular zinc loss accounts for Zip2 upregulation. Upregulating Zip2 with pharmacological or genetic approaches may have a great translational potential for the treatment of patients with acute myocardial infarction. The observation that the factors involved in zinc homeostasis contribute

to STAT3 activation and its subsequent protective action in the setting of myocardial I/R might be conducive to understanding of STAT3's cardioprotection.

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**Disclosure**

None.

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**Figure legends**

**Fig. 1.** *A*, Zip2 mRNA expression at reperfusion in mouse hearts subjected to I/R (n = 5 each group). \*\*  $p < 0.01$ . *B*, Zip2 mRNA expression 2 h after the onset of reperfusion in mouse hearts subjected to I/R (n = 5 each group). ZnCl<sub>2</sub> (300 μg/kg) was infused starting 5 min before the onset of reperfusion and continuing for 30 min. \*\*  $p < 0.01$ . *C*, Zip2 protein expression 2 h after the onset of reperfusion in mouse hearts subjected to I/R (n = 8 each group). ZnCl<sub>2</sub> (300 μg/kg) was infused starting 5 min before the onset of reperfusion and continuing for 30 min. \*\*  $p < 0.01$ . *D*, Zinpyr-1 fluorescence measured with flow cytometry. H9c2 cells were transfected with Zip2 plasmid (n = 4 each group). \*\*  $p < 0.01$ . *E*, Cardiac zinc levels in mouse hearts. Four weeks after the delivery of AAR carrying Zip2 plasmid, cardiac zinc levels were measured with ICPOES. \*  $p < 0.05$ .

**Fig. 2.** *A*, Zip2 mRNA expression at reperfusion in mouse hearts subjected to I/R (n = 5 each group). Stattic (3 mg/kg) was given starting 20 min before ischemia and continuing for 20 min.\*  $p < 0.05$ ; \*\*  $p < 0.01$ . *B*, Zip2 protein expression 2 h after the onset of reperfusion in mouse hearts subjected to I/R (n = 5 each group). Stattic (3 mg/kg) was given starting 20 min before ischemia and continuing for 20 min.\*\*  $p < 0.01$ . *C*, Zip2 protein expression in mouse hearts (n = 5 each group). Four weeks after the delivery of AAR carrying STAT3 plasmid or Y705F, Zip2 expression was detected with Western blotting. \*\*  $p < 0.01$ .

**Fig. 3.** *A*, Western blotting analysis of STAT3 phosphorylation (Tyr<sup>705</sup>) in mouse hearts subjected I/R (n = 4 each group). \*  $p < 0.05$ ; \*\*  $p < 0.01$ . *B*, Western blotting analysis of STAT3 phosphorylation (Tyr<sup>705</sup>) in mouse hearts subjected I/R (n = 5 each group). \*\*  $p < 0.01$ .

**Fig. 4.** *A*, Zip2 protein levels in the mouse heart and skin (n = 5 each group). \*\*  $p < 0.01$ . *B*, Zi2 mRNA levels in the mouse heart and skin (n = 5 each group). \*\*  $p < 0.01$ . *C*, Infarct size of mouse hearts subjected to I/R (n = 6 each group). \*\*  $p < 0.01$ .

**Fig. 5.** *A*, Zip2 protein levels in the mouse heart and skin (n = 5 each group). \*\*  $p < 0.01$ . *B-D*, Serum LDH, cTnl, and CK-MB activities in mouse subjected to myocardial I/R (n = 6 in control; n = 8 in Zip2<sup>-/-</sup>; n = 5 in vector; n = 6 in Zip2 AAV). \*\*  $p < 0.01$ . *E*, Infarct size of mouse hearts subjected to I/R (n = 8 in Zip2<sup>+/+</sup>; n = 10 in Zip2<sup>-/-</sup>; n = 6 in vector; n = 6 in Zip2 AAV). \*\*  $p < 0.01$ .

**Fig. 6.** *A*, Cell viability in H9c2 cells subjected to H/R (n = 4 each group). \*\*  $p < 0.01$ . *B*, Cell viability in H9c2 cells subjected to H/R (n = 4 each group). \*\*  $p < 0.01$ .

**Fig. 7.** *A*, Western blotting analysis of STAT3 expression levels in mouse hearts (n = 4 each group). Vectors were injected through the tail vein. \*\*  $p < 0.01$ . *B*, Western blotting analysis of STAT3 phosphorylation (Tyr<sup>705</sup>) in mouse hearts injected with vectors (n = 4 each group). \*\*  $p < 0.01$ . *C*, Serum cTnl activity in mouse subjected to myocardial I/R (n = 5). \*\*  $p < 0.01$ . *D*, Infarct size of mouse hearts subjected to I/R (n = 5 each group). \*\*  $p < 0.01$ .

**Highlights**

- Zip2 expression was upregulated upon reperfusion in response to zinc deficiency in mouse hearts.
- STAT3 phosphorylation at Tyr<sup>705</sup> by zinc deficiency at reperfusion was responsible for Zip2 upregulation.
- Zip2 gene knockout exacerbated ischemia/reperfusion injury in mouse hearts.
- STAT3 gene delivery failed to ameliorate myocardial ischemia/reperfusion injury in Zip2 gene knockout mice.

ACCEPTED MANUSCRIPT