Mechanisms of [2,3-Butanedione Bis(*N*⁴-Dimethylthiosemicarbazone)]zinc (Zn-ATSM₂)-Induced Protection of Cultured Hippocampal Neurons Against *N*-Methyl-D-Aspartate Receptor-Mediated Glutamate Cytotoxicity

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ABSTRACT—Hyperexcitation of glutamatergic neurons may play a key role in ischemia-related neurodegeneration. Recent studies have suggested that the zinc ion (Zn²⁺), which is present in the central nervous system, has a modulatory role in glutamatergic neuron activity. Zinc ions block glutamate-induced depolarizing currents and neuronal damage by binding with zinc sites on the NMDA subtypes. Therefore, we examined the usefulness of zinc as a therapeutic agent for the prevention of ischemic neuronal damage in the brain. In our previous study, 2,3-butanedione bis(*N*⁴-dimethylthiosemicarbazone) zinc complex (Zn-ATSM₂), with high brain uptake, showed significant neuroprotective effects against cerebral ischemia in rats when administered systemically. In this study, to elucidate the mechanism of the neuroprotective effect of Zn-ATSM₂, we first examined its *in vitro* protective effects against glutamate-, NMDA- and kainite-induced neurotoxicity in primary cultures of hippocampal neurons. Zn-ATSM₂ elicited protective effects against this glutamate- and NMDA-induced neurotoxicity, but did not affect kainite-induced cytotoxicity. In addition, we studied the effects of Zn-ATSM₂ on influx of Ca²⁺, which undergoes modification subsequent to NMDA activation. Zn-ATSM₂ significantly decreased glutamate-induced ⁴⁵Ca²⁺ uptake. Thus, Zn-ATSM₂ protected against glutamate-induced neurotoxicity and its protective effect was, at least in part, due to the blockage of NMDA receptor-mediated Ca²⁺ influx.

Keywords: Zinc, *N*-Methyl-D-aspartate receptor, Neuroprotection, Hippocampal cell culture, Glutamate

It has been postulated that excessive activation of glutamatergic neurons plays an important role in the pathogenesis of the neuronal cell loss associated with several neurological disease states (1, 2). Glutamate neurotoxicity mediated by *N*-methyl-D-aspartate (NMDA) receptors involves Ca²⁺ influx into cells via ligand-gated ion channels. For example, the NMDA subtype of glutamate receptor was suggested to be one of the predominant routes of glutamate neurotoxicity since selective antagonists of NMDA receptors prevent neuronal degeneration both in animal models of ischemia and in cultured neurons (3–5).

The zinc ion (Zn²⁺), a divalent cation, is an important trace element in a variety of metabolic processes. Within the CNS, Zn²⁺ is found in synaptic vesicles in a subgroup of glutamatergic neurons (6). Recent studies have shown that Zn²⁺ plays a role in modulation of synaptic transmis-

sion by interacting with a zinc-specific site on the NMDA receptor (7–10). Previous studies in cultured cortical and retinal neurons further revealed that Zn²⁺ blocks NMDA-induced depolarizing currents and protects neurons from NMDA receptor-mediated glutamate neurotoxicity (5).

Therefore, we examined the usefulness of zinc as a therapeutic agent for the prevention of ischemic neuronal damage in the brain. However, because Zn²⁺ does not readily permeate across cell membranes including the blood-brain-barrier (BBB) due to its polarity (11), peripheral administration is not effective. Thus, we developed a lipophilic zinc complex, 2,3-butanedione bis(*N*⁴-dimethylthiosemicarbazone) zinc complex (Zn-ATSM₂), which was found to result in significantly higher brain uptake of zinc than administration of Zn²⁺ ions themselves (12). Furthermore, receptor binding assays indicated that the inhibitory potency of Zn-ATSM₂ against MK-801 binding to NMDA receptors was similar to that of Zn²⁺ (12).

In addition, systemic administration of Zn-ATSM₂ on

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ischemic neuronal injury after transient middle cerebral artery occlusion in the rat significantly decreased infarct volume (13).

In this study, to further estimate the mechanism of the neuroprotective effect of Zn-ATSM₂, we examined its protective effects against glutamate receptor-mediated neurotoxicity and calcium influx in cultured hippocampal neurons.

MATERIALS AND METHODS

[⁴⁵Ca]Calcium chloride (50 mCi (1.85GBq)/mg Ca) was purchased from Amersham Pharmacia Biotech UK Ltd. (Buckinghamshire, England). All other chemicals used were of reagent grade and were purchased commercially. Sprague-Dawley rats were supplied by Japan SLC Co. (Hamamatsu).

Cell culture

Primary hippocampal mixed neuronal/glial cultures were established essentially according to the methods of Banker and Cowan (14).

Hippocampi were excised from 18-day-old Sprague-Dawley rat embryos, trypsinized, and triturated by pipetting through a narrow-bore pipette. Cultures were plated onto poly-L-lysine (50 µg/ml)-coated 48-well plastic plates at a density of 1.8×10^5 cells per well. The cultures were maintained in Eagle's minimal essential medium (MEM Eagle's salt) containing 10% heat-inactivated fetal bovine serum (1–3 days after plating) or N-2 supplement (4–12 days after plating), 25 U/ml streptomycin, 25 µg/ml penicillin glutamine (2 mM), glucose (total 11 mM), NaHCO₃ (26 mM) and HEPES (10 mM) at 37°C in a humidified 10% CO₂ / 90% air mixture. Only mature cells (9–12 days in vitro) were used in this study. Immunocytochemical analyses of these primary cultures showed that they contained >95% neurons and <5% glia or other contaminating cells.

Measurement of neurotoxicity

Glutamate or kainite exposure was carried out in Locke's solution (154 mM NaCl, 5.6 mM KCl, 1.2 mM MgCl₂, 2.3 mM CaCl₂, 5.6 mM glucose, 3.6 mM NaHCO₃ and 5 mM *N*-[2-hydroxyethyl]piperazin-*N*-[2-ethansulfoic acid] (HEPES), adjusted to pH 7.4 with NaOH). NMDA exposure was carried in the absence of Mg²⁺. The cultures were exposed to 1 mM glutamate or NMDA for 10 min followed by incubation in glutamate-free buffer for 60 min. Kainite treatment was performed for 60 min. The test agents were added to solutions containing excitatory amino acids.

The neurotoxic effect of excitatory amino acids (EAAs) were quantitatively assessed by the examination of cultures

under Hoffman modulation microscopy at $\times 200$. After drug treatment, cell cultures were stained with 1.5% Trypan blue for 4 min at 37°C and then rinsed with physiological saline. Cells stained by Trypan blue treatment were regarded as non-viable. More than 200 cells in each well were counted to determine viability of the cell cultures. In each experiment, 4–8 wells were used.

Measurement of ⁴⁵Ca²⁺ influx

Stimulation of ⁴⁵Ca²⁺ uptake was studied as described by Wroblewski et al. (15). Briefly, 9-day old cultures were washed with Locke's solution (pH 7.4) and preincubated with 1 ml of ⁴⁵Ca²⁺ (2 µCi/ml, 74 kBq/ml) for 10 min to allow equilibration of the radioactive tracer in the various compartments. Cultures were then exposed to 1 mM glutamate for 10 min with ⁴⁵Ca²⁺ (2 µCi/ml, 74 kBq/ml). The test agents were added to the glutamate-containing solutions. ⁴⁵Ca²⁺ uptake was terminated by washing the cultures with an ice-cold solution containing 154 mM choline chloride, 5 mM ethyleneglycol tetracetic acid (EGTA) and 10 mM Tris-HCl, pH 7.2. Cells were dissolved in 0.5 N NaOH, and radioactivity was measured by liquid scintillation counter (2300TR Liquid Scintillation Analyzer; Packard, Meriden, CT, USA).

Preparation of Zn-ATSM₂ complex and effects of addition of Ca²⁺ on Zn-ATSM₂ solution

Zn-ATSM₂ complex was prepared according to the method of Petering (16). Briefly, ATSM₂ dissolved in dimethyl sulfoxide (DMSO) (20 µl) was added to acetate buffer (pH 4.0, 1980 µl) containing zinc acetate in an ATSM₂ / Zn²⁺ molar ratio of 1:1, and the mixture was vortexed. The final concentration of Zn-ATSM₂ was 50 µM. Furthermore, calcium chloride was added to prepared Zn-ATSM₂ solution at a final concentration 2 mM. Calcium chloride was also added to 50 µM ATSM₂ solution at a final concentration of 1 mM. UV absorbance spectra were recorded on a UV spectrometer (Model 330-S; Hitachi, Tokyo) at room temperature.

Statistical analyses

Data are presented as the mean $\pm$ S.E.M. The statistical significance of the data was determined by Student's *t*-test with Dunnett's adjustment for multiple comparisons.

RESULTS

Effects Zn-ATSM₂ on EAA-induced neurotoxicity in cultured hippocampal neurons

Table 1 summarizes the protective effects of Zn²⁺, Zn-ATSM₂ and MK-801 against glutamate, NMDA- and kainite-induced cytotoxicity. Since Kikuchi et al. reported that Zn²⁺ at a concentration of 30 µM reached maximum

Table 1. Effects of ZnCl₂, Zn-ATSM₂ and MK-801 against neurotoxicity induced by excitatory amino acids

Treatment	N	Viability (%)
Control	8	80.4 ± 0.5
Glu 1 mM ^a	8	33.7 ± 0.8
Glu 1 mM ^a +		
ZnCl ₂ 30 μM ^b	8	73.0 ± 1.3**
Zn-ATSM ₂ 30 μM ^b	8	67.0 ± 1.7**
MK-801 30 μM ^b	8	81.4 ± 1.1**
ATSM ₂ 30 μM ^b	4	34.6 ± 1.7
Control	8	81.5 ± 0.7
NMDA 1 mM ^c	8	46.1 ± 0.6
NMDA 1 mM ^c +		
ZnCl ₂ 30 μM ^b	8	72.6 ± 1.2**
Zn-ATSM ₂ 30 μM ^b	8	67.2 ± 1.3**
MK-801 30 μM ^b	8	82.5 ± 1.2**
Control	4	82.6 ± 2.2
KA 1 mM ^d	4	35.4 ± 0.5
KA 1 mM ^d +		
ZnCl ₂ 30 μM ^b	4	29.4 ± 1.1
Zn-ATSM ₂ 30 μM ^b	4	27.6 ± 0.5
MK-801 30 μM ^b	4	42.7 ± 0.6

Values are means ± S.E.M. ***P* < 0.01, compared with glutamate, NMDA or kainate alone. (Student's *t*-test with Dunnett's adjustment for multiple comparisons). ^aCultures were treated with glutamate for 10 min followed by incubation with glutamate-free buffer for 60 min. ^bCultures were treated with NMDA for 10 min followed by incubation with NMDA-free buffer for 60 min. Mg²⁺-free buffer was used. ^cDrugs were added to the buffer containing excitatory amino acids. ^dCultures were treated with kainate for 60 min. Glu: glutamate, NMDA: *N*-methyl-D-aspartate, KA: kainate.

protection against glutamate-induced cytotoxicity on cultured rat retina neurons (5), zinc compounds were used at a concentration of 30 μM in this cytotoxicity study.

Marked reduction of the cell viability was induced by exposure of the cultures to glutamate or NMDA for 10 min. Ten-minute exposure to kainate did not induce significant cytotoxicity, and cytotoxic effects were observed only after

exposure for 60 min. These results agreed with those reported by Tamura et al. (17).

MK-801 and Zn²⁺ induced significant protective effects against this glutamate- and NMDA-induced toxicity. Zn-ATSM₂ also elicited protective effects against this glutamate-, and NMDA-induced neurotoxicity, although Zn-ATSM₂ showed a slightly weaker effect than Zn²⁺. On the other hand, ATSM₂ alone did not affect the cytotoxic effect of glutamate. Furthermore, none of the compounds tested in this study affected kainate-induced cytotoxicity.

Effects of Zn-ATSM₂ on glutamate-induced Ca²⁺ influx

Calcium influx induced by glutamate was determined as the percentage of apparent glutamate-induced influx relative to untreated control cells (Fig. 1). Ten minutes after termination of the treatment with 1 mM glutamate, the uptake of ⁴⁵Ca²⁺ was elevated to about fourfold of that in untreated control cells.

Zn²⁺ significantly reduced glutamate-induced Ca²⁺ influx. Zn-ATSM₂ also elicited a significant inhibitory effect against glutamate-induced Ca²⁺ influx in a concentration-dependent manner, although the effect of Zn-ATSM₂ was weaker than that of Zn²⁺.

Effects of addition of Ca²⁺ to Zn-ATSM₂ solution

Physiological concentration of Ca²⁺ (2 mM) was added to the prepared Zn-ATSM₂ complex solution and the exchange of Zn²⁺ into Ca²⁺ in the Zn-ATSM₂ complex was examined by UV spectrometry. Ca²⁺ did not alter the spectrum of Zn-ATSM₂. Furthermore, ATSM₂ did not chelate Ca²⁺ as determined with UV spectrometry.

DISCUSSION

Zn-ATSM₂ showed a significant protective effect against glutamate- and NMDA-induced toxicity, but did not affect

Fig. 1. Inhibitory effects of Zn-ATSM₂ on glutamate-induced calcium uptake. **P* < 0.05, ***P* < 0.01 versus group treated with glutamate only (Student's *t*-test with Dunnett's adjustment for multiple comparisons). Values are means ± S.E.M. (n = 5 in each group).

the cytotoxicity of kainite (Table 1). In this respect, Zn-ATSM₂-induced protection was similar to that of MK-801 and Zn²⁺, non-competitive antagonists of NMDA receptors. These results indicated that Zn-ATSM₂ inhibits NMDA receptor-mediated glutamate cytotoxicity.

To further assess the protective effect of Zn-ATSM₂ against glutamate-induced neurotoxicity, the influx of Ca²⁺, which undergoes modification subsequent to NMDA activation, was investigated.

The most common method for measuring cytosolic free Ca²⁺ concentrations, [Ca²⁺]_i, is to monitor the fluorescence of Ca²⁺ indicators such as quin 2 and fura-2. These fluorescent indicators are convenient for temporal examination of cytosolic Ca²⁺ concentration in each cell. However, Zn²⁺ binds to these fluorescent Ca²⁺ indicators. For example, fura-2 binds zinc with a dissociation constant of around 2 nM (18). Although dissociation constants of Zn²⁺ complexes are about 100-fold lower than those of Ca²⁺ complexes, Zn²⁺ shifts the fura-2 spectrum in the same way as Ca²⁺, whereas Zn²⁺ quenches quin 2 and therefore acts in the opposite way to Ca²⁺ (19). Therefore, in this study, using the radioactive isotope ⁴⁵Ca²⁺ we examined early glutamate-induced Ca²⁺ elevation, which may be important for cell damage.

As shown in Fig. 1, Zn-ATSM₂ significantly reduced intracellular calcium uptake that was induced by glutamate. Since calcium ions at physiological concentration (2 mM) did not shift the spectra of Zn-ATSM₂ or ATSM₂, neither Zn-ATSM₂ nor ATSM₂ reacts with Ca²⁺, and therefore the concentration of free Ca²⁺ in the incubation medium does not change in the presence of Zn-ATSM₂ or ATSM₂. Thus, the results of blocking studies on Ca²⁺ influx indicate that a protective effect of Zn-ATSM₂ against glutamate-induced neurotoxicity is, at least in part, due to inhibition of Ca²⁺ influx based on the direct reaction of Zn-ATSM₂ with glutamate receptors. However, the inhibitory effect of Zn-ATSM₂ against glutamate-induced Ca²⁺ influx was lower than that of Zn²⁺. Since Zn-ATSM₂ showed a protective effect against NMDA receptor-mediated glutamate-induced neurotoxicity that was similar in degree to that of Zn²⁺ (Table 1), other mechanisms such as the intracellular action of Zn-ATSM₂ based on its high membrane penetrability (12), may be also related to its neuroprotective effect in addition to the direct inhibitory effect on Ca²⁺ influx, although further studies are necessary.

In this study, we tried to examine the mechanism of the neuroprotective effect of Zn-ATSM₂ that decreased infarct volume after transient middle cerebral artery occlusion (13). Therefore, we selected the experimental condition that is valid for evaluation of the protective effect of zinc compounds against glutamate neurotoxicity, that is, exposure of hippocampal cultures to zinc compounds at relatively low concentration (30 μM) for a short time (10 min)

(5). However, many *in vitro* and *in vivo* studies have shown that Zn²⁺ has both neuroprotective and neurotoxic effects. For example, in culture studies, zinc in high micromolar concentrations potentiated the neurotoxicity of alpha-amino-3-hydroxy-5-methyl-isoxazolepropionate (AMPA) and quisqualate on non-NMDA receptors (20, 21). In addition, Zn²⁺ in high concentrations showed direct neurotoxicity by permeating NMDA-, AMPA- and voltage-sensitive calcium channels (22–28). On the other hand, Zn²⁺ in low concentrations has been found to protect cultured cortical neurons against the neurotoxic effects of NMDA on NMDA receptors (21, 29, 30). Therefore, the conditions used in this study may correspond to exposure to zinc in low concentrations described above. In fact, exposure of hippocampal cultures to zinc compounds under these conditions did not induce any reduction of cell viability, compared to the buffer-treated control. More detailed studies including determining neurotoxic effects of Zn-ATSM₂ are necessary before it can be used for *in vivo* treatment of cerebral ischemia.

In conclusion, the results obtained in this study indicated that Zn-ATSM₂ elicited a protective effect against glutamate-induced neurotoxicity by acting on the NMDA receptor complex and its effect was, at least in part, due to the reduction of Ca²⁺ influx that changes following NMDA-receptor activation.

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