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Serofendic acid prevents acute glutamate neurotoxicity in cultured cortical neurons

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Abstract

We have previously reported that a novel neuroprotective substance named serofendic acid was purified and isolated from ether extract of fetal calf serum. In the present study, we investigated the effect of serofendic acid on acute neurotoxicity induced by L-glutamate (Glu) using primary cultures of rat cortical neurons. Exposure of cortical cultures to Glu for 1 h caused a marked decrease in cell viability, as determined by trypan blue exclusion. This acute Glu neurotoxicity was prevented by *N*-methyl-D-aspartate (NMDA) receptor antagonists, extracellular Ca^{2+} removal, nitric oxide (NO) synthase inhibitor and NO scavenger. Serofendic acid prevented acute Glu neurotoxicity in a concentration-dependent manner. Acute neurotoxicity was induced by ionomycin, a Ca^{2+} ionophore, and *S*-nitroso-L-cysteine, an NO donor. Serofendic acid also prevented both ionomycin- and *S*-nitroso-L-cysteine-induced neurotoxicity. Moreover, the protective effect of serofendic acid on acute Glu neurotoxicity was not affected by cycloheximide, a protein synthesis inhibitor, and actinomycin D, an RNA synthesis inhibitor. These results indicate that serofendic acid protects cultured cortical neurons from acute Glu neurotoxicity by reducing the cytotoxic action of NO and de novo protein synthesis is not required for this neuroprotection.

Keywords: Serofendic acid; Fetal calf serum; L-Glutamate; Neurotoxicity; Nitric oxide (NO); Cerebral cortex

1. Introduction

L-Glutamate (Glu) is the most abundant excitatory neurotransmitter in the vertebrate central nervous system (CNS) and plays a crucial role in neurological processes including cognition, learning and memory (Collingridge and Laster, 1989). It has been estimated that approximately 40% of all brain synapses are glutamatergic (Fonnum, 1984; Greenamyre, 1986; Greenamyre and Porter, 1994). Excessive stimulation of glutamate receptors, under pathophysiological conditions, leads to neuronal damage and death. This phenomenon is well known as ‘‘excitotoxicity’’, since neurotoxicity is correlated with excitatory properties of various Glu analogs (Olney et al., 1971). Glu neurotoxicity has also been postulated to play important roles in the pathophysiology of numerous neurological diseases including hypoxic-ischemic brain injury (Choi, 1988; Meldrum and Garthwaite, 1990), epileptic seizures (Olney et al., 1983) and neurodegenerative diseases including Alzheimer’s disease (Koh et al., 1990; Le et al., 1995), Parkinson’s disease (Sawada et al., 1996; Blandini et al., 1996), and amyotrophic lateral sclerosis (Louvel et al., 1997; Urushitani et al., 1998). Accordingly, substances which can prevent Glu neurotoxicity are expected to be potential tools in the therapy of various neurological and neurodegenerative diseases. Considering the pivotal roles of Glu as a major excitatory neurotransmitter despite its robust neurotoxic actions, it is assumed that a variety of regulatory mechanisms should serve to maintain physiological functions and prevent pathogenic effects of Glu. Based on these assumptions, we have previously demonstrated that Glu neurotoxicity was reduced by a variety of endogenous substances

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including nicotinic acetylcholine (Akaike et al., 1994b), prostanoids (Akaike et al., 1994c), neurotrophins (Kume et al., 1997a, 2000) and vitamin B analogs (Akaike et al., 1993).

In a search of novel neuroprotective substances of mammalian origin, we found that conditioned medium of striatal cultures and ether extract of fetal calf serum both reduced Glu neurotoxicity (Kume et al., 1997b). Therefore, we purified a neuroprotective substance from a lipophilic fraction of fetal calf serum based on ability to protect rat primary cortical cultures against nitric oxide (NO) donor-induced cytotoxicity and isolated a novel neuroprotective substance named serofendic acid (Kume et al., 2002a). Mass spectrometry and nuclear magnetic resonance (NMR) spectroscopy revealed the chemical structure of serofendic acid as a sulfur containing atisane-type diterpenoids (15-hydroxy-17-methylsulfinylatisan-19-oic acids), which are epimeric mixtures having the opposite configuration in the sulfoxide group (Terauchi et al., 2002). To our knowledge, serofendic acid is the first atisane derivative found in mammals, although natural atisane derivatives contained in plants have been reported (Lal et al., 1989; Appendino et al., 2000). The discovery of serofendic acid from fetal calf serum provides the possibility that additional unidentified substances which promote survival of CNS neurons exist in mammalian organs.

In the present study, we investigated the effect of serofendic acid on acute Glu neurotoxicity using primary cultures of rat cortical neurons. This study was focused on the acute neurotoxicity induced by Glu because this simple model is suitable to detect neuroprotective agents, acting on either glutamate receptors or reactive oxygen species including NO. An influx of extracellular Ca^{2+} into the cytoplasm via *N*-methyl-D-aspartate (NMDA) receptors and the subsequent formation of reactive oxygen species such as NO and hydroxyl radical are considered to play a crucial role in Glu neurotoxicity (Akaike et al., 1994a; Dawson et al., 1991, 1993; Huang et al., 1994). Here, we used various NMDA receptor antagonists, an NO synthase inhibitor and an NO scavenger to examine the participation of NMDA receptor and NO in acute Glu neurotoxicity. We also examined the effects of serofendic acid on neurotoxicity induced by ionomycin, a Ca^{2+} ionophore, and *S*-nitroso-L-cysteine, an NO donor, as well as Glu to clarify the neuroprotective mechanism of serofendic acid. Moreover, we examined whether de novo protein synthesis was involved in the neuroprotective effect of serofendic acid using cycloheximide, a protein synthesis inhibitor, and actinomycin D, an RNA synthesis inhibitor.

2. Materials and methods

2.1. Materials

Eagle's minimal essential salt medium (Eagle's MEM) was purchased from Nissui Pharmaceutical (Tokyo, Japan). Fetal bovine serum and horse serum were purchased from JRH Biosciences (Kansas, USA). Drugs were obtained from the following sources: L-glutamine (Nacalai Tesque, Kyoto, Japan), D-(+)-glucose (Nacalai Tesque), sodium hydrogen

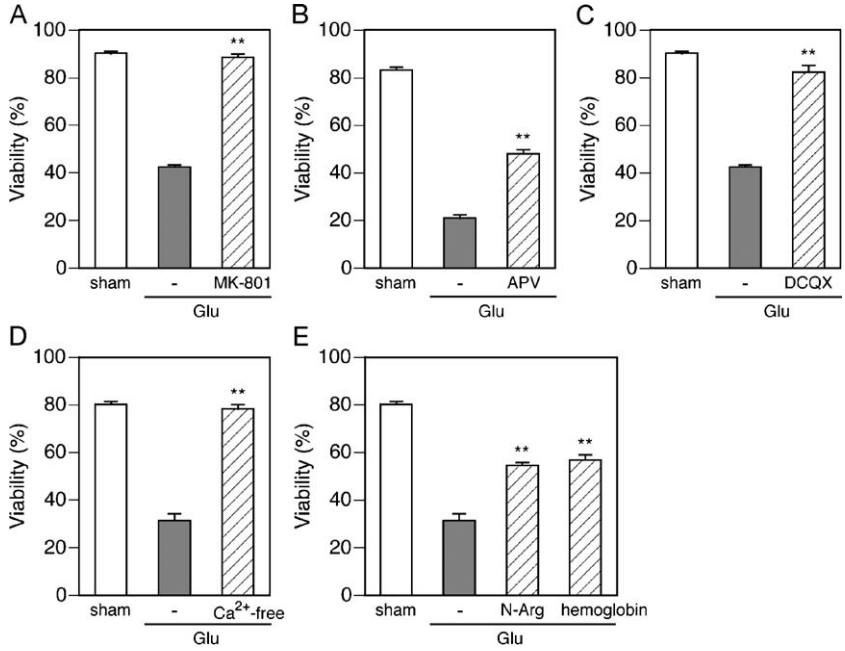

Fig. 1. Effects of various NMDA receptor antagonists, extracellular Ca^{2+} removal, N-Arg and hemoglobin on acute Glu neurotoxicity. Cultures were exposed to Glu (500 μM) for 1 h. MK-801 (1 μM; A), APV (1 mM; B) and DCQX (10 μM; C) were added concomitantly with Glu. Ca^{2+} removal from culture medium was carried out for the 1 h Glu exposure (D). N-Arg (300 μM) and hemoglobin (100 μM) were applied to the culture medium for 30 min before and 1 h during Glu exposure (E). $^{**}P < 0.01$, compared with Glu alone.

carbonate ($NaHCO_3$; Nacalai Tesque), HEPES (Nacalai Tesque), arabinocylcytosine (Kohjin, Tokyo, Japan), L-glutamic acid monosodium salt (Glu; Nacalai Tesque), (+)-dizocilpine maleate (MK-801; Research Biochemicals Internationals (RBI), Natick, USA), DL-2-amino-5-phosphonovaleric acid (APV; Sigma, St. Louis, USA), 6,7-dichloroquinoxaline-2,3-dione (DCQX; RBI), N^{ω}-nitro-L-arginine (N-Arg; Sigma), hemoglobin (Sigma), ionomycin (Calbiochem, San Diego, USA), L-cysteine (Nacalai Tesque), sodium nitrite (Nacalai Tesque), hydrochloric acid (HCl; Nacalai Tesque), cycloheximide (Wako, Osaka, Japan), actinomycin D (Nacalai Tesque) and trypan blue (Wako).

S-nitroso-L-cysteine was prepared according to procedures described previously (Lei et al., 1992). Briefly, L-cysteine and sodium nitrite were combined in equimolar amounts and dissolved in water. To this solution, 10 N of HCl was added to a final normality of 0.5 N and a final *S*-nitroso-L-cysteine concentration of 100 mM. This solution was diluted in Eagle's MEM to yield a final *S*-nitroso-L-cysteine concentration of 200 μM. The *S*-nitroso-L-cysteine solution was added to the cultures within 10 min after the addition of HCl. The cell viability was not affected by the old *S*-nitroso-L-cysteine solution (200 μM), which was kept at 37 °C overnight.

Serofendic acid was synthesized according to procedures reported previously (Terauchi et al., 2002).

2.2. Cell cultures

Primary cultures were obtained from the cerebral cortex of fetal rats (17–19 days of gestation) according to procedures described previously (Nishikawa et al., 2000; Kume et al., 2002b). Briefly, single cells dissociated from the whole cerebral cortex of fetal rats were plated on plastic coverslips which were placed in 60 mm dishes (5.1×10^6 cells per dish). Cultures were incubated in Eagle's MEM supplemented with 10% heat-inactivated fetal bovine serum (1–8 days after plating) or 10% heat-inactivated horse serum (9–14 days after plating), L-glutamine (2 mM), D-(+)-glucose (11 mM), $NaHCO_3$ (24 mM) and HEPES (10 mM). Cultures were maintained at 37 °C in a humidified 5% CO_2 atmosphere. After 6 days of plating, non-neuronal cells were removed by addition of arabinocylcytosine (10 μM). Only mature cultures (10–14 days in vitro) were used for experiments. Immunochemical staining with anti-microtubule associated protein-2 (MAP-2) antibody and anti-glial fibrillary acidic protein (GFAP) antibody revealed that the culture method used in this study provided

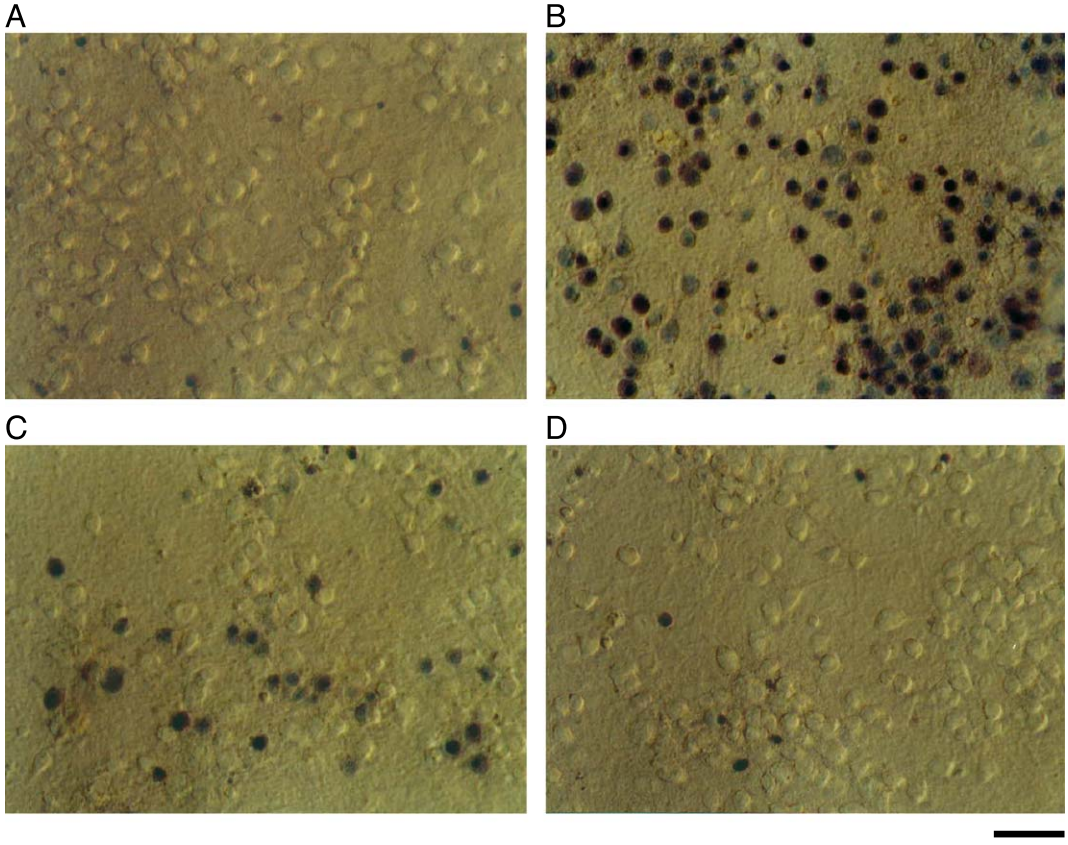

Fig. 2. Hoffman modulation photomicrographs showing serofendic acid-induced protection against acute Glu neurotoxicity. Cell cultures were immediately stained with trypan blue after 1 h Glu exposure. Culture fields were photographed after trypan blue staining followed by formalin fixation. (A–D) show non-treated, Glu (500 μM)-treated, Glu (500 μM) plus serofendic acid (10 μM)-treated and serofendic acid (10 μM)-treated cultures, respectively. In (C), the culture was treated with serofendic acid for 1 h before and 1 h during Glu exposure. Calibration bar = 50 μm.

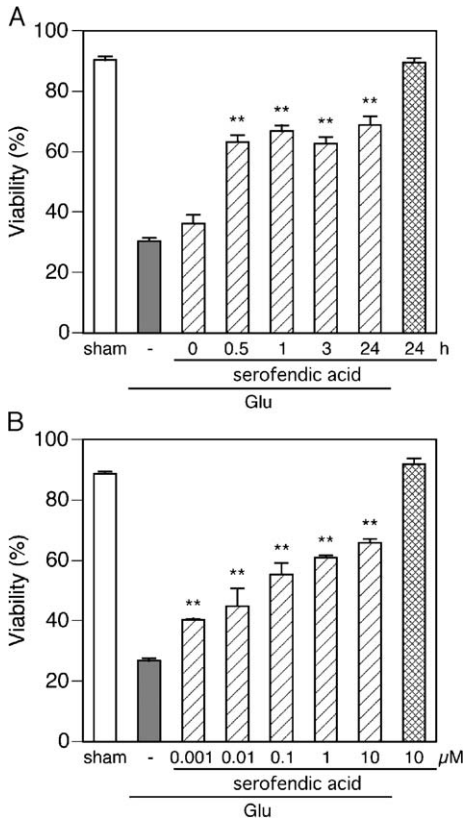

Fig. 3. Time (A)- and concentration (B)-dependency of protective effects of serofendic acid on acute Glu neurotoxicity. Cultures were treated with serofendic acid (10 μM) for 0–24 h before and 1 h during Glu (500 μM) exposure (A). Cultures were treated with serofendic acid (0.001–10 μM) for 1 h before and 1 h during Glu (500 μM) exposure (B). $^{**}P<0.01$, compared with Glu alone.

cell cultures containing about 90% neurons as described previously (Kume et al., 2002b). The animals were treated in accordance with the guidelines of the Kyoto University animal experimentation committee, and the guidelines of the Japanese Pharmacological Society.

2.3. Drug treatment and measurement of neurotoxicity

On the day of the experiment, the maintenance medium was replaced with neurotoxin (Glu, ionomycin or *S*-nitroso-L-cysteine)-containing Eagle's MEM for 1 h. NMDA receptor antagonists such as MK-801, APV and DCQX were added to Eagle's MEM concomitant with Glu. N-Arg and hemoglobin were added to culture medium for 30 min before and 1 h during Glu exposure. Similarly, cycloheximide and actinomycin D were added to culture medium for 1 h before and 1 h during Glu exposure. Unless otherwise indicated, serofendic acid was applied for 1 h before and 1 h during neurotoxin exposure. All experiments were performed at 37 °C.

Neurotoxicity was quantified by examining cultures using Hoffman modulation microscopy as described in our previous report (Nishikawa et al., 2000). Cell viability was assessed by means of trypan blue exclusion. After 1 h neurotoxin exposure, cell cultures were immediately stained with 1.5% trypan blue for 10 min at room temperature, fixed with isotonic formalin, then rinsed with physiological saline. Cells stained with trypan blue were regarded as non-viable. The viability of the cultures was calculated as the percentage of the ratio of the number of unstained cells (viable cells) relative to the total number of cells counted (viable cells plus non-viable cells). Over 200 cells per coverslip were counted to determine cell viability. We represented data obtained from five coverslips derived from distinct culture dishes in one sister culture though we have performed at least three independent experiments to examine the reproducibility of data. In each experiment, the cells on five coverslips were counted to obtain means ± S.E.M. of the cell viability.

2.4. Statistics

Data were expressed as means ± S.E.M. The statistical significance of difference between groups was determined by one-way analysis of variance (ANOVA) followed by Dunnett's two-tailed test using the Instat (Graph Pad Software, San Diego, USA) program. Statistical significance was defined as a probability value of less than 5%.

3. Results

3.1. Mechanism of acute Glu neurotoxicity

Exposure of the cortical cultures to 500 μM of Glu for 1 h markedly reduced cell viability. Concurrent application of MK-801 (1 μM), a non-competitive NMDA receptor antagonist which binds to the phencyclidine binding site, with Glu significantly inhibited acute Glu neurotoxicity (Fig. 1A). Similarly, APV (1 mM), a competitive NMDA receptor antagonist, and DCQX (10 μM), an antagonist of the

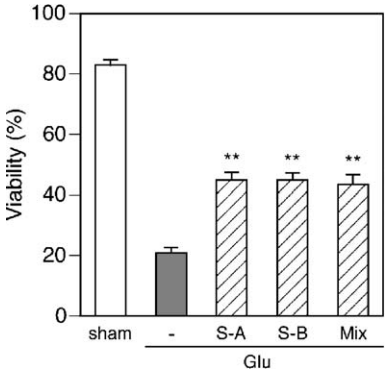

Fig. 4. Comparison with neuroprotective effects of serofendic acid A and B and an epimeric mixture of serofendic acid. Serofendic acid (10 μM) was applied to the culture medium for 1 h before and 1 h during Glu (500 μM) exposure. S-A: serofendic acid A, S-B: serofendic acid B, Mix: epimeric mixture of serofendic acid. $^{**}P<0.01$, compared with Glu alone.

strychnine-insensitive glycine recognition site, significantly attenuated acute Glu neurotoxicity (Fig. 1B,C). Also, neurotoxicity was abolished when Ca^{2+} was removed from the culture medium for 1 h during Glu exposure (Fig. 1D).

Next, we examined the effect of N-Arg, as a selective NO synthase inhibitor, and hemoglobin, as an NO scavenger, on this acute Glu neurotoxicity. Both N-Arg (300 μM) and hemoglobin (100 μM) partially inhibited Glu neurotoxicity when these drugs were applied for 30 min before and 1 h during Glu exposure (Fig. 1E).

3.2. Effect of serofendic acid on acute Glu neurotoxicity

We examined the effect of serofendic acid on acute Glu neurotoxicity. Exposure of the cortical cultures to 500 μM of Glu for 1 h markedly increased the number of trypan blue-stained non-viable cells (Fig. 2B). When serofendic acid (10 μM) was applied for 1 h before and 1 h during Glu exposure, the number of non-viable cells was markedly reduced (Fig. 2C). Thus, we examined the time- and concentration-dependency of the neuroprotective effect of serofendic acid.

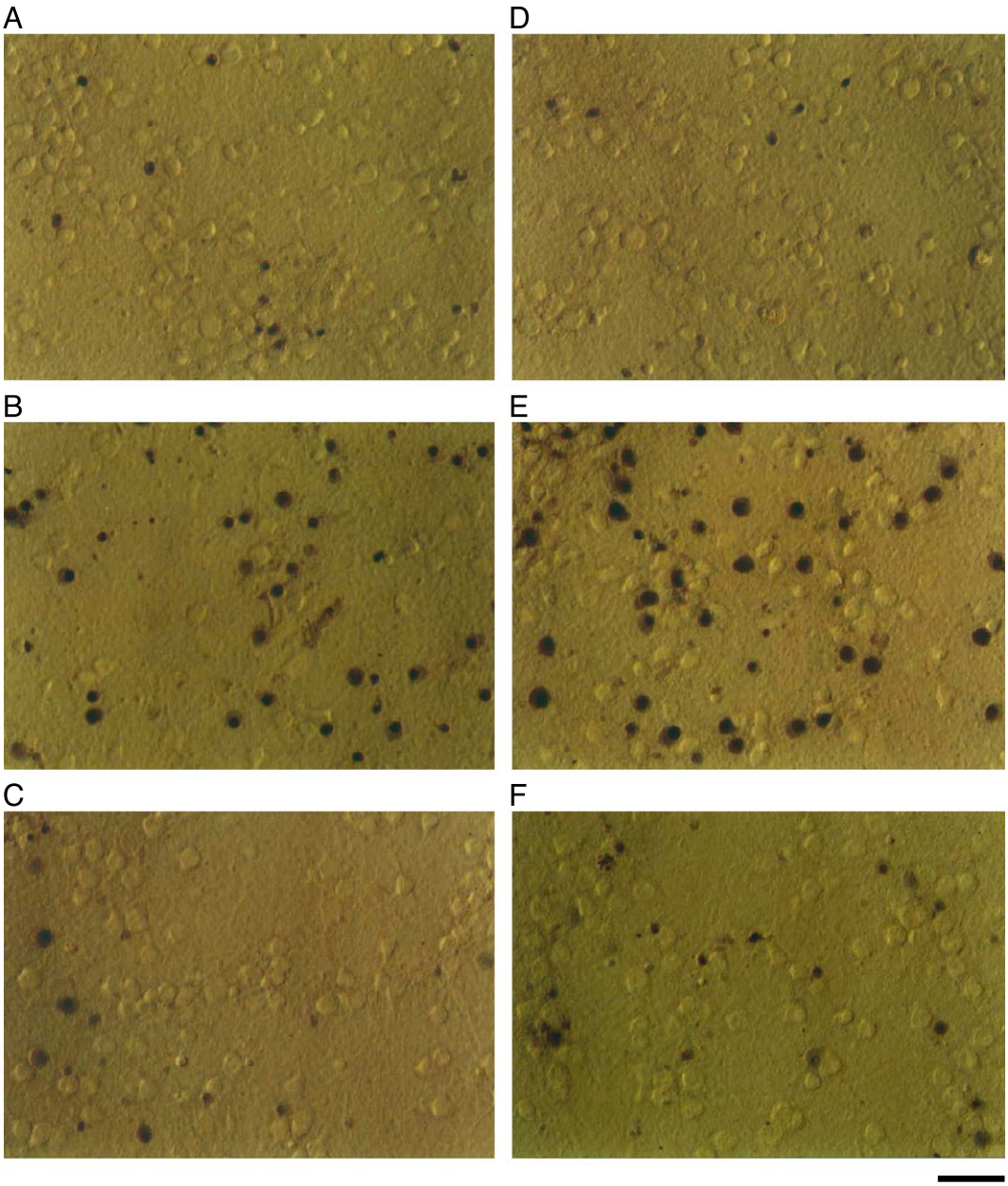

Fig. 5. Hoffman modulation photomicrographs showing protective effects of serofendic acid on ionomycin- and *S*-nitroso-L-cysteine-induced neurotoxicity. Cell cultures were immediately stained with trypan blue after 1 h ionomycin- and *S*-nitroso-L-cysteine exposure. (A–C) show non-treated, ionomycin (10 μM)-treated, and ionomycin (10 μM) plus serofendic acid (10 μM)-treated cultures, respectively. (D–F) show non-treated, *S*-nitroso-L-cysteine (200 μM)-treated, and *S*-nitroso-L-cysteine (200 μM) plus serofendic acid (10 μM)-treated cultures, respectively. In (C) and (F), cultures were treated with serofendic acid for 1 h before and 1 h during neurotoxin exposure. Calibration bar = 50 μm.

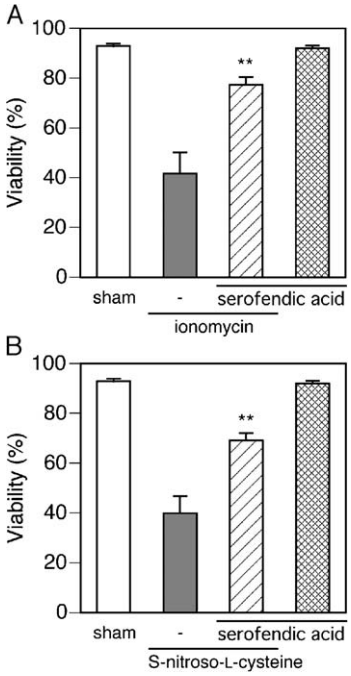

Fig. 6. Protective effects of serofendic acid on ionomycin (A)- and *S*-nitroso-L-cysteine (B)-induced neurotoxicity. Cultures were exposed to ionomycin (10 μM) or *S*-nitroso-L-cysteine (200 μM) for 1 h. Serofendic acid (10 μM) was applied for 1 h before and 1 h during neurotoxin exposure. $^{**}P < 0.01$, compared with neurotoxin alone.

Simultaneous application of serofendic acid with Glu did not affect the cell viability (Fig. 3A). But when we applied serofendic acid for 30 min prior to Glu exposure, we observed a significant protective effect (Fig. 3A). A similar degree of protection was observed when serofendic acid was applied for 1, 3 or 24 h prior to the Glu exposure (Fig. 3A). Also, serofendic acid prevented Glu neurotoxicity in a concentration-dependent manner (Fig. 3B). Interestingly, a significant protective effect of serofendic acid was observed at a concentration as low as 1 nM (Fig. 3B). Serofendic acid (10 μM) alone had no significant effect on cell viability (Figs. 2D and 3B).

It is revealed that serofendic acid exists in fetal calf serum as an epimeric mixture with opposite configurations in the sulfoxide group, and these two isomers were named serofendic acid A and B, respectively (Kume et al., 2002a,b). Thus, we compared neuroprotective activity among serofendic acid A, serofendic acid B and an epimeric mixture of serofendic acid. These compounds showed similar protective activities against acute Glu neurotoxicity (Fig. 4).

3.3. Effects of serofendic acid on ionomycin- and S-nitroso-L-cysteine-induced neurotoxicity

The influx of extracellular Ca^{2+} into the cytoplasm via NMDA receptors and subsequent formation of NO are considered to be key steps in Glu neurotoxicity. Thus, we examined the effects of serofendic acid on neurotoxicity induced by ionomycin and *S*-nitroso-L-cysteine. Exposure of the cortical cultures to 10 μM of ionomycin for 1 h markedly increased the number of non-viable cells (Fig. 5B). Similarly, exposure to 200 μM of *S*-nitroso-L-cysteine for 1 h caused a marked increase in the number of non-viable cells (Fig. 5E). Serofendic acid (10 μM) blocked the neurotoxic action of ionomycin as well as *S*-nitroso-L-cysteine (Figs. 5C,F and 6).

3.4. Effects of cycloheximide and actinomycin D on neuroprotective effects of serofendic acid

Since the neuroprotective effect of serofendic acid required more than 30 min pretreatment prior to the Glu exposure, we investigated the participation of de novo protein synthesis in the neuroprotection. Cycloheximide and actinomycin D were used for this purpose. Neurotoxicity induced by exposure to 500 μM of Glu for 1 h was not affected by cycloheximide and actinomycin D (data not shown), indicating that this acute Glu neurotoxicity is not regulated by de novo protein synthesis. Moreover, the neuroprotecitive effect of serofendic acid was not affected by cycloheximide (1 μg/ml) or actinomycin D (1 μg/ml) (Fig. 7A,B). Cycloheximide and actinomycin D alone had no significant effect on cell viability.

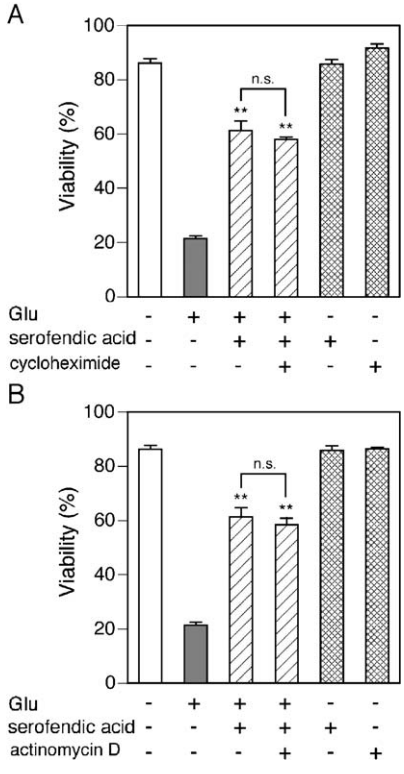

Fig. 7. Effects of cycloheximide (A) and actinomycin D (B) on neuroprotection of serofendic acid against acute Glu neurotoxicity. Cultures were treated with serofendic acid (10 μM) for 1 h before and 1 h during Glu (500 μM) exposure. Cycloheximide (1 μg/ml) and actinomycin D (1 μg/ml) were applied concomitantly with serofendic acid. $^{**}P < 0.01$, compared with Glu alone. n.s.; not significant.

4. Discussion

In the present study, we investigated the mechanism of acute Glu neurotoxicity and the effect of serofendic acid on acute Glu neurotoxicity using primary cultures of rat cortical neurons. Consistent with our previous study (Nishikawa et al., 2000), exposure of the cortical cultures to 500 μM of Glu for 1 h caused a marked decrease in cell viability. Concurrent application of NMDA receptor antagonists such as MK-801, APV and DCQX significantly prevented acute Glu neurotoxicity. The present study demonstrated that serofendic acid prevents acute Glu neurotoxicity in a concentration-dependent manner and maximal protection was observed when serofendic acid was applied for more than 30 min prior to the Glu exposure. We have previously reported that serofendic acid did no affect NMDA-, α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA)- and kainate-evoked currents (Kume et al., 2002a). Taken together, these findings indicate that the NMDA receptor plays a crucial role in acute Glu neurotoxicity, but serofendic acid does not directly inhibit the function of glutamate receptor channels.

The influx of extracellular Ca^{2+} into the cytoplasm via the NMDA receptor and subsequent formation of NO are considered to play a crucial role in Glu neurotoxicity (Akaike et al., 1994a; Dawson et al., 1991, 1993; Huang et al., 1994). We demonstrated that removal of extracellular Ca^{2+} from the culture medium, N-Arg and hemoglobin prevented acute Glu neurotoxicity, supporting this hypothesis. Thus, we used ionomycin, as a Ca^{2+} ionophore, and *S*-nitroso-L-cysteine, as an NO donor, to clarify the neuroprotective mechanism of serofendic acid. Serofendic acid prevented the neurotoxic actions of both ionomycin and *S*-nitroso-L-cysteine. From these results, we considered that serofendic acid protects cultured cortical neurons from cytotoxicity induced by a disruption of intracellular Ca^{2+} homeostasis and the radical stress of NO.

It has been postulated that reactive oxygen species participate in both Glu- and NO-induced neurotoxicity, since the neurotoxicity was prevented by superoxide dismutase, a catalytic enzyme that breaks down superoxide radicals to hydrogen peroxide and molecular oxygen (Lipton et al., 1993; Dawson et al., 1993). NO yields peroxynitrite anion by reacting with superoxide anion, and rapid degradation of peroxynitrite anion produces hydroxyl radical, which in turn induces potent cytotoxicity via lipid peroxidation and DNA damage (Beckman et al., 1990). We have previously demonstrated that serofendic acid inhibited the formation of hydroxyl radicals in a concentration-dependent manner by using electron spin resonance spectroscopy (Kume et al., 2002a). Although further study is required to determine in detail the mechanisms of the neuroprotective action, serofendic acid may protect cultured cortical neurons by scavenging reactive oxygen species including peroxynitrite anion and hydroxyl radical.

Serofendic acid A and B, as well as an epimeric mixture of serofendic acid, significantly attenuated acute Glu neurotoxicity with similar potency. We have previously reported that both isomers showed similar protective activities against *S*-nitroso-L-cysteine-induced neurotoxicity (Kume et al., 2002a). These findings further support that the opposite configuration in the sulfoxide group does not influence the neuroprotective activity of serofendic acid against NO-mediated Glu neurotoxicity.

It is estimated that the concentration of serofendic acid contained in fetal calf serum was in the order of tens of nM (Kume et al., 2002a). In the present study, serefendic acid caused a significant inhibition of acute Glu neurotoxicity when applied at a concentration as low as 1 nM. Therefore, serofendic acid is considered to play crucial roles in the development and maintenance of CNS neurons at embryonic stages by preventing Glu neurotoxicity.

Cell death is generally divided into apoptosis and necrosis. These are believed to be distinct mechanisms of cell death with very different characteristic features, which can be distinguished on the basis of their morphological and biological properties (Wyllie, 1980; Clarke, 1990). Apoptosis is a naturally occurring process of cell destruction, which requires metabolic energy to synthesize de novo RNA and protein (Oppenheim, 1991) and is characterized by cell shrinkage, membrane blebbing, fragmented nuclei with condensed chromatin and the formation of apoptotic bodies (Orrenius and Nicotera, 1994). In contrast, necrosis is non-physiological accidental cell death and is characterized by mitochondrial swelling and a loss of plasma membrane integrity without severe damage to nuclei (Sapolsky, 2001). However, the type of neuronal death related to Glu excitotoxicity is still controversial. Some groups have reported that Glu induced necrosis (Choi et al., 1987; Dessi et al., 1993; Sohn et al., 1998), but others reported features of apoptosis (Kure et al., 1991; Bonfoco et al., 1995; Finiels et al., 1995). Althought detail is unclear, intensity of the Glu insult and difference in brain region may determine the fate of neurons to be either apoptotic or necrotic death. Acute neuronal death under our experimental conditions may be necrotic because neurotoxicity was not affected by cycloheximide and actinomycin D (data not shown). Also, we revealed that the protective action of serofendic acid against acute Glu neurotoxicity was not affected by cycloheximide and actinomycin D. Therefore, these results indicate that serofendic acid prevents necrotic neuronal death induced by acute exposure to Glu and de novo protein synthesis is not required for this neuroprotection.

In conclusion, the present study demonstrated that NO acts as a mediator of acute Glu neurotoxicity and serofendic acid prevented NO-mediated acute Glu neurotoxicity in cultured cortical neurons. This neuroprotective effect of serofendic acid did not require de novo protein synthesis and the opposite configuration in the sulfoxide group does not influence the neuroprotective activity of serofendic acid. Therefore, serofendic acid may prevent neuronal death,

interacting with functional molecules that act in key stages of NO-mediated Glu neurotoxicity and promote neuronal survival by augmenting the resistance to radical stress in CNS neurons.

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References

Akaike, A., Tamura, Y., Sato, Y., Yokota, T., 1993. Protective effects of vitamin B_{12} analog, methylcobalamin, against glutamate cytotoxicity in cultured cortical neurons. Eur. J. Pharmacol. 241, 1–6.

Akaike, A., Tamura, Y., Terada, K., Nakata, N., 1994a. Regulation by neuroprotective factors of NMDA receptor mediated nitric oxide synthesis in the brain and retina. Prog. Brain Res. 103, 391–403.

Akaike, A., Tamura, Y., Yokota, T., Shimohama, S., Kimura, J., 1994b. Nicotine-induced protection of cultured cortical neurons against *N*-methyl-D-aspartate receptor-mediated glutamate cytotoxicity. Brain Res. 644, 181–187.

Akaike, A., Kaneko, S., Tamura, Y., Nakata, N., Shiomi, H., Ushikubi, F., Narumiya, S., 1994c. Prostaglandin E_2 protects cultured cortical neurons against *N*-methyl-D-aspartate receptor-mediated glutamate cytotoxicity. Brain Res. 663, 237–243.

Appendino, G., Belloro, E., Tron, G.C., Jakupovic, J., Ballero, M., 2000. Polycyclic diterpenoids from *Euphorbia characias*. Fitoterapia 71, 134–142.

Beckman, J.S., Beckman, T.W., Chen, J., Marshall, P.A., Freeman, B.A., 1990. Apparent hydroxyl radical production by peroxynitrite, Implications for endothelial injury from nitric oxide and superoxide. Proc. Natl. Acad. Sci. U. S. A. 87, 1620–1624.

Blandini, F., Porter, R.H.P., Greenamyre, J.T., 1996. Glutamate and Parkinson's disease. Mol. Neurobiol. 12, 73–94.

Bonfoco, E., Krainc, D., Ankarcrona, M., Nicotera, P., Lipton, S.A., 1995. Apoptosis and necrosis: two distinct events induced, respectively, by mild and intense insults with *N*-methyl-D-aspartate or nitric oxide/superoxide in cortical cell cultures. Proc. Natl. Acad. Sci. U. S. A. 92, 7162–7166.

Choi, D.W., 1988. Calcium-mediated neurotoxicity, relationship to specific channel types and role in ischemic damage. Trends Neurosci. 11, 465–469.

Choi, D.W., Maulucci-Gedde, M., Kriegstein, A.R., 1987. Glutamate neurotoxicity in cortical cell culture. J. Neurosci. 7, 357–368.

Clarke, P.G., 1990. Developmental cell death: morphological diversity and multiple mechanisms. Anat. Embryol. 181, 195–213.

Collingridge, G.L., Laster, R.A., 1989. Excitatory amino acid receptors in the vertebrate central nervous system. Pharmacol. Rev. 41, 143–210.

Dawson, V.L., Dawson, T.M., London, E.D., Bredt, D.S., Snyder, S.H., 1991. Nitric oxide mediates glutamate neurotoxicity in primary cortical cultures. Proc. Natl. Acad. Sci. U. S. A. 88, 6368–6371.

Dawson, V.L., Dawson, T.M., Bartley, D.A., Uhl, G.R., Snyder, S.H., 1993. Mechanisms of nitric oxide-mediated neurotoxicity in primary brain cultures. J. Neurosci. 13, 2651–2661.

Dessi, F., Charriant-Marlangue, C., Khrestchatisky, M., Ben-Ari, Y., 1993. Glutamate-induced neuronal death is not a programmed cell death in cerebellar culture. J. Neurochem. 60, 1953–1955.

Finiels, F., Robert, J.J., Samolyk, M.L., Privat, A., Mallet, J., Revah, F., 1995. Induction of neuronal apoptosis by excitotoxins associated with long-lasting increase of 12-*O*-tetradecanoylphorbol 13-acetate-responsive element-binding activity. J. Neurochem. 65, 1027–1034.

Fonnum, F., 1984. Glutamate: a neurotransmitter in mammalian brain. J. Neurochem. 42, 1–11.

Greenamyre, J.T., 1986. The role of glutamate in neurotransmission and in neurologic disease. Arch. Neurol. 43, 1058–1063.

Greenamyre, J.T., Porter, R.H.P., 1994. Anatomy and physiology of glutamate in the CNS. Neurology 44 (Suppl. 8), S7–S13.

Huang, Z., Haung, P.L., Panahian, N., Dallara, T., Fishman, M.C., Moskowitz, M.A., 1994. Effects of cerebral ischemia in mice deficient in neuronal nitric oxide synthase. Science 265, 1883–1885.

Koh, J.Y., Yang, L.L., Cotamn, C.W., 1990. β-Amyloid protein increases the vulnerability of cultured cortical neurons to excitotoxic damage. Brain Res. 553, 315–320.

Kume, T., Kouchiyama, H., Kaneko, S., Maeda, T., Kaneko, S., Akaike, A., Shimohama, S., Kihara, T., Kimura, J., Wada, K., Koizumi, S., 1997a. BDNF prevents NO mediated glutamate neurotoxicity in cultured cortical neurons. Brain Res. 756, 200–204.

Kume, T., Kouchiyama, H., Nishikawa, H., Maeda, T., Kaneko, S., Akaike, A., Noda, N., Fujita, T., 1997b. Ether extract of fetal calf serum protects cultured rat cortical neurons against glutamate cytotoxicity. Jpn. J. Pharmacol. 73, 371–374.

Kume, T., Nishikawa, H., Tomioka, H., Katsuki, H., Akaike, A., Kaneko, S., Maeda, T., Kihara, T., Shimohama, S., 2000. p75-mediated neuroprotection by NGF against glutamate cytotoxicity in cortical cultures. Brain Res. 852, 279–289.

Kume, T., Asai, N., Nishikawa, H., Mano, N., Terauchi, T., Taguchi, R., Shirakawa, H., Osakada, F., Mori, H., Asakawa, N., Yonaga, M., Nishizawa, Y., Sugimoto, H., Shimohama, S., Katsuki, H., Kaneko, S., Akaike, A., 2002a. Isolation of a diterpenoid substance with potent neuroprotective activity from fetal calf serum. Proc. Natl. Acad. Sci. U. S. A. 99, 3288–3293.

Kume, T., Nishikawa, H., Taguchi, R., Hashino, A., Katsuki, H., Kaneko, S., Minami, M., Satoh, M., Akaike, A., 2002b. Antagonism of NMDA receptors by σ receptor ligands attenuates chemical ischemia-induced neuronal death in vitro. Eur. J. Pharmacol. 455, 91–100.

Kure, S., Tominaga, T., Yoshimoto, T., Toda, K., Narisawa, K., 1991. Glutamate triggers internucleosomal DNA cleavage in neuronal cells. Biochem. Biophys. Res. Commun. 179, 39–45.

Lal, A.R., Cambie, R.C., Rutledge, P.S., Woodgate, P.D., Rickard, C.E.F., Clark, G.R., 1989. New oxidized ent-atisane diterpens from *Urphorbia fidjian*. Tetrahedron Lett. 30, 3205–3208.

Le, W.D., Colom, L.V., Xie, W.J., Smith, R.G., Alexianu, M., Appel, S.H., 1995. Cell death induced by β-amyloid 1-40 in MES 23.5 hybrid clone, the role of nitric oxide and NMDA-gated channnel activation leading to apoptosis. Brain Res. 686, 49–60.

Lei, S.Z., Pan, Z.H., Aggarwal, S.K., Chen, H.V., Hartman, J., Sucher, N.J., Lipton, S.A., 1992. Effect of nitric oxide production on the redox modulatory site of the NMDA receptor-channnel complex. Neuron 8, 2087–2099.

Lipton, S.A., Choi, Y.B., Pan, Z.H., Lei, S.Z., Chen, H.V., Sucher, N.J., Loscalzo, J., Singel, D.J., Stamler, J.S., 1993. A redox-based mechanism for the neuroprotective effects of nitric oxide and related nitroso-compounds. Nature 364, 626–632.

Louvel, E., Hugon, J., Doble, A., 1997. Therapeutic advances in amyotrophic lateral sclerosis. Trends in Pharmacol. Sci. 18, 196–203.

Meldrum, B., Garthwaite, J., 1990. Excitatory amino acid neurotoxicity and neurodegenerative disease. Trends Pharmacol. Sci. 11, 379–387.

Nishikawa, H., Hashino, A., Kume, T., Katsuki, H., Kaneko, S., Akaike, A., 2000. Involvement of direct inhibition of NMDA receptors in the effects of σ-receptor ligands on glutamate neurotoxicity in vitro. Eur. J. Pharmacol. 404, 41–48.

Olney, J.W., Ho, O.L., Rhee, V., 1971. Cytotoxic effects of acidic and sulphur containing amino acids on the infant mouse central nervous system. Exp. Brain Res. 14, 61–74.

Olney, J.W., deGubareff, T., Sloviter, R.S., 1983. 'Epileptic' brain damage in rats induced by sustained electrical stimulation of the perforant path: II. Ultrastructural analysis of acute hippocampal pathology. Brain Res. Bull. 10, 699–712.

Oppenheim, R.W., 1991. Cell death during development of the nervous system. Annu. Rev. Neurosci. 14, 453–501.

Orrenius, S., Nicotera, P., 1994. The calcium ion and cell death. J. Neural Trans. 43 (S1), 1–11.

Sapolsky, R.M., 2001. Cellular defenses against excitotoxic insults. J. Neurochem. 76, 1601–1611.

Sawada, H., Shimohama, S., Tamura, Y., Kawamura, T., Akaike, A., Kimura, J., 1996. Methylphenylpyridium ion (MPP^+) enhances glutamate-induced cytotoxicity against dopaminergic neurons in cultured rat mesencephalon. J. Neurosci. Res. 43, 55–62.

Sohn, S., Kim, E.Y., Gwag, B.J., 1998. Glutamate neurotoxicity in mouse cortical neurons: atypical necrosis with DNA ladders and chromatin condensation. Neurosci. Lett. 240, 147–150.

Terauchi, T., Asai, N., Yonaga, M., Mano, N., Kume, T., Akaike, A., Sugimoto, H., 2002. Synthesis and absolute configuration of serofendic acids. Tetrahedron Lett. 43, 3625–3628.

Urushitani, M., Shimohama, S., Kihara, T., Sawada, H., Akaike, A., Ibi, M., Inoue, R., Kitamura, Y., Taniguchi, T., Kimura, J., 1998. Mechanisms of selective motor neuronal death after exposure of spinal cord to glutamate: involvement of glutamate-induced nitric oxide in motor neuron toxicity and nonmotor neuron protection. Ann. Neurol. 44, 796–807.

Wyllie, A.H., 1980. Glucocorticoid-induced thymocyte apoptosis is associated with endogenous endonuclease activation. Nature 284, 555–556.