European Journal of Pharmacology 748 (2015) 37–44

Contents lists available at ScienceDirect

European Journal of Pharmacology

journal homepage: www.elsevier.com/locate/ejphar

Neuropharmacology and analgesia

Neuroprotective effect of 3-morpholinosydnonimine against Zn^{2+}-induced PC12 cell death

Jeong Mi An [a,1], Seong Ah Moon [a,1], Soo Young Hong [a], Jeong Wan Kang [b,*], Jeong Taeg Seo [a,**]

[a] Department of Oral Biology, Yonsei University College of Dentistry, Seoul 120-752, Republic of Korea
[b] Department of Oral & Maxillofacial Surgery, Yonsei University College of Dentistry, Seoul 120-752, Republic of Korea

ARTICLE INFO

Article history:
Received 19 June 2014
Received in revised form
3 December 2014
Accepted 5 December 2014
Available online 15 December 2014

Keywords:
SIN-1
Zn^{2+}
PC12 cells
Glutathione reductase
Neuroprotection

ABSTRACT

Excessive intracellular accumulation of zinc (Zn^{2+}) is neurotoxic and contributes to a number of neuropathological conditions. Here, we investigated the protective effect of 3-morpholinosydnonimine (SIN-1) against Zn^{2+}-induced neuronal cell death in differentiated PC12 cells. We found that Zn^{2+}-induced PC12 cell death was reduced in a concentration-dependent manner by pretreatment with SIN-1. The intracellular accumulation of Zn^{2+} was not affected by pretreatment with SIN-1, indicating that SIN-1-induced neuroprotection was not attributable to reduced influx of Zn^{2+} into cells. SIN-1C, the stable decomposition product of SIN-1, failed to prevent Zn^{2+}-induced cell death. Furthermore, the protective effect of SIN-1 against Zn^{2+}-induced PC12 cell death was almost completely abolished by uric acid, a free radical scavenger, suggesting that reactive oxygen and nitrogen species generated by SIN-1 may contribute to the protective effect. SIN-1 prevented the inactivation of glutathione reductase (GR) and the increase in the ratio of oxidized glutathione/total glutathione (GSSG/total GSH) induced by Zn^{2+}. Addition of membrane permeable GSH ethyl ester (GSH-EE) to PC12 cells prior to Zn^{2+} treatment significantly increased cell viability. We therefore conclude that SIN-1 may exert neuroprotective effect against Zn^{2+}-induced cell death in differentiated PC12 cells by preventing inhibition of GR and increase in GSSG/total GSH ratio.

1. Introduction

The divalent cation, zinc (Zn^{2+}), plays an essential role in controlling gene transcription, neurotransmission, and neuromodulation in the central nervous system (CNS) (Aras and Aizenman, 2011; Nakashima and Dyck, 2009; Smart et al., 2004). Zn^{2+} is localized in presynaptic vesicles along with the neurotransmitter, glutamate, and both are released together into the extracellular space (Assaf and Chung, 1984; Qian and Noebels, 2006). By altering the function of several receptors, including *N*-methyl-D-asparate (NMDA) and γ-aminobutyric acid type A ($GABA_A$) receptors, released Zn^{2+} serves as an important modulator of inhibitory and excitatory synaptic transmission (Peters et al., 1987; Sensi et al., 2011; Weiss et al., 1993). In addition to regulating neurotransmission, Zn^{2+} may participate in various neuropathological conditions, including ischemia and Alzheimer's disease (Choi and Koh, 1998; Frederickson et al., 2005; Sensi et al., 2009). Under pathological conditions, Zn^{2+} is released in excess at excitatory synapses and mobilized from intracellular stores, such as metallothionein proteins and mitochondria, causing neuronal death via cytosolic Zn^{2+} overload (Shuttleworth and Weiss, 2011). The precise mechanism underlying Zn^{2+} neurotoxicity is unknown, but Zn^{2+} overload induces an increase in reactive oxygen species and lipid peroxidation (Kim et al., 1999).

3-Morpholinosydnonimine (SIN-1) is a generator of reactive oxygen and nitrogen species. Upon decomposition in aqueous solution, SIN-1 releases superoxide anion (O_2^-) and nitric oxide (NO), which rapidly form peroxynitrite ($ONOO^-$). SIN-1 may also produce highly reactive free radicals, such as nitrogen dioxide and carbonate radicals (Ho et al., 2012). In this regard, SIN-1 has been commonly used to induce oxidative/nitrosative stress in a variety of experimental conditions. Reactive oxygen and nitrogen species generated by SIN-1 have been shown to induce both necrosis and apoptosis. SIN-1 induces an activation of mitochondrial permeability transition (MPT) and poly ADP-ribose polymerase (PARP), leading to the depletion of ATP and causing necrosis (Brown, 2010). SIN-1 may also

* Corresponding author. Tel.: +82 2 2228 3131.
** Corresponding author. Tel.: +82 2 2228 3054.
E-mail addresses: jeongwan@yuhs.ac (J.W. Kang), jeong@yuhs.ac (J.T. Seo).
[1] An JM and Moon SA contributed equally to this work.

http://dx.doi.org/10.1016/j.ejphar.2014.12.003
0014-2999/

activate p38 MAP kinase (Oh-Hashi et al., 2001) and induce ER stress (Kawahara et al., 2001), which results in apoptosis.

Although the cytotoxic properties of SIN-1 are well known, some studies have suggested that SIN-1 also plays a protective role against reactive oxygen and nitrogen species-mediated cytotoxicity. In endothelial cells, for example, $ONOO^-$ generated by SIN-1 activates Nrf2 pathway and exerts cytoprotective effects (Mattart et al., 2012). In addition to this, SIN-1 was also shown to induce the adaptive survival response to cellular stress by upregulating heme oxygenase-1 (HO-1), an important antioxidant enzyme, in PC12 cells (Li et al., 2006). Therefore, given the critical role of reactive oxygen species in Zn^{2+}-induced neuronal cell death, it is tempting to speculate that SIN-1 might protect neurons against Zn^{2+}-mediated cell death.

In the present study, we investigated the protective role of SIN-1 against Zn^{2+}-induced PC12 cell death and found that SIN-1 exerted neuroprotective effect by preventing Zn^{2+}-induced inactivation of glutathione reductase (GR).

2. Materials and methods

2.1. Materials

PC12 cells and human neuroblastoma SH-SY5Y cells were obtained from American Type Culture Collection (Manassas, VA, USA). Fetal bovine serum (FBS), horse serum, Dulbecco's modified Eagle's medium (DMEM), fluoZin-3-AM, hydroxyphenyl fluorescein (HPF), and *N*,*N*,*N*′,*N*′-tetrakis-2-pyridylmethyl ethylenediamine (TPEN) were purchased from Invitrogen (Carlsbad, CA, USA). Mouse nerve growth factor 2.5S (2.5S mNGF) was obtained from Alomone Labs (Jerusalem, Israel). Collagen Type 1 was purchased from Millipore (Billerica, MA, USA). Anti-Nrf2 antibody and anti-Lamin B1 antibody were obtained from Cell Signaling Technology (Danvers, MA, USA). Anti-HO-1 antibody was purchased from NOVUS Biologicals (Littleton, CO, USA). Uric acid was purchased from Merck Chemicals (Nottingham, UK). SIN-1C was kindly provided by Aventis Pharma (Frankfurt, Germany). Other reagents, including $ZnCl_2$, pyrithione, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 4′6′-diamidino-2-phenyindole (DAPI), β-nicotinamide adenine dinucleotide phosphate (NADPH), glutathione (GSH), oxidized GSH (glutathione disulfide, GSSG), GSH ethyl ester (GSH-EE), SIN-1, and anti-GAPDH antibody were obtained from Sigma (St Louis, MO, USA), unless indicated otherwise.

2.2. Cell culture

PC12 cells were maintained in DMEM supplemented with 10% horse serum, 5% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin sulfate. To obtain neuronally differentiated PC12 cells, approximately 1.5×10^4 cells/well were seeded onto collagen-coated 24-well plates and grown for 7 days in medium containing low serum (2% horse serum and 1% FBS) plus 100 U/ml penicillin, 100 μg/ml streptomycin sulfate, and 2.5S mNGF (50 ng/mL). SH-SY5Y cells were maintained in DMEM supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin sulfate. All cultures were maintained at 37 °C in a humidified, 5% CO_2 incubator; medium was replaced every 2 days.

2.3. MTT assay

To determine cell viability, 20 μl of an MTT stock solution (5 mg/ml in sterile phosphate-buffered saline) was added after each indicated treatment and incubated for 1 h at 37 °C, after which 500 μl solubilizing solution (50% dimethylformamide and 20% sodium dodecyl sulfate, pH 4.7) was added. After incubating overnight, absorbance at 570 nm was determined. Viability, defined as percentage survival, was calculated as follows:

$$\text{Percent survival} = \left[\frac{(\text{experimental} - \text{blank})}{(\text{control} - \text{blank})} \times 100\right],$$

where experimental, control and blank are readings obtained from treated cells, untreated cells and wells containing MTT+medium (no cells), respectively.

2.4. DAPI staining

Nuclear morphology was observed by staining with the DNA-binding fluorescent dye, DAPI. PC12 cells, differentiated for 7 days in collagen-coated coverslips, were treated with 10 μM Zn^{2+} and 5 μM pyrithione in the presence or absence of 300 μM SIN-1 for 3 h. The cells were then fixed with 4% paraformaldehyde for 30 min and incubated with 10 μg/ml DAPI for 10 min. Samples were observed under a fluorescence microscope.

2.5. Measurement of intracellular Zn^{2+} concentration ($[Zn^{2+}]_i$)

The $[Zn^{2+}]_i$ in differentiated PC12 cells was measured using a method described previously (Gee et al., 2002). Briefly, cells were loaded with fluoZin-3 by incubating with 10 μM fluoZin-3-AM in a physiologic salt solution (PSS; 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM HEPES pH 7.4, 10 mM D-glucose) for 30 min at 37 °C in a 5% CO_2 incubator. Cells were then rinsed twice and incubated in PSS 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 488 nm and the emitted fluorescence was recorded at 520 nm. FluoZin-3 fluorescence was normalized to starting values and F/F_0 was used as an index of $[Zn^{2+}]_i$.

2.6. Measurement of GR activity

GR activity was measured as previously described (Walther et al., 2003). Differentiated PC12 cells and SH-SY5Y cells were preincubated with 300 μM SIN-1 or SIN-1C for 10 min and treated with 5 μM pyrithione and 10 μM Zn^{2+} for 3 h. The cells were washed and lysed with 200 μl 0.1% Triton X-100. The lysates were added to 700 μl phosphate buffer (0.1 M, 1 mmol/l EDTA, pH 7.0) and then transferred to cuvettes. Absorbance was recorded at 412 nm immediately after 20 μl DTNB (final concentration 30 μg/ml) was added. After the absorbance reached a plateau, NADPH (final concentration 100 μg/ml) and GSSG (final concentration 300 μg/ml) were added and absorbance was recorded again at 412 nm for 3 min at 30 s intervals. The protein contents of the lysates were determined by using BCA reagents (Pierce, Thermo Scientific, Rockford, IL, USA). For each condition, 500 μg of protein were used. All samples were measured in duplicate and expressed as percentages of controls.

2.7. Determinations of cellular GSSG and total GSH

The amounts of total GSH and GSSG were measured as previously described with minor modification (Garcia-Nogales et al., 1999). PC12 cells grown in 60 mm dish were exposed to the indicated reagents. Cells were then collected and immediately dissolved with 250 μl of 1% sulfosalicylic acid. Cell lysates were centrifuged at 13,000*g* for 5 min at 4 °C, and the supernatants were used for total GSH and GSSG measurements. The protein contents of the lysates were determined with BCA reagents. For the measurement of total GSH (GSH+GSSG), 10 μl of the supernatant was transferred into microtiter plate wells and diluted with 90 μl of water. After addition of 100 μl of reaction mixture [1 mM EDTA, 0.3 mM 5,5′-dithio-bis(2-nitrobenzoic acid), 0.4 mM NADPH, and