Seroendic Acid, a Sulfur-Containing Diterpenoid Derived from Fetal Calf Serum, Attenuates Reactive Oxygen Species-Induced Oxidative Stress in Cultured Striatal Neurons

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ABSTRACT

We previously identified a novel endogenous substance, seroendic acid, from a lipophilic extract of fetal calf serum. Seroendic acid protects cultured cortical neurons against the cytotoxicity of glutamate and nitric oxide. Here, we reported the protective effect of seroendic acid on reactive oxygen species-induced oxidative stress using primary rat striatal cultures. In addition, we compared the neuroprotective effect and the radical-scavenging activity of seroendic acid with those of dimethyl sulfoxide (DMSO), because seroendic acid possesses a DMSO structure. Paraquat caused neuronal death, which was inhibited by a cell-permeable superoxide dismutase mimetic, Mn(III)tetrakis(4-benzoic acid)porphyrin chloride (Mn-TBAP); a cell-permeable SOD/catalase mimetic, EUK-134 [manganese 3-methoxy N,N'-bis(salicylidene)ethylenediamine chloride]; and a ferrous ion chelator, 2,2'-dipyridyl, in rat striatal cultures. Seroendic acid (10–100 μM) suppressed the neurotoxicity of paraquat, whereas DMSO (10–100 μM) did not. By contrast, higher concentrations (30–300 mM) of DMSO ameliorated the paraquat-induced cell death. Furthermore, H2O2-induced neurotoxicity, which was prevented by EUK-134 and 2,2'-dipyridyl. Seroendic acid (10–100 μM) also protected striatal neurons against the H2O2-induced toxicity. Higher concentrations (30–300 mM) of DMSO ameliorated H2O2-induced neuronal death, whereas lower concentrations (10–100 μM) did not. Electron spin resonance spectrometry with a spin-trapping technique revealed that seroendic acid and DMSO had approximately the same ability to inhibit the formation of the hydroxyl radical (•OH). These results suggest that the •OH-scavenging activity of seroendic acid is attributable to its DMSO structure and that the remaining components such as the atisane structure play an important role in eliciting neuroprotection at a concentration range of 10 to 100 μM.

We previously purified and isolated a novel neuroprotective factor from an ether extract of fetal calf serum based on an ability to protect cultured cortical neurons against nitric oxide (NO) cytotoxicity (Kume et al., 1997, 2002; Akaike et al., 2003). We named the neuroprotective substance seroendic acid, because it was isolated from serum (sero-), displayed a cytoprotective effect (fend-), and possessed carboxylic acid structure (Fig. 1B). Seroendic acid is a unique low molecular weight substance (mol. wt. 382) among known endogenous substances, because it is the first cyclic diterpenoid found in mammals. Seroendic acid had a marked neuroprotective effect on rat cortical cultures against the cytotoxicity of a nitric oxide donor, a calcium ionophore and glutamate (Kume et al., 2002; Terauchi et al., 2002; Akaike et al., 2003). Interestingly, seroendic acid possesses a dimethyl sulfoxide (DMSO) structure (Fig. 1B). Seroendic acid is 15-hydroxy-17-methylsulfanylatisan-19-oic acid, a sulfur-containing atisane-type diterpenoid (Fig. 1A) (Kume et al., 2002; Terauchi et al., 2002; Akaike et al., 2003). A unique feature of seroendic acid is that it is 15-hydroxy-17-methylsulfanylatisan-19-oic acid, a sulfur-containing atisane-type diterpenoid (Fig. 1A) (Kume et al., 2002; Terauchi et al., 2002; Akaike et al., 2003). Interestingly, seroendic acid possesses a dimethyl sulfoxide (DMSO) structure (Fig. 1B). Seroendic acid is a unique low molecular weight substance (mol. wt. 382) among known endogenous substances, because it is the first cyclic diterpenoid found in mammals. Seroendic acid had a marked neuroprotective effect on rat cortical cultures against the cytotoxicity of a nitric oxide donor, a calcium ionophore and glutamate (Kume et al., 2002; Terauchi et al., 2002; Akaike et al., 2003). At present, however, little is known about the effect of seroendic acid on oxidative stress.

Oxygen is not only vital for life but also is potentially dangerous. Oxygen-derived free radicals such as superoxide...
Fig. 1. Chemical structures of serofendic acid (A) and DMSO (B).

Materials and Methods

Drugs and Chemicals. Eagle’s minimum essential medium was purchased from Nissui (Tokyo, Japan). Fetal bovine serum was obtained from JRH Biosciences (Lenexa, KS). DMSO, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide (MTT), glucose, glutamine, HEPES, methylcellulose, and sodium bicarbonate were from Nacalai Tesque (Kyoto, Japan). 1,1′-Dimethyl-4,4′-bipyridinium dichloride (paraquat), 2,2′-dipyridyl, ferrous sulfate (FeSO₄), saponin, and superoxide dismutase (SOD) were obtained from Sigma-Aldrich (St. Louis, MO). Catalase and H₂O₂ were purchased from Wako Pure Chemicals (Osaka, Japan). Mn(III)tetrakis(4-benzoic acid)porphyrin chloride (Mn-TBAP) was purchased from Cayman Chemicals (Ann Arbor, MI). Diethylenetriamine pentaacetic acid was from Dojindo Laboratory (Kumamoto, Japan). 5,5-Dimethyl-1-pyrroline-N-oxide (DMPO) was obtained from Labotec (Tokyo, Japan). EUK-134 was synthesized according to the method of Baker et al. (1998). Serofendic acid was synthesized as described previously (Terauchi et al., 2002) and supplied by Eissi Co. Ltd. (Tsukuba, Japan).

Cell Culture. The animals were treated in accordance with the guidelines of the Kyoto University animal experimentation committee, and the guidelines of the Japanese Pharmacological Society. Primary cultures were prepared from the anterior striatum of fetal Wistar rats (Nihon SLC, Shizuoka, Japan) at 17 to 19 days of gestation, according to procedures described previously (Osakada et al., 2003). Briefly, the pregnant rats were anesthetized with sodium pentobarbital. The anterior striatum of rat embryos was removed bilaterally, mechanically dissociated using scalpels and blades, and then filtered through a stainless steel mesh. Single-cell suspensions were plated on 0.1% polyethyleneimine-coated 24-well plates (Falcon, Franklin Lakes, NJ) or two-well chamber slides (Nalge Nunc, Naperville, IL) at a density of 3.5 to 4.0 × 10⁵ cells/cm². The culture medium consisted of Eagle’s minimum essential medium supplemented with 2 mM glucose, 11 mM glucose (total), 24 mM sodium bicarbonate, 10 mM HEPES, and 10% heat-inactivated fetal bovine serum. Cells cultures were maintained at 37°C in a humidified atmosphere of 5% CO₂ and 95% air.

Drug Treatment. Three days after plating, cultures were incubated in a medium in the presence or absence of the indicated concentrations of drugs. Cultures were exposed to paraquat or H₂O₂ for 24 h, according to our previous study (Osakada et al., 2003). Serofendic acid, DMSO, SOD, catalase, Mn-TBAP, EUK-134, or 2,2′-dipyridyl was simultaneously applied to the toxic-containing medium for 24 h. After drug treatment, cell viability was determined by immunocytochemistry, MTT assay, or lactate dehydrogenase (LDH) release assay. Because SOD interferes with the LDH release assay system used in the present experiment, the effect of SOD was assessed with the MTT assay. The effects of the other drugs were analyzed using the LDH release assay. In each experiment, cells in four wells received drug treatment to obtain means ± S.E.M. of cell viability. We represented data from four wells in one sister culture, although we performed at least three independent experiments to examine the reproducibility of data.

Immunocytochemistry. Cultures were fixed with phosphate-buffered saline (PBS) containing 4% paraformaldehyde and 7% sucrose for 10 min at room temperature, washed three times with PBS and blocked with 20% BlockAce (Dainihon-Seiyaku, Osaka, Japan) in PBS containing 0.005% saponin for 30 min. Cultures were then incubated at 4°C overnight with primary antibodies diluted in PBS containing 0.005% saponin and 5% BlockAce: mouse monoclonal anti-microtubule-associated protein (2a + 2b) (MAP2ab) antibody (1:400; Sigma-Aldrich) and rabbit polyclonal anti-glial fibrillary acidic protein (GFAP) antibody (1:700; DakoCytomation Denmark A/S, Glostrup, Denmark). After three rinses with PBS, the cultures were incubated for 90 min at room temperature with secondary antibodies diluted in PBS containing 0.005% saponin and 5% BlockAce: Alexa Flour 488-labeled goat anti-mouse IgG (H+L) (1:750; Molecular Probes, Eugene, OR) and Alexa Flour 594-labeled goat anti-rabbit IgG (H+L) (1:750; Molecular Probes, Eugene, OR) for 1 h. For colocalization experiments, cultures were double-labeled with antibodies against glutamate receptor (GluR) subunits and MAP2. Cultures were incubated with primary antibodies, rinsed, and incubated with secondary antibodies. After rinsing, cultures were incubated with DAPI. Cultures were imaged using a Leica TCS SPE confocal microscope equipped with a 63× objective lens. The images were captured with a Hamamatsu Orca-RG High Sensitive Science CMOS camera. Images were analyzed with IPLab software (Scanalytics, Fairfax, VA) and Adobe Photoshop (San Jose, CA).
anti-rabbit IgG (H+L) (1:750; Molecular Probes). Labeled cells were visualized and photographed with a fluorescence microscope (Leica, Wetzlar, Germany). The number of cells stained with anti-MAP2ab antibody and anti-GFAP antibody in a randomly selected field (530 × 530 μm) was counted blind to the experimental treatments. Positively stained cells with developed dendrites that were at least more than twice as long as the cell diameter were considered viable cells.

**MTT Assay.** Cell viability was quantified based on metabolic activity using the MTT assay according to a method described previously (Osakada et al., 2003). The cultures were incubated in serum-free medium containing 0.5 mg/ml MTT tetrazolium salt for 3 h at 37°C. The medium was aspirated and colored formazan products were solubilized by adding isopropanol. The absorbance was photometrically measured at 595 nm with a microplate reader (Bio-Rad, Hercules, CA). The cell viability was expressed as a percentage of the absorbance measured in control cultures.

**LDH Release Assay.** Cell viability was assessed by measuring the amount of LDH released into the culture medium using a Cytotoxicity Detection LDH kit (Kyokuto Pharmaceutical Industrial Corp., Tokyo, Japan) as described previously (Osakada et al., 2003). In this assay, NAD is reduced to NADH through the conversion of lactate to pyruvate by LDH and then NADH reduces tetrazolium dyes to formazan dyes in the presence of diaphorase. Briefly, 25 μl of culture supernatant was mixed with 75 μl of the LDH substrate mixture. After incubation for 1 h at room temperature, the reaction was stopped by adding 100 μl of 1 N hydrochloric acid, and the absorbance was measured at 570 nm with a microplate reader. The reading of background absorbance, consisting of culture medium and each drug in the absence of cells, was subtracted from each value. The cell viability was evaluated relative to the total LDH released by exposure to 1 mM H2O2 for 24 h.

**ESR Spectrometry.** The -OH-scavenging activity of serofendic acid and DMSO was determined by ESR spectrometry. Serofendic acid or DMSO was suspended in 0.5% methylcellulose. Serofendic acid (final concentration ranging from 0.01 to 1 mM), DMSO (final concentration ranging from 0.01 to 1 mM) or vehicle (0.5% methylcellulose) was added to the reaction mixture containing diethylene-triamine pentaacetic acid (100 μM), FeSO4 (25 μM), H2O2 (50 μM), and DMPO (5 mM). The reaction mixture was transferred to a flat quartz cuvette and placed in the cavity of an ESR spectrometer (JEOL, Tokyo, Japan). The -OH produced by Fenton’s reaction between Fe2+ and H2O2 was trapped by DMPO, and the amount of the DMPO-OH spin adduct formed was measured exactly 3 min after the addition of H2O2 by ESR spectrometry. The quantity of -OH generated is determined by the intensity of the ESR signal of the DMPO-OH spin adduct. The signal intensity was evaluated using the relative peak of the second signal of the quartet of the DMPO-OH spin adduct and the intensity of Mn2+ as an internal standard. ESR settings were as follows: magnetic field, 3350 ± 100 G; microwave power, 8 mW; modulation frequency, 100 KHz; modulation amplitude, 1 G; response time, 0.1 s; amplitude, 4 × 100; and sweep time, 2 min.

**Statistical Analysis.** Values were presented as means ± S.E.M. The reproducibility of the results was confirmed in at least three different sets of experiments. Data shown in figures are from a representative set of experiments. All statistical analyses were performed with GraphPad InStat version 3.0 (GraphPad Software Inc., San Diego, CA). The statistical significance of the difference between groups was calculated by one-way analysis of variance and post hoc multiple comparison using Dunnett’s test. A difference was considered significant at P < 0.05.

**Results**

**Effects of Serofendic Acid on Rat Striatal Cultures.** First, we examined whether serofendic acid would affect the survival, proliferation, and morphology of rat striatal cells.

Incubation with serofendic acid alone at up to 100 μM for 24 h did not influence the cell viability of striatal cultures, as assessed by MTT assay (Fig. 2A) and LDH release assay (Fig. 2B). The brain consists of neuronal cells and glial cells. Immunolabeling for the neuron marker MAP2ab and the astrocyte marker GFAP revealed that application of serofendic acid (100 μM) did not affect the number of MAP2ab-positive cells (from 214.9 ± 9.1 to 216.8 ± 15.5 cells/field) and GFAP-positive cells (from 21.5 ± 2.0 to 21.0 ± 2.5 cells/field), compared with control conditions (Fig. 2C). In addition, serofendic acid (100 μM) did not cause marked morphological changes in MAP2ab-positive neurons and GFAP-positive astrocytes in rat striatal cultures. These results suggest that serofendic acid did not have any effect on the survival, proliferation, and morphology of rat striatal cells.

**Characterization of Paraquat-Induced Cell Death in Rat Striatal Cultures.** Paraquat reacts with NADPH to produce the paraquat radical, which reacts with O2 to generate O2−. We clarified the mechanism of the cytotoxic effect of paraquat using primary neuronal cultures prepared from rat striatum. Twenty-four hours of incubation with 200 μM paraquat reduced the cell viability in rat striatal cultures (Fig. 3). To determine the potential involvement of ROS such as O2−, H2O2, and -OH in the toxicity, we investigated the effects of various types of ROS scavengers. SOD is a catalytic enzyme that decomposes O2− to H2O2 and molecular oxygen. Coapplication of SOD (10−100 U/ml) with 200 μM paraquat for 24 h did not affect paraquat-induced cell death (Fig. 3A). Catalase is an enzyme that selectively dismutates H2O2 into H2O and O2. Enzyme treatment with catalase (10−10 U/ml) for 24 h elicited partial but significant protection against the paraquat-induced toxicity (Fig. 3B). Mn-TBAP is a membrane-permeable SOD mimetic responsible for scavenging intracellular O2− (Faulkner et al., 1994). Simultaneous addition of Mn-TBAP (100−300 μM) for 24 h markedly prevented the paraquat-induced cytotoxicity in a concentration-dependent manner (Fig. 3C). EUK-134 is a cell-permeable SOD catalase mimetic (Baker et al., 1998). EUK-134 (3−30 μM) reversed paraquat-induced cell death in a concentration-dependent manner (Fig. 3D). Free chelatable iron reacts with H2O2 to produce -OH. 2,2′-Dipyrpyridyl is a membrane-permeant chelator that favors the binding of Fe2+ (Petrait et al., 1999). 2,2′-Dipyrpyridyl (10−100 μM) also significantly inhibited the cytotoxicity of paraquat in a concentration-dependent manner (Fig. 3E). These results suggest that paraquat-induced toxicity is mediated by production of H2O2 and its subsequent decomposition to -OH.

**Effects of Serofendic Acid and DMSO on Paraquat-Induced Cell Death.** To verify the effect of serofendic acid on the paraquat-induced cytotoxicity in primary striatal cultures, serofendic acid was simultaneously administered with paraquat for 24 h. Immunofluorescence staining with anti-MAP2ab antibody and anti-GFAP antibody showed that treatment with paraquat (200 μM) for 24 h caused a marked decrease in the number of MAP2ab-positive neurons without a decrease in the number of GFAP-positive astrocytes, compared with control experiments (Fig. 4; Table 1). Serofendic acid suppressed the paraquat-induced decrease in the number of MAP2ab-positive cells, without a change in the number of GFAP-positive cells. Marked morphological changes in MAP2ab-positive neurons and GFAP-positive astrocytes were not observed in either group. Next, we examined the
concentration dependence of the neuroprotective effect of serofendic acid by LDH release assay. Serofendic acid afforded a concentration-dependent suppression of paraquat-induced neuronal death, with significant protection at concentrations of 10 and 100 μM (Fig. 5A). Because serofendic acid possesses a DMSO structure (Fig. 1), we compared the effect of DMSO on the paraquat-induced neurotoxicity with that of serofendic acid in the same concentration range. Co-administration of DMSO (1–100 μM) for 24 h failed to prevent paraquat-induced death of rat striatal neurons (Fig. 5B). However, a significant protective effect of DMSO on the paraquat-induced neurotoxicity was observed, when higher concentrations (30–300 mM) of DMSO were concomitantly applied for 24 h (Fig. 5C). DMSO alone at a concentration as high as 300 mM did not affect the survival of rat striatal neurons (data not shown). Notably, the minimal concentration of serofendic acid and DMSO in lowering paraquat-induced neuronal death was 10 μM and 30 mM, respectively.

Characterization of H₂O₂-Induced Cell Death in Rat Striatal Cultures. We next used H₂O₂-induced cytotoxicity to understand the mechanism of the protective effects of serofendic acid and DMSO on paraquat-induced neuronal death. First, we characterized the cytotoxic effect of H₂O₂ on primary cultures of rat striatal neurons. A 24-h exposure to 30 μM H₂O₂ caused cell death in rat striatal cultures (Fig. 6). To verify the involvement of ·OH in the H₂O₂-induced cytotoxicity, catalase, EUK-134, and 2,2′-dipyridyl were each simultaneously applied with H₂O₂ for 24 h (Fig. 6C). DMSO alone at a concentration as high as 300 mM did not affect the survival of rat striatal neurons (data not shown). Notably, the minimal concentration of serofendic acid and DMSO in lowering paraquat-induced neuronal death was 10 μM and 30 mM, respectively.

Effects of Serofendic Acid and DMSO on H₂O₂-Induced Cell Death. We further studied the effect of serofendic acid on the H₂O₂-induced cytotoxicity. After the H₂O₂ challenge (30 μM; 24 h), the number of viable MAP2ab-positive cells was decreased, whereas the number of GFAP-positive cells was unchanged (Fig. 7; Table 1). Serofendic acid at 100 μM rescued the H₂O₂-induced decrease in the number of MAP2ab-positive cells, without affecting the number of GFAP-positive cells. The morphology of MAP2ab-positive neurons and GFAP-positive astrocytes was not markedly changed in either group (Fig. 7). Moreover, the LDH release assay revealed that the neuroprotective effect of serofendic acid on H₂O₂-induced neurotoxicity was concentration-dependent and reached significance at concentrations of 10 and 100 μM (Fig. 8A). To elucidate the role of DMSO in the structure of serofendic acid, we also assessed the effect of DMSO on H₂O₂-induced cell death in rat striatal cultures. Concurrent application of DMSO at lower concentrations (1–100 μM) with H₂O₂ (30 μM) for 24 h did not have any effect on the H₂O₂-induced death of rat striatal neurons (Fig. 8B). However, DMSO at higher concentrations (30–300 mM) protected rat striatal neurons against the H₂O₂-induced toxicity (Fig. 8C). Interestingly, the protective effect of serofendic acid was observed at concentrations greater than 10 μM, whereas the concentration of DMSO required for neuroprotection against H₂O₂-induced cell death was >30 mM.

Effects of Serofendic Acid and DMSO on Hydroxyl Radical Formation. Considering the protective effects of serofendic acid and DMSO on the cytotoxicity caused by
paraquat and H$_2$O$_2$, it is suggested that serofendic acid and DMSO elicited protection by acting downstream of H$_2$O$_2$ production. To investigate the OH-quenching activity of serofendic acid and DMSO, we performed an ESR analysis using a direct detection system for OH. The OH produced from Fenton's reaction involving Fe$^{2+}$ and H$_2$O$_2$ was trapped by DMPO, and the DMPO-OH spectrum was observed, as shown in Fig. 9A. In the presence of serofendic acid, the ESR signal of the DMPO-OH spin adduct was remarkably reduced. The quenching effect of serofendic acid showed clear

**Fig. 3.** Cytotoxic effect of paraquat on rat striatal cultures. Cultures were incubated with 200 µM paraquat for 24 h. SOD (A), catalase (B), Mn-TBAP (C), EUK-134 (D), or 2,2'-dipyridyl (E) was simultaneously added with 200 µM paraquat for 24 h. Cell viability was assessed by MTT assay (A) or LDH release assay (B, C, D, and E). Data represent means ± S.E.M. (n = 4). ***, P < 0.01, compared with paraquat alone.

**Fig. 4.** Paraquat-induced cytotoxicity and its prevention by serofendic acid in rat striatal cultures. Representative photomicrographs showing control (A and B), paraquat treatment (C and D), and paraquat plus serofendic acid treatment (E and F). Cultures were exposed to paraquat (200 µM) with or without serofendic acid (100 µM) and then processed for immunocytochemistry with anti-MAP2ab antibody (A, C, and E) and anti-GFAP antibody (B, D, and F). Scale bar, 100 µm.
TABLE 1
Effects of serofendic acid on H$_2$O$_2$- and paraquat-induced cytotoxicity in rat striatal cultures
Cultures were incubated with H$_2$O$_2$ (30 μM) or paraquat (200 μM) in the presence or absence of serofendic acid (100 μM) and then processed for immunocytochemical analysis with anti-MAP2ab antibody and anti-GFAP antibody. Data represent means ± S.E.M. (n = 6).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of MAP2ab-Positive Cells</th>
<th>No. of GFAP-Positive Cells</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>218.3 ± 10.7</td>
<td>21.5 ± 2.0</td>
</tr>
<tr>
<td>Paraquat (200 μM)</td>
<td>61.8 ± 13.1</td>
<td>22.0 ± 3.8</td>
</tr>
<tr>
<td>Paraquat + serofendic acid (100 μM)</td>
<td>124.2 ± 17.5$^*$</td>
<td>21.5 ± 3.8</td>
</tr>
<tr>
<td>Control</td>
<td>219.7 ± 10.0</td>
<td>19.0 ± 1.3</td>
</tr>
<tr>
<td>H$_2$O$_2$ (30 μM)</td>
<td>73.8 ± 14.9</td>
<td>18.0 ± 1.7</td>
</tr>
<tr>
<td>H$_2$O$_2$ + serofendic acid (100 μM)</td>
<td>141.2 ± 16.0$^*$</td>
<td>18.2 ± 1.4</td>
</tr>
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$^*$ P < 0.05 compared with paraquat alone; $^{**}$ P < 0.01 compared with H$_2$O$_2$ alone.

concentration dependence, and reached significance at concentrations of 0.1 and 1 mM (Fig. 9B). Next, we compared the scavenging effect of serofendic acid with that of DMSO in the same concentration range (0.01–1 mM). In the Fe$^{2+}$-H$_2$O$_2$ reaction system, DMSO markedly diminished the DMPO-OH spectrum (Fig. 10A). The scavenging effect was dependent on concentration, and DMSO at concentrations of 0.1 and 1 mM caused a significant decrease in the intensity of the DMPO-OH signal (Fig. 10B). It should be noted that serofendic acid is as effective as DMSO in scavenging -OH.

Discussion

Neurons are susceptible to oxidative insults caused by excessive ROS production. O$_2^*$ is considered to have limited reactivity per se, and H$_2$O$_2$ seems not particularly toxic at physiological levels (Halliwell, 1992). -OH is the most reactive of ROS and reacts quickly with almost every molecule, including proteins, lipids, and DNA. In our experiments, SOD did not reduce paraquat-induced cytotoxicity, and catalase caused partial inhibition of the paraquat toxicity. O$_2^*$ is not diffusible across the cell membrane, whereas H$_2$O$_2$ can freely diffuse across the cell membrane (Halliwell, 1992). Because SOD and catalase are membrane-impermeable proteins, SOD and catalase exogenously applied affect only extracellular ROS. Thus, it is plausible that exogenous SOD failed to dismutate intracellular O$_2^*$ and exogenous catalase contributed to extracellular H$_2$O$_2$ detoxification. In contrast, we found that membrane-permeable Mn-TBAP and EUK-134 scavenged intracellular ROS, thereby resulting in suppression of the paraquat- and H$_2$O$_2$-induced toxicity. In biological systems, transition metal ions potentiate the formation of extremely toxic -OH through the Haber-Weiss and Fenton reactions (Halliwell, 1992; Barnham et al., 2004), so that chelation of intracellular free iron with 2,2’-dipyridyl blocked -OH production and attenuated the cytotoxicity of paraquat and H$_2$O$_2$. Together, the results of our experiments indicate that intracellular O$_2^*$ generated from paraquat dismutates into H$_2$O$_2$, and then H$_2$O$_2$ causes the ferrous iron-dependent formation of highly toxic -OH inside the cells. Additionally, our data indicate that H$_2$O$_2$ enters the intracellular space and generates -OH through the breakdown of H$_2$O$_2$ in the presence of intracellular ferrous ions. Both paraquat and H$_2$O$_2$ may produce approximately the same level of -OH to cause neuronal death, because the toxicity of paraquat and H$_2$O$_2$ was prevented by various antioxidants and a chelator tested to a similar extent.

We previously reported the discovery of a low molecular weight substance of embryonic metablome, serofendic acid, which is a unique class of atisane-type diterpenoid having a methylsulfoxide moiety (Kume et al., 2002; Terauchi et al., 2002; Akaike et al., 2003). Here, we verified the effect of serofendic acid on ROS-induced neuronal death with special emphasis on the role of DMSO. Seroendic acid at concentrations of 10 to 100 μM significantly protected striatal neurons against the cytotoxicity caused by paraquat and H$_2$O$_2$. We also investigated the -OH-quenching activity of serofendic acid using ESR spectrometry with the spin trapping tech-
nique and found that serofendic acid concentration dependently inhibited the formation of ·OH, which is consistent with our previous study (Kume et al., 2002). These findings suggest that serofendic acid attenuates paraquat- and H₂O₂-induced neuronal damage by reducing the level of ·OH. Next, we studied the possible role of DMSO in the structure of serofendic acid. At lower concentrations (10–100 μM) where serofendic acid showed protection, DMSO failed to modify paraquat- and H₂O₂-induced cytotoxicity. In contrast, higher concentrations (30–300 mM) of DMSO ameliorated this cytotoxicity. Consistent with reports that DMSO has the ability to scavenge ·OH (Reuvers et al., 1973), ESR analysis demonstrated that DMSO elicited a concentration-dependent suppression of the generation of ·OH. It is noteworthy that serofendic acid and DMSO showed comparable potency to prevent the formation of ·OH. These results suggest that the ·OH-scavenging activity of serofendic acid is attributable to its DMSO structure.

DMSO possesses a wide range of biological activities. In addition to its activity as a scavenger of ·OH (Reuvers et al., 1973), DMSO has been reported to regulate cell differentiation (Friend et al., 1971; Collins et al., 1978; Isom et al., 1985), the cell cycle (Darling et al., 1989; Sawai et al., 1990; Srinivas et al., 1991), and apoptosis (Marthyn et al., 1998; Fiore and Degrassi, 1999). Administration of DMSO to rodents and primates can reduce neuronal injury in experimental models of ischemic (Albin et al., 1983; de la Torre, 1983; Phillis et al., 1998) and traumatic brain injury (de la Torre, 1983, 1995), improve cerebral blood flow after injury (de la Torre, 1983), and reduce intracranial pressure after head trauma (Karaca et al., 1991). It is conceivable that the DMSO structure plays important roles in the cellular, molecular, and pharmacological effects of serofendic acid.

Most interestingly, the effective concentration of serofendic acid for a neuroprotective effect was lower than that of DMSO, despite the similar potency in ·OH-scavenging activity. These findings suggest that different mechanisms are involved in the neuroprotective effect of serofendic acid on oxidative stress from that of DMSO. There are putative reasons explaining the neuroprotective effect of serofendic acid.
First, the difference between serofendic acid and DMSO for neuroprotective action derives from the different distribution pattern and reactivity to \( \text{O}_2^* \) and \( \text{H}_2\text{O}_2 \). Serofendic acid may effectively reduce the intracellular level of \( \text{H}_2\text{O}_2 \), because of its localization to the site where \( \text{H}_2\text{O}_2 \) is generated, although we cannot exclude the possibility that serofendic acid affects the levels of \( \text{O}_2^* \) and \( \text{H}_2\text{O}_2 \) in addition to \( \text{H}_2\text{O}_2 \). Second, serofendic acid may up-regulate the expression of endogenous antioxidative molecules such as catalase, SOD, glutathione peroxidase, and glutathione to augment the resistance to oxidative stress. Third, the involvement of other actions such as anti-apoptotic/necrotic or survival-promoting signaling is also possible. Although serofendic acid provided neuroprotection at lower concentrations than DMSO, the underlying reason for the difference between them remains unresolved. Further research is warranted to determine the precise mechanisms of the neuroprotective effect of serofendic acid.

We now proposed that the neuroprotective action by serofendic acid at lower concentrations require its atisane structure, whereas the DMSO structure contributes to the \( \text{H}_2\text{O}_2 \)-scavenging activity of serofendic acid. Li et al. (2001, 2002a) demonstrated that an atisane-derivative, spiramine T, exhibited protective effects on cerebral ischemia-reperfusion injury in gerbils, and its mechanism might be related to reducing calcium accumulation and lipid peroxidation (Li et al., 2001), modulating the activities of endogenous antioxidant enzymes, and inhibiting nitric-oxide synthetase activity (Li et al., 2002a). In addition, recent studies revealed that atisane-derivatives showed antiplatelet aggregation activity (Shen et al., 2000; Li et al., 2002b). These findings suggest that atisane-type diterpenoids have various bioactivities. Further studies should focus on the structure-activity relationship for neuroprotective action.

Notably, this is the first study describing that serofendic acid affords protection against ROS-induced oxidative injury. Together with our previous studies (Kume et al., 2002; Taguchi et al., 2003), it is concluded that the antioxidant property of serofendic acid contributes to its neuroprotective actions.
Increased ROS formation occurs under numerous pathological conditions, including broadly, ischemic, inflammatory processes. For instance, oxidative stress has been postulated to be involved in the pathophysiology of renal, circulatory, pulmonary, retinal, and dermatological disorders, diabetes, and cancer as well as neurodegenerative diseases (Finkel and Holbrook, 2000; Schnackenberg, 2002; Hussain et al., 2003). Hence, the present findings raise the possibility that serofendic acid is a candidate for the cytoprotective therapy of various oxidative stress-mediated disorders.

References
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Fig. 10. Effect of DMSO on OH formation. A, representative ESR spectra of the DMPO-OH spin adduct in the presence or absence of DMSO. B, concentration dependence of OH-scavenging activity of DMSO. DMSO (0.01–1 mM) was added to the reaction mixture containing DMPO (5 mM), FeSO4 (25 μM), and H2O2 (50 μM). The amount of DMPO-OH spin adduct generated was detected exactly 3 min after the addition of H2O2 using ESR spectrometry. Data represent means ± S.E.M. (n = 8), **, P < 0.01, compared with control.