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NO mobilizes intracellular Zn^{2+} via cGMP/PKG signaling pathway and prevents mitochondrial oxidant damage in cardiomyocytes

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Abstract

Objective: Our aim was to determine if NO prevents mitochondrial oxidant damage by mobilizing intracellular free zinc (Zn^{2+}).
Methods: Zn^{2+} levels were determined by imaging enzymatically isolated adult rat cardiomyocytes loaded with Newport Green DCF. Mitochondrial membrane potential ($\Delta\Psi_m$) was assessed by imaging cardiomyocytes loaded with tetramethylrhodamine ethyl ester (TMRE).
Results: *S*-nitroso-*N*-acetylpenicillamine (SNAP) dramatically increased Zn^{2+}, which was blocked by both ODQ and NS2028, two specific inhibitors of guanylyl cyclase. The protein kinase G (PKG) inhibitor KT5823 blocked the effect of SNAP while the PKG activator 8-Br-cGMP mimicked the action of SNAP, indicating that the cGMP/PKG pathway is responsible for the effect of SNAP. The increased Zn^{2+} was prevented by 5-hydroxydecanoate (5HD) but was mimicked by diazoxide, implying that mitochondrial K_{ATP} channel opening may account for this effect. Since chelation of Zn^{2+} blocked the preventive effect of SNAP on H_2O_2-induced loss of $\Delta\Psi_m$ and exogenous zinc (1 μM $ZnCl_2$) prevented dissipation of $\Delta\Psi_m$, Zn^{2+} may play a critical role in the protective effect of NO. The MEK (mitogen-activated protein kinase or extracellular signal-regulated kinase) inhibitor PD98059 blocked the preventive effects of SNAP and zinc on $\Delta\Psi_m$, indicating that extracellular signal-regulated kinase (ERK) mediates the protective effect of both these compounds on mitochondrial oxidant damage. A Western blot analysis further showed that $ZnCl_2$ significantly enhances phosphorylation of ERK, confirming the involvement of ERK in the action of Zn^{2+}.
Conclusions: In isolated cardiomyocytes, NO mobilizes endogenous zinc by opening mitochondrial K_{ATP} channels through the cGMP/PKG pathway. In these cells, Zn^{2+} may be an important mediator of the action of NO on the mitochondrial death pathway.

Keywords: Nitric oxide; Mitochondria; Protein kinase G

1. Introduction

In addition to its important role as a component of numerous structural proteins, enzymes and transcriptional factors [1], free or loosely bound zinc itself has been demonstrated to be involved in various physiological functions [2]. It plays a crucial role in signal transduction by modulating cellular signal recognition, second messenger metabolism, protein kinase and phosphatase activities [3]. In particular, recent studies have proposed that zinc can stimulate the PI3-kinase/Akt signaling pathway [4–6] and inhibits glycogen synthase kinase-3β (GSK-3β) [7]. The PI3-kinase/Akt signaling pathway and GSK-3β have been demonstrated to play important roles in cardioprotection against ischemia/reperfusion injury [8–10]. Thus, zinc may be involved in the mechanism of cardioprotection. A recent report further showed that exogenous zinc suppresses apoptosis in cardiac allografts in a dose-dependent manner [11].

In spite of the important roles of zinc, most of the intracellular zinc is tightly bound to metallothionein and thus the level of intracellular free zinc is very low. Therefore, either the transient release of zinc from the binding sites to cytosol or supplementation of exogenous free zinc ion is required to increase cytosolic free or labile zinc. Nitric oxide (NO) has been shown to induce release of zinc in vascular

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endothelium [12], hippocampus [13], lung fibroblasts [14], and islet cells [15]. NO-triggered zinc release has been associated with reduced sensitivity to lipopolysaccharide (LPS)-induced apoptosis in pulmonary endothelium [16]. NO is cardioprotective [17], and exogenous zinc can protect hearts from reperfusion injury through inhibition of oxidative stress [18]. Therefore, it is highly plausible that NO mobilizes intracellular zinc in cardiomyocytes, which serves as an important mechanism for the cardioprotective effect of NO.

NO at low concentrations stimulates the synthesis of the second messenger cGMP, which in turn regulates various cellular functions by activating downstream targets including protein kinase G (PKG). In contrast, at higher concentrations, NO reacts with O_2 to form reactive nitrogen oxide intermediates such as N_2O_3 [19]. It has been proposed that nitrosylation of metallothionein by N_2O_3 is responsible for the mechanism by which NO (at high concentrations) releases zinc in non-cardiomyocyte cells [12,14,19,20]. However, if NO induces cardioprotection by releasing zinc, it is unlikely that NO mobilizes zinc through nitrosylation of metallothionein in heart cells, since we have found that the cGMP/PKG signal pathway is responsible for the cardioprotective effect of NO [21]. Thus, we chose to determine if NO can release intracellular zinc through activation of the cGMP/PKG pathway. Since PKG has been proposed to open mitochondrial K_{ATP} channels [22], it is worthy to examine whether mitochondrial K_{ATP} channel opening plays a role in the effect of NO on zinc release.

In the present study, we first tested whether exogenous NO can mobilize intracellular zinc by imaging isolated rat cardiomyocytes loaded with the Zn^{2+} specific fluorescence dye Newport Green DCF. We then investigated the mechanism underlying the effect of NO on zinc release. Lastly, we examined if NO prevents mitochondrial oxidant damage via a Zn^{2+}-dependent mechanism.

2. Materials and methods

The investigation conforms with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institute of Health (NIH Publication No. 85-23, revised 1996).

2.1. Chemicals and reagents

Newport Green DCF diacetate and tetramethylrhodamine ethyl ester (TMRE) were purchased from Molecular Probes (Eugene, OR). Type II collagenase was purchased from Worthington Biochemical Corporation (Lakewood, NJ). *S*-nitroso-*N*-acetylpenicillamine (SNAP), 5-hydroxydecanoate (5HD), $ZnCl_2$, and *N,N,N′,N′*-tetrakis-(2-pyridylmethyl) ethylenediamine (TPEN) were obtained from Sigma (St. Louis, MO). ODQ, NS2028, KT5823, and 8-Br-cGMP were purchased from Calbiochem (La Jolla, CA). Phospho-ERK antibody was purchased from Cell Signaling (Beverly, MA).

2.2. Isolation of adult rat cardiomyocytes

Male Wistar rats weighing 200–300 g were anesthetized with sodium pentobarbital (100 mg/kg, i.p.). A midline thoracotomy was performed and the heart was removed and rapidly mounted on a Langendorff apparatus. The heart was perfused in a non-recirculating mode with Krebs–Henseleit buffer (37 °C) containing (in mM) NaCl 118, $NaHCO_3$ 25, KCl 4.7, KH_2PO_4 1.2, $MgSO_4$ 1.2, $CaCl_2$ 1.25, and glucose 10 for 5 min to wash out blood. The buffer was bubbled with 95% O_2/5% CO_2. Then the heart was perfused with calcium-free buffer that contained all the above components except $CaCl_2$. After 5 min of calcium-free perfusion, collagenase (type II) was added to the buffer (0.1%) and the heart was perfused in a recirculating mode for ~15 min. The heart was removed from the apparatus and the ventricles were placed into a beaker containing the calcium-free buffer, and then agitated in a shaking bath (37 °C) at a rate of 50 cycles/min until cells were released. The released cells were suspended in an incubation buffer containing all the components of the calcium-free buffer, 1% bovine serum albumin, 30 mM HEPES, 60 mM taurine, 20 mM creatine, and amino acid supplements at 37 °C. Calcium was gradually added to the buffer containing the cells to a final concentration of 1.2 mM. The cells were filtered through a nylon mesh and centrifuged briefly. Finally the cells were suspended in culture medium M199 for 4 h before the experiments.

2.3. Measurement of intracellular free zinc

Free zinc (Zn^{2+}) in cardiomyocytes was assessed with Newport Green DCF, a Zn^{2+}-selective fluorescence dye. The ester form of Newport Green DCF can diffuse across cellular membranes. Once inside the cells, the ester is hydrolyzed by ubiquitous intracellular esterases to yield a cell-impermeant fluorescence indicator of Zn^{2+}. Cardiomyocytes cultured in a specific temperature-controlled culture dish were incubated with 2 μmol/L Newport Green DCF diacetate in standard Tyrode solution containing (mM) NaCl 140, KCl 6, $MgCl_2$ 1, $CaCl_2$ 1, HEPES 5, and glucose 5.8 (pH 7.4) for 20 min. The cells were then mounted on the stage of an Olympus FV 500 laser scanning confocal microscope. The green Newport Green DCF was excited with the 488 nm line of an argon–krypton laser and imaged through a 525 nm long-path filter. Stage temperature was maintained at 37 °C using a Delta T Open Dish Systems (Bioptechs, Butler, PA). The images recorded on a computer were quantified using Image J (NIH).

2.4. Confocalimaging of mitochondrial membrane potential ($\Delta\Psi_m$)

To measure $\Delta\Psi_m$, myocytes were loaded with 100 nM tetramethylrhodamine ethyl ester (TMRE) (Molecular Probe) for 15 min at 37 °C. The cells loaded with the dyes were imaged with a confocal fluorescence microscope (Olympus FV 500). The probes were excited at 543 nm

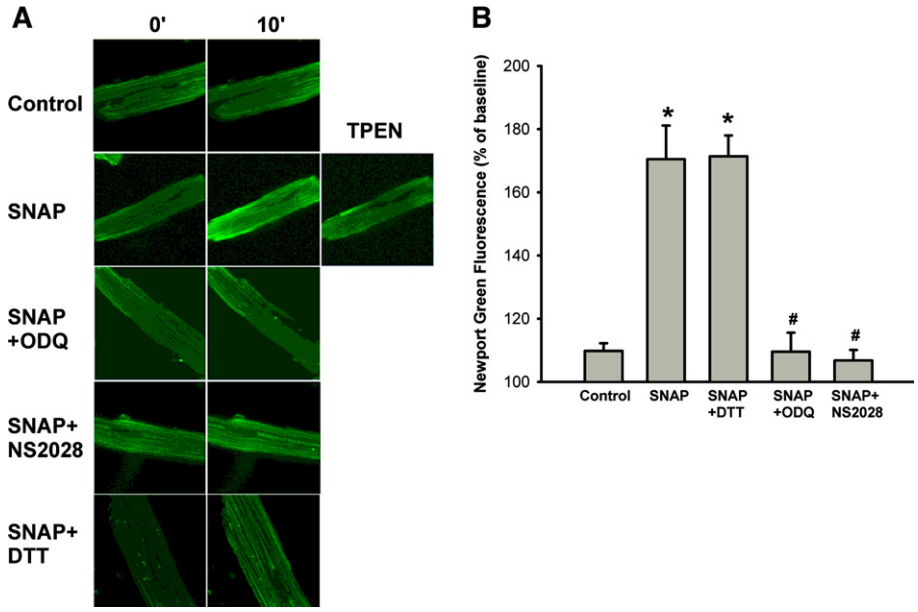

Fig. 1. A, Confocal fluorescence images of Newport Green DCF at baseline and 10 min after exposure to 20 μM SNAP in rat cardiomyocytes. SNAP markedly enhanced the fluorescence intensity, which was reversible by the addition of TPEN (10 μM). Both ODQ and NS2028 but not dithiothreitol (DTT) blocked the action of SNAP. B, Summarized data for Newport Green DCF fluorescence intensity 10 min after exposure to SNAP expressed as percentage of baseline. *$p<0.05$ vs. control; #$p<0.05$ vs. SNAP.

and the red fluorescence image was detected using a 560 nm long-path filter. The images were quantified using Image J.

2.5. Western blotting analysis of phosphorylated ERK activity

After exposure to Zn^{2+} for 10 min, cells were homogenized in ice-cold lysis buffer. Equal amounts of protein (50 μg) were loaded and electrophoresed on 10% SDS-polyacrylamide gel and transferred to a PVDF membrane. The membrane was probed with a primary antibody that recognizes phospho-p42/p44 MAPK (phospho-ERKs). To confirm equal loading, the membrane was stripped and reprobed with an antibody recognizing both phosphorylated and non-phosphorylated forms of p42/p44 MAPK. The primary antibody binding was detected with a secondary anti-rabbit antibody and visualized by the ECL method.

2.6. Experimental protocol

In the experiments monitoring the change in zinc concentration, the agonists were given immediately after baseline (time 0′) measurements, whereas the antagonists were given 10 min prior to the application of the agonists. The measurements were terminated 10 min after the baseline recordings. In the study evaluating the effect of exogenous Zn^{2+} on $\Delta\Psi_m$, cardiomyocytes were exposed to 100 μM H_2O_2 for 20 min to cause mitochondrial oxidant damage. SNAP and $ZnCl_2$ were given 10 min before exposure to H_2O_2. Fluorescence intensity was determined from 5 to 10 cells at each time point.

2.7. Statistical analysis

Data are expressed as mean±SEM. Statistical significance was determined using either one-way ANOVA followed by Tukey's test or Student *t*-test. A value of $p<0.05$ was considered as statistically significant.

3. Results

In the present study, intracellular free zinc (Zn^{2+}) was detected with the Zn^{2+}-selective fluorescence dye Newport Green DCF. Newport Green DCF has moderate zinc-binding

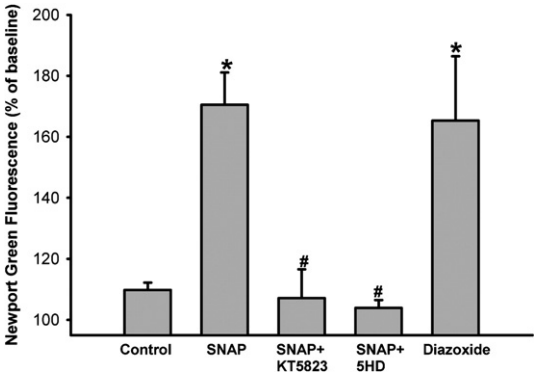

Fig. 2. Newport Green DCF fluorescence intensity 10 min after exposure to 20 μM SNAP in rat cardiomyocytes. Both the PKG inhibitor KT5823 and the mitochondrial K_{ATP} channel closer 5HD abolished the effect of SNAP on Newport Green DCF fluorescence. The mitochondrial K_{ATP} channel opener diazoxide mimicked the action of SNAP by enhancing the fluorescence intensity. *$p<0.05$ vs. control; #$p<0.05$ vs. SNAP.

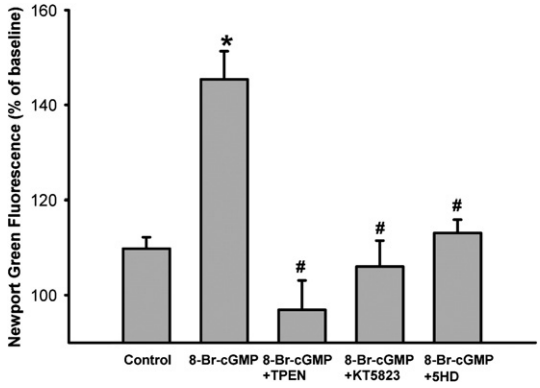

Fig. 3. Newport Green DCF fluorescence intensity 10 min after exposure to 200 μM 8-Br-cGMP expressed as percentage of baseline. The fluorescence intensity was dramatically increased by 8-Br-cGMP, indicating that the direct activation of PKG can mimic SNAP's action to release zinc. Both the PKG inhibitor KT5823 and the mitochondrial K_{ATP} channel closer 5HD abolished the effect of 8-Br-cGMP. *$p<0.05$ vs. control; #$p<0.05$ vs. 8-Br-cGMP.

affinity ($K_D=1$ μM) but is essentially insensitive to Ca^{2+} or Mg^{2+}, making this probe useful for measuring intracellular Zn^{2+} in various experimental conditions [23–25]. As shown in Fig. 1A, no change in Newport Green DCF fluorescence over time was seen in untreated cardiomyocytes. In contrast, cells exposed to the exogenous NO donor SNAP (20 μM) for 10 min had a dramatic increase in Newport Green DCF fluorescence (170.5±10.6% of baseline, $n=16$), indicating that NO can mobilize intracellular Zn^{2+} in isolated rat cardiomyocytes. Importantly, this increase was completely reversed by subsequent addition of the membrane-permeable Zn^{2+}-selective chelator TPEN (10 μM), confirming that this increase is due to changes in Zn^{2+} concentration rather than other ions.

To investigate the mechanisms by which NO induces Zn^{2+} release, ODQ, a potent and selective inhibitor of NO-sensitive guanylyl cyclase, was applied to test if it could alter the effect of SNAP on Newport Green DCF fluorescence. Fig. 1A shows that ODQ (5 μM) was able to prevent the effect of SNAP, implying that cGMP is responsible for the mobilization of Zn^{2+} by NO. The involvement of cGMP in the action of SNAP was confirmed by further experiments in which the action of SNAP was impeded by another specific and irreversible inhibitor of guanylyl cyclase NS2028 (1 μM). To rule out the possibility that SNAP mobilizes Zn^{2+} by nitrosylating metallothionein, we examined if dithiothreitol (DTT), a thiol-reducing agent known to reverse *S*-nitrosylation [26], could block the effect of SNAP. Fig. 1A shows that SNAP still enhances the green fluorescence intensity in the presence of DTT, suggesting that SNAP-induced Zn^{2+} mobilization is not mediated by nitrosylation of metallothionein. Fig. 1B shows Newport Green DCF fluorescence data 10 min after the baseline measurement expressed as % of the baseline. Both ODQ (109.6±6.0% of baseline, $n=6$) and NS2028 (106.8±3.3% of baseline, $n=6$) blocked the effect of SNAP on Zn^{2+} mobilization, indicating that cGMP is essential for the action of NO on Zn^{2+} release.

PKG is an important downstream target of cGMP, and our previous data have shown that it is involved in the cardioprotective effect of SNAP [21]. To determine whether PKG activation is required, the effect of SNAP on zinc release was assessed in the presence of KT5823, a highly specific cell-permeable inhibitor of PKG. As shown in Fig. 2, 1 μM KT5823 prevented the SNAP-induced increase in Newport Green DCF fluorescence (107.1±9.5% of baseline, $n=10$), indicating that the activation of PKG may contribute to the Zn^{2+}-releasing effect of NO. Since PKG can activate mitochondrial K_{ATP} channels in cardiomyocytes [21,27], it is worthy to test if mitochondrial K_{ATP} channel activation is implicated in the action of SNAP. Interestingly, the effect of SNAP was aborted by 500 μM 5HD (103.9±2.6% of baseline, $n=10$), a specific closer of mitochondrial K_{ATP} channels, suggesting that SNAP may release intracellular Zn^{2+} by opening mitochondrial K_{ATP} channels. Further experiments showed that the selective

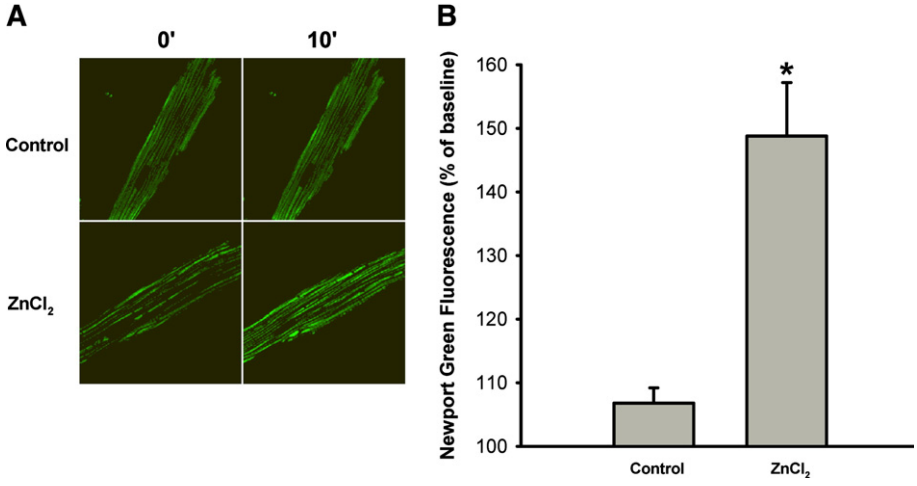

Fig. 4. A, Confocal fluorescence images of Newport Green DCF at baseline and 10 min after exposure to $ZnCl_2$ in rat cardiomyocytes. $ZnCl_2$ (1 μM) markedly increased intracellular free zinc concentration. B, Summarized data for Newport Green DCF fluorescence intensity 10 min after exposure to $ZnCl_2$ expressed as percentage of baseline. *$p<0.05$ vs. control.

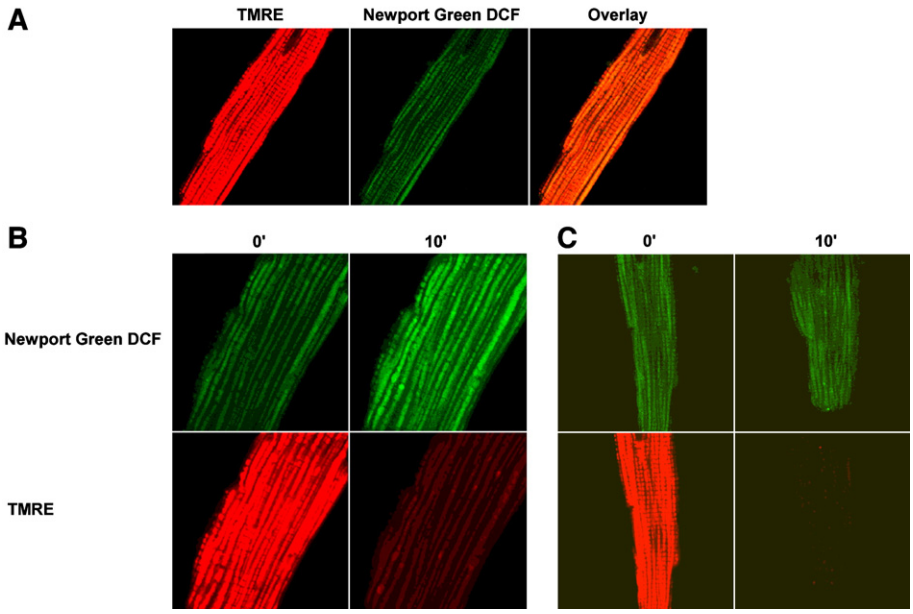

Fig. 5. A, Representative confocal fluorescence images of cardiomyocytes loaded with both TMRE and Newport Green DCF. The overlay image indicates co-localization of the two dyes. B, Confocal fluorescence images of cardiomyocytes loaded with both Newport Green DCF and TMRE. Cardiomyocytes were treated with SNAP in the presence of CCCP. C, Confocal fluorescence images of cardiomyocytes loaded with both Newport Green DCF and TMRE. Cardiomyocytes were treated with CCCP.

mitochondrial K_{ATP} channel opener diazoxide (50 μM) mimicked the effect of SNAP by enhancing Newport Green DCF fluorescence (165.3±21.3% of baseline, $n=6$), confirming the crucial role of mitochondrial K_{ATP} channel.

If PKG is critical for the action of SNAP, direct activation of PKG should result in the release of Zn^{2+} thus mimicking the action of SNAP. As shown in Fig. 3, 8-Br-cGMP (200 μM), a cell-permeable PKG activator, markedly enhanced Newport Green DCF fluorescence 10 min after treatment (145.4±5.9% of baseline, $n=10$), an effect that was reversible by the addition of TPEN (96.9±6.2, $n=5$). These observations confirm that the activation of PKG mediates the action of NO on Zn^{2+} release. Similar to the observation described in Fig. 2, both KT5823 (106.0±5.5% of baseline, $n=6$) and 5HD (113.1±2.8% of baseline, $n=7$) abolished the effect of 8-Br-cGMP on Zn^{2+} release.

Zinc has been reported to have both anti-apoptotic and anti-oxidative effects. In order to determine the potential physiological role of the NO-mobilized Zn^{2+} in cardiomyocytes, the effect of exogenous Zn^{2+} on mitochondrial oxidant damage was assessed in cardiomyocytes. $ZnCl_2$ (1 μM) significantly increased intracellular free zinc. Fig. 4A shows that cells treated with 1 μM $ZnCl_2$ for 10 min in the presence of the zinc ionophore pyrithione (4 μM) exhibited a marked increase in Newport Green DCF fluorescence. Fig. 4B presents the summarized data, and shows a significant increase in the fluorescence intensity (148.8±8.4% of baseline, $n=6$) produced by 1 μM $ZnCl_2$.

In this study, it was noticed that the staining pattern of Newport Green DCF resembled one with the mitochondrial specific dye TMRE. As shown in Fig. 5A, the overlay image indicates that the two fluorescence probes are co-localized, suggesting that Newport Green DCF may be localized in either inside or in close proximity to mitochondria. To rule out the possibility that the changes in Newport Green DCF fluorescence was due to alterations of $\Delta\Psi_m$, cardiomyocytes loaded with Newport Green DCF and TMRE were treated with SNAP in the presence of Carbonyl cyanide *m*-chlorophenylhydrazone (CCCP). Fig. 5B shows that SNAP

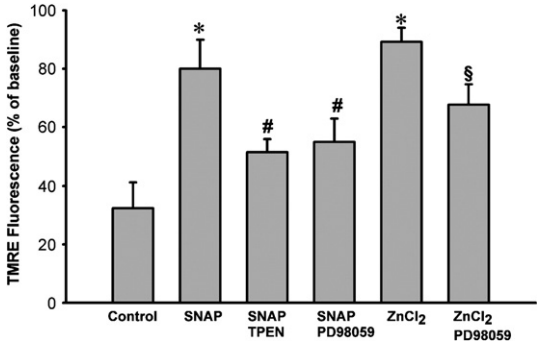

Fig. 6. Summarized data for TMRE fluorescence intensity 20 min after exposure to H_2O_2 (100 μM). Confocal fluorescence images of TMRE were collected at baseline and 20 min after exposure to H_2O_2 in rat cardiomyocytes. Compared to the control, both SNAP (20 μM) and $ZnCl_2$ (1 μM) prevented TMRE fluorescence reduction, indicating that both NO and zinc can prevent dissipation of $\Delta\Psi_m$ caused by oxidant stress. The action of SNAP was partially but significantly blocked by both TPEN and PD98059, implying that NO protects mitochondria via Zn^{2+} and ERK. The protective effect of zinc was blocked by PD98059 (20 μM), indicating an involvement of ERK in the action of zinc. *$p<0.05$ vs. control; #$p<0.05$ vs. SNAP; §$p<0.05$ vs. $ZnCl_2$.

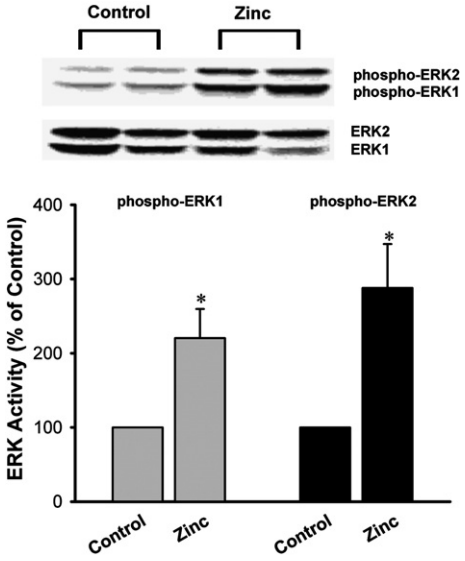

Fig. 7. Western blotting analysis of phospho-ERK activity in rat cardiomyocytes. $ZnCl_2$ (1 μM, 10 min) dramatically increase ERK phosphorylation. *$p<0.05$ vs. control.

was able to significantly enhance Newport Green DCF fluorescence intensity despite the loss of $\Delta\Psi_m$ by CCCP, suggesting that the Zn^{2+} signal is independent of changes in $\Delta\Psi_m$. Further experiments showed that CCCP alone also did not increase the Newport Green DCF intensity (Fig. 5C).

Fig. 6 shows that 100 μM H_2O_2 (20 min) caused a marked decrease in red TMRE fluorescence (32.4±8.8% of baseline, $n=6$), indicating dissipation of $\Delta\Psi_m$. In contrast, 20 μM SNAP attenuated the loss of $\Delta\Psi_m$ (80.0±9.9% of baseline, $n=6$), an effect that was partially blocked by TPEN (51.4±4.5% of baseline, $n=6$), indicating that NO can prevent the mitochondrial death pathway via Zn^{2+}. In support, 1 μM $ZnCl_2$ in the presence of pyrithione (4 μM) given 10 min prior to the exposure to H_2O_2 also prevented the loss of the red fluorescence (89.2±4.8% of baseline, $n=12$), indicating that exogenous Zn^{2+} successfully prevents mitochondrial oxidant damage. Since SNAP-induced cardioprotection against cardiac infarction was mediated by ERK [21], it is interesting to examine whether blockade of the ERK pathway can alter the effect of zinc. As shown in Fig. 6, the effect of $ZnCl_2$ on $\Delta\Psi_m$ was partially but significantly blocked by PD98059 (67.7±7.0% of baseline, $n=6$), indicating that ERK may play a role in the protective effect of Zn^{2+}. Fig. 6 also shows that the protective effect of SNAP was blocked by PD98059 (54.9±8.0% of baseline, $n=5$), corroborating the crucial role of ERK in NO-induced cardioprotection that is mediated by Zn^{2+}. To further confirm the role of ERK in the action of Zn^{2+}, the effect of $ZnCl_2$ on ERK activity was determined. As shown in Fig. 7, cardiomyocytes treated with 1 μM $ZnCl_2$ for 10 min in the presence of pyrithione revealed a significant increase in phosphorylation of both ERK1 (220.4±39.2% of control, $n=5$) and ERK2 (287.4=59.5% of control, $n=5$), indicating that ERK may serve as a downstream signal of Zn^{2+}.

4. Discussion

This is the first study to demonstrate that exogenous NO mobilizes intracellular zinc via the cGMP/PKG signal pathway and the opening of mitochondrial K_{ATP} channels in cardiomyocytes. It appears that Zn^{2+} plays an important role in the protective effect of NO on mitochondrial dysfunction caused by oxidant stress, and that the activation of ERK by phosphorylation may serve as a downstream signal leading to the protective effect of Zn^{2+}.

It is well known that the major intracellular Zn^{2+}-binding protein is metallothionein [28]. Nitrosylation of metallothionein has been proposed to be the mechanism by which NO at high doses (2 mM SNOC or 0.2 and 2 mM DETA/NO) releases Zn^{2+} [12,14,19,20]. In contrast to the well-characterized effect of NO on Zn^{2+} release in these cells, little is known about the impact of NO on Zn^{2+} dynamics in cardiomyocytes. In the present study, we have demonstrated that SNAP at a low dose (20 μM) markedly increased Zn^{2+} release, which was abolished by inhibition of either guanylyl cyclase or PKG. This finding suggests that even at a low concentration NO can trigger Zn^{2+} release through activation of the cGMP/PKG pathway. The involvement of this pathway was also confirmed by the observation that the cell-permeable PKG activator 8-Br-cGMP induced Zn^{2+} release. We further demonstrated that SNAP-induced Zn^{2+} release was not altered by DTT, a thiol-reducing agent known to reverse *S*-nitrosylation [26], indicating that the mechanism of Zn^{2+} release in cardiomyocytes reported here is novel and distinct from the above-mentioned premise that the nitrosylation of metallothionein is responsible for NO-induced Zn^{2+} release. Although the reason for this discrepancy is unclear, it should be noted that compared to those previous studies in which high doses of NO donors were used, we treated cardiomyocytes with a low dose (20 μM) of SNAP. It is well accepted that NO at low concentrations activates soluble guanylyl cyclase to synthesize cGMP, which in turn activates downstream targets such as PKG. Recently, we [21] and others [29] have also found that the activation of PKG is crucial for the cardioprotective effect induced by low doses of SNAP. Thus, it is conceivable that the low dose of SNAP used in this study activated PKG, which in turn induced Zn^{2+} release.

As to the mechanism by which activated PKG causes Zn^{2+} release, we hypothesize that the mitochondrial K_{ATP} channel opening is a potential initiating event that leads to the release of Zn^{2+}, since the activation of PKG has been shown to open the mitochondrial K_{ATP} channels [21,22,27]. In this study, 5HD completely abolished the SNAP-induced release of Zn^{2+} and diazoxide-mobilized Zn^{2+}, indicating that the mitochondrial K_{ATP} channel opening is a required step for the release of Zn^{2+}. Furthermore, the observation that 5HD also blocks the release of Zn^{2+} induced by the PKG activator 8-Br-cGMP suggests that the mitochondrial K_{ATP} channel opening is a downstream event that is needed for the release of Zn^{2+}. Further studies are needed to dissect

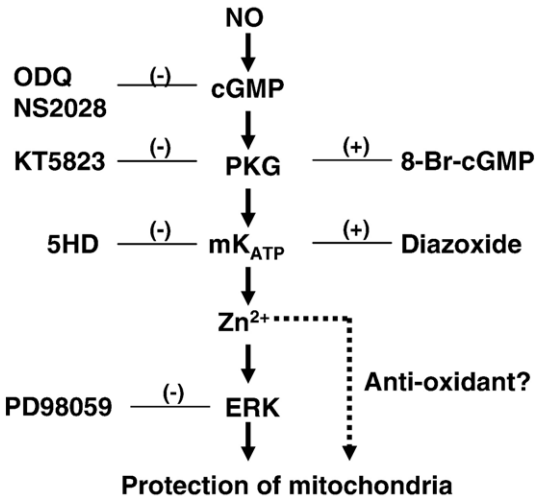

Fig. 8. Signaling mechanisms whereby NO mobilizes intracellular zinc and prevents the mitochondrial oxidant damage.

the detailed mechanisms whereby the mitochondrial K_{ATP} channel opening is linked to Zn^{2+} release. Since the mitochondrial K_{ATP} channel opening is recognized as the central mechanism for the cardioprotective effect of ischemic preconditioning [30], the release of Zn^{2+} by the opening of this channel may serve as an important mechanism for cardioprotection.

In the present study, we found that the Zn^{2+} fluorescence probe Newport Green DCF was co-localized with the mitochondrial membrane potential dye TMRE in cardiomyocytes under normoxic conditions, indicating that Newport Green DCF (thus detected Zn^{2+}) is located inside or in close proximity to the mitochondria. Although the mitochondrial K_{ATP} channel opening leads to the release of Zn^{2+}, it remains unknown whether Zn^{2+} was released from the mitochondria. Further studies using recognized mitochondria-selective Zn^{2+} probes (when available) are needed to ascertain the origin of Zn^{2+} mobilized by NO.

Zinc can modulate apoptosis [2]. In both *in vitro* and *in vivo* models, Zn^{2+} supplementation prevents apoptosis [31,32]. A recent report showed that exogenous Zn^{2+} suppresses apoptosis in cardiac allografts in a dose-dependent manner [11]. The anti-oxidant effect and a direct suppression of caspase-3 may contribute to the anti-apoptotic effect of Zn^{2+} [2,33]. Interestingly, in pulmonary endothelial cells SNAP reduced lipopolysaccharide-induced apoptosis in a zinc-dependent fashion [16]. Since mitochondria are closely associated with both oxidant stress and the development of apoptosis, and NO has been shown to protect cardiomyocytes by modulating mitochondrial functions [34], it is plausible that NO prevents the mitochondrial death pathway by mobilizing intracellular zinc. In the present study, we have demonstrated that SNAP attenuates the loss of $\Delta\Psi_m$ caused by H_2O_2, which was partially but significantly blocked by the Zn^{2+} chelator TPEN, suggesting that the protective effect of NO is mediated by Zn^{2+}. In support, exogenous $ZnCl_2$ increased intracellular Zn^{2+} concentration and prevented the collapse of $\Delta\Psi_m$ caused by H_2O_2. This result suggests that the Zn^{2+} mobilized by NO may play an important role in preventing mitochondrial oxidant damage. Since Zn^{2+} induces mitochondrial dysfunctions in hepatocytes [35] and in neurons [36], we examined whether Zn^{2+} could alter $\Delta\Psi_m$ in cardiomyocytes. $ZnCl_2$ (1 μM) had no effect on $\Delta\Psi_m$ under normoxic condition (data not shown), indicating that Zn^{2+} in low concentrations does not induce mitochondrial dysfunction in cardiomyocytes.

As to the mechanisms by which Zn^{2+} protects mitochondria, we propose that ERK may serve as one of the downstream signals leading to the protection, since the ERK inhibitor PD98059 partially, but significantly, blocked the action of Zn^{2+} on the mitochondrial dysfunction. The involvement of ERK in the effect of Zn^{2+} was further supported by the observation that exogenous Zn^{2+} enhances activation of ERK. In agreement with the current finding, Zn^{2+} was shown to activate ERK in human bronchial epithelial cells [37], PC12 cells [38], and colorectal cancer cell [39]. ERK has been demonstrated to be an essential survival signal blocking cell death caused by ischemia/reperfusion injury [40], and we have demonstrated that ERK plays a role in the protective effect of SNAP on ischemia/reperfusion injury in rat hearts [21]. Also, there is evidence that the activation of the ERK pathway can inhibit apoptosis [41]. Therefore, it is reasonable to propose that NO-mobilized Zn^{2+} activates ERK, which in turn protects mitochondria from oxidant stress. However, it should be noted that the inhibition of ERK activation partially but not completely blocked the protective effect of Zn^{2+}, implying that some additional mechanisms other than the activation of ERK may also be important. As mitochondria have been involved in cell death caused both by necrosis and apoptosis [42], understanding the impact of Zn^{2+} on mitochondrial dysfunction will help explain the cardioprotection of NO in both necrosis and apoptosis.

In summary (Fig. 8), we have demonstrated for the first time that exogenous NO mobilizes intracellular Zn^{2+} via a cGMP/PKG-dependent signal pathway in rat cardiomyocytes, and prevents the mitochondrial death pathway via Zn^{2+}. The opening of mitochondrial K_{ATP} channel may play a role in the effect of NO on Zn^{2+} release. The critical role of Zn^{2+} in the action of NO on mitochondrial oxidant damage may provide novel insights into the mechanism underlying the cardioprotective effects of NO.

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