Neuroprotective Effect of SIN-1 on Zn^{2+}-induced PC12 cell Death

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This certifies that the Master's Thesis

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ABSTRACT

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Peroxynitrite is derived from nitirc oxide (NO) and is a highly reactive cytotoxic molecule. Recently, however, peroxynitrite was also shown to play a neuroprotective role against nitric oxide-mediated apoptosis. In the present study, the role of peroxynitrite in the neuronal cell death caused by intracellular accumulation of Zn^{2+} was investigated in the differentiated PC12 cells.

Intracellular Zn^{2+} concentration was measured using spectrofluorometry in magfura-2-loaded differentiated PC12 cells, and cell viability was assessed by MTT assay. Intracellular accumulation of Zn^{2+}, which was induced by the combined application of pyrithione (5 μM), a zinc ionophore, and

Zn^{2+} (10 μM) for 3 hours, caused cell death in differentiated PC12 cells.

The Zn^{2+}-induced PC12 cell death was prevented by the pretreatment with 3-morpholinsydnonimine (SIN-1), a peroxynitrite donor. The intracellular accumulation of Zn^{2+} was not affected by the pretreatment with SIN-1, suggesting that the neuroprotective effect of SIN-1 was not due to the reduced influx of Zn^{2+} into cells. Since SIN-1 produces nitric oxide as well as peroxynitrite, we examined whether the protective effect of SIN-1 against PC12 cell death was attributed to nitric oxide or peroxynitrite. Pretreatment of cells with various nitric oxide donors, such as SNP and SNAP, was not able to attenuate the Zn^{2+}-induced PC12 cell death. SIN-1C, the stable decomposition product of SIN-1, did not prevent the Zn^{2+}-induced cell death. In addition, the protective effect of peroxynitrite against Zn^{2+}-induced cell death was almost completely inhibited by the peroxynitrite scavengers, such as uric acid, trolox and L-methionine. In conclusion, peroxynitrite exerts neuro-protective effect against Zn^{2+}-induced cell death in differentiated PC12 cells.

Key words : Zn^{2+}, SIN-1, peroxynitrite, PC12 cells, neuroprotection

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Ⅰ. Introduction

The transition metal zinc (Zn^{2+}) plays an essential role in metalloenzyme function, control of gene transcription, neurotransmission and neuromodulation in the central nervous system (CNS)[1]. Zn^{2+} is localized in the presynaptic vesicles along with the neurotransmitter glutamate and is present in many regions of the mammalian CNS[2]. Zn^{2+} is released together with glutamate into the extracellular space[3]. Released Zn^{2+} is an important modulator of inhibitory and excitatory synaptic transmission by altering the function of several receptors including voltage-gated Ca^{2+} channels, N-methyl -D-asparate (NMDA) receptors and Ca^{2+}-permeable α-amino-

3-hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA) receptors[4,5,6]. Electrophysiological studies on cultured neurons found Zn^{2+} to decrease NMDA-receptor-mediated responses through both voltage-dependent and voltage-independent mechanisms, and to potentiate AMPA-receptor-mediated responses[7.8]. Thus, co-release of Zn^{2+} with glutamate might shift excitation from NMDA receptors, and activate AMPA and/or kainate receptors[9]. In addition to regulating neurotransmission, Zn^{2+} may participate in various neuropathological conditions including ischemia, seizure, and trauma[10,11,12].

In pathological conditions, Zn^{2+} is released in excess at excitatory synapses and causes neuronal death via Zn^{2+} overload. The precise mechanism of Zn^{2+} neurotoxicity is unknown, but Zn^{2+} overload induces increase of reactive oxygen species (ROS) and lipid peroxidation in mouse cortical neurons[13]. Another evidences suggest that Zn^{2+} kills neurons through the inhibition of ATP synthesis[9]. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and phosphofructokinase are two glycoytic enzymes that might be impaired when cells experience elevated intracellular free Zn^{2+} [14,15]. Zn^{2+} inhibition of GAPDH could be especially deleterious to the cell, given its critical role in the regulation of glycolysis. Some reports showed that elevated intracellular free Zn^{2+} resulted in build up of glycolysis metabolites upstream of GAPDH (i.e. dihydroxy-acetone phosphate and fructose-1,6-bisphosphate), depletion of

downstream intermediates (1,3-biphosphoglycerate and pyruvate), and reduction of cellular ATP levels in cultured cortical neurons[16]. Some researchers found Zn^{2+} inhibited the a-ketoglutarate dehydrogenase complex (KGDHC) of the tricarboxylic acid (TCA) cycle[17]. Using KGDHC isolated from porcine heart, the site of inhibition was subsequently identified as a catalytic disulfide of the lipoamide dehydrogenase (LADH) subunit[18]. Moreover, LADH inhibition by Zn^{2+} was associated with augmented ROS production. Several studies described Zn^{2+} inhibition of the electron transport chain, possibly at multiple sites, the most sensitive of which was between cytochromes *b* and c_1[19]. Inhibition of the electron transport chain should result in dissipation of mitochondrial membrane potential in intact cells[20]. Given that mitochondria are believed to be the primary source of oxidative stress in neurons, and considering the large body of evidences implicating Zn^{2+} in mitochondrial dysfunction, it is reasonable to speculate that Zn^{2+} augments mitochondrial ROS production. In support of such a mechanism, elevated intracellular Zn^{2+} level via AMPA/kainate receptor activation resulted in mitochondrial free radical production in murine cortical cultures[20]. Zn^{2+} inhibition of TCA cycle may also instigate ROS production[18]. In addition, the superoxide generating enzyme NADPH oxidase is activated by high intracellular Zn^{2+} level in cultured neurons and astrocytes, resulting in elevated cellular ROS and cytotoxicity[21].

Nitric oxide (NO) is a short-lived physiological messenger involved in diverse functions. In neurons and astrocytes, NO is produced through the activation of a constitutive calcium-dependent neuronal nitric oxide synthase and participates in the signaling pathway, leading to rise in cGMP levels[22]. In addition, astrocytes have the ability to form NO in a calcium-independent pathway that requires prior to transcriptional expression of the inducible nitric oxide synthase isoform by lipopolysaccharide (LPS) and certain cytokines[23,24]. NO also contributes to pathological processes, including stroke and neurodegenerative disorders such as Parkinson's disease, Alzheimer's disease, multiple sclerosis and epilepsy[25,26,27]. Pathological levels of NO are generated in neurons by excessive stimulation of the NMDA receptors, in turn leading to Ca^{2+}-mediated activation of neuronal nitric oxide synthase (nNOS)[28]. NO reacts rapidly with endogenous superoxide anion (O_2^-) to form neurotoxic peroxynitrite ($ONOO^-$). The precise mechanisms by which $NO/ONOO^-$ mediates its neurotoxic effect remain unclear, but mitochondrial injury has been suggested, including S-nitrosylation or tyrosine nitration of complex I of the respiratory chain[29,30]. After fulminant exposure to $NO/ONOO^-$, neurons die by necrosis[31]. In contrast, after mild $NO/ONOO^-$ insult, neurons die by apoptosis[31]. Although the cytotoxicity of $ONOO^-$ was well-known, recent study suggests another effect of $ONOO^-$. They reported that $ONOO^-$ elicited a rapid stimulation of pentose phosphate pathway (PPP) activity in both neurons and

astrocytes through the activation of glucose-6-phosphate dehydrogenase (G6PD), followed by NADPH generation through PPP activity. Furthermore, they suggested that such a phenomenon would be involved in the protection of neurons[32].

3-morpholinsydnonimine (SIN-1) has been used, *in vitro*, to simultaneously generate NO and O_2^- . The mechanism of SIN-1 decomposition in oxygenated solution follows the next steps. The sydnonimine ring opens, by a base-catalyzed mechanism, to give SIN-1A. SIN-1A reduces oxygen, in a one electron transfer reaction, to give superoxide and SIN-$1^{\cdot+}$, a cation radical, and SIN-$1^{\cdot+}$ decomposes to form SIN-1C and NO[33]. The product of the reaction between NO and O_2^- is $ONOO^-$, potent oxidizing agent that has been implicated to play a causative role in several pathological conditions. For this reason SIN-1 has been used as an experimental model for the simultaneous generation of NO and superoxide, i.e. a $ONOO^-$ donor, in chemical and biological systems[34]. In this study, we examined the role of $ONOO^-$ in the neuronal cell death caused by intracellualr accumulation of Zn^{2+} on the differentiated PC12 cells.

Ⅱ. Materials and Methods

1. Materials

The rat pheochromocytoma (PC12) cells were obtained from ATCC (Manassas, VA, USA). Fetal bovine serum (FBS), horse serum (HS), Dulbecco`s modified Eagle`s medium (DMEM) were purchased from GIBCO (Grand Island, NY, USA). Mouse nerve growth factor 2.5S (mNGF) was obtained from Almone Labs (Jerusalem, Israel). Collagen Type 1 was purchsed from Upstate (Lake Placid, NY, USA). Magfura-2AM and N,N,N',N'-tetrakis-2-pyridylmethyl ethylenediamine (TPEN) were purchased from Molecular Probes (Eugene, OR, USA). Sodium Nitroferricyanide (III) Dihydrate (SNP), (±)-S-Nitroso-N-acetylpenicillamine (SNAP), Trolox and Uric acid were purchased from Calbiochem (San Diego, CA, USA). SIN-1C was kindly provided from Aventis Pharma (Frankfurt, Germany). Other reagents including $ZnCl_2$, 1-hydroxypyridine-2-thione (pyrithione) and 3-morpholinosydnonimine (SIN-1) were obtained from Sigma (St Louis, MO, USA), unless indicated otherwise.

2. Methods

2-1. Cell culture

PC12 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % horse serum and 5 % fetal bovine serum. The medium was changed every 2 days.

To obtain neuronally differentiated PC12 cultures, cells were

grown on collagen type Ⅰ(10 mg/mL) coated plates for 7 days in DMEM. The cultures were supplemented with 2 % horse serum and 1 % fetal bovine serum containing 50 ng/mL of 2.5S mNGF . The medium, including NGF, was replaced every 2 days. All cultures were maintained at 37 ℃ in a humidified, 5 % CO_2 incubator.

2-2. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay

Approximately 1.5×10^4 cells per 1 well were plated onto collagen-coated 24-well plates and allowed to differentiate to neuronal cells over 7 days. After each indicated treatment, 20 ㎕ stock 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT, 5 mg/ml in sterile phosphate-buffered saline) was added and incubated for 1 h at 37 ℃. Finally, 300 ㎕ of solubilizing solution (50 % dimethylformamide and 20 % sodium dodecyl sulfate, pH 4.8) was added. After incubating overnight, the absorption value at 570 nm was determined. Viability was determined as percentage survival = [(experimental-blank)/(control-blank)×100], where experimental, control and blank were the readings for the treated cells, untreated cells and the MTT added to the medium, respectively.

2-3. Measurement of intracellular Zn^{2+} concentration

Intracellular Zn^{2+} concentration ($[Zn^{2+}]_i$) in differentiated PC12 cells was measured using a modification of a method described previously[35]. Briefly, cells were loaded with magfura-2AM at 37 ℃ in a 5 % CO_2 incubator by including 3 μM magfura-2AM for 20 min in a HCO_3^--buffered solution containing (in mM): 110 NaCl, 4.5 KCl, 1 NaH_2PO_4, 1 $MgSO_4$, 1.5 $CaCl_2$, 5 HEPES-Na, 5 HEPES free acid, 25 $NaHCO_3$ and 10 D-glucose (pH 7.4). Cells were then rinsed twice and incubated in the HCO_3^--buffered solution for at least 20 min before use.

The $[Zn^{2+}]_i$ was measured on the stage of an inverted microscope (Nikon, Tokyo, Japan) by spectrofluorometry (Photon Technology International, Brunswick, NJ, USA). The excitation wavelength was alternated between 340 and 380 nm and the emission fluorescence was recorded at 510 nm.

2-4. Statistical analysis

The statistical significances of differences were determined by using a one-way ANOVA and Bonferroni's test. A P value < 0.05 was considered statistically significant. The results are expressed as means ± SD.

Ⅲ. Results

1. Zn^{2+} induced differentiated PC12 cell death.

Zn^{2+} overload into postsynaptic neurons contributes to neuronal cell death. To achieve rapid and relatively constant elevation of intracellular Zn^{2+} level without depolarizing the PC12 cells, 5μM pyrithione were treated just before treating $ZnCl_2$. Various concentrations of Zn^{2+} were treated to determine the concentration at which cell death induced. As shown in figure 1, Zn^{2+}-induced PC12 cell death increased dose-dependently, and nearly 80 % of cells died at 10 μM combined with 5μM pyrithione. As shown in figure 2, 10 μM Zn^{2+} were treated time-dependently, and cell viability decreased following that time passed.

2. The Zn^{2+}-induced PC12 cell death was prevented by 3-morpholinsydnonimine (SIN-1), a $ONOO^-$ donor.

To investigate the effect of SIN-1 on Zn^{2+}-induced PC12 cell death, cell viability was measured using the MTT assay. As shown in figure 3, various concentrations of SIN-1 were pretreated cells for 10 min. Then I treated 10 μM $ZnCl_2$ with 5 μM pyrithione (Zn^{2+}/pyrithione) on differentiated PC12 cells. After 3 hours, approximately 80 % of Zn^{2+}/pyrithione-treated cells died , while approximately 65 % of 300 μM SIN-1/Zn^{2+}/pyrithione-treated cells survived.

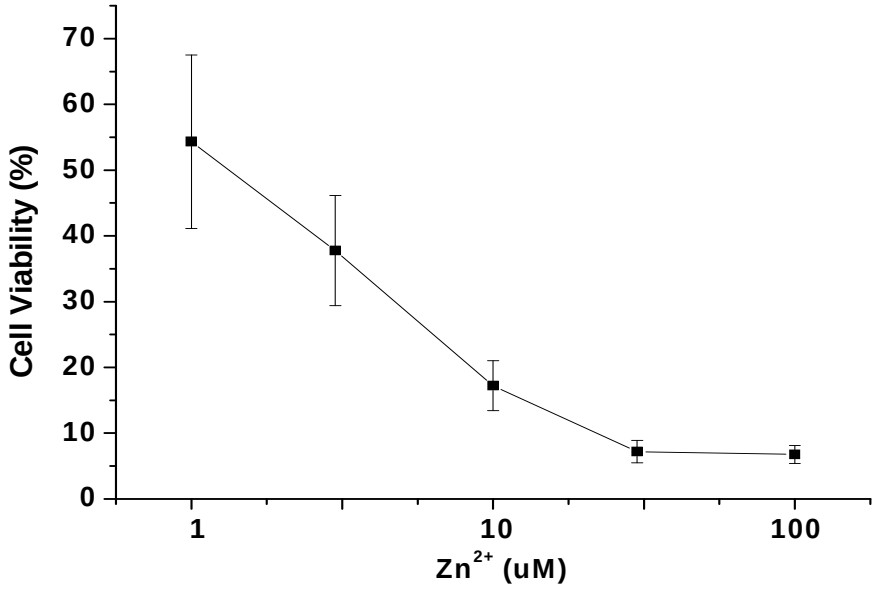

Figure 1. Zn^{2+} dose-dependently induced PC12 cell death.

Differentiated PC12 cells were treated with various concentrations of Zn^{2+} combined with 5 μM pyrithione. After 3 hr, cell viability was assessed by MTT assay. At 10 μM, Zn^{2+} induced cell death successfully when combined with 5 μM pyrithione. Results are presented as means ± SD of five independent experiments.

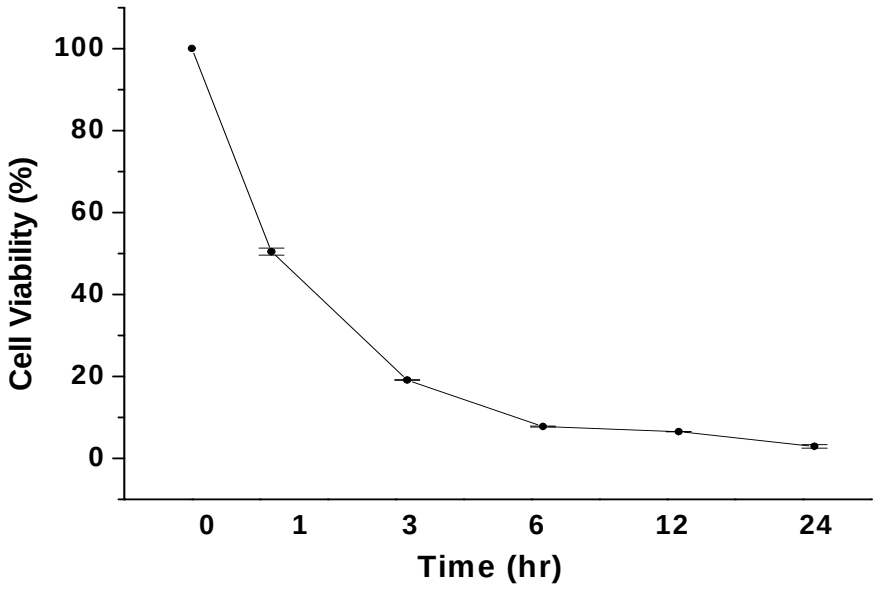

Figure 2. Zn^{2+} time-dependently induced PC12 cell death. Differentiated PC12 cells were treated with 10 μM Zn^{2+} combined with 5 μM pyrithione. After 3 hr, 10 μM Zn^{2+} induced cell death effectively when combined with 5 μM pyrithione. Results are presented as means ± SD of three independent experiments.

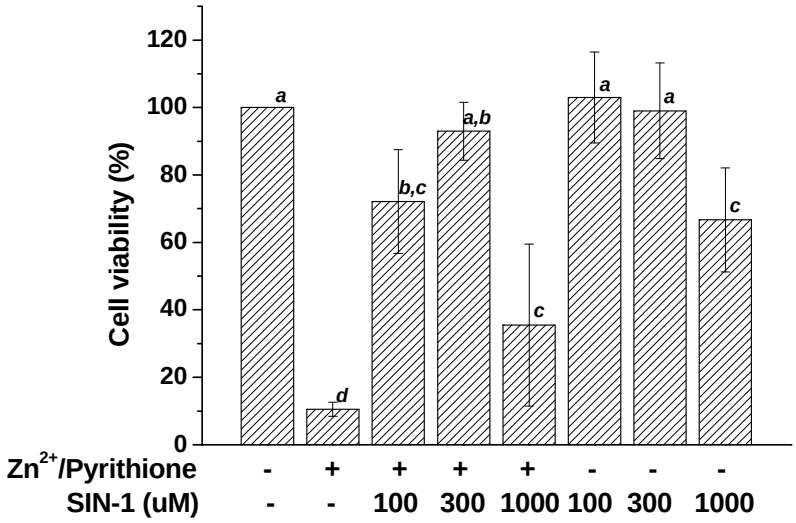

Figure 3. The Zn^{2+}-induced PC12 cell death was prevented by the pretreatment with 3-morpholinsydnonimine (SIN-1), a $ONOO^-$ donor.

Differentiated PC12 cells were preincubated with various concentrations of SIN-1 (100-1000 μM). After 10 min, cells were treated with 10 μM Zn^{2+} and 5 μM pyrithione for 3 hr and cell viability was assessed by MTT assay. At 300 μM, SIN-1 inhibited the Zn^{2+}-induced PC12 cell death almost completely. However, 1 mM SIN-1 induced cell death by itself. Results are presented as means ± SD of ten independent experiments. Means with different lowercase letters are significantly different at $P < 0.05$.

At 300 μM, SIN-1 blocked Zn^{2+}-induced cell death effectively. However 1 mM SIN-1 appeared to induce cell death by itself.

3. SIN-1 did not inhibit the intracellular accumulation of Zn^{2+} in differentiated PC12 cells.

In order to detect the change of $[Zn^{2+}]_i$, we treated differentiated PC12 cells with magfura-2AM, a fluorescent probe sensitive to Zn^{2+}. As shown in figure 4, 300 μM SIN-1 did not alter the Zn^{2+}/pyrithione-induced increase in fluorescent ratio, suggesting that SIN-1 did not inhibit the intracellular accumulation of Zn^{2+}. When the fluorescence ratio reached the peak, 50 μM TPEN, a membrane permeable Zn^{2+} specific chelator, was added and the fluorescence ratio promptly decreased to the basal level. This confirmed that the increased fluorescence ratio was caused solely by the influx of Zn^{2+}.

4. SNP and SNAP did not inhibit Zn^{2+}-induced PC12 cell death.

SNP and SNAP are broadly used as NO donors. Unlike SIN-1, these NO donors do not generate $ONOO^-$. We treated cells with 50 - 300 μM SNP or SNAP to generate NO. According to figure 5. (A) and (B), SNP and SNAP did not exert the neuroprotective effect on the Zn^{2+}-induced PC12 cell death.

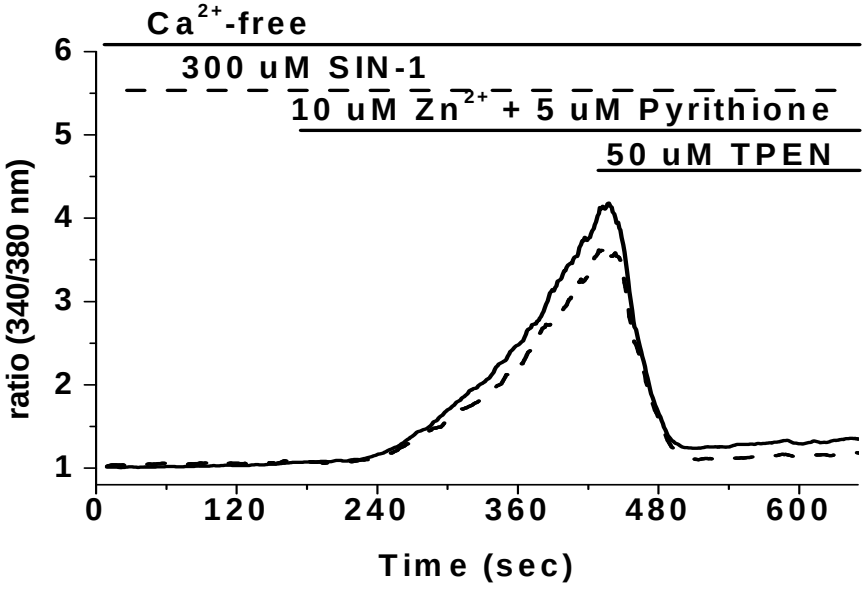

Figure 4. SIN-1 did not blocked the intracellular accumulation of Zn^{2+} in differentiated PC12 cells.

Changes in $[Zn^{2+}]_i$ were measured in magfura-2 loaded differentiated PC12 cells using ratiometric fluorescence recording techniques. Cells were exposed to 10 μM Zn^{2+} and 5 μM pyrithione (solid) or 5 μM pyrithione and 10 μM Zn^{2+} in the presence of 300 μM SIN-1 (dash). To confirm the intracellular accumulation of Zn^{2+}, 50 μM TPEN was treated in the time of $[Zn^{2+}]_i$ elevation. The result is representative of three independent experiments.

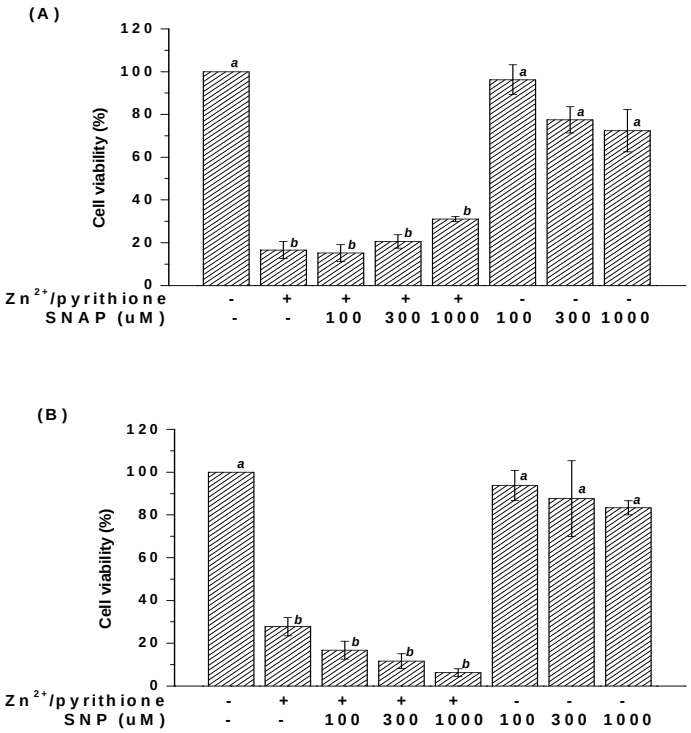

Figure 5. Pretreatment of cells with various nitric oxide donors, such as SNAP and SNP, was not able to attenuate the Zn^{2+}-induced PC12 cell death.

(A) Differentiated PC12 cells were preincubated with various concentrations of SNAP. (B) PC12 cells were preincubated with various concentrations of SNP. Results are presented as means ± SD of eight independent experiments. Means with different lowercase letters are significantly different at $P < 0.05$.

5. SIN-1C, the end product of SIN-1, did not prevent Zn^{2+}-induced PC12 cell death.

As previously mentioned, SIN-1 decomposes to SIN-1C after releasing superoxide and NO. To assess whether SIN-1C was associated with neuroprotective property of SIN-1, PC12 cells were exposed to 100-1000 μM SIN-1C, as shown in figure 6. 300 μM SIN-1C treated with Zn^{2+}/pyrithione caused 80-90% cell death, indicating SIN-1C did not inhibit the Zn^{2+}-induced PC12 cell death.

6. Various $ONOO^-$ scavengers blocked the effect of SIN-1 on Zn^{2+}-induced PC12 cell death.

To investigate the role of $ONOO^-$ on Zn^{2+}-induced PC12 cell death, I used various $ONOO^-$ scavengers, *i.e.* uric acid, trolox and L-methionine. Uric acid, an end product of human purine metabolism, has recently been proposed to fulfil the role as a $ONOO^-$ scavenger and neuroprotective agent. Shown in figure 7 (A), 1 mM uric acid mostly blocked the neuroprotective effect of SIN-1 probably by scavenging $ONOO^-$. Approximately 85 % of cells, treated with uric acid combined with SIN-1/Zn^{2+}/pyrithione, died compared to SIN-1/Zn^{2+}/ pyrithione-treated cells. L-methionine and trolox, the water-soluble vitamin E analogs, were widely used as $ONOO^-$ scavengers too. As shown in figure 7 (B) and (C),

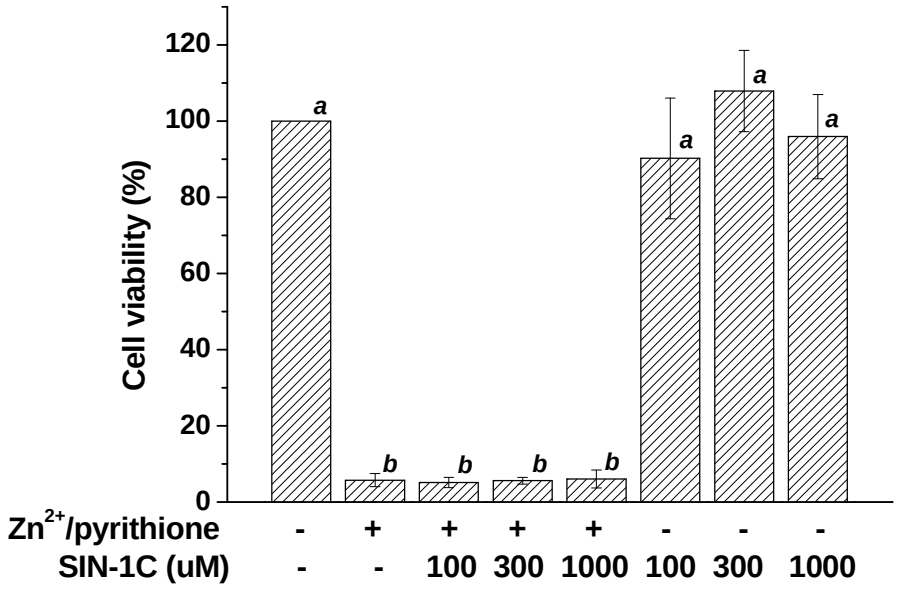

Figure 6. SIN-1C, the stable decomposition product of SIN-1, did not prevent the Zn^{2+}-induced cell death.

Various concentrations of SIN-1C did not prevent the neuroprotective effect of SIN-1 on Zn^{2+}-induced PC12 cell death. Results are presented as means ± SD of four independent experiments. Means with different lowercase letters are significantly different at $P < 0.05$.

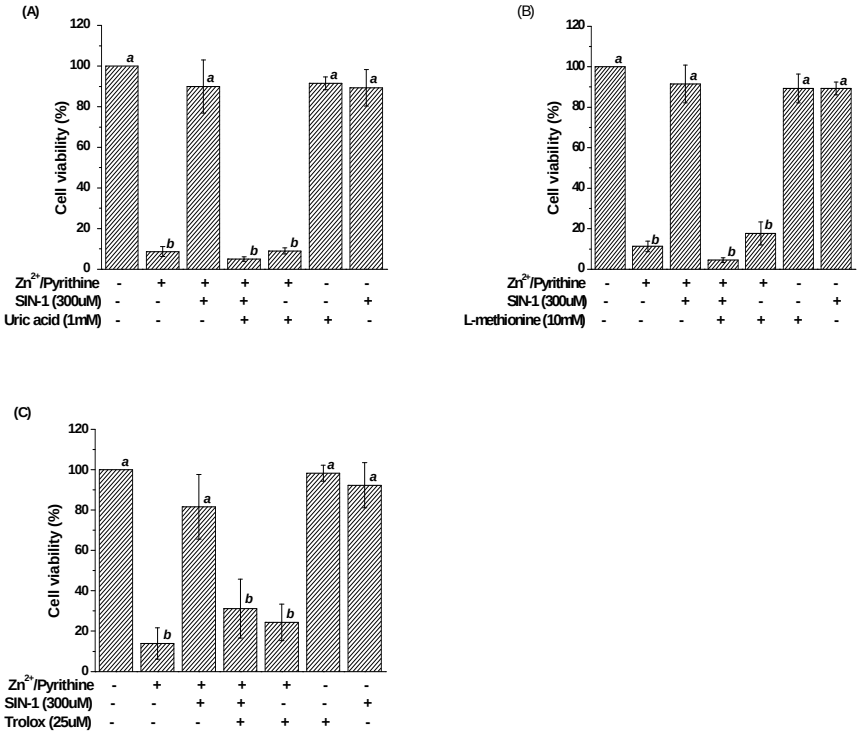

Figure 7. Various $ONOO^-$ scavengers blocked the neuroprotective effect of SIN-1 on Zn^{2+}-induced PC12 cell death.

$ONOO^-$ scavengers, such as uric acid (A), L-methionine (B) and trolox (C), prevented the neuroprotective effect of SIN-1 on Zn^{2+}-induced PC12 cell death. Results are presented as means ± SD of five independent experiments. Means with different lowercase letters are significantly different at $P < 0.05$.

10 mM L-methionine and 25 μM trolox inhibited SIN-1 induced cell survival like uric acid.

7. D-mannitol, a hydroxyl radical scavenger, did not block the Zn^{2+}-induced PC12 cell death.

During the decomposition of SIN-1, a hydroxyl radical (OH•) is also generated in the first step of decomposition. We treated D-mannitol, a OH• scavenger, to reveal the role of OH• on Zn^{2+}-induced PC12 cell death. 20 mM D-mannitol was treated for 10 min prior to SIN-1/Zn^{2+}/pyrithione treatment in differentiated PC12 cells for 3 hours. As shown figure 8, cell viabilities were not affected by D-mannitol.

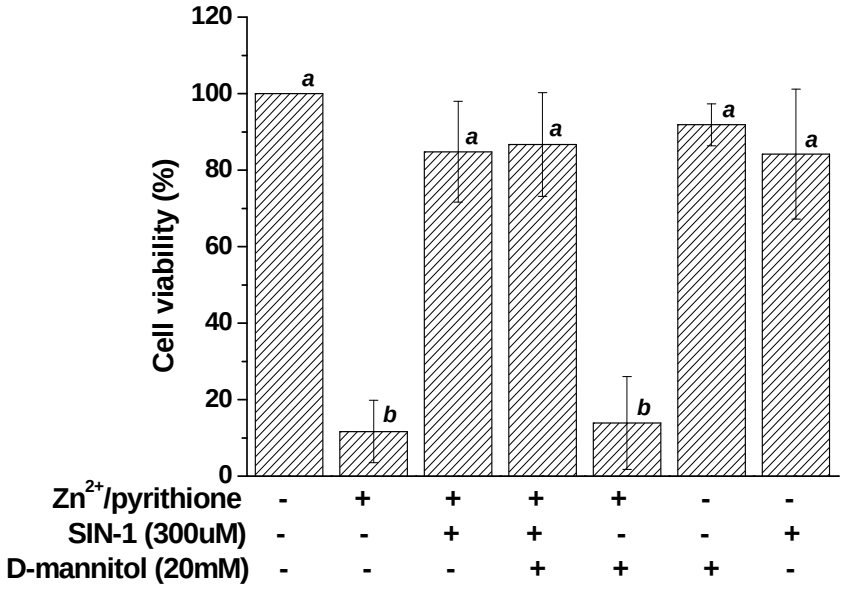

Figure 8. D-mannitol, a hydroxyl radical scavenger, did not block the Zn^{2+}-induced cell death.

20 mM D-mannitol did not abolish the neuroprotective effect of SIN-1 on Zn^{2+}-induced PC12 cell death. OH•, generated from the decomposition of SIN-1, was not related to the neuroprotective effect of SIN-1. Results are presented as means ± SD of five independent experiments. Means with different lowercase letters are significantly different at $P < 0.05$.

Ⅳ. Discussion

The present study was undertaken to investigate the novel effect of SIN-1, a $ONOO^-$ donor, on Zn^{2+}-induced PC12 cell death. 10 μM $ZnCl_2$, with the presence of 5 μM pyrithione for 3 hours, increased intracellular Zn^{2+} level and induced PC12 cell death. These results were consistent with preceding reports that Zn^{2+} induced neurotoxicity after Zn^{2+} overload via various routes, such as voltage-gated Ca^{2+} channels, NMDA receptors and Ca^{2+}-permeable AMPA/kainate receptors in cortical cells[6,36]. In some reports, the cells were exposed to high levels of Zn^{2+} (100-300 μM), and induced cell death[9] by previously mentioned routes. Nevertheless, we used pyrithione, a specific Zn^{2+} ionophore, to achieve rapid and relatively constant elevation of intracellular Zn^{2+} level without depolarizing the cells[37]. When we treated Zn^{2+}/pyrithione for 3 hours, nearly 80 % of PC12 cells died by Zn^{2+} overload compared to control cells, which were not treated Zn^{2+}/pyrithione.

In the CNS, through the activation of NO-sensitive guanylyl cyclase and the generation of cyclic guanosine monophosphate (cGMP)[26,40], NO protects neurons against oxidant stress and damage caused by ROS[41]. Since SIN-1 acts as NO donor as well as $ONOO^-$ donor, various concentrations of SIN-1 (100-1000 μM) were used to examine the effect of SIN-1 on Zn^{2+}-induced PC12 cell death. At 300 μM, SIN-1 effectively decreased Zn^{2+}-induced PC12 cell death. However, lots of

studies suggested SIN-1 induced apoptosis which accompanied depletion of intracellular glutathione (GSH), c-Jun N-terminal kinase activation, mitochondrial membrane depolarization, cleavage of poly (ADP-ribose) polymerase and DNA fragmentation by generating $ONOO^-$[38,39]. In agreement with previous studies, higher concentrations of SIN-1 induced PC12 cell death by itself. However, in the present study, low concentrations of SIN-1 (<300 μM) had a neuroprotective effect, contrary to high concentrations of SIN-1 provoking PC12 cell death. SIN-1 has dual effect as a NO donor and $ONOO^-$ donor and is likely to behave more like an NO donor at specific conditions[48]. To define whether NO could act as the neuroprotective factor, other NO donors, such as SNP and SNAP, were applied. NO released from SNAP and SNP mediates antioxidation and neuroprotection in the brain[49]. However SNP and SNAP had no influence on Zn^{2+}-induced PC12 cell death. Therefore the neuroprotective effect of SIN-1 was caused not by NO but by $ONOO^-$.

Intracellular accumulation of Zn^{2+} is a principle factor causing Zn^{2+} neurotoxicity. In evidence, the cells exposed to high concentrations of Zn^{2+} could be rescued from Zn^{2+} injury by treating CaEDTA, the extracellular Zn^{2+} chelator, in cultured neurons[42]. Another study showed glutathione could form a complex with Zn^{2+} and inhibit Zn^{2+} neurotoxicity[43]. In relation to these results, we confirmed that SIN-1 did not affect intracellular Zn^{2+} accumulation by loading magfura-2,

Zn^{2+}-sensitive fluorescence dye. On the peak of the fluorescence ratio, a Zn^{2+} specific chelator, TPEN was treated and the magfura-2 fluorescence ratio was completely restored to basal level.

As SIN-1 did not block the intracellular accumulation of Zn^{2+}, SIN-1 would affect the Zn^{2+} neurotoxicity in the intracellular mechanism. At pH 7.4, SIN-1 decomposes to produce NO in an oxygen-dependent process involving the sydnonimine ring opening to SIN-1A and further to a more stable SIN-1C[33]. Finally SIN-1 could generate NO, $ONOO^-$, SIN-1C and OH•. SIN-1C, a stable end product of SIN-1, could contribute to the neuroprotective effect of SIN-1, but SIN-1C did not prevent Zn^{2+}-induced cell death. $ONOO^-$, formed during SIN-1 decomposition is probably related to the neuroprotective role of SIN-1 , and this phenomenon contradicted to early reports that $ONOO^-$ mediates neurotoxicity in the brain[38,39]. Diverse $ONOO^-$ scavengers, such as uric acid, trolox, and L-methionine have been used by a large number of research groups[44,45]. Depending on my data, $ONOO^-$ scavengers reduced the neuroprotective effect of SIN-1. More than 50-70 % of cell, cotreated SIN-1 with $ONOO^-$ scavengers, died compared to SIN-1 treated cell. In addition, D-mannitol, a OH• scavenger, did not rescue PC12 cells from Zn^{2+} neurotoxicity.

$ONOO^-$ seemed to have the neuroprotective effect on Zn^{2+}-induced PC12 cell death. But the transient neuroprotective role for $ONOO^-$ is in apparent contradiction with widely held

assumption that $ONOO^-$ would be the NO-derived neurotoxic effector molecule[31,46,47]. Recently, however, one research group indicated $ONOO^-$ protects neurons against NO-mediated cell death and suggested that $ONOO^-$ might be related to the activation of glucose-6-phosphate dehydrogenase in pentose phosphate pathway[32,50]. In agreement of this report, my results showed low concentrations of SIN-1 protected PC12 cells from Zn^{2+}-induced cell death. In conclusion, $ONOO^-$ would play a neuroprotective role against Zn^{2+}-induced PC12 cell death. The mechanism responsible for this phenomenon is presently under investigation.

Ⅴ. References

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<국문 요약>

Zn^{2+}에 의해 유도되는 PC12 세포사에 SIN-1이 미치는 영향

과산화질소 ($ONOO^-$) 는 일산화질소 (NO) 과 활성산소 (O^{2-}) 가 결합되어 생성된 활성 물질로서 체내에서 세포에 해를 끼치는 물질로 알려져 있다. 그러나 최근 과산화질소가 일산화질소에 의한 세포사를 막는다는 사실이 알려졌다.

따라서 이번 연구에서는 Zn^{2+}가 신경세포로 분화된 PC12 세포 내에 축적되어 일어나는 세포사에 과산화질소가 끼치는 영향에 대해서 알아보고자 하였다.

Zn^{2+} (10 μM)를 Zn^{2+} 이온 투과 담체인 pyrithione (5 μM)과 함께 3시간 동안 처리하면 세포 안에 고농도의 Zn^{2+}가 축적되어 세포사를 일으키는데, 과산화질소를 만들어 내는 SIN-1을 함께 처리하면 세포의 사멸이 일어나지 않는 것을 확인하였다.

먼저 SIN-1이 Zn^{2+}가 세포 안으로 유입되는 것을 방해하는지 알아보기 위해 SIN-1을 전처리한 후 Zn^{2+}와 pyrithione을 처리하였다. SIN-1과 Zn^{2+} 및 pyrithione을 처리한 실험군이나 Zn^{2+} 및 pyrithione만 처리한 실험군 모두 세포 내로 Zn^{2+}가 정상적으로 유입되는 것으로 보아 SIN-1이 세포 밖에서 Zn^{2+}와 결합하여 Zn^{2+}가 세포 안으로 들어가는 것을 방해함으로서 Zn^{2+}의 세포사를 막는 것은 아님을 확인하였다.

SIN-1은 과산화질소 뿐만 아니라 일산화질소도 생성하므로, SIN-1이 Zn^{2+}에 의한 세포사를 막는 것이 과산화질소의 영향인지 아니면 일산화질소의 영향인지 알아보고자 하였다. 따라서 또 다른 일산화질소 생성물인 SNP와 SNAP을 처리하였으나, 이들 약물은 SIN-1과 달리

Zn^{2+}에 의한 세포사에 어떠한 영향을 끼치지 않았다. Zn^{2+}에 의한 세포사에 대해서 SIN-1은 uric acid, trolox, L-methionine과 같은 과산화질소 포착제를 함께 처리할 경우에는 거의 효과를 나타내지 않았다. 또한 SIN-1이 분해되어 만들어지는 SIN-1C를 Zn^{2+}와 함께 처리해도 Zn^{2+}에 의한 세포사를 막지 못하였다. 마지막으로 SIN-1이 분해되면서 만들어지는 OH•를 제거하여도 세포사멸을 막는데는 아무런 효과도 나타내지 않았다. 앞의 결과를 토대로 과산화질소가 Zn^{2+}에 의한 세포사를 막는데 중요한 역할을 한다는 것을 확인하였다. 앞으로는 어떤 기전에 의해 과산화질소가 Zn^{2+}에 의한 세포사를 막는지 확인하는 실험이 필요하다고 생각한다.

Key words : Zn^{2+}, SIN-1, 과산화질소, PC12 세포, neuroprotection