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Potential Role for Astroglial D-Amino Acid Oxidase in Extracellular D-Serine Metabolism and Cytotoxicity

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D-Amino acid oxidase (DAO) is a flavoenzyme that catalyzes the oxidation of D-amino acids. In the brain, gene expression of DAO is detected in astrocytes. Among the possible substrates of DAO *in vivo*, D-serine is proposed to be a neuromodulator of the *N*-methyl-D-aspartate (NMDA) receptor. In a search for the physiological role of DAO in the brain, we investigated the metabolism of extracellular D-serine in glial cells. Here we show that after D-serine treatment, rat primary type-1 astrocytes exhibited increased cell death. In order to enhance the enzyme activity of DAO in cells, we established stable rat C6 glial cells overexpressing mouse DAO designated as C6/DAO. Treatment with a high dose of D-serine led to the production of hydrogen peroxide (H_2O_2) followed by apoptosis in C6/DAO cells. Among the amino acids tested, D-serine specifically exhibited a significant cell death–inducing effect. DAO inhibitors, *i.e.*, sodium benzoate and chlorpromazine, partially prevented the death of C6/DAO cells treated with D-serine, indicating the involvement of DAO activity in D-serine metabolism. Overall, we consider that extracellular D-serine can gain access to intracellular DAO, being metabolized to produce H_2O_2. These results support the proposal that astroglial DAO plays an important role in metabolizing a neuromodulator, D-serine.

Key words: D-amino acid oxidase, astrocytes, chlorpromazine, hydrogen peroxide, D-serine.

Abbreviations: 3-AT, 3-amino-1,2,4-triazole; CPZ, chlorpromazine; DAAO, D-amino acid oxidase; FBS, fetal bovine serum; H_2O_2, hydrogen peroxide; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NMDA, *N*-methyl-D-aspartate.

D-Amino acid oxidase (DAO; EC 1.4.3.3) is a flavoenzyme that catalyzes the oxidation of D-amino acids to the corresponding imino acids and hydrogen peroxide (H_2O_2) (*1*). The imino acid is nonenzymatically hydrolyzed to α-keto acid and ammonia. In mammals, DAO is found at the highest concentrations in the kidneys, liver and brain. Previously, we determined the primary structures of the porcine (*2*), human (*3*), rabbit (*4*), and mouse (*5*) kidney DAO mRNAs. We also carried out a series of molecular biological studies on the structure–function relationship of DAO for the porcine (*6*, *7*) and human enzymes (*8*). RNA blot hybridization analysis of porcine tissues showed that three mRNA species were expressed in the kidneys and liver, but only one was detected in the brain, indicating the active biosynthesis of DAO in the brain and the brain-specific regulation of the its expression (*9*). Moreover, we reported the structural organization of the human DAO gene and the regulation of its expression (*10*). We then mapped this gene to human chromosome 12, *i.e.*, to within 1-cM of the Spinocerebellar Ataxia 2 gene locus (*11*), suggesting a genetic link between DAO and neurologic disorders. Recently, we demonstrated the gene expression of DAO in type-1 astrocytes from rat cerebellum and cerebral cortex (*12*).

D-Serine occurs in the mammalian brain (*13*) and is an endogenous agonist of the NMDA receptor (*14*, *15*). The extracellular concentration of D-serine parallels or is higher than that of glycine in the prefrontal cortex and in the striatum, respectively (*16*). Several reports have indicated that D-serine may play a role in pathological conditions related to dysfunction of the NMDA receptor. Massive stimulation of NMDA receptors has been implicated in neural damage following stroke (*17*). Elevation of the extracellular concentration of D-serine was observed after transient cerebral ischemia in animal models (*18*), and drugs that block the glycine sites of NMDA receptors prevented stroke-induced damage (*19*). Hypofunction of the NMDA receptor has also been implicated in the pathology of schizophrenia. D-Serine greatly improved positive, negative and cognitive symptoms in schizophrenic patients (*20*). Mice expressing only 5% of the normal level of the NR1 subunit of the NMDA receptor exhibited behavioral abnormalities related to schizophrenia, including increased motor activity, stereotypy, and deficits in social and sexual interaction (*21*). It is notable that novel human gene G72 was recently implicated in schizophrenia, and the gene G72 product has been shown to bind with DAO

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and to enhance its catalytic activity. DAO is itself associated with schizophrenia, and the combination of the G72/DAO genotypes has a synergistic effect on disease risk (*22*). In this context, it is interesting to note that the inhibitory effect of chlorpromazine, a classical antipsychotic drug, on the activity of DAO through competition with its coenzyme, flavin adenine dinucleotide, was reported by Yagi *et al.* (*23*).

To investigate the potential role of DAO in D-serine metabolism, we established rat glial cell lines (C6) overexpressing mouse DAO and examined the effect of treatment with a high dose of D-serine. Here we show that treatment with a high dose of D-serine induced apoptosis followed by the production of H_2O_2 as a result of DAO's catalytic activity in C6 cells, suggesting that astroglial DAO is involved in regulation of the extracellular level of D-serine, a neuromodulator of the NMDA receptor.

MATERIALS AND METHODS

Reagents and Antibodies—3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and 3-amino-1,2,4-triazole (3-AT) were purchased from Sigma, St. Louis, MO, USA. The DAO inhibitors, *i.e.*, sodium benzoate and chlorpromazine hydrochloride (CPZ), were purchased from Sigma and Wako, Osaka, Japan, respectively. The anti–rat liver catalase IgG was a gift from Dr. S. Yokota (Biology Laboratory, Yamanashi Medical University, Japan). Texas Red–conjugated anti–rabbit IgG and FITC-conjugated anti–mouse IgG were purchased from ICN Pharmaceuticals, Inc., Aurora, OH, USA, and Zymed Laboratories, Inc., San Francisco, CA, USA, respectively. Monoclonal mouse anti–human catalase antibodies were obtained from Sigma. Rabbit polyclonal antibodies raised against cleaved caspase-3 were purchased from Santa Cruz Biotechnology, Santa Cruz, CA, USA.

Cell Culture—Rat C6 cells (Dainippon Pharmaceutical Co., Osaka, Japan) were maintained in DMEM/F12 (Invitrogen Life Technologies, Carlsbad, CA, USA) containing 15% (v/v) horse serum, 2.5% (v/v) fetal bovine serum (FBS), and 1% penicillin-streptomycin at 37°C under a humidified atmosphere containing 5% CO_2. Rat primary type-1 astrocytes were prepared as described previously (*12*). Briefly, mixed glial cultures were prepared from Sprague-Dawley rat cerebral cortex or cerebellum on postnatal days 1–2 in poly-D-lysine–coated culture flasks containing Dulbecco's modified Eagle's medium (Sigma) supplemented with 10% (v/v) FBS. After 10–14 days of culture, astrocytes were isolated by shaking to dislodge microglia and subsequently purified by cytosine arabinoside treatment. The purified astrocytes were replated onto multi-well plates for assays.

Stable Cell Lines Overexpressing DAO—Rat C6 cells were transfected, using Effectene® Transfection Reagent (Qiagen, Valencia, CA, USA), with a plasmid encoding mouse DAO (pEF-BOSneo-mDAO). This plasmid was constructed by subcloning a cDNA fragment of mouse DAO (*5*) into the vector pEF-BOSneo (*24*, *25*). Resistant clones were screened using G418 (400 μg/ml). Subsequently, several clones that overexpressed mouse DAO were selected by Western blotting using a rabbit polyclonal antibody against pig kidney DAO. One of the clones exhibiting the highest level of expression was designated as C6/DAO and analyzed in the present study. Cells were maintained in DMEM/F12 containing 15% (v/v) horse serum, and 2.5% (v/v) FBS supplemented with 250 μg/ml of G418.

Western Blot Analysis—For Western blot analysis, cells were collected by scraping in phosphate-buffered saline (PBS) and then treated with a lysis buffer (137 mM NaCl, 15 mM EGTA, 1 mM Na_3NO_4, 15 mM $MgCl_2$, 0.1% Triton X-100, and 25 mM MOPS) containing Complete™ protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany). Cells were disrupted by sonication, extracted at 4°C for 30 min, and then centrifuged at 16,000 × *g* for 20 min. Protein samples resuspended in denaturating sample buffer were subjected to electrophoresis on 12.5% polyacrylamide gels (Daiichi Pure Chemicals Co., Ltd., Tokyo, Japan), followed by blotting onto Immobilon-P membranes (Millipore, Bedford, MA, USA). Detection of each protein was carried out with an ECL® Western blotting detection system (Amersham Biosciences, Little Chalfont, UK) according to the manufacturer's instructions.

Subcellular Fractionation—C6/DAO cells (2×10^6) were seeded into 100-mm dishes and incubated for 48 h. Cells were harvested by trypsinization, and washed twice in cold PBS. Then, they were divided into two tubes to prepare whole cell extracts and subcellular fractions. For subcellular fractionation, cells were first centrifuged at 200 × *g* for 10 min, and the resulting pellet was resuspended in 400 μl of cold hypotonic buffer (42 mM KCl, 10 mM HEPES, 5 mM $MgCl_2$, and Complete™ protease inhibitor cocktail) and then incubated for 30 min on ice. Cells were then broken by passing the cell suspension through a 30 G needle, followed by incubation for 30 min on ice. The intact nuclei, cell debris, and relatively heavy cellular membranes were initially removed by centrifugation at 1,000 × *g* for 10 min. The supernatants collected were centrifuged at 8,000 × *g* for 10 min to remove heavy membranes. Subsequently, the light membrane and cytosolic fractions were separated by centrifugation at 100,000 × *g* for 10 min. All pellets were resuspended in 400 μl of cold extraction buffer (1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, and Complete™ protease inhibitor cocktail in 2× PBS). After vortexing and incubation for 30 min on ice, extracts were obtained as supernatants by centrifugation at 16,000 × *g* for 20 min.

Cell Viability—The cytotoxic effects of several amino acids including D-serine were examined by means of the MTT assay. Cells (1×10^4) were seeded onto 96-well plates. After incubation for 24 h, cells were treated with several concentrations of amino acids and H_2O_2, respectively. At 21 h after incubation, 50 μl of the MTT (1 mg/ml) solution was added, followed by incubation an additional 4 h. After centrifugation, the supernatant was removed from each well and 150 μl of dimethylsulfoxide was added to dissolve the insoluble formazan crystals. The absorbance was measured at 550 nm.

H_2O_2 Assay—The production of H_2O_2 from C6/DAO cells after D-serine treatment was measured with an Amplex® Red Kit according to the manufacturer's instructions (Molecular Probes, Inc., Eugene, OR, USA). Briefly, cells were seeded at a density of 1×10^4 cells/well onto 96-well plates. After 36 h incubation, the cells were washed once in 50 μl of Hank's balanced salt solution and then incubated

for 1 h with an Amplex Red reagent mixture containing several concentrations of D-serine. Fluorescence was measured with a fluorescence microplate reader with excitation at 530 nm and detection at 590 nm.

Immunocytochemistry—C6/DAO cells (3×10^5) were seeded onto poly-D-lysine–coated coverslips and then incubated for 24 h at 37°C. The cells were washed in 0.1 M PBS and fixed in cold methanol at –30°C. The organellar membranes were then permeabilized in 0.1 M PBS with 0.5% Triton X-100 plus 10% goat serum for 5 min. Subsequently, the cells were blocked for 30 min with 10% (v/v) goat serum, and then incubated with rabbit anti–pig kidney DAO antibodies (1:250) and monoclonal mouse anti-human catalase antibodies (1:2,000) overnight at 4°C. After being washed in cold PBS, the cells were treated with Texas Red–conjugated anti–rabbit IgG (1:100) and FITC-conjugated anti–mouse IgG (1:160). Immunofluorescent images were taken under a confocal laser scanning microscope.

TUNEL Assay—C6/DAO cells were seeded at a density of 2×10^4 cells/well on 8-well chamber slides. After 36 h incubation, the cells were treated with or without 30 mM D-serine for 24 h. They were then subjected to fluorescence-terminal dUTP nick-end labeling (TUNEL) using an *In Situ* Cell Death Detection Kit (Roche). Thereafter, the cells were mounted with propidium iodide (PI) on slides to label nuclei and then examined under a confocal laser scanning microscope.

Statistics—All data were expressed as the means ± SD (8 samples for each set of conditions) for three or more independent experiments. Statistical comparisons between different treatments were made using one-way ANOVA with the post-hoc Scheffé's test. Differences were considered significant if $P < 0.05$.

RESULTS

Incubation of Rat Primary Astrocytes with D-Serine—To clarify whether or not astroglial DAO is able to metabolize extracellular D-serine in the brain, we evaluated the cellular effect of D-serine on rat primary astrocytes. In this study, we hypothesized that extracelluar D-serine is metabolized through astroglial DAO activity, H_2O_2 thereby being produced, which may affect cell viability. Initially, the MTT assay was performed 21 h after D-serine (3 and 30 mM) treatment of rat cerebral cortex- and cerebellum-derived primary type-1 astrocytes. As shown in Fig. 1, cell viability decreased for both types of cells on D-serine treatment. In addition, the level of cell death for cerebellum-derived type-1 astrocytes was slightly higher than that for cerebral cortex–derived ones. This finding was in good accord with our previous report that the gene expression level of DAO is higher in type-1 astrocytes from the cerebellum than in those from the cerebral cortex (*12*). These results show that the application of a high dose of D-serine induce astroglial cell death, implicating cellular DAO activity in extracellular D-serine metabolism.

Overexpression and Subcellular Localization of DAO—In order to enhance the enzyme activity of DAO in cells, we established a stable transformant of rat C6 cells overexpressing DAO (C6/DAO). Western blot analysis revealed that DAO was highly expressed in C6/DAO cells but only weakly expressed in C6 ones (Fig. 2A). H_2O_2 is a ROS produced by oxidases, as well as through spontaneous or

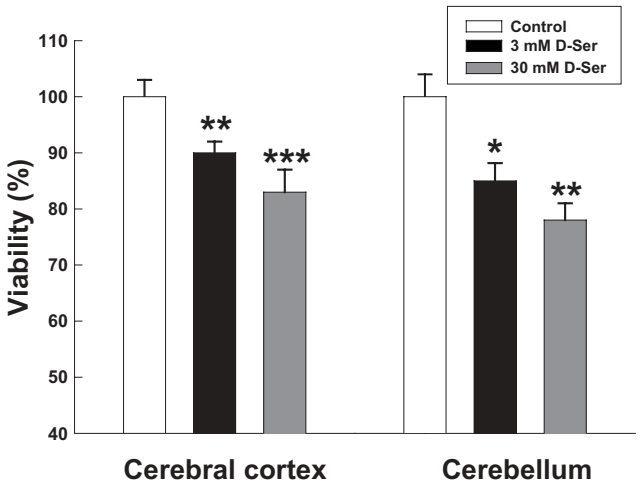

Fig. 1. **Effect of D-serine on viability of primary astrocytes.** The viability of primary astrocytes was examined by means of the MTT assay after treatment with 3 and 30 mM D-serine for 21 h. Type-1 astrocytes from cerebral cortex and cerebellum are shown on the left and right, respectively. The viability of untreated cells was taken as 100%, and the data are means ± SD of the percentage of the control values. The experiments are representative of three independent experiments. *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$, compared with control (ANOVA with post-hoc Scheffé's test).

enzymatic superoxide anion dismutation. A catalase that plays a role in detoxifying H_2O_2 was similarly detected in both types of cells, indicating that the DAO overexpressed in the cells had no influence on the expression of other peroxisomal enzymes.

The subcellular localization of the overexpressed DAO in C6 cells monitored with the peroxisomal marker catalase is shown in Fig. 2, B and C. Western blotting showed that most of the DAO was present in the heavy membrane fraction, which contained numerous peroxisomes, as judged on detection of a peroxisomal marker enzyme (Fig. 2B). This finding is well supported by the presence of a peroxisomal targeting signal at the C-terminus of mouse DAO and other mammalian DAOs (*2*, *3*, *5*, *26)*. Additional evidence of peroxisomal localization of overexpressed DAO in C6 cells was obtained by means of immunocytochemistry. As shown in Fig. 2C, co-localization of DAO and catalase was demonstrated on merging of the confocal images showing DAO (Red) and catalase (Green). Peroxisomes containing DAO in C6 cells appeared as yellow dots in the merged images. Virtually all the dots stained for the peroxisomal marker catalase were also positive for DAO. A similar punctate pattern of the intracellular distribution of peroxisomes was observed with the same monoclonal anti-catalase antibodies in HepG2 cells (*27*). Taken together, these results demonstrated that most of the DAO overexpressed in C6/DAO cells was recruited to peroxisomes containing catalase.

Cell Viability and Production of H_2O_2 in C6 Cells Treated with D-Serine—To confirm the effect of D-serine on primary astrocyte cultures, we also evaluated the viability of C6 and C6/DAO cells after D-serine treatment. The MTT assay was performed 21 h after treatment with 5, 10, 20 or 40 mM D-serine. As shown in Fig. 3A, the cell viability decreased in a dose-dependent manner. Based on

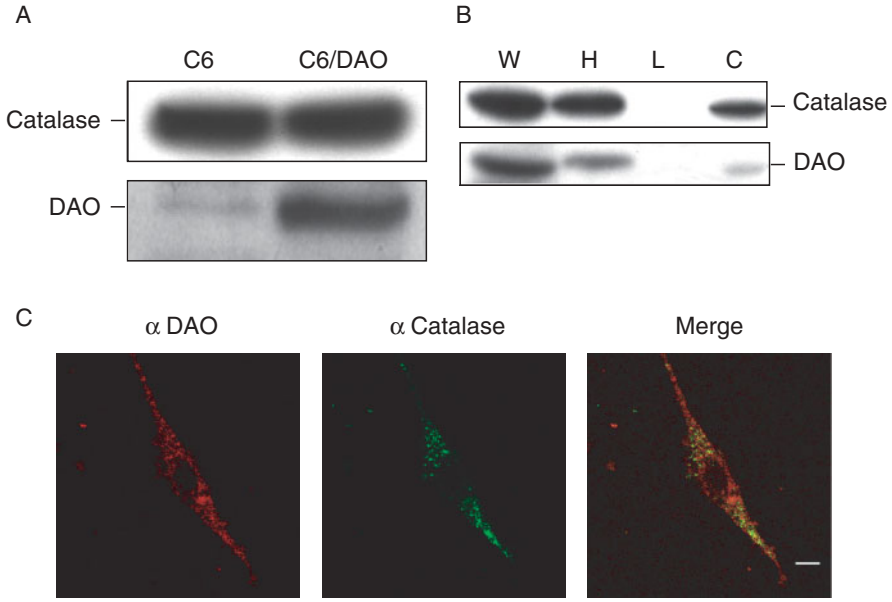

Fig. 2. **Expression and subcellular localization of DAO.** (A) Expression levels of DAO (lower) and catalase (upper) in C6 and C6/DAO cells, as determined on Western blotting. Each lane contains 15 μg of cell extract. The blots were probed with rabbit anti–pig kidney DAO antibodies and anti–rat liver catalase IgG, respectively. Membranes were then incubated with a horseradish peroxidase–conjugated secondary antibody. (B) Subcellular localization of DAO determined by Western blotting. Cellular proteins of C6/DAO were separated into whole cell lysate (W), heavy membrane (H), light membrane (L), and cytoplasmic (C) fractions. Protein samples (20 μl) were separated by electrophoresis on 12.5% polyacrylamide gels. Rabbit anti–pig kidney DAO antibodies (1:1,000) and anti–rat liver catalase antibodies (1:1,000) followed by a horseradish peroxidase–conjugated secondary antibody were used for Western blotting of DAO and catalase, respectively. Major components in the H fraction are mitochondria, lysosomes and peroxisomes, while those in the L fraction are microsomes, endoplasmic reticulum and Golgi apparatus. (C) Subcellular localization of DAO determined by immunocytochemistry. Rabbit anti-pig kidney DAO antibodies and monoclonal mouse anti–human catalase antibodies followed by Texas Red–conjugated anti–rabbit IgG and FITC-conjugated anti-mouse IgG were used for immunostaining of DAO and catalase, respectively. Scale bar, 10 μm.

the data shown in Fig. 3A, the viability of C6 cells after 30 mM D-serine treatment is expected to decrease to the level observed in primary astrocyte cultures. Therefore, we consider C6 cells comparable to primary astrocyte cultures in terms of D-serine metabolism. Moreover, the cytotoxic effect of D-serine on C6/DAO cells was greater than that on C6 cells, indicating that the cytotoxicity of D-serine in glial cells is dependent on the intracellular level of DAO.

We next examined the correlation between the production of H_2O_2 during the metabolism of D-serine and glial cell death. The production of H_2O_2 in C6/DAO cells was measured after treating the cells with various concentrations of D-serine for 1 h. As shown in Fig. 3B, C6/DAO cells treated with 20 and 40 mM D-serine exhibited an increase in H_2O_2 compared with vehicle-treated control cells. These results indicated that one of the possible causes of the astroglial cell death after D-serine treatment is H_2O_2. Although low doses of D-serine (5 and 10 mM) did not result in enhanced H_2O_2 production in this study, this apparent absence of H_2O_2 production might reflect the level of endogenous catalase, as can be seen in Fig. 2A.

Involvement of H_2O_2 in Cytotoxicity—To examine the cytotoxic effect of H_2O_2 on C6 and C6/DAO cells, we evaluated the cell viability at 21 h after H_2O_2 treatment. As shown in Fig. 4A, the cell viability decreased in a dose-dependent manner. To test the hypothesis that the cytotoxicity induced by D-serine in glial cells results from the production of H_2O_2, we evaluated cell viability after pretreatment with a catalase inhibitor, 3-amino-1,2,4-triazole (3-AT) (*28*), followed by D-serine treatment. As shown in Fig. 4B, 3-AT enhanced the cytotoxic effect of D-serine on both C6 and C6/DAO cells, indicating inhibition of cellular catalase activity. These results suggest that H_2O_2 is the causative agent of cell death induced by D-serine.

Effects of DAO Inhibitors Sodium Benzoate and Chlorpromazine on D-Serine–Induced Cytotoxicity—To verify that the cellular DAO activity is involved in the cell death induced by D-serine, sodium benzoate, a competitive inhibitor of DAO, was added to cells 30 min before exposure to 10 mM D-serine. In order to exclude a direct cytotoxic effect of sodium benzoate, but to obtain maximal inhibition, 20 mM sodium benzoate was used in the assay. Although the recovery of C6 cells pretreated with sodium benzoate was minimal, that of C6/DAO cells was significant (Fig. 5A). We did not observe a protective effect of sodium benzoate at 10 mM in C6/DAO cells treated with D-serine (data not shown).

We next examined another DAO inhibitor, chlorpromazine (CPZ), which is a classical antipsychotic drug and has been reported to inhibit DAO in competition with its coenzyme, flavin adenine dinucleotide (*23*). As shown in Fig. 5B, pretreatment with CPZ at a concentration of 1 μM significantly prevented the cell death induced by D-serine (10 mM) in C6/DAO cells. Taken together, these results indicated that astroglial DAO is involved in D-serine–induced cell death due to its D-serine metabolizing activity.

Amino Acid Specificity of Astroglial Cell Death—It has been reported that D-serine could be taken up by C6 cells

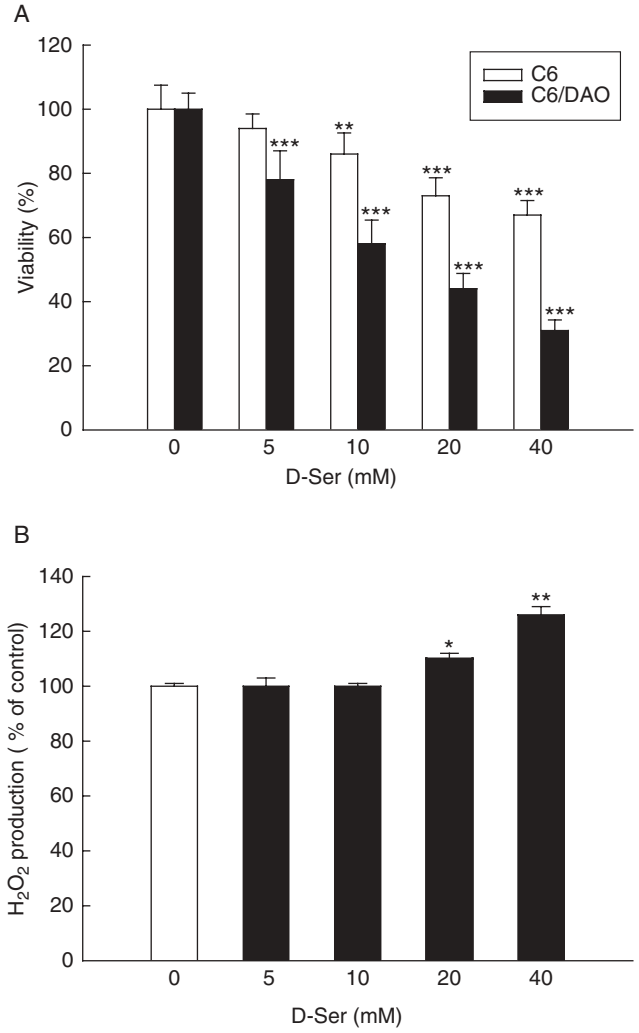

Fig. 3. **Effects of D-serine on cell viability and H_2O_2 production.** (A) The viability of C6 and C6/DAO cells was examined by means of the MTT assay after treatment with the indicated concentrations of D-serine for 21 h. (B) The production of H_2O_2 by C6/DAO cells was measured using a H_2O_2 detection kit after treatment with the indicated concentrations of D-serine for 1 h. The levels of viability and H_2O_2 production in untreated cells were taken as 100%, and the data are means ± SD of the percentage of the control values. The experiments are representative of three independent experiments. $^*P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$, compared with control (ANOVA with post-hoc Scheffé's test).

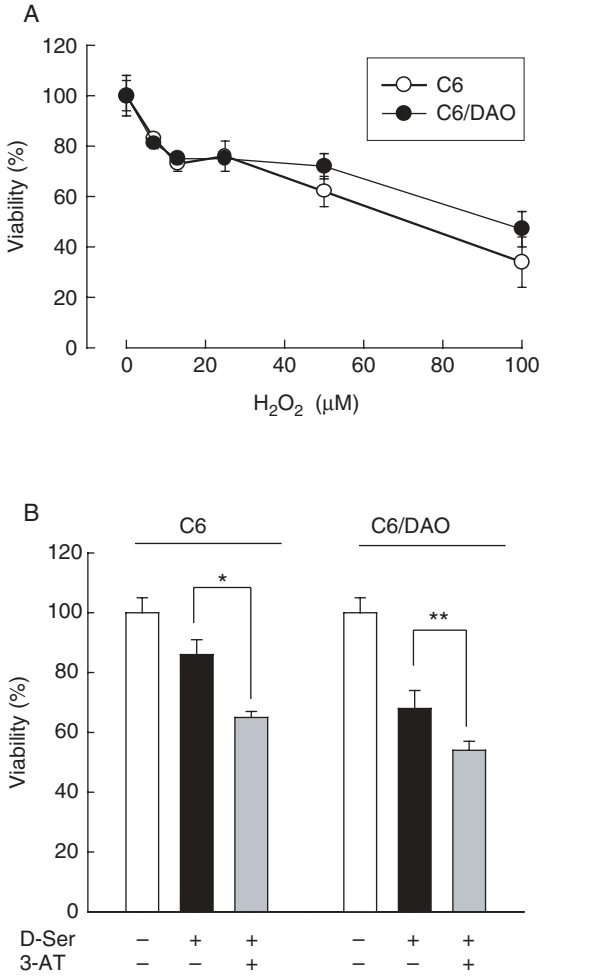

Fig. 4. **Effects of H_2O_2 and 3-AT on cell viability.** (A) The cytotoxic effect of H_2O_2 on glial cells was examined by means of the MTT assay. C6 and C6/DAO cells were treated with the indicated concentrations of H_2O_2 for 21 h. (B) Effects of catalase inhibition by 3-AT on C6 and C6/DAO cells were examined by means of the MTT assay. Both types of cells were pretreated with 20 mM 3-AT for 30 min before D-serine treatment. The cells were then treated with 10 mM D-serine for 21 h. The viability of untreated cells was taken as 100%, and the data are means ± SD of the percentage of the control values. The experiments are representative of three independent experiments. $^*P < 0.05$ and $^{**}P < 0.01$, compared with D-serine treatment (ANOVA with post-hoc Scheffé's test).

and that the properties of the uptake system resembled those of an ASCT2-like neutral amino acid transport system (*29*). Since this transport system showed broad substrate specificity and a higher affinity for L-serine than D-serine, we expected the cell death induced by D-serine to be common to other D- and L-amino acids. To examine this possibility, we determined the effects of various amino acids (3 and 30 mM) on C6 and C6/DAO cells. As shown in Fig. 6, the treatment of C6/DAO cells with 3 mM D-serine and D-serine plus glycine effectively decreased cell viability, whereas the treatment of C6 cells with other amino acids examined did not affect cell viability. In addition, the treatment of C6/DAO cells with 30 mM D-serine and glycine induced a greater increase in cell death compared with 3 mM treatment, whereas other amino acids examined did not exhibit a significant effect on cell viability. Although a previous *in vitro* experiment involving the purified pig DAO enzyme showed that D-alanine and D-proline were better substrates than D-serine (*30*), D-serine and glycine decreased cell viability most effectively in C6/DAO cells. It is of note that glycine, a poor substrate of DAO *in vitro*, exhibited a cytotoxic effect on C6/DAO cells and did not interfere with D-serine–induced cell death. These results suggest that astroglial cells possess an uptake system that preferentially transports D-serine among other D-amino acids, and that astroglial DAO can metabolize extracellular D-serine effectively.

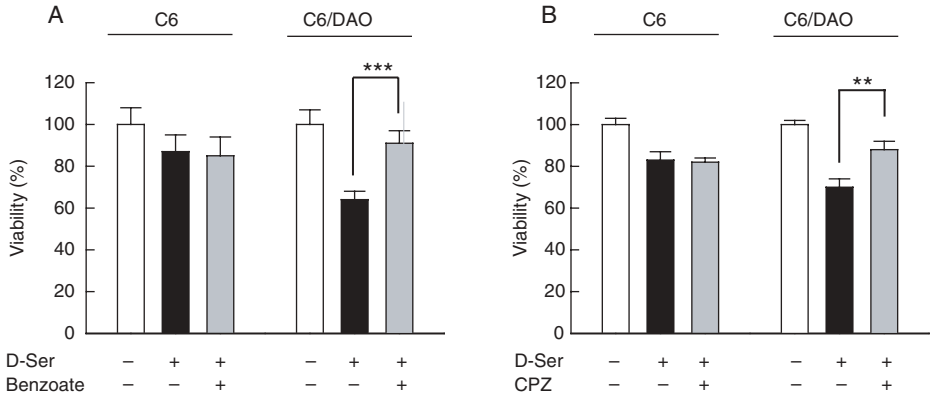

Fig. 5. **Effects of sodium benzoate and chlorpromazine on D-serine–induced cytotoxicity.** (A) C6 and C6/DAO cells were pretreated with 20 mM sodium benzoate for 30 min before D-serine treatment. (B) C6 and C6/DAO cells were pretreated with 1 μM chlorpromazine (CPZ) for 30 min before D-serine treatment. The cells were then treated with 10 mM D-serine for 21 h. Cell viability was measured by means of the MTT assay. The viability of untreated cells was taken as 100%, and the data are means ± SD of the percentage of the control values. The experiments are representative of three independent experiments. $^{**}P < 0.01$ and $^{***}P < 0.001$, compared with D-serine treatment (ANOVA with post-hoc Scheffé's test).

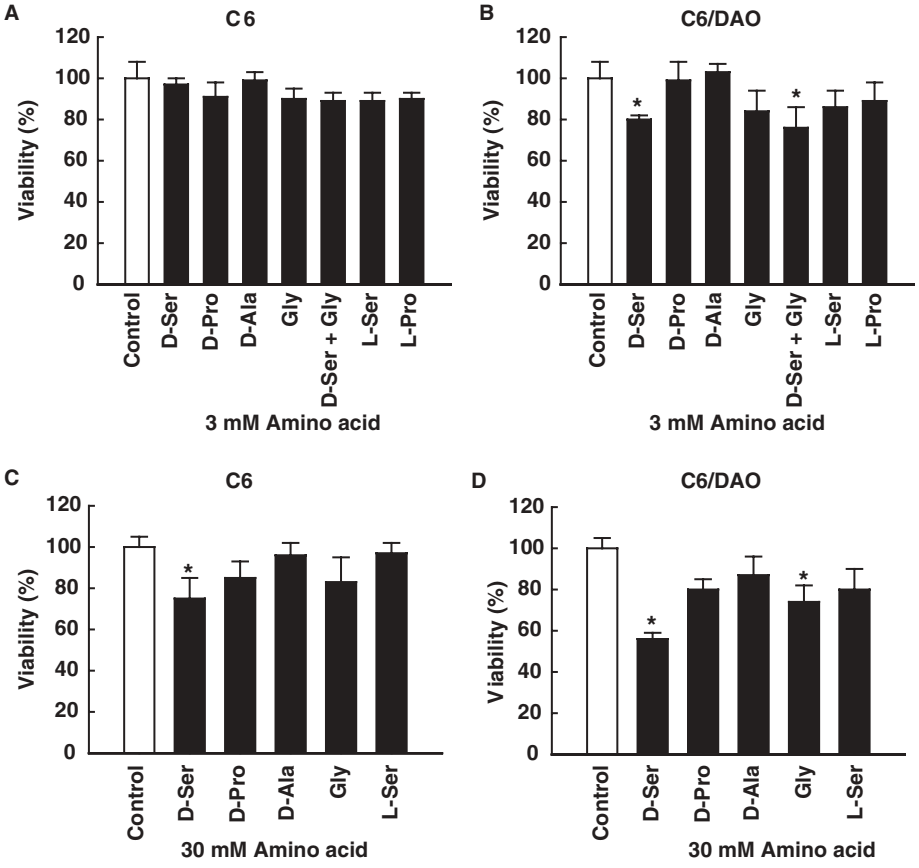

Fig. 6. **Effects of various amino acids on cell viability.** The MTT assay was performed after treatment with 3 mM (A, B) and 30 mM (C, D) amino acids in C6 (A, C) and C6/DAO cells (B, D) for 21 h. The viability of untreated cells was taken as 100%, and the data are means ± SD of the percentage of the control values. The experiments are representative of four independent experiments. $^{*}P < 0.05$, compared with control (ANOVA with post-hoc Scheffé's test).

Apoptosis in Astroglial Cells—We next examined whether or not the decrease in the number of viable cells after D-serine treatment was due to apoptosis, since H_2O_2 has been shown to induce programmed cell death. A TUNEL assay was performed 21 h after treatment with or without 30 mM D-serine. As shown in Fig. 7, D-serine–treated cells clearly showed TUNEL-positive nuclei compared with control cells. Arrows indicate strong TUNEL-positive nuclei.

To confirm the D-serine–induced apoptosis, we investigated the effect of D-serine on caspase-3 activation in C6/DAO cells by Western blot analysis, since the activation of caspase-3 by H_2O_2 has been reported in several cell types such as hippocampal neurons (*31*) and PC12 cells (*32*). As shown in Fig. 7G, treatment of C6/DAO cells with D-serine induced activation of caspase-3, producing an active subunit of about 20 kDa in size. Treatment of C6/DAO cells with H_2O_2 (50 μM) was used as a positive

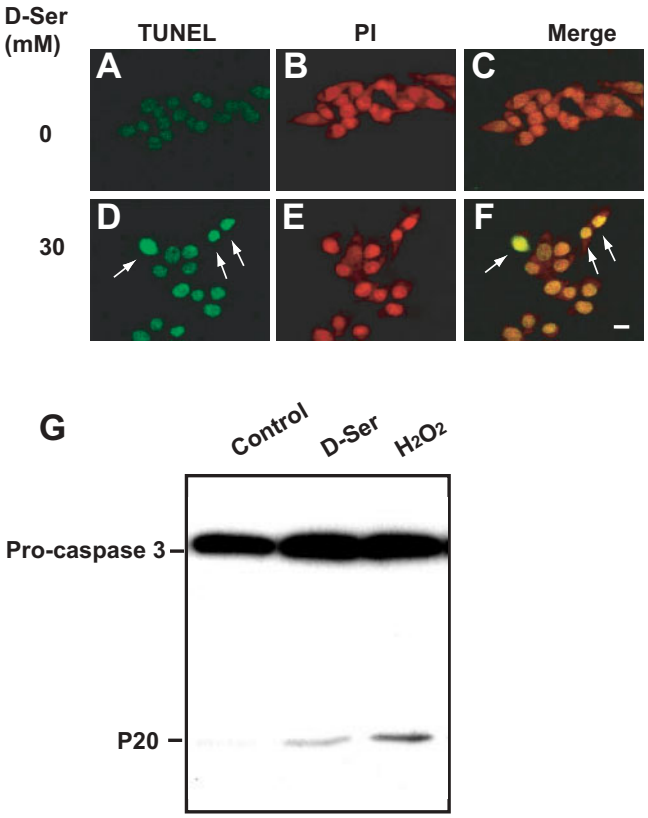

Fig. 7. **Apoptosis in astroglial cells.** (A–F) TUNEL-staining was performed after treatment with or without 30 mM D-serine for 21 h. (A–C) Vehicle-treated cells; (D–F) D-serine–treated cells. Cell nuclei were counterstained with PI (red). Arrows indicate strong TUNEL-positive cells. Scale bar, 10 μm. (G) Western blots show the effect of D-serine on caspase-3 activation. C6/DAO cells were incubated with D-serine (30 mM) and H_2O_2 (50 μM) for 21 h before assay. Cell lysates (20 μg) were subjected to electrophoresis on 15% polyacrylamide gels followed by transfer to Immobilon-P membranes and then incubation with rabbit anti–caspase-3 polyclonal antibodies (1:200). The secondary antibody reaction was performed as in Fig. 2.

control. Taken together, we consider that the astroglial cell death induced by D-serine treatment comprises apoptosis.

DISCUSSION

In the present study, we examined the effect of treatment with an artificially high dose of D-serine on glial cells to investigate the potential role of DAO in the brain system. We extended our studies from primary cultures of astrocytes to C6 cells, because C6 cells have frequently been used as a model of glial function and are considered to correspond to type-1 astrocytes, based on Ran-2 expression (*33*). The advantage of using a high dose of D-serine is that the involvement of astroglial DAO activity in the metabolism of D-serine can be detected through the cytotoxicity of the metabolite, H_2O_2. Previously, histochemical analysis revealed that DAO activity was absent in the forebrain, but present in the brain stem, cerebellum and spinal cord (*34*). However, we have reported that the gene expression of DAO was detected in C6 cells and in type-1 astrocytes from the cerebral cortex as well as the cerebellum (*12*). Moreover, we have observed that high-dose D-serine treatment induced the death of cerebral cortex–derived type-1 astrocytes (Fig. 1), suggesting that astroglial DAO from the cerebral cortex could be involved in the metabolism of D-serine.

Although the substrate of DAO *in vivo* was not known for many years, a substantial amount of free D-serine has been found in the mammalian brain (*13*). D-Serine is an agonist of the NMDA receptor (*15*) and is present at a low micromolar concentration in the mammalian brain (*16*). Moreover, D-serine has been implicated in several pathophysiological conditions related to NMDA receptor dysfunction. Therefore, it is considered that regulatory mechanisms governing the extracellular level of D-serine exist in the brain. Possible mechanisms are as follows.

First, in view of the production of D-serine, the extracellular concentration might be regulated by the activity of serine racemase, which is known to convert L-serine to D-serine. Serine racemase was found to be highly expressed in the brain and to be localized to astrocytes with a similar distribution to that of D-serine (*35*). The pharmacological inhibition of this enzyme decreased the level of D-serine (*36*), suggesting that this enzyme is involved in the regulation of the synaptic concentration of D-serine.

Second, it is possible that D-serine could be removed from the synaptic cleft by specific amino acid transporters. To date, the transport system most associated with serine has been reported to be system ASC, although uptake of D-serine may also occur through system L (*37*). In C6 cells, the D-serine uptake system showed broad substrate specificity and higher affinity for L-serine than for D-serine (*29*). In cortical astrocytes, D-serine has also been reported to be transported *via* a similar pathway (*38*). However, it is unlikely that these transport systems with broad substrate specificity act as regulators of the D-serine level, which apparently requires fine and specific regulation. NMDA receptors are regulated by the endogenous amino acids glycine and D-serine, which bind to an NMDA-associated glycine-binding site. In our experiment, D-serine and glycine decreased cell viability most effectively in C6/DAO cells (Fig. 6), although D-proline and D-alanine are better substrates than D-serine for the pig kidney DAO enzyme activity *in vitro* (*30*). Therefore, our data support the possibility that astroglial cells have a specific uptake system for D-serine with high affinity. Furthermore, it is interesting that D-serine plus glycine had a slightly increased cytotoxic effect on C6/DAO cells, although glycine is a poor substrate of DAO *in vitro*. In a previous uptake study, glycine reduced the accumulation of D- and L-serine in C6 cells (*29*). These observations suggested that glycine may also be transported and can be catalyzed by DAO.

Third, astroglial DAO may play a role in modulating the extracellular level of D-serine in the brain. In this study, we have shown that D-serine treatment induced the death of primary astrocytes and C6 cells. In addition, overexpression of DAO in C6 cells (C6/DAO cells) markedly increased the D-serine–induced cytotoxicity. These results indicate the possible involvement of astroglial DAO in the cytotoxic effect of a high dose of D-serine. Meanwhile, it is unlikely that the changes in cell viability observed with high doses of D-serine are due to non-specific effects such as high osmolality, because other amino acids did not have cytotoxic effects at the same doses (Fig. 6). However, it can not

be completely excluded that decreased cell viability could be partly caused by high doses of amino acids, which may be harmful to cultured cells (39), and by D-serine, which inhibits serine palmitoyltransferase, which is responsible for the initial step of sphingolipid biosynthesis (*40*).

To confirm that astroglial DAO activity was involved in the cytotoxic effect of a high dose of D-serine, CPZ as well as sodium benzoate was examined as a DAO inhibitor. CPZ is the most important antipsychotic compound among the large group of phenothiazine derivatives. It is widely used for treating mental and personality disorders, especially schizophrenia, because of its anti-dopaminergic properties. Interestingly, CPZ was also found to inhibit D-amino acid oxidase through competition with its coenzyme, flavin adenine dinucleotide (*23*). As shown in Fig. 5, not only 20 mM sodium benzoate but also 1 μM CPZ significantly protected C6/DAO cells from D-serine. The concentrations of benzoate and CPZ used seem to be puzzling in view of their affinity to the pure enzyme *in vitro* (the K_d for both ligands are in the micromolar range). However, the efficiency of transport or permeation of benzoate and CPZ across the membrane needs to be taken into account. In fact, CPZ is known to be extremely hydrophobic and to exhibit good cell permeability as well as high serum-protein binding. Since CPZ also exhibits inhibitory effects on various other enzymes, the precise mechanisms underlying the effects of these ligands on C6 and C6/DAO cells remain to be elucidated. In addition, loss of recovery of C6 cells with benzoate and CPZ may reflect the catalytic activity of endogenous catalase and the low level of DAO expression in C6 cells. These results show that astroglial DAO is involved in the metabolism of extracellular D-serine.

H_2O_2, one of the major ROS, is a potentially harmful byproduct of normal cellular metabolism that directly affects cellular functions and survival (*41*). The production of H_2O_2 by 9L*daao*17 cells, a stable transformant of 9L glioma cells expressing red yeast DAO, incubated with D-alanine demonstrated oxidative stress is a mediator of cytotoxicity (*42*). Tumor-targeted delivery of polyethylene glycol–conjugated D-amino acid oxidase together with injection of D-proline generated a potent cytotoxic compound, H_2O_2, at the tumor site (*43*). These findings showed that DAO activity and hence the generation of H_2O_2 can be regulated by the exogenous administration of a D-amino acid. As shown in Fig. 3B, we also found that treatment of C6/DAO cells with D-serine resulted in H_2O_2 production. On the other hand, catalase plays an important role in the antioxidant defense of the organism, as do other enzymes such as superoxide dismutase and glutathione peroxidase, by degrading H_2O_2 to H_2O. As shown in Fig. 4B, inhibition of catalase by 3-AT partially enhanced the cell death induced by D-serine treatment in C6 and C6/DAO cells. We consider the following reasons may explain the possibility that H_2O_2 was able to avoid degradation by catalase. One is that H_2O_2 might have come from the remaining cytosolic DAO that was not recruited to peroxisomes. Another possibility is that all DAO was not colocalized with catalase. Indeed, all dots positive for catalase in C6/DAO cells did not overlap with those for DAO (Fig. 3C). Taken together, these results indicated that H_2O_2 is a causative agent of glial cell death induced by D-serine. Since D-serine is present at a micromolar concentration in the normal mammalian brain, the physiological relevance of this cytotoxic effect may seem unclear. However, it is possible that additional cellular stress or up-regulated biosynthesis and accumulation of D-serine in pathological conditions may influence the cytotoxic effect of D-serine, presumably through the production of H_2O_2.

The finding that D-serine is toxic to glial cells quite resembles the phenomenon observed for the rat kidney. It is well known that D-serine is nephrotoxic in rats. Intraperitoneallly injected D-serine damages the cells in the proximal tubules, leading to proteinuria, glucosuria, and aminoaciduria (*44*). D-Amino acid oxidase is considered to be involved in this process. H_2O_2, a product of the D-amino acid oxidase reaction, is also suspected to be a causative agent (*45*). However, this nephrotoxicity is only observed with D-serine. Other D-amino acids, such as D-alanine, D-threonine and D-cysteine, are not nephrotoxic, although D-alanine is a good substrate of D-amino acid oxidase (*46*). These results are quite similar to the findings described in the present paper. The previous studies so far performed have indicated that renal cells become necrotic. However, we demonstrated that a high dose of D-serine causes apoptosis of glial cells, based on the results of the TUNEL assay and the activation of caspase-3 in the culture system.

In conclusion, we have shown that astroglial DAO contributes to the metabolism of extracellular D-serine, by the detection of cytotoxicity of its metabolite, H_2O_2, with a high dose of D-serine. Detailed studies on the D-serine transport system in astroglial cells are also necessary to clarify the mechanism modulating the extracellular level of D-serine in the brain. Moreover, further study on the D-serine–D-amino acid oxidase system in the brain will help overcome diseases related to hyper- and hypofunctions of NMDA receptor–mediated neurotransmission, such as stroke and schizophrenia.

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